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Faculty of Landscape Architecture, Horticulture and Crop Production Science

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– Utilization of hydroponics and GWAS for identification of QTLs and candidate genes for root traits

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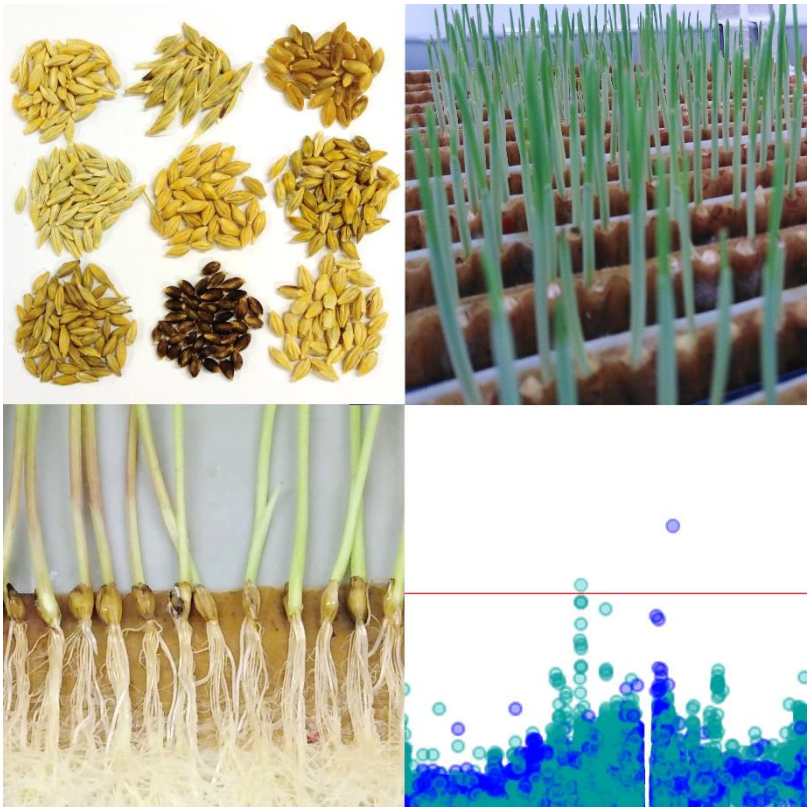

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Screening of early root growth characteristics in seedlings of Nordic spring barley

– Utilization of hydroponics and GWAS for identification of QTLs and candidate genes for root traits

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Preface

Since I started to work as a gardener a little more than 11 years ago, plant breeding has naturally been a topic of curiosity. Although, I never have had the time to immerse myself much in the subject. Therefore, when I got the chance to include a course in plant breeding for my master's studies, I applied for it. One of the classes in the course was lectured by Dr. Therese Bengtsson, who at the end of the lecture advertised an opportunity for a master thesis project connected to a Nordic PPP-collaboration (Public-Private Partnership) focusing on pre-breeding in Nordic spring barley. I thought it sounded interesting to work with a crop that has played, and still is playing, an important role in northern Europe (and as I later learned in several other regions around the world) as well as to learn more about some of the state-of-the-art methods for modern plant breeding. This led to the decision to choose a topic in connection to the ongoing PPP-research project in spring barley.

I want to express my gratitude to my supervisor Dr. Therese Bengtsson (SLU) for sharing her knowledge and time, and for all the support through the whole process of the thesis. Dr. Nils-Ove Bertholdsson has been very helpful, giving inputs and answering questions, while setting up the hydroponic system used for this thesis. Prof. Inger Åhman has been consulted as well, and Ingegerd Nilsson (lab technician at SLU) has been consulted for the practical part of the experiments. I would like to thank the people in the PPP-project, which I had the opportunity to meet on a project meeting at Nordic Seed in Denmark, for their encouragement. My appreciation goes to Dr. Vehbo Hot, for his hospitality during the weeks I shared the laboratory with him. I would like to thank Adam Flöhr, statistician at SLU, who has been consulted while processing the statistics for the experiments. The LTV faculty at SLU are acknowledged for their generous subsidy of the rent of the climate chamber. Boreal, Graminor, Nordic Seed, Sejet Plant Breeding, Lantmännen, NordGen (Nordiskt Genresurscenter), and IPK (Leibniz Institute of Plant Genetics and Crop Plant Research) are acknowledged for providing the genotypes, and SciLifeLab is recognized for conducting the genotyping. Last but not least, I wish to thank my family and friends for the encouragement during the time of compiling the thesis.

