CHAPTER 1

GENETIC AND MOLECULAR ANALYSIS OF GROWTH RESPONSES TO ENVIRONMENTAL FACTORS USING *ARABIDOPSIS THALIANA* NATURAL VARIATION

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Abstract. *Arabidopsis thaliana* natural accessions have been collected in the Northern hemisphere in a wide range of habitats, with a specific environment in each habitat, suggesting that selection for adaptation to these local environments occurred and provided genetic variation of responses to environmental factors.

Plant performance traits are complex traits and are fluctuating under contrasted environmental conditions (e.g., temperature, day length, nutrient nutrition, drought). The genetic architecture of such traits and of their responses to environmental conditions could be analysed by detecting QTL (Quantitative Traits Loci). Accessions from contrasted geographical regions have been used to detect such QTL. QTL analysis and subsequent QTL cloning of genetic variation for growth and plant performance traits in *Arabidopsis* using *Arabidopsis thaliana* natural accessions are powerful tools to understand the genetic and the molecular basis of plant performance in contrasted environments. QTL analysis of growth-related traits and their response to temperature, light and nutrient starvation in hydroponic system are in progress in different populations (recombinant inbred lines, backcross inbred-line populations) Near-isogenic lines or heterogeneous inbred lines are also selected and used to confirm the effect of QTL and to isolate recombinant events in the QTL region in order to fine-map and clone the QTL.

The challenge of these studies is to understand this genetic variation, which is also very relevant for plant breeding, since it involves the traits determining yield and yield stability. It is difficult to predict which processes underlie this genetic variation, but candidate processes are primary and secondary metabolism, nutrient uptake, transport processes and aspects of development etc. This implies that a

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thorough and broad (whole plant) approach needs to be applied to identify the nature of the observed variation. Apart from being relevant for breeding it is assumed that genetic variation for plant performances contributes to the adaptation of specific genotypes to a specific ecological system and therefore has ecological and evolutionary relevance.

ARABIDOPSIS AS MODEL PLANT FOR MOLECULAR STUDIES

Arabidopsis thaliana is a plant species of no economic importance and is not considered to be a problematic weed. However, the progress made in dissecting the genetic and molecular basis of many plant processes by using *Arabidopsis* and its resources, is impressive. The main reasons why so much progress has been made in the past 25 years are the large number of scientists working with this species and the resources that have been made available (Somerville and Koornneef 2002). The choice of *Arabidopsis* was based on some biological characteristics such as the short generation time, it being a self-fertilizer and its small plant size, which all make it very suitable for genetic studies. However, nowadays the large resources which are publicly available for this species might even be more important. Especially the complete genome sequence published in 2000 (Kaul et al. 2000) and the availability of mutations in almost every gene and for which insertion-mutant seeds can be easily ordered through Internet (http://www.arabidopsis.org) are important resources that are still unique to *Arabidopsis*. Nowadays, the amount of genomic resources available is also increasing rapidly for crop plants. For instance, for rice a complete genome sequence is available and accessible through the Internet (see: http://www.cbi.pku.edu.cn/mirror/GenomeWeb/plant-gen-db.html) and other data bases such as Solgenes, Gramene etc. However, completeness of the knock-out mutant collection (> 300.000 mutants, Sessions 2005) in *Arabidopsis* will not easily be met in other plants because their generation in *Arabidopsis* depends on the extremely efficient 'floral dip' transformation procedure.

Based on the technology and resources available in *Arabidopsis*, one can establish a functional analysis of every gene by the analysis of the phenotype of mutants in which a gene of interest is disrupted. This procedure is called reverse genetics (from gene to phenotype). The functional analysis of one gene can be complicated by the fact that many genes are duplicated (redundant). Therefore knock-outs disturbing all the genes of the same family need to be generated to see phenotypes clearly distinct from the wild type. It may also be difficult to detect a mutant phenotype because the effect of the studied gene is not detectable on the phenotypic level. For example, growing plants on soil will not reveal a seed-dormancy phenotype because this requires a specific germination test performed on Petri dishes.

