Environmental tuning of the genetic control of seed performance

A systems genetics approach

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Chapter 1

General Introduction

Seed quality

Over the coming decades, major predicted changes in the climate conditions will result in changes in plant growth and development as well as seed quality which is acquired during seed development on the mother plant (Walck et al. 2011). Such changes will have an impact on plant's life history traits but also consequences for seed companies whose main business is to provide high quality seeds to the growers.

In this thesis, I aimed to extent the understanding of interactions between the maternal environment and genetic factors that regulate seed performance. For this purpose, I used a combination of genetic and ~omics approaches. A detailed description of the biological and technical aspects related to this aim and approach are now being further detailed.

Seed quality is a generic term which definition is a rule-of-thumb determined by the end-user (Ligterink et al. 2012). For instance, quality requirements will differ depending on the if the seeds are intended to be used for consumption or propagation purposes. In agriculture, seed high genetic purity, the absence of seed-borne diseases, low post-harvest dormancy and high storability are often used as quality criteria. Seed performance is also used as a surrogate of seed quality that refers to the germination potential of the seed in terms of rate, speed and uniformity of seed germination. Another key aspect of seed performance is seed vigour which is the ability of the seed to germinate under a wide range of environmental conditions (Dickson 1980; Finch-Savage and Bassel 2016).

Seed quality essentially relies on the inherent properties of the seed acquired during seed development on the mother plant. Seed development is characterized by two main phases; embryogenesis and maturation (Harada 1997; Goldberg et al. 1994). During embryogenesis, the zygote undergoes a series of cell divisions and differentiations. Seed maturation marks the arrest of embryogenesis. Seed maturation is an important phase of seed development. The embryo stops it growth and start accumulating storage products that will be mobilized upon imbibition to support the high energy demanding early phases of seed germination and seedling establishment (Weitbrecht et al. 2011). Seed maturation is also characterized by the acquisition of important seed properties such as dormancy and desiccation tolerance (Goldberg et al. 1994). The dry and dormant state of the seeds provides them an advantage to sustain extended periods of unfavourable conditions and optimize their germination time (Bentsink 2008). Genetic and molecular studies have provided large insights into the regulatory mechanisms associated with seed maturation (Holdsworth et al. 2008; Bentsink and Koornneef 2008; Gutierrez et al. 2007). Important regulators of seed maturation include the LAFL (LEC1, ABI3, FUS3 and LEC2) which can be used to modify seed quality (Fatihi et al. 2016). The hormonal balance between abscisic acid (ABA) and gibberellins (GA) also plays an important role in the control of dormancy and seed germination. ABA is a positive regulator of dormancy while GA counteracts the effect of ABA to release dormancy and to promote germination (Holdsworth et al. 2008). Insights into seed maturation processes have also been gained from omics studies. The different phases of seed development are characterized by specific metabolic signatures (Fait et al. 2006; Angelovici et al. 2010). A high number of changes in gene expression has also been observed during seed development and seed after-ripening (Bassel et al. 2011; Le et al. 2010; Carrera et al. 2008). Further understanding of the control of seed maturation, dormancy and germination is essential to further improve seed quality traits.

Environmental regulation of seed performance

Seed germination is a critical stage in respect to its role in supporting plant fitness. Inopportune environmental conditions can prevent seedling establishment if the timing of germination is not appropriate. Therefore, the sensing and integration of environmental cues by the mother plant as well as the developing seed plays an important role in adjusting the timing of germination (Springthorpe and Penfield 2015). Seed dormancy is a well-known mechanism that contributes to the adaptation of plants to their environment by regulating the timing of germination (Donohue et al. 2005b). The level of seed primary dormancy is largely modulated by genetic and environmental factors (Bentsink et al. 2010; He et al. 2014). The maturation environment of the seed, also called maternal environment, can modulate dormancy and germination in a genotype-dependent manner (He et al. 2014) resulting in genotype-by-environment interactions, further explained. For a wide range of species, as well as Arabidopsis, warmer maternal environments often result in lower seed dormancy as compared to lower temperatures (Fenner 1991; He et al. 2014). The maternal environment also affected other seed traits. For instance, in several studies, high light resulted in an increased seed size (Fenner 1991; He et al. 2014). The nutrient availability, such as nitrate and phosphate, to the mother plant also affect the offspring performance (Galloway 2001; He et al. 2014). It is not well understood how environmental cues are sensed are integrated. Several studies showed the implication of the flowering pathway in integrating environmental cues transmitted to the developing seeds (Penfield and MacGregor 2017). Additionally, phytochromes can also act as important mediators of environmental cues (Donohue et al. 2008). Several other studies indicated that the effect of the maternal environment can be mediated via seed provisioning

(Zas et al. 2013), the maternal tissues such as the seed coat (MacGregor et al. 2015; Chen et al. 2014b) as well as the accumulation of compounds such as storage proteins, metabolites and transcripts (Rosental et al. 2016; He et al. 2016; de Souza Vidigal et al. 2016).

The successful execution of the germination program does also depend on environmental conditions encountered by the seed shed from the mother plant. Light, low temperatures and nitrate are known environmental factors that elicit germination (Holdsworth et al. 2008). Primary dormancy can be alleviated by stratification which consists in imbibing seed in cold and dark prior to germination or by dry natural storage, a process termed 'after-ripening' (Bewley 1997). Unfavourable germination conditions can suspend the germination process and can also induce secondary dormancy (Finch-Savage and Footitt 2017). Germination at high temperature can induce thermo-inhibition that is the inability of the seeds to germinate under high temperature (Hills et al. 2003). Germination in ABA also results in the reversible arrest of germination by preventing water uptake to the embryo (Lopez-Molina et al. 2001). Water availability is essential for seeds to commit germination. Water availability and thus intake becomes limiting under osmotic stress conditions which can be induced by the presence of NaCl, mannitol or Polyethylene glycol in the germination environment (Edwards et al. 2016; Joosen et al. 2012).

Genotype-by-environment interactions

Plants are sessile organisms and must therefore adopt mechanisms enabling them to face fluctuations in their direct environment. One major mechanism to cope with rapid changes is the plant phenotypic plasticity. Phenotypic plasticity which is defined as the ability of plants to produce a range of phenotypes under diverse environmental conditions (Nicotra et al. 2010). The reaction norm, which can be seen as a response to stress, is used as a measure of plasticity and describes the phenotypic expression of a given genotype across a range - or generally two - of environments. Another mechanism of adaptation to environmental fluctuations is genotype-by-environment (G x E) interactions. G x E is the result of differences in plasticity observed across genotypes (El-Soda et al. 2014). Two genotypes can show plasticity, without G x E. This is the case when the reaction norm of these two genotypes is the same. Both plasticity and G x E play an important role in phenotypic diversity and can be used to determine the maximal potential of a given genotype. G x E suggests that the phenotypic variation observed is caused by the effect of the environment on the gene(s) controlling the trait. The mapping of quantitative trait in different environments is a common approach used to identify the genetic basis of

G x E. Knowledge of the molecular basis of G x E can provide insights into underlying mechanisms of plant adaptation (Josephs 2018). Traits providing a fitness advantage in a specific environment might be deleterious in another one and thereby fitness trade-offs can be identified (El-Soda et al. 2014). G x E is of eminent importance in breeding. Multiple-environment testing of the genotypes is needed to identify genotypes suitable for multiple environments (van Eeuwijk et al. 2010).

The genetic basis of such interactions is not well-known. Determining the genetic and molecular mechanisms that give rise to genotype-by-environment interactions is important with many implications in the field of evolutionary ecology (Josephs 2018) and agriculture (breeding) (El-Soda et al. 2014).

Linkage mapping

Genetic traits can be classified as mono-genic, oligo-genic or complex depending on whether these traits are controlled by one, several or multiple genetic factors. Where the genetic factors responsible for regulating mono-genic and oligo-genic traits can be determined with traditional mutant screens, this doesn’t work for complex traits because they are quantitative which means that they are affected by many genes with possibly small effects and often subjected to environmental variation. Therefore, quantitative trait locus (QTL) analysis using linkage mapping is the common tool for determining the genetic factors controlling complex traits.

Linkage mapping is a powerful tool to provide insights into the genetic architecture of segregating genetic traits in many types of mainly bi-parental populations. The power of the QTL mapping relies on three major factors: The complexity of the segregating trait under study, the type and size of the mapping population and the availability of a dense and reliable genetic map (Glazier et al. 2002; Keurentjes et al. 2011). With the advances in high-throughput molecular techniques, such as DNA microarrays and next generation sequencing technologies, it has become feasible to identify a large number of markers distributed across the genome and to genotype these markers for a large sample of individuals, facilitating QTL mapping approaches in many species (Schmidt et al. 2017; Gupta et al. 2008). Recombinant inbred line (RIL) populations are widely used for QTL analyses. This type of population is derived from the F1 of two contrasting parental lines by single seed descend. At the end of several generations (F6-F8), each final recombinant inbred line has a different genotype which consists of a mosaic of parental inherited chromosomal fragments, as a result of meiotic recombination events. The high level of homozygosity of these lines makes them ‘immortal’ and the population suitable to measure multiple traits under different conditions, with no need to genotype them anew. In combination with the high degree of mapping resolution, this type of populations is widely used for QTL mapping. Such populations have been used to investigate natural variation in seed traits such as seed dormancy (Bentsink et al. 2010), longevity (Nguyen et al. 2012) and germination (Joosen et al. 2012).

Other populations that can be used for QTL mapping are F2, double haploid or backcross populations. Genome-wide association studies (GWAS) are also performed to identify genetic factors based on the historical linkage disequilibrium observed in a panel of genetically diverse accessions (Atwell et al. 2010). Many of such mapping populations have been developed using the plant science pioneer plant model *Arabidopsis thaliana* (Alonso-Blanco and Mendez-Vigo 2014).

In this thesis, I used an *Arabidopsis thaliana* Bayreuth-0 (Bay-0) x Shahdara (Sha) core collection of 165 RILs developed by (Loudet et al. 2002). Bay-0 and Sha were originally selected for their known geographical, ecological and genetic distance (Loudet et al. 2002). Bay-0 originates from the low lands and has been collected in Germany, whereas Sha was collected in Central-Asia from the mountains in Tajikistan. The RIL population has been used to identify QTLs for many traits, such as flowering (Botto and Coluccio 2007), root and shoot mass (Bouteille et al. 2012), stress tolerance (Jimenez-Gomez et al. 2010) and seed germination (Joosen et al. 2012).

From G x E to QTL x E

Genotype-by-environment interactions occur when the response to the environment differs across genotypes. To understand the underlying genetics, G x E studies have shifted towards QTL x E, where genotype-by-environment interactions are explained by differences in the expression of the QTLs in relation to the environmental conditions (Boer et al. 2007; van Eeuwijk et al. 2010). Scenarios illustrating such QTL-by-environment interactions are shown in Figure 1.

For the expression of the phenotype, the contribution of genotype-by-environment interactions can be estimated using a simple model. This model includes the expression of a genotype i in an environment j as $P_{ij} = \mu + G_i + E_j + G \times E_{ij} + \varepsilon_{ij}$ where P_{ij} is the expression of the phenotype, μ is the general mean, G_i is the main genotypic effect, E_j is the effect of the environment, $G \times E_{ij}$ the genotype-by-environment interaction term and ε_{ij} the random effects. In QTL studies, the growing interest for investigating the genetic basis of G x E has challenged the development of new QTL mapping approaches. The estimation of the genetic effects can be improved by including multiple environments and also multiple traits in a single QTL analysis. Several mixed models have been suggested that maximize the information gained from genotype-by-environment interactions for multi-traits and multi-environments studies (Malosetti et al. 2013; Boer et al. 2007; van Eeuwijk et al. 2010).

The genetics of ~ omics

QTL mapping and GWAS are commonly used methods to identify genetic loci in bi- and multi-parental populations or in panel of accessions. The advent of high-throughput technologies has granted access to whole biological levels ending in ~omics, which combined with these classical mapping approaches, can speed-up biological insights and gene discovery. These omics data are of various kinds, including metabolomics, transcriptomics, proteomics, methylomics and epigenomics. The major advancements in the metabolic and transcriptomic platforms and analytical methods have promoted their application in plant science.

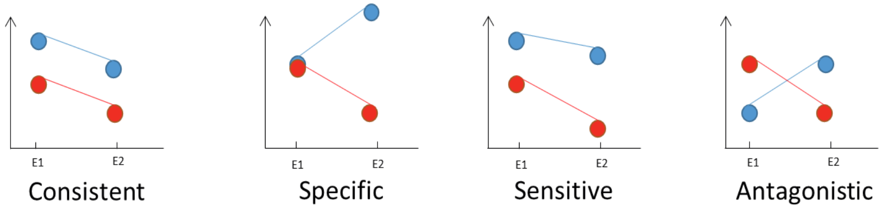

Figure 1: Possible scenarios of G x E associated with QTL x E. The simplified figures depict the allelic effect of the QTL in response to changing environments, from E1 to E2, with in blue and red the allelic effects of two different alleles. In scenario 1, genotypes are plastic but the difference in the allelic effects remains the same; the QTL is consistent across environments; there is no QTL-by-environment interactions (QTL x E). Scenarii 2, 3 and 4 depict cases of QTL x E interactions. In the second scenario, no QTL is identified in E1, while the differences in the allelic effect in E2 indicates an environment specific QTL. In the third scenario, variation in the sensitivity of the alleles to the environment results in exacerbated differences in allelic effects in E2, although the same allele confers higher phenotypic values under both environments. In the final scenario, antagonistic effects are indicated by a change in allele governing higher phenotypic values (modified from (El-Soda et al. 2014)).

Metabolomics

Plants constitute an important source of metabolites. There are two types of metabolites. Primary metabolites include for example organics acids, sugars, and amino acids, all of which are considered essential for the plant and involved in growth and developmental processes. On the other hand, secondary metabolites are considered end-products of primary metabolites and are known to play a major role in the defence mechanisms of plants against their environment, although they have a broader functional range. Currently, several methods are available to detect these metabolites, which opened the door towards comprehensive metabolomics (Carreno-Quintero et al. 2013). Gas chromatography time-of-flight mass spectrometry (GC-TOF-MS) is a powerful tool to study primary metabolites (Lisec et al. 2006). In addition, the possibility of conducting untargeted metabolomics has enabled the simultaneous study of a wide range of metabolites, providing a global view of metabolic changes. The variation in the composition, but also abundance of metabolites is often observed in plants during development, in response to stress, across different organs as well as across genotypes (Kooke and Keurentjes 2012). In seeds, comprehensive metabolic studies have revealed large metabolic changes (Fait et al. 2006). The qualitative and quantitative variation of these metabolites has led to the investigation of the underlying genetic basis. The idea of combining classical QTL analysis with ~omics data was first introduced in 2001 and was termed 'genetical genomics' (Jansen and Nap 2001). In this approach, the quantitative variation of metabolites measured in a segregating mapping population is considered as an 'endo'- or 'molecular phenotype' which is used as a variable for the QTL analysis. The QTL analysis will result in the identification of genomic regions associated with variation for specific metabolites (metabolite QTLs, mQTLs). The co-localization of mQTL might indicate the co-regulation of the metabolic compounds caused by an underlying causal gene(s) (Keurentjes et al. 2006). For Arabidopsis, genes involved in many biosynthetic pathways have been identified (Kanehisa and Goto 2000). Mapping QTLs for metabolites enables to uncover the dynamics of their regulation under different conditions.

Transcriptomics

The transcriptome corresponds to the ensemble of mRNAs present in an organism, organs, tissue or cell. The transcriptome is highly dynamic across tissues, organs, in response to abiotic stresses and across genotypes. Microarrays have been extensively used to simultaneously measure a large number of transcripts matching designed probes. More recently, the advent of high-throughput technologies in combination with their continuously decreasing costs has promoted the use of RNA-sequencing for gene expression profiling (Wang et al. 2009).

In seeds, major shifts in gene expression have been identified between dormant and non-dormant seeds (Bassel et al. 2011; Dekkers et al. 2013), during seed desiccation (Maia et al. 2011; Costa et al. 2015), during seed imbibition and seed germination (Dekkers et al. 2013), (Joosen et al. 2012) and during seedling establishment (Silva et al. 2016).

A popular approach to identify coordinated changes in gene expression is the use of co-expression networks (Bassel et al. 2011; Costa et al. 2015; Silva et al. 2016). These networks are built on the pairwise correlation of genes which expression is measured in a wide range of conditions or time points. The properties of the networks can be exploited to derive biological hypotheses. Highly correlated transcripts cluster together and form modules of co-regulated genes. In these modules, highly connected genes (Hubs) can be identified as master regulators. In a recent study by Silva et al. (2016), a co-expression network was built from gene expression data obtained from several time points during seed germination and seedling establishment. In parallel, dominant patterns of gene expression were identified. By overlaying these dominant patterns on the co-expression network, an homeodomain leucine zipper 1 transcription factor ATHB13 could be identified as regulator involved in the transition from seed to seedling (Silva et al. 2016).

Often changes in gene expression are measured in response to environmental perturbations and limited to a few genotypes. Genetical genomics studies allow the investigation of the effect of genetic perturbations on gene expression. In these studies, variation of transcript abundance in a segregating mapping population is used for mapping expression QTLs (eQTLs). The eQTLs can be classified as cis or trans eQTLs, depending on whether the SNP causal for the eQTL is inside or outside the gene under study, respectively (Figure 2a,b) (Rockman and Kruglyak 2006). eQTL hotpots are prominent features of eQTL studies and offer a great starting point to investigate regulatory interactions (Breitling et al. 2008). eQTL hotspots suggest the presence of a master regulator that potentially affects the expression of multiple genes in trans which can further lead to the construction of regulatory networks (Figure 2c) (Keurentjes et al. 2007b). Several studies in yeast, nematodes, humans and plants (West et al. 2007; Schadt et al. 2003; Brem et al. 2002) have shown that typically expression of a large proportion of genes is influenced by eQTLs.

Generalized genetical genomics

Plants, challenged by their surrounding environments, undergo remarkable changes in their metabolome and transcriptome. Understanding the influence of the environmental variation on the genetic architecture of these traits can bring further insights into the dynamic of gene expression regulation. In contrast to the large QTL x E studies of classical phenotypic QTLs, QTL x E in the field of ~omics often remains limited to one or a few conditions because of the substantial costs associated with molecular profiling of large segregating mapping populations. In the effort to reduce these costs, while optimizing the detection of ~omics QTL x E, (Li et al. 2008b) proposed a generalized genetical genomics (GGG) (Figure 3, GGG) set-up. From an initial mapping population replicated in four conditions, this design consists of selecting equal subsets of non-overlapping RILs for each of the four conditions. In addition, the RILs are partitioned in such a way that there is a balanced allele distribution within each RIL sub-population which allows the newly designed sub-populations to be analysed separately or combined. Separately, the genetic effect is assessed under each condition, while combining the RILs allows including the environment and genotype-by-environment interaction terms in the mapping model. This design was recently applied to investigate changes in the genetic architecture of the seed metabolome at different germination stages in Arabidopsis and tomato (Joosen et al. 2013; Kazmi et al. 2017).

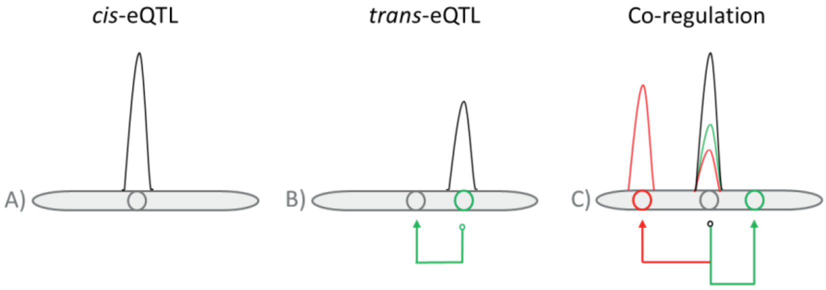

Figure 2: Regulation of gene expression. The circles on the chromosome indicate the physical position of the genes and their respective LOD curves are shown with matching colours. Arrows are draw from the gene with variants to the gene(s) it regulates. Several eQTL patterns can be observed. A) A cis eQTL is an eQTL detected at the physical position of the gene. B) A trans eQTL is an eQTL detected at a distant location of the physical position of the gene, which suggests that its regulation is governed by another gene (green circle) underlying the trans eQTL. C) Cis and trans eQTLs co-locate and form hotspots when the number of co-locating eQTLs reaches a certain significance threshold. The discovery of hotspots can be useful to identify sets of co-regulated genes.

Systems genetics

QTL analyses enable the identification of genomic regions controlling trait variation while the identification of the underlying candidate genes through fine mapping remains a difficult task. Linking genetic components identified by QTL analysis to genomics information can provide deeper insights into the molecular basis of quantitative traits (Paran and Zamir 2003). Genetical genomics studies have paved the way for systems genetics approaches (Hansen et al. 2008). The combination of phenotypic and molecular data generated in similar genetically and environmentally disturbed systems can provide a global and integrative view of the genetic architecture of complex traits (Civelek and Lusis 2014; Ligterink et al. 2012).

The relation between the different phenotypic and ~omics data can be examined using several approaches (Civelek and Lusis 2014), which include correlation analysis of the different traits (Carreno-Quintero et al. 2012) or the identification of genetic markers that affect several traits (co-locating QTLs) (Wentzell et al. 2007). Handling such large data sets makes it difficult to prioritize important links between phenotypes and molecular data, which thus requires the use of appropriate visualization methods. In this respect, correlation and co-expression network analysis provides a useful approach to display, organize, integrate and eventually identify biologically meaningful entities for further in-depth investigations (Langfelder and Horvath 2008).

Arabidopsis thaliana

In many ways, Arabidopsis thaliana is an ideal organism for dissecting complex traits. This small flowering plant belongs to the Brassicacea family. It's genome was the first plant genome sequenced in 2000 (Analysis of the genome sequence of the flowering plant Arabidopsis thaliana 2000). It has a diploid genome which is distributed over five chromosomes spanning 120 Mb and counting approximately 30.000 protein-coding genes. Arabidopsis thaliana offers unique possibilities for genetic studies due to its wide distribution of natural habitat, the great genetic variation and because it is predominantly a self-pollinating plant (Koornneef et al. 2004; Alonso-Blanco et al. 2016). In addition to its broad natural variation, Arabidopsis is also very suitable for linkage mapping. The self-fertilizing nature of the plant facilitates the construction and maintenance of different types of genetic material such as recombinant inbred lines (RILs) (Alonso-Blanco and Koornneef 2003), heterozygous inbred families (HIFs) (Tuinstra et al. 1997) and near-isogenic lines (NILs) (Keurentjes et al. 2007a). In addition to that, the relatively high recombination rate makes it possible to map QTLs to a relatively fine scale with a relative small population as compared to other species (Glazier et al. 2002). Another advantage of using Arabidopsis is that a large number of plants can be grown and replicated under uniform conditions. In combination to this, the plant has a short life cycle (approximately 3 months) which generally ends in a large seed output. These criteria have motivated the use of this plant as a model system in plant science (Koornneef and Meinke 2010). Since its adoption as a model plant, extensive studies using Arabidopsis have generated a wealth of molecular tools and resources to dissect complex traits in Arabidopsis and other species. In the present thesis, another advantage of using Arabidopsis is the availability of, the GERMINATOR, a high-throughput phenotyping method to score germination (Joosen et al. 2010). This method is based on image analysis and relies on the colour contrast between the seed coat and the protruding radicle which marks seed germination (Bewley 1997). The data provided by image analysis are used as input for a curve-fitting module which returns quantitative parameters that describe the germination behaviour of a seed batch. These parameters include the t10 and t50, the time needed to reach 10% and 50% of seed germination, respectively; Gmax, the maximum germination percentage and the AUC, the integration of the area under the germination curve (Joosen et al. 2010). This method has been used previously to investigate the genetic basis of seed performance in large populations (Joosen et al. 2012; Vidigal et al. 2016).

Scope of the thesis

The research presented in this thesis aimed at understanding to what extent the seed maternal environment influences the genetic control of seed performance. In a systems genetics approach, I investigated environmentally and genetically induced changes at the metabolome and transcriptome levels. This work lays the foundation in view of ultimately integrating the different biological scales to provide a system understanding of the control of seed performance.

Chapter 1 introduces the definition and the importance of seed quality and performance. Particular emphasis is given to the environmental regulation of seed performance. Natural variation represents a valuable resource to explore the genetic basis of complex traits. I highlight the potential of genetical genomics to elucidate changes in seeds at the molecular level to ultimately, in a systems genetics approach, get better insights into the control and regulation of seed performance. The general approach I used is summarized in Figure 3 and detailed below.

In **Chapter 2**, I show that the QTL mapping resolution can be improved by high-density genetic maps. Polymorphic markers were derived from RNA-seq data generated from the RILs (Chapter5). As a result I provided a new high-density genetic map for the Arabidopsis RIL Bay-0 x Sha RIL population that is subsequently used for the mapping analyses in the following chapters.

Chapter 3 sheds light into the interplay of genetic and environmental factors determining seed performance. The identified QTLs showed significant QTL x E interactions with both germination and maternal environment contributing to the explained phenotypic variance. The combined analysis of HIFs and expression data was used to narrow down an environment specific QTL and to suggest potential candidate genes.

In **Chapter 4**, I investigated the changes occurring in the seed metabolome of the RILs in response to changes in the maternal environment. This study shows that correlation networks combined to QTL mapping analyses can bring substantial insights into genetically coordinated metabolic changes reflecting metabolic investment strategies in response to stress.

Chapter 5 provides a preview on the dynamics of the genetic basis of gene expression. Using the generalized genetical genomics design, RNA-seq was performed on the dry seed of RILs grown under different conditions. Differential gene expression analysis and eQTL mapping revealed large genotype-by-environment interactions. Comparison of the eQTL features under each condition indicated a highly environment-dependant genetic control of gene expression.

Co-expression networks are an attractive approach to integrate and visualize large data sets.

In **Chapter 6**, I review how co-expression networks can be used to explore and integrate large data sets to address biological questions.
In the general discussion (Chapter 7), I summarize and integrate the key findings of this research in a general discussion providing directions for further investigations.

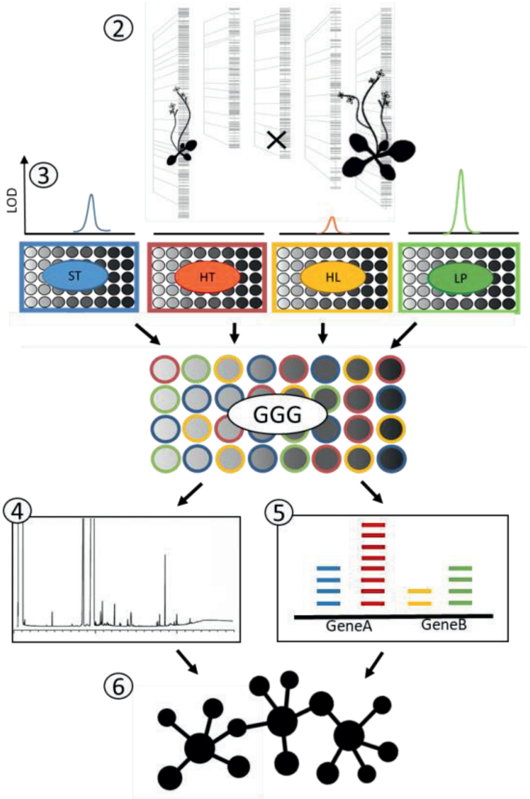

Figure 3. Graphical representation of the approaches used in the different chapters of this thesis aiming at investigating changes at the phenotypic, genetic and molecular level in dry mature seeds of an Arabidopsis RIL population grown under standard (ST), high temperature (HT), high light (HL) and low phosphate (LP) conditions. The circled numbers refer to the corresponding chapters. In Chapter 2, I build a new genetic map for the Bay-0 x Sha RIL population. This map was used in Chapter 3 to identify QTLs with varying effects across conditions (QTL x E) for seed performance. I next use the generalized genetical genomics design (GGG) to perform mQTL analyses in Chapter 4 and eQTL analyses in Chapter 5. In Chapter 6, I review current applications of co-expression networks to integrate large datasets and address biological questions.

Chapter 2

Construction of a high-density genetic map from RNA-Seq data for an Arabidopsis Bay-0× Shahdara RIL population

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Abstract

High-density genetic maps are essential for high resolution mapping of quantitative traits. Here, we present a new genetic map for an Arabidopsis Bayreuth × Shahdara recombinant inbred line (RIL) population, built on RNA-seq data. RNA-seq analysis on 160 RILs of this population identified 30,049 single-nucleotide polymorphisms (SNPs) covering the whole genome. Based on a 100-kbp window SNP binning method, 1059 bin-markers were identified, physically anchored on the genome. The total length of the RNA-seq genetic map spans 471.70 centimorgans (cM) with an average marker distance of 0.45 cM and a maximum marker distance of 4.81 cM. This high resolution genotyping revealed new recombination breakpoints in the population. To highlight the advantages of such high-density map, we compared it to two publicly available genetic maps for the same population, comprising 69 PCR-based markers and 497 gene expression markers derived from microarray data, respectively. In this study, we show that SNP markers can effectively be derived from RNA-seq data. The new RNA-seq map closes many existing gaps in marker coverage, saturating the previously available genetic maps. Quantitative trait locus (QTL) analysis for published phenotypes using the available genetic maps showed increased QTL mapping resolution and reduced QTL confidence interval using the RNA-seq map. The new high-density map is a valuable resource that facilitates the identification of candidate genes and map-based cloning approaches.

Keywords: Arabidopsis, Genetic map, Genotyping-by-sequencing, QTL mapping, RIL population, Resolution, RNA-seq

Introduction

Quantitative Trait Locus (QTL) analysis has successfully identified a large number of genetic loci that contribute to the regulation of quantitative phenotypes. The advent of -omics data has extended the range of usual mapping traits to molecular phenotypes offering new approaches for bridging the gap between genes and their function (Keurentjes et al. 2008). The idea that variation in gene expression can be treated as a quantitative trait, gave rise to the concept of genetical genomics (Jansen and Nap 2001). In combination with a genetic map, quantitative variation in gene expression measured in a segregating population enables the identification of expression QTLs (eQTLs). Many eQTL studies have contributed to our understanding of the genetic architecture of regulatory variation of intricate traits in Arabidopsis (West et al. 2007; Keurentjes et al. 2007b; Lowry et al. 2013; Cubillos et al. 2014; Terpstra et al. 2010; Snoek et al. 2012) (for review see (Joosen et al. 2009)), poplar (Drost et al. 2015), tomato (Ranjan et al. 2016), as well as in other organisms (Li et al. 2006; Li et al. 2010; Vinuela et al. 2010; Rockman et al. 2010; Aylor et al. 2011; King et al. 2014; Sterken et al. 2017; Snoek et al. 2017b).

In essence, the success of QTL mapping is determined by the mapping resolution which mainly depends on the size of the population (and thus the number of recombination events), the complexity of the phenotype and the number of available markers. High-density genetic maps are thus instrumental for accurate mapping of QTLs. Traditional methods used to obtain molecular markers were mainly PCR based (SSR, AFLP, RFLP). New methods to derive molecular markers have recently emerged, together with the advancement of high-throughput technologies. Particularly, single nucleotide polymorphisms (SNPs), represent a rich source of potential markers due to their abundance (Alonso-Blanco et al. 2016). Differences in gene expression measured with microarrays as a result of probe hybridization sensitivity to underlying sequence polymorphisms have been used to derive SNP-based markers (West et al. 2006; Zych et al. 2015; Zych et al. 2017). More recently, next generation sequencing technologies for transcriptome analysis (RNA-seq) have provided unprecedented opportunities for quantitative genetics in plants (Jimenez-Gomez 2011). Becoming a standard for gene expression profiling, RNA-seq has also proven to be an efficient and cost-effective method to identify genome-wide SNPs (Piskol et al. 2013; Markelz et al. 2017). In the context of genetical genomics, RNA-seq on a segregating population can simultaneously provide the molecular phenotype and the sequence information for molecular markers that subsequently provide genotyping information for the population.

Segregating bi-parental populations such as recombinant inbred line (RIL) populations are powerful tools for QTL analysis (Koornneef et al. 2004). These immortal populations capture frequent recombination events in a relatively small sized population, thereby conveniently reducing the costs for genotyping. In this study, we utilized an Arabidopsis thaliana Bayreuth x Shahdara population that has been used extensively for genetic (Loudet et al. 2002; Jimenez-Gomez et al. 2010) and eQTL studies (West et al. 2007; Keurentjes et al. 2007b). The original genetic map for this population consists of 69 markers segregating in 420 F6 RILs (Loudet et al. 2002). Further genotyping efforts on a subset of these RILs have introduced markers derived from gene expression data with microarrays, saturating the original map (West et al. 2006; Zych et al. 2015; Salathia et al. 2007). Here, we present the construction of a high-resolution genetic map from RNA-seq data of 160 RILs. We validate and show the improvements of this new map by performing a QTL analysis with publicly available phenotypic data (Joosen et al. 2012).

Materials and Methods

Plant growth and sample preparation

Seeds from the Arabidopsis thaliana accessions Bayreuth (Bay-0) and Shahdara (Sha) and a Bay-0 x Sha RIL population consisting of 165 lines were used. This population was initially developed by Loudet et al. (2002). As part of a larger experiment aiming to investigate genotype x environment interactions, the parental lines and the RILs were grown under standard and controlled mild stress conditions. In the standard condition, plants were grown under long day (16h light / 8h dark) at 70% RH and 22°C / 18°C (day/night) under artificial light (150 µmol m-2 s-1). The plants were watered with a standard nutritive solution (see supplemental table 1 in He et al. (2014)) three times a week by flooding cycles. The same conditions were used for the stress environments, except for the varying parameter as indicated hereafter: high temperature (25°C day / 23°C night), high light (300 µmol m-2 s-1) and low phosphate (12.5 µM phosphate instead of 0.5 mM in the standard nutritive solution).

The RILs and the parental lines were first grown with three to four plants per environment in a single climate cell under the control conditions mentioned above. When most of the plants flowered, the main stems of all plants were removed to increase the numbers of side branches and thereby seed production, and to ensure that all seeds would complete their development under the specific conditions. Subsequently the plants were transferred to different climate cells to continue their growth under the specific stress conditions. At the time all plants in a given condition produced a sufficient amount of fully matured seeds; the seeds were bulk harvested from the 3-4 plants per line. After drying, a fraction of the freshly harvested seeds were stored at -80°C in sealed 2 ml tubes until RNA-seq library preparation.

RNA isolation and sequencing

RNA was isolated from 4-5 mg of fresh harvested dry seeds that were stored at -80°C. Each of the parents was measured in triplicate per condition *i.e.* 4x3 = 12 replicates per parent. RNA was extracted from the seeds of 160 RILs selected in conformity to the generalized genetical genomics strategy (GGG, Li et al. (2008b) and Table S1). RNA was isolated using the NucleoSpin RNA plant isolation kit (Macherey-Nagel 740949) but adding Plant RNA isolation Aid (Life technologies) according to the manufacturer's protocol and instructions.

RNA-seq reads processing

Strand specific RNA-seq libraries were prepared from each RNA sample using the TruSeq RNA kit from Illumina according to manufacturer's instructions. Poly-A selected mRNA was sequenced using the Illumina HiSeq2500 sequencer, producing strand-specific single-end reads of 100 nucleotides. Reads were trimmed using Trimmomatic (version 0.33, Bolger et al. (2014)) to remove low quality nucleotides. Trimmed reads were subsequently mapped to the Arabidopsis thaliana TAIR10 reference genome (Lamesch et al. 2012) using the HISAT2 software (version 2.0.1, (Kim et al. 2015)) with the "transcriptome mapping only" option. SNPs were called using the mpileup function of samtools (version 0.1.19, Li et al. (2009b)) and bcftools.

SNP identification and RIL genotyping

Variant call format (VCF) files were generated for each of the samples. Since not all SNPs are found in all genotypes, all vcf files were merged to generate a list with all variants present in at least one sample. From this unique list, information regarding the position in base pairs and the chromosome location of each SNP was retrieved and filtered for being consistent across the sequencing data of the parental lines. In order to get a more reliable genotypic score, cancelling out any SNPs miscalls, and to reduce the overall number of markers, SNPs were grouped into bins. 1059 equal size artificial bins of 100 kbp were created along the whole genome. The scoring of the genotype was obtained based on the SNP information within each bin. For regions at the transition between two genotypic blocks, the bin score was rounded up and assigned to the closest genotypic score. The quality of the genotype scoring of the bins was assessed by correlation analysis.

Nomenclature

The bins are ordered based on the genome sequence, thus the unit distance is not expressed in centimorgans (cM) but in bins of 100 kbp. Each bin is used as a marker and the midpoint position of the 100 kbp bin is used as the marker position. Markers were named RSM for RNA-seq markers, followed by the chromosome number of their location and their physical position in mega base pairs (Mbp). As an example RSM_1_0.05 corresponds to the marker at 0.05 Mbp on chromosome 1.

Genetic map construction

The genetic distances in centimorgans of the 1059 markers for 160 RILs were estimated in order to describe and compare the new genetic map to previous maps. The genetic distances were estimated using the “est.map” function with “kosambi” distance from the R/qtl package (Broman et al. 2003)(Arends et al. 2010). The correct order of the markers was verified by pairwise marker linkage analysis using the “est.rf” function. The recombination rate was determined based on the linear relation between the genetic and the physical positions of the marker. The segregation pattern was tested for all markers to identify markers that show significant distortion at the 5% level, after a Bonferroni correction for multiple testing. The statistical programming language R (version 3.3.2) (Team 2008) was used for all analyses. The genetic map and genotypic data are available in table S2.

QTL comparison

To test the effect of increased marker coverage on QTL mapping, we re-mapped 510 published phenotypic traits using the RNA-seq (1059 markers), the pheno2geno (497 markers) (Zych et al. 2015) and the original map (69 markers) (Loudet et al. 2002). In order to compare the mapping resolution, the genetic distances were re-estimated for each map using 145 RILs common to the three studies (Supplemental table 1). The scanone function in R/qtl was used with the default settings for the QTL mapping. LOD score peaks were called by chromosome for each trait, resulting in a total of 2550 (510*5) peak LOD scores. The LOD threshold for the genome-wide significance at the level of 5% was determined after 1000 permutations using each map. The LOD thresholds obtained were 2.36, 2.64 and 2.76 using the original, pheno2geno and RNA-seq map, respectively. The increased LOD thresholds for the Pheno2geno and the RNA-seq map can be explained by the larger number of

markers which will result in a larger multiple testing corrections. We used a stringent LOD threshold of 3 to identify and compare significant QTLs for all maps. The LOD score comparison was performed in a similar way as described in Zych et al. (2015). To be more confident about the comparison, QTLs were considered to have a higher or lower LOD score if the difference between the compared LOD scores was larger or equal to 0.5. The mapping resolution of the RNA-seq map was investigated by comparing the confidence intervals (CIs) of QTLs for the RNA-seq and the original map. LOD-1 CIs were determined for all significant QTLs (LOD >3) for both maps. The genomic positions of the lower and upper limit of each CI were estimated from the equation of the linear relation between genetic and physical position of the markers. Subsequently, the CI width was determined for each QTL in Mbp. The analyses and figures were generated using Microsoft Excel, R/qtl and the R ggplot2 package.
The cross object containing all data for the 510 phenotypes in the 160 RILs for the QTL analysis is available in supplemental table 3. The QTL results for the comparison of the LOD scores and confidence intervals are provided in supplemental table 4 and 5. QTL profiles of the re-mapped 510 traits are available for interactive analysis in AraQTL (www.bioinformatics/araqtl.org, Nijveen et al. (2017)).

Results

Genotyping the RIL population using a SNP binning approach

SNP calling resulted in 185,354 SNPs distributed over the five chromosomes, ranging from 26,514 SNPs for chromosome 2 to 48,151 SNPs for chromosome 1 (Figure 1). Regions with a few or no SNPs correspond to centromeric regions, known to have lower transcriptional density and expression activity (Schmid et al. 2005). Filtering and quality check of the SNPs (as described in Material and Methods) resulted in a final number of 30,049 SNPs covering the whole genome.

The 100 kbp binning approach used, collapsed the 30049 SNPs into 1059 bins distributed over the five chromosomes. Each bin contained on average ~24 SNPs, with a minimum of 2 and a maximum of 130 SNPs per bin (Supplemental figure 1). Overall, 96.7% of the bins could unambiguously be assigned to one of the parental genotypes.

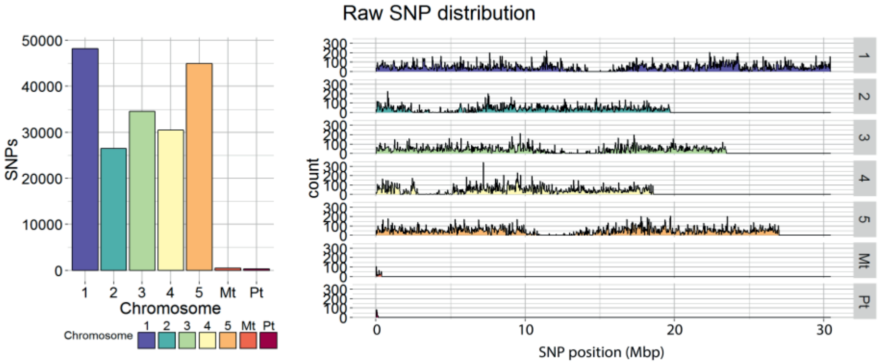


Figure 1. Raw SNP distribution from all genotyped RILs. A. Total SNP count and B. coverage counts of each SNP at each physical position on the chromosome in mega base pairs (Mbp) are displayed for each of the five chromosomes of Arabidopsis thaliana as well as the mitochondrial (Mt) and plastid (Pt) genomes.

Population-based SNPs segregated at the expected allele frequencies as global allelic equilibrium was observed with 49.3 % Bay-0 alleles and 50.7 % Sha alleles. Bias in the segregation ratio between the parental alleles was analysed along the chromosomes (Figure 2). Statistically significant distortion of segregation was observed for 29 consecutive markers on chromosome 4, representing 2.78% of the total number of markers. These distorted markers correspond to the region comprised between the markers RSM_4_12.05 and RSM_4_14.85. The highest distortion was observed at the marker RSM_4_13.05 with 41 (25.6%) lines representing the Bay-0 allele versus 114 (114/160 = 71.25%) lines representing the Sha allele. This deviation from the allelic equilibrium at the chromosome 4 was also reported by Loudet et al. (2002).

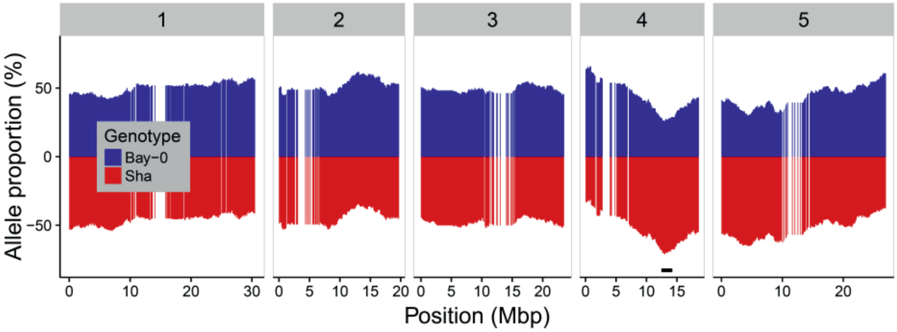


Figure 2. Allele distribution for the 1059 markers along the five chromosomes. Blue and red colors indicated the Bay-0 and the Sha allele percentages, respectively. The black horizontal bar indicates the region on chromosome 4 with 29 markers showing significant segregation distortion (p-value < 0.05 after Bonferroni correction).

