Leaf expansion and biomass allocation in wild wheat (*Aegilops*) species

Bladexpansie en biomassaverdeling
in wilde tarwe (*Aegilops*) soorten

(*met een samenvatting in het Nederlands*)

Proefschrift

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Lieve Jenny Alfons Bultynck

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Promotor	Prof. Dr. J. T. Lambers Plant Ecophysiology, Utrecht University & Plant Sciences, The University of Western Australia

The investigations reported in this thesis were carried out at the Plant Ecophysiology Research Group, Utrecht University, the Netherlands, and at the Department of Plant Sciences, The University of Western Australia, Australia.

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Chapter 1

General Introduction

Cereal crops are often grown in Mediterranean environments, where stored soil-water is limited and the crop needs to get its water from current rainfall. However, in these dry and hot environments, as much as 50% of the rainfall evaporates directly from the soil and is lost for the plants (Leuning *et al.*, 1994). One way to avoid this problem is by increasing the rate of leaf area expansion in the early stages of crop development, also referred to as 'early vigour'. A faster-closing canopy will shade the soil surface earlier, thereby reducing evaporation and increasing water availability for the crop (Siddique *et al.*, 1990). Together with increasing crop water-use efficiency, the faster-expanding leaf canopy increases light interception more quickly (Richards, 2000). In addition, it makes the crop more competitive with weeds for light (Lemerle *et al.*, 2001). For different crop species and cultivars, 'early vigour' has been associated with greater final biomass and grain yield at harvest (Siddique *et al.*, 1990; López-Castañeda & Richards, 1994). The association between early leaf area development, biomass production and yield of cereal crops emphasises the importance of understanding the physiological processes involved in determining leaf expansion and its relationship with whole plant growth. This line of work may highlight traits that can contribute to a higher leaf area expansion and hence increased crop productivity.

Aegilops species

The species we have used for the experiments in this thesis are from the genus *Aegilops* L. (Poaceae). This genus has gained a lot of interest since the discovery of its close relationship with cultivated wheat (*Triticum*). Some of the *Aegilops* species have contributed, in the distant past, to the genome of wheat through natural hybridisation. The hybridisation between tetraploid *T. turgidum* (genomic formula: AABB) and diploid *Ae. tauschii* (DD) resulted in hexaploid *T. aestivum* (genomic formula: AABBDD) (Feldman & Sears, 1981; Van Slageren, 1994).

Over the last decades, the range of genetic variation in cultivated wheat has narrowed dramatically, reducing the possibility for further wheat improvement and making wheat crops more vulnerable to new diseases and climatic changes (Loss & Siddique, 1994). This calls for new approaches to increase the genetic variability in wheat. Due to their genetic link with cultivated wheat species and their adaptations to a wide range of habitats, wild relatives of wheat may be potential donors of valuable traits for future wheat cultivars that are better adapted to drier and warmer conditions, diseases and extreme temperatures (Feldman & Sears, 1981; Damania, 1993).

In order to explore genetic variation related to desirable traits for incorporation in wheat, the physiological variation in such traits needs to be investigated. Villar *et al.* (1998) have demonstrated a wide variation in early growth potential and biomass allocation parameters in 20 *Aegilops* species. Furthering this study, we investigated the control of leaf growth in some of these *Aegilops* species to develop a better understanding of variation in early growth potential, one of the desirable traits for future wheat cultivars.

Cellular processes underlying leaf area expansion in monocotyledonous species

Leaves develop from leaf primordia, which are initiated at the apical meristem of the stem. The rate and duration of expansion of a newly formed leaf determines its mature size. Leaf growth is determined by the processes of epidermal cell division and cell expansion. The epidermis is considered to be controlling organ growth by restricting expansion of the inner organ tissues (Kutschera, 1992; Becraft, 1999). In dicotyledonous leaves, cell division and cell expansion overlap spatially and temporally across the entire leaf blade. However, there is a trend for cell division and cell expansion to cease first in the distal portions of the leaf blade and to continue longest at the leaf base (Dale, 1992). Although the same basipetal age gradient is found in monocotyledonous leaves, their development differs from that of the dicotyledonous leaves. In these leaves, cells are arranged in parallel files, in which cell division and cell expansion are spatially and temporally separated, and occur only in the growth zone at the base of the leaf (Sharman, 1942; MacAdam *et al.*, 1989; Schnyder *et al.*, 1990). The growth zone is enclosed within a whorl of mature leaf sheaths, insulated from

direct light. Cells are produced in the basal meristem, and displaced away from the leaf base by the continuous production and elongation of new cells. When leaving the meristem and entering the 'elongation-only' zone, the cells stop dividing and continue to elongate more rapidly until the cells exit the elongation-only zone and enter the maturation zone. These processes result in a typical cell length distribution with distance from the leaf base. At the start of leaf elongation, only the blade is growing. The onset of sheath growth occurs without any visible change in the rate of leaf elongation or cell length distribution in the growth zone (Kemp, 1980; Schnyder *et al.*, 1990). The unidirectional developmental gradient of cells makes the monocotyledonous leaf an ideal model system to study the cellular basis of leaf expansion.

The kinetics of cell elongation and cell division in growing grass leaves can be studied by means of a kinematic analysis (Erickson & Sax, 1956; Green, 1976; Silk & Erickson, 1979; Gandar, 1983). The data required for such an analysis can be obtained in different ways. The most common way is by marking the growing leaf blade at equidistant locations along the growth zone, and following the displacement of these markers with time (*e.g.*, Schnyder *et al.*, 1987; Bernstein *et al.*, 1993). Because the growth zone of monocotyledonous leaves is enclosed by the whorl of leaf sheaths, the only way to mark it is by piercing the leaf sheath with a fine needle. However, this method is invasive and causes a decrease in leaf growth rate. An alternative, less invasive approach to study cell kinetics is by analysing the cell length distribution along the growth zone during a period of steady leaf growth (*e.g.*, Volenec & Nelson, 1981; Schnyder *et al.*, 1990; Beemster *et al.*, 1996; Fiorani *et al.*, 2000).

Numerous authors have studied environmental effects on cell elongation and cell division in growing leaves (*e.g.*, MacAdam *et al.*, 1989; Ben-Haj-Salah & Tardieu, 1995; Beemster *et al.*, 1996; Fricke *et al.*, 1997; Masle, 2000). These studies have shown that both processes are affected by environmental conditions. Less is known about the cellular processes underlying differences in leaf elongation rates between species (Fiorani *et al.*, 2000) and genotypes (Volenec & Nelson, 1981; Masle, 2000). I have determined the cellular basis of inherent differences in leaf elongation rate between two *Aegilops* species with contrasting leaf elongation rates.

Regulation of cell division and cell expansion

When changes in cell number are part of a growth response, it is more often the number of dividing cells that is affected and not the rate of cell division *per se* (Francis, 1998, and references therein). The mechanisms involved in the activation of non-cycling meristematic cells into the cell cycle are still largely unknown. However, two factors that seem to be required for cell division are a minimum cell size (or a minimum amount of metabolic machinery) and the expression of the *cdc2* gene (John *et al.*, 1993; Jacobs, 1997). Good

evidence now exists that the expression of the *cdc2* gene is controlled by plant growth regulators (Francis & Sorrell, 2001, and references therein). Sucrose is also a strong regulator of the cell cycle (Francis, 1998), possibly through its effect on gene expression (Farrar *et al.*, 2000).

Two essential physical processes work together in an expanding plant cell: extension of the cell wall and uptake of water. Lockhart (1965) was one of the first to formulate an equation describing the relative increase in cell volume (V) with time (t) as a function of cell wall extensibility (m), turgor pressure (P) and yield threshold (Y):

$$\frac{dV}{Vdt} = m(P - Y)$$

Over the last decades, several authors (*e.g.*, Passioura & Fry, 1992) have modified this theory but the basis has remained the same. Cell expansion is driven by the turgor pressure generated by the flux of water into the growing cell. The influx of water in turn is determined by the cell's turgor and osmotic potentials, and the water potential outside the cell. Only when the turgor pressure exceeds the yield threshold of the cell wall will it trigger cell extension. Changes in cell growth rate can be determined by changes in either turgor pressure or cell wall extensibility or both. However, leaf growth rate is correlated more often with cell wall extensibility than with turgor pressure in both dicotyledonous and monocotyledonous species (Van Volkenburgh, 1999, and references therein). The cellular mechanisms controlling changes in cell wall extensibility are only partly known. In bean and pea leaves, light-stimulated leaf expansion and cell wall extensibility are associated with acidification of the cell wall, resulting from increased proton pump activity in epidermal and mesophyll cells (Van Volkenburgh & Cleland, 1980; Linnemeyer *et al.*, 1990; Staal *et al.*, 1994). In water-stressed maize leaves, reduced growth rates are associated with a loss of the cell wall's capacity to extend upon acidification (Van Volkenburgh & Boyer, 1985). It has been suggested that protein molecules located in the cell wall, like expansins (Cosgrove, 2000) and yieldins (Okamoto-Nakazato *et al.*, 2000) which are most active at low pH, are responsible for the acid-induced cell wall loosening. Other cell wall proteins, *e.g.* xyloglucan endotransglycosylases (XET), have also been associated with wall loosening and leaf expansion (Fry, 1995; Palmer & Davies, 1996). These enzymes are most active at pH 6 and they are not thought to play a role in acid-induced wall loosening (Purugganan *et al.*, 1997). In summary, plant cells can differ in their cell wall extensibility due to either a difference in apoplast pH, a difference in the wall's capacity to loosen upon acidification, or other mechanisms that do not involve acidification of the apoplast. In this thesis, I have explored the possibility that the cell walls of *Aegilops* species with contrasting leaf elongation rates differ in their capacity to respond to acidification.

Integration of leaf growth in whole plants

Although leaf elongation rate of individual grass leaves is often assumed to be a major determinant of variation in growth rate, the correlation between a plant's relative growth rate (RGR) and its rate of leaf elongation (LER) is not straightforward (Bultynck *et al.*, 1999). Variation in a plant's *exponential* growth rate cannot be accounted for by variation in a *linear* process such as leaf elongation of individual leaves. However, the mechanisms that underlie variation in a plant's growth potential (*i.e.* relative growth rate, RGR) may be the same that cause variation in the growth potential of its leaves (LER).

Plant growth can be described from two different angles. The first assesses *growth* as increase in dry mass, *i.e.* relative growth rate (RGR), and the second as *development* of meristems. RGR is the product of carbon acquisition by photosynthesis and carbon use in respiration, which are included in the net assimilation rate (NAR; rate of increase in plant mass per unit leaf area), and biomass allocation to leaf area, represented by the leaf area ratio (LAR; total leaf area per unit total dry mass). The search for parameters explaining differences in RGR amongst plant species has been the object of many studies (Lambers *et al.*, 1998a). A recent literature review by Poorter & Van der Werf (1998) shows that LAR is the most important factor in explaining inherent variation in RGR in herbaceous species, including grasses, and that differences in LAR are mainly due to variation in specific leaf area (SLA). However, these conclusions are based on correlations between RGR and its underlying parameters and are not equivalent to mechanisms causing variation in plant growth potential. Rather, these correlations might be the consequence of differences in growth potential between species. This brings us to the second approach of plant growth, *i.e.* development of meristems. RGR might be determined by the number and size of meristematic regions and the rates of cell division and cell expansion in these regions, which are reflected in the number and growth rates of leaves and roots (Lambers, 1998; Nagel, 1998). Since leaf and root meristems are strong sinks for carbon and nitrogen (Skinner & Nelson, 1995; Farrar & Jones, 2000), development is closely linked to the processes of resource acquisition, biomass allocation and dry mass increment. In order to gain better insight into the control of RGR, we need to look at growth from the perspective of meristem development as well as growth analysis. I have determined variation in RGR amongst several *Aegilops* and wheat (*Triticum*) species from both these perspectives.

Regulation of leaf growth and RGR by gibberellins

Several phytohormones have an effect on cell growth, leaf growth, biomass allocation and whole shoot growth. In cereal crop species, the hormones most clearly associated with differences in growth are gibberellins (GAs). GAs are involved in the control of cell growth (Keyes *et al.*, 1990; Sauter *et al.*, 1995; Tonkinson *et al.*, 1995; Wenzel *et al.*, 1997), leaf

growth (Paolillo *et al.*, 1991; Smith *et al.*, 1996; Chandler & Robertson, 1999), as well as RGR (Rood *et al.*, 1990). Most of these studies have been carried out on dwarf genotypes of cereals, which either have reduced sensitivity to GA or reduced levels of endogenous bioactive GA.

In a comparison of wild-type barley with nine barley mutants showing different degrees of dwarfing, Wenzel *et al.* (1997) showed that variation in leaf length was associated with variation in cell number, in cell length, or in both, depending on the leaves, leaf parts, mutants and cell types that were compared. This suggests that both cell division and cell expansion depend on GA for optimal leaf growth in barley. More direct evidence for a regulatory role of GA in cell division and cell expansion is also available. Sauter *et al.* (1995) have shown that GA stimulates cell cycle-related genes in deep-water rice, Keyes *et al.* (1990) have shown that GA regulates cell wall extensibility in wheat and Smith *et al.* (1996) found higher levels of XET activity in GA-treated leaves of barley mutants. On a whole plant level, a highly significant correlation between endogenous GA concentrations and RGR was found in maize hybrids (Rood *et al.*, 1990). Within the species of the genus *Aegilops*, evidence has been found that suggests GA may be involved in determining inherent variation in RGR amongst the species. In a comparison of 20 *Aegilops* species variation in RGR was positively correlated with the relative amount of biomass allocated to the leaf sheaths (Villar *et al.*, 1998), as has been found in a comparison of GA-insensitive wheat mutants with their wild-type (McCaig & Morgan, 1993). A role for GA in the regulation of leaf and whole plant growth of *Aegilops* species with contrasting leaf growth rates has been investigated in this thesis.

Thesis outline

Chapter 2 describes the cellular basis of inherent differences in leaf elongation rates of two *Aegilops* species, by means of a kinematic analysis. In this chapter, it was also tested whether cell walls from the growing zone of leaves of these two species responded differently to cell wall acidification. Chapter 3 reviews the mechanisms that are associated with variation in leaf growth (cell division and expansion) and discusses possible links between variation in the growth rate of the entire shoot and individual leaves. Inherent differences in leaf growth as well as differences that are caused by environmental factors (mainly nutrient supply and temperature) are explored. The links between inherent differences in individual leaf growth and whole shoot expansion are the topic of investigation in Chapter 4, where leaf and shoot growth of three *Aegilops* species and two *Triticum* species are compared. This chapter also gives some insight into the leaf growth potential of wheat and its wild relatives. In Chapters 5 and 6, the role of gibberellins in determining inherent differences in leaf expansion and whole plant growth are investigated. To that end, GA_3 and an inhibitor of gibberellin biosynthesis were supplied exogenously to two *Aegilops* species with contrasting leaf

elongation rates. The effects on leaf growth, leaf area expansion of the entire shoot, and biomass allocation are presented in Chapter 5, while Chapter 6 shows the effects on RGR, investigated by studying the effects on carbon and nitrogen economy. The main results of this thesis are summarised and discussed in Chapter 7.

Chapter 1

Chapter 2

Cellular basis of variation in leaf elongation rate between two *Aegilops* species

Abstract

The twofold difference in final length of leaf 3 on the main stem between the fast-growing *Aegilops tauschii* and the slow-growing *Ae. caudata* is caused entirely by a difference in leaf elongation rate (LER) and not by variation in duration of leaf elongation. In this paper we investigated the cellular basis of inherent differences in leaf elongation rate between these species.

We analysed the dynamics of abaxial epidermal cells along the growth zone of the third leaf on the main stem of both species, by means of a kinematic analysis. The faster LER in *Ae. tauschii* compared with *Ae. caudata* was associated with (i) a larger meristem and cell elongation zone, and (ii) a faster cell production rate due to a larger number of dividing cells. Cell division rate, mature cell size and cell elongation rate did not differ between the two species. The lack of variation in cell expansion rate between the species was supported by a similar capacity of both species to extend their isolated cell walls upon acidification.

These data suggest that differences in the number of dividing cells can bring about differences in the number of simultaneously elongating cells, and hence in LER. Although leaf growth can only occur as a result of cell expansion, differences in LER are not necessarily related to differences in the rate of cell expansion.

Chapter 2

Introduction

Fast expansion of the first few leaves of cereal crops has been shown to benefit yield (Whan *et al.*, 1991; López-Castañeda & Richards, 1994). One advantage of faster-expanding leaves in early development is the faster reduction in evaporation from the soil surface, which results in an earlier increase in water availability for the plant (Richards *et al.*, 1993). Also, rapid leaf area expansion makes the crop species more competitive with weeds for light interception (Lemerle *et al.*, 2001). Several authors have studied the effects of environmental changes on the cellular processes underlying leaf expansion rates in crop species (*e.g.*, MacAdam *et al.*, 1989; Ben-Haj-Salah & Tardieu, 1995; Beemster *et al.*, 1996; Fricke *et al.*, 1997; Masle, 2000). Fewer studies have compared the cellular basis of intra- and interspecific differences in leaf expansion (*e.g.*, Volenec & Nelson, 1981; Fiorani *et al.*, 2000; Masle, 2000).

In monocotyledonous species, growing leaves expand predominantly in length and the rate of leaf elongation (LER) is determined by the number of elongating cells, and the rate and duration of cell expansion in the epidermis. The epidermis is considered to be involved in controlling organ growth (Kutschera, 1992; Becraft, 1999). In leaves, epidermal cell production and cell elongation occur in the growth zone which is located at the base of the leaf and enclosed by the sheaths of older leaves (Volenec & Nelson, 1981; MacAdam *et al.*, 1989; Schnyder *et al.*, 1990). In the most basal part of the growth zone, *i.e.* the meristem, cells are produced in parallel cell files and displaced away from the leaf base as a result of continuous production and elongation of new cells within the same file. As the cells are being displaced through the meristem, they continue to divide and elongate until they reach the elongation-only zone. There, cells stop dividing and continue to elongate until they reach their mature size. These processes result in a typical cell length distribution with distance from the leaf base. The derivative of the cell length distribution along the growth zone is used to determine the distribution of relative cell elongation rates, *i.e.* strain rates, along the elongation-only zone. By studying the cell length distribution and the strain rate distribution along the growth zone during the period of steady-state leaf elongation, we obtain more insight into the cellular processes underlying leaf elongation (Silk *et al.*, 1989).

Differences in cell elongation rates are more often correlated with differences in cell wall extensibility than with differences in cell turgor (Van Volkenburgh, 1999, and references therein). A positive correlation has been shown between leaf growth rate and the capacity of cell walls to expand upon acidification, in dicotyledonous as well as monocotyledonous leaves (Van Volkenburgh & Boyer, 1985; Van Volkenburgh *et al.*, 1985b). Several studies have shown that plants are able to acidify the apoplast, most likely by means of a plasma-membrane proton pump, and thereby make the cell walls more extensible (Rayle & Cleland, 1992). This acid-induced wall extension is mediated by cell wall-loosening proteins, called expansins, that are most active at low pH and in growing

tissues (Cosgrove, 2000, and references therein), including monocotyledonous leaves (Cho & Kende, 1998). It is possible that species differ in their responsiveness of the apoplast to acidification, resulting in a difference in cell elongation rate and hence leaf elongation rate.

The first aim of this study was to determine which cellular growth processes determine inherent differences in leaf elongation rates. We examined cell division parameters, and the cell length and strain rate distributions along the growth zone of leaf 3 on the main stem of two species with contrasting leaf elongation rates. Secondly, we tested whether isolated cell walls from the growing zone of leaves of these two species responded differently to cell wall acidification.

For this study we selected two species of the genus *Aegilops*, which showed a wide range of leaf elongation rates (Bultynck *et al.*, 1999). Moreover, the species of this genus are related to bread wheat (*Triticum aestivum*) and they are becoming increasingly important as potential sources of valuable traits that can be used in wheat breeding (Damania, 1993).

Materials and methods

Plant material and growing conditions

Seeds of *Aegilops caudata* L. and *Ae. tauschii* L. were obtained from ICARDA (International Centre for Agricultural Research in the Dry Areas, Aleppo, Syria). Prior to germination, seeds were surface-sterilised with a 2.5% $NaHClO_3$ solution and stratified (placed on wet filter paper at 4°C in the dark) for 7 days. Seeds were germinated on moistened filter paper in Petri dishes in a germination cabinet (day: 14 h, 50 µmol m^{-2} s^{-1} PAR, 25°C; night: 10 h, 15°C). After germination, seedlings were transferred to trays with washed river-sand, saturated with de-ionised water, and placed in a growth room (day: 14 h, 445 ± 15 µmol m^{-2} s^{-1} PAR, 23 ± 2°C, 70% RH; night: 10 h, 19 ± 2°C, 70% RH). After three days the seedlings were transferred (= day 0) to containers with 20 L of the following aerated nutrient solution: 795 µM KNO_3, 603 µM $Ca(NO_3)_2$, 270 µM $MgSO_4$, 190 µM KH_2PO_4, 40µM Fe-EDTA, 20 µM H_3BO_3, 2 µM $MnSO_4$, 0.85 µM $ZnSO_4$, 0.25 µM Na_2MoO_4 and 0.15 µM $CuSO_4$. The pH of the nutrient solution was adjusted daily to 5.5 with H_2SO_4 and the solution was replenished weekly. Plants were rotated daily within the growth room to minimise the variation in environmental conditions for individual plants.

Individual leaf growth measurements

Individual leaf growth measurements were conducted on leaf 3 of the main shoot for both species, on a first set of eight plants per species. Leaf length was measured daily with a ruler, from the day they emerged from the sheath of leaf 2 until they were fully elongated. Leaf elongation rate (LER, mm day^{-1}) of individual leaves was calculated as the slope of the linear

regression line through the data points within the phase of linear increase in leaf length with time. The linear growth phase of the leaves was determined as the interval between 20 and 80% of final leaf length; outside this interval the increase in leaf length with time was not linear. Leaf elongation duration (DUR, days) of individual leaves was calculated as the ratio between final leaf length and LER.

Cell length measurements

A second set of five plants per species was used for determining epidermal cell length profiles of leaf 3 on the main shoot. The leaves were harvested within the first two days after emergence from the sheath of leaf 2, when the leaves were in the linear growth phase. The leaves were cut at the base of the meristem, immediately transferred to boiling methanol for chlorophyll removal, and subsequently transferred to 90% (w/v) lactic acid for clearing and storage. Leaf length was measured before and after methanol boiling and no tissue shrinkage was observed.

The cleared leaves were mounted on a light microscope (Olympus BX60 F5, Japan), which was connected to a Panasonic CCD camera (model GP-KR222E). Two epidermal cell files, adjacent to stomatal cell files, on the abaxial side of every leaf were selected. Cell lengths of all the cells along those files were measured from video-images (total magnification 100x) using Video Trace image measurement software (Leading Edge, Marion, Australia). Per cell file, a cell length profile along the leaf axis was obtained by plotting the length of each cell versus its distance from the leaf base. The data of the two files per leaf were combined, and then smoothed and interpolated with the procedure described by Beemster & Baskin (1998). From the smoothed data of each leaf, mature cell length (l_m) and the length of the growth zone (L_{gz}) were determined. Mature cell length was estimated as the average cell length of all data points distal to the position where the increase in cell length between successive data points was less than or equal to 0. The length of the growth zone was estimated as the distance from the leaf base to the position where cell length reached 95% of its mature cell length. These values were averaged between leaves and used for subsequent calculations.

Estimation of meristem length

A third set of five plants per species was used to estimate the meristem length in leaf 3 on the main stem. The leaves were harvested within the first two days after emergence from the sheath of leaf 2, when the leaves were in the linear growth phase. The leaves were cut at the base of the meristem and immediately transferred to a 3/1 (v/v) absolute ethanol: glacial acetic acid solution for at least 24 h at 4°C for tissue fixation and chlorophyll removal. Subsequently, the cleared leaves were hydrolysed in 3 M HCl for 20 min and immersed for

at least 2 h in a Feulgen dye prepared with basic fuchsin (rosanilin; Merck, Kilsyth, Australia) for staining the nuclei (method according to Moses *et al.*, 1997).

The stained leaves were transferred to a microscope slide, immersed in a droplet of 0.1 M $Na_2S_2O_5$ dissolved in 0.15 M HCl and covered with a cover slip. The same Image Analysis set-up was used for estimating the meristem length as for the cell length measurements (total magnification 200x). Ten epidermal cell files next to stomatal cell files were selected on the abaxial side of the leaf. The distance between the most distal mitotic figure in a selected cell file and the base of the leaf was measured. We repeated this for ten files per leaf and the length of the 'division' zone (L_{div}) was estimated by the longest distance between the leaf base and the most distal mitosis (method according to Barlow *et al.*, 1991).

Kinematic analysis

The data obtained from the smoothed epidermal cell length profile and the estimated meristem lengths were used in a kinematic analysis, as described by Fiorani *et al.* (2000).

Spatial parameters: Local cell density $\rho(x)$ at a distance x from the leaf base, was defined as the reciprocal of local cell length at position $l(x)$:

$$\rho(x) = \frac{1}{l(x)} \tag{1}$$

where position $x = n \times \Delta x$ with n = 1, 2, 3, … and Δx is a step of 250 μm derived from the smoothing procedure described above.

Local cell density was used to calculate the number of cells per file in the growth zone (N_{gz}) and in the division zone (N_{div}) as:

$$N_{gz,div} = \Delta x \times \sum_{n} \frac{\rho(n\Delta x) + \rho[(n-1)\Delta x]}{2} \tag{2}$$

where the summation was stopped at the distal margin of the growth zone and of the division zone, respectively (Beemster & Baskin, 1998).

The length (L_{ez}) and number of cells (N_{ez}) of the elongation-only zone were determined as:

$$L_{ez} = L_{gz} - L_{div} \tag{3}$$

$$N_{ez} = N_{gz} - N_{div} \tag{4}$$

where L_{gz} and L_{div} were obtained as described above.

Cell division parameters: Due to the continuous production of new cells in the meristem, dividing cells are moved distally through the meristem, until they enter the elongation-only zone where cells stop dividing. During steady-state leaf elongation, the flux of cells through any point in the elongation-only zone is constant and represents the rate of cell production (P, cells day^{-1}), which was estimated as:

$$P = \frac{LER}{l_m} \tag{5}$$

where *LER* is leaf elongation rate and l_m is mature cell length (Silk *et al.*, 1989).

The average cell division rate for the whole meristem ($\overline{D}$; h^{-1}) was calculated from the rate of cell production and the number of dividing cells (Beemster & Baskin, 1998):

$$\overline{D} = \frac{P}{N_{div}} \tag{6}$$

Considering the exponential nature of the cell division process, the average cell cycle duration ($\overline{T}_c$; h) was calculated as (Green, 1976; Dubrovsky *et al.*, 1998):

$$\overline{T_c} = \ln(2) \times \frac{N_{div}}{P} \tag{7}$$

Residence time in division and elongation-only zones: The true residence time of cells in the meristem equals the cell cycle duration, as every meristematic cell only exists from the time it was formed until the time the cell undergoes cytokinesis to form two daughter cells. However, if a constant cell cycle duration over time is assumed, the residence time of the most basal cell wall in the division zone (T_{div}; h) can be estimated as (Beemster & Baskin, 1998):

$$T_{div} = \overline{T}_c \times \log_2(N_{div}) \tag{8}$$

The residence time of cells in the elongation-only zone (T_{el}; h) was determined by the number of cells in the elongation zone (N_{el}) and the flux of cells through that zone which is equivalent to the cell production rate (P) (Beemster & Baskin, 1998):

$$T_{el} = \frac{N_{el}}{P} \tag{9}$$

Strain rate: Once cells exit the division zone and enter the elongation-only zone, they stop dividing and continue to elongate until they reach their mature size. The relative cell elongation rate in the elongation-only zone (strain rate r; h^{-1}), was calculated from the derivative of the cell length profile ($\partial l / \partial x$) and the cell production rate P (Silk *et al.*, 1989):

$$r(x) = P \times \frac{\partial l}{\partial x} \tag{10}$$

The corresponding strain rate profile described the relative cell elongation rate as a function of distance from the leaf base.

