Genetic analysis of the influence of the maternal environment on tomato seed and seedling performance

Nafiseh Geshnizjani

Propositions

1. Although the maternal environment profoundly influences seed- and seedling quality, still genetic background plays a major role.
   (this thesis).

2. The induction of secondary dormancy in tomato seeds at high temperature is a physiological factor within the embryo.
   (this thesis).

3. Quick technologies to fix complex problems inherently lack a *safe-by-design* concept.

4. To solve world hunger, focusing on bulk food waste that we produce must have priority over investing in GM food production.

5. The claim that "science has no borders" is awkward when scientists are limited by geographical borders.

6. Disadvantages of the social media have increased so much that we should reconsider using them.

Propositions belonging to the thesis entitled:

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Contents

Chapter 1

General Introduction

In general, ecological studies show that high genetic heterogeneity in plants should be maintained in agricultural systems. Plant diversity plays a vital role in sustaining ecosystems (Tilman et al., 1997; Loreau, 1998; Litrico and Violle, 2015). On the contrary, most breeding programs focus on monocultures under certain environmental conditions with the main aim to enhance key agronomic characteristics, such as seed performance, biomass and resistance to diseases. However, such an approach might lead to the loss of interaction traits (i.e. key genes in response to modified environments). From a scientific point of view, however, dissecting the underlying (molecular) mechanisms of response of a certain trait to various environmental conditions is more interesting. Further incorporation of those mechanisms in breeding programs will to some extent combine the genetic heterogeneity and the aims of breeding for agricultural traits (Litrico and Violle, 2015).

Seeds are the most important units in the life-cycle of plants for survival. Seeds as dispersal units are used as propagule for the plants which are able to survive adverse conditions and establish the next generation (Bentsink and Koornneef, 2008). In addition to their biological importance for plants, seeds also are used for humans as a source of medicine, oil, feed and food. Furthermore, seeds are considered as an important product across the world and thus producing seeds with high quality and performance is of a great interest for seed producers and breeders.

Seed and seedling quality

The exact definition of seed quality is dependent on the final use of the seed. For example, if seeds are used as a source of food then their oil, protein and starch content become important (Nesi et al., 2008). From a farmer's and producer's point of view high-quality seeds are also those that germinate uniformly and produce healthy seedlings in a wide range of environments, including optimal and sub-optimal conditions (Foolad et al., 2007).

Seed quality related to its role as propagule is a complex trait determined by many different attributes including germination characteristics, seed vigour, storability, genetic purity and lack of dormancy and physical damages (Dickson, 1980; Hilhorst and Toorop, 1997; Hilhorst and Koornneef, 2007; Hilhorst et al., 2010).

The environment under which seeds develop and mature has a huge influence on seed performance since seed quality is mainly obtained at the position where the parent plants grow, and mature their seeds (Delouche, 1980). This influence of the maternal environment probably is an adaptation mechanism of the plants to diverse environments to improve their fitness by the production of seeds that produce healthy vigorous seedlings at the optimal time and with

optimal growth characteristics finally resulting in higher yields (Huang et al., 2010). Thus, many of the parameters determining seed quality such as germinability, dormancy, desiccation tolerance and seed vigour are acquired during seed maturation (Harada, 1997). Among the factors defining seed performance, seed germination characteristics, seed size and subsequently seedling vigour are very important and decisive (Panthee et al., 2005; Foolad et al., 2007).

Seed size related to seedling vigour

An important attribute of seed quality is seed size. It has been reported that during evolution, seed size is a trait that has shown remarkable changes. Large variation was observed for seed size within the different species and populations to the extent that seed size is regarded as the most variable trait of plants (Orsi and Tanksley, 2009). Such a big variation plays an important role in the adaptation of plants to different environments but the reason for that is not exactly clear (Geritz et al., 1999). Most of the domesticated crops (e.g. tomato, soybean, maize and sunflower) produce relatively bigger and heavier seeds in comparison with their wild ancestors. Such a domesticated fitness may have been created by the selection of larger seeds in order to increase (seed) crop yields (Broich and Palmer, 1980; Doganlar et al., 2000; Burke et al., 2002; Fuller, 2007). The production of bigger seeds in a domesticated crop such as tomato may also be related to the positive correlation between seed size and fruit size. Therefore, larger seeds were mostly the results of the selection for bigger fruits which is usually considered a desirable trait (Goldman et al., 1995).

From a seed producer's point of view the ability of the seeds to produce seedlings with good establishment and generate usable plants is one of the major attributes defining seed quality (Ligterink et al., 2012). Seedling vigour has a profound effect on the establishment of seedlings and, subsequently, on the production of useful crops. Therefore seedling vigour may be considered as an important determinant of crop production which is greatly influenced by seed size and seed vigour (Heydecker, 1977; TeKrony and Egli, 1991). The correlation between seed size and seedling establishment has been reported frequently. It has been hypothesized that both in optimal and non-optimal environments larger seeds can survive and establish seedlings better than the smaller ones (Wulff, 1986; Lee and Fenner, 1989; Jurado and Westoby, 1992; Leishman and Westoby, 1994). Partly due to the importance of seed size in determining seed quality, several studies have identified many loci regulating seed size and weight in different species, such as Arabidopsis, rice, soybean and tomato (Alonso-Blanco et al., 1999; Liu et al., 2007; Shomura et al., 2008; Orsi and Tanksley, 2009; Joosen et al., 2012; Khan et al., 2012). Despite these studies, the genetic and molecular basis underlying variation for seed size are still not broadly understood.

Seed germination characteristics

Despite the fact that seed quality is often defined by the germination percentage after a specific period, this factor can hardly describe all aspects of seed quality. Therefore, determining the whole cumulative germination curve and, from this, quantifying the attributes such as onset (t_{10}), rate (t_{50}) and uniformity (U_{8416}) of germination is essential to acquire a more comprehensive understanding of seed quality. Thus, one of the important tools determining the performance of a seed lot is the evaluation of seed germination by assessing different germination characteristics. The stage of seed germination plays a vital role in the plant life cycle of tomato and is strongly affected by various environmental stress factors such as drought, high temperature and salt (Foolad and Chen, 1999; Foolad et al., 2003). The completion of seed germination is defined as the protrusion of the radical through the endosperm and seed coat (Bewley, 1997). It has been previously shown that seed germination is largely dependent on the composition of seed reserves and the balanced level of several hormones including abscisic acid (ABA) and gibberellic acid (GA) (Penfield and King, 2009). Although seed size and/or weight often show significant correlation with seed quality and seedling establishment, they appeared not to be related to the seed germination characteristics under normal maternal conditions for tomato (Fenner, 1991; Khan et al., 2012).

Thermo-inhibition and dormancy

Germination begins with the absorbtion of water by seeds (seed imbibition), is followed by the activation of metabolism and the onset of growth related processes, and, in tomato, ends with radical protrusion through the endosperm and seed coat (Bewley et al., 2012). In response to undesirable environments some seeds postpone their germination until favourable conditions are met and thus show a physiological response which is called dormancy (Hilhorst, 2007). One of the environmental factors with a profound effect on seed germination is temperature, which influences water absorption and activity of metabolic pathways (Weitbrecht et al., 2011; Bewley et al., 2012; Franks et al., 2014). Elevating the environmental temperature has a negative influence on agricultural production (Walck et al., 2011) and its negative effects are expected to become more severe in the future, due to global warming. Therefore, it is essential to investigate the physiological and molecular mechanisms underlying the effect of temperature on seed quality and, more specifically, on seed thermo- dormancy.

Seeds exposed to elevated temperatures during imbibition may display physiological responses such as thermo-inhibition or thermo-dormancy. In the case of thermo-inhibition, seeds do not germinate at high temperature, but germinate immediately upon exposure to optimal temperature. In the case of thermo-dormancy seeds also do not germinate at high temperature, but also not at a subsequent optimal germination temperature (Huo and Bradford, 2015).

Environmental (light and nitrate) and hormonal (GA, ABA and ethylene) factors may influence thermo-inhibition and thermo-dormancy of seeds (Gonai et al., 2004; Kępczyński et al., 2006; Toh et al., 2008). There are many studies indicating that genes functioning in ABA-biosynthesis such as 9-cis-epoxycarotenoid dioxygenase (*NCEDs*) and other *ABA DEFICIENT genes* (*ABAs*) and GA biosynthesis (*GA3ox1*, *GA3ox2* and *GA2ox1*) have a role in thermo-induced dormancy and inhibition of seed germination (Toyomasu et al., 1998; Iuchi et al., 2001). GA and ABA inversely regulate seed germination, in the way that when seed germination is inhibited the regulation of genes involved in GA biosynthesis are suppressed while the ABA biosynthetic pathway genes are up-regulated (Toh et al., 2008). It has also been reported that *FUSCA3* (*FUS3*), which is a B3-domain transcription factor, affects germination of seeds in response to high temperature by increasing and decreasing ABA and GA biosynthesis, respectively (Nambara et al., 2000; Curaba et al., 2004; Holdsworth et al., 2008). Regarding the importance of global warming and its effects on seed germination and final yield of agricultural production, it is imperative to detect more regulators of seed germination and dormancy in response to high temperature.

In general, seed quality is a complex trait regulated by several mechanisms. Although many studies have focused on the variation of seed quality, the mechanism(s) underlying natural variation for seed quality traits and their interaction with the environment are still largely unknown. In this thesis, I attempt to dissect the genetic basis of tomato seed quality by a combined study of physiology, genetics and genomics in relation to the maternal environment.

Natural variation in tomato seed and seedling quality

In many species a wide variation in several seed- and seeding quality traits has been observed, such as seed and seedling size and weight (Doganlar et al., 2000; Vandecasteele et al., 2011; Khan et al., 2012), seed dormancy (Koornneef et al., 2002; Bentsink et al., 2010), seed germination characteristics such as G_{max}, t_{10}, t_{50} and U_{8416} (Foolad et al., 2003; Foolad et al., 2007; Joosen et al., 2012; Kazmi et al., 2012) and thermo-inhibition (Argyris et al., 2008; Huo and Bradford, 2015). Detecting the genes affecting such important traits and their natural variation is essential because existing variation can be used for predicting and improving seed quality. One of the most powerful methods that often has been used for studying the molecular mechanisms underlying natural variation in plants is Quantitative Trait Loci (QTL) analysis. QTL analysis studies the linkage between genetic and phenotypic variation of traits in populations such as recombinant inbred line (RIL) population (Alonso-Blanco and Koornneef, 2000). RIL populations are immortal mapping populations consisting of several almost completely homozygous lines resulting from a cross between two parental lines and subsequent

selfing for several generations. QTLs represent the genomic regions that can explain the observed phenotypic variation and that regulate the analysed traits. Many studies have characterized QTLs affecting complex quantitative seed traits in different species (Alonso-Blanco et al., 2003; Clerkx et al., 2004; Salvi and Tuberosa, 2005; Joosen et al., 2012; Kazmi et al., 2012; Khan et al., 2012).

High quality seeds are those which show uniform and high germination percentage not only in optimal conditions but also under non-optimal germination conditions such as high and/or low temperature and osmotic stress (salt and osmoticum) (Foolad et al., 2007). Therefore, investigating the seed germination response under various germination conditions and determining the genetic basis regulating these responses may lead to the identification of the genes responsible for controlling seed germination. Several QTLs regulating seed germination responses have been detected in foregoing studies many of which were common among different stressful germination conditions (Dudley, 1993; Foolad et al., 2003; Foolad et al., 2007). Such co-locating QTLs may indicate that similar mechanisms are regulating seed germination under different conditions and, thus, detecting the responsible genes for better germination and then improving the seed germination responses in one condition may result in better germination responses in other conditions as well. Although many valuable studies have been carried out and several QTLs governing seed quality traits in different species have been characterized (Koornneef et al., 2002; Argyris et al., 2008; Joosen et al., 2012; Kazmi et al., 2012; Khan et al., 2012), more studies are needed to determine the effect of the maternal environment on the function of responsible genes for final seed performance.

Influence of the maternal environment on seed performance

In addition to the different factors such as physical, physiological, genetic, seed health, method of harvesting and storage of the seeds, the final quality of seeds is also influenced by the maternal environment (Rowse and Finch-Savage, 2003; Sperling et al., 2004). In general, the environment under which plants grow and seeds develop and mature is referred to as the maternal environment and it may affect the phenotype of the progenies (Donohue, 2009). Seeds can alter their phenotype (e.g. metabolite composition, seed size etc.) in response to the environment experienced by either themselves or their parents (maternal environmental effects) (Roach and Wulff, 1987; Sultan, 2000; Snell-Rood, 2013). The environment in which seeds germinate is considered to be a very important factor affecting seed performance. Indeed, the post dispersal environment (experienced by seeds themselves) is thought to be a more accurate predictor of seed quality as compared to the environment that maternal plants are exposed to (DeWitt et al., 1998). Hence, many studies investigated the genetic basis underlying the

response of germinating seeds to various optimal and sub-optimal germination conditions (Clerkx et al., 2004; Ren et al., 2010; Joosen et al., 2012; Kazmi et al., 2012; Khan et al., 2012). However, the environment perceived by the mother plant may predict subsequent responses of the progenies to future competitive conditions which are not yet in existence (Leverett et al., 2016). Therefore, investigating the genetic basis regulating the effects of maternal environments on seed quality is also necessary to improve and predict ultimate seed quality.

There are several reports indicating the influence of various maternal environmental factors such as temperature (Demir et al., 2004; Schmuths et al., 2006; He et al., 2014), photoperiod (Pourrat and Jacques, 1975; Gutterman, 2000; Munir, 2001) and nutrition (Alboresi et al., 2005; He et al., 2014) on final seed performance. For example, in some species including Arabidopsis (Munir, 2001) and *Chenopodium polyspermum* (Pourrat and Jacques, 1975) germinability of seeds that developed under a short day regime was higher than those which were produced under long days. Besides, in several species such as Arabidopsis (Kendall et al., 2011; Penfield and Springthorpe, 2012; He et al., 2014), *Amaranthus retroflexus*, wild oat (Fenner, 1991) and lettuce (Contreras et al., 2009) it has been shown that mother plants grown in warmer environments produce seeds with lower levels of dormancy.

As previously described, nutrient accumulation in the seeds and final levels of seed reserves may be one of the important indicators of seed quality. Accumulation of nutrients in the seeds is a complex process which is not only dependent on genetic but also on environmental factors (Papdi et al., 2009). One of the environmental factors greatly influencing seed reserves and subsequent seedling establishment is the nutritional status of the mother plant (Farhadi et al., 2014). Indeed, fertilizers supplied to maternal plants may alter seed quality traits such as dormancy in progenies. Nitrate, as the major source of nitrogen used in many metabolic pathways such as amino acid biosynthesis, is considered one of the most vital nutrients for plants (Urbanczyk-Wochniak and Fernie, 2004). It has been shown that in some species seeds developed and matured under higher dosage of nitrate may produce seedlings with higher shoot and root weight (Farhadi et al., 2014; Song et al., 2016). Moreover, in some species a higher nitrate regime applied to the mother plant led to the production of larger and heavier seeds (Fenner, 1991). The level and composition of the nutrient supply to the mother plant may also affect dormancy and germinability of the seeds. In several studies dosage of nitrate applied to the maternal plant showed a negative correlation with the level of dormancy in the progenies. For instance, in *Solanum lycopersicum* (Varis and George, 1985), *Nicotiana tabacum* (Thomas and Raper, 1979), *Sisymbrium officinale* (Bouwmeester et al., 1994) and Arabidopsis (Alboresi et al., 2005; He et al., 2014) low nitrate levels in the soil of the mother plant increased the dormancy of its seeds. Alboresi et al. (2005) argued that in Arabidopsis nitrate could decrease dormancy levels of the seeds by affecting hormone (ABA and/or GA) and/or other metabolite

levels. Modi and Cairns (1994) showed that deficiency of molybdenum in wheat mother plants resulted in the production of low dormant seeds. They argued that molybdenum could affect dormancy of the seeds possibly by modifying the seed nitrate content. Since molybdenum acts as a co-factor of nitrate reductase (Mendel and Hänsch, 2002), its deficiency will decrease the activity of nitrate reductase and thus nitrate is accumulated in the seeds, resulting in a lower level of dormancy (Modi and Cairns, 1994).

Phosphate is another important nutrient for plants, being an essential component of some vital molecules including nucleic acids, phospholipids and ATP (Schachtman et al., 1998; Urbanczyk-Wochniak and Fernie, 2004). Arabidopsis seeds from mother plants fertilized with higher levels of phosphate could germinate better under sub-optimal germination conditions (He et al., 2014). Despite the importance of maternal nutrient supply on final seed performance, so far only a few studies have been conducted to identify this effect.

In general, genetics of the seeds and the environment experienced by the mother plants profoundly affect final seed quality. Seed quality is however not only determined by genetic (G) and maternal environments (E), but also by their interaction (G×E).

Genetic by environment interaction

Plants and seeds can perceive their environments and thus engage mechanisms to enable them to face undesirable conditions (such as drought, high temperature, osmotic stress and lack of nutrients) and therefore to be less affected by these conditions. Due to climate changes many seed quality traits, such as seed size and/or weight and seed germination characteristics have been adversely influenced and eventually agricultural productivity and final yield have been reduced (Gornall et al., 2010; Singh et al., 2013). In order to cope with such sub-optimal conditions, plants use several adaptive mechanisms resulting in a significant phenotypic acclimation responses and changes in the plant's physiology and several molecular mechanisms. In many cases the changes due to acclimation varies from one genotype to another indicating genetic variation for the phenotypic acclimation. Such a variation within the genotypes suggests genotype by environment interactions (G×E) (El-soda et al., 2015). To obtain a comprehensive understanding of the effects of the seed maturation environment on seed quality and its regulating genetic mechanisms, G×E can be considered in QTL analysis (QTL by environment (QTL×E)). Testing the genotypes in various environments is needed for identifying G×E factors (van Eeuwijk et al., 2010). By considering G×E effects in genetic variation and QTL mapping, the statistical power of QTL analysis is enhanced and more QTLs are possibly detected (El-Soda et al., 2014). Regarding this effect, QTLs are classified in two categories: first, QTLs detected in all studied environments (regulating a phenotype regardless

of environment) counted as main QTLs; second, QTLs affecting the phenotype in a specific environment which are considered as environment-specific QTLs (El-Soda et al., 2014).

Generalized genetical genomics approach

Several QTLs have been detected for seed quality traits and many causal genes have been characterized and cloned (Salvi and Tuberosa, 2005). Fine mapping is a crucial step for plant breeding in which a regulating genomic region is minimized and putative causal genes are detected by reducing the number of candidate genes. Despite the importance of fine mapping and ultimate cloning of the responsible genes, it is very time-consuming and often not straight forward. This causes difficulties for mapping and characterizing the genes regulating the phenotypic traits such as seed and seedling quality which are complex traits controlled by multiple genes interacting with several environmental factors (Mackay, 2001; Mackay et al., 2009). In general, identifying the molecular mechanisms underlying the genetic variation is not straightforward since each gene is related to many other genes and pathways and thus many linkages between the phenotype and genotype are still unknown.

This problem may be partly overridden by applying an approach called genetical genomics, in which traditional QTL mapping is integrated with omics analysis such as proteomics (which detects the variation of protein abundance in a specific tissue or cell during normal or different sub-optimal conditions), transcriptomics and metabolomics (Jansen and Nap, 2001; Joosen et al., 2013). The variation of transcript abundance, which is measured by microarrays and, more recently, by RNA-sequencing, can be considered as a quantitative trait and may thus be subjected to linkage analysis to identify loci regulating the gene expression levels, which are known as expression QTLs (eQTLs). There are two different eQTLs regarding the location of the marker related to the eQTL and the physical position of the gene under study. When eQTLs are located far from the position of the gene under study they are classified as trans eQTLs. In contrast, the eQTLs which map to the position of the gene under study are considered as cis eQTLs which are known as good candidates for being putative responsible genes for a given trait (Wayne and McIntyre, 2002; Rockman and Kruglyak, 2006).

Metabolomics; dissecting seed and seedling quality

Plants are a rich source of metabolites which are involved in plant development, growth and performance (Binder, 2010). Metabolites, as the final product of the genetic and developmental environmental inputs, determine the plant's developmental status and performance (Kooke and Keurentjes, 2011). Metabolomics is one of the tools that can be applied for detecting, identifying and measuring a range of metabolites (Keurentjes et al., 2006; Verpoorte et al.,

2008). Metabolites may affect phenotypic traits more directly than the transcriptome and proteome.Due to the complexity of data analysis of protein samples, genetical proteomics studies are relatively scarce (Chevalier et al., 2004). Using network analysis of the metabolite contents and integrating that with the phenotypic traits may provide an overall map of metabolic changes and its relation to plant, fruit and/or seed quality traits (Toubiana et al., 2012).

Existing genetic variation is not limited to phenotypic traits such as seed and seedling quality traits. Metabolite content and -composition also show quantitative variation which is regulated by multiple genes and metabolic QTLs (mQTLs) (Windsor et al., 2005; Lisec et al., 2008; Schauer et al., 2008; Toubiana et al., 2012). Many QTL analyses have been conducted and many QTLs affecting metabolite level have been found (Schauer et al., 2006; Kazmi et al., 2017). In general, the quantitative variation of metabolites may have an influence on different phenotypic traits and a relationship between central metabolism and plant growth has been established (Keurentjes et al., 2007; Meyer et al., 2007). Although in several reports a strong correlation has been found between metabolites and phenotypic traits, a lack of overlap between metabolic and phenotypic QTLs has often been observed (Rowe et al., 2008). This might be due to the fact that each of the phenotypes is not correlated with a specific metabolite but with a group of metabolites and, thus, the final metabolic balance between groups of metabolites could affect phenotypic traits (Kazmi et al., 2017).

Approaches such as genetical genomics provide better and more in-depth understanding of the molecular basis regulating complex traits (Kliebenstein et al., 2006; Schauer et al., 2006; Ligterink et al., 2012). As described above, complex traits such as seed and seedling quality are greatly influenced by the environment. Since genetical genomics normally does not take environmental factors into account, an advanced approach is needed to obtain a comprehensive understanding of the regulation of molecular networks that control the phenotypic variation. Therefore, a new experimental setup was designed which is called generalized genetical genomics (GGG) in which environmental cues can be incorporated in the often expensive omics analysis (Li et al., 2008). This approach determines the genetic and environmental effects on detected QTLs by dividing a RIL population into new subpopulations and sampling each of them to a different interesting environment.

Integration of omics analysis

Despite the vast amount of QTLs that have been detected for seed quality, only a few genes have been characterized and functionalized so far (Luo et al., 2005; Bentsink et al., 2006; Song et al., 2007). This could be related to the fact that fine mapping and cloning of the causal genes for the QTLs is time consuming and often more complex than anticipated and thus traditional

QTL analysis may be considered as a less informative and useful technique. Therefore, more advanced approaches are required to provide new information and increase our knowledge on the responses of plants to environmental conditions and mechanisms controlling those responses. Integrating 'omics' data from transcriptomics, proteomics and metabolomics into the genetic dataset could be one of the advanced approaches by which the large number of QTLs may be reduced to a few manageable common QTLs.

Using a combination of these three omics methods may provide a comprehensive dissection of a given biological system at the level of transcription and translation (Tan et al., 2009). In order to analyse the complex correlation of these omics traits, the genetic analysis should be performed in well-structured populations such as a RIL population and such a quantitative analysis should then be subjected to a combined correlation and QTL analysis (Keurentjes et al., 2008). In general, integrating different omics datasets provides beneficial information of how plants cope with and finally adapt to the various environments (Schwarz et al., 2011).

Scope of thesis

The success of growing tomato, like every other agricultural product, depends on many different factors, such as the quality of the soil, water supply, climatic conditions (temperature, light and moisture) and the quality of the seeds that were used. One of the most important factors that affect the success of germination, seedling establishment, growth, development and final yield of crops is seed quality. Quality of seeds is strongly affected by their genetic make-up, the environment during seed development and maturation and also the interaction between genome and environment. Many seed quality traits (e.g. dormancy, germination percentage, rate and uniformity) are quantitative traits that are controlled by many genes. So far, several Quantitative Trait Loci (QTL) analyses have been performed to investigate genetic aspects underlying seed quality in various species including barley, Arabidopsis and tomato (Li et al., 2005; Joosen et al., 2012; Kazmi et al., 2012). Changes in maternal environmental factors are sensed by plants and can be reflected in the performance of their progenies (seeds). Such responses of seeds to the environment of mother plants are probably controlled by several mechanisms which are still largely unknown. The objective of this thesis is to exploit the natural variation of seed and seedling quality by using 'omics' approaches. In addition, I attempt to identify the molecular mechanisms that are involved in the acquisition of seed quality and especially also to investigate how these mechanisms are controlled by adverse maternal nutritional conditions.

In **Chapter 1** I explain the definition and different aspects of seed and seedling quality and the factors affecting them, emphasizing the importance of different maternal environmental factors.

Furthermore, I explore the application of natural variation to detect QTLs for seed quality traits. The genetical genomics approach in several species and its deficiency is described and the alternative GGG approach is introduced. The integration of 'omics' analysis is also suggested and is considered as an advanced approach to reduce the number of relevant QTLs and to facilitate the identification of the causal genes.

The goal of **Chapter 2** is to investigate how different nutritional environments of the mother plant influence the quality of the progenies of different genotypes. I study two different tomato genotypes (*Solanum lycopersicum* (cv. Moneymaker) and *Solanum pimpinellifolium*) under different levels of phosphate and nitrate. The extensive phenotyping of the harvested seeds is done by performing seed germination tests at both optimal and sub-optimal conditions. In order to see whether phenotypic changes of seed and seedling traits are related to the changes of the metabolite content and composition of the seeds, a metabolomics analysis of dry tomato seeds is performed. In addition correlation analysis is conducted between metabolites and seed and seedling quality traits, such as seed germination and seedling growth characteristics, in order to establish links between specific metabolite content of the seeds and physiological performance.

In **Chapter 3** I use a RIL population derived from the two distinct tomato accessions used in Chapter 2. It is of great interest to detect which loci are involved in the response to different nutritional maternal environments. To do so, the RILs are exposed to high phosphate and low nitrate environments during seed development and their seeds are tested for several seed and seedling related traits including maximum germination percentage, rate of germination, thermo-dormancy and -inhibition of seed germination, as well as fresh and dry weight of seedlings. In this chapter the power for detecting the loci influenced by the different maternal environments is increased by performing a QTL×E approach.

In **Chapter 4** metabolic profiling of seeds developed and matured under different nutritional environments is done. Such a metabolic analysis may display more important metabolites regulating plant development and may provide a better understanding of plant acclimation processes. Using a GGG approach I perform metabolic analysis of the RIL population (used in Chapter 3) and the parental lines grown in high phosphate and low nitrate environments. By generating metabolite correlation networks and performing mQTL analysis, more genetic and molecular aspects of seed metabolic changes in response to the maternal environment are discovered.

The objective of **Chapter 5** is to study the genetic variation for thermo-inhibition and thermo-dormancy between the two tomato accessions used in Chapter 2. By applying a candidate gene approach I investigate whether similar molecular mechanisms as in other species such as lettuce and Arabidopsis are regulating thermo-induced inhibition and dormancy in tomato seeds. In

addition, I use a QTL based approach with the RIL population used in Chapters 3 and 4 to identify novel specific regulators that play a role in tomato seed performance under high temperature conditions.

In **Chapter 6** the most important and highlighted findings of this thesis are broadly discussed. In this chapter, I also integrate the obtained results and conclusions from the experimental chapters. In addition, I propose further required investigations and analyses to obtain a comprehensive understanding of the seed and seedling quality, factors that influence them and their underlying mechanisms.

References

Alboresi A, Gestin C, Leydecker M-T, Bedu M, Meyer C, Truong H-N (2005) Nitrate, a signal relieving seed dormancy in Arabidopsis. Plant, Cell & Environment **28:** 500-512

Alonso-Blanco C, Bentsink L, Hanhart CJ, Blankestijn-de Vries H, Koornneef M (2003) Analysis of natural allelic variation at seed dormancy loci of Arabidopsis thaliana. Genetics **164:** 711-729

Alonso-Blanco C, Blankestijn-de Vries H, Hanhart CJ, Koornneef M (1999) Natural allelic variation at seed size loci in relation to other life history traits of Arabidopsis thaliana. Proceedings of the National Academy of Sciences **96:** 4710-4717

Alonso-Blanco C, Koornneef M (2000) Naturally occurring variation in Arabidopsis: an underexploited resource for plant genetics. Trends in plant science **5:** 22-29

Argyris J, Dahal P, Hayashi E, Still DW, Bradford KJ (2008) Genetic variation for lettuce seed thermoinhibition is associated with temperature-sensitive expression of abscisic acid, gibberellin, and ethylene biosynthesis, metabolism, and response genes. Plant Physiology **148:** 926-947

Bentsink L, Hanson J, Hanhart CJ, Blankestijn-de Vries H, Coltrane C, Keizer P, El-Lithy M, Alonso-Blanco C, de Andrés MT, Reymond M (2010) Natural variation for seed dormancy in Arabidopsis is regulated by additive genetic and molecular pathways. Proceedings of the National Academy of Sciences **107:** 4264-4269

Bentsink L, Jowett J, Hanhart CJ, Koornneef M (2006) Cloning of DOG1, a quantitative trait locus controlling seed dormancy in Arabidopsis. Proceedings of the National Academy of Sciences **103:** 17042-17047

Bentsink L, Koornneef M (2008) Seed dormancy and germination. The Arabidopsis Book/American Society of Plant Biologists **6**

Bewley JD (1997) Seed germination and dormancy. The plant cell **9:** 1055

Bewley JD, Bradford K, Hilhorst H (2012) Seeds: physiology of development, germination and dormancy. Springer Science & Business Media

Binder S (2010) Branched-chain amino acid metabolism in Arabidopsis thaliana. The Arabidopsis Book**:** e0137

Bouwmeester HJ, Derks L, Keizer JJ, Karssen CM (1994) Effects of endogenous nitrate content of Sisymbrium officinale seeds on germination and dormancy. Plant Biology **43:** 39-50

Broich SL, Palmer RG (1980) A cluster analysis of wild and domesticated soybean phenotypes. Euphytica **29:** 23-32

Burke JM, Tang S, Knapp SJ, Rieseberg LH (2002) Genetic analysis of sunflower domestication. Genetics **161:** 1257-1267

Chevalier F, Martin O, Rofidal V, Devauchelle AD, Barteau S, Sommerer N, Rossignol M (2004) Proteomic investigation of natural variation between Arabidopsis ecotypes. Proteomics **4:** 1372-1381

Clerkx EJ, Vries BD, Ruys GJ, Groot SP, Koornneef M (2004) Genetic differences in seed longevity of various Arabidopsis mutants. Physiologia Plantarum **121:** 448-461

Contreras S, Bennett M, Tay D (2009) Temperature during seed development affects weight, germinability and storability of lettuce seeds. Seed Science and Technology **37:** 398-412

Curaba J, Moritz T, Blervaque R, Parcy F, Raz V, Herzog M, Vachon G (2004) AtGA3ox2, a key gene responsible for bioactive gibberellin biosynthesis, is regulated during embryogenesis by LEAFY COTYLEDON2 and FUSCA3 in Arabidopsis. Plant Physiology **136:** 3660-3669

Delouche JC (1980) Environmental effects on seed development and seed quality.

Demir I, Mavi K, Oztokat C (2004) Changes in germination and potential longevity of watermelon (Citrullus lanatus) seeds during development. New Zealand Journal of Crop and Horticultural Science **32:** 139-145

DeWitt TJ, Sih A, Wilson DS (1998) Costs and limits of phenotypic plasticity. Trends in ecology & evolution **13:** 77-81

Dickson M (1980) Genetic aspects of seed quality. Hortscience **15:** 771-774

Doganlar S, Frary A, Tanksley S (2000) The genetic basis of seed-weight variation: tomato as a model system. Theoretical and Applied Genetics **100:** 1267-1273

Donohue K (2009) Completing the cycle: maternal effects as the missing link in plant life histories. Philosophical Transactions of the Royal Society B: Biological Sciences **364:** 1059-1074

Dudley J (1993) Molecular markers in plant improvement: manipulation of genes affecting quantitative traits. Crop Science **33:** 660-668

El-Soda M, Malosetti M, Zwaan BJ, Koornneef M, Aarts MG (2014) Genotype× environment interaction QTL mapping in plants: lessons from Arabidopsis. Trends in Plant Science **19:** 390-398

El-soda M, Kruijer W, Malosetti M, Koornneef M, Aarts MG (2015) Quantitative trait loci and candidate genes underlying genotype by environment interaction in the response of Arabidopsis thaliana to drought. Plant, cell & environment **38:** 585-599

Farhadi E, Daneshyan J, Hamidi A, Rad A, Valadabadi H (2014) Effects of parent plant nutrition with different amounts of nitrogen and irrigation on seed vigor and some characteristics associated with hybrid 704 in Kermanshah region. Journal of Novel Applied Sciences **3:** 551-556

Fenner M (1991) The effects of the parent environment on seed germinability. Seed Science Research **1:** 75-84

Foolad M, Chen F (1999) RFLP mapping of QTLs conferring salt tolerance during the vegetative stage in tomato. Theoretical and Applied Genetics **99:** 235-243

Foolad M, Zhang L, Subbiah P (2003) Genetics of drought tolerance during seed germination in tomato: inheritance and QTL mapping. Genome **46:** 536-545

Foolad MR, Subbiah P, Zhang L (2007) Common QTL affect the rate of tomato seed germination under different stress and nonstress conditions. International journal of plant genomics **2007**

Franks SJ, Weber JJ, Aitken SN (2014) Evolutionary and plastic responses to climate change in terrestrial plant populations. Evolutionary Applications **7:** 123-139

Fuller DQ (2007) Contrasting patterns in crop domestication and domestication rates: recent archaeobotanical insights from the Old World. Annals of Botany **100:** 903-924

Geritz SA, van der Meijden E, Metz JA (1999) Evolutionary dynamics of seed size and seedling competitive ability. Theoretical population biology **55:** 324-343

Goldman I, Paran I, Zamir D (1995) Quantitative trait locus analysis of a recombinant inbred line population derived from a Lycopersicon esculentum x Lycopersicon cheesmanii cross. Theoretical and Applied Genetics **90:** 925-932

Gonai T, Kawahara S, Tougou M, Satoh S, Hashiba T, Hirai N, Kawaide H, Kamiya Y, Yoshioka T (2004) Abscisic acid in the thermoinhibition of lettuce seed germination and enhancement of its catabolism by gibberellin. Journal of Experimental Botany **55:** 111-118

Gornall J, Betts R, Burke E, Clark R, Camp J, Willett K, Wiltshire A (2010) Implications of climate change for agricultural productivity in the early twenty-first century. Philosophical Transactions of the Royal Society B: Biological Sciences **365:** 2973-2989

Gutterman Y (2000) Maternal effects on seeds during development. Seeds: the ecology of regeneration in plant communities **2:** 59-84

Harada JJ (1997) Seed maturation and control of germination. *In* Cellular and molecular biology of plant seed development. Springer, pp 545-592

He H, de Souza Vidigal D, Snoek LB, Schnabel S, Nijveen H, Hilhorst H, Bentsink L (2014) Interaction between parental environment and genotype affects plant and seed performance in Arabidopsis. Journal of experimental botany **65:** 6603-6615

Heydecker W (1977) Stress and seed germination: an agronomic view. Physiology and Biochemistry of Seed Dormancy and Germination

Hilhorst H, Koornneef M (2007) Dormancy in plants. *In* Encyclopedia of Life Sciences. Elsevier

Hilhorst HW (2007) Definitions and hypotheses of seed dormancy. Annual Plant Reviews**:** 50-71

Hilhorst HW, Finch-Savage WE, Buitink J, Bolingue W, Leubner-Metzger G (2010) Dormancy in plant seeds. *In* Dormancy and resistance in harsh environments. Springer, pp 43-67

Hilhorst HW, Toorop PE (1997) Review on dormancy, germinability, and germination in crop and weed seeds. Advances in agronomy (USA)

Holdsworth MJ, Bentsink L, Soppe WJ (2008) Molecular networks regulating Arabidopsis seed maturation, after-ripening, dormancy and germination. New Phytologist **179:** 33-54

Huang X, Schmitt J, Dorn L, Griffith C, Effgen S, Takao S, Koornneef M, Donohue K (2010) The earliest stages of adaptation in an experimental plant population: strong selection on QTLS for seed dormancy. Molecular Ecology **19:** 1335-1351

Huo H, Bradford KJ (2015) Molecular and hormonal regulation of thermoinhibition of seed germination. *In* Advances in Plant Dormancy. Springer, pp 3-33

Iuchi S, Kobayashi M, Taji T, Naramoto M, Seki M, Kato T, Tabata S, Kakubari Y, Yamaguchi-Shinozaki K, Shinozaki K (2001) Regulation of drought tolerance by gene manipulation of 9-cis-epoxycarotenoid dioxygenase, a key enzyme in abscisic acid biosynthesis in Arabidopsis. The Plant Journal **27:** 325-333

Jansen RC, Nap J-P (2001) Genetical genomics: the added value from segregation. TRENDS in Genetics **17:** 388-391

Joosen RV, Ligterink W, Hilhorst HW, Keurentjes JJ (2013) Genetical Genomics of Plants: From Genotype to Phenotype. Advances in Genome Science: Probing Intracellular Regulation **2:** 2194

Joosen RVL, Arends D, Willems LAJ, Ligterink W, Jansen RC, Hilhorst HW (2012) Visualizing the genetic landscape of Arabidopsis seed performance. Plant physiology **158:** 570-589

Jurado E, Westoby M (1992) Seedling growth in relation to seed size among species of arid Australia. Journal of Ecology**:** 407-416

Kazmi RH, Khan N, Willems LA, Van Heusden AW, Ligterink W, Hilhorst HW (2012) Complex genetics controls natural variation among seed quality phenotypes in a recombinant inbred population of an interspecific cross between Solanum lycopersicum× Solanum pimpinellifolium. Plant, cell & environment **35:** 929-951

Kazmi RH, Willems LA, Joosen RV, Khan N, Ligterink W, Hilhorst HW (2017) Metabolomic analysis of tomato seed germination. Metabolomics **13:** 145

Kendall SL, Hellwege A, Marriot P, Whalley C, Graham IA, Penfield S (2011) Induction of dormancy in Arabidopsis summer annuals requires parallel regulation of DOG1 and hormone metabolism by low temperature and CBF transcription factors. The Plant Cell **23:** 2568-2580

Kępczyński J, Bihun M, Kępczyńska E (2006) Implication of ethylene in the release of secondary dormancy in Amaranthus caudatus L. seeds by gibberellins or cytokinin. Plant growth regulation **48:** 119-126

Keurentjes JJ, Fu J, De Vos CR, Lommen A, Hall RD, Bino RJ, van der Plas LH, Jansen RC, Vreugdenhil D, Koornneef M (2006) The genetics of plant metabolism. Nature genetics **38:** 842

Keurentjes JJ, Fu J, Terpstra IR, Garcia JM, van den Ackerveken G, Snoek LB, Peeters AJ, Vreugdenhil D, Koornneef M, Jansen RC (2007) Regulatory network construction in Arabidopsis by using genome-wide gene expression quantitative trait loci. Proceedings of the National Academy of Sciences **104:** 1708-1713

Keurentjes JJ, Sulpice R, Gibon Y, Steinhauser M-C, Fu J, Koornneef M, Stitt M, Vreugdenhil D (2008) Integrative analyses of genetic variation in enzyme activities of primary carbohydrate metabolism reveal distinct modes of regulation in Arabidopsis thaliana. Genome biology **9:** R129

Khan N, Kazmi RH, Willems LA, Van Heusden AW, Ligterink W, Hilhorst HW (2012) Exploring the natural variation for seedling traits and their link with seed dimensions in tomato. PLoS One **7:** e43991

Kliebenstein DJ, West MA, van Leeuwen H, Loudet O, Doerge RW, St Clair DA (2006) Identification of QTLs controlling gene expression networks defined a priori. Bmc Bioinformatics **7:** 308

Kooke R, Keurentjes J (2011) Multi-dimensional regulation of metabolic networks shaping plant development and performance. Journal of experimental botany **63:** 3353-3365

Koornneef M, Bentsink L, Hilhorst H (2002) Seed dormancy and germination. Current opinion in plant biology **5:** 33-36

Lee W, Fenner M (1989) Mineral nutrient allocation in seeds and shoots of twelve Chionochloa species in relation to soil fertility. The Journal of Ecology**:** 704-716

Leishman MR, Westoby M (1994) The role of large seed size in shaded conditions: experimental evidence. Functional Ecology**:** 205-214

Leverett LD, Auge GA, Bali A, Donohue K (2016) Contrasting germination responses to vegetative canopies experienced in pre-vs. post-dispersal environments. Annals of botany **118:** 1175-1186

Li J, Huang X, Heinrichs F, Ganal M, Röder M (2005) Analysis of QTLs for yield, yield components, and malting quality in a BC 3-DH population of spring barley. Theoretical and Applied Genetics **110:** 356-363

Li Y, Breitling R, Jansen RC (2008) Generalizing genetical genomics: getting added value from environmental perturbation. Trends in Genetics **24:** 518-524

Ligterink W, Joosen RV, Hilhorst HW (2012) Unravelling the complex trait of seed quality: using natural variation through a combination of physiology, genetics and-omics technologies. Seed Science Research **22:** S45-S52

Lisec J, Meyer RC, Steinfath M, Redestig H, Becher M, Witucka-Wall H, Fiehn O, Törjék O, Selbig J, Altmann T (2008) Identification of metabolic and biomass QTL in Arabidopsis thaliana in a parallel analysis of RIL and IL populations. The Plant Journal **53:** 960-972

Litrico I, Violle C (2015) Diversity in plant breeding: a new conceptual framework. Trends in plant science **20:** 604-613

Liu B, Fujita T, Yan Z-H, Sakamoto S, Xu D, Abe J (2007) QTL mapping of domestication-related traits in soybean (Glycine max). Annals of Botany **100:** 1027-1038

Loreau M (1998) Biodiversity and ecosystem functioning: a mechanistic model. Proceedings of the National Academy of Sciences **95:** 5632-5636

Luo M, Dennis ES, Berger F, Peacock WJ, Chaudhury A (2005) MINISEED3 (MINI3), a WRKY family gene, and HAIKU2 (IKU2), a leucine-rich repeat (LRR) KINASE gene, are regulators of seed size in Arabidopsis. Proceedings of the National Academy of Sciences **102:** 17531-17536

Mackay TF (2001) The genetic architecture of quantitative traits. Annual review of genetics **35:** 303-339

Mackay TF, Stone EA, Ayroles JF (2009) The genetics of quantitative traits: challenges and prospects. Nature Reviews Genetics **10:** 565

Mendel RR, Hänsch R (2002) Molybdoenzymes and molybdenum cofactor in plants. Journal of Experimental Botany **53:** 1689-1698

Meyer RC, Steinfath M, Lisec J, Becher M, Witucka-Wall H, Törjék O, Fiehn O, Eckardt Ä, Willmitzer L, Selbig J (2007) The metabolic signature related to high plant growth rate in Arabidopsis thaliana. Proceedings of the National Academy of Sciences **104:** 4759-4764

Modi A, Cairns A (1994) Molybdenum deficiency in wheat results in lower dormancy levels via reduced ABA. Seed Science Research **4:** 329-333

Munir J (2001) The influence of maternal photoperiod on germination requirements in Arabidopsis thaliana. Am J Bot **88:** 1240-1249

Nambara E, Hayama R, Tsuchiya Y, Nishimura M, Kawaide H, Kamiya Y, Naito S (2000) The role of ABI3 and FUS3 loci in Arabidopsis thaliana on phase transition from late embryo development to germination. Developmental biology **220:** 412-423

Nesi N, Delourme R, Brégeon M, Falentin C, Renard M (2008) Genetic and molecular approaches to improve nutritional value of Brassica napus L. seed. Comptes rendus biologies **331:** 763-771

Orsi CH, Tanksley SD (2009) Natural variation in an ABC transporter gene associated with seed size evolution in tomato species. PLoS genetics **5:** e1000347

Panthee D, Pantalone V, West D, Saxton A, Sams C (2005) Quantitative trait loci for seed protein and oil concentration, and seed size in soybean. Crop Science **45:** 2015-2022

Papdi C, Joseph MP, Salamó IP, Vidal S, Szabados L (2009) Genetic technologies for the identification of plant genes controlling environmental stress responses. Functional Plant Biology **36:** 696-720

Penfield S, King J (2009) Towards a systems biology approach to understanding seed dormancy and germination. Proceedings of the Royal Society of London B: Biological Sciences**:** rspb20090592

Penfield S, Springthorpe V (2012) Understanding chilling responses in Arabidopsis seeds and their contribution to life history. Phil. Trans. R. Soc. B **367:** 291-297

Pourrat Y, Jacques R (1975) The influence of photoperiodic conditions received by the mother plant on morphological and physiological characteristics of Chenopodium polyspermum L. seeds. Plant Science Letters **4:** 273-279

Ren Z, Zheng Z, Chinnusamy V, Zhu J, Cui X, Iida K, Zhu J-K (2010) RAS1, a quantitative trait locus for salt tolerance and ABA sensitivity in Arabidopsis. Proceedings of the National Academy of Sciences **107:** 5669-5674

Roach DA, Wulff RD (1987) Maternal effects in plants. Annual review of ecology and systematics **18:** 209-235

Rockman MV, Kruglyak L (2006) Genetics of global gene expression. Nature Reviews Genetics **7:** 862

Rowe HC, Hansen BG, Halkier BA, Kliebenstein DJ (2008) Biochemical networks and epistasis shape the Arabidopsis thaliana metabolome. The Plant Cell **20:** 1199-1216

Rowse H, Finch-Savage W (2003) Hydrothermal threshold models can describe the germination response of carrot (Daucus carota) and onion (Allium cepa) seed populations across both sub-and supra-optimal temperatures. New Phytologist **158:** 101-108

Salvi S, Tuberosa R (2005) To clone or not to clone plant QTLs: present and future challenges. Trends in plant science **10:** 297-304

Schachtman DP, Reid RJ, Ayling SM (1998) Phosphorus uptake by plants: from soil to cell. Plant physiology **116:** 447-453

Schauer N, Semel Y, Balbo I, Steinfath M, Repsilber D, Selbig J, Pleban T, Zamir D, Fernie AR (2008) Mode of inheritance of primary metabolic traits in tomato. The Plant Cell **20:** 509-523

Schauer N, Semel Y, Roessner U, Gur A, Balbo I, Carrari F, Pleban T, Perez-Melis A, Bruedigam C, Kopka J (2006) Comprehensive metabolic profiling and phenotyping of interspecific introgression lines for tomato improvement. Nature biotechnology **24:** 447

Schmuths H, Bachmann K, Weber WE, Horres R, Hoffmann MH (2006) Effects of preconditioning and temperature during germination of 73 natural accessions of Arabidopsis thaliana. Annals of Botany **97:** 623-634

Schwarz D, Nodop A, Hüge J, Purfürst S, Forchhammer K, Michel K-P, Bauwe H, Kopka J, Hagemann M (2011) Metabolic and transcriptomic phenotyping of inorganic carbon acclimation in the cyanobacterium Synechococcus elongatus PCC 7942. Plant Physiology**:** pp. 110.170225

Shomura A, Izawa T, Ebana K, Ebitani T, Kanegae H, Konishi S, Yano M (2008) Deletion in a gene associated with grain size increased yields during rice domestication. Nature genetics **40:** 1023

Singh RP, Prasad PV, Reddy KR (2013) Impacts of changing climate and climate variability on seed production and seed industry. *In* Advances in agronomy, Vol 118. Elsevier, pp 49-110

Snell-Rood EC (2013) An overview of the evolutionary causes and consequences of behavioural plasticity. Animal Behaviour **85:** 1004-1011

Song J, Zhou J, Zhao W, Xu H, Wang F, Xu Y, Wang L, Tian C (2016) Effects of salinity and nitrate on production and germination of dimorphic seeds applied both through the mother plant and exogenously during germination in Suaeda salsa. Plant species biology **31:** 19-28

Song X-J, Huang W, Shi M, Zhu M-Z, Lin H-X (2007) A QTL for rice grain width and weight encodes a previously unknown RING-type E3 ubiquitin ligase. Nature genetics **39:** 623

Sperling L, Osborn T, Cooper D (2004) Towards effective and sustainable seed relief activities. *In*. Food and Agricultural Organization of the United Nations (FAO)

Sultan SE (2000) Phenotypic plasticity for plant development, function and life history. Trends in plant science **5:** 537-542

Tan KC, Ipcho SV, Trengove RD, Oliver RP, Solomon PS (2009) Assessing the impact of transcriptomics, proteomics and metabolomics on fungal phytopathology. Molecular Plant Pathology **10:** 703-715

TeKrony DM, Egli DB (1991) Relationship of seed vigor to crop yield: a review. Crop Science **31:** 816-822

Thomas JF, Raper CD (1979) Germinability of Tobacco Seed as Affected by Culture of the Mother Plant 1. Agronomy Journal **71:** 694-695

Tilman D, Lehman CL, Thomson KT (1997) Plant diversity and ecosystem productivity: theoretical considerations. Proceedings of the national academy of sciences **94:** 1857-1861

Toh S, Imamura A, Watanabe A, Nakabayashi K, Okamoto M, Jikumaru Y, Hanada A, Aso Y, Ishiyama K, Tamura N (2008) High temperature-induced abscisic acid biosynthesis and its role in the inhibition of gibberellin action in Arabidopsis seeds. Plant physiology **146:** 1368-1385

Toubiana D, Semel Y, Tohge T, Beleggia R, Cattivelli L, Rosental L, Nikoloski Z, Zamir D, Fernie AR, Fait A (2012) Metabolic profiling of a mapping population exposes new insights in the regulation of seed metabolism and seed, fruit, and plant relations. PLoS Genetics **8:** e1002612

Toyomasu T, Kawaide H, Mitsuhashi W, Inoue Y, Kamiya Y (1998) Phytochrome regulates gibberellin biosynthesis during germination of photoblastic lettuce seeds. Plant Physiology **118:** 1517-1523

Urbanczyk-Wochniak E, Fernie AR (2004) Metabolic profiling reveals altered nitrogen nutrient regimes have diverse effects on the metabolism of hydroponically-grown tomato (Solanum lycopersicum) plants. Journal of Experimental Botany **56:** 309-321

van Eeuwijk FA, Bink MC, Chenu K, Chapman SC (2010) Detection and use of QTL for complex traits in multiple environments. Current opinion in plant biology **13:** 193-205

Vandecasteele C, Teulat-Merah B, Morere-Le Paven MC, Leprince O, Ly Vu B, Viau L, Ledroit L, Pelletier S, Payet N, Satour P (2011) Quantitative trait loci analysis reveals a correlation between the ratio of sucrose/raffinose family oligosaccharides and seed vigour in Medicago truncatula. Plant, Cell & Environment **34:** 1473-1487

Varis S, George R (1985) The influence of mineral nutrition on fruit yield, seed yield and quality in tomato. Journal of horticultural science **60:** 373-376

Verpoorte R, Choi Y, Mustafa N, Kim H (2008) Metabolomics: back to basics. Phytochemistry Reviews **7:** 525-537

Walck JL, Hidayati SN, Dixon KW, Thompson K, Poschlod P (2011) Climate change and plant regeneration from seed. Global Change Biology **17:** 2145-2161

Wayne ML, McIntyre LM (2002) Combining mapping and arraying: an approach to candidate gene identification. Proceedings of the National Academy of Sciences **99:** 14903-14906

Weitbrecht K, Müller K, Leubner-Metzger G (2011) First off the mark: early seed germination. Journal of experimental botany **62:** 3289-3309

Windsor AJ, Reichelt M, Figuth A, Svatoš A, Kroymann J, Kliebenstein DJ, Gershenzon J, Mitchell-Olds T (2005) Geographic and evolutionary diversification of glucosinolates among near relatives of Arabidopsis thaliana (Brassicaceae). Phytochemistry **66:** 1321-1333

Wulff RD (1986) Seed size variation in Desmodium paniculatum: II. Effects on seedling growth and physiological performance. The Journal of Ecology**:** 99-114

Chapter 2

The interaction between genotype and maternal nutritional environments affects tomato seed and seedling quality

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Abstract

Seed and seedling traits are affected by the conditions of the maternal environment, such as light, temperature and nutrient availability. In this study, we have investigated whether different maternally applied nitrate and phosphate concentrations affect the seed and seedling performance of two tomato genotypes: *Solanum lycopersicum* cv. Money maker (MM) and *Solanum pimpinellifolium* accession CGN14498 (PI). We observed large differences for seed and seedling traits between the two genotypes. Additionally, we have shown that for nitrate most of the seed and seedling traits were significantly affected by genotype by environment interactions (G×E). The effect of the maternal environment was clearly visible in the primary metabolites of the dry seeds. For example, we could show that the amount of γ-aminobutyric acid (GABA) in MM seeds was affected by the differences in the maternal environments and was positively correlated with seed germination under high temperature. Overall, compared to phosphate, nitrate had a larger effect on seed and seedling performance in tomato. In general, the different responses to the maternal environments of the two tomato genotypes show a major role of genotype by environment interactions in shaping seed and seedling traits.