RNA-seq genotyping identifies new introgressions

Visually, the binning method resulted in the identification of clear genotype blocks (Figure 3). Breakpoints were identified as the point of transition between two genotype blocks. In total, 1455 crossovers were identified with an average of 291 crossovers per chromosome (Table 1). To identify introgressions that were previously not detected, the 1059 new markers together with the 69 'old' markers were first ordered based on their physical positions.

New introgressions were then identified in the RILs as double recombination events occurring within a region spanned by two 'old' flanking markers and of a minimum size of 200 kbp (2 bins) (supplemental figure 2). We could identify 80 unambiguous introgressions with sizes ranging between 200 kbp and 3 Mbp, increasing the number of recombination events detected within the RIL population.

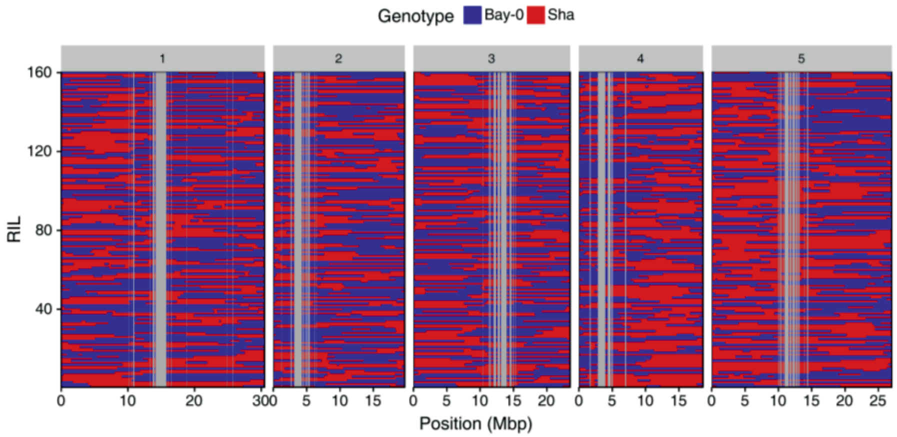


Figure 3. Haplotype representation of the 160 RILs. Each row corresponds to a RIL. Columns represent the 1059 genetic markers physically anchored on the 5 chromosomes. Blue boxes indicate Bay-0 genotype and yellow boxes indicate Sha genotypes.

Table 1: Characteristics of the 1059 marker genetic map using 160 RILs.

Chr	Markers	Total length(cM)	Average marker distance (cM)	Maximum gap (cM)	Cross-overs	Recombination rate (kbp/cM)
chr 1	275	117.87	0.43	2.87	364	258.34
chr 2	171	76.29	0.45	3.23	236	257.58
chr 3	207	82.61	0.40	2.72	255	283.85
chr 4	163	92.12	0.57	4.81	281	201.36
chr 5	243	102.81	0.42	2.34	319	262.13
Total	Total 1059	Total 471.70	Average 0.45	Max 4.81	Total 1455	Average 252.63

High-density genetic map

Using each bin as a marker, the linkage map was calculated in order to validate the order of the markers and evaluate the accuracy of the new map. The characteristics of the new map are reported in Table I. The total length of the genetic map was 471.70 cM. The average genetic distance between two adjacent markers of 0.45 cM represents a great increase in marker density as compared to the 6.1 cM of the 69 markers map for 420 RILs (Loudet et al. 2002). In the new map, the largest gap between two markers is 4.81 cM between the markers RSM_4_1.55 and RSM_4_1.85 on chromosome 4.

Overall, the order of the markers on the genetic map conforms to the physical position of the marker and is also supported by the pairwise marker linkage analysis (Figure 4). The recombination rate was calculated as the relation between the physical and genetic distances. Low recombination was observed at the centromeric regions where the physical distance was greater relative to the genetic distance. On the upper arm of chromosome 3, no recombination events occurred between the markers RSM_3_2.65 and RSM_3_5.25. This was also observed in the 69-markers map as well as in a Sha x Col-0 RIL population (http://publiclines.versailles.inra.fr/page/33). A Sha specific chromosomal inversion in this region was suggested (Figures 4 and 5). The global recombination rate is 252.65 kbp / cM, *i.e.* 4.01 cM per 1 Mbp (Figure 5). This rate is consistent with previously reported recombination rate of 246 kbp / cM (Loudet et al. 2002).

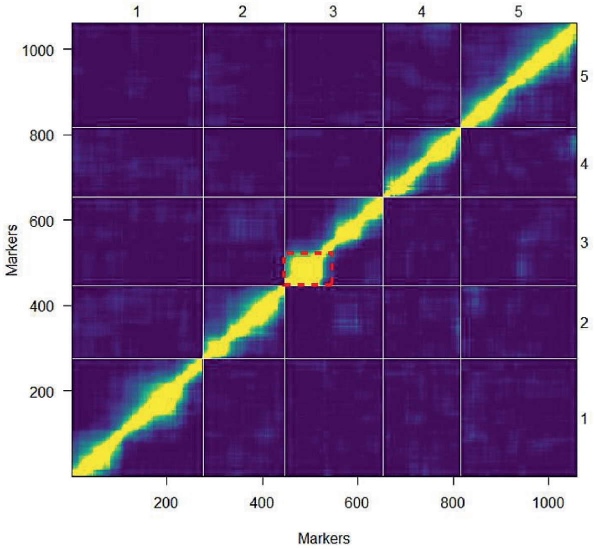


Figure 4. Pairwise marker linkage analysis. The estimated recombination fraction and LOD scores for all pairs of markers are shown in the upper-left and lower-right triangle, respectively. High correlation between markers indicates marker linkage (yellow) while the blue colour shows low correlation values indicating unlinked markers. The grid delineates the five chromosomes. The red dotted frame indicates the region at the top of chromosome 3 with the probable occurrence of an inversion.

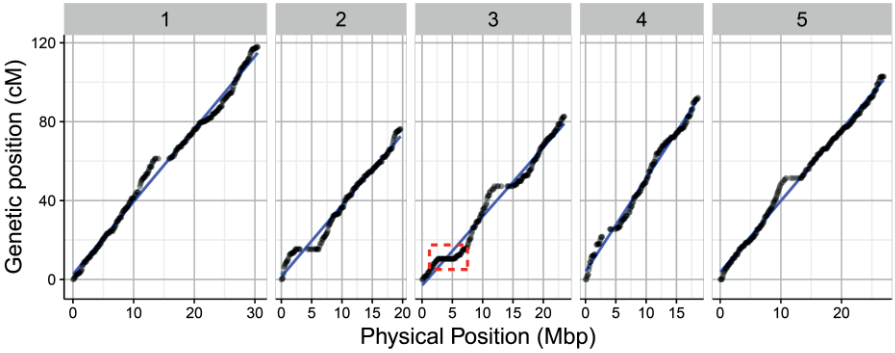


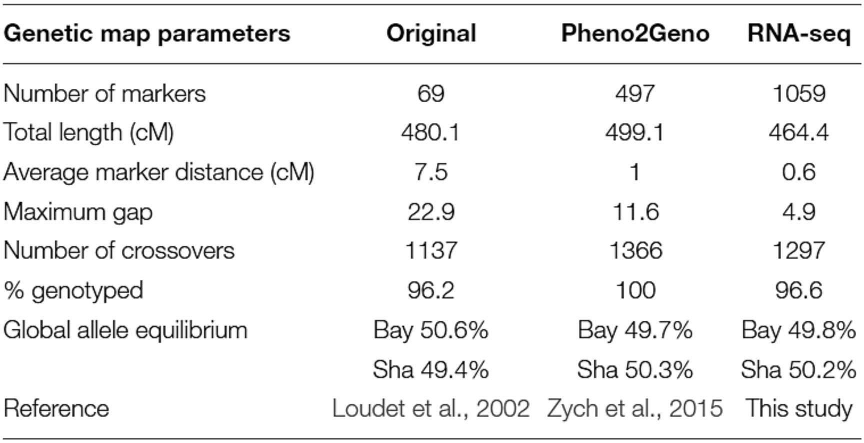
Figure 5. Relation between the genetic length in centimorgans (cM) and the physical length in Mbp for the 1059 markers along the five chromosome using 160 RILs of the Bay-0 x Sha RIL population. The red dotted frame indicates the region on the upper arm of chromosome 3 without recombination events.

QTL mapping comparison

The original genetic map for the analysed Bay x Sha population developed by Loudet et al (2002) comprises 69 PCR-based markers. Recently, Zych et al. (2015) saturated the original map with 497 markers derived from microarray expression data (pheno2geno map). To compare the published maps to the RNA-seq map, the genetic distances were re-estimated using 145 RILs common to the three studies (Table S1).

The RNA-seq map reduces the average distance between markers from 7,5 cM for the 69 marker map and 1 cM for the pheno2geno map to 0.6 cM (Table 2), closing many existing gaps in marker coverage (Figure 6). In addition, the RNA-seq map captures 1297 crossovers as compared to 1137 in the original map. The number of crossovers observed with the pheno2geno map (1366 cross-overs) is likely inflated due to the imputation of the genotypic data to 100% (% genotyped in table 2).

Table 2. Summary of genetic maps for the Bay-0 x Sha RIL population based on 145 RILs



Genetic map parameters	Original	Pheno2Geno	RNA-seq
Number of markers	69	497	1059
Total length (cM)	480.1	499.1	464.4
Average marker distance (cM)	7.5	1	0.6
Maximum gap	22.9	11.6	4.9
Number of crossovers	1137	1366	1297
% genotyped	96.2	100	96.6
Global allele equilibrium	Bay 50.6%	Bay 49.7%	Bay 49.8%
	Sha 49.4%	Sha 50.3%	Sha 50.2%
Reference	Loudet et al., 2002	Zych et al., 2015	This study

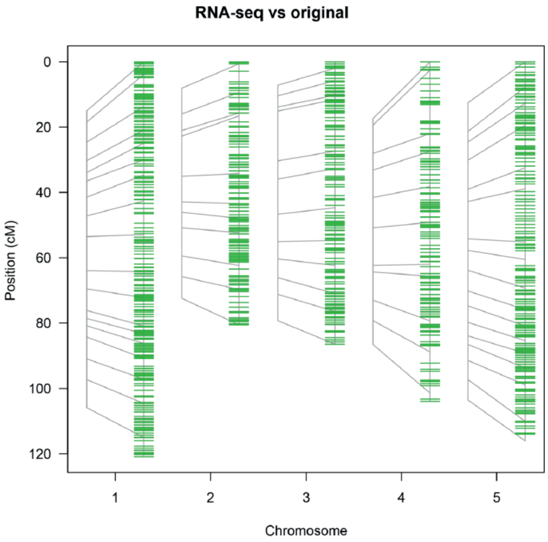


Figure 6. Saturation of the original map (69 PCR-based markers) with RNA-seq derived markers. The position of the original markers is represented on the left of each chromosome in grey and linked to their position in the saturated map (green markers).

QTL mapping was performed to evaluate the mapping resolution of the RNA-seq map as compared to the two other maps. Using a genome-scan single QTL model analysis, 510 published phenotypes were re-mapped using the three maps. The QTL analysis with the RNA-seq map resulted in 754 significant QTLs (LOD > 3), while 684 and 568 significant QTLs were detected using the pheno2geno and the original map, respectively (Table 3, Figure 7). QTLs were considered to have a higher or lower LOD score if the difference between the compared LOD scores was larger than or equal to 0.5. Respectively, 223 and 183 of the total number of significant QTLs in the original map did show an increased LOD score in the pheno2geno map and RNA-seq map (Figure 7A-B, Table 3). When compared to the pheno2geno map, the RNA-seq map resulted in 180 QTLs with a higher LOD score (Figure 7C, Table 3). The pheno2geno map identified 139 new QTLs compared to the original map, while the RNA-seq map added 208 new QTLs. 125 new QTLs were detected in the RNA-seq map as compared to the pheno2geno. In addition, an increase in the LOD scores was observed using the RNA-seq map as compared to the original map (average LOD score differences of 1.74) and the pheno2geno map (1.66) than for the pheno2geno compared to the original map (1.15) (Table 4). Together, these results indicate that the higher marker density of the RNA-seq map provides additional power to detect QTLs.

Table 3. Comparison of LOD scores using the different maps

Genetic map[1] (/compared to)	Significant QTLs (LOD>3)	"New" and "lost" QTLs[2]	Higher LOD QTLs[3]	Lower LOD QTLs[4]
Original	568	-	-	-
Pheno2geno/original	684	**139**/23 (**24%**/0.4%)	223 (39%)	54 (9.5%)
RNA-seq/original	754	**208**/22 (**30%**/0.4%)	183 (32%)	97 (17%)
RNA-seq/pheno2geno		**125**/55 (**18%**/8%)	180 (26%)	185 (27%)

[1] The new maps used for the comparison are indicated in bold. [2] New QTLs are the number of QTLs with a LOD score above 3 in the new map and below 3 in the compared map (bold numbers). These numbers are compared to the number of significant QTLs in the compared map "lost" in the new map. [3] Higher LOD QTLs is the number of QTLs with a higher LOD score in the new map with a difference in LOD score equal or larger than 0.5. [4] Lower LOD QTLs is the number of significant QTLs with a higher LOD score in the new map with a difference in LOD scores equal or larger than 0.5. Percentage of new, lost and lower LOD QTLs in relation to the total number of significant QTLs in the compared map are shown in brackets.

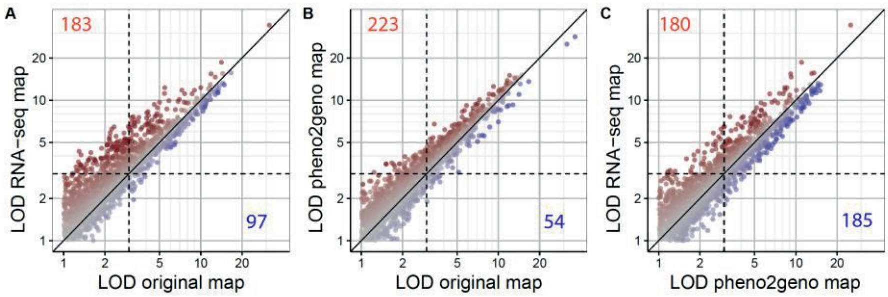


Figure 7. LOD score comparison of QTLs for 2550 QTL peaks of 510 published phenotypes using the original (A, B), the pheno2geno (B, C) and the RNA-seq map (A, C). The significance threshold is indicated by a dashed horizontal and vertical black line. "Stronger" LOD scores are plotted in red. Red and blue numbers correspond to the number of significant QTLs identified on the x-axis map with increased or decreased LOD scores in the y-axis map, respectively.

Table 4. Average LOD score differences across the different maps

	Genetic maps	A		
		Original	**Pheno2geno**	**RNA-seq**
B	Original	-	1.15 (0.04)	1.74 (0.10)
	Pheno2geno	1.45 (0.19)	-	1.66 (0.12)
	RNA-seq	0.98 (0.04)	1.2 (0.04)	-

Numbers indicate the average LOD score difference for QTLs with higher LOD score using map A as compared to map B. Standard errors are indicated in brackets. The numbers of QTLs used for the analysis are reported in **Table 3** (see higher and lower LOD QTLs).

A main factor for the success of QTL experiments is the precision in the estimation of the position of the QTL. We assessed the RNA-seq map resolution by comparing the confidence interval (CI) of QTLs detected in the original map and the RNA-seq map. The CI of 546 QTLs significant in both maps was calculated (LOD >3). 457 (84%) of the QTLs showed a reduced interval in the RNA-seq map (Figure 8). The difference in interval width ranged from 0.08 Mbp to 25.58 Mbp. For example, the QTL for seed circularity at the top of chromosome 5 was delimited to a genomic region of less than 1.12 Mbp using the RNA-seq map compared to more than 26 Mbp using the original map (Figure 9). To verify the consistency of these results, the analysis was also conducted with a LOD threshold of 2 and for QTLs with higher LOD scores using the original map (Supplemental figure 3). 81% (770/952) of the QTLs showed a reduced CI using the RNA-seq map when the significance threshold was lowered to LOD > 2 (supplemental figure 3A). Analysis of 233 significant QTLs in both maps for which the LOD score was higher in the original map as compared to the RNA-seq map, resulted in 72% (169/233) of these QTLs showing a reduced CI using the RNA-seq map (supplemental figure 3B). These results clearly show that the accuracy of the QTL mapping is improved by using the high density SNP bin map.

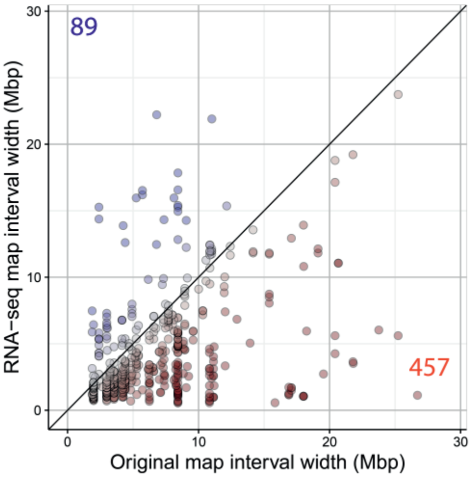


Figure 8. Comparison of the QTL mapping resolution using the original and the RNA-seq map. Confidence intervals (in Mbp) of QTLs detected in the original and the RNA-seq map are shown. Red and blue dots/values indicate the number of significant QTLs (LOD >3) with reduced and increased confidence interval in the RNA-seq map, respectively.

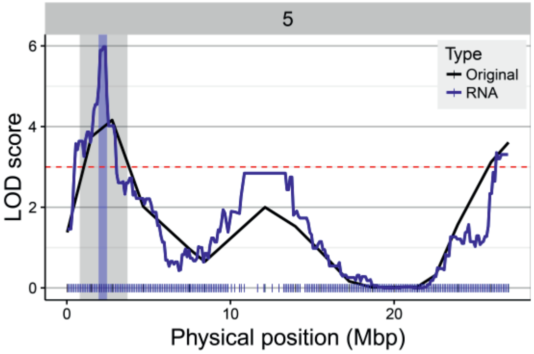


Figure 9. Gain in QTL mapping precision using the RNA-seq map. The figure illustrates the differences in LOD score and confidence interval of the QTL for the trait “Size_circ_D_mei.10” located on the top of chromosome 5 using the original (black line) and the RNA-seq map (blue line). The physical position of the markers in the original and RNA-seq map are represented on the x-axis with black (17) and blue (243) tick marks, respectively. The QTL significance threshold is indicated by a horizontal dashed red line. The grey and blue vertical bars in the region of the QTL of interest indicate the confidence interval of the QTL in the original and RNA-seq map, respectively.

Discussion

High-density genetic map

In this study we showed that RNA-seq data can effectively be used for SNP calling, RIL genotyping and the development of a high-density genetic linkage map. The used binning approach resulted in 1059 high-quality multi-SNP based markers, providing a dense and equal coverage of markers physically anchored to the genome. The high marker density enabled more precise identification of recombination breakpoints and revealed unknown recombination breakpoints within the RIL population (Table 2). As a result, the mapping resolution is no longer limited by the number of markers but rather depends on the number of recombination events captured by the mapping population. This means that the advantages of high-density genetic maps in respect to mapping resolution will be considerably improved in combination with larger and/or more advanced designed populations (Balasubramanian et al. 2009; Kover et al. 2009; Liu et al. 2016). In comparison to the available genetic maps, the RNA-seq map could substantially increase QTLs linkage, eventually resulting in the identification of new QTLs (Table 3). Although, the pheno2geno map showed a larger number of QTLs with higher LOD scores compared to the original map (Table 3), the RNA-seq map considerably increased the LOD scores of significant QTLs compared to both the original and the pheno2geno map (Table 4). Although we focussed in this study on the highest QTL per chromosome and per trait, we expect the RNA-seq map to also increase the overall number of QTLs after a more comprehensive analysis.

Gain in QTL mapping resolution

The detection power and resolution of QTL mapping is significantly improved by high density genetic maps as compared to traditional markers (Yu et al. 2011). With the RNA-seq map, a major improvement was observed in the reduction of the LOD-1 confidence intervals for 74% of the investigated QTLs. As a QTL CI in general encompasses a large number of genes, reduced confidence intervals is of great benefit to narrow down the number of candidate genes for further investigation. In genetical genomics experiments, eQTLs can be identified as being either cis- or trans-regulated. Commonly, the distinction of both is made based on the distance, in cM or Mbp, between the gene and the eQTL peak or from the confidence interval of the eQTL (West et al. 2007; Keurentjes et al. 2007b; Lowry et al. 2013; Cubillos et al. 2014; Terpstra et al. 2010; Snoek et al. 2012; Drost et al. 2015; Ranjan et al. 2016; Li et al. 2006; Li et al. 2010; Vinuela et al. 2010; Rockman et al. 2010; Aylor et al.

2011; King et al. 2014; Sterken et al. 2017; Snoek et al. 2017b). Therefore, gain in mapping precision is also likely to contribute to a more accurate identification of cis- versus trans-eQTLs.

Advantages and limitations of using RNA-seq data

The use of RNA-seq presents several advantages over other methods. Our results show that RNA-seq data is a convenient and cost-effective source of SNP discovery , especially when a population is anyhow subjected to an eQTL analysis with the help of RNA-seq. RNA-seq can also overcome shortcomings identified from expression arrays based studies: while the effect of a SNP on the probe has enabled the identification of new sequence polymorphisms, weakened hybridization on microarrays based on expression studies can also cause the detection of false cis-eQTLs (Alberts et al. 2007; Chen et al. 2009). Furthermore, RNA-seq has the potential to study more complex levels of the genetic control of gene expression, for instance by quantification of alternative splicing (Filichkin et al. 2010; Yoo et al. 2016).

SNPs that are found with RNA-seq are inherently restricted to expressed exons, thus dependent on the developmental stage of the sequenced material and the experimental conditions. This restriction can also cause regions with low gene density or lowly expressed genes to be under represented. However, these disadvantages will often not affect the mapping due to the high number of intermediate to highly expressed genes in any tissue and the SNPs present in those genes. Although our approach finds variants that affect protein-coding sequences, it is largely blind to SNPs in promoters, introns and intergenic regions. Since exons are under high purifying selection pressure, they are evolutionary more conserved than intronic and intergenic regions and therefore harbour less polymorphisms. However, SNPs that are causal for phenotypic variation will often be found in or close to genes and therefore, SNPs in large non-genic regions will hardly result in improvements of quantitative traits mapping (Li et al. 2012). In view of the abundance and saturation of SNPs that were discovered in this study, this does not cause a disadvantage, but might limit SNP detection for crosses from nearly identical parents.

Conclusion

This study demonstrates that RNA-seq data can effectively be used for SNP discovery and the development of high-density genetic linkage maps. Here we provide a new SNP based saturated genetic map for a Bay x Sha RIL population. This saturated genetic map resulted in higher precision QTL mapping with more QTLs and considerably reducing the QTL confidence intervals. Such improvements are of great benefit for the accurate mapping of more complex traits and the identification of causal genes.

The Supplementary material for this chapter can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2017.00201/full#supplementary-material

Chapter 3

Quantitative trait loci associated with G x E for seed performance

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In preparation

Abstract

High quality seeds are required to meet optimal conditions for plant survival. A seed's innate quality is determined during seed development, tightly controlled by the mother plant's genetic make-up and affected by the environment. The interaction between genotype and environment results in substantial phenotypic variation in seed performance. Using natural variation, we aimed to unravel the effect of the seed production environment on the genetic architecture of seed quality traits. An Arabidopsis thaliana Bay-0 x Shahdara recombinant inbred line population was grown in four different seed production environments: standard, high temperature, high light and low phosphate conditions. The seeds harvested were used for an extensive germination phenotyping assay under standard and mild stress conditions. Quantitative trait loci (QTL) analyses identified many environmental sensitive QTLs (QTL x E).Variation in the QTL effects was largely determined by the germination conditions and the effect of the maternal environment was observed at the individual germination QTL level. Using heterogeneous inbred families, we confirmed one QTL strongly affected by high temperature maternal environment and suggested potential candidate genes. Together, our findings highlight the extensive environmental modulation of the genetic influence on seed performance. These data provide a system view of the seed's complex genetic architecture underlying genotype-by-environment interactions.

Keywords: Arabidopsis thaliana, maternal environment, QTL x E, seed performance

Introduction

Seed performance can be defined as the timing and level of seed germination, critical in the early stages of the plant's life cycle as it ultimately determines the survival of the species (Postmaa 2016). Seed performance relies on the quality of the seeds defined by the seed physical and physiological attributes. These attributes are acquired during seed development on the mother plant. Essential seed quality characteristics are the ability of the seed to germinate fast and uniformly under a wide range of conditions (vigour), to possess high genetic purity, to be stored for a long period of time without losing viability and the capability to establish a healthy seedling (Dickson 1980). The timing of germination is also controlled by seed dormancy which allows the seed to overcome unfavourable periods for seedling establishment (Bentsink and Koornneef 2008). Through this mechanism, germination can be put on hold for long periods of time (Finch-Savage and Footitt 2015). Primary dormancy can be released by a period of dry storage termed after-ripening. A quick release of dormancy can be accomplished by stratification which is a dark and cold treatment applied to imbibed seeds prior to germination. Another determinant of the success of germination is the post-dispersal environments of non-dormant seeds. Unfavourable germination conditions can reduce, delay or prevent seed germination (Joosen et al. 2012).

Plants are sessile organisms constantly challenged by fluctuations in their environment and need therefore to adjust their phenotype and/or evolve adaptive mechanisms (Walck et al. 2011). Seed set is a crucial phase in plant's life cycle, yet particularly sensitive to environmental stresses (Springthorpe and Penfield 2015; Bac-Molenaar et al. 2015). Both at the vegetative and reproductive stage, the mother plant as well as the developing zygote process these environmental cues (Penfield and MacGregor 2017). Environmental factors such as temperature (Kendall and Penfield 2012; Penfield et al. 2005; Fenner 1991; Huang et al. 2014), light quality and intensity (Vayda et al. 2018; He et al. 2014; Contreras 2008), photoperiod (Munir et al. 2001) and nutrient availability (He et al. 2014; Hacisalihoglu et al. 2018) have been shown to affect many plant and seed traits. Several studies suggested that the maternal environment provides a mean to adjust the timing of germination (Donohue 2009; He et al. 2014; Leverett et al. 2016; Edwards et al. 2016). As example, high temperature during seed development resulted in reduced seed dormancy (Fenner 1991), while seed maturation under cold conditions can induce strong dormancy (Chiang et al. 2011; Donohue 2009).

The existing natural variation has resulted in many studies investigating the genetic basis of dormancy in Arabidopsis (Bentsink et al. 2010; Silady et al. 2011) and seed germination in Arabidopsis (Joosen et al. 2012; Laserna et al. 2008; Meng et al. 2008; Ren et al. 2010; Vallejo et al. 2010) as well as in other crops (Basnet et al. 2015; Kazmi et al. 2012). Genetic variation was also observed for the effect of the maternal environment in panels of different genotypes (Munir et al. 2001) and the detection of significant genotype-by-environment interactions (He et al. 2014; Nicotra et al. 2010; El-Soda et al. 2014). A few studies have brought insight in G x E at the QTL level, showing that the maternal environment could interact with the QTLs (Postma and Agren 2015; Kerdaffrec and Nordborg 2017). The focus of these studies was mainly on seed dormancy in view of the trait's ecological implications (Donohue 2009). Additional knowledge on seed vigour would provide a more comprehensive understanding of seed performance and its plasticity (Finch-Savage and Bassel 2016; Nicotra et al. 2010; El-Soda et al. 2014).

The genetic and environmental components of seed performance can be determined by replicating genotypes under uniform environments. In classical linkage analysis, the natural variation comprised in recombinant inbred populations is a powerful tool to detect quantitative trait loci (QTLs) (Koornneef et al. 2004). In addition, tools to maximize the information about the environment in large multi-trait and multi-environment experiments can bring substantial insight into QTL x E (Malosetti et al. 2013; Joosen et al. 2012; Boer et al. 2007).

In this study, we used the Arabidopsis recombinant inbred line (RIL) population derived from two Arabidopsis ecotypes: Bayreuth (Bay-0) and Shahdara (Sha) (Loudet et al. 2002). The different natural habitat of these two ecotypes confers those differences in stress sensitivity making the derived Bay-0 x Sha RIL population suitable to investigate the genetic basis of stress responses. The parental lines and the RIL population were grown under standard and different mild stress conditions, from flowering until seed harvested. The conditions tested were high temperature, high light and low phosphate which were shown to significantly affect plant and seed traits (He et al. 2014). The harvested seeds were phenotyped for germination characteristics under a wide range of conditions.

Many of the germination QTLs identified showed QTL x E which was consistent with the large G x E effect observed at the phenotypic level. We used heterogeneous inbred families (HIFs) and RNA-seq expression data to narrow down the region of an interesting environment specific QTL and suggest potential candidate genes.

Material and methods

Plant and growth conditions

In this study, we used the 165 lines of the Arabidopsis Bayreuth (Bay-0) x Shahdara (Sha) recombinant inbred line core collection (Loudet et al. 2002). These RILs, as well as the parental lines, were sown on imbibed filter paper placed in individual petri dishes and stratified (4 days at 4°C in the dark). The sowing of the seeds differed in time based on an estimated flowering time of the RILs from previous experiments (Joosen et al. 2012) in an attempt to synchronize flowering. The seeds were then left to germinate in incubators with continuous light at 22°C. At radicle protrusion, 16 seeds per line were transferred to Rockwool blocks, with one seed per block. The plants were further grown in a controlled climate cell with a day/night cycle of 16h/8h at 22°C/18° with a light intensity of 150 µmol m–2 s–1 and 70% relative humidity The blocks were placed on an ebb and flow table that dispensed standard nutrient solution (Supplemental Table S1) to the plants three times a week.

When all plants reached flowering, their stems and branches were cut short in order to ensure the complete development of the seeds under each controlled mild stress condition. 3 to 4 plants per line were transferred to different climate cells. The controlled stresses applied were high temperature (HT) (25°C/23°C), high light (HL) (300 µmol m–2 s–1) and low phosphate nutritive solution (LP) (12,5 mM). The plants were allowed to grow under the different conditions until seed harvest. At the time all plants in a given condition produced a sufficient amount of fully matured seeds, seeds were bulk harvested from 3-4 plants per line. A fraction of the fresh harvested seeds was dried and stored at -80°C in sealed 2 ml tubes, while the remaining seeds were stored in paper bags placed in a cupboard at ambient room temperature for after-ripening.

Phenotyping

Seed phenotyping was performed as described previously in He et al. (2014). Seed size was determined by taking pictures of approximately 500 seeds on white filter paper using a Nikon D80 camera. Pictures were analysed using ImageJ. The same seeds were carefully transferred into weighing cup and weighed with an AD-4 Autobalance (PerkinElmer, Inc.). Single seed weight was determined by dividing the total weight by the number of seeds.

Germination experiments were performed using the GERMINATOR set-up described in Joosen et al. (2010). Seeds were sown on two layers of blue filter paper (Anchor paper company, St Paul, MN, USA; www.seedpaper.com) with 48 ml of demi-water. Up to 6 seed batches were sown on the same filter paper. Automatic scoring of seed germination was performed using a mounted camera system. Pictures were taken one to three times a day for 5 up to 10 days after sowing, until green cotyledons became visible.

The curve fitting module of the GERMINATOR was used to analyse the general cumulative germination data. Gmax was measured as the total seed germination percentage. The time needed to reach 10 or 50 % of total germination (t10, t50) were calculated when more than 10% of germination was reached. The calculation of the area under the germination curve (AUC) was extended to 300 hours in order to capture the phenotypic variation under all germination conditions. The germination tests were performed using approximately 50 seeds per experiment. Two independent experiments were performed to obtain replicated phenotypic values.

The germination potential of the fresh harvested seeds (Gmax fresh) and release of primary dormancy were determined by performing weekly germination experiments. Seeds were considered fully after-ripened if the percentage of germination reached more than 90% in two consecutive germination experiments. Fully after-ripened seeds were transferred to sealed Eppendorf tubes and stored at -80°C to prevent loss of viability during storage. The DSDS50 was calculated as the number of days of seed dry storage required to reach 50% germination (Bentsink et al. 2010).

The vigour of the seeds was assessed on fully after-ripened seeds by germinating the seeds in twelve different germination conditions. These germination experiments to test seed vigour were started when more than 80% of the lines were fully after-ripened, as described above. Seeds were germinated in demineralized water in standard germination condition. Germination experiments in sub-optimal conditions were conducted at high

(32°C) and low (10°C) temperatures, under osmotic stress (-0.6 MPa mannitol; Sigma Aldrich); under salt stress (100 mM NaCl; Sigma Aldrich) and in ABA (0.25 µM ABA, Duchefa Biochemie). Germination experiments in these conditions were performed with and without stratification. Stratification consisted of storage of the sowed imbibed seeds in the dark for 4 days at 4°C prior to germination. Since the stratification effect can induce variation in stress sensitivity, we adjusted the concentrations for the NaCl and ABA treatments to 125 mM NaCl and 0.5 µM ABA for the experiments with stratification. NaCl, mannitol and ABA stress treatments were performed by adding solutions of the indicated concentrations to the filter paper instead of demi-water prior to stratification.

Seed longevity was assessed by a controlled seed deterioration test. Dry seeds were incubated at 40°C at 85% relative humidity in a closed tank in the presence of a saturated ZnSO4 solution. After five days, seeds were removed and germinated in standard conditions as described above.

Data analysis

Previous studies showed no difference in the QTL mapping performed using transformed and non-transformed germination data (Joosen et al. 2012). Therefore, due to the large number of traits, all analyses were performed on untransformed data. Boxplots were generated with the standard R boxplot function. Spearman correlation coefficients between traits were calculated and displayed in heatmaps using R.

ANOVA analyses

ANOVA analysis was performed for phenotypic mean comparison of the seed traits measured in the RILs grown under the four maternal environments. Post-hoc Tukey test was then used at the confidence level of 0.95 to determine pairwise group significant differences.

Genotype-by-environment interactions

For each germination environment, the extent of the effect of the genotype, maternal environment and genotype-by-environment interaction on the phenotypic variation was determined using the linear model:

$Y = \mu + G + ME + (G \times ME) + \varepsilon$

Where Y is the individual performance, µ is the general mean, G is the effect of the genotype, ME is the fixed effect of the maternal environment, G x ME is the genotype by maternal environment interaction and ε the residual error. The relative contribution of the variance components to the total phenotypic variation was determined as the ratio of the sum of squares of each component to the total sum of squares.

Mapping plasticity QTLs

Trait plasticity was determined as the response to germination in sub-optimal conditions as (germination in standard + stratification) – (germination in stress + stratification) or (germination in standard – germination in stress). For these traits, we mapped plasticity QTLs. The QTL mapping was performed on these values by fitting the following model:

Y = μ + G + (G x ME) + (G x GE) + (G x S) + (G x ME x GE) + (G x ME x S) + (G x GE x S) + (G x ME x GE x S) + ε

Where Y is the performance at each marker, μ the phenotypic mean, G is the effect of the genotype, ME is the effect of the maternal environment, GE is the effect of the germination environment, (ME x GE) is the interaction between the maternal and the germination environment, S is the effect of the stratification treatment and ε is the residual error. Terms in brackets correspond to interactions between the components of the model.

Heritability

For each trait in each maternal environment (ME), the broad-sense heritability (H2) was calculated from estimated variances as H2 = σ^2G / (σ^2G+ σ^2E) where σ2G is the genetic variance and σ2E is the environmental variance. The variance component analysis was analysed using a two-step mixed model approach (REML) from the preliminary single environment analysis in Genstat (18th Edition). Genotype and replicate were set as random effects in the model.

QTL analysis

The mapping was performed using a genetic map for the Bay-0 x Sha population derived from RNA-seq data (Serin et al. 2017). Briefly, the genotyping of 160 RILs resulted in the identification of 1059 polymorphic markers between the two parental lines. In total, the map spans 471 cM. To reduce the computational time and model complexity of the multiple environments QTL x E analysis, the number of markers was reduced to 221 markers (Supplemental Table S1).

QTL x E mapping was performed with the mean phenotypic values per RIL of the seed and germination traits. In order to maximize QTL effect and QTL x E detection, single trait multiple-environments linkage analysis was used. Germination conditions were defined as the different environments tested within each maternal environment. The QTL x E analyses were conducted in Genstat (18th edition). For each analysis, the best variance-covariance model fitting the data was automatically selected. The best model was then used for an initial scan in simple interval mapping. The maximum step size along the genome was set to 5 cM and the minimum cofactor proximity and minimum distance for QTL selection were set to 20 cM. The threshold for the genome-wide significance level was set at α = 0.05. After this first run, markers associated to candidate QTLs were automatically set as cofactors for a composite interval mapping. Resulting QTLs were tested for their interaction with the environment (QTL x E) by selecting the final QTL model.

HIFs

Available heterogeneous inbred lines (HIFs) were selected to validate the strong effect and the high temperature specific QTLs on top of chromosome 1. The HIF198 carrying the Bay-0 (HIFBay-0) or Sha (HIFSha) allele as well as the parental lines were grown under standard and high temperature maternal environments. HIFs were grown under 100 µmol m-2 s-1 light intensity for both ST and HT environments instead of the standard 150 µmol m-2 s-1 reported previously.

Seeds were harvested and stored until primary dormancy was released. After-ripened seeds were used for germination at 32°C with two lines per genotype and two technical replicates. After one week germination at 32°C, Gmax_32 was scored. The germination trays were transferred to 4°C in the dark for four days (stratification), followed by incubation in continuous light at 22°C, to assess the full germination potential of the seeds (Gmax_22). The sensitivity of the genotype to the high temperature germination condition was measured as the difference between Gmax_22 and Gmax_32 (as delta Gmax = Gmax_22-Gmax_32). Paired sample Student's t-test was performed on the replicated phenotypic values of HIFBay-0 and HIFSha.

RNA-seq

Seed RNA isolation and RNA-seq processing is as described in (Serin et al. 2017). Briefly, RNA was isolated in triplicate from fresh harvested and freeze stored seeds of the parental lines matured under the four environments. RNA was isolated using the NucleoSpin RNA Plant isolation Kit (Macherey-Nagel 740949) according to the manufacturer's protocol and instructions.

RNA-seq libraries were prepared from each RNA sample using the TruSeq RNA Kit from Illumina according to the manufacturer's protocol and instructions. Poly-A mRNA was sequenced using the Illumina HiSeq2500 sequencer. Reads were processed using Trimmomatic (Bolger et al. 2014).

Differential gene expression analysis

All reads were mapped to the Arabidopsis thaliana TAIR10 reference genome (Lamesch et al. 2012) using HISAT2 with the 'transcriptome mapping only' option (Kim et al. 2015). The expression levels were normalized using Kallisto (Bray et al. 2016). The edgeR bioconductor package (Robinson et al. 2009) was used to measure differential gene expression between the parental lines in each environment and the response of the genotype to the environment.

Results

To identify the genetic basis of performance of seeds produced under different maternal environments (ME), the Bay-0 x Sha recombinant inbred line (RIL) population was grown in standard (ST) and three controlled mild stress environments: high temperature (HT), high light (HL) and low phosphate (LP) from flowering until seed harvest.

We performed an extensive screening of seed traits. Seed dormancy was measured as the days of seed dry storage required to reach 50% germination (DSDS50, (Bentsink et al. 2010). We measured the germination percentage after a controlled seed deterioration test (Gmax CDT) as a proxy for seed longevity. Seed vigour was assessed by germinating the seeds under 12 different germination environments (GE). Seed performance for each GE was quantified using several parameters: the germination rate (Gmax), the germination speed (t10 and t50) and the area under the germination curve (AUC), summarizing the previous mentioned germination parameters. We report the results of the AUC while data for the other germination traits are available in Supplemental Table S2.

Phenotypic variation of seed traits

Overall, large phenotypic variation was observed for the RILs as a result of differences between the parental lines. Under all maternal environments, Sha had a higher level of dormancy indicated by a lower percentage of germination for fresh harvested seeds (Figure 1A) and higher DSDS50 values (Figure 1B) as compared to Bay-0. Sha had smaller imbibed seed size as compared to Bay-0 (Figure 1F), while Bay-0 was more sensitive to the deterioration treatment as compared to Sha (Figure 1C). In general, seed maturation under the different environments resulted in significant phenotypic differences for seed and germination traits. Maturation under HT resulted in significantly smaller seeds with a lower dormancy levels (Figure 1 A, B, E), while HL increased dormancy as compared to the ST maternal environment (Figure 1 A, B). The HL maternal environment resulted in an increase in dry seed size, dry seed weight and imbibed seed size (Figure 1 D, E, F) as well as significantly higher Gmax CDT indicating a higher tolerance of the RILs to controlled deterioration treatment (Figure 1C). The LP maternal environment resulted in increased dormancy (Figure 1B) and significantly smaller dry seeds (Figure 1 E).

Large phenotypic variation was observed for the parental lines and the RILs for the seed germination phenotypes (Figure 2). For most of the traits, the Bay-0 parent had lower phenotypic values as compared to Sha, indicating higher seed vigour for the Sha parent. This was in line with the higher stress sensitivity reported for Bay-0 as compared to Sha (Joosen et al. 2012; Vallejo et al. 2010). Since the seeds used in the experiments were fully after-ripened, we found, as expected, that most of the lines (> 80%) did germinate at a high percentage in water ('standard' germination condition), while germination in sub-optimal conditions resulted in larger phenotypic variation. Under some conditions, transgression was observed, where substantial parts of the segregating progenies were performing worse or better than both of the parental lines (Figure 2).

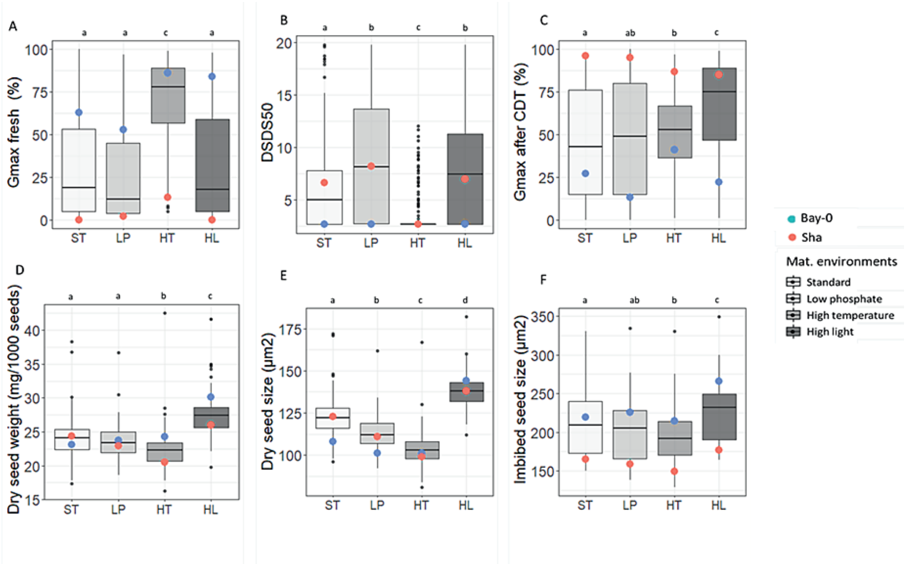


Figure 1. Effect of the seed maturation environment on seed traits. Boxplot shows the distribution of the RILs with significant difference between the maturation environments for several seed traits: A. Fresh Gmax is the maximum of germination of freshly harvested seeds. B. Dormancy is measured as the number of days of storage of dry seeds to reach 50% of dormancy (DSDS50). The values of DSDS50 as show in the plot were square root transformed to fit the scale C. Gmax after controlled deterioration test measured as a proxy for seed longevity. D. The average dry seed weight of 1000 seeds. E. Dry seed size as the average projected seed size of 1000 seeds. F. The average projected size of imbibed seeds. The phenotypic values of the parental lines, Bay-0 and Sha are indicated in blue and red, respectively. Significant differences between maturation environments are indicated by letters above the plots from the ANOVA with post-hoc Tukey HSD test results (p-value < 0.05).