Abstract

In the Nordic countries, the cultivation of barley (*Hordeum vulgare* L.) has a long history, and as one of the major cereals in the world, barley is of global importance for food security. The world population is growing, which naturally will increase demands for agricultural outputs. Simultaneously, there is a need for limiting agricultural caused environmental footprints, including excessive fertilizer usage. It is as well projected that climate change will create challenges for global food security, with higher frequencies of extreme climate events with negative impacts on the agricultural sector. In this light, plant breeding for adapted crop varieties is recognized to be important for maintaining stable yields.

Traditionally, breeding efforts have mostly been focusing on improving above-ground plant traits. Although, in later years the advantages of including root architecture traits in breeding programs have been highlighted, e.g. for enhancing resource efficiency, stress tolerance, and plant adaptation to unstable climates. Previous studies have shown that early root growth can correlate with enhanced nutrient uptake and yield formation. In this thesis, a low-tech hydroponic method was used for screening for root dry weight (RootW) and seminal root length (RootL) in seedlings of spring barley. The plant material comprised 259 genotypes of spring barley, from a Nordic PPP–project, including modern lines as well as historic cultivars and landraces, pre-dominantly from the Nordic countries. Moreover, a Genome-Wide Association Study (GWAS) was performed to examine the underlying genetics of the comprised traits.

The root traits were successfully phenotyped using the hydroponic screening method, and in total 23 significant QTLs (quantitative trait loci) associated with the two traits were identified with GWAS. When comparing the result with other association-studies comprising root-traits in spring barley, several of the QTLs in this thesis were located close to QTLs located in those studies. Besides, some candidate genes mentioned in related studies, associated with RootW and RootL, were found in the vicinity of obtained QTLs in this thesis. The statistical analyses, considering correlations between the replicates and the Analysis of Variance (ANOVA), gave support for the results regarding RootL, but the correlations were lower for RootW and a significant effect of the replicates was shown. While comparing differences in early root growth between historic cultivars/landraces and modern breeding lines, a trend was observed where the modern lines generally had higher values for RootW and RootL. Possible reasons for this are discussed.

Overall, the low-tech hydroponic screening method proved to be useful for screening RootL of many genotypes in a short time, which is a trait that previously has been shown to correlate with nitrogen uptake and yield. This by itself could motivate its usefulness. Moreover, the simplicity of the method compared to other more high-tech solutions might make it useful in situations with a restricted budget, when the screening comprises many genotypes, and/or where modern screening systems are out of reach. Although, regarding RootW, the source of the more variable results must be located for the method to be useful. Other traits of early root growth that could have been interesting to assess are discussed.

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1. Introduction

1.1 Background

Barley is known to be one of the first crops that was adopted to serve as a staple food; therefor its importance for human civilization reaches thousands of years back in history (von Bothmer *et al*., 2003a; Langridge, 2018). Remains of barley, including non-shattering rachises - a morphological feature which likely is a result of human selection - have been found in archaeological sites in the Fertile Crescent, dating back to at least 8000 B.C (Langridge, 2018). Today cultivation of barley is essential for global food security and the crop is grown worldwide in more than 100 countries (International Barley Hub, 2020; Giraldo *et al*., 2019). It is the fourth major cereal in the world in terms of production, after maize, rice, and, wheat (Shahbandeh, 2020). Although today barley is mostly used for animal feed, brewing, and distilling, it still serves as a staple crop in some areas of the world, including regions in North Africa and Asia (von Bothmer *et al*, 2003a; Newman & Newman, 2006).