Introduction

Seeds, as the start point of the life cycle of plants, can be considered as the key life stage in many crops like tomato. High quality and well developed seeds are crucial for a successful life cycle of crops, from seedling establishment through to fruit and seed production, especially under stressful environmental conditions. Seed quality is a complex trait and is composed of different quality characteristics including physical, physiological, genetic and seed health quality (Sperling *et al.*, 2004). In addition, seed quality is influenced by many environmental cues such as drought, light and temperature (Rowse and Finch-Savage, 2003). Establishment of seed quality starts at the position where the plants grow, produce and mature their seeds (Delouche and Baskin, 1971). The maternal environment under which seeds develop and mature, including the climate and growth conditions, has a profound influence on seed quality (Delouche, 1980). Maternal environmental effects are defined as a specific phenomenon in which the phenotype of offspring is influenced by the environment that the maternal plant is exposed to (Donohue, 2009). It has been reported that different temperatures (Demir *et al.*, 2004; He *et al.*, 2014; Schmuths *et al.*, 2006), photoperiod (Munir *et al.*, 2001; Pourrat and Jacques, 1975) and nutrient conditions (Alboresi *et al.*, 2005; He *et al.*, 2014) during seed development and maturation may result in differences in seed performance in plants such as tomato and Arabidopsis.

Seed performance traits, such as seed dormancy and germinability, can be influenced by different environmental conditions. The germinability of a seed batch is defined as the percentage of seed germination during a specific time interval (Fenner, 1991). There are many reports on the influence of environmental conditions under which seeds develop and mature on seed dormancy and germinability. For instance, for *S. lycopersicum* (Varis and George, 1985), *Nicotiana tabacum* (Thomas and Raper, 1979), *Sisymbrium officinale* (Hilhorst and Karssen, 1988), *Arabidopsis thaliana* (Alboresi *et al.*, 2005; He *et al.*, 2014) and *Rumex crispus* (Hejcman *et al.*, 2012) it has been shown that low nitrate levels in the soil of the mother plant results in a decrease in germinability of their seeds. Alboresi et al. (2005) reported that nitrate can reduce dormancy in Arabidopsis seeds by either direct effects or through hormonal and metabolic changes in the seed. These changes probably include interactions with ABA and/or GA synthesis and degradation pathways (Alboresi *et al.*, 2005).

The effects of maternal environmental conditions on seed quality are not restricted to germination characteristics of the seeds, but may also include other seed quality traits such as seed size and seed weight as well as seedling quality characteristics such as root and shoot weight, hypocotyl length and root architecture. In many species a higher level of nutrient supply to the mother plant led to the production of bigger and heavier seeds (Fenner, 1992). Moreover, in some species a higher nitrate regime applied to the mother plant resulted in an enhanced

seedling establishment and higher shoot and root weight of the seedlings (Farhadi *et al.*, 2014; Song *et al.*, 2014). In addition, there are many examples of changed metabolism in seeds in response to the environmental condition of the mother plant (Joosen *et al.*, 2013; Mounet *et al.*, 2007). A better understanding of the influence of the maternal environment on seed and seedling quality can be obtained by performing omics analysis of seeds such as transcriptomics, proteomics and metabolomics.

Fait *et al.* (2006) revealed that seed germination and seedling establishment characteristics are associated with degradation and mobilization of reserves which are accumulated during seed maturation like sugars, organic acids and amino acids. Therefore, profiling the metabolites and finding the ones associated with phenotypes can be regarded as a powerful tool for monitoring seed performance. In general, metabolite contents alter in response to abiotic stress, which is most obvious for primary metabolites such as sugars, amino acids and tricarboxylic acid (TCA) cycle intermediates (Arbona *et al.*, 2013).

In this study, we investigated if different maternal nutritional environments can affect the quality of the progeny of different genotypes. For this purpose we investigated two different tomato genotypes (*S. lycopersicum* and *S. pimpinellifolium*) under different nutrient conditions.

S. pimpinellifolium, the most closely related wild tomato species to the advanced tomato breeding line (*S. lycopersicum* cv. Money maker), has been used in breeding programs for its tolerance to some sub-optimal environments as well as the ability of being naturally crossed with this species. We grew these genotypes in different concentrations of nitrate and phosphate. Phosphate is an important nutrient for plants, making up 0.2% of the dry weight and being an essential part of some vital molecules like nucleic acids, phospholipids and ATP. Nitrate plays a key role in plants as a major source of nitrogen and some signal metabolites (Schachtman *et al.*, 1998; Urbanczyk-Wochniak and Fernie, 2005). Under both optimal and stressful conditions extensive phenotyping by germination tests and metabolite profiling was done after harvesting the seeds. Based on these results we show that different levels of phosphate and nitrate available to the mother plant can influence seed and seedling traits especially under stressful germination conditions. In addition, in order to investigate if physiological changes in seed and seedling performance are influenced by metabolic changes in the dry seed, correlation analysis was performed between physiological traits like seed germination and seedling growth and metabolic changes caused by the different maternal environments in tomato. We showed that several phenotypic traits are either positively or negatively correlated with metabolites.

Results

Several studies have been reported recently about the effect of the maternal environment such as temperature, light and nutrition on seed and seedling quality in plants (Alboresi *et al.*, 2005; He *et al.*, 2014; Hejcman *et al.*, 2012). However there is still a lack of knowledge on the influence of nutritional condition of the mother plant on seed and seedling performance. In order to investigate the effect of maternal nutrient environment on the seed and seedling quality in tomato, two tomato genotypes, *S. lycopersicum* cv. Money maker (MM) and *S. pimpinellifolium* accession CGN14498 (PI) were grown on different nutrient solutions from flowering onwards. Their seeds were harvested and phenotyped for various seed performance traits, including percentage of germination, germination rate and uniformity, under optimal and several stress germination conditions (i.e. high temperature, salt and osmotic stress). Furthermore, seed size and weight were determined. Since final successful and sustainable crop production results from healthy seedlings and good seedling establishment, we also measured some seedling quality traits such as hypocotyl length, root architecture and fresh and dry root and shoot weight.

Table 1. ANOVA analysis of the effect of genotype, maternal environment and genotype-by-environment interactions on seed and seedling quality. Values show the -10 log(*P*) *.

Traits	Nitrate			Phosphate		
	Genotype	Environment	G×E	Genotype	Environment	G×E
SS	25.24	7.81	4.12	23.37	2.41	0.47
SW	27.38	8.88	6.06	24.17	5.71	3.91
FWR	22.90	3.29	1.88	18.22	3.64	1.29
DWR	22.42	4.09	3.85	16.60	3.16	0.75
FWSH	25.07	5.89	3.19	18.45	3.64	2.32
DWSH	22.91	4.67	2.95	20.05	5.07	2.54
MRL	16.23	4.29	1.31	14.61	0.59	0.83
NLR	16.08	0.04	0.43	17.05	0.48	1.49
G_{max} Water	5.74	3.18	3.05	5.59	0.32	0.14
t_{50}^{-1} Water	16.72	0.26	0.20	23.78	0.06	0.93
G_{max} NaCl	13.57	2.25	2.31	10.16	1.55	1.04
t_{50}^{-1} NaCl	16.85	0.11	1.02	27.48	1.89	0.92
G_{max} HT	27.43	3.45	3.63	24.73	1.88	2.27
t_{50}^{-1} HT	29.69	11.88	4.08	23.38	0.89	1.58
G_{max} Mann.	16.27	7.77	8.79	9.98	0.02	0.01
t_{50}^{-1} Mann.	23.71	7.55	10.62	16.50	0.71	2.14
Nit. Content	1.66	0.06	0.77	2.15	3.03	3.39
Phy. Content	12.06	2.74	1.90	16.85	21.16	4.22
ABA Content	6.84	1.04	1.04	4.95	3.21	1.12

SS, Seed size; **SW**, Seed weight; **FWR**, Fresh weight of root; **DWR**, Dry weight of root; **FWSH**, Fresh weight of shoot; **DWSH**, Dry weight of shoot; **MRL**, Main root length; **NLR**, Number of lateral root; **G_{max}**, Maximum seed germination percentage; **t_{50}^{-1}**, Reciprocal of time to respectively reach 50% of maximum germination; **HT**, High temperature; **Mann**, Mannitol; **Nit**, Nitrate; **Phy**, Phytate.
* Coloured cells demonstrate significant levels (Dark green: P<0.001; Light green: P<0.01; Very light green: P<0.05) and non-coloured spots represent non-significant values.

Factors affecting seed and seedling traits

A linear model/ANOVA was used to investigate the effects caused by the different factors like genotype, environment and their interaction (G×E). The results showed that genotype was an important factor, since it had a very pronounced influence on almost all traits in different nitrate and phosphate concentrations (Table 1). Also the environment under which seeds developed had a significant effect which was most prominent for different nitrate conditions (Table 1). Although G×E interactions had a significant effect on some traits for the phosphate environment, most of the seed and seedling traits in the case of different nitrate concentrations were significantly influenced by G×E interactions (Table 1).

The effect of different nutrient regimes of the mother plant on seed quality traits

Seed germination under optimal conditions (water)

Under normal germination conditions only very low nitrate (0 mM) decreased the germination percentage in MM (Figure 1A). Although the rate of the germination (t_{50}^{-1}) was not affected significantly by different amounts of nitrate, it was decreased by higher amounts of phosphate (Supplemental Figure S1A).

Seed germination in stress conditions (high temperature, salt and mannitol)

Our results showed that at high temperature MM seeds from plants grown in 0 mM nitrate, germinated very poorly (4%) while higher concentrations of nitrate resulted in significantly higher germination percentages (40-60%; Figure 1B). These seeds also had a higher t_{50}^{-1} (Figure 1C). In contrast with nitrate, G_{max} was decreased at higher levels of phosphate (Figure 1B).

While seed germination of MM was positively correlated with nitrate concentration in mannitol (Figure 1D), germination rate was contrarily decreased at higher levels of both nutrients (Supplemental Figure S1B). Under salt stress, both phosphate and nitrate had a positive effect on germination percentage of MM seeds and a negative effect on their germination rate (lower $t50^{-1}$ values, Figure 1E, F).

Although different nutritional environments resulted in clear changes in seed quality traits in MM, hardly any effect was seen for PI seeds, indicating that PI was tolerant to the different environments that were tested.

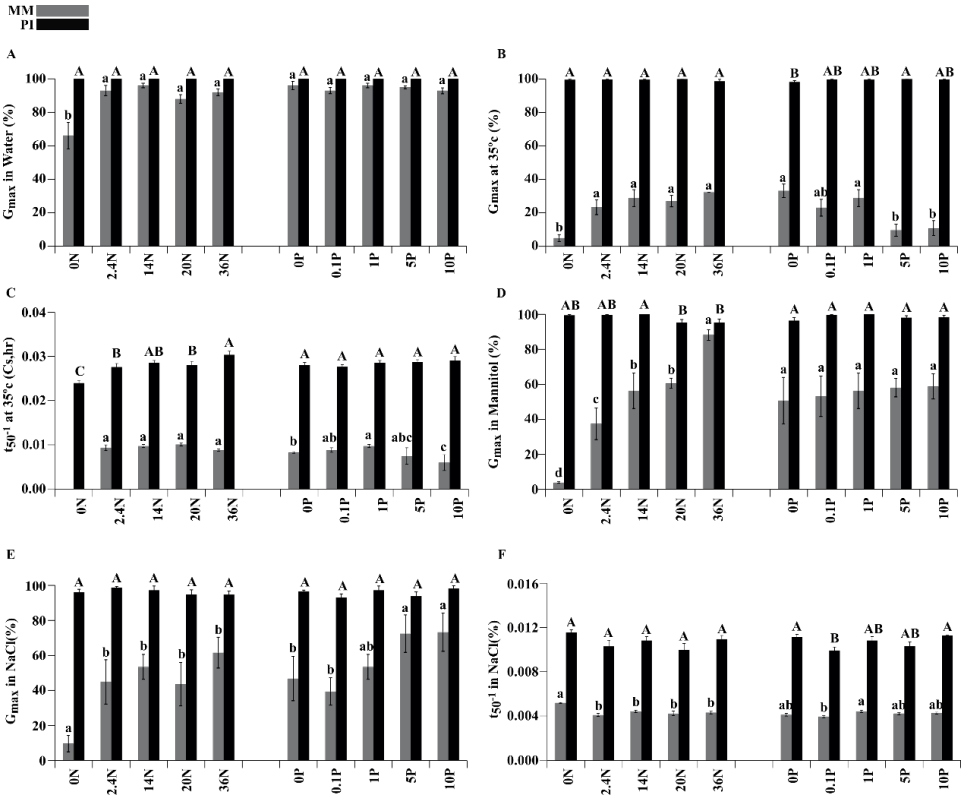

Figure. 1. Effects of maternal nutritional environment on seed germination traits of **MM** and **PI**. **A**, Germination in water (25°C); **B**, Germination at high temperature (Water, 35°C); **C**, t_{50}^{-1} at high temperature (Water, 35°C); **D**, Germination in mannitol (-0.5 MPa, 25°C); **E**, Germination in salt (-0.5 MPa, 25°C); **F**, t_{50}^{-1} in salt (-0.5 MPa, 25°C) in different concentrations of nitrate (0N, 2.4N, 14N, 20N and 36N) and phosphate (0P, 0.1P, 1P, 5P and 10P). Letters above the bars represent significant differences between different concentrations of nitrate or phosphate within each genotype ($p<0.05$).

Seed size and weight

By increasing the nitrate level, seed size and weight of MM plants increased. However, both seed size and weight decreased slightly again at concentrations of 20 mM nitrate or higher. For PI, higher amounts of nitrate and phosphate led to the production of larger and heavier seeds (Figure 2A,B).

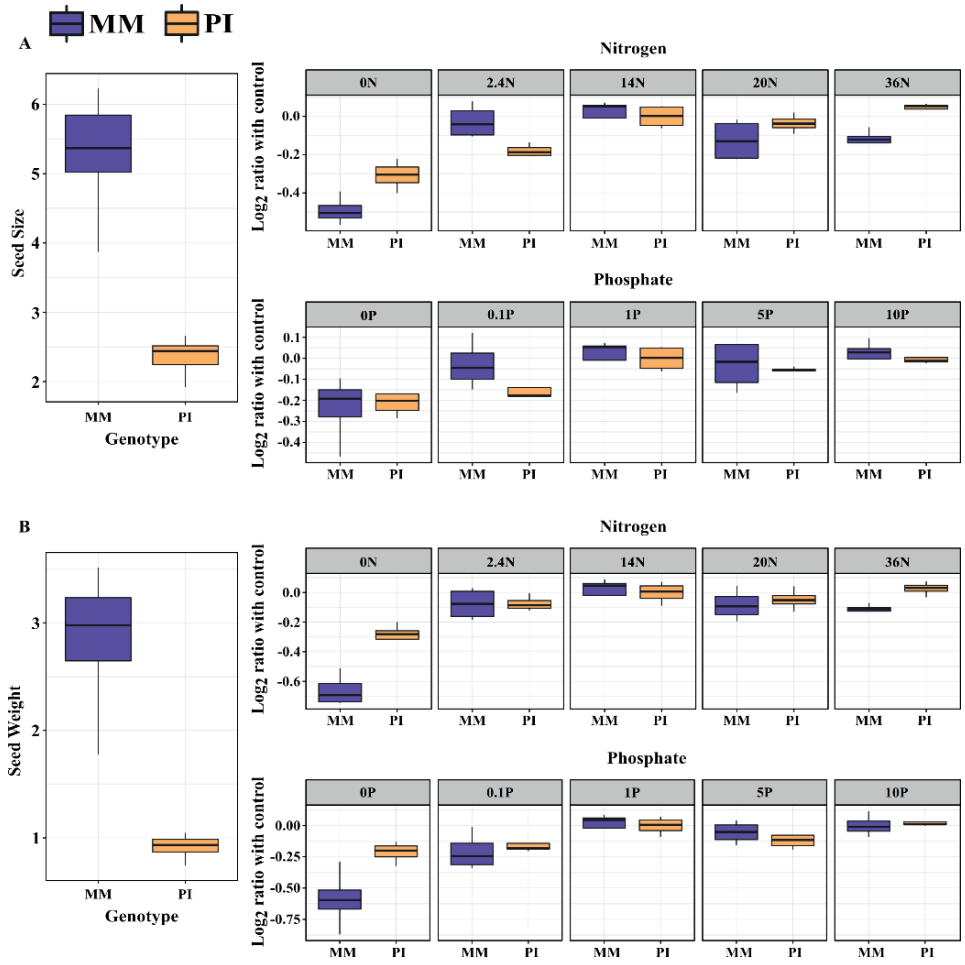

Figure 2. Effects of maternal nutritional environments on seed quality of *Solanum lycopersicum* cv. Moneymaker **(MM)** and *Solanum pimpinellifolium* **(PI)**. **A**, Seed size; **B**, Seed weight of the plants grown in different concentrations of nitrate (0N, 2.4N, 14N, 20N and 36N) and phosphate (0P, 0.1P, 1P, 5P and 10P). On left, the average of seed size and seed weight (regardless of maternal environments) in each genotype are presented.

ABA, nitrate and phytate

ABA content of dry seeds was not significantly influenced by the maternal nitrate concentration, but was increased by application of 1 mM of phosphate. Although ABA showed a relatively consistent increase in PI, concentrations above 1 mM of phosphate resulted in decreased ABA levels in MM seeds (Supplemental Figure S2A). The phytate content of the seeds significantly increased with higher phosphate levels in both genotypes (Supplemental Figure S2B). Application of nitrate up to 14 mM increased phytate levels of MM seeds. However, concentrations above 14 mM led to decreased phytate levels in both genotypes

(Supplemental Figure S2B). In PI seeds nitrate content was not affected by the nutrient nitrate level, while in MM higher levels of nitrate surprisingly led to lower seed nitrate levels.

The effects of different nutrient regimes of the mother plant on seedling quality traits

Fresh and dry shoot and root weight

Both fresh shoot and root weight of seedlings were influenced by different concentrations of nitrate and phosphate for the mother plant. Evidently, raising the dosage of nitrate and phosphate in both MM and PI resulted in heavier seedlings (shoot and root) (Figure 3A, B). Shoot and root dry weight followed the same pattern as that of the fresh weight in different environments in both lines (Supplemental Figure S3A, B).

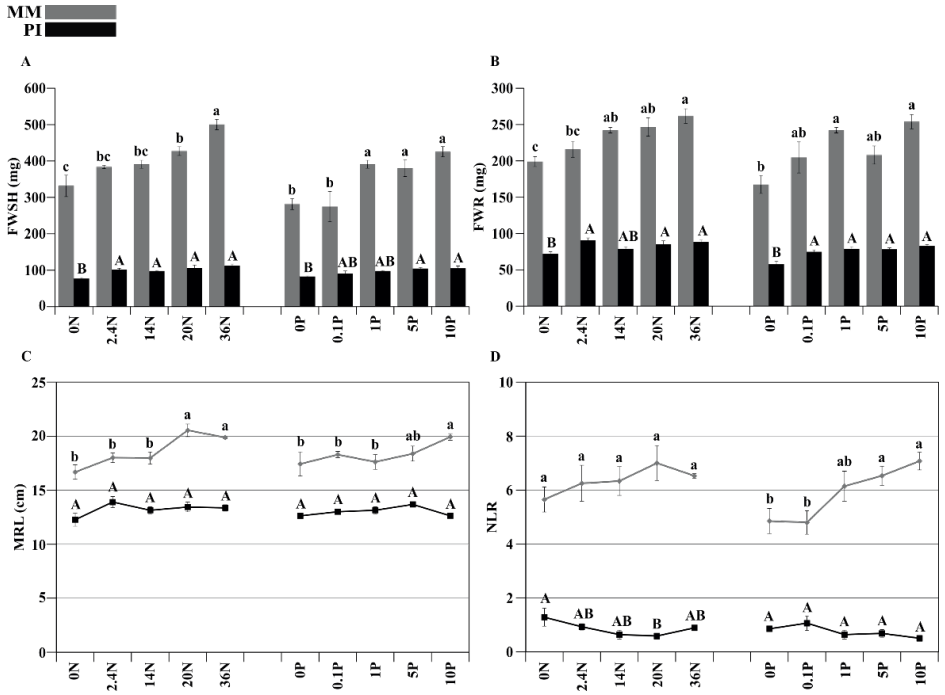

Figure 3. Effects of maternal nutritional environments on seedling quality traits of *Solanum lycopersicum* cv. Moneymaker **(MM)** and *Solanum pimpinellifolium* **(PI)**. **A**, Shoot fresh weight; **B**, Root fresh weight; **C**, Main root length; **D**, Number of lateral roots in different concentrations of nitrate (0N, 2.4N, 14N, 20N and 36N) and phosphate (0P, 0.1P, 1P, 5P and 10P). Letters above the bars (A, B) and lines (C, D) represent significant differences between different concentrations of nitrate or phosphate within each genotype ($p<0.05$).

Root architecture

Although higher amounts of nitrate and phosphate produced a lower number of lateral roots in PI, main root length of these plants were not remarkably influenced by different nutritional

environments (Figure 3C, D). In contrast, MM plants grown in higher regimes of nitrate and phosphate produced seedlings with longer main roots and a higher number of lateral roots (Figure 3C, D). Hypocotyl length of the seedlings was not influenced significantly by the maternal environment (Supplemental Figure S3C).

Trait by trait correlation

In order to investigate how different maternal nutrient environments affected different seed and seedling characteristics in a similar way, a correlation analysis was performed for all pairs of measured traits for either different concentrations of nitrate or phosphate, separately (Figure 4; Supplemental Table S1). For the nitrate environment, nitrate levels were positively correlated with seed and seedling performance traits such as seed size, seed weight and fresh weight of shoot and root, however nitrate content of the seeds was negatively correlated with nutrient nitrate levels (Figure 4). ABA levels had a negative correlation with almost all the measured phenotypes as also has been observed for *A. thaliana* (He *et al.*, 2016). For the phosphate environment, seed size, seed weight, germination in mannitol and salt, fresh root and shoot weight and phytate content were strongly correlated with phosphate levels. Moreover seed size and seed weight also showed a strong positive correlation with fresh root and shoot weight of seedlings for the different phosphate environments (Figure 4).

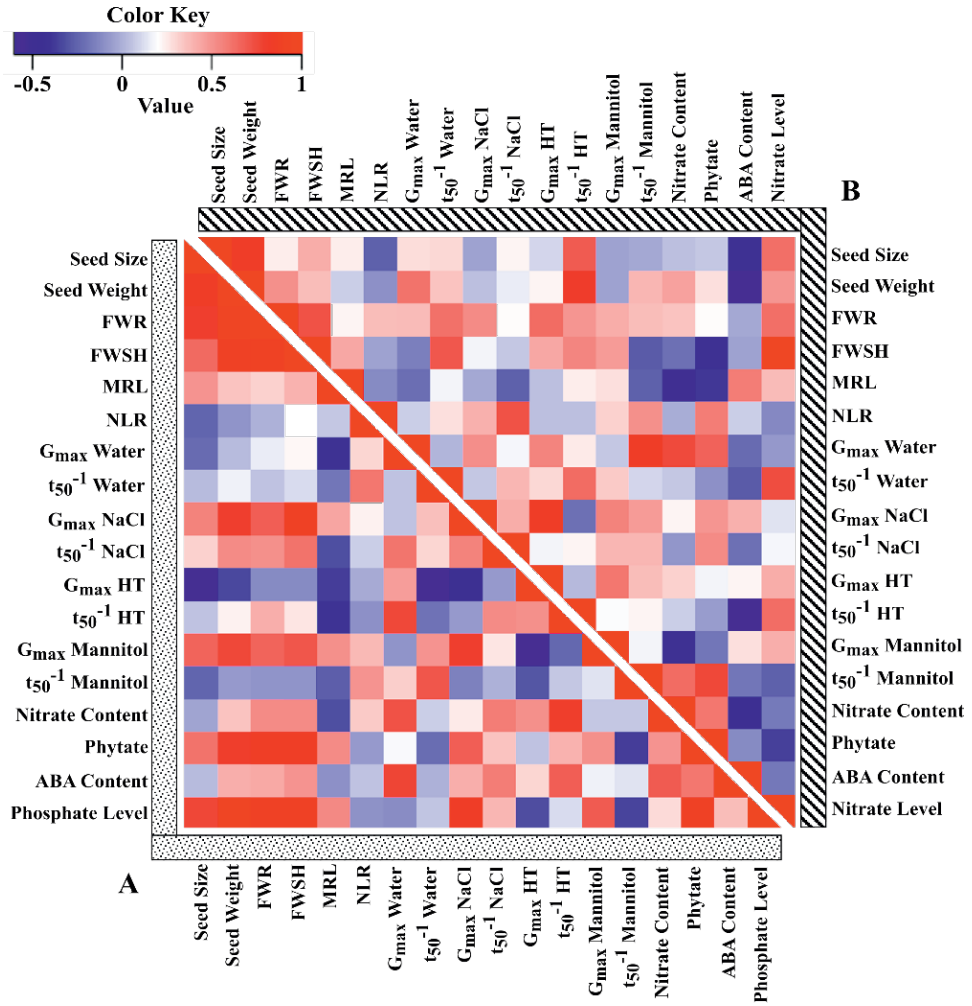

Figure 4. Heatmap of trait by trait correlations of seed and seedling traits in *Solanum lycopersicum* cv. Moneymaker **(MM)** and *Solanum pimpinellifolium* **(PI)**: in response to different concentration of **(A)** phosphate and **(B)** nitrate. **SS,** Seed size; **SW,** Seed weight; **FWR,** Fresh weight of root; **FWSH,** Fresh weight of shoot; **MR Length,** Main root length; **NLR,** Number of lateral roots; **G_{max},** Maximum germination percentage; **t_{50}^{-1},** Reciprocal of time to reach 50% of maximum germination.

Metabolite analysis

Nutritional environments of the mother plant affected seed and seedling performance traits. Since the metabolites in the dry seeds have been built up during seed maturation and drying, the underlying metabolic pathways have been analysed using a metabolomics approach to see if the observed differences in phenotype can be explained by different metabolic content of the mature dry seeds. Dry mature seeds from plants grown in the different nutritional environments have been used for metabolic analysis as it gives a broad overview of the biochemical status of the seeds and helps to better understand the responses to the different environments. This

resulted in the detection of 89 primary metabolites from which 50 could be identified. These could be classified as amino acids, organic acids, sugars and some other metabolites which are intermediates of key metabolic compounds (Supplemental Excel File S1). MM plants grown with 0 mM nitrate produced less seeds which have been used for the germination assays and therefore, metabolites of these seeds could not be measured in this study.

Genetic effects on metabolite profiles

Both PCA and cluster analysis of the metabolites showed that metabolite content was mainly affected by the genetic background of the seeds. A clear separation between samples of the two genotypes in terms of known metabolites was observed in a PCA plot which indicated that the metabolic variation caused by genetic background was larger than the variation caused by the maternal environment (Figure 5). The dendrogram which was created by cluster analysis revealed an obvious segregation between the two genotypes which is already shown by PCA analysis. There are three main clusters for each genotype in which P-Control, P-5P, P-10P and P-2.4N; P-20N and P-36N; and P-0P, P-0.1P and P-0N were grouped together (Supplemental Figure S4). Different environments were clustered with an almost identical pattern for MM seeds (Supplemental Figure S4).

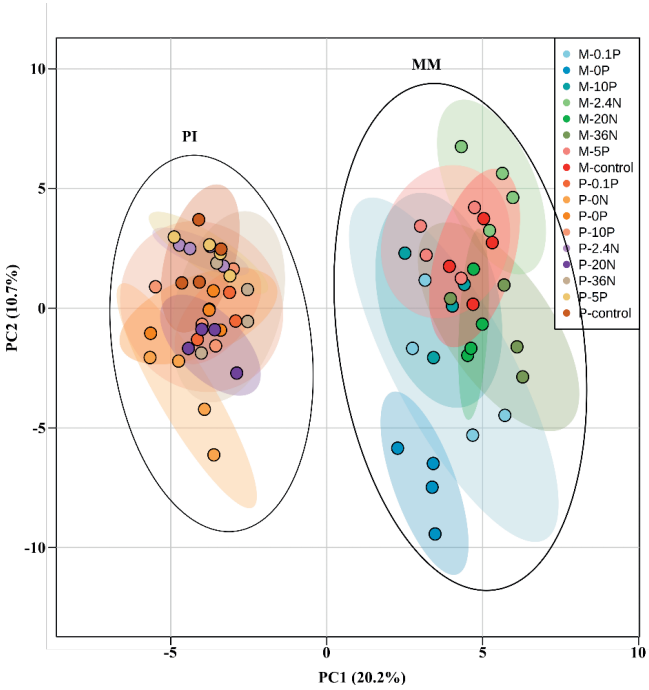

Figure 5. Principle component analysis of known primary metabolites in *Solanum lycopersicum* cv. Moneymaker **(MM, (M))** and *Solanum pimpinellifolium* **(PI, (P))** seeds in response to different concentration of nitrate (0N, 2.4N, 14N, 20N and 36N) and phosphate (0P, 0.1P, 1P, 5P and 10P) during maternal growth.

Metabolic changes in response to the maternal nutrient levels

From the 50 identified metabolites, 46 were successfully mapped to their representative pathways with help of Mapman (http://MapMan.gabipd.org) and this was used to generate a metabolic framework (Figure 6). Changing metabolite contents within the genotypes and different nutritional environments are displayed as heatmap plots below the metabolites which significantly changed in response to at least one environmental factor (Figure 6). In general, contents of nitrogen-metabolism related metabolites such as amino acids (asparagine, alanine and γ-aminobutyric acid (GABA)) and urea were decreased significantly in seeds from plants grown under lower amounts of nitrate for both genotypes. The GABA content of MM seeds was decreased at higher levels of phosphate while it was increased at higher nitrate levels. Galactarate and pyroglutamate which both are precursors of glutamate were also increased by higher amounts of nitrate. Furthermore, some of the glycolysis and TCA cycle intermediates were remarkably affected by the maternal environment. Fructose-6-phosphate (F6P), which is one of the derivatives of glucose in the glycolytic pathway, was reduced by higher phosphate levels. Citrate and malate are two TCA intermediates which were negatively influenced by increasing phosphate levels (Figure 6).

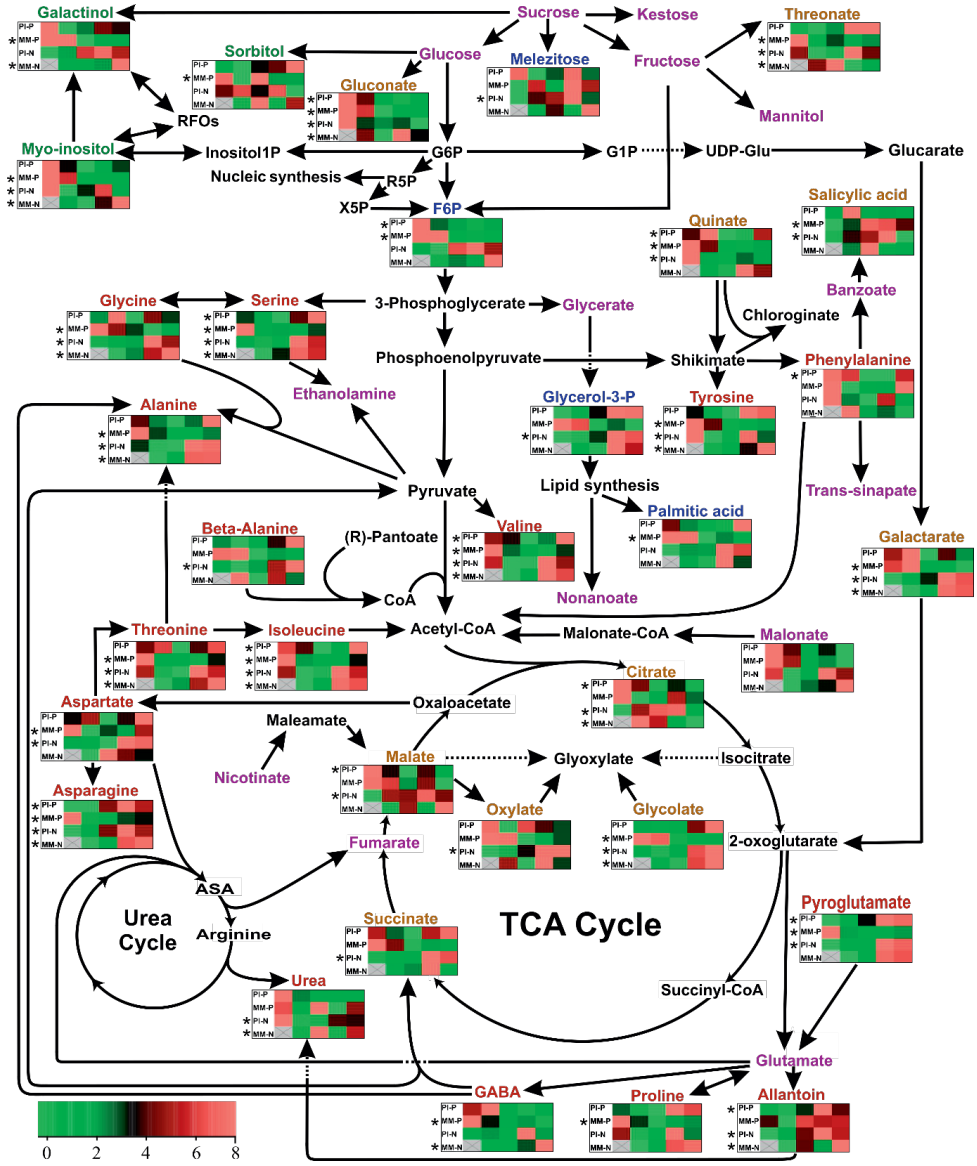

Figure 6. Overview of metabolic changes between the species influenced by maternal nutritional environments. Metabolites are shown in three colours: **Black**, Non detected metabolites; **Purple**, Detected metabolites not significantly influenced by environment; Other colours, Detected metabolites, in different categories, significantly influenced by at least one environment; **Red**, Amino acid; **Light Brown**, Organic acid; **Green**, Sugars and sugar alcohols; **Blue**, Other categories. Heatmaps contain four rows: top two rows represent PI **(PI-P)** and MM **(MM-P)** in different concentrations of phosphate (0, 0.1, 1, 5 and 10 mM from left to right). The bottom two rows represent PI **(PI-N)** and MM **(MM-N)** in different concentrations of nitrate (0, 2.4, 14, 20 and 36 mM). Colour key represents the normalized metabolite content of seeds.

Correlation of seed and seedling quality traits with metabolites

A correlation analysis was performed to find correlations between metabolic changes and seed and seedling performance. In the 9 plots of Figure 7 each plot represents the correlation of metabolites with one specific trait shown in four rows (MM and PI in nitrate and MM and PI in phosphate). Correlation analysis showed that there are some traits which have similar correlation patterns for all four conditions. For example, phytate content is positively correlated with germination characteristics under saline conditions such as Gmax and t_{50} for both environmental factors in both genotypes. Furthermore there is a negative correlation of GABA and gluconate with seed size and seed weight in all four cases. There is also a positive relationship between allantoin and fresh weight of roots and shoots in all four cases (Figure 7).

The correlation plots also indicated that some correlations were specific for either nitrate or phosphate environments. For seeds from plants grown in different phosphate environments, seed size and weight, fresh root and shoot weight and main root length of both genotypes displayed a negative correlation with some TCA cycle intermediates such as citrate, malate and malonate and positive correlation with phytate. Additionally, some correlations showed contrasting trends for the two environmental factors, such as the positive correlation of seed size and weight with citrate for seed batches originating from different maternal nitrate levels while they were negatively correlated in case of different phosphate levels (Figure 7).

There are multiple correlations which were only present for a single condition. For instance in MM plants which were grown in different concentrations of nitrate, fresh weight of root and shoot was positively correlated with a majority of the amino acids. In the same plants, seed germination quality traits under stressful conditions like salt, high temperature and osmotic stress were positively correlated with most amino acids and sugars (Figure 7).

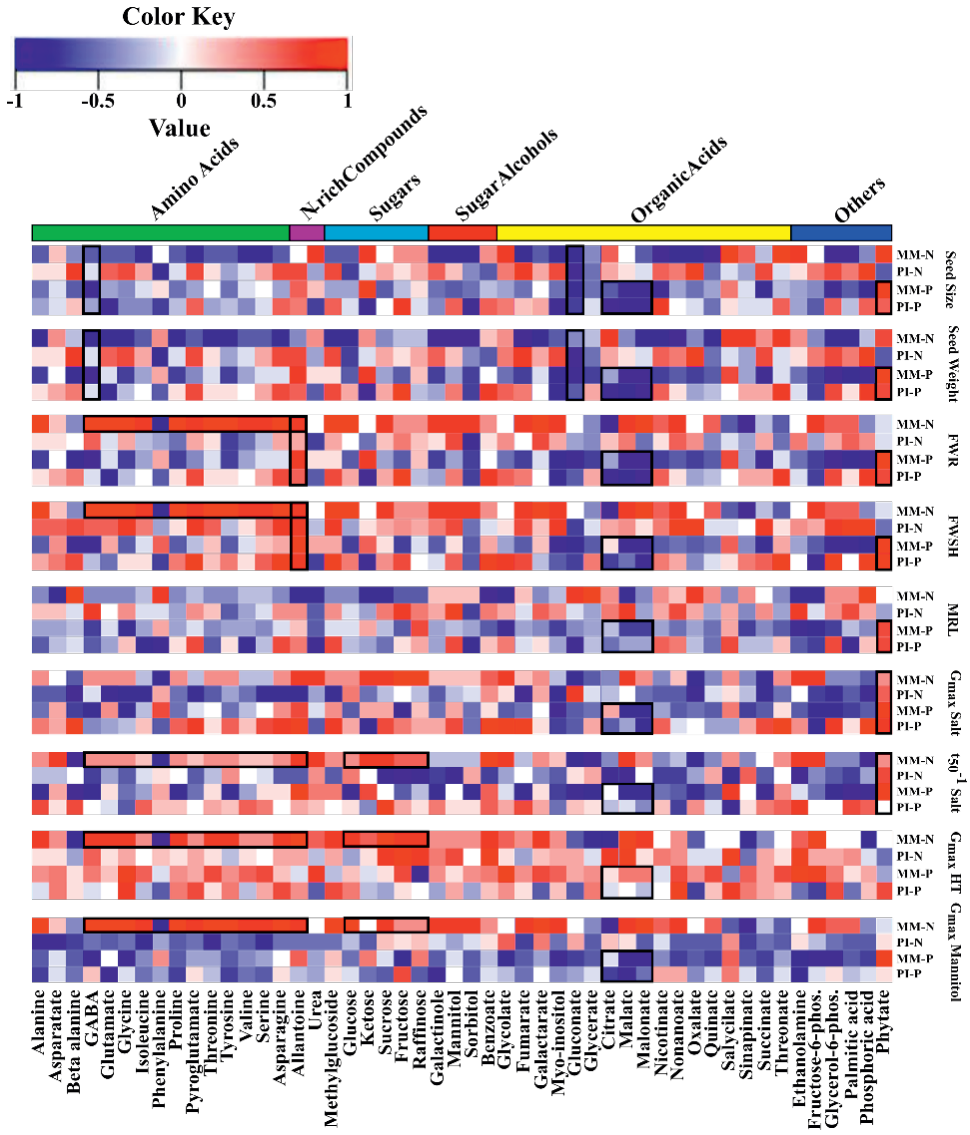

Figure 7. Correlation matrix of metabolites and seed and seedling quality traits. On the right seed and seedling traits of two tomato species: **MM** and **PI** in different concentration of **Nitrate** (**MM-N** and **PI-N**, respectively) and **MM** and **PI** in different concentration of **Phosphate** (**MM-P** and **PI-P**, respectively). At the bottom metabolites are presented in details and on top they are classified as groups of metabolites. Colour key table provides graphical representation of the correlation values of the traits and metabolites. The black rectangles indicate correlations mentioned in the result.

Discussion

Although there are many reports addressing effects of the maternal environment on seed and seedling quality traits in several species, the effect of the maternal nutritional condition on seed

performance has rarely been studied. In general, studying the effects of the maternal environment on seed performance may give insight into the processes that are involved in the adaptability of plants. The influence of the maternal environment on the next generation may be determined by several physiological traits such as germinability, size and weight of the seeds, as well as metabolic traits such as amino acid and sugar content of the seeds. Several studies have shown how the maternal environment affects seed and seedling quality in different crops. There are report on the effect of maternal photoperiod, temperature and nutrient conditions on seed performance (Demir *et al.*, 2004; Donohue, 2009; Munir *et al.*, 2001; Schmuths *et al.*, 2006). The influence of maternal nutrient conditions have been studied in different species such as tomato and Arabidopsis. It appeared that different dosages of maternal nutrients may influence seed characteristics such as seed size, seed weight and seed dormancy (Alboresi *et al.*, 2005; He *et al.*, 2014; Varis and George, 1985; Wulff, 1986). We here report the effect of a maternal environment with different concentrations of nitrate and phosphate on seed and seedling quality of two tomato genotypes. Additionally, we assessed primary metabolite profiles and analysed their correlation with different physiological traits such as seed germination and seedling development.

Genotype by environment interactions (G×E)

We used two different genotypes to see how the nutritional maternal environment may influence seed and seedling quality traits and what is the effect of G×E interactions. We observed that the genotype is profoundly affecting seed and seedling characteristics and, thus, an obvious genotype specific effect was found for some phenotypic traits such as germination at high temperature (G_{max} HT) and metabolite content such as GABA (Table 1, Figure 8). For the future, studying more *S. lycopersicum* genotypes may provide a more robust conclusion on the effect of the genotype. For phenotypic traits such as seed size and seed weight, there is a difference between the two genotypes, but there is no genotype specific effect since both genotypes are significantly influenced by nitrate and phosphate concentration (Figure 8). MM and PI showed almost the same trend for traits such as seed size, seed weight, fresh and dry weight of shoot and root and also main root length of the seedlings. However, MM plants produced generally bigger and heavier seeds and seedlings in all nutritional maternal environments (Figure 2, Figure 3). Furthermore, the highly significant influence of G×E interactions on several seed and seedling performance traits (Table 1) indicates that the phenotypic plasticity of the traits varied in relation to the different nutritional environments. In general, phosphate showed less effect than nitrate and among the nitrate levels, the traits were mostly influenced by 0N which could be an indication of the saturation of the nitrate response at the higher dosages in most of the traits (He *et al.*, 2014).

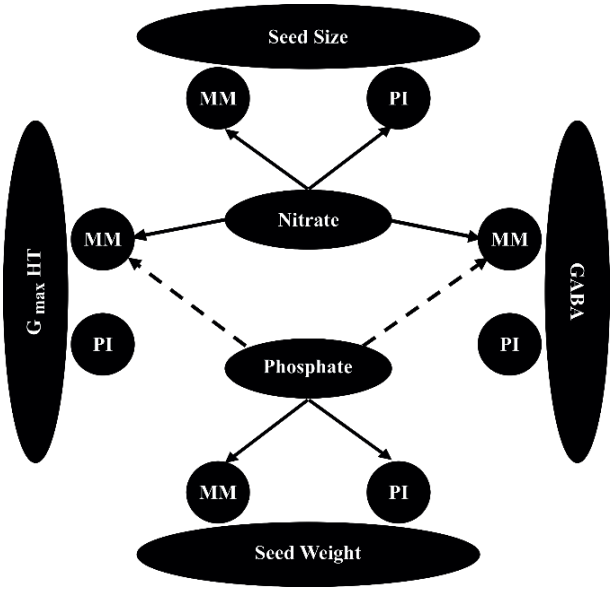

Figure 8. Summarizing the significant effects of nutritional maternal environments on seed size, seed weight, germination at high temperature (G_{max} HT) and the production of GABA in seeds. Nitrate positively regulated seed size for both genotypes while effect of nitrate on GABA accumulation and G_{max} HT was only observed when applied to MM seeds. Seed weight of both genotypes was positively regulated by phosphate content however, the (negative) effect of phosphate on GABA accumulation and G_{max} HT was only observed for MM seeds. Solid blue and dashed red lines indicating the positive and negative effects, respectively.

Relation between the nutritional environments of the mother plant and seed germination

There was also variability in the germination response of MM seeds from plants grown on different nitrate levels. Seeds that developed on higher levels of nitrate germinated better under stressful conditions, such as osmotic, salt and high temperature. These seeds also contained higher contents of amino acids. Several studies have implied that in response to stressful conditions, amino acids can be fed into the TCA cycle and serve as the main substrate for energy generation. This might explain higher seed germination percentages under stress conditions (Galili, 2011). Although different concentrations of nitrate and phosphate altered seed germination percentages under stressful conditions in MM, there was no significant change of seed germination in PI since PI showed almost 100% germination under optimal and the tested stressful germination conditions. PI is a wild tomato species and is often more tolerant to various biotic and abiotic stresses (Kazmi *et al.*, 2012; Kumar, 2006; Rao *et al.*, 2013; Rodríguez-López *et al.*, 2011). Loss of abiotic stress tolerance in tomato cultivars is thought to be the result of genetic bottlenecks during domestication (Bai and Lindhout, 2007; Doebley *et al.*, 2006).

Seed size and seedling growth are strongly influenced by the maternal nutritional environment

As described above, it appears that for both genotypes increasing the nitrate level leads to higher amounts of amino acids in the seeds. Furthermore, proteins are one of the principal storage compounds of seeds that are subsequently used as nutrients and energy source to assert seed germination and seedling establishment (Bewley *et al.*, 2012; Galili *et al.*, 2015). Thus, the higher dosage of nitrate may result in the synthesis of more amino acids during development and this might increase protein content which subsequently might result in bigger and heavier seed and seedling production and eventually successful establishment of seedlings (Castro *et al.*, 2006; Ellis, 1992). Seedling vigour and establishment are two essential parameters that may influence final crop yield and are therefore necessary for profitable crop production. Successful seedling establishment can be considered as the most critical stage of crop development. Such an important stage can be influenced by parameters such as the maternal environment in which the seeds mature and several seed characteristics such as seed size, seed weight and stored organic and mineral nutrients in the produced seeds (Lamont and Groom, 2013; Stevens *et al.*, 2014). Confirming a study by Khan *et al.* (2012), we show that seedling size in tomato is positively correlated with seed size and weight in both genotypes. The positive correlation that we found between seed and seedling size is also in agreement with several other studies (Cornelissen, 1999; Greene and Johnson, 1998; Khan *et al.*, 2012). Additionally, we have shown that increasing the maternal phosphate level enhanced seed size and seed weight which again resulted in increased seedling size. We observed that higher amounts of phosphate decreased the amount of F6P in seeds. Moreover, the level of citrate and malate in the seeds decreased with increasing maternal phosphate levels. Since glycolysis and the TCA cycle are key metabolic pathways by which organisms generate energy, decrease in the level of intermediates of these pathways like F6P, citrate and malate possibly indicates their consumption for energy production. It might suggest that higher utilization of glycolytic and TCA intermediates in seeds of higher maternal phosphate concentrations, results in more production of ATP and consequently more growth of the seedlings (Figure 3, Figure 6). In addition, production of bigger and heavier seedling for seeds developed under higher dosage of phosphate may be related to the higher amount of reserves which could be stored in bigger tomato seeds produced under the same condition.

Role of GABA in plant adaptation

Carbon (C) and nitrogen (N) are two vital factors that help plants to execute essential cellular activities. C and N metabolic pathways are strongly coordinated to ensure optimal growth and development in plants (Zheng, 2009). Several studies have reported that when plants are facing N deficiency, photosynthetic output and, consequently, plant growth is negatively influenced

(Coruzzi and Bush, 2001; Coruzzi and Zhou, 2001). Several studies have implicated a primary role of the GABA shunt in the central C/N metabolism (Fait *et al.*, 2011). In this study we found that the application of lower amounts of nitrate to mother plants resulted in lower production of GABA in the seeds of the progeny. Therefore, the decrease in GABA content in dry seeds as a result of maternal N deficiency could be an indication of GABA usage to alleviate N shortage and, subsequently, to recover the C/N balance (He *et al.*, 2016).

In this study, we observed the highest percentage of seed germination under high temperature conditions in seeds that had developed in high levels of nitrate and/or low levels of phosphate (Figure 1). On the other hand, although enhancing the maternal nitrate level results in an increase in the GABA content of the seeds of MM, enhancing the phosphate levels conversely decreased it (Figure 6). Thus, there is a good correlation for MM seeds between the different GABA levels in the seeds as a result of the maternal environment and the ability to germinate at high temperatures. This is in agreement with many studies in which GABA has been shown to act as an abiotic stress mitigating component in plants (Bouche and Fromm, 2004; Kinnersley and Turano, 2000).

In this study we observed that different dosages of nitrate and phosphate during seed development and maturation may influence the seed and seedling characteristics. We have shown that in tomato, nitrate has a greater effect on seed and seedling performance as compared with phosphate. However, two different tomato genotypes showed different responses to the maternal environment and sometimes genetic specific responses were observed for some traits. Such differential responses may indicate the contribution of different genetic and molecular pathways to the phenotypic adaptation. Further investigating such observations as well as the effect of G×E interaction on the performance of the tomato seed and seedling may ultimately help in predicting and improving seed and seedling quality by controlling production environments and breeding programs.

Materials and Methods

Plant material, growth condition and seed extraction

S. lycopersicum cv. Money maker (MM) and *S. pimpinellifolium* accession CGN14498 (PI) were grown under standard nutrient conditions (Table 2, Supplemental Table S1) with a 16-h light and 8-h dark photoperiod. The temperature was controlled during the day and night at 25°C and 15°C, respectively. From first open flower onwards the plants were transferred to the different nutrient conditions (Table 2, Supplemental Table S1).

Table 2. Nutrient conditions of mother plants after flowering.

Maternal Environment	Nitrate	Phosphate
Standard	14 mM	1.0 mM
Very low nitrate	0.0 mM	1.0 mM
Low nitrate	2.4 mM	1.0 mM
High nitrate	20.0 mM	1.0 mM
Very high nitrate	36.0 mM	1.0 mM
Very low phosphate	14 mM	0.0 mM
Low phosphate	14 mM	0.1 mM
High phosphate	14 mM	5.0 mM
Very high phosphate	14 mM	10.0 mM

For each environment four biological replicates were used. All plants were grown in the greenhouse at Wageningen University, the Netherlands. After harvesting, the seeds were collected from healthy and ripe fruits. In order to remove the pulp attached to the seeds, they were treated with 1% hydrochloric acid (HCl) and subsequently passed through a mesh sieve and washed with water to remove the remaining HCl and pulp. In the following step, seeds were treated with trisodium phosphate ($Na_3PO4.12H_2O$) for disinfection. Finally, seeds were dried at 20°C for 3 days on a clean filter paper in ambient conditions and stored in the paper bags at the room temperature (Kazmi *et al.*, 2012).

Seed phenotyping

Seed size and weight

Seed size was determined by taking photographs of 12-h imbibed seeds on white filter paper (20.2 × 14.3 cm) using a Nikon D80 camera fixed to a repro stand with 60 mm objective and connected to a computer with Nikon camera control pro software version 2.0 (Joosen *et al.*, 2010, Ligterink and Hilhorst, 2017). The pictures were analysed by ImageJ (http://rsbweb.nih.gov/ij/) combining colour threshold with particle analysis. Seed weight was measured by weighing approximately 100 dry seeds and divided by the number of seeds.

Germination assay

Germination assays were performed with four replications of around 50 seeds per sample of both genotypes in a completely randomized design. The seeds were sown in germination trays (21×15 cm DBP Plastics, http://www.dbp.be) on two blue germination papers (5.6′ × 8′ Blue Blotter Paper; Anchor Paper Company, http://www.seedpaper.com) and 50 ml demineralized water in the case of optimal and high temperature germination environments or Sodium Chloride (-0.5 MPa NaCl; Sigma-Aldrich) and mannitol (-0.5 MPa; Sigma-Aldrich) in the salt and osmotic stress conditions, respectively. Each germination tray contained three samples, using a special mask to ensure correct placement. The trays were piled up in different piles with

two empty trays on the top and bottom, containing two white filter papers and 15ml of water and covered by white plastic lids to prevent unequal evaporation and wrapped in a transparent plastic bag and stored at 4°C for 3 days. Subsequently, the bags were transferred to an incubator (type 5042; seed processing Holland, http://www.seedprocessing.nl) in the dark at 25°C except for high temperature which was at 35°C. Germination was scored at 24-h intervals during 14 consecutive days in the case of salt and osmotic stress conditions and at 8-h intervals for one week for the optimal and high temperature conditions.

