On the variation of a functional trait

Mechanisms and consequences of petiole length variation in **Trifolium repens**

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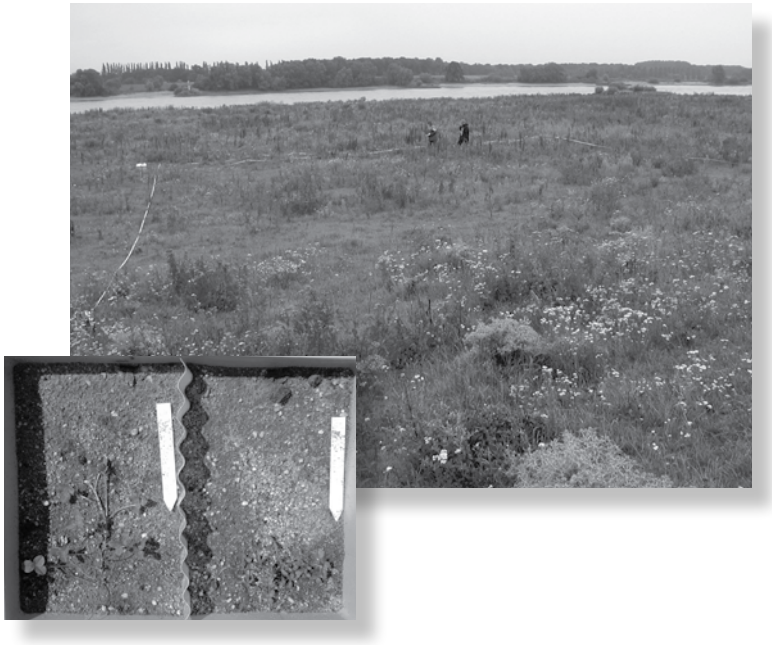
1

Table of contents



General introduction

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Mechanisms and consequences of petiole length variation in *Trifolium repens*

Environmental heterogeneity and genotypic diversity

All organisms need resources (water, carbohydrates, nutrients, etc.) to grow, survive and reproduce. In nature, resources are typically unevenly distributed with favourable and unfavourable patches alternating at various scales within a given habitat (Jackson & Caldwell, 1993; Farley & Fitter, 1999). As a result of local differences in soil conditions, water availability, and disturbance, natural habitats can often be characterized as a mosaic of microhabitats differing in resource availability and growth conditions (Welham *et al.*, 2002).

Plants are sessile and their growth and development depends on conditions characterizing their immediate microhabitat. If plants grow under conditions where resource availability is low, they cannot, like most animals, simply move to places where resource availability is high. Plants must therefore adapt to the local growth conditions or forage for resources by placing leaves or root tips in resource hotspots (de Kroon & Hutchings, 1995; de Kroon *et al.*, 2005; Jansen *et al.*, 2006). The ability of plants to express different phenotypes in response to the immediate environment is called phenotypic plasticity (Bradshaw, 1965; Via & Lande, 1987; Sultan, 1995). In general, phenotypic plasticity refers to any effect of the environment on phenotypic expression of characters.

Plants collected from natural habitats often display large differences in morphological characters. Within a single species, genotypes often intrinsically vary in their phenotypic trait values (Evans & Turkington, 1988; Cain *et al.*, 1995). According to natural selection theory and micro-evolutionary dynamics, small-scaled environmental heterogeneity may favour genotypic variation in traits which are selectively relevant (or associated with plant fitness or performance) (Via & Lande, 1985; Falconer & Mackay, 1996; Kingsolver *et al.*, 2001; Kassen, 2002; Byers, 2005). The occurrence of phenotypically different individuals in the same habitat, for example, may be actively maintained by gradients of selective forces favouring one phenotype at one end of the gradient and another phenotype at the other end of the gradient.

Shade-avoidance

Light is one of the essential resources for plant growth (beside water and nutrients). The amount of light which is available for plants varies strongly at different temporal and spatial scales. Total light availability may vary among years, throughout the seasons, at a daily basis and even from moment to moment (i.e. on half cloudy days or as a result of light flecks in the vegetation). Light availability may differ between habitats (compare for example forest understorey and grasslands) and at small scales due to differences in shading due to variation in species composition or densities within a specific habitat.

In grasslands, light availability does not only vary horizontally (due to the patchiness of the environment) but also vertically. Light quantity is usually low near the soil and pre-

dictably increases towards the top of the canopy. Plants grown under conditions where light availability is (too) low may produce relatively low offspring numbers or may not be able to complete their life cycle. Many plants are able to express so called shade-avoidance responses to shading and crowding in attempting to reduce or avoid the potential negative effects of low light intensities (Schmitt & Wulff, 1993; Schmitt *et al.*, 1999; Pierik *et al.*, 2003).

Shade-avoidance responses are induced by low light intensity, low R:FR ratio of the incident light and accumulation of the gaseous hormone ethylene (Ballare *et al.*, 1994; Pierik *et al.*, 2003). Responses include the elongation of vertical structures (stem internodes and petioles) and increasing leaf area. Elongation of vertical structures can lead to the placement of the light acquiring laminas higher in the canopy where light conditions are more favourable (Ballare *et al.*, 1994; Leeflang *et al.*, 1998). Increased leaf area enhances light interception.

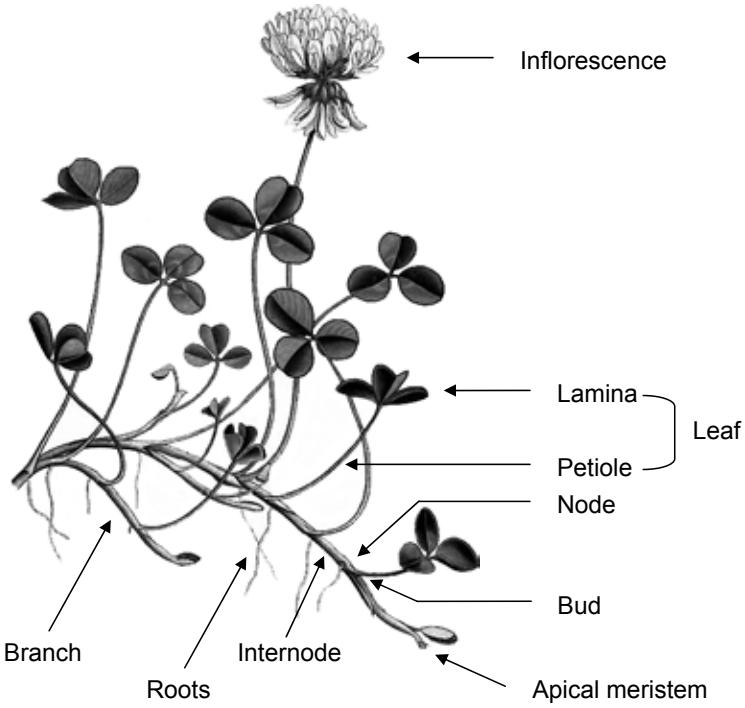

Figure 1: Trifolium repens. *Drawing of White clover* (T. repens). *The plant is composed of repeating modules or ramets, each of which consists of a node, a leaf, two nodal root primordia, a stolon internode that connects the ramet to the next ramet, and a bud positioned in the leaf axil. The bud can stay dormant or develop into either a terminal inflorescence or a branch. A branch consists of a series of new ramets produced by an apical meristem. The horizontal orientation of branches constrains the responses of* T. repens *in competitive conditions. Except for petioles* T. repens *lacks vertical structures that can place laminas higher in the canopy.*

In addition to vertical growth stoloniferous species like *Trifolium repens* can also forage for resources in a horizontal direction (Waite, 1994; Hutchings *et al.*, 1997). This enables stoloniferous species to grow away from local conditions where for example competition is high and light availability is low. Nevertheless, the horizontal orientation of the main shoot axes limits many stoloniferous species in their competitive strength as petioles are the only structures (or plant organs) enabling plants to position the leaves higher in the canopy (see Fig. 1) (Thompson, 1995; Leeflang *et al.*, 1998).

Mechanisms of differences in shade-avoidance traits

In nature, the interplay between genotypic differences and induced plastic responses results in a large variation of morphological traits expressed by individuals of the same species. Up to date, little is known about how the underlying dynamic cellular processes (i.e. cell division and cell expansion) contribute to variation found in trait values among genotypes and to plastic trait variation (Smith, 2000; Sultan, 2004). In addition, little is known about biomechanical consequences associated with both processes.

The size of a plant organ is determined by the number and the size of the cells in that structure (Beemster *et al.*, 2006). Both cell division and cell elongation require considerable amounts of energy and carbohydrates (Voesenek *et al.*, 2004). Cell elongation is considered cheaper since this process only requires the production of extra cell wall material while supplementary cell number production also requires additional DNA-replication.

Plants producing longer petioles need to increase the mechanical strength to carry the weight of the leaves and to avoid physical failure (Givnish, 2002; Anten *et al.*, 2005). This may be better achieved by an increase in cell number than by an increase in cell size as tissue made of more but smaller cells might have a higher density of cell walls providing rigidity and strength, and thus be more resistant to buckling and breaking. However, if plants produce longer petioles under shading (where fewer resources are available) it may be that cell elongation (the cheaper response) drives the main overall response to shading. Consequently, this would result in less rigid petioles which have a higher risk of physical failure.

Consequences of differences in shade-avoidance traits

Whichever mechanism, the benefits of shade avoidance responses are obvious: reduction of the negative effects of low light intensities on plant growth and thus on plant performance. This means that this environmentally induced response should increase the 'match' between the plants and the local growth conditions. Plants which are better able to achieve this match will perform better than plants which are less able to adjust their phenotype to the local growth conditions. Although general responses to shading have been investigated thoroughly (Ballare *et al.*, 1991; Schmitt & Wulff, 1993; Thompson, 1993; Huber *et al.*, 1998), up to date experiments evaluating plasticity and fitness

consequences, especially under natural conditions, are still scarce (Callahan & Pigliucci, 2002; Huber *et al.*, 2004; Weinig *et al.*, 2004) and the assumption that shade-avoidance responses are associated with fitness benefits is therefore still largely hypothetical.

Next to benefits, several types of costs have been hypothesized to be associated with plasticity like maintenance costs, production costs, information acquisition costs, genetic costs, developmental instability costs (DeWitt *et al.*, 1998) and, more recently, biomechanical costs (Givnish, 2002; Anten *et al.*, 2005). Plasticity may for example be costly if the response to the environment does not increase the match between the plant and the environment. This occurs if the cue inducing plastic changes of a phenotype does not accurately describe (future) environmental conditions or if the response to the cue does not lead to increased resource uptake. Under these conditions expression of plasticity is only associated with costs but not with benefits and the resources invested into a specific plastic response could have been saved for other plant functions enabling plants to cope with the low light conditions.

Another type of costs of plasticity is that more plastic genotypes may perform relatively worse than less plastic genotypes under conditions where plasticity is not induced. For example, genotypes that can express higher degrees of plasticity to shading may have a lower fitness under high light conditions than genotypes which express lower degrees of plasticity to shading. Less plastic genotypes would hence benefit in high resource patches since they do not (or to a lesser extent) carry costs associated with the mere ability to respond plastically (van Tienderen, 1991; DeWitt *et al.*, 1998).

The balance between the benefits and costs associated with plasticity within a particular set of environmental conditions ultimately determines whether and to what degree plasticity is evolutionarily favoured.

Study area and study species: river floodplains and *Trifolium repens*

Floodplain areas, along the main river channels such as the River Rhine, are characterized by high spatial and temporal environmental variability. Yearly winter inundations and infrequent summer floods create differences in soil substrate and disturbance regimes (Vervuren *et al.*, 2003; Voesenek *et al.*, 2004; Van Eck *et al.*, 2004). Distance to the river, flow velocity, elevation and water depth, among other things, determine the amount and type of substrate (i.e. clay and sand) that is deposited at a specific site (Voesenek *et al.*, 2004; Thonon, 2006). In addition to these a-biotic growth conditions, the activity of large mammals like cows and horses disturb the standing vegetation.

As a result of this variety in environmental factors the herbaceous vegetation at many places in the floodplain is characterized by a dynamic mosaic of different microhabitats (or sites). Microhabitats range from sites where disturbance has been relatively low and a dense vegetation has developed (competition for light is high), to more open sites where the vegetation has been disturbed or removed (characterized by low above ground competition). Competitive sites are characterized by poor light conditions for plant growth

but the standing vegetation of tall herbs or grasses can offer support for vertical plant structures. Alternatively, disturbed sites are more open and light conditions will be optimal for plant growth. As a consequence of the low vegetation, there will be no vertical support structures.

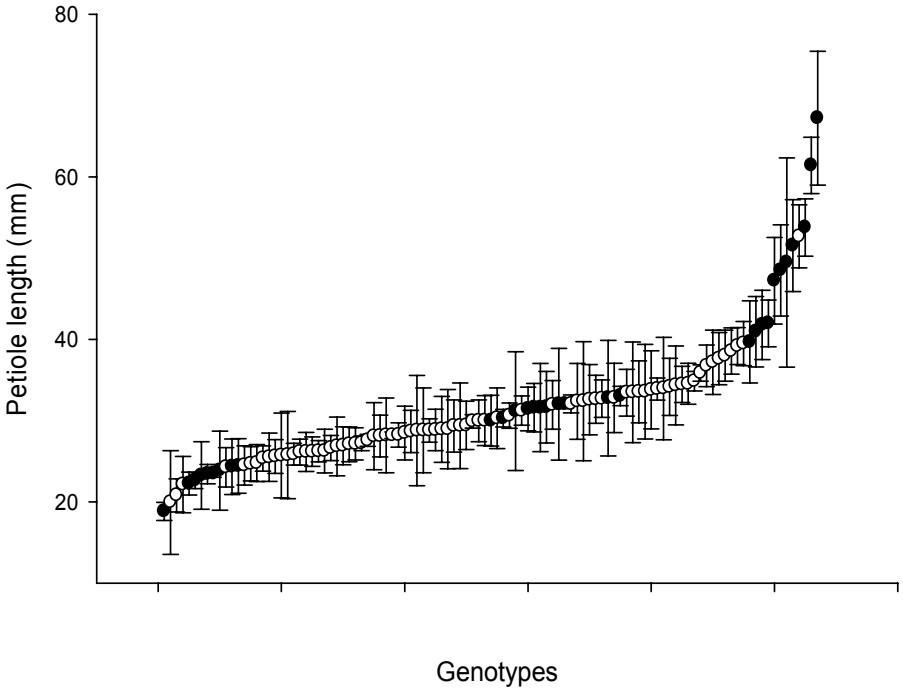

Figure 2: *Selection of the genotypes. From a Dutch floodplain population (along the river Waal near Ewijk, The Netherlands), 107* Trifolium repens *genotypes were collected. All genotypes were subjected to identical growth conditions in a common garden. After one year, all plants were screened for morphological characters, e.g. petiole length. The diagram ranks these 107 genotypes from short to long petiole length. Thirty-four genotypes were selected representing the range of petiole length differences found in the original collection (black dots). Error bars indicate standard errors for each genotype (n=3).*

From this highly heterogeneous riverine habitat along the River Waal near Ewijk (The Netherlands) 107 *T. repens* plants were randomly collected. Molecular techniques (see **Chapter 2**) were used to establish genetic identity of the collected plants. The plants were subjected to identical growth conditions for one year: under homogeneous outdoor conditions in a common garden during the summer and in a non-heated greenhouse during the winter. All plants were then screened for morphological traits like petiole length, stolon internode length and leaf area (H. Huber, unpublished data). The morphological differences among genotypes were rather large: petiole length ranged from 18.8 mm to 67.2 mm; internode length from 7.8 mm to 25.6 mm and leaf length from 7.7 mm to

16.5 mm). From these plants, thirty-four genotypes were selected representing the whole range of petiole length differences (Fig. 2) and these genotypes were used throughout this thesis in a series of experiments.

General aims & outline of this thesis

This thesis aims at unravelling the evolutionary and ecological consequences associated with morphological differences among *T. repens* genotypes and shade-avoidance responses. A starting point in achieving this is **Chapter 2** which investigates the relation of trait values (petiole length and leaf area) and the plasticity in these traits. This is done by comparing petiole length and leaf area of all thirty-four genotypes under high light conditions with the trait values of the same genotypes under two conditions inducing shade-avoidance responses: homogenous low light conditions and a vertical light gradient mimicking natural shade in grasslands. That experimental setup also allowed evaluating the benefits and costs related with plasticity on plant performance. Then, this thesis explores the morphological differences in two directions: the mechanisms underling the differences (**Chapters 3 & 4**) and the evolutionary and ecological consequences of the differences (**Chapters 5 & 6**).

From a mechanistic point of view, little is known about how the underlying dynamic cellular processes (cell proliferation and cell expansion) contribute to variation found in petiole length among genotypes and to shade induced petiole elongation. **Chapter 3** shows that cell number is the main factor explaining petiole length differences among genotypes under high light conditions. By contrast, a single cellular process (cell proliferation or cell expansion) does not explain petiole elongation in response to shading and **Chapter 3** shows that there is a high genetic variation in the relative contribution of changes in cell number and cell length to shade-induced petiole elongation. Moreover, the changes in cell number and cell length due to shading appear to be negatively correlated. **Chapter 4** continues on that observation and examines in the bio-mechanical and plant performance consequences of different contributions of cell proliferation and cell expansions to elongation responses.

From an ecological point of view, **Chapter 5** examines the consequences of vertical and horizontal structures (petioles and internodes) and their plasticity on plant performance in a common garden experiment simulating the main environmental factors creating habitat heterogeneity. This is done by investigating the implications associated with morphological differences among all thirty-four genotypes in competition with *Lolium perenne* (a natural competitive grass species of *T. repens*) and under a disturbance regime. In **Chapter 6**, eight out of our thirty-four genotypes finally return to their home ground. Eight genotypes were explanted into each of 99 microsites in the habitat from which they were originally collected to study the consequences of differences in morphological characters on plant performance under natural field conditions.
The main results of this thesis are discussed and summarized in **Chapter 7**.

Shade avoidance in *Trifolium repens*: costs and benefits of plasticity in petiole length and leaf size

Jelmer Weijschedé, Jana Martínková, Hans de Kroon, Heidrun Huber

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Summary

We tested whether the degree of shade-induced plasticity in petiole length and leaf area is related to the mean trait value expressed under high light conditions, and to what extent trait values expressed under high light and shaded conditions affect plant performance.

Thirty-four *Trifolium repens* genotypes were used which expressed a wide range of petiole lengths and leaf areas. Plants were subjected to a high light environment and two shading regimes, homogeneous shading and a vertical light gradient.

Absolute petiole elongation in response to both shading treatments and absolute leaf area expansion in response to homogeneous shading were independent of the trait values expressed in high light. Consequently, relative plasticity was higher for genotypes with lower high light trait values. Plasticity was associated with enhanced plant performance in a vertical light gradient but not in homogeneously shaded conditions. We also found costs associated with the ability to express plasticity.

Our results suggest that selection can act separately on trait values expressed under high light conditions and on the degree of plasticity.

Introduction

Phenotypic plasticity is the ability of a genotype to produce different phenotypes when exposed to different environments and enables plants to deal with growth conditions that vary in space and time (Bradshaw, 1965; Sultan, 1987; DeWitt *et al.*, 1998). Plasticity enables plants to alter morphological, physiological and developmental traits to match their phenotypes to the environment they are growing in (Ballare *et al.*, 1994; Sultan, 2005), thereby buffering the potentially negative effects of environmental variation on growth and reproduction.

It has been suggested that the degree of plasticity in a trait may intrinsically be coupled to the mean trait value (Pigliucci *et al.*, 2003). This has led to the suggestion that plasticity may have evolved as a by-product of natural selection on mean phenotypic trait values (Via, 1993). Other authors have argued that plasticity may very well be considered a trait in its own right, which evolves separately from the mean value of a character (Scheiner, 1993a; Scheiner, 1993b; Schlichting & Pigliucci, 1993). In the latter case, selection can act on plasticity itself, independent of the trait mean. In spite of the continuing debate on this issue, the relationship between trait means and trait plasticity has not often been investigated systematically (Via *et al.*, 1995; DeWitt *et al.*, 1998; Pigliucci *et al.*, 2003). Previous studies have found correlations between plasticity and trait means across environments (Scheiner, 2002; Pigliucci *et al.*, 2003). However, these studies compared the degree of plasticity with trait means measured across different environments. This analysis may reveal positive correlations between trait means and plasticity, because more plastic genotypes are likely to have higher across environment means as compared to less plastic genotypes. In order to study the relationship between mean trait values and plasticity independently, trait values expressed under conditions not inducing plastic changes (e.g. a high light phenotype) should be compared with trait means expressed under conditions inducing plastic changes (e.g. a shade-induced phenotype).

The independence of the degree of plasticity from the trait could have important evolutionary consequences. If plasticity and trait values were correlated, an evolutionary increase in the mean trait value could lead to an increase in plasticity and potentially also to the accumulation of costs associated with plasticity. The first aim of our study is to provide insight into the relation of trait values and plasticities in these traits. This is done by comparing a range of genotypes that differ in high light trait values in different shading regimes. We use the photo-morphogenic responses such as petiole elongation and the expansion of the leaf surface that many plants from open habitats express in response to crowding and shading. *Trifolium repens* is used in this study because its shade-avoidance structures are limited to petioles and leaf laminas and this species shows high genotypic variability (Gustine & Sanderson, 2001).

Shade-avoidance responses are induced by lower amounts of photosynthetic active radiation, a decreased red to far-red ratio (R:FR) of the incident light, and the perception of ethylene produced by neighbouring plants (Schmitt & Wulff, 1993; Ballare *et al.*, 1994;

Pierik *et al.*, 2003; Schmitt *et al.*, 2003). The induced responses enable plants to reduce the negative fitness consequences caused by competition for light (Aphalo & Ballare, 1995; de Kroon & Hutchings, 1995; Sultan, 1995; Huber & Hutchings, 1997; Van Hinsberg, 1997; Geber & Griffen, 2003). However, plastic responses may also be costly (van Tienderen, 1991; DeWitt *et al.*, 1998; Scheiner & Berrigan, 1998; Scheiner, 2002).

Several types of costs have been hypothesized to be associated with plasticity, such as maintenance costs, production costs, information acquisition costs, genetic costs and developmental instability costs (DeWitt *et al.*, 1998). Whether plasticity is evolutionary favoured depends on the costs and benefits associated with plasticity within a particular set of environmental conditions (Lande & Arnold, 1983; van Tienderen, 1991; Scheiner & Berrigan, 1998; DeWitt *et al.*, 1998). The second aim of this paper is to get a better understanding of the costs and benefits associated with plasticity. We consider three hypothetical situations in which costs and benefits of plasticity can be explored: (1) Induction and expression of plasticity results in net benefits. Under these conditions expression of plasticity results in a better matching of the phenotype with the respective environmental conditions, thereby enhancing plant performance of a highly plastic genotype compared to a less plastic genotype. Under these conditions plastic responses are referred to as adaptive plasticity (Dudley & Schmitt, 1996; Schmitt, 1997; Donohue *et al.*, 2000), leading to homeostasis (Sultan & Bazzaz, 1993; Dorn *et al.*, 2000) as the benefits of plasticity exceed any potential costs associated with plasticity. In order to study these costs and benefits plant performance has to be examined in an environment where plasticity is induced and plastic changes are associated with increased resource acquisition. (2) Plasticity may also be induced without plastic responses being associated with enhanced plant performance resulting in plasticity being non-adaptive or mal-adaptive (Dorn *et al.*, 2000; Weinig, 2000; Poulton & Winn, 2002). This occurs if the cue inducing plastic changes of a phenotype does not accurately describe environmental conditions or if the response to the cue is not an appropriate response and does therefore not lead to increased resource uptake. Under these conditions expression of plasticity is only associated with costs but not with benefits. In order to study these "pure" costs of the expression of plasticity, costs of plasticity have to be studied under conditions where plasticity is induced, but where plastic changes are not associated with increased resource acquisition. (3) The ability to respond plastically may also be costly (due to e.g. maintenance costs (DeWitt *et al.*, 1998)). If such costs of plasticity exist, more plastic genotypes will perform relatively worse than less plastic genotypes under conditions where plasticity is not induced. For example, if grown under high light conditions genotypes that can express higher degrees of plastic changes in response to shading may have a lower fitness than genotypes characterized by lower degrees of plasticity if subjected to shading. Less plastic genotypes would hence benefit under high light conditions since they do not (or to a lesser extent) carry costs associated with the mere ability to respond in a plastic way (van Tienderen, 1991). In order to study the costs of the ability to respond plastically, the degree of plasticity under inductive conditions has to be compared to plant performance under conditions not inducing plastic responses.

We aimed at testing the following specific hypotheses:

(1) The expression of plasticity in petiole length and leaf area depends on the trait value expressed under high light conditions. This implies that larger and smaller structures should change to the same relative extent if plasticity is induced. Consequently, the absolute change would be greater for larger structures than for smaller structures. We therefore expect genotypes with longer petioles and larger leaves in high light conditions to express higher degrees of absolute plasticity than genotypes with shorter petioles and smaller leaf areas. On the other hand, relative plasticity should be equal for genotypes that have higher or lower values in these traits under high light conditions.

(2) Costs and benefits of plasticity depend on the type of shading. In a vertical light gradient, the expression of longer petioles and larger leaves will be associated with benefits. Longer petioles enable plants to reach more favourable light conditions and producing larger leaves increases light capture. In homogeneously shaded conditions, longer petioles will be associated with costs while producing larger leaves will be associated with benefits. If plants are grown in high light conditions and plasticity is not induced, more plastic genotypes (in response to one of the shading regimes) will not perform as well as less plastic genotypes.

We examined trait values and plasticity in three different light regimes, high light conditions, homogeneous shade, and a vertical light gradient. Plastic plant responses to shading are almost always tested in experimental conditions where whole plants are shaded, usually in cages. This provides accurate information about how plants respond to shading, but does not allow investigating the effect of shade-avoidance responses on plant performance (Schmitt, 1997; Leeflang *et al.*, 1998). In homogeneously shaded conditions, like in artificial cages, light quality and quantity at the level of resource acquiring structures do not change while in natural vertical light gradients light interception of the resource acquiring structures systematically increases with height above the ground. Therefore, petiole plasticity, which is hypothesized to be beneficial for plant performance in plants exposed to a vertical light gradient (Leeflang *et al.*, 1998) may only be associated with costs, but not with benefits, if expressed under homogeneously shaded conditions. However, in homogeneously shaded conditions, increasing assimilation rate through physiological changes or increased leaf area or SLA may still be beneficial. By regressing plant performance (total biomass and ramet number produced by a genotype within an environment) on trait values and trait plasticity in these different shading regimes, we are able to test for cost and benefits associated with plasticity expressed in a light gradient and under homogeneous shading (Scheiner & Berrigan, 1998; DeWitt *et al.*, 1998; Relyea, 2002; Scheiner, 2002; Pigliucci, 2005).

Materials and methods

Species and pre-treatment conditions

Trifolium repens is a common stoloniferous perennial herb that shows large variation in morphological traits among plants originating from the same and from different populations (H. Huber, unpublished data). The plant is composed of ramets (or repeating modules (Hay *et al.*, 2001)), each of which consists of a node, a leaf, two nodal root primordia, an internode that connects the ramet to the next, and a bud positioned in the leaf axil. The bud can stay dormant or develop into either a terminal flower or a branch (Turkington & Burdon, 1983; Huber & During, 2000). A branch consists of a series of new ramets produced by an apical meristem. The horizontal orientation of branches constrains the responses of *T. repens* in competitive conditions. Except for petioles *T. repens* lacks vertical structures that can place laminas higher in the canopy.

In the summer of 2001, 107 *T. repens* plants were randomly collected in a floodplain along the river Waal near Ewijk (The Netherlands, 51^0 52'54"N, 5^0 45'00"E). The distance between individual plants was at least five meters. Molecular techniques (AFLP, four primer combinations, 145 markers) were used to establish genetic identity of the collected plants. The plants were grown under homogeneous outdoor conditions in a common garden during the summer and in a non-heated greenhouse during the winter. Twice a year, in autumn and spring, apical cuttings were transplanted into new pots to maintain the collection. In summer 2002, all plants were screened for morphological traits, including petiole and leaf size (Huber *et al*, in prep). Thirty-four unique genotypes were selected which expressed a wide range of petiole lengths ranging from 1.9 to 6.8 cm under outdoor conditions. Under greenhouse conditions petiole lengths varied from 5.0 to 11.0 cm. Cuttings of these plants were pre-grown for two months in a greenhouse on a mixture of sand and potting soil (2:1) receiving 100 ml half strength Hoagland solution once (Hoagland & Arnon, 1950). On January 23rd (2003), 12 cuttings were taken from each genotype. Cuttings consisted of a ramet, a well-developed root system and a lateral stolon with 3-5 ramets. These cuttings were each transferred to 0.18 x 0.22 x 0.05 m trays, filled with a mixture of sand and sieved potting compost (2:1). The cuttings were grown under homogeneous greenhouse conditions for two weeks, during which they received 100 ml half strength Hoagland solution once before the treatments were imposed.

Treatments

All 408 (12 cuttings of each of 34 genotypes) plants were placed into cages (2.70 x 0.95 x 0.40 m) that were covered with transparent plastic film. Control conditions consisted of cages covered with transparent plastic (LEE Colortran International, Andover, UK; no. 130, clear; Photosynthetic Active Radiation transmittance = 80%), subsequently referred to as high light conditions (H). Homogeneous shade (S) was obtained by covering the cages with green transparent plastic (LEE Colortran International; no. 122, fern green) which reduced light quantity to 20% and R:FR ratio to 0.25. To simulate a vertical light gradi-

ent (G), the sides of the cages were covered with the green filter (no. 122) but the top was covered with the transparent plastic (no. 130) used for the control cages. These cages were fitted with vertical 0.30 x 0.90 m sheets of the green plastic every 0.05 m, starting 0.05 m above the soil to allow stolon growth without restraint (Fig. 1a).

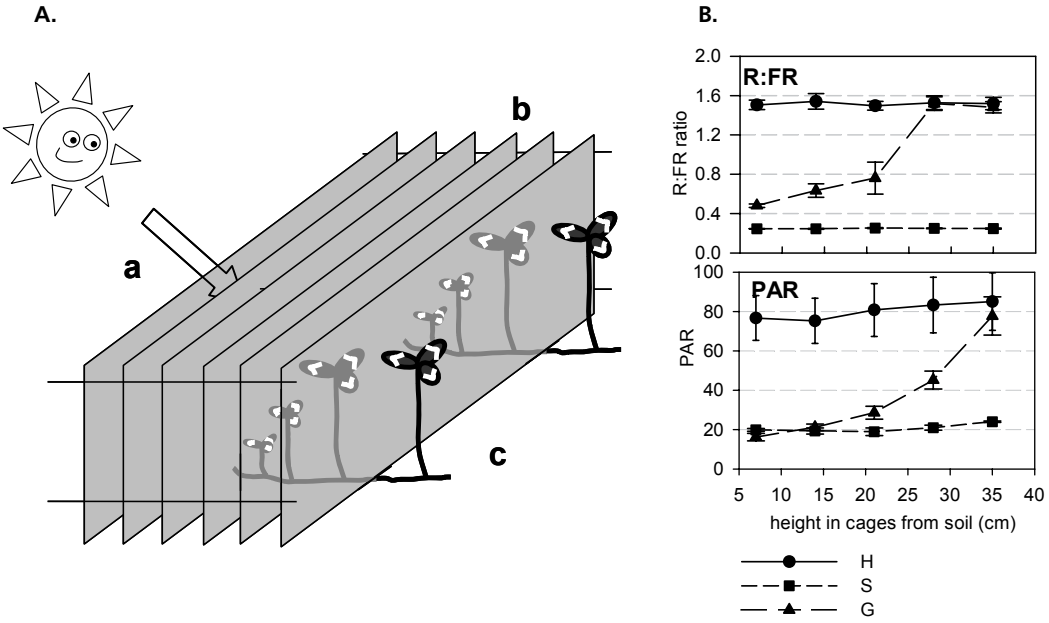

Figure 1a: *Schematic drawing of the vertical light gradient imitating natural shading. (a) Light sources (sun and artificial lamps), the arrow indicates the main direction of the direct radiation; (b) sheets of green transparent filter that were fitted in the cages; (c)* T. repens *plants. Side and top covers of the cages are not drawn.*
1b:*. R:FR and photo active radiation (PAR, means ± 1se) at different heights in the three different treatments. PAR is given relative to the incident light. Treatment abbreviations: H = high light, S = homogeneous shade, G = light gradient.*

To ensure comparable physical characteristics in all treatments, the control and homogeneous shade cages were fitted with vertical transparent plastic every 0.05 m. A spectro radio meter (Li-Cor 1800) was used to measure the amount of light and the spectral light conditions in the cages. Depending on the weather conditions, the light availability in the gradient was 17-20% of the incident PAR and the R:FR ratio was 0.3-0.5 at 5 cm above soil level. At this height, light conditions were similar to light conditions found in the homogeneously shaded cages. In the cages simulating vertical light gradients these PAR & R:FR ratios steadily increased until at 0.35 m above soil level PAR and R:FR reached levels similar to those in high light conditions (Fig. 1b).

The treatments were replicated in 4 blocks, with each genotype being present once per block per treatment, resulting in 4 replicates per genotype per treatment. Within each block, the treatments were arranged in a random sequence and the genotypes within each treatment were arranged randomly as well. At the onset of the experimental treatments, the ramet number was counted in order to be able to correct for initial variation in plant size.

The experiment was performed in a heated greenhouse. Light was supplemented by High Pressure Sodium lamps (Hortilux-Schreder, 600W), whenever irradiance in the greenhouse dropped below 400 $mmol \cdot m^{-2} \cdot sec^{-1}$ between 06:00h and 22:00h. Plants were watered every other day. After 34 days plants were harvested. Primary and lateral ramets were counted and the plants were separated into roots, stolons, petioles and laminas. Dry mass of these structures was determined after the plants had been dried to constant weight at 70°C. In addition to dry biomass, we measured petiole length and leaf area on the third youngest ramet, counted from the apex on the main stolon.

Data analysis

A mixed model multivariate analysis of covariance (MANCOVA) was used to determine the effects of block, treatment, genotypes and treatment x genotype interactions on total biomass and total ramet number and on biomass allocation data (Scheiner, 2001). A mixed model analyses of covariance (ANCOVA) was used to determine the effects of block, treatment, genotype and treatment x genotype on petiole length, leaf area and SLA (specific leaf area ($cm^2 \cdot mg^{-1}$)). In both analyses (MANCOVA and ANCOVA), treatments were considered to be fixed effects whereas genotypes and blocks were considered to be random and the appropriate error terms were used to calculate F-values (Bennington & Thayne, 1994). Initial ramet number was added as a covariate to correct for initial plant size differences. Residuals of the measured variables were tested for normality and homogeneity of variances and log transformed when necessary. A post hoc test (Tukey test, α=0.05) was used to compare the means of the three treatments.

