Plant performance: a physiological and genetic analysis using *Arabidopsis thaliana* natural variation

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To my parents

In the memory of my mother in-law

To my wife, my daughter and my son

To my brother and my sisters

Contents

Chapter 1	General introduction.	7
Chapter 2	Altered photosynthetic performance of a natural Arabidopsis accession is associated with atrazine resistance.	17
Chapter 3	New Arabidopsis recombinant inbred line populations genotyped using SNPWave^{TM} and their use for mapping flowering time QTLs.	35
Chapter 4	Quantitative trait locus analysis of growth-related traits in a new Arabidopsis recombinant inbred population.	51
Chapter 5	Arabidopsis natural allelic variation of carbohydrate-related traits and plant growth in the Landsberg *erecta* x Kondara recombinant inbred line population.	73
Chapter 6	Summarizing discussion.	103
	References	111
	Samenvatting	121
	Publications	125
	Curriculum vitae	126
	Acknowledgements	127
	Arabic summary	

Chapter 1

General introduction

Plant performance

Plant performance depends on the acquisition of raw material (carbon fixation and mineral uptake), the distribution of such materials over the plant organs, and the ability to cope with environmental stresses. Plant performance is viewed as the result of input (photosynthesis and mineral nutrition), allocation, and storage or use (respiration), under a given set of environmental conditions. Functionally a plant can be divided into sources and sinks. Sources are the parts of the plant where net fixation of carbon dioxide occurs, and sinks the sites where assimilates are stored and/or used. Allocation of assimilates between plant parts occurs via transport in the phloem. For total biomass production, photosynthetic carbon dioxide fixation is by far the most important process.

Growth of autotrophic plants depends on photosynthetic activity. Photosynthesis is a metabolic process that is highly integrated and regulated in order to maximize the use of available light, to minimize the damaging effects of excess light and to optimize the use of limiting carbon and nitrogen resources (Paul and Foyer, 2001). Photoassimilates can be either used directly for growth or respiration, or stored for a short period (e.g. in leaves, diurnal) or for a long period (e.g. in seeds or roots). Already in 1868, Boussingault (quoted by Paul and Foyer, 2001), assumed that the accumulation of photoassimilates in leaves has a role in regulating photosynthetic rate. As the accumulation of end products is a function of the balance between photosynthesis and the use by the growth processes of the plant, Boussingault`s hypothesis essentially pointed out that there is an interrelationship between photosynthesis and growth rather than a one-way relationship. A metabolic signaling network involving information on the carbon and nitrogen status of different tissues interacts with phytohormone signaling pathways and redox signals to control photosynthetic gene expression and leaf development. This highly integrated signal transduction network, which forms the basis of the source-sink interaction, regulates photosynthetic activity by determining the amount of photosynthetic apparatus present during leaf development and senescence, overriding direct control of photosynthesis by light and CO_2 (Paul and Foyer, 2001).

Plant growth analysis is a necessary step for the understanding of plant performance and productivity (Leister et al., 1999), which reveals different strategies that plants follow to survive in conditions where certain factors are limiting. The different aspects of the plant performance can be seen as the integration of a wide range of processes, and thus genetic variation for such complex traits is likely to depend on many genes.

Sugars and plant performance/growth

In plants, sugar production through photosynthesis is a vital process, and sugar status modulates and coordinates internal regulators and environmental cues that govern growth and development (Koch, 1996; Sheen et al., 1999; Smeekens, 2000). Not only is sucrose the major stable product of photosynthesis for most plants, but it is also the form in which most carbon is transported in phloem vessels from leaves into sink organs such as roots, flowers, grains

and tubers (Rolland et al., 2002). Interactions of sugars with light, stress and hormone signaling have been reported (Roitsch, 1999; Sheen et al., 1999; Smeekens, 2000; Gazzarrini and McCourt, 2001; Finkelstein and Gibson, 2002), thereby coordinating carbon and nitrogen metabolism (Stitt and Krapp, 1999; Coruzzi and Bush, 2001; Coruzzi and Zhou, 2001).

Soluble sugars have been postulated to act as regulatory molecules that help to control seed and embryo development (Borisjuk et al., 2004; Hills, 2004). During seed development in *Vicia faba*, glucose and sucrose behave in almost opposite manner, with glucose promoting cell division and sucrose being associated with cell expansion and starch synthesis (Borisjuk et al., 2002, 2003). The levels of glucose and other sugars have been shown to affect seed germination and early seedling development.

In addition to mediating early developmental events, soluble sugars also affect the formation of adult structures, such as leaves, nodules, pollen, tubers and roots (Gibson, 2005). For example, growth at elevated CO_2 concentrations, which presumably increases sugar production, sometimes leads to the formation of larger and thicker leaves (Paul and Pellny, 2003).

Soluble sugars may also be involved in the timing with which nutrient-intensive events occur, to ensure an adequate supply of materials and energy for successful completion of the plants life cycle. Levels of sugars, such as sucrose, have been suggested to affect the timing with which at least some plant species flower (Bernier et al., 1993). In *Arabidopsis thaliana*, a species that flowers earlier under long-day conditions, a correlation was found between greater export of carbohydrates from the leaves and increased flower induction (Corbesier et al., 1998). Application of sucrose to the apical part of the plant has also been shown to allow flowering of Arabidopsis in complete darkness (Roldán et al., 1999), giving further support to the hypothesis that sugars promote flowering. Moreover, flower opening may be due to a combination of sugar import and degradation of various polysaccharides (Van Doorn and Van Meeteren, 2003).

In general, leaves are the organs specifically functioning in photosynthesis; their life cycle is optimized for the efficient production of photoassimilates, i.e. sugars. These sugars also act as signaling molecules during leaf senescence (Rolland et al., 2002). At the onset of senescence, sugars accumulate in tobacco leaves (Masclaux et al., 2000), and increased sugar levels have also been reported in senescing leaves of Arabidopsis and other plant species (Yoshida, 2003). The Arabidopsis *hypersenescence1* (*hys1*) mutant is hypersensitive to the inhibitory effects of exogenous sugars on early seedling development and senesces early, providing genetic evidence for a link between sugar responses and senescence (Yoshida et al., 2002).

Starch and plant performance/growth

Starch is one of the most important plant products for the human diet and for numerous technical applications. Photosynthesis produces about 2850 million tons of starch annually (Burrell, 2003). Starch serves as an important storage for carbohydrate residues. Many plants accumulate transitory starch in their leaves.

The extent to which starch accumulates in leaves differs between species. During the light period, a part of the newly fixed carbon is retained within the chloroplast and stored transiently as starch and it may be remobilized during the night. In Arabidopsis the accumulation of transitory starch is relatively constant throughout the light period and occurs concurrently with sucrose synthesis (Zeeman et al., 1998). In the subsequent dark period, starch is mobilized and thereby provides a steady supply of carbon for export to sink organs and for energy metabolism (Zeeman and ap Rees, 1999). The alteration of net biosynthesis and net degradation of starch is reflected by the diurnal variation of leaf starch contents. Both starch synthesis and degradation are tightly controlled to adapt plant metabolism to changing environmental conditions. The importance of storing carbohydrates as starch is reflected in the growth of plants that are unable to synthesize or to degrade fully their transitory starch. The starchless Arabidopsis lines *pgm* and *adg1* (lacking plastidial phosphoglucomutase activity and ADPglucose pyrophosphorylase activity, respectively) grow more slowly in day/night conditions than the wild type. The growth rate of the *sex* (starch excess) mutant, which has a reduced capacity to mobilize starch, is similarly affected (Zeeman et al., 1998).

In most species, the mobilization of storage carbohydrates and/or the import of sucrose accompany flower opening. Young petal cells of many species contain considerable amounts of starch which, shortly before opening, are rapidly converted to glucose and fructose (Van Doorn and Van Meeteren, 2003).

Source-sink relationship

During the plant's life cycle source-sink transitions of organs may occur, as well as changes with respect to the sink strength of individual organs and the number of sink organs competing for a common pool of carbohydrates. In addition, exogenous factors such as abiotic stress or pathogen infection may also influence carbohydrate partitioning. Photosynthesis is active primarily in mature leaf mesophyll cells (the source), and photosynthate is transported via phloem, primarily as sucrose, to developing organs such as tubers, roots, flowers, fruits, or seeds (the sinks) which are characterized by a net import of sugars.

Sugars not only function as substrate to sustain the heterotrophic growth of sink tissues, but are also important signaling molecules that regulate both source and sink metabolism. This ensures optimal synthesis and use of carbon and energy resources and allows for the adaptation of carbon metabolism to changing environmental conditions and to the availability of other nutrients (Stitt and Krapp, 1999; Coruzzi and Bush, 2001; Coruzzi and Zhou, 2001; Grossman and Takahashi, 2001). In general, low sugar status enhances photosynthesis, reserve mobilization, and export, whereas the abundant presence of sugars promotes growth and carbohydrate storage. On the other hand, feedback inhibition of photosynthesis as a result of decreased sink demand is well known and different experimental approaches showed that sugars play a key role in this regulatory mechanism by repressing the expression of photosynthesis genes (Koch, 1996). This feedback inhibition suggests that assimilates might function as link between source and sink tissues.

The circadian clock probably plays an important role in carbon partitioning and

allocation (Harmer et al., 2000). Several photosynthesis genes, for example, peak in expression near the middle of the day, whereas a number of genes involved in sugar consumption, transport, and storage peak near the end of the day. During the night, genes involved in starch mobilization reach their highest expression levels (Harmer et al., 2000). However, although the circadian clock may allow plants to anticipate daily changes, the actual sensing of the quality and quantity of light and especially sugars (as the end products of photosynthesis) ensures an appropriate response of metabolism to specific situations (Rolland et al., 2002). For example, variations in the environment can decrease photosynthetic efficiency and result in sugar-limited conditions in parts of the plant, which downregulate biosynthetic activity to conserve energy and protect cells against nutrient stress while upregulating starch degradation and protein and lipid catabolism to sustain respiration and metabolic activity (Yu, 1999; Fujiki et al., 2000). In general, there is accumulating evidence for crosstalk, modulation and integration between signaling pathways responding to phytohormones, phosphate, light, sugars and biotic and abiotic stress-related stimuli (Roitsch, 1999). These complex interactions at the signal transduction levels and the subsequent coordinated regulation of gene expression seem to play a central role in source-sink regulation.

Plant growth analysis

Plant growth can be defined as the increase in biomass over time. The rate of plant growth depends on the gain of carbon via photosynthesis and the consumption of carbon either during respiration or during building up new tissues. Although many of the physiological mechanisms are similar in all higher plants, the obvious differences observed when different plant species are growing in the same environment indicate that the regulation of the different processes described above varies between species. In many cases these differences can explain why specific plants are better adapted to some environments than others. In addition within species variation, also for physiological traits can be found in nature. Here again one expects that this is related to the environment where plants grow and these environments can be very different especially for cosmopolitan species. Such variation within species allows the genetic analysis of the traits (Maloof, 2003) and has allowed the breeding for varieties with an improved plant performance.

Growth rate can be seen as the integration of a wide range of processes, and thus genetic variation for such a complex trait may depend on many genes. The analysis of plant growth is an important step in the understanding of this genetic variation for plant performance and productivity (Leister et al., 1999) that may reveal different strategies of plants to adapt to certain limiting conditions. Growth rate and, more specifically, relative growth rate (RGR) are comprehensive traits of plants, which characterize to a large extent plant performance and are also important components of fitness (McGraw and Garbutt, 1990). These parameters integrate morphological and physiological traits of plants. RGR is an inherent quantitative trait that may vary among plant species, occurring in a wide range of habitats. Plants in favorable environments often have an inherently high RGR, whereas those

from less favorable habitats have an inherently low RGR, even when grown in the same favorable conditions (Grime and Hunt, 1975; Poorter and Remkes, 1990). In addition, plant growth rate is also affected by developmental changes such as the onset of flowering or the formation of storage organs.

Various parameters have been used to evaluate growth rate, including measurement of fresh or dry weight, root to shoot ratio, shoot number or shoot length (Li et al., 1998; Leister et al., 1999). The measurement of fresh or dry weight is destructive and hence large numbers of plants are required to analyze growth in time. A non-destructive approach would be preferably e.g., using image analysis. For *Arabidopsis thaliana*, which in its vegetative phase grows as a flat rosette with limited leaf overlap, Leister et al. (1999) showed that the use of digital video and image analysis was very effective in the determination of plant growth (rate) non-destructively, even during early developmental stages.

Flowering time

The change from vegetative to reproductive growth has important consequences for the accumulation and distribution of dry matter in plants because the developing flowers, fruits and seeds are important sink tissues. Furthermore in species such as Arabidopsis, flower primordia are formed instead of leaf primordia. This implies that no new source organs are formed although the previously initiated leaves continue to grow and function. Recently, large efforts have focused on the identification of the molecular basis of natural variation for flowering time in the two main model systems, Arabidopsis and rice as well as in crop species of Brassicaceae and Poaceae (Alonso-Blanco et al., 2005). Because much genetic variation is found for flowering time in Arabidopsis, the genetic analysis of plant performance should also consider the flowering time phenotype of the material. Furthermore, analysis of vegetative growth should be performed as much as possible before flowering.

The genetic and molecular dissection of the developmental transition to flowering is being undertaken through the analysis of artificially induced mutants in *Arabidopsis* which identified around 100 genes that affect this transition (Mouradov et al., 2002). These genes are classified in different pathways that integrate the environmental signals (such as photoperiod, light intensity, light quality, or temperature) and the endogenous signals (including hormones and metabolites). Basically, four interacting pathways have been described: the photoperiod response pathway, the vernalization response pathway, the autonomous pathway, and the gibberellin pathway. The integrated signals of these pathways ultimately regulate the expression of genes involved in flower development (Mouradov et al., 2002).

Arabidopsis is a facultative long-day (LD) plant, which means that plants flower earlier under LDs than under short days (SDs), but a LD treatment is not an absolute requirement for flowering. Flowering also occurs rapidly in complete darkness when sufficient carbohydrates are provided to the growing shoot meristem (Roldán et al., 1999). A higher irradiance also promotes flowering probably by its effect on carbohydrate supply

(Bagnall, 1992; King and Bagnall, 1996). Another important treatment promoting flowering is vernalization, which is a transient exposure to low temperatures.

The analysis of variation in the flowering time in the naturally late-flowering accessions has complemented the mutagenesis approach particularly in identifying repressors of the floral transition. A number of genes such as *FRIGIDA (FRI), Flowering Locus C (FLC),* and quantitative trait loci (QTL) that are not represented in the mutant collections have been identified using this approach (reviewed in Mouradov et al., 2002; Koornneef et al., 2004).

Flowering time is one of the traits for which it was shown that natural variation is present also for genes of which the function was established before by mutant analysis (El-Assal et al., 2001; Werner et al., 2005).

Arabidopsis natural variation and QTL analysis

Arabidopsis thaliana (L.) Heyhn. is a small weed plant belonging to the mustard family (Brassicaceae). In nature Arabidopsis grows in a wide range of habitats, distributed over much of the Northern Hemisphere (Hoffmann, 2002) at altitudes from sea level up to 3500 m in the mountains of central Asia. This wild crucifer has become an important model system because it allows combining genetics with molecular biology (Meinke et al., 1998). Arabidopsis has many advantages for genetic and molecular research; it is a small self-fertilizing species with a short life cycle. Furthermore, it has one of the smallest genomes among higher plants, approximately 130 megabases in size, divided over five chromosomes. Its genome has been completely sequenced (Arabidopsis Genome Initiative, 2000) and contains approximately 30.000 genes. Arabidopsis is being used to discover gene functions, mainly by the study of the effects of defective (mutant) genes or by the analysis of overexpression of genes. An important novel tool is also the genome-wide study of gene expression, made possible by the development of gene chips based on the known DNA sequence of this plant.

In addition to the use of mutants it has been realized that the natural variation present among Arabidopsis accessions growing in nature is an interesting source of genetic variation to study. It is expected that the genetic variation for traits in nature reflect their adaptation to specific environments (Koornneef et al., 2004). The traits that show variation in nature includes morphological variation such as the presence or absence of trichomes, resistances to pathogens and many physiological traits such as seed dormancy, flowering time and growth under normal and stress conditions (reviewed by Alonso-Blanco and Koornneef, 2000; Maloof 2003; Koornneef et al., 2004).

The genetic basis of the differences in physiological processes that can be considered as quantitative traits is often complex, because it involves several genes (polygenic) and the expression of these traits depends strongly on environmental factors. Quantitative trait locus (QTL) analysis enables the unraveling of the genetics of such quantitative traits. The genetic identification of the genes controlling natural variation may allow the molecular identification

of these genes and subsequently the processes that are controlled by these genes. Arabidopsis is the best model plant for such an analysis because of the technologies and resources developed by a large research community that allow the efficient molecular analysis of genes. Published and unpublished data have revealed considerable genetic variation for adaptive traits and have shown that this variation can be analyzed at the molecular level; this already resulted in the cloning of a number of QTLs. However, also in other plants such as rice and tomato the molecular basis of natural variation starts to be uncovered (Alonso-Blanco et al., 2005).

For the efficient analysis of QTL in Arabidopsis permanent or immortal mapping populations such as recombinant inbred line (RILs) and near isogenic lines (NILs) are very suitable and such populations can be developed relatively fast due to the short generation time, the self fertilizing nature and the limited space requirement of Arabidopsis (Alonso-Blanco et al. 2000). The advantage of such populations is that experiments can be performed in replicates and in multiple environments and that multiple traits can be analyzed. The use of the same mapping population for different traits allows the comparison of map positions (genetic location) of genes encoding these different traits and will sometimes result in the discovery of one gene controlling several traits. This pleiotropism has been discovered for several genes already (Koornneef et al., 2004).

Scope of the thesis

Plants are sessile organisms that have to cope with highly variable conditions in their direct environment. Most plants have the capacity to modify their phenotype in such a way that the individual plant is better adapted to survive and produce offspring in the prevailing environment. In order to be successful, a plant has to optimize the gain of carbon (photosynthesis), the allocation of carbon between organs and the way it deals with its environment, which can differ also for plants of the same species. To achieve environment-induced changes in phenotype, plants perceive signals that contain information about the direct environment of the plant.

To understand the mechanisms of plant growth and allocation of reserves, insight into the genetic components is essential. In the research described in this thesis a QTL analysis approach is used to gain insight into the various aspects of plant performance, which has been studied at the level of primary production, allocation of assimilates and carbohydrate metabolism.

This thesis deals with the genetic characterization of different aspects of plant performance that is analyzed using *Arabidopsis thaliana* natural variation. For this analysis four new RIL populations were obtained from a cross between the accessions Landsberg *erecta* as a female (L*er*) and Kashmir (Kas-2), Kondara (Kond) and Shakdara (Sha) as pollen parent, while Antwerp (An-1) was reciprocally crossed with L*er*. Chapter 1 gives an overview of the roles of different aspects of plant performance and source-sink relationships in addition to the importance of sugars and starch to plant growth. Chapter 2 describes the screening of

the natural variation for a set of Arabidopsis accessions for chlorophyll fluorescence (a non-destructive measurement for photosynthetic activity) measured as yield. Detailed genetic and physiological analyses of the only deviating accession that exhibited altered photosynthetic characteristics are given and the differences between this accession and the reference strain have been analyzed down to the molecular genetic level. Chapter 3 describes three new recombinant inbred line populations for Arabidopsis, having L*er* as a common parent that was crossed with the accessions An-1, Kas-2 and Kond. Flowering time variation was analyzed in the three recombinant inbred line populations to indicate the usefulness of these new RILs. Chapter 4 describes the variations present for growth-related traits in a set of Arabidopsis accessions. In addition, the genetic variation of the growth-related traits has also been described by QTL mapping using a fourth new RIL population derived from the cross between L*er* and Sha accession, which resulted in the identification of 5 common growth regions. Chapter 5 describes the use of the new RIL population L*er* x Kond in combination with a growth analysis on hydroponics. This population was used to investigate the genetic basis of differences in the carbohydrate content, carbon allocation, growth-related traits and flowering-related traits, and to see if relationships between traits might have a common genetic basis. Finally in chapter 6 the work presented in this thesis is summarized and discussed.

Chapter 2

Altered photosynthetic performance of a natural Arabidopsis accession is associated with atrazine resistance

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Abstract

Natural variation for photosynthetic traits was studied by determining chlorophyll fluorescence parameters in a collection of Arabidopsis accessions. This screen revealed only one single accession (Ely), exhibiting photosynthetic characteristics markedly different from all others, while a few lines showed small but significant variation. Detailed genetic and physiological analyses showed reduced fitness for Ely compared with the standard laboratory strain L*er* for various growth parameters. At low temperature (15°C), Ely had a higher electron transport rate than L*er*, indicating increased photosystem II efficiency under this condition, while at high temperature (30°C) the opposite was observed. Ely had a high sensitivity to UV-B radiation compared with L*er* and was atrazine resistant. This atrazine-resistance and related chlorophyll fluorescence traits were maternally inherited, pointing towards chloroplast-located gene(s). Definite proof that Ely is atrazine-resistant was obtained by sequencing the *psbA* gene, encoding the D1 protein of photosystem II, revealing a point mutation causing the same amino acid change as found in other atrazine-resistant species. Additional nuclear encoded genetic variation was also present, as was concluded from the small but significant differences in phenotype between Ely and its reciprocal crosses with L*er*. It was concluded that the photosynthetic yield is highly conserved and that only severe selection pressure results in marked variations in photosynthetic performance.

Introduction

Photosynthesis is a complex chloroplast-located process, controlled by both nuclear and plastidic genes and considered as a central step in determining plant growth and productivity. Photosynthetic efficiency can be assayed in a non-destructive way by measuring chlorophyll fluorescence (ChlF) (Maxwell and Johnson, 2000). This is an integrative trait, reflecting both light and dark reactions of photosynthesis.

Physiological differentiation among populations showed that evolutionary divergence in photosynthetic traits is common within species. This implies that selection has influenced photosynthetic traits in some way (Arntz and Delph, 2001). Natural variation within a plant species may provide an interesting source of genetic variation to be used for the unraveling of gene functions (Tanksley and McCouch, 1997). Within-species natural variation is the basis for QTL analysis, which has been shown to be useful for the genetic unraveling of complex plant traits (Koornneef et al., 2004). Intraspecific variations in photosynthetic traits may be direct, or indirect via changes in non-photosynthetic traits, and emphasize the importance of viewing the phenotype as an integrated function of growth, morphology, life-history and physiology (Arntz and Delph, 2001). In a previous paper (see chapter 4), substantial variation in growth characteristics has been described among a collection of accessions, based on overall growth characteristics, namely, plant size, relative growth rate, and flowering-related traits (El-Lithy et al., 2004).

In the present study the genetic variation for photosynthetic efficiency has been investigated between Arabidopsis accessions. Remarkably, this screening of a large set of Arabidopsis accessions, using chlorophyll *a* fluorometry, showed that just one single accession exhibited photosynthetic characteristics substantially different from all others. Detailed genetic and physiological analyses showed that the deviating photosynthetic characteristics of this single "natural variant" present in the collection, was caused by a point mutation in the chloroplast *psbA* gene, also leading to atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine) resistance. Atrazine resistance is a trait thought to result from the extreme selection pressure exerted by man-made triazine herbicides at the site of collection. It was concluded that the kinetics of photosynthetic yield is highly conserved within Arabidopsis and that only severe selection pressure results in marked variations in photosynthetic performance.

Results

The identification and genetic analysis of differences in photosynthetic yield

A collection of 127 Arabidopsis accessions was screened for their ChlF, measured as yield ($Y = \Delta F/F_{m'}$; where ΔF = variable fluorescence and $F_{m'}$ = fluorescence yield at zero photochemical and non-photochemical quenching for dark non-adapted leaves). This screening revealed that Ely (CS 6088) was the only accession with a considerably lower yield (Y = 0.54), while the Y for the other accessions ranged from 0.63 to 0.71 with a mean Y of 0.69 (+0.02) (Fig. 2.1).

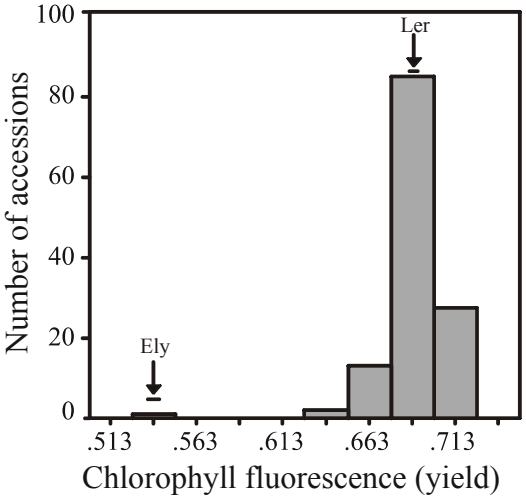

Figure 2.1: Frequency distribution of the chlorophyll fluorescence measured as yield ($Y = \Delta F/F_{m'}$) for 127 Arabidopsis accessions, showing one clearly deviating accession (Ely) with low ChlF. The average value for Ely and L*er* is indicated with an arrow, and the horizontal bars represent the SE for these accessions (based on three independent measurements, one measurement/plant). ChlF was measured at 125 µmol $m^{-2}s^{-1}$ light intensity, 70 % relative humidity and 22°C.

Five other accessions, An-1, Pak-2, Pak-3, Labal and Ws-1, showed reduced photosynthetic Y (Table 2.1), significantly different from both Ely and the rest of the accessions (P = 0.05). The first three accessions showed early senescence that might affect the photosynthetic capacity, although the measurements were carried out on (visually) healthy non-senescing leaves.

Name	Stock No	Yield	SE
Ely	CS 6088	0.544	0.0112
An-1	CS 944	0.634	0.0611
Pak-2	JW 106	0.646	0.0325
Lapal	JW 110	0.654	0.0204
Pak-3	JW 107	0.655	0.0098
Ws-1	CS 2223	0.655	0.0029
L*er*	N 20	0.686	0.0075

Table 2.1: Names and stock numbers of Arabidopsis accessions with low photosynthetic yield ($Y = \Delta F/F_{m'}$) in addition to the reference accession L*er*.

The estimated heritability for all accessions (0.71 and 0.60 with and without Ely, respectively) indicated that despite the small differences for Y, genetic variation for this trait was present.

To determine if nuclear or chloroplast genes control this phenotype, Ely was reciprocally crossed to the laboratory reference accession L*er*. Whenever Ely was used as the

seed-bearing parent, the progeny had a low Y (Y= 0.5 – 0.55), while progeny derived from L*er* as the seed-bearing parent had a high Y (Y= 0.67 – 0.71) (Fig. 2.2). The F_2 progeny of either F_1 did not segregate for Y, with a low Y in the case of Ely maternity and a high Y in the case of L*er* maternity. The differences between both two groups were highly significant (P = 4.94E-13). The differences in Y between plants were maintained after repeated back crossing (BC) of either progeny with L*er* as pollen donor. These findings indicated maternal inheritance of the Y that is probably mitochondrial or chloroplast encoded.

The Y of Ely was significantly lower than that of the F_1 or F_2 plants derived from crosses with Ely as female parent (0.002 > P > 4.94E-13). Moreover, the coefficient of variation (standard deviation as percentage of the mean value) was slightly larger (2.12) among the F_2 plants than among the parent lines, F_1 (Ely x L*er*) or F_1 (Ely x L*er*) x L*er* (1.38, 0.88 and 1.19, respectively) suggesting a segregation of nuclear genes with additional minor effects on Y.

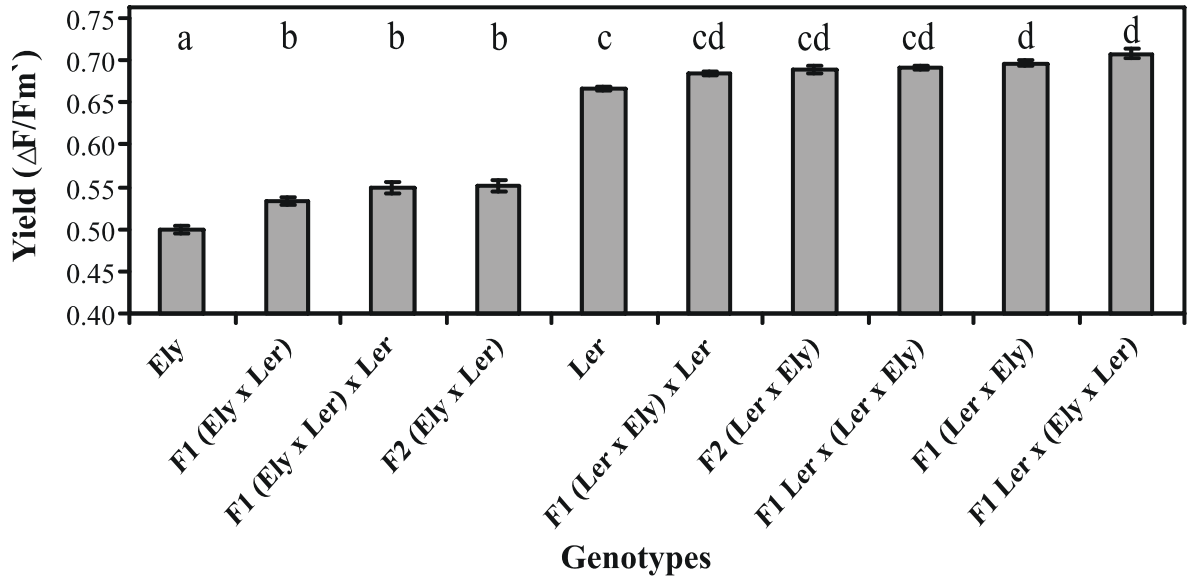

Figure 2.2: Chlorophyll fluorescence measured as yield ($\Delta F/F_{m'}$) for Ely, L*er*, and their reciprocal F_1s and F_2s. Bars labeled with different letters are significantly different at P = 0.002. Error bars indicate SE (based on 24 independent measurements, for Ely, L*er*, F_1s and 120 measurements for the F_2s, both one measurement/plant). ChlF was measured at 125 µmol $m^{-2}s^{-1}$ light intensity, 70% relative humidity and 22°C.

Correlation between photosynthetic yield and plant growth

To quantify the differences observed between the two maternal groups further, the rosette radius of plants was measured on day 20 as a non-destructive way of measuring growth (Fig. 2.3). Plants with low Y had a smaller rosette radius (ranging from 16.6 – 19.4 mm) compared with plants with a high Y (radius ranging from 21.3 – 27.3 mm). The differences between both maternal groups were significant at P < 0.01 (Fig. 2.3). Similar differences have been observed when fresh and dry weights, rosette area, and relative growth rate of L*er* and Ely were compared (El-Lithy et al., 2004). The rosette radius of F_1 (L*er* x Ely)

was significantly larger than that of both parents, suggesting a hybrid vigor effect as shown by Barth et al. (2003). However, the F_1 of the reciprocal cross (Ely x L*er*) did not differ significantly from both parents (Fig. 2.3), which might be due to the low maternally inherited Y of Ely.

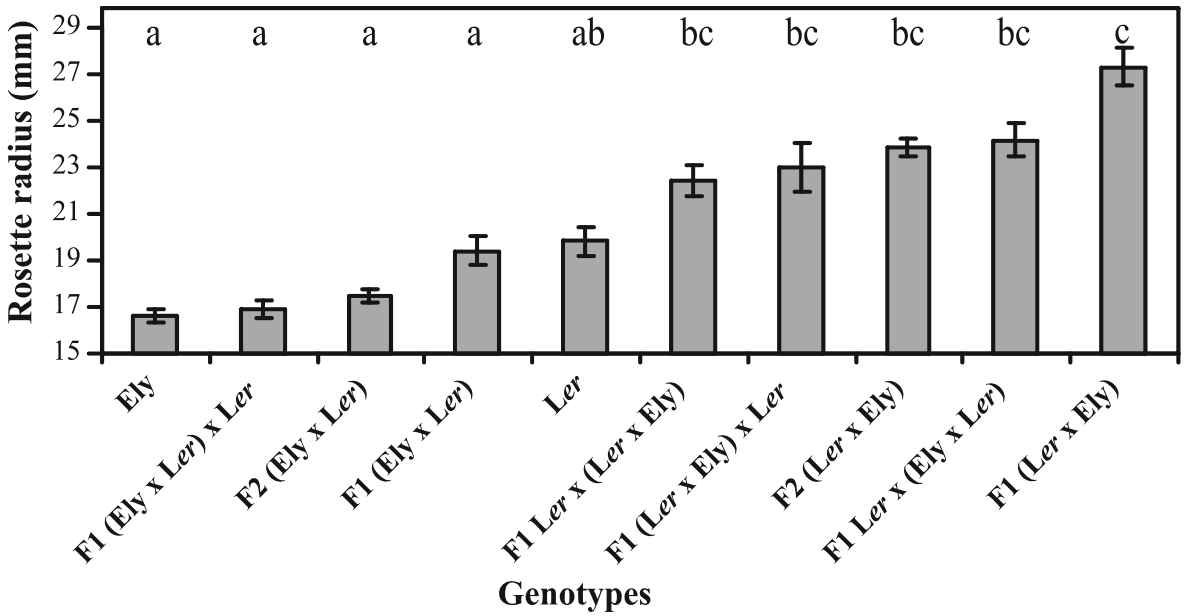

Figure 2.3: Rosette radius at day 20, of Ely, L*er* and their reciprocal F_1 and F_2 plants. Bars labeled with different letters are significantly different at P = 0.01. Error bars indicate SE (based on 24 and 120 independent measurements, one measurement/plant, for Ely and L*er*, and for F_1s and the F_2s, respectively).

Physiological characteristics of the Ely accession and its back cross line

To investigate the differences between the two maternal genotypes, a number of experiments were performed in which photosynthetic characteristics were determined while varying environmental factors known to affect ChlF. The experiments were performed with L*er*, Ely and the progeny of a fourth BC of the hybrid (F2BC4) for which L*er* was always used as the pollen donor. This BC is expected to have mainly L*er* nuclear DNA and Ely cytoplasm.

Effect of temperature on electron transport rate

Chlorophyll fluorescence analysis can be used to monitor the effects of low and high temperatures on photosynthesis, for example, at low temperature increased electron transport to alternative electron sinks was found in maize (Fryer et al., 1998). In this study it was found that, at high temperature (30°C), L*er* had a significantly higher ETR than both Ely and the F2BC4 at PAR-values below 300 µmol $m^{-2}s^{-1}$, which indicated that the overall PSII efficiency of Ely was lower than L*er* at high temperature (Fig. 2.4). By contrast, at low temperature (15°C), Ely had a higher ETR than L*er*, indicating increased PSII efficiency under this condition.

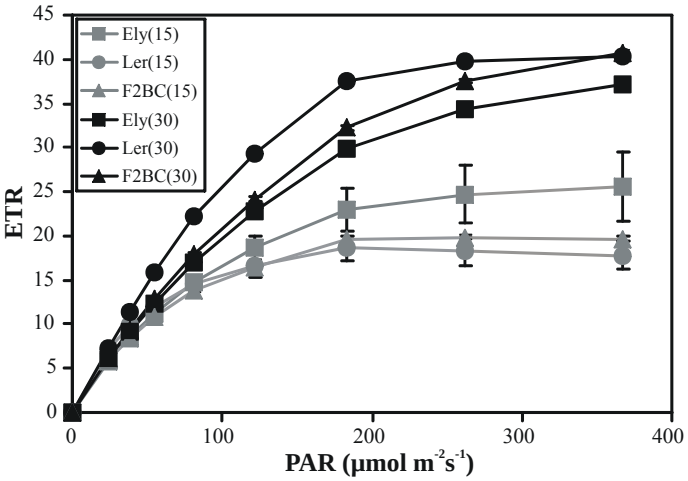

Figure 2.4: Effect of temperature (15 or 30°C) and photosynthetically active radiation (PAR) intensity on electron transport rate (ETR) of Ely, L*er*, and the F2BC4. ETR was measured at 125 µmol $m^{-2}s^{-1}$ light intensity and 70% relative humidity after 10 min adaptation at the proper temperature. Six plants were used for each genotype with one measurement per plant. SE can only be seen when exceeding the size of the symbol.

Effect of UV-B treatment on photosynthetic yield

Using chlorophyll fluorescence measurements, the impact of UV-radiation on photosynthesis was studied. PSII is known to be highly UV-sensitive (Jansen et al., 1998). Arabidopsis genotypes were raised under growth chamber conditions in the absence of UV-B radiation. The UV-sensitivity of these plants was determined by exposing leaf discs to UV radiation, in a low background of photosynthetically active radiation (PAR) (12 µmol $m^{-2}s^{-1}$), and determining any deleterious effects by measuring the photosynthetic efficiency of PSII, (F_v/F_m), where F_v = variable fluorescence and F_m = maximum fluorescence yield at zero photochemical and non-photochemical quenching both for dark-adapted leaves. Prior to UV-treatment, a small difference in the values for F_v/F_m was observed between Ely and L*er*, the latter having on average a 3-5% higher PSII efficiency. After UV-exposure the F_v/F_m values differed significantly. A 5 h exposure of control leaf discs to 11.4 µmol $m^{-2}s^{-1}$ UV-B resulted in a severe decrease in F_v/F_m in Ely and F2BC4, while the leaf discs of L*er* were significantly less affected (Fig. 2.5).