Seedling phenotyping

Seedling characteristics were measured in two separate experiments. In the first 12 × 12 cm petri dishes, filled with half MS medium with agar (1%) were used. The top 4 cm of the medium was removed and the seeds, which were sterilized for 16 h in a desiccator above 100 ml sodium hypochlorite (4%) with 3 ml concentrated HCl, were sown on top of the remaining 8 cm. After sowing the seeds, the plates were stored in the cold room (4ºC) for 3 days and subsequently transferred to a climate chamber and held in a vertical position (70° angle) under 25ºC with 16h light and 8h dark. For each plate 14 seeds were used and the first 7 germinated seeds were kept. Germination was scored during the day at 8-h intervals as visible radical protrusion. After the start of germination pictures were taken at 24-h intervals for root architecture analysis. Five days after germination, seedlings were harvested and hypocotyl length (HypL) was measured. EZ-Rhizo was used to analyse root architecture (Armengaud *et al.*, 2009) and main root length (MRL) and number of lateral roots (NLR) were determined.

In the second experiment, 20 seeds of each seed batch were sown in germination trays and stored for 3 days at 4ºC. Afterwards they were transferred to an incubator at 25ºC. The first 10 germinated seeds were placed on round blue filter papers (9 cm Blue Blotter Paper; Anchor Paper Company, http://www.seedpaper.com) on a Copenhagen table at 25ºC in a randomized complete block design (with 4 biological replicates) for 10 days. To prevent evaporation, conical plastic covers with a small hole on top were placed on top of the filter papers. After 10 days, fresh and dry root and shoot weight of the seedlings was measured (FRW, DRW, FShW and DShW respectively).

Nitrate, phosphate and phytate measurement

To determine the nitrate, phosphate and phytate content of the seed samples, 15-20 mg of dry seeds were frozen in liquid nitrogen and homogenized in a dismembrator (Mikro-dismembrator U; B. Braun Biotech International, Melsungen, Germany), by using 0.6 cm glass beads, at 2500rpm for 1 minute. Fifteen mg of dry homogenized seeds with 1 ml 0.5 N HCl and 50 mg l^{-1} *trans-aconitate* (internal standard) was incubated at 100°C for 15 minutes. After

centrifugation for 3 minutes at 14000 rpm, the supernatant was filtered using Minisart SRP4 filters (Sartorius Stedim Biotech, http://www.sartorius.com) and transferred to an HPLC-vial.

A Dionex ICS2500 system was used for HPLC-analysis with an AS11-HC column and an AG11-HC guard column. The elution was performed by 0–15 min linear gradient of 25–100 mM NaOH followed by 15–20 min 500 mM NaOH and 20–35 min 5 mM NaOH with a flow rate of 1 ml min^{-1} throughout the run. Contaminating anions in the samples were removed by an ion trap column (ATC) which was installed between the pump and the sample injection valve. Conductivity detection chromatography was performed for anion detection, an ASRS suppressor was used to reduce background conductivity and water was used as counter flow. Identification and quantification of peaks was done by using authenticated external standards of nitrate (NaNO3, Merck), phosphate (Na2HPO4.2H2O, Merck) and phytate (Na(12)-IP6 IP6, Sigma-Aldrich) .

ABA determination

For ABA determination, approximately 15 mg of dry weight seed samples were homogenized as described above for nitrate, phosphate and phytate extraction and extracted in 1 ml of 10% methanol/water (v/v) according to Floková et al. (Floková *et al.*, 2014) with modifications. Stable isotope-labelled internal standard of [2H6]-ABA was added to each sample in order to validate ABA quantification. Sample extracts were centrifuged (13000 rpm/10 min/4°C) and further purified by solid-phase extraction using Strata X (30 mg/3 cc, Phenomenex) columns, activated with 1 ml of methanol, water and 1 ml of the extraction solvent. The loaded samples were washed with 3 ml of water and analyte elution was performed with 3 ml of 80% methanol/water (v/v). Samples were evaporated to dryness in a Speed-Vac concentrator and reconstituted in 60 µl of mobile phase prior to the UPLC-MS/MS analysis. The Acquity UPLC® System (Waters, Milford, MA, USA) coupled to a triple quadrupole mass spectrometer Xevo™ TQ S (Waters MS Technologies, Manchester, UK) was employed to measure ABA levels. Samples were injected on a reverse phase based column Acquity UPLC® CSH™ C18; 2.1 x 100 mm; 1.7 µm (Waters, Ireland) at flow rate 0.4 ml min^{-1}. Separation was achieved at 40°C by 9 min of gradient elution using A) 15 mM formic acid/water and B) acetonitrile: 0-1 min isocratic elution at 15% B (v/v), a 1-7 min linear gradient to 60% B, 7-9 min linear gradient to 80% B and a 9-10 min logarithmic gradient to 100% B. Finally, the column was washed with 100% acetonitrile and equilibrated to initial conditions for 2 min. the eluate was introduced to the electrospray ion source of tandem mass spectrometer operating at the following settings: source/desolvation temperature (120/550°C), cone/desolvation gas flow (147/650 l h^{-1}), capillary voltage (3 kV), cone voltage (30 V), collision energy (20 eV) and collision gas flow 0.25 ml min^{-1}. ABA was quantified in multiple reaction monitoring mode (MRM) using

standard isotope dilution method. The MassLynx™ software (version 4.1, Waters, Milford, MA, USA) was used to control the instrument, MS data acquisition and processing.

Analysis of seed metabolites by GC-TOF-MS

For metabolite extraction we used the method as described by Roessner *et al.* (2000) with small modifications. Approximately 30 tomato seeds were homogenized with a micro dismembrator (Sartorius) in 2 ml Eppendorf tubes with 2 iron beads (2.5 mm) precooled with liquid nitrogen and then 10 mg of that material has been used for metabolite extraction. Metabolite extraction was done by adding 700 µl methanol/chloroform (4:3) together with a standard (0.2 mg/ml ribitol) to each sample and mixed thoroughly. Samples were sonicated for 10 minutes and 200 µl Mili-Q water was added, followed by vortexing and centrifugation (5 min, 13500 rpm). The methanol phase was collected and transferred to a new 2 ml Eppendorf tube. Five hundred µl methanol/chloroform was added to the remaining organic phase, kept on ice for 10 min followed by adding 200 µl Mili-Q water. After vortexing and centrifugation (5 min, 13500 rpm), the methanol phase was collected and added to the previous collected phase. Finally, 100 µl of total extract was transferred to a glass vial and dried overnight in a speedvac centrifuge at 35°C (Savant SPD1211).

For each maternal environment four biological replicates were used and the gas chromatography-time of flight-mass spectrometry (GC-TOF-MS) method was used for metabolite analysis which was previously described by Carreno-Quintero *et al.* (2012). Detector voltage was set at 1600 V. The chromaTOF software 2.0 (Leco instruments) was used for analysing the raw data and further processing for extracting and aligning the mass signals was performed using the Metalign software (Lommen, 2009). A signal to noise ratio of 2 was used. Afterwards, the output was further analysed using the Metalign output Transformer (METOT; Plant Research International, Wageningen) and Centrotypes were constructed using MSClust (Tikunov *et al.*, 2012). The identification of Centrotypes was performed by matching the mass spectra to an in-house-constructed library, to the GOLM metabolome database (http://gmd.mpimp-golm.mpg.de/) and to the NIST05 library (National Institute of Standards and Technology, Gaithersburg, MD, USA; http://www.nist.gov/srd/mslist.htm). The identification was based on similarity of spectra and comparison of retention indices calculated using a 3th order polynomial function (Strehmel *et al.*, 2008).

Statistical analysis

Calculation of G_{max}, t_{50}^{-1}, AUC and U_{8416}

Seed performance was determined by calculating maximum germination (G_{max}, %), rate of germination or the reciprocal of time to reach 50% of germination (t_{50}^{-1}), uniformity of

germination or time from 16% till 84% germination (U_{8416}, h) and area under the germination curve (AUC, during the first 100 and 200h for optimal and high temperature respectively and 300h in the case of salt and mannitol stress conditions) using the curve-fitting module of the Germinator package (Joosen *et al.*, 2010).

Analysis of all factors affecting seed and seedling traits: Genotype, Environments and Genotype by environment interactions (G×E)

To identify the factors correlating with seed and seedling traits we used an ANOVA (with linear model trait ~ genotype * treatment). The different treatment regimens (N and P) where studied separately. A significance threshold of 0.05 was used.

PCA, cluster and correlation analysis

Cluster and Principle component analysis (PCA) were performed using the online web tool MetaboAnalyst 3.0; www.metaboanalyst.ca (Xia *et al.*, 2015).

R-packages "MASS", "Hmisc", "VGAM", "gplots" and "graphics" (https://www.r-project.org/) were used for analysis and construction of the correlation between measured traits.

References

Alboresi A, Gestin C, Leydecker MT, Bedu M, Meyer C, Truong HN (2005) Nitrate, a signal relieving seed dormancy in Arabidopsis. Plant Cell Environment **28:** 500-512

Arbona V, Manzi M, Ollas Cd, Gómez-Cadenas A (2013) Metabolomics as a tool to investigate abiotic stress tolerance in plants. International journal of molecular sciences **14:** 4885-4911

Armengaud P, Zambaux K, Hills A, Sulpice R, Pattison RJ, Blatt MR, Amtmann A (2009) EZ-Rhizo: integrated software for the fast and accurate measurement of root system architecture. The Plant Journal **57:** 945-956

Bai Y, Lindhout P (2007) Domestication and breeding of tomatoes: what have we gained and what can we gain in the future? Annals of botany **100:** 1085-1094

Bewley JD, Bradford K, Hilhorst H (2012) Seeds: physiology of development, germination and dormancy. Springer Science & Business Media

Bouche N, Fromm H (2004) GABA in plants: just a metabolite? Trends in plant science **9:** 110-115

Carreno-Quintero N, Acharjee A, Maliepaard C, Bachem CW, Mumm R, Bouwmeester H, Visser RG, Keurentjes JJ (2012) Untargeted metabolic quantitative trait loci analyses reveal a relationship between primary metabolism and potato tuber quality. Plant physiology **158:** 1306-1318

Castro J, Hódar JA, Gómez JM (2006) Seed size. Handbook of Seed Science and Technology. Food Products Press, Binghamton, NY**:** 397-427

Cornelissen J (1999) A triangular relationship between leaf size and seed size among woody species: allometry, ontogeny, ecology and taxonomy. Oecologia **118:** 248-255

Coruzzi G, Bush DR (2001) Nitrogen and carbon nutrient and metabolite signaling in plants. Plant Physiology **125:** 61-64

Coruzzi GM, Zhou L (2001) Carbon and nitrogen sensing and signaling in plants: emerging 'matrix effects'. Current opinion in plant biology **4:** 247-253

Delouche J, Baskin C (1971) Determinants of seed quality. Short Course For Seedsmen **14:** 53-68

Delouche JC (1980) Environmental effects on seed development and seed quality. HortScience **15:** 775-780

Demir I, Mavi K, Oztokat C (2004) Changes in germination and potential longevity of watermelon (Citrullus lanatus) seeds during development. New Zealand Journal of Crop and Horticultural Science **32:** 139-145

Doebley JF, Gaut BS, Smith BD (2006) The molecular genetics of crop domestication. Cell **127:** 1309-1321

Donohue K (2009) Completing the cycle: maternal effects as the missing link in plant life histories. Philosophical Transactions of the Royal Society of London B: Biological Sciences **364:** 1059-1074

Ellis R (1992) Seed and seedling vigour in relation to crop growth and yield. Plant growth regulation **11:** 249-255

Fait A, Angelovici R, Less H, Ohad I, Urbanczyk-Wochniak E, Fernie AR, Galili G (2006) Arabidopsis seed development and germination is associated with temporally distinct metabolic switches. Plant physiology **142:** 839-854

Fait A, Nesi AN, Angelovici R, Lehmann M, Pham PA, Song L, Haslam RP, Napier JA, Galili G, Fernie AR (2011) Targeted enhancement of glutamate-to-γ-aminobutyrate conversion in Arabidopsis seeds affects carbon-nitrogen balance and storage reserves in a development-dependent manner. Plant physiology **157:** 1026-1042

Farhadi E, Daneshyan J, Hamidi A, Rad AS, Valadabadi H (2014) Effects of parent plant nutrition with different amounts of nitrogen and irrigation on seed vigor and some characteristics associated with hybrid 704 in Kermanshah region. Journal of Novel Applied Sciences **3:** 551-556

Fenner M (1991) The effects of the parent environment on seed germinability. Seed Science Research **1:** 75-84

Fenner M (1992) Environmental influences on seed size and composition. Horticultural reviews **13:** 183-213

Floková K, Tarkowská D, Miersch O, Strnad M, Wasternack C, Novák O (2014) UHPLC–MS/MS based target profiling of stress-induced phytohormones. Phytochemistry **105:** 147-157

Galili G (2011) The aspartate-family pathway of plants: linking production of essential amino acids with energy and stress regulation. Plant signaling & behavior **6:** 192-195

Galili G, Avin-Wittenberg T, Angelovici R, Fernie AR (2015) The role of photosynthesis and amino acid metabolism in the energy status during seed development. Advances in Seed Biology**:** 99

Greene D, Johnson E (1998) Seed mass and early survivorship of tree species in upland clearings and shelterwoods. Canadian Journal of Forest Research **28:** 1307-1316

He H, de Souza Vidigal D, Snoek LB, Schnabel S, Nijveen H, Hilhorst H, Bentsink L (2014) Interaction between parental environment and genotype affects plant and seed performance in Arabidopsis. Journal of experimental botany **65:** 6603-6615

He H, Willems LA, Batushansky A, Fait A, Hanson J, Nijveen H, Hilhorst HW, Bentsink L (2016) Effects of parental temperature and nitrate on seed performance are reflected by partly overlapping genetic and metabolic pathways. Plant and Cell Physiology**:** pcv207

Hejcman M, Křišťálová V, Červená K, Hrdličková J, Pavlů V (2012) Effect of nitrogen, phosphorus and potassium availability on mother plant size, seed production and germination ability of Rumex crispus. Weed Research **52:** 260-268

Hilhorst HW, Karssen CM (1988) Dual effect of light on the gibberellin-and nitrate-stimulated seed germination of Sisymbrium officinale and Arabidopsis thaliana. Plant Physiology **86:** 591-597

Joosen RV, Kodde J, Willems LA, Ligterink W, van der Plas LH, Hilhorst HW (2010) germinator: a software package for high-throughput scoring and curve fitting of Arabidopsis seed germination. The Plant Journal **62:** 148-159

Joosen RVL, Arends D, Li Y, Willems LA, Keurentjes JJ, Ligterink W, Jansen RC, Hilhorst HW (2013) Identifying genotype-by-environment interactions in the metabolism of germinating Arabidopsis seeds using generalized genetical genomics. Plant physiology **162:** 553-566

Kazmi RH, Khan N, Willems LA, Van Heusden AW, Ligterink W, Hilhorst HW (2012) Complex genetics controls natural variation among seed quality phenotypes in a recombinant inbred population of an interspecific cross between Solanum lycopersicum× Solanum pimpinellifolium. Plant, cell & environment **35:** 929-951

Khan N, Kazmi RH, Willems LA, Van Heusden AW, Ligterink W, Hilhorst HW (2012) Exploring the natural variation for seedling traits and their link with seed dimensions in tomato. PLoS One **7:** e43991

Kinnersley AM, Turano FJ (2000) Gamma aminobutyric acid (GABA) and plant responses to stress. Critical Reviews in Plant Sciences **19:** 479-509

Kumar N (2006) Breeding of horticultural crops: principles and practices. New India Publishing

Lamont BB, Groom PK (2013) Seeds as a source of carbon, nitrogen, and phosphorus for seedling establishment in temperate regions: a synthesis.

Lommen A (2009) MetAlign: interface-driven, versatile metabolomics tool for hyphenated full-scan mass spectrometry data preprocessing. Analytical chemistry **81:** 3079-3086

Mounet F, Lemaire-Chamley M, Maucourt M, Cabasson C, Giraudel J-L, Deborde C, Lessire R, Gallusci P, Bertrand A, Gaudillère M (2007) Quantitative metabolic profiles of tomato flesh and seeds during fruit development: complementary analysis with ANN and PCA. Metabolomics **3:** 273-288

Munir J, Dorn L, Donohue K, Schmitt J (2001) The influence of maternal photoperiod on germination requirements in Arabidopsis thaliana. American Journal of Botany **88:** 1240-1249

Pourrat Y, Jacques R (1975) The influence of photoperiodic conditions received by the mother plant on morphological and physiological characteristics of Chenopodium polyspermum L. seeds. Plant Science Letters **4:** 273-279

Rao ES, Kadirvel P, Symonds RC, Ebert AW (2013) Relationship between survival and yield related traits in Solanum pimpinellifolium under salt stress. Euphytica **190:** 215-228

Rodríguez-López M, Garzo E, Bonani J, Fereres A, Fernández-Muñoz R, Moriones E (2011) Whitefly resistance traits derived from the wild tomato Solanum pimpinellifolium affect the preference and feeding behavior of Bemisia tabaci and reduce the spread of Tomato yellow leaf curl virus. Phytopathology **101:** 1191-1201

Roessner U, Wagner C, Kopka J, Trethewey RN, Willmitzer L (2000) Simultaneous analysis of metabolites in potato tuber by gas chromatography–mass spectrometry. The Plant Journal **23:** 131-142

Rowse H, Finch-Savage W (2003) Hydrothermal threshold models can describe the germination response of carrot (Daucus carota) and onion (Allium cepa) seed populations across both sub-and supra-optimal temperatures. New Phytologist **158:** 101-108

Schachtman DP, Reid RJ, Ayling SM (1998) Phosphorus uptake by plants: from soil to cell. Plant physiology **116:** 447-453

Schmuths H, Bachmann K, Weber WE, Horres R, Hoffmann MH (2006) Effects of preconditioning and temperature during germination of 73 natural accessions of Arabidopsis thaliana. Annals of botany **97:** 623-634

Song J, Zhou J, Zhao W, Xu H, Wang F, Xu Y, Wang L, Tian C (2014) Effects of salinity and nitrate on production and germination of dimorphic seeds applied both through the mother plant and exogenously during germination in Suaeda salsa. Plant Species Biology

Sperling L, Osborn TC, Cooper HD (2004) Towards effective and sustainable seed relief activities: report of the Workshop on Effective and Sustainable Seed Relief Activities, Rome, 26-28 May 2003, Vol 181. Food & Agriculture Org.

Stevens N, Seal CE, Archibald S, Bond W (2014) Increasing temperatures can improve seedling establishment in arid-adapted savanna trees. Oecologia **175:** 1029-1040

Strehmel N, Hummel J, Erban A, Strassburg K, Kopka J (2008) Retention index thresholds for compound matching in GC–MS metabolite profiling. Journal of Chromatography B **871:** 182-190

Thomas JF, Raper CD (1979) Germinability of tobacco seed as affected by culture of the mother plant. Agronomy Journal **71:** 694-695

Tikunov Y, Laptenok S, Hall R, Bovy A, De Vos R (2012) MSClust: a tool for unsupervised mass spectra extraction of chromatography-mass spectrometry ion-wise aligned data. Metabolomics **8:** 714-718

Urbanczyk-Wochniak E, Fernie AR (2005) Metabolic profiling reveals altered nitrogen nutrient regimes have diverse effects on the metabolism of hydroponically-grown tomato (Solanum lycopersicum) plants. Journal of Experimental Botany **56:** 309-321

Varis S, George R (1985) influence of mineral nutrition on fruit yield, seed yield and quality in tomato. Journal of horticultural science

Wulff RD (1986) Seed size variation in Desmodium paniculatum: I. Factors affecting seed size. The Journal of Ecology**:** 87-97

Xia J, Sinelnikov IV, Han B, Wishart DS (2015) MetaboAnalyst 3.0—making metabolomics more meaningful. Nucleic acids research **43:** W251-W257

Zheng Z-L (2009) Carbon and nitrogen nutrient balance signaling in plants. Plant Signaling & Behavior **4:** 584-591

Supplementary information

Supplementary Tables and Excel files of this chapter can be downloaded from http://www.wageningenseedlab.nl/thesis/ngeshnizjani/SI/chapter2

Supplementary Figures

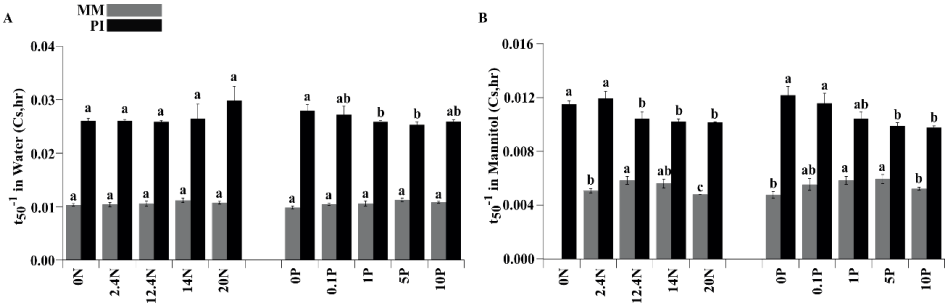

Figure S1. Effects of maternal nutritional environments on seed germination traits of both **MM** and **PI**. **A**, t_{50}^{-1} in water; **B**, t_{50}^{-1} in Mannitol (-0.5 MPa) in different concentration of nitrate (0N, 2.4N, 12.4N, 14N and 20N) and phosphate (0P, 0.1P, 1P, 5P and 10P).

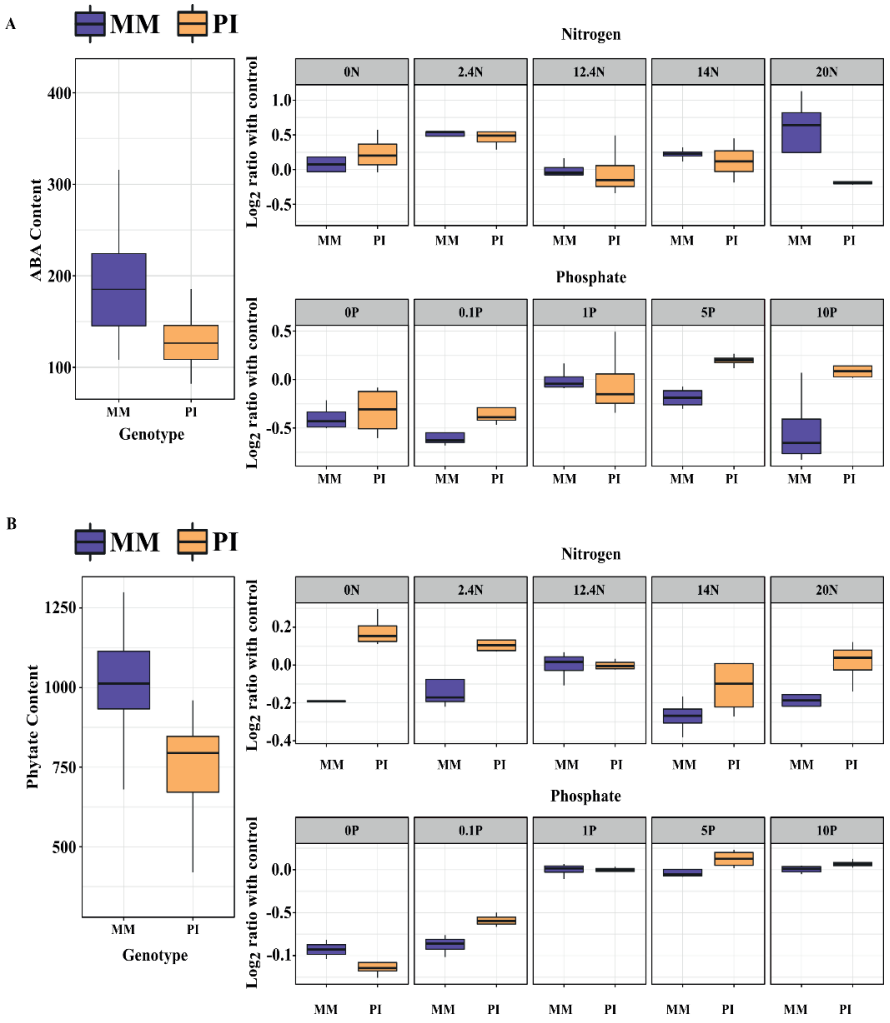

Figure S2. Effects of maternal nutritional environments on ABA **(A)** and Phytate **(B)** content of both **MM** and **PI** seeds developed in different concentrations of nitrate (0N, 2.4N, 12.4N, 14N and 20N) and phosphate (0P, 0.1P, 1P, 5P and 10P).

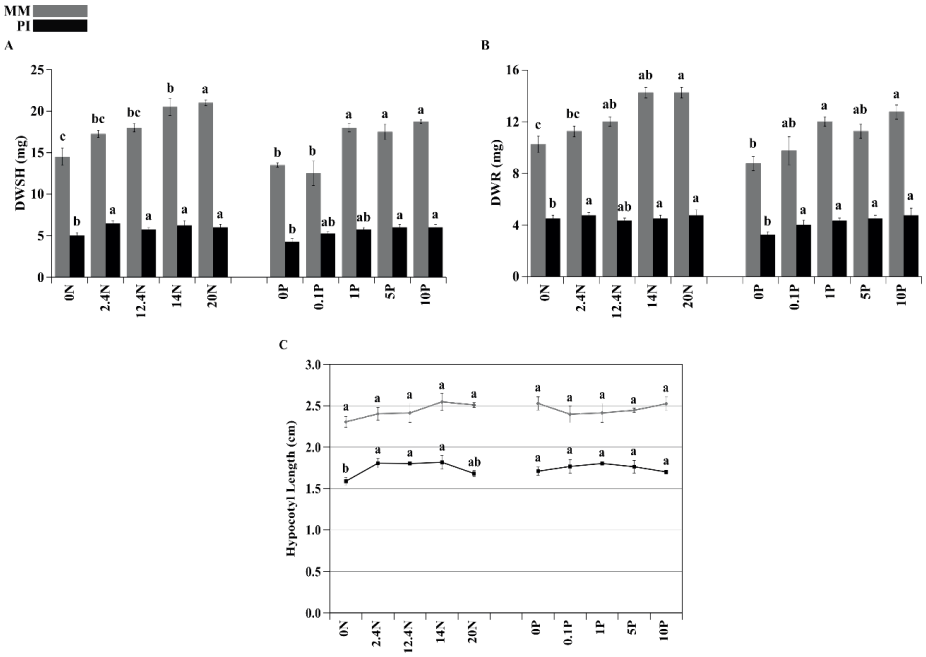

Figure S3. Effects of maternal nutritional environments on seedling quality traits of both **MM** and **PI**. **A**, Dry weight of shoot; **B**, Dry weight of root; **C**, Hypocotyl length of seedlings in different concentration of nitrate (0N, 2.4N, 12.4N, 14N and 20N) and phosphate (0P, 0.1P, 1P, 5P and 10P).

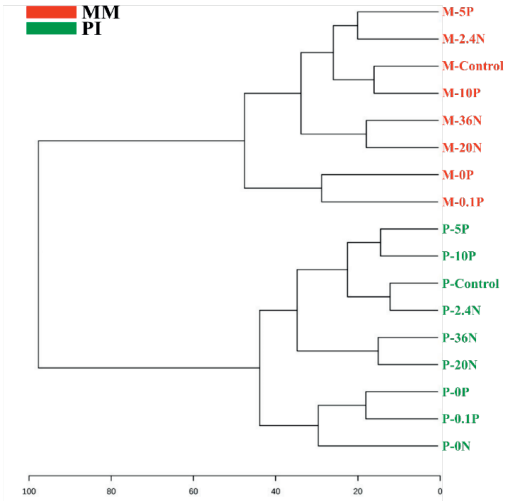

Figure S4. cluster analysis of known primary metabolites in **MM** and **PI** seeds in response to different concentration of nitrate (0N, 2.4N, 12.4N, 14N and 20N) and phosphate (0P, 0.1P, 1P, 5P and 10P) during maternal growth.

Chapter 3

Detection of QTLs for genotype × environment interactions in tomato seeds and seedlings

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Abstract

Many factors influence successful germination, seedling establishment and further growth and development of all plants. Among these factors seed quality is one of the most important with a clear effect on successful crop development. High quality seed will aid successful seed germination and seedling establishment, especially under sub-optimal conditions. Seed quality is acquired during seed maturation and, thus, in addition to the genetic background of the mother plant, it can be strongly influenced by the maternal environment under which the seeds develop. There is little knowledge about the genetic and environmental factors, and their interaction, that influence seed quality and seedling establishment. The aim of this study is to identify the loci and possible molecular mechanisms involved in acquisition of seed quality and how these are controlled by adverse maternal nutritional conditions. For this, we used a tomato recombinant inbred line (RIL) population consisting of 100 lines which during seed development were grown under various nutritional environmental conditions, like high phosphate and low nitrate. Extensive phenotyping of the harvested seeds showed strong variation for most of the seed germination traits such as maximum germination percentage (G_{max}), germination rate (t_{50}) and uniformity (U_{8416}) under different germination conditions. This variation resulted in identification of QTLs which were not only dependent on genetic factors, but also on the maternal environment (QTL×E). Further studies of these QTLs and the environments in which they show up may ultimately help to predict the effect of different maternal environmental conditions on seed quality which will be very useful to improve the production of high-performance seeds.

Introduction

Tomato due to the level of production throughout the world (4.8 million hectares with the average yield of 37 ton per hectare (FAOSTAT2016)) is one of the most important agricultural commodities and being used generally as a fresh vegetable (Heuvelink, 2018). Moreover tomato is of scientific importance as a model organism for fruit-bearing plants (Giovannoni, 2001; Schauer *et al.*, 2006). Tomato producers are attempting to produce plants with high quality fruits as well as with high resistance against stressful environments, such as high temperature and osmotic stress. Since tomato is propagated by seed, the first step to improve tomato production is improving the quality of the seeds.

One of the characteristics of seed quality is the ability of the seed to germinate quickly and uniformly, not only under optimal but especially also under stress-full germination conditions (Foolad *et al.*, 2008). Furthermore, seed quality is not solely determined by germination but also by many other attributes such as genetic purity, vigour, viability and lack of any disease and damages, which all affect seed performance (Hilhorst & Toorop, 1997; Hilhorst & Koornneef, 2007; Hilhorst *et al.*, 2010). Additionally, these quality parameters may severely affect seedling establishment and further growth of the plant and, ultimately, the success of crop production. In general, low quality seeds, for instance seeds with low vigour, lead to poor seedling establishment and finally lower and non-profitable crop yield (Finch-Savage, 1995). An important determinant of seed quality and performance is the maternal environment under which seeds develop and mature. The different environmental factors during seed development, such as temperature, light and nutrients may alter ultimate seed quality. Therefore, seed quality is defined by both the genetics (G) and the environment (E), as well as their interaction (G×E) (McDonald, 1998; Koornneef *et al.*, 2002).

In tomato, as in many other crops, the domestication process has been accompanied by an attrition of genetic variation and, consequently, loss of many potentially desirable traits (McCouch, 2004; Doebley *et al.*, 2006). Therefore, domesticated cultivars are sensitive to non-optimal germination conditions which limits their production to optimal environments (Foolad & Lin, 1997; Foolad & Lin, 1998; Foolad *et al.*, 2008). However, a large source of genetic variation is found within wild species of tomato (*Solanum habrochaitis*, *Solanum pimpinellifolium* and *Solanum pennellii*). As cultivated crops suffer from abiotic stress, such as high temperature, drought and salinity by increased frequency and severity due to climate change, existing genetic variation could be used to reintroduce lost valuable traits in the domesticated cultivars to cope with these environmental stresses (Lippman *et al.*, 2007; Kazmi *et al.*, 2012).

Seed dormancy is profoundly affected by the environment (Huo & Bradford, 2015). Seeds perceive their environment and under undesirable conditions they typically do not germinate and become dormant. Nowadays, due to global warming, high temperature is regarded as one of the most important unfavourable environmental factors affecting seed germination. For instance, the germination of seeds of several species such as carrot (*Daucus carota*), lettuce (*Lactuca sativa*) and Arabidopsis is affected by thermo-inhibition or thermo-dormancy (Toh *et al.*, 2008; Lafta & Mou, 2013; Nascimento *et al.*, 2013). Thermo-inhibition refers to the fact that seeds will stop germination under high temperature, yet will immediately germinate upon facing the optimal temperatures. In the case of seed dormancy, seeds will germinate neither at high temperatures, nor at subsequent lower/optimal germination temperatures (Argyris *et al.*, 2008; Huo *et al.*, 2013). It is previously reported that different maternal environments such as temperature, light, water and nutrient availability during seed development and maturation may affect seed dormancy (Fenner, 1991; Hilhorst, 1995; Holdsworth *et al.*, 2008; Bewley *et al.*, 2012).

Natural variation present in traits such as seed size and weight, as well as dormancy and germination, exhibits a continuous distribution and is considered as quantitative variation likely regulated by multiple quantitative trait loci (QTL) (Koornneef *et al.*, 2002; Argyris *et al.*, 2008). A population of recombinant inbred lines (RILs) may be used for measuring the existing natural variation followed by QTL mapping as a powerful tool to detect loci affecting seed traits (Alonso-Blanco *et al.*, 2009). Many studies have characterized QTLs regulating complex quantitative seed traits in different species (Koornneef *et al.*, 2002; Argyris *et al.*, 2008; Joosen *et al.*, 2012; Kazmi *et al.*, 2012). However, few studies have been conducted to investigate the interaction between the maternal environment and genetic variation (Dechaine *et al.*, 2009; Elwell *et al.*, 2011). In general, final seed performance is determined by the function of several genes and their interaction with the environment. Using high throughput genetic tools, including QTL mapping, to discover the genotype by environment interaction effects on QTLs affecting these seed traits provides a better understanding of how plants adapt to and cope with new stressful environments and is a prerequisite for crop improvement (Des Marais *et al.*, 2013; El-Soda *et al.*, 2014).

In this study we analysed natural variation of several seed and seedling traits including maximum germination percentage, rate of germination, thermo-dormancy and -inhibition of seed germination, as well as fresh and dry weight of seedlings. We have used a RIL population derived from two tomato accessions: *Solanum lycopersicum* (cv. Moneymaker) (MM) and *Solanum pimpinellifolium* (PI) (Voorrips *et al.*, 2000). From the collection of tomato wild cultivars, *S. pimpinellifolium* has been used most frequently in breeding programs. It is the most closely related wild species to the domesticated tomato cultivar (*S. lycopersicum*) and has also

the ability to naturally cross with *S. lycopersicum* (Rick, 1958). To investigate the existing genetic variation of seed and seedling related traits, we specifically focused on the maternal environment in which seeds develop and mature. We investigated how loci with genetic variation respond to different nutritional environments of the mother plant. To do so, the RILs were exposed to high phosphate and low nitrate environments during seed development and their seeds were tested for seed and seedling related traits. In addition we performed a QTL×E approach to increase the power for detecting the loci affected by the different maternal environments (Malosetti *et al.*, 2004; Moreau *et al.*, 2004; Van Eeuwijk *et al.*, 2007; Joosen *et al.*, 2012).

Results

To identify the loci involved in variation in tomato seed and seedling traits in interaction with different maternal nutritional conditions, we used a population of recombinant inbred lines (RILs) derived from a cross between a wild (*Solanum pimpinellifolium*) and a domesticated (*Solanum lycopersicum* (cv. Moneymaker)) tomato species (Voorrips *et al.*, 2000). The RILs were grown under high phosphate (HP) and low nitrate (LN) environments after which the seeds were harvested for seed and seedling trait measurements. We mapped QTLs for 5 seed germination traits under four different germination environments, three seed thermo-dormancy traits, two seed morphology traits and four seedling traits (Table 1). By using the Germinator package (Joosen *et al.*, 2010) seed germination traits including maximum germination (G_{max}), rate of germination (time to reach 50% of germination, t_{50}), uniformity of germination (time between 16% and 84% of germination, U_{8416}) and area under the germination curve (until 200 hrs, AUC) were assessed. In order to quantify seed vigour, we germinated tomato seeds in water and under three suboptimal conditions; NaCl and mannitol solutions, and high temperature (HT). The effect of the environment on seed and seedling traits may be related to the seed germination environment (GE), seed maturation environment (ME) and their interaction. Regarding the importance of global warming and adaptation of seed germination and seedling growth to it, we also mapped QTLs for thermo-tolerance (Th-T), thermo-inhibition (Th-I) and thermo-dormancy (Th-D). Furthermore, seed size (SS) and seed weight (SW) of the RILs were measured and used for QTL mapping. To investigate the loci involved in seedling establishment, we also measured seedling traits such as fresh and dry weight of shoot and root (FWSH, DWSH, FWR and DWR, respectively) (Table 1).

Table 1. Overview of the traits and the germination environments used in this study.

	Traits	Germination Environments	Codes
Seed Germination Traits	G_{max}	Water	G_{max} water
		NaCl	G_{max} NaCl
		Mannitol	G_{max} Mann
		High Temperature	G_{max} HT
	t_{10}^{-1}	Water	t_{10}^{-1} water
		NaCl	t_{10}^{-1} NaCl
		Mannitol	t_{10}^{-1} Mann
		High Temperature	t_{10}^{-1} HT
	t_{50}^{-1}	Water	t_{50}^{-1} water
		NaCl	t_{50}^{-1} NaCl
		Mannitol	t_{50}^{-1} Mann
		High Temperature	t_{50}^{-1} HT
	AUC	Water	AUC water
		NaCl	AUC NaCl
		Mannitol	AUC Mann
		High Temperature	AUC HT
	U_{8416}^{-1}	Water	U_{8416}^{-1} water
		NaCl	U_{8416}^{-1} NaCl
		Mannitol	U_{8416}^{-1} Mann
		High Temperature	U_{8416}^{-1} HT
	Thermo-Dormancy	Thermo-Tolerance	Th-T
		Thermo-Inhibition	Th-I
		Thermo-Dormancy	Th-D
Seed and Seedling Traits	Seed Morphology Traits	Seed Size	SS
		Seed Wight	SW
	Seedling Traits	Fresh weigh of Shoot	FWSH
		Dry weigh of Shoot	DWSH
		Fresh weigh of Root	FWR
		Dry weigh of Shoot	DWR

G_{max}, Maximum seed germination percentage; **t_{50}^{-1}** and **t_{10}^{-1}**, Reciprocal of time to respectively reach 50 and 10% of maximum germination; **AUC**, Area under the germination curve; **U_{8416}^{-1}**, Reciprocal of time between 16 and 84% of maximum germination.

Variability and heritability of seed and seedling traits

In both suboptimal nutritional conditions (HP and LN) most of the traits displayed wide variation for the parental lines (*Solanum lycopersicum* (cv. Moneymaker) (MM) and *Solanum pimpinellifolium* (PI)). Regarding the seed germination traits such as G_{max} and AUC this variation was more evident under suboptimal germination condition such as HT (Figure 1, Table 2). However, for most of the traits MM was affected more by suboptimal conditions than PI, which suggests higher susceptibility of MM to stressful conditions (Figure 1, Supplemental Figure S1). Calculating the $\log_2$ ratio of HP:LN showed that in some traits, notably in SS and SW, different maternal nutritional environments did not affect the parental lines, however in several other traits they have been influenced by different nutrient environments (Figure 2). Considerable phenotypic variation for some of the traits was also reflected in the RILs for each

nutritional environment, with coefficient of variation (CV) ranking from 12% - 120% and 13% - 190% under HP and LN conditions, respectively (Figure 3; Supplemental Table S1).

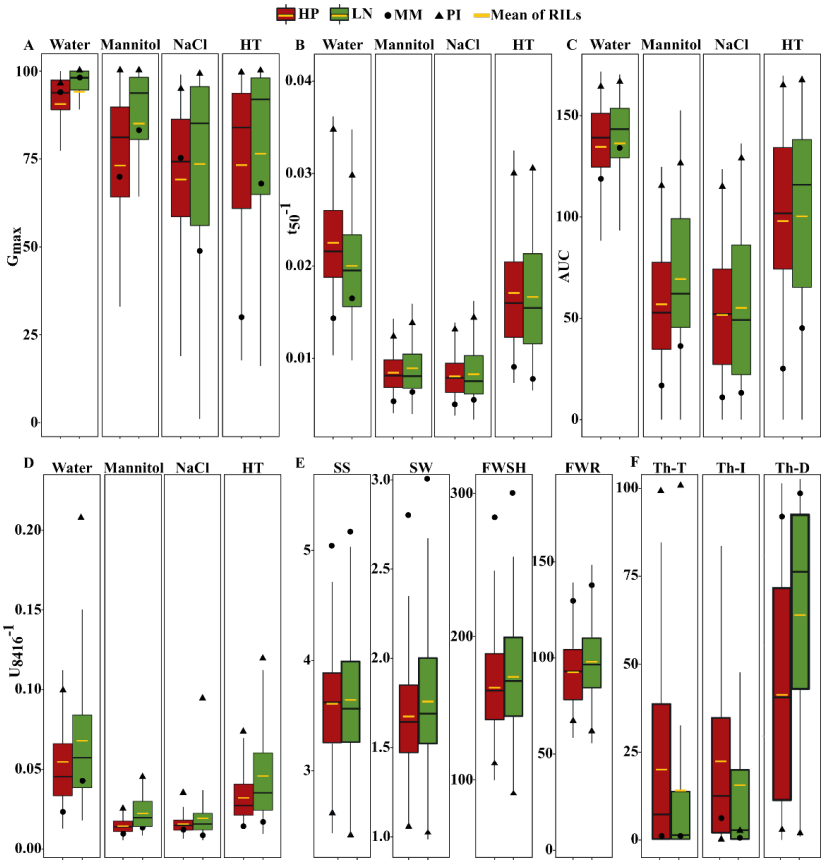

Figure 1. Effect of nutritional maternal environments on seed, seedling and seed germination traits. **A, G_{max}**, Maximum seed germination percentage; **B, t_{50}^{-1},** Reciprocal of time to reach 50% of maximum germination; **C, AUC**, Area under the germination curve; **D, U_{8416}^{-1}**, Reciprocal of time between 16 and 84% of maximum germination; **E,** Seed morphology and seedling traits, **SS**, Seed size; **SW**, Seed weight; **FWSH**, Fresh weight of shoot; **FWR**, Fresh weight of root; **F,** Response of seed germination to high temperature, **Th-T**, Thermo-tolerance; **Th-I**, Thermo-inhibition; **Th-D**, Thermo-dormancy; **HP**, High phosphate (in red); **LN**, Low nitrate (in green); **MM,** *Solanum lycopersicum* (cv. Moneymaker) (black circle); **(PI),** *Solanum pimpinellifolium* (black triangle); Mean of all **RIL**s (Recombinant Inbred Line) (yellow line); **HT**, High temperature.

Regarding the CV values the largest variation was perceived in Th-D followed by AUC and U_{8416} traits indicating high level of variation in these traits. On the other hand, maximum germination percentage (G_{max}) of seeds in water showed the lowest percentage of CV which is as expected since most of the RILs germinated almost 100% in water. The $\log_2$ ratio analysis of HP:LN in RILs exhibited a similar result as the parental line in which several traits like AUC, U_{8416}^{-1}, Th-T, Th-I and Th-D have been differently affected by HP and LN (Figure 2).

Table 2. Averages and broad-sense heritability of seed germination and seedling traits of RILs and their parental accessions *Solanum lycopersicum* (cv. Moneymaker) and *Solanum pimpinellifolium* grown in high phosphate (HP) and low nitrate (LN) conditions.

Trait		HP				LN			
		MM	**PI**	**RIL**	**H^2 (%)**	**MM**	**PI**	**RIL**	**H^2 (%)**
G_{max} (%)	Water	94.1	96.5	90.8	77	98.2	100.0	94.4	93
	NaCl	74.8	94.7	69.2	81	48.4	99.0	73.9	85
	Mann.	69.5	100.0	73.2	90	82.7	100.0	85.3	89
	HT	29.5	99.4	73.8	91	67.5	100.0	76.7	89
t_{50}^{-1} (×100, h^{-1})	Water	1.42	3.47	2.26	90	1.63	2.97	2.00	91
	NaCl	0.48	1.30	0.82	83	0.53	1.43	0.84	70
	Mann.	0.51	1.23	0.86	87	0.63	1.37	0.90	82
	HT	0.89	2.99	1.71	89	0.76	3.04	1.67	92
t_{10}^{-1} (×100, h^{-1})	Water	2.15	4.33	3.08	83	2.11	3.26	2.52	87
	NaCl	0.64	1.65	1.16	83	0.84	1.58	1.15	69
	Mann.	0.76	1.70	1.27	88	0.87	1.68	1.21	84
	HT	1.38	3.92	2.49	88	1.03	3.61	2.23	86
AUC (hrs)	Water	118.0	163.9	135.0	91	133.4	166.3	136.6	93
	NaCl	10.2	114.6	52.0	78	12.4	128.6	55.4	88
	Mann.	15.9	114.8	57.3	85	35.5	126.1	69.7	90
	HT	24.4	164.6	98.6	93	44.4	166.9	100.7	94
U_{8416}^{-1} (×100, h^{-1})	Water	2.22	9.89	5.53	66	4.18	20.8	6.76	75
	NaCl	1.11	3.63	1.63	49	0.76	9.41	1.95	54
	Mann.	0.85	2.47	1.51	71	1.24	4.43	2.28	68
	HT	1.32	7.32	3.27	64	1.59	11.9	4.63	69
Dormancy	Th-T	0.48	95.3	20.5	83	0.50	96.8	13.0	98
	Th-I	5.23	0.00	23.2	50	0.00	2.09	15.0	94
	Th-D	89.3	2.54	44.1	86	95.8	1.56	63.1	92
Seed Traits	SS	4.93	2.59	3.57	89	5.05	2.40	3.60	94
	SW	0.27	0.10	0.16	89	0.29	0.10	0.17	96
Seedling Traits	FWSH	28.2	11.1	16.4	72	30.0	9.00	17.2	78
	DWSH	1.50	0.58	0.92	63	1.66	0.57	0.96	71
	FWR	12.4	6.39	8.94	76	13.2	5.87	9.45	68
	DWR	0.71	0.38	0.53	69	0.79	0.38	0.57	62

HP, High phosphate; **LN**, Low nitrate; **MM**, *Solanum lycopersicum* (cv. Moneymaker); **PI**, *Solanum pimpinellifolium*; **RIL**, Recombinant Inbred Line; **H^2**, Broad-sense heritability (%); **G_{max}**, Maximum seed germination percentage; **t_{50}^{-1}, t_{10}^{-1}**, Reciprocal of time to respectively reach 50 and 10% of maximum germination; **AUC**, Area under the germination curve; **U_{8416}^{-1}**, Reciprocal of time between 16 and 84% of maximum germination; **Mann**, Mannitol; **HT**, High temperature; **Th-T**, Thermo-tolerance; **Th-I**, Thermo-inhibition; **Th-D**, Thermo-dormancy; **SS**, Seed size; **SW**, Seed weight; **FWSH**, Fresh weight of shoot; **DWSH**, Dry weight of shoot; **FWR**, Fresh weight of root; **DWR**, Dry weight of root; **NA**, Not available.

The principal component analysis (PCA) of the RILs and parental lines for all traits in both maternal environments showed that 41 and 11% of the variation was explained by PC1 and PC2, respectively. The PCA plot showed that parental lines in general are flanking the RILs (Figure 4). Similar results have been obtained when considering individual traits where the phenotypes of the RILs are mainly found between the phenotypes of the two parental genotypes; however, some traits show transgression with some RILs displaying more extremes than their parents. This exemplifies the inheritance from both parental lines to the progenies in which one parent has most positive and the other one has most negative alleles. In a few cases, such as

G_{max} water in both nutritional environments, substantial transgression was observed (Figure 1; Table 2).

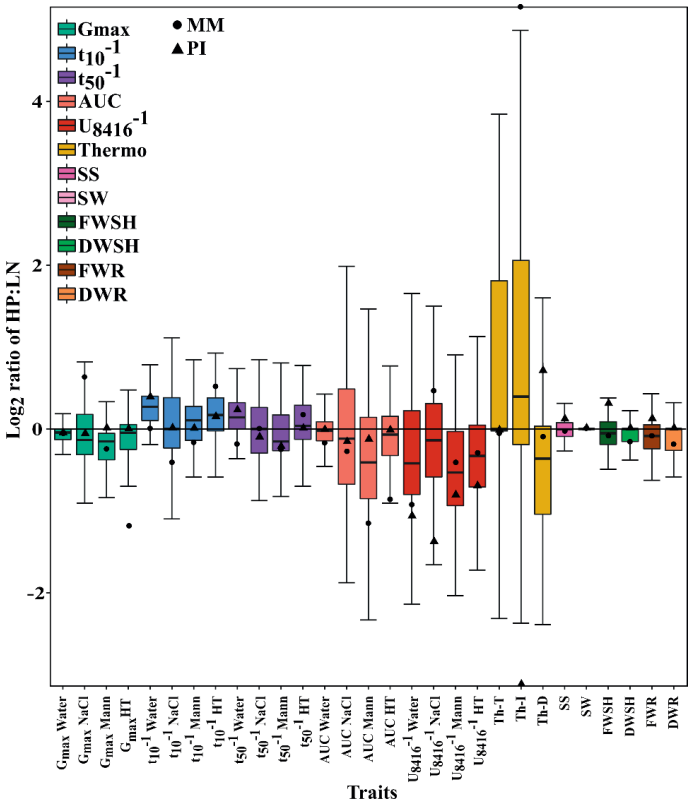

Figure 2. Seed and seed germination trait differences between the maternal environments. Log_2 ratio of HP:LN per line in each trait. Positive and negative values represent the higher effect of HP and LN, respectively. **HP**, High phosphate; **LN**, Low nitrate; **MM**, *Solanum lycopersicum* (cv. Moneymaker); **PI**, *Solanum pimpinellifolium*; $\mathbf{G_{max}}$, Maximum seed germination percentage; $\mathbf{t_{50}^{-1}}$, $\mathbf{t_{10}^{-1}}$, Reciprocal of time to respectively reach 50 and 10% of maximum germination; **AUC**, Area under the germination curve; $\mathbf{U_{8416}^{-1}}$, Reciprocal of time between 16 and 84% of maximum germination; **Mann**, Mannitol; **HT**, High temperature; **Th-T**, Thermo-tolerance; **Th-I**, Thermo-inhibition; **Th-D**, Thermo-dormancy; **SS**, Seed size; **SW**, Seed weight; **FWSH**, Fresh weight of shoot; **DWSH**, Dry weight of shoot; **FWR**, Fresh weight of root; **DWR**, Dry weight of root.

Broad sense heritability (H^2) calculated for each trait in both maturation environments was high for most of the traits (with most traits > 80% in both environments; ranking from 0.49 to 0.91 and 0.54 to 0.93 in HP and LN, respectively) (Table 2). This shows that the phenotypic variation is better explained by genetic variation (between the RILs) than the random variation between different plants (within the RILs) for most traits.

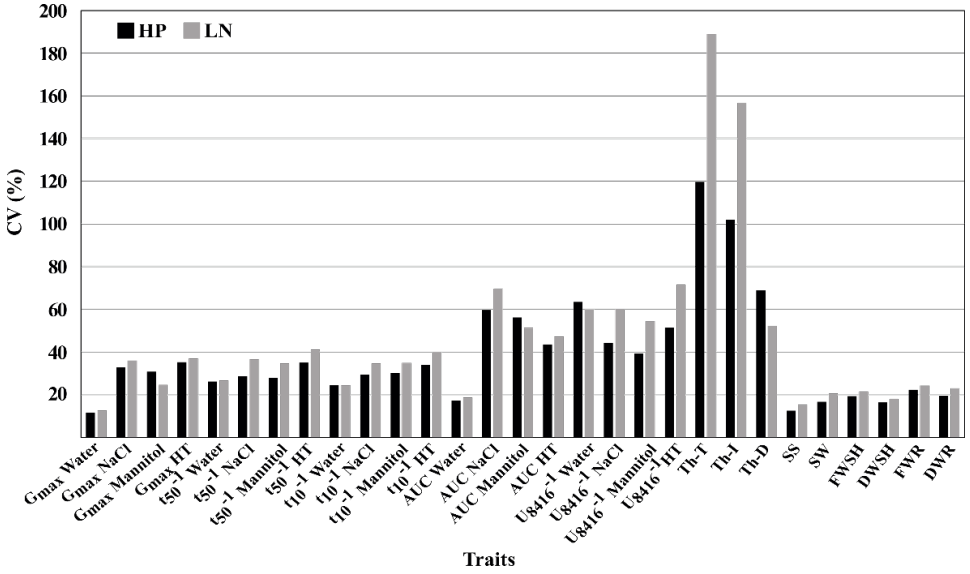

Figure 3. Percentage of coefficient Variation (CV%) across the tomato RIL population containing 100 lines. **HP**, High phosphate; **LN**, Low nitrate; $\mathbf{G_{max}}$, Maximum seed germination percentage; $\mathbf{t_{50}^{-1}}$, $\mathbf{t_{10}^{-1}}$, Reciprocal of time to respectively reach 50 and 10% of maximum germination; **AUC**, Area under the germination curve; $\mathbf{U_{8416}^{-1}}$, Reciprocal of time between 16 and 84% of maximum germination; **HT**, High temperature; **Th-T**, Thermo-tolerance; **Th-I**, Thermo-inhibition; **Th-D**, Thermo-dormancy; **SS**, Seed size; **SW**, Seed weight; **FWSH**, Fresh weight of shoot; **DWSH**, Dry weight of shoot; **FWR**, Fresh weight of root; **DWR**, Dry weight of root.