For each treatment genotypic means were calculated. These means were used to calculate absolute (equation 1) and relative (equation 2) petiole elongation and leaf area plasticity as:

$$plX_i = X_{ij} - X_{ik} \quad (1)$$

$$plX_i = (X_{ij} - X_{ik})/X_{ik} * 100 \quad (2)$$

where plX is the measure of plasticity in a certain trait (X) of the ith genotype in shaded conditions (j) as compared to the trait value of the same genotype grown in high light conditions (k). (j) can be either homogeneous shade or the vertical light gradient. plX thus represents absolute or relative plasticity (in either petiole elongation or leaf area increase) expressed by a single genotype to either the homogeneous shade or to the vertical light gradient. Absolute elongation is an important parameter describing the absolute height gain of the leaves in the canopy. Relative elongation on the other hand is a measure of the investment relative to the trait value in a non-inducing environment.

To test the hypothesis that plasticity is correlated with the high light trait value, we

tested whether petiole elongation was correlated with the petiole length found under high light conditions. In addition we tested whether the relation between high light petiole length and petiole plasticity differed between shading treatments. To do this, we performed an ANCOVA with shade type as main effect. The high light values of petiole length were added into the model as a covariate. If the high light petiole length significantly affects the expression of petiole length in shaded conditions, elongation depends on the length of the petiole expressed under high light conditions. A significant interaction between shade type and high light petiole length would indicate that the effect of high light length on elongation differs between shade treatments. The same analysis was performed for leaf area as well.

Analyses of costs and benefits of plasticity

Costs and benefits of plasticity can be measured by a multiple regression as described by several authors (van Tienderen, 1991; DeWitt *et al.*, 1998; Scheiner & Berrigan, 1998; Callahan *et al.*, 2005; van Kleunen & Fischer, 2005):

$$W_{ij} = X_{ij} + plX_j \qquad (3)$$

where W is the relative plant performance of a genotype (i) within an environment (j) calculated as the total biomass or total ramet number produced by that genotype relative to the mean value of all genotypes in that environment. X_{ij} is the genotypic trait value in the corresponding environment (j) and plX_i is the plasticity of that trait as compared to the trait value of the same genotype grown in high light conditions (calculated as equation 1 or 2).

If plasticity results in net benefits (see introduction; situation 1), we expect to find a positive regression coefficient for the term plX in one of the shading regimes, indicating that a more plastic genotype performed better (produced more biomass or more ramets) than a less plastic genotype with the same trait value in that environment. If plasticity is induced without being associated with enhanced plant performance (situation 2), we expect to find a negative regression coefficient for the term plX (i.e. a negative selection gradient) in one of the shading regimes, indicating inferior performance, and is thus considered to indicate a cost of plasticity. To test if the ability to respond plastically is costly (situation 3), we used the same model (equation 3) slightly modified: W and X are now values found under high light conditions whereas plX still is the plasticity to one of the shading regimes. A negative regression coefficient for the term plX can be considered as the costs of the ability to express plasticity, indicating that the ability to respond plastically to shading reduces plant performance under high light conditions. Furthermore, in this modified analysis, the term X represents costs or benefits associated with high light trait values. A negative regression coefficient for the term X would indicate that, under high light conditions, genotypes with higher high light trait values (i.e. longer petioles or larger leaves) performed worse than genotypes with lower high light trait values.

The analysis was performed for each environment separately. Genotypic trait values were standardized to the means per treatment to allow for direct comparisons of different regression coefficients. SAS (version 9.1) was used for all statistical analyses.

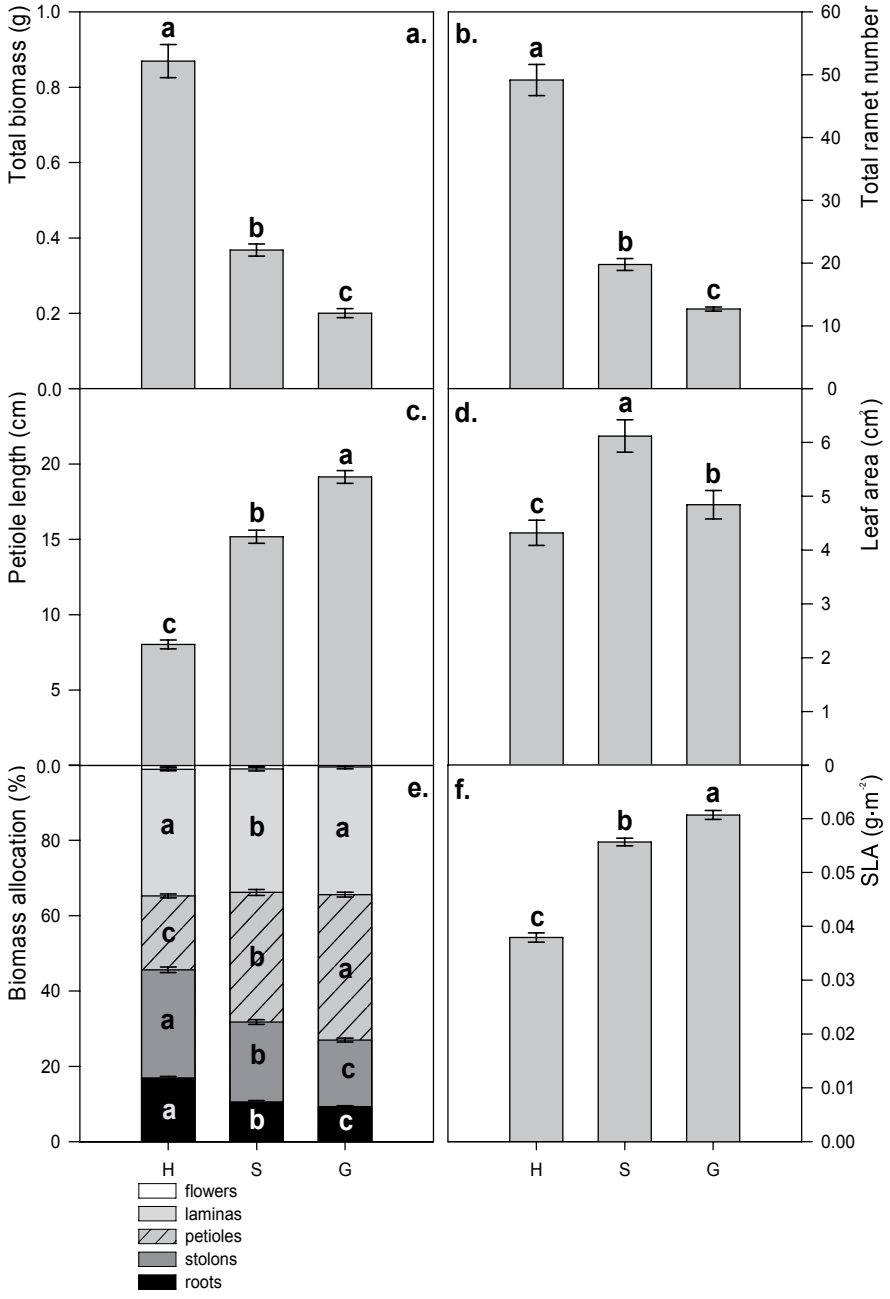

Figure 2: *Mean (± 1se) values of total biomass (a), total ramet number (b), petiole length (c), leaf area (d), biomass allocation pattern (e) and (f) SLA at harvest. Treatment abbreviations: H = high light; S = homogeneous shade; G = light gradient. Different characters indicate significant treatment differences (α<0.05; Tukey's studentized range test).*

Results

Overall treatment effects

Plants elongated their petioles under shaded conditions and this response was more pronounced in the light gradient than under homogeneous shading (Fig. 2). Plants produced larger leaves (laminas) and higher SLA ($cm^2 \cdot mg^{-1}$) in both shading treatments than in high light conditions and the leaves were largest under homogeneous shading (Fig. 2). A significant genotype x treatment effect was found for leaf area (Table 1).

Table 1: *Mixed model ANCOVA results examining the effects of treatments, genotypes, block and initial ramet number on petiole length, leaf area and SLA. Treatment was treated as a fixed effect, genotype and block were treated as random effects. Initial ramet number was used as a covariate to correct for initial variation in plant size. In the table F-values and their significances are presented. Significance levels are as follows: ns: p>0.10; $: 0.10≥p>0.05; *: 0.05≥p>0.01; **: 0.01≥p>0.001; ***: p<0.001.*

Traits	Treatment	Genotype	Treatment x genotype	Block	Initial ramet number	Error
df	2	33	66	3	1	299
Petiole length	530.76 ***	6.84 ***	1.28 $	3.43 *	0.55 ns	
Leaf area	32.49 ***	11.44 ***	1.88 ***	3.25 *	1.70 ns	
SLA	549.73 ***	4.79 ***	0.88 ns	10.72 ***	3.79 $	

There was a marginal genotype x treatment effect for petiole length (Table 1). Total biomass and total ramet number were significantly lower under shaded conditions (Fig. 2). A genotype and genotype x treatment effect was found for total biomass and total ramet number (Table 2) indicating that genotypes differed in their response to the treatments.

Plants allocated relatively more biomass to the petioles under shaded than under high light conditions (Fig. 2), increasing from about 20% under high light conditions to 34% under homogeneous shade and to 38% for plants grown in the light gradient. Genotypes significantly differed in their allocation to petioles and responded differently to treatments (Table 2). Although significant, the change in biomass allocation to the leaves due to the treatments was of a smaller magnitude than the responses of other traits (Fig. 2). Plants subjected to shading invested less biomass into their stolons and roots (Fig. 2), with plants grown in the light gradient allocating significantly less to stolons and roots than plants grown in the homogeneous shade.

High light trait values and their plasticity

On average, all genotypes elongated their petioles by 11 cm if grown in the light gradient and by 7 cm if subjected to homogeneously shaded conditions (Fig. 3). Genotypes

Table 2: *Results of mixed model MANCOVA for effects of treatments, genotype, block and initial ramet number on growth and biomass allocation parameters. Treatment was treated as a fixed effect, genotype and block were treated as random effects. Initial ramet number was used as a covariate to correct for initial variation in plant size. In the table F-values and their significances are presented. Significance levels are as follows: ns: p>0.10; $: 0.10≥p>0.05; *: 0.05≥p>0.01; **: 0.01≥ p>0.001; ***: p<0.001.*

Traits	Treatment	Genotype	Treatment x genotype	Block	Initial ramet number	Error
df	2	33	66	3	1	299
Growth						
Total biomass	429.34 ***	7.87 ***	1.57 **	6.28 ***	41.82 ***	
Total no. of ramets	706.37 ***	7.92 ***	1.52 **	8.18 ***	66.70 ***	
Multivariate test	278.09 ***(4)[a]	10.31 ***(66)[a]	1.78 ***(132)[a]	6.66 ***(6)[a]	33.43 ***(2)[a]	
Allocation						
petiole ratio	838.41 ***	18.40 ***	2.08 ***	9.52 ***	33.65 ***	
leaf ratio	5.51 **	8.93 ***	0.96 ns	0.54 ns	1.73 ns	
stolon ratio	216.71 ***	12.03 ***	1.93 ***	0.15 ns	44.11 ***	
root ratio	434.11 ***	8.50 ***	1.19 ns	7.93 ***	4.27 *	
Multivariate test	231.56 ***(8)[a]	11.5 ***(132)[a]	1.66 ***(264)[a]	4.16 ***(12)[a]	17.03 ***(4)[a]	

[a] Values are multivariate Wilk's λ test statistics, accompanying degrees of freedom are given in parenthesis.

with shorter high light petioles elongated their petioles for up to 250% while genotypes with longer high light petioles elongated their petioles for only 90% (Fig. 3). This means that across the wide variation of petiole lengths expressed under high light conditions absolute elongation response was similar and relative elongation response was negatively correlated with the petiole length under high light conditions (Table 3). The ANCOVA testing whether the relation between petiole length in high light conditions and petiole plasticity differs between shading treatments (Table 4) revealed no interaction between the high light trait value and shading treatments, indicating that the relation between high light petiole length and petiole lengths found in the shading regimes did not differ between the shade treatments.

In homogeneous shade, all genotypes increased their leaf area on average by about 2cm^2 (Fig. 3). Similar to petiole length, genotypes with smaller leaves under high light conditions extended their leaves relatively more as compared to genotypes with larger leaves (Fig. 3). Absolute leaf area increase under homogeneous shading was not correlated with leaf area expressed by the same genotypes under high light conditions (Table 3). Genotypes grown in the vertical light gradient, however, responded very differently:

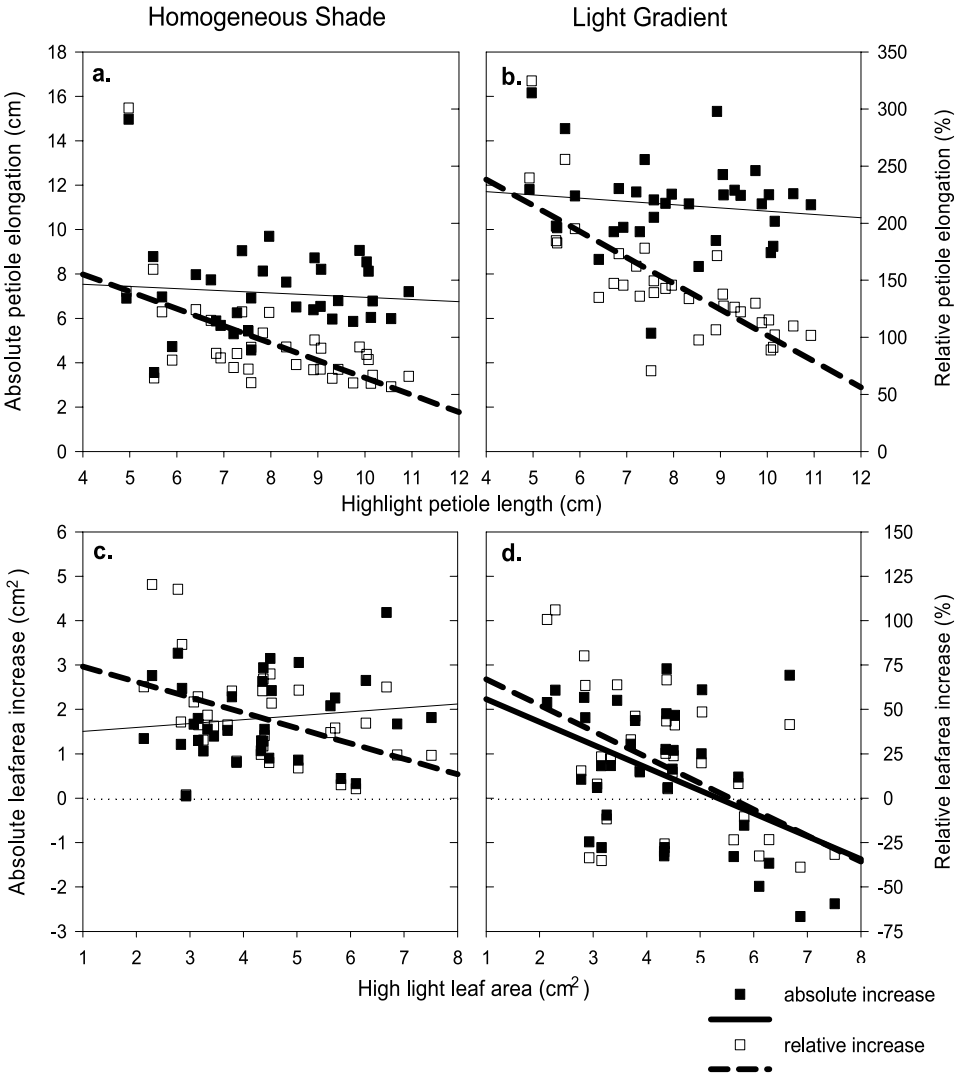

Figure 3: *The graphs indicate the response of petiole length and leaf area to the two treatments relative to the values expressed in high light conditions. Absolute increase (closed squares) in petiole length and leaf area is plotted on the left y-axes and relative increase (open squares) on the right y-axes, under either homogeneous shading (a and c) or the light gradient (b and d). Trait values expressed in high light conditions are plotted on the x-axes. Bold lines represent significant correlations between trait values in high light conditions and trait values found in the respective shading treatments at p<0.05. Positive values indicate an increase of trait values and negative values a decrease in trait values under shaded conditions. Correlation coefficients are given in Table 3.*

Table 3: *Correlation coefficients and their p-values of trait values expressed under high light conditions and their absolute and relative change under shaded conditions (see Fig. 3). Plastic responses to homogeneous shading (S) are the absolute or relative differences of a trait value found under homogeneous shading as compared to the trait value found under high light conditions; Plastic responses to the vertical light gradient (G) are the absolute or relative differences of a trait value found in the light gradient compared to the trait value found under high light conditions. Significant coefficients are highlighted in bold and italic. Genotypic means were used to calculate the correlations.*

	Plastic responses to S		Plastic responses to G	
	absolute	relative	absolute	relative
Petiole elongation	-0.085	***-0.585***	-0.125	***-0.767***
	0.632	<0.001	0.480	<0.001
Leaf area increase	0.129	***-0.437***	***-0.452***	***-0.505***
	0.468	0.010	0.007	0.002

in genotypes with smaller high light leaves, leaf area increased by up to 2cm^2 while in genotypes with larger high light leaves, leaf area decreased by up to 2cm^2 (Fig. 3). In the vertical light gradient, absolute change of leaf area was negatively correlated with leaf area expressed by the same genotypes under high light conditions and the same was true for the relative change of leaf area (Table 3). The ANCOVA testing whether the relation between leaf area in high light conditions and leaf area plasticity differs between shading treatments (Table 4) revealed a significant interaction between the high light trait value and shading treatments, indicating that the relation between high light leaf area and leaf area found in the shading regimes differed between the two shade types.

Table 4: *Two-way ANCOVA of effects of shade type (T) and high light trait values (H) on trait values found under shaded conditions. F-values and their significances are given. Bold values indicate significant values. Genotypic means were used for this analysis. (ns: p>0.10; $: 0.10≥p>0.05; *: 0.05≥p>0.01; **: 0.01≥p>0.001; ***: p<0.001)*

Source	*df*	Petiole length *F-value*	Leaf area *F-value*
T	1	3.38 $	1.85 ns
H	1	**37.11 *** **	**53.06 *** **
T*H	1	0.030 ns	**7.8 ** **
Error	68		

Table 5: *Estimates (standardized to the treatment mean) of the multiple regression coefficients indicating the effects of traits expressed in the respective treatment (trait value) and their plasticity on plant performance (= total biomass and ramet number) following equation (3) in the materials and methods section. In the first part of the table (I.), absolute plasticity was used in the analysis and in the second part of the table (II.) relative plasticity was used in the analysis. a. 'Expressed plasticity' indicates costs and benefits of expressing plasticity in either homogeneous shade (S) or the light gradient (G). A negative regression coefficient indicates costs, a positive coefficient indicates benefits. b. 'The ability to express plasticity' indicates the costs and benefits of the ability to express plastic responses in both shading treatments for plants grown in high light conditions (H). A negative regression coefficient for plasticity indicates costs of the ability to express plasticity; a positive regression coefficient for plasticity indicates that the ability to express plasticity enhances plant performance under high light conditions (H). b. also indicates costs or benefits associated with the high light trait values (H). Genotypic means were used for all analyses. Significant values are represented in bold. ($: 0.10≥p>0.05; *: 0.05≥p>0.01; **: 0.01≥p>0.001; ***: p<0.001)*

		Petiole length		Leaf area	
		S	G	S	G
(I.) Absolute plasticity					
a. Expressed plasticity					
Biomass	Trait value	0.099 ns	0.162 $	0.084 ns	**0.306 *****
	Absolute plasticity	-0.060 ns	0.001 ns	0.059 ns	-0.015 ns
Ramet number	Trait value	-0.124 $	**-0.079 ***	**-0.147 ***	0.010 ns
	Absolute plasticity	0.040 ns	**0.094 ***	0.099 ns	**0.070 ***
b. Ability to express plasticity					
Biomass (H)	Trait value (H)	0.083 $	0.091 $	0.0100 $	**0.162 ****
	Absolute plasticity	-0.075 ns	0.011 ns	0.026 ns	**0.129 ***
Ramet number (H)	Trait value (H)	-0.088 $	-0.089 $	-0.091 $	-0.048 ns
	Absolute plasticity	**-0.114 ***	-0.086 $	0.002 ns	0.094 $
(II.) Relative plasticity					
a. Expressed plasticity					
Biomass	Trait value	0.069 ns	**0.163 ****	**0.118 ****	**0.296 *****
	Relative plasticity	-0.055 ns	-0.020 ns	0.046 ns	0.001 ns
Ramet number	Trait value	**-0.100 ***	-0.012 ns	-0.089 $	0.011 ns
	Relative plasticity	0.020 ns	**0.056 ***	0.078 $	**0.086 ****
b. Ability to express plasticity					
Biomass (H)	Trait value (H)	0.027 ns	0.083 ns	**0.184 *****	**0.155 ***
	Relative plasticity	-0.107 ns	-0.001 ns	**0.160 ****	0.027 ns
Ramet number (H)	Trait value (H)	**-0.167 ****	**-0.173 ***	-0.013 ns	-0.087 ns
	Relative plasticity	**-0.153 ***	-0.125 ns	**0.154 ****	0.007 ns

Costs and benefits associated with plasticity

The data revealed significant costs and benefits of the expression of plasticity and the ability to respond in a plastic way (Table 5). Genotypes that expressed higher levels of petiole plasticity in the light gradient performed better than genotypes that showed less petiole plasticity in terms of ramet number (positive regression coefficient for plasticity in petiole length in Table 5Ia and 5IIa) but not in terms of biomass. Significant benefits were also found for genotypes showing more pronounced increase of the leaf area in the light gradient in terms of ramet number (positive regression coefficient for plasticity in leaf area in Table 5Ia and 5IIa), but these benefits were not present in terms of biomass. Under homogeneous shading, neither benefits nor costs were found for plasticity in petiole length or leaf area (non significant regression coefficients for plasticity in Table 5Ia and 5IIa).

If subjected to high light conditions, genotypes that responded to shading with a larger increase in petiole length performed relatively poorer in terms of ramet number than genotypes that were less responsive (negative regression coefficient for plasticity in Table 5Ib and 5IIb). This result indicates significant costs of the ability to express plasticity in terms of ramet number. No such costs were discernable for biomass (non significant regression coefficient for plasticity in Table 5Ib and 5IIb). No costs were found for the ability to produce larger leaves in a vertical light gradient in terms of lower total biomass production in high light conditions. On the contrary, genotypes that could produce larger leaves in a light gradient had a significantly higher total biomass in high light conditions, and these plants tended also to produce more ramets indicating that there were benefits associated with the ability to respond in a plastic way to a vertical light gradient (positive regression coefficient for plasticity in Table 5Ib). Leaf area plasticity induced by homogeneous shade had no effect on plant performance if the plants were grown under high light conditions (non significant regression coefficients for plasticity in Table 5Ib). When plasticity in leaf area was expressed in relative rather than absolute terms, these trends reversed: the ability to express plasticity in the light gradient had no benefits under high light conditions, while the ability to express plasticity under homogeneous shade had a positive effect on plant performance under high light conditions (Table 5IIb).

Producing longer petioles in high light conditions tended to be associated with benefits in terms of total biomass production (marginally significant positive regression coefficient for trait value in Table 5Ib) and with significant costs in terms of reduced ramet number (negative regression coefficient for trait value in Table 5IIb, marginally significant in 5Ib). Benefits are found for genotypes that have larger high light leaves in terms of total biomass production, but not in terms of total ramet number (positive regression coefficients for trait values in Table 5Ib and 5IIb).

Discussion

Longer petioles and larger leaves are likely to reduce the potentially negative fitness effects of competition for light. Our *T. repens* plants responded to shading by petiole elongation and by increased leaf areas, both of which are typical shade-avoidance responses. Neither absolute petiole elongation in response to both shading treatments nor absolute leaf area increase in response to homogeneous shading depended on the genotypic trait value expressed under high light conditions. Our data showed clear benefits associated with plasticity under more realistic conditions of a light gradient where responses were expected to increase light uptake. Surprisingly, no costs were found if plasticity was expressed in homogeneous shade, where the response was not expected to be associated with immediate benefits. However, genotypes grown under high light conditions experienced significant costs of the ability to respond plastically to homogenous shade.

Light gradient

Petioles elongated more strongly in the vertical light gradient than under homogeneous shading conditions. Increased petiole elongation in a vertical light gradient (as compared to elongation under homogeneous shading) improves leaf placement and should result in enhanced light harvesting and higher carbohydrate production (Ballare *et al.*, 1994; Leeflang *et al.*, 1998). However, this extra supply may directly be used by the petiole carrying the leaf laminas for further elongation. In homogeneous shade leaves were unable to reach better-lit places regardless of the absolute increase of petiole length. The resulting limitation of carbohydrates might have constrained the degree of plastic petiole elongation.

Total plant biomass was lowest in the vertical light gradient, although these plants expressed highest levels of petiole elongation and thus reached better-lit places than plants grown under homogeneous shade. These results are in contrast with results from a similar experiment using *Hydrocotyle vulgaris*, where biomass production was significantly higher in the light gradient than in homogeneous shading (Leeflang *et al.*, 1998). In that experiment leaflets of *H. vulgaris* were able to reach the top of the gradient and could intercept full daylight, while in our experiment leaves of *T. repens* did not reach the top of the gradient. Although the plants elongated their petioles more in the light gradient, and also invested more carbohydrates into petiole elongation than under homogenous shade, the leaves never reached more than 40% of the radiation. This may have been too low to compensate for the increased biomass allocation to the elongating structures. Increased biomass allocation to the petioles has been shown to result in the production of fewer ramets (Huber & Wiggerman, 1997; Stuefer *et al.*, 2002) which, in turn, may have constrained further assimilation. Plants showed stronger morphological responses in the vertical light gradient as compared to plants grown in homogeneously shaded conditions. We expect that the pattern of selection on petiole plasticity under natural conditions will strongly depend on the height and strength of the light gradient and the presence

of mechanical support. We thus believe that mimicking light gradients is a valuable tool improving our understanding of the interplay between plants and their environmental context.

High light trait values and their plasticity

Petiole length expressed under high light conditions did not affect the absolute increase of petiole length in response to shading, neither in the homogeneous shade nor in the vertical light gradient. The same was also true for the relationship between leaf lamina sizes in high light conditions and the absolute leaf lamina increase in response to homogenous shading. Both findings imply that, in contrast to our hypotheses, in *T. repens* absolute petiole and leaf area plasticity, do not depend on the values of these traits expressed in high light conditions. For *Arabidopsis thaliana* it has previously been shown that, between populations, plasticity and character mean in response to foliar shade were positively correlated for traits such as leaf number, number of basal stems and first seed set but not for stem length or rosette diameter, suggesting that it depends on the specific trait of interest whether its character mean and plasticity can evolve independently (Pigliucci *et al.*, 2003). Our results support the notion that trait values and their absolute plasticities can respond to selection independently. This result on the independence of absolute response of a trait and the trait value expressed under high light conditions is in contrast to the relation between high light trait values and their relative response to shading, as relative petiole elongation was negatively correlated with petiole length expressed under high light conditions. Genotypes with shorter petioles under high light conditions showed relatively higher levels of shade induced petiole plasticity than genotypes that have longer petioles under high light conditions. Plants expressing higher levels of relative plasticity may incur higher costs in terms of biomass investment into the elongating petiole or suffer from reduced biomechanical stability (Givnish, 2002; Henry & Thomas, 2002; Anten *et al.*, 2005).

Costs and benefits associated with plasticity

Plasticity results in net benefits.

Under competition for light, induced petiole elongation results in a better matching of the phenotype with the direct environment which will lead to enhanced plant performance and the response can, as often hypothesized but less frequently tested, hence be considered as adaptive plasticity (Dudley & Schmitt, 1996; Schmitt, 1997; Cipollini & Schultz, 1999; Donohue *et al.*, 2000). Consistent with this notion, our data showed that petiole elongation was associated with benefits in the vertical light gradient. In contrast to our expectations we found no benefits of increasing leaf area under homogeneously shaded conditions. Leaves can be expanded relatively easily by changing the SLA which does not require the allocation of more resources (Yano & Terashima, 2004). Other shade induced changes of physiological traits such as changes in photosynthetic characteristics may have contributed to the relatively high fitness of all plants grown in homogeneous shading independent of leaf area changes.

Costs of the expression of plasticity are small.
Contrary to our expectations, petiole elongation was not associated with costs in the homogeneous shade. This means that the plastic petiole response itself is beneficial if expressed under conditions where it leads to enhanced resource acquisition but expressing the response is not costly under conditions where the response cannot increase resource acquisition. Plasticity costs are often thought to be small in magnitude or negligible, suggesting that past selection has minimized these costs (Scheiner & Berrigan, 1998; Sultan & Spencer, 2002). In addition, it also indicates that under natural homogeneously shaded conditions, like forest understories, selection would not act against plastic petioles. However, *T. repens* plants are not often found in these habitats and the ability to respond to homogeneous shading most likely reflects a by-product of selection on elongation responses in natural vertical light gradients, as the same light cues trigger elongation in both types of shading.
Significant costs of the ability to express plasticity.
The ability to express petiole plasticity to homogeneous shading was associated with costs when plants were grown in high light conditions. Apparently, the costs of the ability to respond plastically, including the genetic, signal detection, maintenance and transduction costs (DeWitt *et al.*, 1998; Givnish, 2002), are high enough to reduce plant performance when the response is not induced.
Costs and benefits associated with high light trait values.
The production of larger petioles under high light conditions was associated with significant costs in terms of ramet number production but not with biomass production. The absence of a negative effect on total biomass may be explained by the positive correlation of petiole length and leaf area found under high light conditions (correlation coefficient 0.714; $p<0.001$, data not shown). Genotypes that produce longer petioles under high light conditions also produce larger laminas and this in turn may lead to the production of more biomass thereby compensating for the production costs of longer petioles. These results contradict the results of several other studies (Huber & Wiggerman, 1997; Stuefer *et al.*, 2002) and the general life history theory of a trade-off between ramet size and ramet number which predicts that genotypes with large ramets produce fewer ramets.

Conclusions

For plasticity to evolve, there must be genotypic variation in phenotypic expression of the trait across environments, and natural selection on that trait must differ among environments (Schmitt, 1997; Alpert & Simms, 2002; Schmitt *et al.*, 2003; van Kleunen & Fischer, 2005). Our findings showed genotypic variation in plastic responses in different environments and the selection analyses revealed selection to favour plasticity in one environment and to disfavour it in another. A study with *Ranunculus reptans* showed considerable variation in plasticity of modular architecture among genotypes (Fischer *et al.*, 2004) but the authors found that plasticity for that trait could hardly be selected for. Phenotypic plasticity is likely to evolve in environments that are heterogeneous in space or time (Wijesinghe & Hutchings, 1999; van Kleunen & Fischer, 2005; He *et al.*, 2004). Dynamic competitive grasslands like the river floodplains where the plants were originally collect-

ed from, meet the requirements favouring the evolution of plasticity, as light conditions vary in space and time and the environmental cue indicating shade is predictable. Our results suggest that under these conditions plasticity can evolve as a trait in its own right, independent of the trait value expressed in conditions that do not induce plasticity.

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The effects of cell number and cell size on petiole length variation in a stoloniferous herb

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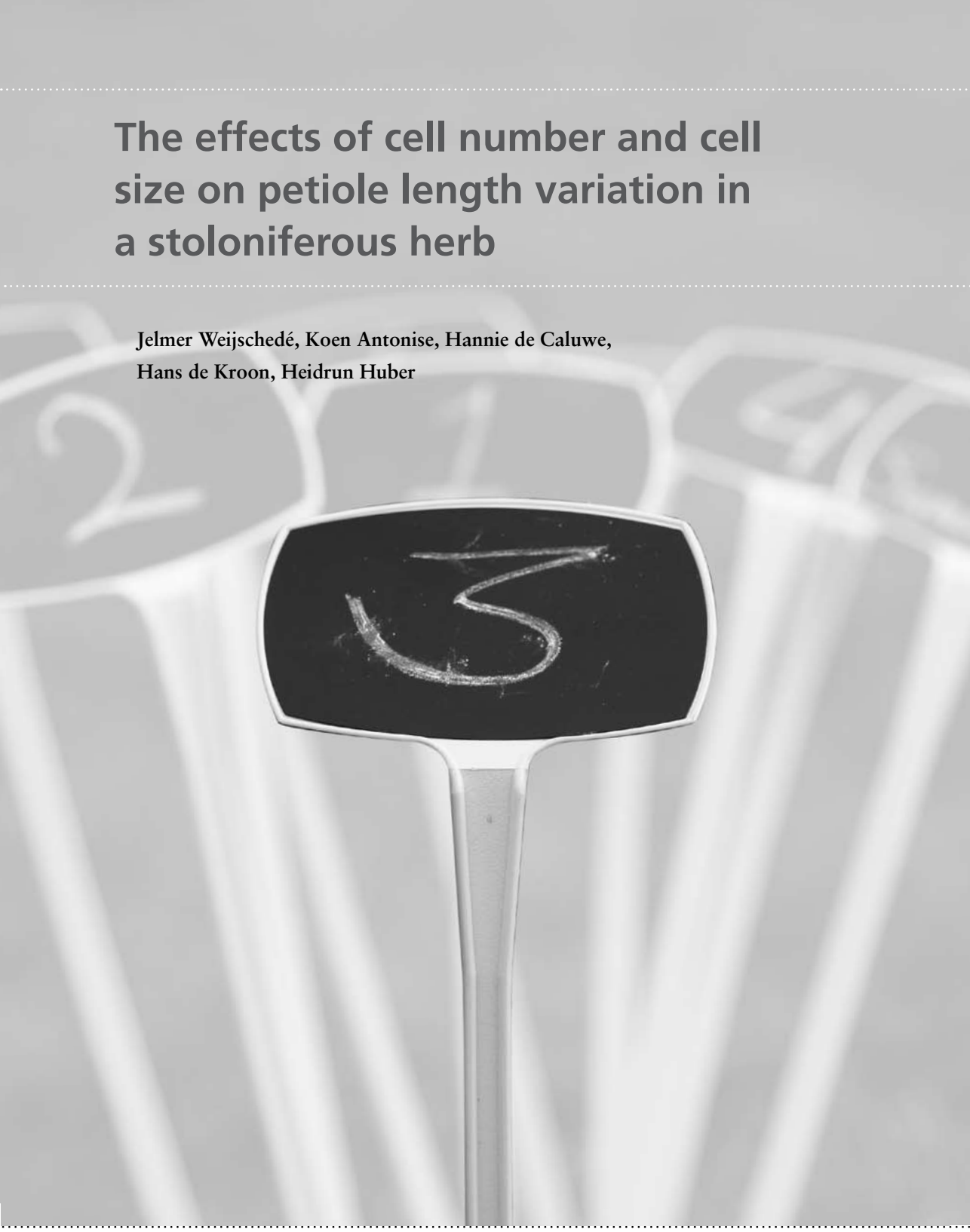

Abstract

In stoloniferous species the length of petioles is of pivotal importance as it determines the position of leaf blades within the canopy. From a mechanistic perspective two developmental processes, cell division and cell elongation are responsible for the length of a given petiole. This study aimed at quantifying the relative contributions of cell division and cell elongation to genotypic and plastic variation in petiole length of the stoloniferous herb *Trifolium repens*. 34 genotypes of *T. repens* were grown under high light conditions and simulated canopy shade. Cell numbers and cell lengths were measured on epidermal prints obtained from fully grown petioles of leaves which had been initiated in the experimental light conditions. Cell number was the main trait explaining petiole length differences among genotypes grown under high light conditions, while both cell number and cell length changed in response to shading. Our study revealed a strong negative correlation between shade-induced changes in cell number and cell length: genotypes that responded to shading by increased cell numbers hardly changed in cell length, and vice versa. Our results suggest that genotypic and phenotypic variation in petiole length results from a complex interplay between the developmental processes of cell elongation and cell division.