Measurement of cell wall extensibility

A fourth set of 12 plants per species was used to determine the capacity of cell walls to extend upon acidification, of main stem leaf 3. The leaves were harvested within the first two days after emergence from the sheath of main stem leaf 2, when the leaves were in the linear growth phase. Leaf sections of 10 mm were excised at a distance from the leaf base where strain rate was around its maximum and the midvein was removed. The sections were frozen to remove turgor pressure and thawed again for cell wall extensibility measurements with a constant stress apparatus (Rayle & Cleland, 1972). The leaf strips were placed between two clamps 3 mm apart and submerged in a 50 mM Na-acetate buffer of pH 6.8. Subsequently, a constant tension of 10 g was applied to the tissue and tissue extension over time was measured with a position transducer. After a constant rate was achieved, the buffer was replaced with a 50 mM Na-acetate buffer of pH 4.5 and the change in extension rate was recorded. The data were expressed as relative extension rate (rate of increase in tissue length over time, per unit of tissue length already present; mm mm^{-1} h^{-1}). Due to occasional breaking of the leaf tissue in *Ae. tauschii*, the sample size was reduced to 8 plants in this species.

Statistics

Data were analysed with SPSS 8.0 for Windows statistical software (SPSS, Inc., Chicago, IL, USA). A one-way ANOVA (at α=0.05) was used to test for significant differences in measured and calculated parameters between *Ae. caudata* and *Ae. tauschii*.

Results

Leaf elongation

In both species, the increase in length of the third leaf on the main stem was approximately linear during the first 4 days after appearance from the encircling leaf sheath (Fig. 1). The change in daily leaf elongation rate (LER) over this period was less than 20% (Fig. 1, inset). This enabled us to calculate a constant LER per leaf over this steady-state growth period. The leaf elongation rates as measured in this experiment are similar to those measured in preliminary experiments (data not shown).

Final leaf length is the product of leaf elongation rate and leaf elongation duration. The more than twofold difference in final leaf length of the third leaf on the main stem between *Ae. caudata* and *Ae. tauschii*, was entirely due to the difference in leaf elongation rate between these species (Table 1 and Figure 1). The duration of leaf elongation of the third leaf on the main stem was the same in both species (Table 1).

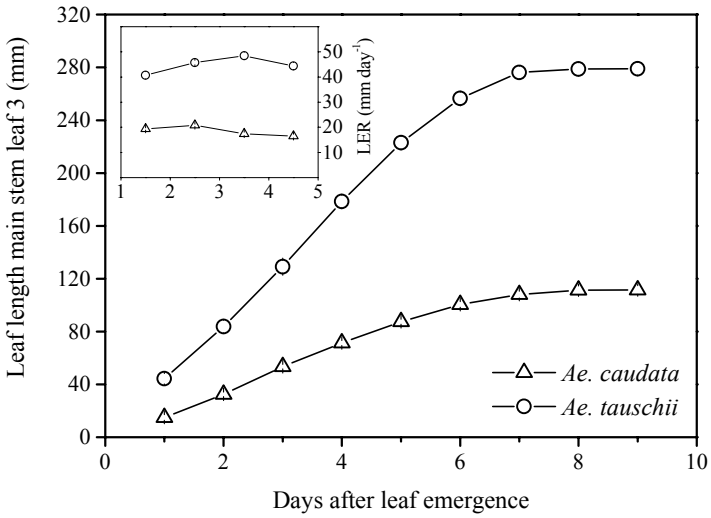

Figure 1.
Leaf length increase of leaf 3 on the main stem of *Ae. caudata* and *Ae. tauschii*, from the day of leaf emergence from the encircling leaf sheath. The inset shows the average daily leaf elongation rate (LER) for the first 4 days after leaf emergence. Data points are means of 8 plants. Standard error bars were smaller than the symbols.

Table 1. Final leaf length, leaf elongation duration, leaf elongation rate, mature cell length, cell production rate and cell division rate of the third leaf on the main stem in *Ae. caudata* and *Ae. tauschii*. Values are means of 8 plants ± SE for final leaf length, leaf elongation duration and leaf elongation rate. Values are means of 5 plants ± SE for mature cell length, cell production rate and cell division rate. Different letters denote significant differences between species.

	Final leaf length	Leaf elongation duration	Leaf elongation rate	Mature cell length	Cell production rate	Cell division rate
	mm	*days*	*mm day^{-1}*	*μm*	*cells day^{-1}*	*cells cell^{-1} day^{-1}*
Ae. caudata	119^{a} ± 3	6.2^{a} ± 0.1	19^{a} ± 0.3	251^{a} ± 21	76^{a} ± 3	1.20^{a} ± 0.15
Ae. tauschii	286^{b} ± 5	6.1^{a} ± 0.1	47^{b} ± 0.7	239^{a} ± 13	198^{b} ± 5	1.42^{a} ± 0.07

Epidermal cell length distribution

Preliminary experiments showed that epidermal cell length distribution along the abaxial leaf axis was constant over the period of steady-state growth (data not shown). Cell length distribution in this experiment was determined within 1 or 2 days after the leaves emerged from the encircling leaf sheath. Figure 2 shows the cell length distribution along the abaxial leaf axis of leaf 3 on the main stem in *Ae. caudata* and *Ae. tauschii*. Both species started off with the same cell size in the division zone (approx. 20 μm), and in both species cell size increased to reach the same mature cell size (Table 1). For the *Aegilops* species in this study, leaf elongation rate was not associated with mature cell size.

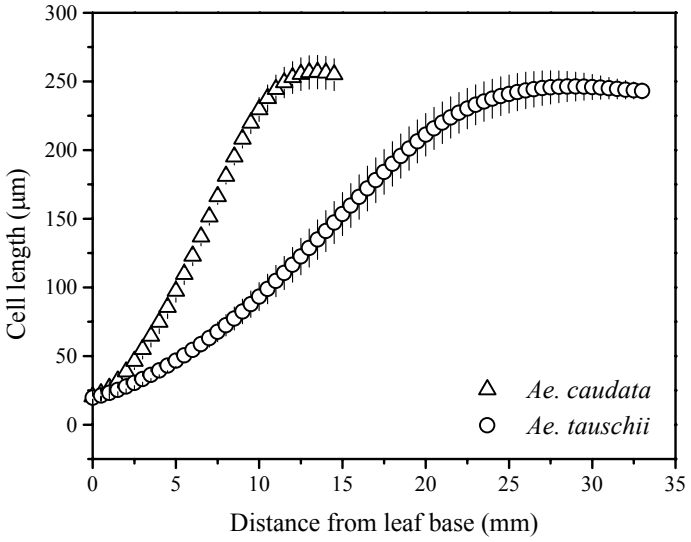

Figure 2.
Spatial distribution of cell lengths in abaxial epidermal cell files adjacent to stomatal cell files, as a function of the distance from the leaf base of leaf 3 on the main stem in *Ae. caudata* and *Ae. tauschii*. Data points are smoothed averages from 5 plants over 0.5 mm intervals. Vertical bars indicate standard error.

The cell length distribution was used to determine size of the growth zone, number of cells in the growth zone, cell residence time in the growth zone, and distribution of relative cell elongation rates in the growth zone. These results are presented below.

Spatial and temporal dimensions of the growth zone

The total growth zone of *Ae. tauschii* (22.3 mm) was twice as long as that of *Ae. caudata* (11.5 mm) (Figs 2 and 3A). The total growth zone comprises a division zone and an elongation-only zone. Also the length of the division zone (L_{div}) and the elongation-only zone (L_{ez}) was twice as high in *Ae. tauschii* (L_{div} = 3.6 mm; L_{ez} = 18.6 mm) as in *Ae. caudata* (L_{div} = 1.6 mm; L_{ez} = 9.9 mm) (Fig. 3A; $p<0.01$). Similar differences between the species were found when the number of cells in the different zones were compared: *Ae. tauschii* had twice as many cells in the division and elongation-only zone than *Ae. caudata* (Fig. 3B; $p<0.01$). Although the length of the division zone was smaller than that of the elongation-only zone, the cells spent more time in the division zone (T_{div}) than in the elongation-only zone (T_{ez}) (Figs 3A, B and C). T_{div} was the same in *Ae. caudata* as in *Ae. tauschii* ($p=0.98$), whereas the T_{ez} was slightly higher in *Ae. caudata* ($p<0.01$). Overall, the two species did not differ in residence time in the total growth zone ($p=0.37$).

In *Ae. caudata* and *Ae. tauschii*, leaf elongation rate was positively correlated with the length of, and the number of cells in, the meristem and elongation-only zone but not with the residence time of the growing cells in these zones.

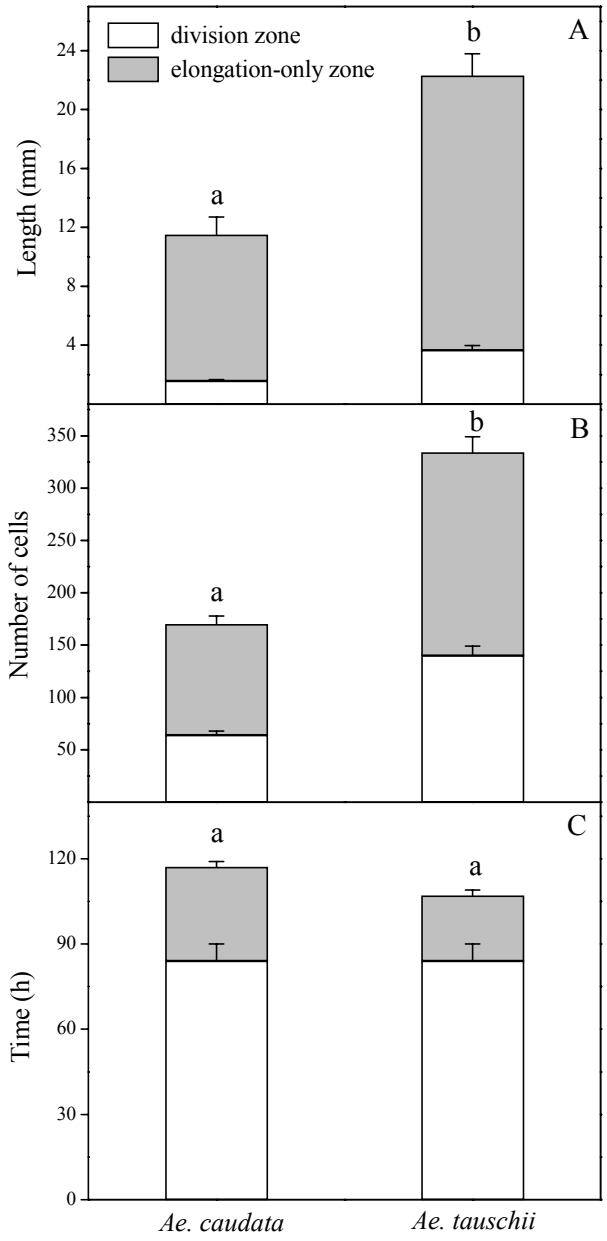

Figure 3.
Average (A) length of, (B) number of cells in, and (C) residence time of cells in, the *division* zone and *elongation-only* zone of the abaxial epidermis of leaf 3 on the main stem of *Ae. caudata* and *Ae. tauschii*. Data are means of 5 plants and vertical bars indicate standard error for the *division* zone and *elongation-only* zone. Different letters indicate a significant difference between the *growth* zones of the species.

Cell production rate, cell division rate and strain rate

During steady-state leaf growth, the flux of cells through any point in the elongation-only zone is constant and equals the cell production rate. The cell production rate within cell files adjacent to the stomatal cell files was 2.6 times higher in *Ae. tauschii* than in *Ae. caudata* (Table 1). Differences in cell production rate are determined by differences in the cell division rate and/or the number of dividing cells. The meristematic cells of *Ae. caudata* and *Ae. tauschii* had similar cell division rates (Table 1), so they only differed in the number of dividing cells (N_{div}) (Fig. 3B).

The derivative of the cell length distribution along the growth zone (Fig. 2) gives the strain rate distribution, *i.e.* the distribution of the relative rates of cell expansion, along the growth zone (Fig. 4). In both species, the strain rate increased sharply with distance from the leaf base, reached a maximum rate in the middle of the elongation-only zone and decreased again to zero at the end of elongation-only zone. The maximum strain rate was the same for both species (± 10% h^{-1}) and was therefore not correlated with the leaf elongation rate.

In *Ae. caudata* and *Ae. tauschii*, LER was positively correlated with cell production rate, whereas it was not correlated with cell division rate or maximum strain rate.

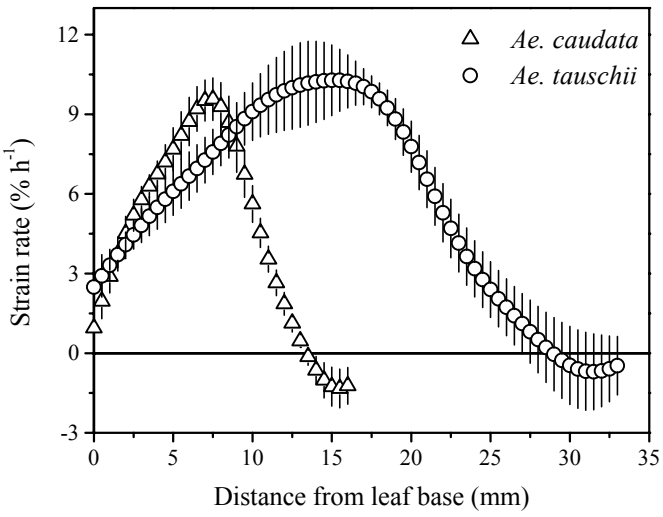

Figure 4.
Spatial distribution of strain rate in abaxial epidermal cell files adjacent to stomatal cell files, as a function of the distance from the leaf base of leaf 3 on the main stem in *Ae. caudata* and *Ae. tauschii*. Data points are derived from the spatial distribution of cell length (Fig. 2 and Eq. 10) and are means of 5 plants. Vertical bars indicate standard error.

Effects of acidification on cell wall extensibility

Figure 5 shows the relative extension rate of isolated cell walls placed under constant stress of 10 g before and after acidification of the apoplast, for *Ae. caudata* and *Ae. tauschii*. The relative cell wall extension rate under constant stress was similar for the two species.

Acidification significantly increased the relative cell wall extension rate in both species. However, the relative cell wall extension rate after acidification was also similar for both species.

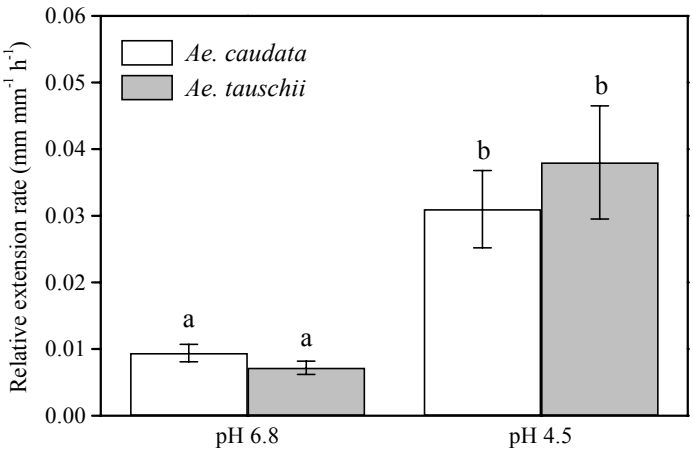

Figure 5.
Relative extension rate of isolated cell walls of leaf 3 on the main stem of *Ae. caudata* and *Ae. tauschii* before (pH 6.8 buffer solution) and after (pH 4.5 buffer solution) acidification. Data are means of 12 *Ae. caudata* leaves and 8 *Ae. tauschii* leaves. Vertical bars indicate standard error.

Discussion

Leaf elongation

This study shows that the considerable difference in final leaf length of leaf 3 on the main stem between *Ae. caudata* and *Ae. tauschii* was entirely due to the difference in leaf elongation rate, whereas leaf elongation duration was similar for the two species. Similar results were found by Fiorani *et al.* (2000) in a comparison of four *Poa* species, which differed in final leaf length. In their study, however, the *visible* leaf elongation duration was estimated (time between appearance of the leaf and end of the growth period), as opposed to the *total* leaf elongation duration in our study. The species in our study also showed a similar visible duration of leaf elongation (data not shown). Similar observations were made by Tonkinson *et al.* (1995) and Calderini *et al.* (1996) for wheat cultivars, which are more related to our *Aegilops* species, where dwarf genotypes showed a lower leaf elongation rate but the same leaf elongation duration than their wild-type.

Leaf elongation of *Ae. caudata* and *Ae. tauschii* was approximately linear with time during the first 4 days after leaf appearance. This is a first indication of the steady-state elongation that is required for performing a kinematic analysis on cell length profiles (Silk & Erickson, 1979). A better indication was given by preliminary experiments that showed an invariable cell length profile for at least 4 days after leaf emergence (data not shown). Recently, Muller *et al.* (2001) have shown that the cell length and relative cell elongation

rate distribution along the growth zone was the same before as after (during steady-state elongation) leaf emergence in maize. Our leaves were sampled within 1 or 2 days after leaf emergence, within the period of steady-state growth. Schnyder *et al.* (1990) have shown for *Lolium perenne* that during this growth period, leaf elongation is almost entirely due to blade (not sheath) elongation. Indeed, in our leaf samples the ligule (boundary between blade and sheath) was either not yet or only just initiated. However, this does not affect the cell length and strain rate profile (Kemp, 1980; Schnyder *et al.*, 1990) and the whole growth zone can be treated as a continuous zone for the derivation of kinematic parameters.

Meristematic activity determines differences in leaf elongation rate

On a cellular level, faster leaf elongation rate in *Ae. tauschii* compared with *Ae. caudata* was associated with a longer growth zone in which more cells are elongating simultaneously. The elongating cells of *Ae. tauschii* had a similar maximum elongation rate and a slightly shorter residence time in the elongation zone than *Ae. caudata*, which resulted in similar mature cell sizes in these species. The longer growth zone of *Ae. tauschii* was correlated with a higher rate of cell production in the meristem, which resulted from a larger number of dividing cells and not from a faster cell division rate. These results agree with those of Fiorani *et al.* (2000) who compared the epidermal cell length distribution of leaf 7 on the main stem in four *Poa* species, and with those of Beemster & Baskin (1998) who studied cell length distribution in *Arabidopsis thaliana* roots with accelerating elongation rates. Similar to the data sets of Fiorani *et al.* (2000), our data suggest that differences in leaf growth between related species are determined by differences in meristematic activity. This suggestion argues against the spatial viewpoint of organ growth, which states that organ growth is determined by the integral of relative cell elongation rate over the length of the growth zone of the elongating organ. From this point of view, cell division is a process that accompanies cell elongation in the meristem and has no other role than subdividing cell volume (Silk & Erickson, 1979). We agree that cell division by itself can not result in leaf elongation. However, we suggest that the higher cell production rate (due to an increase in the number of dividing cells) in the leaf meristem of *Ae. tauschii* increased the number of cells elongating at the same time, which was reflected in a longer elongation zone with more expansion potential in *Ae. tauschii*. This way of looking at leaf growth is more in agreement with the material viewpoint of organ growth. From this point of view, cells behave as individual material particles which have their own developmental program (Silk & Erickson, 1979). The results of our study only provide indirect evidence for a regulatory role of cell production in leaf elongation rate. More direct evidence showing that organ growth rate may be limited by cell production, comes from Doerner *et al.* (1996). These authors activated the cell cycle in transgenic *Arabidopsis thaliana* by over-expressing *cyc 1At*, leading to plants with faster-growing roots and unchanged mature cell size. Numerous studies have investigated changes

in cell production rates in relation to specific growth responses (Francis, 1998, and references therein). In most of these studies, it is a change in the proportion of cycling cells and not in the cell division rate, that correlates with the growth response.

It is interesting to note here that we observed a larger number of parallel cell files in the leaf growth zone of *Ae. tauschii*, the species with the longest meristem, whereas the width of the cell files was similar to those of *Ae. caudata* (data not shown). This indicates not only a difference in the number of proliferative divisions (determine number of cells per file) between the species, but also in the number of formative divisions (determine number of cell files). These differences in the number of formative divisions may reflect differences in size of the shoot apex diameter at leaf initiation between the species (Pieters & Van den Noort, 1988). Alternatively, they may have resulted from differences in cellular processes taking place during the primordial stages that follow leaf initiation (Beemster & Masle, 1996).

Epidermal cell elongation rate

The strain rate distribution shows that *Ae. caudata* and *Ae. tauschii* did not differ in their maximum rates of cell expansion. The fact that both species showed the same capacity to extend their cell walls upon acidification adds to this observation. Numerous studies on cereal plants have shown that treatment- or species-related differences in leaf growth are positively correlated with differences in cell wall extensibility (Van Volkenburgh & Boyer, 1985; Keyes *et al.*, 1990; Matsukura *et al.*, 1998; Lu & Neumann, 1999). Van Volkenburgh *et al.* (1985a) presented evidence of the involvement of an acid-growth mechanism in the control of cell wall extensibility in maize. In these studies however, differences in leaf growth were associated with differences in mature cell length, whereas our species did not differ in mature cell length. Although the capacity of cell walls to extend upon acidification did not play a role in determining differences in cell and leaf elongation rate between *Ae. caudata* and *Ae. tauschii*, acidification of the cell wall may still be an important requirement for cell and leaf expansion in these species, through its effect on the activity of cell wall proteins (Okamoto-Nakazato *et al.*, 2000; Cosgrove, 2001).

These results of the present study show that species-specific differences in leaf growth potential, and possibly also plant growth potential, are not always related to differences in cell expansion but can be related to differences in cell production rate only. Therefore, further investigation of inherent differences in leaf growth of closely related species should focus on the controlling mechanisms behind cell division.

Acknowledgements

We thank Chris Keller for his assistance with the cell wall extensibility measurements. Seeds of *Ae. caudata* and *Ae. tauschii* were kindly provided by ICARDA.

Chapter 2

Chapter 3

Control of leaf growth and its role in determining variation in plant growth rate from an ecological perspective

Abstract

Plants vary widely in their relative growth rate (RGR), be it dependent on environmental conditions or due to their genetic background. In a comparison of the RGR of grasses growing under different environmental conditions, variation in RGR tends to correlate with that in the leaf elongation rate (LER). When different species or genotypes thereof are compared under identical growing conditions, variation in LER may or may not correlate with that in RGR, depending on the comparison. However, since RGR is described by an exponential equation, whereas LER is mainly a linear process, we conclude that any correlation between RGR and LER must be fortuitous. That is, exponential growth must be due to *increases* with time in plant traits like (i) leaf dry mass per unit leaf length invested per unit time, and/or (ii) ΣLER, *i.e*. the total LER of all the growing leaves at one point in time. The latter can be achieved as follows: (i) each subsequent leaf has a higher LER than the preceding one; (ii) leaves appear at an increasing rate; (iii) the duration of the process of leaf elongation increases for subsequent leaves. In this review, we only explore possible factors that account for changes in ΣLER with time, in different genotypes and under different environmental conditions. Inherent variation in LER of individual leaves and variation due to environmental factors may reflect variation in the rate of cell division and/or in cell elongation.

1. Introduction

Plants that grow in harsh environments, *e.g.*, arid or saline areas, alpine or arctic regions, shaded or nutrient-poor habitats, grow more slowly than those growing under more equitable conditions. This is partly due to differences in environmental conditions. However, when the same plants from harsher environments are grown under more favourable conditions, they still have a lower relative growth rate (rate of increment in plant dry mass per unit plant dry mass already present; RGR, mg g^{-1} day^{-1}) compared to plants characteristic of more favourable habitats (Lambers & Poorter, 1992; various chapters in Lambers *et al.*, 1998a). This close link between a species' growth potential and the quality of its natural habitat raises a number of questions, such as: what are the physiological mechanisms that account for the differences in maximum growth rate between species, and what is the ecological advantage, if any, conferred by a plant's growth potential? Several studies have explored the underlying parameters of RGR by means of a growth analysis. Comparisons of species from nutrient-poor or alpine habitats and species from nutrient-rich or lowland sites have shown that variation in specific leaf area (SLA, m^2 kg^{-1}) is strongly correlated with that in RGR (Poorter & Remkes, 1990; Van der Werf *et al.*, 1993; Atkin *et al.*, 1996). Similarly, SLA accounts for much of the differences in RGR between fast-growing annuals and slower-growing congeneric perennial species (Garnier, 1992). These correlating parameters may not actually determine RGR; rather, they may be a result of the RGR of a plant (Lambers, 1998). RGR may be determined by the expansion rates of shoots, that is, the production rate of leaf primordia and/or expansion rate of leaves (Lambers, 1998; Nagel, 1998). In this review we explore mechanisms that are associated with variation in leaf growth (cell division and expansion) and discuss possible links between variation in the growth rate of the entire shoot and individual leaves. We will discuss both inherent differences and differences that are caused by environmental factors (mainly nutrient supply and temperature).

In this paper we mainly restrict ourselves to the shoot growth of grasses. Grasses have been chosen because their leaf growth (cell division and elongation) is restricted to a zone at the base of the leaf; moreover, the leaves only grow in the longitudinal direction. Dicotyledonous leaves, on the other hand, increase both in length and in width and not just at one defined part of the leaf lamina throughout most of their development. The shoot of grasses is, therefore, an attractive model system to study inherent or phenotypic differences in cell division and elongation. By means of a kinematic analysis, which will be discussed in greater detail in section 3, it is possible to accurately study the axial growth of intact leaves as dependent on meristematic activity and on local rate and duration of cell elongation. The restriction to the shoot is justified as long as the plants are in a steady state (*i.e.* the shoot and roots are growing at the same RGR). We implicitly assume that this situation pertains to the examples discussed in this paper, but we will refrain from a further analysis of it.

2. Relative growth rate and leaf elongation rate: is there a link?

It has repeatedly been reported that the rate of elongation of individual leaves in fast-growing grasses is faster than that in slow-growing ones. Differences in elongation due to the supply of nitrogen (MacAdam *et al.*, 1989) or phosphate (Rodríguez *et al.*, 1998a) as well as variation between genotypes (Volenec & Nelson, 1981) or species (Groeneveld & Bergkotte, 1996) have been analysed. Although leaf elongation rate of individual leaves (LER, mm h^{-1}) is often assumed to be a major determinant for variation in growth rate, the correlation between a plant's relative growth rate and its rate of leaf elongation is not absolute. In a comparison of four *Aegilops* species, all grown with free access to nutrients, LER of leaf number four is positively correlated with RGR (Fig. 1, species 1-4). However, the two species *Ae. triuncialis* and *Ae. tauschii* achieve a similar RGR with a considerably different LER. Moreover, when five *Poa* species are included in the comparison (Fig. 1, species 5-8), there is no significant correlation between RGR and LER. Intuitively, it might make sense that fast-growing plants exhibit rapid leaf elongation rates; however, on further scrutiny one comes to realise that the matter is far more complicated, as we will discuss below.

The absolute growth rate (rate of increment in plant dry mass) of plants in their initial stage is appropriately described by exponential curves, and, therefore, the RGR (rate of increment in plant dry mass per unit dry mass already present) is fairly constant until self-shading and ontogenetic effects start to affect the RGR. In contrast to RGR, which describes the exponential increase in plant dry mass, LER describes the linear increase in leaf length. In most of the grass species studied to date, the increase in leaf length with time can be conveniently divided into three phases. In the initial stage of leaf development, leaf length increase is not constant with time (phase1). The next phase is characterized by a virtually constant increase of leaf length with time (phase 2). This phase ends just before the increase

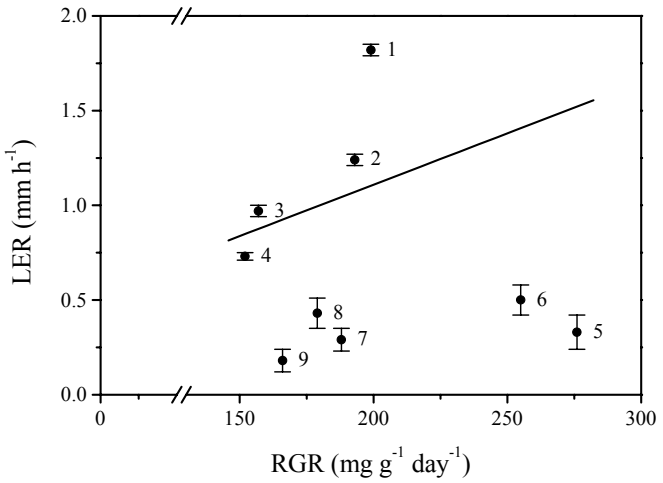

Figure 1. Relationship between RGR and LER of leaf number four of the main shoot, for four *Aegilops* species (1=*Ae. tauschii*, 2=*Ae. triuncialis*, 3=*Ae. juvenalis*, 4=*Ae. umbellulata*) and five *Poa* species (5=*P. annua*, 6=*P. trivialis*, 7=*P. compressa*, 8=*P. pratensis*, 9=*P. alpina*). The line represents the linear regression (r^2=0.32, P=0.4).

in leaf length with time starts to decline and leaves complete their growth (phase 3) (Skinner & Nelson, 1995). Since phase 2 represents the largest proportion of the total time of elongation, LER of a grass leaf can be appropriately described by fitting a linear curve in the phase of nearly constant growth. Assuming that dry mass per unit leaf length produced is constant within the growing leaf, the linear increase in leaf length can be considered as a linear increase in dry mass. In exponentially growing grasses, several leaves are elongating at the same time. Can the linear growth process of individual leaves result in exponential growth of the whole shoot and will a higher LER lead to a higher RGR?