The opposite approach to identify gene function starts with mutant phenotypes and tries to find the corresponding gene(s) by accurate mapping, sequencing and complementation of mutant phenotypes by wild-type DNA; it is called forward genetics (from phenotype to genotype). In addition to its use for reverse genetics the complete DNA sequence also allows the construction of whole-genome micro-arrays (called DNA chip), which allows the study of the expression of all genes at once.

NATURAL VARIATION IN *ARABIDOPSIS*

The above-mentioned approaches rely on mutants often induced in a single genetic background, which are pure lines with favourable properties in the laboratory, usually including earliness and low seed dormancy. However, natural variation is an increasingly important resource for genetic variation in addition to induced mutants. *Arabidopsis* accessions have been collected in a wide range of sites in the Northern hemisphere. Because these accessions can come from different habitats, it is assumed that selection for adaptation to these local environments has occurred and has provided genetic variation in responses to these environmental factors. The traits and genes for which natural variation is present differ from induced mutants by the fact that they survived in nature, implying that natural selection allowed the survival of the various alleles. Furthermore, many of the traits showing natural variation are related to properties that are important for crop plants, including (biotic and abiotic) stress resistance, developmental traits, phenology, seed dormancy and aspects of growth.

The genetic analysis of natural variation

In segregating progenies derived from crosses between diverse accessions such traits can be analysed genetically. Because the genetic differences between two accessions are often determined by more than one gene and because of large environmental effects on the phenotype, methods of quantitative genetics need to be applied. Especially the association of trait phenotypes with the genotype, assayed by molecular markers, is very effective for the analysis of quantitative-trait loci (QTL). QTL analysis reveals the regions on the genetic map where a gene or several closely linked genes are located and their contribution to the total variance of the trait in that experiment. For QTL analysis the use of so-called immortal mapping populations, consisting of homozygous inbred lines is effective because trait values can be obtained from replications and from experiments performed in different environments. In such populations, genotyping (analysis of the marker phenotypes in all lines) needs to be done only once.

Recombinant inbred lines (RILs) are the most frequently used immortal mapping populations in *Arabidopsis*. These are obtained by single-seed descent from F2 plants until the F9 or further generation. The use of RILs is relatively easy in *Arabidopsis*, as it is a self-pollinating plant with a short generation time. A number of introgression lines (ILs) will also become available soon. Another advantage, especially of RIL populations that are used by many researchers, is that an increasing number of traits are mapped in the same populations. Combining the results of multiple studies can lead to the discovery that some loci control more than one trait, sometimes in an unexpected way (Koornneef et al. 2004). Co-location of QTLs can also provide a clue of what pathways might be involved in complex traits. In a recent study this concept was applied and even expanded by combining the results of gene expression analysis with QTL mapping of metabolite levels and enzyme activities (J.J.B. Keurentjes and colleagues, personal communication). Common genetic-map positions of differentially expressed genes and QTLs allow

the construction of genetic networks. A combined analysis of this information within the context of a systems framework is very useful for the future identification of the gene(s) underlying the QTLs because differences in expression that co-locate with the QTLs provide candidates for the QTGs (Quantitative Trait Genes).

The cloning of the genes underlying QTLs

The cloning of genes based on the map position of mutants (positional cloning) is very effective in *Arabidopsis* because of the efficiency with which a segregating population can be analysed and because of the abundance of markers available (Lukowitz et al. 2000). Nowadays the mapping of a mutant down to less than 50 kbp (on average 0.2 centi-Morgan) is sufficient to search for candidate genes. Subsequent DNA sequencing of these genes will rapidly reveal the precise location of the mutations. Complementation of the mutant phenotype after transformation with the wild-type allele provides proof that the causative gene has been positively identified. Similar approaches have been used to clone QTLs. However, because positional cloning is done gene-by-gene, care has to be taken that only one segregating locus is studied at a time. This can be achieved by using so-called near-isogenic lines (NILs) that contain an introgression of one parent's alleles at a QTL position into the genetic background of the other (recurrent) parent. Subsequently, a population of lines with different recombinant events is selected by genotyping the offspring of a cross between the NIL and the recurrent parent with polymorphic markers surrounding the QTL. The QTL is eventually fine-mapped by repeating this process with progeny lines that still show the effect of the QTL on the phenotype. The procedure becomes similar to that of mutant approaches once the QTL has been fine-mapped to a sufficiently small region. Another approach is to make use of residual heterozygosity present at a QTL in RILs after several generations of selfing. The genetic background of the progeny of such lines is a mixture of both parental accessions. This so-called HIF (heterogeneous inbred families) concept (Tuinstra et al. 1997) is effective because one does not first have to create the NILs, which requires several generations of backcrossing and marker-assisted selection (MAS). Using either NILs and/or HIF will allow the validation/confirmation of the presence and the effect of a QTL.