Introduction

The interplay of genotypic differences and induced plastic responses causes plants to express differences in morphological traits (Evans and Turkington, 1988; Aarssen and Clauss, 1992; Stratton, 1995). Evolutionary processes have shaped the morphology a plant displays under a given set of environmental conditions as well as the mechanisms responsible for realizing a given phenotype (Bradshaw, 1965; Via and Lande, 1985; Sultan, 1995). The ultimate outcome of evolutionary processes depends on the relation between costs and benefits associated with the developmental processes leading to a specific phenotype as well as costs and benefits associated with the phenotype itself (Lande and Arnold, 1983; Vantienderen, 1991; DeWitt *et al*., 1998; Pigliucci, 2005).

Plants can increase investment in structures that promote the acquisition of the most limiting resource (for example light) (Bloom *et al.,* 1985). For example, plants show changes in morphological and physiological characters in response to canopy shading (i.e. elongation of stems and stem analogues, increased biomass allocation to shoots and increased chlorophyll content (Schmitt and Wulff, 1993; Ballare *et al.,* 1994; Stuefer and Huber, 1998; Ballare, 1999; Heraut-Bron *et al.,* 1999; Schmitt *et al.,* 2003)) to increase resource capture under low light conditions. Elongation of vertical oriented spacers, like internodes or petioles, result in higher positioning of the light acquiring laminas in the canopy and has therefore been argued to reduce the negative effects of shading caused by neighbouring plants (Huber *et al.,* 1998; Schmitt *et al.,* 1999; Huber *et al.,* 2004). However, elongating of structures requires increased biomechanical strength to carry the weight of the leaves and to minimize the risk of physical failure (Givnish, 2002; Anten *et al.,* 2005; Liu *et al.,* 2007; Huber *et al., in press*). Although shade avoidance responses have a long history in plasticity research, so far it is not known how the underlying dynamic cellular processes (i.e. cell division and cell expansion) contribute to variation found in trait values among genotypes and to plastic trait variation (Smith, 2000; Sultan, 2004). This study provides new information about trait variation and plastic responses to shading at a cellular level and explores plausible evolutionary and functional consequences associated with these issues.

Plant organs, like petioles, develop from one active meristem in which cell division takes place with the meristem activity determining the final cell number in the structure (Mizukami and Fischer, 2000). Newly formed cells that no longer participate in the division process differentiate into their destined function and elongate until they reach their mature sizes (Tsukaya and Beemster, 2006). Cell division and cell elongation are distinctly different developmental processes which are separated in time and place, and, as has been shown for petioles in *Arabidopsis thaliana*, different genes are independently involved in the processes regulating cell proliferation and cell elongation (Tsukaya *et al.,* 2002). Size differences in morphological structures (i.e. petioles) can thus be achieved by differences in the total number of cells, difference in the size of the cells or by a combination of both. Genotypic differences in organ size or differences as a result of environmen-

tally induced plastic responses may not have the same cellular basis and different developmental mechanisms may contribute to genetic and plastic variation in organ size.
To date, different views exist concerning organ size control in plants (Fleming, 2002; Tsukaya, 2003). The classical cell theory states that, since cells are the basic units of a multi cellular organism, the cells are the unit of organogenesis and the final organ size is therefore primarily determined by cell number, but not by cell size (Mizukami and Fischer, 2000). This theory is supported by positive relationships found between final organ size and cell number (Bertin *et al.,* 2003; Cookson, *et al.* 2005). The organismal theory states that organ size is genetically determined and subject to selection, and both cell expansion and cell division can contribute to a different extent to the final organ size. This theory is supported by observations that organ size can, to a certain degree, be maintained when i.e. cell division is reduced, as the effects of decreased cell number can be buffered by increased cell size (Horiguchi *et al.,* 2006). Recently, in the context of understanding leaf morphogenesis, the neo cell theory has been proposed in which the cell is the unit of organogenesis and each cell is controlled by factors that govern the morphogenesis of which that cell (or cell population) is a part (Tsukaya, 2002). This theory suggests that a 'compensatory system' is involved in leaf morphogenesis and that an increase in cell size can be triggered by a decrease in cell number and vice versa. In light of this discussion, our study will present novel results on cell size and cell number contributions to genetically determined petiole length variation expressed under common environmental conditions and in environmentally induced plastically increased petiole lengths.

Trifolium repens genotypes are highly variable in morphological traits (including petioles) when grown under identical conditions (Weijschede *et al.,* 2006). To our knowledge no studies have been carried out explaining these differences at a cellular level. We previously reported that there is considerable variation in shade induced petiole elongation among genotypes while the absolute petiole increment due to shading was independent of the high light phenotype (Weijschede *et al.,* 2006). Investigating the cellular processes may increase our insight in the underlying developmental processes of the genotypic trait differences and the response to shading.

We studied the same 34 *T. repens* genotypes as used in our previous work (Weijschede *et al.,* 2006) to show how cell number and cell size contribute to petiole length differences expressed under high light conditions (genotypic differences) and to study how plasticity in cell number and cell size are involved in shade induced petiole elongation responses. Elongation was induced in all plants by reducing the PAR and the R:FR-ratio of the incident light. Specifically, we aim at answering the following questions: (1) to what extent do cell number and cell size contribute to petiole length differences under high light conditions, (2) to what extent do changes in cell number and cell size contribute to shade induced petiole elongation, and (3) (how) are both processes interrelated in shade induced elongation?

Materials and methods

Plants and pre-growth

T. repens, a very common perennial herb, is known to be highly variable in morphological and developmental traits such as petiole and internode length and leaf area (Jahufer *et al.,* 1997). When shaded, *T. repens* shows typical shade-avoidance responses like petiole elongation and internode elongation (Solangaarachchi and Harper, 1987; Marcuvitz and Turkington, 2000). Due to its stoloniferous growth form, only by adjusting the length of its petioles *T. repens* can place its laminas into upper layers of the canopy. The meristem from where a petiole develops is located directly under the base of the lamina. This site is photoreceptive and a major component in triggering the petiole elongation response (Thompson 1995).

In this study, 34 *T. repens* genotypes were used which expressed a two to three fold variation in petiole length under high light conditions. In 2001, plants were collected from a single natural population occurring at a river floodplain along the river Waal near Ewijk (the Netherlands, 51°52'54"N, 5°45'00"E) and were thereafter grown under common garden conditions (Weijschede *et al.,* 2006). On March 29 (2004), 6 cuttings were made of each of 34 genotypes. Cuttings consisted of a ramet with a well-developed root system and a lateral stolon with 5 ramets. These cuttings were each transferred to 0.18 x 0.22 x 0.05 m trays, filled with a mixture of sand and sieved potting compost (2:1). To ensure sufficient nutrients throughout the experiment, slow release fertilizer (Osmocote exact mini, 3-4M, Scotts International B.V. Heerlen, The Netherlands) was added to the soil mixture (4 grams per litre soil). Trays were filled and moistened two weeks prior to the beginning of planting as nutrient release starts after approximately two weeks.

Experimental set-up

On April 6th (2004), plants were subjected to either homogeneously shaded conditions or to control conditions, which we from here on will refer to as shaded and high light conditions, respectively. The youngest visible leaf was marked at the onset of treatments. To induce petiole elongation, plants were grown in cages covered with green transparent plastic (Lee Colortran International, Andover, UK, no. 122, fern green) which reduced the R:FR-ratio to 0.25 ± 0.01 (1 se) in the cages and the PAR to 31 % of the incident light. Control cages were covered with transparent plastic (Lee Colortran International, Andover, UK, no. 120, clear) which reduced the PAR to 76% and r:fr-ratio in this cage to 1.51 ± 0.02 (1 se). The experiment was conducted in a heated greenhouse. Incident light was supplemented by high pressure sodium lamps (Hortilux Schreder, 600Watt) and was 297 ± 13 (1 se) $\mu mol \cdot m^{-2} \cdot s^{-1}$ during the experiment. In our previous experiment we have shown that this setup was effective in simulating canopy shade and was sufficient to induce shade avoidance responses (e.g. petiole elongation) and to affect plant growth (Weijschede *et al.,* 2006).

Treatments (shade and high light) were applied for two weeks and replicated in

three temporal blocks (for practical reasons) with a one week interval between successive blocks. Each genotype was represented once in each block * treatment combination, leading to a total of 3 replicates per genotype per treatment. In total 204 plants were used for the experiment. During the experiment, plants were watered every other day using regular tap water.

Measurements

After two weeks the first newly developed petiole which was not yet visible at the onset of the experiment was harvested and used for the measurements. In previous experiments petioles have achieved their final length in approximately 10-14 days (pers. obs.). We thus assumed that leaves have finished their main elongation within the two weeks of treatments in this experiment as well. As in some genotypes leaf decay starts earlier in resource poor conditions, we would not have been able to use developmentally older leaves of a comparable developmental stage across genotypes and treatments. Petiole elongation takes place in the uppermost area below the leaf blade. If petiole elongation had not finished in some of the genotype/treatment combinations, the pattern of cell length and cell number response to treatments would have been different among the different segments. However, our results showed that the qualitative results were very similar among the three segments, which further supports the notion that the petioles used in this experiment had finished development.

The length of the petiole was measured and epidermal imprints (Schnyder *et al.*,1990) were made by gently laying the adaxial side of the petiole on liquid rubber (Coltene President Jet Plus, Altstatten, Switzerland). The imprint functioned as a mould and prints of the moulds were made with clear nail polish. Once dried, the prints were carefully removed from the moulds and put on an object glass. These prints showed clear patterns of the upper layer of the petiole under a light microscope (Olympus BX-40, magnification = 200). Epidermal cells were used to represent cell number and size in the petioles (Ridge and Amarasinghe, 1984; Allard and Nelson, 1991). Three zones of the petioles, all three approximately two centimetres long, were used to asses the cell number per millimetre: the top (just beneath the attachment of the laminas), the middle, and the bottom (just above the attachment of the petiole to the stolon). Within each zone, at three different randomly chosen places cell number per millimetre was counted. Areas around stomata were not measured because these cells have markedly different sizes. Average cell number per millimetre differed per zone, but the overall response to shading did not qualitatively differ for the three zones (repeated measures ANOVA, Treatment effect: $F_{1, 66} = 43.20$, $p<0.001$; zone of the petiole effect: $F_{2, 132} = 15.27$, $p<0.001$; Treatment*zone: $F_{2, 132} = 0.64$, $p=0.473$). Total cell number per petiole was estimated as follows: the true length of each zone of the petiole (one third of the total petiole length) was multiplied with the corresponding averaged cell number per millimetre and these three values were summed. We present cell length data (the inverse of the counted cell number per millimetre) for the middle zone, which is the most representative zone to show cell size variation among treatments, as close to the stolon fully developed cells tend to be longer and wider while close to the leaf blade fully developed cells tend to be smaller and narrower (data not shown).

Statistics

A two-way mixed model analysis of covariance was used to test for effects of treatments, genotypes and interactions on petiole length, cell number and cell size. The effects of the treatments were considered fixed factors, genotype and block were considered random. Genotypic means per treatment were used for all further analyses.

To investigate how total cell number and cell length contribute to the variation found in petiole lengths among genotypes under high light conditions, values of cell number and cell length found under high light conditions were correlated using the CORR-procedure (SAS, version 9.1). This procedure was repeated for total cell number and cell length found under shaded conditions.

To investigate how both processes contribute to shade induced petiole elongation we performed a multiple regression analysis with absolute petiole increment as the dependent variable and changes in cell number and cell size as the independent variables. Standardized values were used in this analysis (increase of genotypic means subtracted from the treatment mean and divided by the standard error of the treatment mean) to be able to compare the estimates of the relative change in cell number and cell size.

We investigated the degree of inter-correlation between changes in total cell number and cell length by correlating the relative changes (shaded values as compared to values found under high light conditions) in total cell number and in cell length. A non significant correlation coefficient would indicate that both processes contribute to petiole elongation independently. A significant positive correlation would indicate that both processes are involved in petiole elongation and that they may have coevolved. A significant negative correlation would indicate that a low response of cell number increase was compensated by a high response of cell size increase and vice versa. The program package SAS (version 9.1) was used to perform all statistical operations.

Genotypic differences under high light conditions

Petiole length was on average 98.9 (± 3.0, ise) mm for plants that were grown under high light conditions, with the genotypic means ranging from 67.3 to 136.0 mm (Fig. 1a and 2).

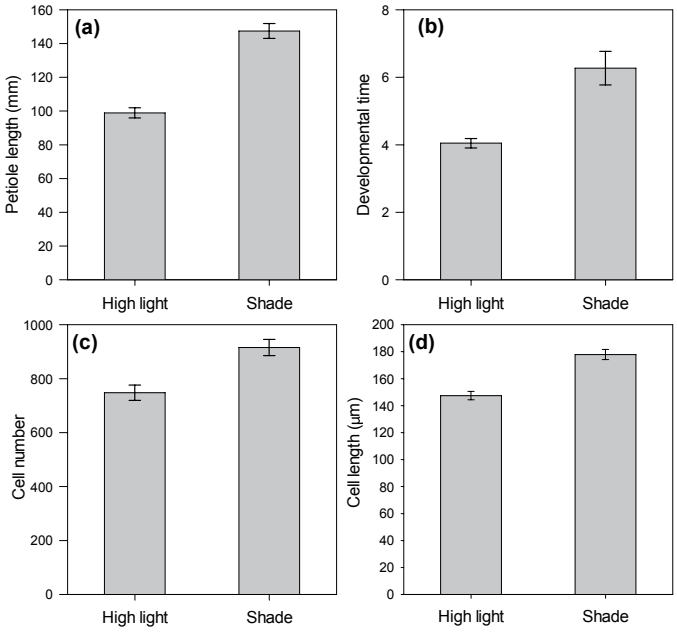

Figure 1. *Average treatment effects (±1 se) on (a) petiole length, (b) developmental time, (c) cell number and (d) cell length. All characters were significantly affected by treatments (Table 1).*

Table 1. *F-VALUES and their significances of mixed-model ANCOVA of the effects of treatments, genotypes and blocks on petiole length, developmental time (days needed to produce one petiole), cell number and cell length. Significances are as follows: ns: p>0.10; $: 0.10≥p>0.05; *: 0.05≥p>0.01; **: 0.01≥p>0.001; ***: p<0.001*

Source	df	petiole length	developmental time	cell number	cell length
Treatment	1	236.0 ***	21.60 ***	62.3 ***	97.6 ***
Genotype	33	5.3 ***	1.4 ns	7.2 ***	3.9 ***
Treatment x Genotype	33	1.1 ns	1.5 $	1.1 ns	0.9 ns
Block	2	1.1 ns	0.7 ns	2.1 ns	10.1 ***
Error	134				

Petiole length was positively correlated with the total cell number per petiole (correlation coefficient r=0.821, p<0.001), showing that under high light conditions longer petioles consist of more cells than shorter petioles (Fig. 2a). Cell length did not correlate with petiole length under high light conditions (r=0.110, p=0.537, Fig. 2b).

Overall shade effects

All plants that were moved from high light to shade conditions responded to shading by producing longer petioles (Table 1, Fig. 1a). Petioles were on average 49% longer under shaded conditions than under high light conditions. Total cell number increased on average with 22% and cells were on average 21% longer in the shade than under high light conditions (Fig. 1c and 1d). Under shaded conditions, petiole length positively correlated with cell number (r=0.788, p<0.001, Fig. 2a) but not to cell length (r=-0.022, p=0.904, Fig. 2b). Under shaded conditions the plastochron index (i.e. the timespan between the production of successive ramets (Birch and Hutchings, 1992a; Birch and Hutchings, 1992b; Huber and Stuefer, 1997; Huber *et al.* 1999)) increased by 55%, indicating that in the time needed to produce 3 new ramets under control conditions, only 2 new ramets could be produced under shaded conditions (Fig. 1b).

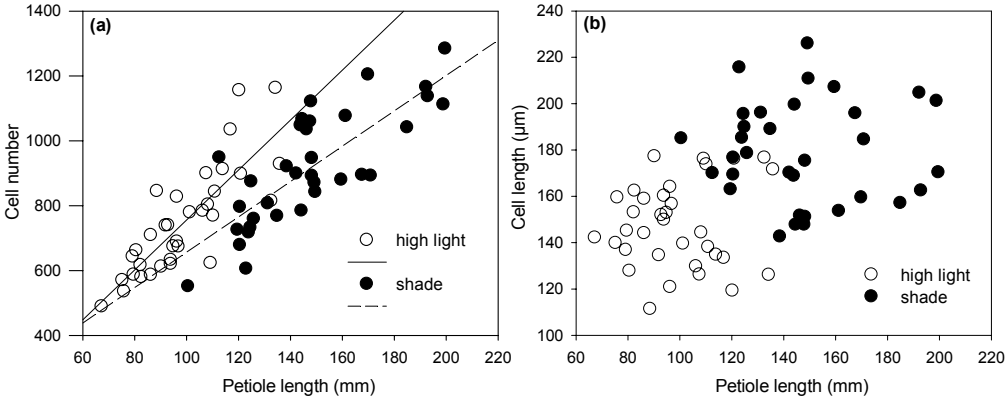

Figure 2. *Relation between petiole length and (a) total cell number and (b) cell length. Points show genotypic mean values. Open circles represent values under high light conditions, closed circles represent shaded values. Significant correlations were found for petiole length and cell number under high light conditions (correlation coefficient r=0.821, p<0.001, solid line) and for petiole length and cell number under shaded conditions (r=0.788, p<0.001, dashed line).*

Cell number and cell size changes

The absolute petiole length increment was not correlate with the petiole length found under high light conditions (r=0.002, p=0.993, Fig. 3a) indicating that shade induced petiole plasticity was independent of petiole length expressed under high light conditions.

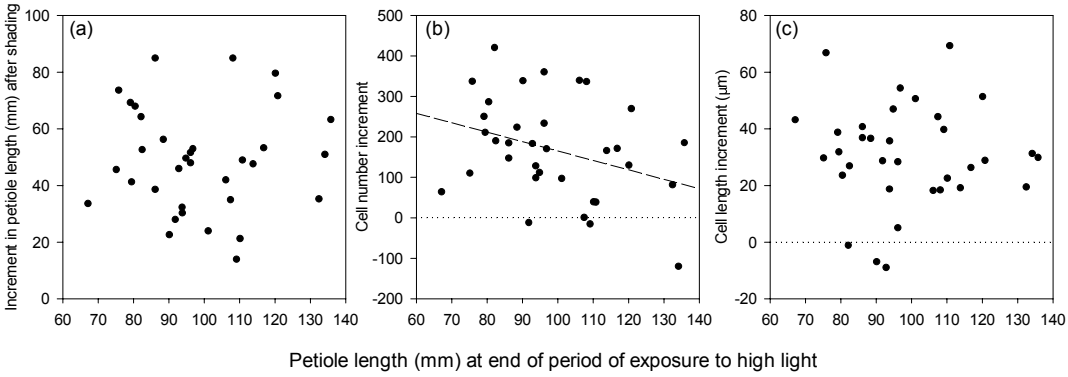

Figure 3. *Relation between petiole lengths found under high light conditions (x-axes) and (a) absolute petiole length increase, (b) absolute cell number increase and (c) absolute cell length increase. The dashed line indicates a marginally significant correlation between high light petiole length and absolute increase in cell number in response to shading (r=-0.331, p=0.056). Increase in petiole length, cell number and cell length was calculated as absolute differences of petiole length, cell number or cell length under shaded conditions and high light conditions. Points represent genotypic means.*

Petiole length found under high light conditions marginally negatively correlated with the increase in cell number in response to shading (r=-0.331, p=0.056, Fig. 3b), suggesting that petioles which are short under high light conditions tended to respond to shading by a stronger increase in cell number than genotypes characterized by longer petioles under high light conditions. There was no correlation between petiole length under high light conditions and the increase in cell size (r=-0.016, p=0.928, Fig. 3c). Table 2 shows the contribution of an increase in cell number and cell length to shade-induced elongation of petioles. Each increase significantly affected petiole elongation.

We found a negative correlation between the relative increase in cell number and the relative increase in cell length in response to shading (r=-0.380, p=0.027, Fig. 4a). Along this negative correlation, the majority of the genotypes showed both an increase in cell

Table 2. *RESULTS of a multiple regression analysis testing the extent to which the increment in petiole length was determined by the increase in cell number and cell length.*

petiole length increment	df	Estimate	st err	t Value	Pr > |t|
Intercept		1.000	0.053	18.8	<.0001
cell number increase	1	0.246	0.059	4.17	0.0002
cell length increase	1	0.151	0.059	2.57	0.0153
Figure legend					

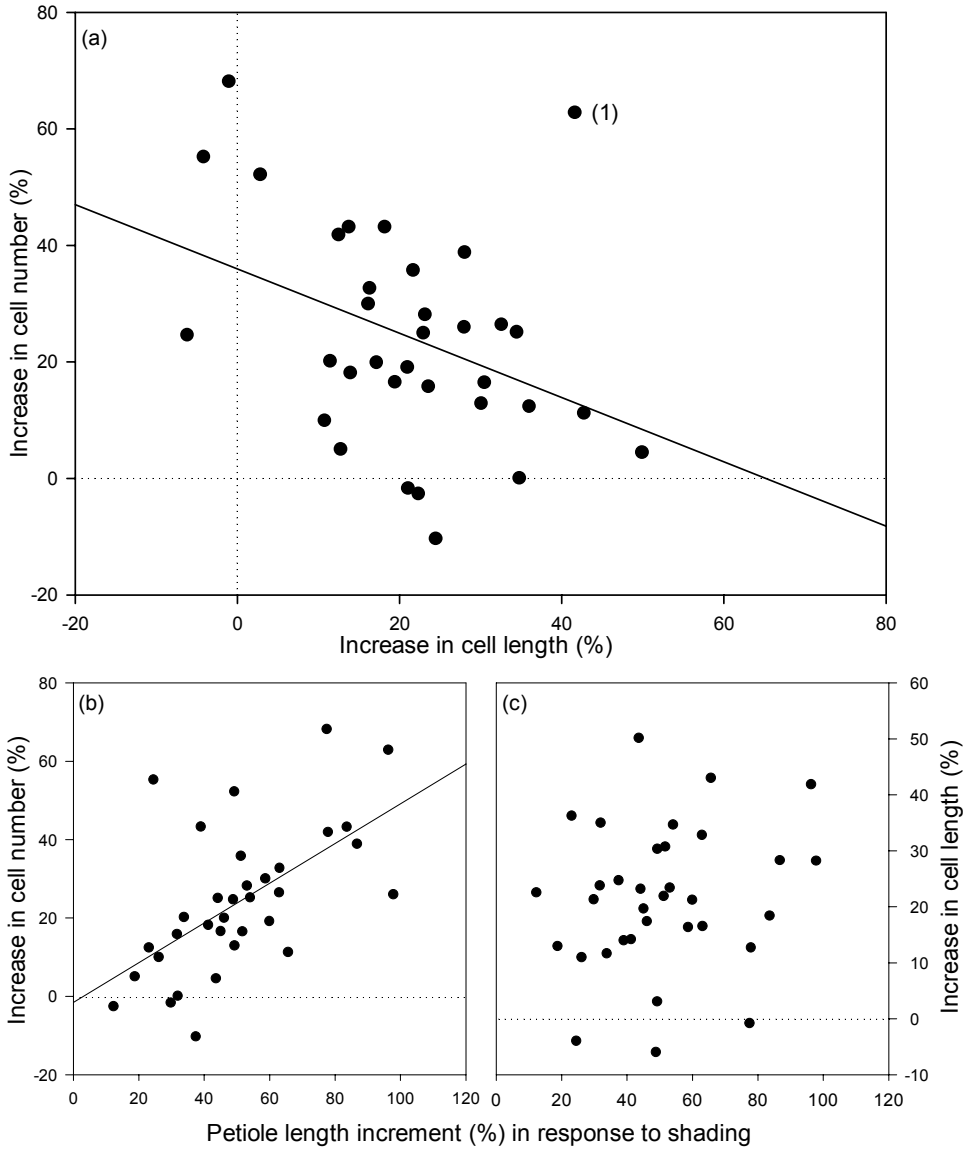

Figure 4. *(a) Relation between relative change in total cell number (y-axes) and cell length (x-axes) in response to shading. Correlation (solid straight line) was calculated with all data points (r=–0.380, p=0.027) and with all data except data point (1) (r=–0.539, p=0.001). (b) and (c) show the relationships between relative increase in petiole length and relative change in cell number (r=0.179, p=0.329) and cell size in response to shading (r=0.179, p=0.329). Dots represent genotypic means.*

number as well as an increase in cell length. Plants that responded to shading mainly by increasing their total cell number per petiole hardly increased their cell length or even produced shorter cells and vice versa. Only one genotype was able to do both a strong (63%) increase in cell number as well as a strong increase in cell length (42%) in response to shading. Since this genotype appeared to be an outlier on figure 4a, analysis was repeated while omitting this data point, which did not qualitatively affect the outcome of the analysis ($r=-0.539$, $p = 0.001$). The relative petiole length increment due to shading positively correlated with cell number increment ($r=0.585$, $p<0.001$, Fig. 4b) showing that genotypes that expressed a stronger elongation response also increased their cell number to a larger extent compared to genotypes that expressed less elongation. By contrast, we found no correlation between petiole elongation and cell length increase ($r=0.179$, $p=0.329$, Fig. 4c).

Discussion

Phenotypic variation can be intrinsic, meaning it is expressed regardless of the environmental conditions or plastic where it varies according to environmental conditions. In this paper we show how the two major determinants of organ size (cell number and size) contribute to intrinsic and plastic variation of petiole length in *T. repens* under high light conditions and in shaded conditions. Our results suggest a complex relationship between the distinctly different processes determining petiole length (cell division and cell elongation). Surprisingly, there was a high genetic variation in the relative contribution of changes in cell number and cell length to plastic petiole elongation, resulting in a trade-off in the change of cell length and cell number under shaded conditions. As both cell elongation and cell division are associated with different cost and benefits, the relatively higher investment into one of the developmental processes is likely to have potentially large evolutionary and ecological implications.

Determinants of genotypic variation in petiole length

Genotypes of *T. repens* display a 2-3 fold variation in petiole length if grown under common garden conditions. Our study revealed that genotypic differences in petiole length can be directly related to differences in cell number: petioles produced under high light conditions that are twice as long contain on average twice as many cells. Although both cell division and cell elongation require considerable amounts of energy and carbohydrates (Voesenek *et al.,* 2004) cell elongation is considered being relatively cheaper since this process only requires the production of extra cell wall material while supplementary cell number production also requires additional DNA-replication as well as additional cell wall material. It is thus surprising that the cost intensive process of cell division mainly contributed to genetic variation in petiole length. One possible explanation may be that biomechanical consequences associated with differences in cell length may have lead to selection against the production of longer petioles by means of increased cell expansion rather than cell division. Longer petioles need increased mechanical strength to carry the

weight of the leaves and to avoid physical failure (Givnish, 2002; Anten, *et al.* 2005). This may be better achieved by an increase in cell number than by an increase in cell size as tissue made of more but smaller cells might have a higher density of cell walls providing rigidity and strength, and thus be more resistant to buckling and breaking. In a study using a smaller set of Trifolium repens genotypes it has been found that increased cell number indeed does lead to increased flexural stiffness (Huber *et al., in press*) which corroborates our interpretation. This indicates that strong selection pressures may have lead to a proportional increase of cell number with increasing petiole length in order to provide sufficient rigidity of the petioles growing under open conditions. Under open conditions plants have been argued to be subjected to additional mechanical forces such as relatively higher wind speed, which requires sufficient investment into organ strength (Anten *et al.,* 2005), thereby selectively favouring investment into expensive cell division rather than relatively inexpensive cell elongation.

Determinants of shade-induced petiole elongation

All genotypes responded to shading by elongating their petioles and the absolute increment was independent of the petiole length found under high light conditions, confirming our earlier observation (Weijschede *et al.,* 2006). Much is known about the molecular basis and the signal transduction pathways of shade-induced elongation responses (Smith, 2000; Chen *et al.,* 2004; Vandenbussche *et al.,* 2005), as well as about their ecological and evolutionary implications (Dudley and Schmitt, 1996; Schmitt *et al.,* 1999; Weinig, 2000; Donohue *et al.,* 2000; Callahan and Pigliucci, 2002; Huber *et al.,* 2004). One may argue that selection will act on the response rather than on the specific cellular mechanism (cf. Calboli *et al.,* 2003). However, the ultimate link between the molecular processes and the expression of stem length involves the control of different developmental processes (Beemster and Baskin, 1998; Tardieu *et al.,* 2000; Francis and Sorrell, 2001; Barrero *et al.,* 2002; Fleming, 2006; Tsukaya and Beemster, 2006). How final organ size is determined by the environment appears to be a complex mechanism which, in fact, we know very little about. The observed large variation in petiole length increment among genotypes, unrelated to the high light petiole lengths, leaves the potential for selection to act specifically on the elongation response.

On average, plastic petiole elongation was achieved by both an increase in total cell number and an increase in cell length. This result contradicts the common view that shade and flooding induced elongation is usually the result of cell elongation (Child *et al.,* 1981; Reed *et al.,* 1993; Peeters *et al.,* 2002; Tsukaya *et al.,* 2002; Cox *et al.,* 2004; Kozuka *et al.,* 2005; Voesenek et al., 2006). However, for some aquatic species variable contributions of cell division and cell elongation in flood-induced elongation have been demonstrated (Ridge and Amarasinghe, 1984; Ridge, 1987). Shade induced change in cell number and cell length were negatively correlated, indicating that these two distinctly different developmental processes (cell division or cell expansion), which operate separately in space and time, both determine in concert the given plastic petiole length increase. These results on the relative contribution of cell number and cell length can be compared with the shade

induced responses of internode length and number in determining stem height. For two *Polygonum* species, Griffith and Sultan found that, in contrast to our results, only the size of internodes, but not the number thereof, responds plastically to shading (Griffith and Sultan, 2006). In contrast to our results the lower degree of internode length plasticity in one of the species was not compensated by higher plasticity in internode number and inevitably resulted in lower height plasticity. The potential to change both cell number and cell length allows *T. repens* to compensate for lower plasticity in one of the traits, thereby ensuring optimal elongation.

Developmental timing

Much research into organogenesis has been conducted on leaf lamina development (Tardieu *et al.,* 1999; Kaplan, 2001; Tsukaya, 2002; Aguirrezabal *et al.,* 2006; Fleming, 2006). Laminas develop and expand as a whole to their final size during which cell division and expansion take place in a coordinated fashion throughout the leaf. This developmental pattern determining leaf expansion is in contrast to the developmental pattern of petiole extension in *T. repens*. Petiole extension is achieved by cell proliferation in one meristem located at the top of petiole near the base of the laminas and subsequent cell extension within the uppermost few centimetres of the petiole (Thompson, 1995). Petiole extension is thus restricted to developmental processes within the upper part of the petiole, while the cells in the lower part of the petioles have already reached their final shape. This implies that each part of the petiole may have a different developmental window in time in which cell proliferation and cell extension can respond to environmental triggers, enabling petioles to fine-tune their final length. In contrast to leaf lamina expansion, in petioles these processes are thus not coordinated throughout the whole organ. This conclusion is supported by the fact that petioles of stoloniferous plants can stop elongation as soon as laminas reach favourable light conditions (Leeflang *et al.,* 1998), implying that both processes are put to a halt as soon as the lamina intercepts a sufficiently high radiation, preventing a plant to invest into elongation which will not increase light interception and may put plants at increased risk of physical failure. Although we know that petiole extension can stop as soon as high light is reached, we do not know yet which triggers determine the halt of cell proliferation and expansion in homogeneous light conditions. A possible trigger may be resource shortage, but further research is needed to answer this question.

Interrelationship of cell number, cell size and organ size

We used multiple genotypes grown under identical conditions and showed that, under high light conditions, longer petioles consist of more cells rather than longer cells. These data are consistent with the classical cell theory stating that final organ size is determined primarily by cell number (Mizukami and Fischer, 2000; Bertin *et al.,* 2003; Tsukaya, 2003; Cookson and Granier, 2006). Petiole length differences in *T. repens* thus appear to have evolved via selection on the correlation between organ size and cell number. In response to shading, petioles elongated and both size and number of cells contributed to the total

petiole plasticity and small contributions (or even a reduction) of one factor was buffered by an increased contribution of the other factor. These results are in line with the organismal theory stating that size (or in this case, the response) is genetically determined and subject to selection, and both cell expansion and cell proliferation can contribute to a different extent to the final size (Hemerly *et al.,* 1995; Kaplan, 2001). The negative correlation between changes in cell number and cell length further suggests that a compensatory system operated beyond the cellular level to ensure sufficient elongation. This is in line with the neo cell theory which suggests that a 'compensatory system' is involved in leaf morphogenesis and that an increase in cell size can be triggered by a decrease in cell number and vice versa (Tsukaya, 2002).