To answer this question, we assume a model plant in which at any time, a constant number of leaves is elongating at the same rate for every subsequent leaf (Fig. 2A). This will result in a linear increase in shoot dry mass (assuming that the invested dry mass per unit leaf

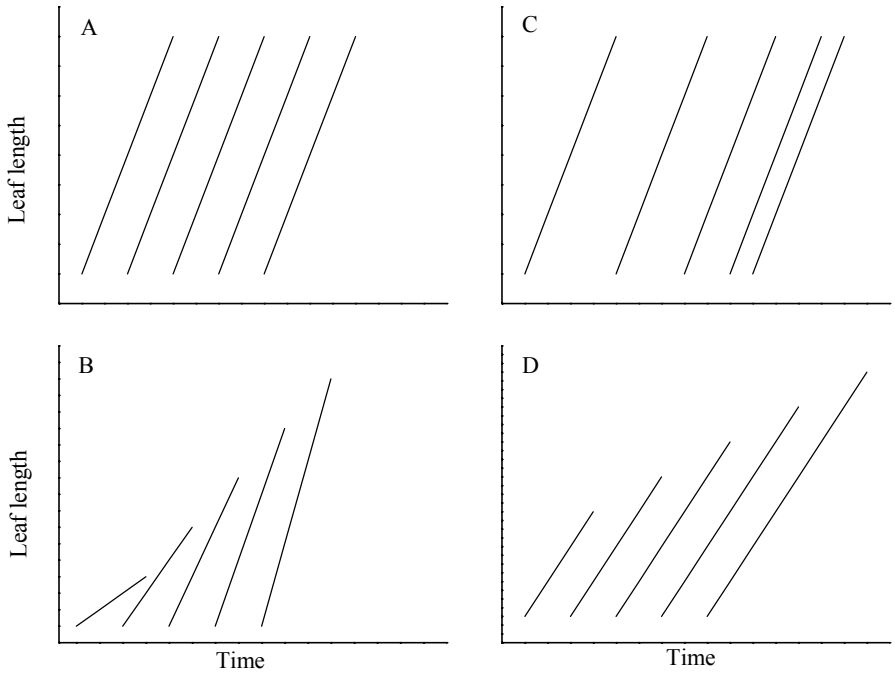

Figure 2. Hypothetical changes in shoot growth parameters resulting in exponential growth. The graphs show the increase in leaf length per unit time for five subsequent leaves. Graph (A) is based on a model grass plant in which at any time, a constant number of leaves is elongating at the same rate. The shoot of this model plant will only grow exponentially if leaf dry mass per unit leaf length increases for subsequent leaves or if ΣLER increases with increasing shoot dry mass due to an increase of (B) LER in subsequent leaves, (C) leaf appearance rate or (D) duration of

leaf elongation in subsequent leaves.

length is constant), and no exponential growth can occur. Taking the same model grass plant but changing the rate of leaf elongation to a higher (albeit constant for all leaves) value, will result in a higher absolute growth rate, still, no exponential growth is achieved. We must therefore conclude that a high LER by itself cannot account for a high RGR. In fact, the linear growth process of an individual leaf cannot possibly lead to the exponential growth of the whole shoot. Linear shoot growth results in a constant increase in shoot dry mass per unit time, whereas for exponential growth to occur, the increase in shoot dry mass should be increasing with time. Exponential growth may be achieved by an increase in leaf dry mass per unit leaf length invested per unit time (due to an increase in leaf width, leaf thickness or leaf mass density). Alternatively, ΣLER may increase with time (i.e. the total LER of all the growing leaves at the same time increases). In the model plant presented earlier, the latter can be achieved by one or more of the following changes: (i) each subsequent leaf has a higher LER than the preceding one (Fig. 2B); (ii) leaves appear at an increasing rate, *i.e.* the time between the appearance of subsequent leaves becomes shorter (Fig. 2C); (iii) the duration of the process of leaf elongation increases for subsequent leaves (Fig. 2D).

In a leaf growth analysis of four *Aegilops* species differing in RGR, an increasing leaf dry mass per unit leaf length was observed in subsequent leaves, mainly due to an increase in leaf width. No difference in LER between subsequent leaves was found, for any of the *Aegilops* species (Fig. 3A). However, in a similar comparison of five *Poa* species, a general trend towards an increase in LER was observed for subsequent leaves (Fig. 3B; i.e. situation Fig. 2B). In *Aegilops* species, the rate of leaf appearance for the plant as a whole increases with increasing shoot dry mass, due to tillering. This results in an increasing number of leaves elongating at one point in time, leading to an increasing ΣLER with time (i.e. the situation described in Fig. 2C). The increase in number of growing leaves with increase in shoot dry mass can also be the result of an increasing duration of leaf elongation for subsequent leaves, assuming that leaf appearance rate has not decreased (i.e. situation in Fig. 2D). The time span of the experiment was too short to see whether the duration of leaf elongation increased in subsequent leaves of *Aegilops* species. However, for the main shoot there is a trend towards a higher duration of leaf elongation in successive leaves. Skinner & Nelson (1994b) demonstrated a co-ordination between duration of leaf elongation and the rate of leaf appearance, but this co-ordination is only valid for the leaves within one tiller. With the production of new tillers, more leaves are growing at one point in time with increasing shoot dry mass. However, duration of leaf elongation of newly produced leaves on tillers is shorter than that of the growing leaves on the main shoot. As we propose for the *Aegilops* species, exponential shoot growth is likely to result from a combination of changes in shoot growth parameters. LER of individual leaves is only one of these parameters and LER by itself can not account for exponential growth. Only by increasing ΣLER with increasing plant dry mass, exponential growth can be achieved and a faster increase in ΣLER

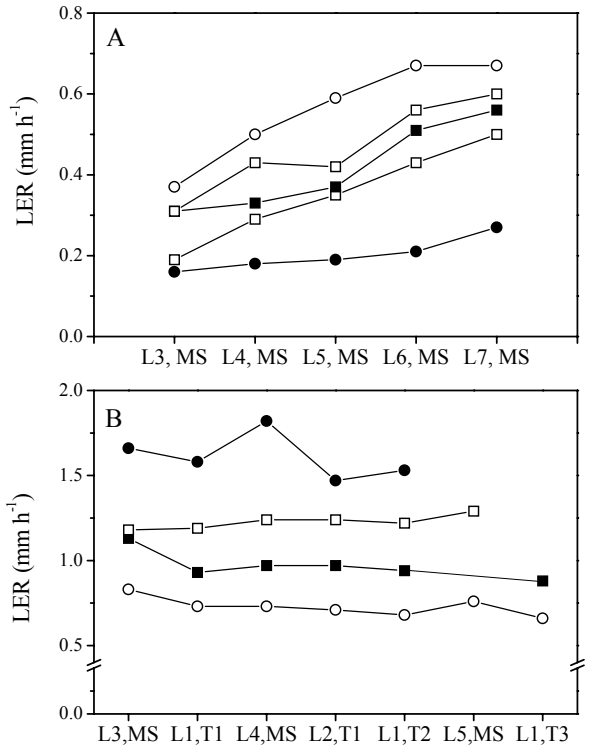

Figure 3.
Leaf elongation rate (LER) in subsequent leaves (Ln, leaf number; Tn, tiller number; MS, main shoot) of (A) five *Poa* species: *P. alpina* (●), *P. compressa* (□), *P. annua* (■), *P. trivialis* (○), *P. pratensis* (◆) and (B) four *Aegilops* species: *Ae. tauschii* (●), *Ae. juvenalis* (■), *Ae. umbellulata* (○), *Ae. triuncialis* (□). For clarity's sake, error bars have been omitted.

with plant dry mass will result in a higher RGR. RGR varies between *Aegilops* species. Which of the changes in the

above-mentioned shoot growth parameters are involved in this variation is variable. Differences in RGR between the 4 *Aegilops* species we compared, are mainly caused by differences in leaf appearance rate (possibly co-ordinated with duration of leaf elongation), sometimes combined with variation in the rate of increase in leaf dry mass invested per unit leaf length. In *Poa* species the rate at which LER increases in subsequent leaves also contributes to differences in shoot RGR.

RGR also varies between plants grown under different environmental conditions. Most studies on effects of controlled environmental changes on plant growth show data on only one or two shoot growth parameters, and this for only a short growing period. Therefore, it is hard to conclude what single parameter or combination of shoot growth parameters determines a change in RGR. Moreover, the progress of every parameter with increasing shoot weight is often not known. Leaf dry mass invested per unit leaf length depends on leaf width and leaf dry mass invested per unit leaf area (LMA, kg m^{-2}). MacAdam & Nelson (1987) investigated the change in leaf width and LMA along a developing leaf blade of tall fescue, grown at two temperature regimes. These data are restricted to the growing leaves at

only one point during the development of the plant. Studies of environmental effects on LER of individual leaves are numerous and these will be discussed in more detail in the following section (Ben-Haj-Salah & Tardieu, 1995; Thomas & Stoddart, 1995; Fricke *et al.*, 1997). Often, these data are limited to only one or two leaves, because the objectives of the investigations were to study cellular processes underlying changes in LER. Hence, no conclusions can be drawn about the effects on LER in subsequent leaves; clearly, shoot growth analyses over longer growing periods are required. Studies with agricultural objectives (crop growth simulation models) often include data on LER and duration of leaf elongation of subsequent leaves, and leaf appearance rate, *e.g.*, Skinner & Nelson (1994a). Generally, these experiments are conducted in the field, where more than one environmental factor changes over time, *e.g.*, temperature and daylength (Kirby *et al.*, 1985; Cao & Moss, 1989). Studies in controlled environments are needed to obtain an accurate picture of the shoot growth parameters determining changes in RGR of the shoot, by specific environmental variables.

3. Variation in LER: cell division and cell elongation

In the previous section we outlined how it is possible, in quantitative terms, to relate leaf growth to shoot growth. A careful analysis of the cellular processes determining inherent variation in LER, *i.e.* cell division and cell expansion, is essential to enhance our understanding of the expansion of individual leaves. To study the relative importance of the two processes and their co-ordination grass leaves are most appropriate, because of the axial, one-dimensional extension of these leaves.

LER in grass leaves is a function of the rate at which cells are supplied by the basal meristem and of the rate and duration at which cells expand (MacAdam *et al.*, 1989). In the epidermis, cells are arranged in files along which a continuum in cell development is found. To understand how this pattern is achieved, it is necessary to consider the structural organisation of the growing zone. In the intercalary meristem at the base of the leaf, cells rapidly divide and are displaced away from their primary location by the addition of new cells in more basal positions, expanding and dividing again until they reach the border of the meristem. Consequently, division and expansion activities spatially overlap in the 'division zone'. Upon entering the next region, the cells stop dividing, but they continue to expand and they expand in the 'elongation-only zone'. At the distal margin of the 'elongation-only' zone, cell expansion ceases and cells reach their mature size. It follows that cells can be considered as particles moving along a given trajectory at a defined rate and, therefore, their motion can be described by means of a kinematic analysis (Erickson, 1976; Green, 1976; Silk, 1984; Gandar & Hall, 1988).

If the condition of steady-state growth is met (linear increase of leaf length with time),

this system can be specified by a spatial analysis (Silk, 1984; Silk, 1992). One approach is to determine the cell length profile along the growth zone for a given cell type (Volenec & Nelson, 1983; Schnyder *et al.*, 1990; Beemster *et al.*, 1996). An alternative approach is to pierce the leaf sheath enclosing the growing zone with fine needles, thus producing equidistant holes through the blade whose separation over a given time is measured (Schnyder *et al.*, 1987). The main parameters that are derived are the cell flux, *i.e.* the number of cells passing a given point of the elongation-only zone per unit time (cells h^{-1}), average cell division rate (cells $cell^{-1}$ h^{-1}) and the local relative cell elongation rate or strain rate (h^{-1}). With these parameters it is possible to analyse the relative contribution and co-ordination of cell division and cell expansion during growth of individual leaves.

The kinematic approach can be used to investigate inherent variation in LER at the cellular level comparing different species grown under the same conditions or to investigate the effect of environmental factors on cell division and cell expansion. Only a few studies have addressed the first question (*e.g.*, Volenec & Nelson, 1981), whereas more information is available about the second aspect. In the following part of this section three environmental factors will be discussed: temperature, nitrogen supply and soil mechanical impedance to root penetration. Not all the investigations cited below contain a complete kinematic analysis; sometimes they focus only on cell expansion or cell division, so that they provide limited information.

In graminaceous leaves, temperature markedly affects the rate of leaf elongation (Watts, 1974; Gallagher & Biscoe, 1979; Kemp & Blacklow, 1980; Thomas & Stoddart, 1995), the leaf base being the site of temperature perception (Peacock, 1975). Ben-Haj-Salah & Tardieu (1995) investigated the effect of temperature on the growth of leaf number six of the main shoot in *Zea mays*. The spatial distribution of epidermal cell length is not affected by temperature in the range 13 to 34^{o}C, as a result of a temperature-dependent increase in cell flux (caused by increased cell production rates) matched by a corresponding increase in cell expansion rate. This causes cells to move faster through the elongation-only zone, but the duration of cell division and elongation is inversely shortened. These effects seem to be caused by a common and spatially uniform effect of temperature on both processes, suggesting co-ordination between the processes of division and expansion (Ben-Haj-Salah & Tardieu, 1995). Contrasting results were found in *Lilium longiflorum* leaves, in which day-night temperature variation affects cell elongation, but not cell division (Erwin *et al.*, 1994). Apparently, the response of cell division and cell expansion to temperature is species-specific. Further insight can be provided by studies on temperature-insensitive mutants, such as the *slender* mutant of *Hordeum vulgare* (Harrison *et al.*, 1998 and references therein) and by an investigation of the short- and long-term effects of temperature on leaf growth.

Increasing the nitrogen supply from a limiting to an optimum level (Thomas, 1983) enhances leaf growth in grasses. In two genotypes of *Festuca arundinacea* that differ in LER, an elevated N supply consistently increases cell flux and also affects the rate and

duration of cell elongation, be it to a minor extent (Volenec & Nelson, 1983; MacAdam *et al.*, 1989). Similar results were found by Fricke *et al.* (1997) for leaves of *Hordeum vulgare*. The LER of seedlings grown at low relative N addition rates was lower than that of control plants (full-strength Hoagland solution) and it can be ascribed to a reduction in both cell flux and relative cell elongation rate. The mechanism that accounts for these effects on cell division and cell expansion, however, is not known. The effects of other nutrients on LER remain to be investigated.

A third environmental factor that may influence leaf growth is soil resistance to root penetration (Masle & Passioura, 1987). Leaves of *Triticum aestivum* show a reduced LER when grown at a high soil mechanical resistance. Beemster *et al.* (1996) found that at the cellular level this effect could be explained in different ways, depending on leaf position on the shoot, thus showing an ontogenetic dependency of perception and responsiveness to stress-generated root signals. In leaf number one LER was reduced due to slower relative cell elongation rate and lower cell flux caused by slower cell division rates. In leaf number five, in contrast, the relative cell elongation rate was unaffected, and lowered cell flux was caused by a greatly reduced number of meristematic cells being present which was partly offset by their cycling time being somewhat shortened.

The results summarised above indicate that several environmental conditions influence LER of individual leaves by affecting both cell division and cell elongation and that the interaction of the two processes in different growth conditions is complex and still poorly understood. When investigating the cellular basis of environmental effects on leaf elongation it should also be taken into account that more than one factor can vary at the same time under natural conditions. Therefore, the actual rates of cell division and cell expansion are likely to be the result of multiple responses to different environmental parameters. Although no obvious pattern seems to be evident, a few general remarks can be made.

Firstly, both cell division and cell expansion activity must be studied together when the aim is to explain how environmental factors influence leaf elongation. Both processes are essential for growth, although only cell expansion contributes to a volumetric increase. In other words, growth can occur with cell division in the 'division zone' (in which cell division and cell expansion spatially overlap) and in the absence of cell division in the 'elongation-only' zone. It remains an open question whether cell expansion that occurs in the 'division zone' and expansion in the 'elongation-only zone' are independent processes. More detailed kinematic studies should provide such information.

Secondly, the responses of leaf elongation of grass species to environmental factors appear to be species-specific and depend on ontogeny. Therefore, it is not necessarily justified to extrapolate results obtained for a plant of one species at a given developmental stage and one set of environmental conditions to other species, developmental stages or environmental conditions.

Thirdly, it is important to study the cellular basis of inherent variation in leaf elongation

(high-LER and low-LER genotypes) of species occurring in contrasting natural environments. This approach might provide information about the relative importance of cell division and cell expansion in determining a specific leaf growth potential and to address the question whether general trends are present in an ecological framework.

4. Perspectives

In this review we have explored if there are any links between a plant's RGR and its LER. We have concluded that the two parameters are correlated when the same genotype is compared under different environmental conditions, but that such a correlation is not invariably found when comparing different genotypes under the same conditions. However, even if there is a correlation, this must be fortuitous, since variation in a plant's *exponential* growth rate cannot be accounted for by variation in a *linear* process such as leaf elongation of individual leaves. Only by considering LER of subsequent leaves and the number of leaves growing at one point in time, more insight can be gained into the involvement of LER in a plant's RGR. It is possible that the mechanisms that underlie variation in RGR are the same that cause variation in LER, but there is as yet no evidence to support this contention.

If we wish to further explore the mechanisms that account for variation in RGR, we have to be aware that LER is only part of the mechanism. Variation in LER of individual leaves may reflect variation in the rate of cell division and/or cell elongation. The data that are presently available do not allow general conclusions; nevertheless, they give the clear indication that both processes must be studied together if our aim is to explain how environmental parameters influence leaf elongation. An analysis of species occurring in contrasting environment and characterised by an inherently high or low LER could provide insights into cellular mechanisms leading to high or low leaf growth potential. A final remark deals with the possibility to analyse variation in leaf expansion of dicotyledonous species at the cellular level. In leaves of Dicotyledonae cell division and cell expansion occur all over the lamina and leaf expansion generally lasts much longer than in Monocotyledonae making the analysis more problematic. Interestingly, Granier & Tardieu (1998) demonstrated that gradients in cell development similar to those in monocotyledonous leaves are found in *Helianthus annuus* leaves.

If we aim to analyse phenotypic or genotypic variation in RGR, a further understanding of the control of leaf elongation and the factors that determine changes in this rate with time are important, even though they may not offer a full explanation of the mechanisms accounting for variation in RGR.

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Chapter 4

From individual leaf elongation to whole shoot leaf area expansion: a comparison of three *Aegilops* and two *Triticum* species

Abstract

A rapid leaf area development is a desirable trait in cereal crops. Differences between crop species or genotypes in individual leaf growth characteristics are well documented, whereas less attention has been paid to differences in the relationships between leaf growth characteristics of successive leaves and tillers. The latter is important in determining differences in leaf area expansion at the whole shoot level and whole plant growth. We investigated the relationships between several leaf characteristics and leaf position on the main stem, tiller 1 and tiller 2, for two wheat (*Triticum*) species and three wild relatives of wheat (*Aegilops* spp.). These relationships were subsequently evaluated in relation to leaf area expansion of whole shoot (RGR_{la}), leaf photosynthetic characteristics, biomass allocation and whole plant growth (RGR_{dm}).

In every species, leaf elongation rate (LER) was strongly and positively correlated with leaf width and sheath length, but to a lesser extent with leaf elongation duration (LED). The leaves of *Aegilops tauschii*, *Triticum aestivum* and *T. durum* elongated at twice the rate as leaves at the same positions in *Ae. caudata* and *Ae. umbellulata*. The species with the fastest-elongating leaves also had the fastest increase in LER and leaf width with leaf position, and hence had the fastest increase in leaf area. Since phyllochron and relative tillering rate did not significantly differ between the species, the faster increase in individual leaf area with leaf position resulted in faster relative leaf area expansion rates (RGR_{la}) in *Ae. tauschii*, *T. aestivum* and *T. durum*. The high RGR_{la} of *Ae. tauschii* in the early growth stage declined with development, to values similar to those of the other *Aegilops* species, because the increase in leaf elongation rate with leaf position slowed down considerably in this species. The high RGR_{la} of *Ae. tauschii*, *T. aestivum* and *T. durum* was reflected in a higher leaf area ratio (LAR) and was associated with more biomass allocated to the leaf sheaths and less to the roots. In contrast with the *Triticum* species, *Ae. tauschii* combined a high leaf area ratio (LAR) with a high rate of photosynthesis per unit leaf area, leading to a higher RGR_{dm} in the early developmental stages of this species.

Introduction

In areas with low rainfall, a rapid early leaf area expansion is a desirable trait in cereal crops. It leads to rapid canopy closure, reducing the evaporation from the soil surface and thus increasing crop water-use efficiency (Richards, 2000). In more favourable conditions, fast development of the canopy will make the crop more competitive with weeds for light interception (Lemerle *et al.*, 2001). Van den Boogaard *et al.* (1996c) showed that in wheat growing under favourable, controlled-environment conditions, a fast leaf area expansion rate was positively correlated with total above-ground biomass and grain yield. Therefore, exploration of variation in leaf area expansion rates in different species or genotypes can give information on valuable traits to select for in wheat breeding.

In order to get a better understanding of the processes underlying variation in relative leaf area expansion rate (RGR_{la}) at the whole shoot level, data on leaf area expansion of individual, successively growing leaves and tillers are needed. The variables determining leaf area expansion of individual leaves are leaf elongation rate (LER), leaf elongation duration (LED) and leaf width. The change in these variables in subsequent leaves, together with the rate at which new leaves and tillers emerge, determine the rate of whole shoot leaf area expansion (Bultynck *et al.*, 1999). In grasses, the initial phase of leaf growth occurs within the whorl of sheaths of the older leaves. The time the growing leaf spends inside this whorl of leaf sheaths, and hence the rate of leaf appearance from it (= inverse of the phyllochron), depends on the length of the whorl together with the LER of the growing leaf. Due to the important relationship between LER, sheath length and phyllochron, as previously found by Skinner & Nelson (1995), changes in sheath length with leaf positions are an important determinant of leaf area expansion. Expansion rates of successive leaves on the main stem of a single genotype in relation to changes in the environment are well documented (*e.g.*, Gallagher, 1979; Gautier & Varlet-Grancher, 1996; Rodríguez *et al.*, 1998a; Masle, 2000). Studies that include several tillers (Bos & Neuteboom, 1998a) or compare a range of genotypes or species are scarce. The first aim of the present study was to analyse inherent variation in individual leaf growth characteristics (LER, leaf width, LED, sheath length) in relation to leaf position (on main stem, tiller 1 and tiller 2) and whole shoot leaf area expansion (RGR_{la}), in five related species.

Variation in RGR_{la} may be closely associated with variation in biomass allocation and biomass production (Chapin *et al.*, 1989; Van den Boogaard *et al.*, 1996a). Moreover, a negative association between leaf area and the rate of photosynthesis per unit leaf area has been found in a comparison of several wheat cultivars and some of its progenitors (Evans & Dunstone, 1970; Rawson *et al.*, 1983; Van den Boogaard *et al.*, 1997; Villar *et al.*, 1998), counteracting the positive effect of an increased leaf area on biomass production. The second aim of this study was to investigate the relationship between RGR_{la}, the rate of

photosynthesis per unit leaf area, the biomass allocation pattern and relative growth rate of dry mass (RGR_{dm}).

For this experiment we chose two wheat species (*Triticum aestivum* and *T. durum*) and three species from the genus *Aegilops* (*Aegilops umbellulata*, *Ae. caudata* and *Ae. tauschii*), which are wild relatives of wheat (Van Slageren, 1994). Some of the *Aegilops* species have contributed in the distant past to the genome of the current bread wheat through natural hybridisation. The hybridisation between tetraploid *T. turgidum* (genomic formula: AABB) and diploid *Ae. tauschii* (DD) resulted in hexaploid *T. aestivum* (genomic formula: AABBDD). Due to their genetic link with the wheat species and their large genetic variation, the species of the genus *Aegilops* are potential donors of valuable traits for future wheat cultivars (Feldman & Sears, 1981; Damania, 1993) that may be better adapted to drier and warmer conditions, diseases and extreme temperatures. The third aim of this study was to evaluate whether the *Aegilops* species have a faster leaf area expansion in their early developmental stage than some of the current wheat species/cultivars, and thus may offer genetic traits to select for and use in wheat breeding.

Materials and methods

Plant material and growing conditions

Three *Aegilops* L. species and two *Triticum* L. species were used: *Ae. caudata, Ae. tauschii, Ae. umbellulata, T. aestivum* cv. Cascades and *T. durum* cv. Tamaroi. *Aegilops* seeds were obtained from ICARDA (International Centre for Agricultural Research in the Dry Areas, Aleppo, Syria) and *Triticum* seeds were obtained from the Department of Agriculture Western Australia (South Perth, Australia). Prior to germination, seeds were surface-sterilised with a 2.5% $NaHClO_3$ solution and stratified (placed on wet filter paper at 4°C in the dark) for 7 days. Seeds were germinated on moistened filter paper in Petri dishes in a germination cabinet (day: 14 h, 50 μmol m^{-2} s^{-1} PAR, 25°C; night: 10 h, 15°C). After germination seedlings were transferred to trays filled with washed river-sand, saturated with de-ionised water, and placed in the growth room (day: 14 h, 420 ± 30 μmol m^{-2} s^{-1} PAR, 23 ± 2°C, 70% RH; night: 10 h, 19 ± 2°C, 70% RH) for 3 days. Thereafter, seedlings were transferred (= day 0) to containers with 20 L of aerated modified Hoagland nutrient solution (2 mM NO_3^-), as described by Poorter & Remkes (1990). The pH of the nutrient solution was adjusted daily to 5.5 with H_2SO_4, and the solution was replenished weekly. Plants were rotated daily within the growth room to minimise the variation in environmental conditions for individual plants. Light competition between plants was avoided through the wide spacing of the plants in the container. As the plants grew larger, the spacing increased because some plants were harvested each week.

Destructive measurements

Eight plants per species were harvested on days 0, 7, 14, 20 (for *T. aestivum* and *T. durum*), 21 (for *Ae. umbellulata*, *Ae. caudata* and *Ae. tauschii*), 26 (for *Ae. umbellulata, T. aestivum* and *T. durum*) and 27 (for *Ae. caudata* and *Ae. tauschii*). The date of the last harvest differed between species depending on when leaf six on the main stem was fully elongated. Plants were separated into leaf blades, leaf sheaths and roots, and fresh weights of every portion were determined. Leaf blades were computer-scanned and analysed for leaf area, leaf length and maximum leaf width using the Win Rhizo V3.9 software (Regent Instruments, Quebec, Canada). Dry weights were determined after all plant material was dried for 48 h at 70°C.

From these data the following parameters were calculated: leaf mass ratio (LMR; leaf blade biomass per unit plant mass, g g^{-1}), stem mass ratio (SMR; leaf sheath biomass per unit plant mass, g g^{-1}), root mass ratio (RMR; root biomass per unit plant mass, g g^{-1}), leaf area ratio (LAR; leaf area per unit plant mass, m^2 kg^{-1}), and specific leaf area (SLA; leaf area per unit leaf mass, m^2 kg^{-1}). Leaf area and plant dry mass data from every harvest were ln-transformed, and relative leaf area expansion rate (RGR_{la}, g g^{-1} day^{-1}), relative growth rate (RGR_{dm}, g g^{-1} day^{-1}) and net assimilation rate (NAR; increase in total plant mass per unit leaf area per day, g m^{-2} day^{-1}) were calculated for each harvest interval, using the equations of Radford (1967).

Plants reserved for the last harvest were used throughout the experiment for daily leaf growth measurements as specified below. From Villar *et al.* (1998) and our own preliminary experiments, we know that handling these plants daily does not affect their growth rates.

Non-destructive individual leaf growth measurements

Leaf growth measurements were conducted on eight plants per species, until leaf six on the main shoot was fully elongated. Leaf and tiller emergence were recorded daily, and leaves and tillers were identified according to Klepper *et al.* (1982). Phyllochron was determined for individual leaves as the time between the appearance of two successive leaf tips from the whorl of leaf sheaths. At every point of the daily non-destructive measurements the number of simultaneously growing leaves and tillers was determined. From the onset of tillering, the relative tillering rate (increase in number of tillers per number of tillers already present; tillers $tiller^{-1}$ day^{-1}) and the relative increase in number of simultaneously growing leaves (increase in number of simultaneously growing leaves per number of leaves already growing; leaves $leaf^{-1}$ day^{-1}) were calculated as the slope of the regression line through the ln-transformed number of tillers and simultaneously growing leaves versus time, respectively.