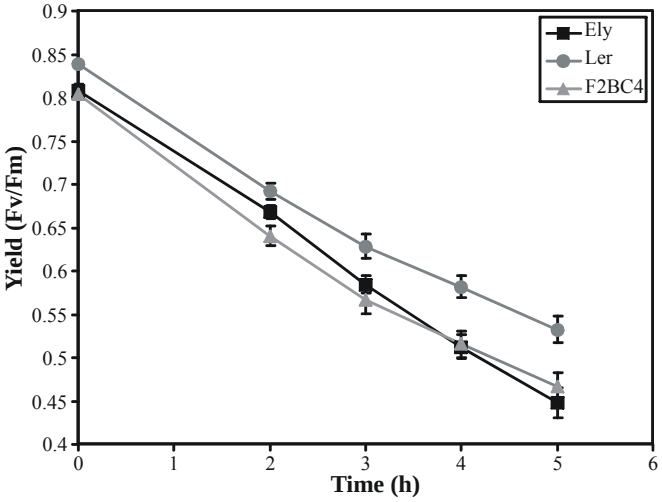

Figure 2.5: Chlorophyll fluorescence of leaf discs of Ely, L*er*, and the F2BC4 measured as the maximum yield (F_v/F_m) after increasing duration of UV-B treatment at 11.4 µmol $m^{-2}s^{-1}$ with additional photosynthetically active radiation (PAR) 12 µmol $m^{-2}s^{-1}$. Data are averages ± SE of 10-14 independent leaves (one leaf/plant); SE can only be seen when exceeding the size of the symbol.

Thus, using F_v/F_m as a sensitive probe for UV-damage to the photosynthetic apparatus, it was noted that both Ely and F2BC4 were more UV-sensitive than L*er*.

Chl a fluorescence rise curves of Ely, F2BC4 and Ler

Chlorophyll *a* fluorescence, in general, is a useful indicator to monitor a wide variety of photosynthetic events. Determining the fluorescence rise curve (from microseconds to a few s), by exposing dark-adapted leaves to saturated light pulses, gives specific information on photosynthesis (Strasser et al., 1995). Chl *a* fluorescence rise curves, and the transients of Ely, F2BC4 and L*er* are presented in Figure 2.6. The L*er* curve had the typical OJIP characteristics as previously described by Strasser et al. (1995, 2000): transients at about 1 ms (J), at about 20 ms (I) and a P-level at about 500 ms. The Ely curve was different: the F_o (chlorophyll fluorescence at origin in dark-adapted reaction centers with maximal photochemical quenching) was slightly higher and the J-level was also increased (Fig. 2.6 A and B).

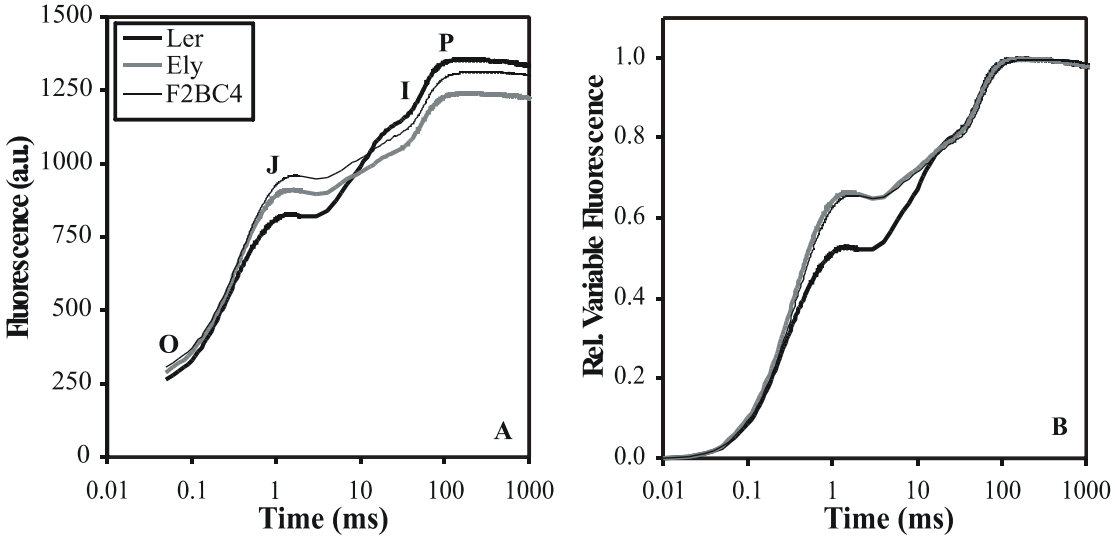

Figure 2.6: Fast fluorescence rise upon excitation of dark-adapted leaves of Ely, F2BC4 and L*er*, plotted on a log time scale. (A) The unnormalized fluorescence, (B) The normalized data plotted as the relative variable fluorescence (F_v/F_m). Each curve is the average of six leaves, two leaves/plant.

Table 2.2: Fluorescence at origin (F_o), fluorescence after 2 and 30 ms of excitation (F2 and F30, respectively), maximum fluorescence (F_m), and potential photochemical yield of PS II (F_v/F_m) measured in dark-adapted leaves of Arabidopsis genotypes L*er*, Ely and the F2BC4. The standard deviations are given between brackets.

Fluorescence parameter	L*er*	Ely	F2BC4
F_o	235 (9)	261 (12)	278 (10)
F2	821 (26)	907 (30)	959 (35)
F30	1162 (28)	1065 (38)	1116 (43)
F_m	1357 (40)	1242 (44)	1315 (57)
F_v/F_m	0.828 (0.004)	0.788 (0.004)	0.789 (0.004)

The curve of the F2BC4 was similar to the one of Ely. Table 2.2 illustrates details of the fluorescence measurements. In Ely and the F2BC4, F_o was slightly higher and the J-level was increased, while there was little difference in the I-transient and in the Fm compared with L*er*. The F_v/F_m values were lower for Ely and F2BC4, as compared with L*er* (Table 2.2), confirming the previous differences found in the UV-B analysis.

Effects of atrazine

The physiological characteristics of Ely and its F2BC4 progeny, namely, low Y, low ETR at elevated temperatures, increased UV-B sensitivity, and altered OJIP characteristics were all found to be maternally inherited, suggesting that this is a chloroplast-encoded trait. A point mutation in the *psbA* gene, which encodes the D1-protein of PSII, is known to cause similar physiological effects in many other species. This point mutation in the *psbA* gene confers atrazine-resistance to the plant (Botterman and Leemans, 1988). This prompted an investigation to find if Ely is resistant to atrazine by measuring Chl *a* fluorescence rise curves in the presence of atrazine and another herbicide DCMU. DCMU inhibits photosynthesis in a similar way as atrazine, but there is no cross-resistance to DCMU in atrazine-resistant plants. The effect was also determined of various concentrations of atrazine on the PSII electron flow in isolated thylakoids as measured by oxygen evolution.

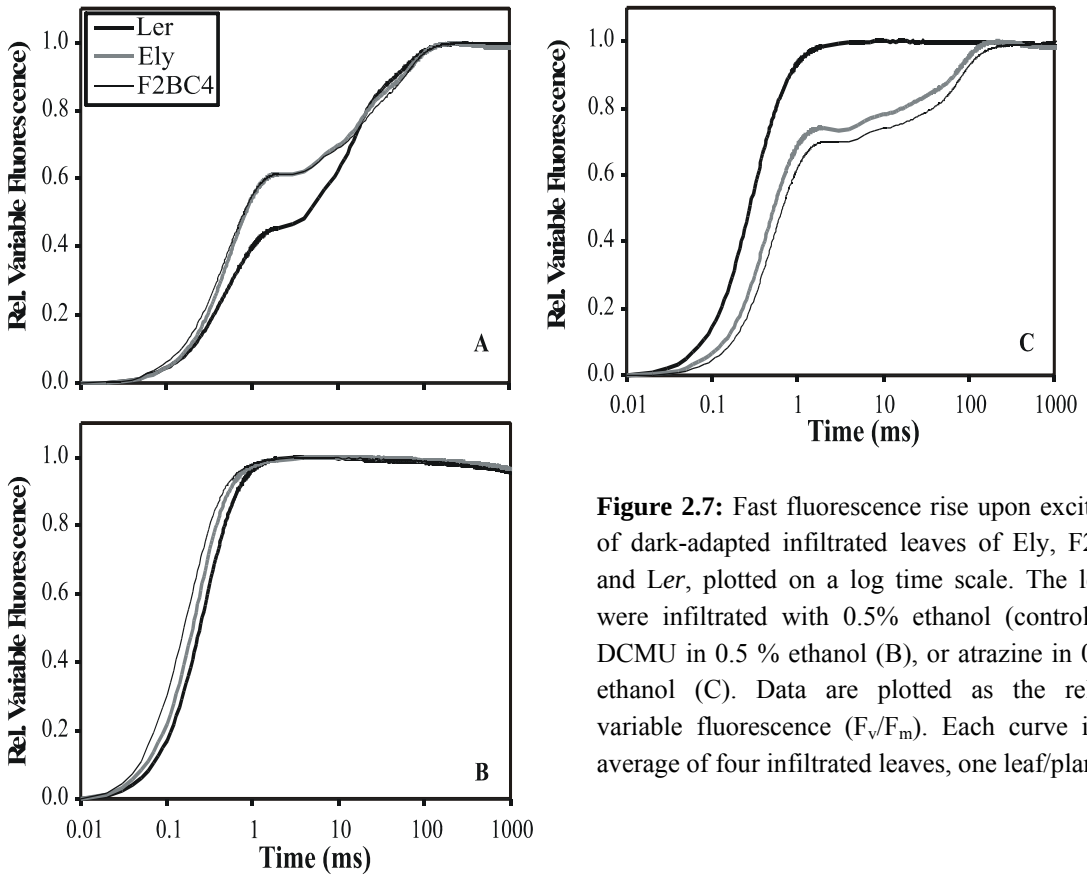

Figure 2.7: Fast fluorescence rise upon excitation of dark-adapted infiltrated leaves of Ely, F2BC4 and L*er*, plotted on a log time scale. The leaves were infiltrated with 0.5% ethanol (control, A), DCMU in 0.5 % ethanol (B), or atrazine in 0.5 % ethanol (C). Data are plotted as the relative variable fluorescence (F_v/F_m). Each curve is the average of four infiltrated leaves, one leaf/plant.

The normalized curves for Chl *a* fluorescence as a function of time are shown in Fig. 2.7 for leaf material infiltrated with DCMU, atrazine, or the solvent 0.5 % ethanol as control. Low concentrations of ethanol have very little effect on the curves (Figs. 2.6B and 2.7A). Infiltration with DCMU caused complete inhibition of electron flow at the acceptor side of PSII, leading to a very fast rise of the fluorescence to the P-level. This increase was equally fast in all three genotypes. However, after infiltration with atrazine this fast rise occurred only in L*er* and not in Ely or the F2BC4, indicating that the latter two genotypes were resistant to atrazine, in contrast to L*er*. The effect of various concentrations of atrazine on PSII electron flow in isolated thylakoids as measured by oxygen evolution are shown for L*er* and Ely in Fig. 2.8. In L*er*, oxygen evolution was inhibited for 50% by 3 µM atrazine, while in Ely a more than ten times higher concentration of atrazine (40 µM) was required to achieve a similar level of inhibition.

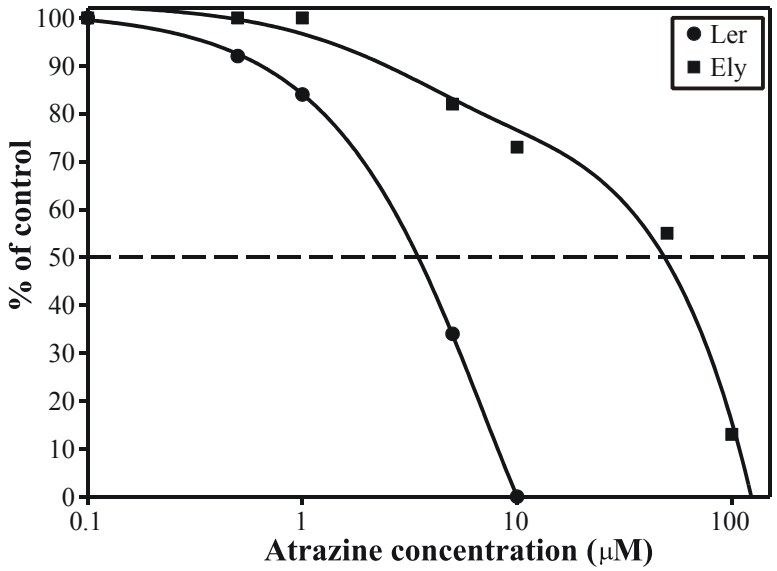

Figure 2.8: Inhibition of oxygen evolution in isolated thylakoids of Ely and L*er* at different atrazine concentrations.

In many species atrazine-resistance is due to a specific nucleotide difference altering codon 264 of the chloroplast *psbA* gene encoding the D1 protein (Oettmeier, 1999). Therefore, the *psbA* gene was PCR-amplified from both L*er* and Ely and the DNA sequence of the PCR fragments was determined. Only one nucleotide difference was found between both accessions, changing the sequence of codon 264 from AGT to GGT, thus changing the predicted amino acid from 264Ser into 264Gly. This conversion is the typical atrazine-resistance conferring mutation found in many other plant species (Botterman and Leemans, 1988; Gronwald, 1994; Sibony and Rubin, 2003).

Discussion

In general, there is ample evidence of genetic variation among and within natural plant populations for photosynthetic traits, although some studies report little or no genetic variation (Arntz and Delph, 2001). The variation found among Arabidopsis accessions for Y was very small. Despite the considerable variation in growth rate characteristics among accessions (El-Lithy et al., 2004), it was found that there is only very limited variation in the underlying photosynthetic reactions. These data are consistent with others, which show strong conservation of the sequences of key PSII proteins (Botterman and Leemans, 1988; Gronwald, 1994; Sibony and Rubin, 2003) and of the kinetics of electron transport near PSII (Jansen and Pfister, 1990) among a wide variety of photosynthetic organisms. A range of man-made PSII mutants has been produced over the last couple of years, some of which were non-photosynthetic while others were only slightly affected in terms of photosynthetic efficiency (Kless et al., 1994; Niyogi et al., 1998; Wu et al., 1999; Keilty et al., 2000; Varotto et al., 2000; Walters et at., 2003). The data clearly shows that photosynthesis appears to be subjected to strong natural selection allowing very few suboptimal genotypes to be maintained in a population. Ely was the only significantly distinct accession within a collection of 127 accessions. Ely showed both reduced photosynthetic yield (described here) and reduced growth (El-Lithy et al., 2004) indicating the penalty for a reduction of photosynthetic yield.

The difference in the photosynthetic traits observed between Ely and L*er* was concluded to be related to the fact that the D1 protein of Ely has been altered because of the *psbA* mutation leading to atrazine-resistance. This conclusion is based on a series of experiments giving direct and indirect evidence.

i. Reduced ChlF is maternally inherited

The pattern of differences in the ChlF between the reciprocal crosses of L*er* and Ely showed that this trait is maternally inherited and likely to be controlled by (a) chloroplast gene(s). This is in agreement with genetic analysis of barley where atrazine tolerance also inherited maternally (Rios et al., 2003).

ii. Ely shows reduced growth

Reduced growth of atrazine-resistant genotypes has been described in other species (Gressel, 2000), and has been attributed to the reduced PSII electron transfer efficiency caused by the *psbA* mutant allele (Holt et al., 1993). Earlier, it was reported that Ely is among the slow-growing Arabidopsis accessions (El-Lithy et al., 2004). These findings are confirmed here, and moreover, comparing the standard laboratory strain L*er* with the F2BC4 backcross population (having L*er* nuclear genome and Ely chloroplasts) shows that the low Y trait is linked with significantly reduced growth.

iii. Temperature affects electron transport rate differently in L*er* and Ely

The sensitive Arabidopsis accession (L*er*) has a higher ETR than the resistant accession (Ely) at 30°C, while at 15°C Ely, surprisingly, has a better ETR than L*er*. Similar results were found for several other species of which triazine-resistance biotypes have been collected (*Polygonum lapathifolium*: Darmency and Gasquez, 1982; *Brassica rapa*: Plowman and Richards, 1997). Triazine-resistant PSII reaction centers were found to be much more sensitive to temperatures above 35°C (Ducruet and Lemoine, 1985; Ducruet and Ort, 1988; Havaux, 1989; Fuks et al., 1992). This implies that the yield penalty observed in the greenhouse and climate chamber conditions used here may have been of less importance in the spring climate of Cambridgeshire (UK) where Ely was found and that these plants might not have a reduced fitness compared to atrazine-sensitive genotypes under those conditions.

iv. Ely is more susceptible to UV-B stress

A lower PSII quantum yield is due to the slower electron transfer between Q_A and Q_B (Jursinic and Pearcy, 1988). It has been speculated that changes in the redox state of PSII directly affect the sensitivity of PSII to UV-radiation (Jansen et al., 1998; Rodrigues et al., unpublished results). It was found that UV-B radiation, when given in the presence of a low background intensity of PAR, resulted in a severe decrease in F_v/F_m in atrazine-resistant genotypes Ely and F2BC4, while the atrazine-sensitive genotype (L*er*) was significantly less affected. Similar findings were reported by Olsson et al. (2000) for an atrazine-resistant cultivar of *Brassica napus*.

v. Chlorophyll *a* fluorescence rise curve of Ely is typical for atrazine resistant genotypes

Chlorophyll *a* fluorescence data show that Ely and also F2BC4 are atrazine-resistant, since the characteristic OJIP curves of these genotypes differ from the one of the atrazine-sensitive biotype (L*er*). Similar results were obtained by Kohno et al. (2000), for wild-type and triazine-resistant *Chenopodium album*. From Fig. 2.6 it is clear that the curves for Ely and F2BC4 are nearly identical, again indicating that the effect was mainly due to differences in the non-nuclear, presumably chloroplastic genome. Moreover, while all three tested genotypes are sensitive to DCMU, only L*er* was inhibited by atrazine (Fig. 2.7).

vi. Oxygen evolution in Ely thylakoids is resistant to atrazine

Chlorophyll fluorescence rise curves were determined using atrazine infiltrated leaf discs and differences in atrazine-resistance between genotypes might thus be due to differences in uptake of the herbicide. Therefore, oxygen evolution in isolated thylakoids and the effect of atrazine was also measured. This revealed that 50% inhibition required about 10 times higher concentration of atrazine in Ely compared with L*er* (Fig. 2.8).

The gene from Ely showed the typical atrazine-resistance mutation changing the sequence of codon 264 from AGT to GGT, thus changing the predicted amino acid from 264Ser into 264Gly (Botterman and Leemans, 1988; Gronwald, 1994; Sibony and Rubin,

2003).

The occurrence of atrazine-resistance in Arabidopsis, not a usual target species for herbicide applications, can be explained by the fact that this resistant accession was collected at a railway station in Ely (UK) in the mid-eighties (P. Williams, personal communication). At that time herbicide applications were common to maintain weed-free railways and atrazine was commonly used until its ban in 1993.

Although, in general, little variation in ChlF was observed between the accessions tested, there is additional nuclear-encoded genetic variation as concluded from the small but significant differences in phenotype between Ely and its progeny. This small-scale variation can still be very amenable for quantitative trait locus (QTL) analysis. It has been observed more frequently that the trait values of segregating populations extend beyond the values of the parents (transgression), implying that more variation is present than can be detected by simply surveying accessions. This will especially be the case with traits that are under selection and for which the optimal phenotype can be obtained by different genetic make-up. A similar situation was observed for the length of the circadian period length (Swarup et al., 1999) and is also suggested by the detection of a ChlF QTL in the L*er* x Sha RIL population (El-Lithy et al., 2004), despite the fact that the parents did not differ.

However, it is very well possible that more variation will be found between accessions when experiments are performed under less optimal conditions. Furthermore, screening for more complex photosynthetic parameters such as qP, NPQ (Niyogi et al., 1998), in addition to the photosynthetic yield (Varotto et al., 2000; Walters et at., 2003), might result in more variation between accessions than observed by only determining yield.

This study demonstrates how screening for natural variation has led to the identification of intraspecific variations in photosynthetic traits in Arabidopsis populations. The variation in photosynthetic traits was linked to growth parameters, revealing the resulting fitness penalty.

A supplementary table is providing at J. Exp. Botany online, listing names and stock numbers of all Arabidopsis accessions used in this study. The averaged data + SE of chlorophyll fluorescence measured as yield (three measurements, one measurement/plant) for the 127 accession are also given in this table.

Materials and Methods

Plant material and growth conditions

Arabidopsis thaliana accessions were obtained from the Arabidopsis stock centers ABRC, NASC and Sendai (www.arabidopsis.org), supplemented with accessions recently collected by members of the Laboratory of Genetics at Wageningen University and currently deposited at ABRC and NASC. Details of all the 127 accessions used in this study are given as supplementary material and can be found at J. Exp. Botany online. The Ely accession (CS6088), provided by ABRC was collected by Dr Paul Williams (University of Wisconsin, Madison, USA) at the railway station of Ely (UK) in 1988 (P Williams personal communication).

All Arabidopsis seeds were pre-sown in Petri dishes on water-saturated filter paper, followed by cold treatment for 4 d at 4°C, and then transferred to a climate room at 25°C and 16 h light for 2 d before planting in 7 cm pots with standard soil. In all the descriptions of the experiments, time is referred to as days after planting. The plants were grown in an air-conditioned greenhouse with 70% relative humidity, supplemented with additional light (model SON-T plus 400W, Philips, Eindhoven, The Netherlands) providing a day-length of at least 16 h light (long day), with a light intensity of 125 µmol $m^{-2}s^{-1}$, and maintained at a temperature between 22-25°C (day) and 18°C (night). For each accession, three plants were selected to measure their photosynthetic yield (Y). Heritability (broad sense) was estimated as the proportion of variance explained by between-line differences using the general linear model module of the statistical package of SPSS version 11.0.1 (SPSS Inc., Chicago, IL). Y and rosette radius were measured on day 20 for 24 plants for each of the parents (Ely and L*er*) and the F_{1s} of the different crosses, while 120 F_2 plants were averaged for the same traits. For measuring electron transport rate (ETR), UV-B effect, chlorophyll *a* fluorescence rise curves and oxygen evolution, plants of L*er*, Ely and the F2BC4 were grown under controlled conditions in a growth cabinet, with 70% relative humidity, 22°C, 12 h day (short day) and a light intensity of 125 µmol $m^{-2}s^{-1}$.

The F2BC4 plants were obtained by crossing Ely (female parent) x L*er* (male parent). Resulting F_1 plants were crossed again with L*er* as the male parent to get the 2nd back cross (BC). The same was done for two further generations until 4th BC plants were obtained. These plants were selfed to get F_2 plants of the 4th BC (F2BC4). The aim was to obtain plants with L*er* nuclear DNA and Ely cytoplasm. Plants were placed on carts and the carts were shuffled daily to avoid an effect of minor local condition differences within the growth cabinet.

DNA isolation and sequencing

DNA was isolated from greenhouse-grown plants, one plant per genotype. The Bernatzky and Tanksley (1986) DNA isolation protocol was adapted for rapid extraction of small quantities. Flower buds were harvested in liquid nitrogen and ground in 330 μL of a preheated (65°C) extraction solution [125 μL extraction buffer: 0.35 M sorbitol, 100 mM Tris, 5 mM EDTA, pH 7.5 (HCl) together with 175 μL lysis buffer: 200 mM Tris, 50 mM EDTA,

2M NaCl, 2% (w/v) cetyl-trimethyl-ammonium bromide, to which 30 μL sarkosyl (10% w/v) was added]. The mixture of crude plant material and extraction solution was incubated for 30 min at 65°C; during this period occasional shaking was applied. Hereafter a solution of 400 μL chloroform/isoamyl alcohol (24:1 v/v) was added and vortexed. After centrifuging for five min at maximum speed in an Eppendorf centrifuge the water phase was transferred to a new tube. An equal amount of cold isopropanol was added to precipitate the DNA by carefully inverting the tube several times. After 10 min centrifugation at maximum speed in an Eppendorf centrifuge the water-alcohol mixture was discarded and the pellet washed with 70% cold ethanol. The pellet was left to dry and dissolved in water containing RNAse A and incubated 30 min at 37°C. Thereafter it was stored at 4°C.

The PCR amplification for the *psbA* gene was carried out using the forward primer 5`-GTGCGCTTGGGGAGTCCCTGATTA-3`, and a reverse primer 5`-TATTTAAAGAAGGCTTATATTGCTCGTT-3`. The PCR product was re-PCRed using another primer combination (forward primer 5`-CTATGCATGGTTCCTTGGTAACTTC-3`, reverse primer 5`-CGTTCATGCATAACTTCCATACCA-3`) flanking the expected point mutation to get a fragment of 400 bp. This fragment was purified for both Ely and L*er* using a PCR purification kit (Roche Diagnostics Corporation Indianapolis, IN, USA).
Both strands of each fragment were sequenced using the same primers. For PCR a protocol of 30 s at 94°C, 30 s at 50°C, and 30 s or 60 s at 72°C (35 cycles), was used.

Chlorophyll fluorescence and electron transport rate measurements

ChlF as a non-destructive marker of photosynthetic efficiency was measured, based on three independent measurements (one measurement/plant), as quantum yield (Y) using a MINI-PAM (Walz Mess- und Regeltechnik, Effeltrich, Germany). The effective photosystem II (PSII) quantum yield of photosynthetic energy conversion was calculated as $Y = F_v/F_m = \Delta F/F_m$, for dark-adapted and non-adapted leaves (Van Kooten and Snel, 1990). The Y data of the 127 accessions are given as supplementary material and at JXB online. ETR was measured using the same MINI-PAM at two different temperatures, 15°C and 30°C in a controlled growth cabinet. Plants were temperature adapted for 10 min before the measurement. Six plants were used for each genotype with one measurement per plant.

UV-B treatment

For UV treatments leaf discs (10-14 independent leaves, one leaf/plant) were exposed to UV-B radiation, generated by Philips TL12 fluorescent tubes (λ_{max} 315 nm). The light emitted by the bulb was filtered through a single layer of cellulose acetate. Exposure times and irradiance conditions were set to obtain a measurable decrease in PSII activity. Leaf discs were exposed for up to 5 h at 11.4 µmol $m^{-2}s^{-1}$. The irradiance level represents radiation in the spectral range between 295 nm and 345 nm. Discs were floated on distilled water, with their adaxial side facing the UV-source. The decrease in photosynthetic activity was attributed to the UV-B wavelengths since the low level of UV-A radiation is ineffective in decreasing PSII activity (Jansen *et al*., 1998). A low level (12 µmol $m^{-2}s^{-1}$) of additional PAR was applied during the UV-treatments. UV-levels were measured using an optometer (United Detector

Technology Inc., Hawthorn, USA) equipped with a probe specific for UV-wavelengths. The photosynthetic efficiency of PSII was determined by the saturating pulse fluorescence technique, using a plant efficiency analyzer (Hansatech, King's Lynn, UK). The minimal fluorescence (F_o), maximal fluorescence (F_m), and the variable fluorescence ($F_v = F_m - F_o$) were all measured according to Van Kooten and Snel (1990). The photochemical yield of open PSII reaction centers, commonly known as the relative variable fluorescence, was calculated as F_v/F_m. It reflects the maximal efficiency of PSII that was measured in tissue dark-adapted for at least 20 min.

Chlorophyll *a* fluorescence rise curves

The fast chlorophyll *a* fluorescence rise curves of dark-adapted leaves were measured with the Plant Efficiency Analyzer fluorometer (PEA, Hansatech Instruments Ltd, King's Lynn, Norfolk, UK). The measurements were performed at room temperature with a 3 s excitation pulse of 100% light intensity, which corresponds to about 600 W m^{-2} of light with a peak at 650 nm (approximately 3000 μmol m^{-2} s^{-1}). After a 10 min dark adaptation, data were recorded for 3 s to generate a fluorescence induction curve. The fluorescence signal at 50 μs, the earliest measurement free of any artifacts related to the electronics of the instrument (Haldimann and Strasser, 1999), was considered as F_o. Of the so-called OJIP fluorescence rise curve the J-level was considered at 1 to 2 ms, the I-level at about 30 ms, and the P-level at about 500 ms. The curves were viewed and averaged with the instrument`s software (WinPea). The curves presented are the averages of measurements on six different leaves from three individual plants.

Infiltration of leaves

Detached leaves (four leaves from four different plants) were vacuum-infiltrated under 0.8 bar with DCMU (diuron) [3-(3,4-dichlorophenyl)-1,1-dimethylurea] and atrazine (both from Sigma Chemical Co., St Louis, USA), using a concentration of 50 μM in 0.5% (v/v) ethanol. Control leaves were infiltrated with 0.5% (v/v) ethanol only. After infiltration, the leaves were dark-adapted at room temperature for 1 hour in Petri dishes lined with filter paper saturated with the infiltrated solution.

Isolation of thylakoids

Details of the isolation of thylakoids were described previously (Van Rensen *et al*., 1977). Leaves from *Arabidopsis* plants were homogenized using a Sorvall Omnimixer in isolation medium containing 0.4 M sorbitol, 20 mM tricine-NaOH (pH 7.8), 10 mM NaCl, 5 mM $MgCl_2$, 2 mM sodium ascorbate and 2 mg ml^{-1} bovine serum albumin. After squeezing through three layers of nylon cloth the chloroplasts were collected by centrifugation for 30 s at 3000 g, washed once in 50 mM sodium phosphate buffer (pH 7.8) to obtain broken chloroplasts collected by centrifugation for 5 min at 1000 g. The chlorophyll content was measured according to Bruinsma (1963), and the chlorophyll concentration adjusted to 2 mg Chl ml^{-1}.

Measurement of photosynthetic electron transport

Details of the measurement of photosynthetic electron transport activity were as described earlier (Van Rensen *et al*., 1977, 1978). Electron transport was estimated as oxygen evolution, which was measured with a Gilson oxygraph provided with a Clark oxygen electrode, at a temperature of 25°C and at saturating white light. The isolated thylakoids were suspended in 2 ml reaction medium containing 0.3 M sorbitol, 50 mM tricine-NaOH (pH 7.6), 5 mM $MgCl_2$, 5 mM NH_4Cl, 1 mM ferricyanide and thylakoids containing 50 µg chlorophyll.

Acknowledgement

We thank Professor Paul Williams (University of Wisconsin, Madison, USA) for providing detailed information on the location where the Ely accession had originally been found.

Chapter 3

New Arabidopsis recombinant inbred line populations genotyped using SNPWave™ and their use for mapping flowering time QTLs

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* The first and second authors contributed equally to this work.

Abstract

The SNPWave™ marker system, based on SNPs between the reference accessions Col-0 and L*er*, was used to distinguish a set of 92 Arabidopsis accessions from various parts of the world. In addition we used these markers to genotype three new recombinant inbred line populations for Arabidopsis, having Landsberg *erecta* as a common parent that was crossed with the accessions Antwerp-1, Kashmir-2 and Kondara. The benefit of using multiple populations that contain many similar markers and the fact that all markers are linked to the physical map of Arabidopsis facilitates the quantitative comparison of maps. Flowering time variation was analyzed in the three recombinant inbred line populations. Per population, 4 – 8 quantitative trait loci (QTLs) were detected. The comparison of the QTL positions related to the physical map allowed the estimate of 12 different QTLs segregating for flowering time for which L*er* has a different allele from 1, 2 or 3 of the other accessions.

Introduction

For the genetic analysis of natural variation, so-called immortal mapping populations are very useful because they allow the localization of many traits that show allelic variation in the same mapping population (Koornneef et al., 2004). Furthermore, they allow replication of experiments and testing in various environmental conditions. Since genotypes differ in their genetic composition, the analysis of similar traits in different populations is required to get insight into the genetic variation of a specific trait within a species.

To allow a proper comparison of the locations of genes, it is important to use the same marker framework and markers should preferentially be anchored to the physical map of the species. Various marker systems have been used to genotype Arabidopsis recombinant inbred line (RIL) populations. In Arabidopsis markers such as single sequence length polymorphisms (SSLPs) and cleaved amplified polymorphic sequences (CAPS) are anchored to the physical map. AFLP markers, as used to genotype the frequently analyzed Landsberg *erecta* (L*er*) x Cape Verde Island (Cvi) RILs (Alonso Blanco et al., 1998b), can be anchored using bioinformatic tools only when it concerns AFLP bands that are characteristic for the sequenced genome of Columbia (Col-0) (Peters et al., 2001). Based on available sequence data of Arabidopsis accessions, many SNP markers have been detected (Schmid et al., 2003; The Arabidopsis Information Resource (TAIR; www.arabidopsis.org); Cereon database (www.arabidopsis.org/Cereon/index.jsp) and http://walnut.usc.edu/2010.html). One of the various SNP detection systems (Cho et al., 1999) is the recently described SNPWave method (van Eijk et al., 2004).

In this study the usefulness of the SNPWave marker system was demonstrated, based on SNPs between the reference accessions Col-0 and L*er*, to distinguish Arabidopsis accessions from various parts of the world. In addition we used these markers to genotype three new sets of RILs derived from crosses between L*er* and Antwerp (An-1), Kashmir (Kas-2) and Kondara (Kond), respectively. The populations studied were developed because their parents showed specific phenotypic differences and represent different geographical origins. To demonstrate their applicability for quantitative traits locus (QTL) mapping as well, we analyzed flowering time (FT) for all lines in the three populations as an example of a quantitative trait. Such analysis using three RIL populations having one common parent allows direct comparison of the loci segregating in these populations and facilitates identification of the different FT loci for which allelic variation is present among Arabidopsis accessions.

Results

Polymorphism between a set of Arabidopsis accessions using SNPWave markers

A 100-plex SNPWave marker set of known SNPs between the two reference Arabidopsis accessions Col-0 and L*er* (van Eijk et al., 2004) was used to genotype 92 Arabidopsis accessions. Among markers that could be amplified in most accessions 37.6 to 62.4 % of the markers were different from the Col-0 allele and 0 to 37.6 % differed from the L*er* allele. The polymorphism data indicated that, for many accessions crosses made with one of the two reference accessions would yield reasonable numbers of polymorphic SNPWave markers.

A few identical genotypes were detected, of which some have been described for other markers systems as well (e.g. Co-1 = C24, L*er* = Di-1, Buckhorn Pass) (Fig. 3.1) (Torjek et al., 2003). For some other accessions (e.g. Co-1 and Es-0, Ct-1 and En-2, Be-0 and Tsu-1) this was not expected in view of their different geographical origins. As reported for many markers systems no obvious structure was detected, related to the geographical origin, using UPGMA cluster analysis. However, a number of accessions from Central Asia and Russia (Fig. 3.1) seem more related to each other than to accessions from other regions as was reported before (Schmuths et al., 2004; Nordborg et al., in press). The data confirm that Kas-1 (N903) and Kas-2 (N1264) are genetically different (Levey and Wingler, 2005), although both cluster in the Central Asian group.

L*er* x An-1, L*er* x Kas-2 and L*er* x Kond linkage maps

The accessions used to construct the RIL populations differed 57.1%, 55.8% and 54.5 % from L*er* for An-1, Kas-2 and Kond, respectively. These markers did not completely cover the genome. To construct genetic maps with equally spaced markers for the three different crosses (L*er* x An-1, L*er* x Kas-2 and L*er* x Kond), either additional SNPWave markers had to be developed or publicly available markers (TAIR database) were used as mentioned in the materials and methods section.

Linkage maps were obtained using 44, 45 and 51 SNP markers supplemented with 20, 31 and 23 SSLP markers for L*er* x An-1, L*er* x Kas-2 and L*er* x Kond, respectively. In addition, the *erecta* mutation segregating in all three populations and the *ga5* - gibberellin deficient mutation (Xu et al., 1995), segregating in the L*er* x Kas-2 population (shown to be present in Kas-2 by the absence of complementation in the cross of the *ga5* mutant and Kas-2) could be scored as morphological markers. This resulted in three genetic maps with 65, 78 and 75 markers for L*er* x An-1, L*er* x Kas-2 and L*er* x Kond, respectively (Fig. 3.2). The markers were assigned to five linkage groups for each population with a total genetic length of 371, 441 and 351 cM for L*er* x An-1, L*er* x Kas-2 and L*er* x Kond, respectively. Most markers were located on the expected linkage groups based on the physical order of the markers in the sequenced Col-0 accession.