Cell number and cell length might have different functions that were selected for to different extents in different genotypes. The genotypes used in this study originate from a Dutch floodplain grassland characterized by high temporal and spatial environmental heterogeneity and species composition (Voesenek *et al.,* 2004; van Eck *et al.,* 2004). The herbaceous vegetation is in fact composed of a dynamic mosaic of different micro-habitats and each single clone of the stoloniferous species *T. repens* may experience different environmental conditions in space and in time. The most prevalent microhabitat conditions experienced by a clone may be one of the forces selecting greater responsiveness of either cell proliferation or elongation. Relatively sparse microhabitats might favour responsiveness in cell number since this character can preserve cell density and thus petiole constructive stiffness (Huber *et al., in press*). On the other hand, genotypes originating from more dense microhabitats might present greater plasticity in cell elongation which may be less costly in a dense canopy where leaves can lean on their neighbours and do not depend on their own petioles' rigidity for preventing physical failure. This study shows that in *T. repens* petiole length variation results from a complex interplay between different developmental processes. Further investigation of the costs and constraints involved with these developmental processes as well as their ultimate effects on plant performance under different environmental settings will enhance our understanding of how selection operates in shaping trait characters under various environmental conditions.

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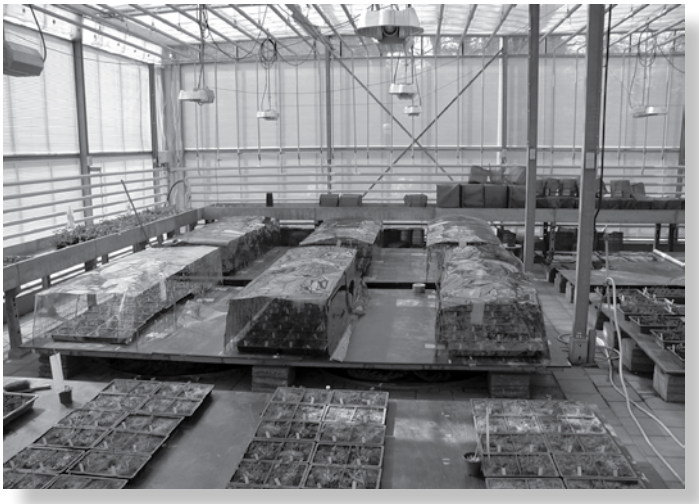

Shade induced changes in biomechanical petiole properties in the stoloniferous herb *Trifolium repens*

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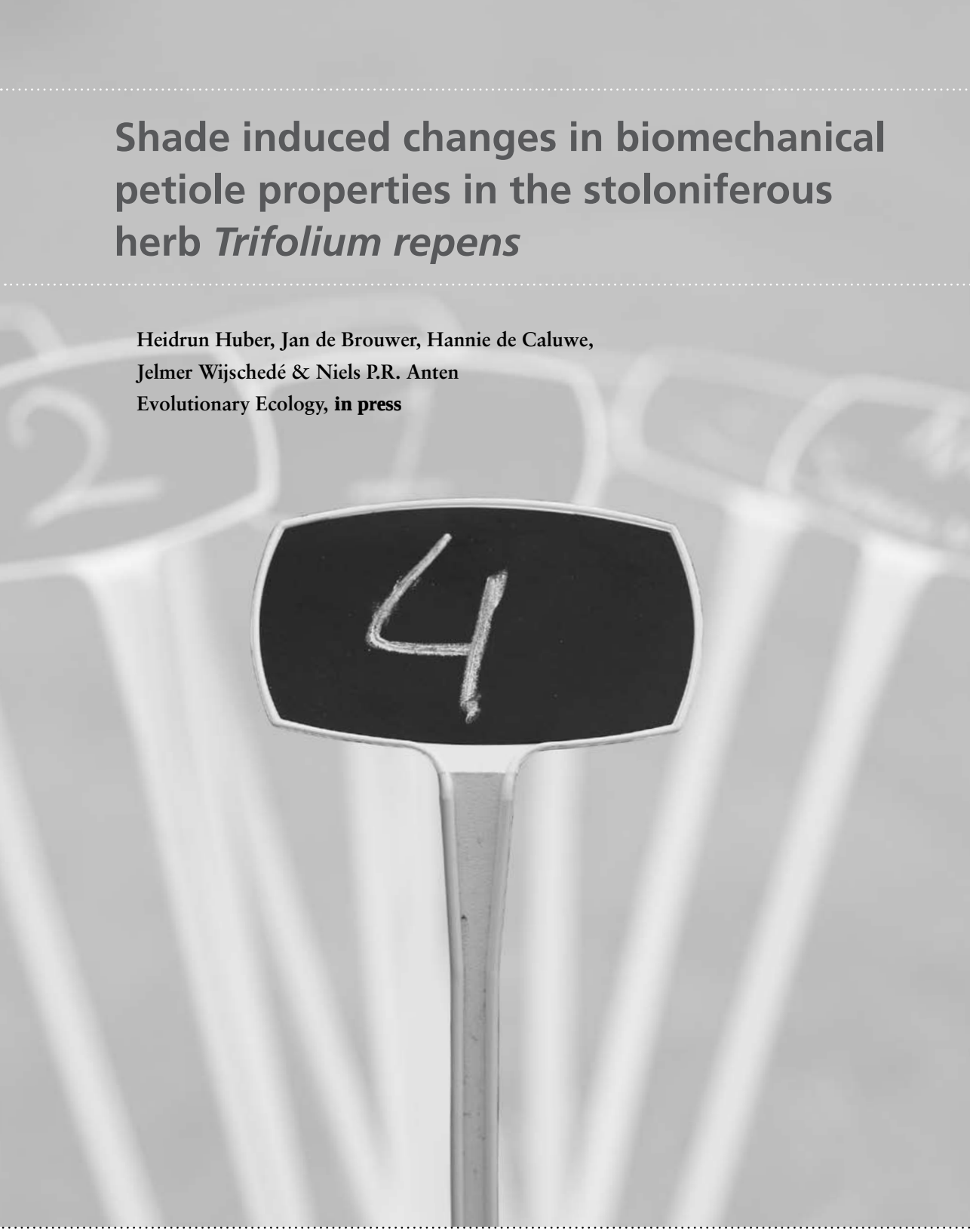

Summary

Increased cell number and cell length both contribute to shade induced elongation of petioles which enables stoloniferous plants to place their leaf lamina higher up in the canopy. Although petiole elongation is assumed to be beneficial, it may also imply costs in terms of decreased biomechanical stability. We test the hypothesis that shade induced elongation changes the biomechanical properties of petioles and that the underlying mechanisms, cell division and cell elongation, differentially affect biomechanical properties.

This was done by subjecting 14 genotypes differing in the relative contribution of cell size and cell number to shade induced elongation responses to high light conditions and to simulated canopy shade. Developmental traits (cell size & cell number), morphological traits characterizing the petioles, as well as biomechanical characteristics were measured. Our results show that, comparable to stems of non-clonal plants, the rigidity of a petiole's tissue (the Young's modulus) increases, leading to increased flexural stiffness of petioles subjected to shading. Increased flexural stiffness proved to be associated to increased performance under shaded conditions. Our results also indicate that cell number affected the material properties and the flexural stiffness of petioles. However, the degree and pattern of the effects differed between light environments. Shade induced increase in cell number translated into shade induced increase of Young's modulus and flexural stiffness. However, genotypes producing relatively larger cells under shaded conditions experienced a decrease in tissue rigidity.

In concert our results indicate that the pattern of selection on flexural stiffness, and thereby also on shade induced changes of cell number and cell size differs among light environments.

Introduction

Most natural environments are characterized by fine-grained temporal and spatial variation in the availability of essential resources such as light, water and nutrients thereby exerting different selection pressures on plant development and morphology (Kalisz, 1986; Stewart and Schoen, 1987; Stratton, 1995; Stratton and Bennington, 1996). If changes in phenotype and/or developmental pattern confer a fitness advantage adaptive plasticity will evolve (Dudley and Schmitt, 1996; Kingsolver, 1995). The photomorphogenetic induction of shade-avoidance responses in crowded plants is a well-studied example of adaptive plasticity (Ballare *et al.*, 1990; Casal and Smith, 1989; Griffith and Sultan, 2006; Morgan and Smith, 1979; Schmitt *et al.*, 2003; Schmitt and Wulff, 1993). Upon shading many herbaceous plants elongate their vertically oriented spacers (i.e. internodes and/or leaf petioles) in order to place their leaves in higher positions of the canopy which results in improved light acquisition (Callaway *et al.*, 2003; Dudley and Schmitt, 1996; Franklin and Whitelam, 2005; Huber *et al.*, 1998; McGuire and Agrawal, 2005; Schmitt and Wulff, 1993; Tsukaya *et al.*, 2002). Plastic spacer elongation has been shown to confer clear advantages in herbaceous canopies and can hence be expected to be under positive selection in a wide range of plant communities (Causin and Wulff, 2003; Donohue *et al.*, 2000; Huber and Wiggerman, 1997; Leeflang *et al.*, 1998; van Kleunen and Fischer, 2003; Weinig, 2000).

Shade induced spacer elongation is associated with other structural changes of the elongating organs. Elongation of internodes and petioles usually implies a change in resource allocation, leading to changed root:shoot ratio of shaded plants (Huber *et al.*, 2004; Maliakal *et al.*, 1999), reduced investment into defense (Cipollini, 2004; Thaler and Bostock, 2004) or to the production of longer, but thinner stems or stem analogous (Anten *et al.*, 2005; Liu *et al.*, 2007). Although the production of thinner stems may reduce cost in terms of the amount of carbohydrates used per unit of stem length, it may also entail significant costs in terms of reduced biomechanical stability, which carries the risk of lodging or breaking of the elongating structures (Anten *et al.*, 2005; Henry and Thomas, 2002; Mitchell, 2003). However, plants have been shown to be able to compensate for the production of thinner stems, by increasing the rigidity of stem tissue (the Young's modulus, *E*) (Hikosaka *et al.*, 2005; Liu *et al.*, 2007). The Young's modulus and the cross sectional area of the stem interact in determining the flexural stiffness of an organ, which describes how easily an organ bends and is thus its ability to carry it's own weight, and resist external forces such as wind (Niklas, 1992; Read and Stokes, 2006). An increase in the Young's modulus can therefore at least in part compensate for a reduction in diameter.

Erect and clonal plants use two distinctively different types of spacers to shift their leaf blades higher up in the canopy. While in erect plants internodes are the main organ showing adaptive elongation responses, in clonal plants the vertically oriented petioles are assuming the same function (Huber, 1996; Huber *et al.*, 1998). From a biomechanical perspective, elongation of internodes and petioles are subjected to different constraints

(Liu *et al.*, 2007). In erect plants each internode has to support its own weight, as well as the weight of the internodes branches and leaves that are formed above it. The extension of a given internode thus affects the positioning of leaves and branches situated on all successive internodes. Although the vertical internodes get thinner under shaded conditions, their rigidity (i.e., Young's elastic modulus) tends to increase (Anten *et al.*, 2005; Liu *et al.*, 2007). Morphological changes of the stem internodes affecting the ratio between stem cavity and the supporting tissue may lead to additional structural stability with limited resource investment. In stoloniferous plants each leaf is supported by a separate petiole, which in terms of biomass use for vertical support is less efficient than producing a single stem; each petiole has to carry its weight in addition to that of the lamina. Extension of petioles, in addition, only affects the lamina placement of a single module, but not of other attached modules. The modular structure of stoloniferous plants and the potential of each module to adjust its own structure to the prevalent environmental conditions make clonal plants very flexible and able to efficiently respond to fine scale variation in light conditions (de Kroon *et al.*, 2005). Yet the biomechanics of stoloniferous plants have hardly been investigated and little is know about the consequences of shade induced petiole elongation for mechanical stability (Liu *et al.*, 2007)

Extension of plant structures can be achieved by either cell extension or cell division (Beemster *et al.*, 2006). In petioles cell division takes place in one meristem (Mizukami and Fischer, 2000), which is situated at the top of the petiole in *Trifolium repens* (H. Huber, pers. obs.). Newly formed cells that no longer participate in the division process differentiate into their destined function and elongate until they reach their mature sizes (Tsukaya and Beemster, 2006). Both, cell division and cell elongation are distinctly different developmental processes which are separated in time and different genes are independently involved in the processes regulating cell proliferation or cell elongation (Tsukaya *et al.*, 2002). As cell division involves additional investment into cell material, a spacer elongation through cell division may be more costly in terms of biomass compared to spacer elongation by means of cell elongation. On the other hand, tissue made of more but smaller cells might have a higher density of cell walls providing rigidity and strength, and thus be more resistant to buckling or rupture. Previous research has shown genetic variation in the relative contribution of both processes to shade induced elongation (Weijschede *et al.*, in press). It is, unclear however, in how far cell size and cell elongation affect biomechanical characteristics of petioles.

In this paper we test the hypothesis that the investment into the production of more, but shorter cells increases the biomechanical stability of petioles. We expect that petioles elongating primarily by increased cell number will be more rigid, and less likely to buckle, than those elongating primarily through (cheaper) cell elongation. This difference will affect the degree to which petioles can independently maintain their vertical position or in which they rely on neighbor plants for support. Such different consequences associated with the relative contribution of cell size and cell number to shade induced petiole extension entails that, depending on the specific environmental conditions and local structure of the vegetation, different developmental processes will be selected for.

We will present data on how shade-induced changes in developmental and morphological traits affect biomechanical characteristics of the petioles in the stoloniferous herb *T. repens*. We aim at providing answers to the following research questions:

1. Do the biomechanical properties of petioles depend on light conditions?
2. Are the biomechanical properties of petioles linked to morphological traits such as petiole length, petiole thickness, leaf area and leaf weight?
3. Do the biomechanical properties of petioles depend on cell length and cell number per unit of petiole length?
4. Do shade-induced changes of cell number and cell length affect the biomechanical properties of petioles?
5. Do biomechanical properties of petioles affect plant performance?

Material and methods

Species description and pre-treatment conditions

T. repens is an abundant species growing in pastures and lawns, on riverbanks and roadside verges throughout temperate Europe and other parts of the world. It produces monopodial above-ground stolons which root on their nodes and form ramets consisting of an internode, a node with one leaf and an axillary meristem, and a root system. The axillary meristem can give rise to either a lateral stolon or an inflorescence (Huber and During, 2000). Plants can produce two to three ramets (i.e. repeated modules) on the primary stolon per week. Petioles are the main structures determining the positioning of light acquiring structures in the canopy (Huber *et al.*, 1998; Huber and Wiggerman, 1997). Genotypes of this species vary greatly in petiole traits, such as constitutive petiole length (Weijschede *et al.*, 2006), plastic petiole elongation (Weijschede *et al.*, 2006), and in the extent to which cell division and cell elongation contribute to shade induced petiole elongation.

All plants used in this experiment were randomly collected in a floodplain pasture along the river Waal near Ewijk (The Netherlands, 51°52'54"N, 5°45'00"E) in 2001. The distance between sampled plants was at least 5 m. The genetic uniqueness of sampled plants was confirmed by molecular fingerprinting (AFLP, four primer combinations, 145 markers). After collection the plants were maintained under uniform outside conditions in the Botanical Garden of the Radboud University in Nijmegen. Plants were grown in individual pots in a substrate consisting of a 1:1 mixture of sand and potting compost. Plants were repotted twice a year. In autumn 2005, 14 genotypes were moved to the heated greenhouse and planted in flat trays filled with a 1:1 mixture of sand and potting compost. These 14 genotypes represent a subset of the 34 genotypes used by (Weijschede *et al.*, 2006; Weijschede *et al.*, *in press*). Genotypes were chosen to represent a wide range of shade induced changes of petiole cell size and cell number and thus to represent the whole variation in developmental mechanisms regulating petiole extension reported by (Weijschede *et al.*, *in press*).

Treatments

In March 2006, two lateral cuttings were made from all genotypes, and these cuttings were planted individually into flat trays (l*b*h:16*14*4), filled with a 1:2 mixture of sand and sieved potting compost and an addition of slow release fertilizer (Osmocote+, Sierra International, 4 gr·l^{-1}) to prevent nutrient limitation. Each lateral cutting consisted of a rooted ramet and a lateral stolon consisting of 3-5 ramets. The cuttings were pinned to the soil with plastic coated wire to ensure good contact with the ground. The plants were covered with a transparent plastic foil for three days to reduce evaporation and minimize negative effects of planting on stolon development. The substrate was kept moist by watering three times a week. This planting was repeated in four temporal plots, with three to four days intermittent individual plantings. The total number of plants used in the experiment was 112 (14 genotypes, 2 treatments, 4 blocks).

Four weeks after planting the plants were subjected to the shading treatments. Shading was induced by placing the plants into shade cages covered by one layer of a green plastic film (Lee filter no 122, fern green, Lee Colortran International, Andover, UK), which reduced light availability to 20%, and the red:far-red ration to 0.25. Control plants were grown in cages covered with a transparent plastic (Lee filter no 130, clear, light transmittance of 80%, red:far-red ratio:1.55) to keep microclimatic conditions comparable between shading treatments (J. de Brouwer, unpublished data). Plants were subjected for 2 weeks to the treatments.

Measurements

All measurements were performed on the third youngest ramet with a fully unfolded leaf lamina. As successive ramets can differ in their petiole length dependent on their developmental stage and the developmental speed can differ among treatments, we measured petioles of the same developmental stage (i.e. a local plastochron index of 3 (Birch and Hutchings, 1992; Huber and Stuefer, 1997).

The third-youngest petiole was detached with a razor blade at its base, and its length and diameter in two perpendicular directions were measured with a caliper to the nearest millimeter and a leaf thickness meter to the nearest 0.01 mm, respectively. Leaf lamina area was measured with a leaf area meter (Licor, LI 3100). The petiole diameter measured perpendicular to the surface of the leaf lamina was used for further calculations.

Young's elasticity modulus (E, MN m^{-2}, which is a measure for the rigidity of a material, was measured with a universal material testing machine (Instron Model 5542, Canton, USA) using a three-point bending method following Liu et a. (Liu *et al.*, 2007). This method has the advantage that it keeps the force perpendicular to the petiole. The middle section of the petiole was placed horizontally over two supports that were 2-3 cm apart. The distance was adjusted such that it was two-thirds of the length of the petiole segment. Vertical applied forces (F, N) and resulting deflections (δ, m) were recorded. Young's modulus was calculated as follows (Gere and Timoshenko, 1999):

$$E = (FL^3) / 48\ \delta I \qquad (1)$$

Where L is the length between the supports (m) and I the second moment of area (m^4), which is a measure for the degree to which the cross sectional area of a support member contributes to mechanical stability (Gere and Timoshenko, 1999). I was calculated from the cross-sectional dimensions of the petiole assuming it to have a parabolic shape (see Fig. 3.3. in (Niklas, 1992)):

$$I = (16/175)\, r_a^3\, r_b \qquad (2)$$

With a length equal to r_a and a width equal to $2*r_b$ (Niklas, 1992). Also the flexural stiffness of the petiole was calculated as the product of E and I (EI, MN m^2).

Immediately after measuring biomechanical characteristics, we made epidermal imprints of each petiole. This was done by gently placing the adaxial side of the petioles on liquid rubber (Coltende President jet Plus, Altstatten, Switzerland). This rubber hardens within 2-3 minutes, after which the petiole can be removed. The dried rubber contains an imprint of the epidermal layer of the whole petiole. This imprint was used as a mould and prints of the moulds were made with clear nail polish. From these prints total cell number and cell length can be estimated (Ridge and Amarasinghe, 1984). Once dried, the prints were carefully removed from the moulds and put on a cover glass. These prints showed clear patterns of the upper layer of the petiole under a light microscope (magnification = 200). Three different randomly chosen places were used to determine the cell number per millimeter. Areas around stomata and directly adjacent cells were not measured because these cells have markedly different sizes.

Leaf and petiole dry mass was determined after drying leaves and petioles to constant weight at 72° for 48 hours.

Statistical analyses

A mixed model ANOVA (SAS Procedures PROC GLM) was used to test for the effects of treatment and genotype on plant traits, with treatment and temporal blocks (see Treatments) being treated as fixed effects and genotypes as random effects. The effects of mean trait value and treatment on mean Young's modulus and mean flexural stiffness were tested with an ANCOVA using within treatment genotypic means. In this analysis a significant effect of traits indicates that in addition to the overall treatment effects, developmental and phenotypic plant traits, which were introduced as covariates in to the model, affect the biomechanical characteristics. Genotypic correlations among all traits were calculated for each treatment separately.

To test for the direct and indirect effects of developmental and phenotypic traits on biomechanical characteristics, we performed a path analysis using the program package AMOS (Arbuckle and Wothke, 1999). Path analytical models can be used to explore and quantify patterns of variation in character correlations (Pigliucci and Kolodynska, 2006) Cell number, cell size, petiole diameter and leaf area were entered in the program as exogenous traits and correlation coefficients among those traits were calculated. Petiole

length, leaf weight, Young's modulus and flexural stiffness (*EI*) were entered as endogenous traits. We tested the effects of the exogenous traits on the endogenous traits as well as the interrelationships between cell number, petiole length, and leaf area and the effects of these traits on biomechanical characteristics. Further we calculated the paths of petiole diameter and elasticity modulus on flexural stiffness of the petioles. This analysis allows for testing which traits exert direct effects on flexural stiffness and which traits affect flexural stiffness via modification of the petiole diameter and the material properties of the petioles (Young's modulus), respectively. For this analysis all plants subjected to a common treatment, and not genetic means, were used.

In order to test for the effects of traits on performance we regressed traits on performance, using the performance data on the same genotypes published in (Weijschede *et al.*, 2006), assuming that the traits measured in the present experiment represents a stable trait characterizing the respective genotypes across experiments and can therefore be used to assess the underlying mechanisms explaining variation in performance across experiments. In the experiment of Wijschede *et al.* (2006) the same genotypes were grown under high light conditions as well as under vertical light gradient and homogeneous shade (Weijschede *et al.*, 2006). We used the performance data (i.e. ramet production) for the control conditions and the plants subjected to homogeneous shade, as these treatments were comparable to the treatments employed in the present experiment. Petiole length was added to the analysis to account for differences in performance inherently correlated to petiole length expression (Weijschede *et al.*, 2006), enabling us to distinguish the effects of petiole length and the traits of interest. From that analysis one can infer in how far the respective traits affect plant performance, whether the effects differs among genotypes expressing different petiole length and the direction of the response.

Results

Individual leaf traits

Plants grown under shaded condition produced significantly longer petioles which tended to be slightly thinner (Figure 1, Table 1). Shading significantly increased allocation to petioles. On average individual ramets of shaded plants invested 68% of their biomass into petioles, while plants grown under high light conditions allocated 42% of their weight into petioles. Lamina area of individual leaves was the same in the two shading treatments, but leaf mass (laminas and petioles together) was negatively affected by shading.

The epidermis of shaded petioles contained more cells, when counted along the petiole length, than that of light-grown plants. Individual epidermis cells were on average 50% longer. The Young's modulus and the flexural rigidity (*EI*) of shaded petioles were higher than that of the high-light ones, indicating that for a given length, the petioles were more resistant to bending. There was a high genetic variation among the 14 genotypes for all traits except Young's modulus (Table 1). In addition, the diameter of petioles and leaf area responded significantly different to shading treatments among genotypes.

Genotypic trait correlations revealed that cell number was significantly positively correlated with petiole length, petiole diameter, leaf area, and leaf weight in both light conditions (Table 2). Under shaded conditions cell size was negatively correlated with area and weight of leaves, as well as with cell number. These correlations were not significant under high light conditions.

This study shows the absence of genetic correlations between the Young's modulus with any of the other measured plant traits in both light conditions (Table 3). However, there was a consistent positive correlation between flexural stiffness and other plant traits. Under both light conditions genotypes producing petioles with greater flexural stiffness were also characterized by longer and thicker petioles, larger and heavier leaves and the petioles consisted of more cells. Under low light conditions, but not under high light conditions, there was a negative correlation between flexural stiffness and cell size.

Influence of leaf traits on biomechanical characteristics

The Young's modulus was mainly affected by shading, while the flexural stiffness was, in addition to treatment effects, also affected by other developmental and morphological traits (Table 3). Flexural stiffness was affected by petiole length, petiole diameter, leaf area, leaf weight, cell number and cell size with the rigidity of petioles decreasing with increasing cell size and increasing with an increase of the other traits.

The data reveal significant correlations between cell number and cell length and the flexural rigidity of the petioles, but not with their tissue properties (Table 2). However, the strength of the correlation between cell size and cell number differed between treatments. Under high light conditions cell number was positively correlated with petiole diameter, thereby also affecting flexural stiffness, indicating that petioles constructed of

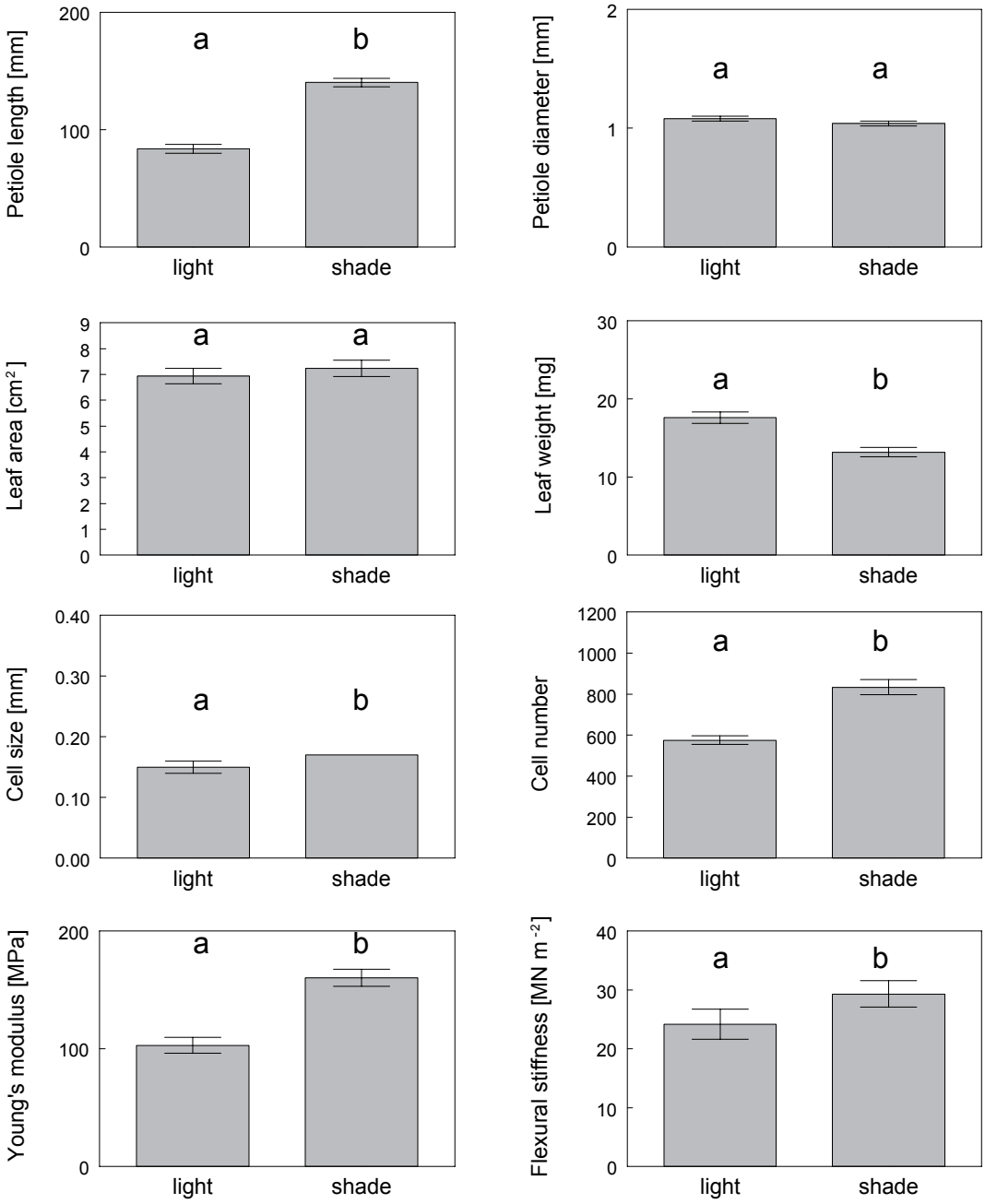

Figure 1: *Mean (± 1se) effect of the two treatments on morphological, developmental and biomechanical plant traits. Different letters indicate significant differences between treatments at $p \leq 0.05$.*

Table 1: *Mixed model ANOVA (SAS Procedure PROC GLM) results on the effects of light treatments and genotypes on morphological, developmental and biomechanical plant traits. The F-values and their significances are given. Significance levels are: ns: p>0.1; $: 0.1>p>0.05; *: 0.05>p>0.01, ** 0.01>p>0.001; ***: p>0.001;*

Source	Treatment	Genotype	Treatment x genotype	Block
d.f.	1	13	13	3
Petiole length	180.59***	4.44 ***	1.52 ns	28.13 ***
Petiole diameter	3.29 $	8.61 ***	1.88 *	6.24 ***
Leaf area	1.04 ns	17.73 ***	2.06 *	14.27 ***
Leaf weight	9.88 ***	12.61 ***	0.90 ns	4.26 **
Cell size	30.52 ***	2.76 **	0.68 ns	6.74 ***
Cell number	47.06 ***	4.88 ***	1.22 ns	9.05 ***
Young's modulus	95.34 ***	0.96 ns	0.31 ns	1.24 ns
Flexural stiffness	5.56 *	0.07 ***	0.72 ns	7.91 ***

Table 2: *Genotypic correlation among morphological, developmental and biomechanic traits. Correlations were calculated for each treatment separately using the genotypic means (n=14). Correlation coefficients above the diagonal indicate correlations expressed under high light conditions, Correlation coefficients below the diagonal indicate genotypic correlations expressed under low light conditions. For significance levels see Table 1.*

		High light							
		Petiole length	**Petiole diameter**	**Leaf area**	**Leaf weight**	**Cell number**	**Cell size**	**Young's modulus**	**Flexural stiffness**
Low light	Petiole length	.	0.85 ***	0.75 **	0.68 **	0.75 **	0.17 ns	0.07 ns	0.85 *
	Petiole diameter	0.78 **	.	0.63 *	0.61 *	0.67 **	0.25 ns	-0.14 ns	0.91 ***
	Leaf area	0.56 *	0.81 ***	.	0.98 ***	0.98 ***	-0.41 ns	0.23 ns	0.81 ***
	Leaf weight	0.65 *	0.91 ***	0.96 ***	.	0.83 ***	-0.44 ns	0.24 ns	0.79 ***
	Cell number	0.80 ***	0.70 **	0.68 **	0.69 **	.	-0.42 ns	0.06 ns	0.76 **
	Cell size	-0.45 ns	-0.45 ns	-0.61 *	-0055 *	-0.86 ***	.	0.15 ns	-0.12 ns
	Young's modulus	-0.06 ns	-0.17 ns	-0.06 ns	-0.09 ns	-0.05 ns	0.11 ns	.	0.48 $
	Flexural stiffness	0.70 **	0.94 ***	0.77 **	0.77 **	0.73 ***	-0.55 *	0.35 ns	.

Table 3: *ANCOVA testing for the effects of treatment and plant traits expressed by a genotype on the average biomechanical characteristics expressed by a genotype. Please note that this analysis has been done on the genotypic means calculated within treatments.*

	Young's modulus			Flexural stiffness		
	r^2	Treatment	trait	r^2	treatment	trait
Petiole length	0.64	9.46 **	0.00 ns	0.57	18.08 ***	31.92 ***
Pet. diameter	0.65	41.23 ***	0.61 ns	0.87	27.35 ***	155.1 ***
Leaf area	0.64	43.75 ***	0.14 ns	0.64	1.28 ns	42.11 ***
Leaf weight	0.64	36.53 ***	0.18 ns	0.62	19.38 ***	37.45 ***
Cell size	0.64	31.29 ***	0.01 ns	0.16	3.92 $	3.65 $
Cell number	0.64	25.01 ***	0.98 ns	0.56	5.50 *	29.63 ***

more cells were thicker and by consequence more resistant to bending. Under shaded conditions this correlation was maintained. In addition cell size was negatively correlated with both diameter and flexural stiffness, indicating that petioles constructed of larger cells tended to be more flexible.

There were significant correlations between shade induced changes of cell number and cell size and shade induced changes in the Young's modulus (Fig. 2). Genotypes increasing their number of cells in response to shading also experienced a relative increase in the tissues rigidity. Shade induced increase in the size of the cells, on the other hand, lead to decreased tissue rigidity. Shade induced increase of cell number lead also to an increased flexural stiffness of the petioles, whereas shade induced increase of cell size did not affect shade induced changes in the flexural stiffness (Fig. 2).

The phenotypic path analysis revealed complex inter-relationships among traits (Fig. 3). The strength and direction of these relationships were affected by the light environment. Independent of light conditions cell size and cell number were negatively correlated and an increase in both lead to the production of longer petioles; though the effect of cell number was greater. Light availability distinctively altered the pattern and direction of the effects of cell number, cell size and petiole length on biomechanical characteristics of the petioles. While under high light condition increased petiole length was associated with a reduction in the Young' modulus (*E*: the rigidity of petiole tissue) and to an increased flexural stiffness of the whole petiole; the increase in diameter and associated *I* more than compensated for the effect of a lower *E*. Under shaded conditions petiole elongation tended to have a positive effect on E and no direct effect on the flexural stiffness. Under both conditions the exogenous variables cell number, petiole diameter and leaf area were positively correlated with each other. Leaf area had a consistent indirect positive effect on flexural stiffness by way of leaf area being positively associated with leaf weight, which in turn positively affected the elasticity modulus. In contrast to high light conditions, where leaf area also directly positively affected the elasticity modulus, this effect was negative under shaded conditions.