Genotype ranking and its stability over different nutritional maternal environments

The ranking of the genotypes per trait was determined using Genstat 18 (Supplemental Table S2, Supplemental Table S3). In order to investigate how consistent the rankings are between the maternal environments and how big the effect is of genotype and environment interactions (G×E) on phenotypic traits, the Spearman rank correlation coefficient (Oury *et al.*, 2006) between two suboptimal nutritional maternal environments was calculated from which the stability of ranking of genotypes over two maternal environments was assessed (Table 3). For phenotypic traits, such as SS and SW, rankings of the genotypes were quite stable from one maternal environment to another and, thus, Spearman rank correlation values were also high for these traits (Table 3). Such high value of correlation suggests a relatively moderate influence of G×E on trait performance.

Table 3. Stability of rankings of the genotypes over the two different nutritional maternal environments.

Traits	Spearman rank correlation
Maximum seed germination **(G_{max})**	0.57
Germination rate **(t_{50}^{-1})***	0.73
Area under the germination curve **(AUC)**	0.64
Uniformity **(U_{8416}^{-1})** **	0.52
Seed size **(SS)**	0.77
Seed weight **(SW)**	0.80
Fresh weight of shoot **(FWSH)**	0.78
Fresh weight of root **(FWR)**	0.66
Thermo-dormancy **(Th-D)**	0.64

*Recirocal time to reach 50% of maximum seed germination
** Recirocal time between 16% and 84% of maximum seed germination

Germination environments versus maternal environments

By germinating the tomato seeds in optimal (water) and suboptimal (salt, osmotic and high temperature stress) conditions, the obtained seed germination traits appeared to be affected by their maternal environment (ME) as well as by their germination environment (GE), and their interaction (ME×GE). In comparison to the optimal GE, seed germination traits showed higher variability in suboptimal GE in both MEs (Figure 3). For instance, CVs for G_{max} and AUC in water were 12% and 17%, respectively, while they showed significantly higher values in salt- (33% and 60% respectively), osmotic- (31% and 56% respectively) and high temperature- (35% and 44% respectively) stress (Figure 3; Supplemental Table S1). We observed the same trend for t_{10}^{-1} and t_{50}^{-1} albeit to a lesser extent. U_{8416}^{-1} showed a pattern which was different from other germination traits, where optimal and suboptimal GE show more similar CVs (Figure 3; Supplemental Table S1). Taken together, the ME affected seed germination traits less than GE. Although ME did not change the germination traits under optimal GE, it caused a small but significant difference under suboptimal GEs. For example, G_{max} exhibited similar CVs under optimal GE in both MEs (HP and LN) whilst under suboptimal conditions they displayed a slight difference in CV (Figure 3; Supplemental Table S1).

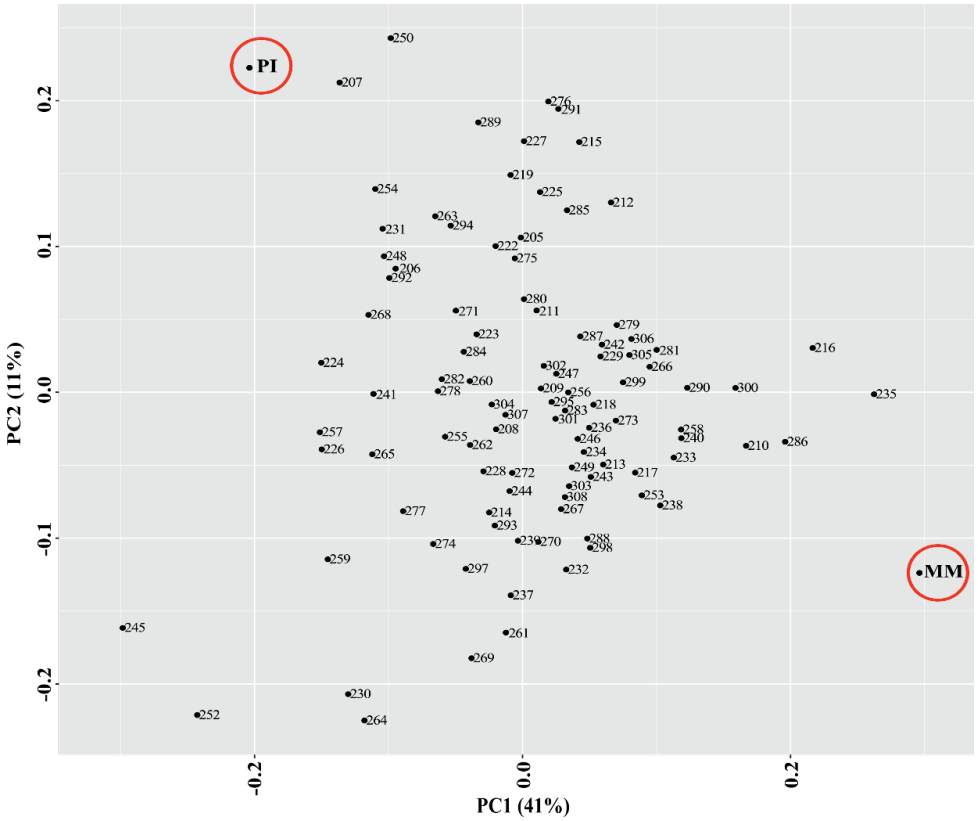

Figure 4. Principle Component Analysis (PCA) of the recombinant inbred and parental lines (*Solanum lycopersicum* (cv. Moneymaker) **(MM)** and *Solanum pimpinellifolium* **(PI)**) for all traits in both nutritional maternal environments. The red circles highlight the parental lines.

Two-way ANOVA shows that the effect of GE on all germination traits was significant (P value ≤0.05). In general, we found that the observed variation in each trait is mostly explained by the existing variation between GEs. For all the germination traits a larger sum-of-squares was found for GE compared to ME and their interaction, showing that GE has a much larger effect. Still, ME also significantly affects the germination traits with the exception of t_{50}^{-1} and AUC. Furthermore, ME×GE significantly affected G_{max} and U_{8416}^{-1} (Table 4).

Table 4. Effect of maternal environment (ME), germination environment (GE) and their interaction (ME×GE) on germination traits of tomato seeds.

Trait	SS			SL		
	ME	**GE**	**ME×GE**	**ME**	**GE**	**ME×GE**
G_{max}	0.84	9.82	0.74	**	**	*
t_{10}^{-1}	0.72	44.29	0.02	*	**	ns
t_{50}^{-1}	0.04	50.96	0.14	ns	**	ns
AUC	0.00	55.62	0.17	ns	**	ns
U_{8416}^{-1}	2.06	37.31	0.99	**	**	**

SS, Sum of square, in each trait represent the proportion of effect of each environmental component (ME, GE and ME×GE) in their total sum of squares; **SL, Significant level,** represent the significance level of the ANOVA test for maternal environment, germination environment and the interaction between them; G_{max}, Maximum seed germination percentage; t_{50}^{-1}, t_{10}^{-1}, Reciprocal time to reach respectively 50 and 10% of maximum germination; **AUC**, Area under the germination curve; U_{8416}^{-1}, Reciprocal time between 16 and 84% of maximum germination.
** *P* value ≤ 0.01; * *P* value ≤ 0.05; **ns**, no significant effect.

Trait by Trait correlation

To obtain a comprehensive visualization of possible correlations among the phenotypic traits, a correlation network has been generated for each maternal environment (Figure 5). In general, the mean value of all phenotypic traits showed a positive significant correlation between the two suboptimal nutrient environments (HP and LN) (Supplemental Table S4). Nevertheless, some differences in trait by trait correlation networks between two environments were observed (Figure 5). Some correlations perceived under high phosphate (Figure 5A) were amplified by the low nitrate condition (Figure 5B). For instance, the positive correlations between seed traits (such as seed size and weight) and seedling quality characteristics (like fresh and dry weight of shoot and root) are stronger under the low nitrate condition. In addition, seed and seedling quality traits showed negative association with seed germination traits including G_{max}, AUC and U_{8416}^{-1}, especially in the HT germination environment, which became visible at the low nitrate condition (Figure 5, Supplemental Excel file S1). On the other hand, in both correlation networks, thermo-dormancy (Th-D) was negatively correlated with most of the germination traits, including G_{max}, AUC and t_{50}^{-1} under different germination environments (such as water, NaCl and HT). However, they were much more correlated under the high-phosphate than the low-nitrate condition (Figure 5, Supplemental Excel file S1).

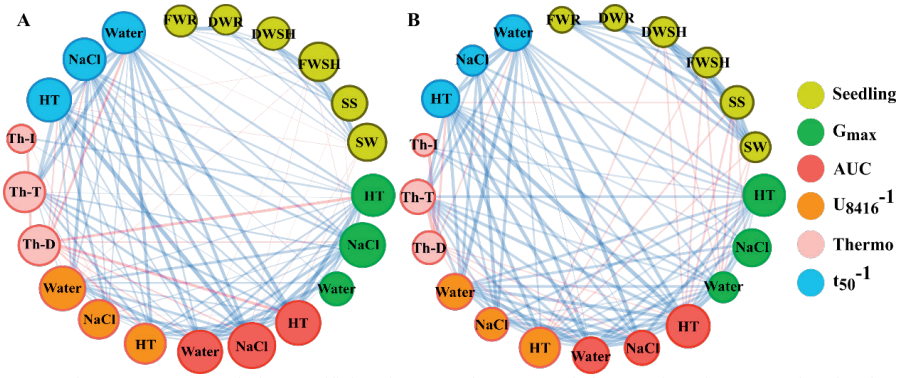

Figure 5. The Spearman correlation coefficient between the means of phenotypic traits assessed under the two maternal environments: **(A)**, High phosphate; **(B)**, Low nitrate. The false discovery rate cut-off was 0.05 (FDR≤0.05). The line colour indicates the direction of the correlation, **Red:** Negative correlation, **Blue:** Positive correlation. The width of lines represents the height of the correlation with wider lines indicating higher correlation values. The size of the circles represents the number of edges, bigger circles indicate that a given trait correlates with a high number of other traits. **G_{max},** Maximum seed germination; **AUC,** Area under the germination curve; **U_{8416}^{-1},** Reciprocal time between 16% and 84% of maximum germination; **t_{50}^{-1},** Reciprocal time to reach 50% of maximum seed germination; **Water**, **NaCl** and **HT** are the seed germination environments water, salt and high temperature, respectively**;** **Th-T**, Thermo-tolerance; **Th-I**, Thermo-inhibition; **Th-D**, Thermo-dormancy; **SS**, Seed size; **SW**, Seed weight; **FWSH**, Fresh weight of shoot; **DWSH**, Dry weight of shoot; **FWR**, Fresh weight of root; **DWR**, Dry weight of root.

QTL identification for each trait

To determine the large effect loci regulating seed, seedling and seed performance traits, QTL analysis of the tomato RIL population was performed using r/qtl software in a single-QTL genome scan approach (Broman *et al.*, 2003). The physical position of the related markers and other characteristics of the QTLs affecting the traits measured for the RIL population grown in the two different maternal environments are summarized in Supplemental Table S5. An overview of the detected QTLs is also provided by the heatmap of the LOD profiles (Figure 6). Concerning all traits, with the exception of chromosomes 2, 3, 5 and 12, all chromosomes contain many QTLs affecting the traits of which many are co-located (Figure 6, Supplemental Table S5). We found 16 QTLs regulating G_{max} under optimal and sub-optimal germination environments of which six were detected in seeds of HP and 10 in LN maternal conditions (Figure 6, Supplemental Table S5). For AUC in all germination environments, 13 QTLs were found of which nine were co-locating with the ones affecting G_{max} on chromosomes 1, 4, 5, 10 and 11. With the exception of two QTLs on chromosome 6 and 10 discovered for the HP environment, all other QTLs regulating AUC were associated with the LN maternal condition. The result showed that t_{10}^{-1} and t_{50}^{-1} in all germination environments and both maternal environments are regulated by almost the same QTLs which is not surprising as they are highly correlated traits. In total 18 QTLs were detected for t_{10}^{-1} and t_{50}^{-1} on chromosomes 2, 4, 6, 7, 8 and 11 which are also largely related to the LN maternal environment (Figure 6, Supplemental Table S5).

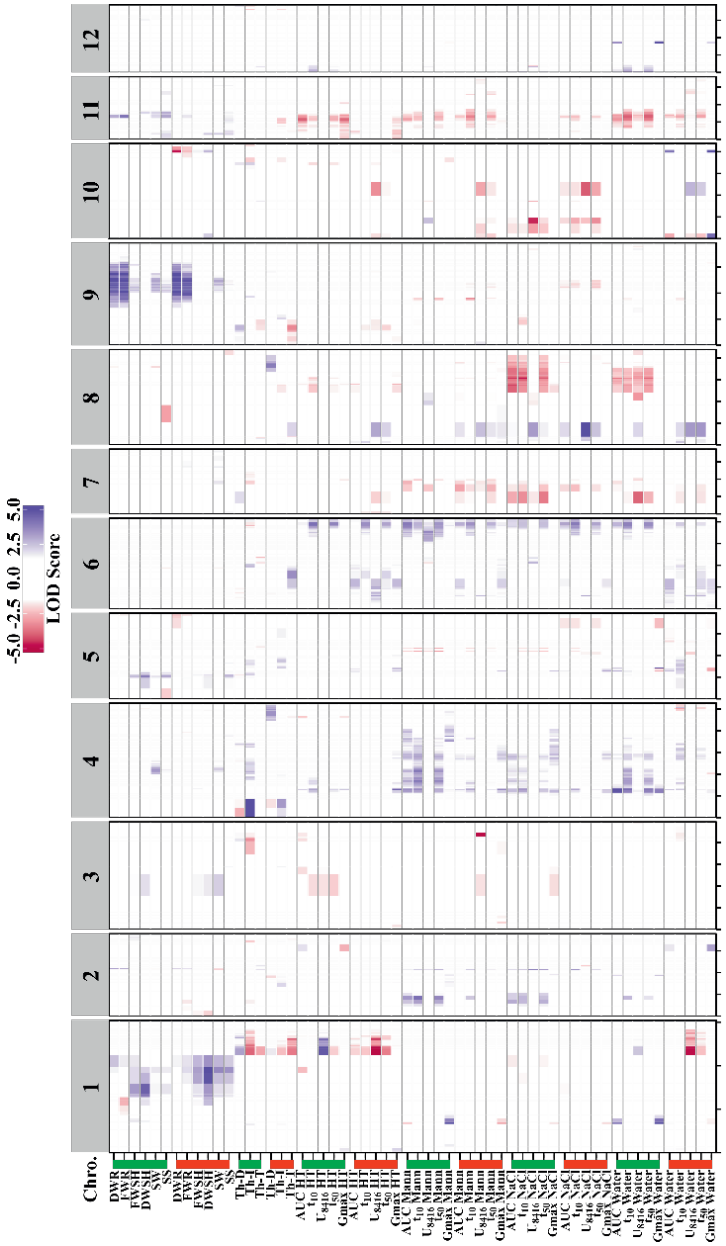

Figure 6. Genomic location of quantitative trait locus (QTL) detected for seed, seedling and seed performance traits. The **green** and **red** thick line next to the traits represent the maternal environment: **LN** and **HP**, respectively. **Chro**, Chromosome; **DWR**, Dry weight of root; **FWR**, Fresh weight of root; **FWSH**, Fresh weight of shoot; **DWSH**, Dry weight of shoot; **SW**, Seed weight; **SS**, Seed size; **Th-D**, Thermo-dormancy; **Th-I**, Thermo-inhibition; **Th-T**, Thermo-tolerance; **AUC**, Area under the germination curve; $\mathbf{t_{10}^{-1}}$ and $\mathbf{t_{50}^{-1}}$, Reciprocal of time to respectively reach and 10 and 50% of maximum germination; $\mathbf{U_{8416}^{-1}}$, Reciprocal of time between 16 and 84% of maximum germination; $\mathbf{G_{max}}$, Maximum seed germination percentage; **HT**, High temperature; **Mann**, Mannitol. The LOD score scale indicates the significant QTLs. Positive (blue) and negative (red) values represent a larger effect of *Solanum lycopersicum* (cv. Moneymaker) and *Solanum pimpinellifolium* alleles, respectively.

For SS and SW, three and four QTLs were found respectively. The co-locating QTLs for these two seed traits for the HP maternal environment were detected on chromosome 1. A co-located QTL was also found for seedling quality in the same maternal environment. Furthermore, another QTL related to seedling quality on chromosome 9 is co-locating with seed traits such as SW (Figure 6, Supplemental Table S5).

There is a strong QTL on chromosome 1 regulating thermo-dormancy traits in both maternal environments. This QTL affects both Th-T and Th-I traits in the same direction, while antagonistically regulating Th-D (Figure 6, Supplemental Table S5). This QTL is co-locating with seed germination traits, such as t_{50}^{-1} and U_{8416}^{-1} under high temperature germination conditions.

For seed germination traits under salt and mannitol germination conditions a co-located QTL is found on chromosome 7. This might be related to the fact that both salt and mannitol cause osmotic stress for seeds and thus seed germination could be regulated by similar mechanisms. On the other hand, we have also identified QTLs on chromosome 8 which are present in the LN maternal environment only. Also, on chromosome 11, a QTL was detected for seed germination in both maternal environmental conditions, which was stronger when maternal plants were cultivated in LN conditions. These QTLs might have been detected as a consequence of genotype by environment interactions (G×E).

QTL by Environment

Generally, when different environments are studied simultaneously, detected QTLs are influenced by several environments. The QTL by Environment interaction (QTL×E) is the term considering the sensitivity of the genetic background to environmental conditions. In this study seeds were grown under two maternal environments and germinated in optimal (water) and three suboptimal conditions including osmotic (NaCl and mannitol) and high temperature stress. Therefore, in each seed germination trait the environmental component of QTL×E can be explained by either the maternal environment (ME) or the germination environment (GE) and their interaction (ME×GE). We identified the QTLs affected by the environments and also decomposed the environmental effect into the different environmental components; GE, ME and their interaction. Figure 7A represents the QTLs regulating the seed germination traits independently from the environments. Those QTLs were detected through all the maternal and germination environments and they are only associated with the genotype. With the exception of chromosomes 5, 9 and 10, the rest of the chromosomes displayed several QTLs strongly regulating seed germination traits including t_{10}^{-1}, t_{50}^{-1}, AUC and U_{8416}^{-1}. As an example, the QTL at the bottom of chromosome 6 significantly affected t_{10}^{-1}, t_{50}^{-1} and AUC regardless of the

different environments under which seeds had developed or were germinated (Figure 7, Supplemental Figure S2). On the other hand, we have shown that some of the QTLs regulating seed germination traits are significantly influenced by the environment. For example, the QTL located near the top of chromosome 2, which regulates AUC, was significantly influenced by GE and to a lesser extent by ME (Figure 7, Supplemental Figure S2). We have observed that GE showed generally more effects on QTLs than the ME. This result is in accordance with the observed variance between ME and GE in which seed germination traits showed higher variance in different GEs in comparison with different MEs (Figure 3; Supplemental Table S1). Germination environment affects QTLs related to t_{10}^{-1} and t_{50}^{-1}, located on chromosomes 3, 6 and 11. Some QTLs affecting U_{8416}^{-1} on chromosomes 8 and 11 were also influenced by the GE (Figure 7, Supplemental Figure S2). In comparison with GE, ME hardly showed an effect on the QTLs. The only effect of the ME was observed for the QTL associated with AUC on top of chromosome 2. Although the detected QTLs were sometimes influenced by either maternal or germination environments, they were not affected by the interaction of GE and ME at all (Figure 7, Supplemental Figure S2).

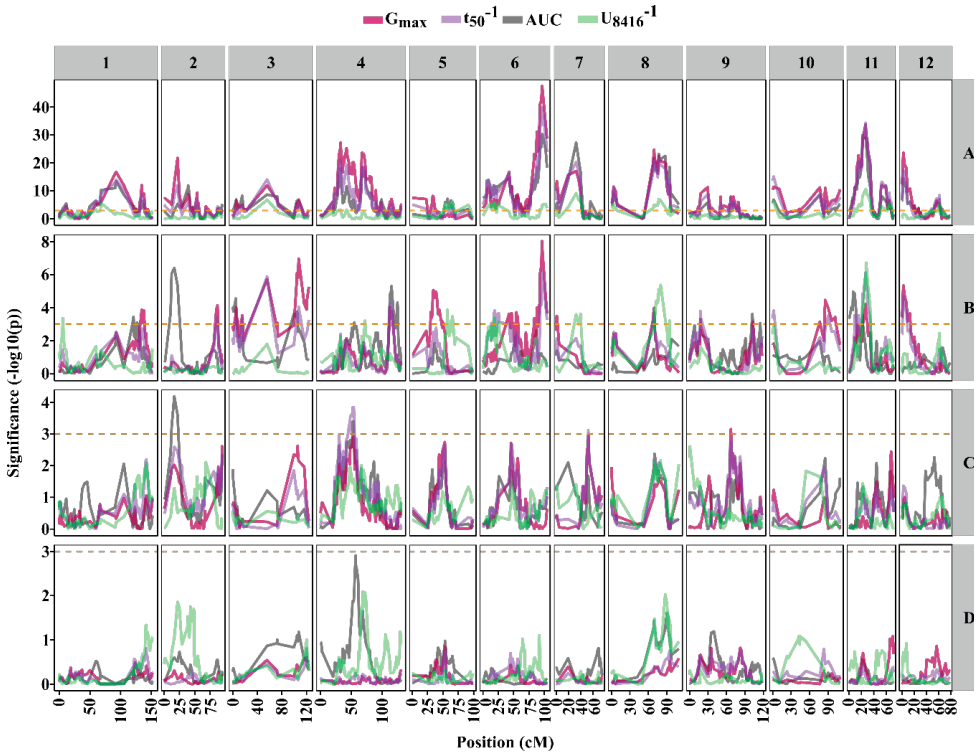

Figure 7. Profiles of the QTLs regulating the seed germination traits. **A,** QTLs detected in all maternal and germination environments; **B,** QTLs with significant effect of germination environment (GE); **C,** QTLs with significant effect of maternal environment (ME); **D**; QTLs with significant effect of GE×ME; $\mathbf{G_{max}}$, Maximum seed germination percentage; $\mathbf{t_{50}^{-1}}$, Reciprocal of time to reach 50% of maximum germination; **AUC**, Area under the germination curve; $\mathbf{U_{8416}^{-1}}$, Reciprocal of time between 16 and 84% of maximum germination.

Discussion

In this study we have used the genetic variation in a tomato RIL population to study how the genotype, maternal environment and their interaction influences seed and seedling quality traits. Although several studies have been conducted previously on the effect of abiotic stresses, such as drought and salt stress on seed quality (Foolad *et al.*, 2003; Foolad, 2004; Asins *et al.*, 2015), studies of the effect of maternal nutritional conditions on the produced seed and seedling traits are scarce. Here we have grown tomato plants in different sub-optimal nutritional conditions and, for the first time, in a genetical approach, studied its effect on produced seed quality and seedling establishment related properties. Nitrogen and phosphorus are two key elements required for plant growth (Schachtman *et al.*, 1998; Urbanczyk-Wochniak & Fernie, 2004). Hence, their non-optimal concentrations in mother plants may seriously affect the produced seed and the seedlings from those seeds. By exploiting the natural variation observed in a tomato RIL population obtained from a cross between *Solanum lycopersicum* (cv. Moneymaker) and *Solanum pimpinellifolium*, we identified several loci controlling seed and seedling traits related to suboptimal nutritional seed maturation conditions.

How are seed and seedling traits correlated?

Breeders and producers often are interested in seed traits such as t_{50}^{-1} and seedling traits such as ability to produce normal and healthy seedlings. Furthermore, traits such as germination percentage and uniformity of germination, may also pose an important focus for breeders. The AUC (combining germination rate (t_{50}) and percentage (G_{max})) will determine how fast seeds will germinate to a certain level, which directly affects further establishment of seedlings. On the other hand, seedling properties such as shoot and root weight determine how fast seedlings can penetrate the soil and start nutrient uptake and how fast the above ground tissues develop to provide required assimilates through photosynthesis. All together these factors determine seed and seedling vigour. Correlation of seed traits (SS and SW) with seed performance (rate of seed germination and uniformity) and with seedling traits have been studied before. Many studies have implied a direct relation between SS and SW and better seedling growth (Nieuwhof *et al.*, 1989; Doganlar *et al.*, 2000; Khan *et al.*, 2012). This can be due to the amounts of reserve food which are deposited in seeds during seed development and maturation. Bigger tomato seeds produce seedlings with higher weight (Nieuwhof *et al.*, 1989; Khan *et al.*, 2012; Chapter 2 of this thesis). Our results confirm the relation of SS and SW with seedling quality and establishment. In both suboptimal nutritional maternal conditions SS and SW were significantly influencing seedling quality traits. However, this correlation was most obvious in the LN nutritional condition. Such a strong correlation between seed and seedling traits suggests a similar genetic architecture, whereas the environment can partially affect such relations. In the former study in which the same RIL population was grown in standard conditions, similar

correlations have been found between seed and seedling size. However, there was no obvious correlation between SS and seed germination traits (Khan *et al.*, 2012). This contradicts our findings in which significant negative correlations were found between SS and seed performance traits such as G_{max}, t_{50}^{-1}, AUC and U_{8416}^{-1} in both nutritional conditions. Such a negative correlation was even more apparent if seeds were germinated at high temperature (HT) (Figure 5, Supplemental Excel file S1). Such a discrepancy may be caused by the maternal environments under which seeds developed and matured. Khan et al., (2012) grew the RILs under optimal environment while suboptimal maturation environments were used in this study. Hence it is postulated that the stressful environments that we used affect the correlation of the seed size and seed germination traits such as G_{max} and t_{50}^{-1}.

The negative correlation that we found between SS and seed performance has been reported previously in tomato. The inheritance of germination time factors (e.g t_{50}^{-1}) was negatively correlated with SS, implying that smaller seeds take longer to germinate (Whittington, 1973). We also have found collocated QTLs for SS and seed performance traits such as G_{max} and t_{50}^{-1} on chromosome 11 which antagonistically affected the traits under study (Figure 6). Such co-locating QTLs might be an indication for the same regulatory mechanism for these traits.

Breeding of crops

In general, a breeding strategy is highly dependent on genotype by environment interactions and the heritability level. Detection of a high correlation between the performance of genotypes in the different maternal environments may simplify the breeding strategy as it is then not required to select different genotypes for implementation into a breeding program. It has been mentioned previously that genotype re-ranking per trait in different environments is an indication of genotype by environment interaction (G×E) (Oury *et al.*, 2006). Considering this, good breeding traits are the ones with the lower G×E effects. The results of the Spearman correlation analysis show that genotype re-ranking for most of the studied traits did not occur, therefore traits were limited affected by G×E. According to the results we would expect a successful breeding process of the traits such as SS, SW, t_{50}^{-1} as well as seedling traits such as FWSH due to their high correlation value. In contrast, breeding for traits like U_{8416}^{-1} with a low correlation value would encounter difficulties because of the feasible influence of the G×E interaction. Furthermore, the genotype ranking per trait demonstrated that from the first 10 genotypes per trait some are consistent between two maternal environments, which is dependent on the trait. Regarding the seed performance traits, with the exception of G_{max}, within the rest of the traits including t_{50}, AUC and U_{8416}^{-1} three genotypes (207, 250 and 289) showed a consistent high ranking level between maternal environments. Hence these high-ranking genotypes may be selected for breeding programs for seed performance (Table 5). Through the seed traits such as SS and SW we also found three stable genotypes (235, 238 and 258) between

two maternal environments which could be considered as good candidates for further breeding of seed traits (Table 5).

Table 5. The 10 genotypes with the highest value per trait within two nutritional maternal environments.

	G_{max}		t_{50}^{-1}		AUC		U_{8416}^{-1}		SS		SW	
	HP	**LN**	**HP**	**LN**	**HP**	**LN**	**HP**	**LN**	**HP**	**LN**	**HP**	**LN**
1	245	225	207	289	207	291	207	291	300	269	235	235
2	289	302	250	291	289	215	219	207	258	258	258	286
3	282	292	289	250	245	250	250	212	235	235	212	257
4	206	206	225	215	225	276	227	250	217	286	290	269
5	276	276	276	276	250	289	289	289	238	257	300	216
6	225	224	291	205	276	227	294	225	269	300	287	258
7	279	250	294	212	219	207	225	215	287	266	217	290
8	207	215	215	227	206	212	291	231	253	238	266	240
9	219	222	245	254	227	222	292	287	266	216	281	238
10	280	291	285	207	294	231	263	227	233	240	238	212

G_{max}, maximum seed germination; **t_{50}^{-1},** Reciprocal of time to reach 50% of maximum seed germination; **AUC,** area under the germination curve; **U_{8416}^{-1},** Reciprocal of time between 16% and 84% of maximum germination; **SS**, Seed size; **SW**, Seed weight. **HP,** High Phosphate; **LN,** Low Nitrate. The consistent genotypes are highlighted.

QTL and QTL×E detection

In general, QTL detection depends on several factors such as trait heritability, population type, number of lines and genetic map quality (Mackay, 2001; Mackay *et al.*, 2009). Controlled growth conditions of the plants together with controlled conditions of performed experiments resulted in identification of traits with high heritability values in our study. Substantial variation found between the parental lines and the 100 RILs provided us with a powerful tool for analysing the genetical background of traits by QTL analysis. QTL analysis ultimately resulted in identification of several interesting QTLs, regulating seed and seed performance traits, as well as seedling characteristics. In this study, we have analysed several traits in two different environments and various regulating QTLs were found for them. According to the results, we have discovered more QTLs with high explained variance at low nitrate maternal environment as compared to high phosphate (Figure 6, Supplemental Table S5). Such a result could indicate that more physiological mechanisms and, subsequently, more genes are involved in plant adaptation to a low nitrate environment. Many of the identified QTLs in this study have been reported previously for the same population, but under standard conditions only (Kazmi *et al.*, 2012; Khan *et al.*, 2012). For example, the QTL that we have found at the end of chromosome 6, predominantly regulating the t_{50}^{-1} trait in both maternal environments, was also detected in the standard condition. In addition, we have identified more environment-specific QTLs which were detected exclusively in one of the environments. These QTLs are more interesting from scientific point of view, however, QTLs detected in all different environments which may be considered as robust QTLs are the most interesting ones for further analysis for breeders and producers. These stable QTLs could regulate the traits independent from the growth

environment. Further analysis, such as fine mapping, would ultimately result in identification of gene(s) regulating the analysed traits. As an example, many studies carried out so far to identify the genetic loci regulating SW in tomato have resulted in the identification of several QTLs (Tanksley *et al.*, 1982; Weller *et al.*, 1988; Grandillo & Tanksley, 1996; Doganlar *et al.*, 2000; Khan *et al.*, 2012). An interesting QTL which is common in different reports is present on chromosome 4 (Orsi & Tanksley, 2009; Khan *et al.*, 2012). This QTL also appeared in our population grown under LN nutritional condition.

Studies of the interactions of QTL by environment have been carried out previously in different crops including tomato and rice taking a relatively simple strategy (Paterson *et al.*, 1991; Lu *et al.*, 1997). Plants were grown in different environments, QTL analysis was performed for individual environments and finally the results obtained from the different environments were compared with each other. Here we report for the first time interactions of the QTL by nutritional environment in a tomato RIL population derived from a domesticated (*Solanum lycopersicum* (cv. Moneymaker)) and a wild-type (*Solanum pimpinellifolium*) tomato species. Here we used a more complex strategy which has been applied previously for other species and/or environments (van Eeuwijk *et al.*, 2010; Des Marais *et al.*, 2013; Li *et al.*, 2015). In this method QTLs are directly studied in several environments. Although there is considerable overlap between the simple and more complex strategies, the second method enhances the statistical analysis resulting in higher LOD values and higher chances of finding significant QTLs (Tétard-Jones *et al.*, 2011). According to our results (Figure 7) we have detected some QTLs with significant QTL×E. The environmental effects are mostly applied by germination environments, which indicates that QTLs are regulating the tomato seed germination traits almost independently from the maternal environments. Therefore, we conclude that in comparison with the nutritional maternal environment, the germination environment must be considered as the more important factor for seed performance in tomato.

Taken together, our results provide the genetical background of the effects of the maternal environment on seed and seedling traits. These results potentially could be further implemented in tomato breeding programs. We also suggest fine mapping of detected QTLs to narrow down the quantitative genetic loci and ultimately identify the causal gene(s). These can be the start to investigate more in-depth details of the molecular regulation of seed germination performance under different maternal and germination environments.

Material and methods

Plant material and growth conditions

The RIL population was derived from a crossing between two parental lines: *S. lycopersicum* cv. Moneymaker and *S. pimpinellifolium* accession CGN14498. The population of 100 lines was genotyped in F_7 using a set of 865 single nucleotide polymorphism (SNP) markers (Voorrips *et al.*, 2000). F_8 seeds of this population were grown under controlled conditions in a greenhouse at Wageningen University, Netherlands with 16h light and 8h dark. The temperature was adjusted to 25°C and 15 °C during day and night, respectively. All the lines were fertilized uniformly by the same dosage of nutrient (standard nutrient condition) until flowering (Table 6, Supplemental Table S6). From the first open flower onwards the lines were transferred to new nutritional conditions and exposed to high and low concentrations of phosphate and nitrate, respectively (High phosphate: 14.0 mM nitrate, 10.0 mM phosphate; Low nitrate: 2.4 mM nitrate, 1.0 mM phosphate; Standard: 14.0 mM nitrate, 1.0 mM phosphate).

Table 6. Nutrient conditions of mother plants after flowering.

Maternal Environment	Nitrate	Phosphate
Standard	14 mM	1.0 mM
Very low nitrate	0.0 mM	1.0 mM
Low nitrate	2.4 mM	1.0 mM
High nitrate	20.0 mM	1.0 mM
Very high nitrate	36.0 mM	1.0 mM
Very low phosphate	14 mM	0.0 mM
Low phosphate	14 mM	0.1 mM
High phosphate	14 mM	5.0 mM
Very high phosphate	14 mM	10.0 mM

Afterwards, healthy and full ripened fruits were collected and seeds were extracted with 1% hydrochloric acid (HCl) to remove the main part of the pulp that is stuck onto the seeds. The seed extract together with diluted HCl was passed through a mesh sieve and then washed with water to remove the residual pulp and HCl. In order to disinfect the seeds, they were soaked in a trisodium phosphate ($Na_3PO_4.12H_2O$) solution and then dried on filter paper at room temperature for 3 days and brushed to remove impurities. At the end, the seeds were stored in small paper bags in a cold (13°C) and dry (30% RH) storage room (Kazmi *et al.*, 2012).

Phenotyping of seeds and seedlings

Seed size and weight

Seed size was measured by using a Nikon D80 camera fixed to a repro stand with 60 mm objective and connected to a PC with Nikon camera control pro software version 2.0 (Joosen *et al.*, 2010). The images of 12-h imbibed seeds on white filter paper (20.2 x 14.3 cm) were

processed by ImageJ (http://rsbweb.nih.gov/ij/) combining colour threshold with particle analysis. For seed weight, a batch of dry seeds was weighed and then divided by the number of the weighed seeds.

Germination experiments

Germination experiments were executed in a completely randomized design with 2 replications of around 50 seeds per line of the RIL population, as well as the parental lines. The seeds were sown in germination trays (21x15 cm DBP Plastics, http://www.dbp.be) containing one layer of white filter paper (20.2 x 14.3 cm white blotter paper; Allpaper BV, Zevenaar, The Netherlands, http://www.allpaper.nl) and 15 ml of demi water for normal and high temperature conditions, or 15 ml NaCl (-0.5 MPa; Sigma-Aldrich) or mannitol (-0.5 MPa; Sigma-Aldrich) for salt and osmotic stress, respectively. Each germination tray was loaded with three samples using a special mask to ensure correct placement of the seeds. The trays were piled up with one empty tray containing one white filter paper and 15 ml of water at the bottom and top of the pile and a white plastic lid at the top. The trays were wrapped in a transparent plastic bag and stored at 4°C for 3 days and subsequently transferred into a dark incubator (type 5042; seed processing Holland, http://www.seedprocessing.nl) at 25°C except for the high temperature condition, which was at 35°C. Germination was scored manually by counting the germinated seeds at 24-h intervals during 14 following days for salt and osmotic stress and at 8-h intervals for one week in the case of normal and high temperature conditions.

Seedling phenotyping

Seedling characteristics were measured by sowing around 20 seeds of each seed batch on germination trays containing two blue germination papers (5.6′ x 8′ Blue Blotter Paper; Anchor Paper Company, http://www.seedpaper.com) and 50 ml demineralized water. The germination trays were stored at 4°C for 3 days. Then, they were transferred to an incubator at 25°C without light. The first 10 germinated seeds were placed on circular blue filter papers (9 cm Blue Blotter Paper; Anchor Paper Company, http://www.seedpaper.com) which were placed on a Copenhagen table at 25°C in a randomized complete block design with 2 biological and 2 technical replicates, for 10 days. Conical plastic covers with a small hole on top were placed on top of each filter paper to inhibit evaporation. At the end of the 10 days, the seedlings were collected and fresh weight of their shoots and roots was measured (FWSH and FWR respectively). The dry weight of shoots and roots was also measured after incubating them in 80°C for 3 days (DWSH and DWR respectively).

Statistical analysis

Calculation of seed quality traits

Seed quality traits like G_{max} (maximum germination), t_{10}^{-1} (time to reach 10% of maximum germination), t_{50}^{-1} (germination rate, time to reach 50% of maximum germination), U_{8416}^{-1} (uniformity, time between 16 and 84% of maximum germination) and AUC (area under the germination curve till 200h) were measured based on the cumulative germination data using the curve-fitter module of the Germinator package (Joosen *et al.*, 2010). The parameters t_{10}^{-1}, t_{50}^{-1} and U_{8416}^{-1} were only determined when germination of more than 80% of the RILs reached 10, 50 and 84%, respectively. The average of two biological replicates of each line was used for subsequent QTL analysis.

Broad sense heritability, coefficient of variation and ANOVA analysis

The total phenotypic variation (V_P) can be influenced by genetic (V_G) and environmental (V_E) variation ($V_P = V_G + V_E$). For each maternal and germination environment the broad sense heritability (H^2) was calculated for individual traits as the proportion of phenotypic variation due to the effect of genetic variation ($H^2 = V_G/V_P$). The calculation was performed in Genstat 18 with the QTL phenotypic analysis tools, using preliminary single environment analysis and considering plant replications as an additional fixed term. Within a population the absolute variation or dispersion per trait is defined as the standard deviation (σ). The relative variation called the coefficient variation (CV) for individual traits is the ratio of the standard variation to the mean (μ) of the lines in the population ($CV = (\sigma/\mu)*100$).

Since tomato seeds were grown in different nutritional maternal environments (ME) and were germinated in several conditions (GE), the seed germination traits were influenced by ME, GE and their interactions (MExGE). To identify the effect of each component on seed performance traits a two way ANOVA analysis was performed using Genstat 18 with a significant threshold of 0.05. The contribution of each environmental component to an individual trait was presented by the sum of squares (SS).

Stability of the genotype rankings over two nutritional maternal environments

For each trait the stability of the genotypes over two nutrient maternal environments was estimated by calculation of Spearman rank correlation. We used the same approach as performed in previous studies to take the G×E interaction affecting traits into account (Becker & Leon, 1988; Oury *et al.*, 2006).

Principle component analysis

A PCA of the RILs and the parents based on the trait measurements was made using the R prcomp function with the scale and center arguments set to TRUE. The first two components of the PCA were plotted using the autoplot function from the ggfortify library.

Correlation analysis

In each maternal environment pairwise Spearman correlation analysis was done between all seed, seedling and seed performance traits using the rcorr R package. The values of the correlation and statistically significant level of the correlations was represented as correlation value and false discovery rate (FDR), respectively. Correlation values with FDR≤0.05 were selected to generate a correlation network using Cytoscape v.3.4.0. The NetworkAnalyser tool in Cytoscape was used to obtain further characteristics of the networks. The correlation between the mean values of each RIL for each trait between two maternal environments was also calculated using the rcorr R package.

QTL and QTL×E analysis

Linkage analysis

At the beginning, 5529 Single Nucleotide Polymorphisms (SNPs) were used to genotype the RIL population and markers with identical values were removed, leaving2251 polymorphic markers. Furthermore, co-segregating markers were also removed and the remaining 865 unique markers were used for generating the genetic linkage map which contains 12 individual linkage groups corresponding to the 12 chromosomes of tomato. The map has been constructed using JoinMap 4 (Van Ooijen and Voorrips, 2001) based on recombination frequency and Haldane's mapping function and integrating the existing SNP marker data set for the RILs (Kazmi *et al.*, 2012).

QTL detection

The values of the seed, seedling and seed performance traits were used for QTL detection. QTL analysis was carried out by genome scan with a single QTL model (scanone) using the r/qtl package (Broman *et al.*, 2003). The Logarithm-of-Odds (LOD), physical position, related marker and additive effects of each detected QTL together with phenotypic variation explained by each QTL (explained variance, EV%) were determined. The genome-wide significant LOD threshold (≥2) was estimated using 10.000 permutation tests (Doerge & Churchill, 1996; Broman *et al.*, 2003).

QTL×E analysis

The QTL by Environment effect was determined by an ANOVA model in which for each germination trait the model includes; the genetic background (GB), GE, ME and marker under study and their interactions (Phenotype ~ ME * GE * marker + GB). All calculations were done in R and visualised using the ggplot2 package (Wickham, 2010). For convenience the commonly used threshold of -log10(p) > 3 was used, to show significant QTLs.

References

Alonso-Blanco C, Aarts MG, Bentsink L, Keurentjes JJ, Reymond M, Vreugdenhil D, Koornneef M (2009) What has natural variation taught us about plant development, physiology, and adaptation? The Plant Cell **21:** 1877-1896

Argyris J, Dahal P, Hayashi E, Still DW, Bradford KJ (2008) Genetic variation for lettuce seed thermoinhibition is associated with temperature-sensitive expression of abscisic acid, gibberellin, and ethylene biosynthesis, metabolism, and response genes. Plant Physiology **148:** 926-947

Asins M, Raga V, Roca D, Belver A, Carbonell E (2015) Genetic dissection of tomato rootstock effects on scion traits under moderate salinity. Theoretical and Applied Genetics **128:** 667-679

Becker H, Leon J (1988) Stability analysis in plant breeding. Plant breeding **101:** 1-23

Bewley JD, Bradford K, Hilhorst H (2012) Seeds: physiology of development, germination and dormancy. Springer Science & Business Media

Broman KW, Wu H, Sen Ś, Churchill GA (2003) R/qtl: QTL mapping in experimental crosses. Bioinformatics **19:** 889-890

Dechaine JM, Gardner G, Weinig C (2009) Phytochromes differentially regulate seed germination responses to light quality and temperature cues during seed maturation. Plant, Cell & Environment **32:** 1297-1309

Des Marais DL, Hernandez KM, Juenger TE (2013) Genotype-by-environment interaction and plasticity: exploring genomic responses of plants to the abiotic environment. Annual Review of Ecology, Evolution, and Systematics **44:** 5-29

Doebley JF, Gaut BS, Smith BD (2006) The molecular genetics of crop domestication. Cell **127:** 1309-1321

Doerge RW, Churchill GA (1996) Permutation tests for multiple loci affecting a quantitative character. Genetics **142:** 285-294

Doganlar S, Frary A, Tanksley S (2000) The genetic basis of seed-weight variation: tomato as a model system. TAG Theoretical and Applied Genetics **100:** 1267-1273

El-Soda M, Malosetti M, Zwaan BJ, Koornneef M, Aarts MG (2014) Genotype× environment interaction QTL mapping in plants: lessons from Arabidopsis. Trends in Plant Science **19:** 390-398

Elwell AL, Gronwall DS, Miller ND, Spalding EP, Durham Brooks TL (2011) Separating parental environment from seed size effects on next generation growth and development in Arabidopsis. Plant, cell & environment **34:** 291-301

Fenner M (1991) The effects of the parent environment on seed germinability. Seed Science Research **1:** 75-84

Finch-Savage W (1995) Influence of seed quality on crop establishment, growth and yield. Seed quality: Basic mechanisms and agricultural implications**:** 361-384

Foolad M (2004) Recent advances in genetics of salt tolerance in tomato. Plant Cell, Tissue and Organ Culture **76:** 101-119

Foolad M, Lin G (1997) Genetic potential for salt tolerance during germination in Lycopersicon species. HortScience **32:** 296-300

Foolad M, Lin G (1998) Genetic analysis of low-temperature tolerance during germination in tomato, Lycopersicon esculentum Mill. Plant breeding **117:** 171-176

Foolad M, Zhang L, Subbiah P (2003) Genetics of drought tolerance during seed germination in tomato: inheritance and QTL mapping. Genome **46:** 536-545

Foolad MR, Subbiah P, Zhang L (2008) Common QTL affect the rate of tomato seed germination under different stress and nonstress conditions. International journal of plant genomics **2007**

Giovannoni J (2001) Molecular biology of fruit maturation and ripening. Annual review of plant biology **52:** 725-749

Grandillo S, Tanksley S (1996) QTL analysis of horticultural traits differentiating the cultivated tomato from the closely related species Lycopersicon pimpinellifolium. Theoretical and Applied Genetics **92:** 935-951

Heuvelink E (2018) Tomatoes. CABI

Hilhorst HW (1995) A critical update on seed dormancy. I. Primary dormancy1. Seed Science Research **5:** 61-73

Hilhorst HW, Finch-Savage WE, Buitink J, Bolingue W, Leubner-Metzger G (2010) Dormancy in plant seeds. *In* Dormancy and Resistance in Harsh Environments. Springer, pp 43-67

Hilhorst HW, Koornneef M (2007) Dormancy in plants. eLS

Hilhorst HW, Toorop PE (1997) Review on dormancy, germinability, and germination in crop and weed seeds.

Holdsworth MJ, Bentsink L, Soppe WJ (2008) Molecular networks regulating Arabidopsis seed maturation, after-ripening, dormancy and germination. New Phytologist **179:** 33-54

Huo H, Bradford KJ (2015) Molecular and hormonal regulation of thermoinhibition of seed germination. *In* Advances in Plant Dormancy. Springer, pp 3-33

Huo H, Dahal P, Kunusoth K, McCallum CM, Bradford KJ (2013) Expression of 9-cis-EPOXYCAROTENOID DIOXYGENASE4 is essential for thermoinhibition of lettuce seed germination but not for seed development or stress tolerance. The Plant Cell **25:** 884-900

Joosen RV, Kodde J, Willems LA, Ligterink W, van der Plas LH, Hilhorst HW (2010) germinator: a software package for high-throughput scoring and curve fitting of Arabidopsis seed germination. The Plant Journal **62:** 148-159

Joosen RVL, Arends D, Willems LAJ, Ligterink W, Jansen RC, Hilhorst HW (2012) Visualizing the genetic landscape of Arabidopsis seed performance. Plant physiology **158:** 570-589

Kazmi RH, Khan N, Willems LA, Van Heusden AW, Ligterink W, Hilhorst HW (2012) Complex genetics controls natural variation among seed quality phenotypes in a recombinant inbred population of an interspecific cross between Solanum lycopersicum× Solanum pimpinellifolium. Plant, cell & environment **35:** 929-951

Khan N, Kazmi RH, Willems LA, Van Heusden AW, Ligterink W, Hilhorst HW (2012) Exploring the natural variation for seedling traits and their link with seed dimensions in tomato. PLoS One **7:** e43991

Koornneef M, Bentsink L, Hilhorst H (2002) Seed dormancy and germination. Current opinion in plant biology **5:** 33-36

Lafta A, Mou B (2013) Evaluation of lettuce genotypes for seed thermotolerance. HortScience **48:** 708-714

Li S, Wang J, Zhang L (2015) Inclusive composite interval mapping of QTL by environment interactions in biparental populations. PloS one **10:** e0132414

Lippman ZB, Semel Y, Zamir D (2007) An integrated view of quantitative trait variation using tomato interspecific introgression lines. Current opinion in genetics & development **17:** 545-552

Lu C, Shen L, He P, Chen Y, Zhu L, Tan Z, Xu Y (1997) Comparative mapping of QTLs for agronomic traits of rice across environments by using a doubled-haploid population. Theoretical and applied genetics **94:** 145-150

Mackay TF (2001) The genetic architecture of quantitative traits. Annual review of genetics **35:** 303-339

Mackay TF, Stone EA, Ayroles JF (2009) The genetics of quantitative traits: challenges and prospects. Nature reviews. Genetics **10:** 565

Malosetti M, Voltas J, Romagosa I, Ullrich S, Van Eeuwijk F (2004) Mixed models including environmental covariables for studying QTL by environment interaction. Euphytica **137:** 139-145

McCouch S (2004) Diversifying selection in plant breeding. PLoS Biol **2:** e347

McDonald MB (1998) Seed quality assessment. Seed Science Research **8:** 265-276

Moreau L, Charcosset A, Gallais A (2004) Use of trial clustering to study QTL× environment effects for grain yield and related traits in maize. Theoretical and applied genetics **110:** 92-105

Nascimento W, Huber D, Cantliffe D (2013) Carrot seed germination and respiration at high temperature in response to seed maturity and priming. Seed Science and Technology **41:** 164-169

Nieuwhof M, Garretsen F, Oeveren J (1989) Maternal and genetic effects on seed weight of tomato, and effects of seed weight on growth of genotypes of tomato (Lycopersicon esculentum Mill.). Plant Breeding **102:** 248-254

Orsi CH, Tanksley SD (2009) Natural variation in an ABC transporter gene associated with seed size evolution in tomato species. PLoS genetics **5:** e1000347

Oury F-X, Leenhardt F, Remesy C, Chanliaud E, Duperrier B, Balfourier F, Charmet G (2006) Genetic variability and stability of grain magnesium, zinc and iron concentrations in bread wheat. European Journal of Agronomy **25:** 177-185

Paterson AH, Damon S, Hewitt JD, Zamir D, Rabinowitch HD, Lincoln SE, Lander ES, Tanksley SD (1991) Mendelian factors underlying quantitative traits in tomato: comparison across species, generations, and environments. Genetics **127:** 181-197

Schachtman DP, Reid RJ, Ayling SM (1998) Phosphorus uptake by plants: from soil to cell. Plant physiology **116:** 447-453

Schauer N, Semel Y, Roessner U, Gur A, Balbo I, Carrari F, Pleban T, Perez-Melis A, Bruedigam C, Kopka J (2006) Comprehensive metabolic profiling and phenotyping of interspecific introgression lines for tomato improvement. Nature biotechnology **24:** 447-454

Tanksley SD, Medina-Filho H, Rick CM (1982) Use of naturally-occurring enzyme variation to detect and map genes controlling quantitative traits in an interspecific backcross of tomato. Heredity **49:** 11

Tétard-Jones C, Kertesz M, Preziosi R (2011) Quantitative trait loci mapping of phenotypic plasticity and genotype–environment interactions in plant and insect performance. Philosophical Transactions of the Royal Society of London B: Biological Sciences **366:** 1368-1379

Toh S, Imamura A, Watanabe A, Nakabayashi K, Okamoto M, Jikumaru Y, Hanada A, Aso Y, Ishiyama K, Tamura N (2008) High temperature-induced abscisic acid biosynthesis and its role in the inhibition of gibberellin action in Arabidopsis seeds. Plant physiology **146:** 1368-1385

Urbanczyk-Wochniak E, Fernie AR (2004) Metabolic profiling reveals altered nitrogen nutrient regimes have diverse effects on the metabolism of hydroponically-grown tomato (Solanum lycopersicum) plants. Journal of Experimental Botany **56:** 309-321

van Eeuwijk FA, Bink MC, Chenu K, Chapman SC (2010) Detection and use of QTL for complex traits in multiple environments. Current opinion in plant biology **13:** 193-205

Van Eeuwijk FA, Malosetti M, Boer MP (2007) Modelling the genetic basis of response curves underlying genotype x environment interaction. Frontis **21:** 113-124

Voorrips RE, Verkerke W, Finkers R, Jongerius R, Kanne J (2000) Inheritance of taste components in tomato. Acta Physiologiae Plantarum **22:** 259-261

Weller J, Soller M, Brody T (1988) Linkage analysis of quantitative traits in an interspecific cross of tomato (lycopersicon esculentum x lycopersicon pimpinellifolium) by means of genetic markers. Genetics **118:** 329-339

Whittington W (1973) Genetic regulation of germination. Seed ecology. Butterworth, London**:** 5-30

Wickham H (2010) ggplot2: elegant graphics for data analysis. J Stat Softw **35:** 65-88

Supplementary information

Supplementary Tables and Excel files of this chapter can be downloaded from
http://www.wageningenseedlab.nl/thesis/ngeshnizjani/SI/chapter3

Supplementary Figures

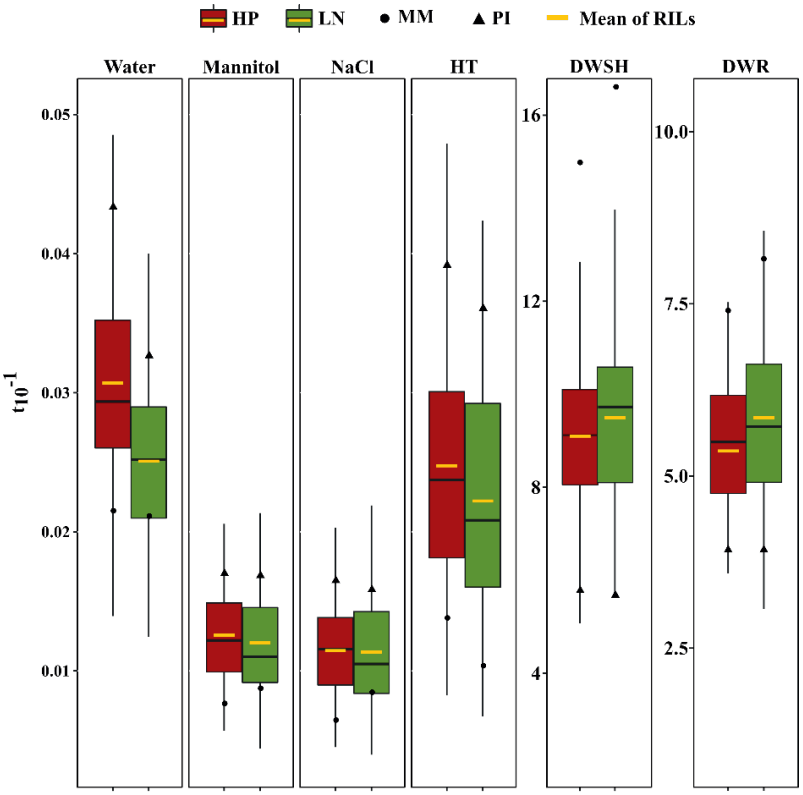

Figure S1. Effect of nutritional maternal environments on seed and seedling traits. t_{10}^{-1}, Reciprocal of time to reach 10% of maximum germination; **DWSH,** Dry weight of shoot; **DWR,** Dry weight of root.