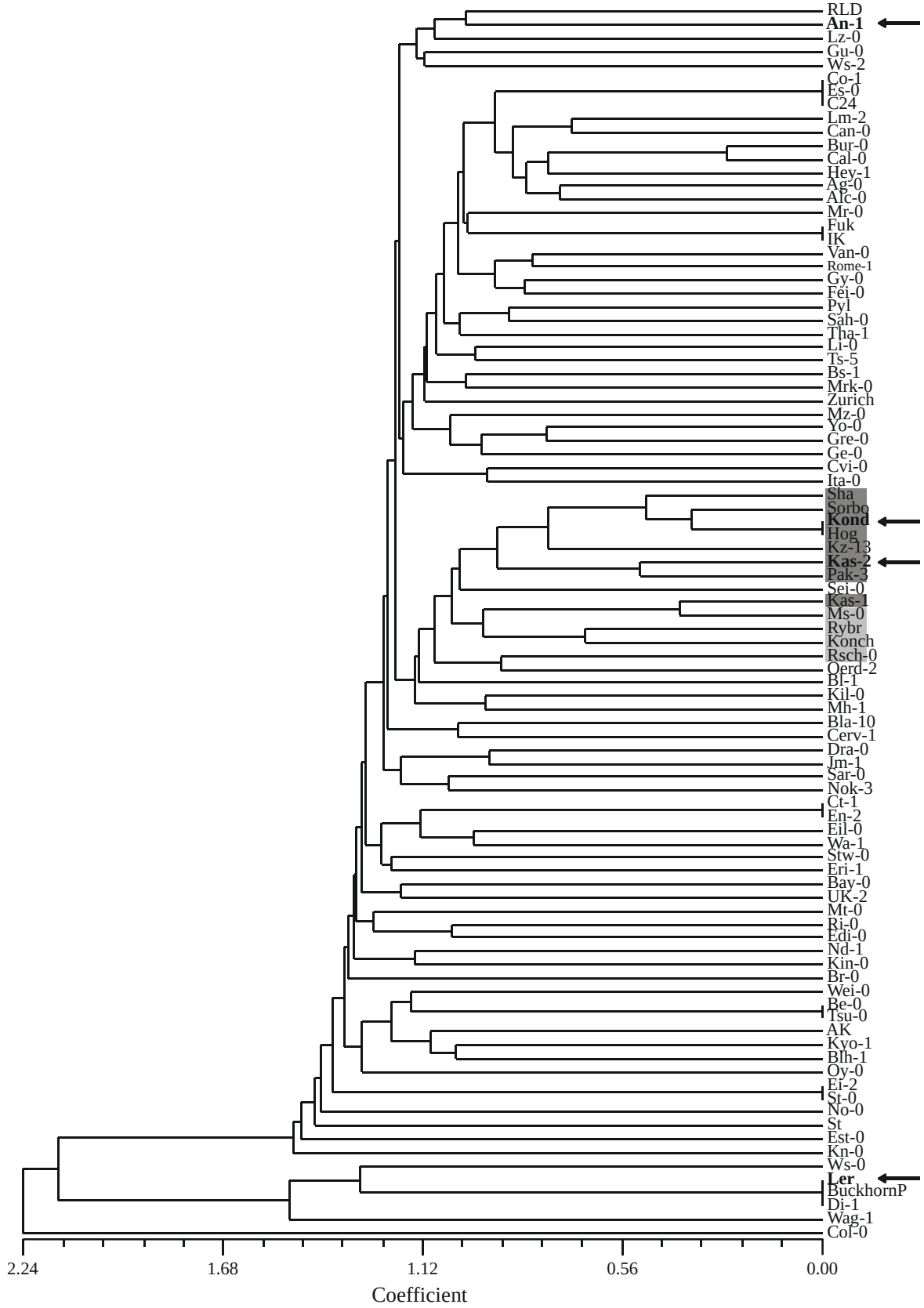

Figure 3.1: Tree plot of the 92 Arabidopsis accessions based on UPGMA cluster analysis using the pattern of polymorphism between 79 SNP markers. The dark gray block indicates accessions from central of Asia, while the light gray block refers to accessions from Russia. The arrows indicate the accessions used to generate the three populations.

Exceptions are SNP395, which was expected on chromosome 4 but mapped to chromosome 3 in L*er* x Kond, and two pairs of makers that are inverted in the L*er* x Kas-2 linkage map, viz., C6L9-78 and SNP395 on chromosome 4 and SNP77 and *FLC* on chromosome 5.

Since in each generation heterozygosity per locus is reduced by half after selfing, the probability that a specific locus is heterozygous is 0.39% for the F9 generation. The average frequency of heterozygosity for all loci is 0.28%, 0.25% and 0.25% for L*er* x An-1, L*er* x Kas-2 and L*er* x Kond respectively, with no locus having a significantly higher value than predicted.

For each marker the expected segregation ratio would be 1:1 for each parental allele, in case of no bias in the selection of individual plants during the maintenance of the populations. Figure 3.2 indicates regions with significantly distorted segregation (at $0.0005 < P < 0.05$) for the three populations. Markers showing significant segregation distortion clustered in a certain region of the genome in the three maps, with ratios ranging from 1.4:1 – 2.1:1. These regions either partially overlap in the different populations as on chromosome 1 but are population specific in other chromosome regions. The distortion in most regions favored L*er* alleles, although in four different regions of the three populations, the non-L*er* alleles were in excess (Fig. 3.2).

Comparison of the L*er* x An-1, L*er* x Kas-2 and L*er* x Kond genetic maps

Figure 3.2 shows the L*er* x An-1, L*er* x Kas-2 and L*er* x Kond genetic maps linked to each other by 41 anchoring markers scored in the three populations. Comparison between the physical map of Col-0 and the three newly generated linkage maps indicate that the overall recombination rates are similar over chromosomes with suppression of recombination observed around the centromeres of chromosomes 2, 3, 4 and 5 (Figure 3.3). The maps are co-linear with the exception of the two inverted pairs of markers in the L*er* x Kas-2 population described above. When comparing recombination frequencies in regions where maps appeared different (Fig 3.2) between the three populations, recombination was found to be significantly higher ($P = 0.001$) in the Kas-2 cross between SNP71 and SNP203 on chromosome 2 compared to Kond cross and recombination was not significantly different when compared to the An-1 cross ($P = 0.2$). In other regions differences were not statistically significant.

The pattern of similarity in recombination described above results in similar genetic lengths of the five chromosomes in the three crosses. The largest differences were observed for chromosome 2 where the genetic map of L*er* x Kas-2 is longer than the other two crosses by more than 20 cM and chromosome 3 where the L*er* x Kond map is shorter than the two other maps.

QTL mapping of flowering time

Flowering time QTLs have been mapped in several Arabidopsis RIL populations (Kowalski et al., 1994; Clarke et al., 1995; Jansen et al., 1995; Kuittinen et al., 1997; Alonso Blanco et al., 1998a; Loudet et al., 2002; El-Lithy et al., 2004; Koornneef et al., 2004 for review).

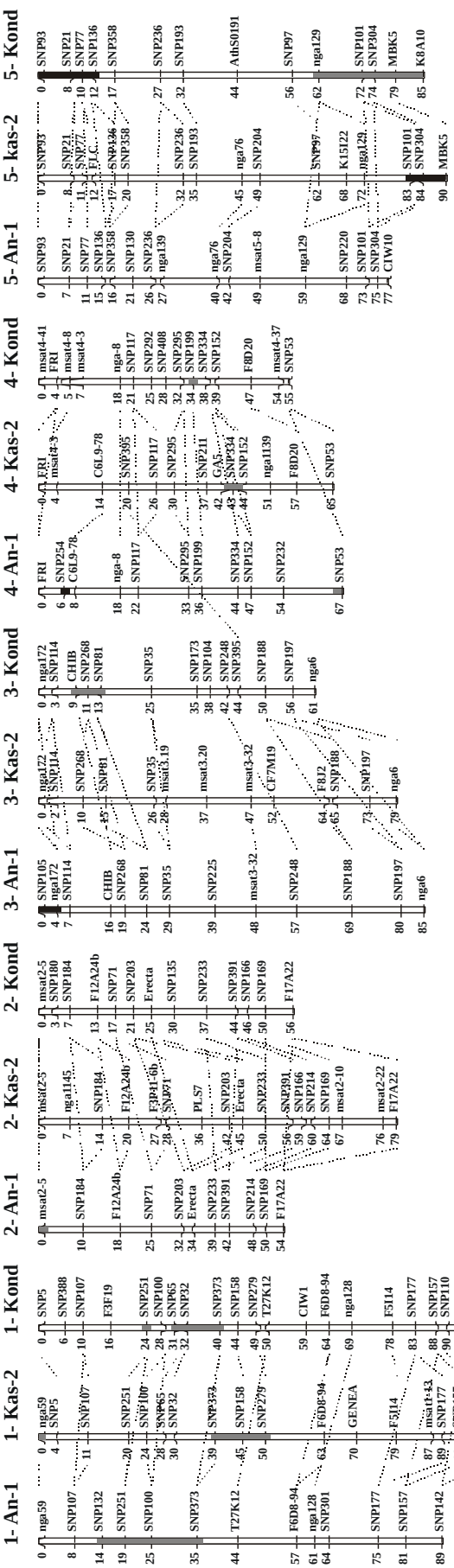

Figure 3.2: Integrated genetic maps linked through anchoring markers scored in the three populations: L*er* x An-1, L*er* x Kas-2 and L*er* x Kond. Distorted regions (regions deviating from the 1:1 ratio ($P < 0.05$)) are indicated by gray boxes (regions with significantly higher number of RILs with L*er* allele), or black boxes (indicate regions with significantly higher numbers of lines in favor of An-1, Kas-2 or Kond alleles).

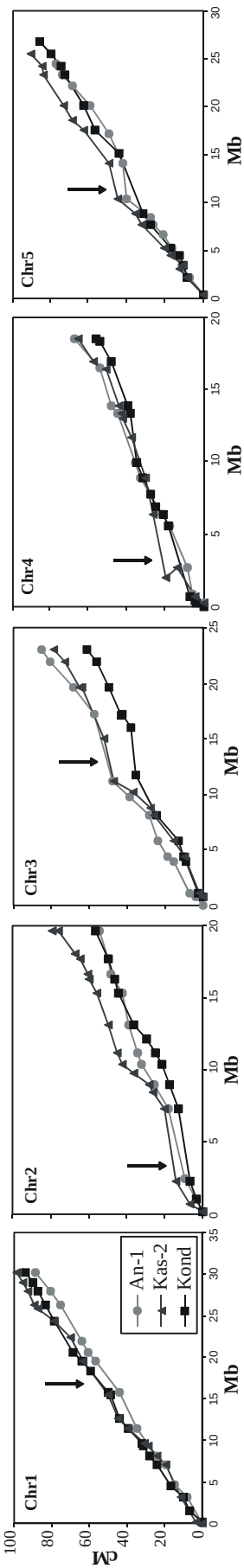

Figure 3.3: The relation between the genetic maps of L*er* x An-1, L*er* x Kas-2 and L*er* x Kond and the physical map along the five chromosomes. The arrows indicate the position of the centromeres.

Since different populations may segregate for different loci depending on the genetic composition of their parental lines, a comparison between multiple crosses is needed to get information about the variation present among Arabidopsis accessions. This allows the description of the so-called global genetic architecture (Symonds et al., 2005) of a trait within a species. Comparison between different crosses can only be done accurately when the same markers are used and/or when these markers are anchored to the Arabidopsis physical map, which acts as a reference map.

To illustrate this approach we analyzed FT in the three RIL populations, which were grown in the same greenhouse under long day conditions but in independent experiments. For all populations heritabilities were high and transgression beyond the parental values was observed both towards earliness and lateness (Table 3.1). In total 4, 6 and 8 QTLs were identified per population (Fig. 3.4).

Table 3.1: Parental values, averages and ranges of flowering time and heritabilities (h^2) in the three populations.

RIL population	FT - L*er*	FT non-L*er*	Average FT RILs	Range RILs	h^2
L*er* x An-1	23.4	22.3	23.2	19.1 – 28.3	0.88
L*er* x Kas-2	33.2	47.0	34.7	24.9 – 54.0	0.86
L*er* x Kond	29.0	49.3	38.7	26.7 – 66.2	0.95

Although the FT differences between L*er* and An-1 were very small (Table 3.1), variation between the RILs is considerable and is explained by four QTLs of which for three the An-1 allele is early flowering (Fig. 3.4 and Table 3.2). In the Kas-2 and Kond populations the parents differed much more and the genetic differences could be explained by six and eight QTLs, for which in three and five cases the L*er* alleles accelerate flowering, respectively (Fig. 3.4 and Table 3.2). The detected QTLs explained 68.3%, 78.8% and 84.8% of the phenotypic variance for the L*er* x An-1, L*er* x Kas-2 and L*er* x Kond populations respectively. Within the three populations significant interactions between several QTLs were detected (Table 3.2).

Relating the map positions and two LOD intervals to the physical map based on the Col-0 sequence allows a comparison between the QTLs in the three populations. In most cases the comparison was relatively straightforward. However, for QTLs with relatively large 2-LOD intervals (top chromosome 1 and 2, for L*er* x An-1 and L*er* x Kond crosses, respectively) and for regions where two linked QTLs were detected only in the same population, interpretation is more complex.

Two FT QTLs are in common among the three populations, viz., the QTLs on top of chromosome 3 (around nga172) for which the L*er* allele delays flowering and the QTLs located around SNP130 (chromosome 5) for which the L*er* allele accelerates flowering.

In addition there are several QTLs common between two of the three populations. These are the QTLs located at the *FRI* locus for L*er* x Kas-2 and L*er* x Kond and around SNP136 (chromosome 5) for L*er* x An-1 and L*er* x Kond and around K8A10 (chromosome 5) for L*er* x Kas-2 and L*er* x Kond. For the L*er* x An-1 population the latter QTL could not be separated from another QTL around SNP236. The presence of two distinct QTLs in this region was clear for the L*er* x Kond population.

Table 3.2: Characteristics of flowering time QTLs detected in the three populations.

RILs	QTL at nearest marker	Map position*	LOD score	% of variance	Additive allele effect (days)****
L*er* x An-1				**68.3*** **	
	SNP107	1-7.9	2.8	4.0	0.6
	SNP105	3- 0.0	16.4	29.4	1.8
	SNP254	4- 6.2	3.3	4.9	0.8
	SNP130	5- 21.0	14.2	24.7	-1.6
	SNP105 x SNP130	P = 0.000045**		6.1	
L*er* x Kas-2				**78.8*** **	
	SNP110	1- 95.5	7.8	6.5	3.0
	nga172	3- 0.0	3.5	2.4	2.0
	FRI	4- 0.0	23.8	24.0	-6.0
	SNP295	4- 30.0	3.8	2.8	-2.6
	SNP358	5- 20.2	8.8	7.2	-3.4
	MBK5	5- 89.6	16.8	15.0	4.8
	SNP110 x MBK5	P = 0.006**		4.7	
	SNP32 x SNP295	P = 0.008**		4.0	
L*er* x Kond				**84.8*** **	
	CIW1	1- 59.2	4.2	2.6	-3.0
	F5I14	1- 78.4	6.2	4.2	3.6
	msat2-5	2- 0.0	5.1	3.4	-3.0
	nga172	3- 0.0	5.0	3.5	3.0
	FRI	4- 4.1	36.5	46.6	-11.4
	SNP136	5- 12.4	3.2	2.0	-2.8
	SNP236	5- 27.4	8.9	6.4	-5.0
	K8A10	5- 84.8	5.4	3.6	3.2
	FRI x SNP136	P = 0.008**		2.3	
	FRI x SNP236	P = 0.009**		2.2	

* Chromosome number is given, followed by the marker position in cM.

** For interactions, the P values are given instead of the LOD scores.

*** Values in bold refer to the total explained variance by the trait.

****The positive and negative values indicate that L*er* and Kond alleles increase the trait value.

In addition, population specific QTLs (Table 3.2) were detected around markers SNP107 and SNP254 for L*er* x An-1, where L*er* alleles delayed flowering. For L*er* x Kas-2, two QTLs with different allele effects were identified around the markers SNP110 and SNP295. In the L*er* x Kond population specific QTLs could be identified around markers CIW1, F5I14 and msat2-5 with different allele effects. The number of co-locating QTLs might be higher since in a few cases suggestive QTLs (LOD between 1.5 and 2.4) were detected in one population at a position where significant QTLs were detected in another population (data not shown). This was true for two suggestive QTLs at SNP301 and GENEA for L*er* x An-1 and L*er* x Kas-2 respectively, which co-locate with a significant QTL in that region in L*er* x Kond (chromosome 1). When taking these suggestive QTLs into account the total number of QTLs did not increase. In total we identified 12 different QTLs for FT for which L*er* has different alleles from the alleles in one, two or three of the other accessions.

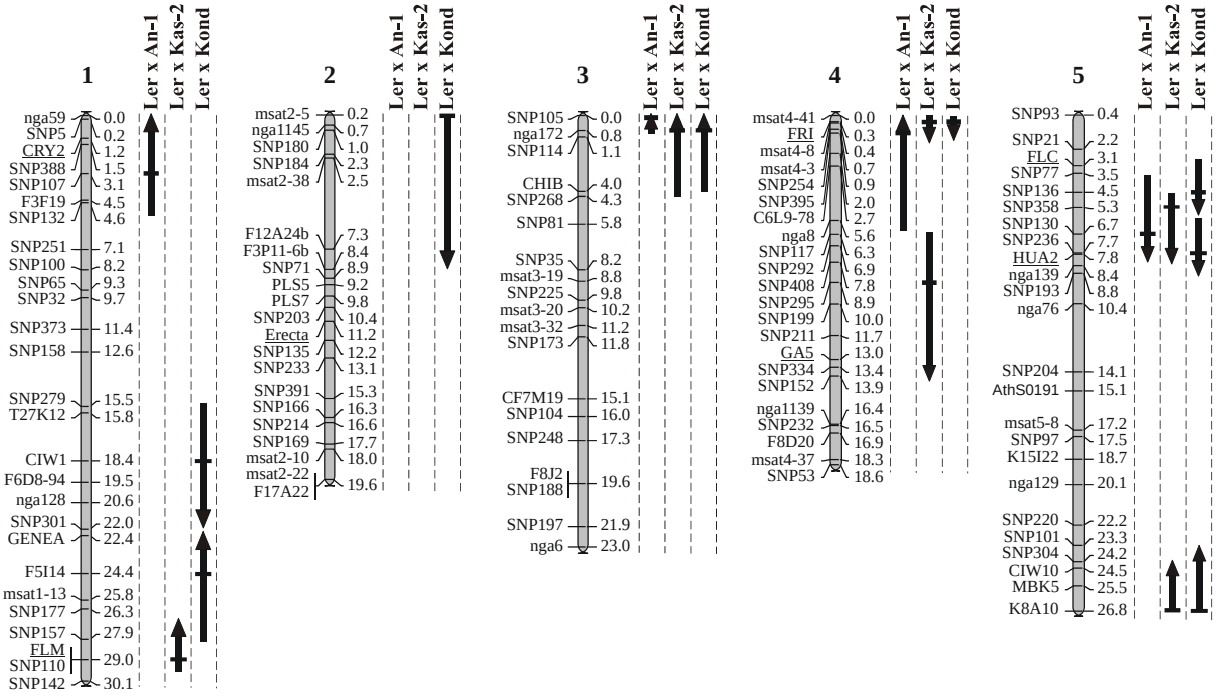

Figure 3.4: Combined physical map of L*er* x An-1, L*er* x Kas-2, and L*er* x Kond containing markers that are used in the present study in addition to several candidate genes (*CRY2*, *FLM*, *HUA2*). Flowering time QTLs are indicated by arrows along the chromosomes. Horizontal dashes in the arrows indicate the marker fixed during the MQM mapping analysis. The lengths of the arrows indicate the 2-LOD support intervals. The direction of the arrows indicates the allelic effect: upward, L*er* increasing the FT and the other allele decreasing; downward, the non-L*er* allele increasing and L*er* decreasing.

Discussion

In the present study we describe three new recombinant inbred line populations for Arabidopsis having L*er* as a common parent. The SNPWave technique was applied to genotype these RIL populations based on SNPs between Col-0 and L*er*. Using the SNPWave markers that are polymorphic between L*er* and Col-0 about 50% (37.6 to 62.5 %) can be used to genotype populations made from crosses with L*er*. Some regions are not covered by the SNPWave markers, such as the upper part of the lower arm of chromosome 1 for all three populations, the middle part of chromosome 3 for L*er* x Kas-2, the top of chromosome 4 for all populations and the middle part of chromosome 5 for L*er* x An-1. As far as this was due to insufficient coverage of the SNPWave markers in the three crosses, this implied that additional markers, such as the common PCR markers used here were required to obtain genetic maps with equally distributed markers.

Since the maps contain many similar markers and, more importantly, all markers are linked to the physical map of Arabidopsis, a quantitative comparison of maps could be performed. This analysis showed that map lengths are quite similar and also in the same range as those published for other populations (Lister and Dean 1993; Alonso Blanco et al., 1998b; Loudet et al., 2002; Clerkx et al., 2004). The two inverted pairs of markers that were detected in the Kas-2 population and the reduced recombination between the two markers on chromosome 2 might also be explained by structural chromosomal inversions between accessions. However differences in local recombination rate as such may exist, as suggested by cytogenetic data (Sanchez-Moran et al., 2002). Structural chromosome variants between accessions have not been studied frequently but are not uncommon (reviewed in Koornneef et al., 2003). Suppression of recombination in specific regions makes map based cloning in such regions difficult. In general a solution for this problem is to perform mapping in a cross with another accession that does not show suppression of recombination.

RIL populations allow the identification of natural genetic variants for which the parents differ. Such populations facilitate the mapping of many traits in the same population (Koornneef et al., 2004). However, since the parents might not be different for a specific QTL for which variation is present within the germplasm pool, additional mapping populations are being developed (www.INRA.fr/NaturalVar/RILSummary.htm). The power of using multiple populations was recently demonstrated by Symonds et al. (2005) who identified nine QTLs for trichome density in a total of four RIL populations, whereas individual population segregated for three to five QTLs.

In the present study similar results were obtained for FT, a frequently studied trait showing large natural variation in Arabidopsis. In this species extreme lateness is mainly due to the presence of dominant alleles at the *FRI* and *FLC* loci (Caicedo et al., 2004; Hagenblad et al., 2004; Koornneef et al., 2004). These large effect loci mask the segregation of other minor effect loci, which are easier to be detected in mapping populations where these large effect alleles do not segregate. This is well illustrated in the present example, where early and middle late accessions are combined and where in total 12 QTLs could be detected. One of

these is probably *FRI*, detected in the L*er* x Kas-2 and L*er* x Kond populations. Kas-2 and Kond have late alleles at the *FRI* locus, which is at least functional in Kond (Gazzani et al., 2003; Hagenblad et al., 2004). Active *FRI* alleles may confer lateness without *FLC*, as was also found in Shakdara (Sha) derived populations (Loudet et al., 2002; El-Lithy et al., 2004). In the L*er* x An-1 population we also identified a novel locus at the top of chromosome 4, for which An-1 is accelerating FT.

The situation around *FLC* is more complicated. We could identify a QTL at the position of *FLC* (marker SNP136) in the L*er* x Kond population, which was expected since Kond has a functional *FLC* allele (Gazzani et al., 2003; Hagenblad et al., 2004). Moreover, the significant interaction between the QTL at *FRI* locus and SNP136 (Table 3.2) is in agreement with the epistatic interaction described before for *FRI* and *FLC* (Koornneef et al., 2004). In the L*er* x Kond population and in the L*er* x Cvi population (Alonso-Blanco et al., 1998a) two linked loci on chromosome 5 were found both conferring lateness, but only when both alleles were derived from the non-L*er* parent. It has been suggested that the upper locus is *FLC* and the lower locus (named *FLG* by Alonso-Blanco et al., 1998a) might encode *HUA2* (Doyle et al., 2005). We could not identify a putative *FLC* QTL in the L*er* x Kas-2 population, although at the molecular level the Kas-2 *FLC* allele in this region was found to be similar in Kond and Sha (Hagenblad et al., 2004). However, the *FLC* locus could also not be detected in the RILs of L*er* x Sha (El-Lithy et al., 2004) and apparently the Sha carries a weak *FLC* allele (Gazzani et al., 2003). The lack of weaker epistatic relationship between the QTL at SNP358/SNP136 and the *FRI* locus suggests that the former one is not the *FLC* locus. On the other hand, for the An-1 cross, the 2-LOD interval includes the *FLC* locus but we could not separate this SNP130 QTL into two QTLs. On the contrary, for total leaf number and rosette leaf number, traits that are known to be correlated to FT (Koornneef et al., 1991; Alonso Blanco et al., 1998a) we could detect two distinct QTLs (data not shown).

The L*er* x Kas-2 and L*er* x Kond populations have common QTLs with the same allelic effects at nga172, at *FRI* and at K8A10. At these positions also the Sha accession from Tadzjikistan carries similar alleles (Loudet et al., 2002; El-Lithy et al., 2004). Kas-2 and Sha may have similar alleles for the QTLs at the bottom of chromosome 1 for which the *FLM* locus is a candidate gene (Werner et al. 2005).

The present analysis shows that for a single trait additional genetic variation is detected when different populations are analyzed for the same trait. However, the accuracy of QTL mapping is such that co-location can also be due to two different closely linked QTLs. Having available different sources for the same type of allelic variation allows the selection of the populations with the strongest alleles for future fine mapping and cloning. In addition these genetic studies provide the basis of the correlation between function and molecular haplotype as has been described for the *FRI* and *FLC* loci (Gazzani et al., 2003; Michaels et al., 2003; Caicedo et al., 2004; Hagenblad et al., 2004). The new RIL populations show segregation for various other traits such as seed dormancy and plant performance (unpublished data) that are currently being analyzed.

Materials and Methods

Plant material and growth conditions

Arabidopsis thaliana accessions were obtained from the Arabidopsis stock centers ABRC, NASC and Sendai (www.arabidopsis.org), supplemented with accessions recently collected by members of the Laboratory of Genetics at Wageningen University and deposited at ABRC and NASC. Arabidopsis seeds were sown in Petri dishes on water-saturated filter paper followed by a 4-days cold treatment at 4 °C, and transferred to a climate room at 25°C and 16h light for two days before planting in 7-cm pots with standard soil. In all descriptions of experiments, time is referred to as days after planting. The plants (12 plants/accession) were grown in an air-conditioned greenhouse with 70 % relative humidity, supplemented with additional light (model SON-T plus 400W, Philips, Eindhoven, The Netherlands) providing a day-length of at least 16 h light (long day), with light intensity 125 µmol $m^{-2}s^{-1}$, and maintained at a temperature between 22-25 °C (day) and 18 °C (night).

New RIL populations were obtained from a cross between the accessions Landsberg *erecta* as a female (L*er*, N20) and both of Kashmir (Kas-2, N1264) and Kondara (Kond, CS6175) as pollen parent, while Antwerp (An-1, N944) was reciprocally crossed with L*er*. The F1 seeds of the different crosses were grown and allowed for self-fertilization to get F2 seeds. From the F2 seeds a set of 120, 164 and 120 RILs, for An-1, Kas-2 and Kond respectively, have been generated by single-seed descent procedure until the F9 generation. In order to minimize any bias in the selection of plants taken to the next generation, 6 individuals per RIL were planted and plant number 3 was selected to go on for the next generation with the fifth one as a back up. Two plots containing six plants per RIL of the F9 generation were planted by the same procedures and under the same conditions mentioned before for growing the accessions. The flower heads of three individuals per RIL were harvested separately for DNA isolation and left for seed harvesting as well as for future use.

DNA isolation and genotyping

Genomic DNA of 92 accessions was isolated from leaf material of individual plants using a modified CTAB procedure (Stewart and Via, 1993). Details of the protocol for genotyping these accessions, using SNP markers, were described previously (van Eijk et al., 2004). For the RIL populations, the flower buds of three F9 plants per genotype were harvested separately for DNA isolation. DNA extraction was performed as described above for the SNP markers. For the SSLP markers, DNA was extracted using the Wizard® magnetic 96 (Promega; #FF3760) DNA isolation kit. SSLP markers were described in Clerkx et al. (2004), the TAIR database or the MSAT database (www.inra.fr/qtlat/msat). Primers used for novel markers that were developed are described in Table 3.3. For practical use the SGCSNP markers are written as SNP. In addition T27K12-SP6 and F5I14-49495 are written as T27K12 and F5I14, respectively. For both markers the physical position can be found in the TAIR database.

All markers used have first been checked to determine if the parental accessions An-1, Kas-2 and Kond were polymorphic with L*er*; thereafter, the polymorphic markers were used to genotype all individual RILs. For SSLP markers a standard protocol of 30 s 94°C, 30 s 50°C, and 30 s 72°C (35 cycles) was used except for *FRI* (54°C annealing, 1-min extension) and *FLC* (52°C annealing, 2-min extension).

Table 3.3: New markers uswd to genotype the three populations.

Marker name	BAC	Primer 1 (5' to 3')	Primer 2 (5' to 3')
F12A24b	F12A24	GGTGTGATGTCGACCGGTAAAG	TGCACAACGTGCTCTCCATG
F17A22	F17A22	ACACACGAATATTGATTGTCTAAGG	TCACTTGTCGGTTTGTGTGG
cF7M19	F7M19	AGCTTGTGTCGTTTCCGATAG	AGTTGCAGAAATAAGCAGTGGC
F8D20	F8D21	CTTAAATGCCGATCCAGTCGAGG	TTCATTCGCGCATTTATTGTTGC
K15I22	K15I22	TCGGTGGTTTACTTTCACTTT	GAATTGTAGCTTCTTCTGAACC

Measurement of flowering time

F10 generation plants (twelve plants/RIL) were grown in the greenhouse in a randomized two-block design to reduce environmental effects. FT for each plant was scored as the number of days from planting until opening of the first flower.

Map Construction and QTL analysis

Initially, the three linkage maps have been constructed using only the SNP markers; gaps between markers that were larger than 13 cM were filled using SSLP markers to obtain uniformly distributed markers. The JoinMap program (version 3.0, www.kyazma.nl) was used to construct the genetic maps.

The software package MapQTL® 5 was used to identify and locate QTLs on the linkage map by using interval mapping and multiple-QTL model (MQM) mapping methods as described in its reference manual (www.kyazma.nl). In a first step, putative QTLs were identified using interval mapping. Thereafter, the closest marker at each putative QTL was selected as a cofactor (van Ooijen and Maliepaard, 1996; van Ooijen, 2000) and the selected markers were used as genetic background controls in the approximate multiple QTL model of MapQTL. LOD threshold values applied to declare the presence of QTLs were estimated by performing permutation tests implemented in MapQTL version 5.0 using at least 1000 permutations of the original data set, resulting in a 95% LOD threshold at 2.4. Two-LOD support intervals were established as 95% QTL confidence interval (van Ooijen, 1999) using restricted MQM mapping implemented within MapQTL. The estimated additive genetic effect and the percentage of variance explained by each QTL and the total variance explained by all the QTLs affecting a trait were obtained using MQM mapping.

Statistical analysis

Using NTSYSpc version 2.10t. (Rohlf, 2001) the tree plot of the 92 Arabidopsis accessions based on UPGMA cluster analysis using the pattern of polymorphism between 79 SNP markers was performed.

Heritability (broad sense) was estimated as the proportion of variance explained by between-line differences using the general linear model module of the statistical package of SPSS version 11.0.1 (SPSS Inc., Chicago, IL) based on measurements of 6-12 plants per genotype.

Differences in recombination were tested using a Chi square test comparing the number of recombinant and parental lines for two identical markers in two populations where differences were observed.

Two-way interactions among the QTLs identified for FT, were tested by ANOVA using the corresponding two markers as fixed factors and the trait as dependent variable, using the general linear model of the statistical package SPSS version 11.5.1. A Bonferroni correction to adjust the 0.05 threshold of significance was applied if multiple tests were performed on the same data set. Only those interactions that were significant after the Bonferroni correction are presented.

Acknowledgement

The SNPWave^{TM} technology is covered by patent applications owned by Keygene N.V. An application for trademark registration for SNPWave has been filed by Keygene N.V.

Chapter 4

Quantitative trait locus analysis of growth-related traits in a new Arabidopsis recombinant inbred population

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Abstract

Arabidopsis natural variation was used to analyze the genetics of plant growth rate. Screening of 22 accessions revealed a large variation for seed weight, plant dry weight and relative growth rate but not for water content. A positive correlation was observed between seed weight and plant area 10 d after planting, suggesting that seed weight affects plant growth during early phases of development. During later stages of plant growth this correlation was not significant, indicating that other factors determine growth rate during this phase. Quantitative trait locus (QTL) analysis, using 114 (F9 generation) recombinant inbred lines derived from the cross between Landsberg *erecta* (L*er*, from Poland) and Shakdara (Sha, from Tadjikistan), revealed QTLs for seed weight, plant area, dry weight, relative growth rate, chlorophyll fluorescence, flowering time, and flowering related traits. Growth traits (plant area, dry weight and relative growth rate) co-located at five genomic regions. At the bottom of chromosome 5, co-location was found of QTLs for leaf area, leaf initiation speed, specific leaf area, and chlorophyll fluorescence but not for dry weight, indicating that this locus might be involved in leaf development. No consistent relation between growth traits and flowering time was observed despite some co-locations. Some of the QTLs detected for flowering time overlapped with loci detected in other recombinant inbred line populations, but also new loci were identified. This study shows that Arabidopsis can successfully be used to study the genetic basis of complex traits like plant growth rate.

Introduction

Analysis of plant growth is an essential step in the understanding of plant performance and productivity (Leister et al., 1999) and may reveal different strategies of plants to survive under limiting conditions.

Growth rate and, more specifically, relative growth rate (RGR) are comprehensive traits of plants, which characterize to a large extent plant performance and are also important components of fitness (McGraw and Garbutt, 1990). These parameters integrate morphological and physiological traits of plants. RGR is an inherent quantitative trait that may vary among plant species, occurring in a wide range of habitats. Plants in favorable environments often have an inherently high RGR, whereas those from less favorable habitats have an inherently low RGR, even when grown in the same favorable conditions (Grime and Hunt, 1975; Poorter and Remkes, 1990). In addition, plant growth rate is also affected by developmental changes such as the onset of flowering or the formation of storage organs.

Various parameters have been used to evaluate growth rate, including measurement of fresh or dry weight, root to shoot ratio, shoot number or shoot length (Li et al., 1998; Leister et al., 1999). The measurement of fresh or dry weight is destructive and hence large numbers of plants are required to analyze growth in time. Although the analysis of growth by measuring the area covered by a plant instead of measuring its weight has been applied successfully (Smith and Spomer, 1987; Smith et al., 1989; Motooka et al., 1991), its use is hampered by complicated experimental designs. A nondestructive approach would be preferable, e.g. using image analysis. For Arabidopsis, which in its vegetative phase grows as a flat rosette with limited leaf overlap, Leister et al. (1999) showed that the use of digital video and image analysis was very effective in the determination of plant growth (rate) nondestructively, even during early developmental stages.

Growth rate can be seen as the integration of a wide range of processes, and thus genetic variation for such a complex trait may depend on many genes. Since also within species heritable differences in growth and morphology can be found (Maloof, 2003) these traits are amenable for genetic analysis. Complex polygenic traits can be studied genetically by quantitative trait loci (QTL) analysis. QTL analysis allows the dissection of quantitative genetic variation to the contribution of different loci. When mechanistically related traits map to similar map positions, this might suggest that variation for these traits at this locus is controlled by the same gene and in genetic terminology is pleiotropic. The extensive natural variation that occurs in Arabidopsis is being exploited increasingly as a source of genetic variation for the analysis of important adaptive traits, e.g. flowering time, plant and seed size, seed dormancy, pathogen resistance and tolerance to abiotic stresses (for review, see Alonso-Blanco and Koornneef, 2000; Koornneef et al., 2004). Recombinant inbred lines (RILs) provide an immortal population, as each individual is practically homozygous, and large numbers of genetically identical individuals can be obtained, allowing repeated measurements of various traits in different conditions (Alonso-Blanco and Koornneef, 2000; Doerge, 2002)

We have used Arabidopsis natural variation to analyze growth rate by image analysis of plant leaf area, and by measuring a series of related parameters. From a greenhouse experiment involving approximately 130 Arabidopsis accessions, from a wide range of habitats, 22 accessions (Table 4.1) were selected based on obvious differences in growth characteristics, carbohydrate content, and/or because they were used in generating Arabidopsis mapping populations (www.natural-eu.org). These accessions were studied to get insight in differences in various growth-related traits, which, when present, can be genetically analyzed further in segregating populations such as recombinant inbred lines (RILs). To investigate the genetic basis of differences in growth and growth-related traits and to see if relationships between traits in the selection of accessions might be due to a common genetic basis, we analyzed growth-related traits by QTL mapping. For this we used a newly developed RIL population derived from the cross between the laboratory accession Landsberg *erecta* (L*er*), originating from northern Europe (Rédei, 1992), and the accession Shakdara (Sha), originating from high altitudes in Tadjikistan (Khurmatov, 1982). These parental accessions were not the extremes in the accession screen, but they showed a considerable variation for various growth traits as was also observed in their progeny.

Since RGR depends on the gain of biomass via photosynthesis and on the starting mass of the plant, i.e. ultimately the seed from which it grows, we determined the seed weight and chlorophyll fluorescence as a nondestructive parameter for photosynthetic capacity. Allocation of biomass within the plant is expected to change upon flower induction and hence flowering time and related parameters were also analyzed in this study.

Results

Variation among the accessions

Screening the 22 accessions revealed a large variation for seed weight, growth rate, and plant fresh and dry weight but less for water content (Table 4.1, Fig. 4.1). The seed weight of these accessions was not correlated with the latitude at which the accessions had originally been collected as suggested before by Li et al. (1998). Seed weight showed a positive correlation with plant area at 10 d after planting, suggesting that seed weight affected plant growth during early phases of development (Fig. 4.1A). During later stages of plant growth this correlation was not significant, indicating that other factors determined plant growth at that phase (Fig. 4.1B). Final plant dry weight correlated with RGR (based on area) especially during the last period (Fig. 4.1 C and D).

Table 4.1: Names, stock numbers, origin, fresh and dry weight, water content and seed weight for 22 Arabidopsis accessions.

