Genetic Dissection of Drought Tolerance in Potato

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This thesis is affectionately dedicated to my beloved parents

Chapter 1

General Introduction

Drought: A Major abiotic stress

Unlike animals, higher plants are sessile and therefore cannot escape from unfavorable conditions such as abiotic stresses. Plant productivity is severely affected by abiotic stress factors such as drought, salinity, flooding, high and low temperatures, UV radiation, excess ozone and heavy metals. Abiotic stress is the primary cause of crop losses worldwide, causing average yield losses of more than 50% for major crops (Boyer 1982). Among the different abiotic stresses, drought is by far the most complex and devastating on a global scale (Pennisi 2008). Agriculture is a major user of water resources in many regions of the world and drought affects agriculture in 45% of the world geographical area. Rainfed agriculture accounts for 80% of cultivated land area in the world and contributes 60% of world food (Rockstrom 2003). The lower yield levels in rainfed crop production are caused by limited and unpredictable rainfall which leads to drought stress. With increasing aridity and a growing world population, water will become an even scarcer commodity in the near future. The growing world population requires more food production but good agricultural land surface is decreasing. The challenge to feed more people with the same or even less agricultural land may be met by enhancing the productivity of crops grown on stress-affected lands, and increasing yield under irrigated agriculture. As yield levels may have already reached a plateau in irrigated agriculture, it is essential to increase the productivity of abiotic stress-affected areas to meet the growing global food demands. Genetic enhancement of drought tolerance crops is one of the important strategies to enhance productivity of crops under less then optimal agricultural conditions.

Drought tolerant mechanisms in plants

Plants have evolved different ways to respond to drought stress namely escape, avoidance and tolerance strategies. An overview of plant adaptive response to water stress is shown in Figure 1. Plants that escape drought exhibit a rapid phenological development and high degree of developmental plasticity, being able to complete their life cycle before physiological water deficit occurs. Escape strategies rely on successful reproduction before the severe stress is perceived. A short life cycle is particularly advantageous in environments with terminal drought stress or where physical or chemical barriers inhibit root growth (Blum 1988, Bidinger and Witcombe 1989).

Drought avoidance refers to the plant’s ability to retain a relatively high level of hydration

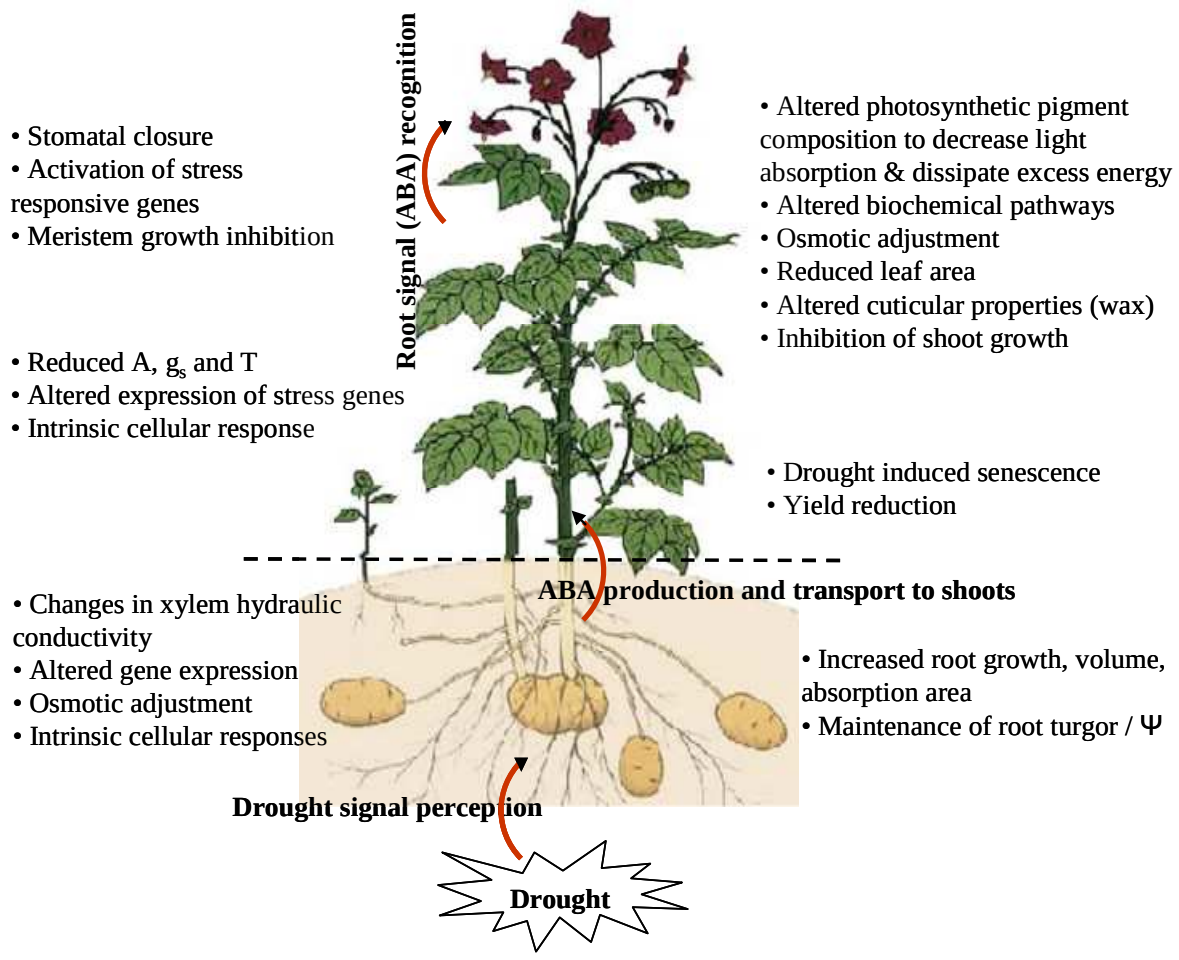

Figure 1 An overview of plant adaptive responses to water stress (modified from Chaves et al 2003)

under conditions of soil or atmospheric water stress (Blum 1998). Drought avoidance has two components: enhanced water uptake and reduced water loss. Improvement of water uptake can be achieved by adapting root traits such as root thickness, root penetration ability through compacted soil layers, and root depth and mass (Price et al 2002). Water loss can be minimized through reduced epidermal conductance, reduced absorption of radiation by leaf rolling and reduced leaf area to minimize evaporative surface.

Drought tolerance is defined as the relative capacity of plants to maintain functional growth under low leaf water status. Drought causes reduction in water potential of the cell, as a result of solute concentration gradients and osmosis, and leads to loss of cell turgor. Some plants have the ability to tolerate dehydration or maintain turgor pressure through an osmotic adjustment via the active accumulation of solutes called osmoprotectants, or compatible

solutes. These molecules, which act as osmotic balancing agents, are accumulated in plant cells in response to drought stress and are subsequently degraded after alleviation of the stress. Osmoprotectants include amino acids, sugar alcohols, polyols and quaternary ammonium and tertiary sulfonium compounds and help in protecting cell components from the adverse effect of water loss through expression of cell rescue mechanisms and through increased capacity of plants to recover after stress.

Drought response is a quantitative trait under complex phenotypic and genetic control (McWiliam, 1989). In many crop species, the capacity for drought escape, avoidance and tolerance has been reported. These strategies are not mutually exclusive and plants may combine a range of response types (Ludlow 1990). This response is probably based on severity of the drought stress but also could involve other factors such as the developmental stage, the level of metabolic reserves and also the ability of plant to predict the nature of the stress it faces using memory (physiological or genetic) and environmental indicators other than the drought stress itself (i.e., light level, temperature, time of year etc). However, these adaptation mechanisms have some disadvantages. Drought escape by reducing growth duration leads to reduced yields. Drought avoidance, by reducing water loss through stomatal closure and leaf area reduction, might result in reduced photosynthetic ability ultimately resulting in reduced carbon assimilates and yield. Increased solute concentrations responsible for osmotic adjustment may have a detrimental effect in addition to energy requirement for osmotic adjustment. Another example of avoidance is decreasing canopy by reducing growth and shedding of older leaves. Accelerated leaf senescence and leaf abscission are associated with drought in nature as means to decrease canopy size; this strategy reduces the yields of annual crops, with concomitant economical loss to farmers. Therefore, adaptation of plants to stress should reflect a balance between escape, avoidance and tolerance while maintaining adequate productivity. Hence drought is a complex trait for breeding, expression of which depends on action and interaction between different characters such as morphological, physiological, biochemical and cellular level processes. Insight in the genetic mechanisms that condition these characters is limited. Improving the tolerance of crops to drought, compared with other abiotic stresses, requires a broader interdisciplinary approach, involving an understanding of the factors determining yield in particular target environments.

Drought response at the molecular level

A better understanding of the effects of drought on plants is vital for improved management practices and breeding efforts in agriculture. Plants activate a diverse set of physiological, metabolic and defense systems to survive and to sustain growth. Any change in the optimal growth conditions is perceived by plants as stress and transduced in the form of signals involving protein phosphorylation and/or dephosphorylation, calcium sensing, protein degradation etc. This activates stress responsive mechanisms either by directly leading to expression of certain genes involved in repair mechanisms or leading to expression of transcription factors which in turn regulate further downstream stress response genes (Bartels and Sunkar 2005). Transcriptomics, proteomics and gene expression studies have identified the regulation and activation of several drought stress-related transcripts and proteins, which are generally classified into two major groups. The first group (*functional proteins*) includes proteins that probably function in stress tolerance. They are protection factors such as chaperones, LEA proteins, and lipid transfer proteins, proteins involved in repair and protection from damages, such as proteinases, detoxification enzymes, protease inhibitors, ferritin and plant defense-related proteins and proteins involved in synthesis of osmoprotectants (proline, glycine betaine, sugars). This group also includes proteins involved in cellular metabolic processes such as carbohydrate metabolism, secondary metabolism, fattyacid metabolism, biosynthesis of plant hormones (ABA, ethylene, IAA and JA), proteins regulated by plant hormones (ABA, auxin and JA), RNA binding proteins, cellular structure and organization-related proteins such as arabinogalactan proteins, senescence related proteins, cytochrome P450, alcohol dehydrogenase, aldehyde dehydrogenase, reproduction development-related proteins such as pollen coat-like protein and respiration related proteins such as flavin-containing monooxygenase.

The second group (*regulatory proteins*) is involved in regulation of signal transduction and transcription as part of the drought response. These are transcription factors of multiple gene families such as DREB, ERF, zinc finger, WRKY, MYB, MYC, HD-ZIP, bZIP and NAC families. Among the regulatory proteins protein kinases such as MAPK, MAPKKK, CDPK, S6K and PRK can be found. This group also includes protein phosphatases such as PP2C, PI turnover related proteins such as PLC, PLD, PIP5K, DGK and PAP, and calmodulin-binding protein and Ca^{2+} -binding proteins. Understanding the mechanisms by which plants perceive environmental signals and transmit such signals to the cellular machinery to activate

responses is a fundamental issue in plant biology and is also vital for the continued development of rational breeding and transgenic strategies to improve stress tolerance in crops.

Potato: Origin and its importance

Potato (*Solanum tuberosum*.L) is the world's 3rd major food crop in terms of food consumption, and 8th in terms of area under cultivation (FAO statistics 2008). The potato tuber is a high energy staple food in many countries around the world and since it provides high productivity per unit area, it can be cultivated intensively. Thus potato represents one of the best candidates for alleviating food shortages. The cultivated potato S. tuberosum is an autotetraploid (2n=4x=48). The domestication of potatoes (*Solanum spp.)* probably started at least 7 000 years ago around Lake Titicaca (in modern-day Peru and Bolivia), when the first inhabitants of this region began selecting edible forms of wild potato species (Simmonds 1995; Spooner et al 2005). The wild species eventually crossed with each other and produced increasingly better varieties. The modern potato (*Solanum tuberosum*) was apparently domesticated from wild potato species of the *Solanum brevicaule* complex (Spooner et al 2005). However, the emergence of agricultural communities, in this and other regions of South America, only occurred some 3800 years ago at the beginning of the Formative Period. Several taxonomical studies suggest that wild solanum species are well spread from North to South America and highly diverse (Hawkes 1990; Spooner and Hijmans 2001). The ploidy level ranges from diploid (2n=2x=24) to hexaploid (2n=6x=72) in which the majority of diploid species are self-incompatible and tetraploids and hexaploids are self-compatible allopolyploids that exhibit disomic inheritance (Hawkes 1990).

The first introduction of potato in Europe was probably around 1570 in Spain (Hawkes 1990). After the potato was introduced, it spread through Europe via three main routes. The potato as an "object of curiosity" was disseminated by a network of scholars and botanists. The potato as an "object of cultivation" spread among the monasteries of Carmelite Friars. The third route of dissemination within Europe was by the continuous trek of the Protestants, who took the potato with them when religious persecution forced them to flee their homes and lands (CIP, Odyssey of potato 2008). By the late 1700s, potato cultivation was widespread in Europe and they were taken from Europe and cultivated in many other parts of world

(Hawkes and Francisco-Oetega 1993; Pandey and Kaushik 2003). In the mid-1800s the devastating potato late blight disease was introduced with the onset of famine in Ireland and other regions of Europe (Fry 2008; Schumann 1991). Nonetheless, potato has persisted as a staple food throughout Europe and currently Europeans have the highest per capita consumption of potatoes in the world.

Today, potato is the most important food crop in the world, after rice and wheat. Potatoes are grown in more than 130 countries and consumed by over a billion people worldwide. In 2005, for the first time, more area was planted with potatoes in the developing nations than in the industrialized nations and the area was around 20 million hectares with a total world production of over 300 million tons (Haverkort et al 2009). At present, the major potato-producing countries are China, Russia, India, USA and Ukraine accounting for 22, 12, 7, 7 and 6% of world production respectively (FAO statistics 2008). Potato is not only used for human consumption but also for industrial purposes mainly for starch in textile, papermaking, glue, flocculating agents and building materials. More uses are anticipated mainly as bioreactors for biopharmaceuticals for encapsulation and controlled release of functional ingredients (Li et al 2009) and designer starches (Davies 1998). Increasingly severe weather events, increasing costs of staple grains, and growing use of food for biofuels suggest that potato production will become even more important as a food security crop in the world.

Impact of drought on potato

Potatoes grow optimally under relatively cool conditions and the formation and tuber bulking depends mainly on day and night temperatures, to enable metabolites produced during daytime to accumulate in the tuber during night (Hooker 1981). One of the major factors that limits potato cultivation is susceptibility to drought and this is mainly due to its shallow root system with a depth ranging from 0.5 to 1.0m (Vos and Groenwold, 1986). About 85% of the total root length is concentrated in the upper 0.3m of soil. Gregory and Simmonds (1992) showed that the potato root system displays a relatively small root length per unit area and this makes the potato plant a poor conductor of water. This is further complicated by the fact that potato extracts less of the available water from the soil compared to other crops (Weisz *et al.*, 1994).

Early growth and tuber formation require large amounts of water and recovery is difficult once potato plants experience water deficit (Harris 1978; Deblonde and Ledent 2001). The

critical level of soil moisture tension for potato yield and quality is around -0.7 bar (Mould and Rutherford 1980) and short periods of water shortage can reduce tuber production and tuber quality (Miller and Martin, 1987). Average tuber yield reduction per mm water deficit has been estimated at 117 kg/ha (Vos and Groenwold 1988). Plants facing drought conditions during the tuber formation stage are more susceptible to scab (*Streptomyces scabies*) and soil cracking can make tubers vulnerable to insect pests such as the potato tuber moth (*Phthorimaea operculella*; Hide and Lapwood 1978). Drought affects transpiration and evaporation which leads to the elevation of soil and plant temperatures. Increased temperatures are detrimental to tuber formation and during the late growth stage, drought and heat stress acting in tandem may cause problems such as brown spots inside the tubers (Hide and Lapwood 1978).

Several studies have shown that drought has a drastic effect on morphological and physiological traits of the potato plant, such as leaf size, leaf number, shoot height (Deblonde and Ledent, 2001), rate of photosynthesis and most importantly tuber number (MacKerron and Jefferies, 1986; Haverkort *et al.*, 1991), tuber yield and biomass (Dalla Costa *et al.*, 1997). The effect of drought on tuber yield depends on the aggregate of morpho-physiological processes, such as photosynthesis, leaf area expansion, leaf senescence, partitioning of assimilates, tuber initiation, bulking and tuber growth (van Loon, 1981). In addition, potato yield under water deficit conditions depends on the timing and duration of the stress within the growth period (Jefferies 1995b) as well as on the climate and soil conditions. Dramatic reduction of yield occurs when stress coincides with the irreversible reproductive processes, making the genetic analysis for drought tolerance at the reproductive stage crucially important.

Potato breeding for drought tolerance

Water availability is crucial to obtaining high yields in the potato crop. Improvements in the tolerance to drought of potato could decrease the input of fresh water in potato cultivation, and increase the cultivation area to drought prone regions in the world. Genetic improvement of potato is mainly hampered by its high level of heterozygosity, tetrasomic inheritance and incompatibility barriers. Classical breeding in potato involves evaluation and selection based on several traits within the clonally propogated progeny of a cross between two tetraploid

clones. These clones can be existing cultivars or clones with introgressions from wild species. Potato relatives range from diploid to hexaploid and although most cultivated potato species are tetraploid, over 74% of naturally occurring species are diploid. Thus, the disadvantages of breeding tetraploid potato plants can be circumvented by using wild type diploid species. Further the diploids can be bred to tetraploids because of their 2n gametes.

The differential response of potato cultivars to water stress indicates that there is genetic variability for drought tolerance in cultivated potato (Harries 1978; Levy 1983). In addition, several wild species of potato growing in its center of origin in South-America have been adapted to harsh environments at high altitudes more than 3,000 meters above sea level and are regularly exposed to water-scarce conditions (Schafleitner et al 2007). This genetic variability within potato and its relatives can be exploited by breeders to improve drought tolerance of the crop. However, selection for drought tolerance while maintaining maximum productivity under optimal conditions is difficult because several plant attributes are involved in drought tolerance mechanisms and because water stress itself varies in time and intensity and is therefore difficult to define. Breeding for drought is further complicated by the fact that several types of abiotic stress such as high temperatures, high irradiance and water deficit can challenge crop plants simultaneously. In addition, successful breeding requires exact information on effective tolerance traits, their heritability and their genotype x environment interaction as well as suitable selection tools for the traits of interest. Selection of useful traits through visible phenotypic traits requires vast and time consuming efforts. Currently two basic genetic approaches utilized to improve stress tolerance are 1) exploitation of natural genetic variation, either through direct selection in stressful environments or through the mapping of quantitative trait loci (QTL) and subsequent marker assisted selection, and 2), generation of transgenic plants to introduce novel genes or alter expression levels of existing genes to affect the degree of drought tolerance.

Molecular markers

Genetic variation is the basis for biodiversity of life (Schlotterer 2004). Variations in the DNA sequence of genes and their regulatory regions underlie most of the phenotypic variation that has been exploited in modern crops (Bryan et al. 2000; Masouleh et al. 2009). Breeding strategies aimed at improving crop agronomical properties have gained momentum

in the last few decades by the use of molecular marker technologies that visualize DNA polymorphisms (Collard et al, 2005). Starting with hybridization-based markers like RFLP, in the late nineteen eighties and early nineties PCR-based markers like AFLP have proven to be quite useful in marker-assisted breeding, for genome-wide screens for variation, genotype identification/fingerprinting, evolutionary and ecological studies. Simple sequence repeats (SSRs), also known as microsatellites and single nucleotide polymorphisms (SNPs) are the modern genetic markers currently being used in plant genetic analysis. With advances in genome sequencing technologies, SNPs that are suitable for high throughput genotyping methods turn out to be markers of choice to extensively map large sets of individuals. The generation of novel markers allows the production of high-density genetic maps and enables the genotype-phenotype link to be defined with greater precision.

Plant breeders often select for and want to track more than one trait including quantitative traits and as the number of genes controlling a trait expands there is a need for rapid, simple, inexpensive, high-throughput genotyping techniques. A large number of individuals must be genotyped with a large number of markers. The ideal genotyping method must possess many attributes like: i) the assay must be easily and quickly developed from sequence; ii) the reaction must be robust, such that even suboptimal DNA samples should yield reliable results; iii) the assay must be easily automated and must require minimal hands-on operation; iv) the data analysis must be simple with automated, accurate genotyping calling; and v) the reaction format must be flexible, scalable and capable of performing a large number of samples per day. Currently several technologies exist which can be used to screen numerous numbers of markers with large number of individuals. Now that the high-throughput marker genotyping methods and the DNA sequences of whole genome for a number of organisms are available. In addition, with advancement in next generation sequencing platforms along with development of very efficient software tools to analyze high dimensional data made the ideal genotyping methods come true.

Recent technological advancements in discovery and detection have made SNP markers attractive for high-throughput use not only in model species, but also in crop plants (Rafalski 2002). In species for which no genome sequence is available, large scale SNP discovery has generally relied on sequence variation found in libraries of expressed sequence tags (ESTs)

(Somers et al. 2003) or on re-sequencing (Choi et al. 2007). The new Next Generation Sequencing techniques (NGS) can produce nucleotide databases that can be mined for SNP with relatively little effort. Several software tools are available for SNP discovery from nucleotide databases, including Polybayes, AutoSNP, and QualitySNP (Marth 1999; Barker et al. 2003; Tang et al. 2006). Along with the development of tools to mine a large number of SNPs from nucleotide databases, new SNP genotyping platforms were developed that could analyze a large number of SNPs in parallel in a large set of individuals (Syvanen 2005). An increasing number of reports indicate that the GoldenGate system of Illumina is a reliable and cost-effective SNP genotyping platform. It is capable of multiplexing from 96 to 1536 SNPs in a single reaction (Fan JB 2003).

The first potato genetic maps were constructed concurrently by following the segregation of RFLP markers in different genetic backgrounds (Bonierbale et al. 1988; Gebhardt et al. 1989b). These maps were then compared and also aligned with the tomato RFLP map (Gebhardt et al. 1991; Tanksley et al. 1992). With the development of new molecular markers the potato map was enriched with more than 350 markers (Gebhardt et al. 2001). Currently, the ultra high density potato map (van Os et al., 2006) for the SHxRH cross is one of the most highly saturated maps with different molecular markers such as AFLP markers (over 10,000), RFLP, SCAR, CAPS recently SSRs and SNPs and is a valuable tool for localizing genes that control the expression of useful traits. The availability of molecular markers in the last decades has allowed potato breeding research to be greatly improved. The use of molecular markers in potato breeding is reported for many purposes, such as cultivar identification (Gebhardt et al. 1989a), analysis of recombination between genomes, identification of genes controlling traits (Gebhardt 1994, Chen et al 2001, Menendez et al 2002, Werij et al 2007,), marker-assisted selection (Hamalainen et al. 1997) and phylogenetic studies (Kardolus et al. 1998; Jacobs et al 2008). In particular with complex traits involving many genes, like drought tolerance, the use of molecular markers to identify and locate different genes and genomic regions which influence drought tolerance may help to gain insight in the factors contributing to this trait. These properties and prospects have initiated an increased interest in the application of marker assisted selection (MAS) for improving drought tolerance in many crops. Molecular marker tools can likewise be used for better understanding of different biochemical, molecular and physiological pathways involved in drought tolerance of potato.

QTL studies

Understanding the genetic networks underlying agronomic trait variation will provide new targets for plant breeders. However as they are generally under the control of many genes, those characters are quantitatively variable and their study requires specific strategies and techniques. Moreover, the large variability in the timing and severity of drought stress and the inadequate understanding of its complexity have made it difficult to characterize the physiological and/or phenotypic traits required for screening and selection in order to improve crop performance under drought stress. Quantitative trait locus (QTL) mapping provides a means to dissect complex phenotypic characters such as drought tolerance into their component traits (QTLs), and allows the identification of molecular markers linked to desirable QTL alleles, so that they can be directly used in marker assisted selection (MAS) (Tanksley 1993, Prioul et al 1997).

Most of the QTL mapping studies in potato have been performed on diploid potato populations for a wide range of traits including late blight resistance, insect, pest resistance and agronomic and quality traits such as leaf senescence (Malosetti et al 1994), dormancy (Freyre et al 1994), tuberization (Fernandez-Del-Carmel et al 2007), yield and starch content (Schafer-Pregl et al 1998), tuber shape, eye depth and flesh color (Sliwka et al 2008; van Eck et al 1994 a,b), cold sweetening (Menendez et al 2002) and enzymatic discoloration (Werij et al 2007). Recently QTLs have been identified in tetraploid populations for traits such as plant height, maturity, crop emergence, tuber size, and for quality traits such as after-cooking darkening, regularity of tuber shape, fry color and yield components (Bradshaw et al 2008; D'Hoop et al 2010). However, knowledge about genetics of drought tolerance in potato is still limited, and hardly any QTLs have been identified for potato drought tolerance traits. The genetic dissection of the quantitative traits controlling the adaptive response of potato to drought stress is a prerequisite to allow cost-effective applications of genomics-based approaches to breeding programs aimed at improving the sustainability and stability of yield under adverse conditions.

Genes for drought tolerance in potato

Genetic modification of successful potato cultivars offers the possibility of targeted improvements which can be achieved using two types of genetic resources: the allelic and genic diversity already present in cultivated potatoes and wild relatives, or genes from any other living organism. A large number of genes are known or thought to be involved in plant responses to drought. These include genes involved in signal transduction and transcriptional regulation, biosynthesis of osmolytes and other protectors, and oxidative stress-related genes. Through genetic engineering several of these genes have been well characterized in potato. Potato transgenic plants over-expressing Arabidopsis CBF3 under a stress-inducible rd29A promoter and the DREB transcription factors and associated genetic components exhibited tolerance to drought and other abiotic stresses (Celebi-Toprak et al 2005; Kasuga et al 1999). Compatible osmolytes such as proline and trehalose, accumulate under water stress and their biosynthetic genes have been introduced into potato plants (Yeo et al 2000; Hmida-Sayari et al 2005). In addition transgenic potato plants that generated a high level of the soluble carbohydrate fructan were developed to investigate whether or not water stress could induce proline synthesis in transgenic potato plants. However, these transgenic potato plants did not accumulate proline under water stress (Knipp and Honermeier 2006), suggesting that modification of carbohydrate metabolism might affect water stress-induced proline accumulation. Gene encoding regulatory proteins like StRD22 are also well characterized in potato (Byun et al 2007).

Though many stress responsive genes are characterized in potato, commercial transgenic potato plants that are tolerant to drought have not yet been successful. This may be due to the quantitative nature and multiple loci of genes involved in plant stress tolerance, it is possible that crop growth and yield may not simply be improved through over expression of a single gene.

Genomics approaches

The limited success of the physiological and molecular breeding approaches until now suggests that a careful rethink is needed of the strategies for better understanding and breeding for drought tolerance. Some of the new plant genomic techniques and platforms may allow us to overcome the previous limitations. The tools of genomics offer the means to produce comprehensive datasets on changes in gene expression, protein profiles and

metabolites that result from exposure to drought. The most commonly used approach is transcriptome profiling using microarrays. Recently a high quality expression profiling platform has been established for potato (Kloosterman et al. 2008) and this platform has been successfully employed to capture drought response in Andean potato genotypes (Vasquez-Robinet et al 2008). Three native Andean potato genotypes (SA2563, Sullu and Negra Ojosa) along with two varieties (Cosanera and Atlantic) were well studied for their response to water stress using transcriptome and metabolite analyses. These studies showed that the *S. andigena* genotypes were more resistant to drought than the *S. tuberosum* genotypes and they reported several candidate genes, such as genes involved in osmotic adjustment, in changes in carbohydrate metabolism, membrane modifications and strengthening of cuticle and in cell rescue mechanisms (Schafleitner et al 2007; Vasquez-Robinet et al 2008). These studies provide insights into potato response to water stress at the transcriptional level, yet the genetic regulation of these transcriptional responses is largely unknown.

The availability of the complete Arabidopsis and rice genome sequences together with several plant ESTs has greatly shifted the focus from determining the sequences to understanding their function. Recent work in functional genomics employing genome-wide strategies, such as expression genomics, proteomics and metabolomics has been widely used in model plants to unravel genetic architecture, complex inheritance, and possible interactions with in and with environmental variables. Extension and refinement of these functional genomics will become possible in crop plants such as rice and others (tomato, potato) which are heading towards completion of genome sequences. In addition, small non-coding RNAs have recently been brought into focus as regulators of transcription and post-translational gene silencing. A few reports are available in which their function has been studied in abiotic stress such as mechanical stress responsive miRNAs in *Populus*, phosphate starvation-responsive miRNAs in Arabidopsis, and dehydration, cold, salt and ABA responsive miRNAs in Arabidopsis (Sunkar and Zhu 2004; Fujii et al 2005; Lu et al 2005). In the future more detailed information will become available using the ~omics techniques together with an integrated bioinformatics system. Genome-wide strategies have accelerated the deciphering of complex stress responsive networks, and will help in the identification of key networks and their associated genes, which can be exploited through breeding strategies and genetic engineering.

Objectives and scope of the thesis

In this thesis we made a first step towards identifying the genetic basis for drought tolerance in potato. For this, we use diploid potato populations that have been genetically well characterized (CxE, SHxRH). The main objectives were i) establishing an advanced genetic map and populate the map of CxE with functional molecular markers like SNPs, ii) screening and understanding of physiological responses of CxE potato population for drought tolerance, iii) identification of QTLs for physiological and growth parameters that are affected by drought and/or may contribute to drought tolerance, and iv) to understand the transcriptome response to drought and to identify candidate genes underlying QTLs by genome-wide transcriptome profiling.

In Chapter 2, we develop a pipeline for effective mining of SNPs from public EST databases using QualitySNP software, selection of reliable SNPs and preparation of the loci for analysis on the Illumina GoldenGate genotyping platform. The applicability of the pipeline was demonstrated using publicly available potato EST data, mine the SNPs, genotype individuals from two diploid mapping populations with a 384 SNP array and subsequently map the SNP markers (putative genes) on the respective genetic maps. Using the same approach a 768 SNP array was composed and successfully applied for genotyping the populations. This array was enriched for markers in genes putatively involved in abiotic stress response.

Chapter 3 investigated the possibility of screening a mapping population (CxE) *in vitro* for PEG-induced water deficit stress and recovery potential. Significant variation was observed for genotype response to drought and recovery potential. Several shoot and root growth parameters or traits were measured. The study showed genetic variation and heritability estimates were high to very high for the measured traits depending on growth condition. In order to identify potato QTLs/genes that contribute to drought tolerance and recovery potential, an SNP marker rich integrated linkage map was used. In total 23 QTLs were detected under control, stress and recovery treatments. Interesting putative candidate genes underlying stress response QTLs were identified. The pros and cons of using *in vitro* plants are discussed as well.

Chapter 4 further explores the genetic basis of drought tolerance and presents a

comprehensive QTL analysis for drought tolerance traits in the CxE potato population. The CxE population was extensively evaluated for drought tolerance in two successive years (2008, 2009) under greenhouse conditions by measuring a number of physiological, growth and yield traits. In this study, physiological parameters like Relative Water Content, chlorophyll content, $\delta^{13}C$, and chlorophyll florescence provide rapid indicators and selectable traits for the study of potato in response to water stress. Multi year, multi treatment QTLs were identified for several traits. QTL x Environment interaction was found for traits like leaf δ13C under drought conditions. The response of potato to drought and recovery, important physiological traits to evaluate drought, QTL analysis and their implications for research and breeding are discussed.

In Chapter 5, genome wide eQTL analysis was performed for the drought of potato using whole genome microarray (POCI array) which contains 42,034 features. The genetic architecture of transcript-level variation for drought response was captured in the diploid potato population CxE and mapped as expression QTLs (eQTLs). Genome wide distributions of eQTLs allowed the identification of regulatory hot spots for drought response. To compare the position of genes and their eQTLs and see whether the genetic variation responsible for eQTLs is *cis*- or *trans*-regulated, we anchored the genes to the physical map and genome sequence of potato. Distribution of important genes known to be involved in drought signal transduction, drought-induced transcriptional regulation, and the cellular response to drought are discussed. Interesting results were obtained by combining QTL analysis of phenotypic traits and gene expression traits and examining co-localization of eQTLs and phenotypic QTLs. The advantages of genome wide expression analysis, the complexity and exciting prospects and possibilities by unlocking the information contained in the genome-wide transcriptome dataset are discussed in this chapter.

In Chapter 6, the results from Chapters 2 to 5 are integrated and the implications further explored. The overall retrospect and prospects of breeding for drought tolerance in potato in relation to our findings are discussed.

Chapter 2

A pipeline for high throughput detection and mapping of SNPs from EST databases

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Abstract

Single nucleotide polymorphisms (SNPs) represent the most abundant type of genetic variation that can be used as molecular markers. The SNPs that are hidden in sequence databases can be unlocked using bioinformatic tools. For efficient application of these SNPs, the sequence set should be error-free as much as possible, targeting single loci and suitable for the SNP scoring platform of choice. We have developed a pipeline to effectively mine SNPs from public EST databases with or without quality information using QualitySNP software, select reliable SNP and prepare the loci for analysis on the Illumina GoldenGate genotyping platform. The applicability of the pipeline was demonstrated using publicly available potato EST data, genotyping individuals from two diploid mapping populations and subsequently mapping the SNP markers (putative genes) in both populations. Over 7000 reliable SNPs were identified that met the criteria for genotyping on the GoldenGate platform. Of the 384 SNPs on the SNP array, 88% of SNPs gave detectable signal. For the two potato mapping populations 165 and 185 SNPs segregating SNP loci could be mapped on the respective genetic maps, illustrating the effectiveness of our pipeline for SNP selection and validation.

Key words: EST database, Illumina GoldenGate assay, QualitySNP, Potato

Introduction

Genetic variation is the basis for the biodiversity of life (Schlotterer 2004). Variations in the DNA sequence of genes and their regulatory regions underlie most of the phenotypic variation that has been exploited in modern crops (Bryan et al. 2000; Masouleh et al. 2009). Breeding strategies aiming to improve crop agronomical performance have gained momentum in the last few decades by the use of molecular marker technologies that visualize DNA polymorphisms (Collard et al. 2005). Molecular markers have proven to be extremely useful in breeding, for genome-wide screens for variation, genotype identification and/or fingerprinting, evolutionary and ecological studies.

In breeding programs that are aimed at transferring genes or alleles within or between different species with the aid of molecular markers several steps can be discerned. The first step in this process is the identification of one or more markers closely linked to or within the traits to be introgressed. For this, a high density map of markers on the genome and/or markers in genes that are likely to be involved in the trait of interest can be an invaluable tool. SNPs are very well suited for this purpose. Their astonishing abundance has been reported in

several discovery projects in many species including humans (Sachidanandam R et al.The International SNP Map Working Group 2001), model species such as *Arabidopsis thaliana* (Jander et al. 2002) and *Drosophila melanogaster* (Hoskins et al. 2001) and in crop plants such as barley (Rostoks et al. 2005), maize (Ching et al. 2002), rice (Shen et al. 2004; McNally et al. 2006), soybean (Zhu et al. 2003) and wheat (Ablett et al. 2006). Recent technological advancements in discovery and detection platforms have made SNP markers attractive for high-throughput use not only in model species, but also in crop plants (Rafalski 2002). In species for which no genome sequence is available, large scale SNP discovery has generally relied on sequence variation found in libraries of expressed sequence tags (ESTs) (Somers et al. 2003) or on re-sequencing (Choi et al. 2007).

Several software tools are available for SNP discovery from nucleotide databases, including PolyBayes, AutoSNP, and QualitySNP (Marth 1999; Barker et al. 2003; Tang et al. 2006). QualitySNP is especially useful in extracting reliable SNPs from EST sequence databases that lack quality information, and is in many cases capable of distinguishing paralogs from allelic sequences effectively (Tang et al. 2006). Along with the development of tools to mine a large number of SNPs from nucleotide databases, new SNP genotyping platforms were developed that can analyze a large number of SNPs in parallel in a large set of individuals (Syvanen 2005). An increasing number of reports indicate that the GoldenGate system of Illumina is a reliable and cost-effective SNP genotyping platform. It is capable of multiplexing from 96 to 1536 SNPs in a single reaction (Fan 2003).

In this paper we describe a bioinformatics pipeline starting from SNP discovery in ESTs to genotyping using the Illumina GoldenGate assay. Following SNP discovery, the SNP loci are further screened for suitability to be analyzed with the Illumina GoldenGate Genotyping platform. We demonstrate the applicability of this pipeline for potato, which is the third most important food crop in the world. Potato is a heterozygous crop, and commercial varieties are generally tetraploid. For potato, approximately 200,000 ESTs mainly from three cultivars are publicly available. We show here that SNPs identified by QualitySNP from this collection of SNPs can effectively be turned into markers that can be mapped in different diploid potato mapping populations, showing the versatility of the pipeline and the produced SNP markers. Our results indicate that the pipeline produces a large number of SNP markers, and that the

selection of SNPs for genotyping on the Illumina GoldenGate genotyping platform yields a high number of reliable functional co-dominant markers that can be easily placed on a genetic map.

Materials and methods

Mapping populations

a) SH×RH: A cross between two diploid heterozygous potato clones SH83-92-488 and RH89-039-16 (SH×RH) resulted in an F1 mapping population of 135 individuals (van Os et al. 2006). Using a Selective Mapping strategy (Vision et al. 2000) 57 individuals were selected which captured the highest number of recombination events.

b) C×E: This diploid backcross population consisting of 250 genotypes was obtained from the cross between clones C [USW5337.3; (Hanneman RE 1967)] and E [originally named 77.2102.37; (Jacobsen 1980)]. Clone C is a hybrid between *S. phureja* PI225696.1 and *S. tuberosum* dihaploid USW42. Clone E is the result of a cross between clone C and the *S. vernei-S. tuberosum* backcross clone VH^{3} -4211 (Jacobsen 1978). A set of 94 randomly selected individuals was used for this study, along with the parents of the cross.

DNA extraction

Genomic DNA was isolated from 50-100 mg of young leaves. After freeze-drying, the leaf material was ground using the MM300 Mixer mill (Retsch Inc., Haan Germany) and DNA extraction was performed using the DNeasy 96 Plant Mini kit (Qiagen, Valencia, California, USA) according to the manufacturer's protocol.

SNP identification pipeline designed for the GoldenGate genotyping platform

For SNP discovery, 219,765 EST reads were downloaded from the EMBL database [(http://www.ebi.ac.uk/embl] (version 88). Functional annotation of the ESTs was obtained from the TIGR gene index (http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=potato or UniGene (Wheeler et al. 2003) and additional BLASTN and BLASTX analyses (Altschul et al. 1990). The ESTs were aligned into contigs and analyzed for true SNPs using the QualitySNP software (http://www.bioinformatics.nl/tools/snpweb), with D-value set at 0.6 and default values for quality regions and other filters as described by Tang et al. (2006). The resulting data are

stored in a 'contig database'. In an additional routine/programme the QualitySNP output was analyzed for SNP loci flanked by 30-50nt reliable sequences on each side to allow for assay development using the Illumina GoldenGate design tool. The output was formatted to fit the requirements for the assay design tool (http://www.illumina.com/downloads/GoldenGateDesign_TechNote.pdf) and stored in the '100 bp fragment' database. As for potato no reference genome sequence is available we performed an additional BLAST analysis to eliminate fragments that have more than 90% homology with each other to maximize the chances of single locus amplification. Only fragments occurring once in the contig database and with less than 90% similarity to all other contigs in the database were maintained and considered for the GoldenGate assay development.