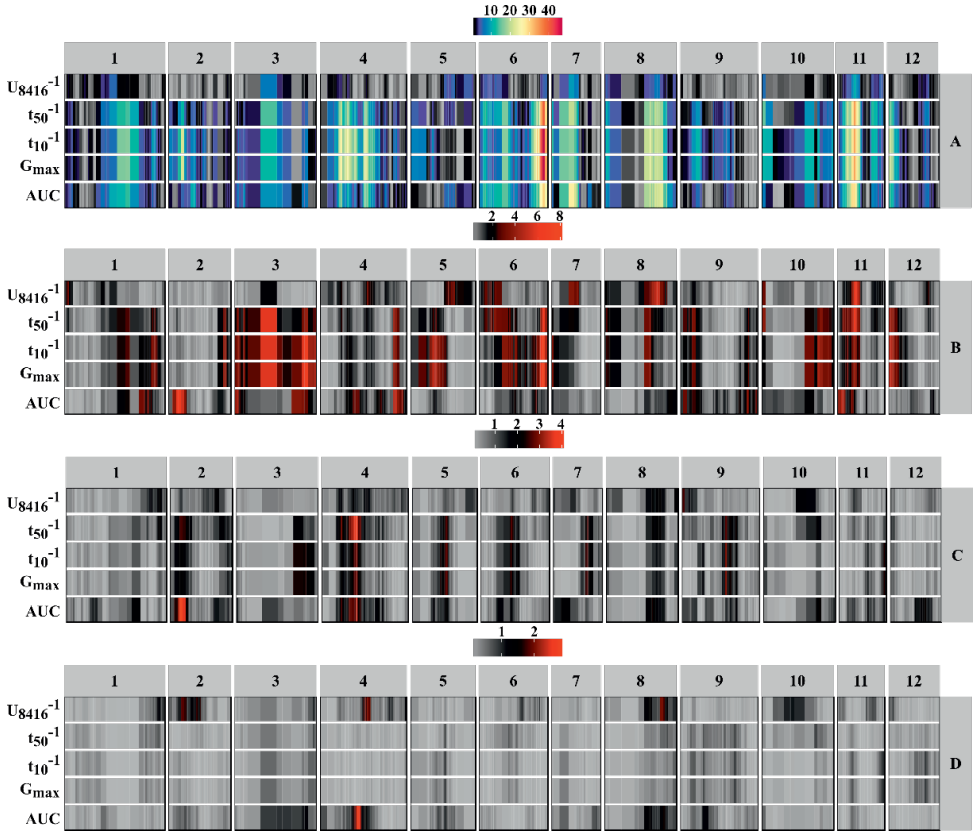

Figure S2. Heatmap of QTLs regulating the seed germination traits. **A,** QTLs detected in all maternal and germination environments; **B,** QTLs with significant effect of germination environment **(GE)**; **C,** QTLs with significant effect of maternal environment **(ME)**; **D,** QTLs with significant effect of **GE×ME;** $\mathbf{G_{max}}$, Maximum seed germination percentage; $\mathbf{t_{50}^{-1}}$ **and** $\mathbf{t_{10}^{-1}}$, Reciprocal of time to reach 50 and 10% of maximum germination, respectively; **AUC**, Area under the germination curve; $\mathbf{U_{8416}^{-1}}$, Reciprocal of time between 16 and 84% of maximum germination.

Chapter 4

Genetic and maturation environmental modulation of tomato dry seed metabolites

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Abstract

Each phenotypic trait of seeds may be influenced by their metabolite composition which varies during seed development and -maturation. Therefore, the metabolic components of dry seeds possibly represent the maternal environment under which seeds developed and matured. The natural variation in seed metabolite composition has been previously studied and several QTLs were detected regulating this variation. In this study, a generalized genetical genomics (GGG) approach has been used for the metabolic analysis of a recombinant inbred (RIL) population which was obtained from a cross between two tomato species: *Solanum lycopersicum* (cv. Moneymaker) and *Solanum pimpinellifolium*. The RILs together with the parental lines were grown in two maternal environments: high phosphate and low nitrate contents. A correlation analysis of metabolite composition and seed phenotypic traits indicated several relations between metabolite contents and seed quality traits such as seed size, seed weight and seed germination percentage. In our study, seed size and -weight exhibited a positive correlation with several amino acids and some intermediates of the TCA cycle, such as succinate, citrate and malate. By performing metabolic correlation analysis and also generating metabolite networks and combining these with QTL analysis, several interesting mQTLs were identified. We showed that the dry seed metabolites and the QTLs regulating them are modulated by genetic factors, maternal environment and their interaction.

Introduction

The maternal environment under which seeds develop and mature have a profound influence on seed properties such as germination vigour. The sink-source connection between the mother plant and the seeds allows the seeds to accumulate reserves required for seed germination and seedling growth (Baud et al., 2008). Metabolites such as amino acids, sugars and organic acids play a vital role in the different stages of seed development such as maturation, desiccation and germination (Borisjuk et al., 2004; Fait et al., 2006). During seed maturation, the content of these metabolites in seeds decreases and storage reserves, including starch, oil and seed storage proteins increase (Fait et al., 2006; Galili et al., 2014). It has also been shown that the subsequent metabolite content and composition of dry seeds may reflect the maturation environments in which they developed (He et al., 2016). For example, in different species it has been reported that nitrogen related metabolites such as asparagine, allantoin and GABA show a lower content in seeds developed under low nitrate maternal environments (He et al., 2016); Chapter 2, this thesis). Although many studies have been performed related to the effect of maternal environments on dry seed metabolic content, more information is required to understand the genetic and molecular mechanisms governing the metabolic changes in response to the maternal environment.

In general, each observed phenotype in plants is the consequence of different cellular processes such as gene transcription, protein translation and, finally, metabolite production (Kooke and Keurentjes, 2011). Therefore, genetic variation is not only confined to phenotypic traits such as seed and seedling quality traits. Many studies revealed that metabolite composition and content, which play a very critical role in plant growth and development, is also controlled by genetic variation within plant species (Windsor et al., 2005). The existing natural variation for both phenotypic traits and metabolite content is displayed by a continuous distribution, considered as quantitative variation. Such variations are often regulated by multiple loci and can be detected in mapping populations like recombinant inbred line (RIL) populations where the different loci are known as phenotypic or metabolite quantitative trait loci (QTLs and mQTLs, respectively) (Lisec et al., 2008; Keurentjes and Sulpice, 2009). Many QTL analyses have been performed in seeds and many QTLs that regulate complex quantitative traits such as seed germination characteristics, seed size, seedling traits as well as seed metabolites have been described (Schauer et al., 2006; Kazmi et al., 2012; Khan et al., 2012; Kazmi et al., 2017).

Plants are a rich source of biochemical compounds that are mainly contributing to plant development, adaptation and final appearance and yield (Binder, 2010). Therefore, the quantitative variation of these metabolites may have an influence on different physiological traits like seed germination and seedling establishment. The integrative analysis of metabolites and genetics has provided valuable information and knowledge on how natural variation

regulates metabolite levels and their subsequent effect on growth of plants and their adaptation and how this knowledge can be used in plant breeding (Kliebenstein, 2009).

Genetical genomics in which QTL analysis is integrated with proteomics, transcriptomics and metabolomics has provided in-depth understanding of molecular mechanisms regulating complex traits (Jansen and Nap, 2001; Keurentjes et al., 2006; Kliebenstein et al., 2006; Schauer et al., 2006). Nonetheless, more advanced approaches are required for further determination of the complexity of quantitative traits. In addition to genotype (G), molecular networks are also influenced by the environment (E) and the interaction between genotype and the environment (G×E). Thus, the incorporation of different environments in genetic studies is a prerequisite for comprehensive perception of the regulation of molecular mechanisms. Li et al. (2008) proposed a new strategy which is called generalized genetical genomics (GGG) by which both genetic and environmental perturbations can be studied. This approach allows QTL analysis governing the interesting molecular traits under consideration of multiple environments. It is a cost-effective method to not only determine the genotype but also the environmental effects and their interaction for detected QTLs (Li et al., 2008). In principle, by creating similar subpopulations of RILs and subjecting each of these to a different environment, G, E and G×E effects can be investigated in a cost-effective experimental design (Joosen et al., 2013).

Although the QTLs governing dry seed metabolite content have been previously detected in many plants, including tomato (Toubiana et al., 2012; Kazmi et al., 2017), the effect of G×E interactions has been studied to a much lesser extent (Albert et al., 2016; Rosental et al., 2016; Kazmi et al., 2017). In this study we used a RIL population derived from a cross between two tomato species: *Solanum lycopersicum* (cv. Moneymaker) and *Solanum pimpinellifolium* (Voorrips et al., 2000). We have exploited the existing natural variation in this population to investigate how QTLs are influenced by the environment to which the mother plants are exposed. Moreover, metabolic profiling of the seeds which have matured in different environments will be useful to illustrate important metabolic differences that regulate the development and adaptation of plants (Joosen et al., 2013). By using a GGG approach we performed metabolite analysis for the RIL population and their parental lines, grown in high phosphate and low nitrate environments. By generating metabolite correlation networks and performing mQTL analysis, genetic and molecular aspects of seed metabolic changes in response to the maternal environments have been discovered.

Results and discussion

In this study we used 100 lines of a tomato recombinant inbred line (RIL) population derived from a cross between *Solanum lycopersicum* (cv. Moneymaker) (MM), and *Solanum*

pimpinellifolium accession G1.1554 (PI) (Voorrips et al., 2000). The RILs, together with the parental lines, were grown in two different nutritional environments; high phosphate and low nitrate. The harvested seeds were used to measure the metabolite content in the dry mature seeds. The metabolites were measured by gas chromatography-time of flight-mass spectrometry (GC-TOF-MS) analysis in a specific GGG design. In total 118 primary metabolites were detected from which 58 could be identified. These identified metabolites were classified as amino acids, organic acids, sugars, sugar alcohols and some other compounds (Supplemental Excel file S1).

Principal component analysis of the primary metabolites in the parental lines (MM and PI) indicated clear genetic effects as the two genotypes displayed different accumulation of metabolites in each maternal environment. In addition, maternal environmental effects were also observed within each genotype as metabolites accumulated differently in HP and LN (the two different maternal environments) (Figure 1).

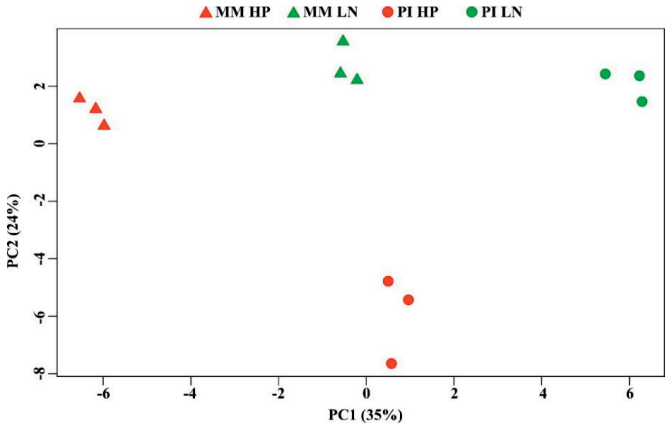

Figure 1. Principal component analysis (PCA) of annotated metabolites in the two parental lines grown in two different nutritional environments. **MM,** *Solanum lycopersicum* (cv. Moneymaker) shown as triangles; **PI,** *Solanum pimpinellifolium* shown as circles; **HP,** High phosphate shown in red; **LN,** Low nitrate shown in green.

Furthermore, analysis of significant changes in dry seed metabolites of the two species (MM and PI) between the different environments revealed that the metabolite contents were influenced to a higher extent in MM compared to PI. In MM the level of 36 metabolites, mostly including amino acids and organic acids, were significantly changed between the environments, while for PI this number decreased to 26 from which 19 were common between the two species (Supplemental Excel file S2). In general PI as a wild tomato species is more tolerant to suboptimal environments (Kumar, 2006; Rodríguez-López et al., 2011; Rao et al., 2013) which might be the reason why it does not need to modify its metabolites to a high extent to cope with

a changing environment. While in the case of MM, which is considered as a domestic sensitive accession, many metabolites should be altered in order to allow it to deal with environmental stresses.

Metabolites are not only influenced by the genetic background of the seeds but also by the environment under which seeds develop and mature and the interaction between genotype and environment (G×E) (He et al., 2016). In our study, a wide range of the metabolites in the parental lines were also significantly influenced by the genotype, nutritional environment and their interaction (Supplemental Table S1). In total 15% of the annotated metabolites did not significantly change in our analysis and 57, 60 and 53% of the metabolites were respectively influenced by genotype, environment and G×E.

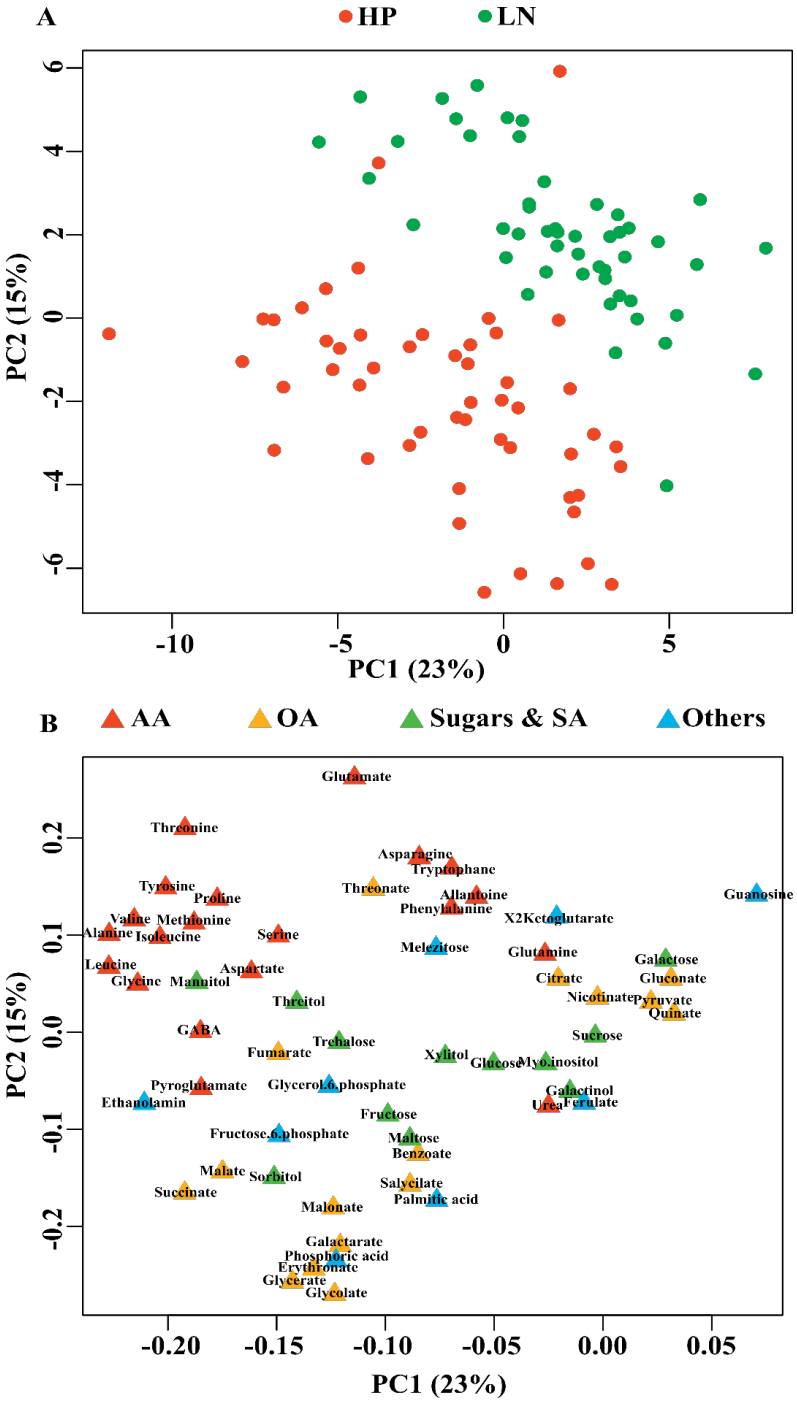

Figure 2. A, Principal component analysis (PCA) of known metabolites of dry seeds of RILs grown in **HP,** High phosphate (green circles) and **LN,** Low nitrate (red circles) conditions. **B,** Loading scores of metabolites for PC1 and PC2. **AA,** amino acids (red triangles); **OA,** Organic acids (yellow triangles); **SA,** Sugar alcohols (green triangles); **Others,** Other components (Blue triangle).

The substantial variation caused by G, E and G×E is probably an indication of a sophisticated regulation of metabolites in developing tomato seeds. Thus, metabolite content of the seeds could be considered as a complex trait which is likely regulated by multiple quantitative trait loci (QTLs) (Lisec et al., 2008; Keurentjes and Sulpice, 2009).

Table 1. Name, category, *p*.value and Log_2 ratio (HP:LN) of the metabolites in the RILs that significantly changed between the two maternal environments: High phosphate (HP) and Low nitrate (LN).

Metabolite	Category	*p*.value*	Log2 ratio (HP:LN)
Sorbitol	Sugar Alcohol	2.15E-20	0.458
Galactarate	Organic acid	1.07E-16	0.222
Glycolate	Organic acid	5.58E-16	0.114
Glycerate	Organic acid	2.11E-15	0.123
Erythronic acid	Organic acid	1.94E-13	0.108
Phosphoric acid	Others	2.24E-13	0.105
Malate	Organic acid	6.44E-12	0.091
Fructose-6-phospate	Others	1.89E-11	0.440
Salycilate	Organic acid	3.08E-11	0.098
Succinate	Organic acid	4.59E-10	0.138
Ethanolamine	Organic acid	1.21E-09	0.133
Guanosine	Others	1.64E-09	-0.103
Malonate	Organic acid	3.32E-08	0.260
Pyroglutamate	Amino Acid	1.76E-06	0.083
Gluconte	Organic acid	3.19E-05	-0.044
Palmitic acid	Others	5.02E-05	0.102
GABA	Amino Acid	0.000105	0.315
Urea	Amino Acid	0.000144	0.277
Glutamate	Amino Acid	0.000286	-0.030
Benzoate	Organic acid	0.000898	0.138
Galactinol	Sugar Alcohol	0.000964	0.037
Glycine	Amino Acid	0.001629	0.129
Aspartate	Amino Acid	0.002707	0.037
Leucine	Amino Acid	0.003873	0.070
Glycerol-6-phosphate	Others	0.004499	0.028
2ketoglutaric acid	Others	0.008428	-0.187
Fumaric acid	Organic acid	0.008688	0.164
Citrate	Organic acid	0.009523	-0.026
Serine	Amino Acid	0.011373	-0.057
Trehalose	Sugar	0.015004	0.138
Phenylalanine	Amino Acid	0.016327	-0.034
Mannitol	Sugar Alcohol	0.020692	0.027
Quinate	Organic acid	0.020719	-0.067
Maltose	Sugar	0.021543	0.127
Threonate	Organic acid	0.021684	-0.035
Alanine	Amino Acid	0.027004	0.042

*p.value shows the significance level of the metabolite changes by ANOVA analysis between the two maternal environments: High phosphate and Low nitrate.

To detect these mQTLs, we analysed metabolite profiles in the dry mature seeds of all the RILs. PCA analysis of this data revealed a clear separation between the known metabolites of the seeds grown in the two nutritional maternal environments (Figure 2A). Twenty-three and 15% of the total variation was explained by PC1 and PC2 respectively. Similar results were obtained after analysis of all 118 detected metabolites (Supplemental Figure S1A). Both PCA plots show that especially PC2 explained the variation related to the maternal environment. The loading

plot of the PCA showed that the main components contributing to PC2 were organic acids including glycolate, glycerate, malonate and succinate and amino acids such as glutamate, serine, threonine and asparagine (Figure 2B, Supplemental Excel file S3).

Metabolite profile and correlation in HP and LN

ANOVA analysis of the metabolites in the two maternal environments indicated that contents of 36 of 58 known metabolites were significantly changed between HP and LN maternal conditions. These metabolites included mostly amino acids such as serine, pyroglutamate and GABA and organic acids including TCA cycle intermediates such as galacterate, malate, succinate and malonate (Table 1).

In general Log2 ratios of HP:LN in the RIL population showed that most of the metabolites had a higher level in seeds from the HP environment as compared to the LN environment (Figure 3, Table 1).

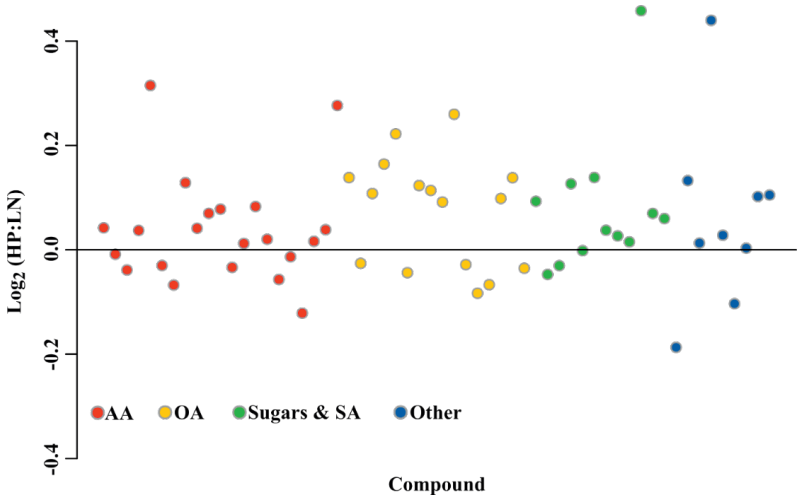

Figure 3. Fold change of the metabolites in two nutritional maternal environments. **HP**, High phosphate and **LN**, Low nitrate. **AA**, Amino acids; **OA**, Organic acids; **SA**, Sugar alcohols.

The metabolite profiles of seeds from the two maternal environments showed that seeds grown in HP conditions contained a higher amount of metabolites, such as many sugars, amino acids and organic acids (Figure 3, 4). A high metabolite level was predominantly observed for GABA, sugars (maltose, fructose and glucose), organic acids including benzoate, salicylate, glycerate and also some TCA cycle intermediates such as galacterate, malate, malonate and succinate. The TCA cycle, including several catabolic reactions, plays a very critical role in energy metabolism in plants. In addition, it contributes in many other ways to the metabolome

by being involved in a large number of metabolic networks (Araujo et al., 2012). In plants, it has been shown that amino acids such as leucine, iso-leucine and valine can be degraded into new products which can be used as precursors for the TCA cycle to provide additional energy for plants. The rate of the degradation of these compounds can be increased due to sub-optimal conditions like abiotic stresses (Binder, 2010).

Both HP and LN maternal environments could be sub-optimal environments for plant growth and seed development. Our results revealed that most of the amino acids were not significantly altered between the two maternal environments; however some of them such as GABA, pyroglutamate, glycine, leucine and aspartate showed significantly lower values in seeds grown in LN. This result was consistent with findings for tomato and Arabidopsis plants that reported a general decrease of amino acid levels under low nitrate conditions (Urbanczyk-Wochniak and Fernie, 2004; Tschoep et al., 2009). GABA is one of the amino acids which frequently shows higher levels under stress conditions (Shelp et al., 1999; Michaeli et al., 2011; Renault et al., 2011). GABA is an amino compound which is produced via the so-called GABA shunt pathway which has a primary role in keeping a balance in central C/N metabolism (Bouche and Fromm, 2004). It has been shown that GABA levels increased rapidly under stressed growth conditions. Thus, GABA is thought to be involved in the tolerance of plants to sub-optimal environments (Kinnersley and Turano, 2000; Fait et al., 2008; Renault et al., 2011). In our study we have observed that the GABA content was lower in seeds developed in LN conditions in comparison with those of the HP maternal condition. Our findings confirmed previous studies, where seeds also showed low amounts of GABA under LN maternal conditions (He et al., 2016); Chapter 2, this thesis).

It has previously been reported that nitrate starvation resulted in a decrease in TCA cycle intermediates in tomato (Urbanczyk-Wochniak and Fernie, 2004) and Arabidopsis (Tschoep et al., 2009) leaves. We also found that in comparison to the HP condition, seeds developed under LN possessed a lower amount of TCA cycle intermediates such as malate, succinate and malonate (Figure 4). This could be an indication of higher consumption of TCA cycle intermediates to produce more energy under LN to allow the plants to survive and continue growth.

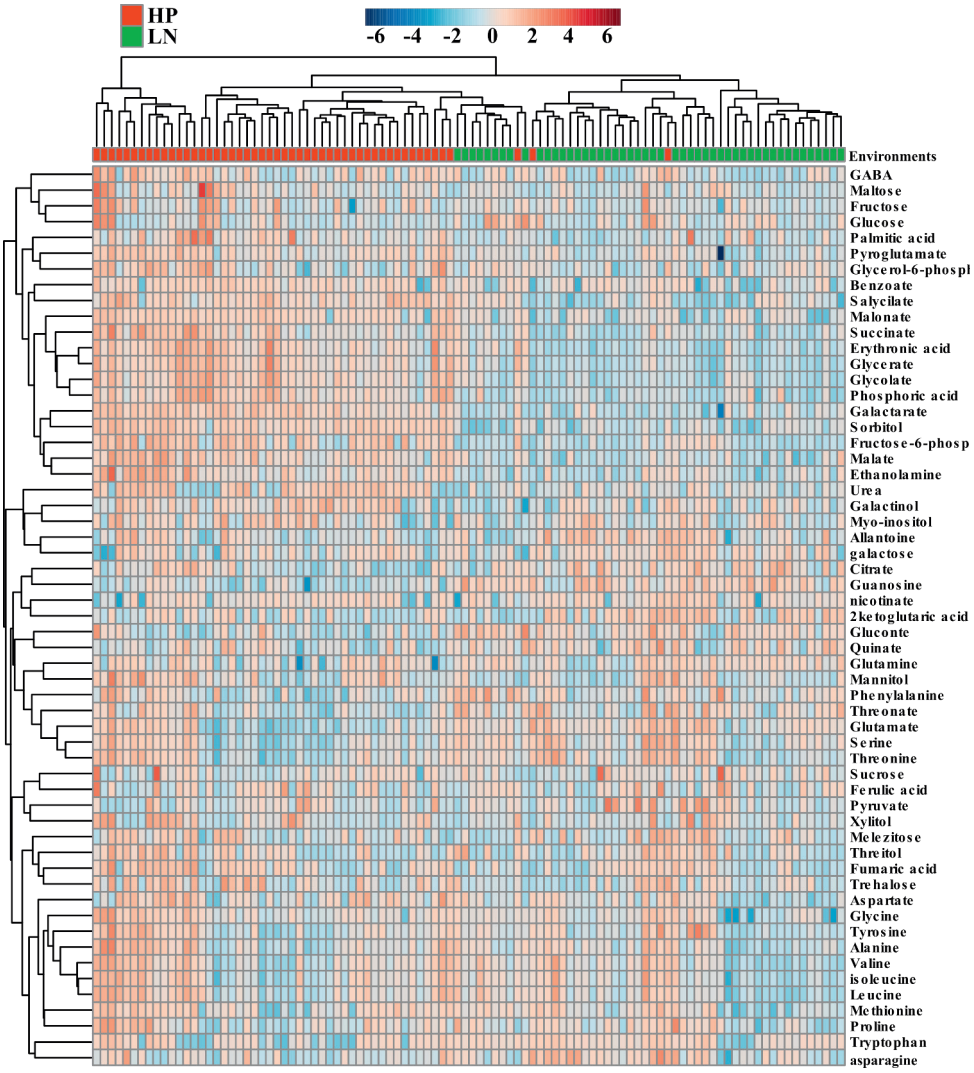

Figure 4. Metabolite profiles in dry tomato seeds. The comparison of metabolite content of the 100 tomato RILs grown in two nutritional maternal environments, **HP**, High phosphate and **LN**, Low nitrate.

Metabolite correlation networks

In general, correlations between metabolites can be used to assist in unravelling the biological basis of variation caused either by different environments or genetic backgrounds (Ursem et al., 2008). In order to understand the correlation between metabolite contents within the RIL sub-populations and how their interaction is influenced by the nutritional maternal environment, pairwise Spearman correlation analysis was performed between the metabolites. For each environmental condition, correlation analysis of all 118 detected metabolites has been

performed and a correlation heatmap was generated (Supplemental Figure S2, Supplemental Excel file S4). The results showed that most of the unknown metabolites are highly correlated with annotated metabolites such as amino acids and organic acids including TCA cycle intermediates. Only known metabolites that showed significant correlations (FDR≤0.05) were selected for constructing correlation networks (Figure 5, Table 2). By using the network approach, the correlation between metabolites within each sub-population as a result of similar genetic regulation can be visualised, while different metabolic patterns in between the different maternal environments could provide more insight into the influence of environment and G×E on regulation of metabolites. Correlation networks have often been used in metabolomics studies (Steuer et al., 2003; Morgenthal et al., 2006) to provide additional information to multivariate approaches which have been described previously (Graffelman and van Eeuwijk, 2005). In our study, the correlation network for the HP maternal environment contains in total 395 significant correlations (edges) between 56 metabolites (nodes). The HP condition resulted in a network with higher density (0.256) as compared to LN, which had in total 238 edges and 51 nodes (Table 2). In general, the network related to the HP environment showed higher levels of some attributes such as range of node degree, number of nodes and edges, network density and average number of neighbours by possessing more metabolite connections and correlations (Table 2). This higher connectivity in the network could be related to the overall higher metabolic levels under this specific condition. In our study dry seed metabolites were connected more under the HP condition, in comparison with LN, which indicates that the regulatory mechanisms under HP conditions induce several changes in metabolism. These metabolic changes could assist plants to cope with sub-optimal growing conditions and may result in acclimation of the plant (Hochberg et al., 2013).

Table 2. Properties of the networks constructed from HP and LN seed metabolite levels.

Attributes	High Phosphate	Low Nitrate
Number of nodes	56	51
Total number of edges	395	238
Number of positive edges	379	232
Number of negative edges	16	6
Range of node degree	1-28	1-21
Average number of neighbours	14.11	9.33
Network density	0.256	0.187

The most highly connected metabolites in each condition can be found in Supplemental Table S2. Under LN, mainly amino acids are highly correlated with each other and thus could be predominantly involved in metabolic changes due to LN conditions (Figure 5A). However, under HP maternal condition, in addition to the amino acids such as alanine, glycine, serine and threonine, some of the TCA cycle intermediates including malate, fumarate and succinate are also highly connected (Figure 5B). In both environments we observed strong correlation between metabolites within the same category such as amino acids. Such a consistent

correlation observed in both environments suggested that these metabolites are mainly under genetic control and not much influenced by the environment or G×E interactions. In our results under HP conditions glycine showed a strong correlation with malate (one of the TCA cycle intermediates, R = 0.6, FDR = 0.00021) while we could not find it back in the LN network. Such different network topologies indicate a strong environmental effect on the correlation between these metabolites. These examples show that the correlation networks and the differences amongst them may provide imperative information to understand the molecular basis of metabolic changes (Schauer et al., 2006).

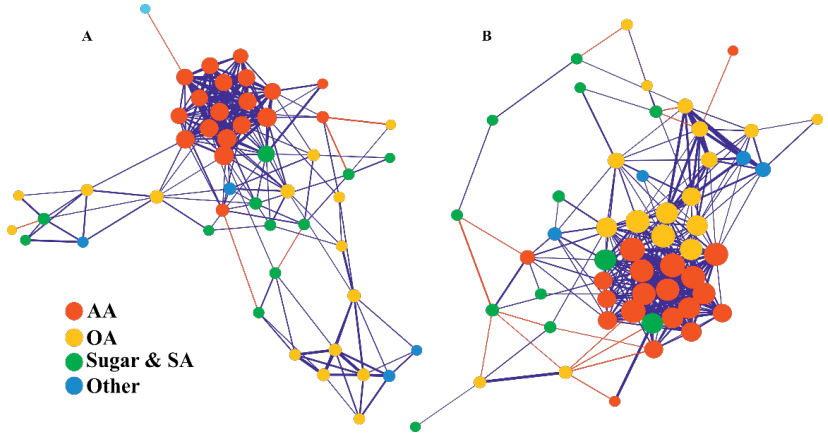

Figure 5. Correlation networks of known metabolites for each maternal environment. **A**, Low nitrate; **B**, High phosphate. The colours of the nodes represent the metabolites category. **AA**, Amino acids; **OA**, Organic acids; **SA**, Sugar alcohols. The **Blue** and **Red** colour of the edges (lines) indicate positive and negative correlations, respectively. The size of the nodes correlates with the number of connections within the network (the degree).

Correlation of metabolites within the whole RIL population

Spearman correlation analysis was performed and a correlation matrix was generated between all pairs of known metabolites across the whole RIL population. The results revealed that some metabolites are highly correlated with each other (Figure 6). Except a few exceptions, all amino acids cluster together. They showed a high degree of correlation of mostly greater than 0.5 with *p*-values of less than 0.001 (Supplemental Excel file S5). Such a high positive significant correlation could be an indication of a preserved metabolism of amino acids in seeds. It has previously been reported that the metabolism of amino acids in seeds might be regulated by post-transcriptional regulators in order to regulate the distribution of nitrogen (Toubiana et al., 2012; Kazmi et al., 2017). We also observed a high number of significant correlations between amino acids and TCA cycle intermediates such as citrate, malate, fumarate and succinate. Such a correlation between amino acids as a nitrogen (N) source and TCA intermediates as carbon

(C) metabolites, indicates a maintained crosstalk between N and C metabolism in the seeds (Figure 6, Supplemental Excel file S5). A similar crosstalk has been previously suggested for different species including Arabidopsis and tomato (Stitt and Fernie, 2003; Gutiérrez et al., 2007; Nunes-Nesi et al., 2010; Kazmi et al., 2017).

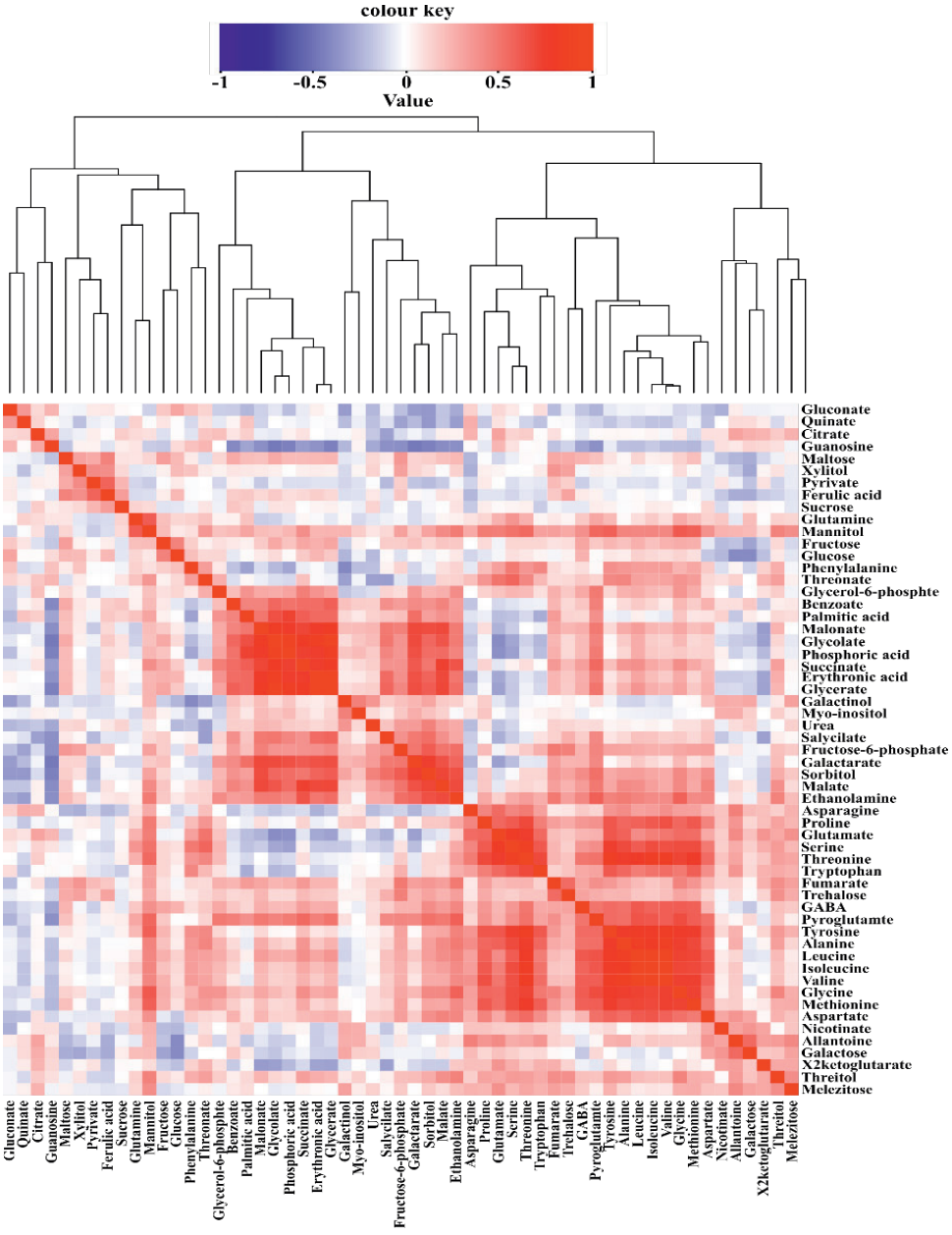

Figure 6. Spearman correlation matrix of all pairs of known metabolites across the whole RIL population derived from *S. lycopersicum* cv. Moneymaker and *S. pimpinellifolium*.

We have also detected significant correlation between galactinol and myo-inositol (R=0.53 and *p*-value=1.06E-6) (Figure 6, Supplemental Excel file S5). These metabolites are classified as sugar alcohols which have been reported to be involved in responses of seeds to stressful environments, such as low temperature (He et al., 2016).

Correlation between metabolites and seed phenotypic traits

In order to assess the relationship between metabolites and seed phenotypic traits, seed performance phenotypes which were previously assessed for the same seeds (Chapter 3), were integrated into the metabolic correlation matrix (Figure 7). We found many positive and negative correlations between metabolites and phenotypic traits. The results revealed that seed size and weight are positively correlated with most of the amino acids and TCA cycle intermediates such as succinate, citrate and malate (Figure 7, Supplemental Excel file S6). The strongest positive correlation was found between seed size and amino acids including pyroglutamate, leucine and isoleucine ($R \geq 0.4$, *p*-value<0.0001). Among all the seed germination traits maximum germination percentage (G_{max}) showed the highest number of significant correlations with metabolites of which most are negative. G_{max} under osmotic stress (mannitol and NaCl) has a significant positive correlation with 2-ketoglutarate which is one of the TCA cycle intermediates, involved in supplying the required energy for seed germination (Supplemental Excel file S6). G_{max} under optimal and sub-optimal germination environments showed strong negative correlation with many of the amino acids (such as pyroglutamate, GABA, methionine and leucine), organic acids (glycerate and malonate) and TCA cycle intermediates (malate and succinate).

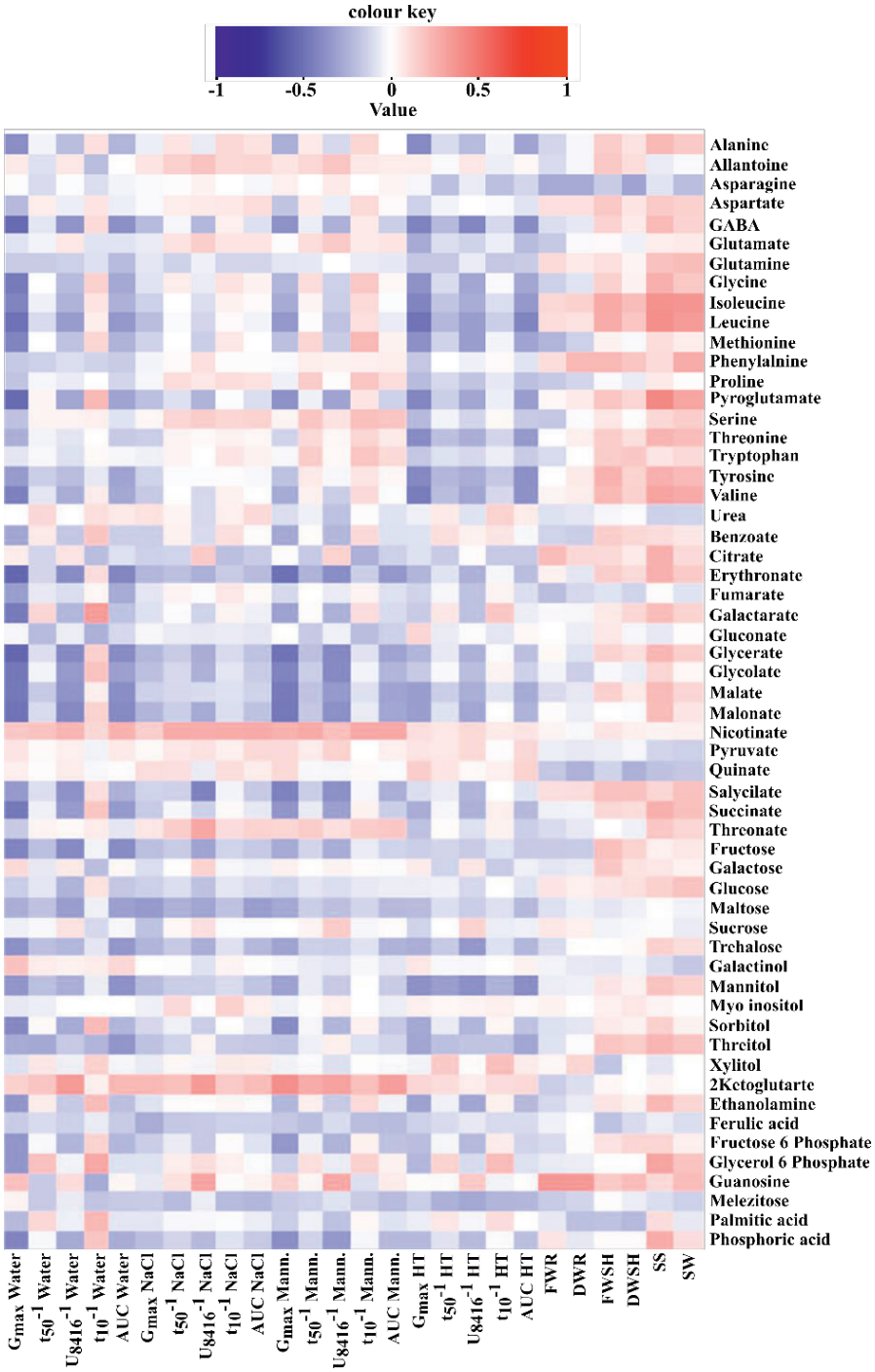

Figure 7. Correlation matrix of metabolites with phenotypic traits within the whole RIL population derived from *S. lycopersicum* cv. Moneymaker and *S. pimpinellifolium*.

Amino acids, are the precursors of protein synthesis and also precursors of some TCA cycle intermediates (e.g. citrate and succinate), serve as energy generation units for embryo growth as well as radicle protrusion (Ratajczak et al., 1996; Lehmann and Ratajczak, 2008; Rosental et al., 2014). Since energy and proteins are two elements supporting germination, such a negative correlation between them and germination of tomato seeds is not expected. However, our results are in accordance with several foregoing studies which reported that accumulation of amino acids, such as methionine, lysine and GABA, may cause inhibition of seed germination (Amir, 2010; Angelovici et al., 2011). In some other reports, amino acids were considered as one of the biological methods to control weeds since the external application of many amino acids decreased the seed germination percentage for some species such as broomrape (Wilson and Bell, 1978; Vurro et al., 2006). Such a negative effect of amino acids on seed germination could be related to accumulation of certain amino acids in the seeds and subsequent reduction of some other metabolites such as TCA cycle intermediates which may play vital roles in seed germination (Angelovici et al., 2011; Rosental et al., 2016). For example, the biosynthetic pathway of lysine uses pyruvate which is the central component of the TCA cycle. Depletion of pyruvate from the TCA cycle will ultimately result in a decrease in the production of TCA cycle intermediates. Hence, such a decrease in TCA cycle input results in declined levels of available energy, which in turn negatively affects seed germination (Shedlarski and Gilvarg, 1970; Day et al., 1994; Angelovici et al., 2011). A strong negative correlation was found between G_{max} in water and methionine content of the seeds ($R=0.42$, p-value<0.001). Similar results have been found in different species such as lettuce (Wilson and Bell, 1978) and tomato (Rosental et al., 2016). Feedback inhibition of increased methionine on the upstream enzymes activity such as cystathionine γ-synthase (CGS) has been reported before (Chiba et al., 2003; Rosental et al., 2016). Hence high methionine content of seeds may limit the synthesis of sulfur-rich proteins which subsequently results in the reduction of seed germination (Amir, 2010). However, our findings seem in contrast with a few other studies in which a high level of methionine did not lead to a decrease of germination which indicated that methionine was not negatively correlated with germination (Gallardo et al., 2002; Amir et al., 2012).

We also performed a correlation analysis between metabolites and seed phenotypic traits within each tomato RIL sub-population and two correlation heatmaps were generated (Supplemental Figure S3). In general, substantial differences were not observed between the two maternal environments (HP and LN); however, correlations appeared stronger within HP as compared to LN conditions and some correlations were specifically observed in one of the environments. For example, the positive correlation observed between many amino acids and seed size and weight were either lost at LN or were not as strong as what was observed at HP (Supplemental Figure S3). In addition, a limited number of metabolites (e.g. galactarate) showed a significant positive correlation with most of the phenotypic traits in LN; however, the same metabolite

showed a weak negative correlation with the same seed phenotypic traits in HP (Supplemental Figure S3). An association of germination percentage and metabolic content of the dry seeds may raise the possibility to predict germination behaviour using the metabolic signature of the dry seeds (Rosental et al., 2014).

mQTL profiling of the tomato RIL population

The calculation of the coefficient of variation (CV) showed that most of the metabolites possess a CV value higher than 40%, which indicates that there is considerable variation within the RIL population for the metabolite levels in the dry seeds (Figure 8, Supplemental Table S3). In order to investigate if such a high level of variability within metabolites could be explained by differences in alleles and genetic factors, a metabolic quantitative trait locus (mQTL) analysis was performed with the obtained metabolite data. Each metabolite is in general controlled by several pathways and regulators. Thus, as expected we hardly identified metabolites for which a single genetic locus significantly explained the metabolite levels.

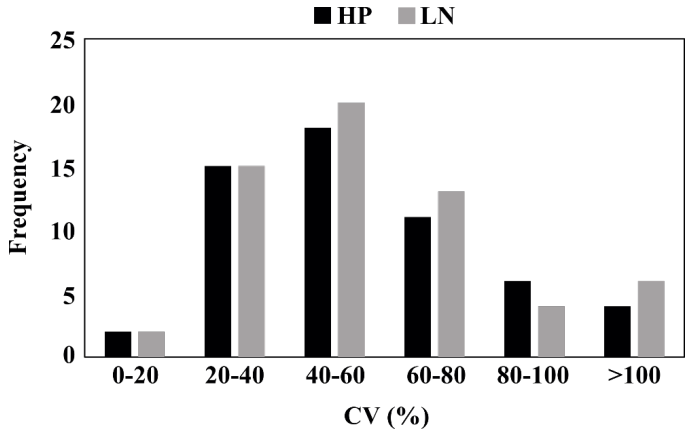

Figure 8. Distribution of metabolite variation within the genotypes for each nutritional maternal environment with high phosphate **(HP)** in black and low nitrogen **(LN)** in grey.

In our study we performed mQTL analysis for each maternal environment to evaluate the genetic variation within each sub-population. Furthermore we used the whole set of RILs to detect mQTLs explained by a genetic component (G) and the genotype by environment interaction (G×E).

Table 3. Number of QTLs identified in each sub-population and for the genetic and genotypei × environment component when analysing the whole population

Condition	Number of mQTLs
High phosphate (HP)	129
Low nitrate (LN)	66
Genetic (G)	382
Genetic by environment interaction (G×E)	146

We identified several mQTLs across all conditions (Table 3). Regarding the sub-populations 66 and 129 mQTLs were detected for seeds from LN and HP environments, respectively. The heatmap of the LOD profiles and characteristics of the mQTLs in each environment are presented in Supplemental Figure S4 and Supplemental Excel file S7, respectively. In both maternal environments several mQTLs were detected which were hardly detected for other metabolites. For example, in the seeds developed under HP conditions a single strong QTL on chromosome 9 was detected, regulating asparagine. Another independent significant QTL was identified on the top of chromosome 11 for phenylalanine under the same environmental conditions (Supplemental Figure S4A, Supplemental Excel file S7). Detection of such specific mQTLs in our data reveals the tight and independent genetic regulation of metabolite biosynthesis in seeds (Keurentjes et al., 2008). Under the same maternal condition some organic acids such as benzoate, gluconate, glycerate and glycolate mapped to a similar position on chromosome 5 (Supplemental Figure S4A, Supplemental Excel file S7). On chromosome 9, we detected mQTLs for TCA cycle intermediates including citrate and malate which were co-locating with the one regulating F6P as one of the precursors of the TCA cycle. There is also a QTL on the top of chromosome 1 affecting amino acids in seeds from the HP environment. Despite the strong correlation that has been found between amino acids and TCA cycle intermediates in seeds from HP conditions, no co-located QTLs were identified for them. This might be due to several smaller QTLs regulating variation of the metabolites, each of them explaining a small part of the variation and therefore not reaching the threshold LOD score. Regarding the seeds grown in the LN maternal environment we found more than one QTL for some of the metabolites such as GABA, citrate and malate. The vital role of these metabolites has been reported in relation with the alleviation of environmental stress effects (Kinnersley and Turano, 2000; Kaplan et al., 2004; Krasensky and Jonak, 2012; Obata and Fernie, 2012). For the LN environment many of the amino acids have co-locating QTLs at the bottom of chromosome 4 and in the middle of chromosome 5 (Supplemental Figure S4B). Such strong co-locating QTLs for amino acids was expected since they showed a high connection in the correlation network of the LN environment (Figure 5A). In general, such co-localizing QTLs for metabolites suggest that in addition to the single independent QTLs regulating metabolite contents, some general regulatory loci and genes are involved in the regulation of metabolite synthesis (Keurentjes et al., 2008).