Name	Stock No	Country	Longitude	Latitude	Fresh Wt (gm)	Dry Wt (gm)	%WC	Seed Wt (mg)
Amel-1	CS 22526	Netherlands	E 5.6	N 53.4	1.43	0.19	87.0	0.026
Nes-1	CS 10041	Netherlands	E 5.8	N 53.3	0.87	0.14	83.9	0.030
Nes-3	CS 10042	Netherlands	E 5.8	N 53.2	0.83	0.11	86.4	0.028
Oerd-2	CS 10299	Netherlands	E 5.9	N 53.2	1.47	0.20	86.4	0.031
Oerd-4	CS 10040	Netherlands	E 5.9	N 53.2	1.03	0.12	88.4	0.030
Cerv-1	CS 22523	Italy	E 12.5	N 41.9	1.70	0.23	86.3	0.020
Rome-1	CS 22524	Italy	E 12.5	N 41.9	1.10	0.16	85.5	0.017
L*er*	N 20	Poland	E 15.2	N 52.7	0.97	0.13	86.2	0.020
Col-2	CS 907	Poland	E 15.7	N 52.7	0.65	0.08	88.5	0.022
Cvi	N 8580	Cape Verde Islands	W 24.4	N 14.9	1.35	0.15	88.9	0.033
An-1	N 944	Belgium	E 4.4	N 51.2	0.37	0.06	84.5	0.021
Bla-10	JA 10185	Spain	E 2.8	N 41.7	0.93	0.12	86.8	0.022
Kond	CS 6175	Tadjikistan	E 68.5	N 38.5	1.50	0.20	86.9	0.019
Ely-1a	CS 6088	UK	W 0.3	N 52.4	0.53	0.07	87.5	0.020
Eri	CS 22548	Sweden	E 15	N 56.4	0.97	0.12	87.2	0.019
Hog	CS 6179	Tadjikistan	E 68.5	N 38.5	1.35	0.16	88.5	0.018
Kas-2	N 1264	India	E 71.8	N 34.3	0.17	0.03	84.0	0.028
Sid-1	CS 6077	UK	E 15.4	N 51.4	0.53	0.06	88.8	0.016
Sha	CS 929	Tadjikistan	E 71.3	N 37.3	0.90	0.10	88.9	0.019
Pak-3	JW 10214	Pakistan	E 73.4	N 33.9	0.15	0.01	93.3	0.032
Ik	JW 10223	Japan	E 135.1	N 35.5	1.30	0.19	85.4	0.030
Kyo-1	JW 10231	Japan	E 135.8	N 35.0	0.70	0.15	79.3	0.021

A positive correlation was observed between area at day 20 and the plant dry weight at day 35 ($R^2 = 0.56$, data not shown). We found a large variation between accessions for their growth rate as well as for their relative growth rate (on the basis of plant area) with Pak-3 having the

lowest and Cerveteri-1, Kyoto-1, Oerd-2, and Kond the highest rates (Fig. 4.2 A and B). Ten days after planting, Pak-3 showed the largest area but gradually its growth rate decreased due to early senescence, which was observed also for Kas-2 (Fig. 4.2A).

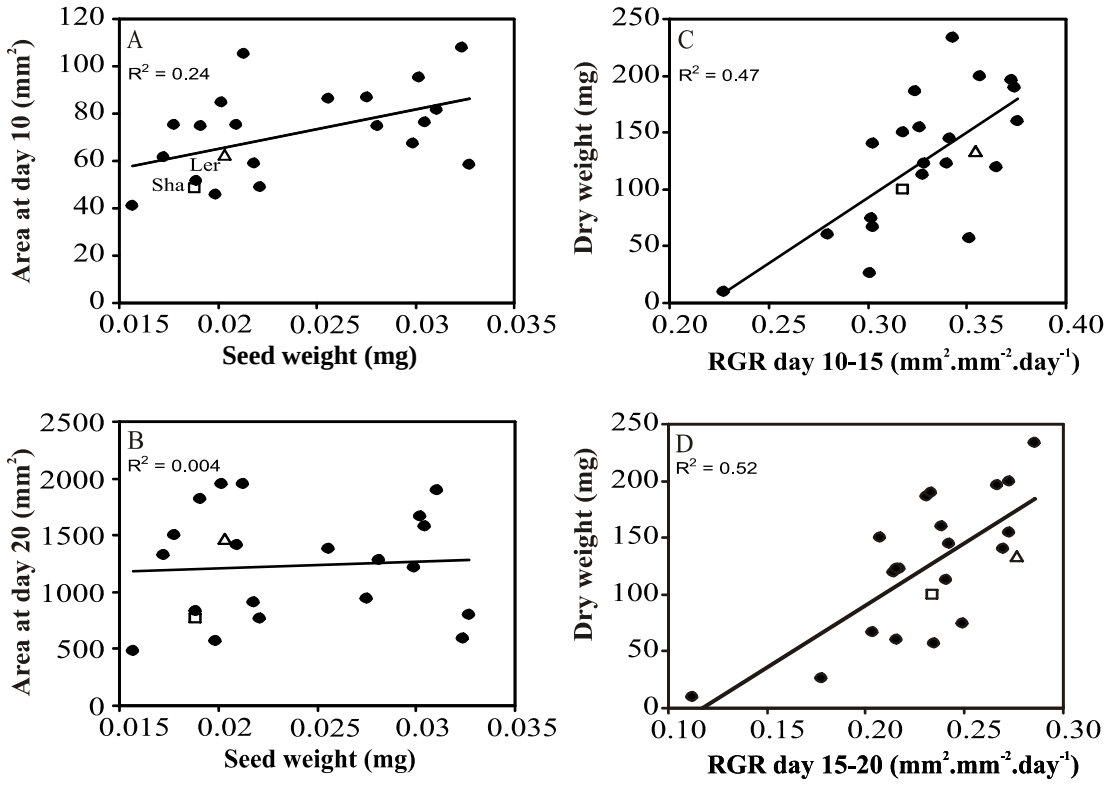

Figure 4.1: Correlation between seed weight and plant area at day 10 (A) and day 20 (B) for 22 Arabidopsis accessions. Correlation between dry weight at 35 days and relative growth rate (RGR) day 10-15 (C) and day 15-20 (D) on the basis of plant area. Δ and □ correspond to L*er* and Sha mean values, respectively.

A principle component analysis indicated that the first three principle components (PCs) explained 97% of the variation for the six traits: fresh weight (FW), dry weight (DW), seed weight (SW), total leaf area 1 (TLA1), total leaf area 2 (TLA2), and total leaf area 3 (TLA3). PC1 showed a large variation between accessions and is mainly determined by growth related parameters (TLA3, TLA2, and DW). On the second function (PC2), TLA1, SW and FW were the most important traits. On the third function (PC3), SW was the main variable discriminating between the accessions. Accessions with a large initial area (TLA1) and high seed weight (SW) were situated on the left side of the graph. The related accessions Oerd2, Oerd4, Nes1 and Nes3, all collected in the dunes of the island Ameland in the north of The Netherlands, as well as IK, had high SW and moderate TLA1, were grouped in the middle (Fig. 4.3). PC1 discriminated between the smallest accession in final plant size (Sid-1) and the largest one (Cerveteri-1), while PC3, which was determined mainly by seed weight, gave the largest seeded accession Cvi a separate position that contrasted most with the low seed weight accession An-1.

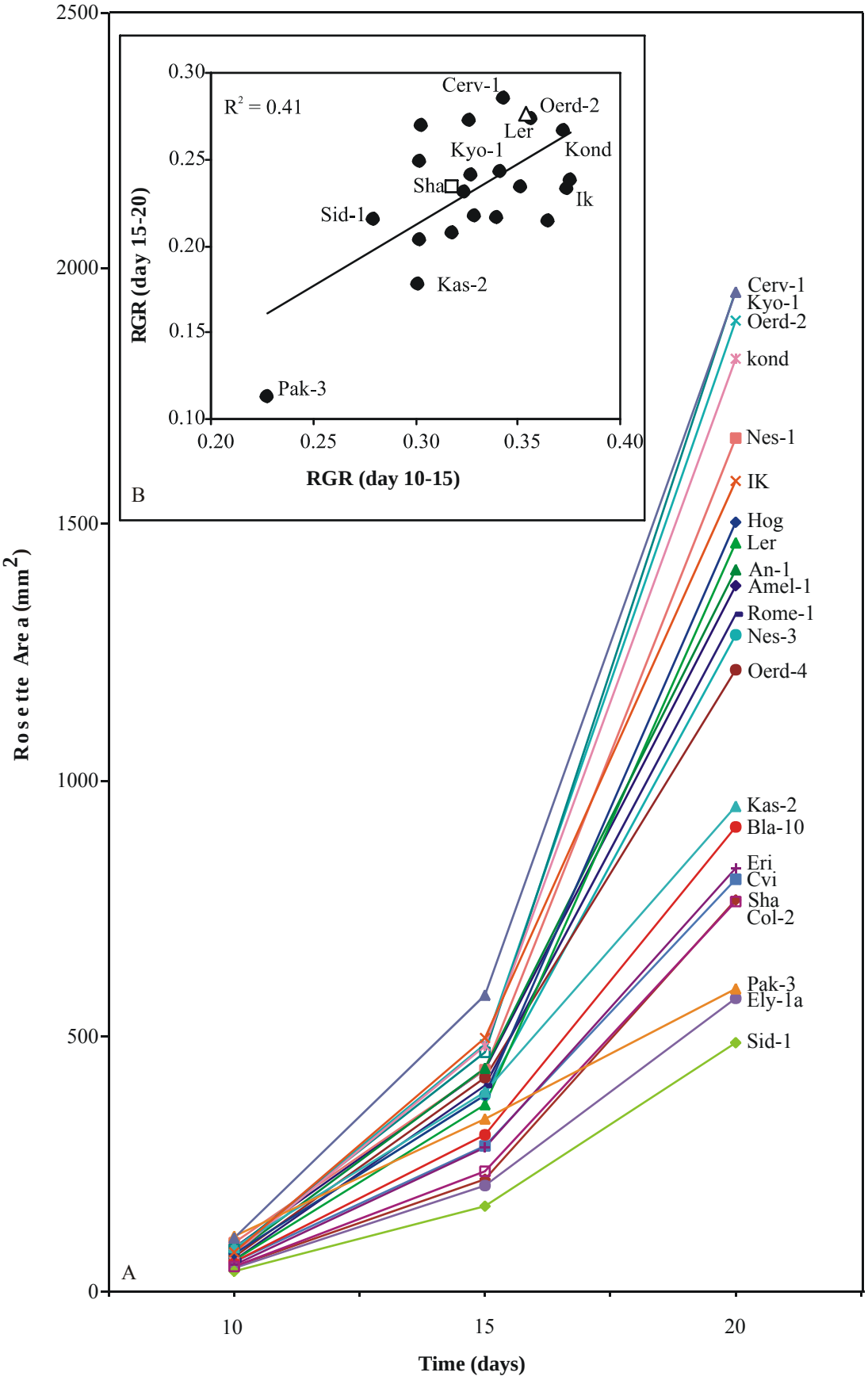

Figure 4.2: Growth rate curves for 22 Arabidopsis accessions determined by plant area (A). The correlation between relative growth rate (RGR) (day 10-15) and RGR (day 15-20) on the basis of plant area (B).

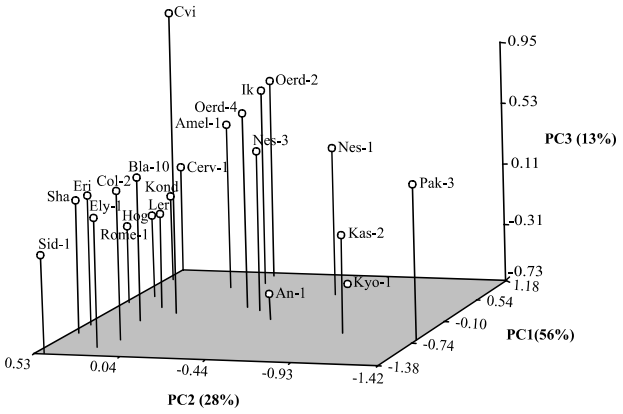

Figure 4.3: Principle component analysis of variables: fresh and dry weight, seed weight, total leaf area1, total leaf area2 and total leaf area3 for the 22 accessions. The first principle component (PC1) is determined by growth-related parameters; total leaf area3, total leaf area2 and dry weight. The second principle component (PC2) is determined mainly by total leaf area1, seed weight, and fresh weight. In the third principle component (PC3), seed weight was the most important variable discriminating between the accessions.

Genetic variation among the L*er* x Sha RILs

For all traits analyzed significant variation was observed between RILs as indicated by the broad sense heritabilities ranging from 0.86 to 0.33 for flowering time traits and number of side branches, respectively (Table 4.2 and Fig. 4.4). Transgression beyond the parental values was observed for all traits including those for which parental values hardly differed, such as chlorophyll fluorescence. This amount of genetic variation indicated that QTL mapping was likely to reveal QTLs for most of the traits.

QTL Mapping

Seed weight

Although the difference in seed weight between the L*er* and Sha parents was small (Fig. 4.4A and Table 4.2), QTL mapping revealed one QTL on chromosome 5 (Fig. 4.5). This QTL, for which the Sha allele increased seed weight, explained 14.3% of the variance.

Plant total leaf area and RGR

Figure 4.5 and Table 4.3 summarize the QTLs found in L*er* x Sha RILs for total leaf area (TLA) (four, five, and four QTLs for TLA1, TLA2, and TLA3, respectively). The detected QTLs showed a total explained phenotypic variance of 34%, 43%, and 37.5% for TLA1, TLA2, and TLA3, respectively (Table 4.3). For TLA1, the L*er* alleles increased plant area at three QTLs (at msat1-10, nga692, and msat5-14), whereas at the CHIB locus the Sha allele increased the area. The Sha alleles at *ER*, CHIB, and nga129 increased the TLA2, whereas the L*er* alleles did so at msat1-10 and SO262. For TLA3, the Sha alleles at *ER*, CHIB, and MBK5 loci, and the L*er* allele at msat1-10 increased the area.

Four, three, and two QTLs were found for RGR2-1, RGR3-2, and RGR3-1, respectively. The detected QTLs showed a total explained phenotypic variance of 34.3%, 18.3%, and 16.7% for RGR2-1, RGR3-2, and RGR3-1, respectively (Table 4.3). For RGR2-1, the Sha alleles at *ER*, CHIB, and MBK5 and the L*er* allele at msat1-10 increased plant growth rate. At the nga361, the Sha allele increased the RGR3-2 values, whereas the L*er* alleles did so at the msat1-10 and nga225 loci. For RGR3-1, at two QTLs (msat1-10 and nga225), the L*er* alleles increased growth rates.

At the top of chromosome 1 (msat1-10), co-location was found of the loci for TLA1, TLA2, TLA3 and for all three RGR parameters, as well as with flowering-related traits, i.e.

flowering time (FT), total leaf number (TLN), cauline leaf number (CL), and plant length until first slique (PLTS). Co-location of QTLs for these different traits could also be observed at the bottom of chromosome 2 at the *ER* locus, at the CHIB marker near the top of chromosome 3 and at the top and bottom of chromosome 5 (Fig. 4.5). Co-location of these QTLs, at the top of chromosome 3, with a QTL for speed of germination (Clerkx et al., 2004) was observed, the Sha allele increasing the speed of germination.

Table 4.2: Parental values, ranges and heritabilities in the L*er* x Sha RILs of all measured traits.

Trait	L*er* value	Sha value	Range	Mean	Heritability
Seed weight (mg)	0.018	0.017	0.014-0.023	0.018	nd
Total leaf area1 (mm^2)	130	153	48-365	161	0.65
Total leaf area2 (mm^2)	436	551	155-1319	555	0.71
Total leaf area3 (mm^2)	2191	2556	576-7418	2703	0.78
Relative growth rate 2-1(area)	0.3	0.32	0.22-0.36	0.31	nd
Relative growth rate 3-2(area)	0.27	0.26	0.08-0.32	0.26	nd
Relative growth rate 3-1(area)	0.28	0.28	0.13-0.33	0.28	nd
Dry weight young plant (mg)	2.5	3.5	1.4-8	3.5	0.68
Dry weight old plant (mg)	37	46	9-74	33	0.74
Relative growth rate (weight)	0.34	0.32	0.15-0.35	0.28	nd
Water content (%)	89	89.4	85.1-92.1	89.5	0.52
Specific leaf area ($mm^2.mg^{-1}$)	175	160	90-350	162	nd
Speed of leaf initiation	13	14	8-19	13.7	nd
Chlorophyll fluorescence	0.72	0.71	0.65-0.75	0.71	0.53
Flowering time SD (days)	33.2	32	21-48.7	35.3	0.86
Flowering time LD (days)	25.2	28.8	21.3-46.3	29.3	0.83
Total leaf number	7.9	9.5	6.6-31.1	10.6	0.86
Rosette leaf number	6	7.7	4.8-24.2	8.4	0.86
Cauline leaf number	1.9	1.8	1.3-6.9	2.3	0.59
Total plant length (cm)	17.5	37.4	11-50.4	26.7	0.85
Plant length till 1st silique (cm)	7.4	8.7	4-21.1	9.5	0.74
Inflorescence length (cm)	10.1	28.7	7-29.3	17.2	0.80
Number of side branches	1.9	2.4	0.4-3.9	1.83	0.33

nd, not determined because only one replica per line was analyzed.

The two detected QTLs for relative growth rate as based on dry weight (RGRdw) co-located with the QTLs for RGR calculated on the basis of plant area.

Plant dry weight and relative growth rate

Despite the small differences in plant dry weight (DW) between L*er* and Sha, large variation was found between the RILs for dry weight of young (DW1) as well as older plants (DW2; Fig. 4.4, D and E). Three QTLs were detected for each parameter, explaining together 25.8% and 24.1% of the total variance, respectively (Fig. 4.5, Table 4.3).

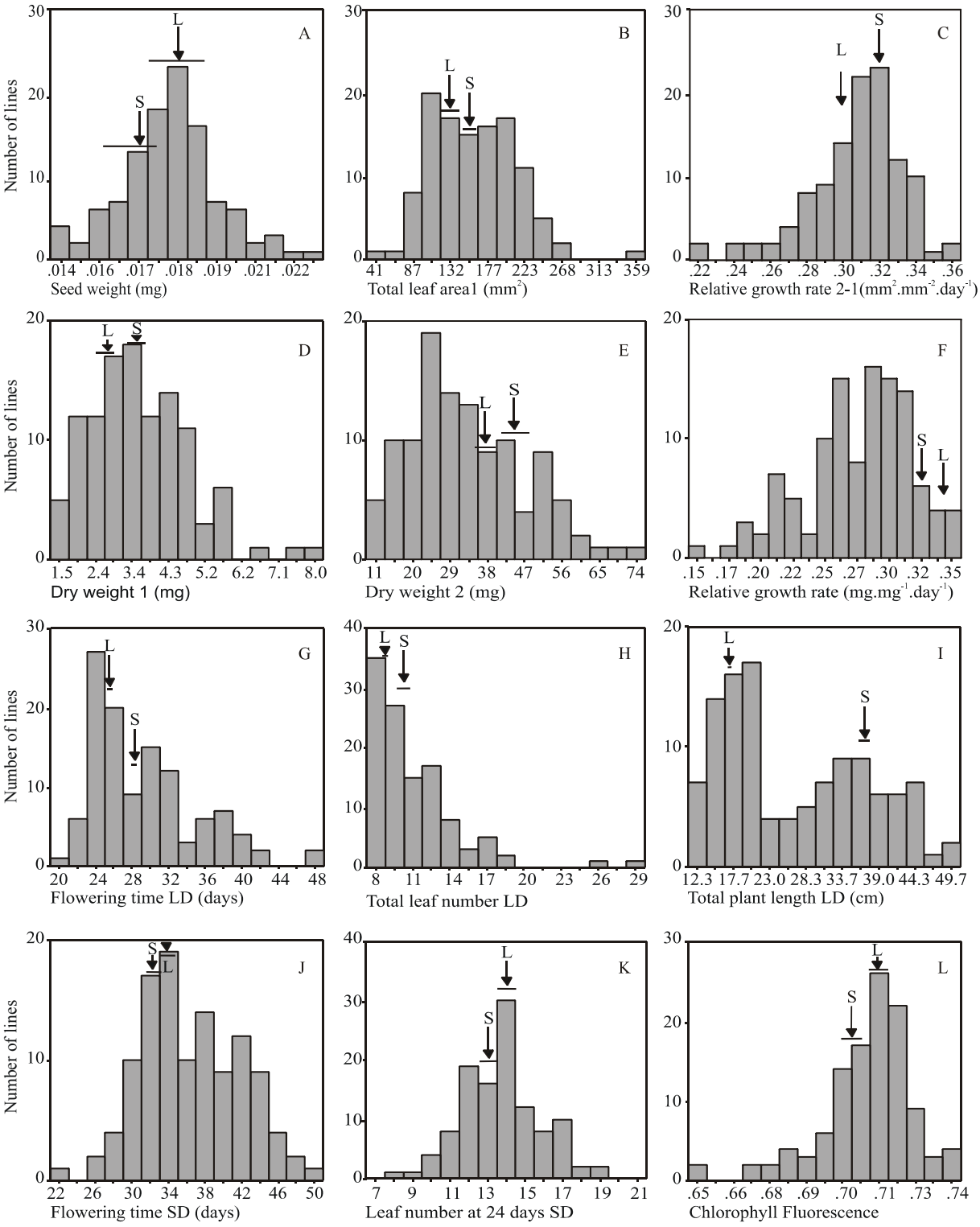

Figure 4.4: Frequency distribution of nonnormalized data of some traits in the L*er* x Sha RIL population. Growth traits: Seed weight (A), Plant area at 10 d (B), Relative growth rate calculated on the basis of plant area (C), Dry weight 1 at 15 d (D), Dry weight 2 at 25 d (E), Relative growth rate calculated on the basis of plant dry weight (F). Flowering traits: Flowering time scored in long day (LD) conditions (G), Total leaf number counted in LD (H), Total plant length measured in LD (I), Flowering time scored in short day (SD) conditions (J), Leaf number counted at 24 d in SD referring to speed of leaf initiation (K), Chlorophyll fluorescence measured in SD (L). The average parental value is indicated with an arrow for both parents, L for L*er* and S for Sha, and the horizontal bars represent the SE for these parental values.

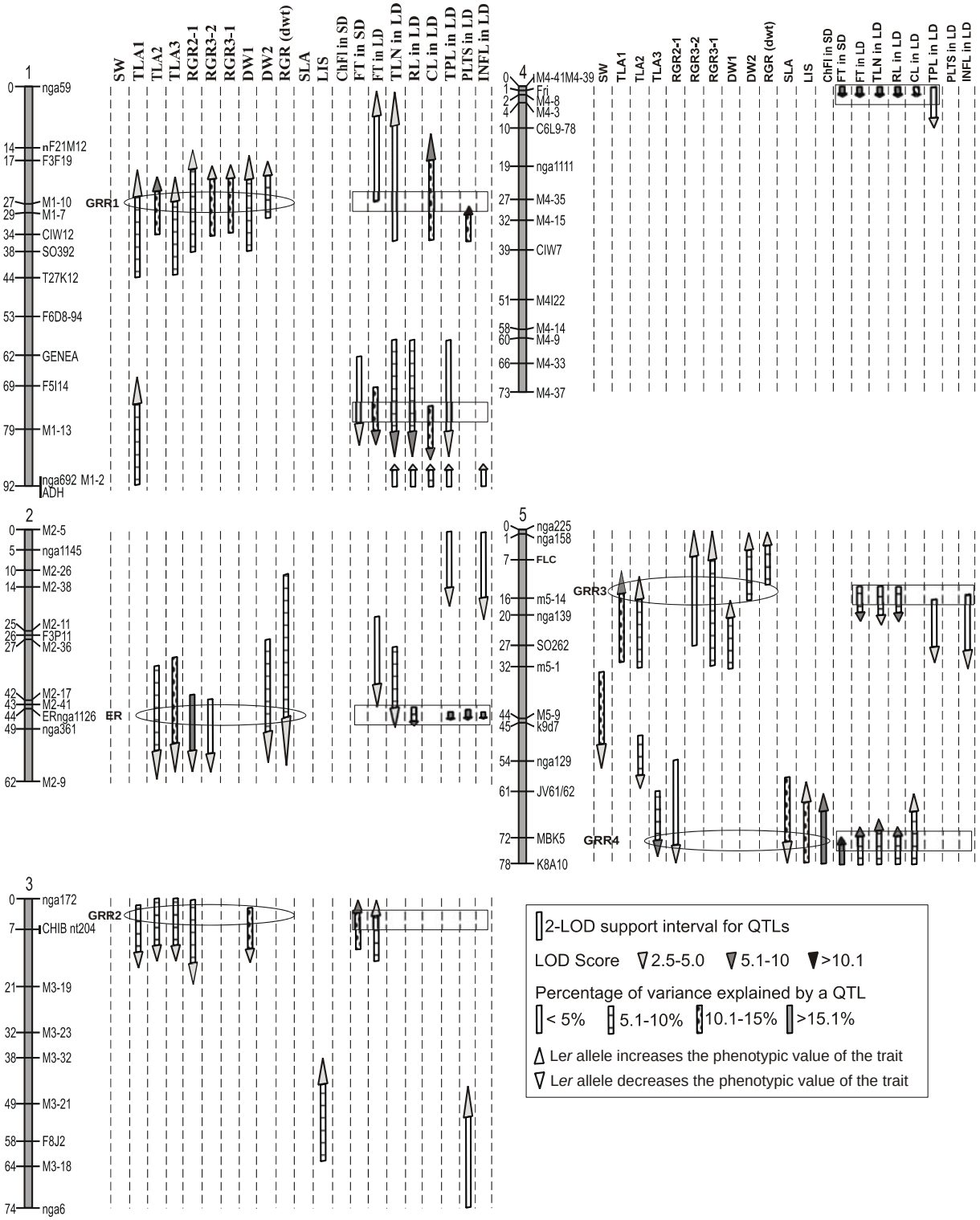

Figure 4.5: The L*er* x Sha linkage map showing the locations of QTLs for the traits analyzed. The lengths of the arrows indicate the 2-LOD support intervals. The direction of the arrow's head indicates the allelic effect: upward, L*er* increasing and Sha decreasing; downward, Sha increasing and L*er* decreasing. The gray scale of the arrow's head indicates the LOD score value. The filling pattern of the arrow's stem refers to the percentage of variance explained by each QTL. The ovals indicate QTLs of growth regions, *GRR1* till *GRR4* and the *ER* region, while the rectangles indicate the flowering time related QTLs.

With the exception of msat1-10, QTLs for DW1 and DW2 were at different positions. For CHIB and SO262 only DW1 QTLs were detected, whereas *ER* and nga225 revealed QTLs for DW2, indicating that growth in different phases of development may be controlled by different genes but also by the same genes. In agreement with the observed transgression QTLs in which either L*er* alleles increased growth (msat1-10, SO262 and nga225) and those for which the Sha alleles (CHIB and *ER*) lead to higher DW were found. Based on DW1 and DW2, the RGR for DW could be calculated, which revealed QTLs at *ER* and nga225, which are the DW2 specific QTLs. In contrast the DW2 QTL at msat1-10 is due to the growth in the first phase, which is continued.

For RGRdw a significant interaction between msat1-10 and CHIB, which represented the DW1 QTL, was found explaining 5.5% of the phenotypic variance.

As might be expected plant total area and plant dry weight, were strongly correlated ($R^2 = 0.61$) at day 15.

Specific leaf area, leaf initiation speed, and chlorophyll fluorescence

For specific leaf area (SLA) only one QTL, located near MBK5 and explaining 13.1% of the phenotypic variance, was detected (Fig. 4.5). This QTL, for which the Sha allele has a higher value, indicating thinner leaves, co-located with QTLs for leaf initiation speed (LIS), TLA, RGR and FT traits.

Two QTLs controlling the speed of leaf initiation were found at the F8J2 and MBK5 (Fig. 4.5) explaining 7.8% and 12.8% of the phenotypic variance, respectively. For the detected QTLs, the L*er* alleles increased the rate of leaf development compared with the Sha alleles. Although the QTL for LIS at MBK5 co-located with one of the flowering time QTLs, this was not the case for the QTL at F8J2. No significant correlation ($R^2= 0.15$) was observed between leaf number at day 24 and FT in short day (SD) indicating that a higher leaf initiation speed does not account for the major variation in flowering time.

One QTL for chlorophyll fluorescence (ChFl), also located near the MBK5 and explaining 21.4% of the phenotypic variance, was detected, which together with the interaction between MBK5 and msat4-14 (a minor QTL) explained 27.6% of the total variance (Fig. 4.5). For the detected QTL, the L*er* allele increased the photosynthetic capacity of the plant compared with the Sha allele.

Flowering time and flowering-related traits including plant length and branching

In *Arabidopsis*, flowering time is often correlated with the number of leaves formed prior to flowering, and one might expect that the mass of the vegetative parts may influence the elongation of the inflorescence and, therefore, may affect total plant height. Furthermore, the number of leaves determines the number of axillary buds that might develop into secondary inflorescence, thus affecting branching. Because of the expected correlation the data obtained for these parameters are discussed together. The expected relationships were indeed observed in the present material as indicated by the strong correlation between flowering time and total leaf number (TLN, $R^2 = 0.86$), rosette leaf number (RL, $R^2 = 0.86$), and cauline leaf number (CL, $R^2 = 0.61$).

Flowering time differences between L*er* and Sha were relatively small and the relative order of both genotypes depended on the day-length condition, Sha being slighter earlier in short day (SD), but later in long day (LD) condition (Table 4.2). However, variation between RILs was considerable and had the same magnitude in LD and SD conditions, with a highly significant correlation ($R^2 = 0.71$) (Fig. 4.4 G and J). Figure 4.5 and Table 4.3 summarize the QTLs found in L*er* x Sha RILs for flowering time in LD and SD conditions (seven and four QTLs, respectively). Detected QTLs showed a total explained phenotypic variance of 66.6% and 52.4%, respectively. In SD, at two of the four detected QTLs, viz, at msat1-13 and *FRI*, Sha alleles delayed flowering, whereas Sha alleles at CHIB and K8A10 accelerated flowering. In LD these four QTLs were also detected and showed the same allelic effects. In addition two QTLs were detected in LD, at msat2-36 and msat5-14, where the Sha alleles delayed flowering and one QTL, at nga 59, where the Sha allele promoted flowering.

Seven QTLs were found for TLN, six of them co-locating with FT QTLs in LD condition, with similar contributions and allelic effect (Fig. 4.5, Table 4.3). At CHIB a minor QTL (at the border of significance) could be detected.

Rosette leaf number and cauline leaf number, being the two components of TLN, showed six and five QTLs, explaining 63.8% and 48.1% of variance, respectively. One QTL, specific for cauline leaf number, co-locating with FT (LD) but not with RL, was found on chromosome 1, near marker F3F19. For RL, two QTLs at chromosome 2 and top of chromosome 5 co-located with FT but not with CL QTLs, indicating that although flowering time is intimately linked with number of leaves initiated before the transition to flowering, the number of elongating internodes is under separate genetic control. The remaining QTLs for RL and CL co-located with each other and with QTLs for TLN and FT (Fig. 4.5).

For total plant length (TPL) and its two length components (length until the first silique and inflorescence length), the total explained variance was relatively high (81.7%, 60.6%, and 79.9%, respectively), which was largely due to the effect of the *ER* locus, explaining 70.1%, 42.6%, and 72.4% of the observed variation. The remaining five QTLs for TPL contribute little and for all loci, except *ADH*, the Sha alleles increased plant length (Table 4.3).

For PLTS and inflorescence length (INFL) fewer QTLs were detected per trait (Table 4.3). One locus at marker CIW12 might be specific for PLTS since it did not co-locate with any other QTLs for TPL or INFL. For INFL, two loci at the bottom of chromosome 1 and near the middle of chromosome 5, co-located with TPL but not with PLTS (Fig. 4.5), suggesting that they might only be responsible for the increase in the internode length between the flowers.

No QTLs could be detected for the number of side branches derived either from the axillary buds of the rosette leaves or the cauline leaves, which may be due to the fact that many genes with small effects segregate in this population or due to the low heritability (0.33).

Table 4.3: Characteristics of the detected QTLs explaining growth traits, flowering time and flowering related traits in L*er* x Sha RIL population.

Trait	QTL	Map position	LOD score	% of variance	Additive allele effect
Seed weight (mg)					
	K9D7	5-45	3.6	14.3	-0.12
Total leaf area1 (mm^2)				**34**	
	M1-10	1-26.7	3.08	6	13
	nga692	1-91.9	3.18	6.1	14
	CHIB	3-6.8	4.55	9	-17
	m5-14	5-16.3	6.13	12.9	19
Total leaf area2 (mm^2)				**43**	
	M1-10	1-26.7	5.53	11.1	68
	ER	2-43.7	3.66	7.1	-55
	CHIB	3-6.8	4.7	9.3	-64
	SO262	5-27.2	4.16	8.1	62
	nga129	5-53.9	3.83	7.4	-65
Total leaf area3 (mm^2)				**37.5**	
	M1-10	1-26.7	3.57	8.1	346
	ER	2-43.7	4.37	10.1	-379
	CHIB	3-6.8	3	6.7	-331
	MBK5	5-72.4	5.36	12.6	-441
Relative growth rate 2-1 (area)				**34.3**	
	M1-10	1-26.7	2.89	7.4	0.07
	ER	2-43.7	3.51	17.4	-0.09
	CHIB	3-6.8	2.99	5.2	-0.06
	MBK5	5-72.4	2.75	4.3	-0.04
Relative growth rate 3-2 (area)				**18.3**	
	M1-10	1-26.7	3.25	10.2	0.11
	nga361	2-48.6	2.58	3.5	-0.09
	nga225	5-0	2.88	4.6	0.10
Relative growth rate 3-1 (area)				**16.7**	
	M1-10	1-26.7	3.45	10.4	0.12
	nga225	5-0	2.69	6.3	0.09
Dry weight 1 (mg)				**25.8**	
	M1-10	1-26.7	3.24	8.3	0.39
	CHIB	3-6.8	4.23	11.1	-0.44
	SO262	5-27.2	2.52	6.4	0.30
Dry weight 2 (mg)				**24.1**	
	M1-10	1-26.7	2.65	6.8	3.73
	ER	2-43.7	3.8	10	-4.58
	nga225	5-0	2.83	7.3	3.68
Relative growth rate (weight)				**20.4**	
	ER	2-43.7	2.75	6.7	-0.11
	nga225	5-0	3.33	8.2	0.12
	M1-10 x CHIB			5.5	
Specific leaf area (mm2.mg-1)					
	MBK5	5-72.4	3.41	13.1	-16

Values in bold refer to the total explained variance by the trait.

Table 4.3: (continued from previous page.)

Trait	QTL	Map position	LOD score	% of variance	Additive allele effect
Chlorophyll fluorescence (SD)				**27.6**	
	MBK5	5-72.4	6.28	21.4	0.008
	m4-14 x MBK5			6.2	
Flowering time SD (days)				**52.4**	
	M1-13	1-78.7	3.77	4.8	-1.19
	CHIB	3-6.8	7.44	11	1.78
	FRI	4-0.8	11.48	18.7	-2.33
	K8A10	5-77.7	11.08	17.9	2.31
Flowering time LD (days)				**66.6**	
	nga59	1-0	3.38	3.6	1.05
	M1-13	1-78.7	8.92	11	-2.13
	M2-36	2-26.6	3.69	4	-1.05
	CHIB	3-6.8	4.83	5.5	1.15
	FRI	4-0.8	17.14	25.5	-2.52
	m5-14	5-16.3	5.84	7	-1.32
	K8A10	5-77.7	8.06	10	1.63
Total leaf number				**63.4**	
	nga59	1-0	2.51	3	0.71
	M1-13	1-78.7	7.24	9.7	-1.54
	ADH	1-92.3	3.63	4.7	1.07
	ER	2-43.7	4.84	6.1	-1.00
	FRI	4-0.8	15.47	25.2	-1.81
	m5-14	5-16.3	4.88	6.4	-0.98
	K8A10	5-77.7	6.13	8.3	1.07
Rosette leaf number				**63.8**	
	M1-13	1-78.7	6.69	8	-1.24
	ADH	1-92.3	3.79	4.5	0.91
	ER	2-43.7	7.45	9	-1.01
	FRI	4-0.8	17	25.9	-1.58
	m5-14	5-16.3	6.13	7.5	-0.88
	K8A10	5-77.7	7.07	8.9	0.93
Cauline leaf number				**48.1**	
	ADH	1-92.3	2.73	5.2	0.20
	FRI	4-0.8	5.86	12	-0.24
	K8A10	5-77.7	2.97	5.7	0.17
Total plant length (cm)				**81.7**	
	F5I14	1-69.1	2.7	1.5	-1.51
	ADH	1-92.3	4.99	2.9	1.70
	M2-26	2-10	3.34	1.8	-1.49
	ER	2-43.7	46.26	70.1	-9.24
	FRI	4-0.8	4.47	2.5	-1.68
	SO262	5-27.2	4.98	2.9	-1.74
Plant length till 1st silique (cm)				**60.6**	
	CIW12	1-34.5	10.93	14.5	1.20
	ER	2-43.7	23.95	42.6	-2.10
	F8J2	3-58	3.14	3.5	0.65
Inflorescence length (cm)				**79.9**	
	nga692	1-91.9	4.14	2.7	1.52
	nga1145	2-5.3	2.61	1.7	-1.09
	ER	2-43.7	43.6	72.4	-7.19
	SO262	5-27.2	4.59	3.1	-1.49

Discussion

Variation specifically for growth of leaves among Arabidopsis accessions as such has not been studied, in contrast to hypocotyl growth (Maloof et al., 2001; Borevitz et al., 2002; Botto and Smith, 2002) and flowering time (for review, see Koornneef et al., 2004). A study by Li et al. (1998) compared growth for a number of accessions and tried to relate growth variation with other parameters such as seed weight and geographical distribution. In addition, growth was studied in genetic analyses of nitrogen-use efficiency (Rauh et al., 2002; Loudet et al., 2003). Ungerer et al. (2002) analyzed leaf size together with developmental traits dealing with flowering and plant architecture, while Pérez-Pérez et al. (2002) used Arabidopsis natural variation for genetic analysis of leaf morphology and leaf area.