Selection of SNPs for the Illumina GoldenGate Assay

A selection of the SNP loci was made based on putative gene functions in abiotic, biotic stress responses, metabolic and biosynthesis pathways. Functional annotations were taken from the EST annotations in the DFCI potato gene index (hosted at http://compbio.dfci.harvard.edu/tgi/ as part of The Gene Index Project). For some genes several SNPs within the same gene were selected. GoldenGate primers were designed using Illumina's design tool and SNP scoring was performed by Service XS (Leiden, The Netherlands), using Illumina's high-density array technology for standard or custom SNP genotyping of 96 samples. For each sample 250 ng of DNA was used for genotyping with the Illumina standard GoldenGate protocol (Shen et al. 2005). Our experimental setup included two separate genotyping runs; one for the SH×RH population, including the parents C and E, and one for the C×E population, again including the C and E parents.. The data was analyzed using Gencall software (Illumina, San Diego, CA) which is integrated in the Illumina bead station package (http://www.illumina.com/Documents/products/technotes/technote_gencall_data_analysis_software.pdf) (Shen et al. 2005).

Additional molecular marker development

AFLP markers were generated according to standard protocols with radioactive labels, using 4 Eco-Mse primer combinations (Vos et al. 1995). Bands were scored as present or absent. AFLP markers were encoded by standard AFLP marker coding, with an ID and a

chromosomal location; for example E39M60-40c10 is a marker from the Eco39 primer and a Mse60 primer, ID number 40 and mapped on Chromosome 10. The SSR markers used in this study were obtained from different sources (Milbourne et al. 1998; Feingold et al. 2005). The CAPS markers were developed for interesting candidate genes with (putative) functions in amongst others quality traits in the CxE population (manuscript in preparation by Werij et al.).

Genetic mapping

The potato SNP markers were first mapped in the two mapping populations using JoinMap 4.0 (Van Ooijen 2006) together with AFLP (only 1:1 segregating markers), SSRs and CAPS as backbone markers. SNP markers were also mapped on the existing SH×RH genetic map using a bin mapping approach (van Os et al. 2006).

Results

Potato SNP array construction

In the 219,765 EST sequences 12,184 reliable SNPs were discovered. A set of 7592 SNPs remained after extra filters were set to select for SNP loci with flanking regions suitable for primer design in Illumina's Goldengate assay (at least 30-50nt flanking sequence on each side, no SNPs detected in the flanking regions and no other sequences that are more than 90% similar present in other contigs/clusters).

The last selection of 384 SNPs for the Illumina array was based on putative functions of the genes containing the SNP loci as deduced from annotations at the DFCI potato gene index website (hosted at http://compbio.dfci.harvard.edu/tgi/). The final selection (hereafter called 384PotSNP array) of SNP markers with their putative functions, locations along with their database ID's (TC numbers) is provided in Supplementary Table.

Evaluation of the 384PotSNP array

The 384PotSNP array was evaluated by genotyping two diploid potato populations and mapping the SNP markers. The quality of each SNP is reflected in the Gencall (GC) score, a value between 0 and 1 (Shen et al. 2005). The Gencall score is a representation of the separation between the heterozygote and homozygote clusters for a particular SNP, and how a SNP score is placed in these clusters. R values below 0.2 generally indicate failed SNP

detection, while scores above 0.5 are considered as highly reliable SNP scores. For C×E, 45 SNPs (12%) did not produce a detectable signal or the signal was too low to use it as a reliable marker. Forty-two of these (11%) were also not successful in SH×RH, indicating that these were SNPs for which the assay was not working. Another 7 SNPs did not produce a good result in SH×RH. Of the remaining 339 SNPs in C×E, 173 were not polymorphic between both parents and did not show a segregating polymorphism. For SH×RH, 149 markers were not polymorphic between both parents. Ninety markers were not polymorphic in both the populations.

Six markers in C×E were homozygous in both parents and polymorphic between the parents with a uniform heterozygous offspring (AA×BB→AB). Another set of 6 markers that were homozygous in both parents C and E, polymorphic between parents and segregated according to a 1:2:1 Mendelian ratio (AB×AB→AA, AB and BB). Table 1 summarizes the results of the 384PotSNP array for both populations.

Table 1: Results of 384 PotSNP array performed in two (C×E and SH×RH) independent assays

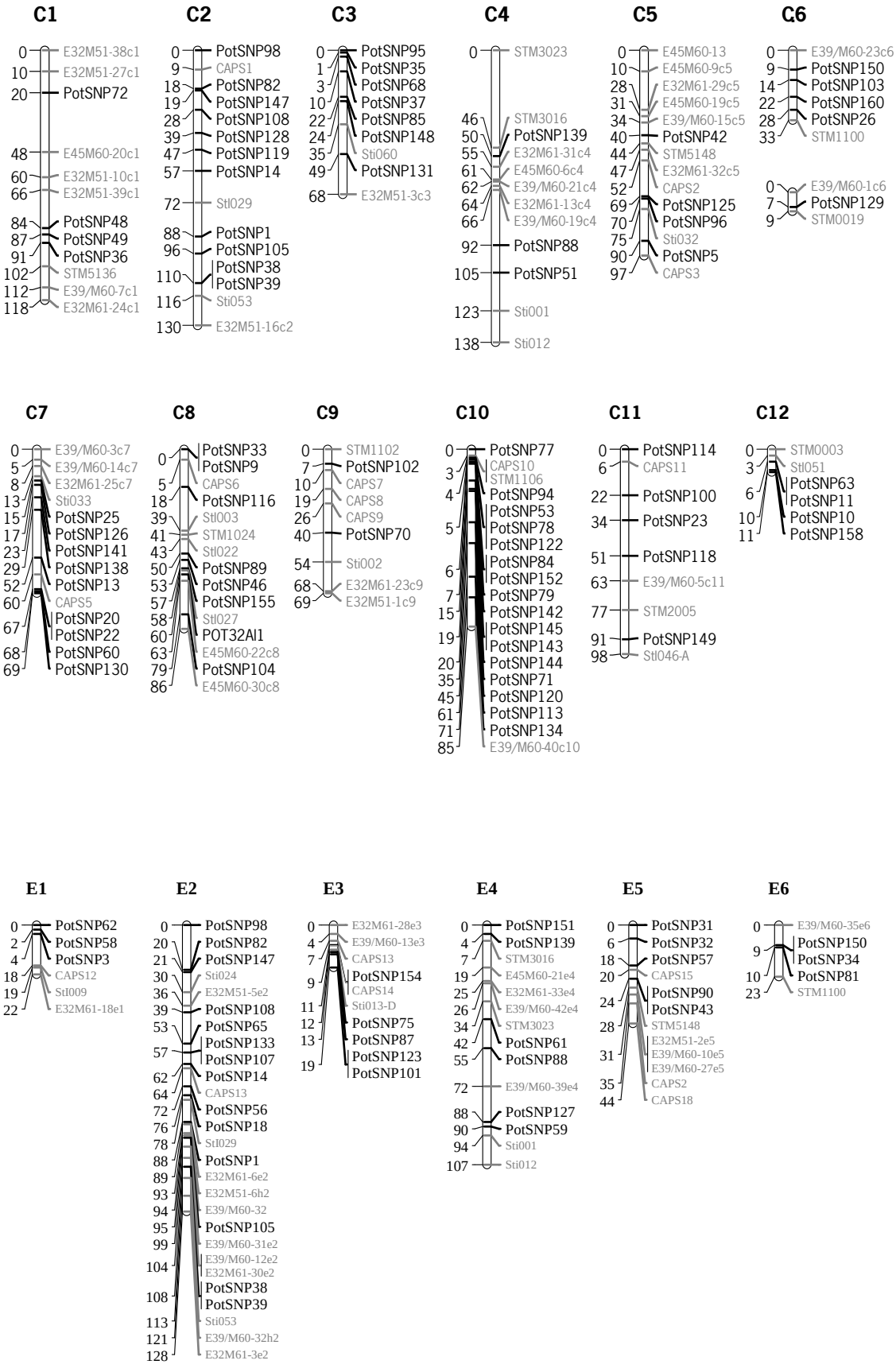
384 PotSNP array	*Mapping*
309 out of the 384 are useful markers (80%) 42 dropped out in any sample (11%) 33 were monomorphic in all material [1](9%)	165 markers could be mapped in C×E 186 markers could be mapped in SH×RH 99 markers could be mapped in both populations

[1] Including a set of 220 tetraploid varieties

Genetic mapping of SNP loci

CxE:

Out of 165 polymorphic SNP markers, fifty were heterozygous only in parent C; 59 were heterozygous only in parent E and 56 segregating markers were heterozygous in both the parents (AB×AB→AA, AB, BB). These 165 SNP markers were placed on parental genetic linkage maps using Joinmap 4.0 (van Ooijen 2006) together with 93 AFLPs, 45 SSRs, and 24 CAPS markers. Only markers with LOD scores of 3.0 and above were considered. Thirteen and 12 linkage groups were obtained in C and E parental maps, respectively (Fig 1). Linkage group 6 was divided into two subgroups in the maternal (C parent) map. Nineteen of the 165 SNP markers could not be assigned to a parental linkage group. The C and E genetic parental map span 1012.4cM and 774.6cM respectively with average distance between adjacent loci 7.2 and 4.5cM.


C1
0 E32M51-38c1
10 E32M51-27c1
20 PotSNP72
48 E45M60-20c1
60 E32M51-10c1
66 E32M51-39c1
84 PotSNP48
87 PotSNP49
91 PotSNP36
102 STM5136
112 E39/M60-7c1
118 E32M61-24c1
C2
0 PotSNP98
9 CAPS1
18 PotSNP82
19 PotSNP147
28 PotSNP108
39 PotSNP128
47 PotSNP119
57 PotSNP14
72 Stl029
88 PotSNP1
96 PotSNP105
110 PotSNP38
PotSNP39
116 Sti053
130 E32M51-16c2
C3
0 PotSNP95
1 PotSNP35
3 PotSNP68
10 PotSNP37
22 PotSNP85
24 PotSNP148
35 Sti060
49 PotSNP131
68 E32M51-3c3
C4
0 STM3023
46 STM3016
50 PotSNP139
55 E32M61-31c4
61 E45M60-6c4
62 E39/M60-21c4
64 E32M61-13c4
66 E39/M60-19c4
92 PotSNP88
105 PotSNP51
123 Sti001
138 Sti012
C5
0 E45M60-13
10 E45M60-9c5
28 E32M61-29c5
31 E45M60-19c5
34 E39/M60-15c5
40 PotSNP42
44 STM5148
47 E32M61-32c5
52 CAPS2
69 PotSNP125
70 PotSNP96
75 Sti032
90 PotSNP5
97 CAPS3
C6
0 E39/M60-23c6
9 PotSNP150
14 PotSNP103
22 PotSNP160
28 PotSNP26
33 STM1100
0 E39/M60-1c6
7 PotSNP129
9 STM0019
C7
0 E39/M60-3c7
5 E39/M60-14c7
8 E32M61-25c7
13 Sti033
15 PotSNP25
17 PotSNP126
23 PotSNP141
29 PotSNP138
52 PotSNP13
60 CAPS5
67 PotSNP20
PotSNP22
68 PotSNP60
69 PotSNP130
C8
0 PotSNP33
PotSNP9
5 CAPS6
18 PotSNP116
39 Stl003
41 STM1024
43 Stl022
50 PotSNP89
53 PotSNP46
57 PotSNP155
58 Stl027
60 POT32Al1
63 E45M60-22c8
79 PotSNP104
86 E45M60-30c8
C9
0 STM1102
7 PotSNP102
10 CAPS7
19 CAPS8
26 CAPS9
40 PotSNP70
54 Sti002
68 E32M61-23c9
69 E32M51-1c9
C10
0 PotSNP77
3 CAPS10
STM1106
4 PotSNP94
PotSNP53
5 PotSNP78
PotSNP122
6 PotSNP84
PotSNP152
7 PotSNP79
15 PotSNP142
19 PotSNP145
PotSNP143
20 PotSNP144
35 PotSNP71
45 PotSNP120
61 PotSNP113
71 PotSNP134
85 E39/M60-40c10
C11
0 PotSNP114
6 CAPS11
22 PotSNP100
34 PotSNP23
51 PotSNP118
63 E39/M60-5c11
77 STM2005
91 PotSNP149
98 Stl046-A
C12
0 STM0003
3 Stl051
PotSNP63
6 PotSNP11
10 PotSNP10
11 PotSNP158
E1
0 PotSNP62
2 PotSNP58
4 PotSNP3
18 CAPS12
19 Stl009
22 E32M61-18e1
E2
0 PotSNP98
20 PotSNP82
21 PotSNP147
30 Sti024
36 E32M51-5e2
39 PotSNP108
53 PotSNP65
PotSNP133
57 PotSNP107
62 PotSNP14
64 CAPS13
72 PotSNP56
76 PotSNP18
78 Stl029
88 PotSNP1
89 E32M61-6e2
93 E32M51-6h2
94 E39/M60-32
95 PotSNP105
99 E39/M60-31e2
E39/M60-12e2
104 E32M61-30e2
PotSNP38
108 PotSNP39
113 Sti053
121 E39/M60-32h2
128 E32M61-3e2
E3
0 E32M61-28e3
4 E39/M60-13e3
7 CAPS13
9 PotSNP154
CAPS14
Sti013-D
11 PotSNP75
12 PotSNP87
13 PotSNP123
19 PotSNP101
E4
0 PotSNP151
4 PotSNP139
7 STM3016
19 E45M60-21e4
25 E32M61-33e4
26 E39/M60-42e4
34 STM3023
42 PotSNP61
55 PotSNP88
72 E39/M60-39e4
88 PotSNP127
90 PotSNP59
94 Sti001
107 Sti012
E5
0 PotSNP31
6 PotSNP32
18 PotSNP57
20 CAPS15
PotSNP90
24 PotSNP43
28 STM5148
E32M51-2e5
31 E39/M60-10e5
E39/M60-27e5
35 CAPS2
44 CAPS18
E6
0 E39/M60-35e6
9 PotSNP150
PotSNP34
10 PotSNP81
23 STM1100

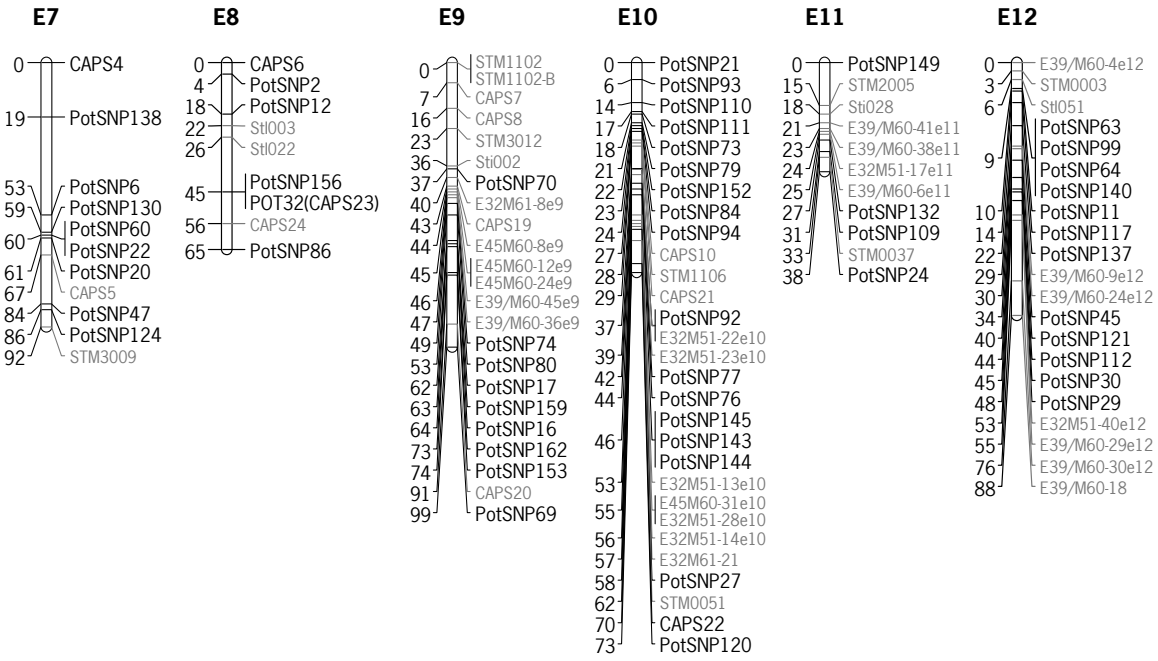


Fig. 1 Location of the SNP markers on parental maps C and E. The number on the left side is the genetic distance in centiMorgans (cM) right side is marker designations. The parental maps were drawn by the MapChart 2.2 program (Voorrips 2002)

SHxRH:

A set of 151 AFLP markers from the same four AFLP primer enzyme combinations used in the C×E population were selected from the ~10,000 available AFLP markers in SH×RH. Parental maps of SH and RH were constructed with 15 SSR and 24 CAPS, 151 AFLP and 186 SNP markers using Joinmap 4.0 (van Ooijen 2006). Out of 186 polymorphic SNP markers, 71 were heterozygous in parent SH; 69 were heterozygous in parent RH and 46 segregating markers were heterozygous in both parents (AB×AB→AA, AB, BB). Table 2 lists the markers used for mapping in both populations.

Twelve parent specific linkage groups were obtained for both SH and RH (Fig. 2) The Linkage group RH01 was divided into two subgroups. In SH the length of the linkage groups ranged from 52.6 cM to 115.9 with the average distance between the loci of 4.05 cM. The RH parental map spans 686.7cM and the average distance between loci is 3.8 cM.

To confirm the SH×RH SNP markers with their bin signatures to calculate error frequency of our mapping results, we compared the marker segregation pattern with the map segregation patterns (bin signatures) and placed these 186 SNP markers in the ultra dense potato map (Van Os et al. 2006). All of the markers were anchored to the bins of the highly saturated parental reference maps and distributed over all linkage groups. Marker order was identical to

Table 2: Number of markers used for construction of C and E parental maps according to marker type.

Marker type	Total markers used in construction of parental maps		Markers on the map	
	C and E	SH and RH	C and E	SH and RH
SNP markers	165	186	146	168
AFLP markers	93	151	82	131
SSR markers	45	16	33	16
CAPS markers	24	21	22	21

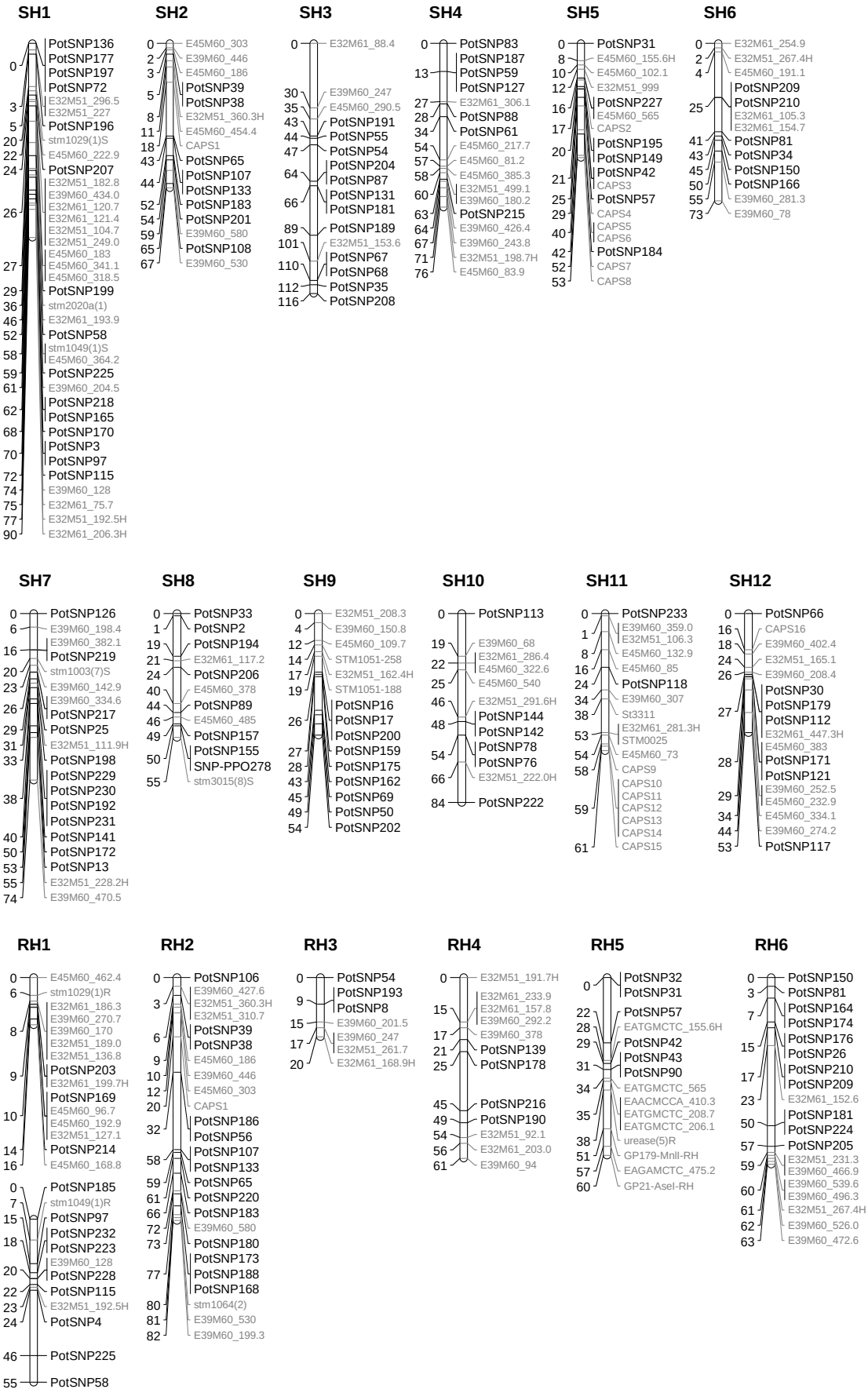
the map positions in the parental maps constructed in this study. Bin mapping procedure not only gives the bin position but also the goodness of fit to that position. Distance to the bin is a measure for the number of singletons or error in the data. Out of 186 markers, 183 showed error scores below 0.1%, the remaining 3 markers had error scores more than 0.1 and showed LOD scores less than 3.

There were 99 markers segregating in both the potato populations. These markers were used to align the C×E with SH×RH maps (Fig. 3), linking the genetic loci of the C×E population are aligned to the ultra dense map and the increasing amount of genomic sequence information of clone RH generated by the Potato Genome Sequencing Consortium (PGSC, http://www.potatogenome.net/).

Discussion

This paper describes the successful development and implementation of a bioinformatics pipeline for the identification of putative SNPs in public EST databases, to convert these SNPs in assays compatible to the Illumina GoldenGate SNP platform, and to map the SNP markers using this genotyping platform. The identification and selection of potato SNPs for the GoldenGate assay results in a score of 89% of working GoldenGate assays, and at least 77% of the full electronic SNP dataset are true SNPs amenable to the GoldenGate genotyping platform.

The first step of this pipeline is the identification of putative SNPs, for which we used QualitySNP. For many SNP assays, including Illumina's GoldenGate assays, the SNP locus needs to be amplified with locus-specific primers that do not amplify any other locus. The paralogous sequences that are placed in separate clusters by QualitySNP may be putative binding targets of the SNP amplification primers designed for a SNP detected in the allelic clusters. The Illumina design tool can eliminate paralogous sequences only when a fully


SH1
0 PotSNP136
PotSNP177
PotSNP197
PotSNP72
3 E32M51_296.5
E32M51_227
5 PotSNP196
20 stm1029(1)S
22 E45M60_222.9
24 PotSNP207
E32M51_182.8
E39M60_434.0
26 E32M61_120.7
E32M61_121.4
E32M51_104.7
E32M51_249.0
E45M60_183
27 E45M60_341.1
E45M60_318.5
29 PotSNP199
36 stm2020a(1)
46 E32M61_193.9
52 PotSNP58
stm1049(1)S
58 E45M60_364.2
59 PotSNP225
61 E39M60_204.5
62 PotSNP218
PotSNP165
68 PotSNP170
70 PotSNP3
PotSNP97
72 PotSNP115
74 E39M60_128
75 E32M61_75.7
77 E32M51_192.5H
90 E32M61_206.3H
SH2
0 E45M60_303
2 E39M60_446
3 E45M60_186
5 PotSNP39
PotSNP38
8 E32M51_360.3H
11 E45M60_454.4
18 CAPS1
43 PotSNP65
PotSNP107
44 PotSNP133
52 PotSNP183
54 PotSNP201
59 E39M60_580
65 PotSNP108
67 E39M60_530
SH3
0 E32M61_88.4
30 E39M60_247
35 E45M60_290.5
43 PotSNP191
44 PotSNP55
47 PotSNP54
PotSNP204
64 PotSNP87
PotSNP131
66 PotSNP181
89 PotSNP189
101 E32M51_153.6
PotSNP67
110 PotSNP68
112 PotSNP35
116 PotSNP208
SH4
0 PotSNP83
PotSNP187
13 PotSNP59
PotSNP127
27 E32M61_306.1
28 PotSNP88
34 PotSNP61
54 E45M60_217.7
57 E45M60_81.2
58 E45M60_385.3
60 E32M51_499.1
E39M60_180.2
63 PotSNP215
64 E39M60_426.4
67 E39M60_243.8
71 E32M51_198.7H
76 E45M60_83.9
SH5
0 PotSNP31
8 E45M60_155.6H
10 E45M60_102.1
12 E32M51_999
PotSNP227
16 E45M60_565
17 CAPS2
PotSNP195
20 PotSNP149
PotSNP42
21 CAPS3
25 PotSNP57
29 CAPS4
40 CAPS5
CAPS6
42 PotSNP184
52 CAPS7
53 CAPS8
SH6
0 E32M61_254.9
2 E32M51_267.4H
4 E45M60_191.1
PotSNP209
25 PotSNP210
E32M61_105.3
E32M61_154.7
41 PotSNP81
43 PotSNP34
45 PotSNP150
50 PotSNP166
55 E39M60_281.3
73 E39M60_78
SH7
0 PotSNP126
6 E39M60_198.4
E39M60_382.1
16 PotSNP219
20 stm1003(7)S
23 E39M60_142.9
E39M60_334.6
26 PotSNP217
29 PotSNP25
31 E32M51_111.9H
33 PotSNP198
PotSNP229
PotSNP230
38 PotSNP192
PotSNP231
40 PotSNP141
50 PotSNP172
53 PotSNP13
55 E32M51_228.2H
74 E39M60_470.5
SH8
0 PotSNP33
1 PotSNP2
19 PotSNP194
21 E32M61_117.2
24 PotSNP206
40 E45M60_378
44 PotSNP89
46 E45M60_485
49 PotSNP157
PotSNP155
50 SNP-PPO278
55 stm3015(8)S
SH9
0 E32M51_208.3
4 E39M60_150.8
12 E45M60_109.7
14 STM1051-258
17 E32M51_162.4H
19 STM1051-188
PotSNP16
26 PotSNP17
PotSNP200
27 PotSNP159
28 PotSNP175
43 PotSNP162
45 PotSNP69
49 PotSNP50
54 PotSNP202
SH10
0 PotSNP113
19 E39M60_68
E32M61_286.4
22 E45M60_322.6
25 E45M60_540
46 E32M51_291.6H
PotSNP144
48 PotSNP142
PotSNP78
54 PotSNP76
66 E32M51_222.0H
84 PotSNP222
SH11
0 PotSNP233
E39M60_359.0
1 E32M51_106.3
8 E45M60_132.9
16 E45M60_85
24 PotSNP118
34 E39M60_307
38 St3311
E32M61_281.3H
53 STM0025
54 E45M60_73
58 CAPS9
CAPS10
CAPS11
59 CAPS12
CAPS13
CAPS14
61 CAPS15
SH12
0 PotSNP66
16 CAPS16
18 E39M60_402.4
24 E32M51_165.1
26 E39M60_208.4
PotSNP30
27 PotSNP179
PotSNP112
E32M61_447.3H
E45M60_383
28 PotSNP171
PotSNP121
29 E39M60_252.5
E45M60_232.9
34 E45M60_334.1
44 E39M60_274.2
53 PotSNP117
RH1
0 E45M60_462.4
6 stm1029(1)R
E32M61_186.3
E39M60_270.7
8 E39M60_170
E32M51_189.0
E32M51_136.8
PotSNP203
9 E32M61_199.7H
PotSNP169
10 E45M60_96.7
E45M60_192.9
E32M51_127.1
14 PotSNP214
16 E45M60_168.8
0 PotSNP185
7 stm1049(1)R
15 PotSNP97
PotSNP232
18 PotSNP223
E39M60_128
20 PotSNP228
22 PotSNP115
23 E32M51_192.5H
24 PotSNP4
46 PotSNP225
55 PotSNP58
RH2
0 PotSNP106
E39M60_427.6
3 E32M51_360.3H
E32M51_310.7
PotSNP39
6 PotSNP38
9 E45M60_186
10 E39M60_446
12 E45M60_303
20 CAPS1
PotSNP186
32 PotSNP56
58 PotSNP107
PotSNP133
59 PotSNP65
61 PotSNP220
66 PotSNP183
72 E39M60_580
73 PotSNP180
PotSNP173
77 PotSNP188
PotSNP168
80 stm1064(2)
81 E39M60_530
82 E39M60_199.3
RH3
0 PotSNP54
9 PotSNP193
PotSNP8
15 E39M60_201.5
17 E39M60_247
20 E32M51_261.7
E32M61_168.9H
RH4
0 E32M51_191.7H
15 E32M61_233.9
E32M61_157.8
E39M60_292.2
17 E39M60_378
21 PotSNP139
25 PotSNP178
45 PotSNP216
49 PotSNP190
54 E32M51_92.1
56 E32M61_203.0
61 E39M60_94
RH5
0 PotSNP32
PotSNP31
22 PotSNP57
28 EATGMCTC_155.6H
29 PotSNP42
PotSNP43
31 PotSNP90
34 EATGMCTC_565
EAACMCCA_410.3
35 EATGMCTC_208.7
EATGMCTC_206.1
38 urease(5)R
51 GP179-MnlI-RH
57 EAGAMCTC_475.2
60 GP21-AseI-RH
RH6
0 PotSNP150
3 PotSNP81
PotSNP164
7 PotSNP174
PotSNP176
15 PotSNP26
PotSNP210
17 PotSNP209
23 E32M61_152.6
PotSNP181
50 PotSNP224
57 PotSNP205
59 E32M51_231.3
E39M60_466.9
60 E39M60_539.6
E39M60_496.3
61 E32M51_267.4H
62 E39M60_526.0
63 E39M60_472.6

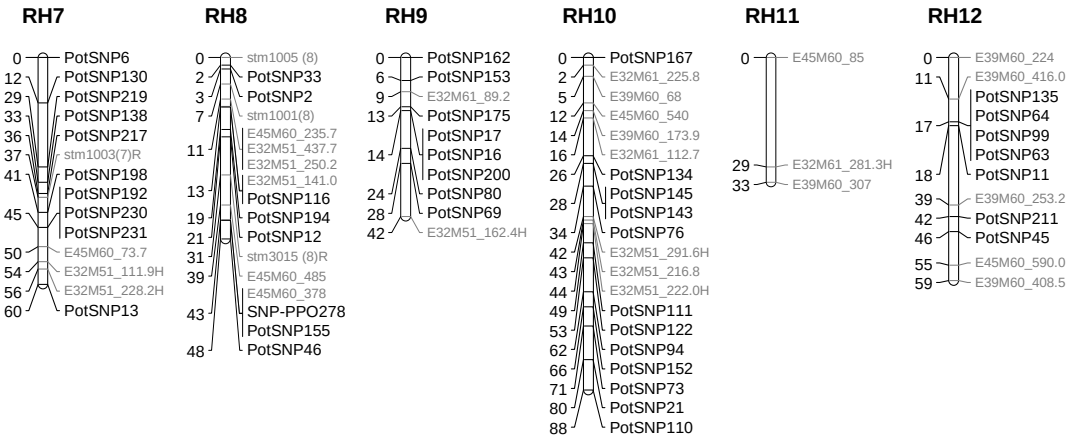


Fig. 2 Location of the SNP markers on parental maps SH and RH. The number on the left side is the genetic distance in centiMorgans (cM) right side is marker designations. The parental maps were drawn by the MapChart 2.2 program (Voorrips 2002)

sequenced reference genome is available. However, this is currently not the case with many crop species like potato. To circumvent this problem of paralogous sequences, our pipeline includes a similarity search using the flanking sequences of the SNP to identify clusters with high similarity to the SNP locus. In this study we eliminate SNPs for which the similarity search found other clusters with more than 90% similarity. This implies that SNPs are eliminated that might be suitable for a SNP assay if the primer binding sites are carefully chosen. If a SNP in a specific gene is required, or only a limited number of SNPs have been identified, it may be worthwhile to look into the SNP loci for which a similarity conflict has been identified, and design primers for these SNPs. However, we intend this pipeline to be used for high through-put analysis of SNPs from databases to produce a genome-wide SNP array. For efficiency purposes, the SNP loci that might be problematic for GoldenGate assays are therefore eliminated from the list that is used for SNP selection for the SNP array rather than evaluated manually.

Performance of the 384PotSNP array

Of the 384 SNPs that we assembled on a GoldenGate SNP genotyping array and used for genotyping two diploid potato mapping populations only 42 SNPs (11%) failed to produce an interpretable output in two separately performed assays. There are several possible explanations for these SNPs to fail. Firstly, failure may be due to incorrect primer synthesis. In other studies it was observed that 10% of validated SNP loci do not give a result in standard GoldenGate assays, pointing to failure as a result from the assay design (Rostoks et

al. 2006, Hyten et al. 2008). Secondly, the SNP frequency in potato is exceptionally high, and therefore SNPs in the primer target sequences could be common. This will likely affect primer annealing as well as signal interpretation. Thirdly, this might have been caused by large introns within the amplicon or introns at the primer sites; the amplification primers were designed on EST sequence information, whereas the SNP assays were performed on genomic DNA. Presence of introns in the SNP amplified region can be detected if genomic sequence information of the SNP locus is available from potato or related species. This was the case for only four of the SNPs with failing assays. Nevertheless, for two out of those four indeed an intron was present within the SNP amplified locus, indicating that in approximately 50% of the failing SNP assays intron presence may be the cause of failure. With the advent of an available genome sequence for potato, the detection of intron-exon boundaries in the vicinity of a SNP will be possible for most if not all SNP loci selected for the array. This information can then be used for primer design, either by filtering out the SNP loci with introns near the SNP site, or by designing primers based on genomic sequence. However, assays with primers based on intron sequences may be more prone to failure than assays with primers in exon sequences, as intron sequences are more variable than exon sequences.

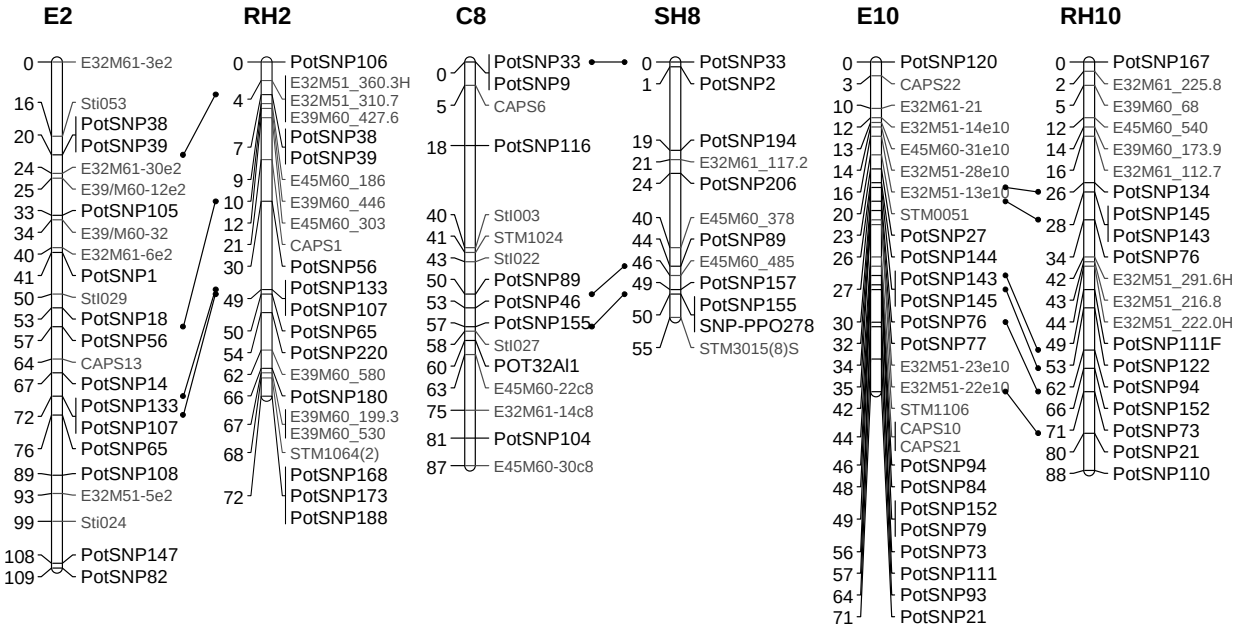


Fig. 3 Alignment of C8 linkage group with the SH8, and E2, E10 with the RH2, RH10 linkage groups respectively using markers common to both populations. Left side number indicates genetic distances in centiMorgan (cM), right side marker designations

We have shown that the SNP GoldenGate assay linked to the pipeline is a proficient strategy for SNP genotyping in potato, with SNP markers successfully mapped in two potato populations. In total, 342 out of 384 SNP account for the 89% success rate of the combination of QualitySNP with the GoldenGate assay which is comparable to the 90% success rate previously reported in barley (Rostoks et al. 2006) and 89% in soybean (Hyten et al. 2008). However, the barley SNP array from Rostoks et al. (2006) is based on resequencing of selected genes with the parents of a mapping population included, whereas the 384PotSNP array contains SNPs from whatever information available in the EST databases, demonstrating the effectiveness of our pipeline in selecting SNPs that are likely to produce reliable data on the GoldenGate genotyping platform.

Level of polymorphism

The PotSNP array permits the rapid generation of a high number of polymorphic markers. Out of 339 SNPs in CxE (342 in SH×RH), 164 could be mapped (186 in SH×RH). In CxE 161 SNP (155 SH×RH) were monomorphic. The high number of monomorphic SNPs is not surprising; the SNPs were discovered in ESTs from only three varieties namely Shepody, Kennebec and Bintje and the parents in the mapping population are not directly related to any of these varieties. Preliminary data obtained using the potato genotyping array with potato cultivars indicated that 60% of these non-segregating SNP loci were in fact polymorphic in a large cultivar set (data not shown) indicating that these are true SNPs. Six markers in C×E were homozygous in both parents and polymorphic between the parents, with heterozygous offspring. For the population C×E this is a highly unexpected result, as C×E is a backcross population (C is a parent of E). At least one allele of parent E should have been inherited from C, so an AA×BB→AB genetic model for these six loci should not be possible. Neither could this be caused by primer annealing polymorphisms creating a null allele (A0×B0) as this would result in a segregating rather than a uniform offspring. Similarly, for nine SSRs four alleles were detected in the C×E population. These markers could be mapped consistent with an AB×CD→AC, AD, BC, BD genetic model for these 9 loci so this is not an artifact. We currently do not have a satisfying explanation for these observations.