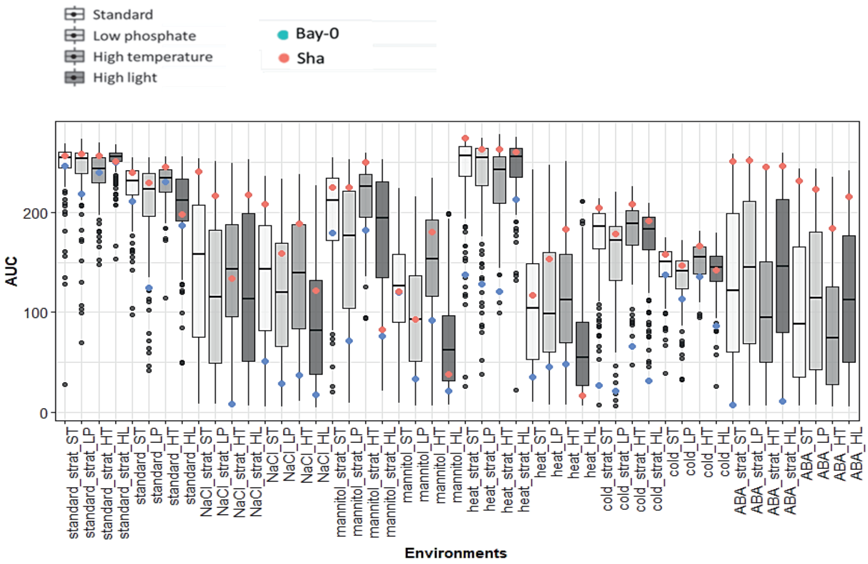


Figure 2. AUC distribution across the germination environments for the parental lines and RILs grown under the four maturation environments. Single environments are represented on the x-axis with the germination condition followed by 'strat' to indicate prior stratification and the maternal environment. Grey colour shades indicate the maternal environments, standard (ST), low phosphate (LP), high temperature (HT) and high light (HL). Center lines show the medians and outliers are represented by dots. The AUC values of the parental lines, Bay-0 and Sha under each condition are indicated by blue and red dots, respectively.

G x E for seed germination traits

As a result of genotype by environment interactions, genotypic values can increase or decrease from one environment to another, which causes the genotypes to rank differently between the environments (El-Soda et al. 2014). This re-ranking eventually results in differences in the correlation coefficients for the same trait measured across different conditions. Considering, each combination of ME and GE as a single environment, spearman correlation analysis of germination traits across the multiple single environments was performed to estimate G x E. Overall, positive correlation of AUC across environments was observed with variation in the correlation values across conditions (Figure 4). The hierarchical clustering of correlated traits indicated the presence of a substantial common genetic basis. The traits were largely clustered based on the germination conditions (*e.g.* germination in ABA; cluster

1) but the clustering was also driven by the maternal environment (*e.g.* HT maternal environment; cluster 2), suggesting additional effects of the maternal over the germination environment.

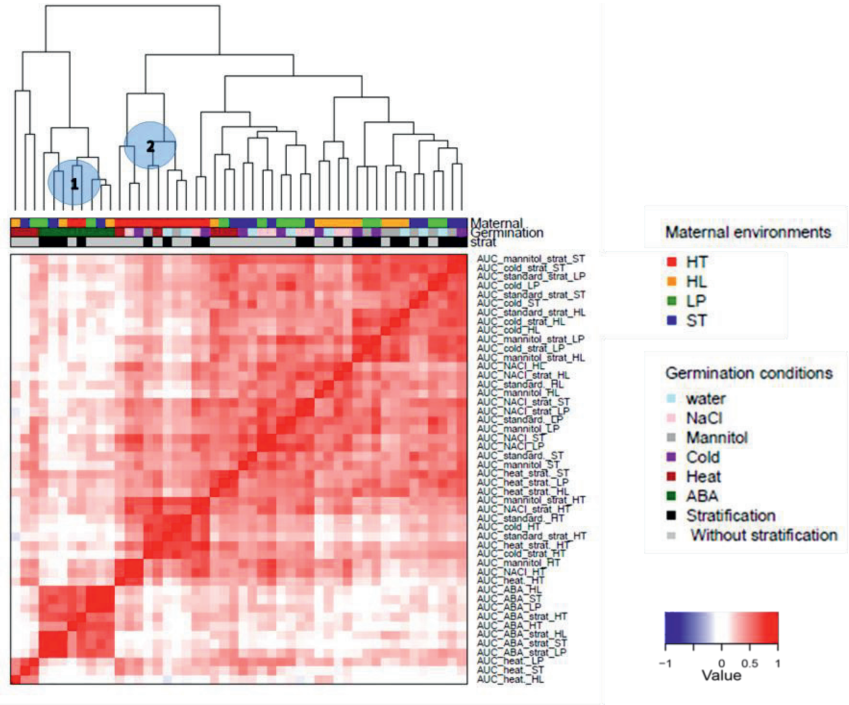


Figure 4. Heatmap of spearman correlations for the AUC measured for the whole population under different maturation environments and germinated in a wide range of conditions. The 48 germination conditions are reported on the y-axis. The name of the germination condition and the maturation environment are concatenated with a "_" symbol and colour coded according to the legend on the x-axis.

To further assess the effect of the ME in each GE, the phenotypic variance was decomposed into the main genetic and maturation environment effect and their interaction. For all germination conditions, a strong effect of the genotype was observed explaining more than 40% of the variance. The phenotypic variance explained by the ME ranged from 0.98 to 26.94% across the different GEs. The largest effect of the ME was observed for germination in mannitol, as expected (Figure 2). Under all conditions, except for germination under mannitol, the effect of G x ME was significant and larger than for the effect of ME alone. The percentage of phenotypic variation explained by G x ME ranged from 16 to 43% across the germination conditions (Table 1).

Table 1. Variance partitioning and heritability for AUC across germination conditions

	Variance components						Heritability (H^2)			
Germination conditions	σ^2_G/σ^2_P (%)		σ^2_{ME}/σ^2_P (%)		$\sigma^2_{G \times ME}/\sigma^2_P$ (%)		ST	HT	HL	LP
Standard_strat	46.04	***	3.56	***	42.95	***	0.87	0.83	0.85	0.89
Standard	49.67	***	8.37	***	33.43	***	0.84	0.80	0.90	0.90
NaCl_strat	73.74	***	4.16	***	16.17	***	0.78	0.85	0.77	0.72
NaCl	68.73	***	7.60	***	17.18	***	0.83	0.85	0.78	0.81
Mannitol_strat	52.56	***	10.71	***	27.88	***	0.87	0.85	0.89	0.89
Mannitol	45.79	***	26.94	***	21.93	***	0.83	0.88	0.86	0.85
Heat_strat	60.81	***	1.99	***	27.57	***	0.85	0.84	0.81	0.87
Heat	54.17	***	11.48	***	29.51	***	0.85	0.86	0.79	0.85
Cold_strat	53.08	***	5.24	***	27.89	***	0.87	0.83	0.93	0.90
Cold	50.23	***	5.62	***	35.28	***	0.86	0.80	0.91	0.88
ABA_strat	39.79	***	1.37	***	13.62	ns	0.00	0.25	0.09	0.30
ABA	17.90	ns	0.98	*	5.12	ns	0.16	0.10	0.00	0.12

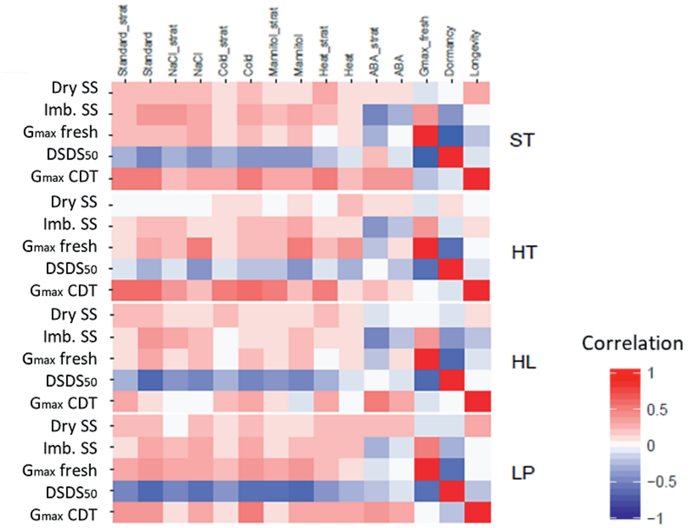
For each germination condition with ("_strat") or without stratification, the results of the two-way ANOVA analysis are shown as variance partitioning where the percentage of the total phenotypic variance (σ2P) explained by the variance components of Genotype (σ2G), the maternal environment (σ2ME) and the interaction between genotype and maternal environment (σ2G x ME) is shown. P-values of the variance analysis < 0.05 *, 0.01 **, 0.001***, ns: not significant. The broad sense heritability (H2) was calculated as the ratio of the genetic variance over the total phenotypic variance. The heritability was calculated for the AUC measured in different germination conditions in the standard (ST), high temperature (HT), high light (HL) and low phosphate (LP) maternal environments.

The strong effect of the genetic component was supported by the overall high heritability values (Table 1). The lowest heritability values were obtained for seed germination in ABA. These low values can be explained by the lack of reproducibility of the ABA treatment, *i.e.* high environmental variation over the genetic variation. Variation in heritability was also observed across the different

MEs, showing that the maternal environment could affect heritable germination traits.

Correlation between seed performance and seed traits

Overall, moderate correlation between seed traits and seed performance was observed (Figure 5). In the four populations, dormancy (DSDS50) was negatively correlated with several germination traits. This negative correlation was exacerbated under LP. The positive correlation between Gmax CDT and seed performance was exacerbated under HT. As expected, strong negative correlation was observed between dormancy and the maximum of germination of fresh harvested seeds with different levels of primary dormancy. Despite the strong effect of the environment on dry seed size (Figure 1 E), no clear correlation was observed between dry seed size and seed performance. Across all environments, negative correlation was observed between imbibed seed size and germination in ABA with stratification specifically. The Sha parent carries a natural mutation in the MUM2 gene (Macquet et al. 2007) involved in seed mucilage production. This absence of mucilage was associated with smaller imbibed seed size (Joosen et al. 2012). The same authors suggested that the absence of mucilage would reduce the water uptake and thus also possibly the sensitivity to germination in ABA.



QTL mapping with QTL x E

Two QTL mapping approaches were used to explore QTL-by-environment interactions (QTL x E) for seed germination phenotypes. Single trait multiple-environments linkage analysis was performed on the mean phenotypic values of the AUC across germination conditions, for each maternal environment (phenotypic QTLs). In the second approach, QTL mapping on the response values measured as the difference between seed performance under stress as compared to its appropriate control. In addition, the plasticity traits were used in the model to identify QTL associated with the genotype main effect as well as the interaction of the genotype at the marker with the germination (G x GE), maternal (G x ME), stratification (G x S) and other possible interactions.

The single trait multiple-environments mapping for the seed germination traits revealed a large number of QTLs distributed over the five chromosomes (Figure 6). The QTLs identified were clustered in distinct regions of the chromosomes, resulting in 15 main QTL clusters. The direction of the QTLs within these QTL clusters was largely consistent across the environments and QTLs with both Bay-0 and Sha as high value allele were identified. Variation in the number of QTLs and the percentages of explained variances of the individual QTLs within these clusters across ME was a first indication for QTL x E. Furthermore, differences observed across the QTL clusters, indicated varying degree of sensitivity of the genetic variants to the environment along the genome. Germination QTLs with certain environment specificity were identified. In HT, a large number of QTLs were identified on top of chromosome 1 and chromosome 3, while QTLs at the bottom of chromosome 1 and top chromosome 5 seem to be specific to LP (Figure 6).

◄**Figure 5:** Heatmap of spearman correlation coefficients of seed and germination traits (AUC) under different germination conditions for the RILs grown in standard (ST), high temperature (HT), high light (HL) and low phosphate (LP). Dry SS: dry seed size; imb. SS: imbibed seed size; DSDS50 is a measure of dormancy and Gmax CDT is the percentage of germination after controlled deterioration test and is a proxy of seed longevity

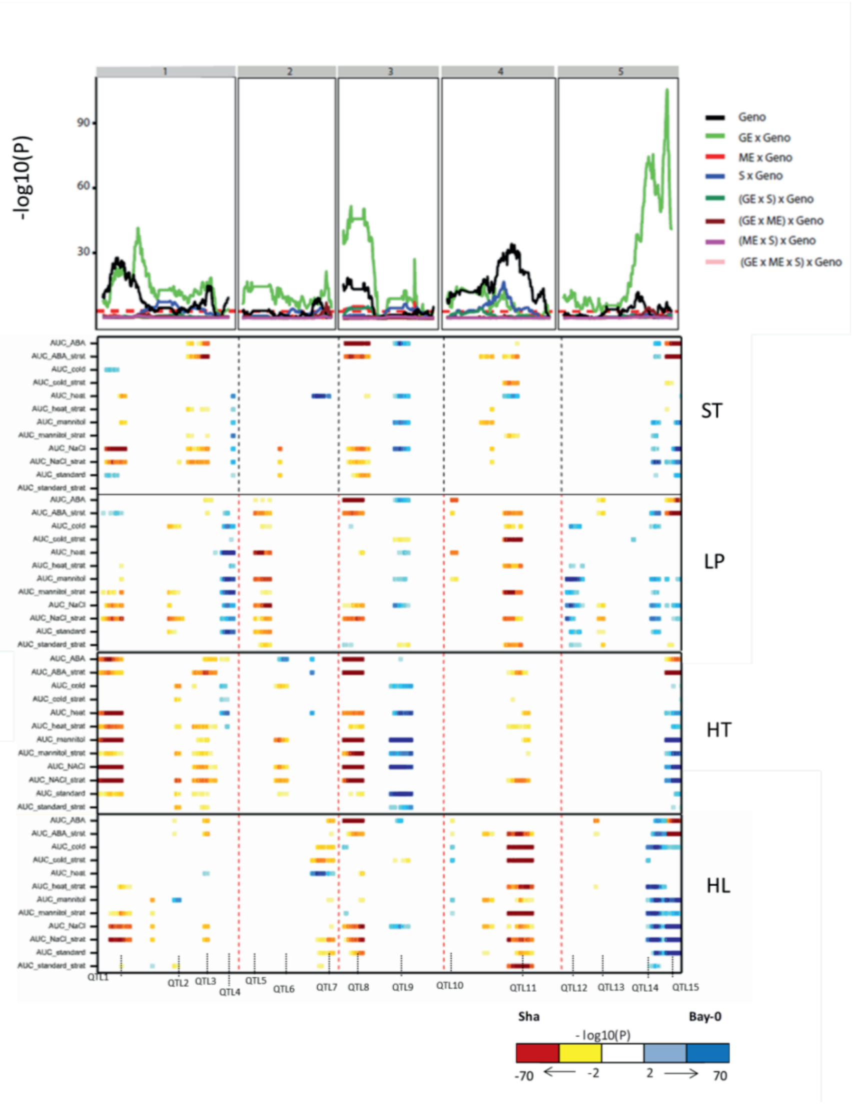
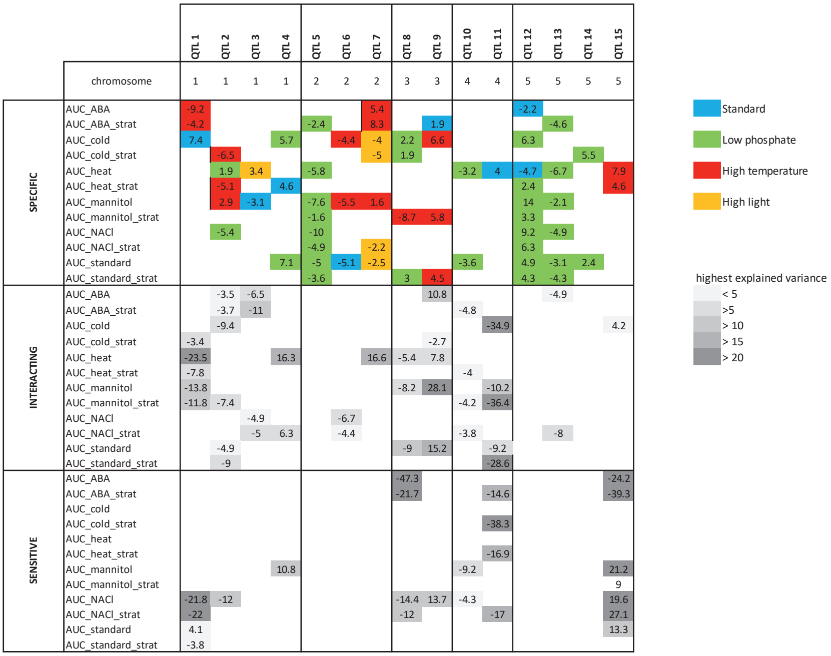


Figure 6: Heatmap of QTL profiles for AUC in a single trait multiple environments linkage analysis approach. The 12 germination conditions are indicated in the rows for each maternal environment (ST: standard, LP: low phosphate, HT: high temperature, HL: high light). The direction and effect of the QTLs is indicated by the gradient false colour scale. Yellow to red indicates QTLs with a higher trait value associated with the Sha allele and blue to dark blue indicates higher allelic effect triggered by the Bay-0 allele. The vertical dashed lines delineate the chromosomes. The upper panel represents the –log(P-value) profile for the different components of the mixed model mapping approach: Geno: genotype, ME: maternal environment, GT: germination environment, S: stratification. Grey dotted lines at the bottom of the figure indicate the position of the QTL clusters identified. The exact position of the QTL clusters is reported in Supplemental Table S3.



		QTL 1	QTL 2	QTL 3	QTL 4	QTL 5	QTL 6	QTL 7	QTL 8	QTL 9	QTL 10	QTL 11	QTL 12	QTL 13	QTL 14	QTL 15
	chromosome	1	1	1	1	2	2	2	3	3	4	4	5	5	5	5
SPECIFIC	AUC_ABA	🟥 -9.2						🟥 5.4					🟦 -2.2			
	AUC_ABA_strat	🟥 -4.2				🟩 -2.4		🟥 8.3		🟦 1.9				🟩 -4.6		
	AUC_cold	🟦 7.4			🟩 5.7		🟥 -4.4	🟨 -4	🟩 2.2	🟥 6.6			🟩 6.3			
	AUC_cold_strat		🟥 -6.5					🟨 -5	🟩 1.9						🟩 5.5	
	AUC_heat		🟩 1.9	🟨 3.4		🟩 -5.8					🟩 -3.2	🟦 4	🟦 -4.7	🟩 -6.7		🟥 7.9
	AUC_heat_strat		🟥 -5.1		🟦 4.6								🟩 2.4			🟥 4.6
	AUC_mannitol		🟥 2.9	🟦 -3.1		🟩 -7.6	🟥 -5.5	🟥 1.6					🟩 14	🟩 -2.1		
	AUC_mannitol_strat					🟩 -1.6			🟥 -8.7	🟥 5.8			🟩 3.3			
	AUC_NACl		🟩 -5.4			🟩 -10							🟩 9.2	🟩 -4.9		
	AUC_NACl_strat					🟩 -4.9		🟨 -2.2					🟩 6.3			
	AUC_standard				🟩 7.1	🟩 -5	🟦 -5.1	🟨 -2.5			🟩 -3.6		🟩 4.9	🟩 -3.1	🟩 2.4	
	AUC_standard_strat					🟩 -3.6			🟩 3	🟥 4.5			🟩 4.3	🟩 -4.3		
INTERACTING	AUC_ABA		-3.5	-6.5						10.8				-4.9		
	AUC_ABA_strat		-3.7	-11							-4.8					
	AUC_cold		-9.4									-34.9				4.2
	AUC_cold_strat	-3.4								-2.7						
	AUC_heat	-23.5			16.3			16.6	-5.4	7.8						
	AUC_heat_strat	-7.8									-4					
	AUC_mannitol	-13.8							-8.2	28.1		-10.2				
	AUC_mannitol_strat	-11.8	-7.4								-4.2	-36.4				
	AUC_NACl			-4.9			-6.7									
	AUC_NACl_strat			-5	6.3		-4.4				-3.8			-8		
	AUC_standard		-4.9						-9	15.2		-9.2				
	AUC_standard_strat		-9									-28.6				
SENSITIVE	AUC_ABA								-47.3							-24.2
	AUC_ABA_strat								-21.7			-14.6				-39.3
	AUC_cold															
	AUC_cold_strat											-38.3				
	AUC_heat															
	AUC_heat_strat											-16.9				
	AUC_mannitol				10.8						-9.2					21.2
	AUC_mannitol_strat															9
	AUC_NACl	-21.8	-12						-14.4	13.7	-4.3					19.6
	AUC_NACl_strat	-22							-12			-17				27.1
	AUC_standard	4.1														13.3
	AUC_standard_strat	-3.8														

🟦 Standard
🟩 Low phosphate
🟥 High temperature
🟨 High light

highest explained variance
□ < 5
▨ >5
▨ > 10
▨ > 15
▨ > 20

Figure 7: Summary of the effect of the maternal environment on germination QTLs. Individual QTLs within the 15 QTL clusters for AUC were investigated for each germination condition and categorized into 'specific' and 'interacting' and 'sensitive' QTLs according to the significance of the QTLs in respect to the maternal environments. QTLs with a significant effect ($p< 0.05$) in only one maternal environment (ME) were considered as 'environment specific' germination QTLs. QTLs with significant effect in several (2 to 3) MEs were categorized as 'interacting'; QTLs with varying but significant effect under all MEs were categorized as 'sensitive'. For each box, the highest explained variance of the QTL(s) is indicated with negative values for Sha and positive values for Bay-0. The colour of the boxes for the environment specific QTLs corresponds to the specific ME in which the QTL is identified. For the interacting QTLs, the colour code corresponds to the highest explained variance found for the QTLs.

Effect of the maternal environment on germination QTLs

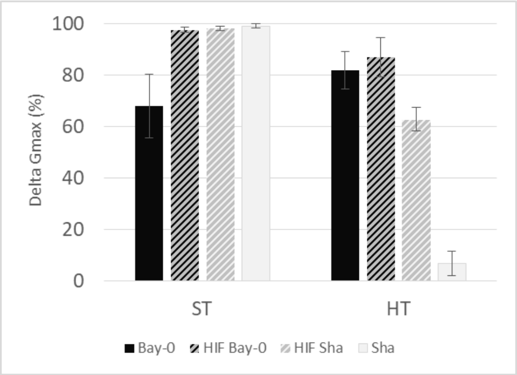
To further understand the nature of the effect of the maternal environment on the germination QTLs identified, an overview of QTL clusters with significant QTL x E for AUC is shown in Figure 7. For each germination condition, the QTLs were classified in three classes according to the significance of the QTL effect ($p< 0.05$) in the different MEs. The QTLs were 'environment specific' when the QTL was detected in only one of the four MEs, 'interacting' when the QTL effect was significant in several MEs and 'sensitive' when the QTL effects were variable but significant across all MEs. A large number of germination QTLs were specific to one ME. Most of these QTLs display low explained variance compared to the 'interacting' and 'sensitive' QTLs. For the 'sensitive' QTLs, the explained variance of the QTL could vary between 5 and 24, showing the large effect of the ME on individual germination QTLs.

Plasticity QTLs

Another approach to investigate the sensitivity of the QTLs to the environment consisted of mapping QTLs for plasticity. The plasticity of a genotype corresponds to the reaction norm which is the response of a given genotype under varying environments. Here, the plasticity was measured as the difference between seed performance in stress conditions and the respective standard (for instance AUC for germination under ABA with stratification was subtracted from AUC for germination in water with stratification). Plasticity QTL results are available in AraQTL(Nijveen et al. 2017). Furthermore, QTL x E was assessed by obtaining QTL profiles for the different components explaining the variation (ANOVA). The QTLs for the different components largely corresponded to the QTL clusters previously identified (upper panel Figure 6). In particular, strong linkage was observed for the genotype and genotype x germination components (upper panel Figure 6). The low linkage for the effect of the maternal environment can be explained by the nature of the plasticity calculation. In the situation where genetic variation for the maternal environment is observed under both standard and stress conditions, calculating the difference between the two germination environments cancels out this variation.

Confirmation of an environment specific QTL

Several germination QTLs with large explained variances were detected on top of chromosome 1 specifically for the HT population (QTL 1) (Figure 6). In addition, a HT specific dormancy QTL co-located with these germination QTLs (Supplemental Table S4). The Sha allele at the position of QTL 1 conferred higher tolerance to germination in sub-optimal germination conditions and lower seed dormancy. For germination at 32°C (AUC_heat), the QTL explained up to 23.5% of the phenotypic variance. To validate this QTL, the parental lines and the HIF198 carrying the parental alleles, HIFBay-0 or HIFSha at the top of chromosome 1, were grown in similar ST and HT maternal environments. Harvested seeds were stored for a few months and germinated at 32°C. To assess the full potential of seed germination, once the germination plateau was reached under germination at 32°C, the seeds were stratified and transferred to 22°C. The difference between the final Gmax and the Gmax under 32°C for the different lines, delta Gmax, is shown in Figure 8. For the seeds grown under ST, the lower delta Gmax for Bay-0 and high delta Gmax for Sha (indicating no germination), likely reflected the degree of primary dormancy of the seeds. Nonetheless, germination at 32°C of the HIFs carrying either parental allele showed significant differences when grown under HT (Figure 8).



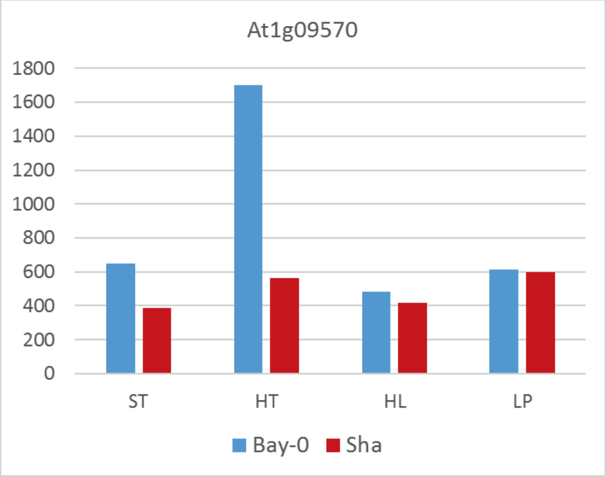
◀**Figure 8.** Confirmation of an environment specific QTL with a HIF approach. Analysis of HIF198 (top of chromosome 1) carrying the Bay (HIFBay) or the Sha (HIFSha) allele. Error bars represent the standard error of 2 and 4 replicates for the parental and HIF lines, respectively. The delta Gmax is shown as the difference of full germination potential and germination at 32°C, as described in the main text. A higher delta Gmax indicates thus a higher sensitivity of the line to germination at 32°C. T-test was performed between the HIFs carrying the Bay-0 or Sha allele grown under standard (ST) and high temperature (HT). * indicates a significant difference by Student t-test (p-value < 0.05).

Mining candidate genes using expression data

The identification of environment specific QTLs suggests the sensitivity of the alleles to the environment. We used gene expression data obtained by RNA-seq on fresh dry seeds of the parental lines, Bay-0 and Sha, grown in the different maternal environments (Serin et al. 2017) Chapter 5), to shortlist candidate genes based on their differential expression.

Overall, the transcriptome of the seeds showed distinct profiles as a result of the difference in their genetic background and the maturation environments (Chapter 5). We focused on the high temperature specific QTL identified on top of chromosome 1 confirmed by the HIF approach (Figure 8). The genomic region defined by the HIF (1.45 – 3.45 Mb) included 578 genes. These genes were stringently filtered for being significantly (p-value after FDR correction < 0.0001) and specifically differentially expressed between Bay-0 and Sha under high temperature and not under the other conditions. The filtering step reduced the initial list to 10 potential candidate genes (Table 2).

Among these genes, the highest fold change was observed for At1g09570 encoding phytochrome A. The known role of phytochromes in the temperature regulation of seed germination strengthens its significance as a candidate gene (Dechaine et al. 2009; Heschel et al. 2007; Donohue et al. 2008; Donohue et al. 2012). The difference in expression is explained by the increased expression of PHYA in Bay-0 in response to HT maternal environment as compared to Sha (Figure 9).



◀**Figure 9:** Genotype and environment dependent gene expression variation. Gene expression levels are shown for Phytochrome A in the dry seeds of the parental lines, Bay-0 and Sha, matured under standard (ST), high temperature (HT), high light (HL) and low phosphate (LP) environments. Significant differential gene expression between the parental lines is indicated by (*) for FDR < 0.0001.

Table 2. List of genes differentially expressed (DEGs) between Bay-0 and Sha underlying the high temperature specific germination QTL on top of chromosome 1. The expression log fold change (logFC) between Bay-0 and Sha under HT and ST are indicated. Negative values indicate higher expression levels in Sha and positive values, higher expression in Bay-0.

Genes	logFC HT	FDR p-value	logFC ST	FDR p-value	Description
AT1G09070	-1.18	7E-19	-0.28	0.12	Soybean gene regulated by cold-2
AT1G05120	-0.98	1E-17	-0.23	0.15	Helicase protein with RING/U-box domain
AT1G07645	-0.70	1E-07	-0.18	0.40	Desiccation-induced 1VOC superfamily protein
AT1G04560	-0.58	9E-10	-0.04	1.00	AWPM-19-like family protein
AT1G06570	0.53	3E-06	0.17	0.37	Phytoene desaturation 1
AT1G07615	0.68	4E-06	0.35	0.07	GTP-binding protein Obg/CgtA
AT1G09640	0.77	2E-12	0.21	0.20	Translation elongation factor EF1B, gamma chain
AT1G07770	1.19	9E-06	0.51	0.19	Ribosomal protein S15A
AT1G07590	1.26	2E-08	0.08	1.00	Tetratricopeptide repeat (TPR)-like superfamily protein
AT1G09570	1.50	3E-05	0.64	0.19	Phytochrome A

Discussion

It is established that the environment experienced during seed development affects seed traits such as dormancy and germination (Penfield and MacGregor 2017). The genotype-dependent response to these effects has further led to the investigation of the interaction between maternal environment and genotype and its effect on seed phenotypes (He et al. 2014; Burghardt et al. 2016; Munir et al. 2001; Edwards et al. 2016). Only a few studies have harnessed natural variation to identify the extent and effect of the maternal environment at the genetic level. Postma and Agren (2015) and Kerdaffrec and Nordborg (2017) identified changes in the effect of QTLs associated to seed dormancy in Arabidopsis populations grown in native field experiments. However, various factors, such as photoperiod and temperature vary simultaneously in the field and therefore experiments under controlled conditions in which individual factors are investigated separately are needed to disentangle these effects. In this study, we grew an Arabidopsis recombinant inbred line (RIL) population under four controlled conditions from flowering until seed harvest. Extensive phenotyping and QTL mapping for the seeds produced under the different conditions revealed the effect of the maternal environment at the phenotypic and genetic level. We further used HIFs and gene expression of the parental lines to narrow down the number of candidate causal genes for an environment sensitive QTL.

Overall, considerable phenotypic variation was observed between the parental lines as well as in the RIL population in response to both the germination and maternal environments. The effect of the maternal and germination environments on seed traits was in line with previous studies. Temperature is a key determinant in the timing and duration of key developmental phases including flowering. Plant morphology and reproductive development are also strongly influenced by temperature (Wigge 2013; Quint et al. 2016). Warmer temperature leads to earlier flowering and subsequently earlier seed set (Balasubramanian et al. 2006; Springthorpe and Penfield 2015). In line with other studies, high temperature maturation environment promoted germination by reducing dormancy levels (Figure 1) (Kendall and Penfield 2012; Huang et al. 2014). The temperature regulation of seed germination is largely influenced by the key hormones, ABA and GA. Kendall et al. (2011) reported that low temperatures during seed development increased abscisic acid (ABA) content and reduced gibberellic acid (GA) levels.

Larger seed size and seed weight was observed under HL as found by (He et al. 2014; Contreras 2008) in different genotypic backgrounds. In several studies, seed size has been associated with seedling vigor and faster germination (Fenner 1991). The low correlation between seed weight and seed performance found in this study shows that this relation is not always verified (Figure 5). We observed that the RILs that matured under HL showed lower AUC under several germination conditions (NaCl, Mannitol and heat). These lower AUC for larger seeds could also be explained by a higher water requirement of these seeds to complete germination compared to smaller seeds for which water absorption can occur faster. These hypotheses are in line with the observation of (Galloway 2001) who found that an increase in resources in the maternal environment, such as high light, decreases the fraction of seeds with early germination. The low phosphate environment also affected seed traits, such as AUC in mannitol (Figure 2), which resulted in LP sensitive QTLs at the top of chromosome 5 (Figure 6). Phosphorus (P) is an essential nutrient that is required for all major developmental processes and reproduction in plants. P can be stored in the plant or mobilised throughout the plant to provide the energy blocks for further reactions, as in the form of ATP. Common responses to P deficiency include delayed plant maturity, reduced leaf size and reduced root growth, which can limit nutrient uptake. Under P starvation conditions, the allocation of P might thus occur at the expense of P storage in seeds, although studies have reported that yield rather than seed quality is affected under resource limiting environments (Hacisalihoglu et al. 2018). Besides the effect on the mean phenotypic values, the effect of the maternal environment also resulted in differences in the response of the RILs, indicating genotype-by-environment (G x E) interactions (Figure 4, Table 1). Such interactions were largely reported in other studies (Munir et al. 2001; Kerdaffrec and Nordborg 2017; He et al. 2014) and supported in this study by the analysis of variance, which estimated that overall approximately 40% of the seed germination variation in the RILs was due to G x E (Table 1). Together, these results show that the maternal environment can effectively modulate the expression of genetic variation. In general, increased variance of AUC values was observed for germination in sub-optimal conditions (Figure 2). These variances reflect differences in the timing of germination of individual seeds in a seed batch. In nature, varying timing of seed germination can be seen as a strategy to ensure the success of seed germination; while in agriculture, seed companies put major efforts to deliver uniformly germinating seed batches.

The quantitative genetic study identified a large number of QTLs detected across multiple environments. Most of these QTLs could be grouped into 15 main QTL clusters. Several studies previously identified common QTLs controlling germination under standard and stress conditions (Foolad et al. 2007; Joosen et al. 2012), suggesting partly shared physiological regulation of seed germination under these conditions. The observed QTL clusters largely corresponded to the major germination QTLs identified in a previous study in the same population (Joosen et al. 2012). The high level of consistency between the previous and present study indicates the reliability of the identified QTLs.

The significant QTL x E corroborated the G x E observed at the phenotypic level. The effect of the environment was mostly observed as a change in allele sensitivity of the QTLs, which is the most common type of interaction for QLT x E (El-Soda et al. 2014). For marker-assisted selection, such QTLs present the advantage of enhancing germination under several conditions. Six QTLs clusters (QTL4, QTL7, QTL9, QTL12, QTL14 and QTL15) were identified with a higher effect provided by the allele of the generally slow and or lower germinating parent, Bay-0. This indicated that alleles promoting germination can also be queried in the parent of apparent opposite phenotype. The presence of QTLs in the direction of both parental alleles is also likely to explain transgressive phenotypes in the RIL populations (Figure 2). Several QTL clusters showed certain specificity to the maternal environments (Figure 6). For instance, QTL 1 was identified for several germination conditions under HT, QTL11 and QTL15 were found mainly in HL, while QTL4, QTL5, and QTL12 showed higher sensitivity to the LP maternal environment. The maternal and germination conditions showed a significant effect on the magnitude change of the QTL effects. Common germination QTLs across maternal environments showed higher LOD scores. ME specific QTLs had generally lower LOD scores. The absence of the germination QTL under the other maternal environments could be due to not reaching the significance threshold (Figure 7).

Despite the use of RILs and a dense genetic map, the identified QTLs had large confidence intervals encompassing a high number of genes. Further strategies need thus to be employed to narrow down these genomic regions and establish a manageable list of potential causal genes. When readily available, heterogeneous inbred lines (HIFs) provide a fast method to validate QTLs (Tuinstra et al. 1997; Joosen et al. 2012). Using this approach, we showed that the effect of the maternal environment on QTLs can be reproduced under controlled conditions, as shown for high temperature ME (Figure 8). The HIFs allowed narrowing down the QTL region although further efforts are necessary to identify the causal gene. With the advent of high-throughput technologies, another popular approach to the identify candidate genes is to combine QTL mapping and gene expression analysis (Wayne and McIntyre 2002; Kloosterman 2010; Albert et al. 2016). In the recent years, RNA-seq has become widely used for transcriptome studies (Wang et al. 2009) presenting many advantages over other methods, such as the large and dynamic range of expression level quantification. In this study, we used RNA-seq data obtained from the mature dry seeds of the parental lines grown under the different maternal environments. The transcriptome of the seeds was largely modulated by the genotypic background and the environment (Figure 9) (He et al. 2016; Chiang et al. 2011). The effect of the maternal environment is likely influenced by gene expression changes and thus we investigated differentially expressed genes between the parental lines matured under high temperature, within the confidence interval validated by the HIF. Using this approach, we drastically reduced the list of candidate genes to 10 genes. Among these genes, we identified At1g07430 encoding for phytochrome A (PHYA). Phytochromes play a known role in regulation of seed germination (Heschel et al. 2007) and can also act as mediator of the environmental cues experienced by the seed during seed maturation (Botto 1996; Dechaine et al. 2009). PHYA is involved in the regulation of germination and dormancy via the ABA/GA hormonal balance (Finch-Savage and Footitt 2017; Cadman et al. 2006), although the effects of PHYA have been shown to vary across genotypes and conditions (Donohue et al. 2008; Donohue et al. 2012; Dechaine et al. 2009). The negative regulation of PHYA on GA levels (Jordan et al. 1995) could be a plausible explanation to link the higher expression of PHYA and the lower seed performance in Bay-0 in HT associated with this QTL. Another study in Arabidopsis showed that an increase in phyA signalling augmented the ABA-mediated inhibition of root growth (Chen et al. 2014a).

Although PHYA is an interesting candidate for the causal gene for this QTL, other genes within the QTL interval are also still candidates. Here we used a stringent threshold (p< 0.0001) to establish a manageable list of candidate genes. In addition, differential expression is not a prerequisite of causality. A QTL in the same region was also identified for germination in ABA and salt in the Ler x Sha population (Ren et al. 2010). The cloning of the QTL led to the identification of RAS 1 (At1g09950). RAS1 has a premature stop codon in Sha which causes a truncated RAS1 protein and improved tolerance to germination in salt conditions (Ren et al. 2010). In our study, analysis of the HIFs showed the HT specificity of the QTL, thus it is likely that besides RAS1, which didn't show HT specific expression, other genes control seed germination at the position of this QTL.

In conclusion, we showed that the maternal environment prominently affects seed germination. The extent of QTL x E observed as a result of genetic interaction with the maternal and germination environments, strengthen the need of multi-environment studies to reveal the genetic mechanisms underlying phenotypic plasticity. Such studies are particularly relevant in a context of climate change (Walck et al. 2011; Huang et al. 2018) to understand plant adaptation. The combined effect of the maternal and germination environment may as well provide a mean to life history evolution (Donohue 2009). From a breeding perspective, the maternal environment should be taken into consideration in order to better predict and fully exploit seed performance potential under either a targeted or wide range of environments.

List of supplemental data

Supplemental Tables below can be found at:
http://www.wageningenseedlab.nl/thesis/earserin/SI/chapter3

Supplemental Table S1: Element concentrations in the standard nutrient solution.

Supplemental Table S2: List of 221 SNP markers used for the QTL x E analysis.

Supplemental Table S3: Average phenotypic values for all seed and germination traits measured in the parents and recombinant inbred lines grown under standard, high temperature, high light and low phosphate.

Supplemental Table S4: output files that summarize the results of the QTL x E analyses for the germination traits.

Supplemental Table S5: output files that summarize the results of the QTL x E analysis for the seed traits.

Chapter 4

Maternal environment and genetic background shape the dry seed metabolome in Arabidopsis

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In preparation

Abstract

Seed set is a crucial event in a plant's life cycle that ultimately determines the plant's fitness. The environment experienced by the mother plant and embryo during seed development is shaping the seed metabolome in a genotype-dependent manner. In seeds, several loci controlling metabolite variation have been identified although only a few studies have investigated the underlying genetic architecture in response to changes in the seed production environment. In the present study, we used an Arabidopsis RIL population grown under different environments to investigate the effect and genetic basis of genotype-by-environment interactions (G x E) on the seed primary metabolism and associated seed germination characteristics. The primary metabolites of the dry seeds of the parental lines and the RILs produced under four contrasting environments were investigated using an untargeted GC-TOF-MS metabolomics approach in a generalized genetical genomics design. A large set of metabolites were affected by G x E. The combined use of network-based metabolite correlation analysis and mQTL analysis indicated an environment-dependent genetic regulation of the dry seed primary metabolome. Overall, 2 known and 2 novel hotspots of metabolic regulation were identified. Although, limited co-locating phenotypic and metabolic QTLs were observed, we found that different sets of metabolites across the environments correlated with seed performance traits, showing that the seed maternal environment can modulate the relation between metabolites and phenotypes.

Keywords: Genetical metabolomics, maternal environment, network analysis, primary metabolism, seeds.

Introduction

Intrinsic seed properties, such as the ability to germinate, are acquired during seed development on the mother plant. Throughout seed development, a sink-source connection between the mother plant and the seeds exists. This connection allows the plant to allocate available resources to support seed growth and the synthesis of seed storage compounds (Ruuska 2002; Baud et al. 2008). Primary metabolites such as sugars and organic and amino acids are key compounds of the metabolic shifts that occur during seed maturation, desiccation and germination (Fait et al. 2006; Angelovici et al. 2010; Galili et al. 2014). Tricarboxylic acid contents decrease during seed maturation while seed desiccation is characterized by an increase in free amino acids (Fait et al. 2006), (Angelovici et al. 2009). At the end of desiccation, fully mature seeds enter a quiescent state nearly devoid of metabolic activity. Upon imbibition, metabolic activity resumes, mobilizing stored metabolites to provide the basis for seed germination; an energy demanding process. As a result, the compounds stored in dry mature seeds reflect the process of seed maturation and can be linked to seed performance (Rosental et al. 2014; Rosental et al. 2016).