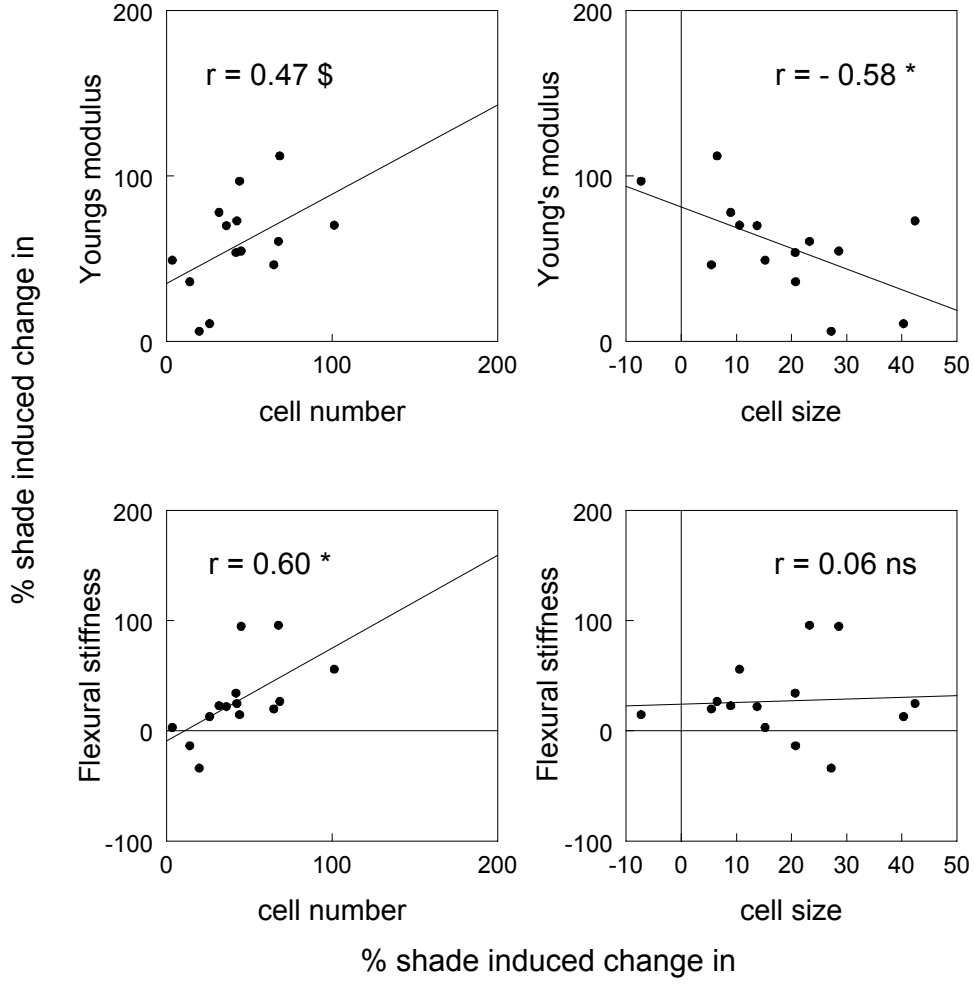

Figure 2: *Effects of shade induced change in cell size and cell number on shade induced change in Young's modulus and flexural stiffness. In the graph the correlation coefficient (r) and its significance is given. Significance levels are as in Table 1.*

Influence of leaf traits on plant performance

The diameter of petioles, the cell number per petiole and the flexural stiffness, but not the Young's modulus was significantly correlated with ramet production of plants subjected to shade (Table 4). There was no effect of leaf traits on performance of plants under high light conditions (data not shown). Plants which produced thicker petioles produced on average significantly more ramets if grown under shaded conditions than plants with thinner petioles. There was also a significant negative interaction between

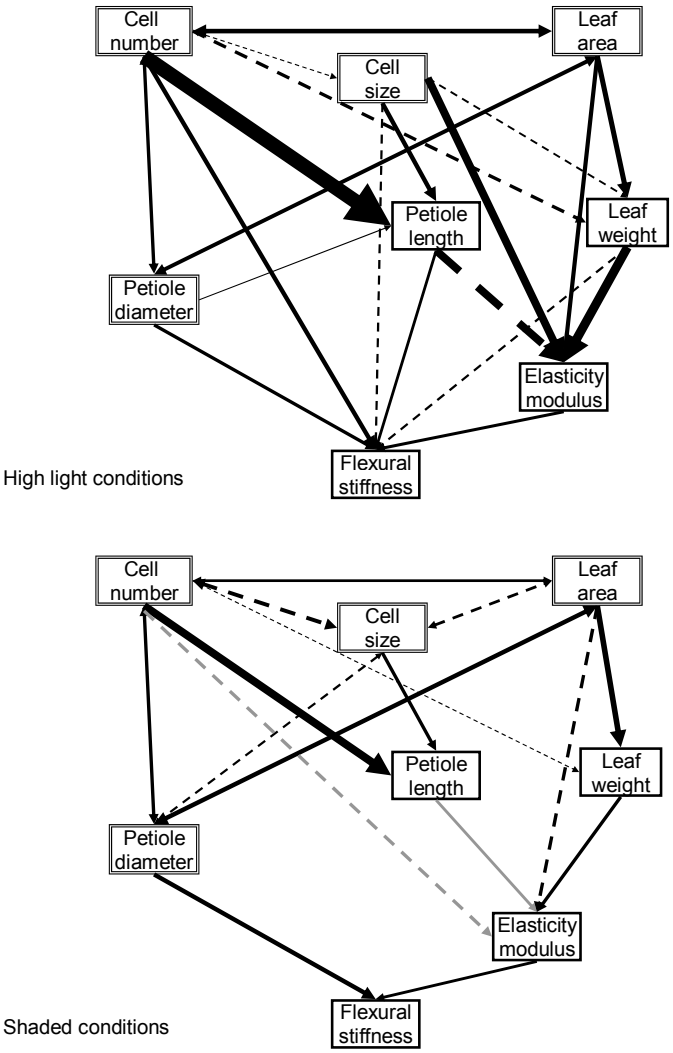

Figure 3: *Results of a phenotypic path analysis depicturing the underlying relationships among morphological and developmental plant traits and their consequence for biomechanical characteristics. Cell number, cell size, diameter and leaf area were assumed to be exogenous traits and are placed in a double lined box, the other endogenous traits, placed in single lined boxes. Correlations among exogenous traits were calculated, indicated by double headed arrows. The thickness of the lines indicates strength of the effects. Black lines indicate significant effects, grey lines non-significant effects with the standardized estimate exceeding 0.5. Non significant paths with a standardized estimate below 0.5 are not represented. Full lines depict positive relationships, dashed lines negative relationships. The analysis was done for plants subjected to high and low light conditions separately.*

Table 4: *Effects of morphological and biomechanical characteristics on plant performance (measured as ramet production) under shaded conditions. Petiole length is added to the analysis as well to take account for the pure effects of petiole length on plant performance. Analyses are done on the genotypic means of plants grown in homogeneous shade. T-values and their significances are given.*

Source	r^2	Petiole length	trait	Length x trait
Petiole diameter	0.49	2.14 $	2.62 *	-2.37 *
Leaf area	0.62	2.15 $	3.40 **	-2.85 *
Leaf weight	0.57	0.01 $	3.08 *	-0.02 *
Cell size	0.55	-3.08 *	-3.03*	2.92 *
Cell number	0.73	3.95 **	4.68 ***	-4.50 **
Young's modulus	0.22	0.30 ns	-0.05 ns	-0.44 ns
Flexural stiffness	0.64	1.65 ns	3.66 **	-3.1 *

petiole length and petiole diameter on plant performance Also petioles containing more cells produced on average more ramets. Comparable to the effects of petiole thickness, there was a significant negative interaction of petiole length and cell number on plant performance. Leaf area and leaf weight followed the same qualitative pattern as petiole diameter and cell number. Overall these results indicate that producing taller more massive leaves, thicker petioles and investing into cell division positively affected plant performance under shaded conditions. Also higher flexural stiffness had a positive effect on plant performance. Genotypes producing stiffer petioles under shaded conditions produced more ramets. This indicates that there are no costs associated to the production of stiffer petioles. However, we also found a significant negative interaction between petiole length and flexural stiffness, indicating that for longer petioles it was favorable to be less stiff, whereas for shorter petioles it was more favorable to be stiffer. There was no effect of the Young's modulus on plant performance.

Discussion

Shade avoidance is very common in many plant species (Morgan and Smith, 1979; Schmitt *et al.*, 2003; Schmitt and Wulff, 1993; Sultan and Bazzaz, 1993; Weinig, 2000). In stoloniferous plants adaptive plasticity to shading is achieved by the production of longer petioles that reach higher positions in the canopy (Huber *et al.*, 1998; Huber and Wiggerman, 1997; Leeflang *et al.*, 1998). Beyond this obvious response, shade induced elongation processes entail a multitude of other structural and developmental changes (Cipollini and Schultz, 1999; Maliakal *et al.*, 1999; Schmitt and Wulff, 1993; Smith, 1982). While the benefits of shade induced elongation responses are beyond doubt, the consequences of the structural changes associated with these responses are still under investigation.

In this paper we show how structural and developmental changes in concert result in the production of more rigid petioles. A better understanding of the effects of structural and developmental changes associated with shade induced elongation responses on biomechanical characteristics and ultimately on plant performance will enhance our understanding of the evolution of shade induced elongation responses in stoloniferous plants. It may also shed light on whether evolutionary trajectories are different for shade induced elongation in vertical spacers of clonal and non-clonal plants.

Biomechanical properties affected by light conditions

In general shaded petioles had a higher Young's modulus (*E*) than unshaded ones, which is consistent with previous findings for both stems of erect plants (Anten *et al.*, 2005) and petioles of stoloniferous plants (Liu *et al.*, 2007). This result could be attributed to a greater turgidity of stem tissue which tends to be greater in shade grown plants (Liu *et al.*, 2007; Niklas and Owens, 1989). The stiffness of herbaceous support structures is largely the result of the rigid epidermis and possibly one or two underlying cell layers being held in tension by a hydrostatically inflated inner core (Hofmeister, 1859; Niklas and Paolillo, 1997). Thus tissue rigidity (*E*) in such structures depends not only on tissue composition but also on cell turgor (Niklas, 1989; Niklas *et al.*, 1999). Direct measurements have shown strong positive correlations between E and stem water potential or water content (Niklas, 1989; Niklas and Paolillo, 1997). For giant petioles of Amorphophallus titatum growing up to several meters in height, a clear positive correlation between turgor pressure and E was also found (Hejnowicz and Barthlott, 2005). Shade induces stem elongation but simultaneously suppresses photosynthesis and thus assimilate supply for growth. An increased turgor pressure may then be an energy efficient way of obtaining the rigidity necessary for self support (Lai *et al.*, 2005). However this mechanism of increased turgor will not change the modulus of rupture, which depends largely on the material properties of cell walls (Niklas, 1994). Thus an increased rigidity (i.e. reduced flexibility) makes petioles more vulnerable to failure under external forces such as wind loading or trampling (Ennos, 1997).

Light environment and plant traits interact in determining Young's modulus and flexural stiffness

The flexural stiffness of a petiole depends both, on the cross sectional area and on the material property of the tissue it is constructed of (Niklas, 1992). Our results show that the mechanical tissue properties are mainly affected by the light environment, as petioles produced under low light conditions consist of more rigid tissue than petioles produced under high light conditions. Leaf area, leaf weight and petiole length interact in affecting the material properties. The direction of the effects of petiole length and leaf area on the material property were, however, distinctly different between light treatments, which may also explain why we did not detect general effects of morphological and developmental traits on tissue rigidity. These results provide evidence that shade induced plasticity of phenotypic traits can alter inter trait correlations (Malausa *et al.*, 2005; Stanton *et*

al., 2004) and that the traits, though interrelated in high light conditions do not respond to shading in concert and that trait correlations may be broken up under resource poor conditions.

A similar, but even more extreme pattern emerged for flexural stiffness, which also increased in shaded plants. While flexural stiffness of the petiole was directly affected by various morphological traits under high light conditions, these effects were, if they were present at all, only indirect in low light conditions. Only petiole diameter, which did not respond to shading, and the Young's elastic modulus, which was increased under shaded conditions directly affected the flexural stiffness of petioles under low light conditions. The strength of correlation was thus generally weaker under low light than under high light conditions, which is in contrast with the notion that the pattern and strength of integration among parameters is stronger in plants experiencing low resource status (Cheplick, 2001; Huber *et al.*, 2004) and other studies that have found that the pattern of phenotypic integration changes little between treatments (Pigliucci and Kolodynska, 2002; Pigliucci and Kolodynska, 2006). In concert these results indicate that the tissue rigidity and the flexural stiffness of the petiole may be fine-tuned depending on other morphological characteristics such as a given length of a petiole, the leaf area a petiole has to support or the weight of the leaf lamina. However, the direction and magnitude of effects differs between light environments. The generally smaller effects of other correlated phenotypic traits on biomechanical characteristics may indicate that shaded conditions lead to a stronger canalization of the expression of mechanical properties.

Surprisingly, petioles were hardly thinner under shaded conditions, which is in contrast to the findings for stems of erect plants. In stems of erect plants internode thickness can be modified throughout ontogenetic development by means of secondary growth (Esau, 1977). If mechanical stability of stem internodes proves not to be high enough to accommodate increasing strain on the stems caused by the acropetal addition of new modules, flexural stiffness of the stems can thus still be adjusted. This continuous ability of internodes to adjust their thickness may enable erect plants to initially invest resources economically into height growth and the production of new modules instead of increased stem thickness. In petioles secondary growth is much less common (Esau, 1977). As soon as petiole length growth and lamina expansion have finished only external forces, but not internally increased biomass load, may exert extra force on the petioles. The limited possibility of secondary growth may necessitate petioles to be constructed of sufficient strength to withstand unpredictable external forces and does thus not allow for the economic production of initially thinner petioles.

Effects of cell size and number on biomechanical properties

Read and Stokes (Read and Stokes, 2006) have argued that fundamental design traits at both the cellular and whole plant level are directly influenced by the immediate environment. Structure, size and alignment of epidermal cells have been argued to affect biomechanical tissue properties (Loodts *et al.*, 2006). To the best of our knowledge the effects of genotypic variation in cell size and cell number on the material properties of petioles

and the resulting flexural stiffness have not been investigated previously. Our data clearly show that biomechanical characteristics of the petioles depend on the developmental mechanisms controlling petiole length. Under high light conditions petioles containing more cells had a higher flexural stiffness. This was achieved both by a direct effect of cell number on petiole diameter and associated second moment of area, and indirectly by a positive effect of cell number on the petiole length, which in turn positively affected flexural stiffness. In *T. repens* traits such as lamina size, petiole length, internode length and petiole thickness are strongly correlated (Weijschede *et al.*, 2006). This may indicate that the same developmental process, i.e. magnitude and speed of cell proliferation, is responsible for within treatment variation in petiole length and thickness, and ultimately for flexural stiffness. However, we did not measure the horizontal extension of cells, and can thus not prove this hypothesis

Contrary to our expectations, between genotypes, there was no negative correlation between the size of epidermal cells and the Young's modulus (*E*) of the petiole. One explanation could be that differences in other petiole characteristics masked the effect of cell size on *E*. First, *E* is largely determined by the turgor pressure exerted by the inner core of the petiole, which in turn is regulated by the maintenance of osmolarity within cells (Lai *et al.*, 2005). Second, the genotypes probably differed with respect to cell wall characteristics of epidermal cells, which may also influence *E* (Niklas, 1994).
On the other hand, in accordance with our prediction, plastic shade induced elongation of cells was negatively correlated with the shade induced increases in *E* (Fig. 2). This suggests that within genotypes increased cell elongation may indeed negatively impact *E* and thus supports the notion that while cell elongation might be an energy efficient way of increasing petiole length as compared to cell division, it can result in lower mechanical stability.

Costs and benefits associated to shade induced elongation responses

Shade induced elongation of spacers has been hypothesized to be associated with costs in terms of decreased biomechanical stability, which may increase the risk of lodging or breaking (Anten *et al.*, 2005; Dudley and Schmitt, 1996; Huber *et al.*, 1998; Huber *et al.*, 2004). Even increased resource allocation to the elongating organ may not be sufficient to match the increased resource demand, resulting in thinner and weaker stem internodes or petioles. However, biomechanical needs can be matched by either changing tissue properties or reallocating tissue in a more efficient way (Niklas, 1992). Our data provide evidence that, cell number and flexural stiffness, but not tissue rigidity confer a selective advantage under shaded conditions. Increased cell size, on the other hand, was associated with decreased ramet production. Interestingly, the same traits did not affect performance under high light conditions. These data show that increasing the number of cells, decreasing the size of cells, or increasing the flexural stiffness is not associated to costs, even in an environment providing homogeneous shade, where the selection pressures are supposed to be lower as lodging will not result in decreased light interception. This indicates that the increased structural demands necessary for producing smaller cells

(i.e. higher number of cell walls) or for producing thicker, and thus stiffer petioles, may not confer costs and lead to reduced plant performance. Plastic or constitutively increased flexural stiffness and the production of more and smaller cells will be selected for in shaded environment and constitutively higher values for those traits will not be selected against under unshaded conditions.

A broader range of genotypes has shown that both strategies, elongating petioles by means of increased cell number and cell size (Weijschede *et al.*, *in press*) are maintained in a population, support the notion that the benefits associated to the production stiffer petioles may outweigh any structural costs potentially incurred under natural conditions. The net benefits associated with increased petiole stiffness differed among light environments. Small scale temporal and spatial heterogeneity may lead to the maintenance of different investment strategies into petiole rigidity under natural conditions. As stolons of stoloniferous plants spread horizontally throughout the vegetation, even successive ramets on an integrated clonal system may experience different selection regimes, and may thus experience advantage, as well as disadvantage of investing into increased cell number or size and the associated costs and benefits.

Conclusions

In short, changes in Young's modulus in response to shading were negatively correlated with changes in cell size while shade induced changes in cell number were positively correlated to changes in Young's modulus and flexural stiffness. A large flexural stiffness in turn was associated to increased fitness in plants under shade but not under high light conditions, which indicates that the pattern of selection on flexural stiffness, and thereby also on shade induced changes in cell size and cell number, differs among light environments.

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Variation in petiole and internode length affects plant performance in *Trifolium repens* under opposing selection regimes

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Abstract

We studied the effects of genotypic and plastic variation in vertical and horizontal spacer lengths on plant performance in a stoloniferous herb subjected to opposing selection regimes. We hypothesized that longer vertical structures are beneficial if plants are subjected to competition, but they should negatively affect plant performance if plants are exposed to aboveground disturbance.

To test these hypotheses we subjected 34 genotypes of *Trifolium repens* to competition and disturbance treatments. Competition was imposed by a grass canopy consisting of Lolium perenne, and disturbance was simulated by regularly clipping the target plants and all the surrounding vegetation at 1 cm above soil level.

Conform to our hypothesis, genotypes with longer vertical structures (petioles) produced fewer ramets than genotypes with shorter petioles in the disturbance treatment. However, genotypes with longer petioles did not perform better under competition than genotypes with shorter petioles. Genotypes with highly plastic vertical structures tended to produce more shoot mass under competition, and they produced fewer ramets if subjected to disturbance.

Unexpectedly, horizontal structures (stolon internodes) expanded in response to competition which, furthermore, was associated with enhanced plant performance. However, producing longer internodes is inherently associated with costs in terms of increased resource allocation to the longer structures, but not to benefits in terms of increased resource capture. Positive correlations among the length and plasticity of vertical and horizontal structures may explain the apparent positive effect of producing longer internodes on plant performance. Our data thus support the notion that trait correlations may weaken selective forces acting on a focal trait in a specific environment if opposing selection pressures act on genetically correlated traits.

Introduction

Plants are often exposed to multiple selective forces. Consequently, species can display considerable variation in morphological traits both among and within genotypes (Galloway, 1995; Via and Lande, 1985). Factors contributing to temporal and spatial heterogeneity in pastures are variation in soil conditions, irregular disturbance due to herbivory and human activity such as mowing (Farley and Fitter, 1999; Jackson and Caldwell, 1993; Waite, 1994). Herbaceous vegetations can be characterized as dynamic mosaics of different microhabitats ranging from sites with low levels of disturbance, and severe competition for light, to more open spots with low above ground competition resulting from high levels of canopy disturbance by grazing or mowing (Evans and Turkington, 1988; Marcuvitz and Turkington, 2000).

Plants have evolved several mechanisms to escape or buffer potentially negative effects of competition (Schlichting and Smith, 2002; Schmitt and Wulff, 1993; Schmitt *et al.,* 1999; Schmitt *et al.,* 2003; Smith, 1982; Smith and Whitelam, 1997). Long vertical structures allow for the positioning of leaves in more favourable light conditions thereby increasing light harvesting and reducing the negative effects of low light availability (Aphalo and Ballare ,1995; de Kroon and Hutchings, 1995; Donohue *et al.,* 2000; Geber and Griffen, 2003; Huber *et al.,* 1998; Sultan, 1995). In competitive sites plants capable of producing long vertical structures perform better than smaller or less plastic plants (Ballare *et al.,* 1994; Dudley & Schmitt, 1996; Griffith & Sultan, 2006; Schmitt *et al.,* 1995; Weijschede *et al.,* 2006; Weinig, 2000a). However, plants with longer vertical structures can be at a disadvantage in grazed or mown sites, because they lose relatively more biomass than plants with shorter vertical structures. Under such conditions investment in long or highly plastic vertical structures can negatively affect plant performance, as the investment into long structures will be associated with costs, but not with benefits (DeWitt *et al.,* 1998; Dorn *et al.,* 2000; Lande and Arnold, 1983; Poulton and Winn, 2002; Weinig, 2000b). Grazing or mowing is therefore expected to favour plants with shorter and less plastic vertical structures, while competition should favour plants with longer and more plastic vertical structures (Stuefer *et al.,* 2002).

As competition for light can mainly be avoided in the vertical direction, plants in dense canopies may prioritize the production of long vertical structures at the expense of horizontal expansion (Hirose and Werger, 1995; Thompson and Harper, 1988). Indeed, comparative studies involving plants with horizontal and vertical stems have revealed that shade-induced spacer elongation is mainly expressed in a vertical direction (Huber and Hutchings, 1997; Huber *et al.,* 1998). In contrast to results obtained under greenhouse conditions, different stoloniferous species have been shown to elongate their horizontally oriented internodes in dense natural canopies, thereby positioning their offspring ramets further away from the parent ramets (Cain, 1994; Hutchings *et al.,* 1997; Kleijn and van Groenendael, 1999; Waite, 1994). As patterns of light availability are usually less predictable in a horizontal than in a vertical direction, elongation of horizontal structures may

not result in increased light capture of newly produced ramets. If increased internode length does not lead to enhanced light capture, the resources needed for internode elongation are lost while they could have been used for other plant functions. It can therefore be expected that plasticity of horizontal structures will be disfavoured in canopies characterized by a low spatial predictability in the horizontal direction and a strong predictable light gradient in vertical direction.

General responses to shading have been investigated thoroughly and the selective advantage of shade avoidance has been shown in a number of studies (Ballare *et al.* 1991; Dudley & Schmitt, 1996; Huber *et al.,* 1998; Schmitt, 1997; Schmitt and Wulff, 1993; Schmitt *et al.,* 2003; Thompson, 1993; Weinig, 2000a). Nevertheless, experiments evaluating plasticity and fitness consequences under multiple contrasting selection regimes are still scarce (but see Anten *et al.,* 2005; Callahan and Pigliucci, 2002; Huber *et al.,* 2004; Weinig *et al.,* 2004). In this study we aim at testing the relationship between plasticity and performance of plants subjected to opposing selection regimes. We expect that genotypes with longer vertical structures or higher density-induced plasticity will perform better in high canopies created by a natural competitor than genotypes with shorter vertical structures or lower density-induced plasticity. Regular disturbance (grazing or mowing) involving the loss of above-ground biomass is expected to disfavour genotypes with longer vertical structures and higher density-induced plasticity. Horizontal structures are expected to remain shorter under competition and - due to resource loss - under grazing or mowing compared to control conditions.

Materials and Methods

Plant material and pre-treatment conditions

During the summer of 2001, 107 *Trifolium repens* plants were randomly collected from a natural meta-population in a riverine grassland close to the river Waal near Ewijk (51°52'54"N, 5°45'00"E, The Netherlands). Due to the activity of cows and horses the herbaceous vegetation consists of a mosaic of different microhabitats ranging from sites with low levels of disturbance and dense vegetation to more open sites where the vegetation has been disturbed or removed (H. van de Steeg and J. Weijschedé, personal observation). In summer 2002, all plants were screened for morphological traits, including petiole lengths and internode lengths. 34 genotypes were selected which expressed a wide range (1.9 cm to 6.8 cm) of petiole lengths under common garden conditions. Molecular fingerprinting techniques (AFLP, four primer combinations, 145 markers) were used to confirm the genetic identity of the collected plants. In a previous experiment we had shown that all 34 genotypes express various degrees of petiole plasticity in response to a vertical light gradient (Weijschede *et al.,* 2006). This elongation response did not depend on the petiole length expressed under control conditions (Weijschede *et al.,* 2006). All 34 genotypes were clonally propagated in a heated greenhouse. 408 Cuttings (12 per genotype) were taken from the stock material and transplanted into 0.29 x 0.19 x 0.19 m trays (one

cutting per tray), filled with a 2:1 mixture of sand and potting soil. All trays were placed outside the greenhouse on an empty field. Every cutting consisted of one ramet with a well-developed root system and a lateral stolon with 3-5 ramets.

Experimental treatments

On June 6th 2003, all 34 selected genotypes were subjected to the following four treatments:

1. No competition and no clipping (referred to as control conditions);
2. No competition and clipping (referred to as clipping);
3. Competition and no clipping (referred to as competition);
4. Competition and clipping (referred to as the clipping + competition treatment).

Under control conditions plants were allowed to grow in an undisturbed manner and without competitors. Plants assigned to clipping treatments were subjected to a simulated grazing regime in which all leaf, but no stolon biomass was clipped 1 cm above the soil level and removed. Clipping treatmens left apical and lateral meristems of all plants intact. Clipping was applied on the 12th, the 18th, and the 32nd day after the onset of the experiment. Plants subjected to competition were grown together with *Lolium perenne* (KenKen, Unifarm, Wageningen, The Netherlands). 310 mg *L. perenne* seeds (app. 220 seeds) were sown per plot 28 days prior to the start of the experiment. When the *T. repens* cuttings were placed in the trays, *L. perenne* plants were about 6 cm high and covered homogeneously the surface of the trays. *T. repens* plants were not able to avoid or escape competition through horizontal expansion. Under undisturbed conditions the grass reached an above ground dry mass density of 173.0 ± 5.6 g·m^{-2}. In the clipping + competition treatment, *T. repens* was subjected to the same clipping regime as in the clipping treatment and to the same competition regime as in the competition treatment. In the clipping + competition treatment, all *L. perenne* biomass higher than 1 cm was removed together with the leaves of *T. repens*. At harvest, the grass had an above ground dry mass density 56.7 ± 1.5 g·m^{-2} in the clipping + competition treatment.

Immediately after planting, ramet number was assessed for each genotype to correct for initial size differences. All genotypes were represented once in each treatment, and treatments were replicated in three temporal blocks. A total of 408 plants were used in the experiment. For practical reasons, blocks (representing every treatment once) were temporally separated by one week intervals.

Harvest

Plants were harvested after 48 days. Roots were not collected because it was impossible to separate the *T. repens* roots from the *L. perenne* roots. For all *T. repens* plants, we measured the length of the primary stolon, counted the number of ramets on the primary stolon, the number of branches on the primary stolon and the total number of ramets. For each plant, the 4th ramet counted from the apex on the primary stolon was used to

measure the petiole length and internode length. Only undamaged leaves were used to measure petiole lengths. Dry mass of these structures was determined after plant parts were dried at 110°C for 48 hours.

Statistical analysis

To test for overall treatment effects, we performed a mixed model ANCOVA (using the GLM procedure in SAS), with genotype, competition and clipping as main factors. Genotype was considered a random factor and competition and clipping were considered fixed factors. Blocks were added as a random factor to the model. This analysis shows how genotype, competition, clipping and their interactions affect various plant traits.

In order to test for the effects of traits on performance, we followed two approaches. First, we used multiple regression analyses based on genotypic means to test for the effects of petiole lengths under high light conditions and competition induced petiole length plasticity on shoot biomass and ramet number in the four treatments separately (see DeWitt *et al.,* 1998; Scheiner and Berrigan, 1998; van Kleunen and Fischer, 2005; van Tienderen, 1991). The same multiple regression model was applied to data on internode length and its plasticity. The absolute differences in petiole and internode length expressed under competitive and control conditions were used to calculate trait plasticities. Genotypic trait values were standardized to the means per treatment to allow for direct comparisons of different regression coefficients. For details about the analyses see Weijschede *et al.,* 2006). We used separate correlation analyses to calculate correlation coefficients of the genetic mean values among the four morphological traits included in the selection analyses.

Thereafter we performed a mixed model ANCOVA (using the MIXED procedure in SAS) with competition and clipping treated as fixed effects and genotype as random effect. The genotypic mean (using least square means to correct for block effects) values of the trait of interest were added to the model as a covariate to test for the effects of traits on plant performance. A significant effect of the covariate indicates that, in addition to genetic variation in performance, plant performance was also affected by the covariate (trait length or trait plasticity). Interactions between the covariate and the treatments were added to the model to test for differential treatment effects of the covariate on plant performance. In other words, this analysis shows whether genotypic differences in a trait (e.g., petiole length) affect plant performance and whether the consequences associated with a given petiole length differ between treatments. A block effect was added to the model to correct for variation among the three temporal blocks. This analysis was performed using mean petiole length and mean internode length produced under high light conditions and mean competition induced petiole length plasticity and mean internode length plasticity. Non significant 3-way interactions were removed from the model. SAS (version 9.1) was used for all statistical analyses.

Results

Overall treatment effects

Genotypes differed in trait expression. However, due to high variation within genotypes and the low number of replicates we failed to detect significant differences in the response of genotypes to the treatments (Table 1). Competition reduced total shoot biomass of *T. repens* by 56% and clipping reduced total shoot biomass up to 32% compared to control conditions (Fig. 1). The combination of competition and clipping reduced total shoot biomass by 62% (significant competition x clipping interaction). Competition reduced total ramet numbers by 59% and clipping by 12% (Fig. 1). The combination of competition and clipping reduced the total ramet number by 47%, indicating that clipping reduced the strong negative effects of competition on the total ramet numbers (significant competition x clipping interaction). Genotypes tended to respond differently to the combination of clipping and competition (marginally significant genotype x clipping x competition interaction).

On average, petioles elongated by 111% in response to competition (Fig. 1). In the clipping treatment, petioles were 16% shorter than under control conditions. In the combined competition + clipping treatment, petioles were 14% longer as compared to control conditions (significant competition x clipping interaction, Table 1). Internodes elongated on average by 18% in response to competition, while clipping did not change internode lengths. The combination regime reduced internode length by up to 14 % (significant competition x clipping interaction, Table 1).

Genetic correlations among morphological traits

Petiole length expressed under control conditions was not significantly correlated with competition induced plasticity in petiole length ($r=-0.14$, $p=0.412$). Internode length under control conditions was negatively correlated with internode length plasticity ($r=-0.51$, $p=0.002$), indicating that genotypes with longer internodes under control conditions exhibited lower levels of internode elongation than genotypes with shorter internodes. Internode and petiole lengths were positively correlated ($r=0.66$, $p<0.001$) under control conditions. Competition-induced plasticity in petiole length was positively correlated with competition-induced internode plasticity ($r=0.48$, $p=0.002$)

Effects of petiole length and plasticity on plant performance

Longer petioles were generally associated with the production of fewer ramets, but had no effects on shoot weight (Table 2a, Fig. 2a, b). Under control conditions long petioles tended to be associated with increased dry weights (Fig. 2a). The ability to elongate petioles in response to competition did not significantly affect plant performance under control conditions (Table 2b, Fig. 2c, d).

Petiole length did not affect performance of plants grown in competitive environments (Table 2a, Fig. 2a, b). The negative effect of petiole length of plants grown under

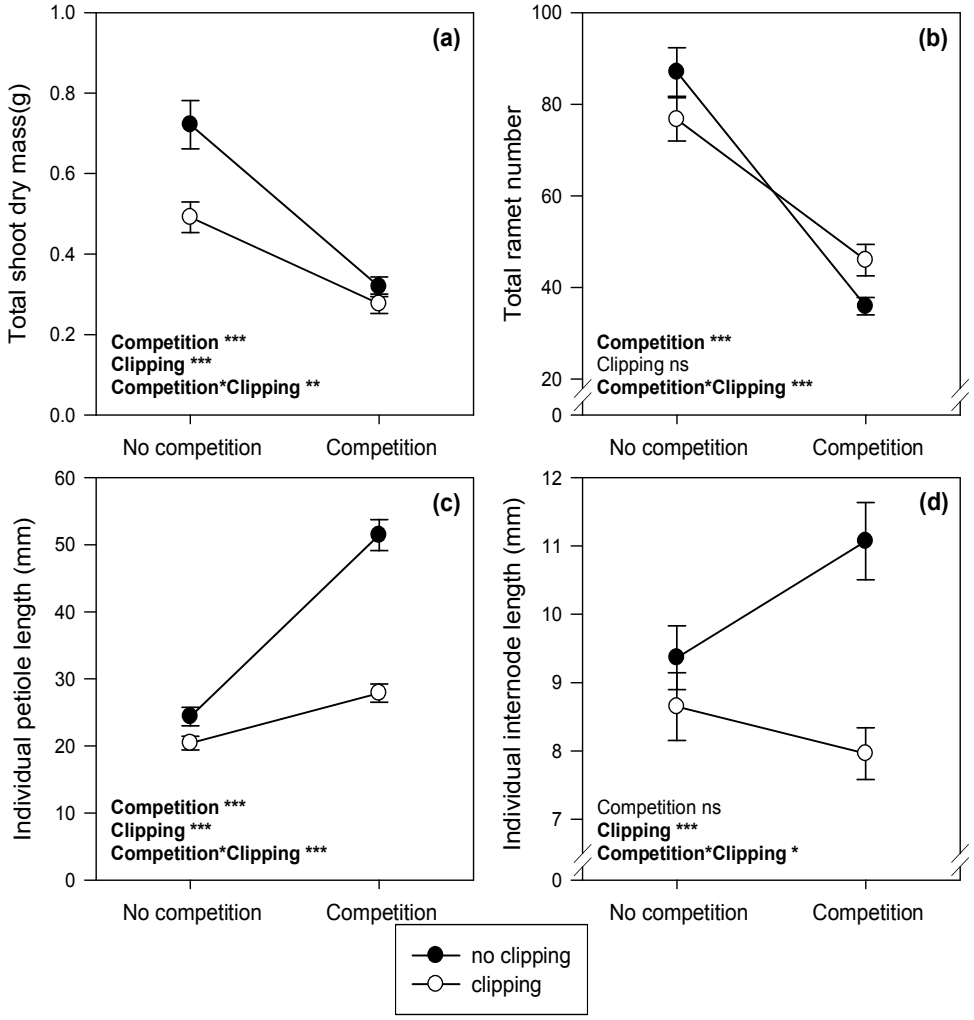

Figure 1. *Responses of (a) total shoot dry mass, (b) total ramet number, (c) petiole length and (d) internode length to grass competition and clipping. Significances of the main effects competition, clipping and competition + clipping on plant characters are inserted in the figures (see Table 1 for the complete statistical). Significant treatment effects are highlighted in bold and are indicated as follows: ns, p>0.05; *, 0.05≥p>0.01; **, 0.01≥p>0.001; ***, p<0.001. Values are means (±1 se) per treatment.*

control conditions on ramet number was thus diminished under competitive conditions. This indicates that benefits associated with producing long petioles in competitive environments buffered costs associated with long petioles apparent in the absence of competition (Fig. 2b). For plants subjected to competition high degrees of petiole plastic-

Table 1. *Results of ANCOVAs examining effects of genotype, competition and clipping on total shoot dry weight, total ramet number, petiole length and internode length. Initial ramet number was added as a covariate to the model. All traits were log transformed to meet ANCOVA assumptions. F-values and their significances are presented. Significance levels are as follows: ns: p>0.10; $: 0.10≥p>0.05; *: 0.05≥p>0.01; **: 0.01≥p>0.001; ***: p<0.001*

source	df	Total shoot dry mass	Total ramet number	Petiole length	Internode length
Genotype	33	**1.65 ***	**4.51 *****	**3.31*****	**2.10 *****
Clipping	1	**18.74 *****	0.26 ns	**150.01 *****	**30.14 *****
Competition	1	**98.64 *****	**208.46 *****	**161.53 *****	0.17 ns
Clipping x genotype	33	1.23 ns	0.99 ns	0.60 ns	0.78 ns
Competition x genotype	33	1.05 ns	1.12 ns	1.08 ns	1.02 ns
Competition x clipping	33	**8.00 ****	**16.67 *****	**21.66 *****	**5.79 ***
Genotype x clipping x competition	33	1.37 $	1.17 ns	1.00 ns	0.98 ns
Block	3	**5.85 ****	2.95 $	**7.52 *****	**32.10 *****
Initial ramet number	1	**6.86 ****	**12.17 *****	0.60 ns	0.73 ns
Error	265				

ity tended to be positively associated with increased shoot dry mass, but not with ramet numbers (Table 2b, Fig. 2c,d).