Another set of 6-8 markers that were homozygous in both parents and polymorphic between parents segregated in the population consistent with e.g. an A0×B0→A0, AB, B0 genetic model. These were found in both populations, and may represent markers with null alleles in

one or both parents. These null alleles may be caused for instance by SNPs or other polymorphisms in one of the alleles that interfere with primer binding and/or subsequent amplification. The results from both runs with the 384PotSNP array were highly comparable, indicating that the reproducibility of the GoldenGate assay is high.

Mapping

The SNP markers in both the potato populations are well distributed over the chromosomes, with minimal clustering. In the parental map of RH linkage group RH01 was divided into two subgroups most likely because the number of markers was not high enough. For some genes more than one SNP within the same open reading frame was selected, for instance SNP38, SNP39 (from ESTs identical to *S. tuberosum* clone transcription factor APFI-like mRNA, TC1649610) and SNP143, 144, 145 (from ESTs identical to *S. tuberosum* StPDC mRNA for pyruvate decarboxylase, TC167230). The SNP markers originating from a single gene all mapped at the same positions. PotSNP156 is located in the coding region of the POT32 gene, and maps at the same position as the CAPS marker POT32A developed for the same gene on chromosome 8 by Werij et al. (2007). For some of the SNP marker loci (genes), the chromosomal location was already known either in potato or in the related species tomato. For each of those markers, the mapping positions agreed with the published mapping positions of the genes. For example PotSNP002 mapped on chromosome 8 in our two populations, and is nearly identical to tomato clone 132639F which also maps on chromosome 8 of tomato. PotSNP009 on chromosome 8 showed a high homology with tomato BiP/grp78 gene, also located on tomato chromosome 8.

In the SH×RH population the positions of 186 markers were confirmed by placing them on the ultra dense bin map (Van Os et al, 2006). Most (98.4%) of the polymorphic markers showed error scores below 0.1%. Hence, the Illumina GoldenGate assay is capable of producing high number of error free markers. These SNP markers can be used not only to align CxE map with SH×RH but also as anchors in the potato physical map (Van Os et al, 2006).

Perspectives

Our bioinformatics pipeline produced over 7500 SNPs using the EST dataset that are amenable to be assayed on the GoldenGate genotyping platform. Therefore, it is reasonable to

expect that more than 7000 remaining SNPs will produce a similar percentage of true and technically scorable SNPs as obtained from the current pilot of 384 SNPs, and are a valuable source for SNP markers in potato populations and cultivars. The EST dataset that was used to mine the SNPs contains sequences from four cultivars: Bintje, Kennebec, Shepody and Kuras. Bintje is an ancient cultivar; Kennebec is a variety from the USA with a pedigree that differs significantly from Bintje. Shepody and Kennebec have a pedigree that is partly overlapping. Especially the parents of the C×E population are only distantly related to these cultivars, but still half of the SNP markers generated by the pipeline are polymorphic in the population. This illustrates the wide usability for mapping, association, marker assisted breeding and biodiversity studies of SNP marker assays such as offered by the GoldenGate platform.

Conclusion

The combined use of Quality SNP and Illumina GoldenGate assay in a pipeline has proven to be an efficient tool for the construction of a genetic linkage map. The pipeline produces a large number of co-dominant, polymorphic loci rapidly with a good distribution of markers over the chromosomes. The SNP markers have been selected from EST sequences which were annotated based on sequence similarity to genes with a known function, or in an isolated case based on gene function in potato. The SNP based genetic map therefore allows a candidate gene-based QTL mapping approach. This SNP array offers markers in genes with a variety of putative functions, including biotic and abiotic stress tolerance. Marker assisted breeding with such SNP markers can accelerate the improvement of potato for important traits.

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Chapter 3

In vitro screening and QTL analysis for drought tolerance in diploid potato

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Submitted

Abstract

Drought stress is a major abiotic constraint limiting crop production worldwide. Screening for drought tolerance and the traits that enhance drought tolerance is not straightforward in large mapping populations. In this study, we investigated the possibility of screening a mapping population *in vitro* for PEG-induced water deficit stress and recovery potential. We have measured several shoot and root growth parameters or traits in the CxE diploid potato mapping population. Significant variation was observed for genotype-specific responses to water deficit and recovery potential. Genetic variation and heritability estimates were high to very high for the measured traits depending on growth conditions. In order to identify potato QTLs for drought tolerance and recovery potential an SNP marker-rich integrated linkage map was used. A total of 23 QTLs were detected under control, stress and recovery treatments explaining 10.3 to 22.4% of the variance for each phenotypic trait. Among these, 10 QTLs were located on chromosome 2. Three QTLs involved in the important trait root to shoot ratio were identified on linkage groups 2, 3 and 8. These loci explained together 41.1% of the variance for this trait, and may be breeding targets for stress tolerance and yield in the field as well. The SNP markers derived from EST sequences underlying these QTLs led to the identification of putative candidate genes for further study in potato. This study constitutes the first knowledge of *in vitro* screening of a mapping population for drought tolerance in potato.

Key words: Drought, *In vitro*, PEG, Potato, QTL, Recovery

Introduction

Drought is one of the most common environmental stresses affecting plant growth and productivity (Boyer, 1982). The consequences of water deficit include adverse effects on plant phenology, phasic development, growth, carbon assimilation, assimilate partioning and plant reproduction. Climate models have indicated that drought episodes will become more frequent because of long-term effects of global warming (Salinger et al 2005; Cook et al 2007) emphasizing the urgent need to develop adaptive agricultural strategies for a changing environment. These strategies range from changes in traditional management and agronomic practices to the use of marker assisted selection for the improvement of drought-related traits and the development of transgenic crops with enhanced tolerance to drought and improved water use efficiency that might minimize drought-related yield losses and ensure food

production for a growing population of humans.

Plants utilize various resistance mechanisms in response to drought stress. These mechanisms range from adapting whole plant characteristics such as life cycle timing (maturity), deep rooting, to cellular-level functions (osmoregulation) (Mohamed et al 2000). A single trait alone may not ensure successful survival; however, it may enhance water stress resistance. Plant cell and tissue culture has been a useful tool to study stress tolerance mechanisms under *in vitro* conditions. *In vitro* techniques can make it possible to screen a large number of genotypes rapidly for stress tolerance since *in vitro* plant cultures even at different stages of development may exhibit their capacity to withstand stress (Gosal and Bajaj 1984; Tewary et 2000). Polyethylene Glycol (PEG) of high molecular weights has been widely used to simulate drought stress in plants as a non-penetrating osmotic agent, lowering the water potential in a way similar to soil drying (Larher et al 1993). Although there are biochemical, genetic and physiological constraints in obtaining stress-tolerant plants through *in vitro* culture, Nabars (1990) pointed out that this technique has been successfully used to produce stress-tolerant plants from several species.

Potato (*Solanum tuberosum*) is the most consumed food crop world wide after wheat and rice, yet it is relatively susceptible to drought (Harris 1978). Several authors have reported that limited soil water availability may affect the potato plant at all developmental stages, resulting in earlier crop maturity and decrease of plant growth, tuber yield, number of tubers per plant, tuber size and tuber quality (MacKerron and Jefferies 1988; Tourneux et al 2003). Recent studies have mainly focused on identifying drought tolerant varieties (varieties with limited decline in tuber yields upon drought) as well as unraveling drought tolerant traits either by physiological (Tourneux et al., 2003) or molecular approaches (Watkinson et al 2006, Schafleitner et al 2007). Many researchers identified Quantitative Trait Loci (QTLs) for different traits like tuber dormancy (van den Berg et al 1996), tuber yield and starch content (Schfer-Pregl et al 1998), tuber shape (Van Eck et al 1994), tuber flesh color (Bonierbale et al 1988), tuber skin color (Gebhardt et al 1989, 1991), yield, agronomic and quality traits (Bradshaw et al 2008) in potato under normal conditions. However, knowledge about genetics of drought tolerance in potato is still limited, and hardly any QTLs have been identified for potato drought tolerance traits.

Potato is highly amenable to tissue culture (Espinoza et al 1986) and *in vitro* techniques like micropopagation and micro-tuberization have become established methods for rapidly multiplying cultivars as well as for germplasm conservation and exchange (Roca et al 1979; Ranalli et al 1994; Gopal et al 2005; Donnelly et al 2003). Simko et al (1999) studied the similarity of QTLs detected for plant height and tuberization earliness under *in vitro* and greenhouse growing conditions. Gopal and Iwama (2007 and 2008) showed that *in vitro* culture of potato cultivars under normal and water limited conditions by raising plantlets from nodal cuttings obtained from *in vivo* grown plants may provide an effective screening system for selecting genotypes with a high root mass production potential under dry field conditions. However, there are no reports on *in vitro* screening and assessment of drought tolerance to dissect the genetic variation for drought tolerance in potato mapping populations. This study was aimed at i) evaluation of a mapping population under *in vitro* control, water-stressed conditions induced with PEG and studying the recovery potential, and ii) identification of QTLs for root and shoot traits at the juvenile stage of potato plant growth under *in vitro* conditions.

Materials and methods

Plant materials

The potato diploid mapping population C×E consisting of 250 genotypes was obtained from the cross between clones C [USW5337.3; (Hanneman RE 1967)] and E [originally named 77.2102.37; (Jacobsen 1980)]. Clone C is a hybrid between *S. phureja* PI225696.1 and *S. tuberosum* dihaploid USW42. Clone E is the result of a cross between clone C and the *S. vernei-S. tuberosum* backcross clone VH^{3} -4211 (Jacobsen 1978). The entire population has been maintained *in vitro*. A set of 94 randomly selected individuals was used for this study, along with the parents of the cross.

Media and water deficit treatment

Water potential was lowered by the addition of polyethylene glycol (PEG) (molecular weight 8000; Sigma St Louis, MO) to the growth medium. The PEG-infused tube system was modified as described by van der Weele et al (2000) and Gopal and Iwama (2007). PEG-infused tubes were made by dissolving solid PEG in a sterilized solution of basal media (half strength Murashige and Skoog salts with 2mM MES buffer) and pH was adjusted to 5.7. This

PEG solution was then overlaid on agar-solidified ($8gL^{-1}$ Bacto agar) basal 10ml media in 15cm x 2.5 cm test tube. The agar media and PEG solution were then allowed to equilibrate for 3 days. The excess PEG solution was removed from the tubes. 400g of PEG was added per liter of overlay solution to achieve -0.7 Mpa water potential of PEG-infused tubes (Gopal and Iwama, 2007).

Explant and culture conditions

From each genotype of the *in vitro* maintained CxE population plants were cut into 1.5 to 2 cm nodal segments. The segments were transferred to a 15 cm x 2.5 cm test tube and cultured on fresh growth medium and 14 replications were made and maintained for 2 weeks. Once the genotype shootlets or nodal segments were adapted and started to grow, they were transferred to PEG-infused tubes. Each genotype with seven replications was maintained under normal and water deficit conditions. The cultured tubes were incubated at 24 ±1ºC growth chambers, under 16 hours photoperiod/day for seven weeks.

Measurement of drought-related traits

After seven weeks, when control plants were fully grown with stout stems and broad leaves, traits like root length, root fresh weight, dry weight, plant height, shoot fresh and shoot dry weight were measured on four replications under control and water deficit treatments. The remaining three replications from each treatment were transferred to fresh growth medium without PEG to study recovery potential of the genotypes. Recovery treatment was maintained for 4 weeks prior to root and shoot traits were measured.

Statistical analysis

The phenotypic data were tested for normality and when the trait values were not normally distributed the data was transformed ($\log_e$). Analyses of variance (ANOVA) of the different traits and other statistical analyses were done with GenStat software 12th edition (VSN international, Hertfordshire HP1 1ES UK). Relative reduction (RR) of each trait was calculated as RR= (control- drought)/control, and expressed in terms of percentage. Broad sense heritability (h^2_m) was estimated on a genotype mean basis as a ratio of $\sigma^2_g / (\sigma^2_g + \sigma^2_e/r)$, where ($\sigma^2_g$) is the genetic variance, (σ^2_e) is the error variance and r is the number of replications.

Genetic map and QTL mapping

The genetic map of CxE as described in Anithakumari *et al.* (2010) was extended with 339 markers from a 768 SNP Illumina GoldenGate genotyping array. The CxE integrated maps were constructed using JoinMap 4.0 (Van Ooijen 2006) for QTL analysis.

MapQTL version 5 (Van Ooijen et al. 2006) software was used to identify QTL for all traits. First the interval mapping procedure was performed to identify the major QTLs. Markers with LOD scores exceeding the threshold (3.5) were used as cofactors in multiple-QTL-model (MQM) mapping procedures. If new QTLs were identified, the linked markers were added to the cofactor list and the analysis was repeated. If the LOD value of a marker dropped below threshold in a new model, it was removed from the cofactor list and the MQM was rerun. This procedure was repeated until the cofactor list became stable. The final LOD scores were determined by restricted MQM. The 2 LOD support interval was calculated to estimate the position of significant QTLs with 95% confidence. For each trait a 1,000 x permutation test was performed to identify the LOD threshold corresponding to a genome-wide false discovery rate of 5% ($P < 0.05$).

Results

96 individuals of the CxE diploid potato mapping population including the parents were grown *in vitro* and subjected to osmotic stress for a period of seven weeks. Following the stress period, 4 replications were harvested, and another 3 replications were allowed to recover for 4 weeks and harvested.

Quantitative variation, heritabilities and correlations

The mean values of different root and shoot traits of the parents C and E under normal, PEG stress and recovery conditions are shown in Table 1. Parents C and E significantly differed in their response to water stress and recovery potential. Parent C had lower mean values under normal conditions when compared to parent E. However, under PEG induced water deficit conditions parent C performed better when compared to parent E which showed drastic reduction in biomass. Parent C had good root growth, root dry weight and root to shoot ratio under stress conditions. The ability of plants to recover completely after stress is crucial to survive and complete their life cycle. In natural situations recovery potential is very important under intermittent drought conditions. Upon alleviation of stress parent E extremely outperformed parent C with higher recovery potential.

Table 1 Mean performance of parents and progeny, estimates of genetic and non-genetic components of variance, heritability and relative reduction of measured traits in different growth conditions.

		Performance					Variance			
		Parents		Progeny						
Trait	Treatment*	C	E	Min	Max	Mean	σ^2_g	σ^2_e	h^2_m	RR (%)
Fresh biomass (mg)	Con1	193.88	389.73	5.70	545.60	153.70	6330.75	1095.00	0.85	
	PEG	131.80	81.55	5.80	184.90	51.75	469.90	202.53	0.70	66.33
	Con2	185.27	1567.20	38.30	2450.00	499.90	0.36	0.05	0.87	
	Rec	97.37	1097.93	18.90	1372.00	312.40	35544.25	2708.25	0.93	37.51
Dry biomass (mg)	Con1	16.45	26.28	0.10	34.30	11.11	28.84	5.00	0.85	
	PEG	15.03	11.30	0.00	24.60	7.28	9.71	3.54	0.73	34.46
	Con2	23.80	89.43	7.20	163.00	45.78	0.27	0.04	0.88	
	Rec	13.30	83.30	2.80	113.10	26.74	220.25	16.09	0.93	41.59
Plant height (cm)	Con1	11.00	17.25	0.50	29.00	14.34	23.88	3.12	0.88	
	PEG	7.75	10.63	0.80	18.00	5.34	3.59	1.33	0.73	62.79
	Con2	12.03	19.83	4.50	34.00	17.70	13.89	2.58	0.84	
	Rec	11.43	18.00	2.70	51.10	13.06	10.08	1.25	0.89	26.21
Root length (cm)	Con1	4.88	12.38	0.00	22.00	6.95	12.12	1.58	0.88	
	PEG	5.63	7.75	0.00	17.50	4.66	7.22	1.34	0.84	32.95
	Con2	7.47	15.33	1.33	30.50	9.99	0.06	0.00	0.93	
	Rec	6.77	14.63	0.50	22.00	8.95	17.40	0.60	0.97	10.36
Shoot dry weight (mg)	Con1	14.80	20.03	0.10	28.00	9.38	15.15	3.56	0.81	
	PEG	12.23	8.40	0.00	20.30	5.88	4.81	2.05	0.70	37.31
	Con2	20.10	59.87	4.60	101.00	33.46	0.20	0.04	0.85	
	Rec	11.17	50.80	2.80	71.30	19.15	84.98	6.55	0.93	42.77
Root dry weight (mg)	Con1	1.65	6.25	0.00	9.20	1.81	3.16	0.36	0.90	
	PEG	2.80	2.90	0.00	8.50	1.44	1.36	0.50	0.73	20.22
	Con2	3.70	29.57	0.00	95.30	12.32	0.14	0.02	0.87	
	Rec	2.13	32.50	0.00	80.90	7.59	32.86	2.90	0.92	38.40
Shoot fresh weight (mg)	Con1	161.50	257.90	3.30	461.80	131.30	4068.25	973.25	0.81	
	PEG	103.35	68.03	5.80	149.80	42.85	326.45	119.43	0.73	67.36
	Con2	165.23	910.23	36.10	1408.00	366.80	0.05	0.01	0.85	
	Rec	92.00	677.77	18.40	706.00	220.50	13543.75	1161.00	0.92	39.89
Root fresh weight (mg)	Con1	32.38	131.83	0.00	109.90	23.89	460.70	60.15	0.88	
	PEG	28.45	13.53	0.00	40.00	7.98	33.07	13.99	0.70	30.00
	Con2	20.03	656.97	1.40	1246.00	133.00	1.06	0.15	0.88	
	Rec	5.37	420.17	0.00	932.90	91.97	5554.25	451.75	0.92	30.85
Root:shoot ratio	Con1	0.11	0.31	0.00	1.00	0.18	0.02	0.00	0.81	
	PEG	0.23	0.35	0.00	1.43	0.22	0.02	0.01	0.65	-20.65
	Con2	0.18	0.19	0.00	2.32	0.32	0.05	0.01	0.83	
	Rec	0.19	0.64	0.00	2.32	0.34	0.03	0.00	0.91	48.65

σ^2_g : genetic variance, σ^2_e: variance that is not explained by genetic effects, h^2_m: broad sense heritability, RR: relative reduction.* Treatments- Con1: control conditions at time point 1, PEG: PEG induced drought stress, Con2: control conditions at time point 2, Rec: Recovery treatment

All the trait distributions were continuous, reflecting their quantitative nature. The mid parent values for all the traits were higher than the population mean and the progeny displayed transgressive segregation, with more extreme values in the progeny than the parents.
Analyses of variance revealed significant differences between the progeny for all the traits measured, indicating that the traits related to the juvenile growth stage under water stress and recovery conditions are genotype-dependent in *in vitro* grown potato and interaction between genotype and treatment was found for all the measured traits. When compared to control conditions, a decrease in mean value of all measured traits was observed as an effect of water stress on traits with the exception of root to shoot ratio. The root to shoot ratio increased under water stress conditions and there was no relative reduction when compared to other traits indicating an increased partitioning of biomass towards roots as an adaptive mechanism. Water deficit conditions had a drastic effect on shoot fresh weight and fresh biomass as indicated by their relative reduction of about 67.4 and 66.3%, respectively. Root dry weight showed less relative reduction of about 20.2% (Table 1). Broad-sense heritabilities were calculated for all traits measured under controlled condition and water stress (Table 1). The estimates were in general high under control conditions and tend to be somewhat higher than those found under water stress. How ever, under PEG induced water deficit conditions, the estimates for root length, dry biomass and shoot fresh weight were higher with values ranging from 73.2 to 84.3%. The root to shoot ratio trait of *in vitro* grown potato seems to be moderately heritable.

After alleviation of stress, the progeny showed considerable variation for recovery potential. Interaction between genotypes and treatment was observed under recovery treatment. *In vitro* potato plants seemed to have good recovery potential, as there was increase in mean values of all measured traits when compared to drought stress. However, when compared to control genotypes, progeny showed decreased trait mean values after recovery (Table 1). Under recovery all the traits showed very high heritabilities compared to control and stress conditions. Root length had a high heritable value of 0.97. Plant height showed the lowest heritability of about 0.89 (Table 1).

The coefficients of correlation among traits under PEG induced water stress are presented in Table 2 with the direction (+ or -). All the shoot and root traits measured under drought stress

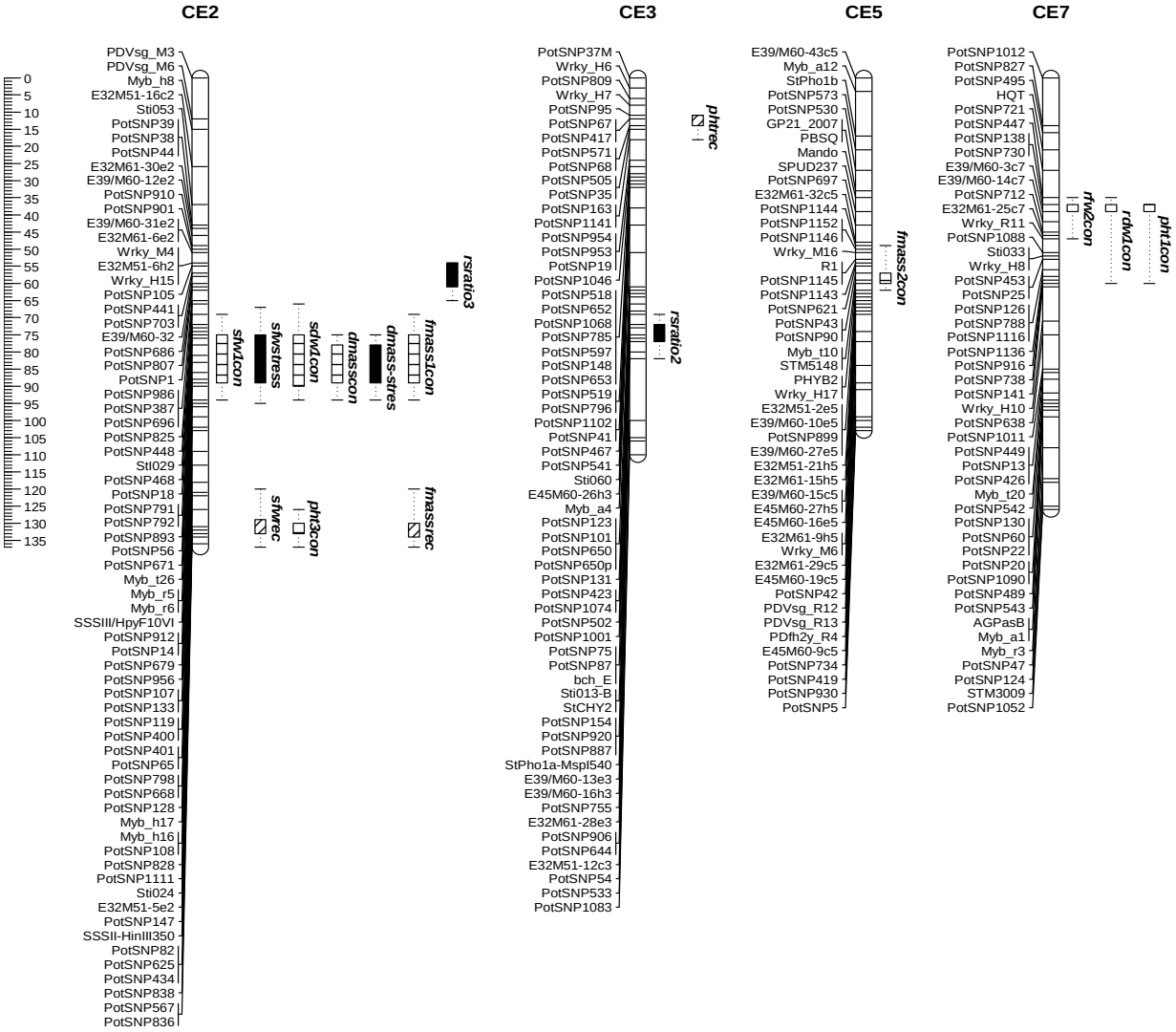
Table 2 Matrix of Pearson coefficients of correlation among the traits under drought stress at significance levels *Significant at P≤ 0.05; ** Significant at P≤ 0.01; *** Significant at P≤ 0.001

Trait	*Fresh Biomass*	*Root fresh weight*	*root length*	*shoot dry weight*	*shoot fresh weight*	*Plant height*	*Dry biomass*	*Root:shoot ratio*
Fresh Biomass (mg)	-							
Root fresh weight (g)	0.73***	-						
Root length (cm)	0.37**	0.60***	-					
Shoot dry weight (g)	0.88***	0.51***	0.22**	-				
Shoot fresh weight (g)	0.87***	0.54***	0.25**	0.89***	-			
Plant height (cm)	0.45**	0.22**	0.23**	0.34***	0.50***	-		
Dry biomass(mg)	0.91***	0.69***	0.37***	0.66***	0.87***	0.36***	-	
Root:shoot ratio	0.32**	0.68***	0.58***	0.16*	0.16*	0.15*	0.40***	-
Root dry weight (g)	0.67***	0.87***	0.58***	0.55***	0.52***	0.27**	0.76***	0.81***

Table 3 Pearson coefficients of correlation between the traits under normal and PEG induced drought stress at significance levels *Significant at P≤ 0.05; ** Significant at P≤ 0.01; *** Significant at P≤ 0.001

Treatment	***PEG induced stress***								
	Traits	Dry biomass	Root dry weight	Root fresh weight	Shoot dry weight	Shoot fresh weight	Fresh biomass	Root:shoot ratio	Root length	Plant height
Control	Root dry weight	0.19*	0.15	0.08	0.18	0.13	0.13	0.14	0.18	0.15
	Root fresh weight	0.16	0.13	0.13	0.15	0.13	0.14	0.14	0.11	0.04
	Shoot dry weight	0.51***	0.28*	0.18	0.53***	0.46***	0.42***	0.08	0.09	0.31**
	Shoot fresh weight	0.48***	0.28*	0.18	0.49***	0.43***	0.40***	0.09	0.06	0.28*
	Fresh biomass	0.45***	0.27*	0.18	0.46***	0.40***	0.38**	0.12	0.08	0.25*
	Root: shoot ratio	-0.02	0.03	-0.03	-0.03	-0.06	-0.06	0.14	0.19	0.03
	Root length	-0.01	0.08	0.21	-0.04	-0.05	0.02	0.17	0.40***	0.02
	Plant height	0.07	-0.09	-0.07	0.12	0.11	0.07	-0.17	-0.10	0.29*
	Dry biomass	0.48***	0.28*	0.17	0.50***	0.43***	0.40***	0.10	0.11	0.31**

conditions positively correlated with each other. Shoot fresh weight was highly correlated with fresh biomass (0.87***). Plant height showed relatively less correlation with root length and root fresh weight. Similar correlations patterns were seen in recovery condition (Data not shown). Phenotypic traits measured under well watered and PEG induced stress correlations are presented in Table 3. The traits related to biomass production (shoot fresh and dry weights, shoot fresh and dry weights) were significantly correlated between control and PEG induced stress. Biomass productivity traits were positively correlated with plant height under stress conditions. However, there is no considerable correlation of the root:shoot ratio with other traits measured under non-stress condition.



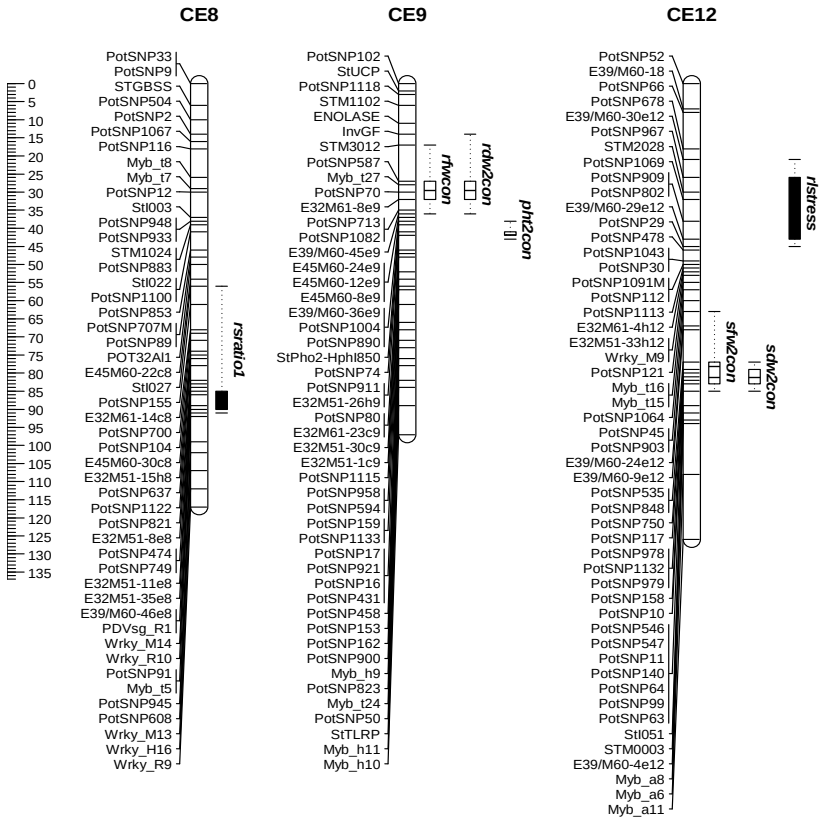


Figure 1 Location of the QTLs on the C x E integrated map. Only the linkage groups (chromosomes) with QTLs are shown. The scale on the left side is the genetic distance in centiMorgans (cM), marker designations are given on the left side of linkage group. QTLs are shown at the right side in vertical bars with trait names in different shades for different treatments (Open with horizontal lines- well watered, Solid filled bars- Stress, and open with cross vertical lines- Recovery). The integrated maps were drawn by the Map Chart 2.2 program. The vertical bar shows the 1LOD support interval and the dotted line 2 LOD interval of the QTL.

QTL analysis

In total 23 QTLs on 7 linkage groups were detected for the normal, PEG induced water stress and recovery conditions (Figure 1). However the number of separate loci may be less, as we found a number of stable co-segregating QTLs over traits and treatments. Map positions, QTL names and effects of these QTLs are summarized in Table 4. Stable QTLs were found for shoot fresh weight, dry biomass and fresh biomass and most of them localized on chromosome CE2. The proportion of the variance explained by single QTLs ranged from 10.3 to 22.4%. Three QTLs involved in the root to shoot ratio were identified: *r:s1* on linkage group 8, *r:s2* on linkage group 3 and *r:s3* on linkage group2. These loci explained altogether 41.1% of the variance for this trait. Three QTLs were detected for plant height under control conditions on linkage groups CE7, CE9 and CE2 together explaining 43% of the total variation. A QTL on linkage group CE3 under recovery treatment was associated with plant

height that explained variance of 19.3%. Two regions of CE2 and CE12 had QTLs (*sfwcon1, sfwcon2)* for shoot fresh weight measured under control conditions. *sfwstress* and *sfwrec* QTLs for shoot fresh weight measured under stress and recovery conditions were found on linkage group CE2. One QTL explaining 16.1% of the variance and located on linkage group 12 was found to be linked with root length under water deficit conditions. Two QTLs on linkage group CE2 and CE12 together explained 37% of the variance for shoot dry weight under control conditions. A total of 10 QTLs were found on chromosome CE2, and QTLs for shoot fresh and dry biomass co-localized at this location. QTLs on linkage group CE2 for fresh biomass and shoot fresh weight after a recovery period co-localized with QTLs for plant height under controlled conditions.

CxE genetic map was populated with functional markers such as SNPs. These SNP markers were mined and developed from EST sequences having putative functions. SNP markers with their putative functions underlying root to shoot ratio trait were presented in Table 6. Several interesting putative candidate genes involved in drought response were identified. SNP markers having sequence homology with genes such as dehydration responsive protein underlies with QTL for root to shoot ratio on chromosome CE2. Root border cell specific protein, transcription factor from AP2 family and protein kinases were identified on chromosome CE3. On chromosome CE8, eight SNP markers with putative functions were detected under third QTL for root shoot ratio. These genes had homology with genes involved in signaling and other functions such as calcium dependent protein kinase, aquaporin protein, vacuolar membrane ATPase. These genes may be putative candidates for PEG induced stress response in potato.

Discussion

Significant genetic variation for all the measured traits was observed under well watered, PEG induced stress conditions and after a four week recovery period. The parents differed in their response to PEG induced drought tolerance and recovery potential. Transgressive segregation was observed for all the traits measured under non-stress, PEG-induced stress and recovery conditions and this may be due to complementary action of alleles of different genes affecting a trait; alleles which were dispersed between the parents but may come together in the progeny (Tanksley 1993). Broad sense heritabilities were high to very high ranging from 0.65 to 0.97 under different treatments. These high heritabilities may be the consequence of *in*

vitro culture techniques, which minimize environmental variation due to defined nutrient media, controlled conditions and homogeneity of stress application. This relatively simple setup enables the study of large plant populations and application of well-defined stress treatments in a limited space and short period of time, irrespective of the crop season and thus holds the potential for pre-selection of genotypes for complex trait evaluation in the greenhouse or in the field.

Table 4 Main characteristics of QTLs with a LOD score >3.5 for the traits under well watered, water stressed and recovery conditions.

Name of the Trait	QTL name	Treatment	Linkage group	LOD score	Marker linked to trait	% variation explained
Shoot fresh weight	*sfwcon1*	Control	2	5.8	PotSNP912	22.3
	sfwcon2	Control	12	4.3	PotSNP750	13.3
	sfwstress	Stress	2	3.8	PotSNP912	15.0
	SFWrec	Recovery	2	3.7	PotSNP838	15.5
Shoot dry weight	*sdwcon1*	Control	2	6.1	PotSNP671	22.4
	sdwcon2	Control	12	4.7	PotSNP1132	14.6
Root fresh weight	*rfwcon*	Control	9	5.1	E32M61-8e9	16.2
	rfwstress	Stress	7	3.5	E32M61-25c7	12.9
Root dry weight	*rdwcon1*	Control	7	5.5	E32M61-25c7	18.8
	rdwcon2	Control	9	3.4	E32M61-8e9	12.3
Plant height	*phtcon1*	Control	7	4.5	PotSNP138	15.3
	phtcon2	Control	9	4.3	PotSNP911	14.9
	phtcon3	Control	2	4.1	PotSNP838	12.8
	phtrec	Recovery	3	4.4	PotSNP67	19.3
Dry biomass	*dmass*	Stress	2	3.8	PotSNP912	16.0
	dmass	Control	2	4.9	PotSNP912	21.4
Fresh biomass	*fmasscon1*	Control	2	4.6	PotSNP912	16.8
	fmasscon2	Control	5	4.1	myb_t10	13.0
	fmassRec	Recovery	2	3.9	PotSNP838	15.5
Root length	*rlstress*	Stress	12	3.8	STM2028	16.1
Root:Shoot ratio	*r:s1*	Stress	8	6.9	Wrky_M14, E32M51-11e8	19.3
	r:s2	Stress	3	5.0	E39M60-13e3	11.5
	r:s3	Stress	2	4.1	WrkyH15	10.3

PEG and mannitol containing nutrient solutions are often used in *in vitro* labs as a medium for inducing osmotic stress in plants and tissue cultures. PEG is an inert, non-ionic, long chain polymer, highly soluble in water and available in a wide range of molecular weights. Because of its properties, PEG has been used in tissue culture studies to simulate drought stress as it

occurs in plants in the field or greenhouse. Previous research revealed that PEG gives more consistent results than mannitol as an external osmoticum in studies of water relations in stressed plants (Hohl and Schopfer 1991; Pandey and Agarwal 1998). Mannitol can be taken up by plants as it is a natural product that is known to accumulate in certain lower and higher plants (Lipavska and Vreugdenhil, 1996). The differences in ability to take up mannitol between plants may affect screening for drought tolerance. Such an osmoticum therefore is considered not to be suitable for the study of plant responses to root medium water status. PEG with molecular weights ≥ 6000 cannot penetrate the cell wall pores (Carpita et al 1979). Therefore, PEG solutions mimic dry soil more closely than solutions of lower molecular weight osmotica, which infiltrate the cell wall as solute (Verslues et al 1998). However, care must be taken when interpreting the results from desiccation experiments using osmolytes such as PEG. Although PEG with high molecular weight was used in many studies to simulate the effect of drought on the root system (Lawlor 1970; Money 1989; van der Weele et al 2000; Nayyar and Gupta 2006) and to differentiate among genotypes (Sanguineti et al 2006, Gopal et al 2008), it should be kept in mind that drought stress in the field does not affect the soil water potential alone. For example, drought goes along with changes in the mechanical resistance and soil-grown plants respond to drought in a complex way. However, PEG-treatment in *in vitro* cultured plants may be used for a first and rapid screening for drought tolerance.

Thirteen QTLs were detected for traits measured under well-watered conditions, as well as seven stress-related QTLs and three recovery QTLs. Six QTLs for shoot fresh and dry biomass co-localized on chromosome CE2 and were highly correlated, QTLs for root fresh weight, dry weight and plant height colocalized on linkage groups CE7 and CE9. Not surprisingly these traits were significantly correlated ($r = 0.27$, $P \leq 0.01$).

A good indicator of drought adaptation is an increased root to shoot ratio under water stress (Begg and Turner 1976; Harries 1978; Jefferies 1993). Partitioning of carbon in favor of roots is a typical response of plants to amongst others water deficit stress and nutrient deficiency. Three genomic regions were associated with variation in root to shoot ratio in the *in vitro* CxE population, together explaining 41% of the variance for this trait. It is imaginable that some of these QTLs may be relevant under field conditions.

Table 5 Functional markers underlying root to shoot ratio QTLs with their putative functions.

Chromosome number	Marker name	Sequence Homology
Chromosome 2	PotSNP105	Homologue to UPST14_SOLTU (Q41495) STS14 protein precursor, complet
	PotSNP441	Similar to RFNP protein binding {Arabidopsis thaliana}
	PotSNP703	Similar to UPQ9SVL6_ARATH (Q9SVL6) Cold acclimation protein WCOR413-like protein
	PotSNP686	weakly Similar to GBAA At5g39890 {Arabidopsis thaliana}
	PotSNP807	Similar to UPQ653G1_ORYSA (Q653G1) Dehydration-responsive protein-like
	PotSNP001	Similar to UPQ3E9C6_ARATH (Q3E9C6) Protein At5g19130
Chromosome 3	PotSNP154	UPQ2XPX4_SOLTU (Q2XPX4) Root border cell-specific protein-like protein
	PotSNP920	UPQ2XPX4_SOLTU (Q2XPX4) Root border cell-specific protein-like protein
	PotSNP887	Homologue to Ethylene-responsive transcription factor 1 (EREBP-1) (ERF1-like protein)
	PotSNP755	Similar to UPQ8HME2_9TELE (Q8HME2) NADH dehydrogenase subunit 2,
	PotSNP906	Similar to UPQ8H2L4_ORYSA (Q8H2L4) ABC1 family protein kinase-like protein,
	PotSNP644	Similar to receptor-like protein kinase {Arabidopsis thaliana}
Chromosome 8	PotSNP155	UPQ41427_SOLTU (Q41427) Polyphenol oxidase, complet
	PotSNP700	Homologue to Vacuolar membrane ATPase subunit c
	PotSNP104	Similar to UPQ69K57_ORYSA (Q69K57) Smr domain-containing protein-like
	PotSNP637	Similar to UPQ8RUF8_ARATH (Q8RUF8) AT5g12040/F14F18_210, partial (83%
	PotSNP1122	Homologue to UPQ948X8_TOBAC (Q948X8) CIG2, complet
	PotSNP821	Similar to aquaporin NIP5.1 (NOD26-like intrinsic protein 5.1)
	PotSNP474	Similar to Calcium-dependent calmodulin-independent protein kinase 5
	PotSNP749	Similar to Vacuolar ATP synthase subunit E (V-ATPase E subunit)
	PotSNP091	Similar to UPQ2PGG3_ARATH (Q2PGG3) Serine racemase

Genes underlying the QTLs may be related to stress response regulation, hormone signaling, transport channels and proteins and the factors regulating partitioning of carbon and the enzymes mediating changes in partitioning under stress conditions. Several putative candidate genes underlying QTLs were detected in the present study. Aquaporins are channel-forming membrane proteins with extraordinary ability to combine a high flux with high specificity for water. In our study putative genes underlying root to shoot ratio QTL was identified. These putative genes may have interest in further study as candidates in potato for drought tolerance, since rice and tobacco plants over expressing an aquaporin (PIP1) gene increased their drought tolerance (Lian et al 2004; Yu et al 2005).Transcription factors and signaling molecules such as protein kinases, ERF1 transcription factor were identified that share a homology to known genes previously studied in other species (Liu et al 1998; Zou et al 2010), proving some clues about putative regulatory and signaling pathways that might be involved

in drought response in potato under *in vitro* conditions.