Barley is cultivated under a wide range of different climatic and environmental conditions in all temperate regions around the world (von Bothmer *et al*, 2003a). It is grown on fertile as well as marginal lands, and under extreme conditions - including high altitudes, in seasonally flooded and arid areas. Europe together with the Russian Federation accounts for more than half of the global barley production today (Langridge, 2018). In 2018, the top five producing countries were the Russian Federation, France, Germany, Australia, and Spain (FAOSTAT, 2020). In the Nordic countries, the cultivation of barley has a long history. The oldest archaeological finding of cereals in Sweden (known to be about 6000 years old) consists of remains of barley (Leino, 2017). It is, alongside wheat, the most dominating cereal crop in the Nordic countries (FAOSTAT, 2020). In 2018, it was the most cultivated cereal in Sweden in terms of area cultivated, and in 2019, it was the second one, just after wheat (SJV, 2019).

Several initiatives have been formed around concerns of current and future global food security (Elsevier, 2021; FAO, 2021; Feed the Future, n.d.). The world population is growing and is expected to reach almost 10 billion by 2050, which naturally will increase demands for agricultural outputs (FAO, 2017). Simultaneously there is a need for limiting agricultural caused environmental footprints (NordGen, 2019). It is projected that climate change will create serious challenges for global food security. This is due to higher frequencies of climate extreme events, such as droughts, floods, and strong winds, together with influxes of new pests and diseases (EAA, 2019). Plant breeding for adapted crop varieties is recognized to be important for maintaining stable yields in the light of already ongoing as well as future challenges (NordGen, 2019; ADAS, 2015).

The Nordic region constitutes a small market for seed companies, due to its unique conditions in terms of climate, temperature, and day length, as well as specific regulations regarding fertilizers and pesticides (Mistra Biotech, 2018; Nilsson *et al*., 2016). Since global plant breeding today largely is carried out by a few large multinational companies, small markets like the Nordic region risk being neglected. With this concern, The Nordic Council of Ministers initiated a Public-Private Partnership (PPP), to strengthen plant breeding in the Nordic region, involving breeding companies and governmental organizations (Nilsson *et al*., 2016). One of the groups in the PPP is working with pre-breeding in spring barley, with a focus on disease resistance and agronomic traits related to earliness for the specific conditions of Northern Europe.

1.2 Taxonomy, characteristics, and academic role of barley (*Hordeum vulgare* L.)

Barley (*Hordeum vulgare* L.) is a member of the grass family (Poaceae) and belongs to the subfamily Pooideae (von Bothmer *et al*., 2003b; Judd *et al*., 2016). There it is located within the monophyletic tribe Triticeae together with other important cereals such as wheat (*Triticum aestivum* L.) and rye (*Secale cereal* L.). Members of Triticeae are known to be distributed in all major temperate areas of the world, and some species of the tribe are to be found in the subtropics. The genus *Hordeum* comprises around 30 species (Stevens, accessed on 2021; von Bothmer *et al*., 2003b). Even if these species share many morphological characteristics, the genus shows a high degree of diversity and comprises annuals, perennials, self-pollinators, self-incompatible species as well as species with a versatile reproductive system. Most species of *Hordeum* are diploid (i.e. have two sets of chromosomes), but the genus includes tetraploid and hexaploid species as well.

The wild progenitor (*Hordeum vulgare* ssp. *spontaneum)* of cultivated barley (*Hordeum vulgare* ssp. *vulgare*) has the center of its origin in the Middle East (von Bothmer *et al*, 2003b). However, it is found in the whole Mediterranean region, and as far to the east as Tibet and Western China (Leino, 2017). There are theories of independent events of domestication of barley in different regions, but the barley that came to be cultivated in Europe is believed to originate from the Fertile Crescent. Some of the characteristics which are used for distinguishing wild barley from the cultivated forms include i) row-type, where ssp. *spontaneum* always is two-rowed while cultivated barley can be either two- or six-rowed, ii) height, where ssp. *spontaneum* often is taller than ssp. *vulgare* of the same area and iii) the rachis, where ssp. *spontaneum* has brittle rachis, while the rachis of ssp. *vulgare* is tougher (von Bothmer *et al*, 2003b; Leino 2017).