Throughout their life cycle, plants experience fluctuating conditions, requiring coordinated changes at the transcriptome, proteome and metabolome levels to adjust the biochemical pathways to facilitate their physiological response. During seed set, changes in the maturation environment of the seed substantially affect seed performance in Arabidopsis (He et al. 2014)(Chapter 3) which is reflected by the metabolome level in a genotype dependent manner (He et al. 2016). Seeds that matured under high light conditions had a higher accumulation of galactinol, which has been shown to positively correlate with Arabidopsis seed longevity (He et al. 2016; de Souza Vidigal et al. 2016). In contrast, low temperature and low nitrate maturation environments resulted in a decrease of nitrogen-metabolism compounds such as GABA, asparagine and allantoine (He et al. 2016). Although fundamental molecular mechanisms of seed maturation have been described (Holdsworth et al. 2008; Gutierrez et al. 2007), more work is needed to understand how environmental factors interact with these processes. Increasing our understanding of the genetic and molecular mechanisms controlling seed metabolome variation in response to stress will provide valuable insights in this direction.

The advancement of tools in metabolomics and genomics has enabled untargeted and large scale exploration of metabolome composition and variation (Kooke and Keurentjes 2012). In particular, GC-MS based methods have largely contributed to the profiling of primary metabolites (Lisec et al. 2006). The extensive diversity in metabolites and their high interconnectivity has led to large metabolite variation revealing the large plasticity and complexity of metabolic networks (Obata and Fernie 2012; Sulpice et al. 2013). Identifying the factors controlling these intricate networks remains an important challenge.

Quantitative trait locus (QTL) analysis is a powerful tool to identify genes responsible for variation in a segregating population. For molecular traits, such as transcript levels, genetical genomics was proposed (Jansen and Nap 2001) and successfully applied (Cubillos et al. 2012; Lowry et al. 2013; Keurentjes et al. 2007b). Since metabolite levels can also be treated as quantitative traits, quantitative trait mapping of metabolite variation enables the identification of metabolic QTLs (mQTLs) (Keurentjes et al. 2006). This "genetical metabolomics" approach has been successfully employed to dissect the genetic basis of metabolism in plant systems (Keurentjes et al. 2006; Knoch et al. 2017; Toubiana et al. 2012; Carreno-Quintero et al. 2012; Wen et al. 2015).

Commonly, the effect of environmental perturbations on the genetic basis of metabolome variation has been resolved by comparing mQTLs of the same population exposed to contrasting conditions (Kliebenstein et al. 2002; Rosental et al. 2016; Wu et al. 2018). Although this kind of approach provides full power for the genetic analysis, often only a few conditions are tested due to practical reasons, consequently limiting insights into the extent of metabolome genetic regulation in response to environmental variation. Recently, the generalized genetical genomics (GGG) design (Li et al. 2008b) was introduced to investigate mQTL x E for metabolome variation at different stages of the seed germination process (Joosen et al. 2013). In this study, several mQTLs controlling seed metabolism were identified.

To extent our understanding on the genetic control of the seed metabolome, we queried the effect of the maturation environment on the genetic architecture of the seed metabolome in an Arabidopsis thaliana Bayreuth-0 (Bay-0) x Shahdara (Sha) recombinant inbred line (RIL) population (Loudet et al. 2002). A core population of 165 RILs and the parental lines were grown in four contrasting environments, including standard, high light, high temperature and low phosphate from flowering until seed harvest. Metabolic profiling of dry harvested seeds of the RILs and the parental lines was performed by GC-TOF-MS in a GGG design. Using metabolite correlation network and mQTL analyses, we shed light on key genetic features of maternal environment induced changes in dry seed metabolome regulation.

Material and Method

Plant Material

Seeds from an Arabidopsis thaliana Bayreuth (Bay-0) x Shahdara (Sha) recombinant inbred line (RIL) population (Loudet et al. 2002) were used in this experiment. Plants were grown on 4x4 cm Rockwool blocks (MM40/40, Grodan B.V.) in a fully randomized set up. In the standard condition, plants were grown under long day (16h light / 8h dark) at 70% RH and 22°C / 18°C (day/night) under artificial light (150 µmol m-2 s-1). The plants were watered with a standard nutritive solution (see Supplemental table 1 in (He et al. 2014)) three times a week by flooding cycles. When all lines reached flowering, the main stem of all plants was cut short to synchronize the developmental stage of the plants prior to stress exposure and to collect only seeds developed under the different conditions. Three to four plants per RIL were transferred to different climate cells with controlled environmental conditions. The same conditions as standard were used for the stress environments, except for the varying parameter as indicated hereafter: high temperature (25°C day / 23°C night), high light (300 µmol m-2 s-1) and low phosphate (12.5 µM phosphate instead of 0.5 mM in the standard nutritive solution). Fully mature seeds from 3-4 plants per RIL were bulk harvested. A fraction of fresh harvested seeds was stored at -80°C one week after harvest. The remaining seeds were stored at room temperature and ambient relative humidity for after-ripening. Fully after-ripened seeds were used to perform germination experiments (Chapter 3).

Metabolites profiling based on GC-TOF-MS

Prior to the measurements, the RILs were selected following the Generalized Genetical Genomics design as used in (Joosen et al. 2013). This resulted in a total of 160 RILs in sub-populations of 41, 40, 39 and 40 RILs for ST, HT, HL and LP conditions respectively. Metabolite abundance was measured in triplicate for the parental lines from all four conditions. Metabolites for GC-TOF-MS were extracted according to the following procedure.

Metabolite extraction

Primary metabolites were extracted as described by Roessner (2000) with minor modifications. In brief, 10 mg seeds for each RIL were taken from freshly harvested seeds which were store in -80°C. Extraction was done in 2mL Eppendorf Safe-lock tubes. Samples were frozen in liquid nitrogen and homogenized with 2 iron balls (1.25mm) using a dismembrator (Retch MM200) at 1500 rpm. A solution of 350µL MeOH/CHCl3 (4:3) was added to the samples followed by 75µL filtered water (MilliQ, Millipore) containing 0.133mg/mL Ribitol as internal standard. After 10 minutes sonication 100µL MilliQ was added, followed by mixing and centrifugation. After centrifugation the water/methanol phase was transferred to a new clean Eppendorf tube and the remaining extract was extracted again with 250µL MeOH/CHCl3 (1:1). After 10 minutes incubation on ice, 100µL MilliQ was added. After centrifugation the water/methanol phase was taken again and combined with the previously collected phase and mixed. Hundred µL of this mix was dried overnight in a speedvac (35°C, Savant SPD121) in a vial with insert (06090357, Grace). The next day the vials were crimp capped with magnetic caps (8618261, Grace) in the presence of argon. The GC-TOF/MS procedure was previously described by Carreno-Quintero et al. (2012). Detector voltage was set at 1900 V.

Data processing

Raw data was processed using the ChromaTOF software to produce netCDF files. A signal to noise ratio of 2 was used. Further processing was done by Metalign sofware (Lommen 2009). First a baseline correction was done with a peak slope factor (x noise) set to 1 and a peak threshold factor (x noise) of 2. All mass signals below 25 were discarded. Different chromatograms were aligned with a maximum shift of 75 scans. This resulted in 66665 mass signals. These mass signals were further processed using Metalign output transformer (METOT, Plant Research International, Wageningen). Mass signals that were present in less than three RILs were discarded. Mass signals that were below background were randomized between 50% and 100%. This resulted in 39418 mass signals. From these mass signals centrotypes were constructed by using the MSClust software (Tikunov et al. 2012) with the following parameters: correlation threshold 0.8 with 0.02 margin and PD reduction 0.9 with 0.01 margin. Criterion was stopped at 3 masses. This resulted in 172 unique centrotypes from which we could identify 71 by comparing the mass spectra in the NIST software (NIST Mass Spectral Search Program version 2.0) to an in-house constructed library and to the Golm library (http://gmd.mpimp-golm.mpg.de/). This identification is based on spectral similarities and comparing the retention indices calculated by using a third polynomial function on the alkanes that were added to the samples (Strehmel et al. 2008).

Statistical analyses

Log10 transformation allows scaling of the variation and making the metabolites comparable. Therefore, all metabolites were log10 transformed before further analysis in order to improve normality (Ursem et al. 2008). Two-way ANOVA was performed on the parental lines, Bay-0 and Sha, grown in each environment. False discovery rate (FDR) correction was applied for multiple testing. FDR < 0.05 was used as a threshold for corrected p-values. Analyses were performed in Metaboanalyst 3.0 (Xia and Wishart 2016).

Principal component analysis was performed on the log10 scaled and centred metabolic values of the RILs and parental lines. Analysing the samples by the batches used for the GC-TOF-MS measurements did not reveal any batch effects.

Correlation analyses

Correlation analysis was conducted for the 71 annotated metabolites in each RIL sub-population, separately; as well as on the combined RIL for the 172 metabolites (Supplemental Table 7). Spearman metabolite correlation was obtained using the rcorr R package.

RV coefficient analysis

RV coefficients were used to calculate the degree of similarity between metabolite correlation matrices. The CoeffRV function of the FactoMineR Rpackage (Husson et al. 2008) was used to statistically compare the correlation matrices for each condition. The RV coefficient varies between 0 and 1, with lower values indicating matrix dissimilarities. The associated p-values provided statistical support to the analysis.

Correlation network construction and analysis

The correlation p-values were converted into q-values, a measure of significance in terms of the false discovery rate (FDR). FDR < 0.05 was applied to identify statistically significant correlations which were used as input for the network. Correlation networks were visualized with Cytoscape v.3.3.0 (Shannon et al. 2003). Significance of the network clustering coefficient was evaluated by comparing the clustering coefficient of the network to the clustering coefficients of 10.000 random networks. Highly clustered metabolites were identified using the walktrap community detection algorithm (igraph, R package). Network properties were calculated using the NetworkAnalyser tool in Cytoscape. Pathway enrichment analysis was performed for the metabolites of the metabolic clusters using Metaboanalyst 3.0 with the default parameters (Xia and Wishart 2016). Pathways with p-value < 0.01 were considered significantly enriched.

mQTL analysis

A high-density genetic map derived from the same population was used for QTL analysis (Serin et al. 2017). The QTL analysis was performed as described in (Joosen et al. 2013). The approach consists of using two models to identify genetic determinants of genetic (G) and genetic x environment (G x E) control of metabolite variation. The single marker model, $Y = G + \mathcal{E}$ was used to determine the genetic component on the metabolic profiles of the RILs in the sub-populations. In this model, the phenotypic variance Y is explained by the genetic (G) and residual (e) variations. The full marker model was used on the combined sub-populations for $Y = G + E + G \times E + \mathcal{E}$ to determine the effect of the genetic background and the interaction between genetic background and the maternal environment on the mQTL profiles. Log10 transformed data were used for the mapping. Thousand permutations were used to estimate the significance threshold at an alpha level of 0.05 for each separate mapping. mQTL analyses were performed with Rqtl v3.3.1 (Broman et al. 2003; Arends et al. 2010).

mQTL hotspot identification

mQTL hotspots were identified using the permutation procedure as described and implemented by Breitling et al. (2008). The genotypic data were permutated between the RILs while conserving their mutual correlation. Metabolic data of the RILs remained unchanged. For each of the 1000 permutations, the number of mQTLs per marker with a LOD score higher than 3 was counted. The maximum number of mQTLs co-locating by chance was determined for each marker across the permutation sets. The distribution of the maximum values enabled the identification of mQTL counts at 95% of the distribution which was used as a threshold for the identification of significant mQTL hotspots. As a result 15, 19, 13, 14, 11 and 11 mQTLs at the same markers for the ST, HT, HL, LP, G and G x E models respectively, were used as significance thresholds.

Results and discussion

In this study, the metabolite profiles of the parental lines and 160 recombinant inbred lines (RILs) of the Bay-0 x Sha population were measured in freshly harvested dry seeds matured under different maternal environments. The RIL population was grown, from flowering until seed harvest, in standard conditions and either kept under these conditions or further exposed to high temperature (HT), high light (HL) or low phosphate (LP) conditions.

In order to determine the metabolic status of the seeds, GC-TOF-MS analysis was performed on the parental and RILs in a generalized genetical genomics set-up (GGG, see material and methods). This design provides RIL sub-populations of equal genetic variation and size for each of the maternal environments studied (Supplemental Figure 1). The samples were analysed in a random order interspaced by controls consisting of pooled samples. In total, 172 primary metabolites were detected. Normalized metabolite data for the parental lines and RILs under the different environments are provided in Supplemental Table 1. Among those, 71 metabolites could unambiguously be identified using in-house available libraries of known metabolites based on their centrotypes and retention times. Most of these metabolites could be classified as organic acids, sugars or amino-acids (Supplemental Table 2).

Natural variation in primary metabolites

Large quantitative differences in metabolite abundance were observed in the seeds of the parental lines, Bay-0 and Sha, matured under the different environments (Figure 1). For the Sha parent grown under different environments, higher levels of sugars (fructose, mannose) were observed compared to Bay-0. In contrast, the Bay-0 parent showed higher levels of amino acids in comparison to Sha. For the parental lines measured in triplicate, variance analysis showed that 113 of the 172 metabolites were significantly (FDR < 0.05) affected by the genotype and 105 were significantly affected by the maternal environment. For 49 metabolites, a significant effect of the genotype, environment and genotype x environment interactions was observed, while 36 metabolites were not affected by any of the genetic or environmental factors (Supplemental Table 2). Metabolite levels in the different RIL subsets showed large variation as well as transgressive segregation, where the metabolite levels were higher or lower than the parental lines (Supplemental Table 2).

Principal component analysis (PCA) was conducted on the data of the 71 identified metabolite levels of the RILs to further explore the effect of the maternal environment (Figure 2). Together, the first two principal components (PCs) explained 34.79% of the total variance. A clear separation of the HT sub-population from the other sub-populations was observed based on PC1. Among the main variables contributing to this component were tricarboxylic acid (TCA) cycle intermediates such as succinate, fumarate, amino acids such as GABA, alanine and glycine, as well as other organic acids (Supplemental Table 3). Similar results were obtained when all 172 metabolites were analysed (Supplemental Figure 2). PCA was also performed on the sub-populations separately (Supplemental Figure 3).

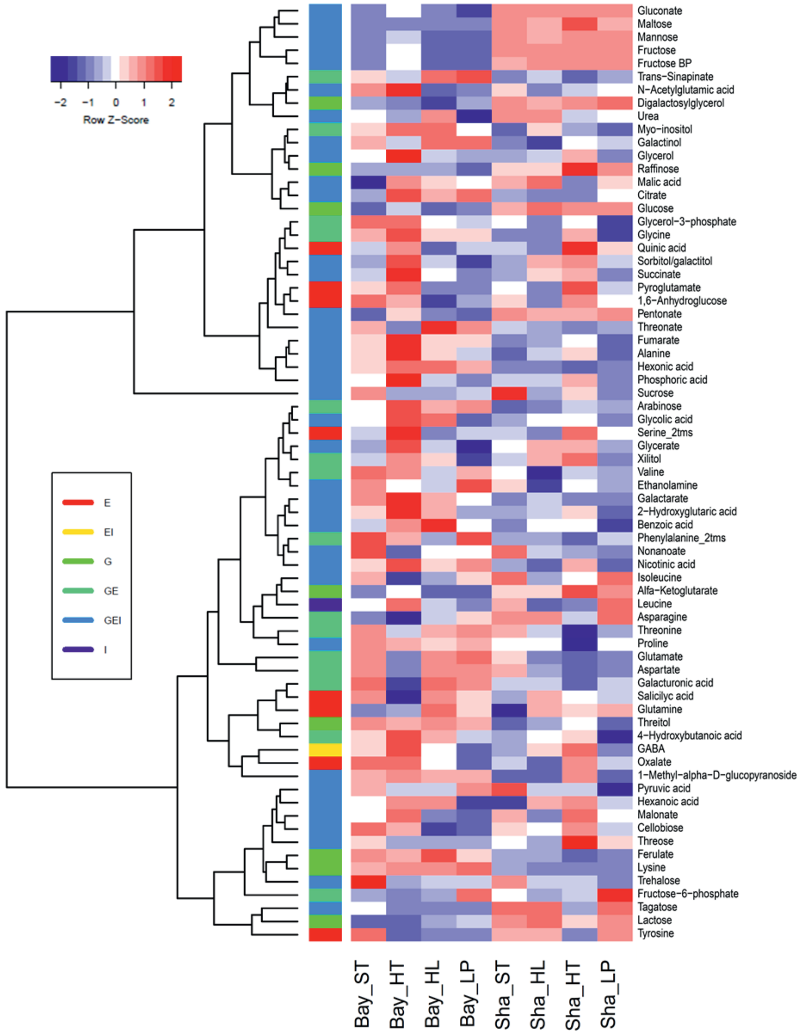


Figure 1. Heatmap showing the major changes in primary metabolites as a result of genotype-by-environment interactions in Bay-0 and Sha. Row scaled and centred Log10 values of the averaged abundance over the three replicates are shown in false colour code for the parental lines, Bay-0 and Sha, grown under standard (ST), high light (HL), high temperature (HT) and low phosphate (LP) conditions. Colour code is assigned to the metabolites according to the significance of the effect of the environment (E), genotype (G), genotype-by-environment interactions (I) by ANOVA. Details of the ANOVA results are available in Supplemental Table 2.

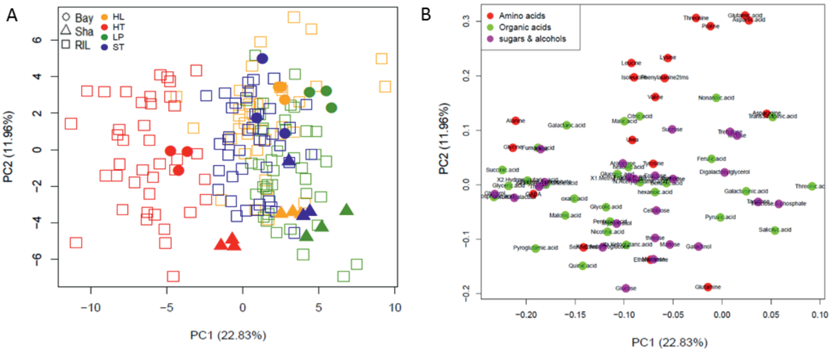


Figure 2. Principal Component Analysis (PCA) of 71 seed metabolites identified in the Bay-0 x Sha RIL population grown under different environments. A) The scores plot indicates differences in the metabolome profiling of the RILs and the parental lines. The data points of the same colour represent RILs from the same maturation environment; standard (ST), high temperature (HT), high light (HL) and low phosphate (LP). B) The loadings plot indicates the contribution of the metabolites to the two first components of the PC plot. Colours of the metabolites correspond to the class of the compounds as indicated in the legend. Loading values for the PCA are available in Supplemental Table 3.

Network correlation analysis reveals metabolic patterns regulated by G and G x E

Metabolite correlation networks are an attractive approach to visualize and investigate coordinated metabolic changes. Several studies have used network approaches to investigate metabolome plasticity and to identify coordinated metabolic changes across different time points, tissues and environmental conditions (Ursem et al. 2008; Toubiana et al. 2013; Fukushima et al. 2011).

To investigate patterns of dynamics of metabolite levels in response to the different maternal environments, correlation networks of metabolites for each RIL set were made. We used condition specific networks to investigate the changes of metabolite patterns as a result of genotype-by-environment interactions. Using this approach, we reasoned that metabolic clustering within a network is driven in part by genetics, while differences in network topologies across conditions would bring insights into G x E.

Pairwise Spearman correlations were calculated between the 71 annotated metabolites for each condition. Across all conditions, the metabolite correlations ranged from |r| = 0.42 to |r| = 0.99. The RV coefficient was used to estimate the degree of similarity between two correlation matrices (Sulpice et al. 2013; Josse and Holmes 2016). Typically the RV coefficient varies between 0 and 1, with an RV of 1 indicating identical matrices. For all matrix comparisons, the RV coefficients varied between 0.43 and 0.59 indicating significant changes in metabolite correlations across conditions (Table 1).

Table 1. Analysis of the RV coefficients between matrices RV coefficients were calculated for each matrix comparison. The RV coefficients vary between 0 (completely different matrices) and 1 (identical matrices). The RV coefficients and their respective p-values are indicated in the upper and lower part of the result matrix, respectively.

	ST	HT	HL	LP
ST	1	0.59	0.52	0.57
HT	2.27E-19	1	0.43	0.51
HL	2.02E-16	1.54E-12	1	0.49
LP	3.34E-20	5.84E-17	7.81E-16	1

We further explored these changes by building condition-specific correlation networks with only the significant pairwise metabolite correlations (FDR corrected p-values < 0.05). The four networks differed in many aspects (Supplemental Table 4), ultimately leading to different network topologies (Figure 3). The highest number of edges was found for the HT network (320), followed by that of HL (280) and ST (248). LP was the sparsest network with 65 nodes and 210 edges. By comparing the networks we found that overall an average of 60% of the correlations was shared in at least two networks. Metabolites of the same pathway were found highly correlated in all networks. This was for example observed for glycerol and glycerol-3-P as well as myo-inositol and galactinol suggesting a strong effect of the genetic component. The correlation between succinate and fumarate, two TCA cycle intermediates, was significant in all networks while their correlation coefficients varied from 0.51 in HL to 0.80 in HT, showing that the metabolite correlations are modulated by the maternal environment.

To further investigate metabolic changes, cluster analysis was performed on the different networks. Non-random metabolite clustering was observed in the different networks (not shown). The metabolic clusters were shown to be environment specific and enriched for specific metabolic pathways (Supplemental Table 5).

The cluster of amino acids was rather consistent across all maternal environments. In the HT network, the largest cluster was enriched for metabolites related to the tricarboxylic acid (TCA) cycle (succinate, citrate, fumarate and malate) and glyoxylate pathway metabolites (GABA, 4-Hydroxybutanoate, alanine)(Supplemental File 1, Supplemental Table 4). The clustering of these metabolites was also observed when considering pairwise correlations between all 172 metabolites (Supplemental Figure 3). The TCA cycle plays a pivotal role in metabolism as a central hub providing the energy necessary to sustain biochemical processes (Sweetlove et al. 2010). The amine compounds GABA, glycine and alanine were found in the same cluster. GABA is a key compound of the GABA shunt that bypasses two steps of the TCA cycle. The close clustering of alanine and GABA, suggests that alanine might be catabolized by GABA transaminase using pyruvate as a co-substrate. In the same cluster, other sugars and organic acids such as fructose, myo-inositol and glycerol were identified, suggesting a close link between the TCA cycle and other pathways of the carbon metabolism. Clustering of these metabolites in response to high temperatures has been observed before (Caldana et al. 2011).

In the HL network, one of two largest cluster identified comprised mostly carbohydrates, such as arabinose, fructose, glucose and mannose. Myo-inositol was also found in this cluster, as opposed to its clustering with TCA cycle intermediates under HT.

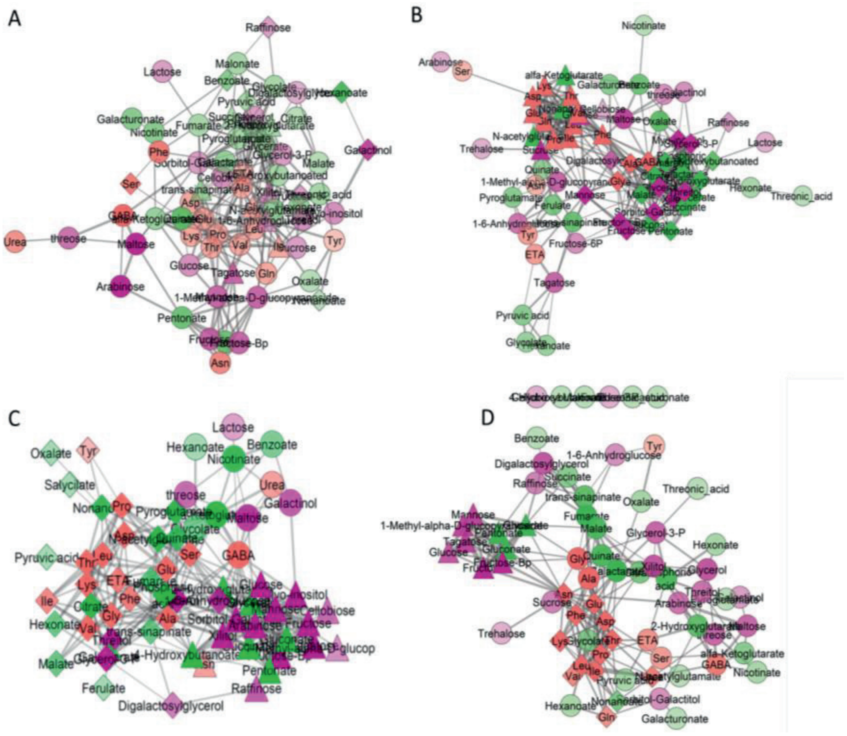


Figure 3. Condition specific correlation networks for A) standard condition (ST), B) high temperature (HT), C) high light (HL) and D) low phosphate (LP). Only significant spearman correlations with FDR corrected p-value < 0.05 were used to build the networks. In the networks, each metabolite is represented as a node and linked to other metabolites by edges; node colours refer to the class of the metabolites with amino acids in red, organic acids in green and sugars in purple. Diamond and triangle shaped nodes indicate membership of the first and second largest detected cluster, respectively. Darker node colour indicates larger node connectivity. Some metabolite names are abbreviated. Ala: alanine, Asn: asparagine, Asp: aspartate, Gln: glutamine, Ile: isoleucine, Leu: leucine, Lys: lysine, Phe: phenylalanine, Pro: proline, Ser: serine, Thr: threonine, Tyr: tyrosine, Val: valine, Eta: ethanolamine. The cytoscape file of these networks is provided as Supplemental File 1.

Genetic basis of the dry seed metabolome

To identify the loci involved in dry seed metabolome variation, mapping of QTLs associated with primary metabolites (mQTLs) in the RIL sub-populations in the aforementioned conditions was performed. For this, two models were used. The single environment, single marker model includes the genetic variation within each sub-population with Y = G + ε. The full environment single marker model Y = G + E+ G x E + ε was used for the combined set of RILs to identify mQTLs explained by the main genetic (G) and the genotype by environment interaction (G x E) components and random effects (ε) of the model.

Table 2. Summary of the mQTLs identified in the separate and combined RIL datasets

					Combined RILs	
	ST	HT	HL	LP	G	GxE
Metabolites with at least 1 mQTL	86	91	80	85	125	42
Total number of mQTLs	127	161	162	168	440	81
Range of mQTLs per metabolite	1-6	1-7	1-7	1-6	1-12	1-5
Average nr of mQTLs per metabolite	1.48	1.77	2.03	1.98	3.52	1.14

A large number of mQTLs was identified (Table 2). In ST, HT, HL and LP, 127,161,162 and 168 mQTLs were identified for 86, 91, 80 and 85 metabolites respectively. The number of mQTLs for each metabolite ranged from 1 to 7 with an average of 1.48 (ST) to 2.03 (HL) across the different environments. Little overlap of mQTLs identified for each metabolite across environments was observed (Supplemental Table 6). Across all conditions, more than 90% of the mQTLs had a LOD score lower than 5. A higher number of mQTLs was identified for the QTL mapping on the combined set of RILs (440 mQTLs) with an average of 3.52 mQTLs per metabolite. For the G x E component, 81 mQTLs for 42 metabolites were identified. The mQTLs detected for the main G effect are shown in Figure 4. The details of the results of all mQTL analyses are given in Supplemental Table 6.

Genetic control of coordinated metabolic changes

Under the four conditions, a high number of mQTLs co-located on the top of chromosome 4 and 5 (Figure 4). In other regions of the genome, mQTLs were identified for specific environments, suggesting distinct genetic basis and metabolic pathways across the different conditions (Figure 4, upper panel).

Conceptually, highly correlated metabolites are likely to share mQTLs (Matsuda et al. 2012; Carreno-Quintero et al. 2012). The high connectivity of the metabolites observed in the different networks lead us to investigate environment specific co-locating mQTLs. We thus investigated whether co-locating mQTLs did correspond to the clustered metabolites identified in the correlation networks (Figure 5). mQTLs for galactinol and myo-inositol co-located on chromosome 2 in ST and HL conditions. In LP, putative mQTLs for these compounds were found in the same region, although these did not reach the significance threshold (LOD > 3.09). In HT, an mQTL for myo-inositol co-located with several mQTLs for TCA cycle intermediates (succinate, fumarate) as well as alanine. These metabolites were found clustered in the HT network (Figure 3), showing that besides their shared pathway they also have a shared genetic architecture. Another significant mQTL for myo-inositol was specifically identified for HL on chromosome 1. This mQTL co-located with several other mQTLs associated with sugar metabolites (mannose, fructose, fructose bisphosphate) and gluconic acid. Their shared genetic basis supported their clustering observed in the HL network (Figure 5). Although the clustering of several amino acids was observed under different conditions (Figure 3, Supplemental Table 5), shared mQTLs for these were only observed under ST.

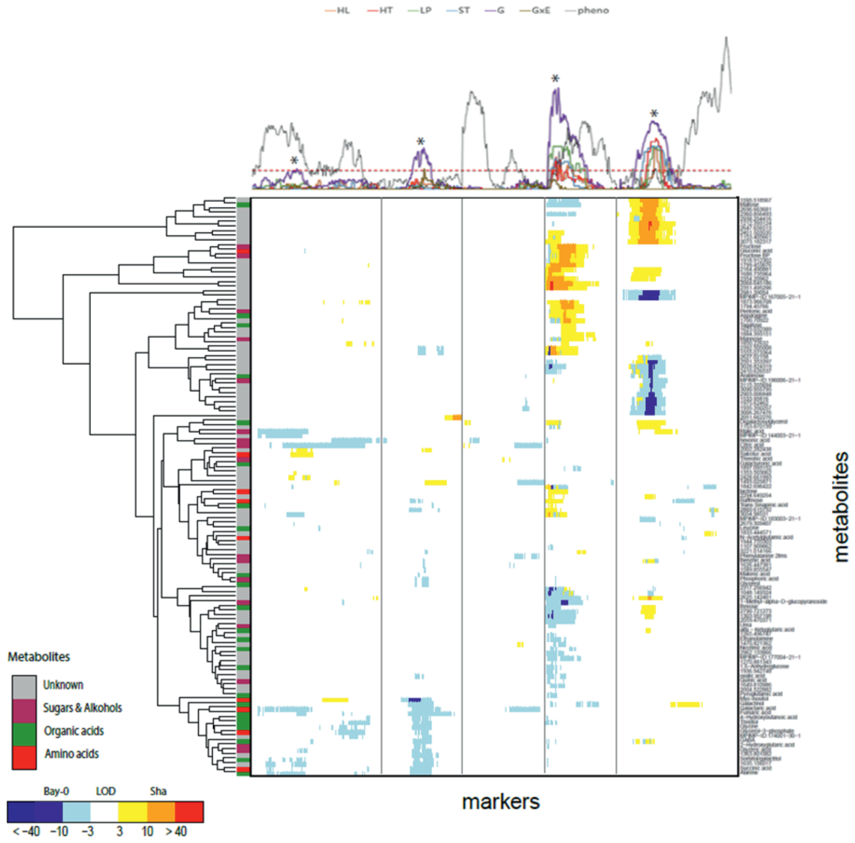


Figure 4. Heatmap showing the position of the mQTL for the G component. The mQTL profiles for metabolites with at least one mQTL are shown in the heatmap. The LOD scores of the identified mQTLs are coloured according to a false colour code with blue indicating higher effect of the Bay-0 allele while yellow and red colours indicate higher effect of the Sha allele. Black vertical lines delineated the five chromosomes. Metabolites are indicated on the left row and ordered by clustering. The vertical coloured bars with grey for unknown metabolites, magenta for sugars, green for organic acids and red for amino acids. The upper panel shows the frequency of the mQTLs across the genome for the different mapping analyses. The black dotted line corresponds to the frequency of the phenotypic plasticity QTLs as reported in Chapter 3. Significant mQTL hotspots above the threshold (dashed red line) are marked with an asterisk.

Hotspots of metabolic regulation

The distribution of the mQTLs for the 172 metabolites was biased towards specific regions in the genome (Figure 4). These so-called "hotspots" count a higher number of co-locating mQTLs than expected by chance and point toward genomic factors likely to directly or indirectly influence a large range of metabolites (Breitling et al. 2008; Keurentjes et al. 2006; Fu et al. 2009; Rowe et al. 2008).

In total 4 distinct significant mQTL hotspots were identified (Figure 4). These hotspots were identified on chromosome 1, 2, 4 and 5. The highest numbers of co-locating mQTLs were observed on top of chromosomes 4 and 5 with 59 and 40 overlapping mQTLs, respectively. These two hotspots have been previously identified for both primary and secondary metabolites measured in Arabidopsis seeds and seedlings (Rowe et al. 2008; Joosen et al. 2013; Keurentjes et al. 2006) (Chan et al. 2011). They correspond to the transcript QTL hotspots of AOP (AOP3, chromosome 4) and Elong (MAM2, chromosome 5) that are involved in the biosynthesis of glucosinolates (Rowe et al. 2008; Wentzell et al. 2007; Chan et al. 2011; Keurentjes et al. 2006). This example of co-location of mQTLs of primary and secondary metabolites might suggest a cross-talk between primary and secondary metabolism regulation (Joosen et al. 2013). In this study, we found a large number of mQTLs for unknown metabolites mapping to these two major hotspots. The other hotspots identified on chromosome 1 and 2 counted 12 and 24 mQTLs respectively, of which respectively 8 and 17 mQTLs were associated with annotated metabolites. mQTLs for several TCA cycle intermediates such as malate, citrate and fumarate clustered in both regions. We noted that a certain number of mQTLs for the G x E component also co-located in the region of the hotspot on chromosome 2, indicating the particular sensitivity of this genomic region for environmental changes (Figure 4, upper panel).

Correlation network predicts common genetic factors

The major cluster in the HT network comprised 25 metabolites which mostly were carbohydrates. In the HT mQTL analysis, 6 of these metabolites co-located on chromosome 2, while for the G analysis mQTLs for 17 of these metabolites co-located in the same region (Supplemental Figure 5). This suggested that the correlation network-based approach on 40 RILs could seize subtle genetic variation, recovered when using large statistical mapping power provided by a large RIL population size.

Several lines of evidence indicate that the G mQTL hotspot on chromosome 2 was driven by the HT RIL dataset. The main G effect QTL analysis excluding the RIL set grown under HT failed to identify this mQTL hotspot. The PCA of the metabolite profiling of the RILs also indicated the remarkable effect of the HT maternal environment on the dry seed metabolome (Figure 2). This was best captured by the first two components of the PCA. QTL mapping using the projected scores for PC1 identified a PC1-QTL coinciding with the position of the mQTL hotspot on chromosome 2 (Supplemental Figure 6). Together, these results indicate that correlation network analysis, even in a context of limited resolution (40 RILs) could help to predict common genetic factors prior to QTL analysis.

Several studies have used network properties to enhance our knowledge of the landscape of metabolic regulation. For instance, other than abundance of the metabolites, variables for mQTL mapping can also be derived from the known inter-relation of the metabolites in a pathway. The mapping is then performed using metabolic ratio or the sum of the metabolites belonging to a same pathway (Kliebenstein et al. 2001; Wentzell et al. 2007; Angelovici et al. 2013). Since, the pathway structure causes metabolites to correlate; using this correlation can facilitate the linkage of metabolic shifts to the impact of the different biochemical pathways (Weckwerth et al. 2004; Steuer et al. 2003). In a recent study, (Angelovici et al. 2017) used network topological properties to derive new metabolic variables. Their approach resulted in the identification of new loci associated with the free amino acid pathways in seeds, showing that network properties can complement classical variables for the identification of genetic variants.

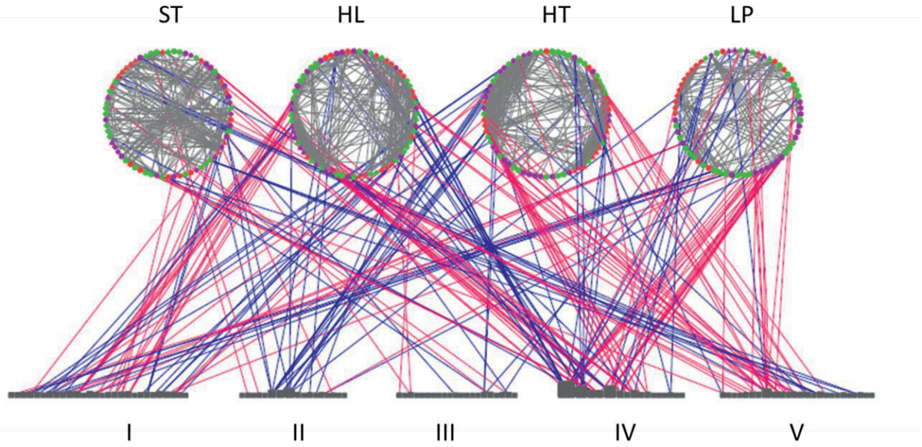


Figure 5. Relation between mQTLs and metabolic correlation networks. The five chromosomes (I-V) are represented in the grey horizontal bars. Bins of 10 markers are represented for each chromosome. The mQTLs identified are linked to their related metabolites on the correlation networks, with the colour of the edges indicating the direction of the additive effect (blue for Bay-0 and red for Sha). The size of the marker is proportional to the number of co-locating mQTLs. Metabolite correlations in each network are represented by grey edges. In each network, metabolites clustering together are grouped and identified by their shared node shape. The Cytoscape file of this network is available in Supplemental File 2.

mQTL hotspot on chromosome II governs coordinated stress responses

The effect of high temperature on physiological and molecular processes in plants is well established (Wahid et al. 2007; Kaplan et al. 2004). In addition, several metabolite profiling studies have also reported changes in the amino acid and carbohydrate metabolisms in response to high temperature (Kaplan et al. 2004; Obata and Fernie 2012).

The mQTL hotspot identified on chromosome II and essentially driven by the high temperature maternal environment, comprised 24 co-locating mQTLs for TCA cycle intermediates (fumarate, succinate), N-compounds (GABA, alanine, glycine) as well as sugars and sugar alcohols (raffinose, galactinol, myo-inositol).

Co-locating mQTLs for fumarate, succinate, GABA, alanine and glycine indicate genetically driven metabolic changes in the TCA cycle and the GABA shunt. The

TCA cycle plays a central role in energy metabolism (Araujo et al. 2012). Under normal conditions, the TCA cycle is used to release stored energy from glucose, fatty acids or amino acids into chemical energy to support other metabolic activities. The GABA shunt bypasses two steps of the TCA cycle; the conversion of 2-oxoglutarate to succyl-coA and subsequently succinate. Instead, 2-oxoglutarate is converted into glutamate and subsequently to GABA. Through the action of a transaminase, GABA is converted into succinate semi-aldehyde which re-enters the TCA cycle in the form of succinate. The accumulation of GABA in response to abiotic stresses has been reported in several studies (Kinnersley and Turano 2000). Although its physiological role remains unclear, GABA has been associated with many physiological responses including stress signalling (Bouche and Fromm 2004). Moreover, a recent study has shown that stress altered function of the TCA cycle can be alleviated by the GABA shunt (Fait et al. 2008). This alternative metabolic route might represent an adaptive mechanism to maintain the metabolic balance under stress conditions.

The co-location of mQTLs for myo-inositol, galactinol and raffinose also suggested coordinated metabolic changes in the regulation of the raffinose family oligosaccharides (RFOs) pathway. In the first step of this pathway, myo-inositol is converted in galactinol and through subsequent steps to raffinose and stacchyose. Several functional roles for these oligosaccharides have been reported. RFOs can act as osmo-protectants of cellular structures to protect the embryo during seed development and in particular seed desiccation (Taji 2002). RFOs are also involved in stress defence mechanisms (reviewed by (ElSayed et al. 2014) by protecting plants from reactive oxygen species (ROS) produced under stress.

For most of these co-locating mQTLs, higher metabolite levels were governed by the Bay-0 allele (Figure 4). Bay-0 and Sha display contrasting sensitivity responses. Sha appears more stress tolerant, while Bay-0 is more sensitive to stresses during seed germination (Chapter 3, (Joosen et al. 2012; Vallejo et al. 2010). Higher temperature is associated with physiological changes such as reduction in plant growth, increased growth rate, earlier flowering and earlier seed set (Springthorpe and Penfield 2015; Wahid et al. 2007). Higher temperature also result in increased respiration and metabolic rate (Wahid et al. 2007). The increase of the TCA cycle intermediates suggests that Bay-0 shift the pool of metabolites towards higher carbohydrate levels and higher energy metabolism to adjust growth and developmental processes to higher temperatures. Under stress conditions elevated cellular metabolic rate can lead to an excessive production of ROS, which are normal products of plant cellular metabolism. ROS can serve as signalling molecules, but also cause oxidative damages, which likely depends on the equilibrium between ROS production and ROS scavenging. The enhanced biosynthesis of RFOs, known osmo-protectant molecules, can prevent such oxidative damages.

MIPS2 as a potential candidate for causing the mQTL hotspot on chromosome 2

The mQTL with the highest LOD score (-16.51) under the hotspot on chromosome 2 was observed for myo-inositol. Myo-inositol is a versatile compound which endorses various roles in plants such as in phytic acid biosynthesis, cell wall biosynthesis and the production of stress-related proteins (Meng et al. 2009), reviewed by Valluru and Van den Ende (2011). Genes underlying the mQTL hotspot were scrutinized for their direct link with the above mentioned metabolic pathways. One of the most likely candidates underlying this hotspot is myo-inositol-1-phosphate synthase 2 (MIPS2, At2g22240) which catalyses the conversion of D-glucose 6-phosphate to 1L-myo-inositol-1-phosphate. MIPS2 is found highly expressed in seeds and in particular during late seed maturation (Supplemental Figure 7). Myo-inositol mQTL co-located with an eQTL for MIPS2 (Nijveen et al. 2017) (http://www.bioinformatics.nl/AraQTL/). Further investigations are needed to measure the impact of MIPS2 on myo-inositol and other metabolite levels, in particular in response to stress conditions.

Maternal environment modulates the metabolite-phenotype relation

In view of the extensive changes occurring at the metabolic level, the question remains whether these changes can explain phenotypic differences. In several studies, the shared genetic basis between metabolic and phenotypic QTLs has been observed (Carreno-Quintero et al. 2012), (Kazmi et al. 2017). Comparison of the mQTL and phQTL distributions was performed with all traits measured for germination in Chapter 3.

Overall, the main mQTL hotspots showed limited overlap with the major hotspots for phQTLs (Figure 4, upper panel). An example of a co-locating mQTL and phQTL was observed on top of chromosome 1. The mQTL hotspot on that chromosome comprising TCA cycle intermediates mQTLs, with higher effect triggered by Bay-0, co-located with a large number of phQTLs. Many of these phQTLs were specific for the population grown under high temperature for several germination conditions (Chapter 3). Higher germination (AUC value) was observed for the Sha allele, suggesting that the higher amounts of TCA cycle intermediates in Bay-0 would negatively affect seed performance. This negative correlation between TCA cycle intermediates and seed performance was also observed for seeds matured under low and high light seed maturation conditions in Arabidopsis (He et al. 2016). On the other hand, low temperature maturation environment altered TCA cycle activity with a significant decrease of fumarate and succinate contents and showed a positive correlation with seed performance traits (He et al. 2016).