Two QTLs were detected for root fresh and dry weight on chromosomes CE9 and CE7. A root length QTL was found on chromosome 12 under stress conditions. Root morphology and architecture are usually not directly accessible traits for breeding. The obvious reason is that roots are located in the soil and cannot be assessed properly without destruction of the plant. QTL analysis in combination with proteome and transcriptome analysis have given insights into the genetic basis of root architecture affecting crop yield under different water regimes (Hochholdinger and Tuberosa 2009). The detection of QTLs for root traits offers an opportunity to use markers and marker-assisted selection (MAS) as a non-destructive alternative approach to root sampling in selection for root traits. Since potato has a smaller and shallower root system than most other field crops (Yamaguchi and Tanaka 1990) screening for root characteristics is considered to be important for the improvement of drought tolerance in potato. Moreover, an improved root system has been associated with increased tuber yield (Iwama 1982).

Obviously, the root environment of *in vitro* cultured plants is quite different from soil conditions. In addition, the plants are grown under highly controlled conditions, and do not go through the same developmental changes as field grown plants. Nevertheless, Gopal and Iwama (2008) have shown that differences in root traits of *in vitro* grown potato cultivars reflected the differences in the same cultivars grown in the field. Morpugro (1991) has shown in another study that highly significant correlation between tuber yields in the field and root fresh weight of the *in vitro* cultured plants. Hence studying root traits under *in vitro* may be of interest for breeding. For plant height three non-stress QTLs were detected on chromosomes CE7, CE9 and CE2 and one single recovery QTL on CE3. Simko et al. (1999) found QTLs for plant height under *in vitro* and greenhouse conditions on the same chromosomes except for chromosome 9. They also found similar QTLs under *in vitro* and greenhouse conditions for tuberization earliness. Plant height trait QTL was consistently found on same linkage groups on different genetic backgrounds and in *in vitro* as well as in field trials. Hence, there may be a possibility of using an *in vitro* system combined with marker-assisted selection for preliminary selection for root traits.

The comparison of QTLs detected under *in vitro* and greenhouse or field conditions could be

a valuable tool to confirm the results obtained in this study. This QTL mapping study suggests the possibility of developing an *in vitro* system that would allow a preliminary screening and selection for drought tolerance traits, of which the root-to-shoot ratio may be the most relevant. From this point of view the use of *in vitro* screening for drought tolerance is of particular interest as it enables large-scale testing of plants in a short period of time. This study constitutes the first knowledge of *in vitro* screening for drought tolerance in potato and has led to the description of important traits for screening and identification of interesting QTLs which may be useful for potato breeding. Several interesting putative candidate genes underlying QTLs were identified. The next step will be the characterization of these genes in potato. Further work is necessary to investigate whether the QTLs identified in this study using *in vitro* plantlets are (stably) expressed in greenhouse environments, multi-locational field trials, as well as in analyses of the variation for these traits at variable growth stages in potato. Further investigations in this CxE population will focus on identifying QTLs for drought tolerance under greenhouse conditions and field conditions, and to compare those to the QTLs in this study.

Chapter 4

Genetic dissection of drought tolerance and recovery potential by QTL mapping of a diploid potato population

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Submitted

Abstract

Potato is the third most important staple food crop in terms of consumption, yet it is relatively susceptible to yield loss because of drought. As a first step towards improving drought tolerance in this crop we set out to identify the genetic basis for drought tolerance in a diploid potato mapping population. Experiments were carried out under greenhouse conditions in two successive years by recording four physiological, seven growth and three yield parameters under stress and recovery treatments. Genotypes showed significant variation for drought and recovery responses. The traits measured had low to moderately high heritabilities (ranging from 22% to 74%). A total of 47 Quantitative Trait Loci (QTL) were identified, of which 28 were drought specific, 17 and two under recovery and under well watered conditions respectively. The majority of these growth and yield QTLs colocalized with a QTL for maturity on chromosome 5. Four QTLs for $\delta^{13}C$, three for chlorophyll content and one for chlorophyll fluorescence (Fv/Fm) were found to co-localize with yield and other growth trait QTLs identified on other chromosomes. Several multi-year and multi-treatment QTLs were detected and QTL x Environment interaction was found for $\delta^{13}C$. The response to drought and recovery and the QTL analysis provide insight in physiological traits that can be used for drought evaluation and breeding for drought tolerance. To our knowledge, this is the first comprehensive QTL study on water deficit and recovery potential in potato.

Key words: Chlorophyll florescence, Chlorophyll content, Drought, Potato, QTL, Recovery, δ13C

Introduction

Potato (*Solanum tuberosum*) is the predominant non-cereal food crop in the world and ranks third in total food consumption after rice and wheat. Yet, this versatile crop is susceptible to drought stress and often considered to be drought sensitive (van Loon, 1981), mainly due to its shallow root system with a depth ranging from 0.5 to 1.0m (Vos and Groenwold, 1986). About 85% of the total root length is concentrated in the upper 0.3m of soil. Gregory and Simmonds (1992) showed that the potato root system has relatively small root length per unit area and this makes the potato plant a poor conductor of water. In addition potato extracts less of the available water from the soil compared to other crops (Weisz *et al.*, 1994). Even short periods of water shortage can reduce tuber production and tuber quality (Miller and Martin,

1987). The relative inability of potato to withstand drought limits its productive range to areas with adequate rainfall or suitable irrigation.

Several studies have shown that drought has a drastic effect on the morphological and physiological traits of the potato plant, such as leaf size, leaf number, shoot height (Deblonde and Ledent, 2001), rate of photosynthesis, tuber number (MacKerron and Jefferies, 1986; Haverkort *et al.*, 1991), tuber yield and biomass (Dalla Costa *et al.*, 1997). The effect of drought on tuber yield depends on the aggregate of morpho- physiological processes, such as photosynthesis, leaf area expansion, leaf senescence, partitioning of assimilates, tuber initiation, bulking and tuber growth (van Loon, 1981). In addition, the timing and duration of the stress within the growth period are factors affecting potato yield (Jefferies 1995b), as well as on the climate and soil conditions. Dramatic reduction of yield occurs when stress coincides with irreversible reproductive processes, making the genetic analysis for drought tolerance at the reproductive stage crucially important.

Potato is a highly heterozygous cross pollinating crop in which many traits show continuous variation. Cultivar-dependent differences of responses to drought have been reported for *S. tuberosum* (Levy, 1983; Jefferies and Mackerron, 1987). In addition, several wild species of potato growing in its center of origin in South-America have been adapted to harsh and water-scarce conditions (Vasquez-Robinet et al 2008). This indicates that genetic variability exists within potato and its relatives that can be exploited by breeders to improve drought tolerance. Successful breeding requires exact information on effective drought tolerance traits, their heritability, the genotype x environment interaction and in addition, suitable selection tools for the traits of interest.

Molecular investigation of complex physiological traits and their genetic relations to agronomic traits has raised a lot of interest. In recent years molecular mapping approaches have been used to dissect agronomically important and physiologically complex traits that are quantitative rather than qualitative. Quantitative trait loci (QTLs) for traits like plant height, maturity, crop emergence, tuber size, and quality traits such as after- cooking darkening, regularity of tuber shape, fry colour and yield components have been identified (Bradshaw *et al.*, 2008). However, insight in genetics and genes underlying quantitative trait loci that are related to drought tolerance is still limited in potato. Locating QTLs for drought tolerance

mechanisms by the use of controlled greenhouse or growth chamber experiments combined with field evaluations under relevant conditions should allow the merits of different drought tolerance mechanisms to be established.

In this study we made use of a diploid potato mapping population to increase our understanding of potato plant performance under water stress conditions, and to establish the nature of phenotypic correlation and genetic association of various physiological and morphological traits. Our main objectives were i) to evaluate physiological and morphological parameters or secondary characters that are correlated with performance and tuber yield under drought stress and subsequent recovery, ii) to determine the heritability of traits under drought and recovery and iii) to identify QTLs for these complex traits in the potato genome as a first step towards identifying candidate genes underlying QTLs of drought related traits.

Material and Methods

Plant material

A population of nearly 250 genotypes was developed from a cross between clones C (USW5337.3) and E (77.2102.37) Clone C is a hybrid between *S. phureja* PI225696.1 and *S. tuberosum* dihaploid USW42. Clone E is cross between clone C and the *S. vernei-S.tuberosum* backcross clone VH34211. Population details can be found in Celis-Gomba B.C (2002).

Phenotyping

For the drought experiment, a core set of 94 CxE progeny and their parents were selected. The experiments were conducted in the greenhouse in two successive years (2008 and 2009) during late spring to summer season at Wageningen UR Plant Breeding, Wageningen University and Research Centre, the Netherlands. The weather conditions and stress period are indicated in Table 1. The greenhouse temperature was matched as much as possible to the external air temperature. The air circulation inside the greenhouse was regulated through openings in the roof. Tubers were planted in pots (19 cm^2 diameter, 3L volume). Eight replications were maintained in a completely randomized design. Each parental line was repeated four times in each replication to monitor position or corner effects. Irrigation was withheld for six replications starting from the stolon initiation stage. Two replications were

maintained as controls with optimal irrigation until the end of each experiment. Three replications out of six were subjected to recovery after three weeks of stress period. Samples were collected and measurements were taken for several traits as follows:

Leaf Relative Water Content (RWC): The uppermost fully expanded leaf was sampled, fresh weight (Wf) was taken as quickly as possible and placed in de-ionized water and left for 12-24 hrs at room temperature. Then turgid weight (Wt) was measured, leaf sample was dried at 85^0C and dry weight (Wd) measured. Leaf RWC was calculated according to the formula RWC (%) = $\{(W_f - W_d) / (W_t - W_d)\}*100$ (Barrs H. D, 1968).

Carbon isotope composition ($\delta^{13}C$): δ^{13}C is a measure of the ratio of stable carbon isotopes ^{13}C:^{12}C, expressed in parts per thousand (per mil, ‰). In C3 plants, δ^{13}C signature is used as a reflection of leaf water-use efficiency (WUE, Condon *et al.*, 2004). Fully expanded mature leaf samples were collected nine days after initiation of stress. Leaves were oven dried at 65˚C. The leaf material was fine powdered using the MM300 Mixer mill (Retsch Inc., Haan Germany) and samples were analyzed using Isotope Ratio Mass Spectrometer (IRMS), (Mamrutha et al., 2010) at the Department of Crop Physiology, University of Agricultural Sciences Bangalore, India and CNR- Institute of Agro environmental and Forest Ecology, Porano (TR), Italy.

Chlorophyll fluorescence (CF): Drought induced decrease in photosynthesis have been associated with photo damage of PSII reaction centres (He *et al.*, 1995). Chlorophyll fluorescence is widely accepted as an indication of the energetic behaviour of PSII (Krause and Weis, 1991). Potential quantum efficiency of PSII (Fv/Fm) can be used as a reliable indicator to evaluate the energetic/metabolic imbalance of photosynthesis and yield performance across genotypes under water deficit conditions. Chlorophyll fluorescence parameters including initial fluorescence (F_0), maximal fluorescence (F_m), variable fluorescence (F_v) and maximum quantum efficiency of PSII (F_v/F_m) were monitored on uppermost fully opened and expanded mature leaves under both well watered and drought stress conditions using an OS-30p handheld chlorophyll fluorometer (Opti- science, Inc. USA) following the manufacturer's instructions. Dark adaptation period for all the measurements was about 30 min, measurements were taken (in two replications) at four day intervals after the beginning of stress and during the recovery period.

Chlorophyll content (CC): In each genotype five leaves were measured (bottom, upper bottom, middle, upper middle and top leaf) using SPAD-502 chlorophyll meter (Minolta Co.,

Ltd. Japan), and the mean of these five values was taken. It was repeated in the biological replicate of the same genotype.

Plant height: At the end of stress period the plant height was measured in centimetres (cm) from the soil surface up to the uppermost leaf. Stems were upheld vertically during the measurement. Measurements were taken on three biological replicates of each genotype.

Root length: Roots were washed with water to remove all soil particles adhered to the roots and the longest root length was measured.

Shoot and root biomass (Fresh and dry): Shoot and root fresh weights were taken immediately after two harvests; one at the end of stress period and another at the end of recovery. Dry weights were taken after complete drying of the plant material in an oven at 105°C.

Stolon, tuber number and weight: Number of stolons was counted for each genotype in three biological replicates. Stolon ends with >1cm diameter were considered tubers; tubers were counted for each genotype and total fresh weight of tubers per plant was measured.

Statistical analysis

All statistical analysis was done with software GenStat 11th edition. Broad sense heritability (H^2) was computed from simple one-way Analysis Of Variance (ANOVA) according to the formula $H^2 = (\sigma^2_G / \sigma^2_G + \sigma^2_e/r)$, where ($\sigma^2_G$) = genetic variance, ($\sigma^2_e$) = environmental variance and r = number of replications. Relative reduction (RR) of each trait was calculated as RR= (control - drought)/ control and expressed in terms of percentage.

Genetic map

The genetic map of CxE as described in Anithakumari *et al.*, (2010) was extended with 339 markers from a 768 SNP Illumina GoldenGate genotyping array. This array is enriched for markers in genes putatively involved in abiotic stress response. The polymorphic markers were first mapped on parental maps using JoinMap 4.0 (Van Ooijen 2006) and parental maps were integrated for QTL analysis.

QTL mapping

MapQTL version 5 (Van Ooijen et al. 2006) software was used to identify QTLs for all traits. First interval mapping was performed to identify the major QTLs. For each trait a 1,000x permutation test was performed to identify the LOD threshold corresponding to a genome-

Table1 Weather data [Temperature and Relative Humidity (RH)] in the greenhouse at time of experimentation in two successive years.

Year	*Temperature (°C)*		*RH (%)*		*Stress period*	*Recovery*
	Minimum	Maximum	Minimum	Maximum	(Days)	(Days)
2008	13.1	33.1	43.2	87.0	21	12
2009	16.5	37.9	45.7	86.8	17	30

wide false discovery rate of 5% ($P < 0.05$). Markers with LOD scores exceeding the threshold were used as cofactors in multiple-QTL-model (MQM) mapping procedures. If new QTLs were identified, the linked markers were added to the cofactor list and the analysis was repeated. If the LOD value of a marker dropped below threshold in new model, it was removed from the cofactor list and the MQM was rerun. This procedure was repeated until the cofactor list became stable. The final LOD scores were determined by Restricted MQM. The 2 LOD support interval was calculated to estimate the position of significant QTL with 95% confidence. The integrated maps and QTLs were drawn using Map Chart 2.2. (Voorrips 2002)

Results:

Effect of water stress on CxE population

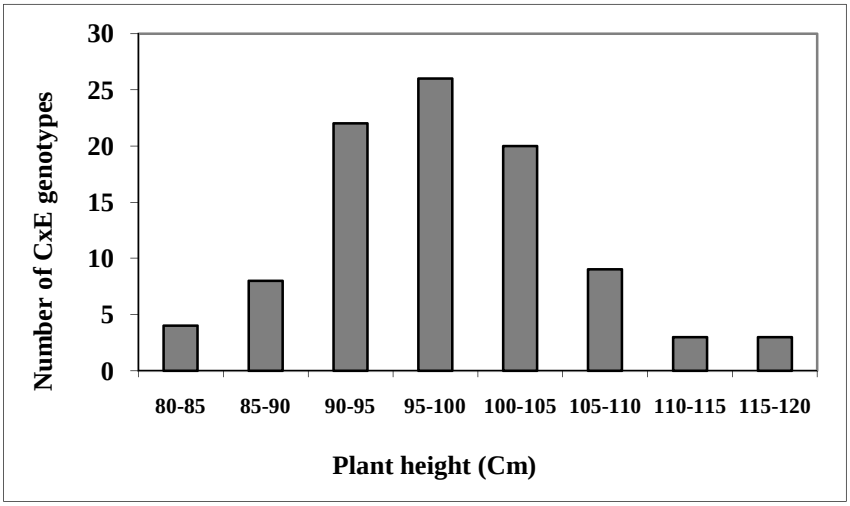
The CxE progeny displayed a wide contrast in drought tolerance, with individuals surviving and recovering completely after three weeks of drought and others completely wilted beyond recovery (Figure 1). The frequency distribution of genotypes for most of the traits evaluated in this study fitted a normal distribution and parents were always in the middle. The progeny displayed extreme performances for all the traits when compared to the parents indicating transgressive segregation, as exemplified by the frequency distribution of the traits plant height and $\delta^{13}C$ (Figure 2). The results revealed that drought affected all the measured traits, although the severity of stress perceived differed as indicated by trait mean values for the population (Table 2). The drought stress had a drastic effect on tuber number and tuber weight as indicated by their relative reduction of about 60 and 80% respectively. Drought had much less of an effect on number of main stems with relative reductions of 4% to 10% in two successive experiments. The root to shoot ratio increased under stress, indicating an increased partitioning of biomass towards root as an adaptive mechanism.

Figure 1 CxE progeny showing contrasting responses after 21 days of drought period and one day recovery.

Genetic variation of traits under water stress and recovery conditions

Analysis of variance showed that there was highly significant variation ($p<0.001$) among the genotypes for all the traits under stress conditions. There were significant differences between well-watered and water stress treatments for all the traits except for number of main stems. Genotypic differences were often specific to the stress response as there was highly significant interaction between treatment and the genotypes for most of the traits. Plant height showed considerable differences between genotypes; however, consistent interaction between genotype and treatment was not noticed. The majority of traits showed moderate to high heritabilities under stress ranging from 41.5 to 79.8% as listed in Table 2.

Drought tolerant plants either seemed to maintain water status of tissues, tolerate a reduction in tissue water content, or recover more completely after re-watering. The ability of plants to recover completely after stress is crucial for plants to survive and complete their lifecycle with optimal yield. Under the recovery treatment all traits varied significantly among progeny. Two-way ANOVA revealed significant differences between the drought and recovery treatments. Growth and yield parameters revealed significant interaction between treatment and genotype except for number of main stems and root length (Table S1). After alleviation of stress, heritabilities for all traits were relatively high when compared to those for the drought treated plants (Table S1).



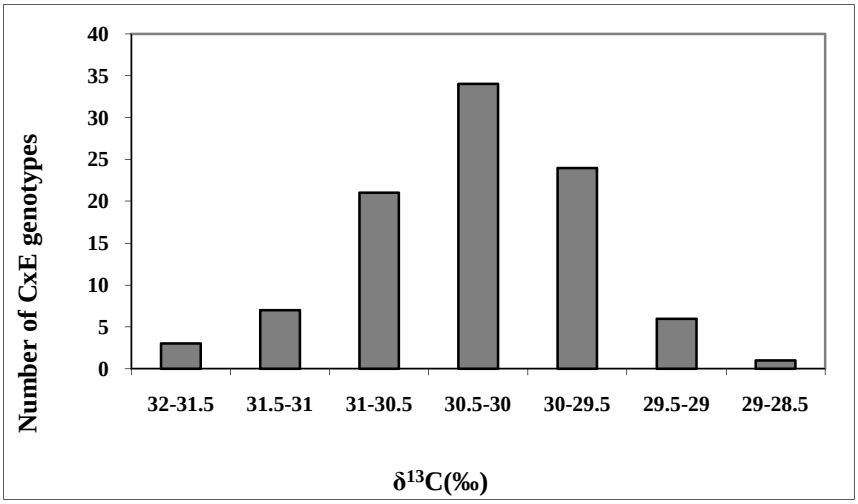


Figure 2 Frequency distribution of the traits, plant height and $\delta^{13}C$ measured under drought.

Evaluation of physiological traits under water stress and recovery

Relative Water Content: RWC is closely related with cell volume. It may more closely reflect the balance between water supply to the leaf and transpiration rate. Drought generally reduced the relative water content of leaves as reflected in the mean population values (Table 2). Although there was significant variation among the genotypes for RWC under drought, no considerable interaction between genotypes and the treatment was observed. RWC was reduced by 30% upon stress induction and showed 37% heritability under drought.

Carbon isotope composition ($\delta^{13}C$): Significant differences in $\delta^{13}C$ among the CxE progeny upon stress were found in two successive experiments. Significant interaction between genotypes and treatment were observed in the 2008 experiment. However, there was no such

interaction in the 2009 experiment. Heritability of $\delta^{13}C$ was 58.2% and 22.6% in 2008 and 2009 trials, respectively. Mean population values over two experiments between well-watered (more negative) and stress conditions (less negative) clearly revealed enrichment of $\delta^{13}C$ under stress (Table 2).

Chlorophyll Florescence (CF): The chlorophyll fluorescence measured as Fv/Fm decreased in the CxE progeny under drought. As expected, under well-watered conditions the mean value of Fv/Fm was 0.8+ and the value reduced as the stress period advanced (Fig. 3). There was significant variation among the genotypes drought and significant differences were observed between treatments. However, significant interaction between genotype and treatment was observed only at 4th and 16th day after stress initiation. Fv/Fm had high heritabilities at one day after stress initiation but heritabilities decreased as severity of stress increased (Table S2). After re-watering plants recovered quickly as reflected by a considerable increase in Fv/Fm over time reaching normal values of 0.81 after 17 days of recovery (Fig 3). Significant variation in Fv/Fm was observed four days after recovery among the progeny tested. We also observed a significant treatment effect over the recovery time period but only at four DAR considerable interaction between genotype and treatment was observed. Heritabilities were low under recovery treatment when compared to stress and inconsistent over the time (Table S2).

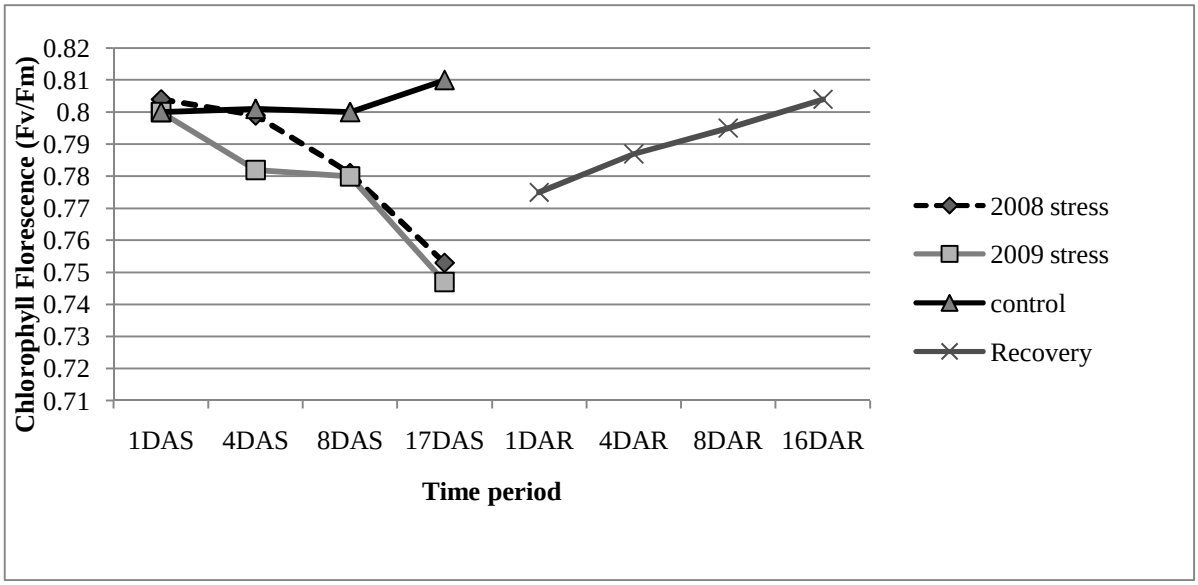


Figure 3 Chlorophyll fluorescence (Fv/Fm) measured during stress and recovery period DAS: days after stress, DAR: days after recovery.

Chlorophyll Content (CC): Water stress typically reduces overall plant chlorophyll content and maintenance of chlorophyll stability is considered an important trait. Our results revealed a linear decrease in chlorophyll content with increasing stress severity (Fig. 4). The progeny showed significant variation for chlorophyll content under stress. Except at day three after stress initiation there was a significant treatment difference. However, genotype by treatment interaction was not observed. There was a decrease (70% to 37%) in heritability of the trait with increasing severity of stress (Table S2).

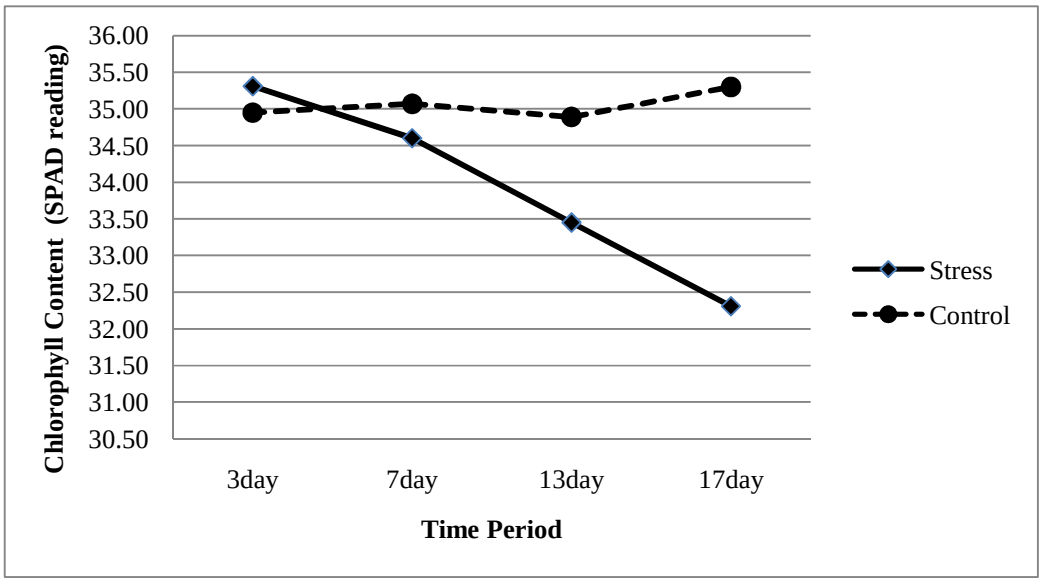


Figure 4 Chlorophyll content measured under drought and well watered conditions at series of time points.

Correlations

Under drought conditions $\delta^{13}C$ showed a highly significant ($P \leq 0.0001$) positive correlation with root dry weight and plant height, significant positive correlation with number of stolons, shoot dry weight and dry biomass, whereas $\delta^{13}C$ did not have significant correlation to any of the measured traits under well watered condition (Table 3 and Table S3). Under well-watered conditions shoot fresh weight, dry weight and root fresh weight showed significant negative correlations with maturity type previously scored in field trials under normal conditions. This implies that genotypes that matured late had higher shoot biomass. However, this correlation was not significant under drought stress (Table 3). Root traits were positively correlated with shoot fresh and dry weight and plant height. Root length was positively correlated with plant height, dry biomass, root to shoot ratio and negatively correlated with number of main stems

under drought stress. After recovery growth parameters were negatively correlated with maturity whereas tuber weight showed a significant positive correlation with maturity type (Table S4).

QTLs

QTL analysis was performed in order to identify the genomic regions contributing to the drought response phenotypes of different physiological, growth and yield traits. Figure 5 is a graphical representation of locations of the QTLs with two-LOD support intervals. Table 4 illustrates the QTL positions at one-LOD interval, LOD scores and percentage phenotypic variance explained by each QTL. A total of 47 significant QTLs were identified on the integrated CE genetic map under well watered, drought, and recovery conditions over two successive years. However the number of separate loci may be less, as we found a number of stable QTLs over treatments and in two successive experiments for growth and yield parameters like number of main stems, plant height, shoot fresh weight, dry weight, tuber number and tuber weight. Two genomic regions on chromosomes 5 and 4 accumulated 31 significant QTLs for different traits in stress, well watered and recovery conditions (Fig 5). Out of 47 QTLs 28 QTLs were detected under stress conditions. Two independent QTLs were detected for plant height under stress on chromosome numbers CE7 and CE2 which explained phenotypic variance of 30% and 21% respectively. Under recovery stable QTLs were found for plant height on chromosome CE5 and one on chromosome CE9. Number of main stems had a stable QTL on chromosome CE4 under stress and recovery treatments. Along with other QTLs on Chromosome CE4, CE9 and CE2 very stable QTLs were found across the treatments for shoot fresh, dry weight, tuber number and tuber weight on chromosome CE5.

For the physiological parameter $\delta^{13}C$ a total of four QTLs were detected, including one on chromosome CE10 under well-watered and stress conditions. When the experiment was repeated new QTLs were found on chromosome CE4, CE1 and CE9. Epistasis between QTLs for $\delta^{13}C$ was analysed by two-way analysis of variance (ANOVA). Significant interaction ($P<0.005$) was detected between the QTLs on chromosome CE1 and CE9, with total variance explained by these two chromosomal regions was 28%. Two independent QTLs on chromosome CE10 and CE4 explained 24% and 19% of variance respectively. On chromosome CE10, the $\delta^{13}C$ QTL co-localized with QTLs for Chlorophyll Content, on chromosome CE1 with dry biomass under stress, on chromosome CE4 with root traits like dry

weight, root length under stress and tuber weight under recovery conditions. On chromosome CE9 the $\delta^{13}C$ QTL was detected in between QTLs for shoot fresh weight under stress and plant height under recovery. Under stress, a QTL on linkage group CE1 for Chlorophyll Fluorescence explained 20% of the phenotypic variation. Three dependent QTLs were detected for Chlorophyll Content. One major QTL was found on chromosome CE10 for Chlorophyll Content at different time points during the stress period, along with two other QTLs on chromosome CE2 and CE4.

Root length and root dry weight had QTLs co-localizing on chromosome CE4 under stress. Under recovery however QTLs for root dry and fresh weight and root length were found on chromosome CE5. Two independent QTLs were detected for dry biomass under stress on chromosome CE5 and CE1 capturing 24% and 15% of phenotypic variation respectively. Dry biomass QTLs co-localized with other traits on chromosome CE5 and on CE1 with $\delta^{13}C$.

Discussion

Genetic variation of parameters under stress and recovery

We screened a diploid potato mapping population for drought tolerance and recovery potential. Most of the drought tolerance traits are quantitative and difficult to measure in a large number of plants and segregating lines. Indeed the genetic part of the phenotypic variation is often masked by the environmental differences acting on the trait, which in turn makes it difficult to manage the trial and perform relevant measurements in a particular window of time. In the present study we measured a number of growth, physiological and yield parameters in the CxE diploid potato mapping population. There was significant variation for drought response among the progeny with clear treatment effects and interaction between treatment and genotypes under water stress and recovery conditions. Progeny showed extreme performances for all the traits when compared to the parents indicating transgressive segregation. The most plausible cause proposed for transgression is accumulation of complementary alleles at multiple loci inherited from two parents in the progeny (Tanksley, 1993). Moderate to high heritability was observed for shoot fresh weight, tuber number, tuber weight, plant height and $\delta^{13}C$ content under stress and recovery conditions. Heritability estimates provide the basis for selection on the phenotypic performance. Therefore, direct or indirect selection based on these traits may be helpful to improve drought resistance and recovery potential in potato.

Table 2 Population mean values of the traits under control and drought treatments, analysis of variance for the traits under stress and well watered condition and relative reduction and heritabilities of the traits under drought condition.

Trait	Year	Control	Drought	Two Way ANOVA (P values)			Relative Reduction (%)	Heritability (%)
				Genotype (G)	Treatment (T)	G*T		
Number of main stem	2008	4.30	4.13	< 0.001	NS	NS	4.03	74.7
	2009	3.46	3.12	<0.001	NS	NS	9.83	59.5
Shoot dry weight (g)	2008	21.03	12.34	<0.001	<0.001	<0.001	41.32	70.1
	2009	17.51	10.36	<0.001	<0.001	<0.001	40.83	61.2
Shoot fresh weight (g)	2008	259.30	102.50	< 0.001	< 0.001	< 0.001	60.47	79.8
	2009	270.11	113.20	<0.001	<0.001	<0.001	58.09	56.5
Plant height (Cm)	2008	137.30	98.28	< 0.001	< 0.001	< 0.001	28.42	64.0
	2009	137.93	98.88	<0.001	<0.001	NS	28.31	49.3
Tuber number	2008	7.17	3.21	< 0.001	< 0.001	0.002	55.23	73.2
	2009	2.60	1.04	0.014	0.003	0.021	60.00	70.6
Tuber weight (g)	2008	33.79	5.53	< 0.001	< 0.001	< 0.001	83.65	69.2
	2009	10.16	1.06	<0.001	<0.001	<0.001	89.57	65.1
$\delta^{13}C$ (‰)	2008	-31.61	-30.28	< 0.001	< 0.001	0.032	4.21	58.2
	2009	-30.48	-29.78	0.034	<0.001	NS	2.29	22.6
RWC (%)	2008	83.95	58.73	0.004	<0.001	NS	30.04	36.8
Root dry weight (g)	2009	1.36	1.07	<0.001	0.012	NS	21.32	41.5
Root length (Cm)	2009	30.62	24.84	<0.001	<0.001	0.011	18.88	45.1
Root: shoot ratio	2009	0.07	0.11	<0.001	<0.001	NS	-43.50	42.3
Number of stolons	2009	5.84	4.09	<0.001	<0.001	NS	29.97	52.3
Dry biomass (g)	2009	18.87	11.43	<0.001	<0.001	<0.001	39.43	60.0

Table 3 Pearson coefficient of correlations among the traits under drought stress at significance levels *Significant at P≤ 0.05; ** Significant at P≤ 0.01; *** Significant at P≤ 0.001

Trait	δ13C	Nr main stems	PM	RDW	R: S wt	SDW	SFW	Tuber wt	Dry biomass	Nr main stems	Nr tubers	Pl ht
Nr stolons	0.400 *	-										
PM	-0.003	0.005	-									
RDW	0.473***	0.319	-0.277	-								
R: S wt	0.109	0.057	-0.022	0.247	-							
SDW	0.370*	0.26	-0.337	0.814***	-0.34	-						
SFW	0.264	0.08	-0.307	0.669***	-0.358*	0.873***	-					
Tuber wt	0.187	0.444*	0.012	0.17	-0.077	0.187	-0.078	-				
Dry biomass	0.385*	0.27	-0.337	0.844***	-0.292	0.867***	0.868***	0.188	-			
Nr main stem	0.031	0.144	0.196	-0.314	-0.302	-0.138	0.048	0.1	-0.156	-		
Nr tuber	0.244	0.542***	0.025	0.188	-0.141	0.246	0.047	0.935***	0.244	0.177	-	
Pl ht	0.525***	0.148	0.024	0.518**	0.125	0.353	0.231	0.156	0.373	-0.193	0.157	-
Root length	0.284	-0.048	-0.206	0.628***	0.443**	0.338	0.265	-0.117	0.369*	-0.410*	-0.21	0.395*

Traits were Number of stolons (Nr stolons), Plant maturity (PM), root dry weight (RDW), root to shoot day weight ratio (R:S wt), shoot dry weight (SDW), shoot fresh weight (SFW), shoot to root length ratio (S:R length), tuber weight (Tuber wt), number of main stem (Nr main stem), number of tubers (Nr tubers) and plant height (Pl ht).

QTL analysis of Physiological and growth parameters

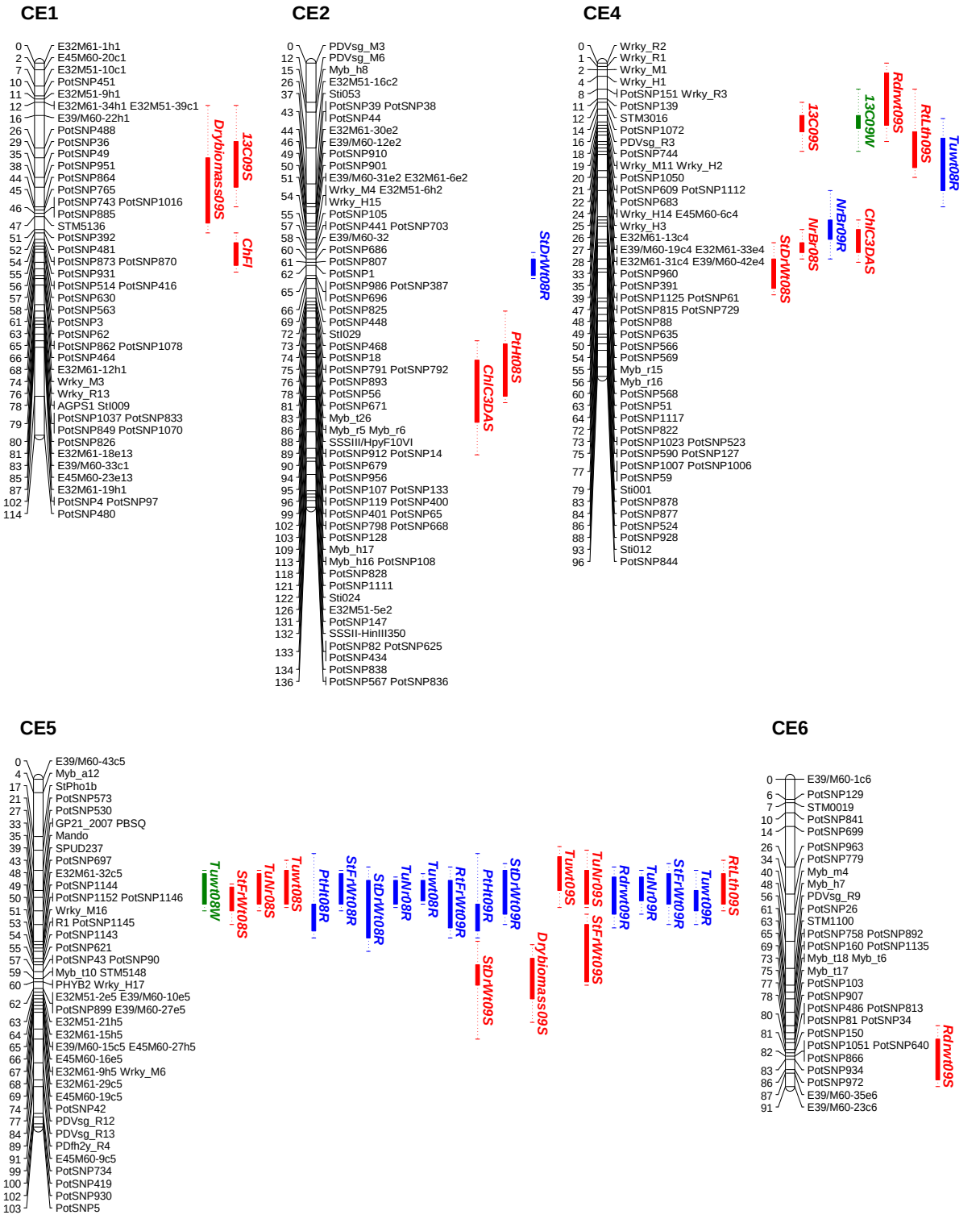
The central objective of this study was to identify QTL regions underlying important physiological and growth parameters under water stress and recovery treatments. We found multi-year as well as multi-treatment QTLs for drought response and recovery potential. We identified several QTLs for carbon isotope discrimination under well- watered and water stress conditions. The results indicate that four genomic regions are involved in $\delta^{13}C$ variation. As would be expected from the complex nature of a trait representing the ratio of two major processes, carbon assimilation (A) and transpiration (T), the control of $\delta^{13}C$ is strongly polygenic in potato. Any gene(s) that affects either A or stomatal conductance (gs) can have an effect on $\delta^{13}C$. No single, large-effect QTL was identified, and QTLs breaking the strong correlation between A and T have not yet been discovered in potato. Moreover, temperature and relative humidity have a significant impact on T, influencing QTL detection for $\delta^{13}C$. Understanding the inheritance of $\delta^{13}C$ is crucial for the development of cultivars with high WUE via selecting high $\delta^{13}C$ lines. Several studies have demonstrated that genetic variation in $\delta^{13}C$ can be attributed to nuclear factors, and QTLs for $\delta^{13}C$ have been reported in many species including Arabidopsis (Juenger *et al.*, 2005; Masle *et al.*, 2005), tomato (Martin *et al.*, 1989), rice (Laza *et al.*, 2006; Takai *et al.*, 2006), soybean (Specht *et al.*, 2001), cotton (Saranga *et al.*, 2004) and barley (Handley *et al.*, 1994; Teulat *et al.*, 2002).