Leaf length of every growing leaf was measured daily with a ruler. Leaf elongation rate (LER, mm day^{-1}) of individual leaves (LER) was calculated as the slope of the linear regression line through the data points within the phase of linear increase in leaf length. After inspection of the data, the linear growth phase of the leaves was considered as the interval

between 20 and 80% of final leaf length for *Aegilops* species, and the interval between 10 and 90% of final length for *Triticum* species.
Leaf elongation duration (LED, days) of individual leaves was calculated as:

$$LED = \frac{L_f}{LER} \quad (1)$$

where L_f (mm) is final leaf length and LER (mm day^{-1}) is leaf elongation rate.

Gas exchange measurements

At the last harvest date, gas exchange was measured on the youngest fully expanded leaf on the main stem of three plants per species. Gas exchange measurements were carried out with a LiCor 6400 system using the red and blue LED light source (LiCor, Lincoln, NE, USA). First, the rate of CO_2 assimilation was measured at ambient CO_2 concentration, at a saturating light intensity (2000 μmol m^{-2} s^{-1}; A_{max}) and then at a light intensity similar to that in the growth room (430 μmol m^{-2} s^{-1}; A_a). Thereafter, the response to intercellular CO_2 was measured at a light intensity of 2000 μmol m^{-2} s^{-1} and at external CO_2 concentrations declining from 1200 μmol mol^{-1} to 50 μmol mol^{-1}.

It was assumed that, at low intercellular CO_2 partial pressures, the assimilation of CO_2 was limited solely by the amount, activity and kinetic properties of Rubisco (Wullschleger, 1993). At these low intercellular CO_2 partial pressure values, Rubisco activity (V_c) was obtained by fitting the following equation to the rates of CO_2 assimilation (Von Caemmerer & Farquhar, 1981):

$$A = \frac{V_c(C - \Gamma_*)}{(C + K_m)} - R \quad (2)$$

where C is the intercellular CO_2 partial pressure (assumed here to be equal to that at the site of carboxylation), Γ_* is the CO_2 compensation point in the absence of dark respiration (R), and K_m is the effective Michaelis-Menten constant for CO_2. The kinetic constants for Rubisco were assumed to be equal to those determined for tobacco (Von Caemmerer *et al.*, 1994), namely 3.69 Pa for Γ_* and 73 Pa for K_m at 25°C. As our data were obtained at growth temperature (23°C), parameter values were calculated using the Arrhenius equation and activation energies given by De Pury & Farquhar (1997). At high intercellular CO_2 partial pressures, it was assumed that CO_2 assimilation was limited by the electron transport activity (J). J was then obtained by fitting the following equation through the rates of CO_2 assimilation at high intercellular CO_2 partial pressures (Von Caemmerer & Farquhar, 1981):

$$A = \frac{J(C - \Gamma_*)}{(4C + 8\Gamma_*)} - R \quad (3)$$

Total nitrogen concentration of the leaves on which photosynthesis was measured, was determined with an automatic C-H-N analyser (Leco CHN 1000, St. Joseph, MI, USA).

Statistical analysis

Data were analysed with SPSS 8.0 for Windows statistical software (SPSS, Inc., Chicago, IL, USA). For each leaf position, differences in LER, leaf width and LED between species were analysed by one-way ANOVA (α = 0.05). The results from this analysis were used to calculate the LSD. Relations between leaf parameters were tested with linear regression equations. Differences between species in biomass allocation parameters were analysed by two-way ANOVA with species and time as factors. Differences amongst species in RGR_{dm} and RGR_{la} were tested by two-way ANOVA of the ln-transformed plant dry mass and leaf area data with species and time as the independent factors (Poorter & Lewis, 1986). A significant interaction between species and time indicates a difference in RGR_{dm} or RGR_{la}. Differences amongst species in gas exchange parameters, relative tillering rate and relative increase in simultaneously growing leaves were analysed with a one-way ANOVA, followed by a Tukey *post hoc* test at α = 0.05.

Results

LER, LED and leaf width of individual leaves

The individual leaves on the main stem, tiller 1 and tiller 2 of *Ae. tauschii*, *T. aestivum* and *T. durum* had a larger leaf area than the ones at similar leaf positions of *Ae. caudata* and *Ae. umbellulata* (Fig. 1A), due to a faster leaf elongation and wider leaves (Fig. 1B and C). No such distinct differences were found in LED between the species (Fig. 1D). Figure 2 shows the relationship between LER and leaf width or LED for all the leaves presented in Figure 1. LER was positively correlated with leaf width within every species ($p<0.001$; r^2 between 0.171 for *Ae. umbellulata* and 0.628 for *T. aestivum*), and the correlation became stronger when all the species were grouped together ($p<0.001$; $r^2=0.71$) (Fig. 2A). The relationship between LER and LED was much less clear, both within and amongst species ($p<0.001$; $r^2=0.049$) (Fig. 2B). In general, irrespective of the species, faster-growing leaves were wider and grew for a slightly longer time and, consequently, had a larger leaf area than slower-growing leaves.

LER, LED and leaf width increased with increasing leaf number on a tiller, in most species (Fig. 1). The increase in LED did not differ significantly amongst most species whereas the increase in LER and leaf width was significantly faster in the two *Triticum* species and *Ae. tauschii* compared with the slower-elongating *Ae. umbellulata* and *Ae. caudata* (Fig. 1). In the latter two species leaf width remained constant in successive leaves. In the fast-elongating *Ae. tauschii* and the two *Triticum* species, LER and leaf width increased by more than 150% from the first to the sixth leaf on the main stem. In the slow-elongating *Ae. umbellulata* and *Ae. caudata*, LER increased by approximately 100% from

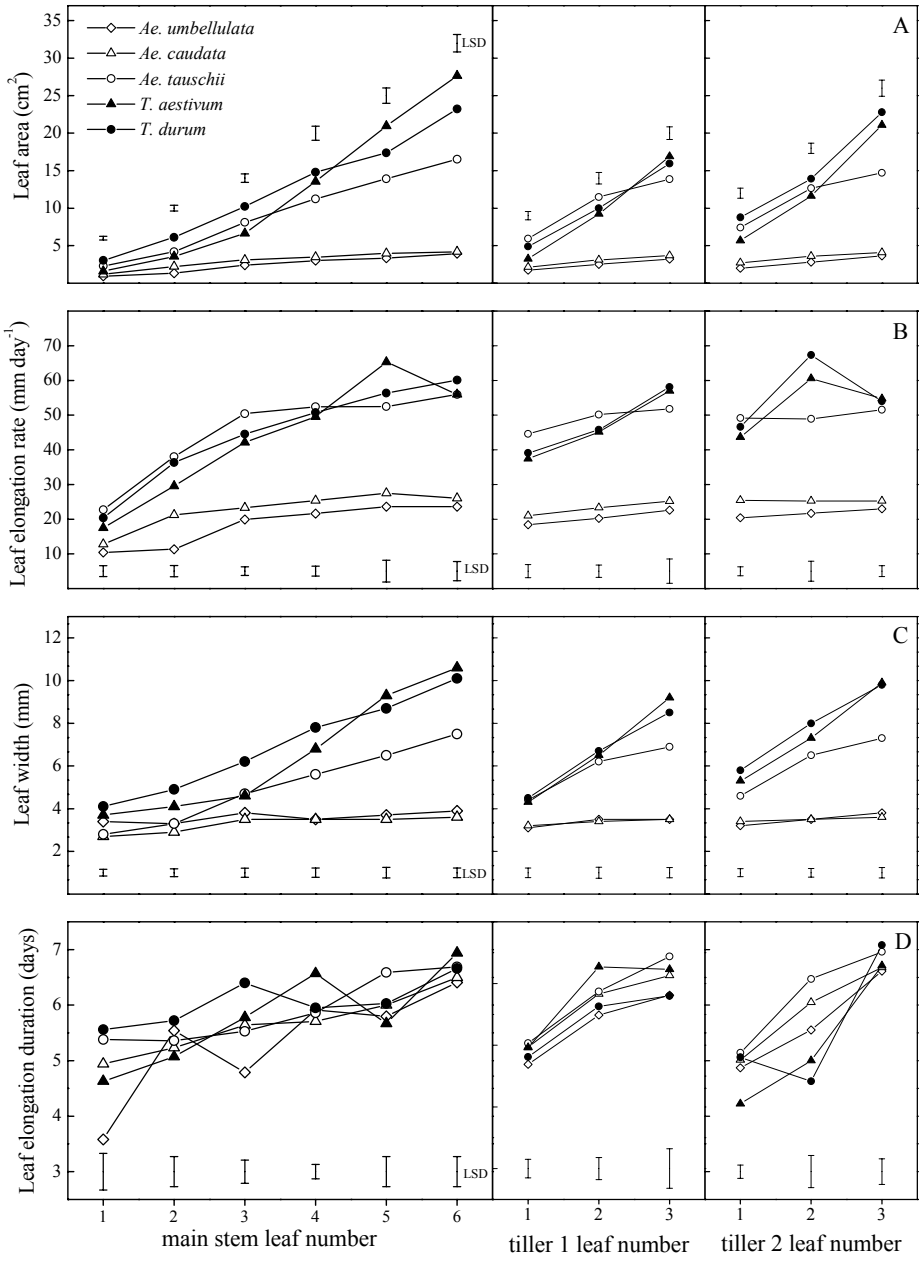

Figure 1. Leaf area (A), leaf elongation rate (B), leaf width (C), and leaf elongation duration (D) of successive leaves on main stem, tiller 1 and tiller 2, of *Ae. umbellulata*, *Ae. caudata*, *Ae. tauschii*, *T. aestivum* and *T. durum*. Symbols denote means of 8 plants per species. Vertical bars represent LSD.

the first to the sixth leaf on the main stem, and leaf width increased by approx. 20%. On tiller 1 and tiller 2, *Ae. tauschii* and the two *Triticum* species showed a higher relative increase in leaf width from the first to the third leaf than *Ae. umbellulata* and *Ae. caudata*. However, the species no longer differed in their relative increase in LER. The relative increase in LED was similar amongst all the species on main stem, tiller 1 and tiller 2, and was generally less than 50%.

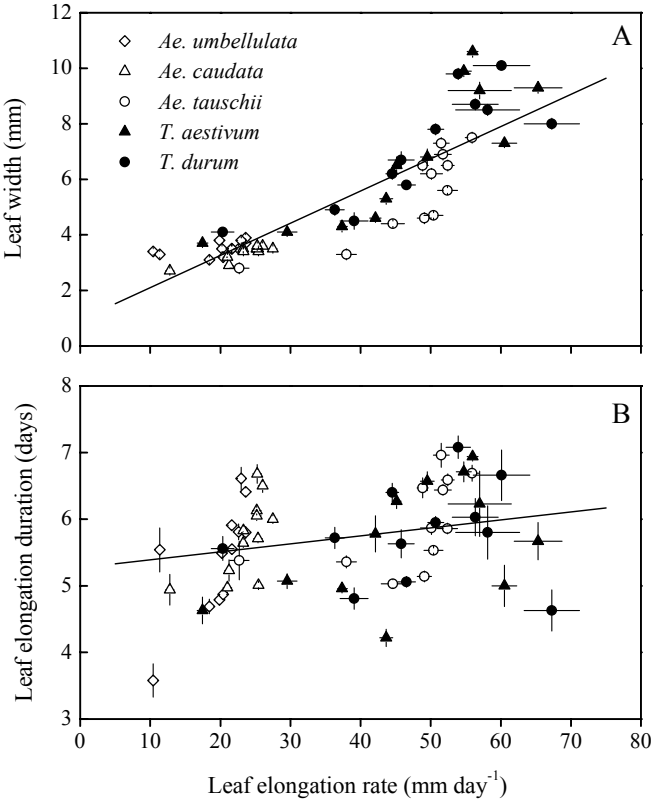

Figure 2. Relationship between (A) leaf elongation rate and leaf width, and between (B) leaf elongation rate and leaf elongation duration, of individual leaves of *Ae. umbellulata*, *Ae. caudata*, *Ae.tauschii*, *T. aestivum* and *T. durum*. Symbols denote mean values (±SE) of 8 leaves per leaf position. The lines indicate the significant linear regressions, derived from the individual values of each leaf.

Leaf appearance rate: leaf and tiller emergence

There were no significant differences in phyllochron (=inverse of leaf emergence) between the different tillers and species (Table 1; two-way ANOVA with tiller and species as factors and α=0.05, data not shown). The timing of leaf emergence depends on the LER of the growing leaf and sheath length of the previously growing leaf (Miglietta, 1991; Skinner & Nelson, 1995). Figure 3 shows that both LER of a specific leaf (leaf n) and sheath length of the preceding leaf (leaf n-1) were greater in *Ae. tauschii* and the two *Triticum* species.

Table 1. Comparison amongst species of phyllochron (days), relative tillering rate (tillers tiller^{-1} day^{-1}), relative increase in number of simultaneously elongating leaves (leaves leaf^{-1} day^{-1}). Means with different letters are significantly different (*post hoc* Tukey; p<.005).

	Phyllochron			Relative tillering rate	Relative increase in simultaneously growing leaves
	Main stem	Tiller 1	Tiller 2		
Ae. umbellulata	4.2	3.8	3.9	0.053^{ab}	0.054^{a}
Ae. caudata	4.7	4.8	4.6	0.051^{a}	0.054^{a}
Ae. tauschii	4.5	4.5	4.4	0.052^{ab}	0.059^{a}
T. aestivum	4.2	4.0	4.2	0.063^{c}	0.064^{a}
T. durum	3.9	4.0	3.7	0.061^{bc}	0.060^{a}

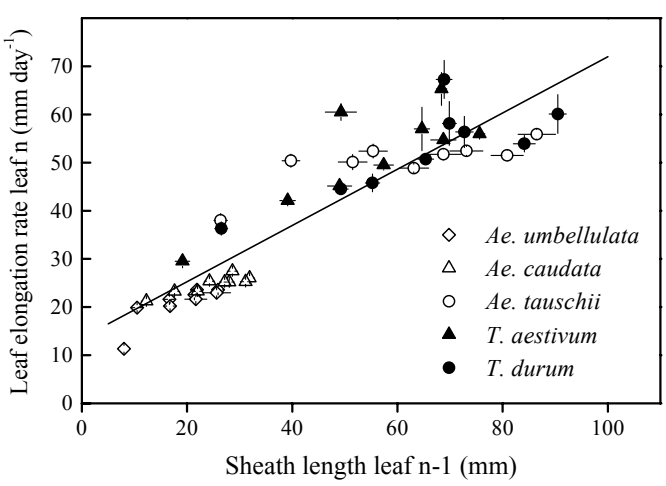

Figure 3. Relationship between leaf elongation rate of leaf *n* and sheath length of leaf *n-1* of individual leaves presented in Figure 1 of *Ae. umbellulata*, *Ae. caudata*, *Ae. tauschii*, *T. aestivum* and *T. durum*. Symbols denote mean values (±SE) of 8 leaves per leaf position. The line indicates the significant linear regression, derived from the individual values of each leaf.

Although *Ae. umbellulata* started to produce tillers earlier (day 6) than the other species (between day 8 and day 10), in all species the first tiller emerged when the third and fourth leaf on the main stem were growing and the tillers emerged in the same sequence (data not shown). From the onset of tillering, no significant difference in relative tillering rate was found between most of the species (Table 1): the two *Triticum* species had a higher relative tillering rate (>0.060 tillers tiller^{-1} day^{-1}) than the *Aegilops* species (<0.055 tillers tiller^{-1} day^{-1}), but the difference was only significant for *T. aestivum* (0.063 tillers tiller^{-1} day^{-1}) (Table 1). The same differences amongst species were observed for the relative increase in

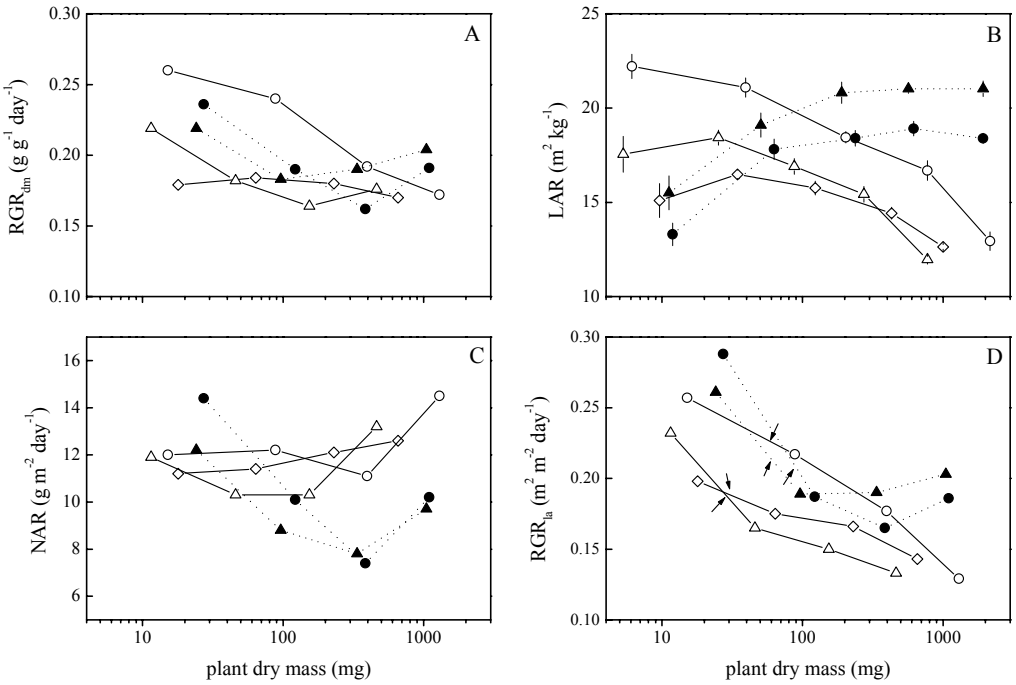

Figure 4. Ontogenetic changes in (A) relative growth rate of plant dry mass (RGR_{dm}), (B) leaf area ratio (LAR), (C) net assimilation rate (NAR), and (D) relative leaf area expansion rate (RGR_{la}), of *Ae. umbellulata*, *Ae. caudata*, *Ae. tauschii*, *T. aestivum* and *T. durum*. Arrows in (D) indicate onset of tillering for each species. Error bars in (B) indicate standard error (n=8). The error bars of RGR were omitted for clarity's sake and for NAR it was not possible to calculate standard error. See Results for statistical evaluation of these parameters.

simultaneously elongating leaves, calculated from the start of tillering, but none of these differences were significant (Table 1). Before the onset of tillering, the number of simultaneously growing leaves on the main stem was constant and similar for all species: it fluctuated between 1 and 2 leaves growing simultaneously (data not shown).

Whole plant growth and biomass allocation

Figures 4 and 5 present biomass allocation, LAR, SLA, NAR, and relative growth rate of dry mass (RGR_{dm}) and leaf area (RGR_{la}) as a function of plant size. Two-way analysis of variance, with species and time as fixed factors, showed highly significant ($p<0.001$) main effects and interactions for all the parameters presented in figures 4 and 5. At the start of the growing period, the relative growth rate (RGR_{dm}) was highest in *Ae. tauschii*, lowest in *Ae. umbellulata* and *Ae. caudata*, and intermediate in *T. aestivum* and *T. durum* (Fig. 4A). The faster RGR_{dm} of *Ae. tauschii* was associated with a higher LAR, whereas the intermediate

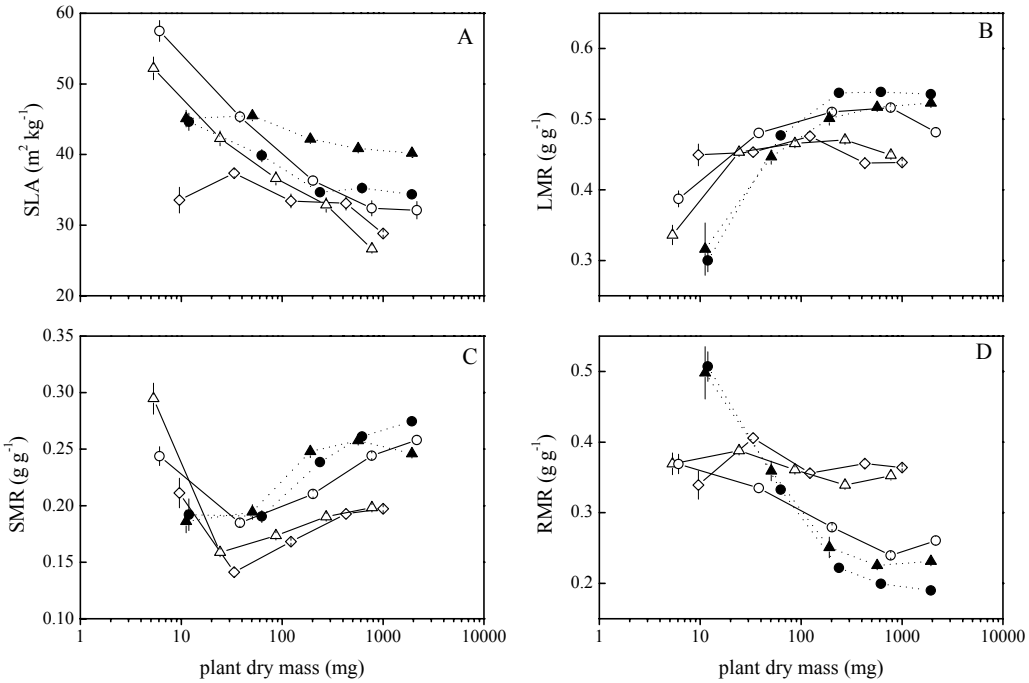

Figure 5. Ontogenetic changes in (A) specific leaf area (SLA), (B) leaf mass ratio (LMR), (C) stem mass ratio (SMR), and (D) root mass ratio (RMR), of *Ae. umbellulata*, *Ae. caudata*, *Ae. tauschii*, *T. aestivum* and *T. durum*. Error bars indicate standard error (n=8). See Results for statistical evaluation of the parameters.

RGR_{dm} of the *Triticum* species was associated with a higher NAR compared with *Ae. umbellulata* and *Ae. caudata* (Figs 4B and C). In *Ae. tauschii*, RGR_{dm} and LAR decreased by approx. 60% over the experimental period and reached values similar to those of the other *Aegilops* species (Figs 4A and B). The two *Triticum* species also decreased their RGR_{dm} initially due to a decrease in NAR. However, the NAR increased again at the end of the experimental period along with a strong increase in LAR, resulting in a faster RGR_{dm} than in the *Aegilops* species (Figs 4A, B and C). The change in RGR_{la} with ontogeny showed a similar pattern as that of RGR_{dm}. RGR_{la} was faster in the two *Triticum* species and *Ae. tauschii* at the start of the growing period, and in the *Aegilops* species it decreased more with increasing plant size (Fig. 4D).

LAR in the *Aegilops* species decreased as a result of the decreasing SLA (Fig. 5A), whereas LAR in the *Triticum* species increased due to a stronger increase in LMR and a lesser decrease in SLA (Fig. 5B). At the start of the experimental period, the biomass allocation pattern of *Ae. tauschii* resembled that of the other *Aegilops* species: they allocated more to the leaf blades (high LMR) and leaf sheaths (high SMR) and less to the roots (low

RMR) than the *Triticum* species did (Figs 5B, C and D). The higher SMR in the *Aegilops* species at the first harvest probably resulted from a relatively longer coleoptile, which was included in the stem fraction. At a later stage of their development, the biomass allocation pattern of *Ae. tauschii* resembled that of the two *Triticum* species: LMR and SMR increased with development while RMR deceased (Figs 5B, C and D).

Photosynthetic characteristics

Table 2 shows the photosynthetic characteristics for the last fully expanded main stem leaf in all the species at the last harvest date. The *Aegilops* species had faster rates of photosynthesis per unit leaf area than the *Triticum* species, both at a PPFD of 430 μmol m^{-2} s^{-1} (A_{430}), the light level at which the plants were grown, and at saturating light levels (A_{max}). The faster rates of photosynthesis per unit leaf area in *Aegilops* were associated with a higher nitrogen concentration, a higher Rubisco activity (V_c) and a higher electron transport activity (J) per unit leaf area than in *Triticum*. Per unit nitrogen, the rate of photosynthesis (A/N), Rubisco activity (V_c/N) and electron transport activity (J/N) did not differ between the *Triticum* and *Aegilops* species, suggesting that similar proportions of leaf nitrogen were allocated to Rubisco and electron transport. *Ae.tauschii*, the *Aegilops* species with the faster-elongating leaves, tended to have a lower leaf nitrogen concentration but a similar rate of photosynthesis per unit leaf area than the *Aegilops* species with the slow-elongating leaves.

Table 2. Photosynthetic parameters of the youngest fully expanded main stem leaf of three *Aegilops* and two *Triticum* species, at the last harvest date. Values of leaf nitrogen concentration per unit leaf area (leaf N), rate of photosynthesis at 430 μmol m^{-2} s^{-1} per unit leaf area (A_{430}), rate of photosynthesis at 2000 μmol m^{-2} s^{-1} per unit leaf area (A_{max}) and per unit leaf nitrogen (A_{max}/N), Rubisco activity at 2000 μmol m^{-2} s^{-1} per unit leaf area (V_c) and per unit leaf nitrogen (V_c/N), and electron transport activity at 2000 μmol m^{-2} s^{-1} per unit leaf area (J) and per unit leaf nitrogen (J/N). Different letters indicate significant differences between species (n=3).

	Ae. umbellulata	*Ae. caudata*	*Ae. tauschii*	*T. aestivum*	*T. durum*
Leaf N (mmol m^{-2})	173^{ab}	189^{a}	153^{bc}	127^{cd}	121^{d}
A_{430} (μmol m^{-2} s^{-1})	22.6^{a}	20.1^{ab}	21.9^{a}	16.7^{bc}	14.9^{c}
A_{max} (μmol m^{-2} s^{-1})	40.0^{a}	34.6^{b}	35.0^{ab}	27.6^{bc}	25.9^{c}
V_c (μmol m^{-2} s^{-1})	174^{a}	167^{a}	159^{a}	119^{b}	120^{b}
J (μmol m^{-2} s^{-1})	285^{a}	237^{ab}	246^{a}	187^{bc}	176^{c}
A_{max}/N (μmol mol^{-1} s^{-1})	234^{a}	176^{b}	222^{ab}	215^{ab}	209^{ab}
V_c/N (mmol mol^{-1} s^{-1})	1.01^{a}	0.87^{a}	1.04^{a}	0.94^{a}	0.99^{a}
J/N (mmol mol^{-1} s^{-1})	1.66^{a}	1.26^{b}	1.60^{ab}	1.48^{ab}	1.45^{ab}

Discussion

Relationship between LER, LED, leaf width and leaf position

In all the species in this study, leaf area increased with leaf position on the main stem, tiller 1 and tiller 2. This increase was strongly associated with an increase in leaf elongation rate (LER) and leaf width, but much less with an increase in duration of leaf elongation (LED). Although increases in leaf length and leaf width with leaf position have been shown before in cereal crops (e.g. Williams & Rijven, 1965; Gallagher, 1979; Bos & Neuteboom, 1998a), comparisons amongst species are scarce. Leaf width and LER increased faster with leaf position in the species with the widest and longest leaves (*Ae. tauschii*, *T. aestivum*, *T. durum*) compared with the species with the smaller leaves (*Ae. caudata*, *Ae. umbellulata*), where an increase in LER and leaf width was often lacking. Contrary to the increase in LER and leaf width with leaf number, the increase in LED was not significantly different amongst the species in the present study and therefore did not contribute to differences in leaf area amongst these species.

In a previous paper, we have shown that the twofold difference in LER and leaf width of the third leaf on the main stem between *Ae. caudata* and *Ae. tauschii* was associated with a twofold difference in length and width of the leaf meristem (*i.e.* the number of dividing cells in length and width) (Chapter 2). Beemster *et al.* (1996) have also shown that the increase in LER in three successive leaves of wheat is associated with an increase in the length of the leaf meristem. Possibly, the faster increase in length and width with leaf position in the fast-elongating species in the present study resulted from a faster increase in leaf meristem size of successive leaves. In several cases, increases in LER and leaf width with leaf position have been associated with increases in apical dome size (Abbe *et al.*, 1941; Kirby, 1974; Pieters & Van den Noort, 1988; Bos & Neuteboom, 1998a). However, some evidence suggests that LER and leaf width are controlled independently and that leaf width is not controlled by the apical dome size. The independent control of LER and leaf width is suggested by our own data as well as those of other authors. Our results show that, at least in some of the species or in some of the tillers, the relative increase in LER with leaf position differed from that in leaf width. Moreover, the AFLP-markers that correlate with LER differ from the ones that are correlated with leaf width, in 46 *Ae. tauschii* accessions (M.W. ter Steege, personal communication). Beemster *et al.* (1996) provide additional evidence for leaves of wheat seedlings grown at different soil resistances. The growing leaves in their study differed in sensitivity of the number of formative divisions (determines number of parallel cell files and is related to leaf width) and of the number of proliferative divisions (determines number of cells along a cell file and is related to LER) to the treatment. Beemster & Masle (1996) also showed that the reduction in leaf width that was induced by

the treatment was not related to a reduction in apical dome size, but to changes in cellular processes that take place after leaf initiation.