Combining the sub-populations and using the whole set of RILs leads to an increase in the number of detected QTLs with 382 and 146 QTLs for G and G×E effects, respectively. An overview of the detected QTLs is provided by the heatmap of the LOD profiles (Figure 9). On the top and bottom of chromosome 4 there are two QTLs that explain the variation for many amino acids such as aspartate, GABA, glutamine, methionine, serine and threonine. Similarly, a co-located QTL was detected for galactarate and malate on chromosome 10 (Figure 9). Co-

localization of these mQTLs is not surprising since galactarate is the precursor of 2-oxoglutarate and 2-oxoglutarate is one of the intermediates of the TCA cycle and is generally converted to malate in a couple of subsequent reactions. Our results show that myo-inositol and galactinol are highly associated with each other and closely grouped together. Therefore, it is not surprising that they both have a co-locating QTL on chromosome two (Figure 6, Figure 9). The robust correlation between raffinose pathway metabolites including galactinol and myo-inositol has also been reported for seeds of other species that developed under environmental stress (Cook et al., 2004; He et al., 2016). These metabolites are known for their protective role for cellular structures of embryos during seed development and desiccation (Taji et al., 2002). Furthermore, they are able to play a key role in protecting plants from the effects of stress resulting from reactive oxygen species (ElSayed et al., 2014). Some of the organic acids including gluconate, glycerate and glycolate, together with two of the TCA cycle intermediates (malate and succinate), had a co-locating QTL on chromosome 9 (Figure 9). Glutamate and GABA showed a shared QTL on chromosome 4 which has previously been detected in the same population developed under standard conditions (Kazmi et al., 2017). Metabolites belonging to the same functional class are often highly correlated and can have co-locating mQTLs (Kazmi et al., 2017). Although several mQTLs were detected at similar positions, in general more co-located mQTLs would be expected due to the strong correlation that has been observed between the metabolites. This could be related to the fact that several small QTLs are involved in regulation of the metabolites and each of them is explaining only a small part of their variation. Such small QTLs are likely to escape the QTL significant threshold in the QTL analysis (Keurentjes et al., 2008).

A few mQTLs co-located with the phenotypic QTLs that have been detected in a previous study (Chapter 3 of this thesis). For instance, the QTLs on the middle of chromosome 10 affecting galactarate and malate co-located with ones influencing uniformity of germination (U_{8416}) at different germination conditions, such as high temperature, mannitol, water and NaCl. In addition, the QTL on chromosome 9, which is specifically regulating methionine, is located at the same position as QTLs affecting seed size, seed weight and fresh and dry weight of the seedlings. Despite the many strong correlations between metabolites and phenotypic traits (Figure 9), we could hardly detect co-locating QTLs for them. This might be due to the fact that each of the phenotypes may not be correlated with a specific metabolite but with a group of metabolites and thus the final metabolic balance between the groups of metabolites could affect phenotypic traits such as G_{max}.

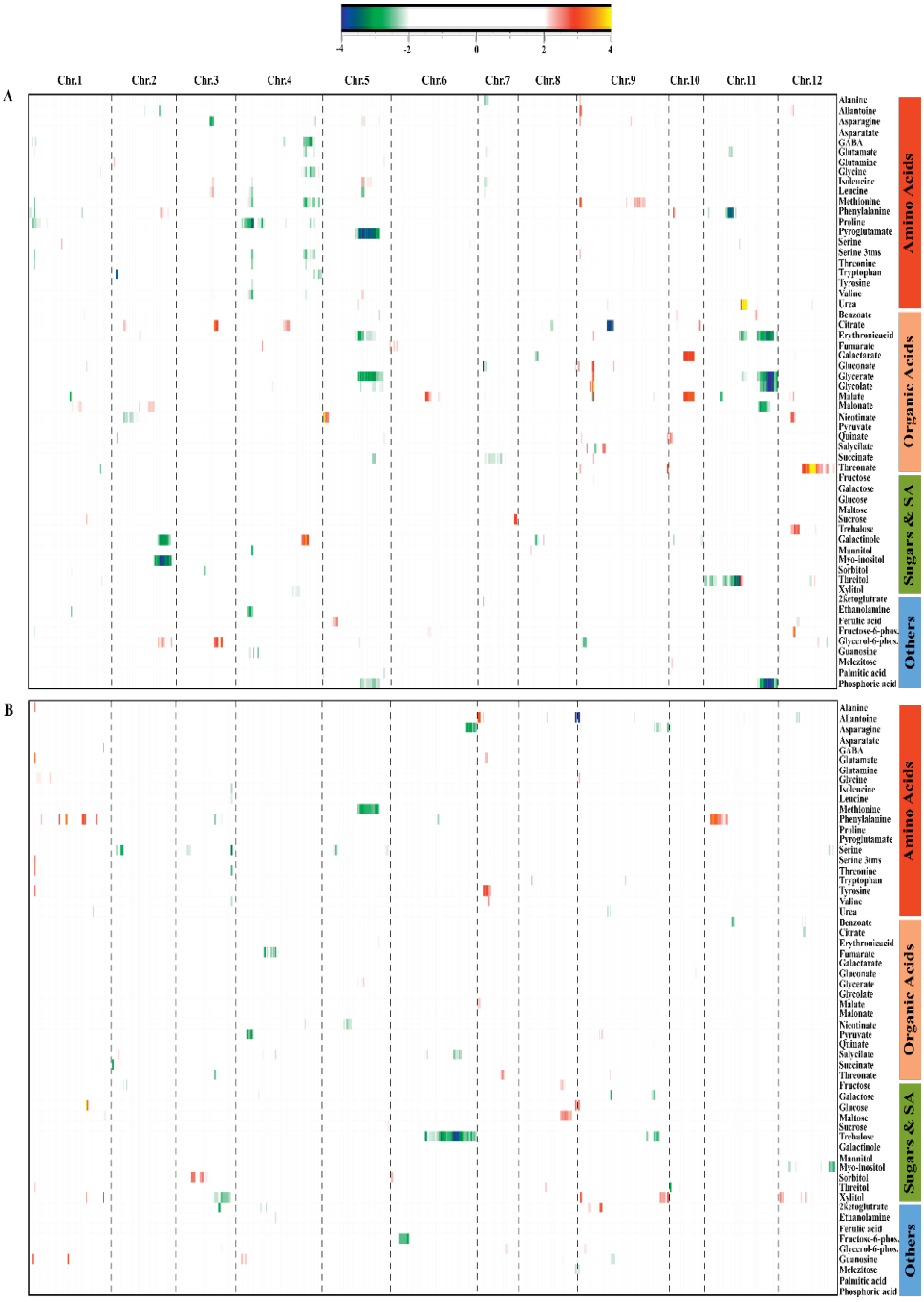

Figure 9. Heatmap of LOD profiles of the mQTLs detected for G and G×E. **A,** Heatmap representing the positions of the mQTLs explained by the genetic component (G); **B,** Heatmap indicating the position of the mQTLs affected by genotype by environment interactions (G×E). The 12 chromosomes of tomato are separated by dashed lines. Coloured spots indicating the significant QTLs. The blue and yellow colours show loci where the *S. pimpinellifolium* and the *S.. lycopersicum* alleles enhance the metabolite levels, respectively. Metabolites and their categories are shown at the right side of the panels.

Conclusion

In this study we performed GC-TOF-MS metabolite profiling of a tomato RIL population and their parental lines grown in high phosphate and low nitrate environments. Our results show clear genetic variation at the metabolite level between the two parental lines, where the maternal nutritional environment was also introducing variation within each genotype. Elucidation of genetic and molecular aspects of metabolic changes of seeds as a response to different maternal environments was carried out by using metabolite correlation networks, followed by mQTL analysis. In general the HP environment induced more metabolic changes as compared to the LN environment. Correlation of metabolites within the whole RIL population revealed a crosstalk between N and C metabolism in which significant correlations were observed between amino acids and TCA cycle intermediates. Besides mQTLs detected in the individual environments and the genetic effects, many mQTLs were detected for G×E. In spite of the strong correlations found between metabolites and phenotypic traits, the detected mQTLs were hardly co-located with the ones affecting phenotypic traits. This might be caused by the fact that not a single metabolite, but a group of metabolites together influence the phenotype. This study has provided novel insights towards better understanding of the effect of maternal environment on tomato seed and seedling performance by combining various physiological, omics and genetical analyses. In addition to the new insights that have been provided in this study, more in-depth investigations are needed to further elucidate the regulation of the dry seed metabolome under different nutritional environments and its influence on seed and seedling performance.

Materials and methods

Maturation conditions and seed collection

One hundred lines of an F_7 RIL population obtained from *S. lycopersicum* cv. Moneymaker (MM) × *S. pimpinellifolium* (PI) accession G1.1554 (Voorrips et al., 2000) have been genotyped with 865 single nucleotide polymorphism (SNP) markers. The F_8 population was grown in two different nutritional maturation environments as previously described (Chapter 3). The fully ripened fruits were collected and the seeds were extracted and dried as previously reported (Chapter 2). Finally, the dry seeds were stored in paper bags at 13°C and 30% RH.

Generalized genetical genomics design (GGG)

The population of 100 tomato lines was divided into two sub-populations based on the distribution of parental alleles. By using the R-procedure DesignGG (Li et al., 2009; Joosen et al., 2013) the tomato lines were allocated to the suitable sub-population in a way that alleles

show a similar distribution in both sub-populations as compared to the whole population (Kazmi et al., 2017).

Extraction and analysis of dry seed metabolites

The dry seed metabolites were extracted using the method as previously described by Roessner et al., (2000) with small changes. In short, 10 mg seeds of each tomato line was homogenized using a micro dismembrator (Sartorius) in a precooled 2 ml Eppendorf tube with 2 iron balls (2.5 mm). A solution of 700 µl methanol/chloroform (4:3) together with a standard (0.2 mg/ml ribitol) was added to each Eppendorf tube and mixed thoroughly. After 10 minutes of sonication 200 µl Milli-Q water was added to the samples followed by vortexing and centrifugation (5 min, 13,500 rpm). Then, the methanol phase was collected and transferred to a new 2 ml tube and the remaining organic phase was extracted again with 500 µl methanol/chloroform. The solution was kept on ice for 10 minutes and afterwards 200 µl Milli-Q water was added. Again after vortexing and centrifugation (5 min, 13,500 rpm), the methanol phase was collected and combined with the former collected phase and mixed well. A solution of 100 µl of this mix was transferred to a glass vial and dried overnight using a speedvac centrifuge at 35°C (Savant SPD1211).

The gas chromatography-time of flight-mass spectrometry (GC-TOF-MS) method which was previously described by Carreno-Quintero et al., (2012) was used for analysis of the dry seed metabolites. Detector voltage was set at 1600 V. Analysis of the raw data was performed using chromaTOF software 2.0 (Leco instruments). Furthermore, the Metalign software was used for further analysis such as aligning the mass signals (Lommen, 2009). The peak threshold for noise was set to 2 and the output was loaded in Metalign Output Transformer (METOT; Plant Research International, Wageningen) and MSClust (Tikunov et al., 2012) was used to construct Centrotypes. The Centrotypes were identified by matching the mass spectra to an in-house-constructed library, to the GOLM metabolome database (http://gmd.mpimp-golm.mpg.de/) and to the NIST05 library (National Institute of Standards and Technology, Gaithersburg, MD, USA; http://www.nist.gov/srd/mslist.htm). The identification was based on spectral similarities and comparing the retention indices calculated by a third order polynomial function (Strehmel et al., 2008).

Statistical analysis (coefficient of variation, PCA and ANOVA analysis)

Within a population the absolute variation or dispersion per trait is defined as the standard deviation (σ). The relative variation called the coefficient variation (CV) for individual traits is the ratio of the standard variation to the mean (µ) of the lines in the population (CV = (σ/µ)*100). In this study we calculated CV for each metabolite in two nutritional maternal environments separately. The metabolomics data were log10 transformed and then used for

further analysis such as ANOVA, principal component and correlation analysis. In order to indicate the effect of the genotype, environment and their interaction, ANOVA analysis was performed on the metabolite content of the parental lines, MM and PI, grown in different nutritional environments. The significance threshold of the *p*-value was set to 0.05. Principal component analysis (PCA) was performed on metabolic values of the RILs and the parental lines using the R-package "pcaplots".

Log2 ratio of metabolites between HP and LN (HP:LN) and metabolite profiles in both maternal environments (HP and LN) were investigated to identify the metabolic differences between two nutritional maternal environments with Metaboanalyst 3.0 (http://www.metaboanalyst.ca/faces/home.xhtml).

Correlation analysis and network construction

R-packages "MASS", "Hmisc", "VGAM", "gplots" and "graphics" (https://www.r-project.org/) were used for analysis and construction of the Spearman correlation between all known metabolites and also between the metabolites and the seed phenotypic traits. In addition, by using the "rcorr" R-package for each sub-population the Spearman correlation between the known metabolites was analysed and the significance level of correlations was described as false discovery rate (FDR). Correlation values with FDR≤0.05 were used to create a correlation network for each maternal environment by using Cytoscape v.3.4.0. The NetworkAnalyser tool of Cytoscape was used to obtain additional characteristics of the metabolic networks.

mQTL analysis

Log10 transformed data together with the tomato linkage map containing 865 SNP markers were used for performing QTL analysis using Rqtl v3.3.1 (Broman et al., 2003; Arends et al., 2010). We performed QTL analysis using the approach as previously described by Joosen et al., (2013) for Arabidopsis and Kazmi et al., (2017) for tomato, with small modifications. A model ($Y = G + E + G\times E + \mathcal{E}$) was used for the whole RIL population to identify the effect of genotype (G), maternal environment (E) and their interaction (G×E). Furthermore, in order to identify the metabolic variation explained by the genetic component we carried out the QTL analysis for known metabolites in each sub-population with simple interval mapping (SIM) using MapQTL® 6.0 (Van Ooijen, 2004). In both QTL analysis (Rqtl and MapQTL) 1000 permutation tests were applied to our data to estimate the LOD threshold at a significance level of 0.05.

References

Albert E, Gricourt J, Bertin N, Bonnefoi J, Pateyron S, Tamby J-P, Bitton F, Causse M (2016) Genotype by watering regime interaction in cultivated tomato: lessons from linkage mapping and gene expression. Theoretical and applied genetics **129:** 395-418

Amir R (2010) Current understanding of the factors regulating methionine content in vegetative tissues of higher plants. Amino acids **39:** 917-931

Amir R, Han T, Ma F (2012) Bioengineering approaches to improve the nutritional values of seeds by increasing their methionine content. Molecular breeding **29:** 915-924

Angelovici R, Fait A, Fernie AR, Galili G (2011) A seed high-lysine trait is negatively associated with the TCA cycle and slows down Arabidopsis seed germination. New Phytologist **189:** 148-159

Araujo WL, Nunes-Nesi A, Nikoloski Z, Sweetlove LJ, Fernie AR (2012) Metabolic control and regulation of the tricarboxylic acid cycle in photosynthetic and heterotrophic plant tissues. Plant, Cell & Environment **35:** 1-21

Arends D, Prins P, Jansen RC, Broman KW (2010) R/qtl: high-throughput multiple QTL mapping. Bioinformatics **26:** 2990-2992

Baud S, Dubreucq B, Miquel M, Rochat C, Lepiniec L (2008) Storage reserve accumulation in Arabidopsis: metabolic and developmental control of seed filling. The Arabidopsis Book**:** e0113

Binder S (2010) Branched-chain amino acid metabolism in Arabidopsis thaliana. The Arabidopsis Book**:** e0137

Borisjuk L, Rolletschek H, Radchuk R, Weschke W, Wobus U, Weber H (2004) Seed development and differentiation: a role for metabolic regulation. Plant Biology **6:** 375-386

Bouche N, Fromm H (2004) GABA in plants: just a metabolite? Trends Plant Sci **9:** 110-115

Broman KW, Wu H, Sen Ś, Churchill GA (2003) R/qtl: QTL mapping in experimental crosses. Bioinformatics **19:** 889-890

Chiba Y, Sakurai R, Yoshino M, Ominato K, Ishikawa M, Onouchi H, Naito S (2003) S-adenosyl-L-methionine is an effector in the posttranscriptional autoregulation of the cystathionine γ-synthase gene in Arabidopsis. Proceedings of the National Academy of Sciences **100:** 10225-10230

Cook D, Fowler S, Fiehn O, Thomashow MF (2004) A prominent role for the CBF cold response pathway in configuring the low-temperature metabolome of Arabidopsis. Proceedings of the National Academy of Sciences of the United States of America **101:** 15243-15248

Day DA, Millar AH, Wiskich JT, Whelan J (1994) Regulation of alternative oxidase activity by pyruvate in soybean mitochondria. Plant Physiology **106:** 1421-1427

ElSayed A, Rafudeen M, Golldack D (2014) Physiological aspects of raffinose family oligosaccharides in plants: protection against abiotic stress. Plant Biology **16:** 1-8

Fait A, Angelovici R, Less H, Ohad I, Urbanczyk-Wochniak E, Fernie AR, Galili G (2006) Arabidopsis seed development and germination is associated with temporally distinct metabolic switches. Plant physiology **142:** 839-854

Fait A, Fromm H, Walter D, Galili G, Fernie AR (2008) Highway or byway: the metabolic role of the GABA shunt in plants. Trends in plant science **13:** 14-19

Galili G, Avin-Wittenberg T, Angelovici R, Fernie AR (2014) The role of photosynthesis and amino acid metabolism in the energy status during seed development. Frontiers in plant science **5:** 447

Gallardo K, Job C, Groot SP, Puype M, Demol H, Vandekerckhove J, Job D (2002) Importance of methionine biosynthesis for Arabidopsis seed germination and seedling growth. Physiologia plantarum **116:** 238-247

Graffelman J, van Eeuwijk F (2005) Calibration of multivariate scatter plots for exploratory analysis of relations within and between sets of variables in genomic research. Biometrical Journal **47:** 863-879

Gutiérrez RA, Lejay LV, Dean A, Chiaromonte F, Shasha DE, Coruzzi GM (2007) Qualitative network models and genome-wide expression data define carbon/nitrogen-responsive molecular machines in Arabidopsis. Genome biology **8:** R7

He H, Willems LA, Batushansky A, Fait A, Hanson J, Nijveen H, Hilhorst HW, Bentsink L (2016) Effects of parental temperature and nitrate on seed performance are reflected by partly overlapping genetic and metabolic pathways. Plant and Cell Physiology **57:** 473-487

Hochberg U, Degu A, Toubiana D, Gendler T, Nikoloski Z, Rachmilevitch S, Fait A (2013) Metabolite profiling and network analysis reveal coordinated changes in grapevine water stress response. BMC plant biology **13:** 184

Jansen RC, Nap J-P (2001) Genetical genomics: the added value from segregation. TRENDS in Genetics **17:** 388-391

Joosen RVL, Arends D, Li Y, Willems LA, Keurentjes JJ, Ligterink W, Jansen RC, Hilhorst HW (2013) Identifying genotype-by-environment interactions in the metabolism of germinating Arabidopsis seeds using generalized genetical genomics. Plant physiology **162:** 553-566

Kaplan F, Kopka J, Haskell DW, Zhao W, Schiller KC, Gatzke N, Sung DY, Guy CL (2004) Exploring the temperature-stress metabolome of Arabidopsis. Plant physiology **136:** 4159-4168

Kazmi RH, Khan N, Willems LA, Van Heusden AW, Ligterink W, Hilhorst HW (2012) Complex genetics controls natural variation among seed quality phenotypes in a recombinant inbred population of an interspecific cross between Solanum lycopersicum× Solanum pimpinellifolium. Plant, cell & environment **35:** 929-951

Kazmi RH, Willems LA, Joosen RV, Khan N, Ligterink W, Hilhorst HW (2017) Metabolomic analysis of tomato seed germination. Metabolomics **13:** 145

Keurentjes JJ, Fu J, De Vos CR, Lommen A, Hall RD, Bino RJ, van der Plas LH, Jansen RC, Vreugdenhil D, Koornneef M (2006) The genetics of plant metabolism. Nature genetics **38:** 842

Keurentjes JJ, Sulpice R (2009) The role of natural variation in dissecting genetic regulation of primary metabolism. Plant signaling & behavior **4:** 244-246

Keurentjes JJ, Sulpice R, Gibon Y, Steinhauser M-C, Fu J, Koornneef M, Stitt M, Vreugdenhil D (2008) Integrative analyses of genetic variation in enzyme activities of primary carbohydrate metabolism reveal distinct modes of regulation in Arabidopsis thaliana. Genome biology **9:** R129

Khan N, Kazmi RH, Willems LA, Van Heusden AW, Ligterink W, Hilhorst HW (2012) Exploring the natural variation for seedling traits and their link with seed dimensions in tomato. PLoS One **7:** e43991

Kinnersley AM, Turano FJ (2000) Gamma aminobutyric acid (GABA) and plant responses to stress. Critical Reviews in Plant Sciences **19:** 479-509

Kliebenstein D (2009) Advancing genetic theory and application by metabolic quantitative trait loci analysis. The plant cell **21:** 1637-1646

Kliebenstein DJ, West MA, van Leeuwen H, Loudet O, Doerge RW, St Clair DA (2006) Identification of QTLs controlling gene expression networks defined a priori. Bmc Bioinformatics **7:** 308

Kooke R, Keurentjes J (2011) Multi-dimensional regulation of metabolic networks shaping plant development and performance. Journal of experimental botany **63:** 3353-3365

Krasensky J, Jonak C (2012) Drought, salt, and temperature stress-induced metabolic rearrangements and regulatory networks. Journal of experimental botany **63:** 1593-1608

Kumar N (2006) Breeding of horticultural crops: principles and practices. New India Publishing

Lehmann T, Ratajczak L (2008) The pivotal role of glutamate dehydrogenase (GDH) in the mobilization of N and C from storage material to asparagine in germinating seeds of yellow lupine. Journal of plant physiology **165:** 149-158

Li Y, Breitling R, Jansen RC (2008) Generalizing genetical genomics: getting added value from environmental perturbation. Trends in Genetics **24:** 518-524

Li Y, Swertz MA, Vera G, Fu J, Breitling R, Jansen RC (2009) designGG: an R-package and web tool for the optimal design of genetical genomics experiments. BMC bioinformatics **10:** 188

Lisec J, Meyer RC, Steinfath M, Redestig H, Becher M, Witucka-Wall H, Fiehn O, Törjék O, Selbig J, Altmann T (2008) Identification of metabolic and biomass QTL in Arabidopsis thaliana in a parallel analysis of RIL and IL populations. The Plant Journal **53:** 960-972

Lommen A (2009) MetAlign: interface-driven, versatile metabolomics tool for hyphenated full-scan mass spectrometry data preprocessing. Analytical chemistry **81:** 3079-3086

Michaeli S, Fait A, Lagor K, Nunes-Nesi A, Grillich N, Yellin A, Bar D, Khan M, Fernie AR, Turano FJ (2011) A mitochondrial GABA permease connects the GABA shunt and the TCA cycle, and is essential for normal carbon metabolism. The Plant Journal **67:** 485-498

Morgenthal K, Weckwerth W, Steuer R (2006) Metabolomic networks in plants: Transitions from pattern recognition to biological interpretation. Biosystems **83:** 108-117

Nunes-Nesi A, Fernie AR, Stitt M (2010) Metabolic and signaling aspects underpinning the regulation of plant carbon nitrogen interactions. Molecular plant **3:** 973-996

Obata T, Fernie AR (2012) The use of metabolomics to dissect plant responses to abiotic stresses. Cellular and Molecular Life Sciences **69:** 3225-3243

Rao ES, Kadirvel P, Symonds RC, Ebert AW (2013) Relationship between survival and yield related traits in Solanum pimpinellifolium under salt stress. Euphytica **190:** 215-228

Ratajczak W, Lehmann T, Polcyn W, Ratajczak L (1996) Metabolism of amino acids in germinating yellow lupine seeds. I. The decomposition of 14 C-aspartate and 14 C-glutamate during the imbibition. Acta physiologiae plantarum **1**

Renault H, El Amrani A, Palanivelu R, Updegraff EP, Yu A, Renou J-P, Preuss D, Bouchereau A, Deleu C (2011) GABA accumulation causes cell elongation defects and a decrease in expression of genes encoding secreted and cell wall-related proteins in Arabidopsis thaliana. Plant and Cell Physiology **52:** 894-908

Rodríguez-López M, Garzo E, Bonani J, Fereres A, Fernández-Muñoz R, Moriones E (2011) Whitefly resistance traits derived from the wild tomato Solanum pimpinellifolium affect the preference and feeding

behavior of Bemisia tabaci and reduce the spread of Tomato yellow leaf curl virus. Phytopathology **101:** 1191-1201

Rosental L, Nonogaki H, Fait A (2014) Activation and regulation of primary metabolism during seed germination. Seed science research **24:** 1-15

Rosental L, Perelman A, Nevo N, Toubiana D, Samani T, Batushansky A, Sikron N, Saranga Y, Fait A (2016) Environmental and genetic effects on tomato seed metabolic balance and its association with germination vigor. BMC genomics **17:** 1047

Schauer N, Semel Y, Roessner U, Gur A, Balbo I, Carrari F, Pleban T, Perez-Melis A, Bruedigam C, Kopka J (2006) Comprehensive metabolic profiling and phenotyping of interspecific introgression lines for tomato improvement. Nature biotechnology **24:** 447

Shedlarski JG, Gilvarg C (1970) The pyruvate-aspartic semialdehyde condensing enzyme of Escherichia coli. Journal of Biological Chemistry **245:** 1362-1373

Shelp BJ, Bown AW, McLean MD (1999) Metabolism and functions of gamma-aminobutyric acid. Trends in plant science **4:** 446-452

Steuer R, Kurths J, Fiehn O, Weckwerth W (2003) Interpreting correlations in metabolomic networks. *In*. Portland Press Limited

Stitt M, Fernie AR (2003) From measurements of metabolites to metabolomics: an 'on the fly'perspective illustrated by recent studies of carbon–nitrogen interactions. Current opinion in biotechnology **14:** 136-144

Strehmel N, Hummel J, Erban A, Strassburg K, Kopka J (2008) Retention index thresholds for compound matching in GC–MS metabolite profiling. Journal of Chromatography B **871:** 182-190

Taji T, Ohsumi C, Iuchi S, Seki M, Kasuga M, Kobayashi M, Yamaguchi-Shinozaki K, Shinozaki K (2002) Important roles of drought-and cold-inducible genes for galactinol synthase in stress tolerance in Arabidopsis thaliana. The Plant Journal **29:** 417-426

Tikunov Y, Laptenok S, Hall R, Bovy A, De Vos R (2012) MSClust: a tool for unsupervised mass spectra extraction of chromatography-mass spectrometry ion-wise aligned data. Metabolomics **8:** 714-718

Toubiana D, Semel Y, Tohge T, Beleggia R, Cattivelli L, Rosental L, Nikoloski Z, Zamir D, Fernie AR, Fait A (2012) Metabolic profiling of a mapping population exposes new insights in the regulation of seed metabolism and seed, fruit, and plant relations. PLoS Genetics **8:** e1002612

Tschoep H, Gibon Y, Carillo P, Armengaud P, Szecowka M, Nunes-Nesi A, Fernie AR, Koehl K, Stitt M (2009) Adjustment of growth and central metabolism to a mild but sustained nitrogen-limitation in Arabidopsis. Plant, cell & environment **32:** 300-318

Urbanczyk-Wochniak E, Fernie AR (2004) Metabolic profiling reveals altered nitrogen nutrient regimes have diverse effects on the metabolism of hydroponically-grown tomato (Solanum lycopersicum) plants. Journal of Experimental Botany **56:** 309-321

Ursem R, Tikunov Y, Bovy A, Van Berloo R, Van Eeuwijk F (2008) A correlation network approach to metabolic data analysis for tomato fruits. Euphytica **161:** 181

Van Ooijen J (2004) MapQTL 5, Software for the mapping of quantitative trait loci in experimental populations. Kyazma BV, Wageningen, the Netherlands. MapQTL 5, Software for the mapping of quantitative trait loci in experimental populations. Kyazma BV, Wageningen, the Netherlands.**:** -

Voorrips RE, Verkerke W, Finkers R, Jongerius R, Kanne J (2000) Inheritance of taste components in tomato. Acta Physiologiae Plantarum **22:** 259-261

Vurro M, Boari A, Pilgeram AL, Sands DC (2006) Exogenous amino acids inhibit seed germination and tubercle formation by Orobanche ramosa (broomrape): potential application for management of parasitic weeds. Biological Control **36:** 258-265

Wilson MF, Bell EA (1978) Amino acids and β-aminopropionitrile as inhibitors of seed germination and growth. Phytochemistry **17:** 403-406

Windsor AJ, Reichelt M, Figuth A, Svatoš A, Kroymann J, Kliebenstein DJ, Gershenzon J, Mitchell-Olds T (2005) Geographic and evolutionary diversification of glucosinolates among near relatives of Arabidopsis thaliana (Brassicaceae). Phytochemistry **66:** 1321-1333

Supplementary information

Supplementary Tables and Excel files of this chapter can be downloaded from
http://www.wageningenseedlab.nl/thesis/ngeshnizjani/SI/chapter4

Supplementary Figures

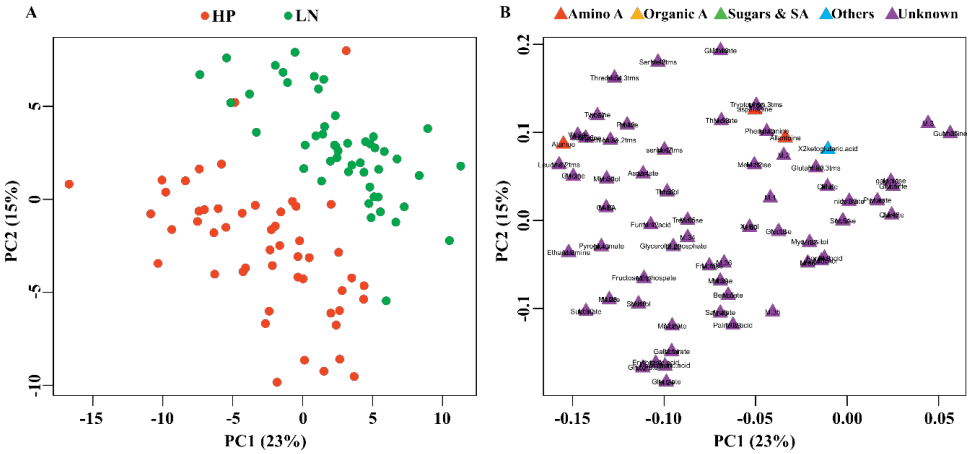

Figure S1. Principle component analysis (PCA) of all known and unknown metabolites of dry seeds grown in **HP,** High phosphate and **LN,** Low nitrate. **A,** Scores for PCA for HP and LN are presented by two components PC1 and PC2. **B,** Loading scores of metabolites for PC1 and PC2. **Amino A,** amino acids; **Organic A,** Organic acids; **SA,** Sugar alcohols.

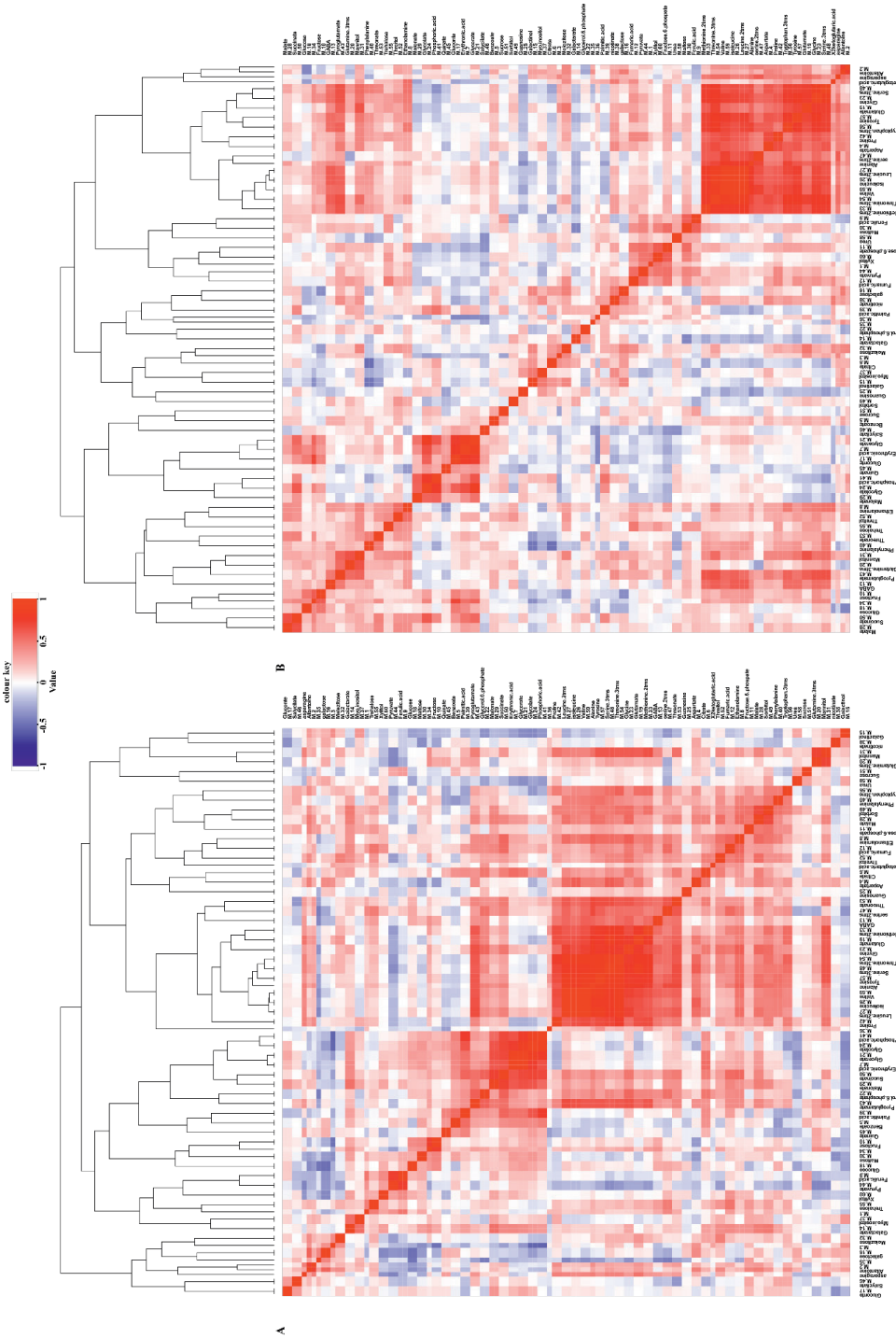

Figure S2. The correlation matrix of all detected (known and unknown) metabolites in tomato RIL population derived from s cross between *Solanum. Lycopersicum* cv. Moneymaker and *Solanum pimpinellifolium* grown in two maternal environments. **A,** High phosphate; **B,** Low nitrate

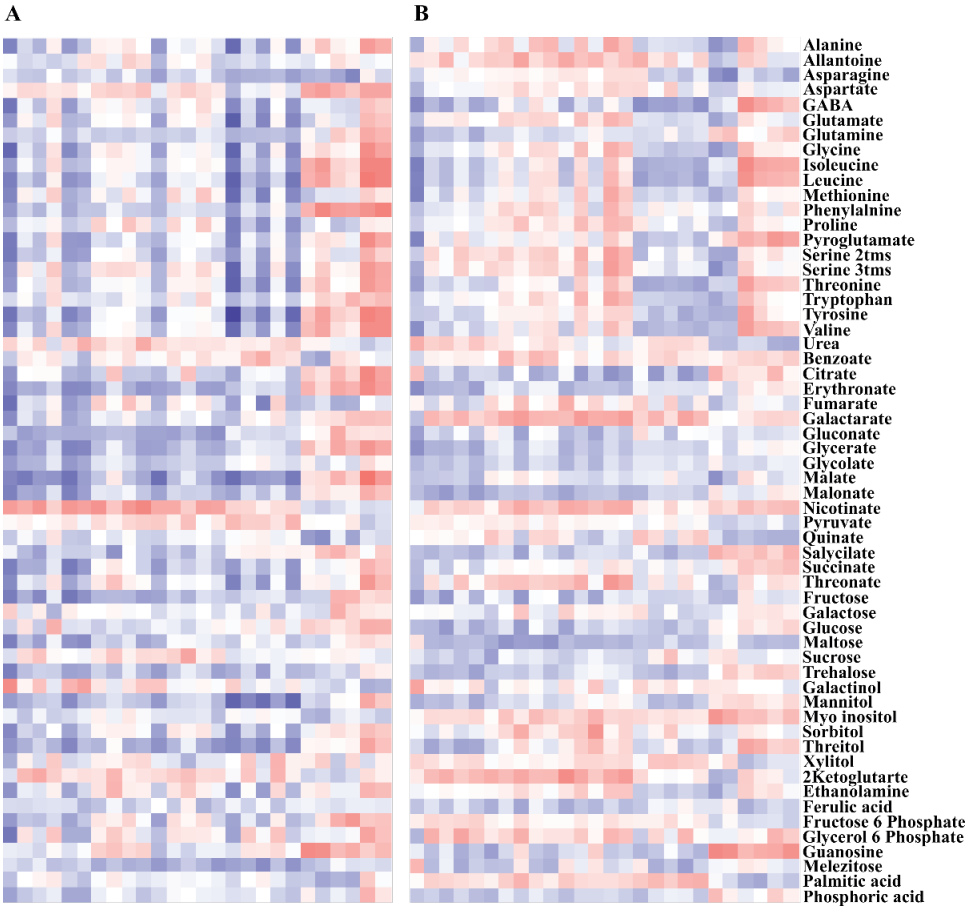

Figure S3. Correlation matrix of metabolites with seed phenotypic traits within each tomato RIL sub-population derived from *S. lycopersicum* cv. Moneymaker and *S. pimpinellifolium*. **A,** High phosphate; **B,** Low nitrate.

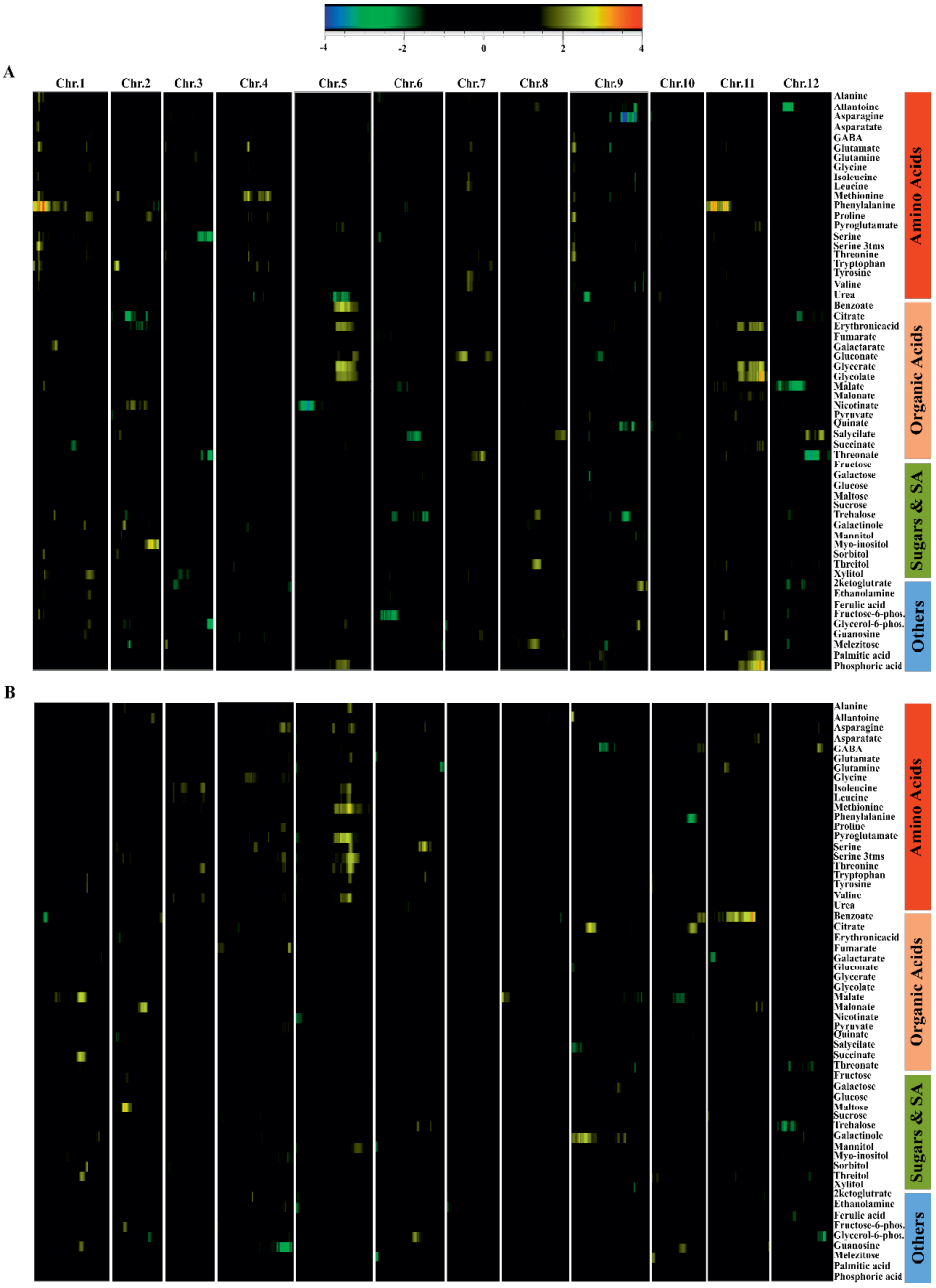

Figure S4. Heatmap of QTL profiles of the mQTLs detected in tomato RIL population derived from s cross between *S. Lycopersicum* cv. Moneymaker **(MM)** and *S. pimpinellifolium* **(PI)** grown in two maternal environments. **A,** High phosphate; **B,** Low nitrate. The 12 chromosomes of tomato are separated by white lines. Coloured spots indicating the significant QTLs. The blue and red colours in the LOD colour scale showing the higher effect of **PI** and the **MM** alleles on metabolite levels, respectively. Metabolites and their categories are shown at the right side of the panels.

Chapter 5

Characterization of and Genetic Variation for Tomato Seed Thermo-inhibition and Thermo-dormancy

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Abstract

Exposing imbibed seeds to high temperatures may lead to either thermo-inhibition of germination or thermo-dormancy responses. In thermo-inhibition, seed germination is inhibited but quickly resumed when temperatures are lowered. Upon prolonged exposure to elevated temperatures, thermo-dormancy may be induced and seeds are not able to germinate even at optimal temperatures. In order to explore underlying physiological and molecular aspects of thermo-induced secondary dormancy, we have investigated the physiological responses of tomato seeds to elevated temperatures and the molecular mechanisms that could explain the performance of tomato seeds at elevated temperature. In order to investigate how tomato seeds respond to high temperature we used two distinct tomato accessions: *Solanum lycopersicum* (cv. Moneymaker) (MM) and *Solanum pimpinellifolium* accession CGN14498 (PI). MM seeds did not germinate under high temperature conditions while seeds of PI reached a maximum germination of 80%. Despite the high germination percentage of PI, germinated seeds did not produce healthy seedling at 37°C. By using a candidate gene approach we have tested if similar molecular pathways (abscisic acid (ABA) and gibberellic acid (GA)) present in lettuce and Arabidopsis, are regulating thermo-inhibition and thermo-dormancy responses in tomato. We showed that the ABA biosynthesis pathway genes *NCED1* and *NCED9* were upregulated whereas two of the GA-biosynthesis regulators (*GA3ox1* and *GA20ox1*) were downregulated in tomato thermo-dormant seeds at elevated temperature. To identify novel regulators of tomato seed performance under high temperature, we screened a Recombinant Inbred Line (RIL) population derived from a cross between the two tomato accessions MM and PI for thermo-inhibition and dormancy induction. Several QTLs were detected, particularly for thermo-dormancy, which may be caused by new regulators of thermo-inhibition and thermo-dormancy in tomato. None of the genes studied in this research were co-locating with the detected QTLs. The new QTLs discovered in this study will therefore be useful to further elucidate the molecular mechanisms underlying the responses of tomato seeds to high temperature and eventually lead to identification of the causal genes regulating these responses.

Introduction

Seed germination is the start and end of the life cycle of most flowering plants and is, therefore, a critical step in plant development and growth. Although rapid seed germination directly after sowing is a desirable trait for seeds from the producer's point of view, it can be an undesirable trait at times (Huo and Bradford, 2015). Generally, seeds are able to sense unfavourable environmental conditions and may, thus, postpone germination until conditions are more favourable. Such a physiological response is called dormancy (Hilhorst, 2007). The occurrence of dormancy is heavily influenced by environmental factors. One of the environmental factors which are becoming more relevant in the light of global warming is elevated temperature (Long and Ort, 2010; Franks et al., 2014). Therefore it is of great importance to study the physiological and molecular background of seed germination and dormancy in response to the adverse effect of high temperatures in more detail. Elevated temperatures not only influence post-germination processes, but may also directly affect germination. Exposure of seeds to high temperature may result in certain physiological responses, called 'thermo-dormancy' and 'thermo-inhibition', in order to prevent seedling damage. Thermo-inhibition refers to the fact that seeds will halt germination at high temperature, but will immediately germinate upon encountering optimal temperatures. Thermo-dormancy is defined as dormancy that is induced by high temperatures (Argyris et al., 2008; Huo et al., 2013). In this case seeds will not germinate at high temperatures, but also not when the seeds are exposed to lower/optimal germination temperatures. This implies that prolonged imbibition at high temperature results in the induction of dormancy.

Many studies have shown that germination of species such as sunflower (*Helianthus L.*), carrot (*Daucus carota*), *Arabidopsis thaliana* and lettuce (*Lactuca sativa*) is inhibited if they are exposed to high temperatures during imbibition (Corbineau et al., 1988; Toh et al., 2008; Lafta and Mou, 2013; Nascimento et al., 2013). Genetic, molecular and physiological analysis have provided insights into the mechanisms of seed germination and dormancy (Holdsworth et al., 2008). Thermo-inhibition and thermo-dormancy can be alleviated by environmental factors such as light and nitrate, or by applying GA, inhibitors of ABA biosynthesis, or ethylene (Gonai et al., 2004; Kępczyński et al., 2006; Hilhorst, 2007; Matilla and Matilla-Vázquez, 2008; Toh et al., 2008; Bogatek and Gniazdowska, 2012).

Combining genetic and physiological analysis may improve the understanding of the molecular mechanisms underlying seed germination and dormancy (Yamauchi et al., 2004; Bentsink et al., 2006). The existing natural variation in plants for seed germination behaviour has been used for genetic analysis and identification of Quantitative Trait Loci (QTLs). Many studies have been reported regarding the genetic aspects of thermo-inhibition and thermo-dormancy in seeds

resulting in several QTLs in both model plants such as Arabidopsis and crops such as rice and lettuce (Alonso-Blanco et al., 2003; Gu et al., 2006; Bentsink et al., 2007).

Abscisic acid (ABA) and gibberellic acid (GA) are phytohormones that play critical roles in the life cycle of plants, including seed germination and dormancy. ABA has been shown to be a major inhibitor of seed germination and it has an important role in the induction of primary and secondary dormancy in seeds. The ABA biosynthesis pathway is sensitive to several environmental factors and this is one way in which different environments result in different levels of dormancy (Kermode, 2005; Finch-Savage, 2013). Conversely, by application of exogenous GA or by increasing the GA synthesis in seeds, dormancy can be alleviated in many plant species (Carter and Stevens, 1998; Gonai et al., 2004). Thus, germination is regulated by a balance between synthesis and catabolism of ABA and GA. Several genes are known which function in ABA (9-cis-epoxycarotenoid dioxygenase (*NCEDs*) and *ABAs*) and GA (*GA3ox1*, *GA3ox2* and *GA2ox1*) biosynthetic pathways and which may, thus, affect the inhibition of germination caused by high temperatures (Toyomasu et al., 1998; Iuchi et al., 2001). In general, when seed germination is inhibited, ABA-associated genes are up-regulated and GA-related genes are suppressed. In Arabidopsis, it has been indicated that high temperature results in the accumulation of ABA in seeds by increased expression of *NCED2*, *NCED5* and *NCED9* and reduced GA content by repression of *GA20ox* and *GA3ox* (Toh et al., 2008). In lettuce, it has been shown that expression of the *LsNCED4* gene, which is involved in ABA biosynthesis, was highly induced under high temperature (Argyris et al., 2008).

Further studies have indicated that the maturation, germination and dormancy of seeds may be regulated by an interaction between phytohormones (e.g. ABA, GA) and a network of transcription factors (Finkelstein et al., 2002; Suzuki and McCarty, 2008). For example, *FUSCA3 (FUS3)* is a B3-domain transcription factor which plays a critical role in hormonal biosynthesis and signalling pathways and, consequently, in the life cycle of plants. Under stress conditions such as high temperature, *FUS3* can induce dormancy in seeds by increasing and decreasing ABA and GA biosynthesis, respectively (Nambara et al., 2000; Curaba et al., 2004; Holdsworth et al., 2008). In Arabidopsis, it was reported that high temperature greatly increased the expression of *FUS3*. Overexpression of *FUS3* results in delayed seed germination at high temperature (32°C), while mutant lines (*fus3*) are tolerant to that high temperature (Chiu et al., 2012). Microarray analysis has revealed the direct effect of *FUS3* on the expression *NCED5* and *NCED9* and several GA biosynthesis genes. This implies that thermo-dormancy may be the consequence of an increase in *NCED5* and *NCED9* expression in Arabidopsis (Yamamoto et al., 2010; Wang and Perry, 2013). A similar effect of high temperature on the expression of *FUS3* was found in lettuce (*LsFUS3*) and it is possible that also in lettuce FUS3 has a direct effect on the expression of *LsNCED4* (Huo and Bradford, 2015).

Ethylene has been shown to be one of the promotors of seed germination (Matilla, 2000; Matilla and Matilla-Vázquez, 2008). It functions in breaking seed coat imposed dormancy in species such as *Rumex crispus* and Arabidopsis (Taylorson, 1979; Siriwitayawan et al., 2003). 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (*ACS*) is one of the genes which is involved in ethylene biosynthesis. In lettuce (Abeles, 1986), chickpea (*Cicer arietimum*) (Gallardo et al., 1991), sunflower (Corbineau et al., 1990) and tomato (Kpczyska et al., 2006) it was reported that either ethylene or its biosynthetic precursor ACC could break thermo-dormancy.

Several reports have presented putative hormonal and molecular mechanisms by which seeds may perceive environmental signals and regulate dormancy and germination (Wigge, 2013; Penfield and MacGregor, 2014). Despite many studies on the mechanisms of thermo-inhibition and thermo-dormancy in crops such as lettuce, little is known about the germination behaviour and regulation of tomato seeds at high temperature. The objective of this study was to investigate the genetic variation of thermo-inhibition between two tomato accessions: *Solanum lycopersicum* (cv. Moneymaker) (MM) and *Solanum pimpinellifolium* (PI). Furthermore, we have used a candidate gene approach to see if similar molecular mechanisms as in lettuce and Arabidopsis are likely to regulate thermo-inhibition and thermo-dormancy in tomato. Finally, a Recombinant Inbred Line (RIL) population derived from a cross between the MM and PI tomato accessions (Voorrips et al., 2000) has been used to perform QTL analysis and detect new QTLs and potential new regulators of seed germination and dormancy in tomato under high temperature.

Results

Germination of the cultivated tomato accession Solanum lycopersicum (cv. Moneymaker) seeds in response to optimal and high temperature

Seeds of MM plants germinated to around 90% at 25°C in both light and dark conditions. However at high temperature (37°C) MM seeds neither germinated at light nor at dark (Figure 1A, B). Thus seeds of the MM genotype were unable to germinate at 37°C.

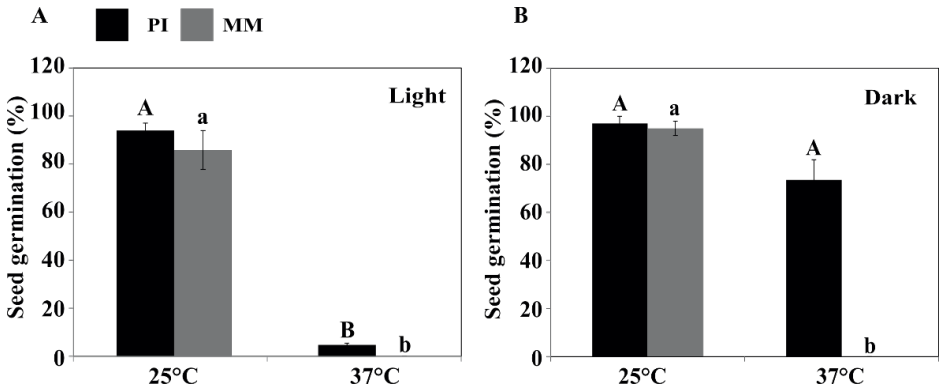

Figure 1. Germination of *Solanum lycopersicum* (cv. Moneymaker) **(MM)** and *Solanum pimpinellifolium* **(PI)** seeds at optimal (25°C) and high temperature (37°C) in the light **(A)** and in darkness **(B)**. Statistical analysis was performed within each genotype.