We found a highly significant positive correlation between $\delta^{13}C$ and plant height under drought. Variation in development and plant height has been shown to affect $\delta^{13}C$ across different plant species. Plant height and flowering date could strongly influence yield dependent variation in $\delta^{13}C$ (Laza *et al.*, 2006; Ehdaie *et al.*, 1991; Hall, 1994; Mckay *et al.*, 2003). Phenology and stature also affect plant growth to change biomass and water use under drought, particularly when drought is terminal (Richards *et al.*, 2002). However, the physiological basis for the relationship between $\delta^{13}C$ and plant height is unclear. In addition, a significant positive correlation was found between $\delta^{13}C$ and dry biomass of the foliage of the plant, in agreement with studies from Jefferies and Mackerron (1994) and Jefferies (1995a) who found a positive correlation between dry matter production and $\delta^{13}C$ in two main crop cultivars of potato.

Carbon isotope composition ($\delta^{13}C$) as a selection criterion for drought tolerance improvement

has been largely documented in cereals, where it has been argued that molecular markers linked to genetic factors controlling $\delta^{13}C$ could enhance selection in breeding programmes (Condon *et al.*, 2004). The recent release and success of two wheat cultivars with high WUE namely Rees and Drysdale for production in rainfed wheat growing regions of Australia demonstrate that it is effective to breed for water-use-efficient cultivars by selecting for $\delta^{13}C$ (Richards, 2006). The major advantage of using $\delta^{13}C$ over instantaneous measurements is that sampling is fast and easy with minimal tissue destruction. Sampling can thus be performed in a short time window, which is more preferable for breeding programs. It is also possible to measure gas exchange parameters directly, which would give more detailed information on the assimilation and transpiration. However, these measurements are time-consuming, and not practically applicable in a large population.

Leaf RWC may be used for indirect selection for drought resistance (Chandrasekar *et al.*, 2000). RWC is a measure of plant water status, which represents also variation in water potential, turgor potential and osmotic adjustment. RWC is closely related with cell volume; it may more closely reflect the balance between water supply to the leaf and transpiration rate (Schonfeld *et al.*, 1988). This influences the ability of plant to recover from the stress and consequently affects yield and yield stability (Lilley and Ludlow, 1996). This parameter can also be easily determined, and therefore be applied for use in large populations. Significant decreases in RWC upon water stress were observed in our study. This result confirms earlier findings in potatoes (Jefferies and Mackerron, 1989; Liu *et al.*, 2005). However, no QTLs were detected for RWC under well-watered and water stress conditions.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16 E39/M60-22h1
26 PotSNP488
29 PotSNP36
35 PotSNP49
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76 Wrky_R13
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85 E45M60-23e13
87 E32M61-19h1
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114 PotSNP480
Drybiomass09S
13C09S
ChFl
CE2
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15 Myb_h8
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37 Stl053
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PotSNP44
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46 E39/M60-12e2
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Wrky_H15
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83 PotSNP671
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88 Myb_r5 Myb_r6
89 SSSIII/HpyF10VI
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95 PotSNP956
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18 PotSNP744
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22 PotSNP683
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25 Wrky_H3
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PotSNP899 E39/M60-27e5
63 E32M51-21h5
64 E32M61-15h5
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66 E45M60-16e5
67 E32M61-9h5 Wrky_M6
68 E32M61-29c5
69 E45M60-19c5
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84 PDVsg_R13
89 PDfh2y_R4
91 E45M60-9c5
99 PotSNP734
100 PotSNP419
102 PotSNP930
103 PotSNP5
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StFrWt08S
TuNr08S
Tuwt08S
PtHt08R
StFrWt08R
StDrWt08R
TuNr08R
Tuwt08R
RtFrWt09R
PtHt09R
StDrWt09R
StDrWt09S
Drybiomass09S
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StFrWt09R
Tuwt09R
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63 STM1100
65 PotSNP758 PotSNP892
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73 Myb_t18 Myb_t6
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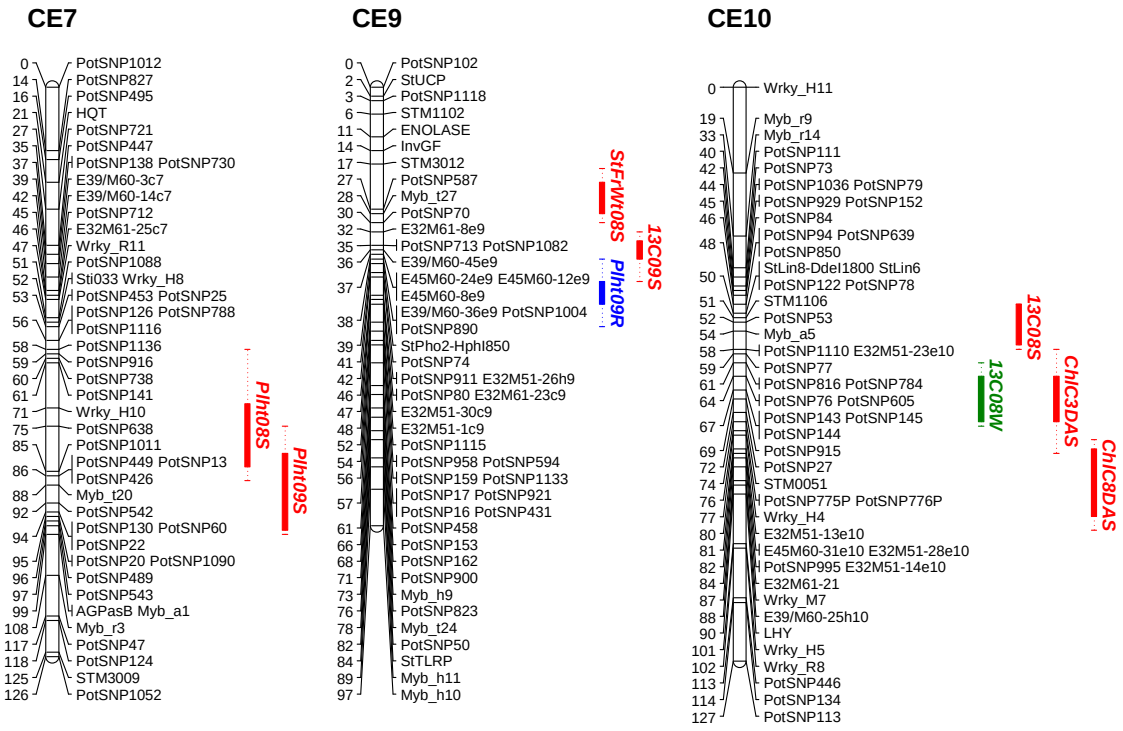

Figure 5 Location of the QTLs on the C x E integrated map. Only the linkage groups (chromosomes) with QTLs are shown. The number on the left side is the genetic distance in centiMorgans (cM), marker designations are given on the right side. QTLs are shown at the right side in vertical bars with trait names in different colors for different treatments (Green: well watered, Red: Stress, and Blue: Recovery).The solid vertical bar shows the 1-LOD interval and the dotted line 2-LOD intervals.

Several studies have reported on a genotype dependent decrease of fluorescence quantum yield (Fv/Fm) in potato under drought and small decreases were associated with drought tolerance, at least in early maturing varieties (Van der Mescht A., 1999). Ranalli *et al.* (1997) showed clone-specific variation in CF in drought-exposed potato with a high association with tuber yield. In the current study drought stress affected chlorophyll fluorescence parameters. The decrease in Fv/Fm may result from photoinhibition under stress (Baker and Horton 1987). A decline in Fo could reflect damage to regulatory processes external to P680 (reaction center of PSII), such as impairment of photoprotective processes that facilitate the dissipation of excess energy with the leaf (Angelopoulous et al., 1996). A reduction of Fv/Fm represents either a reversible photoprotective downregulation or irreversible inactivation of PSII (Baker and Bowyer 1994; Long et al., 1994). Our results revealed significant differences in chlorophyll fluorescence parameters between genotypes and treatments. Although interaction between genotype and treatment was observed in our experiment, the results were not consistent at different time points. Schafleitner *et al.*, (2007) showed that there were no

significant differences in CF between potato clones and treatments in a field trial but the same clones in the greenhouse showed significant difference under stress, demonstrating the environmental impact on CF measurements. We found that as severity of the stress increased the heritability of Fv/Fm was decreased. In agreement with Jefferies (1992) and Zrůst *et al.* (1994) our results also indicated that as severity of the stress increased the Fv/Fm decreased as shown in Figure 3. Hence, CF provides rapid indicators and a method for the study of changes in photosynthetic capacity of the potato in response to water stress. In the present drought response study, an Fv/Fm QTL was detected on chromosome CE1, which is adjacent to QTLs for dry biomass and $\delta^{13}C$ under stress. Whether there is a causal relationship between these QTLs remains to be established.

Chlorophyll is one of the major chloroplast components associated with photosynthesis, and relative chlorophyll content has a positive relationship with photosynthetic rate in barley (Li *et al.*, 2006). Whether a higher chlorophyll content (i.e. stay green trait) contributes to yield under drought conditions is still under debate (Blum, 1998). Many studies in cereals indicate that the stay green trait is associated with improved yield and transpiration efficiency under water limited conditions (Borrell *et al.*, 2000; Haussmann *et al.*, 2002; Verma *et al.*, 2004). Therefore maintaining higher chlorophyll content for a longer period may be one of the strategies for increasing crop production, particularly under water limited conditions. Saranga *et al.*, (2001) detected different chlorophyll content QTLs under well-watered and dry conditions in cotton. In the CxE population, there was substantial decrease in Chlorophyll Content as the severity of water stress increased (Fig 4). Three independent genomic regions were associated with CC, which may contribute to improved chlorophyll stability under drought. One QTL co-localized on chromosome CE10 with $\delta^{13}C$, which also relates to photosynthesis. Other QTLs co-localized on chromosome CE2 with plant height under stress and on chromosome CE4 with number of stems and shoot dry weight under stress, suggesting a relationship of chlorophyll content with growth parameters of plant.

Table 4 Main characteristics of QTLs with a LOD score >4.2 for the traits under well watered, water stressed and recovery conditions.

Name of the trait	Year	Treatment	Linkage group	LOD score	Interval (cM)	% variation explained
Number of stem	2008	Stress	4	4.3	54-64	19.2
	2009	Recovery	4	5.07	47-55	15
Plant height	2008	Stress	2	7.05	77-102	21.9
			7	6.5	59-71	30.9
	2008	Recovery	5	4.83	34-48	26.2
	2009	Stress	7	6.57	93-98	18.3
	2009	Recovery	9	8.8	38-45	56.7
			5	6.09	26-40	6.9
Shoot fresh weight	2008	Stress	5	11.34	26-48	42.3
			9	7.74	16-30	24.6
	2008	Recovery	5	14.37	26-40	57.9
	2009	Stress	5	5.1	47-60	21.6
	2009	Recovery	5	14.95	26-43	60.6
Shoot dry weight	2008	Stress	4	4.99	59-71	22.4
	2008	Recovery	5	8.7	35-48	28.8
			2	4.98	55-65	10.8
	2009	Stress	5	4.6	54-62	20.2
	2009	Recovery	5	7.89	26-44	35.3
Tuber number	2008	Stress	5	10.7	26-39	40.8
	2008	Recovery	5	15.74	26-39	58.3
	2009	Stress	5	6.05	20-39	25.9
	2009	Recovery	5	14.6	32-39	51.1
Tuber weight	2008	Well watered	5	16.4	26-39	66.7
	2008	Stress	5	10.48	20-39	40.2
	2008	Recovery	5	13.19	26-39	40.7
			4	4.96	23-35	9.1
	2009	Stress	5	7.7	20-33	28.8
	2009	Recovery	5	15.12	33-39	52.3
$\delta^{13}C$	2008	well watered	10	5.2	63-74	22.8
		Stress	10	5.62	47-58	24.3
	2009	well watered	4	4.8	14-21	20.7
	2009	Stress	4	5.1	14-22	18.9
			9	4.55	34-40	12.7
			1	4.1	15-37	15.7
Root fresh weight	2009	Recovery	5	6.54	26-48	35.3
Root dry weight	2009	Stress	4	5.62	0-18	24.7
			6	5.35	77-87	17.8
	2009	Recovery	5	8.26	26-43	33.3
Root length	2009	Stress	4	4.48	25-36	19.2
	2009	Recovery	5	8.09	26-35	36.3
CF	2009	Stress	1	4.57	54-63	19.7
CC3day	2009	Stress	2	7.21	89-102	16.4
			10	6.95	64-75	15.4
			4	5.47	47-58	12.2
CC8day	2009	Stress	10	4.76	82-95	20.8
Dry biomass	2009	Stress	5	6.81	51-62	23.9
			1	4.5	29-47	15.3

Association of QTLs of different traits

Tuber yield cannot be assessed properly in the early stages of plant growth within selection processes of a breeding programme. However, physiological and agronomical or morphological parameters measured in the drought-stressed plants may affect tuber formation and bulking at a later stage, and may thus be related to tuber yield performance. Tuber yield was determined by total tuber weight per plant. In our study tuber dry matter content was highly correlated with root and shoot fresh weights, root length and $\delta^{13}C$ under stress conditions. Genetic factors which control plant biomass (shoot, root fresh and dry weights) and $\delta^{13}C$ may affect tuber weight and therefore, tuber yield, which would be reflected by localization of QTLs for these traits in the same genomic regions.

Time to plant maturity and tuberization are related physiological traits, which are controlled by genetic factors as well as day length. In the present study time to plant maturity and tuberization are also significantly correlated with shoot and root dry matter content but only under well-watered conditions and after alleviation of stress. Many researchers (Berg *et al.*, 1996; Schäfer-Pregl *et al.*, 1998; Šimko *et al.*, 1999) identified major QTLs for plant maturity on linkage group V in independent mapping populations. On chromosome CE5 we also found stable QTLs for plant biomass, dry matter content under stress, plant height, tuber number and tuber weight under recovery. The genetic effects were mostly stable over years in a greenhouse environment. These findings suggest that gene(s) with pleiotropic effects on plant growth, tuberization, plant maturity and tuber yield are located on potato chromosome 5.
On chromosome 10, a QTL of $\delta^{13}C$ co-localized with a QTL for Cholorophyll Content under stress as assessed with SPAD meter readings under stress. SPAD meter reading is a good indicator of chlorophyll stability, leaf N and RuBisCo content. These parameters help to evaluate photosynthetic processes, which in turn have possible effects on inter cellular CO_2 concentration (C_i) and $\delta^{13}C$. Several QTLs for $\delta^{13}C$ overlap with the QTLs for other physiological traits and or for yield components.

QTLs for root dry weight or root dry mass and root length were colocalized with independent QTLs for $\delta^{13}C$ under water stress on chromosome CE4, together with a QTL for tuber weight under recovery condition. This co-localization of QTLs is in agreement with previous research showing that tuber yield, reduction of stomatal conductance, photosynthesis and leaf

area significantly correlate to root dry mass under water deficit conditions. Consequently, root mass or traits associated with root dry mass could be used as a selection criterion for enhancing tolerance of potato to drought (Iwama, 2008; Lahlou and Ledent, 2005). The measurement of the root system is tedious and destructive and thus focusing on shoot or other physiological traits highly correlated with root traits and their QTL co-localization may be another possibility of assessing root traits indirectly. We found that root dry mass and root length highly correlated with plant height and shoot fresh and dry masses under water deficit and recovery conditions. Hence, the co-localization QTLs for root dry mass and root length on chromosome CE4 and CE5 with other physiological and growth traits may be of further interest for indirect selection criterion for root traits.

In this study, all these associated traits and their co-localized regions are of interest in terms of plant breeding as they control both important drought-adaptive traits and yield components. Confirmation of the influence of these genomic regions by refining the map or observing similar effects in different populations could help to elucidate biological processes underlying complex traits such as yield or yield stability.

QTL x E interaction

Variations in climatic conditions are expected to have significant influence on $\delta^{13}C$ values (Merah *et al.,* 2001). This was the case in the present study, in the 2008 trial a $\delta^{13}C$ QTL was detected on chromosome CE10. While in the 2009 trial three new QTLs were detected on chromosome CE1, CE4 and CE9 and the QTL on Chromosome 10 was not detected. The fact that the different QTLs were identified for $\delta^{13}C$ in successive years suggests that QTL x Environment interactions influenced the expression of trait. Documented differences in environmental conditions between the two years were observed in the greenhouse particularly in terms of temperature and relative humidity. Whelan *et al.,* (1973) showed increasing discrimination by 1.2‰ per °C rise in temperature. The basis of the biochemical discrimination against ^{13}C in C3 plants lies with the primary carboxylation enzyme ribulose-1,5-bisphosphate (RuP2) carboxylase. At a fixed ambient CO_2 concentration, $\delta^{13}C$ is negatively associated with the intercellular CO_2 concentration (Ci). At any moment in time, the Ci is also negatively correlated with leaf transpiration rate (Hall *et al.,* 1994; Farquhar and Richards, 1984). Under water limitations, leaf transpiration efficiency is the major determinant of long term plant WUE. Under drought conditions a typical response of plants is

simultaneous decrease in photosynthesis and transpiration due to altered leaf conductance (Farquhar *et al.*, 1982). If the supply function (leaf conductance) decreases at a faster rate under stress than the demand function (photosynthesis), this effect should be measurable as either an increase in carbon isotope composition or correspondingly as decrease in carbon isotope discrimination. In the present study an increase in δ values was observed under drought stress. In the 2009 trial severity of drought was higher mainly because of higher temperatures and lower relative humidity when compared to the previous year. We speculate that the $\delta^{13}C$ QTL on chromosome CE10 specific for the 2008 trail is mainly representing the demand function (photosynthesis) which in turn co-localized with QTL for chlorophyll content which is a good indicator of leaf N and Rubisco content. In the 2009 trail, the higher average temperatures and greater vapour pressure deficit of the air may have acted more on stomatal conductance resulting in higher $\delta^{13}C$. The $\delta^{13}C$ QTLs that were detected on chromosome CE4, CE1, and CE9 may therefore represent supply functions. Previous reports in wheat and rice documented that variation in temperature, vapour pressure, stomatal aperture and leaf conductance were identified as driving variation in $\delta^{13}C$ and thereby water use efficiency (Condon *et al.*, 1992; Dingkuhn *et al.*, 1991; Kondo *et al.*, 2004). Further studies are needed to understand precisely how temperature, humidity or vapour pressure, light intensity and other environmental factors contribute to expression of WUE at different stages in potato plant development, to dissect the $\delta^{13}C$ trait in more detail in different components and to confirm whether the QTL identified in this study are stably expressed in other environments.

QTLs and their implications

QTLs identified by genetic dissection of complex characters such as drought tolerance can be used in marker assisted breeding which may ultimately improve selection efficiency for yield, reduce problems associated with genotype x environment interactions, and facilitate combining different tolerance traits into a single genotype. For any trait to be used as an indirect selection criterion in breeding programs, its measurement should be easy, rapid and non-invasive. Such an indirect measurement should have a high genetic correlation with the trait that is being selected for and it should have a high heritability. In our study physiological parameters like RWC, chlorophyll content, $\delta^{13}C$, and chlorophyll florescence provide rapid indicators of drought stress, and methods for the study of the response to water stress of

potato. These physiological parameters as well as plant growth and yield parameters had moderate to high heritabilities and may be of interest to breeders. From our initial QTL studies, response of a potato plant to water stress appears to be strongly quantitative and controlled by many genetic factors rather than a few loci of large effect. The strong multigenic nature of the traits and the transgressive variation observed in our mapping population suggest that even lines that do not themselves have high trait value for WUE, tuber number or tuber weight might still contribute favourable alleles. Related wild species might similarly have unique alleles that would be valuable for improvement of potato for drought tolerance. This study constitutes the first knowledge of genetic determinism of important physiological and growth parameters under drought stress and recovery potential in potato. To confirm whether the QTLs identified in this study are stably expressed in other environments multiple location field trials are necessary as well as analysis of these traits at variable growth stages in potato. Further efforts of QTL mapping in this population will focus on trait x QTL interactions. Sequence data from individual QTL and flanking regions can be compared to the forthcoming genome sequence of the heterozygous diploid line RH (RH 89-039-16) and the doubled monoploid DM1-3 516R44 (DM) potato genome sequence (www.potatogenome.net) to determine the putative candidate genes underlying drought and recovery-specific QTLs.

Supplementary material

Table S1 Population mean values of the traits recovery treatment, analysis of variance for the traits under stress and recovery condition and relative reduction and broad sense heritabilities of the traits under recovery condition.

Table S2 Population mean values of the traits Chlorophyll florescence (Fv/Fm) and Chlorophyll content measured at series of time points during stress and recovery period, analysis of variance for the traits under stress and recovery condition and relative reduction and broad sense heritabilities of the traits under stress and recovery condition.

Table S3 Coefficient of correlations for the traits under well watered condition (harvested at the end of stress period) *Significant at P≤ 0.05; ** Significant at P≤ 0.01; *** Significant at P≤ 0.001

Table S4 Pearson coefficient of correlations for the traits after recovery *Significant at P≤ 0.05; ** Significant at P≤ 0.01; *** Significant at P≤ 0.001

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Chapter 5

Genome wide eQTL analysis for drought response in potato

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To be submitted

Abstract

Potato is a very important food crop grown in many parts of the world but unfortunately it is sensitive to drought. Drought tolerant potato varieties would help to ameliorate the adverse affect of drought on potato yield. Drought tolerance however is a genetically complex trait that involves multiple genes and pathways. Breeding for drought tolerance therefore is a challenge, even more in a crop like potato that is heterozygous, tetraploid and outcrossing. Gene expression studies have shown that the expression of hundreds up to even thousands of genes are altered in response to drought stress. To understand the variation in drought tolerance found within a segregating potato population, a genome-wide transcriptome analysis was performed. For many genes, variation in expression could be explained by expression quantitative trait loci (eQTL). In total 24,571 significant eQTLs could be detected under normal growth and water stress conditions. 67% of the quantitatively controlled transcripts were qualified as *cis* eQTL's, against 23% of *trans*-eQTL's under normal growth conditions. Interestingly the number of *trans* eQTLs strongly increased under stress conditions and revealed genomic regions which were identified as hotspots for transcriptional regulation. Several thousands of eQTLs associated with large phenotypic variation were detected, but *trans* acting eQTLs had small phenotypic effects (R^2 <0.2). Based on gene ontology, eQTLs were classified based on sequence homology with well known drought responsive genes such as transcription factors, signaling molecules, redox genes, chaperones and transporters. A subset of identified eQTLs co-localized with phenotypic trait QTLs measured under drought. To our knowledge, this is the first global eQTL mapping study under control and water stress response in potato. It reveals that the genetic control of transcript level in potato is highly variable and complex in response to drought. This approach yielded information that leads to the genome-wide identification of putative candidate genes involved in drought tolerance their distribution, regulation and identification of putative candidate genes underlying phenotypic trait QTLs for drought response.

Key words:eQTL, drought, genome-wide, potato, transcriptome profiling

Introduction

Genetic variation underlying differences in transcript levels contributes considerably to phenotypic differences and divergence between species and their ability to cope with stress conditions in nearly all organisms studied (Whitehead and Crawford 2006). Drought tolerance is a genetically complex adaptation of plants to adverse conditions that involves multiple genes and pathways (Shinozaki and Yamaguchi-Shinozaki 2007). Several studies have focused on the molecular response of plants to water stress using the model plant *Arabidopsis thaliana* (Ingram and Bartels 1996; Shinozaki and Yamaguchi-Shinozaki 1997). Gene expression studies have shown that expression levels of hundreds up to even thousands of genes are altered in response to drought stress (Ozturk et al 2002; Talame et al 2007; Zhou et al 2007).

Three basic types of transcriptional changes can occur in response to drought stress. A plant suffers the effect of drought physiologically through decreased hydraulic conductance and loss of cell turgor resulting in reduced photosynthetic activity, growth and development (Lu and Neumann 1999; O'Toole and Cruz 1980). The plant adapts the expression of genes encoding the proteins that function in these metabolic processes to the changing conditions (Bray 2002). Secondly, the cellular disequilibria brought about by disrupting normal metabolism causes the accumulation of reactive oxygen intermediates resulting in changes in transcription in defense pathways (Mittler 2002; Ramanjulu and Bartels 2002). A third effect is transcriptional changes that bestow the ability to endure dehydration through a physio-chemical change in cell structure or water potential (Ingram and Bartels 1996; Tripathy et al 2000). These alterations in expression levels are complex and varying widely depending on the magnitude and duration of the drought stress (Drame et al 2007). Such complexity makes understanding the genetic mechanisms of drought tolerance a major challenge.

Improvements in quantitative trait loci (QTL) mapping methodology have led to increased understanding of the genetic complexity and location within plant genomes of genetic determinants of traits conferring drought tolerance (Campos et al 2004; Tuberosa and Salvi 2006). However, despite considerable efforts in QTL mapping the underlying molecular basis of most quantitative traits remains unknown.

Rapid advances in the field of molecular biology and genomics methodologies (transcriptomics, metabolomics, and proteomics) have led to the availability of approaches

that can help to better identify and understand the nature of genes, metabolites or proteins and their networks underlying quantitative traits of interest. One of the more successfully used approaches is transcriptome profiling using microarrays. Several studies of gene expression profiling using cDNA or oligo microarray technology has advanced our basic understanding of gene regulatory networks that are active during the exposure of plants to drought stress (Seki et al 2001; Chen et al 2002; Kawaguchi et al 2004; Swindell 2006; Ma and Bohnert 2007, Zhou et al 2007; Drame et al 2007, Vasquez-Robinet et al 2008).
Another level of integration is added to these genome-wide expression analyses by including genetic information to the expression data as described in the concept of "genetical genomics" (Jansen and Nap 2001). In this concept, variation in gene expression or metabolite content is associated with marker information in mapping populations. Within a population, variation in mRNA transcript abundance can be treated as a heritable trait that is subjected to statistical genetic analysis and can lead to the identification of a so-called expression QTL (eQTL).

Expression variation of a gene can either derive from sequence variation that lies within or in the close proximity of the gene (*cis* eQTL) or indirectly from a distant location on the genome (*trans* eQTL). Genes underlying *trans* eQTLs are assumed to encode trans acting factors like transcription factors that control the expression of the target and potentially the correlated expression of several functionally related genes. Expression QTL studies are gaining importance in plant genetics because they represent a potential approach to shortcut the tedious process of positional cloning, especially for genes underlying quantitative traits (Hansen et al 2008). Several large scale expression profiling studies have shown the potential of the methodology to generate the information required to construct a robust and comprehensive sequence based genetic framework map (West et al 2007; Luo et al 2007; Potokina et al 2008), and provide data for eQTL analysis that is directly coupled to candidate gene identification (Shi et al 2007, Druka et al 2008). Furthermore, the eQTL datasets can be potentially used to explore complex networks between genes and which may significantly lead to unravel the complexity underlying specific biological phenomena (Jansen et al 2009).

Potato is the third most important food crop in the world after rice and wheat but its production is hampered by drought stress in most growing regions. Several wild species of potato growing in its centers of origin in South-America have been adapted to harsh

environments at high altitudes above 3,000 meters above sea level and are regularly exposed to water-scarce conditions (Schafleitner et al 2007). Recently high quality expression profiling platforms have been established for potato (Kloosterman et al. 2008), and Vasquez-Robinet et al (2008) successfully employed microarrays to capture transcriptional changes in drought response in two Andean potato genotypes. Therefore, the tools are available for a genetical genomics approach in potato to elucidate genes and networks that underlay QTLs for drought tolerance traits.

In this study, the concept of eQTL mapping was applied to a diploid potato population that had previously been used for QTL mapping of physiological and morphological traits for drought response (Chapters 3 and 4). Population-wide expression profiles were generated and analyzed in order to i) determine the general changes in gene expression in response to water stress, ii) to asses and report on the genome wide genetic architecture of transcript-level variation under optimal and water deficit conditions iii) to determine the transcriptome-wide expression pattern of genotypes and of the position of eQTLs and iv) to identify candidate genes underlying phenotypic QTLs for drought tolerance.

Material and methods

Plant materials and drought treatment

Tubers of a core set of 94 CxE progeny and their parents were planted in pots under greenhouse conditions. CxE is a diploid potato mapping population of nearly 250 genotypes was developed from a cross between C and E. Clone C is a hybrid between *S. phureja* PI225696.1 and *S. tuberosum* dihaploid USW42. Clone E is the result of a cross between clone C and the *S. vernei-S.tuberosum* backcross clone VH34211 (Celis-Gamboa 2002). Eight replications were maintained under completely randomized design. Irrigation was withheld for 6 replications starting from the stolen initiation stage. Two replications were maintained as controls with optimal irrigation until the end of the experiment. Leaf samples were collected for RNA isolation after 4 at which first wilting symptoms were observed (early response) and 9 (late response) days of drought stress.

RNA isolation

Approximately 0.5g of frozen leaf tissue was ground to a fine powder in liquid nitrogen.

Aliquot of 5mg of grounded leaf tissue was used for RNA extraction. RNA was isolated using the KingFisher Flex system and the MagMAX™-96 Total RNA Isolation Kit according to the manufacturer's instructions. The extracted RNA solutions were treated with DNase I, (Amplification Grade –Invitrogen) and quantified using a NanoDrop ND-100 spectrophotometer (Thermo scientific). RNA quality was visualized with 1% agarose gel electrophoresis and samples were stored at -80ºC until use.

Microarray hybridization

200ng of total RNA was used to synthesize cRNA according to the Agilent two-color microarray-based gene expression analysis protocol. The non-stressed samples were labelled with the Cy3 dye and stress RNA samples labelled with the Cy5 dye. Labeling efficiency was estimated spectrophotometrically using the NanoDrop ND-100 (Thermo scientific). Control (Cy3) and stress (Cy5) labelled cRNA of the same genotype was hybridized to the potato oligo array based on the Agilent 60-mer oligonucleotide platform (Kloosterman et al. 2008) and washed following manufacturer's protocol (Agilent technologies). Hybridized slides were scanned using the Agilent G2505B scanner using the extended dynamic range setting.

Statistical analysis and data mining

Microarray images were imported into Agilent Feature Extraction software (V.9.1.3.1) and normalized using the standard two-color protocol. Different data sets were extracted for all hybridizations and were imported in GeneMaths (V 1.6.1) for visualization and further analysis. Transcript levels of 22,192 and 25,941 unique potato features passed the significance and filtering test in control and stress data sets. Data were treated in three different sets: Control samples (non-normalized (Cy3), stress samples (non-normalized) (Cy5) and control to stress (Cy3/Cy5) ratios (normalized). For control and stress data sets, filtering was applied to remove consistently lowly expressed genes and relevant expression was considered when raw intensities were a factor of 3 above background in at least 20 genotypes of the 94 individuals and parental lines. Using GenStat software (12th edition) quantile normalization was carried out across all arrays for the control and stress samples independently. After quantile normalization, expression values were log10 transformed. Previously calculated log ratio's (control to stress) were filtered to contain only those features that are present in either cy3 or cy5 filtered data sets.

eQTL analysis

Large scale eQTL analysis was performed using the R-program MetaNetwork (Fu et al 2007). Potato is an outcrossing species and therefore separate genetic maps for both parents have to be used in the analysis. Genetic maps used in the analysis were based on the genetic maps presented by Anithakumari et al. (2010) (Chapter 2). Genome-wide 1,000x permutation test was performed to identify the log p threshold corresponding to a genome-wide false discovery rate of 5% ($\alpha < 0.05$). The genome wide log p threshold was 3.4 and 3.1 in C and E parental maps, respectively.

The potato 60-mer oligonucelotides present on the microarray were derived from assembled EST unigene sets (Kloosterman et al 2008). Unigene sequences were blasted against the released potato genome database (www.potatogenome.net) and significant hits with potato scaffolds allowed the mapping of array feature on the genome. The majority of the large genome scaffolds have been anchored to the physical map of potato and were assigned to their respective chromosomes. Using the mapping information, identified eQTL's can be classified as either cis- or trans-acting depending on whether their genetic position coincides with the physical position of the gene itself or not. In current study, as we are in initial stages of anchoring genetic markers to genome sequence, we differentiated *trans* QTLs when the physical location of gene is present on different chromosome. For gene sequences that cannot unequivocally be assigned to any genome scaffold or reside on scaffolds that were not anchored to the physical map, no classification could be made.

Results

A pilot experiment was carried out to understand the variation in transcriptional changes in early and late response to drought stress using the microarray. Three progeny plants of CxE were selected depending on their phenotypic response (wilting) to water stress at 4 and 9 days after stress (DAS) initiation and differentially expressed genes were monitored and analyzed. Several hundred to thousands of genes were differentially expressed (induced and repressed) under stress (Supplementary Table1). The number of differentially expressed genes varied significantly between genotypes. Genotypes showed higher variation at 4 DAS than at 9 DAS. Though a higher number of genes were differentially expressed after 9 DAS, the difference between individual genotypes was lower at that time point. Therefore the early time point (4 DAS) was chosen for genome-wide transcriptional profiling.

Table 1 Summary of eQTLs detected on parental maps under well-watered and water stress conditions.

	Control		Stress	
	C	E	C	E
Total eQTLS	8672	9384	8801	12163
Unique in each map	6420	7132	6514	9876
Common between parental maps	2253		2287	
Unique in each treatment	5893		8766	
Common between control and stress	9912			
Total of distinct eQTLS	24571			

Number of eQTLs detected and their genomic distribution

To determine the effect of genetic factors involved in the regulation of expression in response to well-watered and drought stress conditions, we analyzed genome-wide gene expression in the parents and progeny of the CxE population. The majority of transcripts within the potato genome in well watered and drought stressed plants exhibited heritable variation in gene expression that is attributed to genomic regions visualized by an eQTL. A total of 18,044 and 20,964 eQTLs were detected under well-watered and water stress conditions respectively. After separation of common and unique eQTLs, a total of 24,571 distinct eQTLs were detected and the number of eQTLs detected per transcript varied from 0 to 3. Out of 24,571 expression QTLs 24% of eQTLs were specific to non-stress conditions, 36% specific to drought-stressed plants and 40% of the eQTLs were detected under both conditions (Table 1). The positions of the eQTLs were well distributed across the potato genome.

Cis vs Trans eQTLs

To detect the position of genes and their eQTLs, the sequence of the differentially expressed genes was anchored to the potato genome physical map where possible. Under well-watered condition for 67% of the eQTLs the position overlapped with the position of the gene itself and was thus classified as a *cis*-acting eQTL. In the same manner, identified genes showing eQTLs in genome positions other than their physical map position (different chromosome) were classified as *trans*-acting (Table 2). The number eQTLs were spread almost evenly across the 12 chromosomes of potato with a slightly higher number on chromosome 1. The significance and magnitude of *cis*-eQTLs varied from chromosome to chromosome and their distribution based on log p values is presented in Figure 1. Under drought conditions *cis*

eQTLs were more or less equally distributed over all chromosomes but *trans* eQTLs were non-uniformly distributed across the chromosomes, with the majority of eQTLs localized on chromosome 5 and 2 (43% and 14% respectively) (Table 3). The majority of individual eQTLs accounted for only a small proportion of the associated transcript estimated phenotypic variation (R^2). Around 52% of eQTL had an estimated phenotypic effect R^2 that was lower than 0.2 for each transcript (Figure 2a). *Cis*-eQTLs typically explain more of the observed differential expression than *trans*-acting eQTLs (Figures 2b and 2c). For example, 3,885 (34%) *cis* eQTLs explained phenotypic variation between 10-20%. Around 580 (5%) cis eQTLs explained variation above 70% whereas for *trans* eQTLs a large number (86%) of eQTLs explains observed variation between 10-20% (Figure 2c). A minor number of genes with multiple QTLs showed both *cis* and *trans* eQTLs, and the physical position of 3,898 genes were unknown.

Table 2 Genome-wide eQTL analysis and eQTL distribution under well-watered conditions

	C map (Threshold log p 3.4)			**E map** (Threshold log p 3.1)			
Chromosome	*cis*	*trans*	unknown	*cis*	*trans*	unknown	Total
1	804	521	108	609	146	50	2238
2	528	168	66	642	363	73	1840
3	661	68	60	564	237	82	1672
4	462	140	73	678	181	91	1625
5	468	235	72	525	359	86	1745
6	497	90	67	387	85	42	1168
7	605	141	81	301	36	20	1184
8	461	99	76	260	55	50	1001
9	352	90	50	598	325	104	1519
10	389	44	67	599	194	115	1408
11	494	61	67	344	208	52	1226
12	309	127	59	597	235	91	1418
Total	6030	1784	846	6104	2424	856	18044

eQTL analysis of major drought-responsive genes

A number of eQTLs were detected for known and well-characterized drought responsive genes such as genes encoding members of transcription factor gene families, signaling molecules, late embryogenesis abundant proteins, heat shock proteins, genes involved in protection of cell damage like redox genes such as peroxidases, catalases, super oxide dismutase, and genes involved in the drought inducible hormone ABA pathway. The eQTLs for transcription factors and signaling molecules were well distributed across the genome

(Figure 3a), whereas the eQTLs related to the phytohormone ABA pathway were detected only on chromosome 2, 5, 10 and 11. Genes with eQTLs associated with redox functions were detected on all chromosomes except chromosome 10. For all above-mentioned groups the majority of eQTLs clustered on chromosome 5 (Figure 3a). A most remarkable observation is that more than 90% of these eQTLs are *trans* eQTLs, so transcript variation was detected on chromosome 5 but the physical positions of the genes were found to be on other chromosomes in the genome. In fact, the genes were distributed across all other chromosomes as illustrated in Figure 3b. The highest percentage (18%) of genes whose expression was mapped on chromosome 5 were physically located on chromosome 1 and the lowest percentage was on chromosome 10 and 12 (6%).