Growth-related traits

In this article we provide a genetic analysis of traits related to plant growth. A comparison of the more extreme phenotypes among a collection of Arabidopsis accessions showed that large differences for growth rate exist, which may be different between accessions during consecutive phases of development. Differences in biomass may result from differences in seed mass, emergence time, or variation in RGR (Van Andel and Biere, 1990; Poorter and Navas, 2003). Differences in RGR can be explained by differences in leaf area per unit plant mass (LAR; leaf area ratio) or by differences in the rate of increase in plant mass per unit leaf area (ULR; unit leaf rate; Evans, 1972). In this study we found that RGR and final dry weight were not correlated with seed weight as was described by Li et al. (1998). However, early growth, determined as rosette area, was significantly correlated with seed weight. This correlation weakened during subsequent growth, implying that other factors started to dominate growth rate. A 2-fold difference was observed for seed weight; the low latitude accessions from the Cape Verde Islands and Pakistan but also accessions from the Dutch island of Ameland had heavy seeds, disproving the negative correlation between seed size and latitude suggested by Li et al. (1998). In the latter study this correlation was based almost exclusively on the Cvi accession.

The extensive heritable variation present in natural populations is shown in the analysis of a new RIL population derived from the cross L*er* x Sha, in which we studied a number of traits directly related to biomass production as well as to flowering. For most traits we detected heritable variation and QTLs could be mapped. The highest percentages of explained variation were obtained for flowering time and related traits, which have a high heritability. Less variation could be attributed to specific loci for growth-related traits and even less for parameters that were derived from two measured parameters, for which the variation of both measurements is added up. The usefulness of nondestructive growth measurements is clearly shown by the higher explained variance of leaf area than for dry weight, which is most likely due to the fact that more plants could be measured per genotype.

QTLs that were found for leaf area, dry weight, and RGR co-located in many cases, which is expected since they all measure different, but related, aspects of overall plant growth. However, in several cases no co-locations were found for these growth-related traits.

This indicates that some loci may have an overall effect on plant growth, whereas others specifically regulate certain processes that contribute more to some but less to other of the measured parameters, or act during a specific phase of growth. For example the QTLs on top of chromosome 3 were found mainly for the earlier phases, indicating that this QTL has a development-specific effect.

Co-location of QTLs for traits that are less obviously related might suggest pleiotropy. In case developmental changes such as flowering would be influenced by growth or vice versa, this would be reflected by co-location. Similarly, one could predict that larger late flowering plants would have longer stems. When traits have a causal relationship the allelic effects should also be in the same direction and a high overall correlation of these traits in the RIL population should be observed. Since only two out of the four FT QTLs found in SD, where growth analysis was performed, co-locate with growth QTLs but have opposite allelic effects for the two traits and because the overall correlation between DW2 and FT was not significant (R^2 = 0.03), we conclude that both traits are genetically different. Although a flowering time QTL is found in the *ER* region, we do not consider this a pleiotropic effect because the line with the *ER* wild-type allele in L*er* background does not show this effect (data not shown).

For plant length the strongest effect is due to the *ER* locus, where the Sha allele promotes both growth and length. However, at the top part of chromosome 5 the QTLs for growth and total plant length co-locate but the alleles act in opposite direction, which indicates that at this locus rosette growth might have a trade-off with total plant length. A weak, but significant, overall correlation was found between FT and length when the lines with mutant and wild-type *ER* alleles were treated separately. The highest correlation was between FT and length until the first silique, which was R^2 = 0.49 for *ER* plants and 0.19 for *er* plants. The relationship between both traits is also suggested by co-locations at three positions with allelic effects in the same direction (Fig. 4.5).

For plant growth-related traits we found five regions with QTLs (Fig. 4.5). The effects of the loci were never more than 2-fold. The characteristic of the QTLs around msat1-10 near the top of chromosome 1, which is called *GRR1* (Growth Rate 1), is that it affects all parameters and therefore, growth as such during the vegetative phase of development. This locus might be the same as DM10.1 described by Loudet et al., (2003) in the Bay x Sha population, in which the Sha allele also has a negative effect. An interesting co-location found at this position, but not elsewhere, is between the number of cauline leaves and the length until the first silique. When the two traits are combined, this implies that the L*er* allele promotes formation of cauline leaves and stimulates elongation of internodes between leaves.

The second growth-related QTL region (*ER*) is around the *ERECTA* locus and very likely the *ERECTA* gene itself, since the analysis of a near-isogenic line, having the wild-type *ER* allele in a L*er* genetic background, showed similar differences with L*er* for the same traits (data not shown). Interestingly, the growth effects of this locus were not detected at the earlier phases of development. As shown before for both Col x L*er* and Cvi x L*er* RIL populations (Alonso-Blanco et al., 1999; Pérez-Pérez et al., 2002; Ungerer et al., 2002), this locus always makes a major contribution to plant length and leaf size.

Torii et al. (1996), Yokoyama et al. (1998) and Shpak et al. (2003) provided arguments that the *ER* gene plays a role in coordination of cell growth patterns within the organ primordia initiated from the shoot apical meristem. The gene is predominantly expressed in the shoot apical meristems and in organ primordia. The expression is weak during early plant development but increases with the transition from the vegetative to the reproductive growth phase, in agreement with the absence of effects during the early phase of growth. Douglas et al. (2002) also showed that the *ER* gene influences multiple processes during Arabidopsis development, including internode and pedicle elongation, leaf and silique morphogenesis, and thickness of stem tissue.

A third locus for growth on top of chromosome 3, named *GRR2*, mainly affected early growth. When comparing the accessions it was noted that early plant growth correlated positively with seed weight (Fig. 4.1A). However, in the L*er* x Sha RIL population, the *GRR2* locus affected early growth, but not seed weight. The finding of a QTL for speed of germination at that position (Clerkx et al., 2004) may suggest that the cause of this early growth to be related to seed vigor, giving plants a faster start. Loudet et al. (2003) found a QTL for dry mass, which they named DM3.2 at the same position, for which the Sha allele also increases growth. This effect is rather small and was only observed when plants were grown at low nitrogen (3 mM) conditions (Loudet et al., 2003).

The locus near nga139 on top of chromosome 5 (*GRR3*) has not been described in other populations. It might actually consist of two loci that did not show up as significant in all analyses. A locus on top of this chromosome was described as DM10.7 by Loudet et al. (2003).

Probably the most interesting new QTL region is at the bottom of chromosome 5 (*GRR4*), where possibly two QTLs are located. Besides QTLs for growth rate and FT, also loci affecting LIS, SLA, and ChFl were found in this region, the latter two not being found in the other regions. Interestingly a higher rate of leaf initiation due to the L*er* allele coincided with smaller leaves and lower growth rate, suggesting that the leaves that are formed are smaller and also thinner as indicated by the reduction of SLA by the L*er* allele. The effect on chlorophyll fluorescence suggests that the physiology of these leaves is also different.

Flowering time

In this study we have analyzed the flowering behavior of two early Arabidopsis accessions. They differed slightly in their flowering phenotype (measured as both FT and TLN) and in their response to photoperiod length. However, variation between segregating RILs derived from crosses between these two accessions showed a large variation as shown also in other crosses, viz, between L*er* and Cvi (Alonso-Blanco et al., 1998a) and between Bay-0 and Sha (Loudet et al., 2002), and larger than that between L*er* and Col (Jansen et al., 1995). The significant correlation between flowering time in SD and in LD conditions (R^2 = 0.71) indicates that flowering time in both conditions is predominantly controlled by the same genetic factors in the L*er* x Sha RILs. The flowering behavior differences between the L*er* x Sha lines in both LD and SD conditions can be mainly attributed to QTLs located at msat1-13, CHIB, *FRI*, and K8A10. L*er* alleles at CHIB and K8A10 result in lateness, while at

msat1-13 and *FRI* L*er* alleles are earlier, thus explaining the similar behavior of the parental lines and the transgression in the RILs. Three other loci that were found in LD only are located at nga59, msat2-36, and msat5-14, where L*er* alleles at the first locus give lateness while for the other two loci the L*er* alleles lead to earliness. Previously it was shown that the *FRI* and the *FLC* loci determine flowering time differences between very late, vernalization-responsive accessions and early ones (Johanson et al., 2000; Gazanni et al., 2003; Michaels et al., 2003). Sequence analysis has shown that Sha contains a wild-type *FRI* gene (Gazzanni et al., 2003; Michaels et al., 2003) in contrast to L*er* (Johanson et al., 2000) making it most likely that the *FRI* locus is the gene for this QTL. This is further supported by the flowering time QTL, detected by Loudet et al. (2002) in the Sha x Bay-0 population. We could not find any QTL at the *FLC* locus, probably because both the Sha and L*er* parents carry weak alleles at this locus (Koornneef et al., 1994; Loudet et al., 2002; Gazzani et al., 2003; Michaels et al., 2003). The Sha accession was slightly less sensitive to changes in photoperiod length compared to L*er*. Three of the detected QTLs might be specific to day length and, therefore, affect day length sensitivity viz, at *ADH* (not significant for FT but detected for TLN) and at msat2-36, in LD condition only, and at CIW7 (minor QTL for FT) in SD condition. The first two QTLs might be the same found to be specific for photoperiod in the Bay-0 x Sha population in SD and LD, respectively (Loudet et al., 2002). Some of the flowering-related QTLs that we found in the L*er* x Sha population colocalized with previously published QTLs detected in other populations (Kowalski et al., 1994; Clarke et al., 1995; Jansen et al., 1995; Kuittinen et al., 1997; Alonso-Blanco et al., 1998a; Loudet et al., 2002).

Concluding remarks

Screening a number of Arabidopsis accessions revealed different patterns for growth. In this study we could identify a number of QTLs affecting plant growth. These loci appear to have different physiological functions, as concluded from co-locations of QTLs for different traits. Especially the *GRR4* locus near marker MBK5 looks very interesting because it affects a plethora of physiological effects including speed of leaf initiation, specific leaf area, and chlorophyll fluorescence. However, it should be emphasized that due to the inaccuracy of QTL mapping in a population of this size, it cannot be excluded that independent but linked genes control these apparent pleiotropic effects. This should further be investigated by fine mapping, which is most effectively done when no other QTLs segregate, i.e. using near–isogenic lines (NILs, see Alonso-Blanco and Koornneef, 2000). In addition the loci *GRR1* and *3*, which might be related to nitrogen use efficiency (Loudet et al., 2003) deserve further study. The *GRR3* QTL has the intriguing property that it affects early seedling growth. Because of the complexity of comprehensive traits like growth we cannot speculate on candidates for the QTLs, except for the *ERECTA* locus for which isogenic lines prove the involvement of the *ERECTA* gene. For flowering time several previously detected QTLs were found as well as a few new ones. Natural allelic variation for *FRI* and *FLC* is already studied at the molecular level (Johanson et al., 2000; Le Corre et al., 2002; Gazanni et al., 2003; Michaels et al., 2003). This may indicate the direction for future research, aiming to understand causes and consequences of natural genetic variation.

Materials and Methods

Plant material and growth conditions

The seeds from different accessions were sown in petri dishes on water-saturated filter paper, followed by a 4-d cold treatment at 4°C, and then transferred to a climate room at 25°C and 16 h light for 2 d before planting in 7-cm pots with standard soil. In all descriptions of experiments, time is referred to as days after planting. Details of the selected 22 accessions are given in Table 4.1. These accessions (24 plants/accession) were grown under controlled conditions in a growth cabinet, with 70% relative humidity, 22°C, 12 h day length and light intensity 25 Wm^{-2}, for a detailed growth analysis. Plants were placed on carts, and the carts were shuffled daily to avoid minor environmental differences within the growth cabinet.

F9 plants of a new set of 114 RILs, obtained by single-seed descent of F2 plants derived from the cross L*er* x Sha, were analyzed for flowering time and growth-related traits in two different experiments. The first one was carried out in an air-conditioned green house supplemented with additional light (model SON-T plus 400W, Philips, Eindhoven, The Netherlands) providing a day length of at least 16 h light which is a long day (LD), and maintained at a temperature between 22°C and 25°C (day) and 18°C (night). The second one was carried out in a growth cabinet under 12 h light, which is a mild short day (SD) treatment for Arabidopsis. In the greenhouse experiment 12 plants/RIL were grown in the same conditions as mentioned before (LD), in a randomized two-block design to reduce environmental effects, while 10 plants/RIL were grown in the growth cabinet, in the same conditions as mentioned above, also in a randomized two-block design. A line with the *ERECTA* wild-type allele in the L*er* genetic background, the two reciprocal hybrids, and both parents were included in all experiments.

Digital imaging, computer analysis and RGR determination

The mean total leaf area (TLA) of each accession was obtained by imaging 20 to 24 plants per accession at 10 (TLA1), 15 (TLA2), and 20 (TLA3) d after transferring the seedlings to the pots. Leaf areas were determined with an image processing technique, using a Nikon digital camera (model COOLPIX 950; Nikon Corporation Imaging Products Division, Shinagawa-Ku, Tokyo), and analysis of the pictures using the computer program MetaMorph (version 4.01; Universal Imaging Corporation, West Chester, PA, www.image1.com). The mean TLA for each line of the 114 RILs was obtained by imaging five plants/line at day 10 (TLA1) and four plant/line at 15 (TLA2) and 20 (TLA3) d. The relative growth rate (RGR) was calculated according to the following equation: $(\ln A_x - \ln A_y)/dt_{(x-y)}$. RGR was calculated for each line based on the three measurements of rosette area, resulting in RGR2-1, RGR3-2 and RGR 3-1, referring to RGRs in the intervals 10 to15, 15 to 20, and 10 to 20 d, respectively.

Weight, water content, and SLA determinations

The mean seed weight (SW) for each accession was obtained by weighing two seed lots each of 100 seeds using a Perkin-Elmer microbalance (model AD-4 Autobalance, Boston). SW for each line of the 114 RILs was determined for one batch per line.

The mean fresh weight (FW) of the plants was determined at day 35 by harvesting and weighing the aboveground parts of two plants/accession. The mean FW for each RIL was determined at day 15 and 25, by harvesting and weighing two plants/line, one from each block. Dry weights (DW) were determined after drying the plants at 105^{o}C for 48 h, and the water content (WC) was estimated as the relative ratio between water and dry weight using the formula [(FW-DW)/FW] X 100. The relative growth rate as based on dry weight (RGRdw) was calculated in the same way as RGR based on leaf area.

The specific leaf area (SLA) was calculated as area divided by weight (mm^2mg^{-1}). The relation between the 22 accessions based on seed weight, fresh and dry weights, and areas at 10, 15, and 20 d was described with principle component analysis using NTSYSpc version 2.10t. (Rohlf, 2001) with standardized data, which were converted in a correlation matrix from which three eigenvectors were extracted using the EIGEN function of the NTSYS-pc program.

Chlorophyll fluorescence measurements

Chlorophyll fluorescence as a nondestructive means of photosynthetic capacity was measured using a MINI-PAM (S/N: 0133; WALZ Mess- und Regeltechnik, Effeltrich, Germany), with the determination of the effective quantum yield of photosynthetic energy conversion (Yield = Δ F/Fm').

Measurement of flowering time and related traits in RILs

From the greenhouse experiment, in which 12 plants/RIL were grown in LD condition, FT for each plant was recorded as the number of days from planting until the opening of the first flower. Flowering time was also scored by counting the TLN, i.e. RL plus CL, excluding the cotyledons, since there is a close correlation between leaf number and flowering time (Koornneef et al., 1991). The following traits were also recorded: TPL, PLTS, and total number of side shoots or inflorescences (TSSN) (number of branches in the main inflorescence plus the number of side shoots from the rosette). In the phytotron experiment (SD), flowering time was scored as described above, for six plants/line. In addition, the number of rosette leaves was counted at day 24 from planting (i.e. before flowering) which refers to the leaf initiation speed (LIS).

Genetic mapping

The mapping of the segregating population was done by using 66 molecular markers, including the morphological marker *erecta*, located at a distance from 1 to 15 cM on the genetic map to obtain a regular distribution among the five chromosomes. These markers were used to generate the linkage map; details are published elsewhere (Clerkx et al., 2004). This map was used for QTL analysis of the various traits.

Statistical analysis and QTL mapping

For each RIL, the mean value of the traits under investigations was (log_{10}) transformed to improve normality of the distribution, except for the relative growth rates, rosette areas, and the specific leaf area. Transformed data were used for QTL analysis. The software package MapQTL version 4.0 (van Ooijen, 2000) was used to identify and locate QTL on the linkage map by using interval mapping and multiple-QTL model (MQM) mapping methods as described in its reference manual (http://www.plant.wageningen-ur.nl/products/). In a first step, putative QTLs were identified using interval mapping. Thereafter, the closest marker at each putative QTL was selected as a cofactor and the selected markers were used as genetic background controls in the approximate multiple QTL model of MapQTL. Log of the odds (LOD) threshold values applied to declare the presence of a QTLs were estimated by performing the permutation tests implemented in MapQTL version 4.0 using at least 1000 permutations of the original data set for each trait, resulting in a 95% LOD threshold between 2.4 and 2.6.

Two-LOD support intervals were established as 95% QTL confidence interval (van Ooijen, 1992). The estimated additive genetic effect and the percentage of variance explained by each QTL and the total variance explained by all the QTLs affecting a trait, were obtained using restricted MQM Mapping implemented with MapQTL.

Two-way interactions among the QTL identified for each trait were tested by ANOVA using the corresponding two markers as fixed factors and the trait as dependent variable, using the general linear model of the statistical package SPSS version 11.5.0. A Bonferroni correction to adjust the 0.05 threshold of significance was applied if multiple tests were performed on the same data set. Only those interactions that were significant after the Bonferroni correction are presented.

Heritabilities were calculated based on measurements on 6 to 12 plants.

Chapter 5

Arabidopsis natural allelic variation of carbohydrate-related traits and plant growth in the Landsberg *erecta* x Kondara recombinant inbred line population

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Abstract

Arabidopsis thaliana natural variation was used to study plant performance viewed as the accumulation of photoassimilates, their allocation and storage, in relation to other growth-related features. A set of 123 Arabidopsis accessions revealed large variation for the patterns of sugars and starch accumulation during the day, the majority of the accessions accumulating carbohydrates in the leaves at the end of the photoperiod. No correlation was observed between accumulation patterns and geographical origin. The diurnal pattern of 23 selected accessions in addition to three mutants (*pgm*, *adg1* and *sex)*, was studied in more details and revealed three different groups: i. Accumulating soluble sugars and starch during the day; ii. Having more or less constant levels; iii. Intermediate behavior. The three mutants were apart from all the accessions and showed reduced growth rates. Quantitative trait locus (QTL) analysis using recombinant inbred lines derived from the cross between Landsberg *erecta* (group 2) and Kondara (group 1) grown on hydroponics, revealed QTLs for the different aspects of plant growth-related traits, sugar and starch contents and flowering-related traits. Co-locations of QTLs for these different aspects were detected at different regions, mainly at the *ER* locus, the top of chromosome 3 and 4 and the bottom of chromosome 5. In general, plant growth was more closely linked to leaf transitory starch levels than to the soluble sugar levels. From the significant correlation and the co-locations of the QTLs for these aspects, we conclude that there is a complex relationship between plant growth-related traits, carbohydrate content and flowering-related traits.

Introduction

Growth of autotrophic plants depends on photosynthetic activity. Photoassimilates can be either used directly for growth or respiration, or stored for a short period (e.g. in leaves, diurnal) or for a long period (e.g. in seeds or roots). The accumulation of carbohydrates in leaves has a role in regulating their photosynthetic rate, as the level of these end products is a function of the balance between photosynthesis and the use by growth processes of the plant.

Production of sugars through photosynthesis is an essential process, because sugar status modulates and coordinates internal regulators and environmental cues that control growth and development (Koch, 1996; Sheen et al., 1999; Smeekens, 2000). Sucrose is considered as the major stable product of photosynthesis for most plants and it is also the form in which most carbon is transported in phloem vessels from leaves into sink organs such as roots, flowers, grains and tubers (Rolland et al., 2002).

Besides sucrose, starch serves as an important storage for carbohydrate residues. Many plants accumulate transitory starch in their leaves. It is synthesized from photosynthetically fixed carbon dioxide and serves as a short to medium term carbohydrate reserve. Transitory starch is degraded during the night, or when the rate of photosynthesis is low, to provide substrates for respiration and for the synthesis of sucrose and other translocated metabolites (Zeeman an ap Rees, 1999).

The extent to which starch accumulates in leaves differs between species. In *Arabidopsis thaliana* it is the major carbohydrate that accumulates and it is synthesized throughout the photoperiod (Caspar et al., 1985; Zeeman et al., 1998). The importance of storing carbohydrates as starch is reflected in the growth of plants that are unable to synthesize or to fully degrade transitory starch. The starchless *Arabidopsis* lines *pgm* and *adg1* (lacking plastidial phosphoglucomutase activity and ADPglucose pyrophosphorylase activity, respectively) grow more slowly in day/night conditions than the wild type (Casper et al., 1985; Lin et al., 1988). The growth rate of the *sex* (starch excess) mutant, which has a reduced capacity to mobilize starch, is similarly affected (Casper et al., 1991; Zeeman et al., 1998).

An alternative for laboratory induced-mutants, as described above, is the use of genetic variation that can be found among naturally occurring populations of *Arabidopsis* (Alonso-Blanco and Koornneef, 2000). The development of DNA markers has allowed studying naturally occurring allelic variation underlying complex traits (Tanksley, 1993; Paterson, 1995; Yano and Sasaki, 1997). Such analysis is often referred to as quantitative trait locus (QTL) analysis. QTLs have been identified for various complex traits in several plant species (Paterson, 1995; Yano and Sasaki, 1997; Alonso-Blanco and Koornneef, 2000). The comparison of accessions from different environments allows genetically different parental lines to be selected for further studies. In addition, genetic variation undetectable by accession comparison might be revealed when analyzing segregating populations derived from crosses between accessions. This is the case when segregating individuals display phenotypes outside the range of variation of the parents (transgression), and therefore segregating populations

provide a better estimate of the variation present between two accessions (Alonso-Blanco and Koornneef, 2000).

Here we describe a study in which we analyzed a series of carbohydrate-related traits (e.g. soluble sugars and starch content) in different accessions of *Arabidopsis thaliana*. Furthermore, we use QTL analysis to map these traits in a newly developed RIL population of Arabidopsis, derived from the cross between Landsberg *erecta* (L*er*) and Kondara (Kond), which was grown on hydroponics. The study also includes the analysis and the mapping of additional traits, related to both vegetative and generative development, in order to investigate if these traits show correlations with the carbohydrate status of the plants, which might indicate control by the same genes when this correlation is based on co-location of the respective QTLs.

Results

Carbohydrate natural variation and their diurnal pattern in Arabidopsis

In the light period, leaves carry out photosynthesis and either export photoassimilates (mainly sucrose) to other plant parts to be used for growth or respiration, or they store assimilates transiently as starch. During the night or when the rate of photosynthesis is low, starch is degraded to support leaf respiration and continuous synthesis and export of sucrose. To understand the pattern of sugar accumulation as well as starch turnover we screened 123 Arabidopsis accessions at two time points; two hours before the end of the light period and two hours before the end of the dark period. There was a large variation for the contents of sugars (glucose, fructose and sucrose) and starch (Table 5.1). The majority of the accessions showed higher sugars and starch contents at the end of the light period compared to their contents at the end of the dark period (data not shown). Using cluster analysis, no clear correlation was observed between the origin of the accessions and their pattern of sugars and starch content. However, in some cases related accessions from one area, such as accessions from the Dutch Island of Ameland (Amel-1, Nes2-1, 2, 4, 5, and 6 and Oerd-1 and 3), clustered together (Fig. 5.1).

Table 5.1: Ranges and averages of carbohydrate contents in leaves of 123 Arabidopsis accessions measured before the end of the dark and the light periods

Trait	Range (dark)	Average (dark)	Range (light)	Average (light)
Glucose mg/g DW	2.5 - 27.4	11.2	1.6 - 34.5	13.4
Fructose mg/g DW	0.0 - 7.6	1.1	0.7 - 10.7	3.8
Sucrose mg/g DW	0.0 - 9.1	2.7	0.7 - 14.7	5.3
Starch mg/g DW	0.7 - 166.1	34	0.9 - 274.6	102.1

To study in more details the diurnal pattern of the carbohydrate content, 23 Arabidopsis accessions were selected on the basis of the previous screen and other growth-related traits. In addition three mutants were analyzed, known to have altered starch synthesis and degradation patterns, viz., the starchless lines *pgm* and *adg1* (lacking plastidial phosphoglucomutase activity and ADPglucose pyrophosphorylase activity, respectively) and the *sex* (starch excess) mutant, which has a reduced capacity to mobilize starch. Carbohydrate analysis was carried out at six time points; the first was two hours before the end of the dark period and the last was two hours after the onset of the next dark period. The additional four determinations were carried out with material sampled during the light period. A principle component analysis (PCA) was performed to summarize the diurnal carbohydrates content of the different genotypes, where the first three principle components (PCs) explained 70 % of the total variation for carbohydrate content.

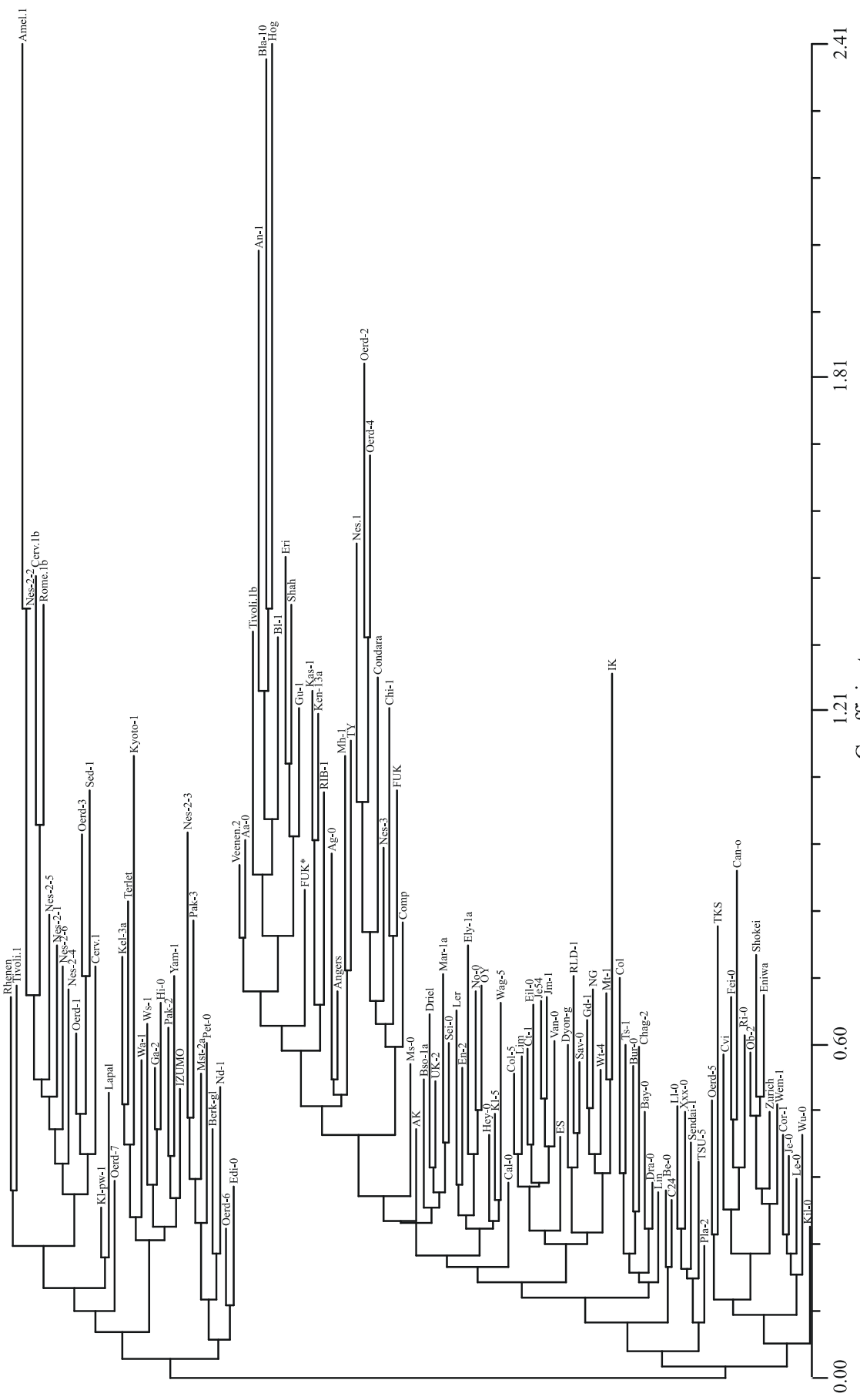


Figure 5.1: Neighbor-joining distance tree of 123 Arabidopsis accessions based on contents of sugars (glucose, fructose and sucrose) and starch in the leaves, determined both at the end of the dark and at the end of the light periods.

For the accessions, the diurnal measurements of sugars and starch contents revealed three groups of genotypes, whereas the mutants were clearly distinct from the accessions (Fig 5.2). The first group of accessions exhibited a nearly constant level of carbohydrates over the day. As an example of this group the diurnal patterns of carbohydrates of L*er* are given (Fig. 5.3A). The second group accumulated high levels of sugars and starch during the day, Kond being an example of this group (Fig. 5.3B). The third group had intermediate levels of carbohydrate during the day.

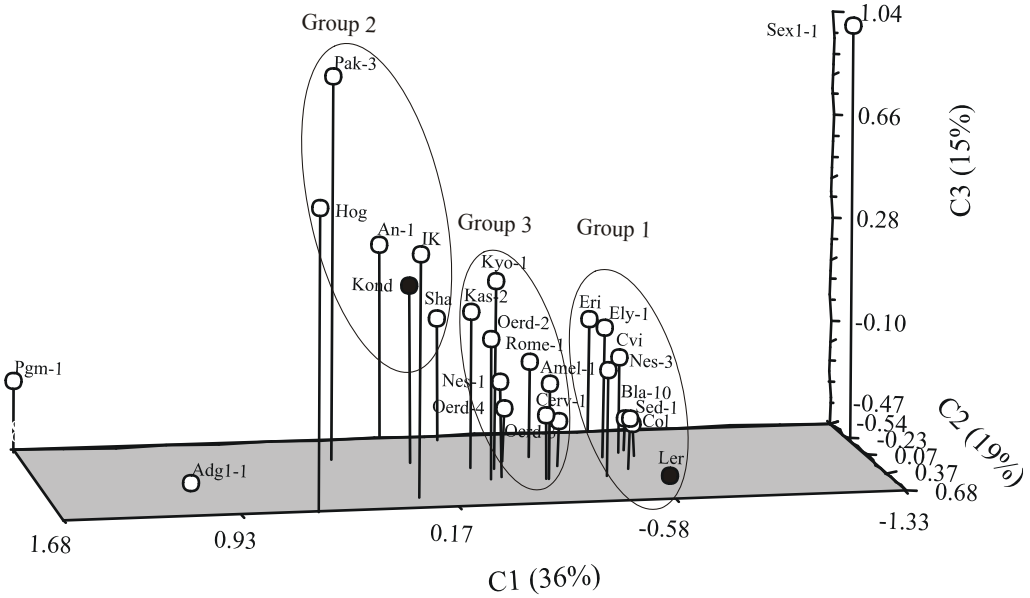


Figure 5.2: Principle component analysis of the 23 Arabidopsis accessions plus three mutants (pgm-1, adg-1 and sex-1), using the diurnal pattern of their carbohydrate content. The ovals indicate 3 different groups; the accessions of each group had more or less a similar diurnal pattern. L*er* and Kond are indicated with closed circles.

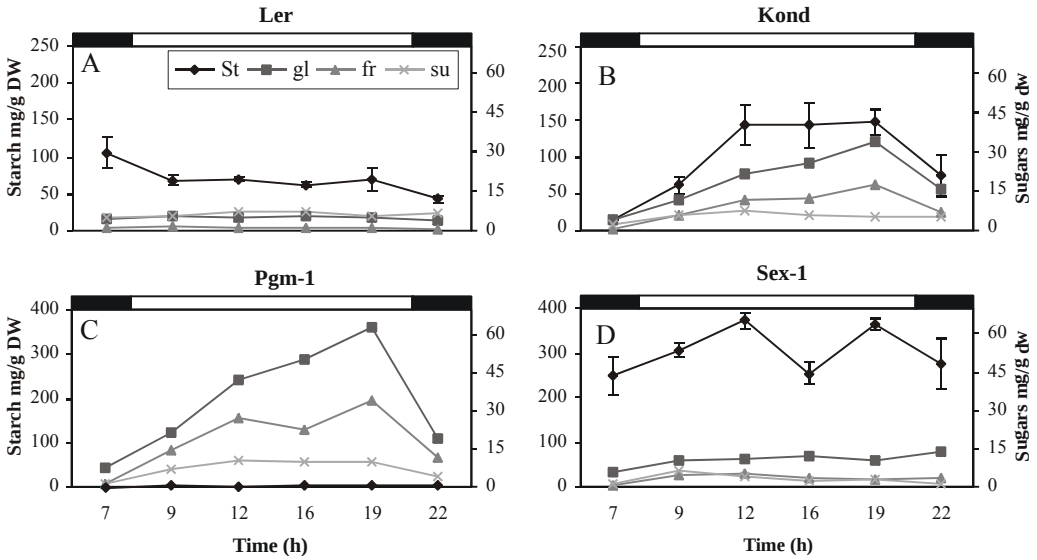


Figure 5.3: Diurnal patterns of carbohydrate accumulation in leaves of L*er* (A), Kond (B), and the mutants pgm-1 (C) and sex-1 (D) grown in 12 h light/12 h dark. The results are the mean ± SE (n = three separate samples, each of one individual plant). st, starch; gl, glucose; fr, fructose and su, sucrose.

As described before, the *pgm-1* mutant accumulated high levels of sugars during the day and hardly any starch, the *adg-1* mutant behaving similarly (data not shown), while the *sex-1* mutant shows high starch content throughout the day/night cycle (Fig. 5.3, C and D). The growth rates of the mutants *pgm-1, adg-1 and sex-1* was severely reduced compared to the Arabidopsis accessions (Fig. 5.4).

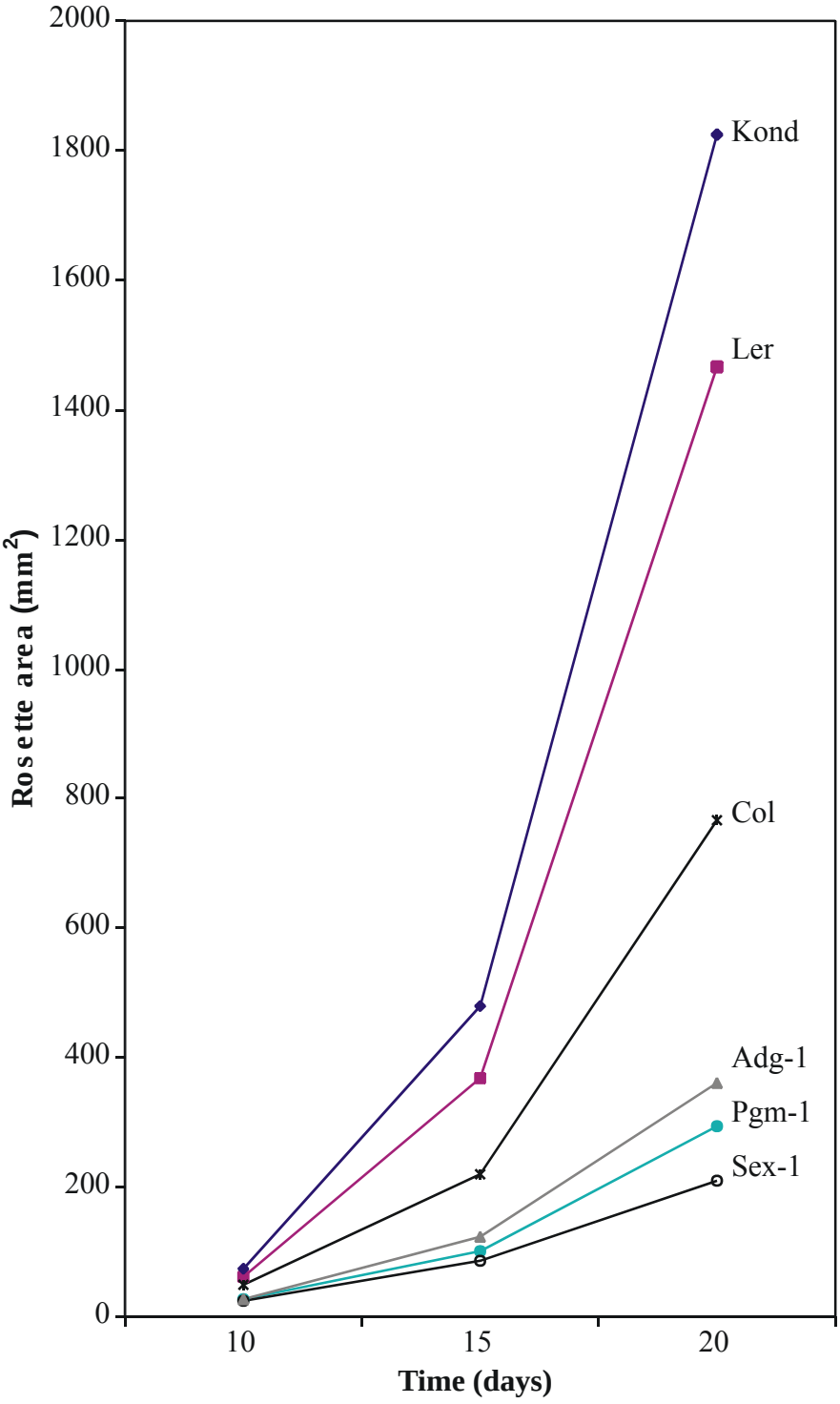


Figure 5.4: Growth rate curves for L*er*, Kond, Col and the three mutants (*pgm-1*, *adg-1* and *sex-1)* as determined by plant rosette area.