Accurate phenotyping of QTLs with small effects and with relatively large environmental influence is difficult. Therefore, it is often more effective to select homozygous recombinants in a genomic region surrounding the QTL and then analyse their phenotype in replicates. Such procedures are also described by Peleman et al. (2005) and are commonly referred to as QTL Isogenic Recombinant Analysis. A complication of QTL cloning compared to positional cloning of mutants is finding the gene(s) responsible for the phenotype among the candidate genes in the region where the QTL was fine-mapped. In case of mutants in a self-pollinating plant such as *Arabidopsis*, any DNA sequence difference that is detected indicates that the gene has been identified. However, among *Arabidopsis* accessions polymorphisms of sequences occur at an abundance of about one in every 350 bp (Schmid et al. 2003), even in coding regions. This implies that several differences

will be found in each gene. As a consequence, sequence information is in most cases not informative for the detection of the QTN (Quantitative Trait Nucleotide). However, sometimes the presence of deletions (Alonso-Blanco et al. 2005a) or mutations in known important parts of the gene (Teng et al. 2005) is informative. Therefore, transformation experiments are crucial to prove which gene(s) is (are) responsible for the phenotypic differences observed between NILs or HIFs. That this concept works in *Arabidopsis* has been shown by the cloning of several QTLs affecting traits such as flowering time, frost tolerance and seed dormancy (for review: Alonso-Blanco et al. 2005b). Mutants are complementary tools for the identification of QTGs.

Fine-mapping and cloning QTLs is also a powerful way to distinguish pleiotropic effects from the effect of linked genes when QTLs of several traits are co-localized. It is also helpful in confirming or disproving unexpected co-location of QTL for diverse traits. Examples are the effect of *FLC* on flowering time and length of circadian periods (Swarup et al. 1999), the effect of an invertase gene on invertase activity and root growth (Sergeeva et al. 2006), the effect of the *ERECTA* locus on plant length and water use efficiency by affecting stomatal densities (Masle et al. 2005) and the resistance to *Plectospaerella cucumerina* (Llorente et al. 2005). The fine-mapping of QTL in combination with candidate genes, expression analysis and the search for mutants with related phenotypes in the QTL region are the tools available at the moment to identify genetic variation at the molecular level. Because some tools are still unique to *Arabidopsis*, QTL cloning is most efficient in this species.

A summary of the various approaches used to identify the molecular basis of genetic variation is shown in Figure 1.

THE GENETICS OF PLANT PERFORMANCE

Plant performance traits (photosynthetic performance, growth performance, yield performance, etc.) are complex traits and their values fluctuate with the variability in environmental conditions encountered in the field. Finding the pertinent environmental variables to which these traits are responding is an important aspect of the genetics of plant performance. It is a challenge to find the genes that underlie variation in plant performance in contrasting environments.

Genes with relatively large effects on phenotype (or trait value) have been identified in *Arabidopsis* using mutant approaches, often accompanied with over-expression of the genes of interest. However, this is often a brute-force approach which requires high-throughput technologies for making transformation constructs, the transformation and, very important, trait analysis. Examples where transgenic approaches resulted in yield enhancement have been reviewed by Van Camp (2005), who also described the high-throughput platforms developed mainly by the industry. Finding the genes controlling the variation present in nature and identifying these genes within the germplasm pool of crop plants will require the application of the technology described above in section *The cloning of the genes underlying QTLs*.