Correlation between seed performance and metabolites

The limited overlap between mQTLs and phQTLs lead us to also investigate the relation between metabolic changes and seed performance (AUC under different conditions) by correlation analysis. Overall, intermediate Spearman correlations were observed between metabolite and seed performance (Supplemental Table 8). Nonetheless, we found different sets of metabolites significantly correlated with seed performance traits across the four conditions. Under HT, negative correlations were observed between metabolites of the HT cluster (glycerate, glycine, glycerol-3-P) and several seed performance traits. Under HL, significant positive correlations were observed between metabolites of the cluster HL (mainly sugars) and seed performance in particular with germination under cold or heat with stratification. This observation is in line with the aforementioned properties of sugars and inositol compounds to act as osmolytes and provide stress protection to ensure germination. In ST, galactinol, myo-inositol and glycerol-3-phosphate were negatively correlated with seed performance. Galactinol synthase (GolS) is considered to be a key regulator of the biosynthesis of Raffinose family oligosaccharides (RFOs) and the accumulation of RFOs has been reported to play a role in protection against abiotic stresses. A recent study showed that the expression of AtGOLS1, encoding a galactinol synthase, decreases when conditions are favourable for seed germination, indicating a negative regulation of seed germination by AtGOLS1 (Jang et al. 2018). Under ST, malate, citrate and sugars (mannose and glucose) and key compounds of the TCA cycle were positively correlated with seed performance supporting the role of the TCA cycle in the control of the energetic status of the seeds as well as the link with seed performance. Together, these results show that the maternal environment can modulate seed metabolism and its link to seed phenotypes.

Conclusion

In this study, we used untargeted GC-TOF-MS metabolite profiling of an Arabidopsis RIL population to explore the effect of genotype and maturation environment on the dry seed metabolism and the implication for seed germination. Our results show that changes in the maturation environment markedly affect the dry seed metabolome in a genotype-dependent manner. The topological relationships among the metabolites extracted from the condition specific networks, combined with mQTL analyses showed that coordinated metabolic changes are environment specific and genetically controlled. A major mQTL hotspot on chromosome 2 was identified and provided insights into the adaptive metabolic responses to stress conditions. The role of MIPS2, identified as a potential candidate underlying this mQTL hotspot, needs to be further investigated. Together, these approaches bring new insights into the understanding of the genetic regulation of dry seed metabolome under stress conditions and its effect on seed performance.

List of supplemental data

If not found below, supporting information can be downloaded from:
http://www.wageningenseedlab.nl/thesis/earserin/SI/chapter4

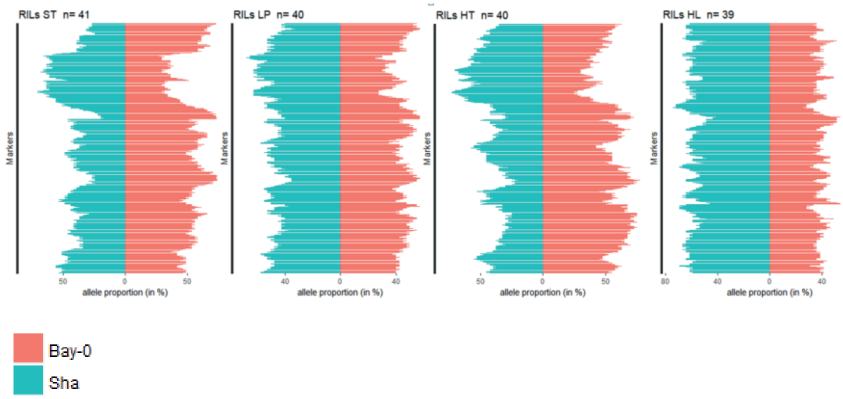
Supplemental Table 1: Raw data for metabolites measured in the dry seeds of parents and RILs grown under the different maternal environments.
Supplemental Table 2: ANOVA and summary statistics for the metabolite data of parents and RILs.
Supplemental Table 3: Loadings for the principal component analysis on the 71 annotated metabolites from the combined RILs.
Supplemental Table 4: Condition specific metabolite correlation network properties.
Supplemental Table 5: Pathway enrichment analysis for the two largest clusters identified in the condition specific correlation networks.
Supplemental Table 6: Results of the mQTL analyses in a GGG design.
Supplemental Table 7: Spearman correlation r and associated p-values for all pairwise correlations between all 172 metabolites from each condition.
Supplemental Table 8: List of significant correlations between seed performance phenotypes and metabolites for the different conditions.

Supplemental Figure 1: Allele distribution across markers for the RILs in the GGG design.
Supplemental Figure 2: PCA with score and loading plots for all 172 metabolites from the combined RILs.
Supplemental Figure 3: PCA of the 71 annotated metabolites measured in the RILs grown under the different maternal environments.
Supplemental Figure 4: Metabolite correlations for all 172 detected metabolites under high temperature.
Supplemental Figure 5: Link between mQTL hotspot on chromosome 2 and the HT correlation network.
Supplemental Figure 6: Heatmap for the mapping of the principal components.
Supplemental Figure 7: Expression profile of MIPS2 at several developmental stages using the eFP browser from the Bio Analytic Resource for Plant Biology.

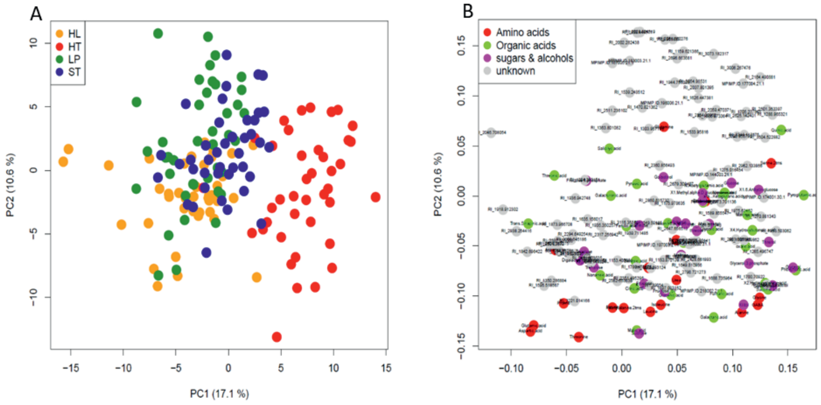
Supplemental File 1: Cytoscape files for the condition specific correlation networks.

Supplemental File 2: Cytoscape file for the link between mQTL mapping and correlation networks.

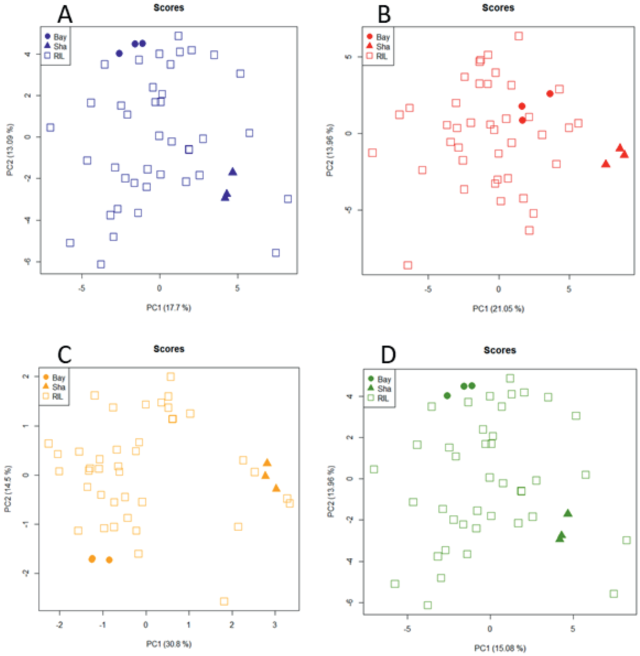
Supplemental Figure 1: GGG design for different RIL subsets (ST, LP, HT and HL). The number of RILs (n) in each subset is indicated above each plot. The distribution of the Bay-0 and Sha alleles across the 662 markers used in the analysis is shown in the different barplots.



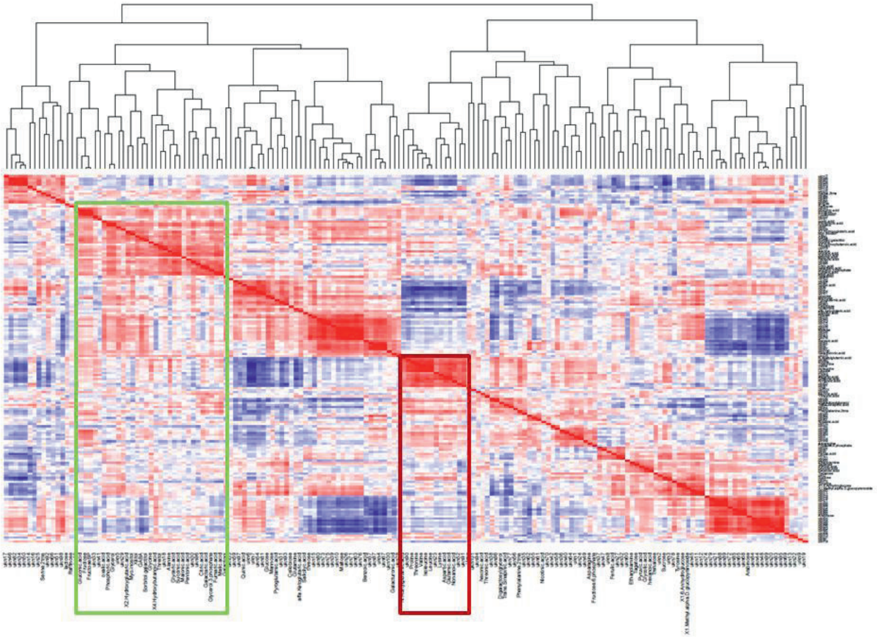
Supplemental Figure 2: Principal component analysis for all 172 metabolites in a GGG design A. score plot showing the RILs from the four environments (HL: high light, HT: high temperature, LP: low phosphate, ST: standard). B. Loading plot showing all the 172 metabolites and their contribution to the first two principal components.



Supplemental Figure 3: PCA for the 71 annotated metabolites measured in the RILs grown under A. standard (ST) B. high temperature (HT) C. high light (HL) and D. low phosphate (LP). Filled symbols correspond to the parental lines.



Supplemental Figure 4: Metabolite correlations for all 172 detected metabolites under high temperature. The heatmap shows the pairwise spearman correlations between all 172 metabolites measured in the HT-RILs. Hierarchical clustering was performed based on the distance dissimilarity matrix. The colour code indicates low (blue) to high (red) correlation values. The two frames indicate clusters of highly correlated sugars and organic acids (green) and amino acids (dark red) as observed in the HT network for the 71 annotated metabolites.



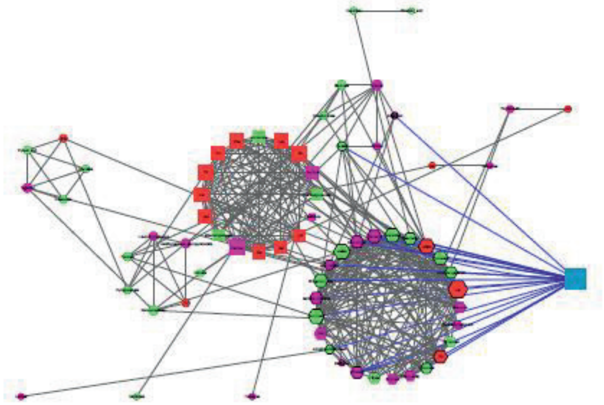
Supplemental Table 4: Condition specific metabolite correlation network properties

Network	Standard	High temperature	High light	Low phosphate
Number of RILs	41	40	39	40
Total Number of nodes	68	68	68	65
Total Number of edges	248	320	280	210
Range Node degree	1-20	1-26	1-20	1-15
Unique edges (%)	29.4	40.9	36.1	21.9
Network density	0.109	0.140	0.123	0.101
Clustering coefficient	0.414	0.477	0.431	0.431
Average number of neighbours	7.294	9.412	8.235	6.462
Number of communities (>4 nodes)	12 (4)	14(5)	9(3)	21(7)
modularity	0.42	0.43	0.41	0.48
Top 5 high degree nodes (degree)	- Leucine (20) - Alanine (17) - Proline (16) - Threonine (15) - 4-Hydroxybutanoate (14)	- Alanine (26) - Malate (20) - GABA (20) - Succinate (19) - Glyceric acid (18)	- Glycerate (20) - Sorbitol/Galactitol (19) - Alanine (18) - Glycerol (17) - Fructose (16)	- Aspartate (15) - Glutamic acid (15) - Proline (14) - Threonine (14) - Asparagine (14)

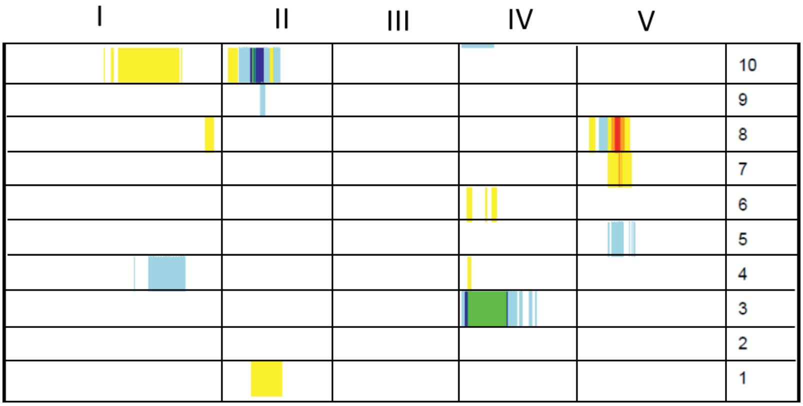
Supplemental Table 5. Significantly enriched pathways for metabolic clusters of condition specific correlation networks. Pathway enrichment analysis was performed for the two largest clusters found in each network. Cluster 1 corresponds to diamond shaped metabolites and cluster 2 corresponds to triangle nodes as depicted in Figure 4. Only clusters with significantly enriched pathways (p-value < 0.01) are shown. Pathways are ranked based on the p-value and n is the number of metabolites in the cluster compared to the total number of metabolites from the pathway, N.

Network	Cluster	pathway name	n	N	p < 0.01
STANDARD	Cluster 1	Glycerolipid metabolism	2	13	0.007996
	cluster 2	Aminoacyl-tRNA biosynthesis	9	67	9.90E-10
		Arginine and proline metabolism	5	38	1.85E-05
		Valine, leucine and isoleucine biosynthesis	4	26	8.27E-05
		Alanine, aspartate and glutamate metabolism	3	22	0.001109
		Valine, leucine and isoleucine degradation	3	34	0.004015
		Lysine biosynthesis	2	10	0.004046
		Nitrogen metabolism	2	15	0.009174
HIGH TEMPERATURE	cluster 1	Aminoacyl-tRNA biosynthesis	12	67	2.41E-10
		Alanine, aspartate and glutamate metabolism	5	22	3.09E-05
		Arginine and proline metabolism	5	38	4.82E-04
		Valine, leucine and isoleucine biosynthesis	4	26	0.001056
		Galactose metabolism	4	26	0.001056
		Nitrogen metabolism	3	15	0.002236
	cluster 2	Citrate cycle (TCA cycle)	4	20	1.13E-04
		Glyoxylate and dicarboxylate metabolism	3	17	0.001381
		Alanine, aspartate and glutamate metabolism	3	22	0.002993
HIGH LIGHT	cluster 1	Alanine, aspartate and glutamate metabolism	5	22	8.94E-06
		Citrate cycle (TCA cycle)	4	20	1.42E-04
		Arginine and proline metabolism	4	38	0.001819
		Carbon fixation in photosynthetic organisms	3	21	0.003064
		Glycine, serine and threonine metabolism	3	30	0.008596
	cluster 2	Galactose metabolism	3	26	0.001084
LOW PHOPSHATE	cluster 1	Aminoacyl-tRNA biosynthesis	12	67	2.11E-12
		Alanine, aspartate and glutamate metabolism	5	22	6.66E-06
		Arginine and proline metabolism	5	38	1.11E-04
		Valine, leucine and isoleucine biosynthesis	4	26	3.30E-04
		Nitrogen metabolism	3	15	9.40E-04
		Carbon fixation in photosynthetic organisms	3	21	0.002607
		Glycine, serine and threonine metabolism	3	30	0.007354
		Lysine biosynthesis	2	10	0.007772
		Cyanoamino acid metabolism	2	11	0.009421

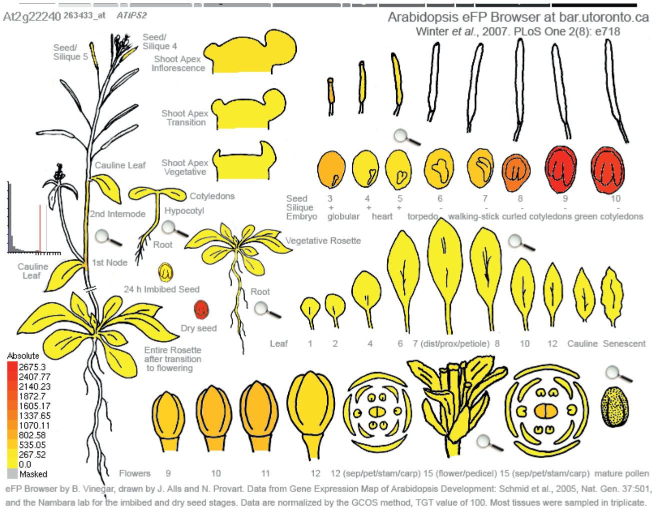
Supplemental Figure 5. Link between the mQTL hotspot detected on chromosome 2 and the metabolite correlations under high temperature. The mQTL hotspot is represented as the blue square and is linked to metabolites/nodes in the HT specific network that have an mQTL at the position of the hotspot. The colour of the nodes indicates the class of the metabolites - with red for amino acids, green for organic acids and pink for sugars and sugar alcohols. The network is displayed with a forced configuration based on the detected metabolite clusters. Most of the metabolites in the largest cluster have co-locating mQTLs.



Supplemental Figure 6. Heatmap of the LOD scores for the 10 first components of the PCA including all annotated metabolites. The heatmap shows for each principal component (1 to 10) the LOD profile along the chromosomes indicated by romans numerals (I – IV). Yellow to red colour indicate significant QTLs with higher effect in Bay-0 while blue to red indicate significant QTls with a higher effect in Sha. A pcQTL is identified on chromosome II for the highest explanatory component, PC1.



Supplemental Figure 7. Expression pattern of MIPS2 (At2g22240) in the developmental series of Arabidopsis thaliana from the eFP browser from the Bio Analytic Resource for Plant Biology.



Arabidopsis eFP Browser at bar.utoronto.ca
Winter *et al*., 2007. PLoS One 2(8): e718

eFP Browser by B. Vinegar, drawn by J. Alls and N. Provart. Data from Gene Expression Map of Arabidopsis Development: Schmid et al., 2005, Nat. Gen. 37:501, and the Nambara lab for the imbibed and dry seed stages. Data are normalized by the GCOS method, TGT value of 100. Most tissues were sampled in triplicate.

Chapter 5

Environmental and genetic effects on gene expression in Arabidopsis seeds

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** These authors contributed equally to this work*

Abstract

Gene expression is largely influenced by environmental and genetic factors. The environment experienced during seed development results in transcriptomic changes in a genotype-dependent manner. A deeper investigation of the genetic basis of these changes and the extent of genotype by environment interactions is needed for a comprehensive understanding of seed quality and its control. In this study, an Arabidopsis Bay-0 x Sha RIL population, consisting of 165 lines, was grown under four different environments; standard, high temperature, high light and low phosphate from flowering until seed harvest. RNA-seq was performed on the dry seeds of the parental and 160 recombinant inbred lines produced under the different environments using a generalized genetical genomics set-up.

A large number of genotype-by-environment interactions (G x E) were identified in the parental lines which were reflected in the RILs. Overall, a large number of eQTLs was identified. Their profile substantially differed between conditions, indicating eQTL x E interactions. Consistent with previous studies, local eQTLs largely overlapped between environments while distant eQTLs were highly variable across environments. The eQTL distribution along the genome showed environment-specific genetic hotspots of transcript regulation enriched for different specific biological processes.

With this study, we show that transcriptional changes found in dry seeds are largely caused by genotype-by-environment interactions. These datasets represent a valuable resource for further research towards understanding the dynamics and mechanisms of gene expression regulation in seeds in response to environmental changes and their link with seed performance.

Keywords: *Arabidopsis thaliana*, eQTL, G x E, maternal environment, RNA-seq, seed performance

Introduction

During seed development, seeds accumulate various macro-molecules, such as proteins and mRNAs. These molecules contribute to seed developmental processes such as seed maturation, desiccation tolerance and dormancy, while others can remain stored in dry seeds for a role during germination. The role of these stored mRNAs in germination has been highlighted by studies showing that germination can take place in the absence of de novo transcription (Rajjou et al. 2004; Kimura and Nambara 2010). This suggests that the early initiation of molecular and physiological processes essentially relies on the activity of stored proteins, enzymes as well as on the translation of these stored mRNAs (Rajjou et al. 2004; Kimura and Nambara 2010). Genome-wide profiling of these mRNAs in Arabidopsis seeds identified more than 10 000 stored mRNAs associated with several biological processes (Dekkers et al. 2013; Nakabayashi et al. 2005; Belmonte et al. 2013). A recent study showed that this pool of stored mRNAs is modulated by the seed production environment in a genotype-dependent manner (He et al. 2016).

Genome wide expression analyses in Arabidopsis seeds have brought large insights into the dynamics of the transcriptome during seed development (Ruuska 2002; Belmonte et al. 2013), dormancy (Bassel et al. 2011), desiccation tolerance (Costa et al. 2015), germination (Dekkers et al. 2013) and seedling establishment (Silva et al. 2016). Naturally occurring genetic variation for seed traits has been largely studied in Arabidopsis. The genetic basis of such variation can be explored using quantitative trait locus (QTL) analysis. Gene expression can be treated as a quantitative trait and thus combining QTL analysis with large scale expression profiling can provide insights into the genetic determinants of gene expression (Jansen and Nap 2001). This genetical genomics approach has revealed the genetic architecture of gene expression variation in plants as well as in other organisms (Joosen et al. 2009; Li et al. 2006; Brem et al. 2002; Schadt et al. 2003). Several eQTL studies have also investigated changes in the eQTL landscape in response to perturbations such as across different populations (Cubillos et al. 2012), in time (Vinuela et al. 2010), in response to abiotic stresses (Snoek et al. 2017b; Cubillos et al. 2014; Lowry et al. 2013) and across different tissues (Drost et al. 2015).

A promising application of eQTL analysis is the insights into regulatory mechanisms that can be gained from the identification of two types of eQTLs regarding their mapping position (Kliebenstein 2009; Rockman and Kruglyak 2006). These are often reported as cis and trans-eQTLs in the literature. We prefer to refer to local versus distant eQTLs since their categorization is based on genomic distances between the position of the eQTL and the physical position of the genes which leave an uncertainty regarding their true nature. Local eQTLs correspond to eQTLs that map at the physical position of the gene investigated. In this case, the local regulation of gene expression can be explained by a sequence polymorphism in the gene itself or in physically neighbouring regions (Rockman and Kruglyak 2006). On the other hand, distant eQTLs map at other positions on the same or on another chromosome. In this case, the distant regulation occurs as a result of a gene's polymorphic variant affecting the expression of one or several other genes (Rockman and Kruglyak 2006). The non-random accumulation of eQTLs in genomic regions leads to the identification of eQTL hotspots. Often observed in eQTL studies, these hotspots may suggest the large pleiotropic effect of an underlying polymorphic variant (Breitling et al. 2008). These hotspots provide a good starting point to identify groups of co-regulated genes with shared biological functions. For Arabidopsis, the extensive knowledge on gene function and pathways (Lamesch et al. 2012; Kanehisa and Goto 2000) provides valuable input to drive such investigations.

The elucidation of the genetic control of genotype-by-environment interactions (G x E) is crucial to understand the regulation of gene expression and ultimately seed performance. In this study, we used an Arabidopsis thaliana recombinant inbred line (RIL) population of 165 lines grown under standard, high temperature, high light and low phosphate environments to provide a genome-wide view of the plasticity of gene expression regulation. We used a generalized genetical genomics (GGG) design to investigate the effect of genetic and multi-environment perturbations on gene expression in a cost-efficient manner (Li et al. 2008b). In such a design, complementary and equal subsets of lines are drawn from the initial population while maintaining a balanced allele distribution in each subset. Subsequently different treatments and/or developmental stages can be used for the different subsets. In contrast to previous eQTL studies in plants using microarrays (Cubillos et al. 2014; Snoek et al. 2012) we use for the first time RNA-seq on dry seeds matured under different conditions to perform eQTL analysis in a GGG design. We showed that the dry seed eQTL landscape is largely influenced by the maternal environment, resulting in several environment-specific eQTL hotspots. In addition, we provide a glimpse on the potential of these datasets to enhance gene discovery in relation to seed performance.

Material and Methods

Plant Material

Plants from the Arabidopsis thaliana Bay-0 x Sha recombinant inbred line population (165 RILs) (Loudet et al. 2002) were grown in standard (ST) conditions, namely long day (16h light / 8h dark) and 22°C/18°C (day / night) under artificial light (150 µm m-2 s-1) in a climate chamber until flowering. Once all plants flowered, the stems were cut short to ensure complete seed development under the four environments. Four plants per RIL as well as the parental lines were transferred to high light (HL)(300 µm m-2 s-1) and high temperature (HT)(25°C/23°C) conditions in different climate cells. RILs grown under standard and low phosphate (LP)(12.5 µM) conditions remained in the same climate cell, with an adjustment of the nutritive solution for RILs grown in the LP condition on a separate flooding table. Once sufficient amounts of fully matured seeds were produced under each environment, fresh dry seeds were bulk harvested from 3-4 plants, dried and stored at -80°C until RNA-seq library preparation.

Sample preparation

The population was divided in four sub-populations optimized for the distribution of the parental alleles as previously described (Serin et al. 2017). Four mg of fresh dry seeds of the parental lines and the RILs grown under the different environments in a GGG design were used to extract total RNA. RNA was isolated using the NucleoSpin RNA plant isolation kit (Macherey-Nagel 740949) adding Plant RNA isolation Aid (Life technologies) according to the manufacturer’s protocol and instructions. RNA from three replicates was isolated for the parental lines, Bay-0 and Sha, grown under the different conditions.

RNA-seq analysis

The processing of the RNA-seq is described in (Serin et al. 2017). Strand-specific RNA-seq libraries were prepared from each RNA sample using the TruSeq RNA kit from Illumina according to manufacturer’s instructions. Poly-A-selected mRNA was sequenced using the Illumina HiSeq2500 sequencer, producing strand-specific single-end reads of 100 nucleotides. Reads were trimmed using

Trimmomatic (version 0.33, (Bolger et al. 2014) to remove low quality nucleotides. Trimmed reads were subsequently mapped to the *Arabidopsis thaliana* TAIR10 reference genome (Lamesch et al. 2012) using the HISAT2 software (version 2.0.1, (Kim et al. 2015) with the “transcriptome mapping only” option.

Differential gene expression analysis

The quantification of the transcripts was done using ‘Kallisto’ (Bray et al. 2016). The Bioconductor package ‘edgeR’ (Robinson et al. 2009) was used to perform the differential gene expression analysis at the isoform level. Transcripts were considered differentially expressed for an FDR corrected p-value < 0.05.

ANOVA analysis

ANOVA was performed for each transcript measured in the parental lines using the model:

$$Pij = Gi + Ej + GEij + \mathcal{E}ij$$

where Pij is the transcript abundance level, Gi is the effect of the genotype i , Ej is the effect of the maternal environment j and GEij the interaction between genotype and environment and Ɛij, the residuals. The sum square of the variance for each component was calculated as a percentage of the total sum of the variances explained by the different terms of the model. Only transcripts for which the summed explained variance for the G, E and G x E components exceeded 50% and with a significant effect (p-value < 0.05) of at least one component were selected for the ternary plot representation (Figure 1B).\

eQTL mapping

For eQTL mapping, we used the 1059 bin based markers derived from the eQTL data as described in (Serin et al. 2017). The eQTL analysis was performed on the combined environments to identify genetic (G) main effect eQTLs. eQTL mapping was also done for the separate environments corresponding to approximately 40 RILs each. The QTL mapping was conducted in R using a single marker analysis. The permutation LOD score at 0.05 FDR (LOD = 4.1) was set as the significance threshold for the eQTL significance for all datasets. The eQTL data were stored in AraQTL to facilitate the exploration of the eQTL data (Nijveen et al. 2017),http://www.bioinformatics.nl/AraQTL/).

Definition of local and distant eQTL peaks

For each gene with at least one significant eQTL, peak detection was performed. The position and marker associated with the highest LOD scores were considered as the peak location for the eQTL. The physical position of the genes were obtained from the TAIR10 database (Lamesch et al. 2012) and compared to the peak location of the eQTLs. A 1Mb cut-off distance was set between the eQTL peak and the physical position of the gene to distinguish between *local* versus *distant* eQTLs. eQTLs within this cut-off distance were classified as '*local*' (*cis*-eQTLs) while eQTLs outside this range were considered as '*distant*' (*trans*-eQTLs). We noted that the peak detection procedure we used sometimes resulted in several *local* eQTLs per genes. This might be due to the detection of two close peaks, which might also be a single QTL. We concede that this might lead to a slight over-estimation of the reported number of both *local* and *distant* eQTLs.

GO enrichment analysis

Gene ontology enrichment analysis was performed using the BiNGO plugin (Maere et al. 2005) in Cytoscape (Shannon et al. 2003). Using the Arabidopsis Genemodel TAIR10 as a reference (Lamesch et al. 2012), hypergeometric testing was performed with a Benjamini & Hochberg False Discovery Rate (FDR) correction at a significance level of 0.01.

Results and discussion

To investigate the effect of the seed maternal environment on the seed transcriptome, an Arabidopsis Bay-0 x Sha RIL population was grown under different environments. Gene expression data were obtained using RNA-seq for fresh dry seeds of the parental lines and 160 RILs grown under the different environments in a generalized genetical genomics design (Li et al. 2008b; Serin et al. 2017)(Supplemental Table 1). The RIL set up used for the transcriptomics was the same as previously used for the metabolomics analysis (Chapter 4).

The main goal of the analysis was to identify genes that change expression in response to seed maternal environments in the parental lines and to map changes in the eQTL landscape across environments and ultimately connect these to differences in seed performance.

Gene expression variation

Gene expression was quantified in the RILs grown under the different environments to explore changes in gene expression in response to genetic and environmental perturbations. Overall 35.386 transcripts were identified, representing 27.416 unique gene models.

In order to get more insights into stress specific responses, we examined the genes differentially expressed between the seeds from the parental lines grown under each environment and within each accession between the different environments (Supplemental Table 2). A large number of transcripts was found differentially expressed (FDR< 0.05) in Bay-0 in response to stress as compared to control conditions. The largest number of differentially expressed genes (DEGs) was observed in response to high temperature (HT) (4402) and the lowest for low phosphate (LP) (144). Only a few genes were differentially expressed in Sha in response to stress, ranging from 18 to 10 for the different environments. Large differences in terms of DEGs were observed between the seeds of the parental lines grown under the different conditions ranging from 5443 (LP) to 7799 (HT), showing that the genetic background has a huge effect on transcript levels. Similar numbers of genes were found up and down regulated for each condition.

To better understand the effect of the genetic background and transcriptional response to environmental variation an ANOVA was performed for each transcript measured in the parental lines. Transcripts with more than 90% of missing data were removed prior the analysis, resulting in 25971 filtered transcripts. Overall a large number of transcripts (13,861/25,971 ≈ 50%) was significantly (p< 0.05) affected by the environment (Figure 1 A). As expected, a large fraction of the transcripts showing both G and E effects did also show G x E effects (≈ 58%). The strong effect of the environment was supported by the overall large proportion of gene expression variance influenced by the environment (50%) (Figure 1 B).

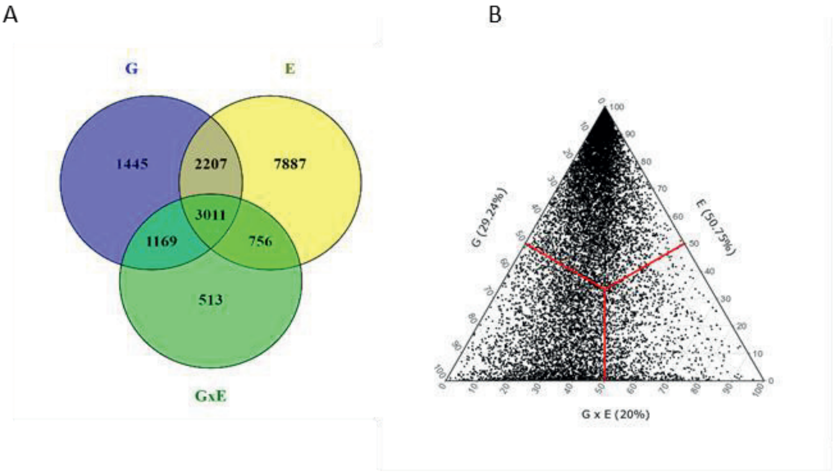


Figure 1. A. Overlap of the transcripts for which the expression levels are significantly ($p < 0.05$) affected by the genotype (G), the maternal environment (E) of the parental lines or their mutual interaction (G x E) B. Composition of the proportion of variance explained by the genetic (G), environment (E) and genotype-by-environment interaction (G x E) components. Transcripts for which summed explained variance for the G, E and G x E components exceeded 50% and with a significant effect ($p< 0.05$) for at least one component were plotted. This corresponded to ≈ 56% (14.574 / 25 971) of all transcripts. The average values of the proportions are indicated in bracket next to their corresponding components.

The recombination of the parental genetic background in interaction with the maternal environment resulted in large differences among the transcriptomes of the RILs (Figure 2). Similar to the metabolome profiling of the RILs shown in Chapter 4, a clear separation was observed for the population grown under high temperature by the first component of the principal coordinate analysis. The clustering in the vicinity of the HT Bay-0 parent might indicate dominant effects of this genotype on the gene expression values of the RILs. A similar pattern was observed for the RILs grown under high light (HL) for which the expression profile resembled the Sha parent. These profiles are in line with the mQTL study (Chapter 4), showing that mQTLs for TCA cycle intermediates are largely driven by the Bay-0 parent in the HT condition while mQTLs associated with sugar metabolites under HL were driven by the Sha parent. Differences in the RIL expression profiling indicate the potential of the dataset to further explore the genetic basis of G x E.

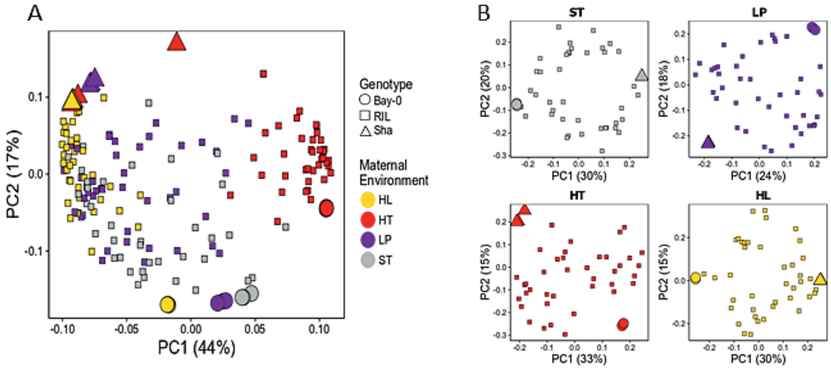


Figure 2. Principal coordinates analysis (PCoA) plot for the whole genome transcriptome in dry seeds of the parental and recombinant inbred lines grown A) under the different environments and B) for each environment separately.

Main effect eQTLs

In a genetical genomics framework, transcript abundance can be treated as a quantitative variable. Linkage analysis of the expression values of the combined set of RILs was performed to identify main effect eQTLs (Supplemental Table 3, 4). A large number of eQTLs was identified (Figure 3). The linkage analysis produces two types of eQTLs classified according to the position of the detected eQTLs compared to the position of the gene investigated. Local eQTLs map to the location of the affected genes and are thought to be caused by polymorphic cis-acting elements, for instance in the promoter of the affected gene (Snoek & Terpstra 2017, Keurentjes et al. 2007). On the other hand, distant eQTLs are regulated by trans-acting elements on other locations in the genome, such as transcription factors that can affect the expression of other genes. The position of the eQTLs with regard to the physical position of the gene affected is shown in figure 3, where local eQTLs are typically found on the diagonal, while off-diagonal points are distant eQTLs. Vertical bands of points indicate a large number of co-locating eQTLs. These 'hotspots' show loci at which a polymorphic regulator is likely affecting the expression of many genes and that can be seen as a master regulator (Breitling et al. 2008). Several regions in particular on chromosome I, IV and V accumulate a high number of eQTLs.

Overall, large numbers of eQTLs were identified along the genome. In AraQTL, for different FDR/LOD thresholds, the number of eQTLs detected was larger than reported in a previous study on the same population based on microarray data (Nijveen et al. 2017). This might indicate the increased power of RNA-seq based eQTL mapping as compared to previous micro-array based eQTL studies.

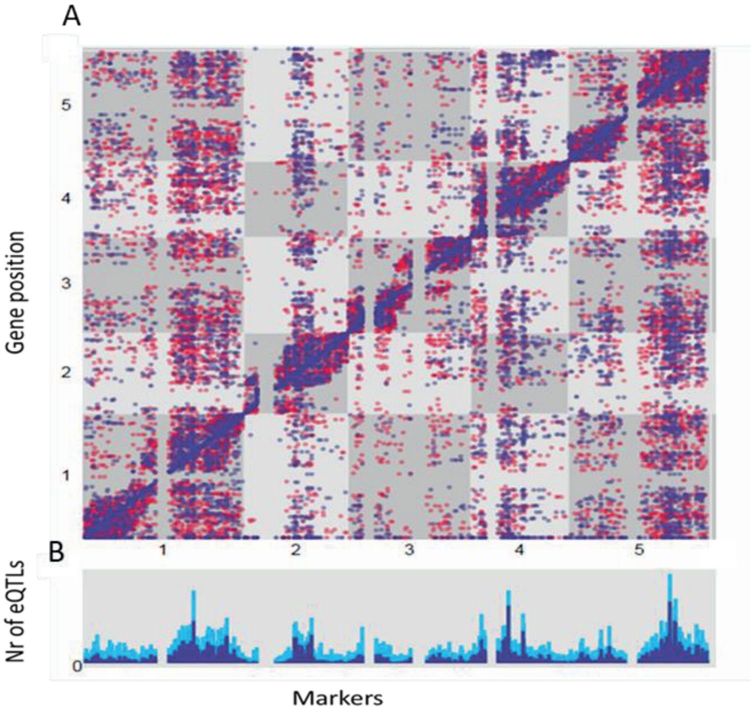


Figure 3. Distribution of eQTLs along the genome. A) Local - distant eQTL plot for the genetic (G) component at LOD > 5 for multiple environments. The x-axis shows the position of the eQTL on each of the five chromosomes; the y-axis indicates the location of the affected gene. Blue dots (eQTLs) indicate that the Bay-0 allele has a positive effect on the expression level of the gene at the position of the eQTL, while red dots indicate eQTLs where the Sha allele has a larger effect. Numbers correspond to the chromosomes. B) The distribution of the local (dark blue) and distant (light blue) eQTLs across the markers.

Environment-specific eQTL hotspots

We also investigated the eQTL distribution for the four environments separately (Supplemental Table 3, 4). For each environment, we represented the significant eQTLs in a histogram showing the physical position of the markers and the number of associated eQTLs (Figure 4). Peaks that did not obviously coincided across conditions are highlighted as condition-dependent eQTL hotspots. These hotspots resulted from the higher accumulation of *distant* eQTLs.

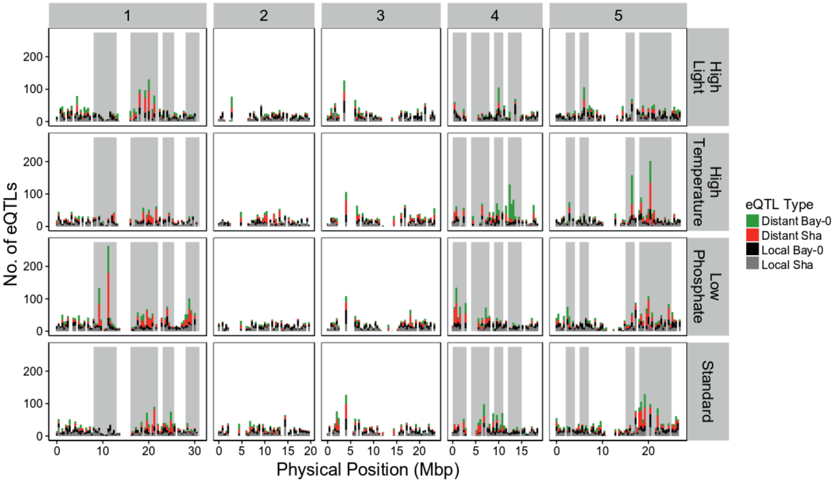


Figure 4. Frequency and distribution of eQTLs associated to the markers along the genome. The distribution of the local and distant eQTLs along the genome is shown for the RILs grown in the four environments. The colour code indicates the direction of the effect (Bay-0 or Sha) and the nature (local versus distant) of the eQTLs. Grey coloured regions indicate regions of the genome with environment dependent eQTL profiles.

Comparison of eQTL landscapes across environments

In total, 8313 to 9977 significant eQTLs were identified in the different datasets. These eQTLs were associated with approximately 6000 genes for each environment. The average number of eQTLs per gene varied between 1.34 and 1.72 showing that often more than one locus controlled the expression level of a single gene. Genes with an eQTL largely overlapped across conditions as indicated by the low number of environment-specific genes (Table 1).

Table 1. Summary of genes with an eQTL.

	total eQTLs	genes with eQTL	eQTLs per gene	Genes with only *local*	Genes with only *distant*	Genes with *local* and *distant*	Environment specific genes
ST	9442	6450	1.46	2774 (43%)	2429 (37.6%)	1247 (19.3%)	15.57%
HL	8318	6206	1.34	2957 (47%)	2179 (35%)	1070 (17%)	15.13%
HT	8708	6120	1.72	2585 (42%)	2432 (39.7%)	1103 (18%)	18.30%
LP	9977	6893	1.45	2913 (42%)	2667 (38.7%)	1313 (19%)	19.24%

ST: standard, HL: high light, HT: high temperature, LP: low phosphate

Distribution of local and distant eQTLs

The eQTL mapping showed overall similar eQTL features for the separate environments (Table 2) (Supplemental Table 5). Across all conditions, a slightly higher number of *distant* eQTLs as compared to *local* eQTLs was identified with a ratio of the number of *distant* to *local* eQTLs varying between 1,0 and 1,27. The direction of the eQTL effects was observed in equal proportion for the Bay-0 and Sha alleles (Table 2).