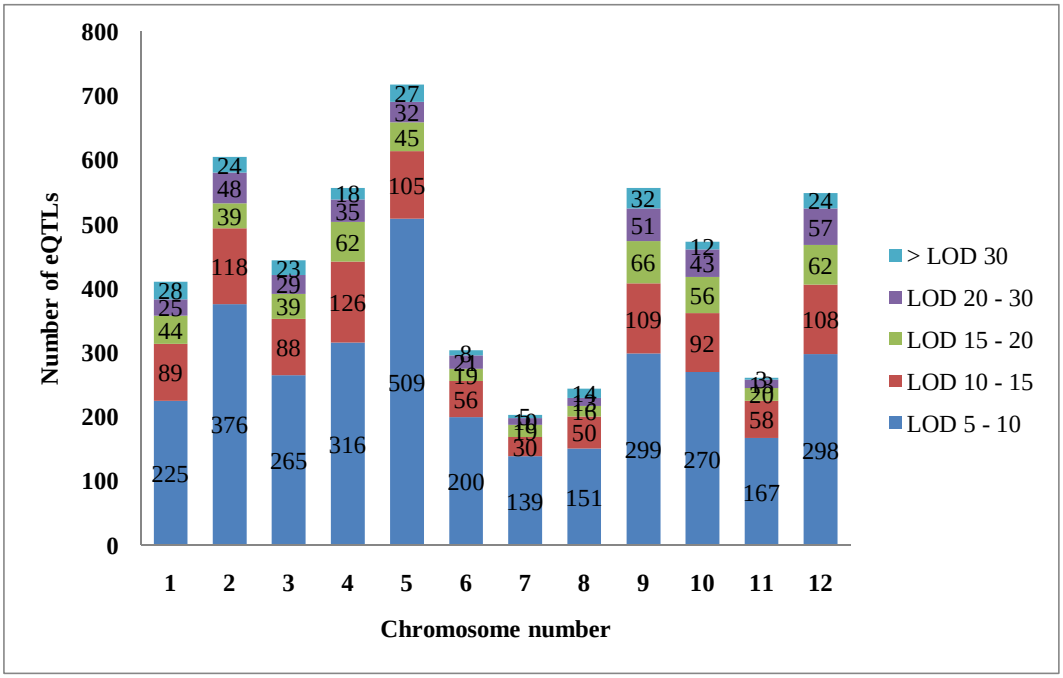


Figure 1 Genome wide distribution of stress eQTLs with log p values. Number of eQTLs for each log p category (different colors) are indicated in the columns.

eQTL analysis of differentially expressed genes (Control/Stress)

The stress specific response of genes was analyzed by calculating the expression ratio of control to stress (control/stress). A total of 25,966 genes were considered to be differentially expressed which accounted for 61.7% of all genes present on the oligo array. From these, 4,393 eQTLs were detected and distributed across twelve chromosomes as shown in Figure 4. Remarkably, a disproportionally large number of the eQTLs (3,119 eQTLs; 71%) were *trans*-

acting. The control/stress ratio eQTLs were grouped into functional categories based on their ontology (Table 4). The functional class RNA processing, regulation of transcription had a high number of eQTLs. The majority of eQTLs belonged to signaling, protein degradation and cell wall, cell wall proteins, cell division functional classes. More than half of the genes could not be functionally annotated.

Table 3 Genome-wide eQTL analysis and their distribution for drought stress.

	C map (Threshold log p 3.5**)**			**E map** (Threshold log p 3.2)			
Chromosome	*cis*	*trans*	unknown	*cis*	*trans*	unknown	Total
1	648	206	96	543	117	50	1660
2	461	67	58	704	935	144	2369
3	628	136	74	502	189	94	1623
4	423	95	71	644	364	125	1722
5	541	1041	156	663	1978	213	4592
6	459	40	67	349	126	44	1085
7	570	106	94	289	42	31	1132
8	429	56	96	269	48	70	968
9	335	185	72	595	317	123	1627
10	354	53	84	551	116	111	1269
11	498	151	91	343	253	75	1411
12	270	39	51	641	399	106	1506
Total	5616	2175	1010	6093	4884	1186	20964

Trans-eQTLs reveal transcriptional Hotspots

Mapping of differentially expressed genes revealed several genomic hotspots for *trans* eQTLs as shown in Figure 4. These hotspots were detected mainly on chromosome 1 and 12 (C-map) and 2, 4, 5 and 12 (E-map). Under control conditions eQTLs were distributed evenly across all chromosomes, but under water stress conditions a large number of eQTLs were detected on chromosome 5. In total 4,592 genes which have variation in expression were mapped on the genetic map of chromosome 5 and more than 65% were *trans* eQTLs (Table 3). Hence this chromosome was considered to be an eQTL hotspot, and possibly a hotspot for transcriptional regulation under drought stress.

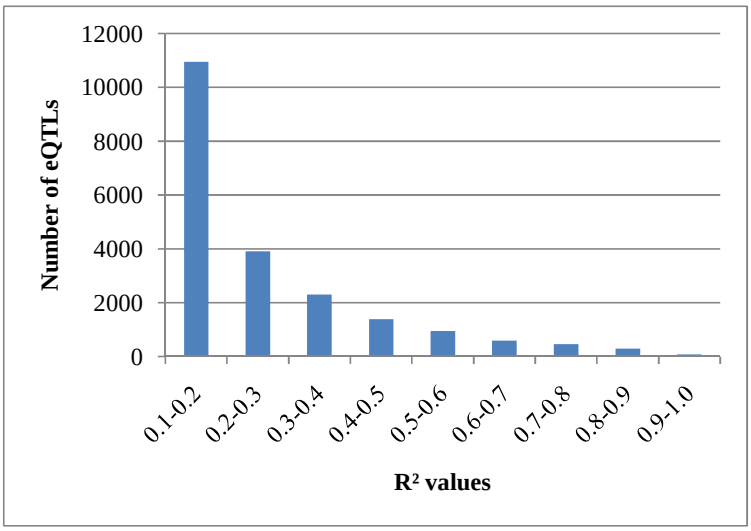
Co-localization of eQTLs with phenotypic trait QTLs for drought response

Several drought responsive phenotype QTLs under *in vitro* and greenhouse conditions have previously been identified in the CxE population (Chapters 3 and 4). A cluster of phenotypic traits colocalized on chromosome 4 and 5. We examined which eQTLs co-localized with

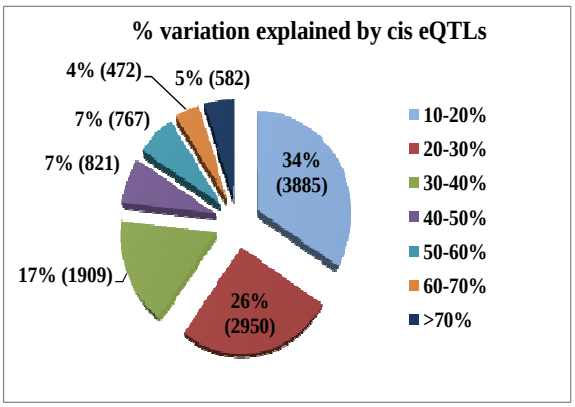
major phenotypic trait QTLs and detected several hundreds of eQTLs co-localizing within phenotypic trait QTL intervals under drought conditions. Table 5 presents QTL regions with each 10 eQTLs mapping to the same region, along with the % of expression variance explained by each eQTL. Several interesting putative candidate genes were detected among these eQTLs. The eQTL for a gene that was annotated with a putative function in the photosystem II light reaction colocalized with trait QTL of chlorophyll florescence (Fv/Fm) on chromosome 1, along with other genes involved in drought response such as heat shock proteins and signaling proteins with known induced expression under stress conditions.
On chromosome 4, around 500 eQTLs colocalized with phenotypic QTLs such as root length, δ13C, number of branches, root dry weight measured under water stress conditions, which covered almost half of the chromosome. Some of these eQTL genes belong to the putative functional classes of signaling, transcription factors of multiple gene families such as AP2, MYB, NAC, WRKY and genes involved in carbohydrate metabolism.

Under water deficit conditions a considerably higher number of eQTLs were detected on chromosome 5 in comparison to control conditions. The majority of growth and yield phenotypic QTLs observed under water deficit and recovery conditions were associated with a region on chromosome 5 known to control plant maturity. Nearly 1,600 eQTLs colocalized with yield and growth trait QTLs. These eQTL genes represent diverse functional classes such as amino acid, carbohydrate and lipid metabolism, transport, signaling, redox, hormones and secondary metabolism.
Phenotypic QTLs for chlorophyll content and $\delta^{13}C$ were located on chromosome 10 and at the same position we found eQTLs for genes involved in carbon partitioning, signaling receptor kinase, transcriptions factors and hormone and lipid metabolism (Table 5). Co-localization of eQTLs was detected for traits such as plant height, root dry weight, shoot fresh weight on other chromosomes as well (data not shown).
The CxE population was evaluated previously under *in vitro* conditions for PEG induced water stress (Chapter 3). Several QTLs were detected under control and under PEG induced stress on chromosome 2. In total 685 eQTLs were detected on chromosome 2 that colocalized with the cluster of QTLs measured under *in vitro* conditions (Chapter 2). Some of these interesting eQTLs are presented in Table 5.

a)



b)



c)

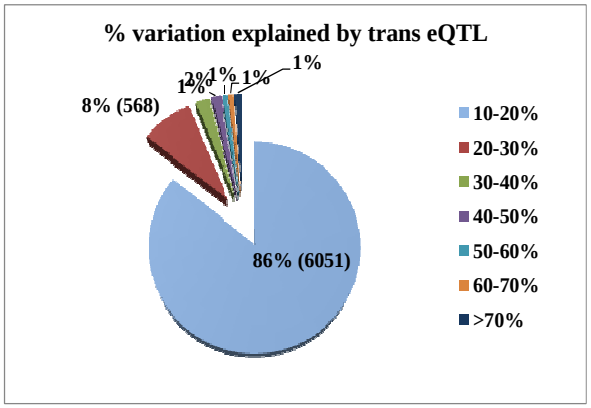


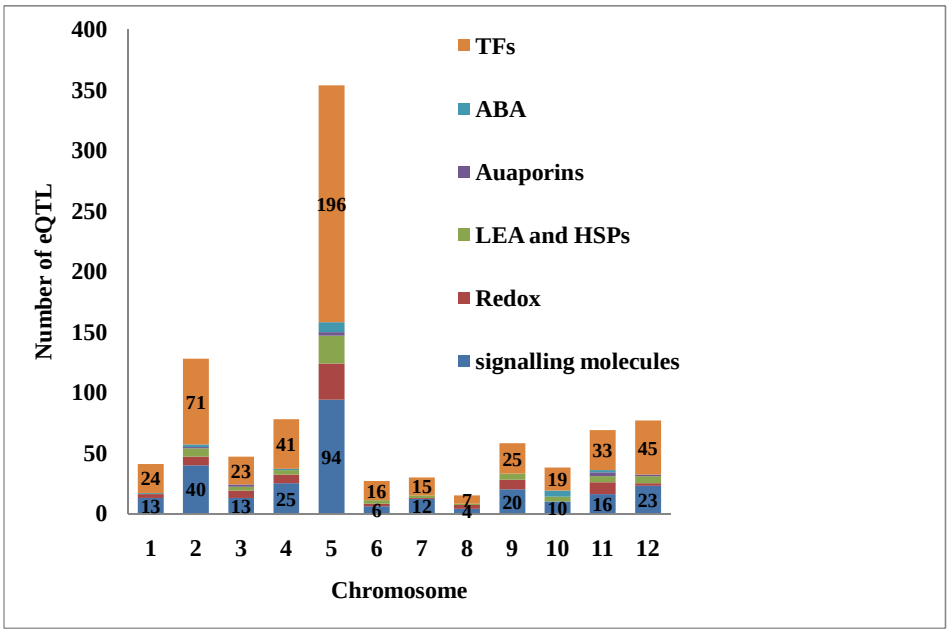
Figure 2. Distribution of estimated phenotypic effect (R^2) for all eQTLs. A distribution of R^2 values for all 20,964 eQTLs is shown in intervals of 0.1, with minimum R^2 of 0.12 and a maximum of 0.97. The two pie graphs (b and c) illustrate the distribution of % variation explained by *cis* (b, total 11,386) and *trans* (c, total 7059). The color scale to the right indicates the color categories for % explained variance.

Discussion

Transcript expression is in many ways an extraordinary phenotype with special attributes that can be of particular importance for genetic studies. The primary potential of genome-wide gene expression genetics is the total number of traits (variation in transcript abundance) that can be assayed simultaneously. In the current study, we unravel the complexity of transcript abundance in potato in relation to the early response to drought. The metabolic changes that occur in plants in response to dehydration stress are described in several reviews (Bray 2002; Ramanjulu and Bartels 2002; Zhu 2002). The large number of drought- or dehydration-induced transcriptome changes underscores the difficulty in understanding the global context

of the drought stress response. As a first step to unravel the complexity of potato transcriptome changes under drought, we performed a genome wide eQTL analysis. A total of 20,968 eQTLs were detected under stress of which 8,766 eQTLs were specific to stress treatment.

a)



b)

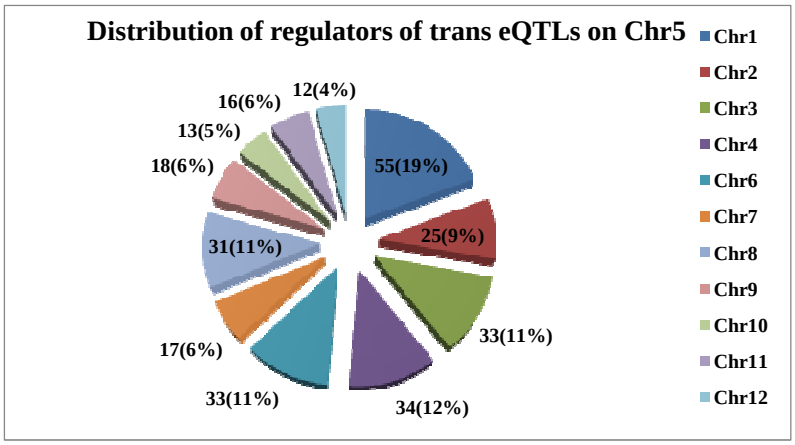


Figure 3 a) eQTL distribution of important drought response genes b) Distribution of gene locations of *trans* eQTLs on chromosome 5.

For differentially expressed genes (control vs stress expression levels) a relatively low number of eQTLs (4,393) were detected when compared to the number of eQTLs identified in the control and water stress data sets. Interestingly, the majority of eQTLs for genes induced

or repressed by drought was *trans*-acting and most of them accumulated on chromosome 2, 4, 5 and 12. The majority of differentially expressed genes showed a quantitative expression profile with complex inheritance patterns. This is because in general genes can be regulated by several independent factors, which may result in a *trans* eQTL. Because of the multiplicity of regulators and often observed epistasis, each *trans* eQTL is expected to have a relatively small effect as confirmed by our results (Figure 2b). In addition, compared to the local regulation of *cis* eQTLs, the variation in the expression of *trans* regulated genes is indirectly also determined by the expression variation of one or more regulators. As a result the detected number of *trans* eQTLs relative to the number of *cis* eQTLs drops when the stringency for detection is increased (Doss et al 2005).

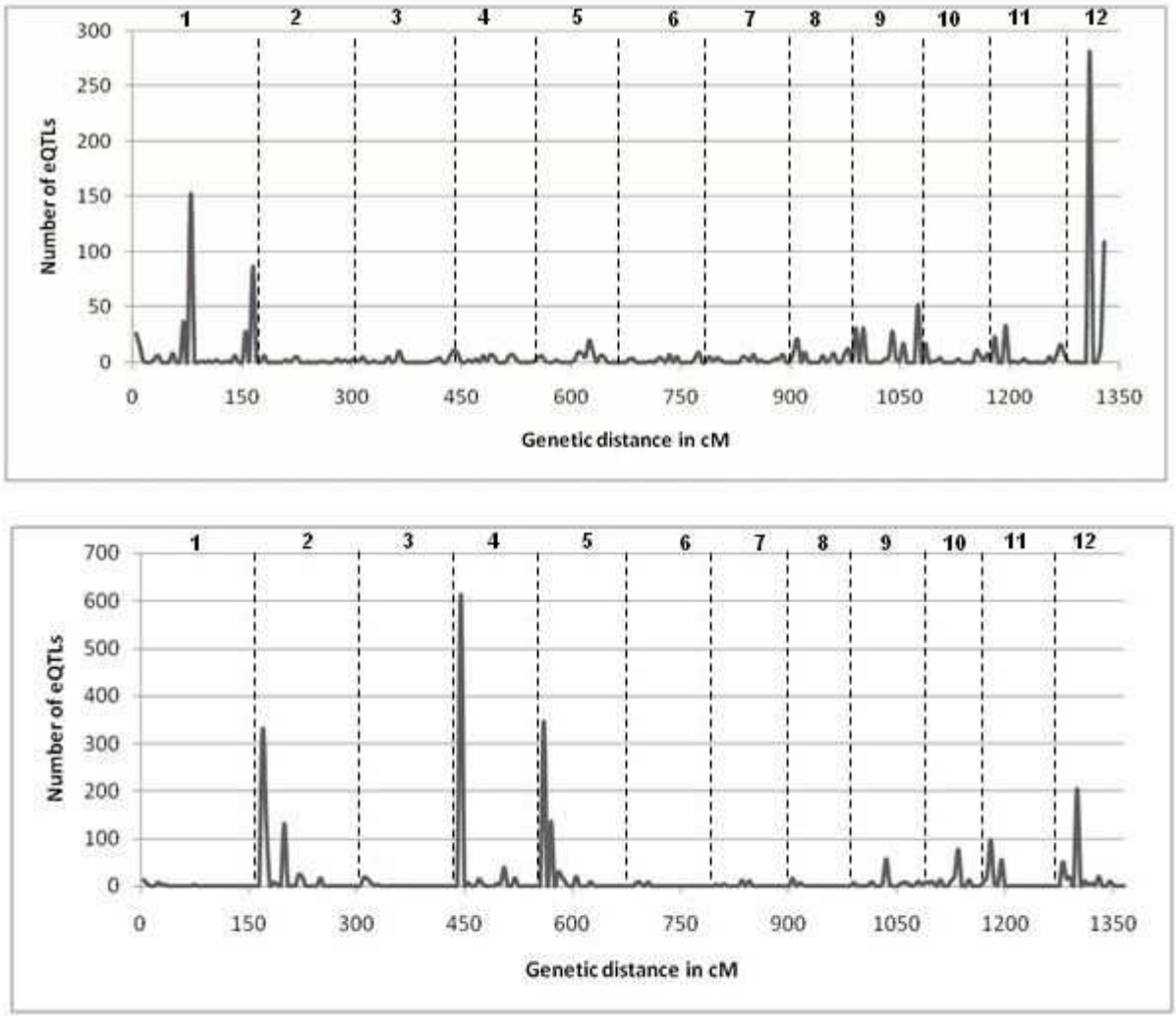


Figure 4 Genome wide eQTL distributions of differentially expressed genes between control and stress on parental genetic maps of C (upper) and E (lower)

In data sets of control and stress separately, we identified 24, 571 significant eQTLs with a genome-wide false discovery rate of 5% ($\alpha < 0.05$) affecting the expression of 18,568 genes. Among the genes with variation, 89% have only one eQTL while the rest have two to three eQTLs; hence we have a higher number of eQTLs than genes. This also implies that genotypic differences in expression are often mainly determined by a single factor. When comparing the location of the eQTL to that of the gene itself in potato, under well watered conditions 67% of the observed eQTLs were *cis* and 23% were *trans*. The majority of the genotypic expression differences may therefore be determined by differences within the gene or its regulatory regions itself. Genetic variation in the promoter region is the most likely underlying reason for these *cis* eQTLs. Under drought stress however the number of *trans* eQTLs detected increased to 34% and *cis* eQTLs accounted for 55%. Several large scale microarray studies on plant eQTLs have been published in different plant species such as Arabidopsis (DeCook 2006, Keurentjes et al 2007; West et al 2007) maize (Schadt et al 2003; Shi et al 2007) wheat (Jordan et al 2007) poplar (Street et al 2006) eucalyptus (Kirst et al 2004) and barley (Potokina et al 2008). These studies report varying numbers of *cis* vs *trans* eQTLs. In one study one third of eQTLs were *cis* eQTLs (West et al 2007) and in another it was 50% *trans* eQTLs and 50% *cis* eQTLs (Keurentjes et al 2007). A study with a barley population found an intermediate value for number of *cis* and *trans* eQTLs when compared with studies in Arabidopsis (Potokina et al 2008). Overall the proportion and number of *cis* and *trans* eQTLs identified in an experiment depends on many factors such as the inherent genetic architecture of the population under study, the number of lines used, the degrees of replication and the external conditions or treatment (Hansen et al 2008). Across the different studies the number of eQTLs identified and their significance varies dramatically. In all these studies a complex inheritance is consistently observed for thousands of transcript traits.

It is important to realize that sequence polymorphisms have been shown to influence the efficiency of hybridization between probe and target on arrays, causing Single Feature Polymorphism (SFP) (Rostoks et al 2005). These SFPs can cause a difference in the estimated transcript abundance of a specific gene (Luo et al 2007). The differences as found on the arrays are then not expression differences, but DNA variations in the region of probe hybridization. This is most likely only a complicating factor for the *cis* eQTLs, as the effect on the expression levels is caused by the gene itself. As potato is highly heterozygous,

variation in gene expression may also occur as the result of different hybridization specificity, making it even more complex in potato than in other species.

Table 4 List of functional classes of differentially expressed gene (control/stress) eQTLs

Functional class	*cis*	*trans*	Unknown	Total
Amino acid metabolism.	19	42	5	66
Cell wall.cell wall proteins, division , organisation	28	131	6	165
Development. lea/storage	12	21	3	36
DNA.synthesis/chromatin structure-histone, repair, unspecific	10	52	3	65
Hormone metabolism.	25	62	5	92
Lipid metabolism.	17	63	5	85
CHO metabolism	16	41	3	60
Metal handling	7	7	1	15
Misc.cytochrome P450	14	22	6	42
Misc. other	34	101	8	143
Nucleotide metabolism.	7	21	1	29
Mitochondrial electron transfer and PS light reaction, calvin cycle	9	44	4	57
Protein. degradation	21	177	11	209
Protein. postranslational modification	16	103	8	127
Protein. synthesis.targeting	14	59	4	77
Redox	9	26	1	36
RNA. process, regulation of transcription	55	267	15	337
Secondary metabolism	24	62	9	95
Signalling	36	109	11	156
Stress. abiotic	13	68	2	83
Stress. biotic	16	20	9	45
Transport	43	90	8	141
Not assigned.unknown	49	2090	24	2163

cis QTLs and *trans* QTL difference in R^2

In the present study *cis* and *trans* eQTLs are associated with different distributions of R^2, which indicates that *cis* QTLs generally have a stronger effect on expression differences than *trans* QTLs. This was also observed in several other studies (West et al 2007, Wayne et al 2004, Hughes et al 2006). A probable explanation for this observation is that the transcript abundance of most genes is regulated by multiple factors at multiple levels, like the expression levels of transcription factors, the posttranslational regulation of these factors, the cellular environment, and the genetic variation in regulatory regions of the target gene itself. Polymorphism in any one of these regulatory levels may be functionally limited to only a

small change in transcript accumulation. *Trans* QTL genes are more likely to be regulated at multiple levels than *cis* QTL genes, explaining the lower average R^2 for *trans* QTLs vs *cis* QTLs. Yet natural mutations in transcription factors can still generate large expression differences in genes regulated in *trans* by the transcription factor, so high R^2 are not exclusive for *cis* QTLs. Polymorphisms in the promoter region of a gene can make the difference between expression or silencing and underlie a strong *cis* QTL with high R^2, but may also have a more modulating effect for instance in efficiency of binding of transcription factors (resulting in lower R^2). The polymorphism underlying a strong *trans*-eQTL does have greater potential to be pleiotropic than a *cis*-eQTL. Large-effect mutations in pleiotropic genes are more likely to be deleterious than mutations in less interconnected genes (Wright 1977; Turelli1988; Wagner 2000; Jeong et al 2001; Yu et al 2004). There may be an evolutionary fitness limitation on the potential genetic effect of polymorphisms that generate *trans*-eQTL hotspots.

Stress response

Among the eQTLs that were detected only under drought conditions, a large number of transcription factors and signaling molecules were identified that share homology with known genes previously studied in other species, proving some clues about putative regulatory and signaling pathways that might be involved in the drought response and drought tolerance in potato. These eQTLs were well distributed across the potato genome (Figure 3a and b).

Expression QTLs for genes involved in biosynthesis and signaling of the stress hormone ABA as well as ABA activated genes were identified on chromosome 2, 5, 10 and 11 (Figure 3). The eQTLs for ABA biosynthesis genes were all *trans*-regulated, whereas signaling, ABA induced and response-regulating genes were *cis*-acting. The involvement of ABA in mediating drought stress has been extensively studied. ABA plays a critical role in regulating growth and plant water status through guard cells that mediate stomatal closure. In addition, genes that encode enzymes and other proteins involved in cellular dehydration tolerance are induced (Zhu 2002). Early work showed that ABA can act as a long distance water stress signal in sensing incoming soil drying (Davis and Zhang 1991). ABA produced in dehydrated roots is transported via xylem and regulates stomatal opening and leaf growth in shoots. Considerable progress has been made in the last decade in identifying ABA dependent and independent pathways (Zhu 2002; Shinozaki and Yamaguchi-Shinozaki 2007). Thus in many

ways ABA plays a pivotal role in whole plant responses to drought stress. Hence, ABA related *cis* eQTLs in our dataset may directly serve to identify potato genes important for ABA mediated drought stress tolerance, whereas *trans* eQTLs can help to identify the regulatory network of the ABA drought response pathway in potato. In addition, the eQTLs related to the ABA-dependent drought response pathway may be linked to metabolic changes, adding yet another functional level to the genes.

Heat Shock Proteins (HSP) are a group of proteins that is induced in plants subjected to water deficit (Joshi and Nguyen 1996; Vierling 1991). HSP function as molecular chaperones that assist in protein folding and prevent protein denaturation (Zhu et al 1993). During stress HSP are necessary to protect proteins against aggregation and denaturation. Like the small HSP, LEA proteins are mainly low molecular weight (10-30kDa) proteins that are involved in protecting higher plants from damage especially during drought (Hong-Bo et al 2005). Several genes with homology to HSP and LEA varied in gene expression within the CxE population. Interestingly several of the identified eQTLs for those genes colocalized with phenotypic QTLs for drought response traits, leaving room for speculation on a role of these chaperones in response to water stress in potato and labeling them as putative candidate genes for drought tolerance.

Drought affects root hydraulic conductivity which is mainly regulated by abundance and/or activity of water channel proteins (aquaporins). Aquaporins are channel-forming membrane proteins with the extraordinary ability to combine a high flux with high specificity for water (Javot and Maurel 2002). Transcript profiles of several aquaporins were identified in our study. These putative genes may be interesting for further study as candidates for drought tolerance; overexpression of an aquaporin (PIP1) gene in rice and tobacco plants conferred drought tolerance (Lian et al 2004; Yu et al 2005). Water deficit may also cause changes in post-transcriptional regulation of aquaporins. Aquaporin activity has been shown to be regulated by phosphorylation, divalent cations and pH (Luu and Maurel 2005). Also it is known that ABA modulates the expression of some PIP genes in roots and leaves (Suga et al 2002; jang et al 2004; Zhu et al 2005).

eQTL Hotspots

As mentioned before, variation in the expression of differentially expressed genes was captured on particular genomic positions of the potato genome (Figure 4). In addition, under

stress conditions the majority of eQTLs accumulate on chromosome 5 (Table 3, Figure 1). Potato chromosome 5 is known to control plant maturity and affects a large number of phenotypic traits. The observed eQTL hotspot does however not coincide with the plant maturity locus. Further genetic dissection of each *trans* eQTL hotspot is needed to understand the biological function of such hotspots. These hotspots may reflect local gene-dense regions, in contrast to cold spots, which may reflect low gene density regions such as centromers. Alternatively, hotspots may contain master regulators such as transcription factors (gene controlling expression of many other genes). In addition, the hotspot on chromosome 5 in particular has a disproportionally high percentage of *trans*-acting QTLs. The hotspot on chromosome 5 may contain key regulators of stress-induced changes. During drought stress and indeed other stresses, the plant needs to adapt its growth, physiology and metabolism in order to survive the adverse conditions. A genome-wide reprogramming is necessary. It is not unlikely that some of the key transcription factors that affect metabolic routes and biosynthetic pathways may present at the locations of the eQTL hotspots. Obviously these hotspots deserve extra attention for abiotic stress tolerance research, and may provide switches for adaptation to adverse conditions of the potato plants.

Co-localization of Phenotype QTLs and eQTLs

Phenotypic traits related to abiotic stress have proven to be quantitative and genetically complex, with multiple underlying genes and interactions among the loci as well as with environmental parameters. Several interesting putative candidate genes are detected in our study with their eQTLs co-localizing with phenotypic trait QTLs (Table 5). One such example is an eQTL on chromosome 1 of a gene encoding a photosystem II reaction center W protein which has a putative function in the photosynthesis light reaction. This eQTL co-localized with a QTL for the chlorophyll fluorescence parameter Fv/Fm. Drought induces a decrease in photosynthetic activity, which has been associated with photo damage of PSII reaction centers (He et al 2005). Chlorophyll fluorescence is widely accepted as an indication of the energetic behavior of PSII (Dau 1994). The potential quantum efficiency of PSII (Fv/Fm) is used as a reliable indicator to evaluate the metabolic imbalance of photosynthesis and yield performance across genotypes under water deficit conditions. Our results suggest that the photosystem II reaction center W gene may play a role in photosynthetic activity and affect the Fv/Fm parameter under drought.

Table 5 List of colocalized eQTLs with phenotypic trait QTLs (10 eQTLs from each linkage group are presented)

Chromosome	Phenotypic trait QTL	QTLmk	log p	% variation explained	QTL type (physical location of gene)	Gene id on array	Functional class
1	Chlorophyll florescence	PotSNP481	4.7	17.9	trans (9)	MICRO.1149.C2	PS.lightreaction.photosystem II.PSII polypeptide subunits
		PotSNP392	15.1	50.8	cis	MICRO.5413.C2	lipid metabolism.lipid degradation.Carnitine racemase
		PotSNP392	3.3	12.6	trans (2)	MICRO.6010.C2	UDP-glucosyltransferase family 1 protein
		PotSNP481	5.6	21.5	cis	SSBN003N09u.scf	not assigned.unknown
		PotSNP392	9.7	35.9	cis	bf_mxlfxxxx_0057g04.t3m.scf	RNA.regulation of transcription.MADS box transcription factor
		PotSNP392	4.2	16.2	trans (8)	MICRO.5912.C2	RNA.regulation of transcription.General Transcription
		PotSNP392	15	50.6	--	MICRO.919.C1	Signalling.G-proteins
		PotSNP481	12.5	43.9	cis	MICRO.11727.C1	not assigned.unknown
		PotSNP392	4	15.2	cis	MICRO.12105.C1	Stress.abiotic.heat shock protein
		PotSNP481	4.9	18.7	cis	STMIF10TV	RNA.regulation of transcription.C2C2(Zn) DOF zinc finger family
4	Tuber wt recovery Root length root dry weight $\delta^{13}C$ number of branches Chlorophyll content	POCI_30301	18.2	57.8	cis	cSTB30E7TH	stress.abiotic.heat
		PotSNP569	17.2	56.1	cis	MICRO.327.C1	TF.APETALA2/Ethylene-responsive element binding protein
		PotSNP1072	17.4	56	--	STMGE18TV	not assigned.unknown
		PotSNP1072	17.1	55.5	--	STMGI80TV	not assigned.unknown
		POCI_30301	16.8	54.7	cis	MICRO.5064.C1	TF-NAC domain transcription factor family
		PotSNP391	13.3	46.2	cis	ACDA00306B06.T3m.scf	stress.abiotic.drought/salt
		PotSNP391	13.2	46	trans (1)	MICRO.171.C2	protein.postranslational modification
		PotSNP609	13.2	46	cis	MICRO.14759.C1	protein.degradation.ubiquitin.E3.SCF.FBOX
		PotSNP569	13	45.8	trans (5)	MICRO.2200.C3	not assigned.unknown
		PotSNP815	13.2	45.8	trans (1)	cSTD9O1TH	not assigned.unknown
5	Tuber weight stress Tuber number stress Tuber nr Recovery Tuber wt recovery Root length Shoot dry wt recovery plant ht recovery	Mando	22.6	66.5	cis	BF_TUBSXXXX_0036H03_T3M.SCF	not assigned.unknown
		StPho1b	21.8	66.3	cis	MICRO.16058.C1	RNA.regulation of transcription.unclassified
		Sti032	21.9	66.1	cis	bf_arrayxxx_0078f11.t7m.scf	not assigned.unknown
		Mando	17.7	57.1	cis	MICRO.12780.C1	Late blight resistance protein homolog R1B-17
		StPho1b	16.9	56.5	cis	bf_swstxxxx_0052e03.t3m.scf	not assigned.unknown
		StPho1b	12.5	45.4	cis	MICRO.765.C7	cell wall.cell wall proteins.RGP
		Sti032	12.5	45.1	cis	MICRO.9523.C1	redox.thioredoxin
		StPho1b	12.3	44.9	cis	MICRO.765.C6	not assigned.unknown
		StPho1b	11.5	42.6	cis	MICRO.763.C1	stress.abiotic.Heat shock protein 90
		StPho1b	11.4	42.4	cis	MICRO.9765.C1	stress.biotic.receptors-Tospovirus resistance protein E
10	Chlorophyll content $\delta^{13}C$	PotSNP776	31.7	78.5	cis	MICRO.9387.C2	RNA.regulation of transcription.Global transcription factor group
		PotSNP27	29.4	75.8	cis	MICRO.10029.C1	RNA.regulation of transcription.putative DNA-binding protein
		PotSNP605	28.3	74.5	cis	POAB769TP	not assigned.unknown
		PotSNP605	29.1	75.5	cis	MICRO.11873.C1	hormone metabolism.gibberelin.induced-regulated-responsive-activated

		StLin8	16.8	56.7	cis	MICRO.4223.C1	major CHO metabolism.degradation.sucrose.invertases.cell wall
		PotSNP53	17.2	55.6	cis	MICRO.7298.C1	transport.amino acids
		PotSNP776	17.1	55.5	cis	MICRO.280.C2	lipid metabolism.Phospholipid synthesis
		PotSNP776	17.1	55.5	cis	MICRO.5814.C1	Co-factor and vitamine metabolism
		PotSNP776	17	55.3	trans (3)	POCAC91TV	signalling.receptor kinases.DUF 26
		PotSNP776	16.8	54.7	cis	SDBN002G04u.scf	development.late embryogenesis abundant
2 (1)	***In vitro***	PotSNP986	49.9	91.3	cis	MICRO.10921.C1	not assigned.no ontology
		PotSNP792	49.8	91.3	cis	MICRO.11641.C1	not assigned.unknown
		PotSNP986	38.8	84.9	cis	STMHK73TV	DNA.repair
	Fresh biomass control	PotSNP18	38.5	84.6	--	MICRO.6663.C7	not assigned.unknown
	Dry biomass control	PotSNP56	37.1	83.5	trans (8)	MICRO.5912.C1	RNA.regulation of transcription.General Transcription
	Dry biomass stress	PotSNP893	29.4	75.9	cis	MICRO.141.C2	redox.glutaredoxins
	shoot fresh weight stress	PotSNP56	27.5	73.4	cis	bf_stolxxxx_0042c03.t3m.scf	TF-bHLH,Basic Helix-Loop-Helix family
		PotSNP986	26.6	72.2	cis	POCAE94TV	nucleotide metabolism.synthesis.pyrimidine.dihydroorotase
		PotSNP56	24	68.4	--	STMHZ36TV	signalling.calcium
		PotSNP893	20.8	63	cis	MICRO.16545.C1	Receptor-like serine-threonine protein kinase
2 (2)	***In vitro***	PotSNP838	88.4	98.7	cis	cSTB2J20TH	transport.metabolite transporters at the envelope membrane
		PotSNP567	49.3	91.1	cis	MICRO.6230.C2	secondary metabolism.wax
		PotSNP567	47.5	90.2	cis	MICRO.12870.C2	not assigned.unknown
	Fresh biomass recovery	Sti024	39.7	87.1	cis	MICRO.16695.C1	not assigned.unknown
	Plant height control	PotSNP838	38.4	84.6	cis	bf_suspxxxx_0030b01.t3m.scf	not assigned.unknown
	Shoot fresh weight recovery	PotSNP838	36.1	82.7	cis	MICRO.12327.C1	protein.folding
		PotSNP838	36.1	82.7	cis	MICRO.7159.C1	transport.potassium
		PotSNP838	35.3	82	cis	MICRO.17393.C1	not assigned.no ontology
		PotSNP838	34.1	80.9	cis	MICRO.8097.C2	protein.postranslational modification (protein kinase)
		PotSNP838	32.8	79.6	cis	MICRO.15171.C1	CONSTANS-like zinc finger protein
		Sti024	29.3	77.6	cis	MICRO.10417.C1	Zinc ion binding protein

Regulation of gene expression influences or controls many of the biological processes in a cell or organism, such as progression through the cell cycle, metabolic and physiological balance and responses and adaptations to the environment. Development is based on the cellular capacity for differential gene regulation and is often controlled by transcription factors acting as switches between regulatory cascades (Scott 2000). In addition, alterations in the expression of genes coding for transcriptional regulators are emerging as a major source of the diversity and changes that underlie evolution (Carroll 2000). In the present study, eQTLs of transcription factor genes of members of multiple gene families co-localized with a cluster of phenotypic trait QTLs on chromosome 4 under water stress conditions. Several of those genes belong to AP2 and NAC domain transcription factor families. Several studies reported that AP2 domain proteins control the expression of amongst others abiotic stress responsive genes, ethylene-responsive genes involved in ethylene, salicylic acid and jasmonic acid responses and disease resistance (Liu et al 1998; Sakuma et al 2002; Gutterson and Reuber 2004; Karaba et al, 2007). The plant specific NAC transcription factors play diverse roles in plant development and stress response. Over-expression of NAC genes as well as AP2 transcription factors in Arabidopsis, brassica and rice showed significant increase in drought resistance (Hegedus et al 2003; Lu et al 2007; Nakashima et al 2007). Hence, eQTL genes of transcription factors may be important genes for drought response and drought tolerance in potato and particular members of these families may be identified as candidate genes by further analysis of our datasets.

In the current study several hundreds of eQTLs co-localized with phenotypic trait QTLs, making it difficult to identify a causal relationship between genes and the phenotypic traits. Though eQTL analysis is potentially a powerful approach for the identification of genes underlying particular biological phenotypes (Chen et al 2010; Kliebenstein et al 2006), for the approach to be applicable to a specific trait, variation in the observed and measured phenotype of the trait is required to visualize the biological manifestation of variation in the expression of causal genes. In order to be able to pinpoint a candidate gene for a specific trait, the variation in expression of a gene in the drought response should correlate with the drought response phenotypic trait. In addition both the causal genes and their eQTLs should co-localize with the phenotypic QTL, which means it is regulated in *cis*. If these criteria are not met it is difficult to identify genes underlying the trait of interest. A fundamental issue in

quantitative genetics is how the genotype determines the quantitative trait phenotype (Mackay 2001). A study of transcriptional variation may only answer part of this question. Determining the actual biological relationship between transcript level variation and phenotype may also require protein and metabolite data that in turn influence trait phenotypes further downstream. Integration of all these datasets is still highly complex but may be very rewarding (Jansen et al 2009).