To investigate if the inhibitory effect of the temperature is either thermo-inhibition or thermo-dormancy, the non-germinated MM seeds were transferred to the optimal germination temperature (25°C) in the presence and absence of light after 4 days (4DI) and 6 days (6DI) of imbibition at 37°C. In the presence of light at 25°C, seeds which had been imbibed at 37°C for 4 days (4DI), germinated more and faster than seeds which had been imbibed for 6 days at 37°C (6DI) (Figure 2A, B). Neither 4DI nor 6DI seeds germinated without light at 25°C. Nevertheless, when 4DI and 6DI seeds after 18 days of dark imbibition were transferred to light conditions, they started to germinate (Figure 2C, D). Interestingly, the 6DI seeds which germinated more slowly and to a lower percentage in dark, could immediately germinate after transfer to light and reached 100% of germination after a few days. However, germination of 4DI seeds was delayed by 3 days after transfer to optimal temperature in the light. It took 9 days for these seeds to reach ~100% of germination, starting from the transfer date. It may be concluded that at high temperature thermo-dormancy was induced concomitantly with the induction of light sensitivity.

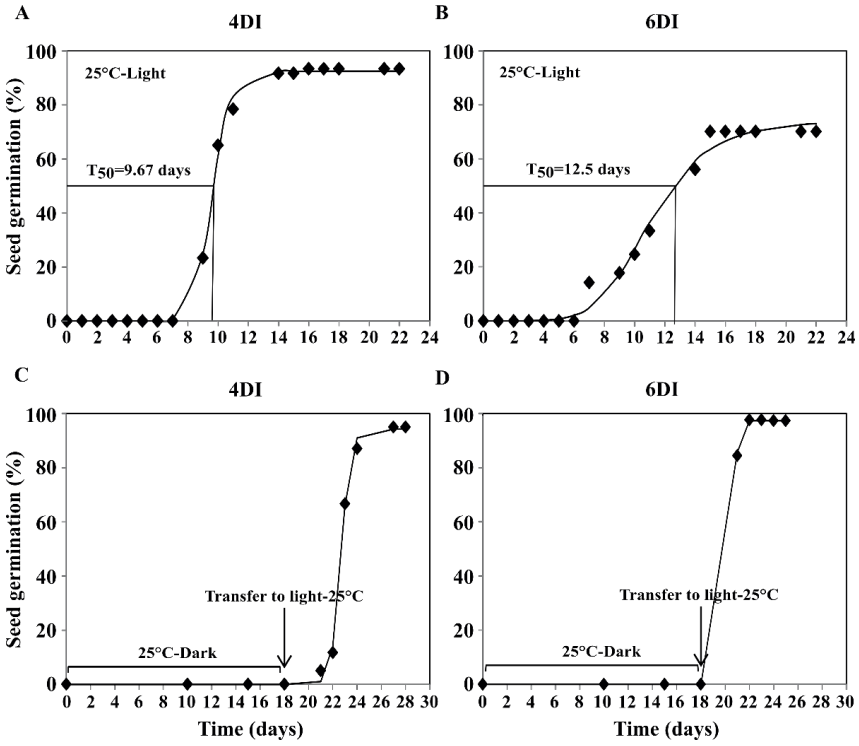

Figure 2. A and **B,** Germination percentage of (*Solanum lycopersicum* cv. Moneymaker) 4DI and 6DI seeds at 25°C with light, respectively; **C** and **D,** germination percentage of the same 4DI and 6DI seeds, respectively, at 25°C without light and post transferring to light after 18 days of dark imbibition.

Endospermic dormancy in MM tomato seeds

Stiffness of the endosperm can be one of the major inhibiting factors in germination of tomato seeds (Kępczyński et al., 2006) and therefore we investigated the role of the endosperm in thermo-dormancy of tomato seeds. Germination of MM embryos which were dissected from the seeds was assessed at 25°C and 37°C. The absence of seed coat and endosperm had some effect on germination speed at 25°C. Embryos showed uniformly and rapid radicle growth in almost 90% of the seeds after three days (Figure 3). However, removing the endosperm did not promote seed germination at high temperature and seeds became dormant in that condition even without seed coat and endosperm (Figure 3, Supplemental Figure S1). Our results suggest that the thermo-dormancy induced in tomato seeds is not related to the inhibition of endosperm weakening but to embryo dormancy. Thus, contrary to general consensus of tomato seed dormancy being coat-imposed we here propose that tomato seed dormancy also has a component of physiological dormancy located in the embryo.

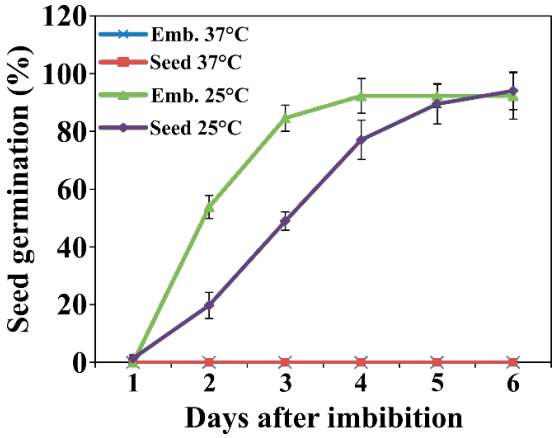

Figure 3. Germination of seeds and separate embryos **(Emb.)** of tomato seeds (*Solanum lycopersicum* cv. Moneymaker) under normal (25°C) and high temperature (37°C).

Required time to induce thermo-dormancy in tomato seeds (MM)

Since we observed that high temperature induced thermo-dormancy in MM seeds, we investigated in more detail how long MM seeds required to be at high temperature to induce dormancy. During the first 33 hours (~1.5 days) seed germination was not influenced by high temperature and thus they germinated to almost 90%. Seeds started to go into thermo-dormancy after 33 hours at 37°C. From this time point onwards seed germination declined sharply until 4 days at which seeds did not germinate anymore (Figure 4).

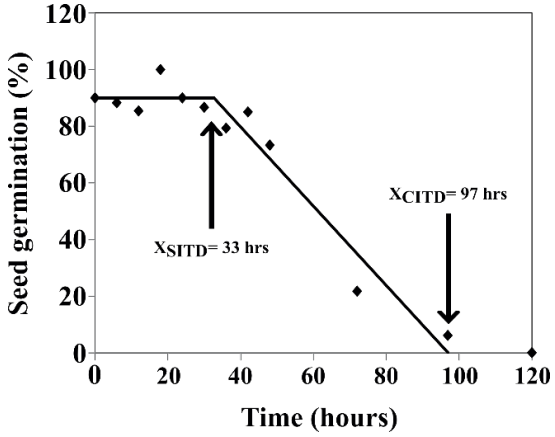

Figure 4. Required time to induce dormancy in tomato seeds (*Solanum lycopersicum* cv. Moneymaker).

Gene expression analysis

Several genes have been implied in the regulation of thermo-inhibition and thermo-dormancy in species such as lettuce and Arabidopsis (Argyris et al., 2008; Toh et al., 2008; Chiu et al., 2012). Since this type of dormancy has not been previously reported in tomato, we selected tomato homologs with the highest identity to Arabidopsis and lettuce genes for ethylene, ABA and GA biosynthesis pathway such as *ACS1*, *NCED9*, *NCED1*, *ABA1*, *GA3ox1*, *GA20ox1* and *GA20ox3*. Additionally, the expression of *FUSCA3* was also analysed due to its direct regulatory effect on the expression of several ABA and GA biosynthesis genes. To test whether *FUS3* is possibly associated with a role in the induction of thermo-dormancy in tomato seeds, expression levels of *FUS3* were measured during imbibition at 25 and 37°C. *FUS3* transcript abundance was very low in dry seeds and in 1 hour and 1 day imbibed seeds at 25°C and 37°C (Figure 5A). However, transcript abundance was significantly increased in 4DI and 6DI seeds which already expressed thermo-dormancy (Figure 5A). Additionally, we measured expression of some genes related to ABA and GA biosynthesis. Although *NCED5* was not expressed in the selected stages, transcript levels of *NCED9* and *NCED1* increased in seeds exposed to 37°C (Figure 5B). In the case of *NCED9*, expression remained fairly constant until 4 days at 37°C (4DI) but displayed increased expression in 6DI seeds. *NCED1* transcript levels started to increase from 2 days of high temperature imbibition onwards with the highest level in 6DI seeds (Figure 5B). Abundance of both *GA3ox1* and *GA20ox1* transcripts was very low in dry and 1 hour imbibed seeds at 25°C but their expression levels peaked at 1 and 2 days of imbibition at 37°C, respectively and decreased thereafter (Figure 5C). We measured the expression level of *ACS* which is one of the genes in the ethylene biosynthesis pathway. *ACS* transcript abundance was decreased upon imbibition at high temperature, but expression did not change further at longer durations of the high temperature treatment (Figure 5D).

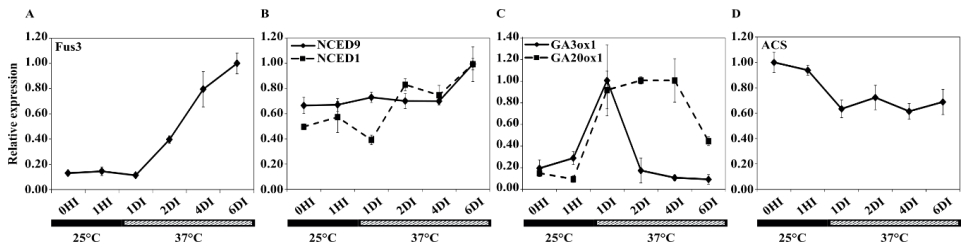

Figure 5. Relative expression of target genes in *Solanum lycopersicum (cv. Moneymaker)* dry seeds **(0HI)**; Seeds imbibed at 25°C for 1 hour **(1HI)** and imbibed at 37°C for 1 **(1DI)**, 2 **(2DI)**, 4 **(4DI)** and 6 **(6DI)** days for *fus3* **(A)**, *NCED1* and *NCED9* **(B)**, *GA3ox1* and *GA20ox1* **(C)** and *ACS1* **(D)**.

A genetic basis of thermo-inhibition and thermo-dormancy in tomato

Similar to MM seeds those of PI germinated also around 90% at 25°C in both light and dark conditions (Figure 1A, B). However, under high temperature (37°C) conditions the two genotypes showed an inverse germination behaviour. As described before, MM seeds neither germinated at light nor at dark conditions at 37°C (Figure 1A, B). At the same temperature light played a very critical role for germination of PI seeds. In the presence of light PI seeds germinated almost 0%, while without light their germination increased to around 90% at 37°C (Figure 1A, B). It is worth noting that despite the high germination percentage of the PI seeds at high temperature, they did not grow into normal healthy seedlings (Supplemental Figure S2). Therefore the PI seeds showed a behaviour which could be called thermo-insensitive germination. Due to the differences observed for thermo-dormancy induction in MM versus PI, we were interested in the differences between these genotypes that cause this contrasting phenotype. To study these, we used a RIL population of 100 lines derived from a cross between MM and PI to investigate the genetic basis of thermo-dormancy in tomato seeds.

The frequency distribution of germination percentage revealed that although germination values of the RILs varied between the two parental lines, at 37°C and 25°C many of the RILs either germinated to a very low percentage or did not germinate at all similar to the MM parental line (Figure 6A, B, Supplemental Table S2). Exposing these non-germinated seeds to GA and stratification at 4°C for three days resulted in almost 100% germination at 25°C (Figure 6C, Supplemental Table S2). Taken together these results illustrate that most of the progenies did not inherit the germination ability at high temperature from the PI parental line. Apparently, these lines do not possess the loci which make PI thermo-insensitive and, thus, they display thermo-dormancy. In our study we considered seeds as thermo-tolerant, thermo-inhibited and thermo-dormant when they germinated at 37°C, at 25°C but not at 37°C and at 25°C after GA and stratification treatment, respectively.

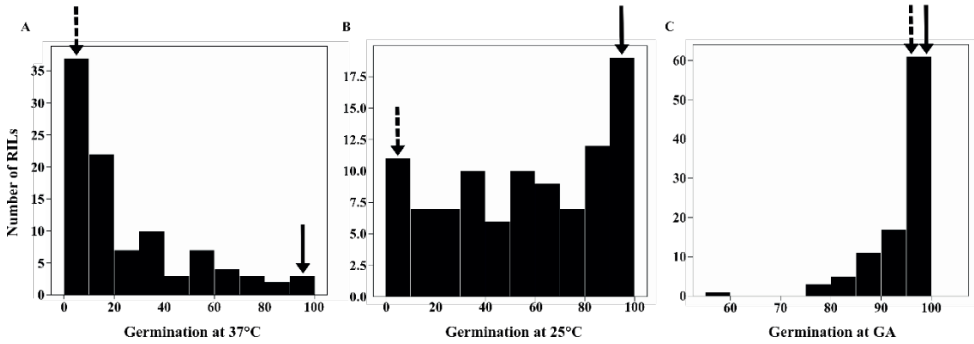

Figure 6. Frequency distribution of non-normalized data of the cumulative germination percentage of *Solanum lycopersicum* and *Solanum pimpinellifolium* RILs. Germination percentage of all lines was assessed in the dark: **A,** at high temperature (37°C); **B,** at 25°C (of the seeds that did not germinated at 37°C); **C,** at 25°C after using GA and stratification for remaining non-germinated seeds. The average germination percentage of parental line is indicated by a solid arrow **(PI)** and dashed arrow **(MM)**.

Identification of QTLs for thermo-inhibition and thermo-dormancy in the tomato RIL population

In order to identify the loci regulating the existing diversity of seed thermo-inhibition and thermo-dormancy in MM and PI, we performed a QTL analysis using the RIL seed germination percentages at 37°C, the following incubation at 25°C, or further incubation at 25°C with a pre-treatment of GA and stratification. The position, related marker, LOD score and other characteristics of the identified QTLs for thermo-inhibition and thermo-dormancy are listed in Table 1. The heat map of LOD profiles visualizes QTLs as hot spots across the 12 chromosomes of tomato (Figure 7).

Table 1. Characteristics of all QTLs associated with thermo-tolerance, thermo-inhibition and thermo-dormancy in a *Solanum lycopersicum* x *Solanum pimpinellifolium.* RIL population.

Trait	Chromosome	Marker[1]	LOD[2]	Supporting interval (CM)	Explained variance[3] (%)	Additive[4]
Th-T*	1	67512259-1	3.53	53.737-89.311	13.6	-0.104
	6	43761285-6	2.3	94.955-100.821	8.7	0.78
Th-I**	1	83852566-1	3.66	119.883-134.3	14.1	-0.094
	12	62576889-12	2.27	74.454-77.989	8.5	-0.074
Th-D***	1	86171125-1	5.45	119.883-139.883	16.3	-0.122
	3	58231771-3	3.24	103.485-111.308	9.2	0.092
	8	50811756-8	2.41	48.872-57.589	6.7	-0.081
	10	63534969-10	2.96	98.017-107.47	8.3	0.088
	11	47411518-11	3.29	20.24-25.426	9.3	0.094

*Thermo-tolerance, **Thermo-inhibition, ***Thermo-dormancy.
[1]Physical position of closest marker to the QTL peak.
[2]LOD score illustrating significant threshold (P=0.05) calculated by a permutation test.
[3]Percentage of variation explained by each QTL.
[4]Positive and negative effect respectively representing the contribution of alleles of *Solanum lycopersicum* and *Solanum pimpinellifolium* to increase the traits at that specific locus.

In total 9 putative QTLs were detected which were predominantly related to thermo-dormancy. For this trait we found specific QTLs on chromosomes 3, 8, 10 and 11. Furthermore, a robust QTL on chromosome 1 co-located with the QTL detected for thermo-inhibition. We found one QTL regulating the thermo-tolerance trait on the same chromosome but at a different location (Table 1, Figure 7).

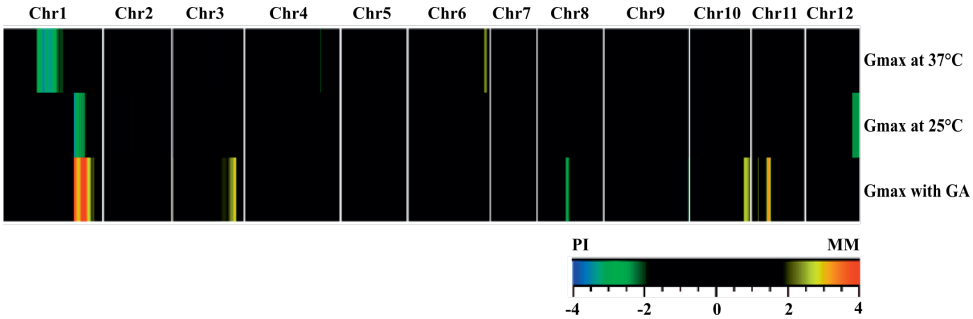

Figure 7. Genomic location of QTLs detected for thermo-tolerance, thermo-inhibition and thermo-dormancy of a *Solanum lycopersicum* and *Solanum pimpinellifolium* RIL population. The 12 chromosomes of tomato are separated by white lines. Centimorgan positions increase from left to right. 37°C, 25°C and GA represent germination at 37°C (thermo-tolerance), 25°C (thermo-inhibition) and 25°C after GA and stratification treatments (thermo-dormancy), respectively. Colours across the chromosomes display significant QTLs (P= 0.05) based on the LOD colour scheme in which green and blue represent a larger effect of *S. pimpinellifolium* (PI) alleles on the traits and yellow and red of the *S. lycopersicum* (MM) alleles.

Discussion

There are many reports on adverse effects of high temperature on seed germination of several crops such as sunflower (Corbineau et al., 1988), carrot (Nascimento et al., 2013) and lettuce (Lafta and Mou, 2013). Tomato, however, as one of the most important crops worldwide, has not been investigated for this adverse effect so far. Here we report on the molecular basis and mechanisms regulating secondary dormancy induced by high temperature in tomato seeds.

MM seeds displayed thermo-induced secondary dormancy regardless of light conditions (Figure 1A, B). In addition, we found that induction of secondary dormancy in MM seeds is highly dependent on the duration of exposure to high temperature. Our results show that short term (≤33 h) exposure of MM seeds to high temperature is not sufficient for induction of secondary dormancy. Induction starts after 33 hours, resulting in decreased seed germination percentages, reaching 0% after ~100 h.

Generally, thermo-dormancy prevents seeds from germination which needs to be relieved to allow germination. Light is one of the factors which can pre-dominantly affect thermo-dormancy. There are several reports on how light can alleviate secondary dormancy induced by

different factors (Bewley, 1997; Argyris et al., 2008; Gubler et al., 2008). We showed that after induction of secondary dormancy in MM seeds by high temperature, light plays a vital role in breaking it. Germination of thermo-dormant seeds was promoted upon encountering an optimal temperature (25°C) and permissive light conditions (Figure 2). Interestingly, light is showing a dual function depending on the genetic background: i) induction of secondary dormancy in PI seeds at high temperature (Figure 1A) and ii) breaking of secondary dormancy of MM thermo-dormant seeds at optimal temperatures (Figure 2). Apparently light, in the presence of high temperature activates different mechanisms in MM and PI. We can speculate that light might act as an additional stress factor in PI resulting in the secondary dormancy. Contrarily light in a different genetic background (i.e. MM) works as a dormancy terminating factor at high temperature condition.

It has previously been reported that endosperm weakening is one of the basic inhibiting factors for tomato seed germination (Kępczyński et al., 2006). Furthermore, in some crops like lettuce and tomato it has been suggested that secondary dormancy might be related to the seed coat and endosperm tissues which could be circumvented by removing either or both (Bewley, 1997; Bonina, 2005). However, we show that the endosperm does not play a role in secondary dormancy induction in MM tomato seeds, since thermo-induced secondary dormancy was not released upon removal of the endosperm and seed coat (Figure 3). This indicates that thermo-induced secondary dormancy in tomato is a physiological process which supposedly is regulated by physiological blocks in the embryo. Despite results from previous studies on secondary dormancy in tomato pointing towards the physical inhibitory role of the seed coat (the strength of which is regulated by the physiology of the seed) in this phenomenon (Bonina, 2005; Kępczyński et al., 2006), our results suggest that the thermo-dormancy induced in tomato seeds is not related to the inhibition of endosperm weakening but to embryo dormancy.

Our results suggest that induction of secondary dormancy by high temperature in tomato is significantly affected by the genetic background as MM seeds are more sensitive to high temperature than those of PI (Figure 1). However, exposure of PI seeds to the combination of high temperature and light also resulted in the induction of secondary dormancy (Figure 1).

Expression analysis of genes previously described to be involved in thermo-induced secondary dormancy in other species (i.e. Arabidopsis and lettuce) provided useful information on the molecular background of thermo-induced secondary dormancy in tomato. Among all investigated genes *FUS3*, as one of the master regulators of both GA and ABA biosynthetic pathways, showed a very distinct expression profile (Figure 5A). This is in accordance with Chiu et al., (Chiu et al., 2012) who demonstrated a similar regulatory role of *FUS3* in thermo-induced secondary dormancy in Arabidopsis. It has been reported that downstream genes of ABA and GA biosynthesis and catabolism (*NCED*s and *GA3ox*s and *GA20ox*s) are being

regulated in accordance with the gene expression profile of *FUS3* (Yamamoto et al., 2010; Wang and Perry, 2013). Interestingly, in the present study *GA3ox1* and *GA20ox1*, involved in biosynthesis of GA, were downregulated upon upregulation of *FUS3*. On the other hand, ABA biosynthesis pathway genes were upregulated with upregulation of *FUS3*, albeit to a lesser extent. However, in case of *NCED9*, one of the downstream ABA biosynthetic pathway genes, the expression pattern is not exactly matching to what was observed for the upstream master regulator, *FUS3*. This could be due to delayed expression of downstream genes, regulated by this transcription factor. We propose GA and ABA biosynthetic pathway genes and their master regulator(s) as putative targets to obtain further insights in this process.

Our findings regarding the effect of several GA and ABA metabolic pathway genes on high temperature-induced dormancy in tomato seeds was in accordance with previous expression analysis of similar genes in Arabidopsis (Cadman et al., 2006; Finch-Savage et al., 2007; Toh et al., 2008; Chiu et al., 2012).

In order to further identify genes involved in the regulation of dormancy induction in tomato seeds, we have used a QTL analysis approach with a RIL population derived from a cross between the two tomato accessions *S. lycopersicum* cv. Moneymaker and *S. pimpinellifolium* accession CGN14498. Some interesting QTLs were identified (Figure 7). The same population has previously been screened for germination QTLs under control conditions [50], but except for the QTL on chromosome 6 there was no overlap with the QTLs found in this study. It is intriguing that none of the genes (*FUS3*, *NCED9*, *NCED1*, *GA3ox1* and *GA20ox1*) commonly considered to be involved in induction of thermo-dormancy in other species (Huo and Bradford, 2015) (Figure 5) were co-locating with these identified QTLs. Similar holds true for the tomato homolog of ETHYLENE RESPONSE FACTOR1 (ERF1) which has been found to have a role in thermo-dormancy in lettuce (Yoong et al., 2016). Evidently, other mechanisms and/or genes may be involved in the regulation of thermo-dormancy in tomato seeds. Furthermore, the high number of identified QTLs suggests a complex multi-genic trait. QTLs identified in this research can pave further routes towards detailed investigations of the mechanism of action of thermo-dormancy in tomato seeds. Combination of fine mapping of detected QTLs combined with RNA-sequencing data will result in better understanding of this process in tomato seeds.

Conclusion

Global warming is an undeniable phenomenon which affects slowly, but continuously, agricultural commodities. Hence understanding the underlying mechanisms in plants by which plants/seeds tolerate suboptimal temperatures is of a great importance. In this study we showed that thermo-induced secondary dormancy in tomato seeds is genotype-dependent. We observed

that secondary dormancy was only induced in MM seeds encountering high temperature (37°C) and not in PI. The induced dormancy in MM seeds was not related to the physical inhibitors such as seed coat and endosperm. A candidate gene approach has been used to check whether the molecular pathways involved in thermo-induced dormancy in tomato are similar to the ones in other species such as lettuce and Arabidopsis (ABA and GA). Upregulation of ABA biosynthesis pathway genes (*NCED1* and *NCED9*) and on the other hand downregulation of two of the GA-biosynthesis genes (*GA3ox1* and *GA20ox1*) in tomato thermo-dormant seeds at elevated temperature implies similar mechanisms as the reported ones of lettuce and Arabidopsis involved in thermo-dormancy in tomato seeds. Besides, QTL analysis showed genomic regions involved in thermo-dormancy regulation. Intriguingly, the mentioned regulatory molecular elements in thermo-dormancy (*NCED*s and *GAox*s) were not co-located with our detected QTLs. This finding points towards additional mechanisms involved in tomato seeds thermo-dormancy regulation. Identification of genes causal for these QTLs and their functional characterization will pave the route towards identification and characterization of those mechanisms.

Materials and Methods

Plant material

The RIL population was obtained from a cross between two parental lines: *S. lycopersicum* cv. Moneymaker and *S. pimpinellifolium* accession G1.1554 (Voorrips et al., 2000). This population was provided and produced by Adriaan W. van Heusden of Wageningen UR Plant Breeding, Wageningen, The Netherlands. *S. lycopersicum* cv. Moneymaker and *S. pimpinellifolium* accession G1.1554 were obtained from the Centre Genetic Resources: the Dutch genebank for plant genetic resources for food and agriculture under a mandate of the Netherlands government (reference CGN14330 and CGN14498, respectively). A total of 727 single nucleotide polymorphism (SNP) markers was used for genotyping the population in F_7 and seeds of F_8 plants were used for the phenotyping. The RIL population, together with the parental lines, were grown under standard conditions in a greenhouse at 25°C and 15°C during day and night and 16 and 8 hours of light and dark, respectively. The fully ripened fruits were harvested and the seeds were extracted using 1% hydrochloric acid (HCl) to remove all sticky parts of the seed's pulp. Afterwards, the seeds were soaked in a trisodium phosphate ($Na_3PO_4.12H_2O$) solution to disinfect the seed batches. Finally, the seeds, which had been dried at room temperature for 3 days, were stored in paper bags under cool and dry conditions (13°C and 30% RH) (Kazmi et al., 2012).

Seed germination assay

In order to study how temperature and light regulate seed germination of the two parental lines (MM and PI), three replications of around 50 seeds were sown on germination trays (21x15 cm DBP Plastics, http://www.dbp.be). Each tray contained two layers of blue germination paper (5.6′ x 8′ Blue Blotter Paper; Anchor Paper Company, http://www.seedpaper.com) and 50 ml demineralized water. Trays were piled with one empty tray consisting of one germination paper and 50 ml of water at the bottom and top of the pile to prevent unequal evaporation. The piles were transferred to either optimal (25°C) or high temperature (37°C) in both presence and absence of light. Germination was scored manually twice per day for one week at optimal as well as high temperature.

Germination of tomato embryos

MM seeds were imbibed at 25°C for 3-4 hours and subsequently seed coat and endosperm layers were removed using forceps and a sharp blade. The extracted embryos were immediately placed on new germination trays with 2 blue germination papers and 50 ml water. The trays were transferred to optimal (25°C) and high temperature (37°C) in dark. The growth of the radicles was evaluated manually once a day for 6 days.

Time required to induce dormancy

Seeds of MM were subjected to 37°C for 6, 12, 18, 24, 30, 36, 42, 72, 96, 120 and 144 hours. Subsequently, they were transferred to 25°C for 14 days. Afterwards, the normal germinated seeds were counted manually for each time point. A segmented model was fitted to seed germination (percentage) versus time (hours) using the NLIN procedure of the SAS software (Ghaderi-Far et al., 2011):

$y=a+b*x$	for	$x>x_0$
$y=a+b*x_0$	for	$x<x_0$

where y is germination percentage, x is days at 37°C, b is model slope line, x_0 is the start time of dormancy induction.

Effect of light on thermo-dormancy alleviation

MM seeds were imbibed at 37°C for 4 and 6 days and then transferred to 25°C under light and dark conditions. Germinated seeds were counted every day for 18 days. Since the seeds did not germinate in the dark, they were transferred to light after 18 days of dark imbibition and seed germination was scored every day for 10 days. For each condition a logistic model was fitted to the cumulative seed germination (percentage) versus time (days) with the NLIN procedure using SAS software (Ghaderi-Far et al., 2012).

$$y(\%)=G_{max}/(1+(x/D_{50})^{\wedge}G_{rate})$$

where y is the total germination (%) at time x, G_{max} is the maximum germination (%), D_{50} is the time to 50% of the maximum germination and G_{rate} indicates the slope of the curve at D_{50}.

Germination assays for QTL analysis

The seeds of the RIL population were sown in 4 replications as described above for the germination assay of the parental lines. Following stratification at 4°C, the trays were transferred to 37°C and kept there for five days. After this incubation the healthy germinated seeds were counted and considered thermo-insensitive. Thereafter, the non-germinated seeds were incubated at 25°C for one week after which the geminated seeds were manually scored and considered thermo-inhibited. Remaining non-germinated seeds were transferred to new trays containing two layers of blue germination paper and 10 µM GA which were incubated at 4°C for 3 days. After this stratification the trays were incubated at 25°C and eventually the final germinated seeds were scored again after 5 and 7 days and considered thermo-dormant. QTL detection for the traits under study was carried out with simple interval mapping (SIM) using mapping software MapQTL® 6.0 based on the linkage map of the RIL population (Supplemental File S1) containing 727 SNP markers (Van Ooijen, 2004). Thousand permutation tests of our data were implemented in MapQTL® 6.0 and resulted in a 95% LOD threshold of 2.0. Therefore, we adjusted the LOD threshold to 2.0 to determine putative QTLs related to thermo-inhibition and thermo-dormancy (Doerge and Churchill, 1996).

RNA extraction and cDNA synthesis

Total RNA was extracted from 30 seeds of each sample. Dry seeds, imbibed seeds at 25°C for 1 hour and imbibed seeds at 37°C for 1, 2, 4 and 6 days were used for RNA extraction. The seeds were frozen in liquid nitrogen and ground by a dismembrator (Mikro-dismembrator U; B. Braun Biotech International, Melsungen, Germany), with the help of 1 1/8 inch RNAse free metal bullet at 2000 rpm for 1 minute. Then, 1.5 ml of buffer A containing 681 µl of a mix of TLE grinding buffer and β-mercaptoethanol, 681 µl phenol and 138 µl chloroform, was added to each sample and mixed immediately. The TLE grinding buffer consisted of Tris (0.18M) (Trizmabase Fulka 3362), LiCl (0.09M) (Sigma L0505), EDTA (4.5mM) (Sigma E-5134), SDS (1%) (natriumlaurylsufaat Sigma L3771). The mixture of homogenized sample and buffer A was centrifuged for 10 minutes at maximum speed (14000 rpm) and the supernatant was collected subsequently and placed in a new 2 ml microfuge tube. One ml of 1:1 phenol:chloroform was added, followed by vortexing and centrifugation for 2 minutes at 14000 rpm. Afterwards, 1 ml of chloroform was added to the collected supernatant, which was then placed in a new microfuge tube, and mixed and centrifuged at 14000 rpm for 2 minutes. The new supernatant was collected again and transferred to a new tube and thereafter 100 µl of 10

M LiCl was added, mixed well, and stored at 4°C on ice overnight. The following day, thawed samples were centrifuged at 4°C for 30 minutes at 14000 rpm, followed by pipetting off the supernatant, adding 250 µl of 70% cold ethanol to the remaining pellet and shaking. After 5 minutes centrifugation at 14000 rpm at 4°C, the supernatant was removed and the remaining pellet was air dried for 10 to 15 minutes in a fume hood. The dried pellet was dissolved in 30 µl RNAse free water and stored at -80°C. RNA was quantified spectrophotometrically using a QIAxpert device (www.qiagen.com/goto/TechSupportCenter). RNA integrity was further qualified by checking the ribosomal RNA bands on a 1% agarose gel. Samples with sharp and clear bands without obvious degradation were selected for subsequent steps. Five µg of RNA from each sample was treated with DNAse by adding 10 µl of DNAse enzyme (Promega) and DNAse buffer, filled to 100 µl with RNAse free water and incubated at 37°C for 30 minutes. Subsequently 100 µl of phenol:chlorophorm (1:1) was added and the solution was transferred to phase lock tubes and centrifuged for 5 minutes at 14000 rpm. After centrifugation the supernatant (~90 µl) was collected, placed in a new tube and 9 µl of 3M NaAc and 250 µl of 100% ice-cold ethanol were added and kept at -20°C for 2 hours. After 2 hours the tubes were centrifuged for 30 minutes at 4°C and 14000 rpm and, subsequently, the supernatant was removed and the remaining pellet was washed with 250 µl of 70% cold ethanol and centrifuged for 5 minutes at 4°C. In the final step, after removing the supernatant, the pellet was air dried for 10-15 minutes and dissolved in 20 µl of RNAse free water. cDNA was synthesized from 500 ng of total RNA according to the manufacturer's protocol (iScriptTM cDNA synthesis kit, Bio-Rad) and diluted 20 times with miliQ water and stored at -20°C.

Reference and target gene selection, primer design and RT-qPCR analysis

TIP41-like (SGN-U584254) and *PP2Ac1* (SGN-U567355) were used as reference genes (Dekkers et al., 2012). CLCbio software (CLCbio, Aarhus, Denmark) was used to design the primers with melting temperature of 58-62°C, a length of 18-22 bp and a template length of 80-200 bp. The efficiency of the primers was evaluated with a two-fold series dilution of a pooled cDNA of all samples. Gene names and their homologs and annotation and primer sequences are described in Supplemental Table S1. The RT-qPCR was performed using 2.5 µl of cDNA, 0.5 µl of primer mix (forward and reverse), 5 µl of iQ SYBR Green Supermix (Bio-Rad) and 2 µl of miliQ water according to the manufacturer's instructions (CFX, Bio-Rad). The RT-qPCR protocol used for the analysis was 95°C for 3 minutes, continued with 40 cycles of 95°C for 15 seconds and 60°C for 30 seconds and melting curves were recorded (Ribeiro et al., 2014).

References

Abeles FB (1986) Role of Ethylene in Lactuca sativa cvGrand Rapids' Seed Germination. Plant Physiology **81:** 780-787

Alonso-Blanco C, Bentsink L, Hanhart CJ, Blankestijn-de Vries H, Koornneef M (2003) Analysis of natural allelic variation at seed dormancy loci of Arabidopsis thaliana. Genetics **164:** 711-729

Argyris J, Dahal P, Hayashi E, Still DW, Bradford KJ (2008) Genetic variation for lettuce seed thermoinhibition is associated with temperature-sensitive expression of abscisic acid, gibberellin, and ethylene biosynthesis, metabolism, and response genes. Plant Physiology **148:** 926-947

Bentsink L, Jowett J, Hanhart CJ, Koornneef M (2006) Cloning of DOG1, a quantitative trait locus controlling seed dormancy in Arabidopsis. Proceedings of the National Academy of Sciences **103:** 17042-17047

Bentsink L, Soppe W, Koornneef M (2007) Genetic aspects of seed dormancy. Annual Plant Reviews Volume 27: Seed Development, Dormancy and Germination**:** 113-132

Bewley JD (1997) Breaking down the walls—a role for endo-β-mannanase in release from seed dormancy? Trends in Plant Science **2:** 464-469

Bewley JD (1997) Seed germination and dormancy. The plant cell **9:** 1055

Bogatek R, Gniazdowska A (2012) Ethylene in seed development, dormancy and germination. Annual Plant Reviews, The Plant Hormone Ethylene **44:** 192

Bonina JR (2005) A Rapid, Simple, Inexpensive and Reproducible Endo-beta-mannanase Assay Test for Determining Optimal Hydrothermal Timing of Commercial Priming of Lettuce Seed. University of Florida

Cadman CS, Toorop PE, Hilhorst HW, Finch-Savage WE (2006) Gene expression profiles of Arabidopsis Cvi seeds during dormancy cycling indicate a common underlying dormancy control mechanism. The Plant Journal **46:** 805-822

Carter AK, Stevens R (1998) Using Ethephon and GA3 to Overcome Thermoinhibition inJalapeño M'Pepper Seed. HortScience **33:** 1026-1027

Chiu RS, Nahal H, Provart NJ, Gazzarrini S (2012) The role of the Arabidopsis FUSCA3 transcription factor during inhibition of seed germination at high temperature. BMC plant biology **12:** 15

Corbineau F, Bagniol S, Côme D (1990) Sunflower (Helianthus annuus L.) seed dormancy and its regulation by ethylene. Israel Journal of Botany **39:** 313-325

Corbineau F, Rudnicki R, Côme D (1988) Induction of secondary dormancy in sunflower seeds by high temperature. Possible involvement of ethylene biosynthesis. Physiologia Plantarum **73:** 368-373

Curaba J, Moritz T, Blervaque R, Parcy F, Raz V, Herzog M, Vachon G (2004) AtGA3ox2, a key gene responsible for bioactive gibberellin biosynthesis, is regulated during embryogenesis by LEAFY COTYLEDON2 and FUSCA3 in Arabidopsis. Plant Physiology **136:** 3660-3669

Dekkers BJ, Willems L, Bassel GW, van Bolderen-Veldkamp R, Ligterink W, Hilhorst HW, Bentsink L (2012) Identification of reference genes for RT–qPCR expression analysis in Arabidopsis and tomato seeds. Plant and Cell Physiology **53:** 28-37

Doerge RW, Churchill GA (1996) Permutation tests for multiple loci affecting a quantitative character. Genetics **142:** 285-294

Finch-Savage B (2013) Seeds: Physiology of development, germination and dormancy JD Bewley, KJ Bradford, HWM Hilhorst H. Nonogaki. 392 pp. Springer, New York–Heidelberg–Dordrecht–London 2013 978-1-4614-4692-7. Seed Science Research **23:** 289-289

Finch-Savage WE, Cadman CS, Toorop PE, Lynn JR, Hilhorst HW (2007) Seed dormancy release in Arabidopsis Cvi by dry after-ripening, low temperature, nitrate and light shows common quantitative patterns of gene expression directed by environmentally specific sensing. The Plant Journal **51:** 60-78

Finkelstein RR, Gampala SS, Rock CD (2002) Abscisic acid signaling in seeds and seedlings. The Plant Cell **14:** S15-S45

Franks SJ, Weber JJ, Aitken SN (2014) Evolutionary and plastic responses to climate change in terrestrial plant populations. Evolutionary Applications **7:** 123-139

Gallardo M, del Mar Delgado M, Sánchez-Calle IM, Matilla AJ (1991) Ethylene production and 1-aminocyclopropane-1-carboxylic acid conjugation in thermoinhibited Cicer arietinum L. seeds. Plant Physiology **97:** 122-127

Ghaderi-Far F, Alimagham S, Kameli A, Jamali M (2012) Isabgol (Plantago Ovata Forsk) Seed Germination and Emergence as Affected by Environmental Factors and Planting.

Ghaderi-Far F, Soltani A, Sadeghipour H (2011) Changes in seed quality during seed development and maturation in medicinal pumpkin (Cucurbita pepo subsp. pepo. convar. pepo var. styriaca Greb). Journal of herbs, spices & medicinal plants **17:** 249-257

Gonai T, Kawahara S, Tougou M, Satoh S, Hashiba T, Hirai N, Kawaide H, Kamiya Y, Yoshioka T (2004) Abscisic acid in the thermoinhibition of lettuce seed germination and enhancement of its catabolism by gibberellin. Journal of Experimental Botany **55:** 111-118

Gu X, Kianian S, Foley M (2006) Isolation of three dormancy QTLs as Mendelian factors in rice. Heredity **96:** 93-99

Gubler F, Hughes T, Waterhouse P, Jacobsen J (2008) Regulation of dormancy in barley by blue light and after-ripening: effects on abscisic acid and gibberellin metabolism. Plant Physiology **147:** 886-896

Hilhorst HW (2007) Definitions and hypotheses of seed dormancy. Annual Plant Reviews Volume 27: Seed Development, Dormancy and Germination**:** 50-71

Holdsworth MJ, Bentsink L, Soppe WJ (2008) Molecular networks regulating Arabidopsis seed maturation, after-ripening, dormancy and germination. New Phytologist **179:** 33-54

Huo H, Bradford KJ (2015) Molecular and hormonal regulation of thermoinhibition of seed germination. *In* Advances in Plant Dormancy. Springer, pp 3-33

Huo H, Dahal P, Kunusoth K, McCallum CM, Bradford KJ (2013) Expression of 9-cis-EPOXYCAROTENOID DIOXYGENASE4 is essential for thermoinhibition of lettuce seed germination but not for seed development or stress tolerance. The Plant Cell **25:** 884-900

Iuchi S, Kobayashi M, Taji T, Naramoto M, Seki M, Kato T, Tabata S, Kakubari Y, Yamaguchi-Shinozaki K, Shinozaki K (2001) Regulation of drought tolerance by gene manipulation of 9-cis-epoxycarotenoid dioxygenase, a key enzyme in abscisic acid biosynthesis in Arabidopsis. The Plant Journal **27:** 325-333

Kazmi RH, Khan N, Willems LA, Van Heusden AW, Ligterink W, Hilhorst HW (2012) Complex genetics controls natural variation among seed quality phenotypes in a recombinant inbred population of an interspecific cross between Solanum lycopersicum× Solanum pimpinellifolium. Plant, cell & environment **35:** 929-951

Kępczyński J, Bihun M, Kępczyńska E (2006) Implication of ethylene in the release of secondary dormancy in Amaranthus caudatus L. seeds by gibberellins or cytokinin. Plant growth regulation **48:** 119-126

Kermode AR (2005) Role of abscisic acid in seed dormancy. Journal of Plant Growth Regulation **24:** 319-344

Kpczyska E, Pikna-Grochala J, Kpczyski J (2006) Hormonal regulation of tomato seed germination at a supraoptimal temperature. Acta Physiologiae Plantarum **28:** 225-231

Lafta A, Mou B (2013) Evaluation of lettuce genotypes for seed thermotolerance. HortScience **48:** 708-714

Long SP, Ort DR (2010) More than taking the heat: crops and global change. Current opinion in plant biology **13:** 240-247

Matilla A, Matilla-Vázquez M (2008) Involvement of ethylene in seed physiology. Plant Science **175:** 87-97

Matilla AJ (2000) Ethylene in seed formation and germination. Seed science research **10:** 111-126

Nambara E, Hayama R, Tsuchiya Y, Nishimura M, Kawaide H, Kamiya Y, Naito S (2000) The role of ABI3 and FUS3 loci in Arabidopsis thaliana on phase transition from late embryo development to germination. Developmental biology **220:** 412-423

Nascimento W, Huber D, Cantliffe D (2013) Carrot seed germination and respiration at high temperature in response to seed maturity and priming. Seed Science and Technology **41:** 164-169

Penfield S, MacGregor D (2014) Temperature sensing in plants. Temperature and Plant Development**:** 1-18

Ribeiro PR, Dekkers BJ, Fernandez LG, de Castro RD, Ligterink W, Hilhorst HW (2014) Identification of reference genes for gene expression studies during seed germination and seedling establishment in Ricinus communis L. Seed Science Research **24:** 341

Siriwitayawan G, Geneve RL, Downie AB (2003) Seed germination of ethylene perception mutants of tomato and Arabidopsis. Seed Science Research **13:** 303-314

Suzuki M, McCarty DR (2008) Functional symmetry of the B3 network controlling seed development. Current opinion in plant biology **11:** 548-553

Taylorson R (1979) Response of weed seeds to ethylene and related hydrocarbons. Weed Science**:** 7-10

Toh S, Imamura A, Watanabe A, Nakabayashi K, Okamoto M, Jikumaru Y, Hanada A, Aso Y, Ishiyama K, Tamura N (2008) High temperature-induced abscisic acid biosynthesis and its role in the inhibition of gibberellin action in Arabidopsis seeds. Plant physiology **146:** 1368-1385

Toyomasu T, Kawaide H, Mitsuhashi W, Inoue Y, Kamiya Y (1998) Phytochrome regulates gibberellin biosynthesis during germination of photoblastic lettuce seeds. Plant Physiology **118:** 1517-1523

Van Ooijen J (2004) MapQTL® 5. Software for the mapping of quantitative trait loci in experimental populations. Kyazma BV, Wageningen

Voorrips RE, Verkerke W, Finkers R, Jongerius R, Kanne J (2000) Inheritance of taste components in tomato. Acta Physiologiae Plantarum **22:** 259-261

Wang F, Perry SE (2013) Identification of direct targets of FUSCA3, a key regulator of Arabidopsis seed development. Plant physiology **161:** 1251-1264

Wigge PA (2013) Ambient temperature signalling in plants. Current opinion in plant biology **16:** 661-666

Yamamoto A, Kagaya Y, Usui H, Hobo T, Takeda S, Hattori T (2010) Diverse roles and mechanisms of gene regulation by the Arabidopsis seed maturation master regulator FUS3 revealed by microarray analysis. Plant and cell physiology **51:** 2031-2046

Yamauchi Y, Ogawa M, Kuwahara A, Hanada A, Kamiya Y, Yamaguchi S (2004) Activation of gibberellin biosynthesis and response pathways by low temperature during imbibition of Arabidopsis thaliana seeds. The Plant Cell **16:** 367-378

Yoong F-Y, O'Brien LK, Truco MJ, Huo H, Sideman R, Hayes R, Michelmore RW, Bradford KJ (2016) Genetic Variation for Thermotolerance in Lettuce Seed Germination Is Associated with Temperature-Sensitive.

Supplementary information

Supplementary Tables and Excel files of this chapter can be downloaded from http://www.wageningenseedlab.nl/thesis/ngeshnizjani/SI/chapter5

Supplementary Figures

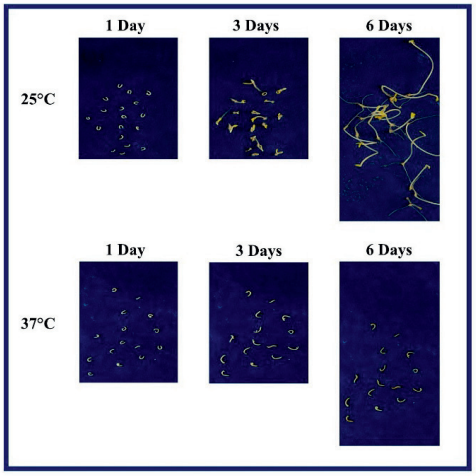

Figure S1. Germination of tomato embryos (*Solanum lycopersicum* cv. Moneymaker) at normal (25°C) and high temperature (37°C) at first, third and sixth day after sowing.

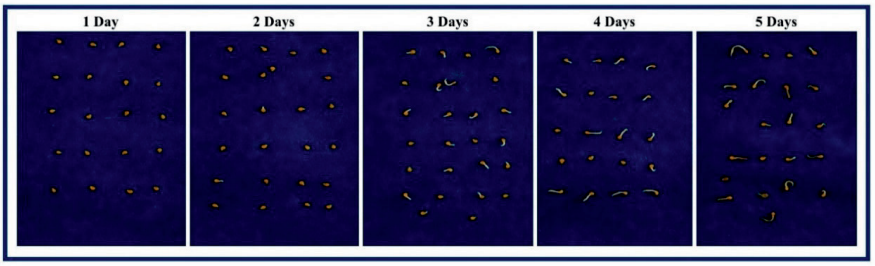

Figure S2. Germination of *Solanum pimpinellifolium* seeds at 37°C at first, second, third, fourth and fifth day after sowing.

Chapter 6

General discussion

Physical, physiological and genetic factors, *inter alia,* influence final seed quality. Seed quality however, is also greatly influenced by the environment in which the seeds have developed and matured (Rowse and Finch-Savage, 2003; Sperling et al., 2004). Such an influence is thought to be reflected as an adaptation mechanism of plants in response to various environmental conditions (Huang et al., 2010). In general, the environment in which plants grow and produce seeds is called the maternal environment. The maternal environment of the plants plays an important role in the response of the progeny to future competitive (stress) conditions (Leverett et al., 2016). Such responses may be expressed in various ways such as reproduction ability, susceptibility to plant diseases and other developmental properties. Responses of progeny which are associated with the maternal sensing of the growth environment may be enduring over time (Auge et al., 2017). Hence, from an ecological point of view they may help the progeny to withstand adverse conditions (e.g. dormant seeds in stress conditions) and/or best use the upcoming desired incidents (e.g. flowering time) (Auge et al., 2017).

The aim of this study was to determine the influence of the maternal environment on seed- and seedling quality and to elucidate the underlying molecular regulation and/or mechanisms. For this purpose an advanced combination of phenotypic, genetic and omics analysis to study seed and seedling quality of seeds developed and matured under different maternal environments was used. Natural variation for seed and seedling quality traits has been used together with molecular-genetic methods in order to obtain better understanding of the mechanisms regulating these traits. In this thesis I have shown the effect of genotype, maturation environments and their interaction on phenotypic traits of tomato seeds and seedlings. In this chapter the main outcomes of the thesis will be discussed, the results from different experimental chapters integrated and future perspectives for this research explained.

Seed and seedling quality

Seed quality is a complex trait largely representing the conditions under which seeds have developed and matured. Seed quality is determined by several features such as viability, genetic and physical purity, lack of physical damages, germination capacity and dormancy (Hilhorst and Toorop, 1997; Hilhorst and Koornneef, 2007; Hilhorst et al., 2010). The definition of seed quality may alter depending on the different usages of the seeds. For example, oil producers are looking for seeds with high fatty acid compositions (i.e. seed as the final commodity) but farmers and seed producers are interested in seeds with rapid and uniform germination together with the highest yield in various growth conditions (i.e. seed as propagule). Seed quality is traditionally determined by attributes mostly related to seed function such as maximum germination, dormancy and storability. However, nowadays growth and establishment of

healthy seedlings is also considered as one of the important seed quality characteristics. Seed quality may then be defined as the ability of the seeds to germinate under various optimal and sub-optimal conditions and to grow into normal and healthy seedlings (Finch-Savage, 1995; Foolad et al., 2007). Thus, the quality parameters of seeds may affect seedling performance and growth of the plant and, eventually, the success of crop production.

Maternal environment regulating seed and seedling performance

Seed quality is mainly acquired during seed development and maturation. Therefore, the maternal environment under which seed develop may have a profound effect on seed and, subsequently, seedling performance. Undesirable conditions, such as sub-optimal temperature, light and nutrient dosage may diminish seed and seedling performance (Bewley et al., 2012). Despite the importance of the maternal environment in governing seed quality, the regulating mechanisms are still largely unknown. In **Chapter 2** I focus on the seed and seedling quality of two tomato species, *Solanum lycopersicum* cv. Moneymaker (MM) and *Solanum pimpinellifolium* accession CGN14498 (PI), which were grown under maternal environments with different dosages of nitrate and phosphate. PI is the most closely related wild tomato species to the advanced tomato breeding line MM, it has the ability of being naturally crossed with domesticated species (i.e. MM) and it has been used in breeding programs for its tolerance to some sub-optimal environments. Furthermore, in compare with other wild species, PI has much less undesirable horticultural properties which makes it good genetic material to be used in breeding programs.

Effect of maternal environment on seed and seedling size

Seed and seedling size were affected by the maternal environment. Bigger and heavier seeds and seedlings were obtained from higher maternal levels of nutrients, which is in agreement with foregoing studies (Ellis, 1992; Castro et al., 2007). I have illustrated that higher levels of nitrate and phosphate, applied to the mother plant, triggered the production of bigger tomato seeds and seedlings by increasing the level of seed reserves through different mechanisms (Figure 1). Higher levels of nitrate resulted in an increased level of amino acids in the mature seeds. The effect of amino acids, as the building blocks of proteins which are considered as one of the major tomato seed reserves (Khan et al., 2012) on increased seed size could be explained by increase of the amino acid reservoir and subsequently protein levels of the seeds. Increased seed size in response to higher phosphate levels may be due to alteration in fructose-6-phosphate (F6P) and TCA cycle intermediates. Consumption of these intermediates (i.e. depletion of F6P and TCA cycle intermediates such as citrate and malate) at higher phosphate levels may be attributed to elevated energy production units (e.g. ATP) being continuously used

during the seed filling/expansion process. Although energy production units were not measured, I hypothesize that high levels of energy may be directed towards accumulation of more reserve and storage compounds in the seeds as a result of high phosphate levels. It is noteworthy to mention that seedling size was also affected by higher levels of nitrate and phosphate. Increased seedling size may be a reflection of a higher quality seed due to bigger seeds and, therefore, more seed reserves. Effect of seed size on seedling growth and quality characteristics (e.g. seedling size) has previously been reported and discussed (Lamont and Groom, 2013; Stevens et al., 2014).