We found that *local* eQTLs were highly replicable across the environments, since only ~29% of the eQTLs was specific for one environment (Figure 5). In contrast, a large proportion of *distant* eQTLs was specific for a single environment (Figure 5). This was in line with several eQTL studies reporting the plastic nature of *distant* eQTLs in response to environmental fluctuations (Li et al. 2006; Smith and Kruglyak 2008; Vinuela et al. 2010; Cubillos et al. 2014; Cubillos et al. 2012; Snoek et al. 2017b; Drost et al. 2010; Lowry et al. 2013)

Table 2. local and distant eQTLs across the different environments.

	Number of eQTLs	ST	Fraction (%)	HT	Fraction (%)	HL	Fraction (%)	LP	Fraction (%)
local	Bay	2137	51.7	1918	49.6	2183	52.8	2284	52.1
	Sha	2047	47.9	1952	50.4	1954	47.2	2106	48
	total	4184		3870		4137		4390	
distant	Bay	2333	44.4	2286	47.25	2310	51	2555	46
	Sha	2925	55.6	2552	52.7	1871	44.7	3032	54.3
	total	5258		4838		4181		5587	
	Distant/local ratio	1.26		1.25		1.0		1.27	
total nr of eQTLs		9442		8708		8318		9977	

ST:standard, HL: high light, HT: high temperature, LP: low phosphate

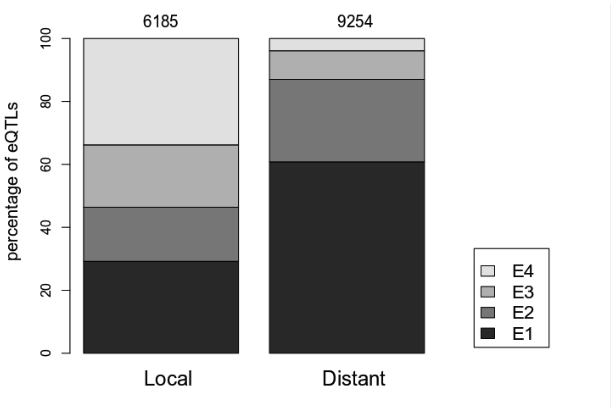


Figure 5. Overlap of genes with local or distant eQTLs across the different environments. Barplot showing the percentage of genes with local or distant eQTLs that are unique (E1) or found for 2 (E2), 3 (E3) or all (E4) environments. The total number of genes with local or distant eQTLs is indicated above their respective columns.

Effect of the environment on regulation of gene expression

We performed a genome wide comparison of the eQTL effect profile of each gene across the different environments. A strong correlation between eQTL profiles would indicate that the gene is regulated by the same loci while differences in the number, type and effect of the eQTLs would result in lower correlation values. Overall intermediate correlation coefficients were observed, suggesting high frequency of changes in response to the environment (Figure 6).

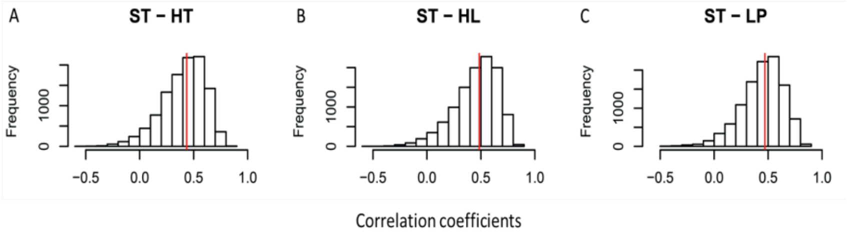


Figure 6. Distribution of correlation coefficients for the genome wide comparison of the eQTL profiles of each gene across environments. Correlation coefficients were calculated for 10,913 genes that had at least one significant eQTL in one condition, by comparing their eQTL profiles between standard (ST) and A) high temperature (HT) B) high light (HL) and C) low phosphate (LP). The red line indicates the median value.

In order to examine the nature of the changes more closely, we investigated changes in the direction (Bay-0 versus Sha) and magnitude of the effect of the eQTLs across conditions. We first examined 2088 genes for which a local eQTL was detected across all environments. For all these eQTLs, we found that the directions (for either Bay-0 or Sha) as well as the magnitude of the effect were highly correlated and thus consistent across all conditions (Figure 7).

The comparison of the distant eQTLs is more delicate, since several distant eQTLs mapping to different regions of the genome can be detected per gene. Therefore, we selected a subset of genes to analyse the dynamics of changes between the type of eQTL (local versus distant) and the direction of the effect (Bay-0 versus Sha) at the individual gene level. To keep the comparison simple, we selected 769 genes showing a single eQTL under each environment. Most of these eQTLs (77%) were consistent in their effect and type. For only three (distant) eQTLs, the direction of the effect changed between environments. Variation in local versus distant eQTL was observed for 162 genes while the direction of their effect was consistent across environments. Fourteen eQTLs showed variation in the type of eQTL and the direction of the effect across the different conditions (Table 3).

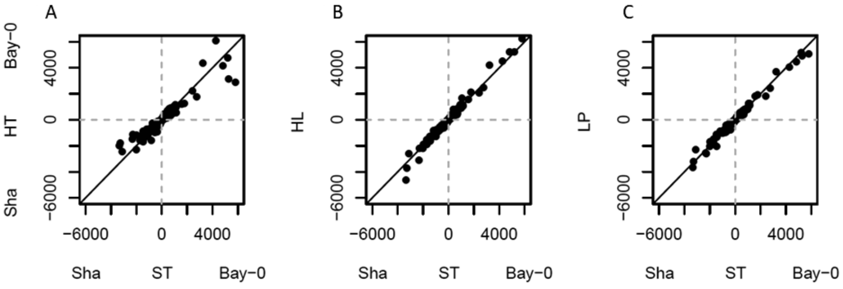


Figure 7. Consistency in the direction and magnitude of the effect of local eQTLs across environments. The effect of 2085 local eQTLs in standard environment (ST) was plotted against their corresponding effect in A) high temperature (HT) B) high light (HL) and C) low phosphate (LP) environments. Positive and negative values correspond to eQTL effects in the Bay-0 and Sha direction, respectively. Extreme values were left out for clearer graphical representation.

For 8 of these 14 genes, the HT maternal environment explained the change in the type and direction of the eQTL, which is in line with the strong effect of the HT environment on the transcriptome profile of the RILs (Figure 2). Among these genes were isocitrate dehydrogenase (At4g35260) and alanine glyoxylate aminotransferase (At4g39660). These two genes encode enzymes that are involved in the tricarboxylic acid cycle and glyoxylate pathway, respectively. These observations were in line with the metabolic changes in response to HT, since HT specifically resulted in coordinated changes of metabolites enriched for the TCA cycle and glyoxylate pathway in the mQTL study (Chapter 4).

One compelling example of a gene showing G x E in its eQTL profiles is shown in Figure 8. Under ST, expression of At2g24570 is regulated by a significant local eQTL with a larger allelic effect for the Bay-0 allele, while under HT, the expression of the gene is regulated by a significant distant eQTL detected on chromosome IV. The Sha allele at the eQTL on chromosome 2 for LP and on chromosome 5 for HL, resulted in higher expression levels of At2g24570 (Figure 8).

Table 3. List of 14 genes with one eQTL peak showing different effects (Bay-0 vs Sha) and type of eQTL (local (L) vs distant (D)) across different environments. ST: standard, HT: high temperature, HL: high light, LP: low phosphate.

Genes	Description	ST	HT	HL	LP
At1g01920	SET domain-containing protein	Sha L	Bay-0 D	Sha L	Sha L
At1g03080	Kinase interacting (KIP1-like) family protein	Sha L	Bay-0 D	Sha L	Sha L
At1g22970	Cyclin-D1-binding protein	Sha L	Sha D	Bay-0 D	Sha L
At1g26400	FAD-binding Berberine family protein	Sha L	Sha L	Bay-0 D	Sha L
At2g24570	AtWRKY17, WRKY DNA-BINDING PROTEIN 17, WRKY17	Bay-0 L	Bay-0 D	Sha D	Sha D
At3g50060	MYB DOMAIN PROTEIN 77, MYB77	Sha L	Bay-0 D	Sha L	Sha L
At3g61450	Syntaxin of plants 73	Sha D	Bay-0 D	Bay-0 L	Bay-0 D
At4g22360	SWIB complex BAF60b domain-containing protein	Sha L	Bay-0 D	Sha L	Sha D
At4g30150	Urb2/Npa2 family protein	Bay-0 L	Sha D	Bay-0 L	Bay-0 L
At4g35260	IDH-I, IDH1, ISOCITRATE DEHYDROGENASE 1I	Sha L	Bay-0 D	Sha L	Sha L
At4g39660	Alanine:glyoxylate aminotransferase 2	Bay-0 L	Sha D	Bay-0 L	Bay-0 L
At4g39690	MIC60	Bay-0 L	Sha D	Bay-0 L	Bay-0 L
At5g16290	AHASS2, AIP3, ALS-INTERACTING PROTEIN3, VALINE-TOLERANT 1, VAT1	Bay-0 L	Bay-0 L	Bay-0 L	Sha D
At5g48570	Encodes one of the 36 carboxylate clamp (CC)-tetratricopeptide repeat (TPR)	Bay-0 L	Sha D	Bay-0 L	Bay-0 L

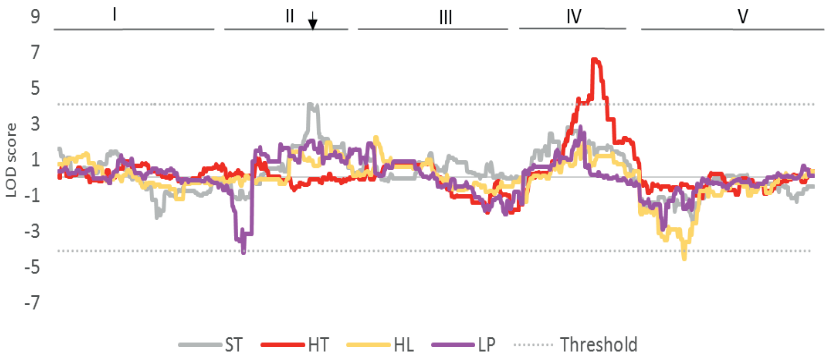


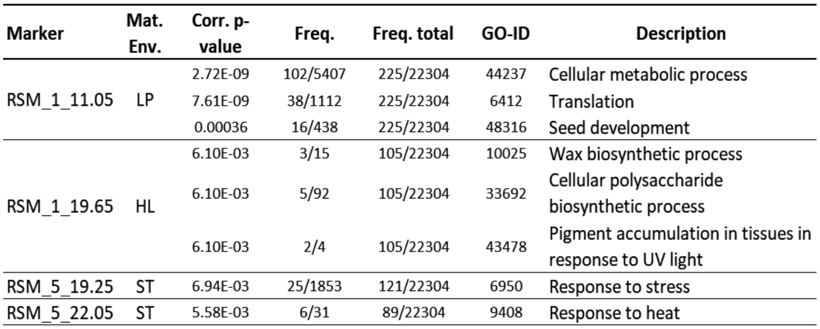
Figure 8. eQTL profile for At2g24570 for the different environments. The y-axis shows the LOD score. Positive and negative LOD score values indicate the direction of the effect for Bay-0 and Sha respectively. The x-axis represents the genomic position in Mbp. Dotted lines indicate the significance threshold for eQTL detection (LOD = 4.1, FDR corrected p-value < 0.05). The colour of the eQTL profile refers to the environment as indicated in the legend where ST is standard, HT is high temperature, HL is high light and LP is low phosphate. The physical position of the gene investigated on chromosome 2 is indicated by an arrow.

GO enrichment analysis

In order to investigate the biological function of the genes affected by the environment-specific eQTL hotspots, a gene ontology enrichment analysis was performed for single marker hotspots identified in Figure 4 containing more than a 100 genes (Table 4) (Supplemental Table 6). The large hotspot on chromosome 1 in LP was enriched for the GO terms ‘seed development’ and ‘translation’. The translation GO term includes essentially ribosomal proteins. During seed development, the precise control of the mRNA translation is also fundamental to cell homeostasis in particular in response to physiological stress. In dry seeds, ribosomes are inactive, but they can form polysomes upon water uptake when they are recruited to translate stored mRNAs (Bai et al. 2017). Translation of stored mRNA plays an important role in the completion of germination (Rajjou et al. 2004; Galland and Rajjou 2015; Kimura and Nambara 2010). Proteomic profiling of dormant and non-dormant imbibed seeds showed that maintenance of dormancy is associated with the repression of germination related processes to maintain dormancy (Arc et al. 2012). The enrichment for the translation machinery might thus reflect the need for selective mRNA translation of genes involved in stress response as well as the maintenance of seed dormancy, for which higher levels were observed for the RILs grown under LP (Chapter 3).

In HL, the major hotspot on chromosome 1 was enriched for ‘polysaccharide biosynthetic process’, which includes genes involved in cell wall synthesis (Table 5). The differential expression of cell wall genes was also observed in a previous study in response to a high light maternal environment (He et al. 2016). We also noted that this eQTL hotspot (with a peak at marker RSM_1_19.65) was identified in the vicinity of the dry seed size phenotypic QTL (peaking at marker RSM_1_24.25). Therefore this eQTL hotspot might have a function in regulating seed size, but further investigations are needed to understand the effect of high light during seed development, the role of the cell wall in interaction with the environment and their possible involvement in regulating seed size.

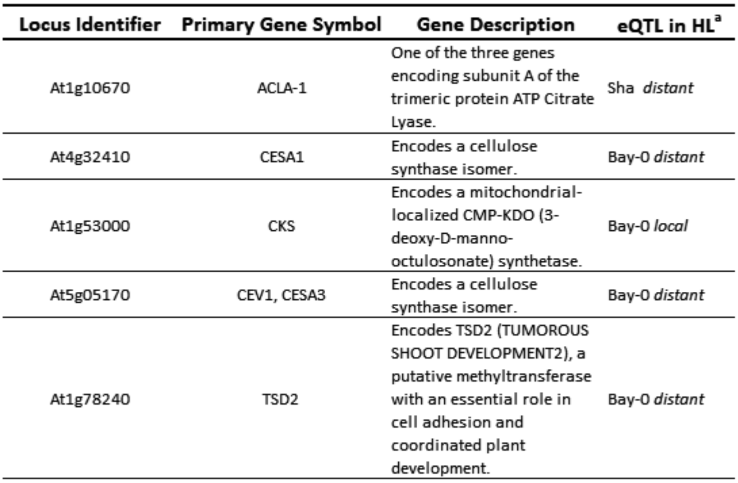
Table 4. GO terms associated with environment specific eQTL hotspots



Marker	Mat. Env.	Corr. p-value	Freq.	Freq. total	GO-ID	Description
RSM_1_11.05	LP	2.72E-09	102/5407	225/22304	44237	Cellular metabolic process
		7.61E-09	38/1112	225/22304	6412	Translation
		0.00036	16/438	225/22304	48316	Seed development
RSM_1_19.65	HL	6.10E-03	3/15	105/22304	10025	Wax biosynthetic process
		6.10E-03	5/92	105/22304	33692	Cellular polysaccharide biosynthetic process
		6.10E-03	2/4	105/22304	43478	Pigment accumulation in tissues in response to UV light
RSM_5_19.25	ST	6.94E-03	25/1853	121/22304	6950	Response to stress
RSM_5_22.05	ST	5.58E-03	6/31	89/22304	9408	Response to heat

Hypergeometric test was performed for the genes of the hotspots against the Arabidopsis TAIR10 genome database using FDR corrected p–values. Only the most significant (corrected p-values < 0.01) and representative GO terms are reported in the table. Full results of the GO enrichment analysis and associated lists of genes are available in Supplemental Table 6.

Table 5. Description of the genes associated with the 'Cellular polysaccharide biosynthetic process' GO term for the HL specific eQTL hotspot on chromosome 1.



Locus Identifier	Primary Gene Symbol	Gene Description	eQTL in HL[a]
At1g10670	ACLA-1	One of the three genes encoding subunit A of the trimeric protein ATP Citrate Lyase.	Sha *distant*
At4g32410	CESA1	Encodes a cellulose synthase isomer.	Bay-0 *distant*
At1g53000	CKS	Encodes a mitochondrial-localized CMP-KDO (3-deoxy-D-manno-octulosonate) synthetase.	Bay-0 *local*
At5g05170	CEV1, CESA3	Encodes a cellulose synthase isomer.	Bay-0 *distant*
At1g78240	TSD2	Encodes TSD2 (TUMOROUS SHOOT DEVELOPMENT2), a putative methyltransferase with an essential role in cell adhesion and coordinated plant development.	Bay-0 *distant*

[a] Bay-0 and Sha indicate the direction of the eQTL effect (higher expression value at the position of the eQTL for the Bay-0 or Sha parental allele). Distant and local refer to the type of eQTL identified under HL for the corresponding gene.

Conclusion and future prospects

In this study, we investigated changes in the genetic control of gene expression measured in dry seeds of an Arabidopsis thaliana Bay-0 x Sha RIL population grown in different maternal environments. A high number of genes were found differentially expressed between the parental lines and in response to the different environments. This led us to investigate changes in the genetic architecture of gene expression.

Large numbers of eQTLs were identified for each environment. Interestingly, genes with eQTLs largely overlapped across conditions, indicating that the environment essentially changes the regulation of a defined set of genes rather than triggering the expression of new genes (Table 1). This was also observed for other organisms such as C.elegans (Snoek et al. 2017a). A slightly higher number of distant eQTLs over local eQTLs were identified in all environments. Furthermore, consistent with other studies, we found that local eQTLs were consistent across environments, while distant eQTLs showed a high degree of environment specificity which is likely due to their versatile nature (Snoek et al. 2017a; Cubillos et al. 2014).

These variable distant eQTLs led to the identification of several environment-specific eQTL hotspots which we found enriched for specific biological processes. For a high light specific hotspot, we found a subset of genes enriched for cell wall synthesis. Although the role of these genes in mediating high light cues in seeds remains to be investigated, this example shows that without prior knowledge on the trait investigated, eQTL studies can be used to enhance gene discovery for complex traits.

In this study, the threshold applied for eQTL significance (FDR < 0.05, LOD = 4.1) resulted in eQTL hotspots of around a hundred co-locating eQTLs. To further investigate these hotspots, one might consider relaxing this threshold to increase the number of genes (Keurentjes et al. 2007b) or to expand this number of genes by including genes with eQTLs peaking at neighbouring marker bins. Such approach could lead to the identification of master regulators within eQTL hotspots (Wu et al. 2008). For this purpose, gene co-expression networks can be used to prioritize candidate genes as it was shown in several studies (van Muijen et al. 2016; Basnet et al. 2016; Drost et al. 2010) and partly reviewed in Chapter 6.

These data complement previous phenotypic (Chapter 3) and metabolic (Chapter 4) datasets derived from the same plant material using the same GGG set-up. Similar effects of the maternal environment, in particular for HT, on the metabolome and transcriptome (Chapter 4 and Figure 2, Table 3) indicated that these eQTL data are a valuable source for further investigations in a systems genetics approach, to the extent to which transcriptomic changes can explain metabolic and ultimately phenotypic differences.

List of supplemental data

Supporting information can be downloaded from:
http://www.wageningenseedlab.nl/thesis/earserin/SI/chapter5

Supplemental Table 1: Gene expression data for the parental and recombinant inbred lines in a generalized genetical genomics set-up
Supplemental Table 2: Lists of differentially expressed genes in the parental lines
Supplemental Table 3: eQTL LOD scores for the separate and combined environments
Supplemental Table 4: eQTL effects for the separate and combined environments
Supplemental Table 5: Summary of all *local* and *distant* eQTLs
Supplemental Table 6: GO enrichment for the environment specific eQTL hotspots (> 100 genes)

Chapter 6

Learning from co-expression networks: possibilities and challenges

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Abstract

Plants are fascinating and complex organisms. A comprehensive understanding of the organization, function and evolution of plant genes is essential to disentangle important biological processes and to advance crop engineering and breeding strategies. The ultimate aim in deciphering complex biological processes is the discovery of causal genes and regulatory mechanisms controlling these processes. The recent surge of omics data has opened the door to a system-wide understanding of the flow of biological information underlying complex traits. However, dealing with the corresponding large data sets represents a challenging endeavor that calls for the development of powerful bioinformatics methods. A popular approach is the construction and analysis of gene networks. Such networks are often used for genome-wide representation of the complex functional organization of biological systems. Network based on similarity in gene expression are called (gene) co-expression networks. One of the major applications of gene co-expression networks is the functional annotation of unknown genes. Constructing co-expression networks is generally straightforward. In contrast, the resulting network of connected genes can become very complex, which limits its biological interpretation. Several strategies can be employed to enhance the interpretation of the networks. A strategy in coherence with the biological question addressed needs to be established to infer reliable networks. Additional benefits can be gained from network-based strategies using prior knowledge and data integration to further enhance the elucidation of gene regulatory relationships. As a result, biological networks provide many more applications beyond the simple visualization of co-expressed genes. In this study we review the different approaches for co-expression network inference in plants. We analyse integrative genomics strategies used in recent studies that successfully identified candidate genes taking advantage of gene co-expression networks. Additionally, we discuss promising bioinformatics approaches that predict networks for specific purposes.

Keywords: co-expression, gene expression, gene networks, gene prioritization, transcriptomics

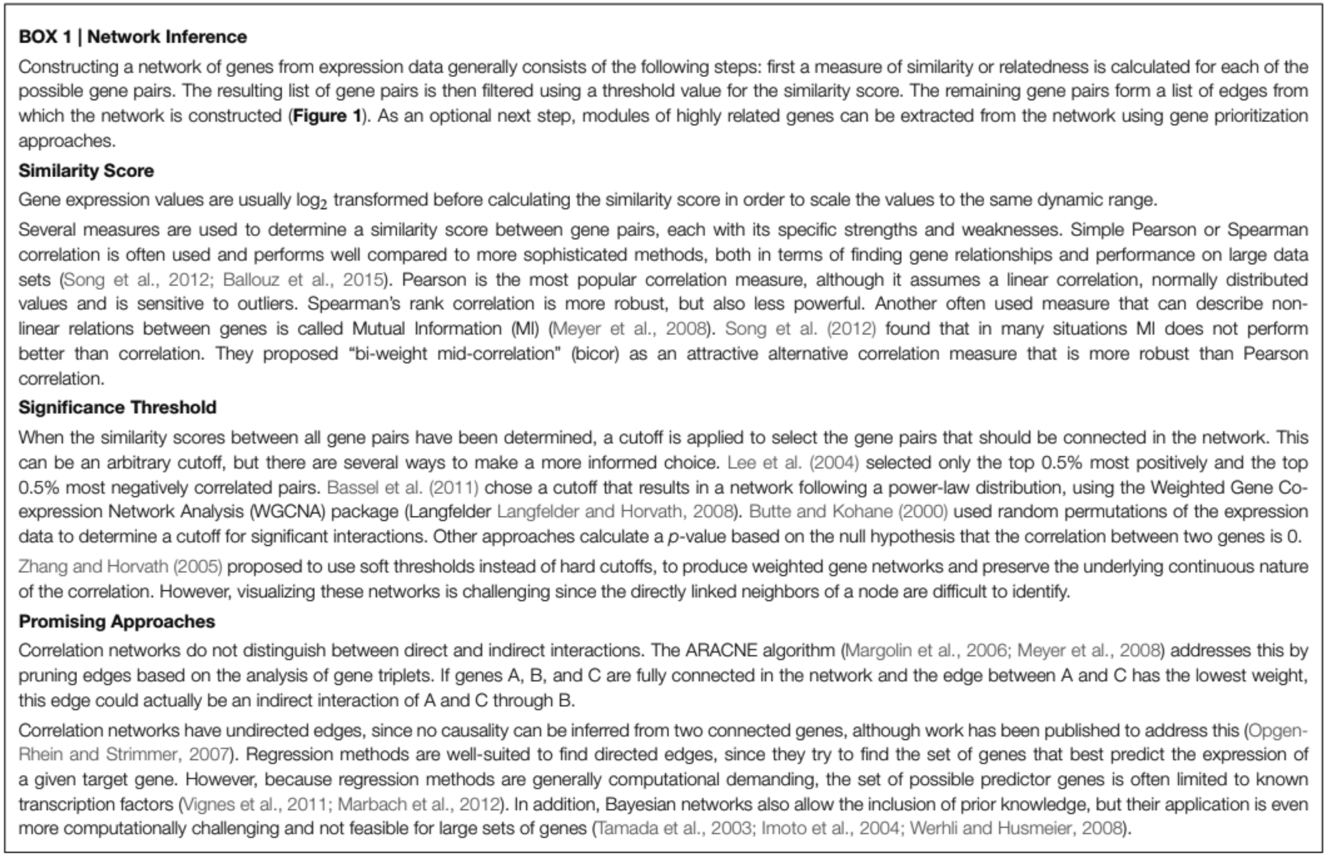
Introduction

In plants, the age of systems biology has accelerated the investigation of complex molecular mechanisms underlying intricate developmental and physiological processes. Since plants are anchored to their environment, they cannot escape from stresses by simply moving away. Instead, plants have developed a wide range of mechanisms to cope with environmental fluctuations. This plasticity generally involves changes at the level of DNA, RNA, protein and metabolites, resulting in complex phenotypes governed by multiple genes. Advanced genetic and molecular tools have led to tremendous progress in revealing the genetic architecture but also the regulatory mechanisms of complex traits (Mochida and Shinozaki 2011). The development of molecular profiling techniques nowadays enables the high-throughput and affordable acquisition of large omics data sets, such as for transcriptomics, proteomics and metabolomics.

While substantial efforts are being made to generate large omics data sets, there is a growing need to develop platforms to integrate these data and derive models describing biological interactions in plants. In this context, networks have rapidly become an attractive approach to manage, display and contextualize these large data sets in order to obtain a system level and molecular understanding of biological key processes (Costa et al. 2015)(Silva et al. 2016; Barabasi and Oltvai 2004; Usadel et al. 2009).

Biological networks are generally classified by the nature of the compounds and interactions involved. These networks can be derived from various molecular data resulting in, *e.g.*, gene expression networks (correlation or co-expression networks), protein-protein interaction (PPI) networks, metabolic networks and signaling networks. Graphically, networks are represented as an ensemble of components (nodes or vertices) and interactions depicted by links (edges) connecting pairs of nodes. Such interaction maps provide an attractive framework to study the organizational structure of complex systems and have found many applications in plants (Jiménez-Gómez 2014).

The fast development of transcriptomic technologies, as compared to other analytical platforms, has supported a range of studies on genetic and environmental perturbations at the transcriptome level in many organisms. Co-expression networks have grown in popularity in the last years as they enable the integration of large transcriptional data sets (Li et al. 2015)(Liseron-Monfils and Ware 2015). Co-expression network analysis allows the simultaneous identification, clustering and exploration of thousands of genes with similar expression patterns across multiple conditions (co-expressed genes). The main procedure for co-expression network inference is explained in Box 1 and illustrated in Figure 1. Briefly, a similarity score (*i.e.*, correlation coefficient) is calculated from the pairwise comparison of the gene expression patterns for each possible pair of genes. Above a certain threshold, genes and gene pairs form a list of nodes and corresponding edges from which the network is constructed. As a rule, the guilt-by-association principle is applied stating that genes sharing the same function or that are involved in the same regulatory pathway will tend to present similar expression profiles and hence form clusters or modules in the network (Wolfe et al. 2005). Thus, within the same module, genes of known function can be used to predict the function of co-expressed unknown genes (Rhee and Mutwil 2014).



BOX 1 | Network Inference

Constructing a network of genes from expression data generally consists of the following steps: first a measure of similarity or relatedness is calculated for each of the possible gene pairs. The resulting list of gene pairs is then filtered using a threshold value for the similarity score. The remaining gene pairs form a list of edges from which the network is constructed (**Figure 1**). As an optional next step, modules of highly related genes can be extracted from the network using gene prioritization approaches.

Similarity Score

Gene expression values are usually $\log_2$ transformed before calculating the similarity score in order to scale the values to the same dynamic range.

Several measures are used to determine a similarity score between gene pairs, each with its specific strengths and weaknesses. Simple Pearson or Spearman correlation is often used and performs well compared to more sophisticated methods, both in terms of finding gene relationships and performance on large data sets (Song et al., 2012; Ballouz et al., 2015). Pearson is the most popular correlation measure, although it assumes a linear correlation, normally distributed values and is sensitive to outliers. Spearman's rank correlation is more robust, but also less powerful. Another often used measure that can describe non-linear relations between genes is called Mutual Information (MI) (Meyer et al., 2008). Song et al. (2012) found that in many situations MI does not perform better than correlation. They proposed "bi-weight mid-correlation" (bicor) as an attractive alternative correlation measure that is more robust than Pearson correlation.

Significance Threshold

When the similarity scores between all gene pairs have been determined, a cutoff is applied to select the gene pairs that should be connected in the network. This can be an arbitrary cutoff, but there are several ways to make a more informed choice. Lee et al. (2004) selected only the top 0.5% most positively and the top 0.5% most negatively correlated pairs. Bassel et al. (2011) chose a cutoff that results in a network following a power-law distribution, using the Weighted Gene Co-expression Network Analysis (WGCNA) package (Langfelder Langfelder and Horvath, 2008). Butte and Kohane (2000) used random permutations of the expression data to determine a cutoff for significant interactions. Other approaches calculate a *p*-value based on the null hypothesis that the correlation between two genes is 0.

Zhang and Horvath (2005) proposed to use soft thresholds instead of hard cutoffs, to produce weighted gene networks and preserve the underlying continuous nature of the correlation. However, visualizing these networks is challenging since the directly linked neighbors of a node are difficult to identify.

Promising Approaches

Correlation networks do not distinguish between direct and indirect interactions. The ARACNE algorithm (Margolin et al., 2006; Meyer et al., 2008) addresses this by pruning edges based on the analysis of gene triplets. If genes A, B, and C are fully connected in the network and the edge between A and C has the lowest weight, this edge could actually be an indirect interaction of A and C through B.

Correlation networks have undirected edges, since no causality can be inferred from two connected genes, although work has been published to address this (Opgen-Rhein and Strimmer, 2007). Regression methods are well-suited to find directed edges, since they try to find the set of genes that best predict the expression of a given target gene. However, because regression methods are generally computational demanding, the set of possible predictor genes is often limited to known transcription factors (Vignes et al., 2011; Marbach et al., 2012). In addition, Bayesian networks also allow the inclusion of prior knowledge, but their application is even more computationally challenging and not feasible for large sets of genes (Tamada et al., 2003; Imoto et al., 2004; Werhli and Husmeier, 2008).

The two main applications for co-expression network analysis are to find novel genes involved in the biological process under investigation and to suggest the biological process a gene is involved in. Intuitively, reliable networks are needed to infer meaningful gene function predictions. Such networks heavily depend on a combination of decisions taken throughout the network inference process. From the quality, type and availability of the input data, the correlation coefficient and inference algorithm used, to the prior knowledge, the experimental and computational resources, any negligence can result in unreliable networks and subsequent misleading biological interpretations.

Caveats and opportunities of co-expression network analyses have been discussed previously (Usadel et al. 2009). When handling large data sets, co-expression networks can become very complex which limits their biological interpretation (Usadel et al. 2009). In addition, in contrast to regulatory networks, and because of their static representation, co-expression networks do not provide per se information on the nature of the regulatory relationship of connected genes (Stuart et al. 2003). Careful application of network analysis tools and strategies is thus important to maximize the information extraction, to disentangle reliable network connections and to infer true biological meaning.

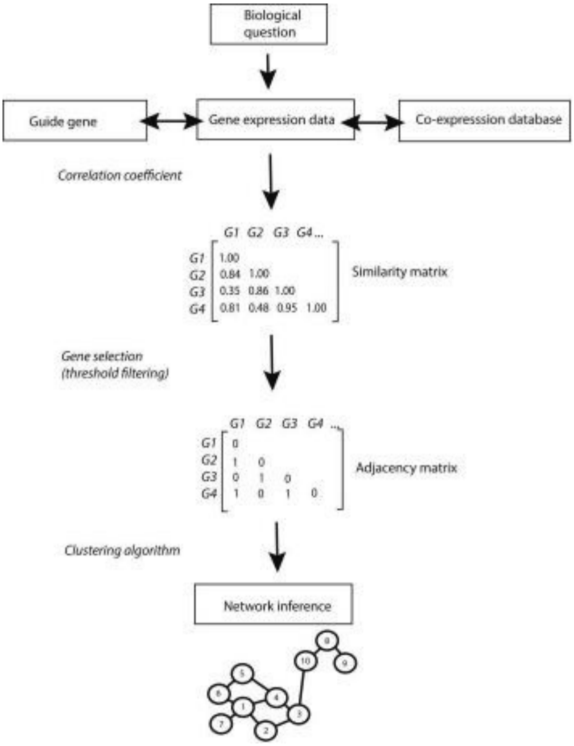
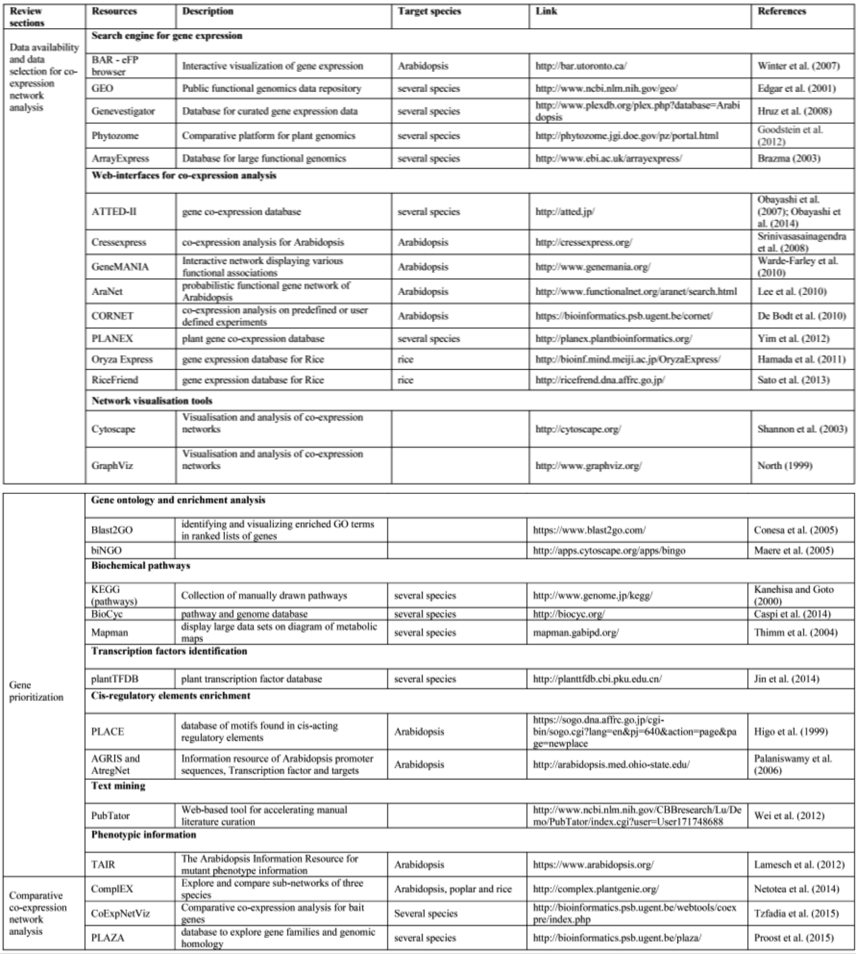


Figure 1. Co-expression network inference pipeline. The biological question addressed drives the strategy for the co-expression network analysis: prior knowledge can be used to identify guide-genes and co-expression databases can be queried to investigate gene co-expression patterns across multiple conditions. Similarity in gene expression patterns is calculated using correlation coefficients (Pearson, Spearman…). A user defined threshold (in this example set at 0.8) enabled the selection of genes with high co-expression scores. Significantly co-expressed genes are reported in the binary adjacency matrix as 1. A clustering algorithm is applied on the adjacency matrix to infer networks of significantly co-expressed genes. In the resulting network, significantly co-expressed genes are depicted as numbered nodes (vertices) linked by edges (links). The length of the edges is relative to the expression similarity of the connected gene, with a short edge corresponding to high co-expression value. A "path" corresponds to the number of edges connecting two nodes (the shortest path from node 9 to 4 is 4 edges). Hubs are identified as highly connected nodes (node 1) and group of connected genes from modules (nodes 1-7). Network properties can be described by different parameters such as:

- The **connectivity** of a network corresponds to the total number of links in the network
- The **node degree** corresponds to the number of connections of a node with other nodes in the network (node 4 has a node degree of 3).
- The **betweenness** of a node corresponds to the sum of the shortest paths connecting all pair of nodes in the network, passing through that specific node. The betweenness of node 8 corresponds to the sum of the shortest path connecting the nodes 10-9,3-9,4-9, etc…).

In this review, we aim to provide an overview of the different strategies to employ during or after the co-expression network construction with the common aim of exploiting the full predictive potential of co-expression networks. The application of these strategies is illustrated by examples of recent studies. Particular attention is given to available and promising bioinformatics tools. Finally, we will speculate on network aspects worth developing in the near future to strengthen their inference power for a comprehensive understanding of the regulation of important biological processes.

Table 1: Overview of available resources for co-expression network analysis



Review sections	Resources	Description	Target species	Link	References
Data availability and data selection for co-expression network analysis	**Search engine for gene expression**				
	BAR - eFP browser	Interactive visualization of gene expression	Arabidopsis	http://bar.utoronto.ca/	Winter et al. (2007)
	GEO	Public functional genomics data repository	several species	http://www.ncbi.nlm.nih.gov/geo/	Edgar et al. (2001)
	Genevestigator	Database for curated gene expression data	several species	http://www.plexdb.org/plex.php?database=Arabidopsis	Hruz et al. (2008)
	Phytozome	Comparative platform for plant genomics	several species	http://phytozome.jgi.doe.gov/pz/portal.html	Goodstein et al. (2012)
	ArrayExpress	Database for large functional genomics	several species	http://www.ebi.ac.uk/arrayexpress/	Brazma (2003)
	Web-interfaces for co-expression analysis				
	ATTED-II	gene co-expression database	several species	http://atted.jp/	Obayashi et al. (2007); Obayashi et al. (2014)
	Cressexpress	co-expression analysis for Arabidopsis	Arabidopsis	http://cressexpress.org/	Srinivasasainagendra et al. (2008)
	GeneMANIA	Interactive network displaying various functional associations	Arabidopsis	http://www.genemania.org/	Warde-Farley et al. (2010)
	AraNet	probabilistic functional gene network of Arabidopsis	Arabidopsis	http://www.functionalnet.org/aranet/search.html	Lee et al. (2010)
	CORNET	co-expression analysis on predefined or user defined experiments	Arabidopsis	https://bioinformatics.psb.ugent.be/cornet/	De Bodt et al. (2010)
	PLANEX	plant gene co-expression database	several species	http://planex.plantbioinformatics.org/	Yim et al. (2012)
	Oryza Express	gene expression database for Rice	rice	http://bioinf.mind.meiji.ac.jp/OryzaExpress/	Hamada et al. (2011)
	RiceFriend	gene expression database for Rice	rice	http://ricefrend.dna.affrc.go.jp/	Sato et al. (2013)
	Network visualisation tools				
	Cytoscape	Visualisation and analysis of co-expression networks		http://cytoscape.org/	Shannon et al. (2003)
	GraphViz	Visualisation and analysis of co-expression networks		http://www.graphviz.org/	North (1999)
Gene prioritization	**Gene ontology and enrichment analysis**				
	Blast2GO	identifying and visualizing enriched GO terms in ranked lists of genes		https://www.blast2go.com/	Conesa et al. (2005)
	biNGO			http://apps.cytoscape.org/apps/bingo	Maere et al. (2005)
	Biochemical pathways				
	KEGG (pathways)	Collection of manually drawn pathways	several species	http://www.genome.jp/kegg/	Kanehisa and Goto (2000)
	BioCyc	pathway and genome database	several species	http://biocyc.org/	Caspi et al. (2014)
	Mapman	display large data sets on diagram of metabolic maps	several species	mapman.gabipd.org/	Thimm et al. (2004)
	Transcription factors identification				
	plantTFDB	plant transcription factor database	several species	http://planttfdb.cbi.pku.edu.cn/	Jin et al. (2014)
	Cis-regulatory elements enrichment				
	PLACE	database of motifs found in cis-acting regulatory elements	Arabidopsis	https://sogo.dna.affrc.go.jp/cgi-bin/sogo.cgi?lang=en&pj=640&action=page&page=newplace	Higo et al. (1999)
	AGRIS and AtregNet	Information resource of Arabidopsis promoter sequences, Transcription factor and targets	Arabidopsis	http://arabidopsis.med.ohio-state.edu/	Palaniswamy et al. (2006)
	Text mining				
	PubTator	Web-based tool for accelerating manual literature curation		http://www.ncbi.nlm.nih.gov/CBBresearch/Lu/Demo/PubTator/index.cgi?user=User171748688	Wei et al. (2012)
	Phenotypic information				
	TAIR	The Arabidopsis Information Resource for mutant phenotype information	Arabidopsis	https://www.arabidopsis.org/	Lamesch et al. (2012)
Comparative co-expression network analysis	ComplEX	Explore and compare sub-networks of three species	Arabidopsis, poplar and rice	http://complex.plantgenie.org/	Netotea et al. (2014)
	CoExpNetViz	Comparative co-expression analysis for bait genes	Several species	http://bioinformatics.psb.ugent.be/webtools/coexpre/index.php	Tzfadia et al. (2015)
	PLAZA	database to explore gene families and genomic homology	several species	http://bioinformatics.psb.ugent.be/plaza/	Proost et al. (2015)

Data availability for co-expression network analyses

In the post-genomic era, the reduction of costs for large scale and high-throughput measurement technologies, such as for transcriptomics, has to the extensive collection of gene expression profiles capturing changes in gene expression during development, between different treatments or tissues, etc. DNA-microarrays are widely used to measure simultaneously the expression level of a large number of genes in species with a genome sequence available. In addition, the sequenced genomes of model plants (*e.g.,* Arabidopsis, medicago, and poplar) and economically important crops (*e.g.,* tomato, potato, tobacco, rice, and soybean) strongly improve our understanding of transcriptional dynamics.

The compendia of generated data led to the development of publicly available gene expression databases (Table 1). These databases still largely contain microarray data and many of them are related to the model plant Arabidopsis. In recent years, RNA-sequencing, using next- generation high-throughput sequencing technologies (RNA-seq) has proven to be a powerful tool for whole transcriptome profiling with enhanced sensitivity for the discovery of new transcripts and enhanced specificity such as for the examination of allele-specific expression. The power of these sequencing technologies has enabled co-expression network analysis in species without a sequenced genome and, as a result, has opened the way for new applications (see Section Comparative Co-expression Network Analysis). RNA-seq based co-expression network construction is still in its infancy (Iancu et al. 2012; Ballouz et al. 2015) but the foreseen predominance of next generation sequencing tools in the coming years will certainly enrich existing databases for the benefit of network studies. Microarrays are still commonly used for transcriptome analysis because they are relatively cheap and their analysis is highly standardized. Comprehensive microarray gene expression sets are available in public repositories such as the Gene Expression Omnibus (GEO, (Edgar et al. 2001), Genevestigator (Hruz et al. 2008) or Array Express (Parkinson 2004). Other tools, such as the online bio-analytical resource for plant biology (BAR, (Winter et al. 2007), provide interactive interfaces for the exploratory visualization of gene expression variation.