Based on their different pattern of carbohydrate accumulation, the two accessions L*er* and Kond were chosen to generate a RIL population for QTL analysis to map these and other traits. This population was grown on hydroponics, since it allows more uniform growth and the analysis of roots.

Genetic variation within the L*er* x Kond RIL population

In total 45 traits were studied in the RILs of (L*er* x Kond) which can be grouped in three main categories: vegetative growth traits both for shoots and roots, carbohydrate contents and flowering-related traits. Traits and abbreviations used are listed in Table 5.2, and the numbers 1 or 2 after the trait abbreviations refer to samples from 15 and 25 days old plants, respectively; L stands for light and D for dark. For all traits analyzed significant variation was observed between RILs. The broad sense heritabilities ranged from 0.92 for TLN and RLN to 0.30 for shoot Ra2L (Table 5.2). Transgression beyond the parental values was observed for all traits including those for which parental values hardly differed, such as RGR3-1, WC2 and St1L (Fig. 5.5, A, C and J). This amount of genetic variation indicated that QTL mapping was likely to reveal significant loci for most traits.

Table 5.3 presents Pearson correlation coefficients between all studied traits in the L*er* x Kond RIL population: significant positive correlations are shown above the diagonal and the negative correlations below the diagonal. The correlation co-efficient (R^2), ranging from 20% to 90% at –Log P values ranging from 1.6 to 87, are grouped in three categories represented by a gray scale in Table 5.3.

QTL mapping

The ultimate aim of this study is to unravel (genetic) relationships between carbohydrate-related traits and overall growth of plants. Therefore, we first analyzed growth of the RILs in terms of size, weight and related parameters.

Vegetative growth

Shoot-related traits

At the *ERECTA* (*ER*) locus, co-location was found of QTLs for TLA1, TLA2, and TLA3 and for RGR parameters on the basis of plant area, as well as for shoot DW1 and DW2 and for RGR on the basis of weight. Co-locations occurred also with SLA, RLL, all with the same allele effect, the Kond allele increasing the trait value (Fig. 5.6). The explained variance for each individual QTL ranged from 12.4% to 37.3% for TLA1 and shoot DW2, respectively (Table 5.4). At the *ER* locus also QTLs for carbohydrate-related and flowering-related traits were detected, that will be described later in more detail.

At the top of chromosome 4, around *FRI*, there is a co-location for QTLs of WC1 and 2 with a QTL for RGR 3-1, with the same direction of the allele, L*er* increasing the trait values (Fig. 5.6). Few other QTLs were detected for TLA1 and 2 (top chromosome 5) and WC2 at the bottom of chromosome 4 (Fig. 5.6).

Table 5.2: Parental values, ranges, means and heritabilities in the L*er* x Kond RILs for all measured traits

Trait	L*er* value	Kond value	Range	L*er*+	Mean	h^2
Shoot-related traits						
Total leaf area 1 (mm^2) (TLA1)	8.9	32.6	8.9 - 52.7	35.2	33.3	0.64
Total leaf area 2 (mm^2) (TLA2)	64.1	117.5	42.1 - 165.8	130.1	106	0.76
Total leaf area 3 (mm^2) (TLA3)	301.6	606.2	141.6 - 1007.9	578.5	502.5	0.80
Relative growth rate 3-2 (area) (RGR3-2)	0.31	0.33	0.21 - 0.37	0.30	0.31	nd
Relative growth rate 3-1 (area) (RGR3-1)	0.30	0.29	0.21 - 0.31	0.28	0.27	nd
Dry weight 1 (mg) (DW1)	2.0	2.3	1.08 - 3.2	2.5	2.1	0.62
Water content 1 (%) (WC1)	88.0	89.3	86.1 - 90.1	88.8	88.9	0.36
Dry weight 2 (mg) (DW2)	34.1	49.6	13.1 - 71.4	49.4	40.2	0.73
Water content 2 (%) (WC2)	89.8	89.6	88.0 - 91.2	89.6	89.6	0.48
Relative growth rate (DW)($RGR_{(DW)}$)	0.29	0.31	0.24 - 0.34	0.30	0.29	0.45
Specific leaf area ($mm^2.mg^{-1}$) (SLA)	32.5	50.5	31.2 - 75.9	52.6	51.7	nd
Rosette leaf length (cm) (RLL)	5.2	7.1	3.2 - 10.3	5.6	5.7	0.77
Root-related traits						
Root dry weight 2 (mg) (RoDW2)	7.4	8.3	3.1 - 14.7	10.3	8.1	0.58
Shoot/Root ratio (DW2) (Sh/Ro)	4.6	6.0	3.7 - 9.0	4.8	5.1	0.49
Root length 1 (cm) (RoL1)	14.9	10.4	4.0 - 16.2	13.9	11.7	0.56
Root length 2 (cm) (RoL2)	25.9	20.9	15.9 - 29.9	24.5	23.2	0.42
Chlorophyll fluorescence (ChlF)	0.743	0.758	0.614 - 0.772	0.736	0.728	0.39
Chlorosis (Chlo)	2.0	1.5	1.0 - 6.5	1.5	3.3	0.77
Shoot carbohydrate content						
Glucose 1 at light (mg/g DW) (Gl1L)	0.9	4.6	3.8 - 14.6	3.0	3.6	0.61
Fructose 1 at light (mg/g DW) (Fr1L)	6.6	8.5	0.8 - 21.8	7.9	6.9	0.68
Sucrose 1 at light (mg/g DW) (Su1L)	31.0	34.1	14.5 - 40.1	27.4	29.1	0.59
Raffinose 1 at light (mg/g DW) (Ra1L)	0.8	1.3	0.1 - 3.7	0.7	1.0	0.48
Glucose 2 at dark (mg/g DW) (Gl2D)	0.7	2.6	0.7 - 4.3	1.7	1.5	0.72
Fructose 2 at dark (mg/g DW) (Fr2D)	1.3	3.1	1.1 - 5.8	3.1	2.2	0.66
Sucrose 2 at dark (mg/g DW) (Su2D)	4.0	5.6	4.0 - 10.0	5.8	5.7	0.64
Raffinose 2 at dark (mg/g DW) (Ra2D)	0.2	0.3	0.1 - 0.7	0.3	0.2	0.63
Glucose 2 at light (mg/g DW) (Gl2L)	0.8	4.6	0.7 - 5.1	1.1	1.9	0.41
Fructose 2 at light (mg/g DW) (Fr2L)	1.3	2.9	0.4 - 7.1	1.9	1.9	0.59
Sucrose 2 at light (mg/g DW) (Su2L)	6.9	8.5	5.3 - 9.9	8.2	7.8	0.66
Raffinose 2 at light (mg/g DW) (Ra2L)	0.5	1.3	0.4 - 2.0	0.6	0.8	0.30
Starch 1 at light (mg/g DW) (St1L)	186.1	189.0	102.8 - 285.1	186.5	197.7	0.68
Starch 2 at dark (mg/g DW) (St2D)	44.0	58.9	8.6 - 106.0	32.4	51.1	0.52
Starch 2 at light (mg/g DW) (St2L)	102.5	124.1	63.0 - 140.2	74.0	100.7	0.45
Root carbohydrate content						
Fructose 2 at light (mg/g DW) (Fr2L)	2.7	2.6	1.0 - 9.5	4.5	3.1	0.43
Sucrose 2 at light (mg/g DW) (Su2L)	10.7	22.6	1.0 - 33.2	20.4	18.7	0.43
Raffinose 2 at light (mg/g DW) (Ra2L)	3.3	1.7	0.4 - 5.3	2.4	2.4	0.52
Flowering-related traits						
Flowering time (days) (FT)	35.5	48.3	24.7 - 71.5	42.3	42.0	0.89
Total leaf number (TLN)	17.5	41.0	8.5 - 64.3	30.0	24.8	0.92
Rosette leaf number (RLN)	13.0	37.0	6.5 - 53.3	22.0	20.4	0.92
Cauline leaf number (CLN)	4.5	4.0	1.5 - 9.9	8.0	4.6	0.81

Table 5.2: (continued from previous page.)

Trait	L*er* value	Kond value	Range	L*er*+	Mean	h^2
Total plant length (cm) (TPL)	33.6	46.2	11.8 - 80.4	62.6	45.1	0.90
Plant length till 1st silique (cm) (PLTS)	13.0	10.4	4.4 - 25.8	22.6	13.8	0.85
Inflorescence length (cm) (INFL)	20.6	35.8	5.9 - 59.9	40.1	31.3	0.87
Rosette branches (RB)	6.5	3.3	0.0 - 13.4	1.8	5.1	0.59
Stem branches (SB)	5.2	4.0	1.7 - 11.0	8.3	5.1	0.78

nd, not determined because these traits are calculated only on the basis of averages.
L*er*+ is *ERECTA* wild type allele in the L*er* genetic back ground.

Root-related traits

The measurement of root dry weight of 15 days old plants was difficult because of the very low weight of the roots, resulting in relatively large errors in the determinations. Despite the small differences in root dry weight at day 25 (RoDW2) between L*er* and Kond, large variation was found between the RILs for this trait (Table 5.2). Around the *ER* locus, two QTLs were detected for RoDW2 and for Sh/Ro ratio on the basis of dry weight at day 25, each explaining 17.2% and 20.3% of the total variance (Fig. 5.6 and Table 5.4). These two QTLs co-located with the earlier mentioned shoot-related traits around *ER*, with similar allele effect.

At the top of chromosome 3, QTLs for RoL1 and Sh/Ro ratio co-located, with opposite allele effect. In addition a QTL for RLL with similar allele effect as the QTL for Sh/Ro ratio was located at that position.

At the top and at the bottom of chromosome 4, two and one QTLs were found for RoL1 and RoL2, respectively, with the same allele effect, L*er* increasing root length. These QTLs co-located with QTLs for WC1 and WC2 with a similar allele effect (Fig. 5.6). Moreover, a significant positive correlation between RoL1 and WC1 and between RoL2 and WC2 (Table 5.3) was found.

At the top of chromosome 5, around marker SNP77, QTLs for RoDW2 and RoL1, for which the L*er* allele increased the trait value, co-located with QTLs for TLA1, TLA2 and TLA3 (suggestive QTL, LOD between 1.5 and 2.4) and shoot DW1 (suggestive QTL) with similar allele effects (Fig. 5.6). For the RoDW2 significant positive correlations were found with plant biomass at two stages (DW1 and DW2), $RGR_{(DW)}$, the plant total leaf areas (TLA) and RGR 3-2 and 3-1 (Table 5.3). No QTLs for RoL1 and 2 were found at the *ER* locus.

Chlorophyll fluorescence (ChlF) and chlorosis (Chlo)

Two QTLs for ChlF were detected, located on chromosome 4 and 5 at SNP408 and SNP 236 and explaining 24.1% of the phenotypic variance (Fig. 5.6). For these QTLs, the Kond allele increased the photosynthetic capacity of the plant compared with the L*er* allele (Table 5.4). The ChlF QTL at SNP408 at the middle of chromosome 4 co-located with sugars QTLs with similar allele effect. A significant positive correlation was found between the photosynthetic yield and the shoot Fr2D, Ra1L and Ra2L and the FT traits (Table 5.3).

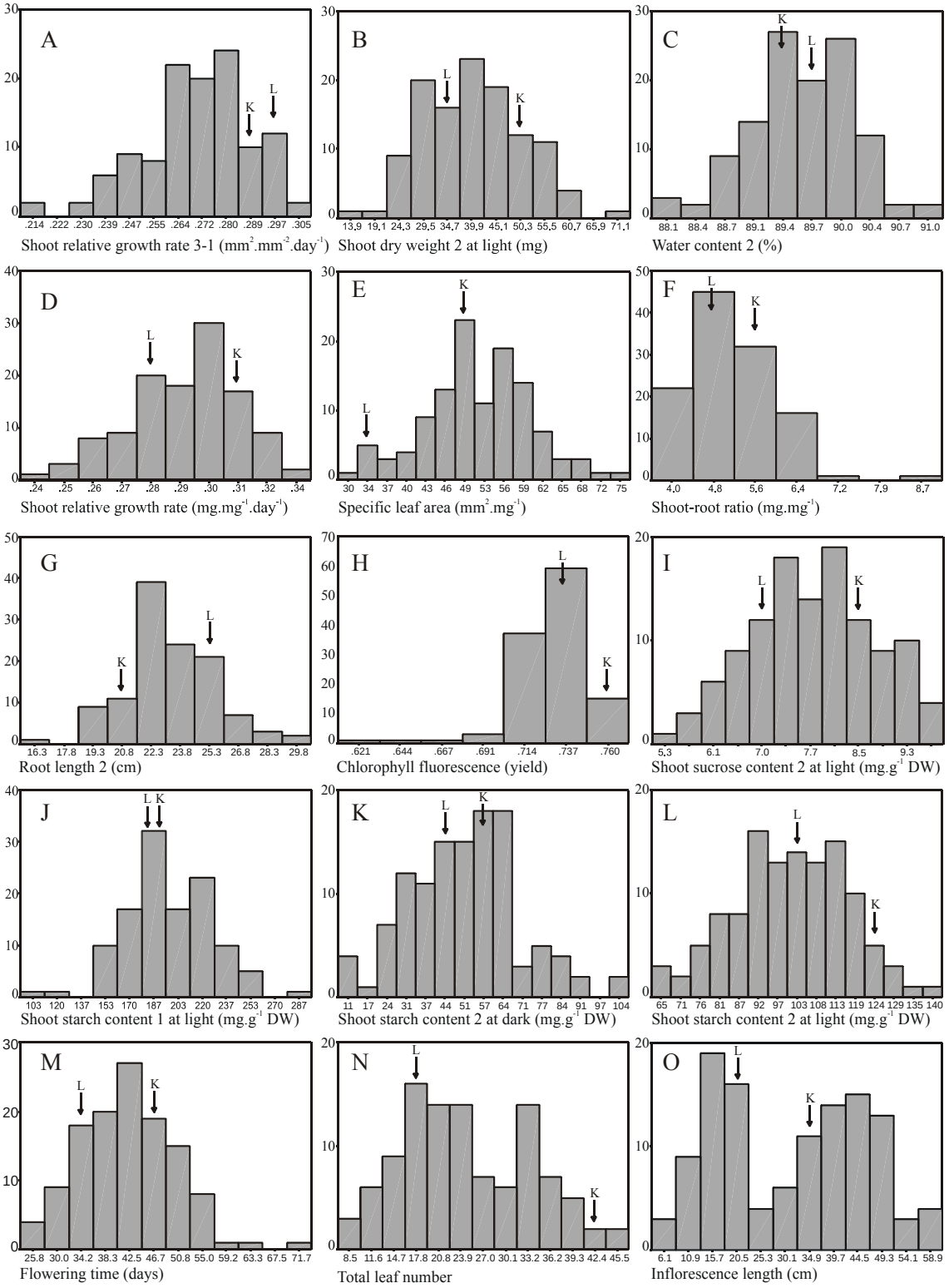


Figure 5.5: Frequency distribution of non-normalized data of selected traits in the L*er* x Kond RIL population. Vegetative traits: Shoot relative growth rate 3-1 calculated on the basis of plant area (A), Shoot dry weight 2 (at 25 days) at light (B), Water content 2 (at 25 days) (C), Shoot relative growth rate calculated on the basis of plant dry weight (D), Specific leaf area (E), Shoot-root ratio (F), Root length 2 (at 25 days) (G). Chlorophyll fluorescence measured as yield (H). Carbohydrate traits: Shoot sucrose content 2 (at 25 days) at light (I), Shoot starch content 1 (at 15 days) at light (J), Shoot starch content 2 (at 25 days) at dark (K), Shoot starch content 2 (at 25 days) at light (L). Generative traits: Flowering time (M), Total leaf number (N), Inflorescence length (O). The average parental value is indicated with an arrow for both parents, L for L*er* and K for Kond.

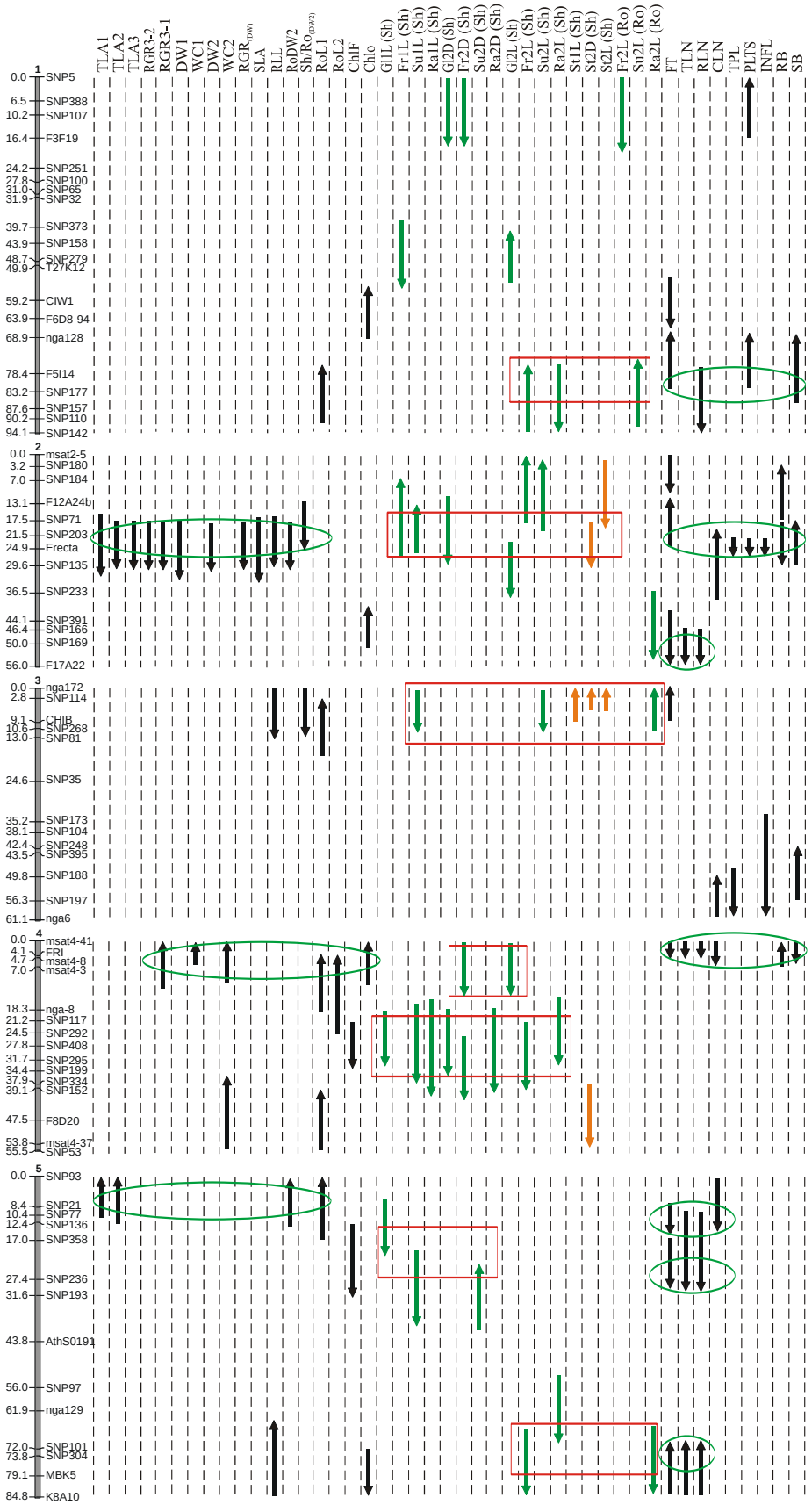
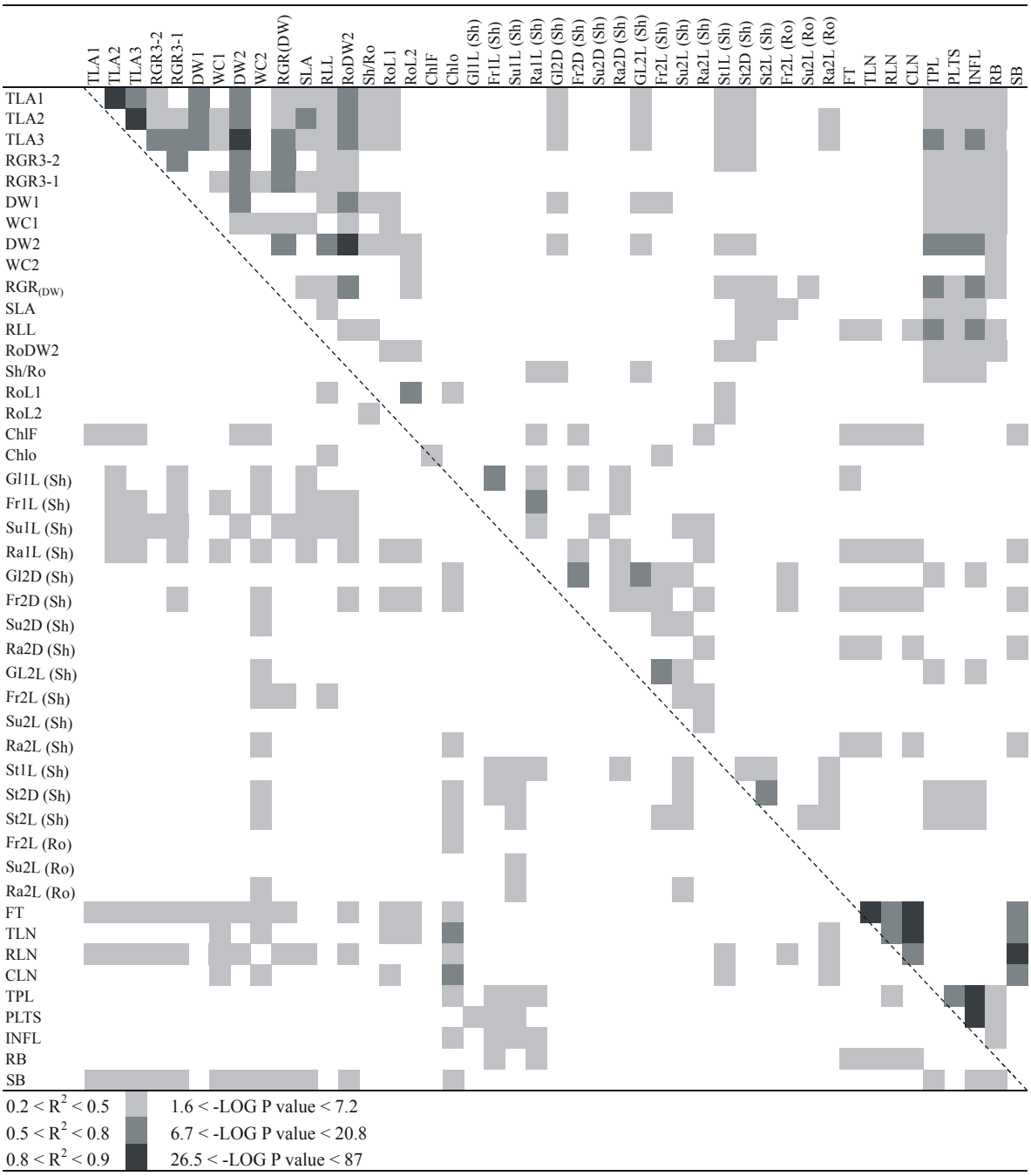


Figure 5.6: The L*er* x Kond linkage map showing the locations of QTLs for the traits analyzed. The lengths of the arrows indicate the 2-LOD support intervals. The direction of the arrow's head indicates the allelic effect: upward, L*er* increasing and Kond decreasing; downward, Kond increasing and L*er* decreasing. The ovals indicate QTLs of growth and flowering regions, while the rectangles indicate the carbohydrate-related QTLs.

For chlorosis four QTLs, explaining 51.0% of the phenotypic variance, were detected (Fig. 5.6). At three of the four QTL positions, the L*er* alleles accelerated the leaf senescence (Table 5.4). A significant negative correlation was found between chlorosis and photosynthetic yield, sugar levels, starch content and flowering-related traits (Table 5.3). This negative correlation between Chlo and FT traits was consistent with the opposite allele effect of the three co-locating QTLs for Chlo with other QTLs for FT traits at three different places, at the bottom of chromosome 2 and 5 and the top of chromosome 4 around *FRI* (Fig. 5.6).

Table 5.3: Pearson correlation between the studied growth-related traits, carbohydrate content and flowering-related traits in the L*er* x Kond RIL population.



Cells above the diagonal represent the positive correlations between the traits, while the ones below the line represent the negative correlations.

Carbohydrate-related traits

Levels of glucose, fructose and sucrose were determined since these are important products of photosynthesis and are involved in many metabolic processes in the plant life cycle. In addition we measured the starch content as it has a crucial role in plant respiration, metabolism and storage of reserves. The analyses were carried out for shoots and roots, at two time points, at two hours before the end of the dark or light periods with 25 days old plants, while for 15 days old plants the analysis was done only at the end of the light period.

Shoots carbohydrate content

Figure 5.6 and table 5.4 show the QTLs detected for sugars and starch in the leaves at two different stages of plant development and at two different time points during the day/night cycle. One of the most interesting regions is the one around the *ER* locus, where QTLs for Fr1, Su1, Gl2 and St2 all in light and Gl2 in darkness co-located with other vegetative growth QTLs as well as with flowering time related QTLs. Also at the top of chromosome 3 carbohydrate QTLs for Su1, Su2 St1 and St2 all in light and St2 at darkness co-located with QTLs for RLL, Sh/Ro ratio, RoL and with FT. The directions of the allele effects of the various co-locating QTLs are not all in the same direction.

At the top of chromosome 4, two QTLs for Fr2 in darkness and Gl2 in light co-located with vegetative growth-related traits with opposite allele effects and with other QTLs for flowering time-related traits with the same direction, Kond alleles increasing the trait values. Another interesting region is in the middle of chromosome 4 where almost all sugar parameters showed a QTL. However, at this position no QTL was found for starch content. In all cases the Kond allele increased the accumulation of sugars. These QTLs co-located with a QTL for ChlF with the same direction of the allele effect. At F8D20 on chromosoe 4 a QTL for St2 at darkness co-located with a QTL for WC2, with opposite allele effects (Fig. 5.6), explaining the significant negative correlation between these two traits (Table 5.3).

Both at the middle part and at the bottom of chromosome 5 several sugars QTLs co-located with other QTLs for growth-related and flowering-related traits. The explained variance for individual QTL detected for carbohydrates ranged from 6.4% to 31.1% of the total variance (Table 5.4).

Roots carbohydrate content

Growing this population on hydroponics allowed investigation of carbohydrates accumulation in roots, being an important sink in still vegetative Arabidopsis plants, together with growth-related traits of roots. For Ra2L two significant QTLs were detected at the bottom of chromosome 2 and at the top of chromosome 3, with explained variance of 9.6% and 24.1%, respectively. Two other QTLs for Fr2 and Su2 content in light were detected at the top and bottom of chromosome 1 (Table 5.4 and Fig. 5.6). The root Ra2L QTL at the top of chromosome 3 co-located with QTLs for Su1L and Su2L in the shoot with opposite allele effects, while it also co-located with QTLs for St1L, St2D, St2L and FT with the same allele effect (Fig. 5.6).

Table 5.4: Characteristics of the detected QTLs explaining growth traits, carbohydrate levels, flowering time and flowering related traits in L*er* x Kond RIL population.

Trait	QTL	Map position*	LOD score	% of variance	Additive allele effect**
Shoot-related traits					
Total leaf area 1 (mm^2)				**28.6**	
	Erecta	2 - 24.874	4.09	12.4	-2.67
	SNP77	5 - 10.425	4.07	12.7	2.81
Total leaf area 2 (mm^2)				**29.7**	
	Erecta	2 - 24.874	6.26	19.7	-10.83
	SNP77	5 - 10.425	2.68	7.9	7.05
Total leaf area 3 (mm^2)				**39.6**	
	Erecta	2 - 24.874	11.02	32.7	-86.05
Relative growth rate 3-2 (area)				**26.9**	
	Erecta	2 - 24.874	7.04	23.4	-0.015
Relative growth rate 3-1 (area)				**35.4**	
	Erecta	2 - 24.874	9.01	27.8	-0.010
	msat4-41	4 - 0	3.43	9.5	0.006
Shoot dry weight 1 (mg)				**18.3**	
	SNP135	2 - 29.562	3.03	10.6	-0.127
Water content 1 (%)				**22.3**	
	msat4-41	4 - 0	4.72	16.1	0.31
Shoot dry weight 2 (mg)				**42.6**	
	Erecta	2 - 24.874	12.66	37.3	-6.474
Water content 2 (%)				**31.9**	
	msat4-41	4 - 0	3.31	9.9	0.19
	F8D20	4 - 47.45	3.63	10.8	0.20
Relative growth rate (ShDW)				**42.6**	
	Erecta	2 - 24.874	12.1	35.4	-0.012
Specific leaf area ($mm^2.mg^{-1}$)				**11.1**	
	Erecta	2 - 24.874	2.98	11.1	-2.87
Rosette leaf length (cm)				**54.5**	
	Erecta	2 - 24.874	11.17	30.1	-0.60
	FRI	4 - 4.097	2.41	5.3	-0.26
	MBK5	5 - 79.141	2.52	5.5	0.26
Root-related traits					
Root dry weight 2 (mg)				**37.3**	
	Erecta	2 - 24.874	6.15	17.2	-0.808
	SNP77	5 - 10.425	3.13	8.5	0.587
Shoot : Root (DW2)				**34.2**	
	SNP203	2 - 21.475	6.68	20.3	-0.029
	CHIB	3 - 9.059	3.18	9.1	-0.021
Root length 1 (cm)				**48.0**	
	SNP157	1 - 87.624	4.74	10.8	0.58
	SNP268	3 - 10.62	2.62	5.7	0.43
	msat4-3	4 - 7.01	3.9	8.8	0.52
	msat4-37	4 - 53.759	4.09	8.9	0.53
	SNP77	5 - 10.425	3.21	7.1	0.48
Root length 2 (cm)				**19.8**	
	nga-8	4 - 18.332	2.59	8.8	0.71
Photosynthetic yield (ChlF)				**24.1**	
	SNP408	4 - 27.75	3.67	12.1	-0.008
	SNP236	5 - 27.372	3.31	10.9	-0.007

Table 5.4: (continued from previous page.)

Trait	QTL	Map position*	LOD score	% of variance	Additive allele effect**
Chlorosis				**51.0**	
	F6D8-94	1 - 63.935	8.45	20.1	0.71
	SNP166	2 - 46.428	3.57	7.6	0.43
	FRI	4 - 4.097	5	10.8	0.52
	MBK5	5 - 79.141	3.33	7.1	-0.43
Shoot carbohydrate content					
Glucose 1 at light (mg/g DW)				**25.9**	
	SNP292	4 - 24.537	3.34	10.5	-0.761
	SNP77	5 - 10.425	2.71	8.4	-0.700
Fructose 1 at light (mg/g DW)				**24.5**	
	SNP373	1 - 39.655	3.19	10.4	-1.125
	SNP71	2 - 17.471	3.41	10.9	1.139
Sucrose 1 at light (mg/g DW)				**42.6**	
	Erecta	2 - 24.874	3.68	8.9	1.331
	SNP114	3 - 2.791	8.9	24.2	-2.191
	SNP292	4 - 24.537	3.07	7.4	-1.202
	SNP193	5 - 31.59	3.05	7.4	1.223
Raffinose 1 at light (mg/g DW)				**20.4**	
	SNP408	4 - 27.75	2.74	9.3	-0.188
Glucose 2 at dark (mg/g DW)				**32.7**	
	F3F19	1 - 16.437	4.31	12.5	-0.227
	Erecta	2 - 24.874	4.2	12.2	-0.224
	SNP292	4 - 24.537	2.93	8.3	-0.185
Fructose 2 at dark (mg/g DW)				**38.9**	
	F3F19	1 - 16.437	3.47	9	-0.242
	msat4-8	4 - 4.707	4.41	11.5	-0.290
	SNP152	4 - 39.058	2.48	6.4	-0.212
Sucrose 2 at dark (mg/g DW)				**27.0**	
	SNP193	5 - 31.59	3.83	12	0.365
Raffinose 2 at dark (mg/g DW)				**19.4**	
	SNP117	4 - 21.217	4.35	15.3	-0.045
Glucose 2 at light (mg/g DW)				**36.9**	
	SNP279	1 - 48.684	3.16	8.6	0.269
	SNP135	2 - 29.562	4.89	13.6	-0.335
	msat4-41	4 - 0	3.16	8.6	-0.267
Fructose 2 at light (mg/g DW)				**28.9**	
	SNP142	1 - 94.068	2.49	7.4	0.302
	F12A24b	2 - 13.078	2.49	7.4	0.302
	SNP152	4 - 39.058	2.78	8.2	-0.318
	MBK5	5 - 79.141	2.45	7.2	-0.304
Sucrose 2 at light (mg/g DW)				**39.6**	
	SNP180	2 - 3.194	2.59	6.4	0.256
	nga172	3 - 0	10.49	31.1	-0.557
Raffinose 2 at light (mg/g DW)				**32.1**	
	SNP177	1 - 83.238	2.78	7.9	-0.070
	SNP117	4 - 21.217	3.65	10.6	-0.082
	AthS0191	5 - 43.816	3.23	9.4	-0.077
Starch 1 at light (mg/g DW)				**31.0**	
	nga172	3 - 0	8.85	29.1	15.692

Table 5.4: (continued from previous page.)

Trait	QTL	Map position*	LOD score	% of variance	Additive allele effect**
Starch 2 at dark (mg/g DW)				**57.0**	
	Erecta	2 - 24.874	8.02	16.2	-7.764
	nga172	3 - 0	13.53	30.9	10.766
	F8D20	4 - 47.45	4.24	8	-5.514
Starch 2 at light (mg/g DW)				**40.2**	
	SNP184	2 - 7.012	3.05	8	-4.689
	nga172	3 - 0	7.46	20.7	7.712
Root carbohydrate content					
Fructose 2 at light (mg/g DW)				**17.6**	
	F3F19	1 - 16.437	3.34	11.6	-0.519
Sucrose 2 at light (mg/g DW)				**16.0**	
	SNP157	1 - 87.624	2.5	8.6	1.470
Raffinose 2 at light (mg/g DW)				**43.5**	
	SNP391	2 - 44.120	3.88	9.6	-0.315
	SNP268	3 - 10.620	8.81	24.1	0.515
	SNP101	5 - 72.009	2.86	7	-0.285
Flowering-related traits					
Flowering time (days)				**80.6**	
	F6D8-94	1 - 63.935	7.57	7	-2.68
	F5I14	1 - 78.426	4.62	4.2	2.04
	SNP180	2 - 3.194	3.04	2.6	-1.57
	SNP203	2 - 21.475	5.62	4.7	2.17
	F17A22	2 - 56.043	5.84	5.2	-1.97
	SNP114	3 - 2.791	3.82	3.5	1.59
	FRI	4 - 4.097	29.28	44.6	-5.70
	SNP136	5 - 12.417	3.69	3.2	-1.85
	SNP236	5 - 27.372	5.64	5.2	-2.34
	MBK5	5 - 79.141	6.13	5.4	2.02
Total leaf number				**69.8**	
	F17A22	2 - 56.043	5.51	9.4	-2.78
	FRI	4 - 4.097	18.8	44.1	-6.29
	SNP236	5 - 27.372	8.31	15	-3.58
	MBK5	5 - 79.141	3.57	5.6	2.18
Rosette leaf number				**74.0**	
	SNP110	1 - 90.161	3.32	4.5	-1.76
	F17A22	2 - 56.043	5.51	7.9	-2.29
	FRI	4 - 4.097	20.24	40.4	-5.39
	SNP236	5 - 27.372	8.05	12	-2.85
	K8A10	5 - 84.777	5.23	7.3	2.24
Cauline leaf number				**46.8**	
	SNP135	2 - 29.562	2.98	7.7	0.46
	nga6	3 - 61.134	4.28	11.9	0.57
	FRI	4 - 4.097	5.35	15.4	-0.67
	SNP21	5 - 8.404	3.69	10.3	-0.54
Total plant length (cm)				**84.7**	
	Erecta	2 - 24.874	42.76	82.2	-16.66
	SNP197	3 - 56.324	2.99	2	-2.55
Plant length till 1st silique (cm)				**76.2**	
	SNP388	1 - 6.465	3.74	4.2	0.90
	F5I14	1 - 78.426	11.46	14.9	1.74

Table 5.4: (continued from previous page.)