Prospective

Genome wide identification of genes regulated by drought conditions has many benefits. Firstly, it provides a more comprehensive understanding of the transcriptional response to drought. Secondly, it provides novel candidate genes that can be the subject of further research. Thirdly, it aids in the identification of regulatory networks based on *cis*- and *trans*-acting QTLs that can serve as a basis for novel plant breeding strategies and crop engineering. The current study demonstrates that the genetic control of transcript levels is highly variable and multifaceted. Based on gene ontology, a number of eQTLs were detected for genes which have homology to very well known drought responsive genes such as transcription factors of multiple families, signaling molecules, redox genes, chaperones and transporters. This eQTL approach led to the genome-wide identification of putative candidate genes involved in drought tolerance, their distribution and regulation as well as identification of putative candidate genes underlying phenotypic trait QTLs for drought response. However, due to complexity of the required statistical analyses involving both large number of tests and a large number of eQTL, we have only touched the surface of the information contained in the transcriptome dataset combined with the phenotyping data. Processes like epistasis will be investigated in a subsequent effort. Knowledge of and insight in regulation and interaction of genes contributing to specific phenotypes is often limited. Further analyses will be focusing on construction of regulatory networks which may narrow down the number of candidate genes in an eQTL interval and to select the best candidate gene.

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We would like to thank Marian Oortwijn for help in labeling the probs.

Chapter 6

General discussion

Introduction

Drought is one of the most common abiotic stresses that limits crop growth and productivity worldwide and its effects on crop plants can range from minor reductions in yield to the destruction of crops leading to famine (Hetherington 1998). Research into the plant response to water stress is becoming increasingly important, as most climate change scenarios suggest an increase in aridity in many areas of the globe (Petit et al 1999). This increase in arid land and the world's growing population will have direct impact on water resources and water availability. The response to drought at the whole plant and crop level is complex because it reflects the integration of stress effects and responses at all underlying levels of organization over space and time (Bray 1997). Breeding activities have led to some yield increase in drought environments mainly in cereal crop plants. Meanwhile, fundamental research has provided significant gains in the understanding of the physiological and molecular responses of plants to water deficits, but there still is a large gap between yields in optimal, sub-optimal and stress conditions. Minimizing the yield gap and increasing yield stability under different stress conditions is of strategic importance for food security in the near future.

Potato is the most important non-cereal food crop in the world and cultivated worldwide under various environmental conditions. Besides being important in human diet, potatoes are also used as animal feed and as raw material for several industrial purposes. This versatile crop is sensitive to water stress. This thesis describes the initial efforts made in dissection of drought tolerance in potato by genetic and genomics approaches using a diploid mapping population.

Molecular markers

An important requirement for genetic dissection of a complex trait like drought is a good quality genetic map. Single nucleotide polymorphisms (SNPs) are used as molecular markers for a variety of tasks in crop improvement including quantitative trait loci (QTL) discovery, assessment of genetic diversity, association analysis and marker assisted selection. SNPs have two main advantages over other molecular markers; they are the most abundant form of genetic variation within genomes (Zhu et al 2003), and a wide array of technologies have now been developed for high-throughput SNP analysis.

The initial phase of the potato SNP discovery effort described in Chapter 2 resulted in the identification of over 7000 reliable SNPs from public EST databases that met the criteria for

high-throughput genotyping on the Illumina GoldenGate platform. The 384 SNPs that we assembled on a GoldenGate SNP genotyping array were used in the two diploid mapping populations CxE and SHxRH and we achieved an 89% assay success rate. In total 165 and 185 polymorphic markers (for CxE and SHxRH respectively) were successfully mapped on genetic linkage maps (Chapter 2). Another array with 768 SNPs from the same potato SNP database was used to further populate the genetic map. A total of 343 polymorphic markers were added on the CE genetic map. Using 163 markers that were heterozygous in both parents an integrated map was constructed (Figure 1). In both arrays 10-11% of SNPs failed to give a result in the standard GoldenGate assay. In Chapter 2 we discussed the possible explanations for SNP assay failures, such as SNPs in the primer target sequence and presence of introns in the SNP amplified regions. With the availability of the genome sequence of doubled monoploid potato DM1-3 516R44 (DM) and of the RH89-039-16 clone, it was possible to examine the SNP marker loci for paralogs and intron spanning sequences using BLAST analysis (www.potatogenome.net) (Dr. H. van Eck, Wageningen UR Plant Breeding, personal communication). From the 384 SNP array 279 SNP sequences had a unique hit on the DM scaffolds. However 105 SNPs had on average 2.5 hits. The reason for two or more hits could be either the presence of multiple paralogs, or introns within the SNP locus. Based on the similarity between the query length of 101 basepairs (or less) and the sequence match a distinction between paralogs and intron spanning could be suggested. When the intron/exon boundary was between 28 and 73bp from either end of the query sequence the SNP amplification was concluded to be intron spanning. When the query sequence and the match differed less than 14bp the duplicates were concluded to be paralogs. From the 105 SNPs with multiple hits, about half were concluded to have introns in the SNP amplified locus, and the other half most likely had paralogous sequences interfering with the SNP assays. The possibility of screening potential markers for their usefulness in marker assays is one more example of the many ways in which the potato genome sequence can enhance and improve genetic research. In total 732 SNP marker loci were unique in the potato genome sequence many of these SNP markers not only served as landmarks on the genetic map but also as putative genes that may underlie quantitative traits (Chapter 3). In addition these SNP markers are now utilized as anchors in the potato physical map.

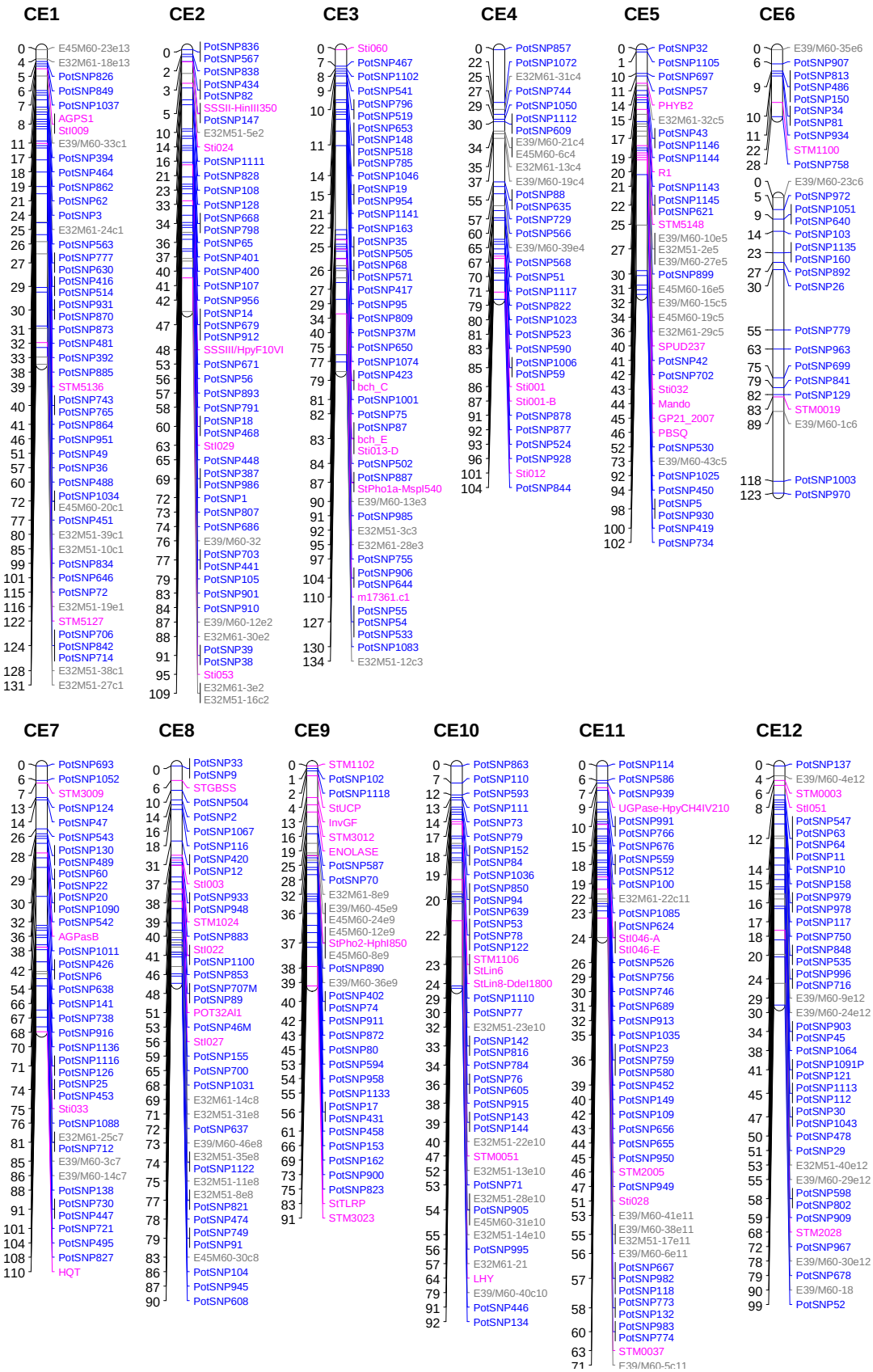

Figure 1 Location of the SNP markers on the integrated map of CxE. The numbers on the left side are the genetic distances in centiMorgans (cM) right side shows the marker designations. Color indicates different marker types (blue: SNPs, pink: SSRs and CAPs, grey: AFLP markers).

QTL analysis

Molecular markers can be used to explore germplasm through segregation and association mapping and identify useful alleles in both cultivated and wild relatives. Most of the data available on drought tolerance are based on segregation mapping and QTL analysis. During the last decade, the application of QTL analysis has provided unprecedented opportunities to identify chromosome regions regulating the physiological, morphological and developmental changes observed during plant growth in water limiting conditions. Particular attention has been paid to: i) genetic variation of osmotic adjustment (Teulat et al 1998; Robin et al 2003); ii) genetic basis of phenological traits such as the stay-green phenotype (Sanchez et al 2002; Verma et al 2004); iii) the ability of roots to exploit deep soil moisture to meet evapo-transpirational demand (Nguyen et al 2004); iv) the limitation of water use by reduction of leaf area and shortening of growth period (Anyia and Herzog 2004); v) isotope discrimination (Saranga et al 2004; Juenger et al 2005); vi) the limitation of non-stomatal water loss from leaves through the cuticle (Lafitte and Courtois 2002) and vii) the response of leaf elongation rate to soil moisture and evaporative demand (Reymond et al 2003).

Drought tolerance of genotypes can be assessed by several parameters, namely yield or biomass under drought, biomass under drought as a percentage of yield (biomass) in control (relative yield) and drought susceptibility index (Fischer 1978). We evaluated the potato CxE mapping population for drought response under *in vitro* and greenhouse conditions (Chapters 3 and 4). Several physiological traits as well as root, shoot and yield parameters were studied under control and stress conditions. In addition, we estimated the relative reduction for all measured traits to study the severity of stress effects. Many significant multi-year, multi-treatment QTLs were detected. However, the QTLs for the estimated relative reduction traits were below the threshold levels. This may be due to the presence of more random variation resulting from the estimation of relative reduction traits when compared to absolute measurements taken under stress and control conditions.

Many of the QTLs for growth traits measured both in the greenhouse and *in vitro* were specific to either of the growth conditions. Obviously the large difference between growing plants *in vitro* and in pots in the greenhouse has a large effect on the genetic factors determining growth under both well-watered and water-deficit conditions. In addition several studies show that in many species shoot culture itself is quite stressful for the plants (De Klerk

2007; Van Staden et al 2006; Desjardins et al 2009). *In vitro* plants grow under unnatural

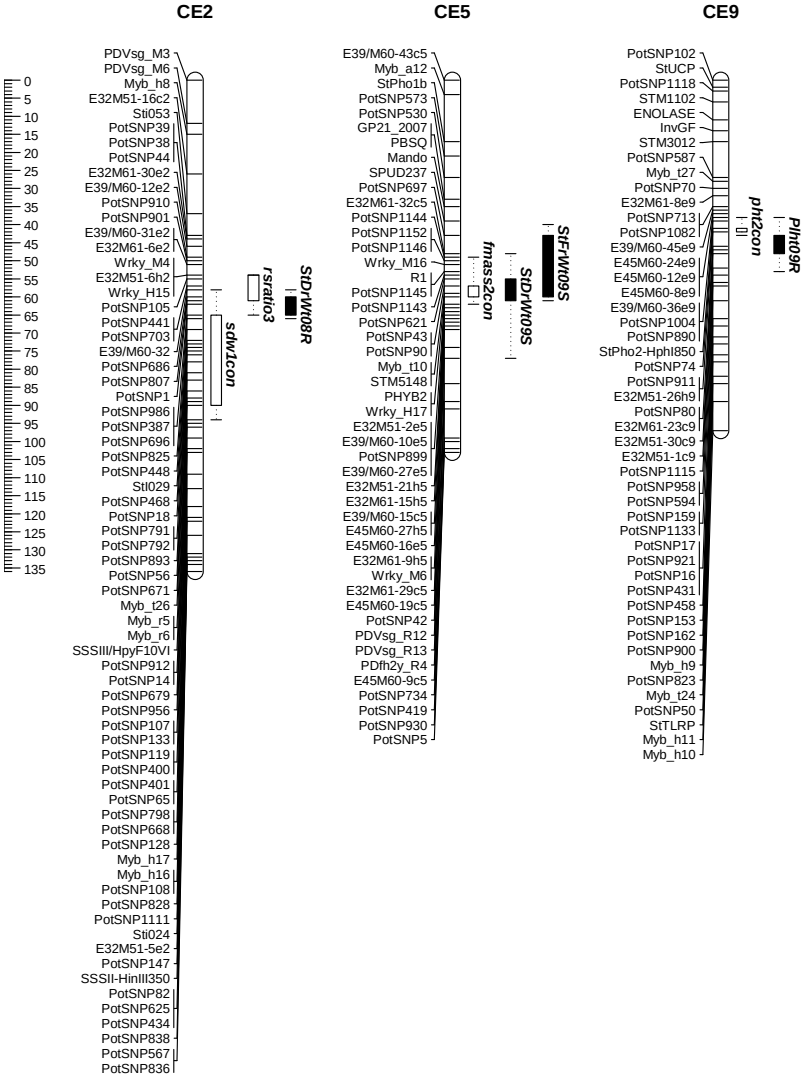

Figure 2 Co-localization of QTLs from *in vitro* (open bars) and QTLs from greenhouse (solid/filled bars). Only the linkage groups (chromosomes) with co-localized QTLs are shown. The left side ruler is the genetic distance in centiMorgans (cM); marker designations are given on the left side of the linkage group, QTLs are shown at the right side in vertical bars with trait names.

conditions: plantlets are wounded first, they receive sugar from the nutrient medium as a replacement of photosynthesis in the leaves, and water balance is disturbed by the very high humidity in the tissue culture containers. Hence, the *in vitro* plants do not go through the same developmental changes as greenhouse or field grown plants. Therefore, depending on growth conditions there may be a different genetic regulation of traits. In addition, the method for inducing water stress in both environments is different. The *in vitro* plants experienced a

constant osmotic stress through PEG treatment, while for the greenhouse plants the pot soil was completely dried down.

Despite these differences significant QTLs that were detected for plant height, shoot dry weight, fresh biomass for plants grown in the greenhouse were also found when the population was grown *in vitro* (Figure 2). These QTLs may be less affected by environmental influences, and we may expect that some of these QTLs will be relevant under field conditions as well. This also suggests that the *in vitro* system may be used for a first and preliminary selection in breeding programmes for specific performance-related traits.

Identification and measurement of secondary traits associated with yield provides a guide to specific mechanisms that contribute to final yield under drought. Water depletion patterns and canopy temperatures are indicative of root exploration, water extraction capacity and transpiration characteristics, and chlorophyll content is a measure of functional stay-green (Baker et al 2004). Some secondary traits such as photosynthetic rate are indicative of plant growth. Ideally secondary traits should be correlated with yield under stress, highly heritable, easy to measure and stable over time and location. Several studies have addressed yield under drought stress as a function of single physiological traits in attempts to understand which metabolic processes or morpho-physiological traits are crucial in ensuring high yield performance under a wide range of environments. We also studied several physiological traits such as leaf relative water content (RWC), chlorophyll fluorescence (Fv/Fm), chlorophyll content (SPAD meter reading) and carbon isotope discrimination ($\delta^{13}C$) under stress conditions (Chapter 4). Significant QTLs were detected for these parameters that provide rapid indicators of drought stress and can be used as methods for studying the response to water stress of potato.

It is important to note that QTL x Environment interaction (year 2008 and 2009 experiments) does exist for these traits. We found QTLxE interaction for the trait $\delta^{13}C$ and we speculated that the function of $\delta^{13}C$ was genetically split into a stomatal and non-stomatal component (Chapter 4). The QTL proposed for the stomatal component on chromosome 10 was mapped on a SNP and CAPs marker within a gene annotated as the carbon partitioning enzyme invertase. In the absence of stress, sucrose converted by invertases into hexoses is the source for starch accumulation in tubers. Water deficit inhibits photosynthesis by closing the stomata and thereby the photosynthetic flux decreases, a condition associated with loss of invertase activity and a depletion of starch and sugars in sink tissues (Zinselmeier et al 1995). Thereby

more sugar accumulates in source tissues, which in turn leads to the feedback inhibition of photosynthesis. The $\delta^{13}C$ trait depends on several factors including carbon partitioning and transport. Carbon portioning between photosynthetically active source tissues and photosynthetically less active or inactive sink tissues such as roots, tubers and fruits is essential for plant growth and development. The two key enzymes (invertase and sucrose synthase) involved in sucrose metabolism are very important both in phloem unloading and for the import of sucrose into sink organs (Ho et al 1991). The extracellular invertases are well studied for role in source and sink regulation (Roitsch et al 2005). In addition extracellular invertase was shown to be an essential component of cytokinin-mediated delay of senescence (Balibrea Lara et al 2004). Drought induced senescence is one of the factors that reduces plant biomass under water limited conditions. Delayed leaf senescence is often associated with drought tolerance and it has been well studied trait in many crops (Campos et al 2004; Jiang et al; Rivero et al 2007; Agbicodo 2009). To understand more about the essential role of carbon partitioning enzymes to unravel the relationship between $\delta^{13}C$, source and sink relationship and drought response we are further focusing on invertases, sucrose synthases and sucrose phosphate synthases in the CxE population for drought response. The expression and activity of these genes and of associated metabolites will be studied.

In general, the complexity of drought tolerance explains the slow progress in yield improvement in drought prone environments. Despite all the advanced work in quantitative genetics for drought tolerance, the overall contribution to the breeding of drought-tolerant cultivars has so far been marginal. Direct selection for yield traits under water stressed conditions is hampered by low heritability, polygenic control, epistasis, significant genotype by environment (GxE) interactions, and QTLxE interactions (Piepho 2000). An important pitfall of most QTL studies in drought is that the parental lines have mainly been chosen based on differences in target traits rather than on their overall agronomic value, which is often poor. Although this approach maximizes the possibility of identifying QTLs for the target traits, it doesn't guarantee any real progress in terms of field performance (Tuberosa and Salvi 2006). In the current study we used a diploid mapping population mainly because both drought and cultivated potato genetics are complex in nature. The CxE population has other advantages. It has been well characterized genetically for several quality traits. It also has the wild relative *S. phureja* as a parent, which is likely to harbor interesting stress

tolerance alleles. CxE shares a parent in its pedigree with the clone RH89-039-16 for which the complete genome sequence will be available soon (http://www.potatogenome.net/), allowing optimal exploitation of genome sequence information. In addition it segregates for drought response. Dissection of the genetics of drought response in this population especially with the integration of genomics approaches helps to zoom in onto the gene level (Chapter 5), and once the candidate genes for particular traits are identified, allelic variation for these genes can be exploited in tetraploid potato cultivars with high agronomic value.

Genomics approaches

The tools of genomics offer a means to produce comprehensive datasets on changes in gene expression, protein profiles and metabolites in response to drought for further understanding of the genetic basis of drought tolerance in crops. Regulation of gene expression at the level of transcription influences or controls many of the biological processes in a cell or organism, such as progression through the cell cycle, metabolic and physiological balance and the adaptive response to environmental changes. The genetic architecture of transcript-level variation for the drought response was captured in the diploid potato population CxE and mapped as expression QTLs (eQTLs) (Chapter 5). To identify whether the genetic variation responsible for eQTLs is *cis*- or *trans*-regulated, we anchored the genes to the genome sequence of potato. Initial results from our study revealed that genome-wide distributions of eQTLs allowed the identification of regulatory hot spots for drought response. Based on gene ontology, a number of eQTLs were detected for genes known to be involved in drought signal transduction and drought-induced transcriptional regulation, and for redox genes, chaperones and transporters. Although interesting results were obtained by examining co-localization of eQTLs and phenotypic QTLs, we have only studied the top layer of the information contained in the transcriptome dataset combined with the phenotyping data and there is more information that can be mined from this dataset in subsequent efforts. For instance, an *in silico* BSA-QTL analysis can be performed. Kloosterman et al (2010) demonstrated the identification of genes by a pooling strategy: genotypes with contrasting phenotypes for a specific trait were pooled, and transcriptome analysis was performed on the pooled samples. Expression differences observed between the pools are expected to be most likely linked to the selection criteria of the genotypes in the pool, which is the trait effect. BSA-QTL analysis is done on pools of genotypes selected for the presence specific alleles/haplotypes of markers

specifying a target QTL. Differences in expression between these pools in transcriptome analysis will reveal the genes underlying the QTL. With the dataset presented and discussed in Chapter 5, it is possible to do a BSA-QTL analysis for all interesting QTLs by pooling the data for genotypes selected for the presence of the different QTL marker haplotypes. This would give additional information on genes specifying the QTL effect. In addition, further analyses will be focusing on construction of regulatory networks which may narrow down the number of candidate genes in an eQTL interval and to select the best candidate gene. In addition processes like epistasis will be investigated in a subsequent effort.

Several interesting genes with putative functions as transcription factors such as AP2, NAC, MYB, MYC and B-Zip genes were colocalized with phenotypic QTLs. The genes from the AP2/ERF transcription factor family were well characterized for their involvement in abiotic stress. The two *Arabidopsis* genes *HARDY* (*HRD*) and *SHINE* (*SHN*) belonging to AP2 family were well studied in *Arabidopsis* and rice for drought and salt tolerance (Karaba et al 2007 and Dixit 2008). Both genes confer enhanced drought stress tolerance to Arabidopsis and rice. The effect of over-expression of the *HRD* gene in rice is especially interesting as it has no yield (biomass) penalty under well-watered conditions either. *HRD* expression both in rice and Arabidopsis results in an increased root system, while *SHN* over-expression affects leaf properties like wax deposition. We are currently characterizing the consequences of expression of these interesting genes in potato. Potato cultivar Desiree was used for transgenic studies with *HRD* and *SHN* genes under the control of different promoters (pCaMV35S, pRD29A and pAKT1 promoters for *HRD* and pCaMV35S, pRD29A, pSsuAra promoters for *SHN)*. Initial results show better performance of several transgenic plants when compared to non-transformed Desiree plants under water stress conditions (data not shown). Experiments are continuing for detailed phenotype and expression analysis.

Recent progress in functional genomics employing genome-wide strategies is widening our knowledge for better understanding of genetic complexities. The successful exploitation of genomics to enhance drought tolerance will only be possible within a coherent, interdisciplinary context that enables integrated analysis of several levels of regulation, with the potential to gain a thorough understanding of the factors limiting potato yield in drought-prone environments. In potato further emphasis is needed on following research areas:

1) High throughput precision phenotyping for drought tolerance

The importance of characterizing and understanding the plant phenotype can not be overemphasized. Especially when dissecting complex traits into genetic parameters through a QTL mapping approach, it is often useful if not essential to be able to dissect the phenotype into parameters which are more heritable and can be measured under normal and stress conditions. To unravel the genetic basis of complex traits, genotypic information is associated with the corresponding phenotypic data. Despite the spectacular progress in DNA marker assays and sequencing technologies during the past decade, the implementation of accurate, high-throughput phenotyping for drought tolerance traits remains a major challenge as part of plant genomics and genetic (quantitative/population genetics) studies on drought tolerance. There is a need for high throughput precision phenotyping that would allow the researcher to obtain detailed information of plant characteristics that collectively provide reliable estimates of trait phenotypes for many of the underlying genotypes that comprise a typical plant breeding population. The ideal precision phenotyping traits should be easily adaptable to field conditions, as drought tolerance that impacts field crop yield can only be assessed reliably in multi-location field trials. An ideal phenotyping technique should also enable to measure dynamic traits such as biomass accumulation in relation to drought tolerance. These traits are often only measured once, and ignoring their dynamic nature and progress in time entails a tremendous loss of information regarding the analysis of gene networks that are active at different phases of plant development but may impact end harvest yield, and their interaction with environmental stresses.

Precision phenotyping might be useful in effective exploitation of the potential benefits deriving from QTL discovery. In addition, when genetic and genomics approaches are integrated, precision phenotyping would possibly enable identification of only few candidate genes related to a trait. Dissection of complex traits into individual gene functions will enhance our understanding of the physiology and genetic regulation of the genes and alleles that contribute to drought tolerance.

2) Importance of other omics

With the ever decreasing costs on sequencing technologies, it may be affordable in the near future to sequence the full genomes or parts of the genome from the different offspring plants or from association panels. This would greatly help us in understanding genetic organization

and regulation at transcript level. However, genome sequence information alone is insufficient to reveal how genes function in developmental/regulatory pathways and the biochemical kinetics of plants to adapt under stresses and consequently to determine the exact responsive mechanism. To investigate this, more comprehensive approaches that include quantitative and qualitative analyses of gene expression products are necessary not only at the transcriptome level but also at proteome and metabolome levels.

Several studies in recent years have shown that metabolomics research can be an invaluable tool for generating information of use in many research areas. Metabolomics information can assist in the establishment of a deeper understanding of the complex interactive nature of plant metabolomic networks and their responses to environmental and genetic change. It will provide unique insights into the fundamental nature of plant phenotypes in relation to development, physiology, tissue identity, resistance, biodiversity and other processes (Shinozaki and Sakakibara 2009; Keurentjes 2009).

Proteomics is also becoming a powerful tool to analyze biochemical pathways and the complex response of plant to environmental stimuli. In particular, comparative proteomic investigations of plants before and after specific or interactive stresses will allow us to obtain information on how tolerance mechanisms are adopted from plants (Timperio et al 2008). In addition, proteomics also provides an essential link between the transcriptome and metabolome (Cook et al 2004; Gray and Heath 2005), complementing genomics research.

Systematic analyses of data from different omics are important for integrative biology. For thorough understanding of biological function or plant response to stress it is necessary to integrate omics data at various levels. Despite the challenges, scientists are making progress in identifying, extracting and interpreting biological insights from omics datasets. For example, transcriptomics and metabolomics data was integrated to analyse the central plant metabolism of the *Arabidopsis* by biochemical networks and epistasis (Rowe et al 2008). In the future more emphasis will be on integration of omics along with modeling predictions and this will lead towards a system biology insight, which will paint a complete picture of the plant response at several levels leading to its final phenotype.

3) Association mapping

Most quantitative approaches used to study complex traits such as drought tolerance have been conducted in a limited number of mapping populations, which harbor a very small part

of the existing allelic variation. This identifies only a fraction of the loci involved in the control of the traits. There is need to be able to add multiple alleles to genetic analyses to get insights into the broad genetic architecture of traits. An association mapping (linkage disequilibrium mapping) approach is well suited for this because it scrutinizes the results of thousands of generations of recombination and selection (Syvanen, 2005). Association mapping is increasingly being adopted as a genetic method complementary to traditional QTL mapping. The main advantages of association mapping are exploitation of allelic diversity from a collection of various more or less related cultivars and breeding materials, and providing generic results. In addition, a higher mapping resolution may be reached as many more meiotic recombination events are sampled compared to a bi-parental segregating mapping population. Application of association mapping also has more advantages particularly in crops that are limited to no more than one generation per year (Flint-Garcia et al 2003; Gaut and Long 2003). Association mapping has been successfully applied for quality traits in tetraploid potato (D'hoop et al 2010). Very recently association mapping was also used to dissect the genetic basis of drought-adaptive traits and grain yield in a collection of 189 elite durum wheat accessions evaluated in 15 environments highly differing in water availability (Maccaferri et al 2010). In view of the advantages and applications of association mapping, it's worthwhile to apply it in potato to dissect drought tolerance. It should be noted that in an association mapping population, multiple alleles of the genes underlying traits can contribute to that trait. This often results in relatively minor effects exerted by many identified QTLs. Therefore, it is even more necessary to dissect complex drought traits into individual genetic parameters, and to use precision phenotyping of these traits for better analysis and understanding. Along with the evaluation of drought tolerance of the diploid mapping population CxE we included several commercial tetraploid cultivars namely Bintje, Bildstar, Biogold, Desiree, Mona Lisa, Mondial, Mozart, Nicola, Premiere and Russet Burbank. The cultivars displayed a lot of genetic variation for traits related to drought tolerance, indicating that even in commercial germplasm, ample genetic variation for drought tolerance is available that can be explored and possibly utilized with an association mapping approach.

4) Interaction between drought and other abiotic and biotic stresses

Water deficit is caused not only by a simple lack of water in the soil, but also by other stresses like low temperature or salinity that limit water availability for the plant; thus it is not

surprising that the responses to these various stresses involve many shared molecular components. For example, at molecular level 40% of the genes that are induced by salinity or drought are also induced by cold stress (Shinozaki and Yamaguchi-Schinozaki 2007). Some responses or mechanisms may have opposing effects under different stresses. Therefore tackling tolerance to one stress may lead to sensitivity to another. For example, closing the stomata helps to minimize transpiration to decrease the loss of water and maintain turgor under water deficit conditions, which has a favorable effect on water use efficiency. However, plants can avoid heat stress by increasing stomatal conductance, and consequently cooling of the leaf and canopy through transpiration. These mechanisms of stomatal control may be conflicting when drought and high temperature stress occur simultaneously. Another example is the osmo-protectant proline which may be accumulated under osmotic stress to adjust osmotic potential, but has a toxic effect under heat stress. Proline accumulation may therefore not be an appropriate tolerance mechanism in field conditions when heat and drought stress are combined (Rizhsky et al 2004; Salekdeh et al 2009).

Abiotic and biotic stress response pathways may also interact. Abuqamar et al (2009) have shown that the ABA responsive MYB transcription factor SlAIM1 modulates ABA responses, thereby integrating the plant response to pathogens as well as abiotic stresses. Similarly, several transcription factors from the NAC family have been shown to be up-regulated following pathogen infection, as well as under abiotic stresses (including drought), and crosstalk between pathogen defense and abiotic stress pathways is also mediated by members of the AP2 transcription factor family (Yoshioka and Shinozaki 2009). Interestingly, we found MYB, NAC and AP2 transcription factors to co-localize with phenotypic QTLs as described in Chapter 5, making these genes putative targets for studying the interaction between drought and biotic stress resistance in potato.

Although the simplified approach of studying isolated stresses has considerably increased our knowledge of tolerance mechanisms, interaction between multiple stresses and stress combinations should be studied to make even more progress in identifying traits and genes that are relevant to the field conditions.

5) The role of small RNAs and epigenetics in drought stress

Stress induced changes in epigenetic processes have been shown to regulate stress responsive gene expression and plant development under stress (Chinnusamy and Zhu 2009). In addition,

functional analyses have demonstrated that several plant miRNAs play vital roles in plant resistance to abiotic as well as biotic stresses (Kawaguchi et al 2004; Sunkar et al 2007). *In silico* identification of miR395, miR398 and miR399 homologues in diverse plant species suggest that these miRNAs are conserved across species. Conservation of these mRNAs implies that they have conserved biological functions. Appropriate manipulation of miRNA target genes should help to overcome posttranslational gene silencing (Sunkar et al 2006, 2007; Aung et al 2006; Bari et al 2006). Small RNAs and epigenetic changes add one more level of regulation in determining the actual biological relationship between transcript level variation and phenotype. Therefore, it is crucial to understand small RNA-guided stress regulatory networks and epigenetic variation and this might produce new tools for the genetic improvement of plant stress tolerance.

In summary, the results presented in this thesis provide valuable results for screening and evaluation for drought tolerance in potato. Chromosomal regions responsible for regulation of drought response were identified by QTL mapping. The application and advantages of integration of genetic and genomics approaches to unravel the molecular components underlying interesting drought response traits were demonstrated. Further exploration of the data collected in this thesis and additional focus on specific traits, QTLs and associated genes will allow identification of genes and alleles that can be exploited in cultivated potato to improve drought tolerance of this important food crop.

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Supplementary Material

Chapter2

SNP markers with their putative functions, sequences along with their database ID's (TC numbers) is provided in table and can be downloaded from below link.

http://www.springerlink.com/content/p0r40lx1131l372u/supplementals/

Chapter 4

Table S1. Population mean values of the traits recovery treatment, analysis of variance for the traits under stress and recovery condition and relative reduction and broad sense heritabilities of the traits under recovery condition.

Trait	Year	Recovery Mean	Two Way ANOVA (P values)			Relative Reduction (%)	Heritability (%)
			Genotype (G)	Treatment (T)	G*T		
Number of main stem	2008	3.9	<0.001	NS	NS	3.2	65.4
	2009	2.7	<0.001	NS	NS	-7.5	47.4
Shoot dry weight (g)	2008	14.2	<0.001	<0.001	NS	32.6	82.1
	2009	27.4	<0.001	<0.001	<0.001	7.1	41.1
Shoot fresh weight (g)	2008	145.1	<0.001	<0.001	<0.001	44.0	85.4
	2009	298.8	<0.001	<0.001	<0.001	14.5	64.7
Plant height (Cm)	2008	107.0	<0.001	<0.001	0.018	22.1	68.3
	2009	149.6	<0.001	<0.001	NS	15.5	54.7
Tuber number	2008	2.7	<0.001	NS	NS	53.2	81.1
	2009	5.4	<0.001	<0.001	<0.001	47.1	85.8
Tuber weight (g)	2008	6.4	<0.001	0.019	0.011	81.1	87.9
	2009	33.2	<0.001	<0.001	<0.001	73.6	77.7
Root dry weight (g)	2009	1.9	<0.001	0.012	0.04	16.2	64.6
Root length (Cm)	2009	33.5	<0.001	<0.001	NS	18.0	50.4
Root:shoot ratio	2009	0.1	<0.001	<0.001	0.001	10.4	44.4
Number of stolons	2009	8.1	<0.001	<0.001	0.002	7.8	66.1
Dry biomass (g)	2009	29.4	<0.001	<0.001	<0.001	7.7	41.6

Table S2. Population mean values of the traits Chlorophyll florescence (Fv/Fm) and Chlorophyll content measured at different time points during stress and recovery period, analysis of variance for the traits under stress and recovery condition and relative reduction and broad sense heritabilities of the traits under stress and recovery condition.

Trait	*Time*	*Mean Values*	*Two Way ANOVA (P values)*			*Relative Reduction (%)*	*Heritability (%)*
			Genotype (G)	**Treatment (T)**	**G*T**		
Chlorophyll florescence (CF)	1DAS	0.8	NS	NS	NS	0.00	96.2
	4DAS	0.782	< 0.001	< 0.001	< 0.001	2.25	35.2
	8DAS	0.78	NS	< 0.001	NS	2.50	32.7
	17DAS	0.747	< 0.001	< 0.001	< 0.001	6.63	14.8
	1DAR	0.77	0.028	0.04	0.01	3.75	22.2
	4DAR	0.78	NS	0.034	NS	2.50	33.7
	8DAR	0.79	NS	0.04	NS	1.25	7.0
	16DAR	0.81	< 0.001	< 0.001	NS	-1.25	14.1
Chlorophyll content (CC)	3DAS	35.31	<0.001	NS	NS	-1.03	70.8
	7DAS	34.60	<0.001	<0.001	NS	1.34	47.4
	13DAS	33.45	<0.001	0.028	NS	4.13	48.3
	17DAS	32.31	0.006	<0.001	NS	8.47	37.2

DAS: days after stress; DAR: days after recovery

Table S3. Coefficient of correlations for the traits under well watered condition (harvested at the end of stress period) *Significant at P≤ 0.05; ** Significant at P≤ 0.01; *** Significant at P≤ 0.001

Traits	δ13C	Nr stolons	PM	RDW	RFW	R: S dry wt	SDW	SFW	Tuber wt	Nr stems	Nr tubers	Pl ht
Nr stolons	-0.140											
PM	-0.287	-0.211	-									
RDW	0.268	0.486*	-0.370	-								
RFW	0.138	0.453*	-0.515*	0.762***	-							
R: S dry wt	0.149	0.364	-0.07	0.723***	0.5623**	-						
SDW	0.257	0.376	-0.468*	0.785***	0.5579**	0.1633	-					
SFW	0.045	0.442*	-0.437*	0.659***	0.54**	0.0818	0.8768***	-				
Tuber wt	0.339	0.505*	-0.031	0.236	0.2297	-0.0355	0.3849	0.3699	-			
Nr stems	-0.377	-0.1042	0.228	-0.4434*	-0.1768	-0.4216*	-0.3651	-0.0839	-0.2174	-		
Nr tubers	0.265	0.543**	0.085	0.2247	0.2615	0.0175	0.303	0.2757	0.8006***	-0.1501	-	
Pl ht	0.326	-0.401*	-0.157	0.2092	0.1146	0.1817	0.0989	-0.0693	-0.2515	-0.3952*	-0.2358	-
Root length	0.040	0.031	-0.172	0.5384**	0.275	0.32	0.5534**	0.5176**	-0.1087	-0.3638	-0.2189	0.308

Traits were Number of stolons (Nr stolons), Plant maturity (PM), root dry weight (RDW), root to shoot day weight ratio (R:S dry wt), shoot dry weight (SDW), shoot fresh weight (SFW), tuber weight (Tuber wt), number of main stem (Nr stems), number of tubers (Nr tubers) and plant height (Pl ht).

Table S4. Pearson coefficient of correlations for the traits after recovery *Significant at P≤ 0.05; ** Significant at P≤ 0.01; *** Significant at P≤ 0.001

Traits	Nr tubers	Nr stolons	PM	RDW	RFW	R: S dry wt	SDW	SFW	Tuber wt	Nr stems	Pl ht
Nr stolons	0.279	-									
PM	0.485***	0.021	-								
RDW	0.051	0.437***	-0.354**	-							
RFW	0.000	0.454***	-0.372**	0.975***	-						
R:S dry wt	0.176	0.443***	-0.147	0.780***	0.779***	-					
SDW	-0.099	0.247*	-0.378**	0.731***	0.672***	0.174	-				
SFW	-0.190	0.297*	-0.496***	0.722***	0.678***	0.288*	0.863***	-			
Tuber wt	0.774***	0.072	0.419***	-0.102	-0.165	0.010	-0.168	-0.28*3	-		
Nr stems	0.030	0.572***	0.005	0.117	0.161	0.115	0.080	0.176	-0.230*	-	
Pl ht	-0.405***	0.087	-0.421***	0.306*	0.326**	0.132	0.321**	0.444***	-0.469***	-0.004	-
Root length	-0.114	0.056	-0.224	0.474***	0.416***	0.313**	0.489***	0.548***	-0.079	-0.138	0.358**

Traits were Number of stolons (Nr stolons), Plant maturity (PM), root dry weight (RDW), root to shoot day weight ratio (R:S dry wt), shoot dry weight (SDW), shoot fresh weight (SFW), tuber weight (Tuber wt), number of main stems (Nr stems), number of tubers (Nr tubers) and plant height (Pl ht).