Regulation of leaf and tiller appearance

The relationship between LER of a growing grass leaf and sheath length of the preceding leaf determines the time needed for the growing leaf to appear from the whorl of sheaths, and hence the phyllochron (Skinner & Nelson, 1995). The fast-growing species in our study had both longer leaf sheaths and faster LER, resulting in similar phyllochron values for all species. Within each species, however, sheath length increased faster than LER increased whereas phyllochron remained constant for successive leaves (Fig. 3). Since the interval between initiations of successive leaves seems to be constant under constant environmental conditions (Hay & Kemp, 1990, Rodríguez *et al.*, 1998b), the most likely explanation is that the initiation of leaf elongation was progressively earlier in successive leaves. Skinner & Nelson (1995) showed that initiation of leaf elongation in the youngest leaf primordium of tall fescue was synchronized with ligule initiation in the second youngest leaf, cessation of cell division in the sheath of the third oldest leaf, and initiation of tiller elongation at the axillary bud associated with that leaf. This observation shows a close association between the development of successive leaves and their associated tillers, as well as between the timing of leaf and tiller appearance. That may explain why we found very little variation in both phyllochron and relative tillering rate between the species in this study. Although the different species had similar relative tillering rates and the first tiller emerged at the same stage of development, the species differed in the time at which they reached this stage of development. Especially *Ae. umbellulata* reached the four-leaves stage at which tillering commences earlier than the others. This was either due to a difference in the timing of germination or in the rate of shoot development. We have no data on the exact timing of germination, but the phyllochron gives an approximation of the developmental rate (McMaster, 1997). The phyllochron tended to be shorter in *Ae. umbellulata* compared with the other *Aegilops* species, but was similar to that of the *Triticum* species. Therefore, differences in the timing of germination also played a role.

Whole shoot leaf area expansion

Differences in individual leaf growth affected the relative leaf area expansion rate (RGR_{la}), mainly during the very early growth stages. The faster increase in leaf width and leaf length with leaf position on the main stem of the fast-expanding *Ae. tauschii*, *T. durum* and *T. aestivum* resulted in a faster RGR_{la} during early growth compared with the slow-expanding *Ae. umbellulata* and *Ae. caudata*. As the increase in leaf elongation rate with leaf position slowed down, RGR_{la} quickly declined in all species, which clearly showed that a linear increase in leaf area cannot lead to exponential growth without tillering (Van Loo, 1992;

Groeneveld, 1998; Bultynck *et al.*, 1999). In accordance with other studies on wheat (Longnecker *et al.*, 1993; Rodríguez *et al.*, 1998b; Miralles & Richards, 2000), the first tiller started to appear on all the species in this study when the third and fourth leaf on the main stem were growing. Tillering reduced the rate of decline in RGR_{la} in *Triticum* but not in *Aegilops*. This was partly due to the slower increase in LER and leaf width with leaf position on the newly formed tillers of the *Aegilops* species compared with the *Triticum* species. The slightly higher tillering rates of both *Triticum* species probably also played a role in this.

We have focused in this experiment on the RGR_{la}. However, the absolute size of leaf area during early growth is also important in determining a high ground cover, final biomass and yield (López-Castañeda *et al.*, 1996). The species in the present study that had a higher RGR_{la} (*Ae. tauschii, T. aestivum* and *T. durum*) also achieved a higher total leaf area early in development. Since total leaf area increased slower in *Ae. tauschii* than in the two *Triticum* species, the advantage of a large leaf area in *Ae. tauschii* disappeared as plants grew older and larger. Several studies have shown that seed or embryo size is more important than RGR in determining plant and leaf size of seedlings (e.g. Chapin *et al.*, 1989; López-Castañeda *et al.*, 1996; Van Rijn *et al.*, 2000). We did not measure seed size in this experiment but, assuming leaf width of the first seedling leaf is a good measure of embryo size (López-Castañeda *et al.*, 1996), this would suggest that the *Triticum* species had larger embryos than the *Aegilops* species (Fig. 1C). Despite its supposedly small embryo, *Ae. tauschii* achieved a larger total leaf area than the other *Aegilops* species early in development due to a faster RGR_{la}, showing that a high RGR_{la} can be important during early development.

Whole plant growth and biomass allocation

The faster increase in leaf area expansion with leaf position in *Ae. tauschii*, *T. aestivum* and *T. durum*, compared with *Ae. umbellulata* and *Ae. caudata*, was reflected in the higher leaf area ratio (LAR) in these species. Initially, the *Triticum* species had a low LAR, even lower than that of the slow-expanding *Ae. umbellulata* and *Ae. caudata*, because they invested proportionally more carbon in their roots (high RMR). However, upon elongation of the first few leaves of the *Triticum* species, the RMR quickly dropped below that of the *Aegilops* species, whereas the LAR increased to values considerably above those of the *Aegilops* species. It is likely that the high demand for carbon in the division and expansion zones of the growing leaves (Hu *et al.*, 2000; Schäufele & Schnyder, 2001) resulted in more carbon being used in the shoot instead of going to the roots. The relatively high RMR for *Aegilops* compared with *Triticum* has been found before (Van den Boogaard & Villar, 1998) and may be an adaptation to growth in dry and nutrient-scarce environments (Villar *et al.*, 1998). In contrast with the *Triticum* species, *Ae. tauschii* had a high LAR at the start of the growing period, which quickly dropped below that of the *Triticum* species due to a decrease in SLA with development. SLA in the *Triticum* species however, remained rather constant over the

growing period. Species occurring in dry environments, like the *Aegilops* species, probably benefit from making thicker leaves (lower SLA) (Lambers & Poorter, 1992).

In the early developmental stage, *Ae. tauschii* was able to combine a high LAR with a relatively high NAR, and this led to a considerably higher biomass production (high RGR_{dm}). This growth advantage disappeared as the plants grew larger and LAR dropped below that of the *Triticum* species. At the end of the experimental period, the higher LAR of the *Triticum* species was associated with a lower NAR and lower rates of photosynthesis per unit leaf area than in the *Aegilops* species. A negative association between leaf area and the rate of photosynthesis per unit leaf area has been found by several authors in wheat cultivars as well as wheat ancestors (Evans & Dunstone, 1970, Rawson *et al.*, 1987: individual leaf area; Van den Boogaard *et al.*, 1997: leaf area ratio; Villar *et al.*, 1998: total leaf area). In the present study, most of the differences in photosynthetic rate per unit leaf area were explained by the lower leaf nitrogen concentration per unit leaf area of the *Triticum* species, as the photosynthetic rate expressed per unit leaf nitrogen was similar to that of the *Aegilops* species. Similarly, Evans (1985) showed that variation in photosynthetic rate per unit leaf area amongst three wheat species and one *Aegilops* species was strongly related to variation in leaf nitrogen concentration per unit leaf area. The lower leaf nitrogen concentration of the species in the present study was a consequence of both the higher SLA and the lower leaf nitrogen concentration per unit mass.

The fast-growing *Ae. tauschii*, *T. aestivum* and *T. durum* have a higher SMR than the slow-growing species in this study, a relationship that was also found by Van den Boogaard & Villar (1998) in a comparison of 22 *Aegilops* species with 10 *Triticum* cultivars. These authors suggested that gibberellins might be involved, as gibberellins have previously been associated with differences in RGR and SMR (Nagel *et al.*, 2001a). Our results support such a hypothesis, since we have shown that the species with the highest SMR also have the fastest-elongating leaves, while several authors reported on the important stimulating effect of gibberellins on leaf elongation rate (Tonkinson *et al.*, 1995; Chandler & Robertson, 1999). In a forthcoming paper (Chapter 5), we explore the influence of GA on leaf expansion and biomass allocation in these *Aegilops* species.

Future use of Aegilops?

From the *Aegilops* species used in the present study, only *Ae. tauschii* was able to reach the same high RGR_{la} as the *Triticum* species. Moreover, in the early growth stages *Ae. tauschii* had an even higher LAR than the *Triticum* species. Since the development of a large leaf area has been shown to be related to high yield in several cereal crops (López-Castañeda *et al.*, 1996), it is worthwhile to further explore this trait in *Ae. tauschii*, the wild relative from which wheat inherited the D-genome (Feldman & Sears, 1981). In further studies, more accessions of *Ae. tauschii* need to be investigated under field conditions. From this study, we

can also conclude that there is a large variation in leaf growth characteristics within the *Aegilops* genus, which makes this genus ideal for future experiments on the regulation of leaf growth.

Acknowledgements

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Chapter 4

Chapter 5

Effects of exogenous supply of gibberellic acid and paclobutrazol on leaf expansion and biomass allocation in two *Aegilops* species with contrasting leaf elongation rates

Abstract

The role of gibberellin (GA) in leaf elongation has long been known, however, its involvement in whole shoot growth and biomass allocation is much less clear. We studied the effects of exogenously supplied GA_3 and paclobutrazol, an inhibitor of GA biosynthesis, on these processes in *Ae. caudata* and *Ae. tauschii*, species with contrasting leaf growth characteristics.

In both species, addition of GA_3 increased leaf elongation rate (LER) through its promoting effect on both cell size and cell number, while paclobutrazol decreased it. Similarly, GA_3 increased biomass allocation to the leaves, mainly leaf sheaths, at the cost of allocation to the roots, whereas paclobutrazol had the opposite effect in both species. Despite the increase in LER and biomass allocation to the shoot upon GA_3 application, the relative growth rate (RGR) remained constant. Specific leaf area (SLA) was only temporarily affected by GA_3 addition.

Our results show that the inherent differences in LER and biomass allocation between the slow-elongating *Ae. caudata* and the fast-elongating *Ae. tauschii* are considerably reduced by the exogenous supply of GA_3 to the slow-elongating species, or paclobutrazol to the fast-elongating one. This suggests a role for gibberellins in explaining inherent differences in leaf area expansion and biomass allocation between the two species in this study.

Introduction

Several authors working with dwarf genotypes of cereal crops have suggested a role for endogenous gibberellins (GA) in the control of leaf growth (Pinthus *et al.*, 1989; Keyes *et al.*, 1989; Paolillo *et al.*, 1991; Tonkinson *et al.*, 1995; Chandler & Robertson, 1999). Research on dwarf mutants of wheat (Pinthus *et al.*, 1989) and barley (Chandler & Robertson, 1999) indicates that slower leaf growth, compared with that of wild-type plants, can be due to either reduced sensitivity to GA or reduced levels of endogenous bioactive GA. In a comparison of slow-growing GA-deficient tomato mutants with the fast-growing wild-type, Nagel *et al.* (2001b) showed that the reduced leaf size in the mutant is accompanied by an increased biomass allocation to the roots at the cost of the stem. The authors suggest that lack of GA causes a reduction in epidermal mature cell size and number in the mutants' leaves and stems, thereby restricting expansion of these organs and consequently reducing the flux of assimilates towards them. As a result, the relative flux of assimilates to the roots may be increased, resulting in an increased root mass ratio (RMR) and a reduced relative growth rate (RGR). From this point of view, the RGR of a plant is determined by the rates of cell production and cell expansion in the shoot.

In a comparison of all species of the *Aegilops* genus, Villar *et al.* (1998) found a wide variation in RGR and biomass allocation, while our preliminary research showed a wide variation in leaf elongation rates (LER) in several species within this genus (L. Bultynck, unpublished results; see also Chapter 4). Interestingly, within the *Aegilops* genus, a faster growth (high RGR) is associated with faster leaf expansion (high RGR_{la}), a higher proportion of biomass allocated to the stem (which consists mainly of leaf sheaths), and a lower proportion of biomass allocated to the roots. This leads to the hypothesis that GA is involved in differences in LER and allocation patterns between *Aegilops* species.

Since differences in leaf expansion and biomass allocation between species may be associated with differences in cell production and cell expansion in the shoot, GA may be involved in these processes. In a comparison at the leaf cellular level of the fast-elongating *Aegilops tauschii* with the slow-elongating *Ae. caudata*, we found that differences in LER were associated with differences in cell production rate, while mature cell size was similar for the two species (see Chapter 2). In contrast, the differences in LER and final leaf length between the wild-type and dwarf mutants in cereals are mainly a result of the effect of GA on cell expansion (Keyes *et al.*, 1989; Sauter & Kende, 1992; Tonkinson *et al.*, 1995; Matsukura *et al.*, 1998). However, several authors reported that next to its effect on cell elongation, GA also increases the rate of cell division and the size of the meristem (Hoffman-Benning & Kende, 1992; Sauter *et al.*, 1995). In a comparison of wild-type barley with nine barley mutants showing different degrees of dwarfing, Wenzel *et al.* (1997) found that variation in leaf length was associated with either variation in cell number, cell length, or both, depending on the leaves, leaf parts, mutants and cell types that were compared.

For the *Aegilops* species, we suggest that inherent differences in leaf expansion rate, biomass allocation and cell production rate is mediated by GA. In this paper, as a first step to test this hypothesis, we used the slow-elongating *Ae. caudata* and the fast-elongating *Ae. tauschii* to study the effects of exogenously supplied GA_3 and paclobutrazol, an inhibitor of GA biosynthesis (Lenton *et al.*, 1994), on LER, leaf elongation duration, leaf appearance, leaf cell size and cell production rate, and biomass allocation. Although numerous authors have studied the relationship between LER and GA, very few of them have identified the links between GA, LER, biomass allocation and whole shoot growth. We need this information to better understand the regulation of whole plant growth.

Materials and methods

Plant material and growing conditions

Seeds of *Aegilops tauschii* L. and *Ae. caudata* L. were obtained from ICARDA (International Centre for Agricultural Research in the Dry Areas, Aleppo, Syria). Prior to germination, seeds were surface-sterilised with a 2.5% $NaHClO_3$ solution and stratified (placed on wet filter paper at 4°C in the dark) for 7 days. Thereafter, seeds were germinated on moistened filter paper in Petri dishes in a germination cabinet (day: 14 h, 50 μmol m^{-2} s^{-1} PAR, 25°C; night: 10 h, 15°C). After germination, seedlings were transferred to trays, filled with washed river-sand and saturated with de-ionised water, and placed in a growth room (day: 14 h, 420 ± 40 μmol m^{-2} s^{-1} PAR, 22 ± 1°C, 70% RH; night: 10 h, 10 ± 1°C, 70% RH). After three days the seedlings were transferred to containers filled with 32 L of aerated modified Hoagland nutrient solution (2mM NO_3^-), as described by Poorter & Remkes (1990).

Two experiments were carried out. In the first experiment (Experiment I), the effect of two concentrations (1 μM and 10 μM) of either GA_3 or paclobutrazol on growth was investigated. Plants were randomly assigned to one of five treatments: (1) control nutrient solution, (2) nutrient solution with 1 μM GA_3, (3) nutrient solution with 10 μM GA_3, (4) nutrient solution with 1 μM paclobutrazol, (5) nutrient solution with 10 μM paclobutrazol. Based on the results of the first experiment (see Fig. 1 and Results section), we repeated the experiment (Experiment II) with the lower concentration of GA_3 and paclobutrazol. In Experiment II, plants were randomly assigned to one of four different treatments: (1) control nutrient solution, (2) nutrient solution with 1 μM GA_3, (3) nutrient solution with 1 μM paclobutrazol, (4) nutrient solution with 1 μM GA_3 + 1 μM paclobutrazol. The fourth treatment was used to determine whether there were any effects of paclobutrazol, other than inhibition of gibberellin biosynthesis.

The pH of the nutrient solution was adjusted daily to 5.5 with H_2SO_4, and the solution was replaced weekly. Plants were rotated daily within the growth room to minimise the variation in environmental conditions for individual plants.

Leaf growth measurements

Leaf growth was measured on a set of five (Experiment I) and six (Experiment II) plants per species. Leaf length (from leaf tip to the base of the whorl of leaf sheaths) of either leaf 3 on the main stem (L3MS) or leaf 1 of the first tiller (L1T1) was measured daily with a ruler. Leaf elongation rate (LER, mm day^{-1}) was calculated as the slope of the linear regression line through the data points within the phase of linear increase in leaf length. The linear growth phase of the leaves was determined as the interval between 20 and 80% of final leaf length. No distinction was made between the elongation in the leaf sheath and leaf blade, because most of the elongation in the linear growth phase of the leaf is due to blade elongation (Schnyder *et al.*, 1990). Leaf elongation duration (LED, days) was calculated as:

$$LED = \frac{L_f}{LER} \tag{1}$$

where L_f (mm) is final leaf length and LER (mm day^{-1}) is leaf elongation rate. When the leaf was fully expanded, the length of the leaf sheath and the leaf blade were measured and the ratio of sheath length to total leaf length was calculated.

Leaf emergence was recorded daily during 2 weeks after transfer to nutrient solution, and leaves were identified according to Klepper *et al.* (1982). Phyllochron (= time between the appearance of successive leaves) for the main stem was estimated as the slope of the regression line through the Haun index against time, where the Haun index is defined as (Haun, 1973):

$$\text{Haun index} = (n-1) + \frac{L_n}{L_{n-1}} \qquad \left(0 < \frac{L_n}{L_{n-1}} \leq 1\right) \tag{2}$$

where n is the number of visible leaves on the main shoot, L_{n-1} (mm) is the length of the penultimate leaf and L_n (mm) is the length of the youngest visible leaf on the main shoot.

For Experiment I only the LER data are shown. The other data are similar to the data from the first experiment, for the same hormone concentrations used in the first experiment. This confirms the reproducibility of the experiment.

Cell length measurements

A second set of 3 plants per species was used for determining mature epidermal cell length of the third leaf on the main stem. The leaves were harvested when the third leaf was fully expanded. Leaves were cut at the base of the leaf blade, immediately transferred to boiling methanol for chlorophyll removal, and subsequently transferred to 90% (w/v) lactic acid for clearing and storage. Leaf length was measured before and after boiling in methanol and no tissue shrinkage was observed (data not shown).

The cleared leaves were observed under a light microscope (Kontron/Zeiss fitted with Plan 6.3x and Optovar 1.6x objectives, Eching, Germany) that was connected to a CCD camera

(Panasonic WC-CD50). Lengths of more than 100 cells in epidermal cell files, adjacent to stomatal cell files, on the abaxial side of the leaf blades were measured from video-images (total magnification 100x). The measurement software was developed by Dr. M. Terlou (Image Processing and Design, Faculty of Biology, Utrecht University, the Netherlands). There was no significant difference in mature cell size at different positions in the leaf blade (results not shown). Therefore, the mature cell length was averaged for the whole leaf blade. We estimated the cell production rate in the meristem of the leaf blade as follows (P; cells day^{-1}):

$$P = \frac{LER}{l_m} \tag{3}$$

where LER (mm day^{-1}) is the average leaf elongation rate of the whole leaf and l_m (mm) is the mature cell length of the blade (Silk *et al.*, 1989). The use of the average LER of the whole leaf is justified, since most of the elongation in the linear growth phase of the leaf is due to blade elongation (Schnyder *et al.*, 1990).

Biomass allocation and whole shoot growth

In Experiment II, a first set of 6 plants was harvested at 7 days after transfer to nutrient solution and a second set of 6 plants (i.e. the plants used for leaf growth measurements) was harvested after 2 weeks on nutrient solution. Roots, leaf sheaths and leaf blades were separated, and their fresh mass and leaf area were determined. After drying for 48 h at 70°C, dry mass of the different plant parts were determined. From these data the relative growth rate based on leaf area (RGR_{la}) and dry mass (RGR_{dm}) were calculated over the period from 7 to 15 days after transfer to nutrient solution, according to the equation of Radford, 1967. For both harvest days, the following parameters were calculated: leaf mass ratio (LMR; leaf blade biomass per unit plant mass, g g^{-1}), stem mass ratio (SMR; leaf sheath biomass per unit plant mass, g g^{-1}), root mass ratio (RMR; root biomass per unit plant mass, g g^{-1}) and specific leaf area (SLA; leaf area per unit leaf mass, m^2 kg^{-1}).

For another set of 6 plants, those that were used for the leaf growth measurements, fresh mass of the whole plant (after blotting the roots gently with tissue paper) was measured at 7 and 15 days after transfer to the nutrient solution. From these fresh mass data, we calculated the relative growth rate of individual plants (RGR_{fm}) over a period from 7 to 15 days after transfer to nutrient solution.

Statistical analysis

Data were analysed with SPSS 8.0 for Windows statistical software (SPSS, Inc., Chicago, IL, USA). Differences in individual leaf growth parameters, phyllochron and RGR_{fm} were analyzed using a two-way analysis of variance with species and treatments as fixed factors. To be able to compare each species-treatment combination separately, a one-way analysis of

variance followed by a Tukey *post hoc* test at $\alpha = 0.05$ was applied for each parameter. Differences in specific leaf area and biomass allocation parameters were analyzed using a three-way analysis of variance with species, treatment and time as fixed factors. For some parameters, data were transformed to ensure homogeneity of variances. Differences in RGR between species and treatment were tested by a three-way analysis of variance of the ln-transformed leaf area and plant dry mass data. A significant interaction between species or treatment and time indicates a difference in RGR between species and between treatments (Poorter & Lewis, 1986).

Results

Individual leaf growth

The leaves of *Ae. tauschii* elongated approximately twice as fast as those of *Ae. caudata* (Figs 1 and 2A, Table 1). In Experiment I, we evaluated changes in leaf elongation rate (LER) at two leaf positions (leaf 3 on the main stem, L3MS and leaf 1 on tiller 1, L1T1) in response to the supply of GA_3 or paclobutrazol, at two concentrations (Fig. 1 and Table 1).

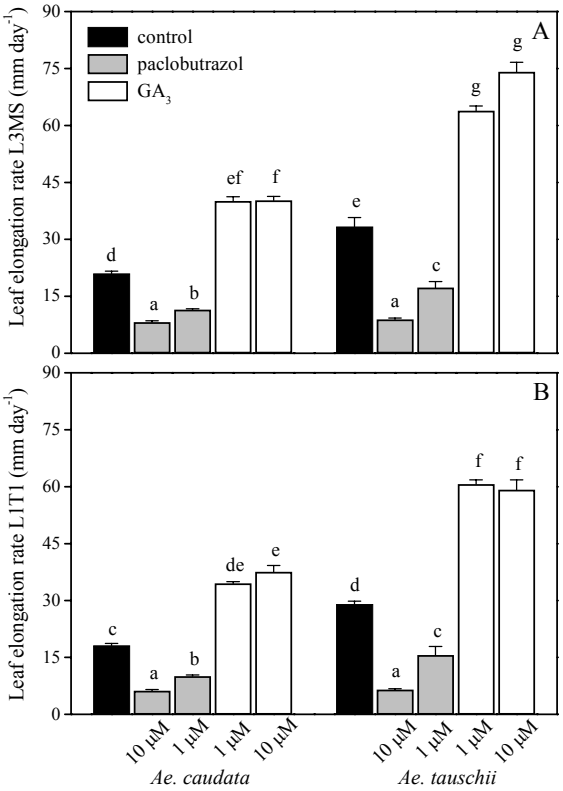

Figure 1.
The effects of 1 µM GA_3, 10 µM GA_3, 1 µM paclobutrazol and 10 µM paclobutrazol (Experiment I) on leaf elongation rate of (A) leaf 3 on the main stem (L3MS) and (B) leaf 1 on tiller 1 (L1T1) of *Aegilops caudata* and *Ae. tauschii*. Error bars denote standard error (n=5). In each graph, different letters indicate significant differences (P<0.05).

GA_3 stimulated LER, in both leaves and in both species, at a supply of 1 μM. A supply of 10 μM did not further increase LER for either of the leaves in either of the species. 1μM paclobutrazol decreased the LER in both leaves of both species and more so for *Ae. tauschii* than for *Ae. caudata*. Supply of 10 μM paclobutrazol reduced leaf elongation even further, resulting in similarly low LERs for both species. Since the inherent difference in LER between the species was reduced most after exogenous supply of 1 μM GA_3 to the slow-elongating *Ae. caudata* or supply of 1 μM paclobutrazol (and presumably a reduction of endogenous GA concentration) to the fast-elongating *Ae. tauschii*, we used only these concentrations in Experiment II. In the latter experiment, we also restricted our measurements to L3MS, since L1T1 showed similar changes in LER upon addition of GA_3 and paclobutrazol.

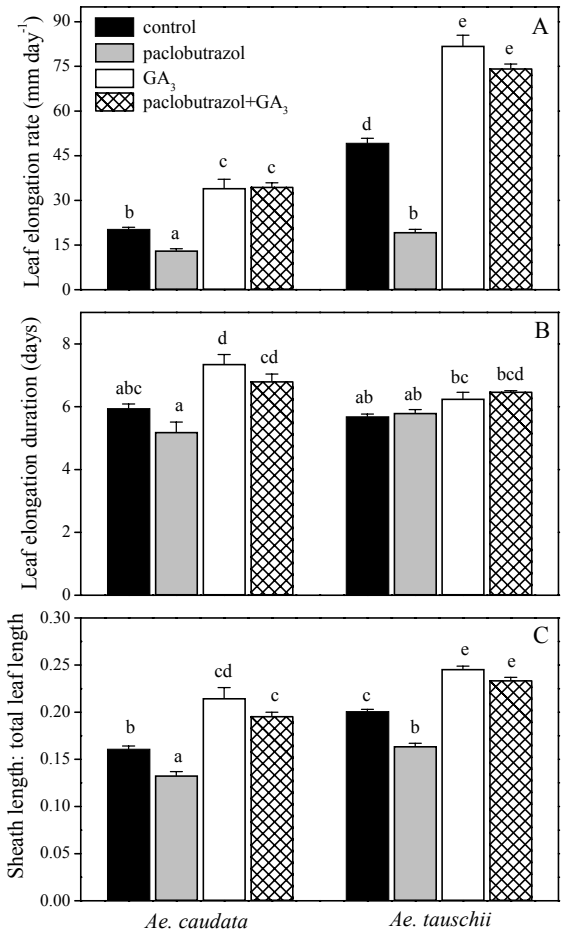

Figure 2.
The effects of 1 μM GA_3, 1 μM paclobutrazol, and 1μM GA_3 + paclobutrazol (Experiment II) on (A) leaf elongation rate, (B) duration of leaf elongation, and (C) sheath to total leaf length ratio of leaf 3 on the main stem of *Ae. caudata* and *Ae. tauschii*. Error bars denote standard error (n=6). In each graph, different letters indicate significant differences (P<0.05).

As observed in Experiment I, addition of GA_3 significantly increased LER, while addition of paclobutrazol decreased it in both species (Fig. 2A, Table 1). The relative increase in LER upon addition of GA_3 was similar for both species (66 – 68 %), whereas the relative decrease in LER upon addition of paclobutrazol was different (treatment × species interaction, Table 1). The fast-elongating *Ae. tauschii* decreased its LER to a greater extent than the slow-elongating *Ae. caudata* (60% compared to 45%, respectively). The effect of the GA_3 + paclobutrazol treatment on LER was similar to the effect of the GA_3 treatment, suggesting that addition of paclobutrazol did not have any side effects.

Under control conditions, there was no significant difference in leaf elongation duration (LED) of L3MS between the species (Fig. 2B, Table 1). Addition of GA_3 increased LED significantly in *Ae. caudata* but not in *Ae. tauschii*. Addition of paclobutrazol did not significantly change LED. In both species, the addition of GA_3 or paclobutrazol affected the growth of the leaf sheath more than they affected blade elongation (Fig. 2C, Table 1). Under control conditions, the fast-elongating *Ae. tauschii* had a 25% higher sheath to total leaf length ratio than the slow-elongating *Ae. caudata*. Upon addition of the plant growth regulators, the relative difference between the two species in sheath to total leaf length ratio remained the same, but paclobutrazol decreased the ratio whereas GA_3 increased it. For LED and sheath to total leaf length ratio, the effect of the GA_3 + paclobutrazol treatment was similar to the effect of the GA_3 treatment (Figs 2B and 2C; Table 1).