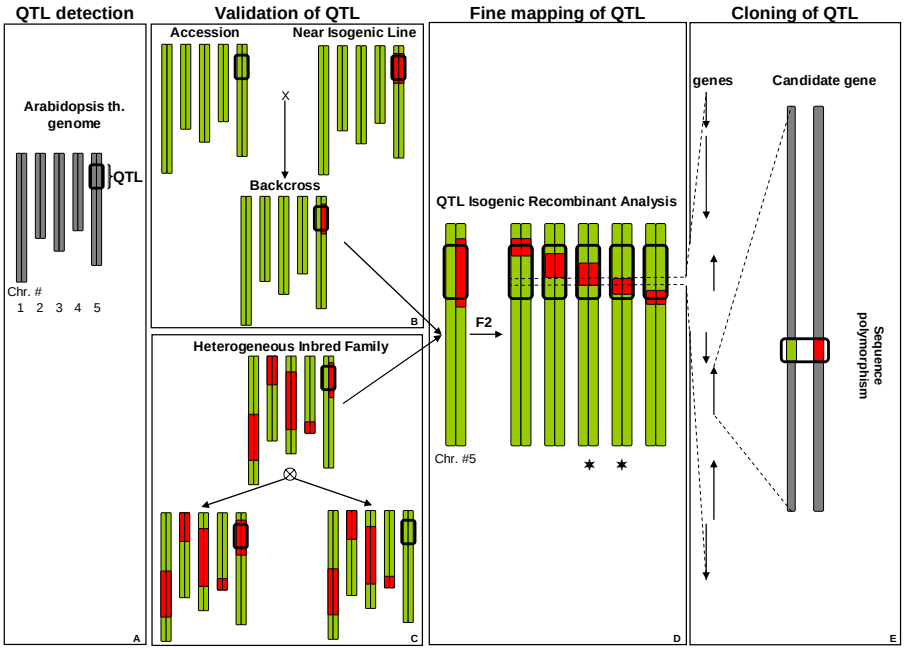

Figure 1. *Various approaches used to identify the molecular basis of a QTL.* **(A)** *QTL detection. QTLs could be detected using several types of populations (Recombinant Inbred Lines, F2, Backcrossed lines,…). For this example on* Arabidopsis thaliana*, a QTL on chromosome 5 (Chr.#5) has been detected.* **(B and C)** *QTL validation. Validation of a QTL could be done by comparing either* **(B)** *a Near Isogenic Line (NIL) at the position of the detected QTL and the corresponding accession (having the same genetic background) or* **(C)** *lines from a Heterogeneous Inbred Line (HIL at the position of the detected QTL).* **(D)** *Fine mapping of a QTL. Fine mapping could be performed by making use of recombinant events from selfed heterozygote lines at the position of the detected QTL. These recombinant lines are then phenotyped. In this example, asterisks indicate lines showing a phenotype significantly different from the accession having the same genetic background. The dotted traits indicate the fine position of the QTL.* **(E)** *Cloning a QTL. Recombinant events occurring during the fine-mapping of the QTL are usually not enough to get a recombinant event between each gene within the QTL. Hence, once the QTL is fine-mapped, several genes are included in the highlighted regions (arrows represent predicted Open Reading Frames within the region of the fine-mapped QTL). Candidate-gene approach could then be performed. In the case of obvious candidate gene(s), this approach could already be performed just after the QTL detection*

QTL analysis for yield in crop plants has been performed in many studies. In *Arabidopsis*, natural variation for growth-related traits has been detected in various studies but the number of studies where QTL analyses have been done is limited. El-Lithy et al. (2004) identified QTLs for growth-related traits under normal (laboratory) conditions. Many of these QTLs co-localized with QTLs of flowering