Mixed model Ancova revealed a significant interaction in the effects of clipping and petiole length on plant performance (Table 2a, b). Genotypes with longer petioles produced less biomass and fewer ramets if subjected to disturbance, but not if grown under control conditions (Figs. 2a, b). Selection analyses revealed that under disturbance regimes (both, with and without concurrent competition) petiole length expressed under control conditions had a significant negative effect on ramet production (Fig. 2b). The potential to elongate petioles under competitive environments tended to have a negative effect on ramet production if plants were simultaneously subjected to clipping as well, indicating that the production of long petioles and high degrees of petiole length plasticity will be selected against in disturbed environments (Fig. 2d).

Effects of internode length and plasticity on plant performance

Internode length had a slight negative effect on ramet production, but not on shoot dry mass. Plasticity in internode length had a slight overall negative effect on shoot dry mass, but not on ramet production. Under control conditions, longer internodes tended to be associated with increased shoot dry mass (Fig. 3a), while the ability to produce longer internodes under competition had negative effects on dry mass and ramet production under control conditions (Table 3d, Fig. 3c, d).

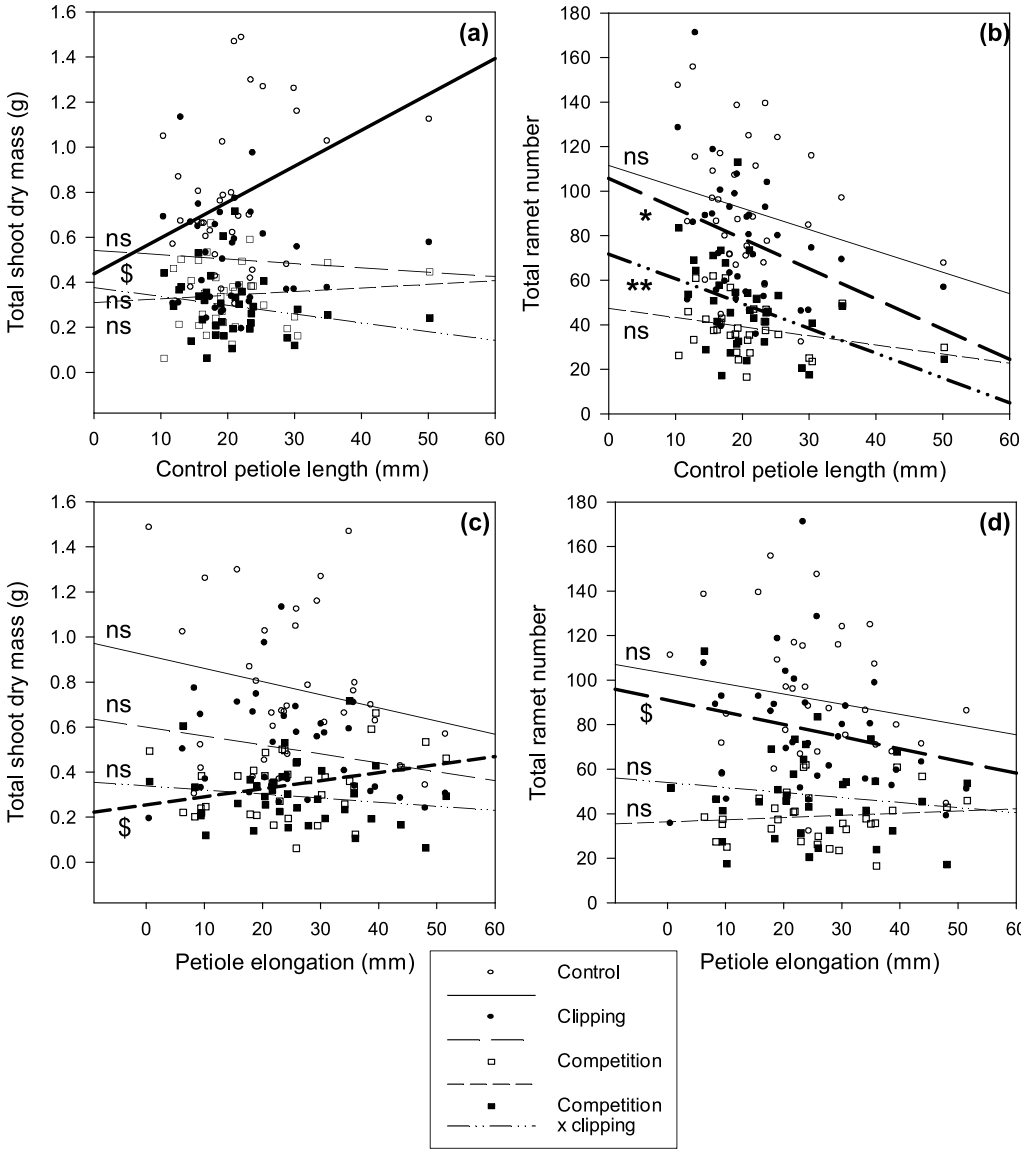

Figure 2. *The relationship between petiole length found under control conditions (x-axes) and (a) total shoot biomass and (b) ramet number in the four treatments. Graphs (c) and (d) show relations between petiole elongation (measured as the absolute difference in petiole length between the competition treatment relative to control conditions (x-axes)) and total shoot biomass (c) and ramet number (d) per treatment. Bold lines indicate a significant or marginally significant effect of the trait on performance as indicated by selection analyses (multiple regression on performance, see Material & Method section) revealed an effect of petiole and petiole plasticity on plant performance. For significance levels see Table1.*

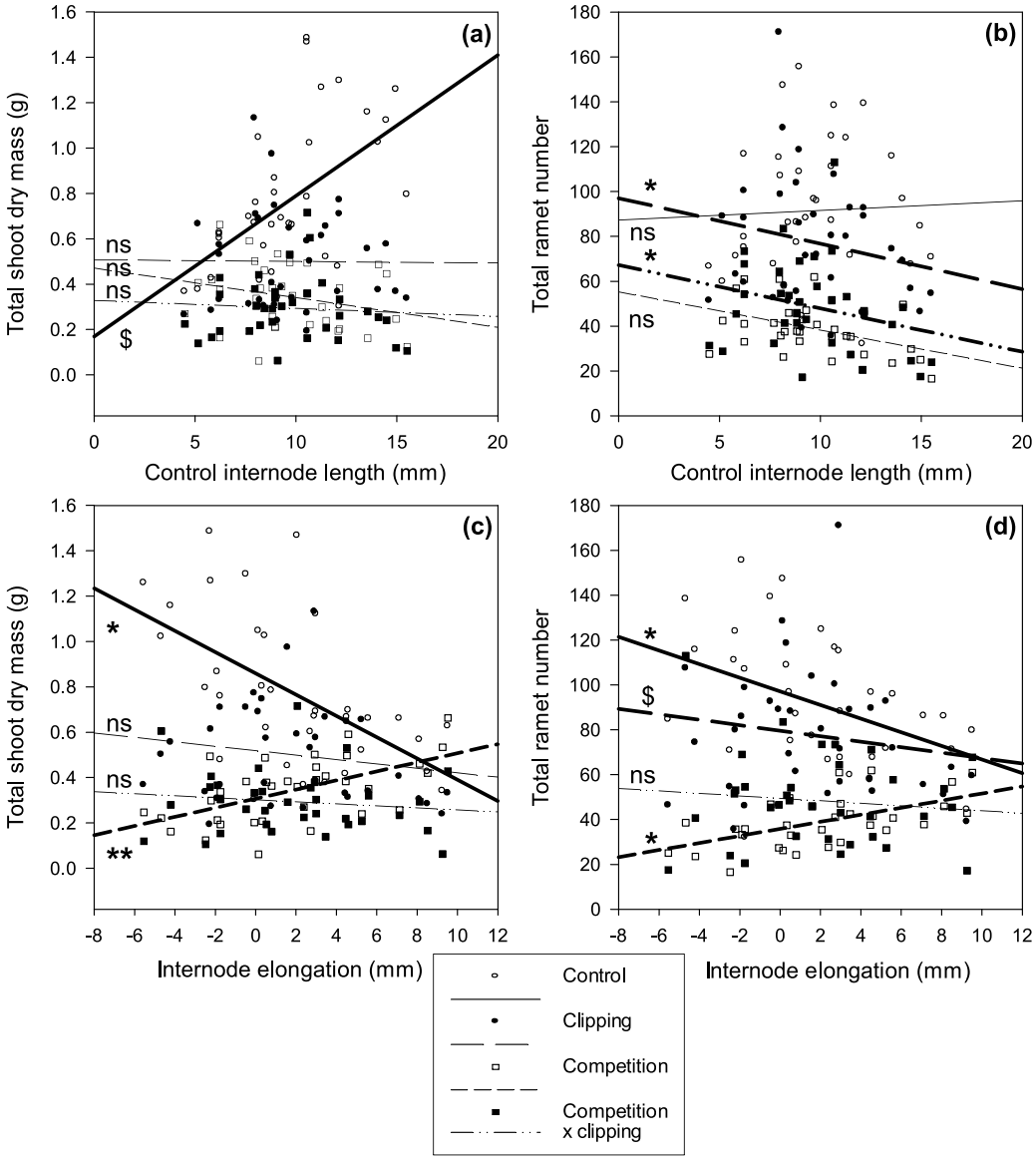

Figure 3. *The relationships between internode length found under control conditions (x-axes) and total shoot biomass (a) and ramet number (b) per treatment. Graphs (c) and (d) show relations between internode elongation (measured as the absolute difference in internode length between the competition treatment relative to control conditions (x-axes)) and total shoot biomass (c) and ramet number (d) per treatment. Bold lines indicate that selection analyses revealed a significant and marginal significant effect of internode length and internode plasticity on performance. For significance levels see Table 1.*

These relationships reversed if plants were grown in competition with Lolium perenne. Mixed model Ancova revealed a significant interaction between competition and internode length, indicating that genotypes producing longer internodes performed relatively worse if grown in competition than if grown under control conditions (Table 2c, Fig. 3a, b). However, there was no direct selection on internode length in competitive environments (Fig. 3a,b). Genotypes responding to competition by shortening their internodes performed significantly worse if subjected to competition than if grown in competition-free environments (Table 2d, Fig. 3c,d).

In contrast to control conditions, plants could not benefit from producing longer internodes if subjected to clipping (Table 2c, Fig. 3a). Selection analyses revealed a negative effect of internode length and internode length plasticity on ramet production (Figs. 3b,d). Increased internode length was also associated with reduced ramet production in plants subjected to concurrent competition and clipping (Fig. 3b), indicating that under disturbed conditions internode length and competition induced internode length plasticity will be selected against.

Discussion

Longer vertical structures may buffer negative effects of light limitation within herbaceous canopies (Schmitt and Wulff, 1993; Schmitt *et al.,* 2003; Van Hinsberg, 1997). Conversely, grazing or mowing should select against the production of long vertical spacers. Extension of horizontal structures should be disfavoured by both, competition and clipping as producing longer horizontal structures is unlikely to enhance light capture and biomass production, while it incurs costs in terms of inefficient biomass allocation (Thompson and Harper, 1988). Contrary to our expectations genotypes with long petioles under control conditions were not favoured under competition. As predicted, higher degrees of petiole plasticity tended to increase plant performance under competition. Regular clipping disfavoured genotypes with longer petioles and genotypes expressing higher degrees of petiole plasticity were marginally disfavoured. Longer internodes were disfavoured under clipping but did not negatively affect plant performance under competition. By contrast, plastic internode elongation in response to competition was associated with enhanced plant performance. This was unexpected as plastic internode elongation did not result in enhanced light harvesting in this experiment.

Consequences of genotypic trait differences

The length and competition-induced plasticity of vertical and horizontal structures were highly correlated in our experiment. We also found strong positive relationships between total shoot dry mass, petiole length and internode length under control conditions. In a previous experiment we showed that petiole length was also positively correlated with leaf area (Weijschede *et al.,* 2006). Natural populations of *T. repens* genotypes consist thus of a continuum of morphologies ranging from genotypes with large modules (longer

Table 2. *Results of mixed model of ANCOVAs examining the effects of competition and clipping (fixed effects) and the covariates (a) control petiole length, (b) petiole length plasticity, (c) control internode length and (d) internode length plasticity on plant performance (shoot biomass and ramet production). Internode and petiole length plasticity were calculated as the absolute difference of internode and petiole length in control as compared to competition treatment. Genotype and block were added as random effects. F-values and significances are presented. For significance levels see Table 1.*

a. Source	total shoot drymass	total ramet number
Competition	**3.85 $**	**23.78 *****
Clipping	0.52 ns	**2.94 $**
Competition*Clipping	**7.16 ****	**14.02 *****
Petiole length	0.73 ns	**5.72 ***
Competition*Petiole length	1.93 ns	0.00 ns
Clipping*Petiole length	**5.75 ***	**2.82 $**
Block[1]	0.94 ns	0.92 ns
Genotype[1]	**1.47 $**	**2.92 ****
Residual[1]	**13.48 *****	**13.44 *****

b. Source	Total shoot drymass	total ramet number
Competition	**33.15 *****	**57.13 *****
Clipping	0.74 ns	1.75 ns
Competition*Clipping	**7.08 ****	**14.02 *****
Petiole length plasticity	1.40 ns	0.95 ns
Competition*Petiole plasticity	**3.16 $**	2.07 ns
Clipping*Petiole plasticity	1.46 ns	1.61 ns
Block[1]	0.93 ns	0.92 ns
Genotype[1]	**1.40 $**	**3.05 ****
Residual[1]	**13.48 *****	**13.43 *****

c. Source	total shot drymass	total ramet number
Competition	0.04 ns	**4.43 ***
Clipping	0.45 ns	1.70 ns
Competition*Clipping	**4.06 ***	0.03 ns
Internode length	1.35 ns	**3.44 $**
Competition*Internode length	**9.43 ****	**4.10 ***
Clipping*Internode length	**4.21 ***	1.54 ns
Competition*Clipping* Internode length	**8.30 ****	0.78 ns
Block[1]	0.94 ns	0.92 ns
Genotype[1]	**1.46 $**	**2.99 ****
Residual[1]	**13.45 *****	**13.42 *****

d. Source	total shoot drymass	total ramet number
Competition	**118.54 *****	**224.09 *****
Clipping	**18.78 *****	0.67 ns
Competition*Clipping	**15.86 *****	**19.75 *****
Internode length plasticity	**3.53 $**	0.12 ns
Competition*Internode plasticity	**17.24 *****	**11.92 *****
Clipping*Internode plasticity	0.01 ns	1.04 ns
Competition*Clipping* Internode plasticity	**11.94 *****	**5.31 ***
Block[1]	0.94 ns	0.93 ns
Genotype[1]	**1.36 $**	**3.10 ****
Residual[1]	**13.45 *****	**13.42 *****

[1] Z-values and significances are given for random effects.

petioles, longer internodes, and larger leaf areas), higher biomass production and a more linear morphology (less branching) to genotypes with smaller modules (shorter petioles, shorter internodes, and smaller leaf areas), lower biomass production and a more branched morphology. Our data explore the consequences of these genotypic trait differences for plant performance. Genotypes with longer petioles and internodes under high light conditions were disfavoured under clipping regimes. In contrast to our hypothesis, however, producing long petioles under high light conditions was not beneficial if plants were subjected to competition. Since petiole and internode length were positively correlated, the expected benefits of producing longer petioles under competitive, low light conditions may have been counteracted by ineffective resource allocation to production of longer internodes (note the similarity between figures 2 and 3). These trait correlations may hence explain the apparent discrepancy between our prediction and results. Our data support the notion that trait correlations may weaken selective forces acting on a focal trait in a specific environment if opposing selection pressures act on genetically correlated traits (Garland and Kelly, 2006; Pigliucci and Kolodynska, 2002; Pigliucci *et al.,* 1998).

Internode elongation

In contrast to our hypothesis, plants subjected to competition produced 18% longer internodes than plants grown alone. These results are in line with some observations reported in the literature (de Kroon and Hutchings, 1995; Thompson, 1995; van Kleunen and Fischer, 2001; Waite, 1994) but are contradictory to many others (Huber *et al.,* 1998; Leeflang, 1999; Solangaarachchi and Harper, 1987; Thompson and Harper, 1988) including our previous work with the same *T. repens* genotypes (Weijschede *et al.,* 2006) in which low levels of plasticity or a shortening of horizontal structures were observed under low light conditions. Our data obtained on the same set of genotypes grown in different experimental conditions suggest that internode elongation may not only be triggered by decreased light availability, a reduction of the red to far-red ratio (Schmitt and Wulff, 1993) or a reduction in blue light (Gautier *et al.,* 1998). Plant resource status and other cues intercepted by plants under field conditions like for example, ethylene concentrations (Pierik *et al.,* 2003) or relative humidity (Price and Hutchings, 1996) may interact in determining final internode length.

Consequences of plastic responses

In line with our hypothesis, genotypes which expressed higher degrees of plasticity in vertical structures performed (marginally) better under competition compared to less plastic genotypes. Expressing higher degrees of petiole elongation was associated with higher shoot biomass (but not increased vegetative propagation) under competition. Genotypes which produced the highest shoot dry mass under competition were also characterized by the highest degrees of internode elongation. The positive genetic correlation among internode and petiole length plasticity may explain the unexpected positive relationship between internode elongation and shoot biomass production, as internode length plas-

ticity does not directly affect resource capture and can thus also not affect biomass production. Pleiotropic effects may have caused the positive relationship between internode and petiole length plasticity. Alternatively, the increased resource capture in genotypes characterized by high petiole length plasticity may also be allocated to the internodes, leading to increased internode growth.

Similar to our earlier results on petioles (Weijschede *et al.,* 2006) genotypes that expressed higher degrees of shade induced internode plasticity produced lower shoot biomass and fewer ramets under control conditions. We found that the potential to express higher degrees of internode plasticity was associated with decreased plant performance under conditions in which plasticity was not induced. Observations in both studies suggest that the potential to express shade-induced elongation per se is costly for plants that grow in non-inductive environments (DeWitt *et al.,* 1998; van Tienderen, 1991).

Implications for selection

If trait correlations constrained the benefits of plastic responses, selective pressures would consequently be weakened. Shade-avoidance responses should result in benefits under poor light conditions but pleiotropic effects of correlated traits may lower these benefits. Other trait correlations such as changes in root-shoot allocation between treatments or genotypes may also have reduced the expected benefits of petiole length and petiole length plasticity (Cahill, 2002; Cahill, 2003; Zobel and Zobel, 2002). This suggests that selection on shade-avoidance traits may be weaker or virtually absent in some systems, while they are undoubtedly prevalent in a number of other systems (Dorn *et al.,* 2000; Dudley and Schmitt, 1996; Schmitt *et al.,* 1999; Schmitt *et al.,* 2003). In *T. repens* the evolutionary consequences of trait correlations will depend on the spatial and temporal scale of environmental heterogeneity. In homogeneous grasslands, escaping the shade of neighbouring plants can only be achieved in the vertical direction and concurrent length increase of correlated horizontal structures may constrain the benefits of producing longer vertical structures. Alternatively, in highly patchy environments plants are likely to frequently encounter different micro-sites during their life span. In this case, escape in both vertical and horizontal direction will be beneficial and increased plasticity in the length of vertical and horizontal structures will result in enhanced plant performance (Cain, 1994; Waite, 1994). Under these conditions, trait correlations may not counteract the benefits associated with longer or more plastic vertical structures.

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Does environmental heterogeneity favour trait variation? A field study using *Trifolium repens* under divergent microhabitat conditions

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Summary

We studied the consequences of genotypic and plastic variation in morphological characters on plant performance in a stoloniferous herb under natural field conditions. We hypothesized that plants characterized by longer vertical structures and larger leaves would be favoured in higher vegetation and under higher degrees of light reduction.

Eight floodplain genotypes of *Trifolium repens* consistently expressing different petiole lengths and leaf areas were each explanted in 99 plots in their original habitat with each genotype representing each of the plots. The 580 out of 792 surviving plants were harvested and measured at the end of the summer. Mean vegetation height and shading (light reduction at soil level) differed considerably per plot during the experiment and plant performance correlated with microhabitat conditions, as expected.

Genotypes responded differently to vegetation height and shading. In addition to light, herbivory proved to be a major player affecting plant performance under natural conditions.

In contrast to our hypothesis genotypic differences (measured under high light greenhouse conditions) in petiole length and leaf length did not explain variation in plant performance in the field. However, phenotypic differences in petiole length and leaf length were associated with differences in plant performance: producing longer petioles reduced the negative effects of higher vegetation and shading on total shoot dry mass and producing longer leaves reduced the negative effect of light reduction on total shoot dry mass and ramet production.

Our results indicate that, in our system, phenotypic petiole and leaf length values are more important for *T. repens* growth under natural conditions than genotypic differences therein. In addition, we found no evidence that environmental heterogeneity favours petiole length or leaf length differences among genotypes.

Introduction

In natural vegetations, growth conditions for plants vary temporally and spatially and resources for plant growth (i.e. light, nutrients, moist) are distributed heterogeneously rather than homogeneously (Bell *et al.*, 1991; de Kroon & Hutchings, 1995; Huber *et al.*, 2004). Grasslands are in fact composed of numerous patches (or microhabitats) each with specific growth conditions and resource availability. Many vegetations are developped into a mosaic of microhabitats varying in canopy height and thus light availability as a result of local differences in soil conditions, water availability or disturbance (mammal activity) (Waite, 1994; Welham *et al.*, 2002).

Plants display a wide variety in morphological characters among species but also within a single species individual genotypes can differ considerably (Evans & Turkington, 1988; Cain *et al.*, 1995; Weijschede *et al.*, 2006). Genotypes can differ in their fitness (or general plant performance, measured as total biomass or total ramet number production) as well as in their morphological characters (i.e. internode length, petiole length or leaf size) (Fischer & van Kleunen, 2001; Pan & Price, 2001). Theory predicts that high environmental heterogeneity favours high genotypic variation: genotypes with different trait values are hypothesized to be selected for in different microhabitats (Via & Lande, 1985; Falconer & Mackay, 1996; Kingsolver *et al.*, 2001; Kassen, 2002; Byers, 2005). If this assumption is true, plants with for example longer vertical structures (i.e. stems and stem analogues) perform better in microhabitats with higher vegetation relative to plants with shorter vertical structures. Plants with longer vertical structures can position their leaves higher up in the canopy which enables them to capture considerably more light (Ballare *et al.*, 1994; Leeflang *et al.*, 1998; Weijschede *et al.*, 2006). Plants with shorter vertical structures, on the other hand, invest fewer resources in vertical structures and may perform relatively better in microsites with lower vegetation compared to plants with longer vertical structures since the saved resources can be used for other plant functions (i.e. storage, sexual or vegetative reproduction or defensive mechanisms (Weiner, 2004)).

In addition to morphological differences among species or genotypes, phenotypic plasticity can be assumed to be important component in microhabitat selection (Ballare *et al.*, 1994; Sultan, 2005). For example, in attempting to reduce the negative effects of lower light availability caused by crowding and shading by neighboring plants, most plants express so called shade-avoidance-responses (Schmitt & Wulff, 1993; Schmitt *et al.*, 2003). Triggered by changes in the amount and quality of the incident light, plants can elongate their vertical structures to reach higher and thus more favourable places in the canopy or increase their leaf area to enhance light harvesting under lower light intensities (Aphalo & Ballare, 1995; Ballare, 1999; Vandenbussche *et al.*, 2005). Phenotypic plasticity may buffer the effects of environmental heterogeneity on genotypic variation if genotypes can alter their phenotypes in a way to maintain constant performance under a variety of microhabitat conditions (van Tienderen, 1991).

Trifolium repens individuals originating from natural grasslands often display a large

range in morphological trait values among genotypes (Aarssen & Turkington, 1985; Evans & Turkington, 1988; Hutchings *et al.*, 1997; Weijschede *et al.*, 2006). In this study, we aim at relating plant characters to performance variation in response to different natural microhabitats. First, we investigate whether the study area contains various microsites featuring different environmental characteristics. Secondly, we examine if plant performance and morphological characters differ among genotypes and correlate with microsite conditions. Finally, we test if performance profiles among genotypes differ across microsites to examine if microsite conditions favour specific trait values. We transplanted our previously collected genotypes (Weijschede *et al.*, 2006) back into their original habitat and hypothesized that plants characterized by longer vertical structures (petioles) and larger leaves perform better in microsites with higher vegetation and more light reduction than genotypes with shorter petioles and smaller leaves.

Materials and methods

Plants

T. repens is a very common perennial herb and genotypes differ considerably in morphological traits such as petiole length, internode length and leaf area (Jahufer *et al.*, 1997; Weijschede *et al.*, 2006). Plant individuals are composed of repeating modules (or ramets, (Hay *et al.*, 2001)), each consisting of a node, a leaf, two nodal root primordia, an internode which connects the modules and a bud positioned in the leaf axil. The bud can develop into either a branch or a terminal inflorescence, or stay dormant (Turkington & Burdon, 1983; Huber & During, 2000). A branch consists of a series of new modules produced by an apical meristem. Due to the stoloniferous growth form, petioles are the only structures that allow *T. repens* to position the laminas higher in the canopy.

107 *T. repens* plants were collected from a natural grassland population along the river Waal near Ewijk (the Netherlands, 51°52'54"N, 5°45'00"E) in the summer of 2001. Molecular techniques (AFLP, four primer combination, 145 markers) were used to verify genetic identity of the collected plants. Thirty-four unique genotypes were previously selected according to their range in petiole length and used in our preceding experiment (Weijschede *et al.*, 2006). From these 34 genotypes, we select a sub-sample of 8 genotypes representing the whole range in petiole length produced under high light greenhouse conditions (see Table 1 for detailed genotype characteristics).

To provide sufficient material for the experiment, each genotype was clonally propagated in a greenhouse. On May 13th, 99 cuttings of each genotype were transplanted into 'Forestry Pellets' (diameter: 3.5 cm, length: 7.0 cm, Jiffy International A/S, Ryomgård, Denmark). Each cutting consisted of a ramet, a well-developed root system and a lateral branch with 3-4 ramets. All 792 plants were left to grow for four days under greenhouse conditions and subsequently for seven days under outdoor conditions.

Table 1: *Morphological characteristics and standard errors per genotypes found under high light greenhouse conditions (see Weijschede et al., 2006). Genotypes are ranked according to their petiole length. Ramet weight includes the dry masses of the petiole, internode and lamina of the fourth ramet counted from the apex.*

Genotype	Petiole length mm	1 se	Internode length mm	1 se	Leaf area cm2	1 se	Ramet weight mg	1 se	Ramet production per day	1 se
E1	56.8	2.69	17.3	2.02	22.9	2.42	19.0	3.21	2.1	0.26
H2	64.3	5.57	23.8	1.11	28.7	1.36	21.5	1.51	1.3	0.10
A3	72.3	5.74	18.3	2.06	33.5	2.52	25.6	2.31	0.7	0.21
F4	75.8	5.36	25.8	2.50	34.4	0.79	23.9	0.57	1.0	0.13
B5	78.8	3.42	23.8	1.25	50.8	5.42	34.6	2.61	0.9	0.09
D6	89.3	8.86	32.0	1.63	50.4	8.80	32.4	3.67	0.9	0.13
G7	94.3	3.09	23.5	1.50	37.8	2.80	28.2	1.41	1.3	0.22
C8	105.5	7.24	32.3	1.65	44.8	8.03	31.6	1.72	1.0	0.12

Study area

The study was performed in the river floodplain along the river Waal from which the plants were originally collected. This area is characterized by yearly winter floods and the presence of human and herbivore activity (horses and cows). As a result, the vegetation develops into a mosaic of different microhabitats (or microsites) ranging from more open microsites to more crowded microsites. In this habitat we selected an area of 20x50 m. This area was overlain with a 2x2 m grid. The grid created 216 dissections which we used to randomly select 99 plots. On May 24th, we explanted each of our 8 genotypes in each plot in a circle with a 0.10 m diameter with the apex of each plant growing away from the centre to avoid interactions between the developing plants (see Fig. 1). The genotype order and growth direction was randomly chosen for each plot. From May 24th until August 16th all 792 plants (8 genotypes, 99 plots) were left to grow. All plants were watered directly after explanting and again after 1, 3 and 7 days to avoid planting shock and facilitate establishment. Thereafter plants were not artificially watered for the rest of the experiment.

Measurements and harvest

The vegetation height was measured on the 9th, 26th, 46th, 60th, 72nd, and 85th day after transplanting in each plot. The mean of these six measurements were used as an indication for the average vegetation height per plot. Light reduction (extinction of photosynthetic active radiation (PAR) measured at 1 cm above the soil relative to the top of the vegetation) was recorded on the 26th, 46th, 60th, 72nd and 85th day in each plot using a Skye SKP 200 equipped with a Skye quantum sensor (Skye instruments, Llandrindod Wells, UK). The mean of these five measurements were used as an indicator of light reduction (or shading) per plot in the analyses.

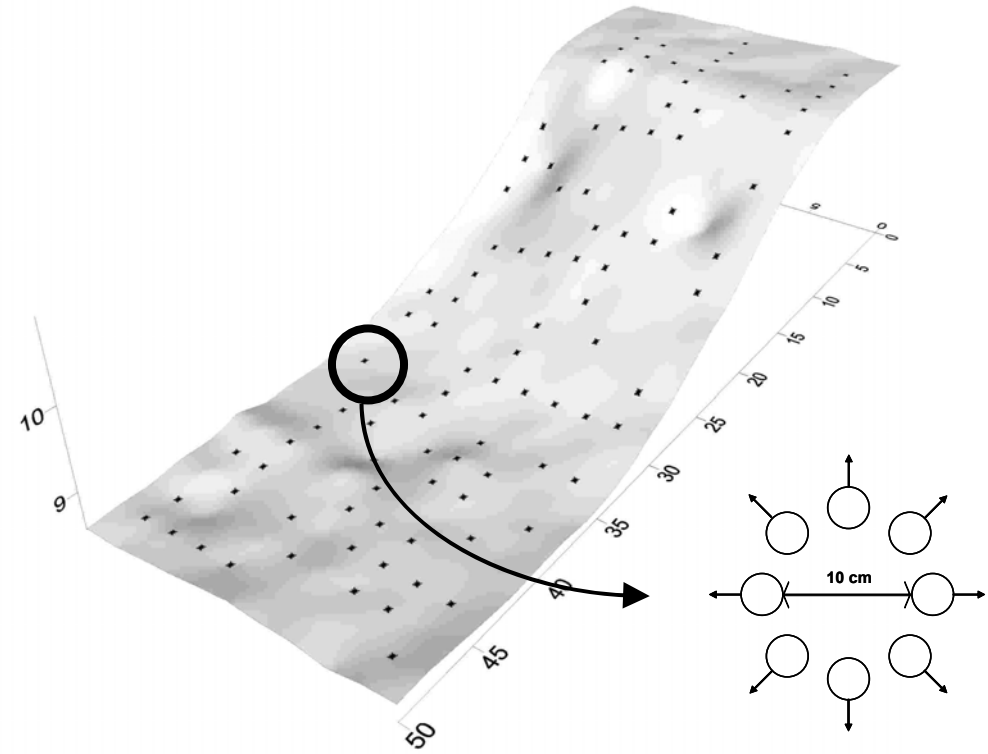

Figure 1: *Overview of the experimental area and the 99 selected microsites. One site is enhanced and shows how the genotypes were planted into each microsite. Growth direction and order of the genotypes per site were randomly assigned.*

On August 16th, all surviving plants were harvested. The total ramet number was recorded and petiole length, internode length and leaf length were measured at the 4th youngest ramet at the main stolon (or the 5th if the 4th was damaged). Plants were dried at 70°C for two days before measuring the total shoot dry mass.

During harvest, most of the plants showed clear marks of damage (i.e. damaged leaves, apices, stolons) caused by small herbivores (mostly slugs, pers. obs. J. Weijschedé). We therefore recorded the amount of damage for each plant according to 4 categories (0-3): 0, no damage, no visible marks of damage to the leaves, petioles or stolon; 1, little damage, some laminas of the plant were damaged but in total no more than five complete leaves were missing; 2, average damage, up to half of the leaves were damaged or missing but the apex on the main axis was intact; 3, heavy damage, more than 50% of the leaves were damaged or missing and the apex on the main stolon was damaged or absent.

Statistics

To test if the high mortality in this experiment (580 out of 792 plants survived) differed among genotypes we performed a logistic regression (using the GENMOD procedure in

SAS). The mean vegetation height throughout the experiment was added as a covariate to the model as well as the interaction between vegetation height and genotype to test if genotypic specific survival depends on the vegetation height. Plants that did not survive the experiment were excluded from all further analyses.

To test if plant performance depends on vegetation height or light reduction, we calculated correlations between mean total shoot biomass and ramet number production per plot (sum of eight genotypes per plot) and mean vegetation height and light reduction. Significant correlations indicate that different habitat conditions per plot are associated with differences in mean plant performance.

We performed a multivariate analysis of covariance (MANCOVA) to determine the effects of vegetation height, light reduction, and genotype on total shoot dry mass, ramet number, petiole length, internode length, and leaf length. Genotypes were considered fixed effects since we specifically chose these genotypes for their morphological characteristics. To account for the effects of damage on plant growth, we added the damage categories as a covariate to the model.