Table 1. Two-way analysis of variance of the effect of treatment (control, GA_3, paclobutrazol) and species (*Ae. caudata*, *Ae. tauschii*) on leaf elongation rate of leaf 3 of the main stem (LER_{L3MS}) and leaf 1 on tiller 1 (LER_{L1T1}) in Experiment I, and on leaf elongation rate (LER), leaf elongation duration (LED), sheath to total leaf length ratio (ShL: L_f), mature cell size (l_m), cell production rate (P), phyllochron and relative growth rate on fresh mass basis (RGR_{fm}) in Experiment II. For each independent variable the figures indicate the percentage of the total sum of squares explained by the model, which could be attributed to that effect. Level of significance: *: P<0.05, **: P<0.01, ***: P<0.001. Absence of an asterisk denotes a non-significant effect.

		Species	Treatment	Species x Treatment	Error
Experiment I	LER_{L3MS}	7***	91***	1**	1
	LER_{L1T1}	6***	92***	1***	1
Experiment II	LER	34***	60***	3***	3
	LED	2	50***	15**	33
	ShL: L_f	19***	73***	0	8
	l_m	0	91***	2	7
	P	69***	26***	2	3
	Phyllochron	1	69***	7**	23
	RGR_{fm}	42***	34***	1	23

Epidermal cell length and cell production rate

No significant difference between the species was found in the mature epidermal cell length (approx. 250 μm in both species) of L3MS (Tables 1 and 2). The estimation of cell production rate, based on the LER and mature epidermal cell length, suggests that differences in LER between the two species were due to the large difference in cell production rate (Table 2). This confirms our previous findings (see Chapter 2).

GA_3 increased both mature cell size and cell production rate by approximately 30% in both species, while addition of the GA-biosynthesis inhibitor decreased mature cell size and cell production rate more in *Ae. tauschii* (41% and 33% respectively) than in *Ae. caudata* (22% and 17% respectively) (Table 2).

Table 2. Effect of GA_3, paclobutrazol and GA_3 + paclobutrazol on mature cell length (l_m, μm) and cell production rate (P, cells day^{-1}) of leaf 3 of the main shoot of *Ae. caudata* and *Ae. tauschii*. Values are means (± SE) of 3 plants. Different letters indicate significant differences (P<0.05).

			% change relative to control	
	l_m	P	l_m	P
Ae. caudata				
Control	232^{bc} ± 15	88^{ab} ± 6		
Paclobutrazol	180^{ab} ± 6	73^{a} ± 3	-22	-17
GA_3	316^{d} ± 10	108^{bc} ± 3	36	23
Paclo + GA_3	306^{d} ± 4	113^{bc} ± 2	32	28
Ae.tauschii				
Control	253^{cd} ± 10	196^{d} ± 8		
Paclobutrazol	149^{a} ± 8	131^{c} ± 7	-41	-33
GA_3	316^{d} ± 27	263^{e} ± 24	25	34
Paclo + GA_3	315^{d} ± 2	236^{de} ± 2	25	20

Leaf appearance, growth and biomass allocation

Leaves on the main stem appeared at similar rates in *Ae. caudata* than in *Ae. tauschii* as can be derived from the similar phyllochron (time between appearance of two successive leaves on the main stem) (Tables 1 and 3). Addition of GA_3 decreased the rate of leaf appearance in *Ae. caudata* whereas addition of paclobutrazol increased this rate in both species (Tables 1 and 3).

Table 3. Effects of GA_3, paclobutrazol and GA_3 + paclobutrazol on phyllochron, relative leaf area expansion rate (RGR_{la}, day^{-1}) and relative growth rate on dry mass basis (RGR_{dm}, day^{-1}) and on fresh mass basis (RGR_{fm}, day^{-1}) of *Ae. caudata* and *Ae. tauschii*. Values are means (± SE) of 6 plants. Different letters indicate significant differences per parameter (P<0.05). See Table 4 for statistical evaluation of RGR_{la} and RGR_{dm}.

	Phyllochron	RGR_{la}	RGR_{dm}	RGR_{fm}
Ae. caudata				
Control	4.6^{bc} ± 0.2	0.165 ± 0.011	0.189 ± 0.012	0.175^{b} ± 0.005
Paclobutrazol	3.4^{a} ± 0.2	0.132 ± 0.012	0.160 ± 0.016	0.146^{a} ± 0.006
GA_3	5.6^{d} ± 0.3	0.169 ± 0.011	0.198 ± 0.014	0.178^{b} ± 0.005
Paclo + GA_3	5.5^{d} ± 0.1	0.166 ± 0.016	0.194 ± 0.019	0.187^{bc} ± 0.012
Ae. tauschii				
Control	5.3^{cd} ± 0.1	0.198 ± 0.014	0.217 ± 0.016	0.213^{cd} ± 0.002
Paclobutrazol	3.9^{ab} ± 0.1	0.147 ± 0.011	0.177 ± 0.013	0.179^{b} ± 0.003
GA_3	5.7^{d} ± 0.1	0.195 ± 0.009	0.229 ± 0.013	0.221^{d} ± 0.004
Paclo + GA_3	4.9^{cd} ± 0.3	0.206 ± 0.007	0.254 ± 0.010	0.214^{d} ± 0.003

Figure 3 shows the effects of GA_3 and paclobutrazol on specific leaf area (SLA), leaf mass ratio (LMR), stem mass ratio (SMR) and root mass ratio (RMR) at the two harvest days, as a function of ontogeny. There were significant differences between species and treatments of these variables (when tested on a time-scale) (Table 4). The effects of the GA_3 + paclobutrazol treatment did not differ from those of the GA_3 treatment and were omitted from Fig. 3 for clarity's sake. *Ae. tauschii* had a higher SLA than *Ae. caudata* (Fig. 3A). GA_3 increased SLA in both species, but this was a transient effect. In contrast, paclobutrazol did not affect SLA initially, but decreased SLA later on. The two species differed significantly in the amount of biomass allocated to the roots and leaf sheaths, whereas LMR was similar (Fig. 3B, C and D; Table 4). The fast-elongating *Ae. tauschii* allocated significantly more biomass to the leaf sheaths (higher SMR) and less to the roots (lower RMR), compared with the slow-elongating *Ae. caudata*. Addition of GA_3 increased SMR and decreased RMR. LMR was increased in *Ae. caudata*, but not in *Ae. tauschii*. Addition of paclobutrazol increased RMR and decreased both SMR and LMR.

The relative growth rate (RGR_{dm}) and the relative leaf area expansion (RGR_{la}) differed significantly between species (three-way ANOVA, species*time interaction: $p<0.01$) and between treatments (three-way ANOVA, treatment*time interaction: $p<0.01$) (Table 4). Addition of paclobutrazol to *Ae. tauschii* decreased RGR_{dm} and RGR_{la} to rates that were more similar to those of *Ae. caudata* (Table 3). However, addition of GA_3 did not increase RGR_{dm} or RGR_{la} in either of the species (Table 3). The differences in RGR_{dm} were confirmed by the differences in RGR_{fm} between treatments and species (Tables 1 and 3).

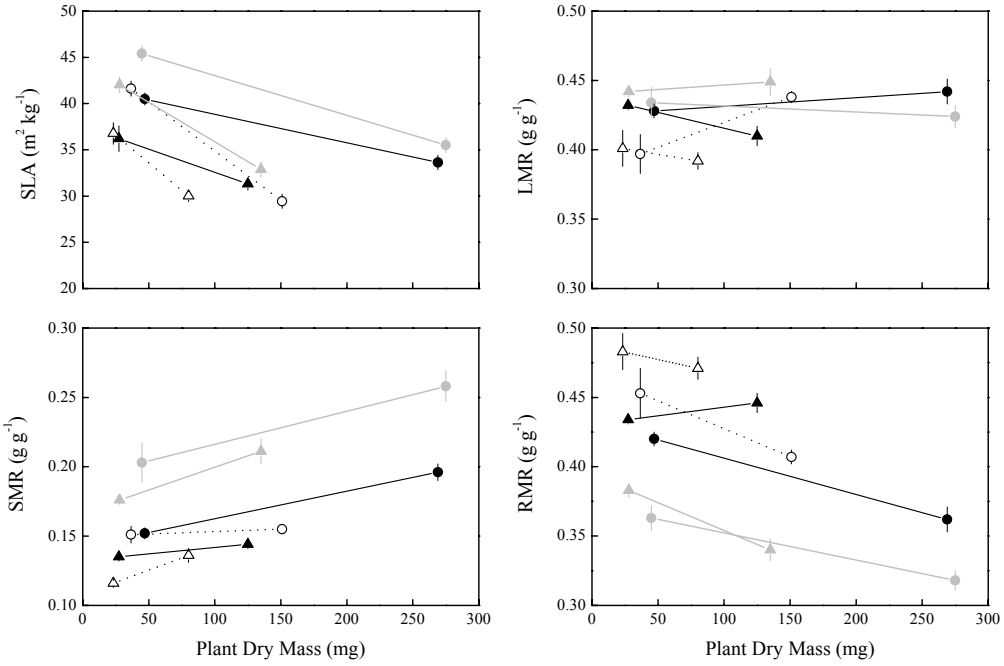

Figure 3. The effects of 1 µM GA_3 (grey symbols), and 1 µM paclobutrazol (open symbols), compared to the control treatment (filled black symbols), on (A) specific leaf area (SLA), (B) leaf mass ratio (LMR), (C) stem mass ratio (SMR) and (D) root mass ratio (RMR) of *Ae. caudata* (triangles) and *Ae. tauschii* (circles), as a function of plant dry mass. Error bars denote standard error (n=6).

Table 4. Three-way analysis of variance of the effect of species (Sp), treatment (Tr) and time on the ln-transformed values of leaf area (lnLA) and total plant dry mass (lnDM), specific leaf area (SLA), leaf mass ratio (LMR), stem mass ratio (SMR) and root mass ratio (RMR). For each independent variable the figures indicate the percentage of the total sum of squares explained by the model, which could be attributed to that effect. Level of significance: *: P<0.05; **: P<0.01; ***: P<0.001. Absence of an asterisk denotes a non-significant effect.

	Sp	Tr	Time	Sp × Tr	Sp ×Time	Tr × Time	Sp × Tr × Time	Error
lnLA	16***	8***	71***	0	1**	1***	0	3
lnDM	9***	3***	82***	0	1**	1**	0	4
SLA	12***	13***	55***	3***	3***	2**	1*	11
LMR	1	27***	1	6*	2	9**	7*	47
SMR	17***	53***	13***	1	1	2	2	11
RMR	13***	64***	6***	1	3***	1	0	12

Discussion

Ae. caudata and *Ae. tauschii* differed in their leaf growth characteristics and biomass allocation pattern when grown under identical conditions. *Ae. tauschii* had fast-elongating and long leaves with a relatively high sheath to total leaf length ratio, while *Ae. caudata* had slow-elongating and shorter leaves with a lower sheath to total leaf length ratio. The differences in leaf elongation rate between the species were caused by differences in cell production rate, whereas mature cell size was the same for both species. *Ae. tauschii* allocated more biomass to the stem compared with *Ae. caudata*, at the cost of biomass allocation to the roots. At the whole shoot level, *Ae. tauschii* had significantly higher relative growth rate of leaf area (RGR_{la}) and plant dry mass (RGR_{dm}) compared with *Ae. caudata*.

Our results show that all of these inherent differences in biomass allocation and leaf growth between *Ae. tauschii* and *Ae. caudata,* can be greatly reduced by exogenous supply of either paclobutrazol to the fast-elongating *Ae. tauschii* or GA_3 to the slow-elongating *Ae. caudata*. We also showed that the effects of addition of the GA biosynthesis inhibitor paclobutrazol, can be entirely reversed by subsequent addition of GA_3. Even though we have not measured endogenous GA levels in these species, this last observation suggests that paclobutrazol had a specific effect on gibberellin biosynthesis and that exogenous supply of GA_3 indeed results in an increase in endogenous GA levels. Therefore, we think it is acceptable to assume that exogenous supply of either GA_3 or paclobutrazol manipulates endogenous GA levels, suggesting that GA plays an important role in determining differences in leaf area expansion and biomass allocation between the two species in this study.

A role for GA in the regulation of LER has been reported by numerous authors in several grass species (Paolillo *et al.*, 1991; Sauter & Kende, 1992; Tonkinson *et al.*, 1995; Chandler & Robertson, 1999), and our results on *Aegilops* species confirm these data. The difference in LER between the slow-elongating *Ae. caudata* and the fast-elongating *Ae. tauschii* can be greatly reduced by exogenous supply of GA_3 to *Ae. caudata* or paclobutrazol to *Ae. tauschii*. This strongly suggests that differences in GA metabolism (i.e. differences in endogenous levels or sensitivity) may be involved in explaining the large difference in LER between these species. The difference in leaf growth response to increasing concentrations of GA_3 between *Ae. caudata* and *Ae. tauschii* (Fig. 1) resembled the difference in dose-response curves between barley mutants with reduced GA sensitivity and wild type barley (Chandler & Robertson, 1999). Despite this resemblance, it is impossible to conclude from the present results whether differences in endogenous GA concentrations or in GA sensitivity are responsible for the observed differences in leaf growth between *Ae. caudata* and *Ae. tauschii*. It is likely that both processes contribute (Weyers *et al.*, 1995) and the relative importance of each of them can only be determined by measuring endogenous GA levels and GA dose-response curves. Unlike LER, the duration of leaf elongation was not greatly affected by GA.

This is in accordance with the results from Calderini *et al.* (1996) and Tonkinson *et al.* (1997) who found a significant difference in LER between semidwarf (i.e. GA-insensitive genotype) and wild-type wheat plants, while duration was similar for the leaves of both genotypes.

Although adjusting GA concentrations could reduce the differences in LER between *Ae. caudata* and *Ae. tauschii*, it could not fully make the differences underlying LER disappear. The two species differ in cell production rate, whereas they have the same mature cell size. Exogenous supply of GA_3/paclobutrazol stimulated/reduced both mature cell size and cell production rate to almost the same extent (Table 2). This is consistent with the results of Sauter & Kende (1992) which show that application of GA_3 to internodes of deepwater rice first promotes cell elongation in the intercalary meristem, which subsequently stimulates cell division. This result does not necessarily mean that differences in cell production rate and LER between the *Aegilops* species are determined by another factor than GA. Exogenous supply of GA_3 probably changed the physiological GA levels and distributions among the tissues or cell types (like dividing and elongating cells) drastically, and may have exceeded the subtle differences in GA concentrations between the cell division and cell elongation zone. In wheat leaves, Tonkinson *et al.* (1997) have shown higher concentrations of GA_1 and GA_3 (thought to be the principal active growth promoting GAs) in the most basal third of the growth zone, which includes the cell division zone. Apart from having different concentrations of GA, the various parts of the leaf growth zone can also differ in their sensitivity to GA (Bradford & Trewavas, 1994). Whether differences in GA levels or sensitivity between the cell division and cell expansion zones contribute to the differences leaf growth between *Ae. caudata* and *Ae.tauschii*, needs more detailed studies.

In both species, leaf sheath elongation is more responsive to GA_3 than leaf blade elongation (Fig. 1C). A greater effect of GA_3 supply on sheath length relative to blade length was also shown by Smith *et al.* (1996) in the third leaf of dwarf barley, whereas Ogawa *et al.* (1999) showed that leaf sheath meristematic tissue of maize had high transcript levels of GA-responsive genes. Since the difference in sheath to total leaf length ratio between *Ae. caudata* and *Ae. tauschii* could be overcome by manipulating the GA levels, GA may be responsible for this difference. The length of the leaf sheath partly determines the phyllochron (= time between appearance of successive leaves), along with the LER within the whorl of sheaths and the time when elongation is initiated (Skinner & Nelson, 1995). The longer leaf sheath of *Ae. tauschii* was not entirely compensated by its higher LER, and hence *Ae. tauschii* tended to have a higher phyllochron than *Ae. caudata*. Since GA could account for differences in sheath length and LER between *Ae. caudata* and *Ae. tauschii*, it was expected that GA could also account for differences in phyllochron of these species. Indeed, addition of GA_3 increased and addition of paclobutrazol decreased the phyllochron in these species.

The relatively greater effect of GA on sheath growth compared to leaf growth might explain the difference in stem mass ratio (SMR) between *Ae. caudata* and *Ae. tauschii*.

Addition of GA_3 to the slow-elongating species increased its SMR to the level of the fast-elongating species at the cost of allocation to the roots. Similarly, Nagel *et al.* (2001a) observed that GA-deficient mutants of tomato allocated more biomass to their roots and less to stems, compared with the wild-type. These slow-growing GA-deficient tomato mutants had a lower SLA than the fast-growing wild-type, just like the slow-elongating *Ae. caudata* had a lower SLA which could be increased by addition of GA_3 that of the fast-elongating *Ae. tauschii*. However, this effect was transient, similar to the transient increase in SLA of a slow-growing inbred line of *Plantago major* supplied with GA_3 (Dijkstra *et al.*, 1990). SLA is determined by the rate of leaf expansion and the carbon budget of the leaves (net photosynthesis and net fluxes of carbon). These two processes are independent and changes in either of them will affect SLA (Tardieu *et al.*, 1999). We have shown that the growing leaves of the *Aegilops* species respond to GA_3 with an increased expansion rate. It seems that the increased leaf area expansion rate is not accompanied by increases in net photosynthesis and/or carbon influx in these leaves, resulting in an increase in SLA. Why does the increase in SLA decrease over time almost to the level of the control plants? One explanation is that the carbon budget of the leaves changes in a delayed manner. Alternatively, the proportion of young leaves with a higher SLA may decrease with plant age, and the effect on the SLA of the whole plant becomes less obvious. The faster leaf expansion in GA_3-treated plants, increases the sink strength in the shoot of these plants, resulting in more carbon being allocated to the shoots (higher LMR and SMR) and less to the roots (lower RMR). The opposite reasoning applies to paclobutrazol-treated plants. Paclobutrazol reduces the rate of leaf expansion, thereby reducing the sink strength of the leaves and increasing the carbon flux to the roots. Initially, the SLA is not affected, probably because both expansion and carbon budget of the growing leaves are altered. However, these leaves have smaller cells, and as they mature more cell wall material can be deposited and SLA of these leaves will be lower. As the plant develops, the number of mature leaves with reduced cell size increases and SLA will decrease faster in paclobutrazol-treated plants than in control plants.

Together with an increase in RMR and a decrease in SMR and SLA, a decrease in endogenous GA concentration also reduced RGR of both leaf area and dry mass. However, increasing GA concentration decreased RMR and increased SLA and SMR, without any significant effect on RGR. This is in accordance with the results of M. Berrevoets and O.W. Nagel (pers. comm.) who added 1 μM $GA_{4/7}$ to wild-type tomato plants and also found an increase in SMR without an accompanying increase in RGR, while addition of the same concentration of $GA_{4/7}$ to GA-deficient tomato plants did result in an increase in both SMR and RGR. These observations show that GA is one of many parameters determining RGR: lack of GA will reduce RGR, while extra supply of GA, without changing all the other parameters involved in RGR, is not sufficient to increase RGR. Moreover, the data from GA-treated *Aegilops* plants clearly show that a large increase in LER of individual leaves does not necessarily result in an increasing RGR.

By manipulating the endogenous gibberellin concentrations in the slow-elongating *Ae. caudata* and the fast-elongating *Ae. tauschii*, it was possible to strongly decrease most differences in biomass allocation and leaf growth. Gibberellins appear to play a role in the partitioning of biomass to roots and shoots, especially stems, and in determining leaf growth rates, especially in the leaf sheath. The large difference in LER between *Ae. caudata* and *Ae. tauschii*, and the possibility to reduce these differences by manipulating endogenous GA levels makes these species ideal for future experiments on the role of GA in LER.

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Chapter 5

Chapter 6

Effects of exogenous supply of gibberellic acid and paclobutrazol on the carbon and nitrogen economy of two *Aegilops* species with contrasting leaf elongation rates

Abstract

Results of a previous study showed that differences in leaf growth characteristics and biomass allocation pattern between *Aegilops tauschii* and *Ae. caudata* can be reduced by manipulating the GA levels in these species, whereas differences in the relative growth rate (RGR) between these species cannot. The aim of the present study was therefore to further investigate the effects of exogenous supply of GA_3 and paclobutrazol, an inhibitor of GA biosynthesis. We analysed physiological parameters underlying RGR by studying the carbon and nitrogen economies of *Ae. tauschii* and *Ae. caudata*.

The fast-growing *Ae. tauschii* assimilated more carbon per unit plant mass, respired a smaller proportion of its assimilated carbon, and invested proportionally more biomass in leaf area and leaf sheaths than the slow-growing *Ae. caudata*. *Ae. tauschii* had a lower leaf nitrogen concentration per unit leaf area but used its leaf nitrogen more efficiently for photosynthesis than *Ae. caudata*. The paclobutrazol-induced reduction in RGR of the fast-growing *Ae. tauschii* was associated with reductions in leaf area ratio (LAR) and rate of photosynthesis per unit leaf nitrogen. In contrast, GA_3 supply to the slow-growing *Ae. caudata* increased LAR but not RGR, due to the simultaneous reduction in the rate of photosynthesis per unit leaf area, which was associated with a reduction in leaf nitrogen concentration per unit leaf area. Differences in the proportion of carbon used for root respiration between *Ae. caudata* and *Ae. tauschii* were reduced by manipulating the endogenous GA levels in these species. These results confirm previous findings that GA plays a key role in determining differences between *Ae. caudata* and *Ae. tauschii* in the investment of biomass in leaf area, leaf sheaths and roots. GA indirectly affects the carbon and nitrogen economies in these plants, and hence their RGR.

Introduction

Species from the genus *Aegilops* (wild relatives of wheat) show a wide variation in leaf elongation rate (LER) and in relative growth rate (RGR) (Villar *et al.*, 1998; Bultynck *et al.*, 1999). Numerous authors have suggested that endogenous gibberellins (GAs) are involved in the control of LER in cereal crop species (Pinthus *et al.*, 1989; Keyes *et al.*, 1989; Paolillo *et al.*, 1991; Tonkinson *et al.*, 1995; Chandler & Robertson, 1999). Similarly, in several studies a positive correlation has been found between endogenous GA levels and RGR (Dijkstra *et al.*, 1990; Rood *et al.*, 1990; Nagel *et al.*, 2001a). In Chapter 5, it was shown that differences in leaf growth and biomass allocation between the fast-elongating *Ae. tauschii* and the slow-elongating *Ae. caudata* can be reduced either by the exogenous supply of GA_3 to *Ae. caudata*, or by paclobutrazol, an inhibitor of GA biosynthesis (Lenton *et al.*, 1994), to *Ae. tauschii*. Reduction of the endogenous GA concentration in *Ae. tauschii* resulted in a decrease in RGR to the level of that of *Ae. caudata*. However, addition of GA_3 to *Ae. caudata* was not sufficient to increase its RGR to the level of that in *Ae. tauschii*. In order to get more insight into the causes for this apparent discrepancy, we studied the carbon economy of these species.

The RGR is the product of leaf area ratio (leaf area per unit plant weight; LAR) and net assimilation rate (rate of biomass increase per unit leaf area; NAR). The NAR depends on carbon gain in photosynthesis, carbon use in respiration and the carbon allocation to the different plant organs (Lambers & Poorter, 1992). Analysing a plant's carbon economy can provide insight into the causes of variation in RGR between plants. For several species comparisons, it was found that RGR is positively correlated with the rate of daily carbon gain per unit plant dry mass, which was positively correlated with specific leaf area (SLA) and negatively correlated with the proportion of carbon used in respiration (Poorter & Remkes, 1990; Van der Werf *et al.*, 1992; Atkin *et al.*, 1996). Similar results were found in a comparison of slow-growing GA-deficient tomato mutants and their fast-growing wild-types (Lambers *et al.*, 1998b).

The first aim of the present study was to compare the effects of GA_3 and paclobutrazol supply on the carbon economy of the fast-expanding *Ae. tauschii* and the slow-expanding *Ae. caudata* in order to get a better understanding of (1) the causes of differences in RGR between these species, and (2) the reasons for a lack of effect of GA_3 supply on RGR of these species. For this purpose we measured the rates of photosynthesis of whole shoots, respiration of shoots and roots, and the carbon concentrations of the different organs.

A plant's carbon economy is closely linked with its nitrogen economy because rates of photosynthesis and leaf respiration correlate with leaf nitrogen concentrations (Field & Mooney, 1986; Evans, 1989). Therefore, the second aim of this study was to compare the nitrogen economy of *Ae. caudata* and *Ae. tauschii* as well as the effect of gibberellin and paclobutrazol on the nitrogen economy.

Materials and methods

Plant material and growing conditions

Seeds of *Aegilops tauschii* L. and *Ae. caudata* L. were obtained from ICARDA (International Centre for Agricultural Research in the Dry Areas, Aleppo, Syria). Seeds were germinated and plants were grown as described in Chapter 5. Plants were randomly assigned to one of four nutrient solutions: (1) control nutrient solution, (2) nutrient solution with 1 μM GA_3, (3) nutrient solution with 1 μM paclobutrazol, (4) nutrient solution with 1 μM GA_3 + 1 μM paclobutrazol. The latter treatment was included to check whether any effects of paclobutrazol, other than inhibition of gibberellin biosynthesis, could be excluded.

Gas-exchange measurements

For each treatment, six plants were harvested on day 7 and on day 15 (for further details, see Chapter 5). Four of the six plants, reserved for the final harvest, were used for the gas-exchange measurements. These plants were placed inside transparent, airtight cuvettes with the shoots and roots in separate compartments (Poorter & Welschen, 1993). The root compartments were filled with continuously aerated nutrient solutions similar to that of the treatments. The irradiance, light period, temperature and vapour pressure deficit in the cuvettes were similar to those in the growth room. Net photosynthesis and respiration of intact plants were measured as CO_2 exchange. CO_2 and H_2O exchange were measured differentially with infrared gas analysers (ADC, model 225 MK3, Hoddesdon, UK) in an open system.

CO_2 exchange measurements started at the end of day 13, when the lights were switched off. During the first 10 h dark period, shoot dark respiration was measured continuously on half the plants and root respiration on the other half. In the following 14 h light period (day 14), photosynthesis and root respiration were measured on all the plants. During the second 10 h dark period, shoot dark respiration was measured continuously on half the plants that were used for root respiration measurements during the first 10 h dark period, while root respiration was measured continuously on the other half. Net photosynthetic rate per unit leaf area (A_a) and per unit leaf mass (A_m), shoot dark respiration (SR) and root respiration during the light (RR_{light}) and the dark (RR_{dark}) period were calculated according to Von Caemmerer & Farquhar (1981). To calculate the rates of photosynthesis or respiration per unit of organ mass or area, we calculated the mass or area at the time of the measurement from the RGR of the respective organ (derived from growth analysis data of Chapter 5), the mass or area at the time of the harvest, and the difference in time between measurement and harvest.

Biomass allocation, relative growth rate and net assimilation rate

At the end of the gas-exchange measurements, plants were separated into leaf blades, leaf sheaths and roots. Leaf area, fresh and dry mass were determined as described in Chapter 5. From these data, the following parameters were calculated for the four plants used in the gas-exchange measurements: leaf mass ratio (LMR; leaf blade biomass per unit plant mass, g g^{-1}), stem mass ratio (SMR; leaf sheath biomass per unit plant mass, g g^{-1}), root mass ratio (RMR; root biomass per unit plant mass, g g^{-1}), leaf area ratio (LAR; leaf area per unit plant mass, m^2 kg^{-1}) and specific leaf area (SLA; leaf area per unit leaf mass, m^2 kg^{-1}). The relative growth rate (RGR; mg g^{-1} day^{-1}) and the net assimilation rate (NAR; increase in total plant mass per unit leaf area per day, g m^{-2} day^{-1}) at the time of the gas exchange measurements were calculated from the physiological data, the biomass allocation data and the plant carbon concentration, according to the equation used by Poorter & Pothmann (1992):

$$RGR = \frac{[A_a \times SLA \times LMR - SR \times (LMR + SMR) - RR \times RMR]}{C} \qquad (1)$$

where A_a is the rate of photosynthesis per unit leaf area, SLA is specific leaf area, LMR is leaf mass ratio, SR is the rate of shoot respiration per unit shoot mass, SMR is stem mass ratio, RR is the rate of root respiration per unit root mass, RMR is root mass ratio, and C is the plant carbon concentration.

Chemical analyses

Total carbon and nitrogen concentrations of all dried plant material were determined with an element analyser (Carlo Erba, model 1106, Milan, Italy).

Carbon budget

Gross carbon assimilation per day was calculated as the sum of the average net photosynthesis and the average shoot respiration during 14 h, assuming that shoot respiration rate during the day was similar to that measured during the night. Gross carbon assimilation per day minus root and shoot respiration per 24 h gave the proportion of carbon that was used for growth. The proportions of carbon used for growth of leaf blades, leaf sheaths and roots were calculated using biomass partitioning data and the carbon concentration of each organ.