Trait	QTL	Map position*	LOD score	% of variance	Additive allele effect**
	Erecta	2 - 24.874	28.83	52.7	-3.33
Inflorescence length (cm)				**77.3**	
	Erecta	2 - 24.874	35.68	75.8	-12.69
	SNP197	3 - 56.324	2.45	2.3	-2.21
Rosette branches				**41.5**	
	F12A24b	2 - 13.078	2.84	7	0.90
	Erecta	2 - 24.874	6.54	17.4	-1.42
	msat4-8	4 - 4.707	5.35	13.8	0.93
Stem branches				**54.6**	
	F5I14	1 - 78.426	4	8.1	0.60
	Erecta	2 - 24.874	4.46	8.5	0.54
	SNP188	3 - 49.83	3.44	6.6	0.47
	FRI	4 - 4.097	9.52	21.3	-0.83

* Chromosome number is given, followed by the marker position in cM.
** The positive and negative values indicate that L*er* and Kond alleles increase the trait values respectively.

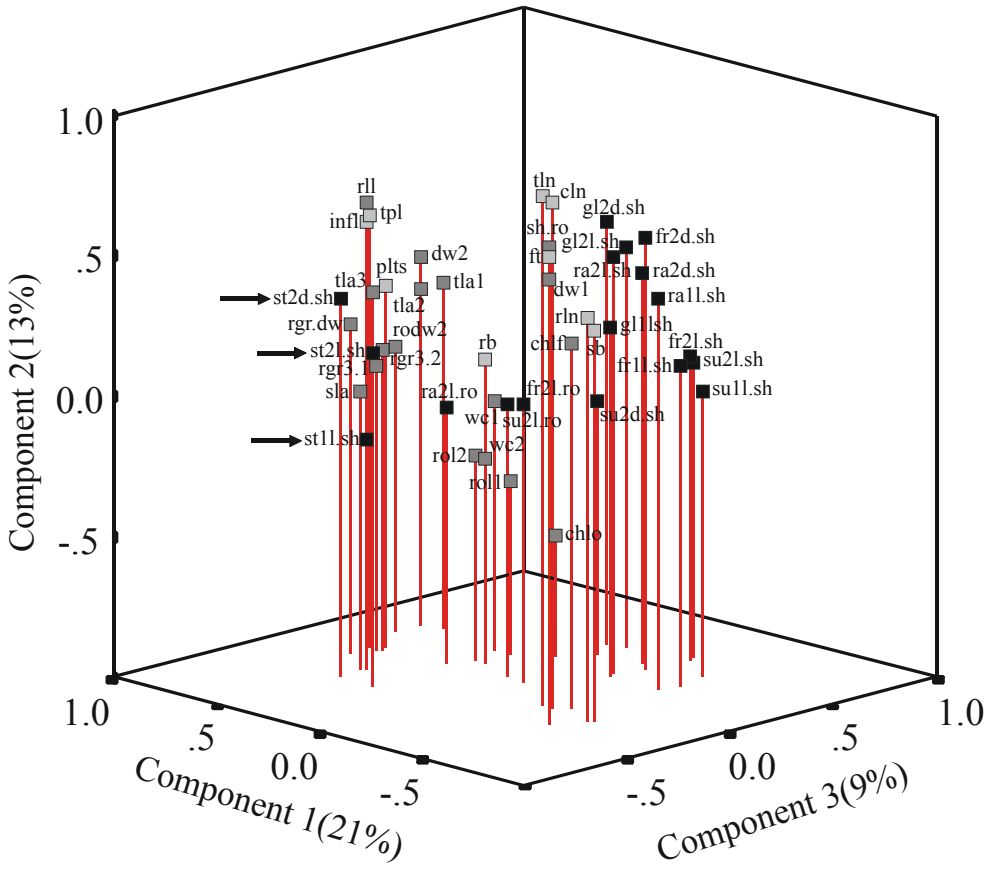


Figure 5.7: Principle component analysis of the 45 studied traits in the RIL of the (L*er* x Kond) population, using the trait values of the RILs. The black squares refer to sugars and starch traits, the dark gray squares refer to the growth traits and the light gray squares refer to the flowering-related traits. The black arrows refer to leaf starch traits at the different time points.

No QTLs were found for starch content in roots at day 25 when measured before the end of the photoperiod. The levels of starch in roots were very low, ranging from 0.32 to 5.6 mg per g dry weight.

Flowering time and flowering-related traits including leaf numbers, plant length and branching

In Arabidopsis, a high correlation is usually observed between flowering time and total leaf number formed prior to flowering and one might also expect to find correlations between rosette leaf number and rosette branches and between cauline leaf number and stem branches. Furthermore, plant height might be affected by the vegetative mass (rosette) and this might also have an effect on both stem leaves and on branches. Therefore, these traits are presented together.

The highly significant correlation between FT and TLN, RLN, CLN, RB and SB (R^2 = 0.85, 0.70, 0.88, -0.41 and 0.73 respectively) and between CLN and SB (R^2 = 0.67) confirm the expected relationships between these traits (Table 5.3). A positive correlation between plant length traits (TPL, PLTS and INFL) and rosette branching was observed, and a negative correlation was found with stem branching.

On average L*er* flowered about 13 days earlier than Kond and transgression beyond the parental values was observed (Table 5.2, Fig. 5.5M). A large portion of the variation between lines (80.6%) could be explained by ten QTLs, for six of which the L*er* allele promoted flowering (Fig. 5.6 and Table 5.4). In LD conditions (Chapter 3 of this thesis) eight out of the ten QTLs detected here in SD conditions were also observed and showed the same allele effects. The two QTLs only detected in SD condition were located at SNP203, where L*er* allele delayed flowering, and at F17A22, where the L*er* allele promoted flowering, both at chromosome 2.

Four QTLs were detected for TLN, which co-located with the FT QTLs with the same allele effects (Fig. 5.6 and Table 5.4) explaining 69.8% of the total variance. Rosette leaf number (RLN) and cauline leaf number (CLN), being the two components of TLN, showed five and four QTLs, explaining 74.0% and 46.8% of the variance, respectively. One QTL at SNP110 (chromosome 1), specific for RLN, did not co-locate either with FT, TLN or CLN QTLs, while for CLN another specific QTL was detected at nga6, at the bottom of chromosome 3. The remaining QTLs for RLN and CLN either co-located with each other or with QTLs of TLN or FT (Fig. 5.6).

For total plant length and its two components (length till first silique and inflorescence length), the total explained variance was relatively high (84.7%, 76.2% and 77.3%, respectively), which was largely due to the effect of the *ER* locus, explaining 82.2%, 52.7% and 75.8% of the observed variation (Table 5.4). The frequency distribution of the RILs for TPL and INFL (Fig. 5.5O) shows two classes, which could be explained by the detection of only two significant common QTLs, viz., at *ER* and SNP197 at chromosome 3. Kond alleles increased length for both traits.

For PLTS, three QTLs were found; two at chromosome 1 at SNP388 and F5I14, both being specific for PLTS with L*er* alleles increasing the length, suggesting that they might only be responsible for the increase in length of the stem internodes. The third QTL at the *ER* locus co-located with the other plant length traits (Fig. 5.6).

No QTLs were found for the total number of branches, because all the detected QTLs for rosette branches co-located either with significant or suggestive QTLs detected for stem branches but with opposite allele effects. Rosette and stem branches, being the two components of TNB, showed three and four QTLs, explaining 41.5% and 54.6% of the variance, respectively. Two QTLs at *ER* and around *FRI* co-located for both traits with opposite allele effects (Fig. 5.6 and Table 5.4). The two remaining QTLs for SB with L*er* alleles increasing the number of branching co-located with suggestive QTLs for RB around F5I14 (bottom chromosome 1) and around SNP188 (bottom chromosome 3) with opposite allele effects.

Relationships between all analyzed traits in L*er* x Kond RILs

The 45 traits analyzed in this study represented growth-related traits, sugar and starch content and flowering-related traits that might be correlated because these traits affect each other in a direct or an indirect way. Table 5.3 represents the significant negative or positive correlations that exist between the studied traits and the P values corrected for the multiple use of the same date set. Highly significant positive correlations within the different growth traits were found. There is also, a positive correlation between plant biomass traits (areas, RGRs, WC, DW, SLA, RLL, RoDW and Sh/Ro) on the one hand and plant length traits (TPL, PLTS and INFL) and RB from the other. Sugar content at 15 days correlated negatively with plant biomass traits, whereas starch content correlates positively. FT, TLN, RLN, CLN and SB exhibited a negative correlation with plant biomass traits.

A principal component analysis was carried out to further describe the predicted relationships between these traits. The first 13 components with Eigen values not less than one have 80.8% of the total variance explained by these traits. In the first component, the plant areas, RGRs, DW2, RoDW and plant length traits were the most important traits determining the pattern of clustering. FT, TLN, CLN and RLL mainly determine the second component. While, on the third component sugars content and DW1 were the main traits discriminating between the others (Fig. 5.7). Starch clustered more with growth traits than with soluble sugars. Sugars traits clustered together with other traits determining their production and consumption or use as sink including TLN, RLN, CLN, SB and FT and with DW1 and ChlF as well.

Discussion

In this study we investigated plant performance by analyzing different aspects of plant growth-related traits, sugars and starch content and flowering-related traits, trying to explain the correlations that were found between these different aspects. Growing the RILs of the L*er* x Kond population on hydroponics allowed the study of the source-sink relationship including the roots.

Natural variation of carbohydrate contents and diurnal pattern in leaves

Screening of a large set of Arabidopsis accessions for their carbohydrate content at two different points during the day/night cycle revealed a large variation between these accessions. Moreover, studying the diurnal pattern of 23 Arabidopsis accessions at moderate short day conditions (12h light/12h dark), showed three different groups: the first group had relatively constant levels of carbohydrates, the second group accumulated high levels of carbohydrates during the light period, while the last group was in between. Apart from this grouping on the basis of the overall diurnal pattern of carbohydrate accumulation, some more differences were observed within each group: for example, the time at which the carbohydrate levels peaked during the day varied.

In this screen, the starch-deficient mutants *pgm* and *adg1*, which lack the ability for starch synthesis, showed a reduced growth rate and a smaller biomass during the vegetative phase that can be explained by changes in respiration caused by a higher turnover of soluble sugars (Schulze et al., 1991, 1994). Similarly, in the *sex* mutant, which does not mobilize starch, the growth rate and plant biomass accumulation are also hampered because part of the fixed carbon is not available for export from the leaves (Zeeman et al., 1998). This indicates the importance of transitory starch for plant growth.

The relationship between plant growth-related traits and carbohydrate content in the RILs of L*er* x Kond

Plant growth as well as carbohydrate content are complex polygenic traits; therefore, we used QTL analysis to clarify the genetic basis of the variation for both traits, which might reveal a common genetic basis. The overall significant positive correlations between plant rosette areas and plant biomass are presented in Table 5.3. This shows that non-destructive measurements of plant area well represent plant biomass accumulation, at least in the early stages of development (Leister et al., 1999; Chapter 4). The co-location of QTLs for plant areas, RGR (areas), DW1, DW2, RGR (DW), SLA, RLL, RoDW2 and Sh/Ro ratio with similar allele effects confirm that these parameters all represent (different) aspects of plant growth. The *ER* locus harbors a major QTL for these traits. El-Lithy et al., (2004) also found QTLs for leaf areas, RGR and DW that co-located at the *ER* locus in the RILs of (L*er* x Sha) which might suggest that *ER* is the likely candidate gene that plays an important role in plant growth regulation. This is confirmed by the phenotype of the NIL containing the wild type *ER* allele (Table 5.2). There are also other regions at the top of chromosome three, four and five

where several growth-related traits co-located. Four out of the five growth regions (GRR) that had been identified in the RIL of L*er* x Sha population (El-Lithy et al., 2004) are also detected in the present study, although some differences exist between similar regions, concerning the traits affected by the various regions. SLA, considered as a key factor for plant growth (Poorter, 2002), shows a significant positive correlation with RGR3-1(area) and RGR(DW) and a significant negative correlation with sugar content at 15 days, which might indicate that the plants invested more assimilates to build new tissues. The overall negative correlation between TLA2, TLA3, RGR3-1, SLA, RLL and RoDW2 from one side and the sugar levels of 15 days old plants might suggest that there is an imbalance between the source and the sink (roots at this stage) at the whole plant level (Paul and Foyer, 2001) or that the source activity outpaces the growth and/or the sink capacity (Geiger et al., 2000). This in agreement with a study done on *Lolium perenne* seedlings, where carbohydrate accumulation was inversely related to constitutive growth rate (Turner et al., 2001).

In the Bay-0 x Sha RIL population a QTL for WC has been found at the top of chromosome 4 (Loudet et al., 2003) that co-located with the QTL detected for WC1 and 2 at the same region in the present study. Water content at 15 and 25 days correlated negatively with sugars and starch contents, suggesting a link between carbohydrate content and the water availability in plant cells, although Poorter and Nagel (2000) stated that changes in carbon allocation are smaller in the case of limited water supply. In barley, the accumulation of sugars during the light period was accompanied by a rise in the tissue osmotic pressure (Koroleva et al., 2002). The two QTLs detected for WC1 and WC2 at top of chromosome four co-located with two QTLs for sugars with opposite allele effect that might partly explain this negative correlation. The positive correlation between RoL1 and WC1 and between RoL2 and WC2 in combination with the co-location of the QTLs for these two traits confirms the assumption that water turgor pressure in root cells might lead to cell expansion thus increasing root length.

It had been shown that the *ER* gene is involved in cell growth patterns in shoot apical meristems and in organ primordia initiated from the shoot apical meristem (Torii et al., 1996; Yokoyama et al., 1998; Shpak et al., 2003). In the present study, no QTLs could be detected at the *ER* locus for RoL, which indicates that the *ER* gene might not be involved in root elongation.

Chlorophyll fluorescence (ChlF) showed a positive correlation with Ra1L and Ra2L and one of the two detected QTLs for ChlF at the middle of chromosome four co-located with several sugar QTLs with similar direction, which confirm the relationship between the photosynthetic efficiency and sugar accumulation. Leaf chlorosis correlated also negatively with sugars content, which could suggest that a high level of sugars, mainly sucrose, induces leaf senescence (Yoshida, 2003). The alternative hypothesis is that when the leaves started to senesce there is a remobilization of sugars from senescing leaves which are transported into young leaves (Himelblau and Amasino, 2001). Starch content correlated positively with plant areas, RGR based on area or dry weight, DW2, SLA, RLL, RoDW2, RoL1 and 2, pointing out to the importance of starch synthesis and mobilization for plant growth. In agreement with

earlier studies (Caspar et al., 1985), the *sex* mutant, which has a changed synthesis and degradation of transitory starch, showed a reduction in plant growth.

The relationship between flowering-related traits and carbohydrate content in the RILs of L*er* x Kond

Earlier studies have shown that flower induction and opening might be due to a combination of sugar import and mobilization of various polysaccharides (Corbesier et al., 1998; Van Doorn and Van Meeteren, 2003). In this study we indeed found a significant positive correlation between carbohydrate content and flowering-related traits, although it should be stressed that the carbohydrate measurements were done before flowering. Flowering time and/or flowering-related QTLs co-located with sugars and/or starch QTLs at the bottom of chromosome one, two and three, around the *ER* locus and at the top of chromosome three, four and five. However, not all QTLs for these traits have similar allele effects, indicating that the correlations between carbohydrates and flowering time and flowering-related traits are complex.

The relationship between plant growth-related traits and flowering related traits in the RILs of L*er* x Kond

From the co-location of the QTLs detected for growth-related traits with QTLs found for flowering-related traits, three main regions where QTLs might explain this relationship will be discussed. The first one is around the *ER* locus, where several growth traits co-located with TPL, PLTS, INFL and RB with similar allele effects while they co-located with CLN and SB but with opposite allele effects. Similarly, in the L*er* x Sha RILs (El-Lithy et al., 2004) co-location between QTLs for growth-related traits with QTLs for TPL, PLTS, INFL, TLN and RLN were detected. From the previous findings we can conclude that *ER* has an effect on plant growth and flowering-related traits but not on the flowering time itself. This relationship can be seen clearly from the significant positive correlation between the majority of the growth-related traits with all plant length traits and with rosette branching (Table 5.3). This might suggest that large plants allow a larger increase in length and in the number of their rosette branches. Earlier studies showed that the *ER* gene is involved in a series of processes during Arabidopsis development, like internode and pedicle elongation, leaf and silique morphogenesis and thickness of stem tissue (Douglas et al., 2002).

The second and third regions are located at the top of chromosomes four and five where the growth-related QTLs co-located with opposite allele effect with QTLs detected for flowering-related traits. This co-location of the QTLs with opposite allele effect of these two sets of traits was supported also by the significant negative correlation found between FT, TLN, RLN, CLN and SB with the majority of growth traits (Table 5.3). Such correlation is understandable since early flowering plants have fewer leaves and fewer branches, which means less area and biomass as well. It is interesting that some FT QTL do not co-locate with growth (bottom 1, 2 and 5).

In general, plant growth was more closely linked to leaf transitory starch levels than to levels of soluble sugars. From the correlation and the co-locations of the QTLs for growth,

carbohydrate and flowering traits, we conclude that there is a complex relationship. The co-location of QTLs for different traits does not prove pleiotropy, because of the limited accuracy of QTL mapping. It could also be explained by the presence of a set of closely linked genes, each affecting different traits, which cannot be separated by QTL mapping. For confirmation of pleiotropic effects further fine mapping is required.

Materials and Methods

Plant material and growth conditions

Arabidopsis thaliana accessions were obtained from the Arabidopsis stock centers ABRC, NASC and Sendai (www.arabidopsis.org), supplemented with accessions recently collected by members of the Laboratory of Genetics at Wageningen University and deposited at ABRC and NASC. Details of all accessions used in this study are given as supplementary material. For the screening of the 123 accessions for their sugar and starch content at two time points, seeds were sown in Petri dishes on water-saturated filter paper followed by a 4-days cold treatment at 4 °C. They were then transferred to a climate room at 25°C and 16h light for two days before planting in 7-cm pots with standard soil. The plants (12 plants/accession) were grown in an air-conditioned greenhouse with 70 % relative humidity, supplemented with additional light (model SON-T plus 400W, Philips, Eindhoven, The Netherlands) providing a day-length of at least 16 h light (long day), with light intensity 125 µmol $m^{-2}s^{-1}$, and maintained at a temperature between 22-25 °C (day) and 18 °C (night).

To study the diurnal pattern of sugar and starch accumulation and for growth analyses, 23 accessions and three mutants (24 plants/accession) were selected and grown under controlled conditions in a growth cabinet, with 70 % relative humidity, 22^{o}C, 12h day length and light intensity 25 Wm^{-2}. Plants were placed on carts and the carts were shuffled daily within the growth cabinet to avoid minor environmental differences within the cabinet. In all descriptions of experiments, time (days) is referred to as days after planting.

The new RIL population was obtained from a cross between the accessions Landsberg *erecta* as a female (L*er*, N20) and Kondara (Kond, CS6175) as pollen parent (details of genotyping and map construction are mentioned in Chapter 3). The F1 seeds were grown and allowed for self-fertilization to get the F2 seeds. From the F2 seeds a set of 120 RILs have been generated by single-seed descent procedure until the F9 generation that had been used for DNA extraction and genotyping. In order to minimize any bias in the selection of plants taken to the next generation, 6 individuals per RIL were planted and plant number 3 was selected to go on for the next generation with the fifth one as a back up.

For the analysis of sugars, starch, growth-related traits and flowering-related traits, the F10 seeds were transplanted in agar filled tubes and grown on hydroponics solution (Tocquin et al., 2003). The agar filled tubes were prepared by cutting a small part of the conical end of the 0.5 ml eppen-dorf tubes (SARSTEDT, 72.698.200) then filled by 0.55% DAISHIN agar (DUCHEFA BIOCHEMIE, D1004.1000) and left to solidify. The agar filled tubes were placed in holes prepared in black plastic sheets used as lids for the gray containers (46 x 31 x 8 cm, FAVORITE, 36.140) containing 8 liters of the hydroponics solution. The experiment was carried out in a randomized two-block design containing 18 plants per RIL of the F10 seeds. Seeds were sown in Petri dishes on water-saturated filter paper followed by a 4-days cold treatment at 4 °C, and then transferred to a climate room at 25°C and 16h light for one day before transplanting in the agar filled tubes. The experiment was also carried out in a growth cabinet under the same growth conditions mentioned before for growing the 23

Arabidopsis accessions. A line with the *ERECTA* wild type allele in the L*er* genetic background and both parents were included in the experiment.

Measurement of sugars and starch

For analyses and quantification of soluble sugars (glucose, fructose and sucrose) and starch, for the screening of 123 Arabidopsis accessions or for determining the diurnal pattern of the 23 accessions plus the three mutants, three leaves per plant were sampled together and immediately frozen in liquid nitrogen. This analysis was carried for three plants per accession at each time point. For the analysis of carbohydrate levels in the RILs of (L*er* x Kond), whole rosette leaves and roots were harvested separately for all the measurements, and then freeze-dried. The freeze-dried materials were ground, and samples between 5-10 mg were weighed. Two plants per block for each RIL were harvested for the analysis of sugars and starch.

Sugars were extracted by boiling in 80% methanol and quantified as described by Bentsink et al. (2000), with one exception, that the soluble sugars were separated by elution in an increasing linear gradient concentration of NaOH (20-150mM), with a flow rate of 1mL per minute. Starch was determined from the pellets of the sugar extractions, after extensive washing with water. Starch is determined as glucose, using a commercially available kit (EnzyPlus starch test kit, EZO 942).

Digital imaging, computer analysis and RGR determination

The mean total leaf area (TLA) of each accession was obtained by imaging 20 to 24 plants per accession at 10 (TLA1), 15 (TLA2) and 20 (TLA3) days after transferring the seedlings to the pots. The mean total leaf area (TLA) of each RIL was obtained by imaging 5 plants per RIL at the same time points after transferring the seedlings to the agar filled tubes. Leaf areas were determined with an image processing technique, using a Nikon digital camera (model COOLPIX 950) (Nikon Corporation Imaging Products Division, Shinagawa-Ku, Tokyo, Japan), and analysis of the pictures using the computer program MetaMorph (version 4.01) (Universal Imaging Corporation, West Chester, USA, www.imagel.com). The relative growth rate (RGR), on the basis of rosette areas, was calculated according to the following equation: $(\ln A_x - \ln A_y)/dt_{(x-y)}$, where "A" is the rosette area measured at "x" and "y" the second and the first time points, and "dt" is the time difference in days between these two points. RGR was calculated for each line based on the three measurements of rosette area, resulting in RGR2-1, RGR3-2 and RGR 3-1, referring to RGRs in the intervals 10 to15, 15 to 20, and 10 to 20 days, respectively.

Weight, water content, shoot/root ratio, RGR, SLA and root length determinations

The mean fresh weights (FW) of shoots for each RIL were determined at day 15 and 25, by harvesting and weighing two plants per line from each block. Dry weights (DW) of shoots (at day 15 and 25) and roots (only at day 25) were determined after drying the plants at 105^{o}C for 48 h. The water content (WC) of shoots was estimated as the relative ratio between water and dry weight using the formula [(FW-DW)/FW]*100. The shoot/root ratio (Sh:Ro) was calculated as shoot dry weight divided by root dry weight at day 25. The relative growth

rate as based on dry weight of shoots (RGRsdw) was calculated in the same way as RGR based on leaf area. The specific leaf area (SLA) was calculated as area divided by weight ($mm^2.mg^{-1}$). Plant root length was measured at day 15 and 25 using a ruler for two plants for each RIL from each block.

Chlorophyll fluorescence, chlorosis and rosette leaf length measurements

Chlorophyll fluorescence as a non-destructive means of photosynthetic capacity was obtained for each RIL by measuring three different leaves per plant for three plants for each RIL from each block. The measurements were done by using a MINI-PAM (S/N: 0133) (WALZ Mess- und Regeltechnik, Effeltrich, Germany), with the determination of the effective quantum yield of photosynthetic energy conversion (Yield = Δ F/Fm') for dark non-adapted leaves (Van Kooten and Snel, 1990). ChlF was measured at 125 µmol $m^{-2}s^{-1}$ light intensity, 70 % relative humidity and 22 °C.

Chlorosis was estimated at day 48, by scoring the color of the whole rosette leaves on a scale ranging from 1 (dark green) to 7 (yellow). Rosette leaf length was measured upon flowering using a ruler from the plant stem till the tip of the three largest leaves per plant for three plants for each RIL from each block.

Measurement of flowering time and related traits in RILs

From the hydroponics experiment, in which 18 plants/RIL were grown in SD condition, FT for three plants was recorded as the number of days from planting till the opening of the first flower. Flowering time was also scored by counting the total leaf number (TLN) i.e., rosette leaf number (RLN) plus cauline leaf number (CLN), excluding the cotyledons, since there is a close correlation between leaf number and flowering time (Koornneef et al., 1991). The following traits were also recorded: total plant length (TPL), plant length till first silique (PLTS), inflorescence length (INFL), rosette branches (RB), stem branches (SB) and total number of side shoots or inflorescences (TNB) (number of branches in the main inflorescence plus the number of side shoots from the rosette).

Genetic mapping

The mapping of the segregating population was done by using 51 SNPWave, 23 SSLP markers and the morphological marker *erecta*, located at a distance from 0.5 –13 cM on the genetic map to obtain a regular distribution among the five chromosomes. These 75 markers were used to generate the linkage map; details are given in Chapter 3. This map was used for QTL analysis of the various traits used in this work.

QTL mapping and statistical analysis

The software package MapQTL® 5 was used to identify and locate QTL on the linkage map by using interval mapping and multiple-QTL model (MQM) mapping methods as described in its reference manual (www.kyazma.nl). In a first step, putative QTLs were identified using interval mapping. Thereafter, the closest marker at each putative QTL was selected as a cofactor (van Ooijen and Maliepaard, 1996; van Ooijen, 2000) and the selected

markers were used as genetic background controls in the approximate multiple QTL model of MapQTL. LOD threshold values applied to declare the presence of QTLs were estimated by performing the permutation tests implemented in MapQTL version 5.0 using at least 1000 permutations of the original data set, resulting in a 95% LOD threshold at 2.4. Two-LOD support intervals were established as 95% QTL confidence interval (van Ooijen, 1999) using restricted MQM mapping implemented with MapQTL. The estimated additive genetic effect and the percentage of variance explained by each QTL and the total variance explained by all the QTLs affecting a trait were obtained using MQM mapping.

Heritability (broad sense) was estimated as the proportion of variance explained by between-line differences using the general linear model module of the statistical package of SPSS version 11.0.1 (SPSS Inc., Chicago, IL) based on measurements on 4-18 plants.

The relation between the 123 and the 23 Arabidopsis accessions was described with the neighbor joining (NJ) and the principle component analysis (PCA) respectively, using NTSYSpc version 2.10t. (Rohlf, 2001) with standardized data. For the PCA the standardized data were converted in a correlation matrix from which three eigenvectors were extracted using the EIGEN function of the NTSYS-pc program. The relations between the 45 studied traits in the RILs of (L*er* x Kond) were performed by using the Pearson Correlation coefficients and PCA of the statistical package of SPSS version 11.0.1 (SPSS Inc., Chicago, IL).

Chapter 6

Summarizing discussion

Improving agricultural production in a sustainable way is a challenge for scientists nowadays. Plant breeding has been and will continue to be an important technology to achieve this goal. Plant scientists are using the available genetic and technological resources to improve yield, quality of plant products and plant tolerance to biotic and abiotic stresses. The development in molecular genetics has allowed the use of information about genes for breeding. Applications of molecular techniques include the transfer of genes but also the use of genetic markers for marker assisted selection. The latter is attractive for complex traits such as yield, for which environmental factors strongly influence the expression of these often polygenic traits. Preferably markers should be in the genes controlling the traits that one selects for (Andersen and Lubberstedt, 2003). Knowledge about the genes underlying complex traits can be based on the genetic mapping of the genes underlying the observed genetic variation followed by the identification of these genes. The cloning of genes controlling natural variation has started (Alonso-Blanco et al., 2005) but is still difficult in crop plants where the identification of functional markers is based mainly on candidate genes that co-locate with QTLs. An unbiased approach for the identification of genes controlling quantitative traits (QTL) is easier in well studied model plants because of the efficiency of the experimental approaches to be used, and the already available knowledge and resources.

For these reasons, *Arabidopsis thaliana* has been chosen as a major model plant suitable for genetic and molecular research, because of its small size, short life cycle, and small genome of about 130 megabases, organized in 5 chromosomes and estimated to contain approximately 30,000 genes. This has led to the establishment of a large research community with important biological and molecular resources available (Meinke et al., 1998). The existing genetic variation among and within naturally occurring populations of Arabidopsis, collected from different geographical regions (Rédei, 1970; Alonso-Blanco and Koornneef, 2000; Koornneef et al., 2004) provides an important source of genetic variation that can be used to study the function of genes.

The main aim of the work presented in this thesis was to increase the knowledge about the physiology and the genetics of plant performance. For that reason, we have focused on traits associated with the gain and the allocation of carbon aiming to investigate if there is relationship with various growth parameters as well as with flowering-related traits.

Plant performance depends mainly on the acquisition of raw material (via photosynthesis and mineral uptake), the allocation of such materials to the plant organs, their storage and/or use (respiration) and the ability to cope with environmental stresses. Photosynthesis is a complex chloroplast-located process, controlled by both nuclear and plastidic genes and considered as a central step in determining plant growth and productivity.

The natural variation for photosynthetic characters within Arabidopsis accessions is described in Chapter 2. Natural variation for chlorophyll fluorescence, a property that is characteristic for photosynthetic performance was studied in 127 Arabidopsis accessions using a non-destructive measurement of photosynthetic yield. This screen revealed one

accession (Ely), exhibiting photosynthetic characteristics markedly different from all others, whereas the other accessions showed only small, although sometimes significant differences.

Detailed physiological analyses showed reduced fitness for Ely compared to the standard laboratory strain L*er* and also for genotypes with mainly L*er* nuclear background but with Ely derived organelles. At low temperature (15 °C), Ely had a higher electron transport rate than L*er*, indicating increased photosystem II efficiency under this condition, while at high temperature (30 °C) the opposite was observed. Ely had an increased sensitivity to UV-B radiation and a high resistance to atrazine compared to L*er*. The findings that Ely was atrazine-resistant and that the chlorophyll fluorescence related traits were maternally inherited pointed towards chloroplast-located gene(s). Sequencing the *psbA* gene, encoding the D1 protein of photosystem II, revealed a point mutation causing the same amino acid change as found in other atrazine-resistant species (Oettmeier, 1999) providing the genetic cause of the atrazine resistance and the changes in the photosynthetic characteristics of Ely.

The significant differences in phenotype (the quantum yield) between Ely and the reciprocal crosses with L*er* suggested additional nuclear encoded genetic control for the trait. Since the major difference between L*er* and Ely was due to non-nuclear genes, it was decided not to pursue further identification of the nuclear variation by making a segregating population using these two accessions as parents. However, nuclear genetic variation could be shown by detecting one and two quantitative trait loci (QTLs) for quantum yield mapped in two different population (L*er* x Sha, Chapter 4) and (L*er* x Kond, Chapter 5), respectively.

The variation we found among Arabidopsis accessions for quantum yield was relatively small. Therefore, we conclude that the photosynthetic yield is highly conserved and that only severe selection pressure results in marked variations in photosynthetic performance. However, it is still possible that more variation can be found between accessions when experiments are carried out at different conditions and/or by screening for more complex photosynthetic parameters such as photochemical quenching (qP), nonphotochemical quenching (qN), CO_2 uptake and O_2 evolution in addition to the photosynthetic yield.

Based on the natural variation that we found within Arabidopsis accessions for different traits that related to plant growth, carbohydrate content and flowering time, four new recombinant inbred line (RIL) populations of Arabidopsis were developed. The reference laboratory accession Landsberg *erecta* (L*er*) was crossed as a female with Kashmir (Kas-2), Kondara (Kond) and Shakdara (Sha) as pollen parent, while Antwerp (An-1) was reciprocally crossed with L*er*. The construction and the genotyping of the three populations (L*er* x An-1, L*er* x Kas-2 and L*er* x Kond) using SNPWave and SSLP markers are described in Chapter 3. Flowering time (FT) was analysed to indicate the usefulness of these three new populations. The use of similar markers that are anchored to the physical map of Arabidopsis allowed a direct comparison of QTL map positions in the different populations.

When screening 92 Arabidopsis accessions, it appeared that about 50% (37.6 to 62.5 %) of the SNPWave markers that are polymorphic between L*er* and Col-0 can be useful to

genotype populations made from crosses with the L*er* accession. Because the SNPWave markers did not cover some regions in the three populations, standard PCR markers were added to obtain genetic maps with equally distributed markers. This analysis showed that map lengths are quite similar and also in the same range as those published for other populations (Lister and Dean 1993; Alonso-Blanco et al., 1998b; Loudet et al., 2002; Clerkx et al., 2004).

Flowering time variation was analyzed in the three recombinant inbred line populations under long day (LD) conditions (16h light/8h dark). Per population, 4, 6 and 8 QTLs were detected. Relating the map positions and the two-LOD intervals to the physical map based on the Col-0 sequence allowed the comparison between the QTLs detected for FT in four populations at LD (L*er* x An-1, L*er* x Kas-2, L*er* x Kond and L*er* x Sha) and at short day (SD) (L*er* x Kond and L*er* x Sha) conditions (Chapter 3, 4 and 5). The comparison of the QTL positions that related to the physical map allowed the estimation of approximately 12 - 16 different QTLs segregating for flowering time (Fig. 6.1). In two regions FT QTLs were detected in the four populations in LD condition, viz., the QTLs on top of chromosome 3 (around nga172) for which the L*er* allele delayed flowering and the QTLs located around SNP130 (chromosome 5) for which the L*er* allele accelerated flowering. In the latter region on chromosome 5, two QTLs have been detected in the RILs of the (L*er* x Cvi) population (Alonso-Blanco et al., 1998a) and these are most likely the *FLC* and *HUA* genes (Doyle et al., 2005). From the present map positions it is not clear whether Kas-2 and An-1 contain an active *FLC* allele (Fig. 6.1). However the presence of *FLC* in Kond is most likely. A FT QTL was detected around the *FRI* locus for three populations and *FRI* might be the gene encoding for this QTL (Gazzani et al., 2003; Michaels et al., 2003). The slightly different map position of the An-1 QTL from the *FRI* locus and the reverse allele effect suggest that this QTL represents a different locus. At the bottom of chromosome 5 around K8A10 a QTL where the L*er* allele delayed flowering was found in the populations with the three Asian accessions. In addition there are several QTLs common between two of the four populations or present in only one population (Fig. 6.1). Some of the QTLs detected for flowering time overlapped with loci detected in other RIL populations, but also new loci were identified.

From these data we can conclude that different populations might segregate for different loci depending on the genetic composition of their parental lines. A comparison between multiple crosses is needed to get an impression about the total genetic variation present among Arabidopsis accessions for a complex trait such as flowering time.

To study growth-related traits, we compared approximately 127 Arabidopsis accessions for carbohydrate content and a subset was analysed in more detail. We decided to study RIL populations derived from crosses of L*er* with the Sha and Kond accessions to perform QTL analysis for growth and growth-related traits. Because of the expected consequences of flowering on growth this trait was also analysed in the same material.