To correct for overall genotypic differences in performance we repeated the same analyses with relative plant performance which was calculated as the performance of a genotype in a plot relative to the mean performance of that specific genotype measured over all plots. If specific microhabitat conditions (i.e. vegetation height) favour specific genotypes, we expect to find significant genotype x environment interactions.

To test whether differences in trait values (petiole length, internode length or leaf length) contribute to plant performance in the field, we used the following regression model:

$$W_{ij} = E_j + T_{ij} + E_j*T_{ij} + D_{ij} \qquad (1)$$

with Wij as the relative plant performance (either total shoot dry mass or total ramet number) of genotype i in plot j; E_j, the environmental factor (either vegetation height or light reduction) in plot j; T_{ij}, the trait value (either petiole length, internode length or leaf length) of genotype i in plot j; E_j*T_{ij}, the environment x genotypic trait value interaction; D_{ij}, damage category for genotype i in plot j (added as covariate to the model). A significant positive partial regression coefficient of T indicates that genotypes with higher values in that trait perform relatively better than genotypes with lower trait values in the same environment. A significant E*T interaction indicates that the effect of trait T on plant performance depends on microhabitat conditions.

To test if genotypic differences in trait values (measured under high light greenhouse conditions) contribute to plant performance under field conditions, we used the same model with the trait values found under field conditions (T_{ij}) being replaced by the genotypic trait values found under homogeneous high light greenhouse conditions (as in Table 1). In this modified analysis, a positive regression coefficient for the term T indicates that genotypes which have higher trait values (i.e. longer petioles) under controlled conditions perform better under field conditions than genotypes with lower values in that trait.

Results

Microsite conditions and general plant performance

The vegetation height median over all microsites was 10.5 cm and ranged between 7.5 and 18.2 cm in 82% of the plots. In 10% of the microsites the vegetation height was lower than 7.5 cm and in 8% higher than 18.2 cm (Fig. 2). The median of the light reduction was 79.5% (at soil level relative to the top of the vegetation) and was between 65.0% and 91.8% in most plots (86%). In 6% of the microsites the reduction was higher than 91.8% and in 8% it was lower than 65.0% (Fig. 2).

27% of the plants did not survive the experiment. The vegetation height was the main factor correlating with survival and survival did not differ among genotypes (Vegetation height effect: $\chi^2_{df=1}$=152.6, p<0.001; Genotype effect: $\chi^2_{df=7}$=5.72, p=0.573; Vegetation height x genotype effect: $\chi^2_{df=7}$=3.90 p=0.791).

In most plots (82%), surviving plants produced on average between 0.121 and 0.585 gram total shoot dry mass per plot, in 9% of the plots plants produced more than 0.585 g biomass and in 9% less than 0.121g (Fig. 2). Plants produced on average between 22.5 and 72.0 ramets in most microsites (82%), in 9% of the plots plants produced more than 72.0 ramets and in 9% fewer than 22.5 (Fig. 2). Figure 2 also shows that there were only a few plots where the average plant performance was relatively high (up to 1.0 gram shoot dry weight and up to 180 ramets) indicating that, generally, the growth conditions in the plots were not favourable for plant growth.

In most plots (84%) the average damage recorded on the plants ranged between missing at least five leaves up to missing more than half of the leaves (damage category per plot was between 1.3 and 2.8). In 7% of the microsites damage was less than 1.3 and in 9% more than 2.9. There were no plots where no damage was recorded (Fig. 2) and table 2 shows that damage strongly affected plant performance and trait values.
The average shoot dry mass and ramet number production per plot was significantly lower with increasing vegetation height and decreasing light quantity while damage increased with increasing vegetation height and decreasing light availability (see Fig. 3 for correlation coefficients). On average, plants produced longer petioles and longer laminas in higher vegetation and under low light conditions (positive correlations between the average petiole length and leaf length and the mean vegetation height and light reduction, Fig. 3), while internode length did not correlate with either vegetation height or light total.

Genotypic responses to vegetation height and light quantity

In the microsites, genotypes differed in their growth characteristics as well as in their morphology (Table 2). Genotypes tended to respond differently to vegetation height with respect to total shoot dry mass (marginal genotype x vegetation height interaction, Table 2) but not regarding ramet production. Genotypes responded differently to vegetation height with respect to their petiole length and leaf length (significant vegetation height

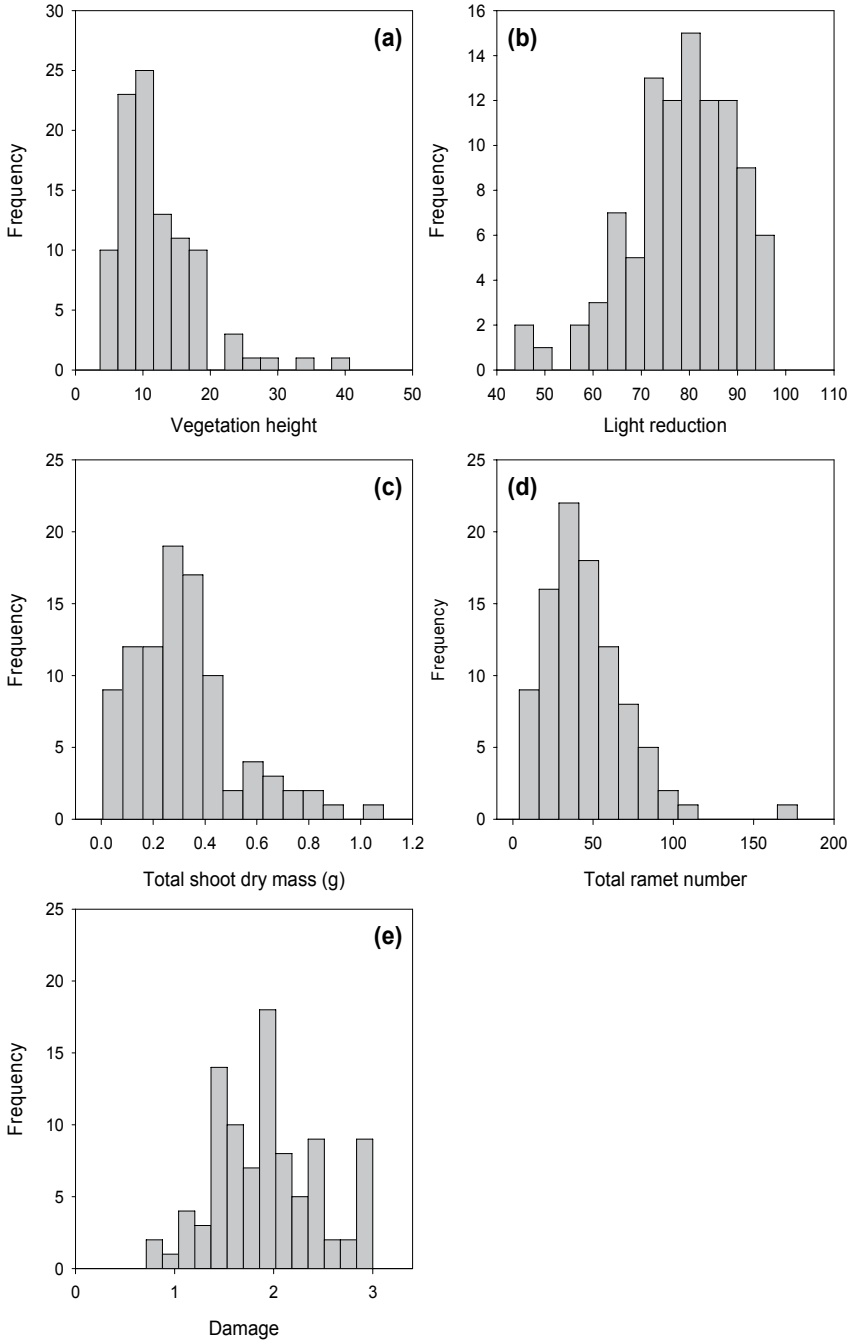

Figure 2: *Frequency distributions of growth conditions and general plant performance per microsite: (a) vegetation height, (b) light reduction, (c) total shoot dry mass, (d) total ramet number and (e) amount of damage recorded on the plants (for damage categories see Materials and Methods section).*

x genotype interactions) but not regarding their internode length.

Decreased light availability reduced the total ramet number and affected plant morphology (Fig. 3). Light quantity also reduced total shoot dry mass (correlation coefficient (r)=-0.305, p=0.003, Fig. 3), but the MANCOVA (Table 2), in which other interacting factors are added (like damage), did not show effects of light reduction on total shoot dry mass. Genotypes differed in their response to reduced light availability with total shoot dry mass and marginally with total ramet number (significant light reduction*genotype interactions, Table 2A, see Fig. 4).

Although the MANCOVA revealed environment and genotype and genotype x environment effects on the absolute values of plant performance (Table 2A), the same analyses performed on the relative shoot dry mass and ramet number did not reveal significant effects of vegetation height and light reduction (Table 2B).

Trait contributions to plant performance

Genotypes characterized by longer petioles, longer internodes and longer leaves in the microsites produced relatively heavier shoots than genotypes characterized by shorter petioles, internodes and leaves (significant positive regression coefficients for (field) trait value in Table 3A). Genotypes producing longer internodes and longer leaves under field conditions produced relatively more ramets (Table 3A). Shoot biomass from genotypes producing longer petioles was less affected by vegetation height compared to genotypes producing shorter petioles (marginal vegetation height x petiole length interaction, Table 3A). Producing longer internodes or longer leaves in higher vegetation did not change the effect of vegetation height on plant performance. The negative effect of increased shading on total shoot dry mass was less for genotypes producing longer petioles, internodes and leaves (significant light reduction x trait value interactions in Table 3A). Total ramet number from genotypes producing longer leaves was less reduced under lower light intensities compared to genotypes producing shorter leaves.

Genotypic differences in morphology (found under homogenous high light greenhouse conditions) did not determine relative differences in plant performance among microsites (non significant regression coefficients for (high light) trait value or environment x trait value in Table 3B), indicating that genotype specific petiole, internode and leaf size values were not favoured or disfavoured in our microsites.

Table 2: *(A) Results of MANCOVA for the effects of vegetation height (left side of the table) or light reduction (right side) and genotype on growth and morphological characters. (B) Results of the same analysis but with the absolute genotypic performance values (total shoot biomass and ramet number) per microsite being replaced by genotypic values relative to the mean performance per genotype across all microsites. Damage was added as a covariate to the analysis to correct for variation caused by herbivore damage. F-values are given. Significance levels are as follows: ns, p>0.10; $, 0.10 ≥p>0.05; *, 0.05≥p>0.10; **, 0.01≥p>0.001; ***, p<0.001.*

	Vegetation height (Veg.h.)	**Genotype**	**Veg.h. x genotype**	**Damage**	**Error d.f.**	**Light reduction (L.red.)**	**Genotype**	**L.red. x genotype**	**Damage**	**Error d.f.**
(A) Absolute values										
Growth										
Total shoot biomass	**10.55 ****	**5.05 *****	**1.85 $**	**18.53 *****	559	1.16 ns	**3.34 ****	**2.35 ***	**21.78 *****	559
Total ramet number	**44.59 *****	**2.80 ****	1.12 ns	**10.95 *****	559	**24.46 *****	**2.34 ***	**1.81 $**	**1154 *****	559
Multivariate test	**23.17 *****	**2.91 *****	1.15 ns	**10.59 *****		**16.40 *****	**2.34 ****	**1.85 ***	**11.42 *****	
Traits										
Petiole length	**120.83 *****	**3.16 ****	**2.32 ***	**4.64 ****	480	**114.73 *****	0.79 ns	0.54 ns	**2.38 $**	480
Internode length	**16.11 *****	**5.91 *****	**2.07 ***	**2.78 ***	480	**15.45 *****	**2.47 ***	1.47 ns	**2.19 $**	480
Leaf length	**35.03 *****	**3.46 ****	**1.69 ns**	**6.01 *****	480	**31.00 *****	1.54 ns	1.05 ns	**4.71 ****	480
Multivariate test	**43.52 *****	**3.98 *****	**1.80 ***	**3.17 *****		*41.58* ***	1.38 ns	1.04 ns	**2.55 ****	
(B) Relative values										
Growth										
Relative shoot biomass	**9.47 ****	0.86 ns	1.01 ns	**16.67 *****	559	0.37 ns	**1.88 $**	**1.97 $**	**20.39 *****	559
Relative ramet number	**49.35 *****	0.73 ns	0.76 ns	**10.47 *****	559	**25.20 *****	1.38 ns	1.42 ns	**11.27 *****	559
Multivariate test	**26.11 *****	0.68 ns	0.79 ns	**10.10 *****		**18.52 *****	1.49 ns	**1.56 $**	**11.04 *****	
d.f.		1	7	7	3		1	7	7 3	

Table 3: *Effects of vegetation height (left side of the table) or light reduction (right side) and genotypic trait values (found under either (A) field or (B) high light conditions, see Materials and Methods) on relative plant performance. Numbers are partial regression coefficients and significant values indicate positive or negative (indicated by the sign of the estimate) contributions of that specific character to relative plant performance. Note that the estimates are not standardized and that the magnitude of the estimates cannot be compared to each other. Significance levels are as follows: ns, p>0.10; $, 0.10≥p>0.05; *, 0.05≥p>0.10; **, 0.01≥p>0.001; ***, p<0.001.*

	Vegetation height (Veg.h.)	Trait value	Veg.h. x trait value	Damage	Error d.f.	Light reduction (L.red)	Trait value	L.red. x trait value	Damage	Error d.f.
(A) Trait values found under field conditions										
Petioles										
Relative shoot biomass	-0,018 ns	**0,010 *****	**-0,000 $**	**-0,267 *****	506	0,008 ns	**0,022 ***	**-0,000 ***	**-0,280 *****	506
Relative ramet number	**-0,071 *****	-0,003 ns	0,000 ns	**-0,193 *****	507	-0,010 ns	0,003 ns	-0,000 ns	**-0,204 *****	507
Internodes										
Relative shoot biomass	-0,014 ns	**0,052 *****	-0,001 ns	**-0,249 *****	514	0,007 ns	**0,091 ****	**-0,001 ***	**-0,272 *****	514
Relative ramet number	**-0,035 ***	**0,024 ***	-0,001 ns	**-0,204 *****	515	-0,006 ns	**0,057 ***	**-0,001 $**	**-0,211 *****	515
Laminas										
Relative shoot biomass	0,006 ns	**0,140 *****	-0,004 ns	**-0,232 *****	493	**0,030 ***	**0,322 *****	**-0,003 ***	**-0,256 *****	493
Relative ramet number	-0,050 ns	0,028 ns	-0,001 ns	**-0,185 *****	494	0,018 ns	**0,232 ***	**-0,003 ***	**-0,196 *****	494
(B) Trait values found under high light conditions										
Petioles										
Relative shoot biomass	-0,033 ns	0,000 ns	0,000 ns	**-0,278 *****	574	0,004 ns	0,008 ns	-0,000 ns	**-0,311 *****	574
Relative ramet number	-0,044 ns	0,002 ns	-0,000 ns	**-0,218 *****	574	-0,005 ns	0,012 ns	-0,000 ns	**-0,238 *****	574
Internodes										
Relative shoot biomass	-0,024 ns	0,002 ns	-0,000 ns	**-0,277 *****	574	0,005 ns	0,026 ns	-0,000 ns	**-0,310 *****	574
Relative ramet number	-0,035 ns	0,006 ns	-0,001 ns	**-0,217 *****	574	-0,002 ns	0,043 ns	-0,001 ns	**-0,237 *****	574
Laminas										
Relative shoot biomass	**-0,077 ***	-0,014 ns	0,001 ns	**-0,282 *****	574	-0,012 ns	-0,018 ns	0,000 ns	**-0,311 *****	574
Relative ramet number	**-0,077 ****	-0,008 ns	0,001 ns	**-0,220 *****	574	-0,015 ns	0,001 ns	-0,000 ns	**-0,238 *****	574

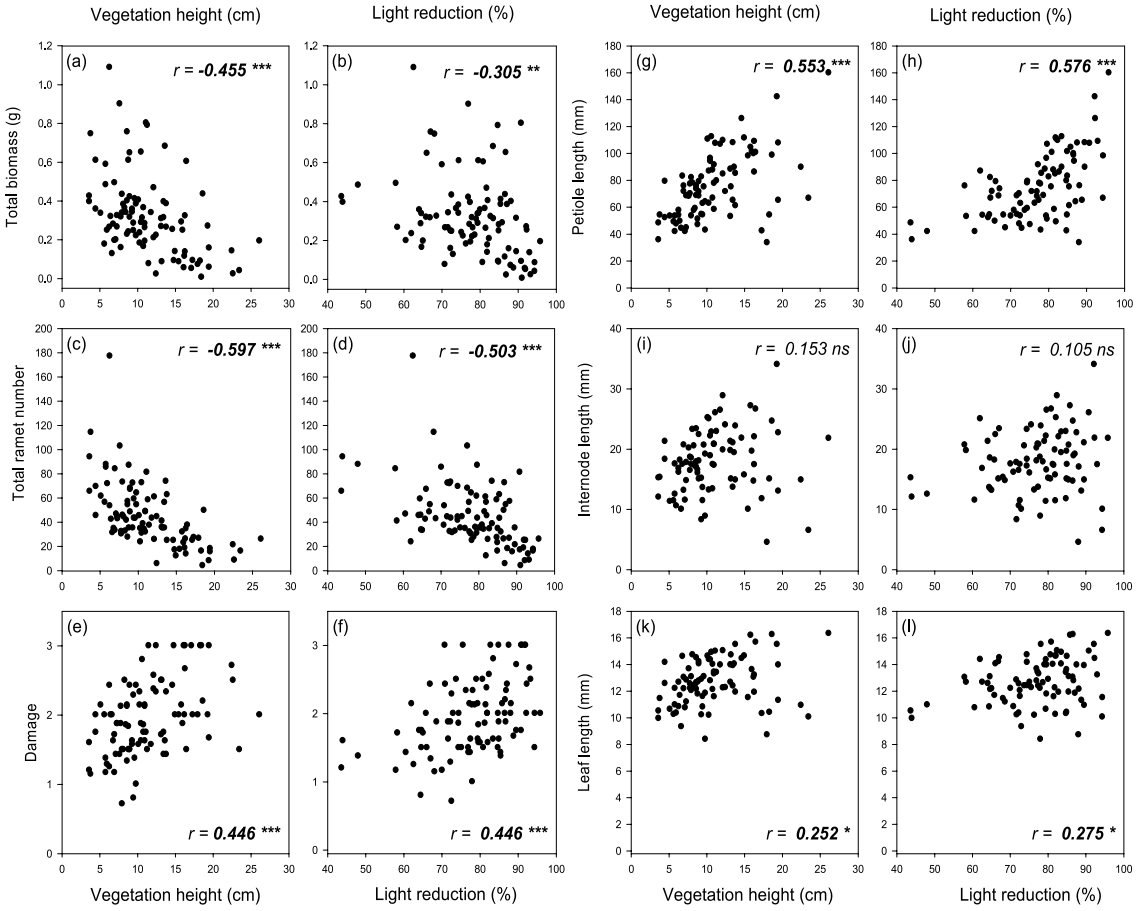

Figure 3: *Graphs (a) – (d) show the correlations between environmental conditions (vegetation height and light reduction) and plant performance (average performance of all genotypes per site). Graphs (e) and (f) show the correlations between environmental conditions and the average amount of damage recorded on the genotypes per site. Graphs (g)-(l) show correlations between the microsite growth conditions and the average trait values recorded on the genotypes per site. Pearson correlation coefficients (r) are inserted in the graphs and highlighted in bold and italic at P < 0.05. Significance levels are as follows: ns, p>0.10; $, 0.10≥p>0.05; *, 0.05≥p>0.10; **, 0.01≥p>0.001; ***, p<0.001.*

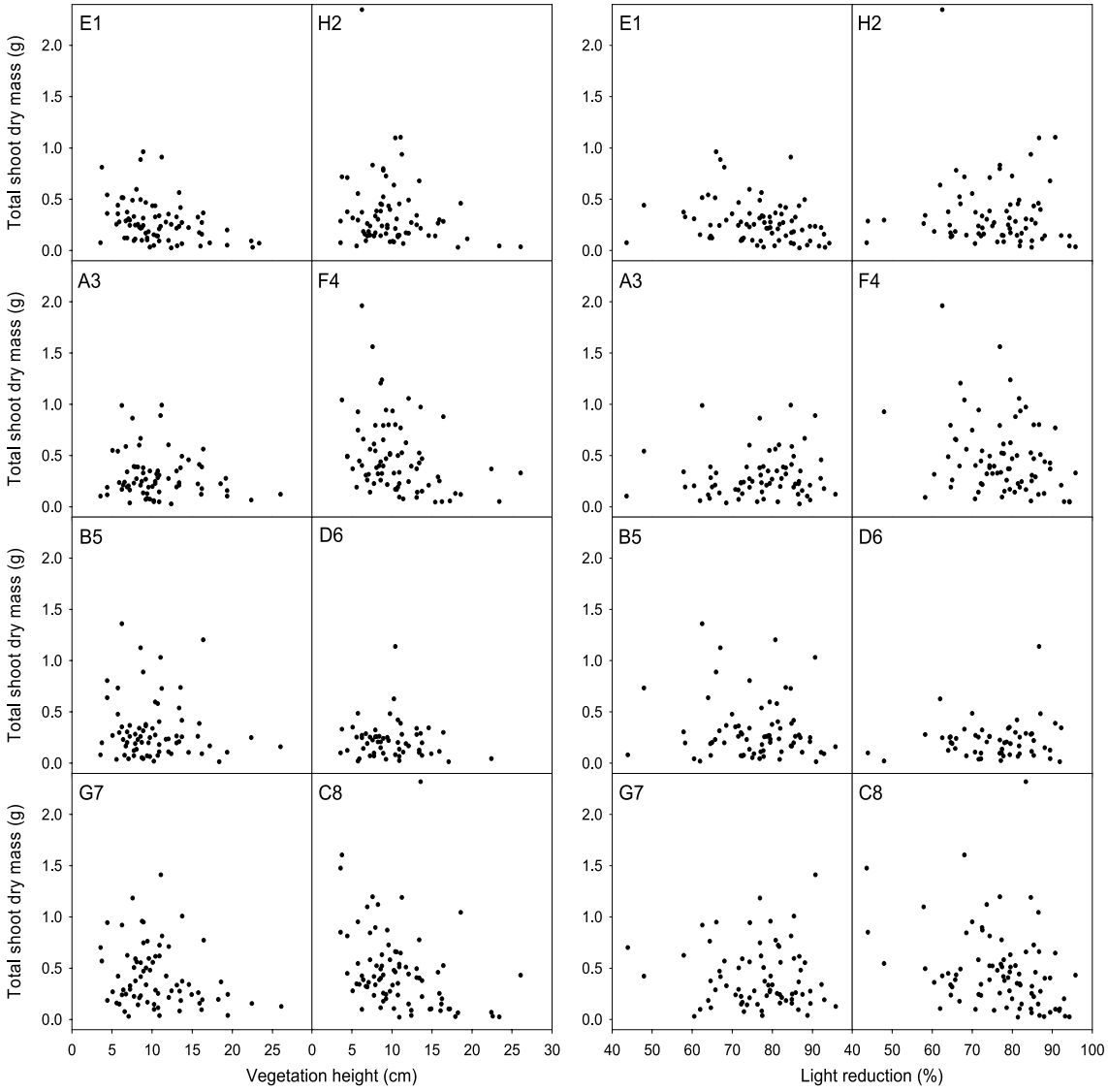

Figure 4: *Correlations between general plant performance (total shoot dry mass and total ramet number production) and microsite conditions per genotype.*

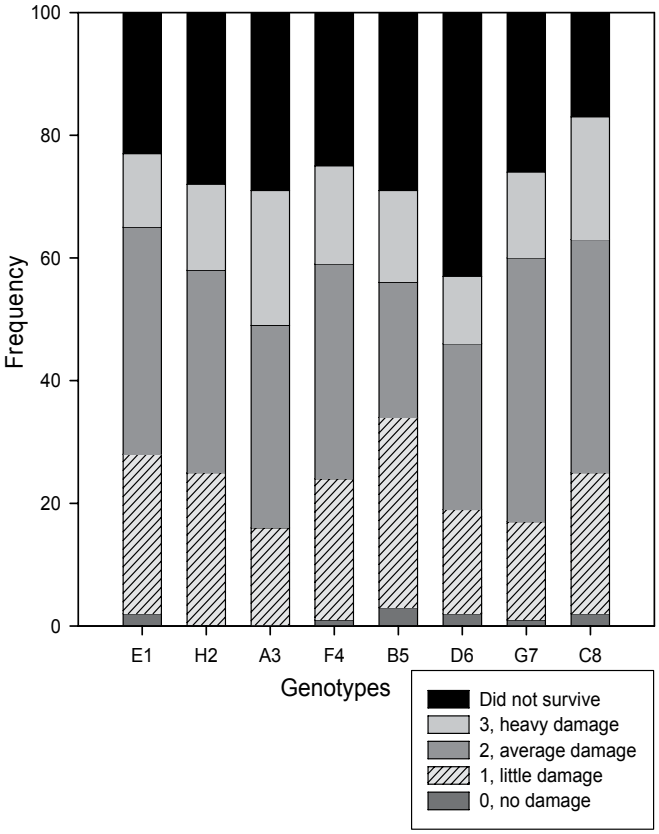

Figure 5: *Frequency distribution of damage recorded on the genotypes: 0, no damage, no visible marks of damage on the plants; 1, little damage, some laminas of the plants were damaged but less than five complete leaves missing; 2, average damage, up to half of the leaves damaged or missing but the apex on the main axes intact; 3, heavy damage, more than 50% of the leaves damaged or missing and the apex on the main stolon damaged or absent.*

Discussion

Microhabitat variation has often been proposed to favour genotypic variation (Via & Lande, 1985; Falconer & Mackay, 1996; Kassen, 2002; Byers, 2005). In the floodplain grasslands along the river Waal, we observed a large variation in microsite conditions due to variation in vegetation height and light reduction. The magnitude of this variation was relevant for the performance of *T. repens* plants: vegetation height and light reduction significantly affected total shoot dry mass and ramet number production. Our explanted genotypes, originating from this area, responded as expected to these growth conditions: plants produced longer petioles and larger leaves in higher vegetation and under higher

degrees of light reduction. Longer petioles enables stolonferous species such as *T. repens* plants to position their leaves higher in the canopy and larger leaves increased the light interception (Huber *et al.*, 1998). Our data are one of the few field experiments showing that longer vertical structures and larger leaves are indeed beneficial for plants in microsites characterized by higher vegetation. However, despite our predictions, our data show no advantages of genotypes intrinsically characterized by longer petioles or larger leaves if subjected to higher vegetation. Our results therefore suggest that, in our system, genetically determined intrinsic specific petiole length or leaf length values are not selected for under natural microsite conditions, but that environmentally modified actual expression of petiole length is of major importance for plant performance under heterogeneous conditions. These results also indicate that plasticity of leaf traits is indeed adaptive under natural conditions and will therefore be selected for.

As expected, we found strong correlations between the plant phenotypes and microsite growth conditions. Microsites did not only differ in vegetation height and light reduction but also in the amount of plant damage caused by small herbivores. The amount of damage differed per microsite and increased with vegetation height and light reduction. This may have directly and indirectly affected plant growth. Herbivore damage directly affected plant performance by the loss of tissue, subsequently reducing future plant growth. In addition, herbivory indirectly affected plant performance by reducing the plants potential to respond to shading (Kurashige & Agrawal, 2005). Increasing severity of damage reduced petiole and leaf lengths (measured on undamaged plant parts) which are both essential for present and future resource uptake (Weijschede *et al.*, 2006). Recently Gomez and coworkers also showed that in *T. repens* herbivory led to reduced petiole length on connected clonal fragments which were subjected to competition but not to direct herbivory (Gomez *et al.*, in press). These results indicate a negative impact of herbivory on overall competitive ability. In general it has been recognized before that the beneficial effects of shade avoidance responses may be masked by other interacting environmental factors like water availability (Huber *et al.*, 2004) or herbivory (Kurashige & Agrawal, 2005). Our experiment shows that in higher vegetation we find both, plants with longer (shade-induced) petioles as well as higher severity of herbivore damage. Contrasting selection pressures excerted by damage and shading may interact in determining plant performance. Some authors have recently argued that there may be a trade-off between shade avoidance responses and herbivore susceptibility (Cipollini, 2004; Kurashige & Agrawal, 2005). Herbivore induced reduction of petiole length can therefore be assumed to have negative effects on plant growth, exceeding the cost incurred by the pure loss of plant tissue and reducing the potential for selection on petiole length.

We found no evidence that microsite selection under the heterogeneous conditions of our field sites contributes to maintaining high genotypic variation in morphological traits like petioles. Our genotypes ranged in petiole length from 5.5 cm up to 10.5 cm under high light growth conditions. In most sites, the vegetation height was between 7.5 cm and 18.2 cm. If petiole length is an important parameter in determining performance profiles among genotypes, genotypes with longer petioles should perform relatively better

in higher vegetation than genotypes with shorter petioles. However, our data does not confirm this hypothesis. In addition, we could also not reveal performance advantages for *T. repens* genotypes with longer petioles in our previous common garden experiment in the presence or absence of competitors (using dense *Lolium perenne* monocultures) (Weijschede *et al.*,*in press*). The genotypic differences in petiole length may not be that important in higher vegetation especially as the absolute petiole elongation response to shading is not correlated to the genotypic petiole length (Weijschede *et al.*, 2006). In our previous greenhouse study we showed that genotypes producing shorter petioles as well as the genotypes producing longer petioles under high light conditions, could increase their petiole lengths up to 12 cm (thereby reaching heights of 17 cm up to 22 cm) in a vertical light gradient (Weijschede *et al.*, 2006) which, in this field experiment, was sufficient to place the laminas in the upper parts of the canopy in most microsites. Plasticity may therefore have buffered the effects of microsite conditions on plant performance rather than genotypic variation being favoured by microsite selection. In addition, herbivory may, directly or indirectly, have masked the effects of morphological differences among our genotypes.

In conclusion, this field study shows that, although all prerequisites for finding microhabitat selection were met (we had high morphological variation among genotypes, high environmental variation across microsites and every genotype was present in each microsite), we found no evidence for microhabitat selection for different trait values. Adaptive plasticity appeared to be more important in buffering environmental variation. In adition, other environmental factors present in nature like herbivory (Kurashige & Agrawal, 2005), water availability (Huber *et al.*, 2004) or nutrient availability (Pigliucci *et al.*, 1998) may overrule or interfere with the selective pressures acting on specific trait values. Input of genetic material from other populations or neutral selection may also contribute to the high observed variation in petiole length among genotypes (Ellstrand & Elam, 1993; Ouborg *et al.*, 1999). How natural selection acts in favouring genotypic variation under natural conditions remains a complex and interesting topic that still requires further research.

Acknowledgements

We thank 'Stichting Ark' for their permission to conduct this experiment in the river floodplain. We are seriously grateful to G. Bögemann, G. Clements, N. Cornips, S. 'Coco' Gomez, C. Jansen, T. van Mölken, L. Mommer, A. Smit-Tiekstra and E. Visser for their practical support.

Summarizing discussion

Jelmer Weijschedé

Genotypes, plasticity and environmental heterogeneity

Different genotypes of the same species may differ in morphological characters such as petiole length, internode length and leaf area. Plants of a single genotype can also give rise to different phenotypes in response to different environmental conditions (phenotypic plasticity) (Bradshaw, 1965; Sultan, 1987; DeWitt *et al.*, 1998). Plasticity enables plants to alter morphological traits (like petiole length, internode length and leaf area) and is adaptive if it increases the match between the phenotype and the conditions at which the plant grows, thereby increasing resource uptake (Dudley & Schmitt, 1996; Schmitt, 1997; Cipollini & Schultz, 1999; Donohue *et al.*, 2000). If plants grow close together, plants shade each other and competition for light occurs. Plants have evolved a whole suite of traits to respond to shading and increase light interception, the most prominent being elongation of vertical structures like stem internodes and petioles (Huber *et al.*, 1998; Huber & Hutchings, 1997). These responses enable plants to position their leaves higher in the canopy where more light is available. Stoloniferous plants have been argued to also respond in a horizontal direction by elongating horizontal structures (stolon internodes) which may lead to an escape from unfavorable microhabitat conditions (i.e. locally high desities)(de Kroon & Hutchings, 1995; Waite, 1994).

Resource distribution in natural habitats is rather patchy. In natural grasslands, growth conditions for plants vary temporally and spatially on a variety of scales (Bell & Lechowicz, 1991; de Kroon & Hutchings, 1995; Charpentier & Stuefer, 1999; Huber *et al.*, 2004) and grasslands are composed of numerous patches (or microhabitats) each with specific growth conditions and resource availability (Waite, 1994; Welham *et al.*, 2002). Grasslands in river floodplains for example show high environmental variation due to river floods, differences in soil conditions and irregular disturbance caused by the activity of small and large herbivores (Vervuren *et al.*, 2003; Voesenek *et al.*, 2004; van Eck *et al.*, 2004). As a result of these diverse growth conditions the herbaceous vegetation is characterized by a dynamic mosaic of microhabitats varying in canopy height and thus light availability. Microhabitats range from sites where the vegetation has developed and competition for light is high (disturbance has been relatively low) to more open sites where the vegetation has been disturbed or removed (characterized by low above ground competition).

Trifolium repens (white clover) is a very common stoloniferous herb and abundant in river floodplains. Individuals show a large variation in morphological traits such as petiole length, internode length and leaf area (Aarssen & Turkington, 1985; Evans & Turkington, 1988; Hutchings *et al.*, 1997; Jahufer *et al.*, 1997). Thirty-four genotypes originating from floodplains of the River Rhine and selected from a larger collection of 107 genotypes, were used in this thesis representing a large range of petiole length differences among genotypes. Petiole length is of vital importance for *T. repens* since this is the only structure allowing the plant to increase light interception in a vertical direction.

Although a vast number of studies have found that low light conditions generally induce plastic elongation responses in plants, the mechanistic determinants and as well as

the ecological consequences of phenotypic variation on plant performance is still largely unknown. The main aim of this thesis was to provide insight into the evolutionary and ecological consequences associated with morphological differences among *T. repens* genotypes and shade-induced responses. In particular, this thesis investigated how genetically determined variation as opposed to environmentally induced differences in trait values (i.e. petiole length) affect plant performance. The first step in achieving this was to examine whether the degree of shade-induced plasticity differs among genotypes and to what extent trait values expressed under different light regimes affect plant performance (**Chapter 2**). Second, cellular mechanisms were examined to show how cell number and cell size contribute to variation found in petiole length among genotypes and to shade-induced petiole elongation as well as the associated consequences for biomechanic characteristics and plant performance (**Chapters 3 & 4**). Third, fitness consequences of different trait values were investigated in a multifactorial common garden experiment (**Chapter 5**) and under natural conditions in a field experiment (**Chapter 6**).