Wild barley is generally more open flowering compared to the cultivated forms, which leads to a higher degree of cross-pollination (von Bothmer *et al*, 2003b). Even if barley mostly is self-pollinating introgression is known to occur where cultivated and wild barley exist together. Often used characters for distinguishing between different types of cultivated forms of barley include the kernel row-type (two-rowed or six-rowed), the kernels being hulled or hulless, and the adaption for spring or winter sowing (i.e. spring or winter barley) (Leino, 2017). In high latitudes, as in the Nordic region, cultivars adapted for spring sowing are most common since cold winters can cause crop damage (von Bothmer *et al*, 2003b). In Sweden in the years 2000-2019, 90% of the barley areal consisted of spring barley (SJV, 2019).

The differences in row-type stems from the spikelets in the ears of the plant being arranged in triplets, where there are differences in the number of fertile spikelets per triplet (Leino, 2017; Komatsuda *et al*., 2007). In the two-rowed varieties, only the middle spikelet of each triplet is fertile. This results in fewer and bigger kernels compared to the six-rowed cultivars, where all three triplets develop into kernels. The six-rowed type of barley is known to originate from mutations in a single gene (*Vrs1*) in two-rowed barley (Komatsuda *et al*., 2007). It is believed that six-rowed plants were selected during early domestication to increase kernel yield (Komatsuda *et al*., 2007).

Two-row barley to the left, and six-row barley to the right (CC Public Domain).

Barley has played an important role as a model species in plant research (von Bothmer *et al*, 2003a; Langridge, 2018). Many concepts and tools in modern crop research have been developed through early studies on the species, and later used for studying its close relative wheat. Barley has a diploid genome with a low chromosome number (2n=14), and even if the genome is large (5.1 Gb), it is still only about a third of the size of the hexaploid genome of wheat (Langridge, 2018; IBGSC, 2012). Today since techniques for genome analysis have improved, barley does not serve the same purpose in research, but other properties of barley are believed to keep it useful as a model species (Langridge, 2018). As it is one of the hardiest of the cultivated cereal crops and a highly diverse species, it is useful for studying the adaptation of crops to new environmental conditions and for developing techniques for expanding the germplasm base for crops.

1.3 Hydroponic screening for early-stage root growth

More than providing anchorage for the plant, roots play several vital roles for plant growth, including nutrient and water uptake, energy storage, and biosynthesis of plant hormones (Zhu *et al*., 2011). Traditionally, breeding efforts have mostly focused on improving above-ground plant traits, but in later years research on root systems has gained a lot of attention. The advantages of including genetic information of root traits in breeding programs have been highlighted for enhanced resource efficiency, stress tolerance, and plant adaptation to unstable climates (Jia *et al*., 2019; Zhu *et al*., 2011). It is known that individual root traits can correlate with e.g. enhanced nutrient uptake, water use efficiency, and yield formation (Karunarathne *et al*., 2020; Manschadi *et al*., 2006; Robinson *et al*., 2018). Studies have shown that early root growth can be predictive for crop performance in later stages (Bertholdsson & Kolodinska Brantestam, 2009; Li *et al*., 2015). Screening for early root traits in the seedling stage has therefore become a method of interest in crop research, to gain knowledge with the potential to be implemented in breeding programs (Jia *et al*., 2019; Abdel-Ghani *et al*., 2019; Wang *et al*., 2017).

While phenotyping roots in field conditions can have the advantage of giving an accurate representation of root growth in an authentic setting, complications arise due to the soil obscuring the root system and high throughput can be difficult (Jia *et al*., 2019). Even if methods have been developed for phenotyping plants in the field, it is not optimal for efficiently screening a high number of seedlings with a fine root system at an early growth stage (Trachsel *et al*., 2011; Jia *et al*., 2019). Different high throughput laboratory and greenhouse methods have been developed, including the use of substrate-filled rhizoboxes, seedlings grown on agar, and hydroponic systems where seedlings are grown in a nutrient solution (Jia *et al*., 2019; Wang *et al*., 2017; Zhu *et al*., 2011; Bertholdsson & Kolodinska Brantestam, 2009).