Chapter 5

S Table Differentially expressed genes of genotypes which showed different phenotypic response to water stress

Genotype	Early response (4 DAS)		Late response (9 DAS)	
	wilting symptom	Nr of genes	wilting symptom	Nr of genes
CE017	-	265	-	5182
CE084	-	1992	+	4612
CE782	+	3742	+	6483

Summary

Drought is the most important cause of crop and yield loss around the world. Breeding for drought tolerance is not straightforward, as drought is a complex trait. A better understanding of the expression of drought traits, the genes underlying the traits and the way these genes interact will significantly increase the success of breeding for drought tolerance.

Potato is an important food crop, yet it is relatively susceptible to drought. As a first step towards identifying the genetic basis for drought tolerance in potato, we make use of diploid potato populations that have been genetically well characterized (CxE, SHxRH). The CxE population was extensively evaluated for drought tolerance *in vitro* and for two successive years (2008, 2009) under greenhouse conditions and the data were used for QTL mapping.

For optimal QTL mapping, we expanded the CxE and SHxRH genetic maps with 499 SNP markers (two arrays 384 and 768SNP arrays respectively, enriched for putative stress tolerance candidate genes). The SNPs were discovered in public EST databases using QualitySNP software and detected with the Illumina GoldenGate assay. About 300 SNPs served as bridge markers between the CxE and SHxRH maps. This will enable us to make use of the extensive genetic and sequence information of the SHxRH population and the RH genome sequence. With the availability of the potato genome sequence of the doubled monoploid DM1-3 516R44 (DM) (www.potatogenome.net), it was possible to further examine the SNP marker loci for paralogs and intron spanning sequences. In total 732 SNP marker loci were found to be unique in the potato genome sequence. Many of these SNP markers not only served as landmarks on the genetic map but may also as putative genes underlying quantitative traits. In addition the validated SNP markers are now utilized as anchors in the potato physical map.

We investigated the possibility of screening potato for relevant drought traits in *in vitro* cultures and evaluated the CxE population for the response to PEG-induced water deficit stress and recovery potential after stress. Significant genetic variation was observed for the response to drought and for recovery potential. Several shoot and root growth traits were measured. In this study the genetic variation and heritability estimates were high to very high for the measured traits under control and recovery condition. In total 23 QTLs were detected in plants under control, stress and recovery treatments. Interesting putative candidate genes that may underly stress response QTLs were identified.

The drought tolerance evaluation of the CxE population in pots in the greenhouse included traits like leaf Relative Water Content, $\delta^{13}C$ as a measure of Water Use Efficiency, Chlorophyll Fluorescence, Chlorophyll Content, shoot and root biomass and tuber yield. The progeny displayed a wide contrast for drought tolerance, with individuals surviving and recovering completely after 3 weeks of drought, and others completely wilted beyond recovery. Most of the traits had high heritabilities. QTLs effective in multiple treatments and years were detected for tuber number, tuber weight, plant height, shoot fresh and dry weight. Other QTLs were found to be dependent on the environment: QTL x Environment interaction was found for leaf δ13C under drought conditions and we speculate that the function of $\delta^{13}C$ was genetically split into a stomatal and non-stomatal component.

Many of the QTLs for growth traits measured both in the greenhouse and in *in vitro* cultures were specific to either of the growth conditions. Yet significant QTLs that were detected for plant height, shoot dry weight, fresh biomass for plants grown in the greenhouse were also found when the population was grown *in vitro.* These QTLs may be less affected by environmental influences, and we may therefore expect that some of these QTLs will be relevant under field conditions as well. This also suggests that the *in vitro* system may be used for preliminary selection in breeding programmes for specific performance-related traits.

The genetic architecture of transcript-level variation for drought response was captured in the potato population CxE and mapped as expression QTLs (eQTLs). We anchored the differentially expressed genes to the genome sequence of potato, and this enabled us to determine whether the transcription of these genes (the eQTLs) is in *cis* or in *trans* regulated.
The combined use of genome-wide detection of eQTLs in combination with genome sequence information for gene location has enables us to detect regulatory hot spots for drought response in the CxE population. Based on gene ontology annotation, a number of eQTLs were detected for genes known to be involved in drought signal transduction and drought-induced transcriptional regulation, and for redox genes. Examination of co-localization of eQTLs and phenotypic QTLs identified several interesting eQTLs for genes that may be involved in specifying the phenotypic QTL, for instance, the eQTL for a gene that was annotated with a putative function in the photosystem II light reaction colocalized with trait QTL of chlorophyll florescence (Fv/Fm) on chromosome 1, along with other genes involved in

drought response such as heat shock proteins and signaling proteins with known induced expression under stress conditions. On chromosome 10, eQTLs for genes involved in carbon partitioning, signaling receptor kinases, transcription factors and hormone and lipid metabolism were colocalized with phenotypic QTLs for chlorophyll content and stomatal component of $\delta^{13}C$. As we have only touched the surface of the information contained in the transcriptome dataset combined with the phenotyping data, continued efforts on mining the dataset and in depth analysis will most likely reveal more putative candidate genes for QTL effects.

This thesis constitutes the first knowledge of *in vitro* and greenhouse screening for drought tolerance in potato and has led to the description of important traits for screening and selection in breeding for drought tolerance. The QTLs identified in this thesis may be interesting targets for potato breeding to improve drought tolerance of the potato crop. Furthermore, our results illustrate the power of application of integrated genetic and genomics approaches to unravel the molecular components underlying abiotic stress tolerance traits.

Samenvatting

Droogte is de belangrijkste oorzaak van wereldwijde gewas- en opbrengstverliezen. Veredelen voor droogtetolerantie is echter niet eenvoudig, want droogte is een complexe eigenschap. De kans van slagen van een veredelingsstrategie voor droogtetolerantie zal toenemen bij een beter begrip van de expressie van eigenschappen betrokken bij de droogterespons van planten, van de genen die verantwoordelijk zijn voor de eigenschappen en de manier waarop deze genen elkaar beïnvloeden.

Aardappel is een belangrijk voedselgewas, maar het is relatief gevoelig voor droogte. Dit proefschrift beschrijft de eerste stappen om de genetische basis voor droogtolerantie in aardappel op te helderen. Daarvoor hebben we gebruik gemaakt van diploïde karteringspopulaties die genetisch goed zijn gekarateriseerd (CxE, SHxRH). De CxE populatie is uitgebreid getest voor droogtetolerantie, zowel in vitro als gedurende twee opeenvolgende jaren in de kas, en de verzamelde gegevens zijn gebruikt voor het in kaart brengen van zgn. Quantitative Trait Loci (QTL).
Om een optimale QTL kartering te kunnen uitvoeren zijn de bestaande genetische kaarten van de CxE en SHxRH populaties uitgebreid met 499 Single Nucleotide Polymorphism (SNP) merkers (gebruikmakend van 384 en 768 Goldengate SNP arrays die verrijkt zijn met kandidaatgenen voor stress tolerantie). De SNP zijn opgespoord in publieke databases met behulp van het computerprorgamma QualitySNP, en gedetecteerd in de populaties met de illumina GoldenGate assay. Ongeveer 300 SNP merkers dienen als brugmerkers tussen de kaarten van CxE en SHxRH. Dit maakt het mogelijk om gebruik te maken van de uitgebreide genetische en sequentie informatie van de SHxRH populatie en van de sequentie van het RH genoom. Met het beschikbaar komen van de aardappel genoomsequentie van de verdubbelde haploid SM1-3 516R44 (DM) (www.potatogenome.net) werd het ook mogelijk om de SNP loci te onderzoeken op de aanwezigheid van paralogen en aanwezigheid van intronen in de geamplificeerde fragmenten. Er werden in toaal 732 SNP merkers gevonden die uniek waren in het genoom van de DM aardappel. Veel van deze merkers dienen niet alleen als markeringen op de genetische kaart maar ook als mogelijke kandidaatgenen die verantwoordelijk kunnen zijn voor kwantitatieve eigenschappen. Daarnaast worden de gevalideerde SNP merkers nu ook gebruikt om de fysieke kaart van aardappel (RH) te verankeren.

Wij hebben ook onderzocht of het mogelijk is om aardappelplanten in vitro te screenen voor relevante droogte-eigenschappen. Hiertoe is de respons van de CxE populatie op PEG-geïnduceerde stress als gevolg van watertekort onderzocht, en ook de mate van herstel na droogtestress, waarbij verschillende scheut- en worteleigenschappen werden gemeten. Er werd significante genetische variatie gevonden met betrekking tot de droogterespons en de mate van herstel. De genetische variatie en mate van overerving was met name hoog tot zeer hoog voor de gemeten verschillen tussen controle en droogte-behandelde planten. In totaal zijn 23 QTLs gedetecteerd voor verschillende eigenschappen in zowel de controle, droogte-behandelde en herstellende planten. Voor enkele van deze QTLs zijn mogelijke kandidaatgenen geïndentificeerd.

Tijdens de evaluatie van droogtetolerantie van de CxE populatie in potten in de kas zijn een aantal eigenschappen gemeten waaronder: relatieve waterhoeveelheid van het blad (Relative Water Content, RWC), δ13C als maat voor efficiënt watergebruik (Water Use Efficiency, WUE), chlorofyl fluorescentie, chlorofyl hoeveelheid, scheut- en wortelbiomassa, en knolopbrengst. Het nakomelingschap was sterk contrasterend voor tolerantie voor droogte, waarbij een aantal individuen de droogteperiode van 3 weken overleefden en volledig herstelden, en andere individuen aan het eind van de droogteperiode volledig verwelkt waren en niet meer herstelden. De meeste gemeten eigenschappen hadden een hoge vererfbaarheid. QTLs voor aantallen knollen, knolgewicht, plantlengte, versgewicht en drooggewicht van de scheut werden gevonden in beide jaren en in zowel controle planten als planten onder. Andere QTLs waren juist sterk afhankelijk van de omgeving. QTL x Omgeving interactie werd gevonden voor δ13C van het blad in droogte gestresste planten. De functie van δ13C bestaat genetisch mogelijk uit twee componenten een stomataire en een niet-stomataire component, die een verschillende rol spelen in de beide jaren.

Veel van de QTLs voor groei-eigenschappen die zowel in de kasexperimenten in potten en in vitro werden gedetecteerd waren specifiek voor één van beide experimentele omstandigheden. Desalniettemin werden enkele significante QTLs voor plantlengte, drooggewicht van de scheut, en versgewicht van de scheut zowel in de kasexperimenten als in het in vitro experiment gedetecteerd. Deze QTLs lijken minder afhankelijk van de experimentele condities en de omgeving, en daarom zouden deze QTLs mogelijk ook relevant kunnen zijn

onder veldcondities. Dit resultaat laat ook zien dat in vitro cultures mogelijk ook kunnen worden gebruikt voor voorselectie in veredelingsprogramma's voor specifieke eigenschappen die gerelateerd zijn aan de prestaties van de plant.

De genetische verschillen in de respons van planten op droogte stress worden in belangrijke mate bepaald door verschillen in expressie van genen. Deze expressieverschillen zijn gemeten in de CxE aardappelpopulatie, en genetisch gekarteerd als expressie-QTLs (eQTLs). De genen waarvan de expressieverschillen zijn bepaald zijn met behulp van de genoom sequentie van aardappel verankerd op de genetische kaart, en dit stelt ons in staat aan te geven of de differentiële expressie van deze genen (de eQTLs) in cis of in trans gereguleerd wordt. Door combinatie van genoom-brede identificatie van eQTLs gecombineerd met informatie over de fysieke locatie van deze genen zijn zgn. hotspots van aansturing van genen voor de droogte-respons gedetecteerd. Bovendien zijn aantal eQTLs geïdentificeerd voor genen waarvan bekend is dat ze betrokken zijn bij signaal transductie en bij transcriptionele regulatie na droogtestress, en voor genen betrokken bij redox processen. Op grond van co-localisatie van de bijbehorende eQTLs en van QTLs voor fenotyische eigenschappen konden verschillende interessante genen worden aangewezen die mogelijk betrokken zijn bij het effect van deze QTL. Zo viel een eQTL voor een gen geannoteerd als een gen coderend voor een eiwit dat deel uitmaakt van de lichtreactie van het fotosyteem II samen met een QTL voor de chlorofyl fluorescentie parameter Fv/Fm (capaciteit van fotosynthese) op chromosoom 1. Op deze locatie werden ook eQTLs gevonden voor andere genen betrokken bij de droogte respons, zoals heat shock eiwitten, en eiwitten betrokken bij signaaloverdracht onder stress omstandigheden. Op chromosoom 10 werden eQTLs voor genen betrokken bij koolstofverdeling over de plant en hormoon en vetzuurmetabolisme, en genen coderen voor signaal receptor kinases en transcriptiefactoren op dezelfde locatie gevonden als fenotypische QTLs voor chlorofylhoeveelheden in de bladeren en voor de stomataire component van δ13C. Een uitgebreidere analyse van de dataset met expressieverschillen en eQTLs in combinatie met de fenotypische informatie en QTLs zal hoogstwaarschijnlijk nog meer mogelijke kandidaatgenen voor aan droogtetolerantie gerelateerde eigenschappen opleveren.

Dit proefschrift beschrijft voor het eerst een uitgebreide analyse van droogtetolerantie in aardappel in in vitro cultures en in planten in de kas. Dit heeft geleid tot een beschrijving van

belangrijke eigenschappen voor screening en selectie voor droogtetolerantie in de veredeling. De QTLs die zijn geïdentificeerd in dit proefschrift kunnen gebruikt worden als speerpunten in de aardappelveredeling ten behoeve van verbetering van droogtetolerantie in dit gewas. Onze resultaten laten ook de meerwaarde zien van een aanpak waarin genetische en “genomics” methoden worden geïntegreerd om de moleculaire mechanismen op te helderen die ten grondslag liggen aan eigenschappen die bijdragen tot tolerantie voor abiotische stress.

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Today as a grown up sapling I am turning back to look at that day when I landed as a dormant seed in the Wageningen soil. Besides the watering and nurturing from **Dr. Gerard van der Linden** and **Prof. Richard G. F Visser** as PhD committee, my project cotyledons opened up and grew faster under favorable conditions in Wageningen UR Plant Breeding. While growing, my skills to acquire, adapt and advance were challenged. Stressful times primed my stress tolerance and adaptation for advantageous genes. Having not been in a multi-cultural society was not a problem at all, since WU favored my exposure into a wide spectrum of culture in a friendly way. A wonderful opportunity to taste a bit of every other culture on and off the formal environment. Set only the sky as the limit to grow and establish, feeling positive about where I am standing at present, but not complacent. So I strongly believe growth will inspire further growth.

First of all I would like to thank my promoter Prof**. Richard G. F. Visser** for giving me the opportunity to pursue my doctoral degree in department of Plant Breeding under his promotion. This opportunity brought me not only academic degree but also considerable personal development. It was complete package of science, colleagues, new culture, families, and friends. Dear **Richard**, I am very glad for your constant support especially letting me to do experiments that I was interested in, inspite of their expensive nature. I heartily appreciate the fact that inspite of your busy schedule you visited all my experiments and provided good discussions. Apart from science, you also supported me for extra curricular activities. Thank you very much.

I am very grateful to my co-promoter **Dr. Gerard van der Linden** for his constant scientific guidance, support, care and concern through out my PhD period. Dear **Gerard**, you are my mentor. From day one till the end of my PhD you were always there for me. Your friendly nature made it easier for me to get quick acquaintance with you and project. I enjoyed all our stimulating scientific conversations. At every stage you gave me freedom to take decisions, to explore my limitations, which boosted my confidence, and helped me to be an independent and self-reliant researcher. I am very glad and lucky to have had the opportunity to be your student. I truly believe that you are the best supervisor I could have had to pursue my doctoral studies. **Gerard**, I never forget the letter you gave to my parents on my first visit back home, that was the turning point, it convinced me to come back to NL and continue my studies and I am very grateful for that. I am very proud to say that you are not only my co-promoter but also a good friend. Being a friend you were always there to share my happiness and stood next to me in my tough times. Thank you very much for your advice and or opinions. You are a wonderful person and have ability to bring positive difference in others life. Be the person as you are!! And thanks for being a part of my life.

Being away from home and family was not easy in the beginning, but soon I was introduced to a **Family van der Linden**. In a short time I became part of the family because of the warmth of the family members. I am very proud to write about “my Dutch family”. I am grateful to **Gerard van der Linden, Esther van der Meulen,** kids **Ruben** and **Lieke** for their constant love, care and concern. Each visit to Boerenbond 18 was familiar, comfortable, and

joyful and I felt homely. **Esther** you are kind, friendly and generous. You treated me not as Gerard's student but as a friend of your family which made it easier for me to integrate into the family. My sweet little friends **Ruben** and **Lieke**, though I thought language might be a barrier to communicate but without any problem every time we enjoyed hours together and I never got bored to spend a time with you. To be a member of family in foreign land means lot to me. I am very grateful to all of you, I don't know how to express my gratitude, and all I can say is thank you very much for everything. I would like to extend my thanks to **Cees van der Linden, Annie van der Linden, Gerrit van der Meulen (Pake), Hanna van der Meulen (Beppe), Roeland van der Meulen** and **Martha Jones** and their little cute twin girls **Anna and Marieke**. I enjoyed all your company and I am very thankful to the entire family.

I would like to thank **Dr. Eric van de Weg** for his support and care. Dear **Eric** in the very beginning, I started learning mapping with you, that was a start but it never ended. Though you are not directly involved in my project, you always had time to discuss and to know the progress of my project. I also appreciate concern and care you showed towards me especially when I was not doing well with my health and during my huge phenotyping experiments. Eric I never forget the day when you called me and I told you that I was not well and unable to stand; with in ten minutes you were there in front of my door with food. Thank you very much for everything. Eric you also introduced me to your family. I sincerely appreciate the affection and care shown to me by your family. I cherished each visit to your home and the company of **Henriet Wape Naar,** children **Woulter, Elsje** and **Marieke**. Dear **Henriet** you are such a kind-hearted person and have lot of serenity, I adore you for that. Thanks for the affection and care you showed to me. **Marieke** I enjoyed your company very much; all those sessions of bead making, playing games, ticklings are unforgettable.

I would like to thank all the members of the abiotic stress group for their support and valuable discussions at group meetings. Especially I would like to thank **Dr. Oene Dolstra** for his input. Dear **Oene** you are kind and spent your valuable time with me to discuss and give suggestions on data analysis whenever it was needed and thanks for critical comments on one of my manuscripts. I extend my gratefulness to **Dr. Ben Vosman** for his help and support during SNP genotyping and preparation of the manuscript on it and **Dr. Herman van Eck** for nice discussion on markers, maps and extension of analysis of SNP markers. I would like to acknowledge **Dr. Chris Maliepaard** for statistical advice. I would like to thank **Dr. Rene Smulders, Dr. Paul Arens** and **Dr. Sjaak van Heusden** for their support.

In the last year I had a big experiment with microarrays, which was quite new to me. Without the help and advice from **Dr. Bjorn Kloosterman** it wouldn't have been possible to finish the experiment and analysis in such a short time. **Bjorn** thanks for your help and I hope to publish this work soon. **Marian Oortwijn** thanks for the help in labelling the probes. My acknowledgments to **Mr. Dirkjan Huigen, Dr. Ronald Hutten, Mr. Rene Alles, Mrs. Gerda van Engelenhoven** and all my friends for their timely help and support during phenotyping experiments.

I would like to thank our secretaries **Annie, Letty, Mariame Gada, Janneke** and **Nicole** for their help with administrative things especially **Annie** who helped me to settle all the bills after conferences. I would like to thank all the lab staff members (molecular biology and tissue culture lab) for their timely help. During my PhD I had a chance to supervise students and I would like to thank **Wosene, Marianne, Berend-Jan, Addisu, Nelson** and **Octavio** for

their input.

Dear officemates **Dirkjan Huigen** and **Gerard Bijsterbosch** I really enjoyed your company in the office. Dirkjan, I enjoyed our conversations about weather, culture, weddings and social life. Gerard though in the beginning there were no conversations, soon I realized that you have great knowledge about history, classical music, political issues and current news. I enjoyed our conversations on these topics including scientific talks.

The first person I met on my arrival to Netherlands was **Dr. Aarathi Karaba**, who helped me to settle down in Wageningen. It was like a crash course to introduce everything to me from train, strippenkaart for the bus to super market. Dear Aarathi mam I really enjoyed your company though it was very short but sweet. I would like to thank my teacher **Dr. Nataraj Karaba**. Sir, thank you for your support, encouragement and care you showed to me. I am very grateful for your help in carbon isotope measurements and preparation of manuscript on it.

I would like to thank **Prof. V.R. Sashidhar** for his support and encouragement. I extend my thanks to all the faculty members at the department of Crop Physiology, University of Agricultural Sciences, Bangalore, India for their moral support. My special thanks to **Dr. N Rama** for support, affection and care.

In the last four years I have made many friends and met many people across the world. First among them was **Dr. Paola Pollegioni**. Ciao bella, thank you very much for unconditioned friendship. You also treated me as member of your family, I am grateful for that. My sincere thanks to **Maria Rita Radicchio** (Mamma) and **Domenico Pollegioni** (Babbo) for their hospitality, care and love. Mamma and Babbo I had a wonderful time in Italy, each time while coming back it was hard to say good bye to you. You both are wonderful people. I hope to visit you again. **Hulya Yilmaz Temel**, and **Serdal Temel** thanks for your friendship and nice hospitality and I had great time in Turkey especially with **Efe**. Then, a bunch of wonderful international friends were added to my list **Nasim, Arwa, Natalia Carreno, Paula, Cesar, Luis, Mirjana, Shiva, Leila, Freddy, Anna, Bjorn D‘ Hoop, Brana.** Dear friends’ time flew away with all your great company. The weekend (mmm…week days as well) parties were just unforgettable! I also enjoyed colleagues at work namely **Reza, Thierry, Nadeem, Sabaz, Ningwen, Collete, Antoine, Anoma, Estelle, Nico, Krissana, Mathieu, Pierre, Ram, Alireza, Stefano, Luigi, Zheng, Yusuf, Marcel, Eugene, Brigitte, Virginia, Dora, Long, Paweena, Efstathios, Xingfeng, Alvaro, Ricardo, Robab, Marleen, Thijs** and many more……

My special thanks to **Cesar Andres Ospina Nieto** and **Marian Oortwijn** for being my paranymphs and I am looking forward to my big day with you!!. I would like to thank my friend **Deepu kumar** for the cover page design.

I cherished my moments with all my Indian friends **Madhuri and Manoj, Animesh and Sharmistha, Sameer and Vani, Prasad, Kishore, Arun, Senthil, Feana, Ganeshan, Srinivas, Rama Bangera, Shital and Basav, Ram and Veda, Nagesh, Aparna and Arvind, Abhishek, Anand, Rajshekar Korada, Mam Singh, Sachin and Pradnya, Uttara, Urmila, Geo, Sushmitha, Kuppu, Partha and Vidyadhar** and many more with whom I celebrated Indian festivals, dinners, birthday parties, graduation ceremonies and many more…

I would like to thank all my global Indian friends for their support and concern. Irrespective of time differences, I enjoyed all our everlasting conversations on Skype, Messenger, and Google chat to share life abroad!!

Special thanks to **Bhoomika Troop** (A group of Mesmerizing artists). This is a platform where I learnt to stand on the stage and to face hundreds of audience and it gave a lot of confidence. All troop members were just amazing with great talent. Though I lived 4 years away from theatre activities, missed lot of events, still my interest and thirst haven't went down just because you all made memorable events when ever I visited India. **Pavianna** (Back bone of the troop) thank you very much for your support and weekend updates about troop and friends.

This endeavour would not have been complete with out my best friend **Girish G**. Hi **Kanna** you are the person who made such a big move in my life. You are the one who asked 'why not a PhD abroad'? That's it! Now I am done. Though there were many tough times… and life moved on…, thanks for being there for me with all your everlasting advice, support, care and encouragement. I wish you a very happy life with your wife **Priyanka** and son **Gunin**.

Most importantly, it is my immense pleasure to thank my family members for their constant and endless support, care and love. My father is the true inspiration of my life. **Appa** and **Amma** I am very proud to be your daughter, you have always supported, encouraged and believed in me for every thing, even when I took the big step to go abroad to pursue my PhD which was out of your imagination. Each step I claimed in my life I knew there were your blessings. My elder sisters **Suma, Poornima**, younger sister **Saritha** and brother **Praveen** brought colours into my life. Your unconditioned love, care and support is incredible. I would also thank my brothers-in-law **Dr. Nagaraj** and **Dr. Raghunath** for their support and encouragement. The kids of our family **Ankitha Raghunath**, **Likith Raghunath** and **Nairuthya Nagaraj**, **Ammu Nagaraj** have kept me motivated as they have always looked up to their Aunt. Especially Ankitha (my budri) being a first kid in the family, I had chance to spend more time with you, I am always amazed by you, I cherished every moment I spent with you and I realized incredible, innocent, contentment to be with kids is speechless. I love you all and I am sure I made everyone in family proud.

With Love,
Anitha
March 2011, Wageningen, The Netherlands

About the author

Anithakumari Arabikothanuru Muniyappa was born on 1st June, 1981 in Arabikothanuru, a village in Karnataka, India. After completing her schooling in 1999, she joined for Bachelors in Agriculture (1999-2003) at the University of Agricultural Sciences (UAS), Bangalore, India. On completion of bachelors, she continued in the same university to pursue her Masters in Agriculture (2003-2005) with specialization in Crop Physiology. As a part of her MSc, she worked on thesis project entitled 'Enhanced calcium homeostasis through over expression of the vacuolar Ca^{2+}-ATPase gene (ACA4) and its role in salt tolerance in tobacco' in the department of Crop Physiology for one year. On completion of MSc in November 2005, she worked as a technical assistant in the same department for a period of six months on a project 'Cloning and characterization of Epicuticular wax synthesizing genes from Mulberry (*Morus alba*): A potential option to reduce post harvest water loss'. From May 2006 to February 2007 she worked as senior research fellow on a project **'**Development of drought stress specific ESTs in pea nut (*Arachis hypogea)'*. In March 2007 she started her PhD program at the Laboratory of Plant Breeding, Wageningen University and Research Center (WUR), The Netherlands. This thesis presents the outcome of her four years PhD research work on "Genetic Dissection of Drought Tolerance in Potato".

List of Publications:

A. M. Anithakumari, Jifeng Tang, Herman J. van Eck, Richard G. F. Visser, Jack A. M. Leunissen, Ben Vosman and C. Gerard van der Linden (2010), A pipeline for high throughput detection and mapping of SNPs from EST databases. Mol Breeding 26:65-75.

A. M. Anithakumari, Nataraja N Karaba, Richard G.F. Visser, C.G. van der Linden (2011) Genetic dissection of drought tolerance and recovery potential by QTL mapping in diploid potato. (Submitted)

A. M. Anithakumari, Oene Dolstra, Richard G.F. Visser, C.G. van der Linden (2011) *In vitro* screening and QTL analysis of drought tolerance in potato. (Submitted)

A. M. Anithakumari, Bjorn Kloosterman, Chris A Maliepaard, Richard G. F. Visser, C. Gerard van der Linden (2011) Genome wide eQTL analysis for drought response in potato (In preparation)

Conference Abstracts:

A. M. Anithakumari, Richard G.F. Visser, C.G. van der Linden (2009) QTL mapping for drought tolerance in potato In: 6th Solanaceae genome workshop New Delhi, India. Abstracts p45

A. M. Anithakumari, Richard G.F. Visser, C.G. van der Linden (2009) Molecular breeding for drought tolerance in Potato In: Interdrought- III, Shanghai, China. Program and Abstracts p160

A. M. Anithakumari, Karaba N. Nataraja, Dolstra. O, Maliepaard C, Richard G. F. Visser, C. G. van der Linden (2009) Characterization of Drought Tolerance Traits in Potato In: International conference on Plant abiotic stress tolerance, Vienna, Austria. Abstracts p163

A. M. Anithakumari, Richard G.F. Visser, C.G. van der Linden (2008) Molecular tools for genetic dissection of potato for drought tolerance. In: 5th Solanaceae genome workshop Cologne, Germany. Abstracts p91

A. M. Anithakumari, Marcel van Culemborg, Richard G.F. Visser, C.G. van der Linden (2008) Breeding for drought and salt tolerance in potato. In: 17th Triennial conference of the EAPR, Abstracts of papers and posters, Brasov, Romania. Abstracts

A. M. Anithakumari, Richard G.F. Visser, C.G. van der Linden (2008) Mapping of potential drought related markers in potato by Motif-Directed Profiling. In: International conference on Molecular mapping & marker assisted selection in plants Vienna, Austria. Abstracts p23

A. M. Anithakumari, Rama N, Lokesha A.N., V.R. Sashidhar (2006) Enhanced calcium homeostasis through over expression of the vacuolar Ca^{2+}-ATPase gene (ACA4) and its role in salt tolerance in tobacco. In: International conference on Frontiers in Genetics and Biotechnology – Retrospect and Prospect. Osmania University, Hyderabad, India. Abstracts p93.

Education Statement of the Graduate School
Experimental Plant Sciences

The Graduate School EXPERIMENTAL PLANT SCIENCES

Issued to: **Anithakumari A. M.**
Date: **4 March 2011**
Group: **Laboratory of Plant Breeding, Wageningen University**

1) Start-up phase	*date*
► **First presentation of your project**	
Genetic dissection of drought tolerance in potato	Jun 01, 2007
► **Writing or rewriting a project proposal**	
Genetic dissection of drought tolerance in potato	Mar-Jun 2007
► **Writing a review or book chapter**	
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	***7,5 credits****

2) Scientific Exposure	*date*
► **EPS PhD student days**	
EPS Ph.D. student day 2007, Wageningen University	Sep 13, 2007
EPS Ph.D. student day 2009, Leiden University	Feb 26, 2009
► **EPS theme symposia**	
EPS Theme symposia "Genome plastisity", Leiden University	Dec 07, 2007
EPS Theme symposia "Metabolism and adaptations", Amsterdam University	Feb 18, 2009
EPS Theme symposia "Metabolism and adaptations", Leiden University	Feb 19, 2010
EPS Theme symposia"Genome plastisity", Wageningen University	Dec 10, 2010
EPS Theme symposia "Metabolism and adaptations", Wageningen University	Feb 10, 2011
► **NWO Lunteren days and other National Platforms**	
ALW meetings Experimental Plant Sciences	Apr 07-08, 2008
ALW meetings Experimental Plant Sciences	Apr 06-07, 2009
ALW meetings Experimental Plant Sciences	Apr 19-20, 2010
► **Seminars (series), workshops and symposia**	
Flying seminar by Prof. Dr. Jim Carrington, "Diversification of small RNA pathways in plants"	Mar 26, 2007
Flying seminar by Prof. Dr. Adreas Graner, "Comparative Genomics of Barley & Rice:Promises kept and pending"	May 10, 2007
Seminar by Dr. Anne Osbourn, John Innes Centre (UK) "The evolution of metabolic diversity in plants"	Sep 12, 2007
Seminar by Prof.dr. R. Scott Poethig, "Regulation of phase change in plants by miRNAs and trans-acting siRNAs"	Sep 21, 2007
Seminar by Professor Jaakkko Kangasjärvi, University of Helsinki (Finland), "ROS and stomatal regulation"	Mar 13, 2008
Seminar by Prof. Jian-Kang Zhu, University of california (USA), "Mechanism and function of active DNA demethylation in Arabidopsis"	Nov 03, 2008
Lecture by Pamela Hines Senior Editor of Science, Washington (USA), "Science from an Editor's view; Science organization, tips about being an author and a referee etc. "	Nov 06, 2008
Seminar by Prof.dr. Sjef Smeekens Utrecht University, Molecular Plant Physiology "Sweet connections – Reprogramming metabolism in response to stress"	Nov 27, 2008
Seminar by Dr. Keatinge general director of AVRDC	Jun 18, 2009
Seminar by Dr. Wallace Cowling, "Association mapping :Overcome the paradox of modern plant breeding"	Jun 26, 2009
Seminar by Prof. Fenny Dane, Auburn university (USA), "Unravelling drought and cold tolerance mechanisms in watermelon and Citrus"	Sep 22, 2009
Seminar by Dr. Justin Borevitz, University of Chicago (USA), "Genetics of adaptaion: from model organism to model ecosystem"	Jan 12, 2010
Seminar by Dr. Christiane Gebhardt, Max Planck Cologne (Germany), "The molecular basis of quantitative traits in potato"	Feb 05, 2010
Seminar by Prof. Kazuto Iwama, Hokkaido University (Japan), "Varietal difference in potato root system and its implications in Drought tolerance"	Feb 22, 2010
Seminar by Dr. Heribert Hirt, University of Vienna (Austria), "Engineering protein kinase signalling cascades for improving stress tolerance in plants"	Sep 08, 2010
Seminar by Dr. Adam Price, university of Uberdeen (UK), "Studying the genetics of root growth in rice"	Sep 17, 2010
► **Workshops**	
Research day Plant breeding dpt WUR	Sep 27, 2007
Research day Plant breeding dpt WUR	Jun 17, 2008
Research day Plant breeding dpt WUR	Mar 03, 2009
QTL MAS WICC Wageningen	Apr 20-21, 2009
Research day Plant breeding dpt WUR	Feb 08, 2010
► **Symposia**	
Plant Roots: From genes to ecosystems Radboud University Nijmegen	Oct 23, 2008
Statistical genetics Biometris Dpt, WUR	Nov 05, 2008
Advances in Life-science technologies, CBSG, Wageningen, Netherlands	Nov 25, 2010
► **Seminar plus**	

	Seminar by Prof. Jian-Kang Zhu, University of california (USA), "Mechanism and function of active DNA demethylation in Arabidopsis"	Nov 03, 2008
	Seminar by Prof. Kazuto Iwama, Hokkaido University (Japan), "Varietal difference in potato root system and its implications in Drought tolerance"	Feb 22, 2010
►	**International symposia and congresses**	
	Molecular maaping & Marker assisted selection in plants Vienna (Austria)	Feb 03-06, 2008
	The 5th solaneceae genome workshop SOL 2008 Cologne (Germany)	Oct 12-16, 2008
	Abiotic stress tolerance in plants Vienna (Austria)	Feb 08-11, 2009
	Inter drought III Shanghai (China)	Oct 11-16, 2009
	The 6th solaneceae genome workshop SOL 2009 New Delhi (India)	Nov 08-13, 2009
	Gordon conference (Switzerland)	Jun 13-18, 2010
►	**Presentations**	
	Molecular maaping & Marker assisted selection in plants Vienna austria - Oral	Feb 03-06, 2008
	ALW meetings Experimental Plant Sciences Lunteren The Netherlands - Poster	Apr 07-08, 2008
	Research day Plant breeding WICC wageningen - Oral	Jun 17, 2008
	17th triennial conference of the european association for Potato research. Brasov, Romania - Poster	Jul 06-10, 2008
	The 5th solaneceae genome workshop SOL 2008 Cologne Germany - Oral	Oct 12-16, 2008
	STW-INCAS meeting - Oral	Nov 27, 2008
	Abiotic stress tolerance in plants Vienna Austria - Poster	Feb 08-11, 2009
	STW-INCAS meeting - Oral	Jun 11, 2009
	CNR-IBAF Porano Italy- Oral	Sep 03, 2009
	Inter drought III - Poster	Oct 11-16, 2009
	The 6th solaneceae genome workshop SOL 2009 New Delhi India - Oral	Nov 08-13, 2009
	ICRISAT India - Oral	Nov 24, 2009
	Department of crop physiology, university of agricultural sciences, Bangalore, India - Oral	Dec 08, 2009
	STW-INCAS meeting - Oral	Jan 19, 2010
	Gordon conference - poster	Jun 13-18, 2010
	STW-INCAS meeting - Oral	Oct 07, 2010
►	**IAB interview**	Feb 17, 2011
►	**Excursions**	
	McCain, Hoofddorp, The Netherland	Sep 15, 2008
	Syngenta,Enkhuizen, The Netherland	Sep 25, 2008
	C. Meijer, Rilland,The Netherland	Jul 14, 2009
	DLF - Tripolium, Moerstraten, The Netherland	Jul 14, 2009
	Visit to ICRISAT, Hyderabad, India	Nov 23-26, 2009
	Subtotal Scientific Exposure	***36,8 credits****

3) In-Depth Studies		*date*
►	**EPS courses or other PhD courses**	
	Natural variation in Plants WUR	Aug 26-29, 2008
	System biology course: Statistical analysis of ~omics data	Dec 08-11, 2008
►	**Journal club**	
	Participated in literaure discussion group 'Plant Breeding'	2007-2010
►	**Individual research training**	
	Carbon Isotope analysis, CNR-IBAF Porano, Italy	Aug 28-Sep06, 2009
	Subtotal In-Depth Studies	***8,9 credits****

4) Personal development		*date*
►	**Skill training courses**	
	Scientific Writing (CENTA), WUR	2008
	PhD competence assesment	Jun 04, 2009
	EPS career day (ExPectationS)	Nov 19, 2010
►	**Organisation of PhD students day, course or conference**	
	Organized biweekly Phd colloquiums at plant breeding WUR	Sep 2008 -Oct 2009
	Organized international food tasting evening at plant breeding department	Jun 24, 2010
	Organized plantbreeding department one day outing	Sep 16, 2010
►	**Membership of Board, Committee or PhD council**	
	Member of EPS PhD Council	2009-2011
	Subtotal Personal Development	***6,8 credits****

TOTAL NUMBER OF CREDIT POINTS*	**60**

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

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

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PROPOSITIONS

1) SNP markers derived from ESTs not only serve as landmarks on genetic maps but also as candidate genes underlying quantitative traits (This thesis)

2) Co-localization of QTLs under different growth conditions such as greenhouse and *in-vitro* makes the *in vitro* system a reliable alternative for preliminary selection in a breeding program for specific drought tolerance-related traits (This thesis)

3) Genome-wide eQTL analysis opens multiple avenues for network analyses that will lead to identification of molecular components underlying abiotic stress tolerance traits (This thesis)

4) Comparative studies on stress-responsive epigenomes together with ~omics provide the missing link in our understanding of stress adaptations in plants

5) There is no magical single trait to evaluate drought tolerance as it is as complicated and difficult to plant biology as cancer is to mammalian biology

6) To explore the genetic basis of complex traits in quantitative genetics the same emphasis should be put on high-throughput precision phenotyping platforms as has been put on high-throughput sequencing technologies and marker assays.

7) Adaptation to Dutch culture and integration into a multi-cultural society is an additional pleasure in doing a PhD in Wageningen UR Plant Breeding.

8) Social discrimination is part of human nature; It can be taken out of language dictionaries but it will always be there in one or the other way.

Propositions belonging to the thesis, entitled:

"Genetic Dissection of Drought Tolerance in Potato"

Anithakumari Arabikothanuru Muniyappa

Wageningen, 4th March 2011