Statistics

Data were analysed with SPSS 8.0 for Windows statistical software (SPSS, Inc., Chicago, IL, USA). Differences between cultivars and treatments were analysed using two-way analysis

of variance. When a significant treatment effect was found, differences between treatments were tested using a Tukey *post hoc* test at $\alpha = 0.05$.

Results

Rates of photosynthesis and respiration

Rates of photosynthesis and respiration were measured over a total period of 14 h light and 20 h dark. During the light period, the rates of photosynthesis were constant, whereas the rates of root respiration increased significantly by approx. 10% in *Ae. caudata* and approx. 20% in *Ae. tauschii* for all treatments except the control (repeated measures ANOVA, α=0.05; data not shown). During the dark period, root respiration decreased by approx. 20% in *Ae. caudata* and 10% in *Ae. tauschii*, again for all treatments except the control treatment. Shoot respiration decreased in all treatments by approx. 25% in *Ae. caudata* and by 10% in *Ae. tauschii* (repeated measures ANOVA, α=0.05; data not shown).

Figure 1. Effects of GA_3, paclobutrazol and paclobutrazol + GA_3 on the rate of (A) net photosynthesis per unit leaf area, (B) root respiration during the light period, (C) root respiration during the dark period and (D) shoot respiration of *Ae. caudata* and *Ae. tauschii* after 15 days of growth. Vertical bars indicate standard error (n=4).

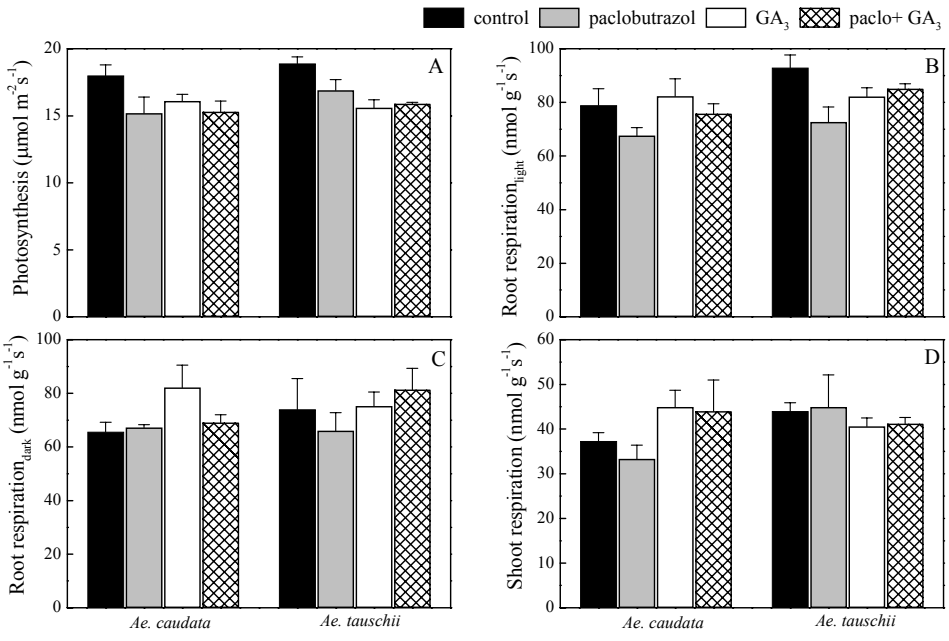

Table 1. Two-way analysis of variance of the effect of species (Sp) and treatment (Tr) on the mean rates of photosynthesis per unit leaf area (A_a) and per unit leaf mass (A_m), shoot respiration (SR), and root respiration during the light (RR_{light}) and the dark (RR_{dark}) period. For each independent variable the figures indicate the percentage of the total sum of squares explained by the model, which could be attributed to that effect. Level of significance: *: p<0.05; **: p<0.01; ***: p<0.001. Absence of an asterisk denotes no significant effect.

	Species	Treatments	Sp × Tr	Error
A_a	4	47***	6	44
A_m	37***	40***	4	19
SR	4	4	19	73
RR_{light}	11*	30*	6	53
RR_{dark}	2	13	9	77

Figure 1 shows the mean rates of whole shoot net photosynthesis and root respiration measured during the light period, and whole shoot and root respiration measured during the dark period. *Ae. tauschii* tended to have a slightly higher mean rate of photosynthesis per unit leaf area (A_a) than *Ae. caudata* (Fig. 1A; Table 1). However, on a leaf mass basis, the difference in photosynthetic rate (A_m) between the species increased to 19%, due to the significantly higher SLA in *Ae. tauschii* compared with *Ae. caudata* (Tables 1 and 2). All treatments significantly decreased the mean rates of photosynthesis per unit leaf area and per unit leaf mass (Fig. 1A; Table 1). *Ae. tauschii* had a 9% higher root respiration rate during the light period (RR_{light}) than *Ae. caudata*, whereas the root respiration rate during the dark

Table 2. Effects of GA_3, paclobutrazol and paclobutrazol + GA_3 on root mass ratio (RMR; g g^{-1}), stem mass ratio (SMR; g g^{-1}), leaf mass ratio (LMR; g g^{-1}), specific leaf area (SLA, m^2 kg^{-1}), leaf area ratio (LAR; m^2 kg^{-1}), net assimilation rate (NAR; g m^{-2} day^{-1}) and relative growth rate

	RMR	SMR	LMR
Ae. caudata			
Control	0.450 ± 0.006	0.143 ± 0.004	0.407 ± 0.007
Paclobutrazol	0.469 ± 0.004	0.143 ± 0.002	0.387 ± 0.006
GA_3	0.344 ± 0.010	0.221 ± 0.009	0.435 ± 0.005
Paclo + GA_3	0.371 ± 0.006	0.202 ± 0.001	0.430 ± 0.007
Ae. tauschii			
Control	0.368 ± 0.008	0.202 ± 0.006	0.430 ± 0.005
Paclobutrazol	0.409 ± 0.006	0.157 ± 0.004	0.434 ± 0.004
GA_3	0.323 ± 0.007	0.252 ± 0.013	0.425 ± 0.009
Paclo + GA_3	0.304 ± 0.013	0.267 ± 0.008	0.430 ± 0.008

period (RR_{dark}) did not differ between the species (Figs 1B and C; Table 1). Most treatments did not significantly affect root respiration rate, except paclobutrazol that reduced RR_{light} (Fig. 1B; Table 1). No differences were found in mean rate of shoot respiration (SR) between species or treatments (Fig. 1D, Table 1). The effects of paclobutrazol + GA_3 on photosynthesis and respiration were not significantly different from those of GA_3.

Biomass allocation and growth

Tables 2 and 3 show the effects of GA_3 and paclobutrazol on biomass allocation, NAR and RGR. In the control treatment, *Ae. tauschii* had a 6% higher LMR and a 41% higher SMR than *Ae. caudata*, at the expense of allocation to the root. Of the biomass allocated to the leaf blades *Ae. tauschii* invested more in leaf area (13% higher SLA) than *Ae. caudata* Together with a 20% higher LAR (product of SLA and LMR), *Ae. tauschii* also had an 11% higher NAR than *Ae. caudata*, resulting in a much faster growth rate (33% higher RGR). Addition of GA_3 to the slow-growing *Ae. caudata* and addition of paclobutrazol to the fast-growing *Ae. tauschii* significantly reduced the differences in the biomass allocation pattern, SLA and LAR. NAR on the other hand, was reduced in both species after addition of either GA_3 or paclobutrazol, mainly due to the reduction in the net rate of photosynthesis in both treatments (Fig. 1). As a result of the effects on LAR and NAR, paclobutrazol clearly reduced RGR whereas GA_3 slightly reduced RGR only in *Ae. tauschii*. The effects of paclobutrazol + GA_3 on biomass allocation and growth were not significantly different from those of GA_3.

(Table 2. continued)
(RGR; mg g^{-1} day^{-1}) of *Ae. caudata* and *Ae. tauschii* after 15 days of growth. Values are means (± SE) of 4 plants.

SLA	LAR	NAR	RGR
30.7 ± 0.7	12.49 ± 0.60	16.05 ± 1.15	199 ± 7
30.2 ± 0.8	11.69 ± 0.65	11.94 ± 1.09	138 ± 5
32.0 ± 1.0	14.17 ± 0.45	13.47 ± 0.16	191 ± 5
32.9 ± 0.9	13.89 ± 0.68	12.67 ± 1.03	175 ± 12
34.6 ± 0.6	14.87 ± 0.34	17.83 ± 0.77	265 ± 10
30.5 ± 0.5	13.25 ± 0.24	14.52 ± 0.71	192 ± 8
36.3 ± 0.7	15.41 ± 0.45	14.53 ± 0.72	223 ± 5
36.6 ± 0.8	15.69 ± 0.20	15.03 ± 0.23	236 ± 5

Table 3. Two-way analysis of variance of the effect of species (Sp) and treatment (Tr) on root mass ratio (RMR), stem mass ratio (SMR), leaf mass ratio (LMR), specific leaf area (SLA), leaf area ratio (LAR), net assimilation rate (NAR) and relative growth rate (RGR). For each independent variable the figures indicate the percentage of the total sum of squares explained by the model, which could be attributed to that effect. Level of significance: *: p<0.05; **: p<0.01; ***: p<0.001. Absence of an asterisk denotes no significant effect.

	Species	Treatment	Sp × Tr	Error
RMR	25***	63***	4*	8
SMR	20***	66***	5*	9
LMR	16***	14	28**	42
SLA	28***	37***	7	28
LAR	34***	34***	2	23
NAR	20***	45***	2	33
RGR	49***	39***	3	9

Carbon economy

The effects of GA_3, paclobutrazol and paclobutrazol + GA_3 on the carbon economy of *Ae. caudata* and *Ae. tauschii* are presented in Fig. 2 and Table 5. The rate of daily carbon gain per total plant mass was 28% higher in *Ae. tauschii* than in *Ae. caudata*, due to the 19% higher rate of photosynthesis per unit leaf mass, the 6% higher LMR and the 41% higher SMR. Paclobutrazol reduced the rate of daily carbon gain in both species mainly due to a reduction in photosynthesic rate. GA_3 reduced the rate of daily carbon gain in *Ae. tauschii* but not in *Ae. caudata* because in *Ae. caudata* the reduction in the rate of photosynthesis was less, and the increase in biomass allocated to the shoot was greater.

In the control treatment, *Ae. caudata* used 4% more of its carbon for respiration than *Ae. tauschii*, leaving a smaller proportion of carbon for growth in this species. *Ae. caudata* used a larger fraction of its carbon (22%) for root respiration than *Ae. tauschii* (17%) due to its higher RMR, whereas they both used approx. 15% for shoot respiration. Addition of paclobutrazol to the fast-growing *Ae. tauschii* slightly increased the fractions of carbon used in root respiration and shoot respiration. Addition of GA_3 to the slow-growing *Ae. caudata* tended to decrease the fraction of carbon used in root respiration but increased that used in shoot respiration. This was largely due to increases in SMR and, to a lesser extent, increases in LMR. Therefore, the total fraction of carbon used in respiration did not change upon GA_3 addition to the slow-growing species.

Since the differences in carbon concentrations between species and treatments were small (less than 3%) (Table 4), differences in the proportion of carbon used for growth of the different organs were largely determined by the biomass allocation pattern (Fig. 1; Table 1).

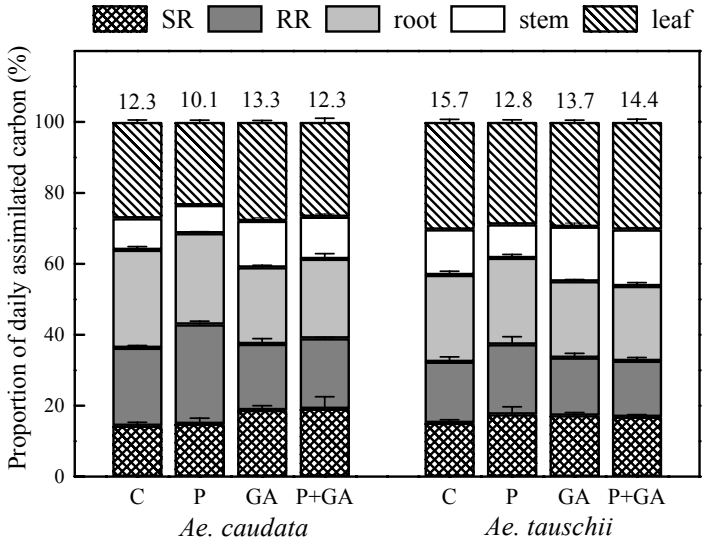

Figure 2. Effects of GA_3, paclobutrazol and paclobutrazol + GA_3 on the proportion of daily assimilated carbon used in shoot respiration (SR), root respiration (RR), leaf growth (leaf), stem growth (stem) and root growth (root) in *Ae. caudata* and *Ae. tauschii* after 15 days of growth. Vertical bars indicate standard error (n=4). The values above the stacked bars give the daily gross photosynthesis per unit plant mass (nmol g^{-1} day^{-1}). C: control, P: paclobutrazol, GA: GA_3.

In the control treatment, *Ae. tauschii* used 4% more of its carbon for stem growth than *Ae. caudata*. The higher RMR of *Ae. caudata* was not reflected in the absolute fraction of carbon used for root growth due to the larger fraction of carbon used in respiration in this species. Addition of paclobutrazol to *Ae. tauschii* reduced the fraction of carbon used for stem growth but did not increase the proportion of carbon used for root growth because a higher proportion of carbon was used in respiration. Addition of GA_3 to *Ae. caudata* increased the fraction of carbon used in shoot growth at the expense of that used for root growth. The effects of paclobutrazol + GA_3 on the carbon budget were not significantly different from the effects of GA_3.

Nitrogen economy

The nitrogen concentrations in the different organs and species are presented in Table 4, and Table 5 shows the results of the two-way analysis of variance on these data. For both species the nitrogen concentration was highest in the leaf blades. *Ae. caudata* and *Ae. tauschii* did not differ significantly in nitrogen content per unit mass in any of the organs. However, when expressed on an area basis, leaf nitrogen content was 16% higher in *Ae. caudata* than in *Ae. tauschii* due to the lower SLA in *Ae. caudata* (Fig. 3A; Table 5). Addition of GA_3 to *Ae. caudata* reduced the nitrogen content per unit stem mass, per unit leaf area and per unit leaf

Table 4. Effects of GA_3, paclobutrazol and paclobutrazol + GA_3 on plant carbon concentration (plant C; mmol C g^{-1}), leaf nitrogen concentration (leaf N; mmol N g^{-1}), stem nitrogen concentration (stem N; mmol N g^{-1}) and root nitrogen concentration (root N; mmol N g^{-1}) of Ae. caudata and Ae. tauschii after 15 days of growth. Values are means (±SE) of 4 plants.

	Plant C	Leaf N	Stem N	Root N
Ae. caudata				
Control	33.47 ± 0.03	4.96 ± 0.06	4.07 ± 0.05	3.50 ± 0.06
Paclobutrazol	33.51 ± 0.05	5.10 ± 0.04	4.05 ± 0.03	3.38 ± 0.04
GA_3	34.15 ± 0.08	4.34 ± 0.05	3.42 ± 0.14	3.31 ± 0.04
Paclo + GA_3	33.93 ± 0.08	4.24 ± 0.01	3.66 ± 0.01	3.37 ± 0.06
Ae. tauschii				
Control	34.27 ± 0.08	4.82 ± 0.09	3.86 ± 0.06	3.39 ± 0.02
Paclobutrazol	33.73 ± 0.12	5.07 ± 0.07	4.06 ± 0.07	3.40 ± 0.09
GA_3	33.49 ± 0.08	4.46 ± 0.07	3.79 ± 0.08	3.38 ± 0.06
Paclo + GA_3	33.57 ± 0.06	4.48 ± 0.11	3.82 ± 0.02	3.43 ± 0.03

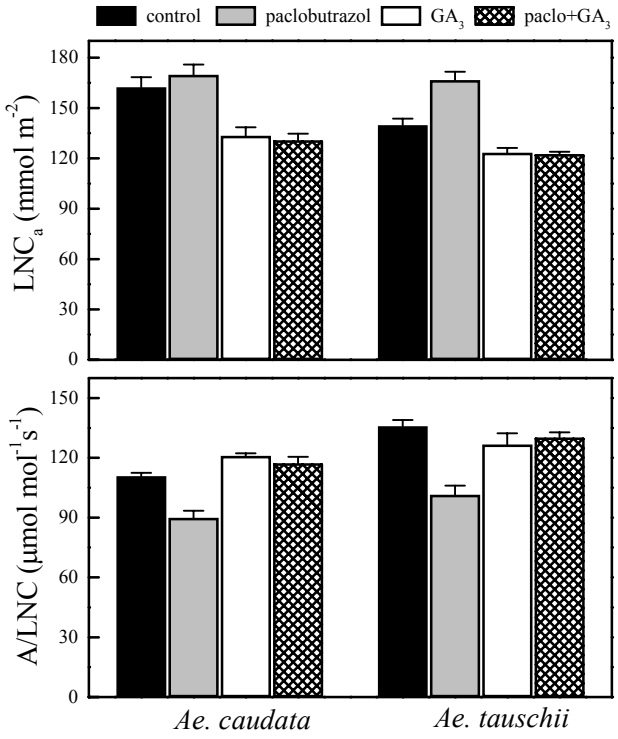

Figure 3.
The effects of GA_3, paclobutrazol and paclobutrazol + GA_3 on (A) the nitrogen concentration per unit leaf area (leaf N_a) and (B) the rate of photosynthesis per unit leaf nitrogen (A/N) in *Ae. caudata* and *Ae. tauschii* after 15 days of growth. Vertical bars indicate standard error (n=4).

mass, whereas the nitrogen contents were increased upon addition of paclobutrazol to *Ae. tauschii* (Fig. 3A; Tables 4 and 5). Root nitrogen concentrations were not affected by the treatments.

The rate of photosynthesis per unit leaf nitrogen (A/N, μmol CO_2 mol^{-1} N s^{-1}) was 23% higher in *Ae. tauschii* compared with *Ae. caudata* (Fig. 3B; Table 5). Addition of paclobutrazol to *Ae. tauschii* reduced A/N by 25% whereas addition of GA_3 to *Ae. caudata* increased A/N by only 10% (Fig. 3B; Table 5).

The effect of the paclobutrazol + GA_3 treatment on the nitrogen concentrations and A/N was similar to that of GA_3 treatment.

Table 5. Two-way analysis of variance of the effect of species (Sp) and treatment (Tr) on C- and N-economy parameters and C and N concentrations. Abbreviations as in Figures 2 and 3 and Table 4. For each independent variable the figures indicate the percentage of the total sum of squares explained by the model, which could be attributed to that effect. Level of significance: *: p<0.05; **: p<0.01; ***: p<0.001. Absence of an asterisk denotes no significant effect.

	Species	Treatment	Sp × Tr	Error
C-economy				
gross A_{daily}	40***	36***	11**	13
SR	0	21	11	68
RR	33***	37***	8	22
Root growth	8*	58***	5	29
Stem growth	25***	62***	3	10
Leaf growth	51***	22***	9*	18
Plant C	0	9**	78***	13
N-economy				
Root N	0	13	12	75
Stem N	3	54***	19**	24
Leaf N	1	83***	4	12
Leaf N_a	8**	75***	3	14
A/N	20***	61***	5	14

Discussion

Differences in carbon and nitrogen economy between *Ae. caudata* and *Ae. tauschii*

The fast-growing *Ae. tauschii* had a greater daily photosynthate production per unit plant mass and respired a smaller proportion of its assimilated carbon than the slow-growing *Ae. caudata*. The greater photosynthate production in *Ae. tauschii* was associated with a higher leaf area ratio (LAR), which was mainly due to a higher specific leaf area (SLA), and not with a higher rate of photosynthesis per unit leaf area. This is in agreement with most of the literature on the carbon economy of fast- and slow-growing grass species (e.g. Poorter & Pothmann, 1992; Poorter *et al.*, 1995; Atkin *et al.*, 1996; Van den Boogaard *et al.*, 1996b). The faster RGR of *Ae. tauschii* was also associated with more biomass allocated to the leaf sheaths (higher SMR). Villar *et al.* (1998) and Van den Boogaard & Villar (1998) found a similar relationship between RGR and SMR among 20 *Aegilops* species and in a comparison of 10 wheat cultivars with these 20 *Aegilops* species. These authors suggested two explanations for the positive correlation between a higher biomass allocation to the leaf sheaths and a faster growth rate. Firstly, the architecture of plants with a higher SMR might be more beneficial for light interception and whole shoot photosynthesis. Since we found similar rates of photosynthesis per unit leaf area on a whole shoot level and on a leaf level (see Chapter 4 for rates of leaf photosynthesis) in *Ae. caudata* and *Ae. tauschii*, differences in light interception between these species is unlikely. Secondly, the greater investment in leaf sheaths (higher SMR) might invariably be associated with more investment in the leaf blade area (higher LAR), which subsequently increases light interception and hence whole shoot photosynthesis. Although this can explain the correlation between SMR and RGR in the grass species of this study, a correlation between SMR and LAR is not invariably found in comparisons of grass species (Garnier, 1992, Atkin *et al.*, 1996).

Despite its considerably lower nitrogen concentration per unit leaf area, *Ae. tauschii* achieved similar whole shoot photosynthetic rates per unit leaf area as *Ae. caudata* because *Ae. tauschii* used its nitrogen more efficiently for photosynthesis. Also at the leaf level, *Ae. tauschii* tended to have a higher photosynthetic nitrogen-use efficiency than *Ae. caudata* (see Chapter 4). This result is in accordance with the general finding that photosynthetic nitrogen-use efficiency at the leaf level is representative for that at the whole plant level (Pons *et al.*, 1994), and with the observation that faster-growing species have a higher photosynthetic nitrogen-use efficiency (van der Werf *et al.*, 1993; Poorter & Evans, 1998). The lower photosynthetic nitrogen-use efficiency of *Ae. caudata* may suggest that this species invests a larger proportion of its nitrogen in nitrogen-containing molecules that are not associated with photosynthesis (*e.g.*, Westbeek *et al.*, 1999). Alternatively, *Ae. tauschii* might use its photosynthetic apparatus more efficiently than *Ae. caudata*, due to less internal shading, a

higher internal CO_2 concentration, a higher Rubisco activity, or less feedback inhibition (Pons *et al.*, 1994). Greater limitation by Rubisco activity can be excluded as one of the factors contributing to differences in photosynthetic nitrogen-use efficiency because the limitation was similar in the two species (see Chapter 4).

Although *Ae. tauschii* had a faster rate of root respiration than *Ae. caudata*, *Ae. tauschii* used a smaller fraction of its carbon for root respiration than *Ae. caudata*, due to its lower proportion of biomass allocated to the roots. The proportion of assimilated carbon used in shoot respiration was similar for both species. As a result, the slow-growing *Ae. caudata* respired a larger proportion of its total assimilated carbon than the fast-growing *Ae. tauschii*. Although the differences in respiratory carbon use between the fast-growing *Ae. tauschii* and the slow-growing *Ae. caudata* resemble those between other fast- and slow-growing species (*e.g.*, Poorter *et al.*, 1990; Atkin *et al.*, 1996), the differences in the proportions of carbon used for growth of the different organs between these species do not. Whereas Poorter *et al.* (1990) and Atkin *et al.* (1996) found that fast-growing species allocated a smaller to similar proportion of carbon to the stems as the slow-growing ones, we found that the fast-growing *Ae. tauschii* allocated a greater proportion of its carbon to the stems than the slow-growing species at the expense of allocation to the roots. A similar trade-off between biomass allocation to roots and stems was found in a comparison of tall, semi-dwarf and dwarf near-isogenic wheat lines (McCaig & Morgan, 1993) and also in a comparison of GA-deficient mutants and the wild-type of tomato (Nagel *et al.*, 2001a). These studies suggest a role for gibberellins in the relationship between RGR, SMR and RMR through their effect on cell division and cell expansion: the reduction in shoot growth (lower RGR, lower SMR) in GA-deficient wheat and tomato allows more carbon to be translocated to the roots (higher RMR). We showed that differences in the biomass allocation pattern between *Ae. tauschii* and *Ae. caudata* can indeed be reduced by the exogenous supply of GA_3 to the slow-growing *Ae. caudata*, or of paclobutrazol supply to the fast-growing *Ae. tauschii* (see Chapter 5). Although reduction of the endogenous GA concentration in *Ae. tauschii* resulted in a decrease in relative growth rate (RGR) to the level of that of *Ae. caudata*, addition of GA_3 to *Ae. caudata* was not sufficient to increase its RGR tot the level of that in *Ae. tauschii*. Therefore, we investigated the effects of GA_3 and paclobutrazol on the carbon budget.

Effects of exogenously supplied GA_3 and paclobutrazol on gas exchange, carbon and nitrogen economy

Changes in plant growth, gas-exchange parameters, and carbon and nitrogen economy upon addition of the GA biosynthesis inhibitor can be reversed entirely by subsequent addition of GA_3. This observation indicates that paclobutrazol has a specific inhibiting effect on gibberellin biosynthesis, and that exogenous supply of GA_3 resulted in an increase in endogenous GA levels.

Inhibition of gibberellin biosynthesis in the fast-growing *Ae. tauschii* reduced RGR and the daily photosynthate production per unit plant mass to that of the slow-growing *Ae. caudata*. The reductions in RGR and photosynthate production are associated with reductions in LAR and in the rate of photosynthesis per unit leaf area. This is in contrast with the results of Morgan *et al.* (1990), who found an increase in leaf photosynthetic rates in dwarf genotypes of wheat, which they attributed to a greater amount of photosynthetic machinery per unit leaf area. Despite the increase in nitrogen per unit leaf area in paclobutrazol-treated *Ae. tauschii*, the rate of whole shoot photosynthesis decreased due to a reduction in the efficiency with which nitrogen was used for photosynthesis. A reduction in photosynthetic nitrogen-use efficiency was also found in GA-deficient tomato mutants (Nagel, 1998). Possibly the rate of photosynthesis was adjusted to the low requirement for growth in the GA-deficient plants by down-regulation of photosynthetic machinery upon accumulation of photosynthates (Farrar *et al.*, 2000). Alternatively, GA levels may have affected internal shading within the leaves, the proportion of nitrogen associated with non-photosynthetic leaf components, Rubisco activity or internal CO_2 concentration (Pons *et al.*, 1994). Light interception may also have been decreased in paclobutrazol-treated *Ae. tauschii* as a result of smaller leaf sheaths and leaf blades. Reduced light interception and whole shoot photosynthesis has also been found in reduced-height isolines of wheat lower compared with tall isolines (Gent, 1995).

Despite its increasing effect on LAR, GA_3 did not increase RGR and daily photosynthate production of the slow-growing *Ae. caudata* to the level of that of the fast-growing *Ae. tauschii*. This was due to the decreasing effect of GA_3 on the rate of photosynthesis per unit leaf area. Similarly, Heide *et al.* (1985) and Dijkstra *et al.* (1990) found that spraying GA_3 on the foliage of *Poa pratensis* and a slow-growing inbred line of *Plantago major*, respectively, increased leaf growth but decreased photosynthetic activity per unit leaf area. The decrease in photosynthetic rate upon GA_3 addition, observed in both *Ae. caudata* and *Ae. tauschii*, was accompanied by lowered plant nitrogen concentrations. Since GA_3 addition reduced the RMR in both species, a smaller amount of roots has to take up more nitrogen to achieve similar plant nitrogen concentrations as in the controls. In addition, leaves and stems contain higher nitrogen concentrations than roots. Apparently both species did not increase their specific nitrogen uptake rate upon GA_3 addition, to the extent needed to keep their nitrogen concentrations constant. Upon addition of GA_3, the nitrogen concentration per unit leaf area of the slow-growing *Ae. caudata* resembled that of the fast-growing *Ae. tauschii*. However, the photosynthetic nitrogen-use efficiency of the GA_3-treated *Ae. caudata* plants did not increase enough to achieve the high photosynthetic nitrogen-use efficiency of *Ae. tauschii* plants.

The main difference in carbon use between the species was that the slow-growing *Ae. caudata* used more carbon for root growth and respiration and less for stem growth than the fast-growing *Ae. tauschii*. Our results have shown that these differences in carbon economy

can be greatly reduced by increasing GA levels in the slow-growing species or by decreasing them in the fast-growing species. Changes in GA levels mainly affected the partitioning of biomass between roots and leaf sheaths and thereby indirectly affects the proportion of carbon used in respiration of these organs. This confirms the results of Nagel (1998) in GA-deficient mutants of tomato.