The screening and the QTL analysis for growth are described in Chapter 4. Screening of 22 accessions revealed a large variation for seed weight, plant dry weight and relative growth rate but not for water content. A positive significant correlation was observed between

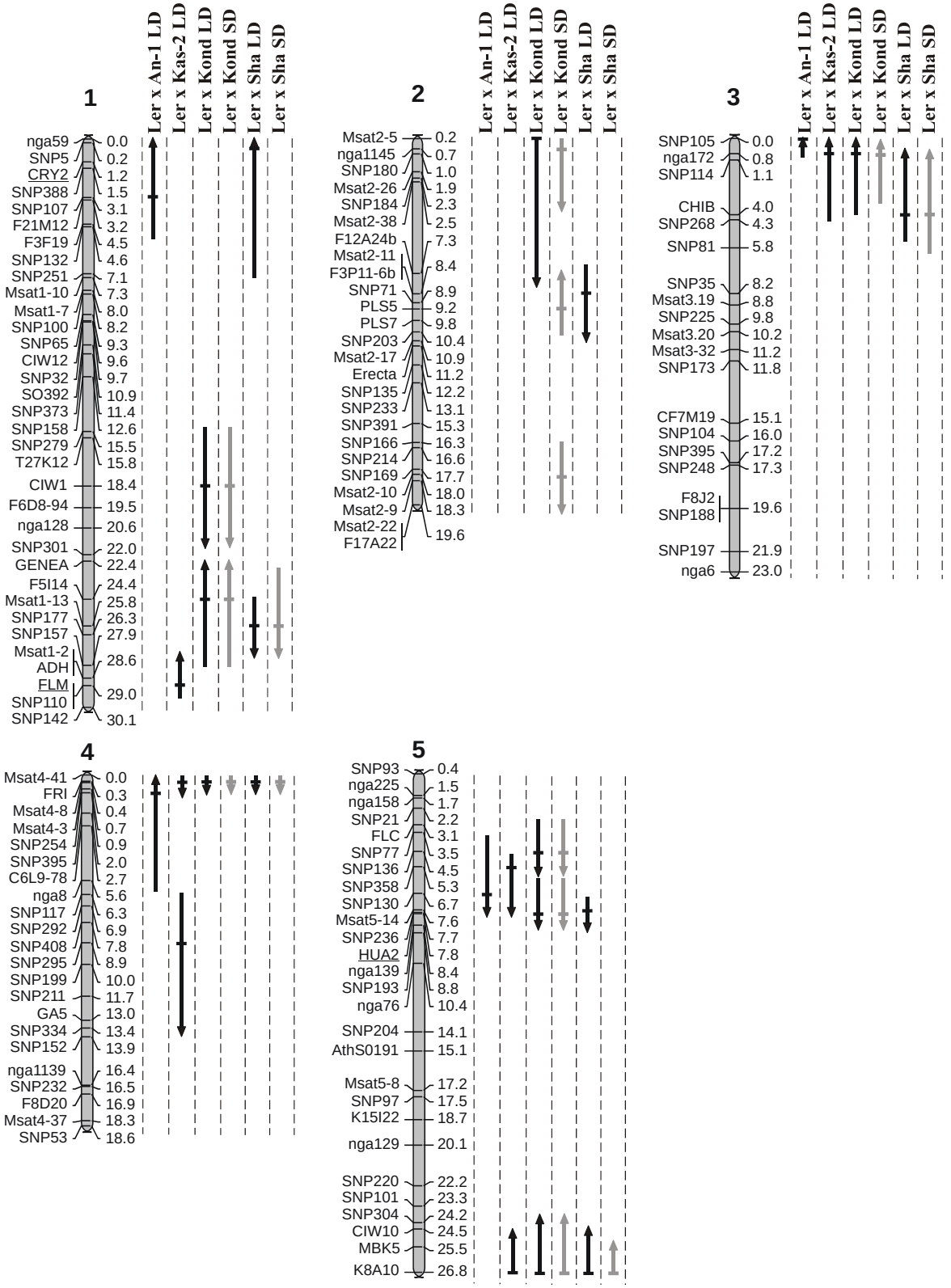


Figure 6.1: Combined physical map of L*er* x An-1, L*er* x Kas-2, L*er* x Kond and L*er* x Sha containing markers that are used in genotyping these populations, in addition to several candidate genes (*CRY*2, *FLM*, *HAU*2). Flowering time (FT) QTLs are indicated by arrows along the chromosomes. The black and gray arrows indicate the FT QTLs detected in long (LD) and short (SD) day conditions, respectively. Horizontal dashes in the arrows indicate the marker fixed during the MQM mapping analysis. The lengths of the arrows indicate the 2-LOD support intervals. The direction of the arrows indicates the allelic effect: upward, L*er* increasing the FT and the other allele decreasing; downward, the non-L*er* allele increasing and L*er* decreasing.

seed weight and plant area at 10 days but not at later stages, suggesting that seed weight affects early plant growth while during later stages other factors might determine plant growth rate.

QTL analysis, using the recombinant inbred lines (RILs) derived from the cross between L*er* and Sha, revealed five genomic regions where QTLs for growth traits (plant areas, dry weight and RGR) are located. The first region was around msat1-10 (chromosome 1), which affects all the growth parameters, while in L*er* x Kond RILs (Chapter 5), around this region no growth QTLs were found. In the Bay-0 x Sha RIL population a locus for dry mass at the same place had been identified as DM10.1 (Loudet et al., 2003). The second growth region was around *ER* where the majority of the growth traits in L*er* x Sha and L*er* x Kond were detected. It has been shown before in both the Col x L*er* and L*er* x Cvi populations that the *ER* locus usually contributes to plant length and leaf size (Alonso-Blanco et al., 1999, Ungerer et al., 2002, Pérez-Pérez et al., 2002).

The third growth region was found at the top of chromosome 3, mainly affecting early growth. Loudet et al. (2003) also found a QTL for dry mass named DM3.2 at the same position. The fourth and the fifth regions where growth QTLs were detected in the L*er* x Sha population are at the top and the bottom of chromosome 5. Loudet et al. (2003) identified a locus on top of chromosome five described as DM10.7.

In the L*er* x Kond population few QTLs for growth traits could be detected in the same regions, except for the *ER* region. This can be due to the differences in genotype and/or the use of the cultivation system.

However, in the L*er* x Kond population an additional growth region was found at the top of chromosome 4 that did not show up in the L*er* x Sha population. In this region the flowering time QTL, which most likely is encoded by the *FRI* locus was detected in both populations suggesting that this locus may not encode the growth QTL in Kondara. Considering the two populations L*er* x Sha and L*er* x Kond, no consistent relation between growth traits and flowering time was observed despite some co-locations.

Because it was expected that growth differences would be related to the primary metabolism of the plant, sugar and starch accumulation was analysed in a set of 123 Arabidopsis accessions (Chapter 5). The majority of the accessions accumulate carbohydrates in their leaves at the end of the light period as expected but a large variation was observed in the levels of the accumulated carbohydrates and the data of some accessions suggested differences in their diurnal pattern. Studying in more detail the diurnal pattern of 23 selected Arabidopsis accessions in addition to three mutants (*pgm*, *adg1* and *sex)*, revealed three different groups of genotypes, whereas the mutants were clearly distinct from the accessions and showed reduced growth rates. The first group of accessions, including L*er*, exhibited a nearly constant level of carbohydrates over the day. The second group accumulated high levels of sugars and starch during the day. Kond was an example of this group. The third group had intermediate levels of carbohydrates during the day.

Growing the RILs of L*er* x Kond on hydroponics allowed the study of the accumulation of photoassimilates in leaves and in roots, thus revealing data on shoot-root ratios, in combination with other growth-related traits (Chapter 5).

QTL analysis revealed QTLs for the different aspects of plant growth-related traits, sugars and starch contents and flowering-related traits. Co-locations of QTLs for different traits were detected in different regions, mainly at the *ER* locus, at the top of chromosome 3 and 4 and at the bottom of chromosome 5. In general, plant growth was more closely linked to leaf transitory starch levels than to the soluble sugar levels. This is consistent with earlier studies based on starch deficient mutants whose growth was reduced severely (Schulze et al., 1991, 1994). From the significant correlations and the co-locations of the QTLs for these aspects, we conclude that there is a complex relationship between plant growth-related traits, carbohydrate content and flowering-related traits.

This study shows that *Arabidopsis thaliana* natural variation can successfully be used to study the genetic basis of complex traits like plant growth, carbohydrate levels and flowering related traits. In this study we could test if there is a correlation between these different aspects of plant performance based on co-locations of QTLs for these traits. The present study showed that QTLs for all of these traits can be identified and that they may co-locate using genetically diverse material (comparing this study with that of Loudet et al., 2003). However, the QTLs detected indicated a complex relationship between growth, carbohydrate content and flowering traits and further studies will be required to unravel these relationships. Changing environmental parameters and sampling at other time points might increase the physiological relevance of this analysis. Using near isogenic lines (NILs) to confirm these QTLs and their fine-mapping are the next steps which might lead to the cloning of the genes that underlie the allelic variation for the QTLs detected here. Unraveling the molecular functions of these genes will help understanding their physiological effects. It then remains to be elucidated whether similar genes control genetic variation for growth and yield in crop plants.

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Samenvatting

De opbrengst van een gewas hangt af van de totale hoeveelheid biomassa die er tijdens het groeiseizoen wordt geproduceerd en de manier waarop dit in de plant wordt verdeeld over de verschillende organen. Biomassaproductie is, behalve van water- en mineraalvoorziening, grotendeels afhankelijk van fotosynthese. De verdeling van de drogestof, gevormd via fotosynthese, bepaalt in hoge mate hoeveel oogstbaar product er uiteindelijk wordt gevormd, bijvoorbeeld de hoeveelheid graankorrels ten opzichte van de hoeveelheid stro.

Deze processen zijn enerzijds afhankelijk van genetische eigenschappen van de plant en anderzijds van omgevingsfactoren, zoals temperatuur, bemesting, en waterbeschikbaarheid. Daarnaast spelen biotische en abiotische stress en de reactie van de plant hierop een belangrijke rol. De hier geschetste eigenschappen van de plant worden naar verwachting bepaald door een groot aantal genen. Het ophelderen van de (functie van) genen betrokken bij een bepaald proces is mogelijk door bestudering van de natuurlijke variatie voor dat proces, die aanwezig kan zijn binnen een soort. Tot voor kort was dit moeilijk, zo niet onmogelijk, voor complexe kwantitatieve en polygene eigenschappen zoals groei. De aangewezen benadering hierbij is QTL analyse: analyse van Quantitative Trait Loci. Door de recente ontwikkeling van moleculaire merkers zijn dit soort eigenschappen sinds een aantal jaren veel beter bestudeerbaar geworden. Moleculaire merkers worden gaandeweg ook steeds belangrijker in de veredeling. Voor het begrijpen van complexe processen en het ontwikkelen van geschikte merkers is de beschikbaarheid van nauwkeurige genetische kaarten van een gewas van groot belang. Om de haalbaarheid te testen van een genetische en later een moleculaire analyse van plant performance, is voor het onderzoek, beschreven in dit proefschrift, gebruik gemaakt van een modelplant, de zandraket (*Arabidopsis thaliana*). De voordelen van deze plant zijn: de geringe afmetingen, een korte generatieduur en een relatief klein genoom, dat bovendien geheel ‘gesequenced’ is. Vanwege deze voordelen wordt deze plant zeer veel gebruikt in fundamenteel onderzoek, waardoor al veel kennis beschikbaar is waarop kan worden voortgebouwd.

Het doel van het onderzoek, beschreven in dit proefschrift, is het bestuderen van de fysiologie en de genetica van ‘plant performance’, d.w.z., groei en ontwikkeling van de plant, inclusief verdeling van biomassa binnen de plant. Hierbij is gebruik gemaakt van natuurlijke variatie binnen Arabidopsis voor eigenschappen die relevant geacht werden: fotosynthese, groeisnelheid, koolhydraatmetabolisme en bloei.

In hoofdstuk 2 is de natuurlijke variatie voor fotosynthese in Arabidopsis bestudeerd door de fluorescentie van chlorofyl te meten. Dit is een eenvoudige en niet-destructieve maat voor de efficiëntie van fotosynthese. Vergelijking van de fluorescentie van 127 accessies (ook wel ecotypen genoemd) resulteerde in slechts één accessie (Ely) met een sterk afwijkend gedrag. Uit een nauwkeurige fysiologische en genetische analyse van deze lijn, in vergelijking

met de standaardlijn Landsberg erecta (L*er*) bleek dat het afwijkend fotosynthese-gedrag overerfde via het cytoplasma, en niet via de kern, hetgeen erop wees dat deze eigenschap waarschijnlijk gecodeerd wordt door het genoom van de chloroplast. Verdere analyse toonde aan dat Ely sterk leek op mutanten van andere plantensoorten die ongevoelig zijn voor het onkruidverdelgingsmiddel atrazine. Dit vermoeden werd bevestigd door enerzijds de gevoeligheid van Ely voor atrazine te bepalen (veel minder gevoelig dan L*er*) en anderzijds door de basenvolgorde van het gen, coderend voor het D1 eiwit van fotosysteem II te bepalen. Dit vertoonde een mutatie ten opzichte van het wildtype, waarvan bekend is dat deze kenmerkend is voor atrazine-resistentie.

Naast dit verschil waren er ook andere, kleinere, verschillen in efficiëntie van fotosynthese tussen L*er* en Ely, die in de kern gecodeerd werden. Omdat het voornaamste verschil veroorzaakt werd door een chloroplast-gen, is besloten om verder identificatie van natuurlijke variatie voor fotosynthese niet te onderzoeken door het maken van een splitsende populatie uitgaande van Ely en L*er*. Dit is wel gedaan in twee andere populaties (L*er* x Sha, hoofdstuk 4 en L*er* x Kond, hoofdstuk 5), waarbij bleek dat deze variatie klein was. Desondanks zijn er wel QTLs voor chlorofyl-fluorescentie gevonden. De conclusie uit dit deel van het onderzoek is dat er slechts een geringe mate van natuurlijke variatie is voor eigenschappen gerelateerd aan fotosynthese. De variatie die gevonden is (in Ely) was zeer waarschijnlijk het resultaat van sterke niet-natuurlijke selectie door herhaald bespuiten met herbiciden.

Op basis van natuurlijke variatie voor een groot aantal eigenschappen, gerelateerd aan groei, koolhydraatgehalte en bloeitijdstip, zijn vier nieuwe zogenaamde RIL (recombinant inbred line) populaties gemaakt. De standaard lijn L*er* is gekruist met de accessies Kashmir (Kas-2), Kondara (Kond), Shakdara (Sha) en Antwerpen (An-1). De genotypering van de populaties die hieruit ontstonden is gedaan met de SNPWave techniek en met PCR-gebaseerde merkers, en is beschreven in hoofdstuk 3. De gevonden genetische kaarten van deze populaties hadden ongeveer dezelfde lengte als eerder beschreven kaarten.

Voor alle populaties is de eigenschap ‘bloeitijdstip’ bepaald. Omdat de genotypering van alle populaties met identieke merkers is gedaan, die gekoppeld konden worden met de fysische kaart, kunnen de QTLs, gevonden voor de verschillende populaties, direct vergeleken worden. In totaal werden 12-16 verschillende QTLs gevonden voor bloeitijdstip, sommigen in alle populaties, en andere slechts in een of twee. Uit dit onderzoek blijkt dat meerdere splitsende populaties gemaakt moeten worden om een indruk te krijgen van de totale genetische variatie binnen een soort voor complexe eigenschappen zoals bloei.

Voor de analyse van groei-gerelateerde eigenschappen hebben we ruim 120 Arabidopsis accessies vergeleken. Een deel hiervan is in meer detail bestudeerd ten aanzien van koolhydraat-eigenschappen. Op basis hiervan is besloten de RIL populaties (L*er* x Sha) en (L*er* x Kond) te gebruiken voor QTL analyses.

In hoofdstuk 4 bleek uit een vergelijking van 22 accessies dat er grote variatie is voor de eigenschappen ‘zaadgewicht’, ‘plant drooggewicht’ en ‘relatieve groeisnelheid’, maar niet voor ‘watergehalte’. De positieve correlatie tussen zaadgewicht en plantgrootte (op dag 10) suggereert dat zaadgewicht de initiële groei van de plant beïnvloedt, maar dat andere factoren verantwoordelijk zijn voor de groei in latere stadia.

In de (L*er* x Sha) RIL populatie werden 5 chromosomale gebieden gevonden die QTLs bevatten voor groei-gerelateerde eigenschappen. Een hiervan, rond het erecta-gen op chromosoom 2, wordt zeer waarschijnlijk veroorzaakt door dit gen. Deze QTL werd ook gevonden in de L*er* x Kond populatie (hoofdstuk 5). Verder was er slechts geringe overlap tussen groei-QTLs, gevonden in de twee RIL populaties. Dit kan veroorzaakt zijn door genetische verschillen tussen de populaties, maar kan ook veroorzaakt zijn door verschillende kweekmethoden: de L*er* x Sha populatie is opgekweekt in grond, terwijl de L*er* x Kond populatie in watercultuur is gekweekt.

Er werd vanuit gegaan dat groei van de plant gerelateerd zou zijn met primair metabolisme, en daarom zijn suiker- en zetmeelgehalten in 123 accessies van Arabidopsis bepaald (hoofdstuk 5). Zoals verwacht, vertoonde het merendeel van de accessies een ophoping van koolhydraten aan het eind van de lichtperiode, maar er was een grote variatie in de gehalten, en het leek erop dat sommige accessies een dag-nacht patroon vertoonden dat verschilde van de meeste andere. In een beperkte set accessies is dit dag-nacht patroon verder bestudeerd, waarbij ook het gedrag van drie mutanten, die een verstoring in het zetmeelmetabolisme hebben, is bekeken. De bestudeerde accessies vielen in drie groepen uiteen, die elk verschilden van de mutanten. De eerste groep, waartoe ook L*er* behoorde, heeft een vrijwel constant gehalte aan koolhydraten in de bladeren gedurende een 24-h cyclus. De tweede groep vertoont een sterke ophoping van koolhydraten gedurende de dag, terwijl de derde groep een tussenliggend patroon heeft.

De (L*er* x Kond) RIL populatie is opgekweekt in watercultuur, zodat ook de groei en koolhydraatgehalten van de wortels gemeten konden worden. Voor vrijwel alle eigenschappen die gemeten zijn konden QTLs worden bepaald. Op verscheidene posities vielen QTLs voor de diverse eigenschappen samen, wat kan wijzen op pleiotropie. In het algemeen bleek dat groei van de plant meer gerelateerd was aan de accumulatie van zetmeel in de bladeren, dan aan de ophoping van oplosbare suikers (hexoses en sucrose).

Uit het hier gepresenteerde onderzoek blijkt dat Arabidopsis met succes gebruikt kan worden om de genetische basis van complexe eigenschappen zoals groei, koolhydraatmetabolisme en bloei te bestuderen. Voor de meeste eigenschappen werden QTLs gevonden, die soms samenvielen. Bovendien werden loci geïdentificeerd die mogelijk identiek zijn aan loci gevonden door anderen, in andere populaties. De gegevens wijzen erop dat de relaties tussen groei, koolhydraatmetabolisme en bloei, ingewikkeld zijn. Voortgezet onderzoek is nodig om na te gaan of de hier gesuggereerde relaties inderdaad relevant zijn. Dit kan het best gebeuren met zogenaamde bijna-isogene lijnen (near-isogenic lines, NILs), waarbij een klein deel van het genoom van een van de accessies is ingekruist in de standaard

lijn L*er*. Tevens kan hiermee de exacte positie van de QTL beter worden bepaald, uiteindelijk resulterend in het kloneren van het betreffende gen en de analyse van de fysiologische functie. Daarna resteert nog de vraag of deze processen in economisch interessante gewassen ook door soortgelijke genen worden gestuurd.

Publications

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Curriculum vitae

The author of this dissertation, Mohamed Ezzat Mahmoud El-Lithy, was born on January 1st, 1968 in Shebin El-Kom, Menoufia, Egypt. After finishing high school in 1985, he joined the Faculty of Sc. (Botany Department), Menoufia University, Egypt. He obtained his B.Sc. degree in Botany in May 1989, where he became the first among all his colleagues and he graduated with the general grade Very Good with Honor degree. In 1990, he was nominated to work as a demonstrator in the same department, and after joining the Egyptian army for 14 months, he took the responsibility of doing his research and helping in teaching within the University. He started his M. Sc. post-graduate courses in September 1991 for one year. In September 1996, he got his M. Sc. degree in Botany (Plant Ecology) entitled “Study on the development and chemical composition of some laticiferous plants”. From that date till now he is appointed as assistant lecturer in Botany Department, Faculty of Sc., Menoufia University, Egypt. He was also involved in helping teaching practical courses in the University. In 1999, the author was awarded a fellowship by the Egyptian Ministry of Higher Education to obtain his Ph.D. degree. In August 2000, he joined two groups; the Laboratory of Genetics and the Laboratory of Plant Physiology, Plant Sc. Department, Wageningen University, The Netherlands, where he finished his Ph.D. thesis entitled “Plant performance: a physiological and genetic analysis using Arabidopsis thaliana natural variation”.

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As Egypt is proud and famous by the three Pyramids, I do believe that Wageningen University should be proud by having three huge worldwide scientists like Maarten, Linus and Dick.

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I would like to take this opportunity to express my deep heartily thanks and appreciation to my lovely parents, to the spirit of my parent's in-law "mercy from ALLAH goes to them". Very deep gratitude to my brother Sherif and his wife Asmaá, my sisters Eman and Neivein and their husbands Mohsin Gonim and Aiman El-Hadad, for their emotional support and kind wishes. Thanks also to my uncles Mr. Tawfik El-Lithy, Mr. Moktar El-Mahdy, Mr. Mohamed Showman, Mr. Mohamed, Abd El-Aziz and Kamis El-Ngar and their families. Special thanks to my brothers in-law Magdy and Emad, and my sister in-law Salwa and her husband Mohamed Mesilhy and my wife's uncles Mr. Abd-Elmapod and Mr. Abd-Elzaher Shálan. Especial thanks to our great friends, Mrs. Amal El-Bakry, Mr. Mohamed El-Azmy, Mr. Said El-Mikanin and their families for their moral encouragement and support, and also my thanks to all my friends in Egypt. To all of you I dedicate this thesis.

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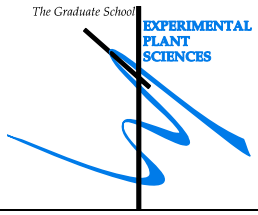
Mohamed E. El-Lithy, June 2005.

Education Statement of

the Graduate School

Experimental Plant Sciences



1) Start-up phase	*date*
First presentation of your project	August 2001
Writing a project proposal	2001
Subtotal Start-up phase	*5 credits**
2) Scientific Exposure	*date*
EPS PhD student days (5x)	2001/2002/2003/2004/2005
EPS theme symposia (3x)	2001/2002/2003
Seminars (series), workshops and symposia	
Flying Seminar (9x)	2001/2002
Workshop (Auxins and Cytokinins in WICC)	2002
Symposium	2002
Workshop (Ecological and Evolutionary Genomics in WICC)	2005
International conferences	
14th International Conference on Arabidopsis Research , Madison	2003
15th International Conference on Arabidopsis Research , Berlin	2004
Natural EU meeting, Wageningen	2001
Natural EU meeting, Versailles	2002
Natural EU meeting, Nottingham	2003
Natural EU meeting, Golm	2004
Natural EU meeting, Madrid	2005
Presentations	
Oral presentations (4x) in Plant Physiology seminar series	2001/2002/2004/2005
Oral presentation in EPS theme 3 (UvA)	2003
Poster presentation (2x)	2002/2003
Oral Presentation in Natural EU meeting, Versailles	2002
Oral Presentation in Natural EU meeting, Nottingham	2003
Oral Presentation in Natural EU meeting, Golm	2004
National meetings	
ALW Lunteren	2001/2002/2003/2004
Araned	2001/2002/2003
Subtotal Scientific Exposure	*12 credits**
3) In-Depth Studies	*date*
EPS courses or other PhD course	
Environmental signalling: Arabidopsis as a model (Summer School)	2001
The analysis of natural variation within crop and model plants (Int. Sum. Sch.)	2003
Ecophysiology of Plants (Summer School)	2002
Bioinformatics course	2004
3rd Bioinformatics course (Bioinformation Technology-1)	2004
Subtotal In-Depth Studies	*15 credits**
4) Personal development	*date*
Skill training courses	
Working with EndNote	2001
Guide to Digital Scientific Artwork	2002
Subtotal Personal Development	*2 credits**
TOTAL NUMBER OF CREDIT POINTS*	**34**

** A credit represents a normative study load of 28 hours of study*

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تحليل السلوك النباتي: دراسة فسيولوجية ووراثية باستخدام التباين الطبيعي لنبات *Arabidopsis thaliana*

رسالة دكتوراة مقدمة من

محمد عزت محمود الليثي

جامعة فاخننجن – قسم علوم النبات

معمل الوراثة و معمل فسيولوجيا النبات

فاخننجن – هولندا

إهداء

أهدي هذه الرسالة .....

الي نبع العطاء و الحنان.....

أبي و أمي و الي روح والدة زوجتي

الي شركائي في الحياة.....

أمال و رانيا و كريم

و إخوتي

الملخص العربي

يعد تحسين المنتج الزراعي بطريقة فعالة التحدي الحقيقي للعلماء هذه الأيام. و لهذا يعد إكثار النباتات من أهم التقنيات لتحقيق هذا الهدف حيث يستخدم علماء النبات كل المصادر التقنية و الوراثية المتاحة لتحسين الناتج النباتي من الناحية الكمية و الكيفية. هذا بالإضافة الي زيادة مقاومة النباتات للإجهادات الحيوية و الغير حيوية. و لقد ساعد التقدم في علم الوراثة الجزيئية في إستخدام معلومات المورثات (الجينات) في إكثار النباتات إما بنقلها أو بإنتقائها عبر الأجيال المتعاقبة. و يعد إختيار العلامات الوراثية المرتبطة ببعض الصفات الوراثية و التي قد تتأثر بالنواحي البيئية من الأهمية بمكان. و بمعرفة الخريطة الوراثية للجينات من خلال التباين الوراثي يمكن التعرف علي هذه الجينات و التي أحيانا ما تتحكم في صفات مركبة. و لقد بدأ بالفعل فصل العديد من الجينات التي تتحكم في التباين الطبيعي في العديد من النباتات البرية و التي تستخدم كنموذج للدراسة الوراثية لتوافر الكثير من المعلومات عنها و لكن ذلك مازال صعبا الي حد ما في نباتات المحاصيل.

و لهذا تم إختيار نبات *Arabidopsis thaliana* ليكون نموذج تجريبي للدراسات الوراثية و الجزيئية لصغر حجمه و قصر دورة حياته و لصغر حجم الهيئه الوراثية حيث أنها تحتوي علي حوالي ٣٠٠٠٠ جين موزعة علي ٥ كروموسومات. و يعد التباين الوراثي الموجود فيما بين العشائر الطبيعية لهذا النبات و التي جمعت من مناطق جغرافية مختلفة مصدر مهم يسمح بدراسة وظائف الجينات.

ويعد الهدف الرئيسي للبحث المقدم في هذه الرسالة الحالية هو محاولة لزيادة المعلومات الوراثية و الفسيولوجية المتوافرة عن السلوك النباتي. و لهذا السبب قمنا بالتركيز علي دراسة الصفات المرتبطة باكتساب و نقل الكربون في النبات مستهدفين من ذلك إستيضاح العلاقة فيما بين صفات النمو المختلفة من جانب و بينها و بين صفات التزهير من جانب آخر.

السلوك النباتي يعتمد أساسا علي إكتساب المادة الخام (عبر البناء الضوئي و التغذية المعدنية) ثم نقل هذه النواتج عبر أجزاء النبات المختلفة ثم تخزينها أو إستهلاكها (من خلال التنفس)، و المقدرة علي التكيف مع الإجهادات البيئية. و يعد البناء الضوئي عملية معقدة تلعب دورا مركزيا في نمو النبات و إنتاجيته و التي تجري أحداثها في الكلوروبلاست (اليخضور)، حيث يتحكم فيها مجموعة من الجينات التي تقع في النواة و في البلاستيدات الخضراء.

يقدم الفصل الثاني من هذه الرسالة وصف للتباين الطبيعي الموجود بين ال Arabidopsis لصفة البناء الضوئي. حيث تم دراسة الكلوروفيل فلوريسنت كمؤشر للبناء الضوئي في عدد ١٢٧ سلالة لهذا النبات للتعرف علي التباين الطبيعي الموجود. و لقد أوضح هذا المسح سلالة واحدة فقط (Ely) و التي تمتلك صفات مختلفة تماما عن بقية السلالات. فيما يعد التباين بين بقية السلالات صغير الي حد ما و إن كان في بعض الأحيان معنوي. و لقد أوضحت الدراسة الفسيولوجية المستفيضة إختزال في كفاءة Ely و كذلك الطرز الجينية التي تحتوي علي سيتوبلازم Ely و نواة *L*er و ذلك مقارنة بالسلالة المرجعية *L*er . فعند درجة حرارة ١٥ْ م أظهرت سلالة Ely زيادة في معدل إنتقال الإلكترونات مقارنة بالسلالة المرجعية، و الذي يدل علي زيادة في مقدرة ال PS II تحت هذه الظروف، بينما عند درجة حرارة ٣٠ْ م وجد العكس. أيضا أظهرت Ely زيادة في الحساسية لأشعة UV-B و مقاومة عالية للأترازين مقارنة بالسلالة المرجعية. و يعد إكتشاف السلالة Ely بأنها مقاومة للأترازين و أن الصفات المرتبطة بالكلوروفيل فلوريسنت تورث عن طريق البويضة (السيتوبلازم)، إشارة لجين أو جينات تقع في الكلوروبلاست. و بدراسة التتابع الوراثي لجين *psbA* و المسئول عن تكوين بروتين D1 of PS II وجد أن هناك طفرة تسببت في تغيير

الحامض الأميني المتكون شبيهه بما وجد في أجناس أخري مقاومة للأترازين، معطية بذلك تفسيرا وراثيا لكون Ely مقاومة للأترازين وتمتلك خصائص ضوئية مختلفة.

و يقترح الإختلاف المعنوي في صفة الكلوروفيل فلوريسنت بين Ely و التلقيحات العكسية مع *Ler* وجود تأثير إضافي لجينات تتواجد في النواة. و لقد أمكن إستيضاح التأثير الناتج عن جينات النواة بتعيين موقع وراثي (QTL) لصفة الكلوروفيل فلوريسينت في عشيرة (*Ler* x Sha) و الذي ورد بالفصل الرابع، و موقعين وراثيين آخرين لنفس الصفة و ذلك في عشيرة (*Ler* x Kond) و الذي ورد بالفصل الخامس.

ويعد التباين الذي تم تعيينه في سلالات ال Arabidopsis لصفة الكلوروفيل فلوريسنت صغيرا الي حد ما. و لهذا نخلص الي أن هذه الصفة محفوظة و أن إنتقاء مؤثر نتج عنه هذا التباين لسلالة Ely. علي أية حال، ربما لا يزال من الممكن إكتشاف تباين أكثر بين السلالات عندما تجري التجارب تحت ظروف مختلفة، أو بإجراء تجارب أخري أكثر تعقيدا لدراسة الخواص الضوئية المختلفة.

و لقد تم تكوين أربعة عشائر جديدة بناء علي التباين الذي وجدناه بين عدد ١٢٧ سلالة لهذا النبات لصفات متعلقة بالنمو، و محتوي الكربوهيدرات، ووقت التزهير. حيث تم تلقيح السلالة المرجعية *Ler* كمصدر للبويضة مع سلالات (Kas-2, Sha and Kond) كمصدر لحبة اللقاح، و مع (An-1) بكلا الإحتمالين. حيث يقدم الفصل الثالث من هذه الرسالة وصف لكيفية تكوين ثلاثة عشائر هي (*Ler* x An-1, *Ler* x Kas-2 and *Ler* x Kond) مرباه داخليا لمدة تسعة أجيال حتي نضمن عدم وجود مواقع خليطة وراثيا. كما يقدم هذا الفصل أيضا كيفية إستخدام تقنية جديدة و حديثة من العلامات الوراثية لتكوين الخرائط الوراثية لهذه العشائر الثلاثة. و لقد تم تسجيل وقت التزهير لهذه العشائر الثلاثة لإثبات جدوي إستخدامهم لدراسة صفات كمية أخري. و لقد أثبتت هذه الدراسة أهمية إستخدام نفس العلامات الوراثية للعشائر المختلفة لسهولة مقارنة النتائج. و لقد أوضحت هذه الدراسة أيضا أن طول الخرائط الوراثية لهذه العشائر الثلاثة متماثل الي حد ما فيما بينها و أيضا مع العشائر المكونة سابقا.

و لقد أظهر قياس وقت التزهير في هذه العشائر الثلاثة تحت ظروف إضاءة لمدة طويلة (١٦ ساعة ضوء / ٨ ساعات إظلام) وجود أربعة، و ستة، و ثمانية مواقع وراثية (QTLs) علي الترتيب لكل عشيرة. و بمقارنة هذه المواقع الوراثية التي تم تعيينها في هذه العشائر الثلاثة بالإضافة لما تم تعيينة في عشيرة (*Ler* x Sha) بالفصل الرابع، أمكن تحديد ما يقرب من ١٢ إلي ١٦ موقع وراثي تتحكم في صفة التزهير. حيث أنه في بعض هذه المواقع توجد جينات تم تعريفها مسبقا بكونها تتحكم في وقت التزهير مثل *FLM* and *CRY2* عند أعلي و أسفل الكروموسوم الأول علي الترتيب، و جين *FRI* عند أعلي الكروموسوم الرابع، و جين *FLC* عند أعلي الكروموسوم الخامس. أما المواقع الأخري فلا تزال طي البحث.

من هذا نخلص الي أن دراسة نفس الصفة بالعشائر المختلفة من الأهمية بمكان، حيث أظهرت مواقع وراثية مختلفة، لصفة كمية مثل وقت التزهير، إعتمادا علي التكوين الوراثي للأبوين لكل عشيرة.

و لدراسة الصفات المتعلق بالنمو قمنا بمقارنة عدد ١٢٧ سلالة لنبات Arabidopsis من حيث محتوي الكربوهيدرات خلال فترتين زمنيتين، ثم خلصنا الي دراسة عدد ٢٢ سلالة بإستفاضة أكثر. و من هذه الدراسة قررنا تكوين العشيرتين النباتيتين (*Ler* x Sha) و (*Ler* x Kond) للقيام بدراسة و تحديد المواقع الوراثية للصفات الكمية (QTL-mapping) و التي تتحكم في صفات النمو و محتوي الكربوهيدرات و التزهير، و لقد ورد ذكر نتائج هذه الدراسة في الفصل الرابع و الخامس.

و لقد أوضح هذا المسح لعدد ٢٢ سلالة وجود تباين كبير بين السلالات في وزن البذور، و الوزن الجاف للنبات، و معدل النمو النسبي، و لكن ليس للمحتوي الرطوبي. و لقد وجد أن هناك علاقة طردية معنوية بين وزن البذرة و مساحة سطح النبات عند عمر عشرة أيام و تختفي هذه العلاقة عند الأعمار المتقدمة، مما يدل علي أن وزن البذرة يؤثر في النمو الأولي للنبات عنه خلال الأعمار المتقدمة حيث توجد عوامل أخري تحكم نمو النبات.

و بإستخدام البرنامج الإحصائي الخاص بتحديد المواقع الوراثية للصفات الكمية (QTL-mapping) أمكن تحديد خمسة مواقع وراثية لعشيرة (L*er* x Sha) تتحكم في صفات عديدة للنمو و منها (مساحة السطح، و الوزن الجاف، و معدل النمو النسبي للنبات). حيث تقع المنطقة الوراثية الأولي عند أعلي الكروموسوم الأول، بينما تقع المنطقة الوراثية الثانية حول جين *Er في منتصف* الكروموسوم الثاني. و تقع المنطقة الوراثية الثالثة عند أعلي الكروموسوم الثالث. بينما تقع المنطقة الوراثية الرابعة و الخامسة أعلي و أسفل الكروموسوم الخامس علي الترتيب.

و حيث أنه من المتوقع أن ترتبط صفات النمو المختلفة بمحتوي الكربوهيدرات، قمنا بقياس نسب السكريات و النشا في عدد ١٢٣ سلالة لنبات *Arabidopsis* حيث ظهر أن غالبية هذه السلالات تخزن الكربوهيدرات في أوراقها قرب نهاية فترة الإضاءة كما هو متوقع ، و لكن لوحظ أن هناك تباينا كبيرا بين هذه السلالات (الفصل الخامس). و أوضحت دراسة محتوي الكربوهيدرات علي مدار اليوم لعدد ٢٣ سلالة مختارة بالإضافة الي ثلاثة طفرات هي (*pgm*, *adg1*, and *sex*) وجود ثلاثة مجموعات: المجموعة الأولي و تشمل السلالة المرجعية L*er* و تحتوي علي معدل منخفض و ثابت من الكربوهيدرات خلال اليوم. أما المجموعة الثانية، فتخزن نسب عالية من الكربوهيدرات علي مدار اليوم حيث تنتمي سلالة Kond لهذه المجموعة. أما المجموعة الثالثة فتحتوي علي معدلات متوسطة من الكربوهيدرات علي مدار اليوم.

و بزراعة عشيرة (L*er* x Kond) علي محلول مغذي أمكن دراسة محتوي الكربوهيدرات في المجموع الخضري و كذلك الجذري ثم مقارنتها مع صفات النمو و التزهير المختلفة و التي تم قياسها في نفس التجربة. و بإستخدام البرنامج الإحصائي الخاص بتحديد المواقع الوراثية للصفات الكمية (QTL-mapping) أمكن تحديد مواقع وراثية تتحكم في الصفات المرتبطة بالنمو ، و محتوي السكريات و النشا ، و الصفات المرتبطة بالتزهير. و لقد وجد تراكم للصفات المختلفة عند عدة مواقع وراثية منها حول جين *Er* . و لقد أوضحت هذه الدراسة وجود علاقة مركبة بين صفات النمو للنبات و محتوي الكربوهيدرات و الصفات المرتبطة بوقت التزهير.