Co-expression networks allow the simultaneous investigation of multiple gene co-expression patterns across a wide range of conditions. As a result, publicly available transcriptome data sets represent valuable resources for such analysis. It has been reported that nearly one in four studies uses public data to address a biological problem without generating new raw data. The reuse of such data strengthens the need for reliable expression studies. A correct experimental design, the proper execution of the wet lab experiments and thorough annotation of the data are essential prerequisites for successful subsequent reuse (Brazma 2003).

Several gene co-expression databases are available to help researchers in their investigations (reviewed in (Brady and Provart 2009) and (Usadel et al. 2009) (Table I)). These databases provide user-friendly interfaces to facilitate access to the data and most of them also offer integrated data processing tools. ATTED-II (Obayashi et al., 2007, 2014) allows condition specific searches for co-expressed genes in several plant species. For Arabidopsis, CressExpress (Srinivasasainagendra et al. 2008) in addition allows selection of data sets based on a quality score to filter out "bad" microarrays. GeneMANIA (Warde-Farley et al. 2010) uses a large set of functional data of various types (predicted interactions, correlations, physical interactions and shared protein domains) to display all predicted interactions for a query gene list in an interactive network. The probabilistic functional gene network AraNet (Lee et al. 2015b) provides a measure to assess the connectivity of the query genes used in regard to the generated network. Additionally, AraNet integrates enrichment analysis tools for network components for gene ontology terms and biochemical pathways (Mapman, BioCyc and KEGG) (see section "Gene prioritization"). A popular platform for network inference is Cytoscape (Shannon et al. 2003). This open source program with its many plugins and apps allows the integration, visualization and analyses of network data (Saito et al. 2012).

Data selection for co-expression network analyses

Publicly available gene expression databases can be queried using two main approaches. These approaches are reported in the literature as "non-targeted" (or "global") and "targeted" (or "guided-gene") approaches (Aoki et al. 2007). The use of one or the other approach is largely determined by the biological question addressed and the available knowledge.

The non-targeted approach provides a global overview of co-expression patterns of multiple genes across many conditions. This approach is also termed knowledge-independent or condition-independent, as no a priori information is used to construct the network. As an example, Mao et al. (2009)

built an Arabidopsis gene co-expression network using gene expression data from 1094 non-redundant Affymetrix ATH1 arrays from the AtGenExpress consortium. This data set represented nine categories of experimental conditions, such as environmental stresses, hormonal treatments and developmental stages. The resulting network consisted of 6206 nodes and 512,936 edges. These “global” networks are generally used to describe the overall set of connections predicted to occur between gene pairs. Separated modules of functionally related genes can be identified and enable further gene prioritization (see Section Gene Prioritization).

In these global networks, also designated as condition- independent, weak interactions or interactions only occurring under specific conditions are easily missed. This can be circumvented by specifically selecting data from experiments that are relevant to the biological question addressed (Saito et al. 2008; Usadel et al. 2009). The resulting condition-dependent networks provide insights on specific biological processes (Atias et al. 2009). Illustratively, by selecting 138 samples from publicly available gene expression data sets exclusively from mature imbibed Arabidopsis seeds, Bassel et al. (2011) established a seed specific network. This SeedNet enabled the identification of modules associated with seed traits such as germination and dormancy. Childs et al. (2011) reported the improved predictive power for gene functional annotation of such condition-dependent networks. One of the limits of this approach is that the elucidation of system wide properties, such as intersecting biological pathways and genes exhibiting pleiotropic effects, might be overlooked.

An alternative approach allows to mimic condition-dependent data set selection, while using the full potential of gene expression data sets. This approach consists of pre-clustering the samples prior to network construction. In this case, a clustering algorithm is directly applied to the normalized expression matrix (genes × conditions) to partition the input samples into a defined number of groups based on their overall expression similarity. Co-expression networks are then built from each of the clusters obtained. Using this technique, Feltus et al. (2013) have shown that such an unsupervised pre-clustering approach improved capturing of co-expressed genes and the representation of unique biological terms in the derived network modules.

When experimental data have elucidated key components of specific pathways, a guide-gene approach can help to identify novel members of the same pathway in a more targeted manner (Itkin et al. 2013). These known genes, also called bait or seed genes, are used as input genes to build a seeded co-expression network. For example, Yang et al. (2011) used this approach to identify new candidate genes involved in cell-wall biosynthesis. They first established a list of 121 genes known to be involved in cell-wall biosynthesis and by querying available data sets with these seed genes, the initial list was extended to 694 potential candidate genes.

Strategies combining guide-gene queries and condition-dependent approaches may empower the predictive power of co-expression networks. For instance, Li et al. (2009a) implemented a pipeline based on QUBIC, a QUalitative BIClustering algorithm, to select the conditions under which seed genes of the plant cell-wall biosynthesis pathway in Arabidopsis were found to be co-expressed among a total set of 351 conditions. These conditions were then used to generate networks of co-expressed gene modules.

Gene prioritization

Once a co-expression network is obtained, biological relevant information can be mined by gene prioritization. This process consists of integrating diverse data sources to allow the ranking of the nodes in the network and to identify groups of functionally related genes, down to important putative regulatory genes. A panel of databases and tools are available to facilitate the integration of gene information in the network (Table 1).

In nature, a variety of biological networks have displayed evidence of scale-free behaviour (Barabasi and Oltvai 2004; Albert 2005; Atias et al. 2009). Such networks are characterized by a distribution of nodes following a power law distribution. Graphically, this type of network displays a relatively large number of low-connected nodes and a few nodes with a high connectivity, the so called “hubs.” Even though, the assumption of a power law distribution is stated in numerous studies, statistical analyses have also refuted this approach (Khanin and Wit 2006; Lima-Mendez and van Helden 2009).

The network topology encodes preliminary evidences for the understanding of the underpinning biological organization and reveals biological relevant information on the functional importance of individual nodes (Atias et al. 2009). Parameters derived from network local properties such as clustering coefficient, node degree (number of connected nodes), betweenness and centrality are commonly used for node ranking (Pavlopoulos et al. 2011). Nodes with a higher rank, *i.e.* with a high degree of connection and a high clustering coefficient, are identified as major hubs and are also likely associated to essential genes in the network (Provero 2002; Carlson et al. 2006). The phenomenon, describing the link between connectivity and essentiality, is termed the 'lethality-centrality rule' (Jeong et al. 2001). Several studies have associated the non-trivial topological features of scale free networks to an essential buffering system for biological networks robustness and environmental responses (Levy and Siegal 2008; Fu et al. 2009; Lachowiec et al. 2015).

Groups of highly connected genes in a network tend to form modules. Extracting modules from the network is thus a commonly used approach to generate manageable graph subunits for further study (Aoki et al. 2007; Mao et al. 2009). For this purpose, several clustering algorithms are available. These algorithms can be categorized into hierarchical and non-hierarchical algorithms. Hierarchical clustering algorithms identify clusters by iteratively assigning nodes to clusters. In a first step, weights are assigned to the network vertices, using for instance the calculated correlation coefficient. Clusters are then built from high weight vertices and progressively expanded by including neighboring vertices. The number of final clusters varies, for instance depending on a chosen threshold. A variety of hierarchical clustering methods are available including Weighted Gene Correlation Network Analysis (WGCNA) (Langfelder and Horvath 2008), Markov Cluster Algorithm (MCL) (Enright et al. 2002; Mao et al. 2009), Normalization Engine for Matching Organizations (NeMo) (Rivera et al. 2010) and Improved Principal Component Analysis (IPCA) (Li et al. 2008a; Fukushima et al. 2012). Mutwil et al. (2010) suggested a novel Heuristic Cluster Chiseling Algorithm (HCCA). For each node in the network, this algorithm generates node vicinity networks by collecting all nodes within n steps away from the seed node. Non-hierarchical approaches, such as K-mean clustering (Stuart et al. 2003), identify a certain number of modules given the input cluster criteria instead.

The performance of the different clustering algorithms can be assessed by evaluating the functional coherence of the predicted modules and inform, in return, the user on the best clustering algorithm to use (Lysenko et al. 2011). MORPH, an algorithm developed by (Tzfadia et al. 2012), combines a guide-gene approach with data set selection and clustering to enable finding the best combination of gene expression data and network clustering to optimally associate candidate genes with a given target pathway.

Modules are often used as the starting point for more detailed studies as they considerably reduce the global network complexity. A panel of tools can be employed to further mine these modules (Table 1). These tools enable the functional annotation of nodes and modules and to unravel the nature of the gene-gene relationships.

Enrichment analysis for the genes within a module is the most widely used technique to associate modules with particular functions. Under the 'guilt-by-association' rule, these functional modules provide a powerful framework for the identification of new genes relevant to biological processes and their functional annotation in the absence of strong a priori knowledge. These enrichment analyses mostly rely on annotation databases (Table I). The most popular ones are the gene ontology (GO) database (Ashburner et al. 2000) and manually curated databases for metabolite pathways such as the Kyoto Encyclopedia for Genes and Genomes (KEGG) (Kanehisa and Goto 2000), Mapman (Thimm et al. 2004), or BioCyc (Caspi et al. 2014).

Phenotypic data can also be used with the a priori expectation that clustered genes collaborate to control the same phenotypic trait. For example, Mutwil et al. (2010) successfully associated an individual cluster with a specific biological function using phenotypic data and tissue-dependent expression profiles for each gene in the cluster. Similarly, Ficklin et al. (2010) used phenotypic information of rice mutant lines to identify clusters of genes enriched for mutant phenotypic terms such as "sterile" or "dwarf". In another study, Lee et al. (2010) showed that genes whose disruption is associated with embryonic lethality and pigmentation were significantly more interlinked in the AraNet network than expected by chance, corroborating the aforementioned centrality-essentiality theory.

Other available data can help to unravel the nature of the links connecting genes in the network. Co-expression networks are undirected networks as the edges between two genes do not indicate the direction of the interaction. Additionally, the co-expression link between two connected genes might also indicate an indirect interaction. To further unravel the gene regulatory dynamics in such modules, known gene-gene interactions can be displayed on the network and help to identify gene regulatory relationships (Ulitsky and Shamir 2009).

One of the common approaches to identify regulatory relationships is to focus on known transcription factors and their known targets in the network. As transcription factors regulate the expression of many genes in the genome, one might also expect to find them as highly connected nodes in the network or connected to hub genes. The range of interactions of a transcription factor is defined by its binding capacity to specific cis-regulatory elements (motifs) identified in the promoter region of its target genes. Consequently, the search for such motifs in the nodes located in the vicinity of identified transcription factors can be a complementary source to functionally annotate genes and infer potential gene regulatory relationships (Vandepoele et al. 2009).

In their approach, Ma et al. (2013) used a bottom-up approach by first creating sub networks of genes based on motif enrichment for specific cis-regulatory elements and then identifying co-expression modules in those sub-networks.

Gene interaction information can also be retrieved from other data sources. The development and application of genome-wide methods for detecting protein-protein interactions, such as yeast two-hybrid (Bruckner et al. 2009) or affinity purification methods coupled to mass spectrometry (Morris et al. 2014) have increased available interactome data. The InterProScan (Quevillon et al. 2005) or STRING (Szklarczyk et al. 2014) databases can be investigated to retrieve known physical interactions, both structurally resolved and experimentally validated. Knowledge on genetic interactions enables further inferring of functional relationships between genes and pathways. Besides data storage in databases, information on gene function and interactions can also be found embedded in textual data (Hakala et al. 2015). Text mining methods applied to literature resources, such as PubMed articles, help to extract additional information using manual curation efforts (Szakonyi et al. 2015) or semi-automated tools such as PubTator (Wei et al. 2012).

Previously mentioned data mining approaches essentially rely on available knowledge. Ample knowledge is available for Arabidopsis, but for other less well-studied plant species, the lack of knowledge regarding gene annotation and interactions severely limits network analysis using gene prioritization. Comparing networks from different species can provide an additional source of knowledge for gene functional annotation and gene connectivity using gene orthologs information and network alignment (see Section Comparative Co-Expression Network Analysis). As an example, Lee et al. (2015a) used conserved functional gene associations from networks inferred for Arabidopsis, worm, human and yeast as an additional source of data for the RiceNet, which was initially limited to rice-specific data sets.

The availability of these complementary data has opened the way to integrated approaches for function prediction studies. Multiple independent lines of evidence provide confidence for network functional gene associations. Kourmpetis et al. (2011) employed the Bayesian Markov Random Fields (BMRF) model to integrate protein sequence information, gene expression and protein-protein interaction data in their function prediction approach in Arabidopsis. They demonstrated that the model for network integration had the best performance when all of these data sources were used. One of the best examples of data integration is provided by GeneMANIA. This prediction server relies on a Gaussian Markov Random Fields-based method for protein function prediction combining multiple networks (Warde-Farley et al. 2010). Together with computational methods, these tools, mobilizing and integrating prior knowledge and network features, have contributed to the establishment of diverse strategies to prioritize candidate genes for further experimentation (Table 2).

Table 2: Examples of strategies used for co-expression network analysis in regard to the respective biological question addressed

Review sections	Biological question	Species	Strategy	References
Data availability for co-expression network analysis	Identify functional modules associated to germination and dormancy	Arabidopsis	Use of a condition dependant approach	Bassel et al. (2011)
	Build a comprehensive and functional co-expression network	Arabidopsis, rice	Integration of multiple sources of data in the network construction to support functional gene linkage	Lee et al. (2010) Lee et al. (2011)
	Gene functional annotation	rice	Comparison of condition dependant and condition independent network based approach.	Childs et al. (2011)
	Maximize the capture of gene co-expression relationship	Arabidopsis	Pre-clustering of input expression samples to approximate condition dependant approach	Feltus et al. (2013)
Gene prioritization	Explore the modular biological organization	Arabidopsis	Arabidopsis gene co-expression network based on 1000 microarrays. Modules were extracted using the Markov Clustering Algorithm (MCL)	Mao et al. (2009)
	Infer gene regulatory relationships in gene co-expression modules	Arabidopsis	Identify gene expression modules driven by known *cis*-regulatory motifs	Ma et al. (2013)
	Gene functional annotation	Arabidopsis	Module enrichment for known cis-regulatory elements	Vandepoele et al. (2009)
	Identify co-expression modules	Arabidopsis	Development of an Heuristic clustering algorithm	Mutwil et al. (2010)
eQTL based co-expression networks	Identify causal genes responsible for glucosinolate variation	Arabidopsis	Use co-expression network as non-genetic (independent) filter to prioritize GWA mapping candidates	Chan et al. (2011)
	Identify candidates for shade avoidance	Arabidopsis	Prioritize genes underlying phenotypic QTL using co-expression network analysis, eQTL information and functional classification	Jimenez-Gomez et al. (2010)
	Examine natural variation in circadian clock function	Arabidopsis	eQTL mapping using *a priori* defined phase groups and comparison with metabolomics QTLs	Kerwin et al. (2011)
	Examine transcriptional network response to biotic interactions	Arabidopsis	Perform a network eQTL analysis from *a priori* defined gene expression networks	Kliebenstein et al. (2006)
	Identify novel abiotic stress genes	Arabidopsis	Network guided genetic screen: gene ranking combined to co-expression network analysis	Ransbotyn et al. (2014)
Temporal resolution for co-expression network	Resolve the chronological regulatory mechanisms involved in the response to pathogen infection	Arabidopsis	Temporal clustering by combining extensive time series data and co-expression network analysis	Windram et al. (2012)
	Identify key genes regulating the acquisition of longevity during seed maturation	Medicago Arabidopsis	Developmental time course data and cross species comparison for co-expression network analysis	Righetti et al. (2015)
Spatial resolution for dynamic co-expression network	Identify cell-specific molecular mechanisms	maize	Combine Laser-capture microscopy with RNA-seq	Zhan et al. (2015)
Comparative co-expression network analysis	Knowledge transfer between species	maize rice	Global co-expression network alignment using both gene homology and network topology	Ficklin and Feltus (2011)
	Identify conserved modules across species	Several species	Co-expressed node vicinity networks (NVNS) compared across species.	Mutwil et al. (2011)

Co-expression network applications

eQTL Based Co-Expression Networks

Advances in "genetical genomics" have greatly benefited the elucidation of the genetic loci controlling transcription and the inference of regulatory mechanisms underlying complex phenotypic traits. The concept of 'genetical genomics' was first introduced by Jansen and Nap in 2001 (Jansen and Nap 2001), marking a new turn in genetic studies. The basic idea of this approach is to join classical genetic linkage analysis (Quantitative trait Loci (QTL) analysis) with gene expression studies (Keurentjes et al. 2007b). The variation in gene expression is regarded as a quantitative trait for which the genetic basis (expression QTL, eQTLs) is investigated in mapping populations, such as recombinant inbred line (RIL) populations. In plants, "genetical genomics" has proven to be a successful strategy to dissect complex traits in a number of studies (for reviews see (Joosen et al. 2009; Kliebenstein 2009; Ligterink et al. 2012)).

Detected eQTLs for a specific gene can be classified into "local" or "distant" eQTLs depending on whether they co-localize with the physical position of the studied gene or are located elsewhere in the genome, respectively (Rockman and Kruglyak 2006). eQTLs can also be classified as cis- or trans-acting based on the location of the associated causal polymorphism in the gene under study or elsewhere in the genome, respectively. Consequently, distant eQTLs are always trans-acting, while local eQTLs can be cis-acting, if the associated causal polymorphism resides in the gene under study, or trans eQTLs when they are caused by a closely linked allelic variation in a trans-acting factor. Allele specific expression analysis can specifically determine whether a local eQTL is trans or cis-acting (for review see (Kliebenstein 2009)).

A common feature of global eQTL studies is the identification of trans eQTL hotspots (West et al. 2007; Keurentjes et al. 2007b). These eQTL hotspots correspond to a high number of co-locating trans eQTLs in one region of the genome, indicating a hotspot for transcriptional regulation (Kliebenstein 2009). Due to their analogy to high degree nodes in a network, cis eQTLs located in these hotspots are sought as candidate master regulators affecting the expression of genes with a trans eQTL in that same region (West et al. 2007). A regulatory relationship can be inferred by correlating gene expression profiles between the cis eQTL candidate regulators and their potential downstream trans regulated genes. An iterative group analysis can be used to detect significant associations (Breitling et al. 2004; Keurentjes et al. 2007b; Wang et al. 2014). Keurentjes et al. (2007b) established a regulatory network for genes involved in the transition of flowering based on eQTL data. The GIGANTEA (GI) protein, known to be involved in the circadian clock controlled flowering time pathway, was identified as a regulator. Phenotypic QTLs associated with flowering and the circadian clock were also identified at the genetic locus of GI. Similarly, Wang et al. (2014) identified eight regulatory groups and their target genes for heading time in rice RILs. One regulatory group centered on Ghd7, an important regulator in heading time and yield potential in rice, was identified with a cis eQTL connected to nine genes with trans eQTLs. The network was validated by inspecting the transcript abundance of downstream-regulated targets and supported by co-localizing phenotypic QTLs for yield and heading time. These studies illustrate the usefulness of eQTL based co-expression analysis to guide the identification of candidate genes controlling quantitative traits. Other studies combined eQTL with co-expression analysis to identify regulator candidates underlying eQTLs (Terpstra et al. 2010; Flassig et al. 2013).

Interestingly, eQTL studies have also reported noteworthy properties of eQTLs in regard to their regulatory and evolutionary significance. Cis eQTLs were found to be highly inheritable with a larger genetic effect when compared to trans eQTLs (West et al. 2007; Petretto et al. 2006; Kloosterman et al. 2012). Cis eQTLs were also found to be more consistent across different genetic backgrounds (Cubillos et al. 2012) and more robust to environmental perturbations (Cubillos et al. 2014), while genes with trans eQTLs were more frequently reported as tissue or organ specific (Drost et al. 2010; Kloosterman et al. 2012).

QTLs tend to cover large regions of the genome, typically spanning hundreds of genes, and finding the actual gene that causes the observed trait variation is a formidable task. The capacity of gene co-expression networks to handle genome-wide data and filter out genes based on their correlation coefficients offers an attractive approach to prioritize genes. This strategy was successfully applied in the identification of EARLY FLOWERING 3 (ELF3), and its implication in shade avoidance response (Jimenez-Gomez et al. 2010). In this study, a network was built for each of the 363 candidate genes underlying the main phenotypic QTL for shade avoidance, connecting each candidate gene to co-expressed genes across 1.388 (selected) experiments. The eQTLs available for the investigated RIL population allowed pruning of the networks to keep only the co-expressed genes with a cis eQTL, which is indicative of a regulatory relationship (Hansen et al. 2008). In a similar approach, Chan et al. (2011) used co-expression analysis to prioritize candidate genes resulting from a genome wide association study (GWAS). Alternatively, co-expression networks can be used prior to eQTL analysis (Kliebenstein et al. 2006; Kerwin et al. 2011). Kliebenstein et al. (2006) implemented an a priori network eQTL approach by calculating the mean expression value of the genes within each pre-determined network and using this as a quantitative trait in a subsequent QTL analysis.

One main advantage of eQTL analysis is that regulatory insights can be gained without prior knowledge. Information on the nature of the inferred interaction in such an approach, combined with co-expression network analysis, can substantially accelerate understanding of molecular regulatory interactions (Figure 2). However, the link between phenotype and transcript variation is not always straightforward as changes are also likely to occur at the protein or metabolite levels. The additional integration of other omics data available as QTLs for protein (pQTL) or metabolite (mQTL) variation (Wentzell et al. 2007; Kerwin et al. 2011) can bridge the gap between genotype and phenotype, providing an in-depth understanding of causal mechanisms. As an example, Kerwin et al. (2011) identified overlapping eQTLs and mQTLs for circadian time and glucosinolate variation in Arabidopsis. Specifically, AOP2, a 2-oxoglutarate-dependent dioxygenase, was identified as a potential regulator. Altered AOP2 function resulted in changes in expression of clock output genes, suggesting a causal relationship between changes in clock function and metabolite content.

High resolution co-expression networks

Co-expression networks offer a conceptual framework to study gene interactions. However, their static representation does not capture all possible gene relationships as these do not operate simultaneously due to spatial and temporal variation in gene expression.

Temporal Resolution for Dynamic Co-Expression Networks

In response to developmental or environmental stimuli, plants undergo global transcriptional reprogramming. Monitoring transcriptional changes over time can provide more insight into the cascade of biological processes involved in the signal perception, transduction and final response. Using time series data sets throughout seed development, Le et al. (2010) identified seed specific transcription factors active in different compartments and tissues of the seed at unique moments of seed development, suggesting a chronology of specific regulatory programs triggering seed development.

Time series experiments are often used to examine the dynamics of gene expression. Wei et al. (2013) used six time points during growth of poplar roots in low nitrogen conditions. GO categories associated with signal transduction were identified for differentially expressed gene sets in the early time points of the response (6h and 24h), while categories associated with organ morphogenesis were prevalent throughout the later time points (48 and 96h).

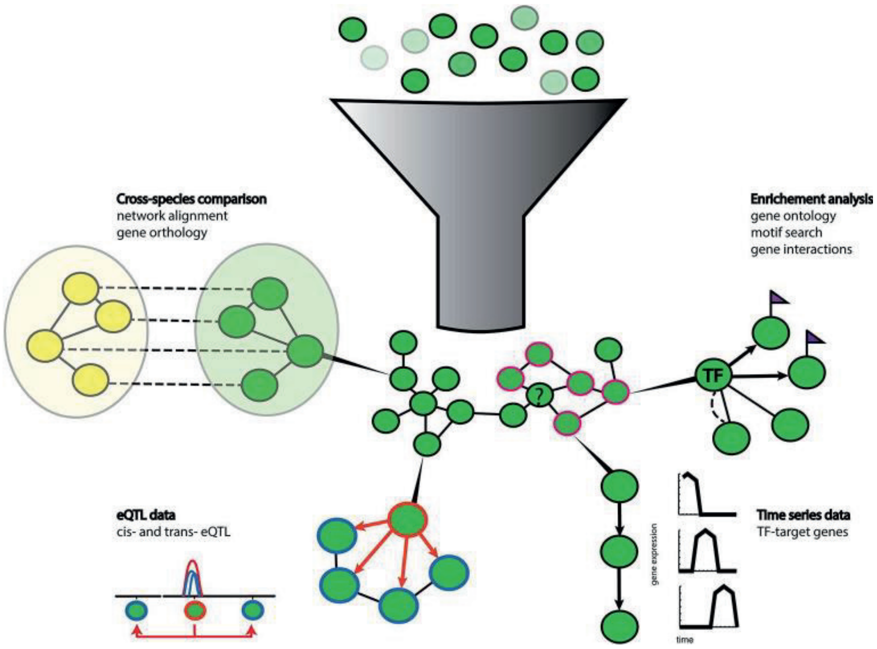


Figure 2: Schematic representation of gene prioritization strategies. Gene sets of different expression values (Shades of green) are used to co-expression network inference. Genes with co-expression values above a user define threshold (dark green nodes) form nodes and edges in the network. Various additional data can then be used to enrich and extract biological relevant information from the network. Enrichment analysis tools such as gene ontology terms (pink contour nodes) can be used to functionally annotate unknown genes (question marked genes) clustered in the vicinity. Prior knowledge can also help to highlight known gene-gene interactions (dotted line) and cis-regulatory motif (purple flags) can suggest local regulatory interactions (arrows) between transcription factors (TF node) and their target genes (flagged nodes). Gene

regulatory relationships can also be extracted from time series data. Algorithms can extract causal regulatory relationship between genes with a cis-eQTL (orange contour node) and genes with trans-eQTLs (blue contour node). Additionnal information can be gained from comparisons with networks of other species (yellow node) by onthology and network alignment (dotted lines).

By reducing the time scale to minutes, Krouk et al. (2010) observed that within three minutes following nitrate addition in Arabidopsis, functional categories such as ribosomal proteins were over-represented, suggesting the rapid activation of key elements of the translation machinery to synthesize proteins required for nitrogen acquisition.

Combining time series and co-expression network analysis can unveil gene interactions associated with the dynamics of transcriptional programs. Global expression patterns can be obtained from the expression similarity calculated across samples collected at different time points. This approach is well suited to find modules of simultaneous expressed genes and gene interactions but is not well suited for time lagged regulations since all genes influencing the expression of downstream target genes are not necessarily captured within a same time point (experiment). This results in complex relationships between co-regulated genes, including co-expression, time shifted and inverted relationships (Zhang et al. 2005): an activated transcription factor gene first has to be transcribed and the resulting mRNA translated before it in turn can activate its downstream targets. The delay further depends on the dynamics of the regulation, and for instance the presence of network motifs like feed forward or negative feedback loops (Alon 2007).

Windram et al. (2012) dissected the infection response of Arabidopsis to Botrytis cinerea using 48 time points with 2h intervals. To capture the chronological establishment of the associated transcriptional events and to predict their regulation, the differentially expressed genes were first clustered based on the similarity of gene expression patterns over time or based on the timing of differential expression of each gene. Regulatory predictions were made using a discrete-time causal structure identification algorithm. The expression means of the clusters and Botrytis cinerea growth information were used to build a regulatory network. In this network, a NAC transcription factor identified in one cluster connected to two downstream clusters enriched for the NAC binding motif in their promoter sequence, suggesting a regulatory relationship.

This example shows that causality information of time series on a fine temporal scale can provide valuable information on the directionality of gene interactions. Several algorithms have been proposed to perform time delayed correlation analysis in time series data (De Smet and Marchal 2010). For instance, Lavenus et al. (2015) proposed a time delay correlation algorithm (TDCor) that includes minimal prior knowledge on the nature of the genes, with transcription factors categorized as repressor, activator, regulator or non-regulator, to build a network of plausible interactions from time series data. Krouk et al. (2010) used a noise reduction state-space modeling algorithm to build a dynamic linear model defining the rate of change in expression between time points t and t + 1. This model was then used to predict the influence of transcription factors on the genes they regulated (influential rate). The authors reasoned that the observed low influential rate of the transcription factors could be due to the functional redundancy that is often observed in biological networks and is consistent with a proposed global buffering system counteracting stresses and evolutionary forces (Fu et al. 2009). Polanski et al. (2014) suggested a module identification procedure based on the Wigwams algorithm capable of mining multiple time series for condition dependent co-expression across a subset of time series. Using such an approach, the reconstruction of co-expression networks can be directed to time specific modules of co-regulated genes.

Together, these studies suggest that new regulatory insights can be gained from integration of co-expression networks with data from time series, for the identification of “subtle” gene clusters, showing condition dependent regulation. Time series are valuable for further disentangling of real co-regulatory gene relationships from co-expression links. For application in more studies, new challenges have to be addressed such as the judicious selection of time points (Vashishtha et al. 2015), the development of performant inference algorithms, the reliable detection of direct and indirect gene interactions and most importantly the connection with their real biological meaning (reviewed by (Bar-Joseph et al. 2012). We believe that this approach will offer new venues for deeper insights into the fine-tuned regulation and predictive analysis of gene expression behavior in future studies.

Spatial Resolution for Dynamic Co-Expression Networks

Plants are multicellular organisms whose vegetative and reproductive organs are composed of complex tissues and cell types. Cell differentiation is a fundamental process required to acquire cell identity and consequently ensure the correct execution of essential structural and biological functions. Genome-wide transcriptome and gene network analyses have mostly been conducted on whole plant organs, severely limiting the identification of more specific regulatory interactions occurring at the tissue or single cell level. The development of new highly selective methods has enabled the collection of expression profiles at unprecedented resolution (Nelson et al. 2008; Tang et al. 2011; Belmonte et al. 2013) offering new insights into the various biological levels of transcription regulation. As an example, laser capture microdissection (LCM) enables isolation of specific tissues at cell level while fluorescent activated cell sorting (FACS) allows separation of specific cell types expressing green fluorescent protein (GFP) under control of cell specific promoters.

These techniques were used to get insight into single cell transcriptomic data for well-studied and specialized organs such as roots or pollen (Becker et al. 2014; Slane et al. 2014; Aya et al. 2011; Efroni et al. 2015).

A fluorescent cell sorting technique was used to obtain a high-resolution map of spatiotemporal expression profiles of Arabidopsis roots (Brady et al. 2007). In this study, transcriptome analysis of root transverse sections revealed 51 dominant root radial expression patterns among which 17 showed enrichment in a single cell type, whereas 34 expression patterns were found across 2–5 cell types to 5 cell types (Brady et al 2007). In the same study, the longitudinal root section expression profiling to analyse different developmental stages in root cell-type formation, enabled the identification of specific expression patterns. Transcriptional changes may also occur in response to environmental shifts. Interestingly, a close link was observed between development and stress responses at the cell-type specific level in the Arabidopsis root showing developmental plasticity (Gifford et al. 2008) while adding a layer of complexity, *i.e.* environment specific effects, to an already intricate system. Together, these results highlight the spatiotemporal transcriptional complexity down to the cellular level and suggest cell-specific transcriptional programs.

Integrating tissue- or cell-type specific high-resolution datasets by co-expression network analysis is a promising approach for the regulatory dissection of specific biological functions. Illustratively, Zhan et al. (2015) combined LCM and RNA-seq to isolate and profile filial and maternal cell types of maize kernels at eight days after pollination. From the resulting gene co-expression network, 18 endosperm-associated co-expression modules were identified among which 10 were found to be highly compartment- or cell-type-specific. The comparison of these spatial co-expression modules with temporally upregulated gene data sets showed that genes within co-expression modules are regulated both in time and space. Collectively, these results support the effectiveness of co-expression networks analysis to uncover the temporal and spatial organization of specific differentiation processes.

On-going developments to further improve single-cell RNA-seq analysis (Buettner et al. 2015) should strongly benefit the establishment and interpretation of specialized co-expression networks in the coming years. Furthermore, the advancement of computational tools able to manage the increasing amount of data as well as the development of robust and efficient algorithms to analyse large-scale data will be needed to tackle the increasing complexity added to gene regulatory networks.

Comparative co-expression network analysis

Classic research in evolutionary developmental biology (“evo-devo”) has focused on comparative analysis with the help of mutant analysis, heterologous mutant complementation, comparative gene expression studies and phylogenetic analysis. These analyses mostly rely on gene and protein sequence information; however the increasing number of gene expression data in many different species is opening up new perspectives. Cross-species comparison of co-expression networks is a promising approach to understand the interplay between regulatory function and evolution (Movahedi et al. 2012; Hansen et al. 2014).

There are several advantages of cross-species network comparisons. Networks of well-studied plants such as Arabidopsis can enrich sparse networks, such as for crops, reducing the need of extensive functional genomic and phenomic resources. Cross-species comparison can accelerate the functional annotation of genes and the discovery of gene-gene interactions, consequently hastening the gene prioritization process for targeted mutational studies.

There is evidence that networks are shaped by major evolutionary features, such as by neo- or sub-functionalization following whole genome duplications (Conant and Wolfe 2006; De Smet and Van de Peer 2012). These adaptive processes may result in an evolutionary functional gene network partitioning associated with a rewiring in the gene regulatory circuitry (Conant and Wolfe 2006). In this context, co-expression network comparison can be used to identify functionally conserved network patterns and to study their evolution.

Different methods have been proposed to compare co-expression networks. Leal et al. (2014) compared gene co-expression networks obtained for several plant species in response to different pathogens using a multivariate analysis. Each network was characterized by eight graph variables which were then summarized in a principal component analysis. Clustered networks identified in the principal component analysis plot suggested similar pathogen specific responses across species.

An obvious method to align networks and to get better insight into the degree of network conservation is to link orthologous genes between different species. The effectiveness of such comparative analysis essentially relies on the consistency of the orthologous information as well as the quality of the underlying co-expression networks. Orthologous gene information can be obtained through various methods (Kuzniar et al. 2008). Simple approaches use best Blast hits or reciprocal hit blast (RHB) for closely related species (Yang et al. 2011). More advanced tools such as the OrthoMCL clustering algorithm (Li et al. 2003) or OrthoFinder (Emms and Kelly 2015) enable differentiation of true orthologous from paralogous genes. Zarrineh et al. (2011) proposed a cross-species co-clustering approach (COMODO). Network comparisons can be done at the global scale or focused on specific gene modules. In a global approach, Ficklin and Feltus (2011) used an alignment algorithm, IsoRank, that incorporates both gene homology and network topology to compare networks in rice and maize. They identified aligned modules enriched for similar functional terms, suggesting their potential evolutionary conservation.

In another study, Obertello et al. (2015) used orthologous information from OrthoMCL and BlastP, to align genes between Arabidopsis and rice co-expression networks. The authors observed that integrating rice data in an Arabidopsis network did not improve the available interaction knowledge, while Arabidopsis could substantially enrich rice network interactions. This study illustrates the usability of network comparisons to promote translational discoveries. It shows that well-known networks, such as those from model plants like Arabidopsis, can enrich more sparse networks of crops, such as rice, although Lee et al. (2011) demonstrated a higher accuracy for a rice network, RiceNet, derived from data of diverse species (with 15.5% of true positive linkages) than for a rice network derived solely from orthology with AraNet, the Arabidopsis network (with 6.5% true positive linkages).

In a more targeted approach, Yang et al. (2011) investigated conserved co-expression of cell-wall associated genes between Arabidopsis and poplar. An initial list of known cell-wall related genes was used to build a co-expression network with 22 clusters. The orthologous clusters of co-expressed genes identified in poplar did not all correlate in gene expression pattern with the clusters in Arabidopsis (gene expression pattern correlated for 9 of 22 clusters). Additionally, conserved co-expression clusters referred to plant essential biological functions, such as cell-wall formation. More comprehensively, Movahedi et al. (2011) implemented an expression context conservation score (ECC) to quantitatively estimate the degree of conservation of expression similarity between orthologous genes and their co-expression partners. The overall ECC scores revealed that for 4.630 orthologs in rice-Arabidopsis gene pairs, 77% had a conserved expression context. In another study, Netotea et al. (2014) performed an extensive examination of network properties, like node degree distribution and gene centrality, to compare co-expression networks of Arabidopsis, poplar and rice. They analyzed the degree of conservation of gene co-expression links and neighborhood (connected genes) among all orthologs in the three networks and showed that genes with high centrality, typically hubs, were significantly conserved while local regulatory motifs were relatively less well conserved across species.

Additionally, they noted that sequence similarity did not always predict gene regulation conservation. Beyond simple gene sequence comparison, the integration of co-expression networks to cross-species data provides a new dimension in evolutionary studies, revealing conservation and divergence in the regulation of genes.

At the moment, several integrative platforms are available to enquire, display and compare co-expression networks. Examples of these are PLANEX (Yim et al. 2012) , ComPLex (Netotea (Netotea et al. 2014), CoExpNetViz (Tzfadia et al. 2015), PLAZA (Proost et al. 2015) and the "NetworkComparer" pipeline on the PlaNet platform (Mutwil et al. 2011) that integrates genomics, transcriptomics, phenomics and ontology analyses to compare seven plant species.

Conclusion and perspectives

Co-expression networks are a powerful approach to accelerate the elucidation of molecular mechanisms underlying important biological processes. Importantly, network based strategies are largely determined by the biological question addressed and the prior knowledge available.

We anticipate that the increase in available experimental data, driven by new molecular techniques, will enrich existing databases. In addition, the shift from microarrays to next generation high-throughput sequencing technologies will provide further insights into genome scale functional networks of many species. Together with the increased sensitivity of high-resolution technologies enabling the acquisition of cell-specific transcriptome profiles, novel biological insights can be gained. The extensive accumulation of data will require further efforts for their storage, accessibility and processing. One of the common strategies for all co-expression network studies is the integration of disparate data sources for the biological interpretation of networks. As a result, the development of integrative web interfaces such as CressInt (Chen et al. 2015) are needed to facilitate the integration of available genomics data. Furthermore, the development of computational tools, such as machine learning based algorithms, although computationally intense, will support the optimal integration and exploitation of prioritization strategies (Radivojac et al. 2013). In such a scenario, the collaboration of bio-informaticians and biologists is highly desirable and will become increasingly important.

To fully describe the link between genotype and phenotype and to understand the underlying gene regulation, coordination of networks at different molecular levels (gene, protein, metabolite) is needed (Gaudinier et al. 2015). Additionally, genetically anchored gene expression profiles (eQTLs) have proven to be powerful tools to reveal causal regulatory variants. The genetical genomics approach provides a multifactorial design to study the simultaneous effect of gene perturbations. (Kliebenstein 2012) demonstrated that shallow sequencing depth in transcriptomics experiments enables capturing most of their genomic information. The result of their study suggested that 10% of the transcripts would detain more than 80% of the information present in a variety of transcriptomics experiments. In another study, Li et al. (2008b) introduced the generalized genetical genomics design to optimally study genetic by environment interactions. These findings suggest that there is room for improvement in the design of transcript sequencing for large-scale factorial analysis in which the size of the population studied or the number of conditions to be tested can be increased in a cost-effective manner.

Co-expression networks are an attractive framework for gene interaction analysis and offer a diverse range of applications, from the gene functional annotation to the comparison of co-expression networks across species. Improved and enriched co-expression network analyses will further empower the predictive power of networks and their translational application by circumventing the need of additional extensive functional genomic and phenomic resources. This approach will further contribute to the elucidation of important biological processes and provide a valuable predictive tool for contemporary molecular breeding and crop engineering strategies.

Chapter 7

General discussion

Introduction

In the coming years as a result of climate change, plants will experience shifts in developmental phase transitions with consequences for their life history strategies. Fundamental knowledge of the factors and mechanisms influencing these shifts can help to understand the importance of these changes in a biological, agricultural and ecological context. Environmental factors influence vegetative aspects of plant development as well as the transition to reproductive development, namely flowering. Beyond flowering, environmental factors can be experienced by the developing offspring and as a result, influence the intrinsic properties of the seeds produced. At the stage of seed dispersal or harvest, the quality of the seeds is instrumental for the reproductive success of the plant or for seed companies to obtain a marketable product.

Seed quality is a generic term, since it describes many seed characteristics, including seed size, seed weight, as well as the seed performance traits associated with the rate, percentage, uniformity and vigour of germination. In nature, the timing of germination is critical for the success of seedling establishment. One of the well-known mechanisms controlling the timing of germination is seed dormancy. Seed dormancy is influenced by genetic loci as well as environmental factors (Bentsink et al. 2010; He et al. 2014; Donohue et al. 2005a). The response to the environmental cues during seed development, also termed 'maternal environment', has been described for several seed traits including dormancy and germination in Arabidopsis (He et al. 2014; Donohue et al. 2005a). These effects also differ across genotypes, suggesting genotype-by-environment interactions (G x E). In spite of the potential phenotypic plasticity induced by maternal environments and the large genetic variation reported for seed traits (Bentsink et al. 2010; Joosen et al. 2012), only a few studies have investigated the influence of the maternal environment on the genetic basis of seed quality traits (Postma and Agren 2015; Kerdaffrec and Nordborg 2017). The advent of high-throughput of post-genomics technologies can provide in-depth insights in the plastic, genetic and molecular bases of the process of seed germination.

The work presented in this thesis aimed to extend studies on the effect of maternal environments by providing insights into the genetic basis of seed performance at the phenotypic, metabolic and transcriptome levels and the changes induced by different seed maturation environments. For this purpose, I used an Arabidopsis thaliana Bay-0 x Sha recombinant inbred line population of 165 lines which was grown under controlled standard (ST), high temperature (HT), high light (HL) and low phosphate (LP) conditions from flowering until seed harvest. A classical quantitative trait locus (QTL) mapping approach was used to investigate changes in the genetic architecture of phenotypic and molecular traits in response to the maternal environment. I generated a new saturated genetic map (Chapter 2) which was subsequently used to identify a large number of QTLs displaying significant QTL-by-environment interactions for seed quality traits (Chapter 3). In Chapter 4 and 5, in a genetical ~omics approach, I showed that the maternal environment triggers profound changes in the genetic basis of the dry seed metabolome and transcriptome. This thesis's research lays the foundation for seed systems genetics studies towards the future integration of the separate studied biological strata. In this respect, networks represent an attractive approach to reduce data complexity and reflect on the underlying molecular mechanisms (Chapter 6). In this last chapter, I discuss the importance and implications of the maternal environment in an evolutionary, ecological and breeding context. I further elaborate on tools and methods that are beneficial for gene discovery and the understanding of the regulation of complex traits.