The results of this paper confirm those of the previous study, that GA plays a key role in determining differences between *Ae. caudata* and *Ae. tauschii* in the investment of biomass in leaf area, leaf sheaths and roots. Although LAR increased upon addition of GA_3, RGR did not due to a decrease in NAR, which was associated with a reduction in the rate of photosynthesis and nitrogen concentration per unit leaf area.

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Chapter 6

Chapter 7

Summary and General discussion

Fast expansion of leaf area is an important trait to select for in cereal crop species, especially in arid environments. It is associated with higher crop water-use efficiency, higher above-ground biomass production and yield (Siddique *et al.*, 1990; López-Castañeda & Richards, 1994; Van den Boogaard *et al.*, 1996c), and increased competitive ability (Lemerle *et al.*, 2001). This thesis examined the physiological basis of differences in leaf expansion rate, starting at a cellular level and scaling up to the whole plant level, in species with contrasting leaf growth characteristics. The species that were used for this study are from the genus *Aegilops* L. (Poaceae), the wild progenitors and relatives of wheat (Van Slageren, 1994). Species of this genus show a wide variation in traits relevant to survival in harsh environments (Villar *et al.*, 1998) and can be crossed with the current wheat cultivars, making them good candidates for wheat improvement (Damania, 1993).

Cellular basis of differences in leaf growth

In monocotyledonous species, growing leaves increase predominately in length, and the cellular processes determining leaf growth are restricted to a growth zone at the base of the leaves. In closely related species or genotypes, the time taken by leaves to complete expansion is rather constant (Calderini *et al.*, 1996; Harrison *et al.*, 1998; Fiorani *et al.*, 2000; Chapter 2). The rate at which leaves expand, however, can differ greatly amongst species and genotypes, resulting in a wide variation of mature leaf size (Volenec & Nelson, 1981; Groeneveld & Bergkotte, 1996; Fiorani *et al.*, 2000; Chapters 2 and 3). Chapter 2 shows that the faster leaf elongation rate of the third leaf on the main stem of *Aegilops tauschii* compared with that of *Ae. caudata* was associated with a higher meristematic activity in the leaf growth zone. The meristem of *Ae. tauschii* produced more cells per unit time than that of *Ae. caudata*, because it had a larger number of cycling cells, and not because the cells divided faster. The maximum cell elongation rate and cell elongation duration were not associated with differences in leaf growth between the species, nor was mature cell size. These results agree with those of Volenec & Nelson (1981) who compared two *Festuca arundinacea* genotypes with contrasting leaf elongation rates, those of Fiorani *et al.* (2000) who compared leaf growth of four *Poa* species, and those of Beemster & Baskin (1998) who studied accelerating root elongation rates in *Arabidopsis thaliana*. These data suggest that cell production can regulate leaf and root growth rate.

There are two views on the regulation of organ growth rate: the spatial view and the material (or cellular) view (reviewed by Silk, 1984). According to the spatial view, leaf (or root) growth rate is determined by the integral of relative cell elongation rates (*i.e.* strain rates), over the length of the whole growth zone, whereas cell division is nothing more than a process that accompanies cell expansion. This approach indicates that leaf growth is dependent on positional (biochemical or biophysical) mechanisms that control cell elongation. For example, the profile of relative cell elongation rate along the growth zone may be determined by gradients of cell wall enzyme activities (Wu & Cosgrove, 2000). According to the material view, leaf growth rate is determined by the integral of the growth activities of the individual cells. From this point of view, the leaf growth zone is determined by the number of cells that are growing and by the developmental program of these cells. Although the data presented in Chapter 2 cannot exclude spatial regulation of leaf growth, they do propose a more important role for material regulation of organ growth in determining differences in leaf elongation rate between *Ae. caudata* and *Ae. tauschii*. The larger number of dividing cells in *Ae. tauschii* resulted in more cells entering the elongation zone per unit time, and thereby increased the expansion potential of the elongation zone of *Ae. tauschii*, without any change in cell elongation rate.

A literature review on the effects of environmental conditions on cell production showed that changes in cell number are more often associated with changes in the number of dividing cells than with changes in the rate of cell division *per se* (Francis, 1998, and

references therein). When differences in cell production rate explain differences in leaf growth amongst closely related species, this also seems to be determined mainly by differences in number of dividing cells rather than in cell division rate (Fiorani *et al.*, 2000; Chapter 2). Apart from the number of proliferative divisions (determines number of cells in length of the leaf), the number of formative divisions (determines number of cells in width of the leaf) was also greater in *Ae. tauschii* than in *Ae. caudata*. The larger leaf meristem size (*i.e*. greater number of dividing cells in length and width of the leaf) of *Ae. tauschii* may result from a larger shoot apex (Kirby, 1974; Pieters & Van den Noort, 1988) or, alternatively, the larger number of dividing cells may have been generated during primordial or post-primordial stages that follow leaf initiation (Beemster & Masle, 1996).

Leaf position effects and coordination of growth amongst successive leaves

The finding that differences in meristem activity play a key role in determining differences in leaf growth rate amongst species, leads to the question whether variation in meristematic activity can also be responsible for determining differences in whole plant growth amongst species. More specifically, does meristematic activity determine the growth rate and the number of simultaneously growing organs (leaves and roots), and thereby the relative growth rate of a plant? In Chapter 3, I have pointed out that linear growth of grass leaves can only lead to exponential growth of the whole shoot by increasing (i) leaf elongation rate in successive leaves, (ii) leaf appearance rate with plant development, (iii) leaf elongation duration in successive leaves, and/or (iv) leaf mass invested per unit length (*i.e.* leaf width or specific leaf area) in successive leaves. Each of these growth characteristics was found to be associated with early exponential growth of grasses (*e.g*., Van Loo, 1992; Bos & Neuteboom, 1998b; Groeneveld, 1998; Rodríguez *et al.*, 1998a; Gunn *et al.*, 1999; Duru & Ducrocq, 2000, Plénet *et al.*, 2000).

In Chapter 4, I showed for two *Triticum* and three *Aegilops* species, that the faster-growing species had a faster leaf elongation rate and leaf width, a faster increase in leaf elongation rate and leaf width in successive leaves, and a slightly faster increase in the number of simultaneously growing leaves. No differences were found in leaf elongation duration or its rate of increase in successive leaves. In Chapter 2, differences in leaf elongation rate and leaf width between two *Aegilops* species were shown to be positively correlated with differences in length and width of the leaf meristem (*i.e*. the number of dividing cells). Moreover, Beemster *et al.* (1996) has shown that the increase in LER in three successive main stem leaves of wheat is associated with an increase in length of the meristem. Possibly, the faster increase in length and width with leaf position in the fast-elongating species of Chapter 4 resulted from a faster increase in leaf meristem size of successive leaves. Several studies suggested that leaf size is determined by apical dome size at the time of leaf initiation, and that the increase in leaf elongation rate and leaf width in successive leaves may be related to an increase in apical dome size with plant age (Abbe *et*

al., 1941; Kirby, 1974; Pieters & Van den Noort, 1988; Bos & Neuteboom, 1998a). For the species of Chapter 4 this would imply that species with faster-expanding leaves have a larger shoot apex, which also increased more in size with plant age than that of the species with slow-elongating leaves. However, the regulation of leaf expansion is probably more complicated than this. For some of the species in Chapter 4, the pattern of increase in leaf elongation rate with leaf position differed from that in leaf width. Moreover, recent work on 46 *Ae. tauschii* accessions has revealed that the molecular markers correlated with leaf elongation rate differ from the ones that are correlated with leaf width (M. W. ter Steege, pers. comm.). These results suggest that leaf elongation rate and leaf width are controlled independently. This is supported by the results of Beemster *et al.* (1996) for leaves of wheat seedlings grown at different soil resistances: leaf elongation rate and leaf width showed different levels of sensitivity to the treatment. Moreover, leaf width does not invariably correspond with apical dome size (Kirby, 1974; Beemster & Masle, 1996), and hence other factors are contributing to the control of leaf width.

The main cause of exponential leaf area expansion in grasses is the increase in the number of simultaneously growing leaves due to an exponential increase in the number of growing tillers. The tillering rate can differ widely amongst species and genotypes, and is closely associated with the timing of leaf appearance in grasses (Davies & Thomas, 1983). Duration of leaf elongation seems to play an important role in the association between leaf and tiller appearance. Skinner & Nelson (1994b) have shown in tall fescue (*Festuca arundinacea* Schreb.) that cessation of cell division in the leaf sheath is associated with the initiation of elongation of the tiller in the axil of that leaf. At the same time, they observed ligule initiation in the first younger leaf and initiation of leaf elongation in the second younger leaf. Skinner & Nelson (1994a) presented evidence that suggests that the synchrony of leaf and tiller initiation is not regulated by nutrient supply (although it can affect which tillers elongate). Rather, they suggested that it is regulated by an internal timer which may depend on the accumulation of a critical number of cells in the apex. Chapter 4 showed only minor or no variation at all amongst the species in tillering rate, leaf appearance rate (inverse of phyllochron) and duration of leaf elongation. If cessation of growth in each leaf is a prerequisite for associated tiller buds and younger leaves to start elongating, the similar time intervals between appearance of successive leaves and tillers amongst the species may be a consequence of their similar leaf elongation duration. Of course, the opposite is also possible, namely that the initiation of leaf and tiller elongation causes other leaves to stop growing. Although differences in apical development might explain differences in leaf and tiller appearance amongst species grown under optimal conditions, it does not explain the effects of changing environmental conditions on tillering rate. At suboptimal conditions, tillers are still initiated but tiller appearance is delayed or some tillers do not emerge at all, due to nutrient limitation or changes in the sink-source relationships (Longnecker *et al.*, 1993, Mosaad *et al.*, 1995, Rodríguez *et al.*, 1998b).

Since the relative tillering rate did not differ significantly amongst the three *Aegilops* and two *Triticum* species of Chapter 4, the species with the fastest increase in leaf area (LER and leaf width) with leaf position had the highest relative leaf area expansion rates (RGR_{la}). The differences in RGR_{la} were mainly present during the very early growth stages. With plant age, the increase in leaf area with leaf position slowed down in all species, but more so in the species with faster-elongating leaves, resulting in a decreasing RGR_{la}.

Relationship between leaf area expansion, biomass allocation and RGR

Elongating leaves are strong sinks for carbon and nitrogen. Nitrogen is deposited mainly in the cell division zone (Gastal & Nelson, 1994, Schäufele & Schnyder, 2001), while carbon deposition is highest in the zone of rapid cell expansion (Hu *et al.*, 2000, Schäufele & Schnyder, 2001). Therefore, shoots with faster-elongating leaves or increased numbers of elongating leaves need more resources than those with slower-elongating or less elongating leaves. Chapter 4 showed that the species with faster-elongating leaves invest more carbon in shoots (higher leaf mass ratio and stem mass ratio) and less in roots (lower root mass ratio). Lambers & Atkin (1995) and Nagel (1998) suggested that the flux of carbon to the roots depends on the rate at which carbon is used for shoot growth, and that shoot growth is regulated by endogenous signals rather than the supply of assimilates (assuming that the supply of assimilates is not limiting the expansion rate of the shoot). The data presented in Chapter 5 support this contention. Decreasing sink strength (cell division and cell expansion) of the growing leaves by addition of a GA biosynthesis inhibitor increased root mass ratio (RMR), whereas RMR was decreased when sink strength of the leaves was increased by GA_3 supply. However, in a recent literature review, Farrar & Jones (2000) re-examined a wide range of experiments designed to determine whether the flux of carbon into the roots is controlled by the shoot (push hypothesis) or by the root (pull hypothesis). They concluded that each of these two hypotheses is too simple to account for the control of carbon flux into the roots. Instead, they suggested that carbon acquisition by the roots is a result of the relative sink strength of both roots and shoots, and the transport pathways between the source leaves and these sinks.

The species with the faster-elongating leaves invested more of their shoot biomass in leaf area, *i.e.* they had a higher specific leaf area (SLA), than the species with slower-elongating leaves (Chapter 4). This higher SLA was the main cause of the higher relative growth rate (RGR_{dm}) of the species with faster-elongating leaves, as has often been found in grass species (Garnier, 1992; Atkin *et al.*, 1996; Van den Boogaard *et al.*, 1996b). The high RGR_{dm} of the species of Chapter 4 was also associated with a high stem mass ratio (SMR), *i.e.* a larger investment of biomass in leaf sheaths. Although a positive correlation between SMR and RGR is not common in grass species (Garnier, 1992; Atkin *et al.*, 1996), it has been found in a comparison of 20 *Aegilops* species with 10 wheat cultivars (Van den Boogaard & Villar, 1998). In this study there was only a weak relationship between RGR_{dm}

and SLA, whereas the correlation between RGR_{dm} and SMR was much stronger. Variation in SMR was also found in a comparison of tall and reduced-height isolines of wheat (McCaig & Morgan, 1993), which were obtained by incorporation of 'dwarfing genes' with antigibberellin action (Gale & Youssefian, 1985; Ross *et al.*, 1997). Gent, 1995 showed that these tall isolines intercepted more light than the reduced-height isolines, leading to greater canopy photosynthesis and greater biomass. Possibly, the correlation between SMR and RGR in the species of Chapter 4 was due to the association of SMR with plant architecture and light interception. However, Chapter 6 showed that two *Aegilops* species with contrasting leaf elongation rates and SMRs, had similar rates of photosynthesis per unit leaf area when measured on the whole shoot as well as on the individual leaf level. Therefore, it was concluded that light interception did not contribute to differences in RGR_{dm} between these species. Instead, the higher RGR_{dm} of *Ae. tauschii* with the fast-expanding leaves, compared to *Ae. caudata* with the slow-elongating leaves, was mainly due to a higher leaf area ratio (LAR) which led to a greater assimilate production per unit plant mass.

Are differences in leaf growth and biomass allocation between *Aegilops* species regulated by gibberellins?

Since the difference in leaf growth and biomass allocation between the fast- and slow-elongating *Ae. tauschii* and *Ae. caudata* resembled that amongst wild-type and GA-insensitive mutants of wheat (*e.g.*, Keyes *et al.*, 1989; McCaig & Morgan, 1993), we explored the possibility that gibberellins are involved in determining these differences between the *Aegilops* species.

Exogenous supply of GA_3 to the slow-elongating *Ae. caudata* or exogenous supply of paclobutrazol, an inhibitor of GA biosynthesis (Lenton *et al.*, 1994), to the fast-elongating *Ae. tauschii* greatly reduced the inherent differences in leaf growth and biomass allocation between these species (Chapter 5). The inherent difference in LER could be overcome completely by the treatments, indicating an important role for GA in the regulation of LER in these species. This is in agreement with the literature on GA-deficient or GA-insensitive mutants, which have lower rates of leaf elongation than the wild-types (*e.g.*, Calderini *et al.*, 1996; Tonkinson *et al.*, 1997; Chandler & Robertson, 1999). The difference in leaf growth response between *Ae. caudata* and *Ae. tauschii* to increasing concentrations of GA_3 resembled the difference in dose-response curves between barley mutants with reduced GA sensitivity and wild-type barley (Chandler & Robertson, 1999). Despite this resemblance, it is impossible to conclude from the present results whether either endogenous GA levels or GA sensitivity are responsible for the differences in leaf growth between these species. Both processes probably contribute to the variation in leaf growth, and the relative importance of each can only be determined by measuring endogenous GA levels and making GA dose-response curves.

Although differences in LER between *Ae. caudata* and *Ae. tauschii* could be overcome by manipulating endogenous GA levels, the differences in cell growth underlying differences in LER could not. The differences in LER between *Ae. caudata* and *Ae. tauschii* were associated only with differences in the cell production rate, whereas GA_3 and paclobutrazol addition affected both the cell production rate and cell size. It is obvious from the reducing effect of paclobutrazol that GAs are needed for cell production and cell elongation. This is in agreement with the numerous studies on GA-deficient and GA-insensitive mutants (Ross *et al.*, 1997 and references therein). The role of GAs in determining differences in the cellular processes underlying differences in LER between species is less straightforward. Tonkinson *et al.* (1997) have shown that the levels of GA_1 and GA_3 are highest in the most basal third of the growth zone of wheat leaves. Supplying GA_3 exogenously, as was done in the experiments described in Chapters 5 and 6, might have changed the physiological GA levels and distributions in the growth zone drastically. For example, the species might differ in active GA levels in the cell division zone only, whereas adding GA_3 probably increased the GA levels in both the cell division and cell expansion zone. In order to get more insight into the role of GA, or any other hormone for that matter, in determining differences in leaf growth between species, the relative contributions of different GAs to leaf growth and the parts of the leaf where they are active need to be clarified.

Manipulating the LER and leaf length with GA_3 and paclobutrazol changed the sheath length to total leaf length ratio and the SMR. This result supports the suggestion of Villar *et al.* (1998) that GA might be involved in determining the wide variation in SMR found in 20 *Aegilops* species. GA_3 also increased LAR but did not increase RGR_{dm} of the slow-growing *Ae. caudata* to the level of that of the fast-growing *Ae. tauschii*, despite LAR being the main cause of differences in RGR between these species (Chapter 6). This was due to a reduction in the rate of photosynthesis per unit leaf area, caused by a reduction in nitrogen concentration per unit leaf area. Since GA_3 addition reduced the RMR in both species, a smaller amount of roots has to take up more nitrogen to achieve similar plant nitrogen concentrations as in the controls. In addition, leaves and stems contain higher nitrogen concentrations than roots. Apparently the GA_3-treated plants did not increase their specific nitrogen uptake rate, to the extent needed to maintain their control nitrogen concentrations and rate of photosynthesis.

From the results of Chapters 5 and 6, I conclude that GA is an important determinant of leaf growth and biomass allocation in *Ae. caudata* and *Ae. tauschii*, and the observed differences in leaf growth and biomass allocation between these species might result from differences in endogenous GA levels or sensitivity.

Aegilops versus *Triticum*

Are there further possibilities for using *Aegilops* in wheat breeding? *Ae. tauschii*, the *Aegilops* species that shares the D genome with bread wheat (*T. aestivum*), has a high leaf

area ratio (LAR) and a rapid leaf area expansion rate (RGR_{la}) early in development (see Chapter 4). These traits have been related to increased crop yields in arid environments (*e.g.*, Siddique *et al.*, 1990; López-Castañeda & Richards, 1994) and to survival in competitive environments (Lemerle *et al.*, 2001). Moreover, Richards (2000) suggested that a further increase in the rate of crop photosynthesis, is more likely to be achieved by selecting cultivars with a more rapid leaf area expansion early in the growing season and not by selecting for the rate of photosynthesis *per se*. A large leaf area is frequently associated with low rates of photosynthesis per unit area in wheat (*e.g.*, Evans & Dunstone, 1970; Rawson *et al.*, 1987; Van den Boogaard *et al.*, 1997; Villar *et al.* 1998). In contrast with the *Triticum* species in this study, *Ae. tauschii* was able to combine a large leaf area with a high net assimilation rate (NAR) during the early stages of development (see Chapter 4). The large leaf area in the early growth stage, the high NAR, and the close genetic relationship with *Triticum* make *Ae. tauschii* an interesting species for further exploration of these traits in a wide range of *Ae. tauschii* accessions.

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Samenvatting

Een snelle groei en ontwikkeling van bladoppervlak in het vroege groeistadium van graangewassen kan de produktie bevorderen, vooral wanneer deze groeien in een droge of competitieve omgeving. Als gevolg van het snel strekken van de eerste bladeren van het graangewas, wordt het bodemoppervlak sneller bedekt waardoor het verlies van grondwater door verdamping vermindert en de hoeveelheid beschikbaar water voor opname door de plant toeneemt. Bovendien kunnen planten die hun bladerdak snel ontwikkelen beter concurreren met onkruid voor licht.

In dit proefschrift werden de onderliggende oorzaken voor soort-specifieke verschillen in bladexpansiesnelheid onderzocht. In meer detail werd gekeken naar (i) de verschillen in bladexpansie op cellulair niveau, (ii) het verband tussen expansie van individuele bladeren en groei van de hele plant, en (iii) de rol die het fytohormoon gibberellinezuur (GA) speelt bij soort-specifieke verschillen in bladstrekking. De planten die voor dit onderzoek gebruikt werden zijn afkomstig van het genus *Aegilops*. Deze soorten behoren tot de wilde tarwe-soorten en hebben in het verleden bijgedragen aan het genetisch materiaal van de huidige broodtarwe. Deze soorten vertonen een grote variatie in fysiologische eigenschappen. Gunstige eigenschappen kunnen dan door middel van kruisingen gebruikt worden voor het ontwikkelen van tarwerassen die beter aangepast zijn aan ongunstige omgevingsfaktoren en bijgevolg meer opbrengen.

Soort-specifieke verschillen in bladstrekking op celniveau

In hoofdstuk 2 werden de cellulaire processen in groeiende bladeren van *Aegilops caudata*, een soort met traag-strekkende bladeren, vergeleken met die van *Ae. tauschii*, een soort met snel-strekkende bladeren. De bladeren van grassoorten strekken vooral in lengte en de cellulaire groeiprocessen vinden plaats in een groeizone aan de basis van het blad. De celdelings- en celstrekkingssnelheid zowel als het aantal delende en strekkende cellen in deze groeizone werden geschat met behulp van een kinematische analyse. Deze analyse heeft aangetoond dat de snellere bladstrekking van *Ae. tauschii* vergeleken met die van *Ae. caudata* gepaard gaat met een groter aantal delende en strekkende cellen, terwijl de

celstrekkings- en celdelingssnelheid en de lengte van uitgestrekte bladcellen gelijk waren in beide soorten.

Deze resultaten laten duidelijk zien dat, ondanks dat celstrekking en niet celdeling vereist is voor bladgroei, verschillen in bladstrekkingssnelheid teweeg gebracht kunnen worden door verschillen in het aantal delende cellen zonder verschillen in celstrekkingssnelheid. Er werden ook aanwijzingen gevonden dat dezelfde verklaring geldt voor verschillen in bladbreedte tussen de onderzochte soorten. De bredere bladeren van *Ae. tauschii* hadden meer cellen in de breedte dan die van *Ae. caudata*, terwijl beide soorten dezelfde celbreedte vertoonden.

Verband tussen groei van individuele bladeren en groei van de hele plant

Een lineaire toename in lengte van individuele grasbladeren, zoals besproken in hoofdstuk 2, kan enkel leiden tot een exponentiele toename in bladoppervlak van de hele plant (gedefinieerd als relatieve groeisnelheid van bladoppervlak) door een toename in (i) de snelheid waarmee opeenvolgende bladeren strekken, (ii) de snelheid waarmee jonge bladeren verschijnen aan de groeiende plant, (iii) de duur van strekking in opeenvolgende bladeren, en/of (iv) bladbreedte van opeenvolgende bladeren (hoofdstuk 3).

De voornaamste oorzaak voor een exponentiele toename in bladoppervlak bij grassen is een toename in de snelheid waarmee zijscheuten en jonge bladeren verschijnen aan de groeiende plant. Zijn verschillen in relatieve groeisnelheid van bladoppervlak dan ook volledig te wijten aan verschillen in de snelheid waarmee nieuwe scheuten en bladeren worden geproduceerd? In een vergelijking van twee tarwesoorten (*Triticum*) en drie wilde tarwesoorten (*Aegilops*) die variëren in relative groeisnelheid van het bladoppervlak (hoofdstuk 4), werd aangetoond dat de snelheid waarmee jonge bladeren verschijnen slechts weinig verschilde tussen deze soorten. Daarentegen hadden de soorten met de hoogste relatieve groeisnelheid van het bladoppervlak de breedste en snelst strekkende individuele bladeren en bovendien de snelste toename in bladbreedte en bladstrekkingssnelheid in opeenvolgende bladeren. De duur van strekking in opeenvolgende bladeren was niet verschillend tussen de soorten.

De soorten, bestudeerd in hoofdstuk 4, met een hoge relatieve groeisnelheid van bladoppervlak investeren een grotere fractie van hun totale biomassa in bladeren (die bestaan uit bladschijven en bladschedes) ten koste van de wortels, investeren relatief meer van hun bladbiomassa in bladoppervlak, en hebben een hogere relative groeisnelheid van biomassa dan de soorten met een lage relatieve groeisnelheid van bladoppervlak. Een positieve correlatie tussen relatieve groeisnelheid en bladoppervlak per eenheid bladbiomassa wordt algemeen gevonden wanneer verschillende soorten die variëren in relatieve groeisnelheid met elkaar worden vergeleken. In hoofdstuk 4 werd bovendien een positief verband met bladstrekkingssnelheid aangetoond binnen een groep van vijf (gecultiveerde en wilde) tarwesoorten.

Is GA betrokken bij de verschillen in bladstrekking en biomassaverdeling tussen *Aegilops* soorten?

Variatie in bladstrekking, relatieve groeisnelheid en biomassaverdeling wordt wel vaker in verband gebracht met gibberelline-zuur (GA). In hoofdstukken 5 en 6 werd onderzocht of GA ook betrokken is bij de verschillen in bladstrekking, relatieve groeisnelheid en biomassaverdeling tussen *Aegilops* soorten. Daartoe werden *Ae. caudata* en *Ae. tauschii* gekweekt op een voedingsoplossing waaraan ofwel GA_3 ofwel paclobutrazol, een remmer van de GA biosynthese, was toegediend. Het remmende effekt van paclobutrazol op bladstrekking, relatieve groeisnelheid en biomassaverdeling in *Ae. caudata* en *Ae. tauschii* toont duidelijk aan dat GA een vereiste faktor is bij deze processen in beide soorten. Bovendien namen de verschillen in bladstrekkingssnelheid en biomassaverdeling tussen de soorten af door toediening van GA_3 aan *Ae. caudata*, de soort met de traag strekkende bladeren, of van paclobutrazol aan *Ae. tauschii*, de soort met de snel strekkende bladeren. Dit suggereert dat het GA metabolisme inderdaad verschilt tussen beide soorten, ofwel door een verschil in GA concentratie, in GA gevoeligheid of in beide.

Hoewel de meeste verschillen in groei tussen beide soorten afnamen door toediening van GA_3 aan *Ae. caudata* of paclobutrazol aan *Ae. tauschii*, kan GA slechts een deel van de verschillen verklaren. Op cellulair niveau hadden GA_3 en paclobutrazol zowel een effekt op het aantal bladcellen dat per tijdseenheid geproduceerd werd als op de totale lengte van volledig uitgestrekte bladcellen (hoofdstuk 5), terwijl *Ae. caudata* en *Ae. tauschii* enkel verschillen in het aantal cellen dat geproduceerd werd per tijdseenheid. Eén van de verklaringen hiervoor kan zijn dat de fysiologische GA concentratie tussen de soorten alleen verschilt in de celdelingszone terwijl het uitwendig toedienen van GA_3 en paclobutrazol de GA concentratie heeft beïnvloed in zowel de delings- als de strekkingszone. Ook de verschillen in relatieve groeisnelheid tussen de soorten kunnen niet enkel verklaard worden door GA. De afname in relatieve groeisnelheid na toediening van paclobutrazol toont aan dat GA vereist is voor een snelle groei maar niet voldoende is voor een toename in relatieve groeisnelheid. Toediening van GA_3 had geen effekt op de relatieve groeisnelheid omdat de fotosynthesesnelheid per eenheid bladoppervlak afnam als gevolg van een afname in stikstof concentratie per eenheid bladoppervlak (hoofdstuk 6).

Aegilops versus *Triticum*

Ae. tauschii, de *Aegilops* soort die het sterkst verwant is met broodtarwe (*Triticum aestivum*), heeft een hoge relatieve groeisnelheid van bladoppervlak, een hoge relatieve hoeveelheid bladoppervlak per plant en een hoge netto-assimilatiesnelheid per eenheid bladoppervlak in het vroegste groeistadium. Al deze eigenschappen zijn belangrijk voor een hoge opbrengst, wat deze *Aegilops* soort interessant maakt voor verder onderzoek.

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Lieve

Curriculum Vitae

Lieve Bultynck was born on 27 May 1973 in Antwerp, Belgium. After passing secondary school in 1991, she started to study Biology at the University of Antwerp (Belgium) in that same year. During her undergraduate studies, she went to Utrecht University (the Netherlands) to carry out a research project in the Department of Molecular Biophysics on 'Physical and chemical properties of plasma membranes of P*hsp* 70-*ipt* transformed tobacco (*Nicotiana tabacum*)'. She graduated in September 1995.

In January 1996 she was granted a Ph. D. scholarship by the Faculty of Biology of Utrecht University. The research, of which the results are presented in this thesis, was carried out at the Plant Ecophysiology Group of Utrecht University (the Netherlands), the Botany Department of the University of Washington (USA) and the Plant Sciences Department of the University of Western Australia (Australia).