time, although growth was analysed during the vegetative phase. Loudet et al. (Loudet et al. 2003b) showed that growth QTLs (biomass accumulation) differ depending on the growth conditions. Another example, obtained when analysing the same population, is root growth under phosphate starvation (Reymond et al. 2006). In experiments in which the supply of nutrients is varied, their concentration in plant tissue can also be analysed (Loudet et al. 2003a). QTLs for the accumulation of nitrogen in several cases co-located with QTLs for growth. El-Lithy (2005) also found co-locations of loci for starch accumulation in the leaves and for plant growth. These co-locations suggest a functional relationship between traits. However, due to the inaccuracy of QTL mapping, co-locations can be due to different but linked genes. An analysis of a number of trait co-localizations was described by El-Assal et al. (2004). The authors made use of a transgenic line different from the reference genotype in one of the confirmed alleles conferring a flowering-time QTL encoded by the Cryptochrome-2 (*CRY2*) gene. They revealed that some co-locating QTLs were pleiotropic effects of *CRY2* (flowering time, ovule number and fruit length) but others (seed dormancy and invertase activity) were due to allelic variation at linked genes.

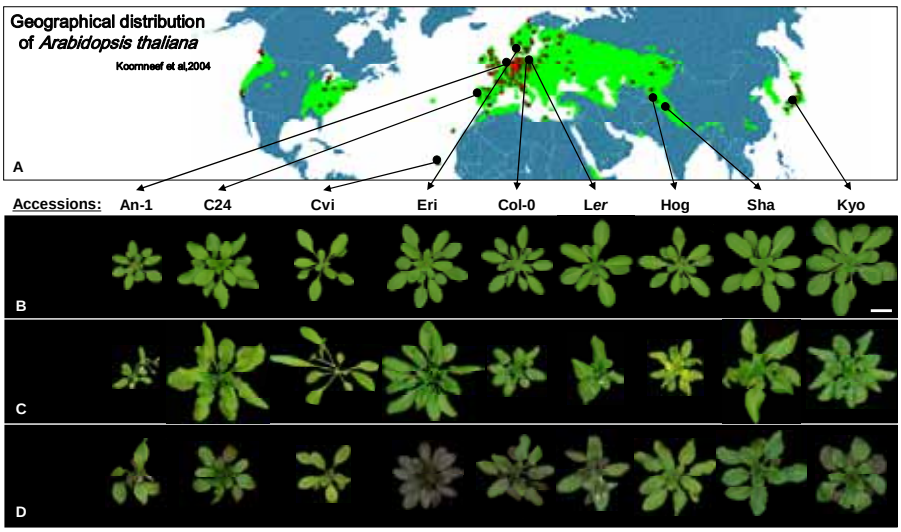

Figure 2. *Example of 'Genotype × Environment' interactions using accessions of* Arabidopsis thaliana **(A)** *Geographical distribution of* Arabidopsis thaliana *accessions (after Koornneef et al. 2004). Black points on the map indicate the location where accessions have been collected for this example.* **(B, C and D)** *Rosette shape and colour of the selected accessions growing in a range of temperature and light conditions (white bar represents 2 cm)* **(B)** *Photoperiod: 12 h*; *temperature: 16/14 °C (day/night); light intensity:150 (±30) µmol* m^{-2} s^{-1}
(C) *Photoperiod: 16 h*; *temperature: 4/4 °C (day/night); light intensity: 15-20 µmol* m^{-2} s^{-1}
(D) *Photoperiod: 16 h*; *temperature: 13/4 °C (day/night); light intensity: 200-400 µmol* $m^{-2}s^{-1}$