Maternal environment regulating G_{max} at high temperature; putative role of GABA

I have shown that seed germination percentage (G_{max}) was affected by high levels of nitrogen and phosphate applied to the mother plant (**Chapter 2**), albeit in a contrasting way. According to the metabolomics results, this difference is likely to come from different GABA contents of the mature seeds. Such an effect could be attributed to the increased levels of nitrate (applied to mother plant) which consequently results in increased GABA levels. Increased GABA levels in turn, may lead to increased G_{max} at high temperature conditions (Figure 1). In contrast to nitrate, increased phosphate levels results in decreased GABA levels in dry seeds. Interestingly, decreased GABA content correlated with a lowered G_{max} at high temperature (Figure 1). According to what is described in **Chapter 2**, which is summarized in Figure 1 (red route), and foregoing reports on the regulatory role of GABA in response to abiotic stress (i.e. mitigating stress responses (Kinnersley and Turano, 2000; Bouche and Fromm, 2004)) I also propose a similar regulatory effect during high temperature stress in tomato seeds. In order to identify the role of GABA in increased seed germination during heat stress, additional experiments (e.g. exogenous application of GABA to heat stressed seeds) need to be done to confirm such an effect. Nevertheless, it is crucial to consider the fact that high nitrate or low phosphate levels (of mother plants) not only influenced GABA levels but also other activated/deactivated complex metabolic regulatory pathways in tomato plants and seeds. Therefore the exact molecular pathway(s), that lead from changes in maternal environment to altered seed quality are to be proven in future studies.

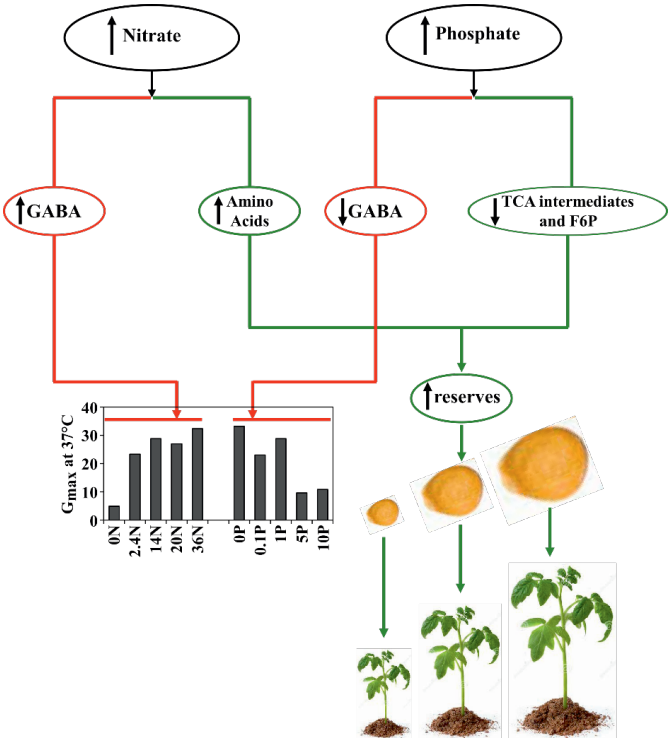

Figure 1. Schematic picture representing the effect of high nitrate and phosphate levels, applied to maternal plants, on tomato seed germination percentage **(G_{max})** at high temperature (37°C) **(Red route)** and on seed and seedling growth (seed and seedling size) **(Green route)** and the possibly involved metabolites.

In general, according to the obtained data in **Chapter 2**, I have observed that the effect of the maternal environment on some seed and seedling traits is dependent on the genetic background of the seeds (MM versus PI). For example, the above mentioned regulatory role of GABA on G_{max} of heat stressed seeds was only observed for MM and not for PI seeds. This indicates that a more detailed analysis of seed and seedling traits is required to better understand the underlying mechanisms (e.g. at phenotypic, metabolic and transcriptomic levels). Hence studying the genetic variation across a population resulting from a cross between MM and PI (for example a RIL population) can provide a comprehensive dataset on the effect of the genetic background, based on the differences observed within this type of populations.

Application of natural variation

In this study I have used a RIL population (consisting of 100 lines) which is derived from a cross between *Solanum lycopersicum* cv. Moneymaker and *Solanum pimpinellifolium* accession CGN14498. Our group (Joosen et al., 2012; Kazmi et al., 2012; Khan et al., 2012; Kazmi et al.,

2017) and other research groups (Alonso-Blanco and Koornneef, 2000; Argyris et al., 2008; Toubiana et al., 2012) have previously shown that natural variation in a RIL population can be used as a powerful tool to study the genetic and physiological mechanisms governing seed quality traits, as well as metabolic differences. Using such a population containing intrinsic genetic differences for phenotypic and molecular traits, like gene expression and metabolite levels, may ultimately lead to the identification of processes and associated genes regulating the existing differences (Ligterink et al., 2012). Identification of these genes (or genomic regions) may be the most interesting piece of research for breeders and commercial producers. This interesting piece of information (i.e. causal genes) could be used in prediction of seed and seedling quality traits and further improvement of the most desired ones, ultimately resulting in a higher final yield.

Although genetic variation for tomato seed quality has been reported before (Kazmi et al., 2012; Khan et al., 2012; Toubiana et al., 2012), in the current research I not only studied the genetic aspects of seed quality but also evaluated the effect of the nutritional maternal environment (**Chapter 3 and 4**). Here first the phenotypic QTLs influenced by the genetic background and their interaction with the nutritional maternal environment are discussed (**Chapter 3**). I will then discuss the obtained results from metabolite QTLs and the generalized genetical genomics approach used for identification of molecular aspects of seed and seedling quality traits (**Chapter 4**).

Phenotypic QTLs

Plant adaptation is a process in which plants adjust themselves to their environment, even at suboptimal/stressful conditions (Körner, 2016). Natural variation (as a result of spontaneous mutations arising during evolution) allows the adaptation process to the suboptimal conditions which results in the survival of only the best performing plants (Alonso-Blanco et al., 2009). The molecular basis regulating the phenotypic differences in plants adapting themselves to suboptimal conditions and mechanisms involved in plant adaptation can be identified using the natural variation present in segregating mapping populations such as RIL populations but also in diversity panels for genome-wide association studies (GWAS), grown under different maternal conditions. In **Chapter 3** of this thesis I studied the response of the tomato plant progenies to suboptimal maternal growth environments (high phosphate and low nitrate) and attempted to understand the molecular and genetic components involved in their acclimation. By performing extensive phenotyping including the use of the Germinator (Joosen et al., 2010), seed and seedling quality traits were evaluated. Measurement of the quality traits reported in **Chapter 3** resulted in an extensive phenotypic dataset. In order to obtain a deeper insight into the loci that were responsible for the observed variations of the different traits in this population, this dataset was used to perform QTL analysis.

The genetic variation of QTLs (G) was studied by application of the r/qtl package for the statistical language R (Broman et al., 2003) and mapQTL software (Van Ooijen, 2004). Furthermore, incorporation of different maternal environments allowed us to further investigate the environmental effect (E) as well as the effect of G by E interaction (G×E) on QTLs. Studying the genetic landscape of different traits such as seed and seedling performance resulted in the detection of many QTLs in Arabidopsis and tomato (Koornneef et al., 2002; Alonso-Blanco et al., 2003; Foolad et al., 2007; Argyris et al., 2008; Joosen et al., 2012; Kazmi et al., 2012; Khan et al., 2012). However, only a limited number of these studies was followed up by cloning and functional characterization of the causal gene such as *DELAY OF GERMINATION* (*DOG1*) in Arabidopsis (Bentsink et al., 2006) and an ABC transporter in tomato (associated with seed weight) (Orsi and Tanksley, 2009). Identification of causal genes involved in seed and seedling quality, followed by their functional characterization is a powerful tool to be implemented in breeding programs. The characterized causal genes will then be used in breeding practices such as modification and improvement of seed and seedling quality.

As described in **Chapter 3,** many QTLs regulating seed and seedling traits were identified. Some of the identified QTLs were common among the studied maternal environments, while some were specific to only one maternal environment. I have also identified overlapping QTLs with what was reported before for the same RIL population (Kazmi et al., 2012; Khan et al., 2012), but grown in an optimal maternal environment. One of the QTLs detected in our research, as well as in former studies is a QTL for seed weight located on chromosome 4 (Orsi and Tanksley, 2009; Khan et al., 2012). Detection of this QTL in both optimal and suboptimal maternal conditions indicates that this locus is regulating the seed weight regardless of the seed maturation environment. Hence identification of the responsible gene for this QTL is of great importance for the improvement of seed weight, which might result in enhanced seedling quality and final yield. Seed size and seed weight are traits which are greatly influenced by the domestication process. It has been shown that in many crops such as tomato and sunflower domesticated varieties produce larger and heavier seeds compared to their wild type species (Doganlar et al., 2000; Burke et al., 2002). Although the reason why seed size is increased during the domestication process in plants which are not used for their seeds is not exactly known, it has been suggested that such an effect is likely due to the selection for bigger fruits (like in tomato) which usually is considered as a desirable trait (Goldman et al., 1995). Larger seed size may then provide increased reserve during the germination process, hence producing more vigorous seedlings with enhanced establishment characteristics, resulting in increased final yield (Harlan et al., 1973; Doganlar et al., 2000). Despite the important role of seed size and seed weight, the genetic and molecular basis regulating their variation still remains unknown in many species. Although some interesting QTLs regulating these traits have been

identified and cloned from some species, identification of causal genes is still cumbersome and often not straight forward (Salvi and Tuberosa, 2005).

In **Chapters 2** and **3** I have detected a significant positive correlation between seed size/weight and seedling characteristics which were in line with previous findings (Baker, 1972; Nieuwhof et al., 1989; Jurado and Westoby, 1992; Khan et al., 2012). Khan et al., (2012) also reported that seed size/weight shows a positive effect on seedling traits using the same population as was used in Chapter 3. They have argued that such a positive correlation could again be due to the increased seed reserve which is used during the very early stages of seedling growth and development, hence producing bigger and heavier seedlings. Besides correlation between seed size/weight and seedling characteristics (fresh and dry shoot and root weight), Khan et al., (2012) also detected collocating QTLs for these traits which is in accordance with our results as described in **Chapter 3**.

A significant negative correlation between seed size and weight and seed germination traits (such as G_{max}, t_{50} and AUC) was identified in the low nitrate maternal environment which has not been reported before. For example in the study of Khan et al. (2012), in which the same RIL population grown in optimal maternal environment was used, this correlation was not detected. Apparently, the correlation of seed size/weight with seed germination traits depends on the maternal growth conditions, as this correlation varies within the same RIL population grown in different maternal environmental conditions. I have detected collocating QTLs which are regulating seed traits such as seed size/weight and seed germination traits (at different germination conditions such as high temperature, mannitol and water). Detection of these collocated QTLs in the low nitrate maternal environment confirms the correlation of these traits. Hence this could imply that seed size/weight and seed performance are regulated by common genetic loci in low nitrate maternal environments only.

One of the studied traits in **Chapter 3** regarding seed performance was maximum seed germination (G_{max}). G_{max} is the determining factor of the overall germination potential which can also be used for estimation of final crop yield. However, it should be noted that there are several factors other than G_{max} that are responsible for determining the final yield. It is also important to consider and calculate other germination factors in time, rather than the final G_{max} at the end of the experiment. Other important factors are t_{10} (onset of germination), t_{50} (rate of germination) as well as uniformity (U_{8416}) which are relatively independent from G_{max}. Besides, the cumulative germination response from the rate and percentage of germination can be summarized in the parameter AUC (area under the germination-time curve). Hence, it is important to take into account all different aspects of germination under different environmental conditions. According to Kazmi (2013) cumulative identification of germination properties, as

a phenotypic attribute in a seed lot, strengthens the approach by which genetic variation of RIL populations are studied (Kazmi, 2013).

One of the characteristics determining seed quality traits is the ability to germinate at both optimal and stressful (e.g. high temperature and osmotic stress) conditions (Foolad et al., 2007). Therefore in this study, I have germinated tomato seeds in different germination conditions such as water, NaCl, mannitol and high temperature which was followed by analysing the genetic variation of seed germination traits. Many crop seeds, such as tomato, have lost their germination ability in stressful germination environments during the domestication process. These crops are not able to germinate and/or produce healthy seedlings in these stressful environments, which limits their growth and development to optimal conditions (Foolad and Lin, 1998; McCouch, 2004; Doebley et al., 2006). By exploring the existing genetic variation in populations and identification of loci regulating these variations, re-introduction of valuable characteristics into domesticated species may be facilitated. Such an approach will ultimately result in modification of these species to be able to cope with stressful environments (Lippman et al., 2007; Kazmi et al., 2012). In this study, QTL analysis resulted in the identification of several stable QTLs which were detected for different seed germination traits in all germination conditions. This however was expected since these traits show a high correlation to each other (**Chapter 3**). Such robust QTLs which are identified in response to different conditions indicate the genetic correlation of seed germination responses in different stressful germination environments and involvement of identical gene(s) controlling these traits regardless of the germination environment. Therefore, identification of those genes and their implementation into breeding programs may result in production of seeds (progenies) which not only have enhanced germination response to one specific stressful environment but also alternatively possess enhanced responses in different stressful conditions (Dudley, 1994; Foolad et al., 2003; Foolad et al., 2007).

Although many QTLs regulating seed and seedling quality traits have been detected in my thesis (**Chapter 3**) as well as in previous studies, identification of causal genes for these QTLs is not well studied. This is due to the large genomic regions that these detected QTLs cover which sometimes may reach up to 2000 annotated genes (especially for low-significant QTLs) underlying specific QTLs. Narrowing down the QTLs with the often used fine mapping technique is a time consuming and labour intensive process. Therefore, alternative advanced approaches are required to facilitate the identification of causal genes for interesting QTLs. This could be done by performing genetic analysis of other molecular traits such as proteins, metabolites and even at the gene expression level.

GGG approach

The huge revolution in DNA and RNA sequencing techniques, as well as metabolite analysis, has helped scientists to generate large scale sets of data and analyse many parameters such as time-line analysis of transcriptomes or to measure and quantify cellular molecules (e.g. metabolites). These technologies known as 'omics technologies' provide us with rapid and high throughput data generation (Mosa et al., 2017). Omics technologies such as proteomics, metabolomics and transcriptomics are approaches that help to take steps forward towards better understanding of the molecular mechanisms underlying the genetic variation of seed and seedling quality. In relation to the use of omics technologies in QTL studies to better understand complex traits, a sophisticated approach called genetical genomics has been used. In the genetical genomics approach the traditional QTL analysis is combined with omics data such as gene expression (transcriptomics) and/or metabolomics to unravel the underlying mechanisms (Joosen et al., 2009; Ligterink et al., 2012). Many studies have been conducted using omics technology where genetic variation of molecular traits was explored and associated with genetical analysis of phenotypic traits such as seed and seedling quality traits (Jansen and Nap, 2001; West et al., 2007; Keurentjes et al., 2008; Rowe et al., 2008; Joosen et al., 2013).

Traits such as seed germination and seedling establishment are complex traits which are the output of various environmental and genetic signals. However, nowadays by using different quantitative genetic tools we can better study the genetic architecture of quantitative traits. One of these tools, which was also used in our study, was the biparental RIL population from MM and PI. Identification of quantitative traits such as seed germination and seedling growth by using a biparental population has a huge advantage compared with natural variation populations (e.g. GWAS). This advantage is due to the fact that detection of variations associated with a trait is not explained by many loci within the population with average low explained variation per locus (as in GWAS) but, alternatively, by a limited number of genes which are different for the two parents used in a generated RIL population. Complex traits are often controlled and regulated by groups of genes. Using a well-structured RIL population together with high throughput phenotyping, as well as genetic analysis of molecular traits (omics analysis) will provide additional information to help in clarifying the genetic and molecular basis of these complex traits. Molecular traits are influenced by environmental conditions, hence in order to obtain a comprehensive understanding of specific traits, genetic analysis should be performed across multiple environmental conditions (Li et al., 2008; Ruffel et al., 2010).

Application of omics analysis for huge populations is laborious and costly. As an alternative, a novel experimental design, called generalized genetical genomics (GGG) was used. GGG is an approach in which the genetic background and the environmental conditions are combined which, ultimately, and similar to genetical genomics, results in identification of QTLs

responsible for certain molecular traits. GGG is defined as the same approach as genetical genomics but in a more cost-efficient version in multi-environment studies (Li et al., 2008). In a GGG design, a RIL population is divided into subsets, where each subset is exposed to a certain environmental condition. The division in subsets is done by considering the optimal distribution of parental alleles over all available markers (i.e. a similar distribution of parental alleles for all the markers between/in the subsets) (Li et al., 2009). The GGG design has already been used in other studies where genetic and environmental regulation of primary metabolites were studied during different seed germination stages in Arabidopsis (Joosen et al., 2013) and tomato (Kazmi et al., 2017). In general, application of this method provides the possibility to address the question "Why do various traits of an organism behave differently in different environmental conditions?".

I have studied the application of the GGG design in **Chapter 4** of this thesis. The tomato RIL population from the cross between MM and PI was divided into two well-balanced subsets. Each subset contained 50 lines which were grown either in HP or LN conditions. Metabolites were extracted and primary metabolites of dry seeds were analysed by GC-TOF-MS. Out of 118 detected metabolites, 58 could be annotated. In this chapter I was able to trace the changes and variation of metabolites in our RIL population by performing the GGG design in different maternal environmental conditions combined with the genetic analysis. This also resulted in dissection and mapping of the variation resulting from genetics (G) and the G×E component. By analysing the genetic variation of the annotated metabolites in each sub-population, 129 and 66 mQTLs were detected for HP and LN environmental conditions, respectively. Genetic variation analysis of only one of the sub-populations at a time, limits the detected QTLs to the ones that control the metabolites in that specific environment. However, the power of mQTL detection is increased if the two sub-populations are combined, and the whole RIL population is used for the genetic variation analysis. In this way, the effect of the environment and the genetic by environment interaction is integrated into the model, hence detecting more QTLs compared with the approach in which only the genetic background is studied (e.g. in a single environment). This explains why in **Chapter 4** the number of detected mQTLs in the whole RIL population was higher than the sum of detected QTLs in each individual sub-population.

Compared to another study performed with the same RIL population, but grown in normal (control) conditions (Kazmi et al., 2017), the number of detected metabolites of tomato dry seeds in normal conditions was higher than the detected ones in this study (LN and HP maternal environmental conditions). In our study, according to the mQTL analysis (**Chapter 4**), the number of detected mQTLs was higher in HP than in the LN condition. In general, although both HP and LN are stress conditions for the plants, I speculate that the HP condition affects the plant to a lesser extent, hence being closer to the normal conditions as reported by Kazmi

et al., (2017). Although the number of detected mQTLs for the LN condition was lower compared to the HP condition some interesting QTLs were identified in the LN condition. For example, two mQTLs for GABA were detected for LN while such a QTL was not present for HP conditions. GABA has previously been shown to be involved in processes for coping with stress conditions (Kinnersley and Turano, 2000; Bouche and Fromm, 2004).

Since each metabolite can be controlled by different pathways, and each pathway is regulated and controlled by different genes, detecting a single mQTL affecting metabolite levels is not common. This was also observed in our results where many metabolites were controlled by different genetic regions (**Chapter 4**). Furthermore, many of the detected mQTLs in this study were overlapping for different metabolites which might indicate that these metabolites are regulated through common regulatory pathways. Such collocated QTLs regulating different metabolite levels may play an important role in central metabolism of maturing seeds. In order to figure out whether these loci are involved in the regulation of seed phenotypic traits the genetic variation of metabolites (**Chapter 4**) and genetic variation of seed and seedling traits (**Chapter 3**) were compared. Although in **Chapter 4** several correlations were detected between metabolites and seed and seedling phenotypic traits, no collocating hotspot QTL was detected for these traits. For example, in **Chapter 4** a positive significant correlation was found between most of the amino acids and seed size and seed weight. This was in accordance with previous findings where seed size and seed weight, and consequently seedling vigour and good establishment of the seedling were related to seed food reserves (Fait et al., 2006; Bewley et al., 2012; Khan et al., 2012).

Genetic analysis of thermo-dormancy and thermo-inhibition in tomato

Global warming and climate change are phenomena affecting agricultural commodities (Walck et al., 2011). Effect of high temperatures on various crops has been investigated. For example, in lettuce different genotypes perform differently in their germination ability (rate and percentage) at high temperature (Lafta and Mou, 2013). Also in a crop such as sunflower, imbibing seeds at high temperature is associated with progressive decrease of seed germination at subsequent optimal temperatures (Corbineau et al., 1988). Thermo-induced dormancy in seeds is one of the consequences and negative effects of exposure to high temperatures (Long and Ort, 2010; Franks et al., 2014). In order to prevent destructive effects of high temperatures, seeds exposed to high temperatures can respond in two different manners namely (i) with thermo-inhibition or (ii) induction of thermo-dormancy. Thermo-inhibition is a condition in which seeds do not germinate at high temperatures but their germination starts as soon as the optimal temperature is provided. On the other hand, thermo-dormancy is a condition in which

seeds are not germinating at high temperatures. This dormancy remains even if seeds are provided with optimal temperatures after the exposure to high temperatures. Thermo-dormancy however could be released upon application of certain treatments such as cold (i.e. stratification) and light (Argyris et al., 2008; Huo et al., 2013).

In **Chapter 5** of this thesis, a study is presented in which the genetic variation of two tomato species (MM and PI) for the response of seeds to high temperatures was investigated and in which the molecular basis of mechanisms controlling these variations are explored. First, I found out that the two genotypes are responding differently to high temperature conditions. MM was showing no germination at high temperature, regardless of light conditions. When the inhibitory element (high temperature) was removed, MM seeds still did not germinate, except when seeds at normal temperatures were simultaneously exposed to light. Therefore it could be concluded that high temperature induces thermo-dormancy in MM seeds which can be broken by exposure to light. This is in accordance with several other studies that reported the positive effect of light on alleviating secondary dormancies (Bewley, 1997; Gubler et al., 2008).

Although there is no previous report on induction of secondary dormancy in tomato seeds at high temperature conditions, there are reports (Bewley, 1997; Bonina, 2005) stating that secondary dormancy in tomato seeds is related to the seed coat and the endosperm tissue. Hence, removal of these inhibiting elements (seed coat, endosperm) released their secondary dormancy. However, in **Chapter 5** I showed that removal of seed coat and endosperm did not alleviate the secondary dormancy induced by high temperature in MM tomato seeds. Therefore, although a physical inhibitor (seed coat or endosperm) has been mentioned as the inhibitory element, here I argue for the first time that the induction of secondary dormancy in tomato seeds is a physiological factor within the embryo.

In general, our results showed that induction of secondary dormancy by high temperature in tomato seeds is related to the genetic background of the seeds. MM seeds at high temperature showed dormancy induction, whereas PI seeds at high temperature, even without exposure to light, germinated. Although PI seeds germinated at high temperature, those seeds did not generate healthy seedlings. Thus, although PI seeds showed thermo-insensitive behaviour and could germinate at high temperature, they did not develop into a healthy seedling afterwards.

In order to better understand the physiological factors controlling thermo-induced dormancy in MM tomato seeds, I performed a gene expression analysis. The tomato homologues of several genes which had already been reported to be involved in induction of thermo-dormancy in other species, such as lettuce and Arabidopsis (Argyris et al., 2008; Toh et al., 2008) were identified (9-cis-epoxycarotenoid dioxygenases (*NCEDs*), *ABAs, GA3oxs* and *FUS3*) and their expression was analysed using qPCR. Our results showed that *NCED1* and *NCED9* (two ABA biosynthesis

pathway genes) were upregulated while *GA3ox1* and *GA20ox1* (GA biosynthesis pathway genes) were downregulated during imbibition at high temperature in MM seeds. It has been shown that plant hormones, especially GA and ABA, are involved in regulation of seed germination at high temperatures. In general, the genes involved in GA biosynthesis and signalling are repressed in thermo-inhibited seeds. (Huo and Bradford, 2015). ABA is playing a key role in the induction of dormancy in seeds and prevents germination. It has been shown that ABA levels are increased and maintained in thermo-induced seeds (Argyris et al., 2008; Toh et al., 2008; Huo et al., 2013). Such an increased accumulation was suggested to be due to the *de novo* ABA biosynthesis in thermo-induced seeds (Huo and Bradford, 2015).

Furthermore, in **Chapter 5** I have also used a QTL analysis approach of the tomato RIL population generated from the cross between PI and MM to identify the genetic loci involved in thermo-dormancy induction. Several QTLs were detected which were not collocating with any of the genes studied by qPCR. Hence it seems that there are mechanisms other than the presently known ones involved in thermo-induced dormancy in tomato seeds. Fine-mapping of these detected QTLs combined with analysis of expression data would result in a better perspective of genes and mechanisms involved in such a complex multi-genic trait.

Future perspectives

Maternal environment

In this thesis I used different nutritional maternal conditions and the results indicated that different nutritional environments differently affect seed and seedling performance. In this thesis a crop with a relatively long growth period was studied, hence time limitation, together with lack of sufficient space (due to the relative big size of tomato plants), did not allow us to study other maternal environmental conditions besides the nutritional environment. Based on our knowledge and according to the literature our study on the effect of maternal nutritional environment on seeds of tomato is the first of its kind. In this thesis altered levels of phosphate and nitrate were used as the maternal nutritional environment. Phosphate is an important nutrient for plants (constituting up to 0.2% of plant dry weight) which is present in vital molecules such as ATP, phospholipids and nucleic acids. Nitrate as the major N source of plants is also present in many compounds and molecules in plants such as proteins and signalling metabolites. However, many different maternal environments have been studied before which may have more profound effects on progenies as compared to the nutritional environment. He et al., (2014) showed in Arabidopsis that among selected maternal environments (such as various temperatures, light intensity, photoperiod, nitrate and phosphate) nitrate and phosphate had a less pronounced effect on seed performance as compared to other selected maternal

environments. Similar (profound) effects could also be expected on progenies in our study if other maternal environments such as high temperature and light conditions had been applied and studied. Since global warming is a serious upcoming issue, especially studying the effect of high temperature as a maternal environmental condition in other crops, as well as in tomato, is highly desired. Furthermore, combining different maternal environments and studying their effect on the progenies could result in additional very interesting findings. For example, it has been shown that when seeds are grown in a heterogeneous maternal environment (e.g. in an open air condition/field) the effect of the maternal environment is much less than when they are grown in a certain specific modified environment (He et al., 2014). Another example could be the effect of different phosphate levels, significantly changing plant and seed performance (He et al., 2014). Interestingly, this effect on plant and seed performance disappeared in maternal environments where not only phosphate levels but also temperature was altered. He et al., (2014) argued that combinatorial maternal environments could buffer the effect of the maternal environment on plant performance compared with an maternal environment with individual alterations. Therefore, using combinatorial maternal environments in tomato and studying their effect on plants and performance of their progenies will provide more insight into their interaction and has the advantage that these kinds of maternal environments are a closer resemblance of natural conditions.

Integration of QTL mappings

I have shown that the maternal environment is playing an important role in the control of responses (i.e. phenotypic responses) of the genetic basis of seed and seedling performance. By using different maternal nutritional environments and comparing different QTL types such as phenotypic and metabolic QTLs, it was shown that controlling the phenotypic responses of the genetic basis of seed and seedling performance is strongly regulated by the maternal environment (**Chapter 4**). Application of a metabolic profiling approach and subsequently building up a metabolic network together with correlation of those metabolites with seed and seedling traits helped us to better identify the variations of these traits at a molecular level. In general the effects of maternal environments have been studied extensively (He, 2014; Rosental et al., 2016) while the underlying mechanisms of action are not well studied yet. Another type of QTL analysis which provides additional information at the molecular level is the transcriptomic QTL (also known as expression or eQTL). eQTL analysis combines gene expression and genetic analysis in order to refine the understanding of involvement of molecular and biosynthetic pathways. These pathways are often complex, hence combining these two datasets (may) allow identification and elucidation of their general and conserved mechanisms (Joosen et al., 2009). The same GGG design used for the metabolomics data (**Chapter 4**) can be used to perform gene expression profiling in the studied RIL population.

Although many QTLs have been detected in this study and other similar studies (Kazmi et al., 2012; Khan et al., 2012; Kazmi et al., 2017), discovery of the gene(s) responsible for the traits is still complicated and time consuming. In order to facilitate the gene discovery and to narrow down the detected QTLs, the identified QTLs in this thesis (phenotypic and metabolic) can be integrated and compared with eQTLs. Integration of these QTLs and detection of overlapping regions between the phenotypic and the omics data (e.g. metabolomics and transcriptomics) will provide more insights towards identification of molecular mechanisms controlling seed traits which can be used in future studies and by breeding companies.

References

Alonso-Blanco C, Aarts MG, Bentsink L, Keurentjes JJ, Reymond M, Vreugdenhil D, Koornneef M (2009) What has natural variation taught us about plant development, physiology, and adaptation? The Plant Cell **21:** 1877-1896

Alonso-Blanco C, Bentsink L, Hanhart CJ, Blankestijn-de Vries H, Koornneef M (2003) Analysis of natural allelic variation at seed dormancy loci of Arabidopsis thaliana. Genetics **164:** 711-729

Alonso-Blanco C, Koornneef M (2000) Naturally occurring variation in Arabidopsis: an underexploited resource for plant genetics. Trends in plant science **5:** 22-29

Argyris J, Dahal P, Hayashi E, Still DW, Bradford KJ (2008) Genetic variation for lettuce seed thermoinhibition is associated with temperature-sensitive expression of abscisic acid, gibberellin, and ethylene biosynthesis, metabolism, and response genes. Plant Physiology **148:** 926-947

Auge GA, Blair LK, Neville H, Donohue K (2017) Maternal vernalization and vernalization-pathway genes influence progeny seed germination. New Phytologist **216:** 388-400

Baker HG (1972) Seed weight in relation to environmental conditions in California. Ecology **53:** 997-1010

Bentsink L, Jowett J, Hanhart CJ, Koornneef M (2006) Cloning of DOG1, a quantitative trait locus controlling seed dormancy in Arabidopsis. Proceedings of the National Academy of Sciences **103:** 17042-17047

Bewley JD (1997) Breaking down the walls—a role for endo-β-mannanase in release from seed dormancy? Trends in Plant Science **2:** 464-469

Bewley JD (1997) Seed germination and dormancy. The plant cell **9:** 1055

Bewley JD, Bradford K, Hilhorst H (2012) Seeds: physiology of development, germination and dormancy. Springer Science & Business Media

Bonina JR (2005) A rapid, simple, inexpensive and reproducible endo-beta-mannanase assay test for determining optimal hydrothermal timing of commercial priming of lettuce seed. University of Florida, [Gainesville, Fla.]

Bouche N, Fromm H (2004) GABA in plants: just a metabolite? Trends in plant science **9:** 110-115

Broman KW, Wu H, Sen Ś, Churchill GA (2003) R/qtl: QTL mapping in experimental crosses. Bioinformatics **19:** 889-890

Burke JM, Tang S, Knapp SJ, Rieseberg LH (2002) Genetic analysis of sunflower domestication. Genetics **161:** 1257-1267

Castro J, Hodar J, Comez J (2007) Seed size, Handbook of seed Science and Technology. Chapter **14:** 397-427

Corbineau F, Rudnicki R, Côme D (1988) Induction of secondary dormancy in sunflower seeds by high temperature. Possible involvement of ethylene biosynthesis. Physiologia Plantarum **73:** 368-373

Doebley JF, Gaut BS, Smith BD (2006) The molecular genetics of crop domestication. Cell **127:** 1309-1321

Doganlar S, Frary A, Tanksley S (2000) The genetic basis of seed-weight variation: tomato as a model system. Theoretical and Applied Genetics **100:** 1267-1273

Dudley J (1994) Molecular Markers in Plant Improvement: Manipulation of Genes Affecting Quantitative Traits. Crop Science **34:** 322-322

Ellis R (1992) Seed and seedling vigour in relation to crop growth and yield. Plant growth regulation **11:** 249-255

Fait A, Angelovici R, Less H, Ohad I, Urbanczyk-Wochniak E, Fernie AR, Galili G (2006) Arabidopsis seed development and germination is associated with temporally distinct metabolic switches. Plant physiology **142:** 839-854

Finch-Savage W (1995) Influence of seed quality on crop establishment, growth and yield. Seed quality: Basic mechanisms and agricultural implications**:** 361-384

Foolad M, Lin G (1998) Genetic analysis of low-temperature tolerance during germination in tomato, Lycopersicon esculentum Mill. Plant breeding **117:** 171-176

Foolad M, Zhang L, Subbiah P (2003) Genetics of drought tolerance during seed germination in tomato: inheritance and QTL mapping. Genome **46:** 536-545

Foolad MR, Subbiah P, Zhang L (2007) Common QTL affect the rate of tomato seed germination under different stress and nonstress conditions. International journal of plant genomics **2007:** 10-15

Franks SJ, Weber JJ, Aitken SN (2014) Evolutionary and plastic responses to climate change in terrestrial plant populations. Evolutionary Applications **7:** 123-139

Goldman I, Paran I, Zamir D (1995) Quantitative trait locus analysis of a recombinant inbred line population derived from a Lycopersicon esculentum x Lycopersicon cheesmanii cross. Theoretical and Applied Genetics **90:** 925-932

Gubler F, Hughes T, Waterhouse P, Jacobsen J (2008) Regulation of dormancy in barley by blue light and after-ripening: effects on abscisic acid and gibberellin metabolism. Plant Physiology **147:** 886-896

Harlan JR, De Wet J, Price EG (1973) Comparative evolution of cereals. Evolution **27:** 311-325

He H (2014) Environmental regulation of seed performance. Wageningen University

He H, de Souza Vidigal D, Snoek LB, Schnabel S, Nijveen H, Hilhorst H, Bentsink L (2014) Interaction between parental environment and genotype affects plant and seed performance in Arabidopsis. Journal of experimental botany **65:** 6603-6615

Hilhorst H, Koornneef M (2007) Dormancy in Plants. *In* eLS,

Hilhorst HW, Finch-Savage WE, Buitink J, Bolingue W, Leubner-Metzger G (2010) Dormancy in plant seeds. *In* Dormancy and resistance in harsh environments. Springer, pp 43-67

Hilhorst HW, Toorop PE (1997) Review on dormancy, germinability, and germination in crop and weed seeds. Advances in agronomy (USA) **61:** 112-165

Huang X, Schmitt J, Dorn L, Griffith C, Effgen S, Takao S, Koornneef M, Donohue K (2010) The earliest stages of adaptation in an experimental plant population: strong selection on QTLS for seed dormancy. Molecular ecology **19:** 1335-1351

Huo H, Bradford KJ (2015) Molecular and Hormonal Regulation of Thermoinhibition of Seed Germination. *In* JV Anderson, ed, Advances in Plant Dormancy. Springer International Publishing, Cham, pp 3-33

Huo H, Dahal P, Kunusoth K, McCallum CM, Bradford KJ (2013) Expression of 9-cis-EPOXYCAROTENOID DIOXYGENASE4 is essential for thermoinhibition of lettuce seed germination but not for seed development or stress tolerance. The Plant Cell**:** tpc. 112.108902

Jansen RC, Nap J-P (2001) Genetical genomics: the added value from segregation. TRENDS in Genetics **17:** 388-391

Joosen RV, Arends D, Li Y, Willems LA, Keurentjes JJ, Ligterink W, Jansen RC, Hilhorst HW (2013) Identifying genotype-by-environment interactions in the metabolism of germinating Arabidopsis seeds using generalized genetical genomics. Plant physiology **162(2):** 553-566

Joosen RV, Kodde J, Willems LA, Ligterink W, van der Plas LH, Hilhorst HW (2010) germinator: a software package for high-throughput scoring and curve fitting of Arabidopsis seed germination. The Plant Journal **62:** 148-159

Joosen RV, Ligterink W, Hilhorst HW, Keurentjes JJ (2009) Advances in genetical genomics of plants. Current Genomics **10:** 540-549

Joosen RV, Ligterink W, Hilhorst HW, Keurentjes JJ (2013) Genetical Genomics of Plants: From Genotype to Phenotype. Advances in Genome Science: Probing Intracellular Regulation **2:** 2194

Joosen RVL, Arends D, Willems LAJ, Ligterink W, Jansen RC, Hilhorst HW (2012) Visualizing the genetic landscape of Arabidopsis seed performance. Plant physiology **158:** 570-589

Jurado E, Westoby M (1992) Seedling growth in relation to seed size among species of arid Australia. Journal of Ecology **80(3):** 407-416

Kazmi RH (2013) Genes for seed quality : integrating physiology and genetical genomics to mine for seed quality genes in tomato. [Wageningen]

Kazmi RH, Khan N, Willems LA, Van Heusden AW, Ligterink W, Hilhorst HW (2012) Complex genetics controls natural variation among seed quality phenotypes in a recombinant inbred population of an interspecific cross between Solanum lycopersicum× Solanum pimpinellifolium. Plant, cell & environment **35:** 929-951

Kazmi RH, Willems LA, Joosen RV, Khan N, Ligterink W, Hilhorst HW (2017) Metabolomic analysis of tomato seed germination. Metabolomics **13:** 145

Keurentjes JJ, Sulpice R, Gibon Y, Steinhauser M-C, Fu J, Koornneef M, Stitt M, Vreugdenhil D (2008) Integrative analyses of genetic variation in enzyme activities of primary carbohydrate metabolism reveal distinct modes of regulation in Arabidopsis thaliana. Genome biology **9:** R129

Khan N, Kazmi RH, Willems LA, Van Heusden AW, Ligterink W, Hilhorst HW (2012) Exploring the natural variation for seedling traits and their link with seed dimensions in tomato. PLoS One **7:** e43991

Kinnersley AM, Turano FJ (2000) Gamma aminobutyric acid (GABA) and plant responses to stress. Critical Reviews in Plant Sciences **19:** 479-509

Koornneef M, Bentsink L, Hilhorst H (2002) Seed dormancy and germination. Current opinion in plant biology **5:** 33-36

Körner C (2016) Plant adaptation to cold climates. F1000Research **5**

Lafta A, Mou B (2013) Evaluation of lettuce genotypes for seed thermotolerance. HortScience **48:** 708-714

Lamont B, Groom P (2013) Seeds as a source of carbon, nitrogen, and phosphorus for seedling establishment in temperate regions: a synthesis. American Journal of Plant Sciences **4:** 30-40

Leverett LD, Auge GA, Bali A, Donohue K (2016) Contrasting germination responses to vegetative canopies experienced in pre-vs. post-dispersal environments. Annals of botany **118:** 1175-1186

Li Y, Breitling R, Jansen RC (2008) Generalizing genetical genomics: getting added value from environmental perturbation. Trends in Genetics **24:** 518-524

Li Y, Swertz MA, Vera G, Fu J, Breitling R, Jansen RC (2009) designGG: an R-package and web tool for the optimal design of genetical genomics experiments. BMC bioinformatics **10:** 188

Ligterink W, Joosen RV, Hilhorst HW (2012) Unravelling the complex trait of seed quality: using natural variation through a combination of physiology, genetics and-omics technologies. Seed Science Research **22:** S45-S52

Lippman ZB, Semel Y, Zamir D (2007) An integrated view of quantitative trait variation using tomato interspecific introgression lines. Current opinion in genetics & development **17:** 545-552

Long SP, Ort DR (2010) More than taking the heat: crops and global change. Current opinion in plant biology **13:** 240-247

McCouch S (2004) Diversifying selection in plant breeding. PLoS biology **2:** e347

Mosa KA, Ismail A, Helmy M (2017) Omics and system biology approaches in plant stress research. *In* Plant stress tolerance. Springer, pp 21-34

Nieuwhof M, Garretsen F, Van Oeveren J (1989) Maternal and genetic effects on seed weight of tomato, and effects of seed weight on growth of genotypes of tomato (Lycopersicon esculentum Mill.). Plant Breeding **102:** 248-254

Orsi CH, Tanksley SD (2009) Natural variation in an ABC transporter gene associated with seed size evolution in tomato species. PLoS genetics **5:** e1000347

Rosental L, Perelman A, Nevo N, Toubiana D, Samani T, Batushansky A, Sikron N, Saranga Y, Fait A (2016) Environmental and genetic effects on tomato seed metabolic balance and its association with germination vigor. BMC genomics **17:** 1047

Rowe HC, Hansen BG, Halkier BA, Kliebenstein DJ (2008) Biochemical networks and epistasis shape the Arabidopsis thaliana metabolome. The Plant Cell **20:** 1199-1216

Rowse H, Finch-Savage W (2003) Hydrothermal threshold models can describe the germination response of carrot (Daucus carota) and onion (Allium cepa) seed populations across both sub-and supra-optimal temperatures. New Phytologist **158:** 101-108

Ruffel S, Krouk G, Coruzzi GM (2010) A systems view of responses to nutritional cues in Arabidopsis: toward a paradigm shift for predictive network modeling. Plant physiology **152:** 445-452

Salvi S, Tuberosa R (2005) To clone or not to clone plant QTLs: present and future challenges. Trends in plant science **10:** 297-304

Sperling L, Osborn TC, Osborne T, Cooper HD (2004) Towards Effective and Sustainable Seed Relief Activities: Report of the Workshop on Effective and Sustainable Seed Relief Activities, Rome, 26-28 May 2003, Vol 181. Food & Agriculture Org.

Stevens N, Seal CE, Archibald S, Bond W (2014) Increasing temperatures can improve seedling establishment in arid-adapted savanna trees. Oecologia **175:** 1029-1040

Toh S, Imamura A, Watanabe A, Nakabayashi K, Okamoto M, Jikumaru Y, Hanada A, Aso Y, Ishiyama K, Tamura N (2008) High temperature-induced abscisic acid biosynthesis and its role in the inhibition of gibberellin action in Arabidopsis seeds. Plant physiology **146:** 1368-1385

Toubiana D, Semel Y, Tohge T, Beleggia R, Cattivelli L, Rosental L, Nikoloski Z, Zamir D, Fernie AR, Fait A (2012) Metabolic profiling of a mapping population exposes new insights in the regulation of seed metabolism and seed, fruit, and plant relations. PLoS Genetics **8:** e1002612

Van Ooijen J (2004) MapQTL® 5, Software for the mapping of quantitative trait loci in experimental populations. Kyazma BV, Wageningen **63**

Walck JL, Hidayati SN, Dixon KW, Thompson K, Poschlod P (2011) Climate change and plant regeneration from seed. Global Change Biology **17:** 2145-2161

West MA, Kim K, Kliebenstein DJ, Van Leeuwen H, Michelmore RW, Doerge R, Clair DAS (2007) Global eQTL mapping reveals the complex genetic architecture of transcript-level variation in Arabidopsis. Genetics **175:** 1441-1450

Summary

Quality of seeds is strongly affected by their genetic make-up, the environment during seed development and maturation and also the interaction between the genome and the environment. In this thesis, I tried to dissect the genetic basis of tomato seed quality by a combined study of physiology, genetics and genomics in relation to the maternal environment. **Chapter 1** of the thesis (General introduction) describes the definition and different aspects of seed and seedling quality and the factors influencing them, with the emphasis on the maternal environment. Additionally background on the used genetic tools with emphasis on the generalized genetical genomics (GGG) approach is provided. Finally, integration of large 'omics' technology driven datasets is suggested as an approach to assist in the identification of the genes underlying QTLs.

Seed performance traits, such as seed dormancy and germinability, can be influenced by different maternal nutritional environmental conditions. In **Chapter 2** I investigated the effect of different maternal nutritional environments on the quality of seeds from two tomato species (*S. lycopersicum* and *S. pimpinellifolium*). I showed that different phosphate and nitrate levels available for mother plants influences seed and seedling quality traits especially in stressful germination environments. Correlation analysis was done between physiological traits and metabolic changes caused by the different maternal environments in tomato and interesting positive and negative correlations were identified which shed light on the molecular regulation of seed quality in different maternal environments.

High quality seed is defined by high levels of seed germination and seedling establishment, especially under sub-optimal conditions. Seed quality however, is acquired during seed maturation and therefore in addition to the mother plant's genetic background can be strongly influenced by the maternal environment in which the seeds develop. **Chapter 3** of the thesis focuses on QTL analysis for seed quality with help of a tomato recombinant inbred line (RIL) population consisting of 100 lines to better understand the possible molecular mechanisms involved in acquisition of seed quality as there is little knowledge about the genetic and environmental factors, and their interaction, that influence seed quality and seedling establishment. For this analysis the RILs originating from a cross between *Solanum lycopersicum* (cv. Moneymaker) and *Solanum pimpinellifolium* together with these parental lines were grown in two maternal environments: at high phosphate and low nitrate contents. By analysing the interaction of QTLs with the maternal environment (QTL×E) I tried to enhance and extend detection of loci affected by the different maternal environments.

In **Chapter 4** I describe the metabolic analysis of the seeds from the experiments described in Chapter 3. Correlation analysis of metabolite composition and seed phenotypic traits revealed several relations between metabolite contents and seed quality traits such as seed size, seed

weight and seed germination percentage. A positive correlation was observed between seed size and -weight and several amino acids and some intermediates of the TCA cycle, such as succinate, citrate and malate. In this chapter I have also identified interesting mQTLs by performing metabolic correlation analysis and metabolic networks generation combined with QTL analysis.

I have investigated the physiological and the genetic variation of two tomato accessions (*Solanum lycopersicum* (cv. Moneymaker) and *Solanum pimpinellifolium*) in response to thermo-inhibition of tomato seeds to elevated temperatures in **Chapter 5**. I have also studied the putative molecular mechanisms that could explain the performance of the seeds at elevated temperature. This was done by analysing the expression levels of genes that have been reported to have a role in thermos-inhibition and dormancy in lettuce and Arabidopsis. Additionally, a QTL analysis on the previous described RIL population has been performed and this resulted in new QTLs and potential new regulators of seed germination and dormancy in tomato under high temperature conditions.

Chapter 6 of the thesis is where I discuss the main outcomes of the thesis. I have also made conclusions by combining and integrating the obtained data from the experimental chapters. Finally I draw a perspective for future research and explain the further required experiments/studies to obtain a comprehensive knowledge of seed and seed quality and their influencing factors. These suggestions will expectantly result in the further identification of the controlling mechanisms regulating seed and seedling quality.

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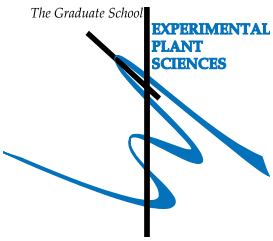
About the Author

Nafiseh Geshnizjani was born on 21st September 1986 in Shahrekord, Iran. in September 2005 she was admitted to the bachelors program of Shiraz University (Shiraz, Iran) in the field of agricultural engineering and Horticultural Sciences. Later in 2009 she was graduated as the top student and immediately started in a Master's program in the field of physiology of ornamental plants in the same university. She performed her master thesis under supervision of Prof. M. Khosh-Khui with the main focus on postharvest physiology of ornamental plants, especially in Gerbera. Later on she moved to Wageningen and started her PhD project in the Laboratory of Plant Physiology of Wageningen University and Research (The Netherlands) under supervision of Dr. HWM. Hilhorst and Dr. JW. Ligterink. During her PhD she was working on genetic aspects of tomato seed and seedling performance under control of maternal environment. From her thesis she has two publications so far in the BMC Plant Biology and Journal of Experimental Botany.

Education Statement of the Graduate School

Experimental Plant Sciences



Issued to: **Nafiseh Geshnizjani**
Date: **19 June 2019**
Group: **Laboratory of Plant Physiology**
University: **Wageningen University & Research**

1) Start-Up Phase	*date*	*cp*
► **First presentation of your project**		
Genetic and environmental control of seed quality and seedling establishment	14 Nov 2014	1.5
► **Writing or rewriting a project proposal**		
Genetic and environmental control of seed quality and seedling establishment	19 May 2014	6.0
► **Writing a review or book chapter**		
► **MSc courses**		
Subtotal Start-Up Phase		7.5

2) Scientific Exposure	*date*	*cp*
► **EPS PhD student days**		
EPS PhD student days 'Get2Gether', Soest, NL	28-29 Jan 2016	0.6
EPS PhD student days 'Get2Gether', Soest, NL	15-16 Feb 2018	0.6
► **EPS theme symposia**		
EPS theme 1 symposium 'Developmental Biology of Plants', Wageningen, NL	24 Jan 2014	0.3
EPS theme 3 symposium 'Metabolism and Adaptation', Wageningen, NL	11 Mar 2014	0.3
EPS theme 3 symposium 'Metabolism and Adaptation', Wageningen, NL	10 Feb 2015	0.3
EPS theme 3 symposium 'Metabolism and Adaptation', Amsterdam, NL	23 Feb 2016	0.3
EPS theme 3 symposium 'Metabolism and Adaptation', Wageningen, NL	14 Mar 2017	0.3
► **Lunteren Days and other national platforms**		
STW meeting, Bejo company, Warmenhuizen, NL	03 Oct 2014	0.2
STW meeting, Enza Zaden Company, Enkhuizen, NL	13 Oct 2015	0.2
STW Annual Congress ´Tech Stage´, Nieuwegein, NL	05 Nov 2015	0.3
STW meeting, Wageningen University, Wageningen, NL	17 Jun 2016	0.2
STW meeting, Wageningen University, Wageningen, NL	09 May 2017	0.2
STW meeting, Rijk Zwaan Company, Fijnaart, NL	10 Oct 2017	0.2
Annual meeting 'Experimental Plant Sciences', Lunteren, NL	11-12 Apr 2016	0.6
Annual meeting 'Experimental Plant Sciences', Lunteren, NL	10-11 Apr 2017	0.6
► **Seminars (series), workshops and symposia**		
EPS Flying Seminar Ortrun Mittelsten Scheid	19 Nov 2014	0.1
EPS Flying Seminar Dr. Siobhan Brady	09 Sep 2015	0.1
4th Dutch Seed Symposium	06 Oct 2015	0.3
5th Dutch Seed Symposium	04-05 Oct 2016	0.6
6th Dutch Seed Symposium	03 Oct 2017	0.3
Seminar “From QTLs to routine DNA-informed breeding: prospects, advances, & needs …and experiences in apple at Washington State University” by Dr. Cameron Peace	16 Nov 2016	0.1
Seminar 'Regulators of Reproductive Development in Rice' by Dr. Sanjay Kapoor	29 Aug 2017	0.1

Seminar 'Extending the range of temperature adaptation for high-quality broccoli' by Prof. Thomas Bjorkman	29 Sep 2017	0.1
Seminar 'Pelargonidin in flowers - why not? Gerbera and petunia flowers block pelargonidin biosynthesis in a different way' by Prof. Teemu Teeri	14 Mar 2018	0.1
Seminar 'Rewiring starch metabolism for plant environmental adaptation' by Dr. Diana Santelia	1 Nov 2018	0.1
Seminar 'The role of the endosperm in Arabidopsis early post-embryonic development' by Dr. Luis Lopez-Molina	25 Jan 2019	0.1
► **Seminar plus**		
► **International symposia and congresses**		
Molecular Aspects of Seed Dormancy and Germination, Vancouver, Canada	31 May-03 Jun 2016	1.2
► **Presentations**		
Talk: STW meeting, Bejo company, Netherlands	03 Oct 2014	1.0
Talk: STW meeting, Rijk Zwaan Company, Fijnaart, NL	10 Oct 2017	1.0
Talk: Tubingen University - PhD trip	29 Apr 2015	1.0
Poster: Annual meeting 'Experimental Plant Sciences', Lunteren, NL	11-12 Apr 2016	1.0
Poster: Annual meeting 'Experimental Plant Sciences', Lunteren, NL	11-12 Apr 2016	1.0
Poster: Molecular Aspects of Seed Dormancy and Germination, Vancouver, Canada	31 May-03 Jun 2016	1.0
Poster: Annual meeting 'Experimental Plant Sciences', Lunteren, NL	10-11 Apr 2017	1.0
Poster: The 12th Triennial Conference of The ISSS, Monterey, California, USA	10-14 Sep 2017	1.0
► **IAB interview**		
► **Excursions**		
PhD Trip: Netherlands, Germany, Switzerland	22 Apr-01 May 2015	1.8
Subtotal Scientific Exposure		18.2

3) In-Depth Studies	*date*	*cp*
► **Advanced scientific courses & workshops**		
Data Analysis and Visualizations in R	11-12 May 2017	0.6
Introduction to R for statistics	23-24 Oct 2017	0.6
► **Journal club**		
Literature discussions in Plant Physiology	2014-2017	3.0
► **Individual research training**		
Subtotal In-Depth Studies		4.2

4) Personal Development	*date*	*cp*
► **General skill training courses**		
WGS PhD Workshop Carousel	08 Apr 2016	0.3
Scientific Writing	06 Sep-08 Nov 2016	1.8
Career Development Plan	03 May 2019	0.9
► **Organisation of meetings, PhD courses or outreach activities**		
► **Membership of EPS PhD Council**		
Subtotal Personal Development		3.0

TOTAL NUMBER OF CREDIT POINTS*	**32.9**

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

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

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