Plasticity of seed performance

The adaptive significance of maternal environments

Changes in the environmental conditions are expected to be more common as a result of climate change. Organisms can respond to these fluctuations in several ways such as shifting their distribution, adaptation to new environments or acclimating via plasticity. Phenotypic plasticity is the result of the expression of a genotype in response to environmental variation, and enables plants to cope with fast changing environments. Such plasticity brings the opportunity for individuals to reach phenotypic optima in novel environments and is thereby likely to contribute to genetic adaptation of a species in the long term (Donohue et al. 2005). As for many organisms, early life history stages of plants are the most sensitive to environmental cues. In the plant life cycle, seed germination is a critical phase determining the success of the establishment of the next generation. Plasticity for seed germination has been largely investigated in the immediate environment, *i.e.* within one generation (Joosen et al. 2012). Environmental cues experienced by the environment of the parental plants can also alter seed traits. Arguably, the influence of the maternal environment is often referred to as a form of trans-generational plasticity (Vayda et al. 2018). However, in this set-up, the maternal environment is concomitant with the environment experienced by the mother plant and the developing seed itself. The maternal environment is often viewed as a key for promoting offspring performance against adverse environmental cues. However, upon seed dispersal, germination frequently occurs under a wide range of conditions (Bewley 1997; Baskin and Baskin 2014) and thus differences between the maternal and offspring environments may result in unpredictable phenotypic performance (Leverett et al. 2016), Chapter 3) questioning the adaptive significance of the maternal environment. Two types of strategies might explain the response to the maternal environment. Early seed germination response in adverse environments can be seen as a competitive advantage that allows the young plant to outgrow potential competitors or overcome temporary unfavourable germination conditions. On the other hand, a reduced germination rate can be viewed as a strategy aiming to delay the timing of germination by promoting prolonged primary dormancy, germination arrest or facilitated induction of secondary dormancy. In Chapter 3, I investigated germination performance, measured as the area under the germination curve (AUC), as a response to both maternal and germination environments. Under most adverse germination conditions, an increased variation in the AUC values was observed across the lines, suggesting non-uniformity of germination in the seed batch. In nature, non-uniformity of germination can be seen as a bet-hedging strategy to scatter germination in time thereby providing a mean to better cope with uncertain and fluctuating germination environments (Springthorpe and Penfield 2015). The ecological importance of non-uniform germination contrasts with the quest for uniformity of germination and seedling establishment in the selection process of high quality seeds for agricultural practices.

In this thesis, I investigated the effect of the maternal environment experienced by the mother plant and developing seeds from flowering until seed harvest. However, the sensing and signalling of environmental cues at the vegetative stage, *i.e.* before flowering, also determines seed quality characteristics, such as seed dormancy (Springthorpe and Penfield 2015). Flowering marks an important transition from the vegetative to the reproductive stage. The major regulator of flowering, FLOWERING LOCUS C (FLC) operates in one of the best characterized molecular pathways with known pleiotropic effects. Several studies have reported relations between the flowering-FLC pathway and the control of seed germination (Blair et al. 2017;

Huo et al. 2016; Chiang et al. 2009). A recent study brought mechanistic insights on how FLC affects seed dormancy in response to high temperature (Chen and Penfield 2018). It would be interesting to further investigate how regulatory mechanisms, *e.g.* associated with flowering, integrate environmental cues and interact with regulatory mechanisms acting during seed development and maturation.

Implications for breeding

The fact that performance of seeds in response to the maternal environment affects final seed quality is of eminent importance for breeding purposes (Chapter 3). In seed companies, large variation in quality of seed lots is expected, since the production sites are often located in different countries around the world. One way to alleviate such hindrances could be to establish a growing protocol based on the predicted environmental effects in such a way that the effect of the environment is stabilized across generations. Alternatively, knowing about the effects of a given growing environment could also be exploited to manipulate seed production environments to predict and improve seed quality.

Since it is not always possible to control greenhouse conditions or even use greenhouses, so far major efforts to improve seed quality have been carried out using post-harvest treatments. The downside of such treatments, such as seed priming, is the often drastic reduction of storage time of the seeds (Hussain et al. 2015). In addition, such treatments remain expensive, crop specific and subjected to trial and error for the optimization of protocols. Including the genetic components of seed quality in breeding programs might thus provide an upstream solution to improve seed quality. In Chapter 3, I found that the maternal environment largely interacts with the genetic basis of seed performance. One of the most striking examples was observed for a high temperature specific QTL identified on the top of chromosome 1 and validated using a heterogeneous inbred families (HIFs) approach. From a breeder’s perspective, selecting varieties that show a stable response to different maternal cues can ensure certain stability in the quality of the seed produced. Alternatively, genotypes could be selected for their predicted performance in terms of seed quality traits for targeted growing environments. The large variation in the effect of seed performance QTLs under the different maternal environments emphasizes the need to consider maternal environments in genetic studies. In general, knowing the potential consequences of maternal environments might help in the development of tools to accurately predict crop performance.

Closing in on complex traits

Complex traits are influenced by the natural variation within multiple genes in interaction with the environment. The study of natural variation continues to reveal new biology (Weigel 2012). In parallel, the recent advances in sequencing technologies and high-throughput phenotyping facilities for phenomics, transcriptomics, proteomics and metabolomics have enabled plant biologists to elucidate genetic and molecular mechanisms of complex traits (Keurentjes et al. 2008). Below I discuss in more detail the methods and tools that can help to identify causal variants of complex traits.

QTL mapping

Many tools for quantitative genetics have been developed aiming at studying the genetic architecture of quantitative traits. Quantitative trait locus (QTL) analysis remains among the most popular approaches. The success of QTL mapping relies on the power and the resolution of the QTL analysis, mainly determined by the type and size of a population as well as on the availability of a dense genetic map (Chapter 2). In this thesis, I used an Arabidopsis Bay-0 x Sha core population of 165 recombinant inbred lines (RILs) to investigate the genetic basis of seed performance. RILs have proven useful for QTL analyses. The number of generations created to obtain the RILs, results not only in almost completely homozygous lines, but also in the accumulation of crossovers. RILs provide thus material for high resolution QTL mapping, although QTLs often still include a large number of genes (Chapter 2, Chapter 3). A limitation of QTL analyses in bi-parental populations is that only the genetic variation present between the two parents and that segregates in the derived population can be investigated. Several strategies exist to increase the genetic variation under study. One approach is the generation and investigation of artificial populations with multiple parents, the so-called Multi-parent Advanced Generation Inter-Cross (MAGIC) populations (Cavanagh et al. 2008; Kover et al. 2009). In contrast, genome-wide association studies (GWAS) use historical recombination events in a large panel of natural accessions to identify marker-trait associations. GWAS can overcome the limitations of traditional bi-parental populations and dissect complex traits with high mapping resolution at the SNP level (Atwell et al. 2010). Therefore, several studies combine QTL and GWAS approaches to validate their results and identify potential causal genes (Mammadov et al. 2015).The GWA approach has been used to investigate the genetic basis of seed traits in Arabidopsis as well as in other species (Yan et al. 2017). In Chapter 3, the QTL x E approaches resulted in a high number of QTL showing certain sensitivity to the maternal and germination environments. Kerdaffrec and Nordborg (2017) investigated the genetic basis of dormancy under two different environments using GWA approach. The power of GWAS relies on the allele frequency in the population and in a GWA x E set-up, a main limitation is that low frequency in the population of QTL alleles with substantial G x E might not be captured (Korte and Farlow 2013; Asimit and Zeggini 2010; El-Soda et al. 2014). The high plasticity of seed traits in particular in response to maternal environments makes such genetic studies particularly challenging.

The advancement in sequencing technologies has provided many possibilities to explore genetic variation. Single nucleotide polymorphisms (SNPs) are commonly identified from genomic data as costs of available technologies are constantly decreasing. In Chapter 2, I showed that RNA-seq, commonly used for the quantitative measurement of gene expression, can also be used to identify SNPs. The discovery of SNPs using RNA-seq data is useful for several reasons. First, by targeting the transcriptome, RNA-seq reduces the search of causal variants of complex and/ or unsequenced genomes. Furthermore, the SNPs identified are located in genic regions. These regions are the most likely to be involved in the regulation of complex traits. Lastly, RNA-seq provides a representation of the transcriptome that can be useful for other analyses as shown in Chapter 5 where I used the same RNA-seq data to identify expression QTLs (eQTLs). In Chapter 2, the SNPs identified from the RNA-seq data were used to saturate the initial map of the Bay-0 x Sha population. The comparison of different available genetic maps showed that increasing the numbers of markers provided higher mapping resolution by reducing the size of the QTL intervals. The ability to derive exponential numbers of markers, through sequence data, shows that the availability of genotypic data no longer represents a limiting factor for QTL studies, thus shifting efforts to enhance QTL mapping towards the use of efficient mapping population designs exploiting large genetic variation, such as the previous mentioned multi-parental populations (Wijnen and Keurentjes 2014). A major challenge in multiple environment studies remains that a large number of plants need to be accurately phenotyped in order to have the statistical power to identify marker-trait associations. The success of these methods thus also requires advanced methods that provide high-throughput phenotyping, which is currently recognized as a major bottleneck in genetic studies.

Phenotypes

Another major aspect for the success of QTL approaches relies on the input data, capturing essential stages of phenotypic variation. In Chapter 3, I used a high-throughput phenotyping method, the GERMINATOR (Joosen et al. 2010), that enables fast and standardized scoring of germination for a large number of seed batches. I also generated omics data to investigate changes at the metabolome (Chapter 4) and transcriptome levels (Chapter 5) in dry mature seeds coming from plants grown under different maternal environments. The large number of mQTLs and eQTLs identified showed that molecular traits can effectively be used for the molecular dissection of complex traits (Chapter 4, 5). These new, also called intermediate, phenotypes increase the chance of finding marker-trait associations, while providing substantial biological insights.

For practical reasons, namely the study at the population level and, thus, the large number of lines to phenotype, I made the choice of investigating omics changes in dry mature seeds. Dry mature seeds are the end product of seed development, thus providing suitable biological material to investigate and compare changes at the genetic and molecular levels in response to environmental variation. Apart from these considerations, the choice for dry mature seeds was supported by the hypothesis that dry seeds are packaged with all components needed to initiate germination. At the end of seed development seeds enter a dry and quiescent enzymatic and metabolic state, accumulating all components (proteins, enzymes, mRNA, metabolites, etc.) that will support the start of the germination machinery. As a result, I expected that differences in seed performance could be related to the seed content at the dry mature state. However, metabolic and transcriptional shifts are also observed during the course of seed imbibition, germination and seedling establishment (Silva et al. 2017; Joosen et al. 2013; Rosental et al. 2014). Therefore, it can be argued that insights into molecular changes occurring at later stages might be more closely related to differences in seed germination.

In a broader prospect, the development of tools and methods that enable high-throughput and high-dimensional acquisition and modelling of phenotypic data will play an important role in identifying the underlying genetic structure of complex traits. For certain visible traits for which the phenotyping methods are limited, the development of 'phenomics' will provide a better definition of the investigated phenotype (Houle et al. 2010). The efforts put in developing imaging systems to provide automated, quantitative and non-destructive phenotyping for a large number of plants, can be exploited to effectively combine genetics and phenomics approaches (Cooper et al. 2014).

For complex traits controlled by multiple small effect genes, the use of the final phenotype might limit the power of classical genetic approaches in identifying all marker-trait associations. In these cases sub-phenotypes can be used as traits and enhance the chance on finding associations. The access to the phenome – which refers to the ensemble of a plant's phenotypes - will provide these sub-phenotypes. These combined sub-phenotypes can provide better understanding of functional relationship between plant physiology and environment and this information can be used to increase the accuracy of whole genome prediction methods (Technow et al. 2015). Additionally sub-phenotypes can also be derived from molecular data, such as omics data, to understand the functioning of the genes underlying complex traits. Sub-phenotypes, such as metabolic profiles, can increase the chances of finding marker-trait associations. This approach was taken in Chapter 4 and Chapter 5. Nonetheless, often knowledge about and detailed study of the function of individual genes will also be needed to identify the right combination of genes explaining specific phenotypes.

From QTL to causal variants

QTL analyses often result in large genomic regions, harbouring a large number of genes. Therefore additional strategies are needed to validate and fine map QTLs and to identify causal genes. In Chapter 3, I used a heterogeneous inbred family (HIF) approach (Tuinstra et al. 1997) to validate a high temperature QTL. Although this approach narrowed down the QTL, many candidate genes remained. For QTLs of reasonable size, gene expression data can also provide a fast approach for mining causal genes for QTLs (Chapter 3) (Wayne and McIntyre 2002). Genes in the region of the QTL can be scrutinized for differences in their expression level (Price 2006) (Chapter 3).

Using these strategies, the region of a high temperature QTL identified on chromosome 1 was refined and the number of candidate genes was reduced to

10 candidate genes. PHYA was identified as a specifically interesting candidate. Despite several indications for the role of PHYA in mediating environmental cues, the role of PHYA in response to high temperature remains unclear. Further studies would be needed to confirm the possible role of PHYA. Gene expression difference is however not a pre-requisite of gene causality, since mutations that induce protein modifications might also result in a QTL and such differences are mostly not captured at the transcriptional level. Protein modification may also result in expression differences due to feedback regulation. For this reason, the functional validation of the candidate gene with help of genetic mutants remains the strongest evidence to prove causality. These mutants can be made by knocking-out genes through mutagenesis or lately by genome-editing technologies such as CRISPR-CAS (Bortesi and Fischer 2015), by ectopic/over-expression approaches or ideally by expressing the gene variant from one parent in the background of the other parent with its own gene variant knocked-out.

Generalized genetical genomics

The emergence of the ~omics technologies has offered new venues to study complex traits. The concept of genetical genomics proposed by (Jansen and Nap 2001) is a useful approach that can effectively capture the effect of genetic perturbations on biological systems at the molecular level (Joosen et al. 2009). Initially applied to transcriptomic (Jansen and Nap 2001) and further to metabolomic and proteomic data sets (Keurentjes 2009), genetical genomics has brought new insights into the genetic basis of complex traits (Joosen et al. 2009). Genetical genomics studies have also been successfully applied to enhance a direct strategy to identify causal relationships (Jimenez-Gomez et al. 2010).

For a comprehensive understanding of biological systems, additional knowledge of the effect of environmental perturbations is needed. To address this, Li et al. (2008b) proposed a generalized genetical genomics (GGG) design for cost-efficient multi-environment genetical genomics studies (Li et al. 2008b; Joosen et al. 2013; Kazmi et al. 2017). In this design, a RIL population is divided into several complementary subsets of equal size and with equal allele distribution (Li et al. 2008b). I used this design to split the RIL population into four subsets to explore the influence of the maternal environment on the genetics of seed performance through omics datasets and linking them to phenotypic differences. Although not fully explored in this thesis, another advantage of the GGG design is to compare the direction of changes at the metabolic, transcriptomic and phenotypic levels across the conditions. For instance, one would expect the direction of changes across environments at the phenotypic level to be reflected in the corresponding datasets. Overall, I found that the maternal environment caused tremendous perturbations at the molecular level (Chapter 4, 5). In the next paragraphs, I discuss the findings of both approaches and the potential of combining these datasets to obtain a seed systems perspective.

Re-programming of the metabolome

In Chapter 4, large scale untargeted profiling of the dry seed primary metabolome was performed in a GGG design using GC-TOF-MS. In total, 172 metabolites were identified of which 71 could be annotated. The levels of the metabolites were predominantly influenced by the genotypic background and the maternal environment. The genotype-by-environment interactions were further explored with condition-specific correlation networks. These networks revealed genetically and environmentally coordinated metabolic changes (Chapter 4). Specifically under the high temperature maternal environment, coordinated changes in metabolites associated with the tricarboxylic acid cycle (fumarate, succinate, citric acid) and GABA pathway (GABA, alanine), suggested the role of energy status of the seed metabolism in response to abiotic stresses. A large number of mQTLs associated with metabolite changes were identified under several conditions. The largest power for detecting mQTLs was obtained by combining the different datasets. A large number of co-locating mQTLs were identified pointing at genomic regions involved in the control of metabolites of similar metabolic function or involved in shared metabolic pathways. The uneven distribution of the mQTLs along the genome resulted in four major mQTL hotspots, indicating that the response to environmental cues triggers targeted genomic regions, likely master regulators, to provide a coordinated metabolic response.

Re-programming of the transcriptome

In contrast to previous eQTL studies in Arabidopsis using microarrays, I performed gene expression profiling of the parental lines and the RILs using RNA-seq. At the transcriptional level, changes in the maternal environment resulted in distinct seed transcriptomes across genotypes with up to hundreds of genes differentially expressed ((He et al. 2016)Chapter 3, Chapter 5). I used the same GGG design as for the metabolomics study to investigate the genetic control of gene expression variation. The large number of eQTLs and the overall high LOD scores reflected the accuracy of RNA-seq in determining the expression levels of specific genes. The plasticity of the genetic control of gene expression in response to the maternal environment was assessed by comparing eQTL features across the four environments. I found that genes with eQTLs were highly consistent across conditions. However, distant eQTLs were more specific to the environment in contrast to local eQTLs that were overall consistent across conditions. This suggested that in response to environmental cues, a core set of genes is evoked by condition specific regulatory mechanisms. Several 'obvious' environment-specific eQTL hotspots were identified which were enriched for specific biological processes (Chapter 5). In this explorative study, such enrichments were rather limited and decreasing the stringency of the threshold for eQTL identification might provide greater biological insights. Nonetheless, these hotspots are also easy targets as starting point for further analysis which can be enhanced by the integration of different datasets as discussed in the next section.

Data integration

The functional relation between different biological layers remains the greatest challenge to meet the high expectations of systems approaches. In this study, substantial interactions between genotype and environment were observed at the different molecular levels. Efforts to link these datasets to the phenotype were made by investigating genetic correlations between traits, resulting in co-locating QTLs. Overall, there was limited overlap between the hotspots observed at the molecular (Chapter4, 5) and phenotypic level (Chapter 3) (Figure 1). However, the functional link between the different datasets was suggested by several indications. In Chapter 4, the hotspot mQTL on chromosome 2, including an mQTL for myo-inositol, co-located with a local-eQTL for myo-inositol phosphatase (MIPS2). In addition, I found that different sets of metabolites were significantly correlated with seed phenotypic traits in response the different maternal environments. In Chapter 5, changes in eQTL profiles under HT were observed for genes involved in the TCA cycle, which coincided with a metabolic shift for the same pathway under the same conditions (Chapter 4). Data mining remains an arduous task and therefore there is an evident need for tools to assist this endeavour. During my PhD, the AraQTL workbench was developed which provides a user-friendly interface for the navigation through the integrated datasets (Nijveen et al. 2017). The development of statistical methods will enhance the integration of large-scale biological data sets into relevant biological networks that will eventually lead to new biological insights (Chapter 6).

An instrumental tool for these gene discovery approaches is the availability of prior information. In Chapters 3, 4 and 5, available gene annotation was used to identify candidate genes from transcriptomic to metabolomics and allowed the identification of over-represented gene ontology terms and metabolic pathways. In this direction, further efforts to increase metabolite annotation will contribute to a better representation of underlying metabolic networks. Years of research on Arabidopsis have provided comprehensive gene annotation information that can be investigated by identifying correlation across the omics data types. This will certainly uncover many more links between the different biological levels (Figure 1). In the last decades, the unprecedented growth in type, size and complexity of the biological datasets has brought along new challenges for data integration. Adjustments to this data overload require the development of advanced data integration methods, to tackle the complexity and high dimensionality of the multi-level and multi-environment datasets (Chapter 6). Multivariate analyses are now accessible and open the way towards omics data integration (Rohart et al. 2017).

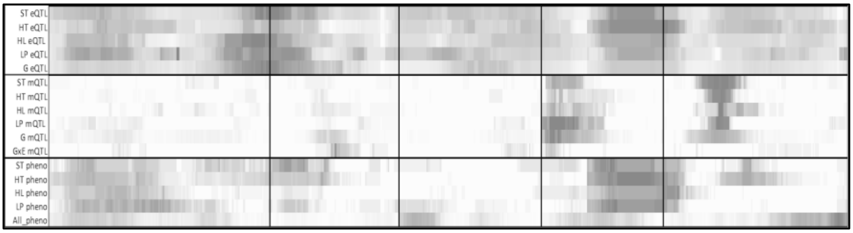


Figure 1. Heatmap representing the frequency of QTLs identified along the genome for the different biological levels and under the different conditions, ST: standard, HT: high temperature, HL: high light, LP: low phosphate. The five vertical blocks represent the five Arabidopsis chromosomes. The three horizontal blocks represent eQTLs, mQTLs and phenotypic QTLs respectively.

Further considerations

Transcript and metabolite accumulation, investigated in this thesis, are not the only regulatory levels affecting the phenotype of an individual. The vast field of omics can be further explored and the combined use of structural genomic, proteomic, translatomics and epigenetic analyses is expected to be required to gain a full understanding of causes of phenotypic variation. To gain a better understanding of the mechanisms responsible for seed performance, the different regulatory levels that lead to the phenotype need to be assessed by integrating these 'omics' approaches.

Fine-tuning environmental cues

The response to the maternal environment can be spatially and temporally resolved. In this study, we investigated the effect of the maternal environment in dry mature seeds, reflecting changes that occurred during seed development in response to environmental cues. It would be interesting to dissect these effects, by applying the stress at different stages of seed development, to identify the most sensitive stages or to investigate changes in the metabolome and transcriptome during seed development. Additionally, since seeds are composed of several tissues, including maternal tissues, identifying the sensitivity of the separate tissues to environmental cues would provide deeper insight into the nature of the response. This approach can also be done at the cellular level. A recent study used reporter constructs and a digital single-cell atlas of the Arabidopsis embryonic radicle to investigate the spatial distribution of hormone responsive components in response to temperature variation (Topham et al. 2017).

Leveraging the power of RNA-seq data

To take a step further in the understanding of the molecular regulation of complex traits, it would be interesting to further exploit the potential of the generated RNA-seq data. The sensitivity of the RNA-seq technology for gene expression quantification provides the advantage to distinguish known as well as new splicing forms and thus provide insights into the effect of the maternal environment on alternative splicing events. The splicing machinery contributes to the re-arrangement of introns and exons creating the opportunity for the mRNA transcripts to be translated into different proteins. In Arabidopsis, alternative splicing is widespread with 42% up to 61% of intron containing genes exhibiting alternative splice isoforms (Filichkin et al. 2010; Reddy et al. 2013; Laloum et al. 2018). These splicing events represent an important post-transcriptional regulatory mechanism in response to different environmental conditions (Laloum et al. 2018). In seeds, several splicing forms for genes involved in developmental processes have been reported. For instance, the two variants of PIF6 expressed during seed development showed opposite effects on the regulation of seed dormancy (Penfield et al. 2010). I expect that changes in splicing machinery would also occur in seeds in a genotype-by-environment fashion.

Another application of RNA-seq is the study of allele-specific expression (ASE). ASE uses fully heterozygous F1 background to assess the differential transcript abundance in an allelic specific manner. In an eQTL approach, the local or distant type of regulation of the eQTL is estimated based on genomic distance of the detected eQTL with the physical position of the gene, while ASE can reveal true cis eQTLs. Several studies have reported little overlap between ASE and local eQTLs, suggesting that local eQTLs might actually often be local trans-acting eQTLs (Hasin-Brumshtein et al. 2014). The differences between the two types of eQTL approaches might thus facilitate sorting local eQTLs into true cis and trans eQTLs. During my PhD study, RNA-seq data for F1 Sha x Bay-0 seed embryos were generated and further efforts are needed to compare the ASE and eQTL results.

RNA-seq can also be used to study long non coding RNAs (lncRNAs). These lncRNAs have gained attention in the last years as a potential new layer of biological regulation. This class of transcripts has been suggested to play a role as regulator of transcriptional control in response to stress (Nejat and Mantri 2018). This was recently demonstrated by a study showing that the regulation of seed dormancy by FLC in response to temperature occurs through the action the FLC antisense lncRNA COOLAIR (Chen and Penfield 2018). Further investigations will contribute to our understanding of the range of actions of these lncRNAs.

Post-transcriptional regulation

Some seed mRNAs produced during seed development are stored in the dry seed. Upon seed imbibition, the stored mRNAs undergo translation. The importance of these mRNA has been demonstrated by the indispensable commitment of translation, while transcription can be inhibited without affecting germination (Rajjou et al. 2004). Since gene expression can be regulated at the point of mRNA translation, it would also be interesting to investigate the dynamics of the 'translational status' of the transcriptome in response to the different maternal environments. Translatomics studies exploit genome-wide polysome occupancy on the mRNA as an indicator of mRNA translation. In seeds, extensive translational dynamics has been observed during seed imbibition and seed germination in Arabidopsis (Bai et al. 2017) indicating another layer of regulation in respect to seed performance. Further insights could be gained from proteomics studies and in this respect it would be interesting to investigate changes at the proteome level. However, proteomic studies remain scarce because of the limited output and technical limitations as compared to the metabolomic and transcriptomic approaches. Using big data approaches, methods are being developed to predict protein structure (Soeding 2017). Protein structure often determines its function. Thus, the information derived from predicted protein structure could in turn be used for proteomic QTL mapping and complete the picture together with eQTL, mQTL and in the future maybe translatomics QTL data.

Epigenetic regulation

The role of epigenetics in the regulation of phenotypic plasticity is attractive, since epigenetic modifications are more versatile than DNA sequence variation and thus also reversible (Kooke et al. 2015). It is known that epigenetic regulation proceeds during the seed life span (Wollmann and Berger 2012). In plants, more than 130 gene encoding proteins involved in epigenetic regulation have been identified (Pikaard and Mittelsten Scheid 2014). In this study, many of these genes (~50%) were found differentially expressed in the dry seeds of the parental lines, Bay-0 and Sha, grown in the different environments. This indicates that epigenetic regulation is also likely to take place in mediating environmental cues. It would thus be interesting to pursue investigations in the direction of the effect of environmental signals perceived during seed maturation on epigenetic mechanisms.

From model to crop

An instrumental tool for gene discovery approaches is the availability of prior information which facilitates the validation of the findings. In chapter 3, 4 and 5, gene annotation enabled the identification of candidate genes and allowed the identification of over-represented gene ontology terms and metabolic pathways. In this direction, further efforts on increasing metabolite annotation will contribute to a better representation of underlying metabolic networks. Years of research on Arabidopsis have provided comprehensive gene annotation information which can be queried through several databases, such as TAIR. This provides a clear advantage for using Arabidopsis as a model for proof-of-concept approaches. Nonetheless, the increasing availability of sequenced genomes and other resources for other species will enlarge their toolbox. Genetical ~omics approaches have most often been applied to model species for which the availability of molecular and genetic data facilitates the approach and the validation of the findings (Rowe et al. 2008; Keurentjes et al. 2006; Morreel et al. 2006; Keurentjes et al. 2007b; Terpstra et al. 2010). However, this approach is not limited to model species and has also been applied in several economically important species as, among others (Joosen et al. 2009), melon (Galpaz et al. 2018) and lettuce (Harper et al. 2012). Undeniably, the increasing power of next generation sequencing will unlock genomic sequence information for a large number of species and increase regulatory insights gained from identification of local and distant eQTLs.

Making sense of the data at the system level remains however largely driven by prior knowledge and thus validation of the findings through physiological and wet lab experiments remains essential.

Concluding remarks

In this thesis, I showed that the maternal environment plays an important role in modulating the genetic basis of seed performance. The combination of genetic and omics approaches provided insight into changes at the molecular level. The different datasets generated provide a basis for many opportunities for further research. The influence of the maternal environment is largely acknowledged, although limited studies have identified the mechanisms mediating these effects. The integration of the data generated in this thesis will provide deeper insights of the molecular networks underlying seed phenotypic plasticity. The last section of the discussion suggests also lines of research as follow-up of this work to ultimately lead to a comprehensive understanding of molecular mechanisms, the function of the genes and their biological role in the control of seed quality aspects.

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Summary

Seed traits are largely influenced by the maternal environment and differences across genotypes in the response to the maternal environment suggest significant genotype-by-environment interactions. Genetic variation for such a response has been reported, however only a few studies have investigated the genetic basis of seed traits in response to the maternal environment. This thesis aimed at providing an overview of changes occurring at the phenotypic and molecular level in response to different maternal environments. This plasticity was investigated at the genetic level in a quantitative trait locus (QTL) x environment (E) approach. The results obtained, the tools developed and insights gained in this thesis are briefly summarized below.

In **Chapter 1**, I provide a general introduction to this thesis and review current knowledge on seed quality and resources available to get a better understanding of the molecular networks controlling seed quality traits with an emphasis on the influence of the maternal environment.

In **Chapter 2** we used RNA-seq data from the parental lines and recombinant inbred lines (RILs) of an Arabidopsis Bay-0 x Sha population to identify sequence polymorphisms. The filtering and binning of the detected single-nucleotide polymorphisms (SNPs) resulted in 1059 genetic markers. These markers were used to establish a new genetic map for the population. The new saturated genetic map was compared to two previous genetic maps and it showed an increased mapping resolution. We showed that RNA-seq data can effectively be used to derive new markers and at the same time for eQTL analysis (**Chapter 5**). This new map was then used in **Chapter 3**, **4** and **5** to investigate the genetic basis of dry seed phenotypic, metabolic and transcriptomic changes in response to different maternal environments.

In **Chapter 3** the performance of seeds from the parental lines and RILs grown under four different maternal environments (standard (ST), high temperature (HT), high light (HL) and low phosphate (LP)) was assessed under several germination conditions. Significant interactions were observed between genotype, maternal and germination conditions. We identified a large number of QTLs which displayed significant QTL-by-environment interactions. Overall, this study showed that the maternal environment plays an important role in the genetic control of seed performance. We further used heterogeneous inbred families (HIFs) to validate a QTL specific for the high temperature maternal environment. In addition, we used expression data for the parental lines to mine for potential candidate genes in the region of the QTL. *Phytochrome A* (*PHYA*) was found specifically differentially expressed between Bay-0 and Sha under high temperature. Its known role in the control of germination and in mediating environmental cues highlighted it as an interesting candidate for this QTL. Using this approach we showed that expression data can be used to efficiently narrow down the number of candidate causal genes for QTLs.

In the following chapters, we investigated changes in the dry seed metabolome and transcriptome in response to the maternal environment.

In **Chapter 4** we explored the dry seed primary metabolome of the parental and RILs in a generalized genetical genomics (GGG) design. G x E interactions were investigated using correlation network analysis which revealed condition specific and coordinated metabolic changes. These genetically driven changes were linked to mQTLs. A novel mQTL hotspot was found on chromosome 2. This hotspot indicated a high level of genetic control over carbohydrate metabolism, with changes in the raffinose oligosaccharides families and TCA cycle intermediates in the dry seed metabolism in response to stress. We identified *MYO-INOSITOL PHOSPHATASE 2 (MIPS2)* as a strong candidate causal gene for the QTL hotspot involved in myo-inositol synthesis. Overall, the main mQTL hotspots identified showed limited overlap with the clusters of phenotypic QTLs identified in **Chapter 3**. However, the maternal environment resulted in differences in correlation between phenotypes and metabolites, suggesting a relation between seed metabolome changes and phenotypic expression in response to the maternal environment.

Using the same GGG design as in Chapter 4, the transcriptome profiling of the mature dry seeds of parental lines and RILs was performed by RNA-seq. The further analysis of this RNA-seq data is described in Chapter 5. Significant G x E interaction was observed for the transcripts in the parental lines. eQTL analysis was performed on each RIL subsets defined by the GGG design. The eQTL features were compared across conditions and we found that *local* eQTLs were largely consistent, whereas *distant* eQTLs were more versatile across conditions. We noted that the eQTLs, in general, were identified for the same set of genes across conditions. In addition we found that many eQTL hotspots were specific for a certain condition. GO enrichment analysis for the set of

genes with an *distant* eQTL within these hotspots revealed over-represented biological processes, which could be linked to seed phenotypic differences.

In **Chapter 6**, I provide a survey of the literature and discuss the use of co-expression networks as tool to complement genomics studies and for faster discovery of genetic variants. The application of this method is promising for the integration and visualization of high-dimensional datasets as the one generated in this thesis.

Finally, in **Chapter 7**, I integrate and discuss the findings of this research with a particular emphasis on the influence of the maternal environment, the mechanisms and implications. I also discuss new possibilities for research as well as the use of additional data to get a more comprehensive understanding of molecular mechanisms underlying seed traits plasticity in the future.

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Environment matters and I would like to emphasize it one more time in this last part of my thesis. I had a great time during my PhD and I would like to thank everyone that was part of my life and created such a positive working environment for the achievement of this thesis.

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successful career and I am sure our paths will cross again in this small world and even smaller country (NL). **Bea**, you are a big-hearted person. I admire your kindness and attitude towards life, living life like there's no tomorrow. Thank you for everything you have done for me, especially changing my mind off work with great talks and the trip to Alicante. I would not know how this all would have ended, without you offering spontaneously your help for printing my thesis. Many, many thanks again.

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About the author

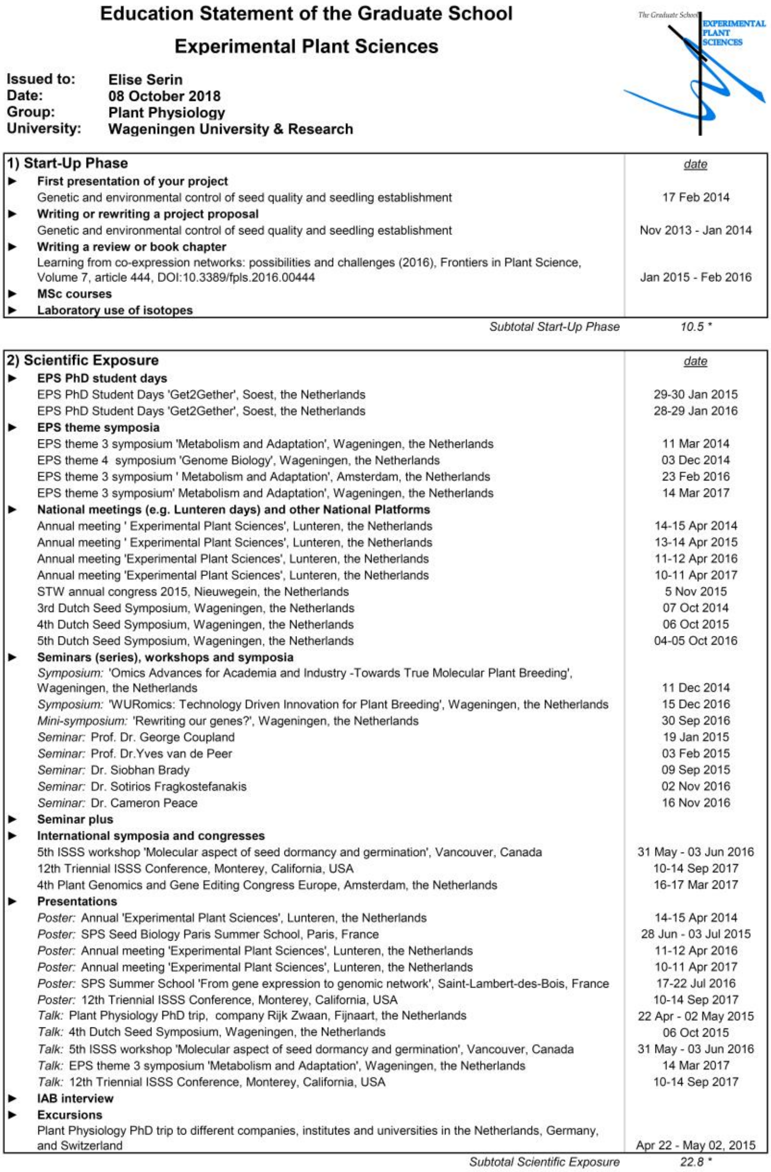
Elise Anna Renee SERIN was born on 29th July 1989 in Angers, France. She started her BSc studies in the field of biology, ecology and population genetics in 2007 at the University of Orléans, France. A summer internship at the University of Agriculture in Florence, Italy, revealed her interest in molecular biology and genetic diversity in plants. She decided that same year to join the Jagiellonian University in Kraków, Poland and completed a first year of master in plant biotechnologies. Back to France, she pursued a master in Integrative Plant Biology in Angers and in Rennes with a specialization in quantitative genetics. She did her master thesis at the INRA in Orléans where she investigated the genetic architecture of wood property traits in Poplar using quantitative trait loci mapping approaches. She graduated in 2013 and started a PhD the same year at the Wageningen University, the Netherlands. During her PhD at the laboratory of plant physiology, she explored the influence of the maturation environment on seed performance using genetical genomics approaches on the model plant Arabidopsis. The results of her PhD are described in this thesis.

Publications

Serin , E. A. R., Nijveen, H., Hilhorst, H. W., & Ligterink, W. (2016). Learning from co-expression networks: possibilities and challenges. *Frontiers in plant science, 7, 444*.

Serin, E. A. R., Snoek, L. B., Nijveen, H., Willems, L. A., Jiménez-Gómez, J. M., Hilhorst, H. W., & Ligterink, W. (2017). Construction of a High-Density Genetic Map from RNA-Seq Data for an Arabidopsis Bay-0× Shahdara RIL Population. *Frontiers in genetics, 8, 201*

Education Statement



Education Statement of the Graduate School Experimental Plant Sciences

The Graduate School EXPERIMENTAL PLANT SCIENCES

Issued to:	**Elise Serin**
Date:	**08 October 2018**
Group:	**Plant Physiology**
University:	**Wageningen University & Research**

1) Start-Up Phase	*date*
► **First presentation of your project**	
Genetic and environmental control of seed quality and seedling establishment	17 Feb 2014
► **Writing or rewriting a project proposal**	
Genetic and environmental control of seed quality and seedling establishment	Nov 2013 - Jan 2014
► **Writing a review or book chapter**	
Learning from co-expression networks: possibilities and challenges (2016), Frontiers in Plant Science, Volume 7, article 444, DOI:10.3389/fpls.2016.00444	Jan 2015 - Feb 2016
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-Up Phase	*10.5 **

2) Scientific Exposure	*date*
► **EPS PhD student days**	
EPS PhD Student Days 'Get2Gether', Soest, the Netherlands	29-30 Jan 2015
EPS PhD Student Days 'Get2Gether', Soest, the Netherlands	28-29 Jan 2016
► **EPS theme symposia**	
EPS theme 3 symposium 'Metabolism and Adaptation', Wageningen, the Netherlands	11 Mar 2014
EPS theme 4 symposium 'Genome Biology', Wageningen, the Netherlands	03 Dec 2014
EPS theme 3 symposium ' Metabolism and Adaptation', Amsterdam, the Netherlands	23 Feb 2016
EPS theme 3 symposium' Metabolism and Adaptation', Wageningen, the Netherlands	14 Mar 2017
► **National meetings (e.g. Lunteren days) and other National Platforms**	
Annual meeting ' Experimental Plant Sciences', Lunteren, the Netherlands	14-15 Apr 2014
Annual meeting ' Experimental Plant Sciences', Lunteren, the Netherlands	13-14 Apr 2015
Annual meeting 'Experimental Plant Sciences', Lunteren, the Netherlands	11-12 Apr 2016
Annual meeting 'Experimental Plant Sciences', Lunteren, the Netherlands	10-11 Apr 2017
STW annual congress 2015, Nieuwegein, the Netherlands	5 Nov 2015
3rd Dutch Seed Symposium, Wageningen, the Netherlands	07 Oct 2014
4th Dutch Seed Symposium, Wageningen, the Netherlands	06 Oct 2015
5th Dutch Seed Symposium, Wageningen, the Netherlands	04-05 Oct 2016
► **Seminars (series), workshops and symposia**	
Symposium: 'Omics Advances for Academia and Industry -Towards True Molecular Plant Breeding', Wageningen, the Netherlands	11 Dec 2014
Symposium: 'WURomics: Technology Driven Innovation for Plant Breeding', Wageningen, the Netherlands	15 Dec 2016
Mini-symposium: 'Rewriting our genes?', Wageningen, the Netherlands	30 Sep 2016
Seminar: Prof. Dr. George Coupland	19 Jan 2015
Seminar: Prof. Dr.Yves van de Peer	03 Feb 2015
Seminar: Dr. Siobhan Brady	09 Sep 2015
Seminar: Dr. Sotirios Fragkostefanakis	02 Nov 2016
Seminar: Dr. Cameron Peace	16 Nov 2016
► **Seminar plus**	
► **International symposia and congresses**	
5th ISSS workshop 'Molecular aspect of seed dormancy and germination', Vancouver, Canada	31 May - 03 Jun 2016
12th Triennial ISSS Conference, Monterey, California, USA	10-14 Sep 2017
4th Plant Genomics and Gene Editing Congress Europe, Amsterdam, the Netherlands	16-17 Mar 2017
► **Presentations**	
Poster: Annual 'Experimental Plant Sciences', Lunteren, the Netherlands	14-15 Apr 2014
Poster: SPS Seed Biology Paris Summer School, Paris, France	28 Jun - 03 Jul 2015
Poster: Annual meeting 'Experimental Plant Sciences', Lunteren, the Netherlands	11-12 Apr 2016
Poster: Annual meeting 'Experimental Plant Sciences', Lunteren, the Netherlands	10-11 Apr 2017
Poster: SPS Summer School 'From gene expression to genomic network', Saint-Lambert-des-Bois, France	17-22 Jul 2016
Poster: 12th Triennial ISSS Conference, Monterey, California, USA	10-14 Sep 2017
Talk: Plant Physiology PhD trip, company Rijk Zwaan, Fijnaart, the Netherlands	22 Apr - 02 May 2015
Talk: 4th Dutch Seed Symposium, Wageningen, the Netherlands	06 Oct 2015
Talk: 5th ISSS workshop 'Molecular aspect of seed dormancy and germination', Vancouver, Canada	31 May - 03 Jun 2016
Talk: EPS theme 3 symposium 'Metabolism and Adaptation', Wageningen, the Netherlands	14 Mar 2017
Talk: 12th Triennial ISSS Conference, Monterey, California, USA	10-14 Sep 2017
► **IAB interview**	
► **Excursions**	
Plant Physiology PhD trip to different companies, institutes and universities in the Netherlands, Germany, and Switzerland	Apr 22 - May 02, 2015
Subtotal Scientific Exposure	*22.8 **

3) In-Depth Studies	*date*
► **EPS courses or other PhD courses**	
An Introduction to Mass Spectrometry-based Plant Metabolomics, Wageningen, the Netherlands	09-13 Dec 2013
System Biology: Statistical analysis of ~omics data, Wageningen, the Netherlands	15-19 Dec 2014
SPS Seed Biology Paris Summer School, Paris, France	28 Jun - 03 Jul 2015
Genotype by environment interaction, uniformity and stability, Wageningen, the Netherlands	19-23 Oct 2015
SPS Summer School 'From gene expression to genomic network', Saint-Lambert-des-Bois, France	17-22 Jul 2016
12th International Master Class on Seed Technology, Wageningen, the Netherlands	11-15 Oct 2015
Plant Phenotyping Workshop, Aberystwyth, UK	09-13 Mar 2015
► **Journal club**	
Participation in the Plant Physiology journal club	2013-2017
► **Individual research training**	
Subtotal In-Depth Studies	*12.4 **

4) Personal Development	*date*
► **Skill training courses**	
ExPectationS Day 'Communication and Ethics in Science', Wageningen, the Netherlands	28 Mar 2014
PhD competence assessment, Wageningen, the Netherlands	Mar - Apr 2015
Wageningen Graduate Schools PhD workshop Carousel, Wageningen, the Netherlands	17 Apr 2015
Data management, Wageningen, the Netherlands	05 Oct 2015
Brain training, Wageningen, the Netherlands	16 Dec 2015
Scientific writing, Wageningen, the Netherlands	Nov - Dec 2016
Career perspectives, Wageningen, the Netherlands	Nov - Dec 2017
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*4.9 **

TOTAL NUMBER OF CREDIT POINTS	***50.6 ****

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits.

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

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