For the analysis of the genetics of plant performance RIL populations derived from crosses between *Arabidopsis* accessions from diverse origins are used. These include Ler (from Poland), Kas-2 (from Kashmir), Sha (from Tajikistan) and Cvi (from the Cape Verde Islands). QTL analysis of growth-related traits and their response to light, temperature and nutrient starvation (using hydroponics) are performed in different populations. An example of the large genotype × environment interaction in *Arabidopsis* is shown in Figure 2. NILs are also selected and used to confirm the effect of QTL and to isolate recombination events in the QTL region in order to fine-map and clone the QTL. In addition to QTLs for growth, QTLs can also be detected for metabolites, enzyme activities and gene expression. The combined QTL analysis of all such traits now provides the possibility to correlate these various traits on the basis of their common map position, thereby unravelling the processes and pathways underlying plant performance traits. Examples are the control of root length by a QTL encoding an invertase (Sergeeva et al. 2006). These combined studies will help to dissect variation for integrative traits (such as plant growth) into variation of various underlying processes and component traits. Candidate processes are primary and secondary metabolisms, nutrient uptake, transport processes, aspects of development, etc. It is of interest that in rice two major yield QTGs that have been cloned deal with hormone metabolism (Ashikari et al. 2005). A cytochrome oxidase was found to underlie a major QTL for grain number per panicle and the yield enhancing allele was conferred by a loss of function mutation. A major QTL for seed length called GS3 revealed a gene for which the function, and thereby the pathway involved, could not be predicted with any certainty from its sequence (Fan et al. 2006). Interestingly, also here the allele positive for yield is a loss-of-function allele. A thorough and broad (whole plant) physiological and biochemical approach is needed to identify the nature of the observed variation in addition to the molecular study of genes involved.

THE TRANSLATION FROM MODEL TO CROP PLANTS

Based on the common molecular basis of many processes in plants, it is now becoming more obvious that many genes, for which the function is discovered in *Arabidopsis*, also underlie the genetic variation that is exploited by plant breeders in crop plants. However, in more distantly related species similar processes may just as well be regulated by unrelated genes. The best known example is flowering time, where QTL cloning in rice revealed a number of genes that, based on mutant analysis, previously had been shown to control flowering time in *Arabidopsis* (Hayama and Coupland 2004). However, the two major genes controlling flowering-time variation in nature in *Arabidopsis* (*FRI* and *FLC)* are not found in monocots. In wheat, in which genes based on phenotype and physiology were predicted to be similar to *FLC*, another regulatory gene was found (Yan et al. 2004). For the major determinant of the day-length response in barley a gene was found that was not identified as a major player in *Arabidopsis* and rice (Turner et al. 2005), although the process in which the gene was involved (circadian rhythm) was known from

Arabidopsis research. However, especially when dicot species are compared many examples of similar genes controlling similar processes have been described.

Another possibility for exploitation of the vast amount of research done on *Arabidopsis* and the availability of its genome sequence is to make use of synteny between genomes. With the complete genome sequence of rice now available it has become clear that there are reasonably sized blocks of highly conserved synteny between this Graminea and *Arabidopsis*, even though both species are estimated to have diverged around 200 million years ago. The largest of these regions spans no less than 119 *Arabidopsis* genes (Goff et al. 2002). The level of synteny can be much higher between more related species. For instance, highly conserved genetic synteny has been reported up to the multi-megabase scale between *Arabidopsis* and other members of the Brassicaceae (Lukens et al. 2003; Parkin et al. 2005). In an older study, comparative mapping had already demonstrated genetic co-linearity of vernalization-responsive flowering-time loci in *Brassica napus* with the top of chromosome IV and V in *Arabidopsis*, the location of *FRI* and *FLC* (Osborn et al. 1997). Evidence that FLC is indeed central to the vernalization response in *B. napus* was provided some years later (Tadege et al. 2001), demonstrating the feasibility of comparative genetics.

In cereals, much has been expected from the synteny among Gramineae. However, not many cases in which similar map positions of QTLs are due to variation at the same genes have been published. Especially, for complex traits controlled by many genes one cannot expect that variation in the same gene set will determine variation in different species. There are too many candidates in the genome.

CONCLUSIONS

From the research done in *Arabidopsis* and rice the procedures for the identification of genes underlying QTLs are well developed. The methodology has been shown to be successful also in plants with large genomes such as wheat and maize for some major-effect QTLs. The need for this knowledge is large because when transgenic approaches are used, these genes are needed. Also for marker-assisted breeding, markers within the gene to follow are preferred over linked markers, because the latter can be separated from the target locus by recombination and therefore do not predict the desired phenotype in 100% of the cases. Research on *Arabidopsis* will contribute to the finding of the genes underlying QTLs, also in crop plants because the candidate genes and pathways for crop-plant QTLs will be identified. The analysis of larger-effect genes has already started for flowering time but will also become available for more complex traits dealing with growth and genotype × environment interactions.

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