I. Trait characteristics and consequences

Genotypic trait values and their plasticity

The relationship between trait values and plasticity was studied by comparing the trait values of thirty-four unique *T. repens* genotypes grown under high light conditions (where plasticity is not induced) to the plasticity in the same traits in response to two shading regimes (homogeneous shading and a vertical light gradient). **Chapter 2** shows that absolute petiole elongation in response to both shading treatments and absolute leaf area expansion in response to homogeneous shading did not depend on the trait values expressed in high light conditions. These results support the notion that trait values and their plasticity can respond to selection independently, an issue which has been strongly debated in the literature (Via, 1993; Scheiner, 1993; Schlichting & Pigliucci, 1993; Via *et al.*, 1995; Scheiner, 2002; Pigliucci *et al.*, 2003). Consequently, genotypes with shorter petioles under high light conditions showed relatively higher levels of shade induced petiole length plasticity than genotypes that have longer petioles under high light conditions. Plants expressing higher levels of relative plasticity may incur higher costs in terms of biomass investment into the elongating petiole or suffer from reduced biomechanical stability (Givnish, 2002; Henry & Thomas, 2002; Anten *et al.*, 2005). The observation that *T. repens* genotypes differ in petiole length and that the shade induced elongation response does not depend on the trait values expressed in high light conditions raises new questions: How do the underlying cellular processes (i.e. cell division and cell expansion) contribute to variation found in trait values among genotypes and to plastic trait variation. These issues are discussed later on in this discussion (II. Mechanisms).

Benefits and costs of plasticity

The use of two shading treatments in **Chapter 2**, homogeneous shading and a vertical

light gradient, allowed for evaluating costs and benefits associated with shade-avoidance responses. In a vertical light gradient light availability increases towards the top, just as in natural grasslands. If plant leaves reach higher places in a vertical light gradient they can harvest more light. By contrast, growing taller in homogeneous shade will not result in increased resource uptake. **Chapter 2** shows that higher degrees of petiole elongation expressed in a vertical light gradient indeed positively affected plant performance. These results support the notion that shade-induced petiole elongation response can, as often hypothesized but less frequently tested, be associated with benefits and hence be considered as adaptive plasticity (Dudley & Schmitt, 1996; Schmitt, 1997; Cipollini & Schultz, 1999; Donohue *et al.*, 2000).

Plasticity can only be associated with costs which are not balanced by benefits if plasticity is expressed while the response can not lead to enhanced resource uptake. For example, petiole elongation expressed under homogeneous shading can not result in improved light interception. As plants have only limited resources available to invest in different functions, resource investment into expensive petiole elongation will limit the amount of resources available for alternative responses. For that reason, petiole elongation was hypothesized to be costly in homogeneous shading where the production of longer petioles does not result in improved light interception. However, **Chapter 2** shows that petiole elongation was not associated with costs if expressed under homogeneous shading. The ability to respond to homogeneous shading most likely reflects a by-product of selection on elongation responses in natural vertical light gradients, as the same light cues trigger elongation in both types of shading.

Another potential cost of plasticity occurs if the more plastic genotypes perform relatively worse than less plastic genotypes under conditions where plasticity is not induced (van Tienderen, 1991; DeWitt *et al.*, 1998). In order to study these costs, the degree of petiole elongation expressed in any one of the two shading treatments was compared with the performance under high light conditions. **Chapter 2** shows that the more plastic genotypes (i.e. genotypes that express higher degrees of petiole elongation in response to shading) indeed grew worse under high light conditions. Apparently, the costs associated with the ability to respond plastically, including the genetic, signal detection, maintenance and transduction costs (DeWitt *et al.*, 1998; Givnish, 2002), are high enough to reduce plant performance under high light conditions which do not induce plastic elongation responses.

In combination, these data showed high variation in petiole length among *T. repens* genotypes and found benefits and costs associated with plasticity. The next aim of this thesis was to investigate the relationship between plant characters and plant performance in different natural microhabitats. Later on in this discussion two experiments are discussed that were conducted to investigate these issues (III. Ecological consequences).

II. Mechanisms

Genotypic differences in petiole length

Two distinct developmental processes, cell division and cell elongation are ultimately responsible for the size of a given plant organ. Plant organs, like petioles, basically develop from one active meristem. The meristem activity determines the final number of cells in the structure (Mizukami & Fischer, 2000) and newly formed cells differentiate into their destined function and elongate until they reach their mature sizes (Tsukaya & Beemster, 2006). Both cell division and cell elongation require considerable amounts of energy and carbohydrates (Voesenek *et al.*, 2004). Cell elongation is considered as being relatively cheaper since this process requires only the production of extra cell wall material while supplementary cell number production also requires additional DNA-replication. Chapter 3 shows that cell division is the main process explaining petiole length differences among *T. repens* genotypes under high light conditions. It is surprising that the cost intensive process of cell division mainly contributed to genetic variation in petiole length. One possible explanation may be that biomechanical consequences associated with differences in cell length may have lead to selection against the production of longer petioles by means of increased cell expansion rather than cell division. Longer petioles need increased mechanical strength to carry the weight of the leaves and to minimize the risk of physical failure (Givnish, 2002; Anten *et al.*, 2005). This may be better achieved by an increase in cell number than by an increase in cell size, as tissue made of more but smaller cells will have a higher density of cell walls providing rigidity and strength, and thus be more resistant to buckling and breaking.

Shade-induced petiole elongation

Petioles elongate in response to shading and **Chapter 3** shows that both changes in cell number as well as changes in cell size contribute to achieving this. Moreover, **Chapter 3** shows a negative correlation between shade-induced changes in cell number and cell size. Genotypes that responded to shading by increasing cell proliferation hardly increased (some even decreased) in cell size while genotypes responding to shading by means of increasing cell size produced fewer cells. These two distinct developmental processes, which operate separately in space and time, both determine in concert the given plastic petiole length increase. The potential to change both cell number and cell length allows *T. repens* to compensate for lower plasticity in one of the traits, thereby ensuring optimal elongation.

Chapter 4 shows that there are biomechanical consequences of producing either more or longer cells in response to shading: *T. repens* genotypes that elongate their petioles mainly by producing more cells have more rigid petioles than genotypes that elongate their petioles primarily by increasing their cell length. More solid petioles will have a lower risk of physical failure. Vegetation types which offer little structural support are therefore hypothesized to favour plants with more rigid petioles. If the vegetation

structure offers sufficient structural support for all plants regardless their petiole rigidity, plants with less rigid petioles (which elongated their petioles by means of the cheaper cell elongation process) may be favoured since these plants can use the saved investment for other plant functions to cope with competition. It would be interesting to further investigate the consequences of different mechanisms involved in petiole elongation. The specific vegetation structure, in addition to differences in costs involved with cell proliferation or cell elongation, may determine whether cell proliferation or cell elongation will be favoured in the shade induced petiole elongation response under different microhabitat conditions.

III. Ecological consequences

Plants in grasslands are often exposed to multiple selective forces operating simultaneously on the vegetation (Via & Lande, 1985; Galloway, 1995). Natural grasslands can be characterized by mosaic of microhabitats varying in i.e. canopy height (and thus light availability) and disturbance (mammal activity) (Waite, 1994; Welham *et al.*, 2002). Competition for light mainly occurs in a vertical direction (Hirose & Werger, 1995) favouring plants with long stems and stem analogues (Ballare *et al.*, 1994; Leeflang *et al.*, 1998). By contrast, longer or plastic vertical structures may not be beneficial or be even disadvantageous for plant performance if the site is disturbed (grazed or mown). For example, plants that grow in poor light conditions allocate more resources to vertical structures (**Chapter 2**). If disturbance occurs, plants with longer and more plastic vertical structures lose relatively more biomass than shorter or less plastic plants. General responses to shading have been investigated thoroughly (Ballare *et al.*, 1991; Schmitt & Wulff, 1993; Thompson, 1993; Huber *et al.*, 1998) but experiments evaluating plasticity and fitness consequences under multiple and more natural factors (i.e. competition with naturally co-occurring species, herbivory, grazing/mowing) are still scarce (Callahan & Pigliucci, 2002; Huber *et al.*, 2004; Weinig *et al.*, 2004).

In this thesis, the consequences of traits characterizing vertical and horizontal spacers (petioles and internodes, respectively) on plant performance were investigated in a two-way factorial common garden experiment (**Chapter 5**). In a competition treatment, *T. repens* genotypes grew together with a natural co-occuring grass species (*Lolium perenne*). Repeated clipping at 1 cm height simulated disturbance in another treatment. Genotypes with longer petioles under high light conditions were disfavoured by disturbance but these genotypes did not perform better under competition than genotypes with shorter petioles. *T. repens* genotypes expressing higher degrees of plasticity in the vertical direction tended to produce more biomass under competition and were marginally disfavoured by regular disturbance. The benefits of shade induced elongation under competition were only marginal and therefore not as high as expected (recall that we did find significant benefits of petiole elongation in a vertical light gradient, **Chapter 2**). Producing longer vertical structures appeared to be correlated with other traits that were

not associated with positive effects. For example, horizontal structures (stolon internodes) expanded in response to competition. Extension of horizontal structures could not have lead to increased light capture in the experiment conducted in **Chapter 5** as plants were not allowed to escape competition in a horizontal direction. The costs of producing these elongating internodes may have impeded the net benefits associated with shade-induced petiole elongation.

In addition to the costs of correlated plant responses, **Chapter 5** shows that genotypes that expressed higher degrees of internode elongation in response to competition grew worse under high light conditions than less plastic genotypes. These results are in line with the observation in **Chapter 2**, where the ability to express petiole plastically was also high enough to reduce plant performance under conditions where the response was not induced. Both chapters show that selection favours plasticity in one environment and disfavours it in another.

Selection takes place in the field and theory predicts that high environmental heterogeneity favours high genotypic variation: genotypes with different fitness related trait values are hypothesized to be selected in different microhabitats (Via & Lande, 1985; Falconer & Mackay, 1996; Kingsolver *et al.*, 2001; Kassen, 2002; Byers, 2005). In this thesis, this prediction was tested in a field experiment by relating plant characters to variable performance in response to different natural microhabitats (**Chapter 6**). This was done by, first, investigating whether the study area contains various microsites featuring different environmental characteristics. Second, examining whether plant performance and morphological characters correlate with microsite conditions, and finally, by testing whether performance profiles among genotypes differed across microsites to examine if microsite conditions favour specific trait values. In order to do so, eight out of the original thirty-four genotypes were transplanted back to their original habitat, each in one of 99 randomly chosen sites (microhabitats). The eight genotypes were chosen to display a high genotypic variation in petiole length and ramet size. **Chapter 6** shows that there was a large variation in microsite conditions (vegetation height and light availability) in the floodplain grasslands. This variation was relevant in magnitude for the performance of *T. repens* plants: vegetation height and light reduction affected total shoot dry mass and ramet number production. The explanted genotypes, originating from this area, responded as expected to these growth conditions: plants produced longer petioles and larger leaves in higher vegetation and under stronger light reduction. **Chapter 6** shows that genotypes which produced longer petioles and larger leaves in microsites with higher vegetation and more intense shading produced relatively more shoot biomass and ramets than genotypes that were less able to adjust their phenotype to the microhabitat conditions. This shows that genotypes that could best plastically match their phenotype to the present growth conditions were fitter than genotypes that were less able to do so. By contrast, under the same microhabitat conditions, genotypic values in petiole length or leaf size (as expressed under high light conditions) were much less important. Plasticity may thus have buffered the effects of microsite conditions on plant performance rather than genotypic variation being favoured by microsite selection.

In addition, microsites did not only differ in vegetation height and light reduction but also in the amount of plant damage caused by small herbivores. The amount of damage differed per microsite and increased with vegetation height and light reduction. Herbivore damage directly affected plant performance by the total amount of biomass loss reducing future plant growth. Additionally, herbivory indirectly affected plant performance by reducing the plant's potential to respond to shading. Some authors have recently argued that there may be a trade-off between shade avoidance responses and herbivore susceptibility (Cipollini, 2004; Kurashige & Agrawal, 2005). Herbivore induced reduction of petiole length can therefore be assumed to have negative effects on plant growth, exceeding the cost incurred by the pure loss of plant tissue. All prerequisites for detecting variable selection among microhabitats were met in the experiment described in **Chapter 6**: there was high morphological variation among genotypes, high environmental variation across microsites and every genotype was present in each microsite. Yet, no evidence for microhabitats favouring different trait values was found. Interacting plant responses to simultaneous multiple selective forces can provide new insight in the fascinating interplay between plants and their environmental context.

IV. Conclusions

This thesis shows that, in controlled experiments, selection favours specific trait values and plasticity in one environment and disfavours the same values and plasticity in another. This thesis also shows that, in nature, it is hard to find evidence for microhabitat selection for differences in petiole length among *T. repens* genotypes, although a field experiment was conducted that met all prerequisites for finding it (**Chapter 6**). There are several reasons why microhabitat selection for morphological traits was hard to observe. First, phenotypic plasticity was more important for plant growth under field conditions than the genotypic differences and may have buffered microhabitat selection on specific trait values (Ballare *et al.*, 1994; Sultan, 2005). This thesis shows both significant benefits and costs associated with plasticity in several studies (**Chapters 2, 5** and **6**) as well as independence of plasticity of the intrinsic genotypic trait value (**Chapters 2** and **3**). Phenotypic plasticity is likely to evolve in environments that are heterogeneous in space or time (Wijesinghe & Hutchings, 1999; van Kleunen & Fischer, 2005; He *et al.*, 2004). Dynamic competitive grasslands like the river floodplains where the plants were originally collected from, meet these requirements. All chapters considered this thesis show that plasticity can evolve in river floodplains as a trait in its own right.

Second, trait correlations may weaken selective forces acting on a specific trait. If a trait is favored in a specific environment, this character may not increase plant performance if a correlated trait negatively affects plant performance in that same environment (Pigliucci *et al.*, 1998; Pigliucci & Kolodynska, 2002; Garland & Kelly, 2006). Third, other environmental factors present in nature like herbivory (Kurashige & Agrawal, 2005), water availability (Huber *et al.*, 2004) or nutrient availability (Pigliucci *et al.*, 1998) may also

interfere with the selective pressures acting on specific trait values. Finally, input of genetic material from other populations or neutral selection may also contribute to the high variation in petiole length observed among genotypes (Ellstrand & Elam, 1993; Ouborg *et al.*, 1999). All together, several additional factors easily operate for microhabitat selection to subside in the noise of nature. It is therefore interesting to further investigate the specific combinations of environmental factors that are required to find microhabitat selection in natural habitats.

While it is intuitively clear that differences in petiole length should be beneficial in dense rather than open microhabitats, this thesis does not support that notion. Shade induced petiole elongation proved to be beneficial in controlled experiments and under field conditions. Nevertheless, this thesis also indicates that a multitude of other factors can interfere with the adaptive significance of trait values and plasticity under natural conditions.


99

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Nederlandse samenvatting

Jelmer Weijschedé

Planten en milieu heterogeniteit

Belangrijke hulpbronnen voor de groei en ontwikkeling van planten (bijvoorbeeld de hoeveelheid licht) zijn in natuurlijke milieus vaak ongelijkmatig verdeeld. Dit houdt in dat niet overal evenveel van een bepaalde hulpbron voor handen is. In graslanden, zoals te vinden in de uiterwaarden, is bijvoorbeeld de vegetatie hoogte niet op elke plek hetzelfde. De dynamiek van de rivier zorgt voor een ongelijkmatige verdeling in de structuur en het substraat van de bodem waardoor de vegetatie zich per plek anders kan ontwikkelen. Tevens wordt de vegetatiehoogte beïnvloed door de activiteiten en interacties van grote en kleine dieren (waaronder paarden, koeien, muizen en slakken).

Op plekken waar de vegetatie hoog is beschaduwen planten elkaar en vindt concurrentie om licht plaats. De hoeveelheid en kwaliteit van het licht neemt van onder naar boven in de vegetatie toe en de concurrentie om licht speelt zich dan ook voornamelijk in de verticale richting af. Veel planten kunnen met hun morfologie op licht concurrentie reageren, een fenomeen wat fenotypische plasticiteit wordt genoemd. De meest uitgeproken reacties zijn de strekkingsreacties van verticale structuren zoals stengels en bladstelen. Deze aanpassingen stellen planten in staat hun bladeren hoger in de vegetatie te brengen waar meer licht beschikbaar is. Hoe een organsime (bijvoorbeeld een bepaalde plant) er uiteindelijk op een bepaald plaats uitziet (het fenotype), wordt dus voor een belangrijk deel door fenotypische plasticiteit (kortweg plasticiteit) bepaald. Een ander deel van het uiteindelijke fenotype wordt bepaald door de unieke genetische informatie (opgeslagen in het DNA) van bepaalde eigenschappen die elk individu bij zich draagt (het genotype).

Morfologische variatie bij Witte klaver

In de uiterwaarden langs de rivier de Waal zijn in de zomer van 2001 verschillende individuen van Witte klaver (*Trifolium repens*) verzameld. Witte klaver is een zeer algemene soort en heeft een liggende groeiwijze: de stengel 'kruipt' als het ware over de grond en de bladstelen dragen de bladeren omhoog (zie figuur 1 in hoofdstuk 1). Onderling bleken de individuen sterk in morfologische kenmerken (zoals bladsteellengte) te verschillen. Zo heeft het ene individu bijvoorbeeld langere bladstelen en bladeren dan het andere individu. De genetische identiteit van de individuen is vastgesteld met behulp van de moleculaire techniek AFLP. Voordat de variatie tussen de individuen in kaart is gebracht, zijn alle planten een jaar lang blootgesteld aan identieke groeiomstandigheden (met gunstige lichtomstandigheden). Hierdoor kon geen morfologische variatie meer worden toegeschreven aan de reactie op verschillende omstandigheden waarin de planten zich hebben ontwikkeld (plasticiteit). Deze morfologische verschillen zijn daarom genotypisch van aard.

Dit proefschrift

De feiten dat 1) belangrijke hulpbronnen voor de groei en ontwikkeling van planten (bijvoorbeeld de hoeveelheid licht) in natuurlijke milieus vaak ongelijkmatig verdeeld zijn

en dat 2) individuen van één plantensoort in sterke mate in morfologische kenmerken (waaronder bladsteellengte) verschillen, vormen de basis van dit proefschrift. In dit proefschrift is onderzocht hoe genotypische variatie en plastische variatie (ten gevolge van schaduw) van morfologische kenmerken de groei van een specifieke individu op een specifieke plaats beïnvloeden. Omdat bij Witte klaver de bladsteel de belangrijkste morfologische structuur is om de bladeren hoog in de vegetatie te krijgen, is er hoofdzakelijk naar deze structuur gekeken. Daarnaast is ook onderzocht welke cellulaire processen (celdeling of celstrekking) ten grondslag liggen aan de genotypische- en schaduwgeïnduceerde variatie in bladsteellengte en wat de eventuele consequenties van deze verschillende processen voor de plantengroei zijn.

Het eerste experiment

Relatie genotype en plasticiteit

Het centrale startpunt van dit proefschrift is **hoofdstuk 2** waarin onderzocht is of de mate van plasticiteit voor elk genotype hetzelfde is. Er is onderzocht of 34 verschillende genotypen, die onder gunstige licht omstandigheden in bladsteellengte van elkaar verschillen, in dezelfde mate met hun bladsteellengte op beschaduwing reageren. Het blijkt dat er variatie is in de mate van plasticiteit; het ene genotype reageert sterker dan het andere. Er wordt tevens aangetoond dat de mate van bladsteelstrekking niet gekoppeld is aan de genotypische verschillen onder gunstige lichtomstandigheden. Er is dus genotypische variatie in de mate waarin bladstelen langer worden in reactie op schaduw. Echter, deze variatie is niet gekoppeld aan de verschillen in bladsteellengte onder gunstige lichtomstandigheden. Omdat beide eigenschappen (langere of kortere bladstelen onder gunstige lichtcondities en de mate van plasticiteit onder beschaduwing) niet gekoppeld blijken te zijn, ondersteunt dit de opvatting dat natuurlijke selectie kan inwerken op één van de eigenschappen, onafhankelijk van de ander.

Baten en kosten van plasticiteit

In **hoofdstuk 2** worden de baten en kosten van plastische reacties op basis van twee schaduwbehandelingen onderzocht; een verticale lichtgradiënt en homogene beschaduwing. In een verticale lichtgradiënt neemt de beschikbare lichthoeveelheid van onder naar boven toe, net als in natuurlijke vegetaties. Hoe langer een plant zijn bladstelen maakt, hoe hoger de bladeren in de gradiënt geplaatst kunnen worden. Hierdoor kan de plant meer licht ontvangen waardoor deze plant relatief beter zal groeien (hogere productie van biomassa en potentiele nakemelingen) dan een plant die zijn bladstelen minder lang heeft kunnen maken. Planten die hun bladstelen meer strekten in de verticale licht gradiënt bleken inderdaad beter te groeien dan planten die in mindere mate hun stengels strekten. Deze observatie ondersteunt de hypothese dat de stengelstrekkingsreactie een aanpassing (of adaptatie) is aan beschaduwing. Hoewel deze hypothese voor de hand liggend is, zijn er slechts weinig studies die dit ook daadwerkelijk hebben aangetoond.

Onder homogene beschaduwing is de hoeveelheid licht (of in dit geval schaduw) overal hetzelfde en het maken van langere bladstelen zal daarom niet leiden tot een hogere lichtopname. Sterker nog, omdat de reactie op schaduw uitgelokt wordt (er heerst immers een schaduw klimaat) terwijl de reactie niet kan leiden tot een hogere lichtopname (de bladeren kunnen wel hoger komen maar dit levert niet meer licht op) zouden de kosten van de schaduwgeïnduceerde strekking zichtbaar moeten worden. Een plant investeert immers bouwstoffen in de strekkingsreactie. Deze verwachte kosten werden echter niet gevonden: planten die een sterkere strekkingsreactie lieten zien groeiden niet slechter dan planten die in mindere mate hun bladstelen strekten. Dit betekent dat de kosten van plasticiteit, als ze al bestaan, heel klein zijn of moeilijk aan te tonen.

Een ander type kosten werd wel gevonden: de meer plastische planten (planten die sterker op schaduw reageren) groeiden slechter onder gunstige lichtcondities (waar de strekkingsreactie niet geïnduceerd was). Blijkbaar zijn de kosten die geassocieerd zijn met de mogelijkheid om plastisch te kunnen reageren (bijvoorbeeld genetische kosten, signaalperceptiekosten, kosten voor het behouden van de respons en transductiekosten) hoog genoeg om planten minder goed te laten groeien op plaatsen waar de respons niet tot uitdrukking komt.

Kortom, de resultaten laten een hoge variatie in bladsteellengte zien, zowel tussen genotypen wanneer de lichtcondities identiek zijn, als ook in schaduwgeïnduceerde bladsteellstrekking. Tevens blijken er naast de baten van strekking ook kosten aan plasticiteit te zitten.

Vanaf dit punt wordt er ingegaan op de volgende twee vragen: 1) welke cellulaire mechanismen (celdeling of celstrekking) liggen ten grondslag aan de genotypische- en schaduwgeïnduceerde variatie in bladsteellengte en 2) wat zijn de ecologische gevolgen (implicaties) hiervan voor de uiteindelijke plantengroei in (meer) natuurlijke situaties?

Onderliggende mechanismen

Genotypische verschillen in bladsteellengte

Twee processen zijn uiteindelijk verantwoordelijk voor de grootte van een bepaalde plantenstructuur: celdeling en celstrekking. Dit wordt beschreven in **hoofdstuk 3**. De verschillen in bladsteellengte tussen genotypen onder identieke groeiomstandigheden hangen vooral samen met verschillen in celaantal: langere bladstelen hebben meer cellen. Dit is een opmerkelijke vondst omdat het produceren van meer cellen een kostbaarder proces is dan het produceren van langere cellen. Aan de andere kant moeten langere bladstelen sterker zijn om het gewicht van de bladeren te kunnen dragen en dit zou wel eens beter bereikt kunnen worden door de bladsteel op te bouwen uit meer cellen in plaats van langere cellen.

Schaduw-geïnduceerde bladsteelstrekking en biomechanische gevolgen

Bladstelen worden langer in de schaduw en dit blijkt zowel een gevolg te zijn van ve-

randering in celaantal als van celstrekking. Een opmerkelijk resultaat is echter dat er een negatief verband bestaat tussen de verandering in celaantal en celstrekking ten gevolge van beschaduwing. Er zijn genotypen die hun bladstelen langer maken door meer cellen te produceren (en nauwelijks tot geen verandering hebben in cellengte) en er zijn genotypen die hun bladstelen met name door celstrekking langer maken (en nauwelijks verandering hebben in celaantal). Deze resultaten laten zien dat er een complexe relatie bestaat tussen strekkingsreactie en verandering in celaantal en cellengte ten gevolge van schaduw.

In **hoofdstuk 4** wordt verder ingegaan op deze relatie en het blijkt dat het produceren van meer of juist langere cellen bij bladsteelstrekking consequenties heeft: genotypen die hun bladstelen strekken door meer cellen aan te maken blijken sterkere bladstelen te hebben dan genotypen die hun bladstelen voornamelijk door celstrekking langer maken. Planten met minder stevige bladstelen zouden hierdoor afhankelijker kunnen zijn van ondersteunende structuren (zoals van een dichte vegetatie) dan planten met steviger bladstelen. Aan de andere kant kunnen de planten die minder stevige bladstelen maken relatief meer investeren in andere structuren of functies om met beschaduwing om te gaan (celstrekking is relatief 'goedkoper'). De specifieke vegetatiestructuur, in combinatie met verschillende kosten die ten grondslag liggen aan celdeling of celstrekking, zouden dus bepalend kunnen zijn in de mate waarin bladsteelstrekking door middel van celdeling of celstrekking bevoordeeld wordt.

Ecologische implicaties

Concurrentie en verstoring

Natuurlijke graslanden kunnen vaak gekarakteriseerd worden als een mozaïek van microhabitats die bijvoorbeeld in vegetatiehoogte (en dus lichthoeveelheid) en verstoring van elkaar verschillen. Concurrentie om licht speelt zich met name in het verticale vlak af waardoor planten met langere verticale structuren bevoordeeld zouden moeten worden. Aan de andere kant kunnen langere structuren nadelig zijn wanneer verstoring in de groeiplaats optreedt (de plek wordt bijvoorbeeld begraasd of gemaaid): deze planten verliezen meer materiaal dan planten met kortere structuren. In **hoofdstuk 5** worden deze hypotheses in een tuinexperiment getoetst door de verschillende genotypen bloot te stellen aan een concurrentieregime (waarbij concurrentie gecreëerd werd door Engels raaigras, *Lolium perenne*) en een verstoringregime (waarbij regelmatig al het plantenmateriaal 1 cm boven de grond werd verwijderd). Genotypen die onder gunstige lichtcondities langere bladstelen maken blijken inderdaad nadeel te ondervinden in het verstoringregime, maar de lagere bladstelen (gemeten onder gunstige lichtcondities) leverden de genotypen geen voordeel op in de concurrentie om licht. Genotypen die een sterkere strekkingsreactie hebben laten zien presteerden iets beter onder concurrentie ten opzichte van minder plastische genotypen. Dat de verwachte baten van de strekkingsreactie lager zijn uitgevallen dan verwacht heeft waarschijnlijk te maken met het feit dat de (horizontaal groeiende) stengels ook langer zijn geworden in de concurrentiebehande-

ling. Dit brengt alleen maar extra kosten met zich mee (verlengen van de structuur) terwijl deze reactie de planten geen voordeel heeft kunnen brengen (de bladeren konden hierdoor niet in gunstiger lichtcondities terecht komen).

Terug naar de uiterwaarden

Uiteindelijk vindt natuurlijke selectie, op welke (planten-) eigenschap dan ook plaats in de natuur. Theorie voorspelt dat een hoge mate van milieuheterogeniteit (veel afwisseling in microhabitats, zie inleiding van dit hoofdstuk) een hoge genotypische variatie kan bevoordelen: genotypen met verschillende eigenschappen (bijvoorbeeld verschillende bladsteellengtes) zouden specifiek bevoordeeld of benadeeld kunnen worden in verschillende microhabitats. In **hoofdstuk 6** wordt deze hypothese getest in een veldexperiment. Dit werd gedaan door 8 van de tot dusver 34 gebruikte genotypen op 99 plekken (microhabitat of microsite) terug te plaatsen in de natuurlijke situatie waar de planten oorspronkelijk vandaan kwamen (de uiterwaarden langs de rivier de Waal tussen Ewijk en Beuningen). De vegetatiehoogte (en lichthoeveelheid) was per microsite verschillend en heeft een negatief effect gehad op de plantengroei. Alle genotypen reageerden als verwacht op de groeicondities van de microsites: in hogere vegetaties werden langere bladstelen geproduceerd. Genotypen die in hogere vegetaties een sterkere strekkingsreactie lieten zien groeiden beter dan genotypen die minder op de groeicondities reageerden. Dit laat zien dat de planten die het beste hun morfologie aan kunnen passen aan de actuele groeiplaatscondities, beter presteren dan planten die dit minder goed kunnen. De genotypische verschillen (langere of kortere bladstelen) zoals deze aan het begin van dit proefschrift gevonden zijn onder gunstige lichtcondities, blijken van veel minder belang te zijn in verschillende microhabitats.

Tevens blijkt ook dat de microsites niet alleen verschilden in vegetatiehoogte maar ook in de hoeveelheid schade die door kleine herbivoren (met name slakken) aan de genotypen was toegebracht. Deze schade heeft een direct effect op de plantengroei, simpelweg omdat de planten biomassa verliezen, waardoor de toekomstige plantengroei wordt gereduceerd. Hier komt een indirect effect bij, omdat aangegeten planten gereduceerde bladsteelstrekking lieten zien. Door deze afname in bladsteelstrekking wordt de plantengroei negatief beïnvloed doordat de beschadigde planten minder goed in staat zijn zich aan de vegetatiehoogte aan te passen.

Conclusie

De resultaten van dit proefschrift laten door middel van gecontroleerde experimenten zien, dat specifieke morfologische eigenschapwaarden en plasticiteit in het ene milieu de plantengroei voordelig beïnvloedt, terwijl deze specifieke eigenschapwaarden en plasticiteit in het andere milieu de plantengroei negatief beïnvloedt. Het blijkt echter dat het in de 'echte' natuur moeilijk is om bewijs te vinden voor microhabitat selectie op verschillende bladsteellengtes (zoals deze geobserveerd werden tussen genotypen van Witte klaver). Verschillende redenen kunnen hieraan ten grondslag liggen:

Ten eerste blijkt dat voor de groei, fenotypische plasticiteit in de natuur belangrijker is dan de genotypische verschillen (gemeten onder identieke groeicondities). Ten tweede kunnen gecorreleerde eigenschappen of reacties de selectie op een specifieke eigenschap verzwakken of teniet doen. Tevens kunnen andere milieufactoren, die in de natuur aanwezig zijn (bijvoorbeeld herbivorie), interfereren met de selectieve kracht op een bepaalde eigenschap. Ten slotte kunnen invoer van genetisch materiaal uit andere populaties en neutrale selectie ook bijdragen aan de hoge genotypische variatie zoals deze is waargenomen tussen genotypen van Witte klaver. Al met al kan microhabitat selectie moeilijk worden gevonden door de hoge ruis aan vele overige natuurlijke factoren waar planten in hun directe omgeving mee te maken hebben.

Terwijl het vanzelfsprekend is dat genotypen met langere bladstelen (zoals geobserveerd onder identieke lichtcondities) een voordeel zouden moeten hebben in hogere vegetaties, kan dit proefschrift deze hypothese niet bevestigen. Schaduwgeïnduceerde bladsteelstrekking blijkt wel voordelig, zowel in onafhankelijke experimenten (zie **hoofdstukken 2** en **5**) als onder veldcondities (**hoofdstuk 6**). Genotypisch of plastisch van aard, dit proefschrift laat tevens zien dat een scala aan overige factoren gemakkelijk roet in het eten kan gooien wanneer men op zoek gaat naar de adaptieve waarde van specifieke eigenschappen in de natuur.


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Bedankt

Allemaal bedankt!

Het zou mooi zijn als ik het bij de volgende twee woorden kon laten:"Allemaal bedankt!" dan ben ik tenminste niemand vergeten. Maar omdat dit proefschrift niet alleen mijn verdienste is, ga ik toch een serieuze poging wagen iedereen te bedanken voor zijn of haar bijdrage. Bij voorbaat mijn excuses voor eventuele over- of onderwaarderingen.

Hans, terwijl er op een stralende dag langs een slootkant ergens op de Veluwe een ijsvogel voorbij vloog en de insecten in mijn netje stierven aan de dampen van de ethylacetaat, ging mijn mobiele telefoon en vertelde jij dat ik het je moeilijk had gemaakt tijdens een sollicitatie. 'Da's mooi' zei ik en het resultaat heb je nu in handen. Ik ben je ontzettend dankbaar voor je enthousiasme, hulp en betrokkenheid, niet alleen in wetenschappelijke zin, maar in de meest algemene zin van deze woorden. Het duurde weliswaar even voordat je mijn humor begreep (over Tsjechische studenten en Skoda's), maar de blokkades in mijn schrijven voelde je feilloos aan en daar kon je goed in sturen. Geen versie teveel, altijd keek je weer met een heldere en verfrissende blik naar mijn manuscripten, heel erg bedankt daarvoor!

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CV

Jelmer Weijschedé werd geboren op 1 februari 1974 te Groningen. Hij haalde in 1993 zijn VWO-diploma aan het Cornelis Drebbel College in Alkmaar. In 1995 begon hij aan de lerarenopleiding Biologie aan de Hogeschool van Utrecht. Tijdens deze studie liep hij stage in het Noord-Hollands Duinreservaat en bestudeerde daar het territoriale gedrag van vossen. In 1998 werd het lerarendiploma behaald en begon hij in Wageningen aan de studie Biologie. Tijdens deze studie specialiseerde hij zich in de ecologie. Bij de Leerstoelgroep Natuurbeheer en Plantenecologie verdiepte hij zich in transportsystemen van veenmossen, onder begeleiding van dr. J. Limpens. In 2001 studeerde hij af als systeemecoloog. Aan de toenmalige Katholieke Universiteit Nijmegen begon hij in 2002 aan dit promotieonderzoek bij de vakgroep Expirimentele Plantenecologie.
Op dit moment is hij werkzaam als docent Research aan de NHTV internationale hogeschool van Breda.