Using hydroponic methods for examining root characteristics under controlled conditions has several advantages. This includes the possibility to investigate root traits of large numbers of lines in a short time, the precise control of nutrient concentrations, and the exclusion of environmental interferences, which can facilitate repeatability (Wang *et al*., 2017; Kumar *et al*., 2014). A variety of traits related to root system architecture, such as root system depth, root spreading angle, root number, and root dry weight, have previously been included in research on seedling characteristics of barley (Jia *et al*., 2019; Wang *et al*., 2017; Bertholdsson & Kolodinska Brantestam, 2009). Considering the two traits included in this

thesis, the root length of seedlings has been shown to correlate with total N-uptake and grain yield in previous hydroponic studies (Bertholdsson & Kolodinska Brantestam, 2009). In a hydroponic study by Karunarathne *et al*. (2015), root dry weight was shown to correlate with nitrogen use efficiency. Hydroponic methods have successfully been used in association studies revealing genetic associations for these traits (Karunarathne *et al*. 2015; Wang *et al*., 2017).

1.4 Quantitative genetics & marker-assisted selection (MAS)

The field of quantitative genetics comprises studies on quantitatively inherited traits that commonly show a continuous rather than a distinct variation across individuals (Gai & Lu, 2013; Xu, 2012). Such traits are called quantitative or complex, and they are known to be controlled by interactions of multiple genes. A quantitative trait locus (QTL) is a genetic locus that affects the variation of a quantitative trait (Members of the Complex Trait Consortium, 2003). Quantitative traits can be controlled by one or many QTLs, as well as by environmental factors independent of genotype or through gene-environment interactions. Generally, quantitative traits are normally distributed among individuals in a population (Mulualem & Bekeko, 2016). Most agronomic traits in plant breeding are of quantitative nature, e.g. yield formation, forms of disease resistance, and various quality traits (e.g. malting quality in barley or baking quality in wheat) (Hayes *et al*., 2003; Mulualem & Bekeko, 2016; Nelson *et al*., 2006).

Over the last decades, the field of quantitative genetics has benefited from the evolution of DNA markers together with rapid advances in computer technology (Hayes *et al*., 2003; Muñoz-Amatriaín & Mascher, 2018). The development of a complete reference genome for barley has been important and can provide information about candidate genes for identified QTL (Smith *et al*., 2018). A variety of molecular markers, such as RAPD (Random Amplified Polymorphic DNA), AFLP (Amplified fragment length polymorphism), and SSR (Simple sequence repeats) have been developed and used in genetic research. Improved high-throughput genotyping techniques together with the development of SNP (single nucleotide polymorphism) markers have increased the precision and made large-scale genome-wide comparative studies possible. This has enabled the identification of markers sufficiently closely linked for effective use in marker-assisted breeding programs (Waugh *et al*., 2014).

In contrast to traditional breeding which solely relies on phenotypic information, marker-assisted selection (MAS) allows individual plants to be selected based on marker scores for the traits of interest (Smith *et al*., 2018). Some advantages attributed to MAS are the increased selection accuracy for traits that are complicated or costly to phenotype traditionally, and the possibility for selection at the seedling stage for traits that only develop later, which reduces time and cost (Smith *et al*., 2018; Collard & Mackill, 2008). Furthermore, MAS allows screening of traits that require specific environmental conditions to be visible (e.g. expression of various diseases) and it can be utilized for increased efficiency in backcrossing, where genes are incorporated into elite varieties, as well as for marker-assisted pyramiding - the process of incorporating several genes into one genotype. MAS has successfully been applied in barley as well as in wheat, for breeding for disease resistance of various diseases (Miedaner & Korzun, 2012).

1.5 Genome-Wide Association Study (GWAS)

Genome-Wide Association Study (GWAS) has become popular for identifying QTLs of plant traits (Smith *et al*., 2018; Waugh *et al*., 2014). It is based on statistical associations between SNP-markers and phenotypic variation between individuals in a population, i.e. comparison between the genotypic and phenotypic data sets. In contrast to QTL mapping (another method for identifying QTL), which requires the construction of balanced populations with known recombination history, GWAS allows analyses of genetically diverse populations where the recombination history is unknown (Smith *et al*., 2018). GWAS has been used to locate QTLs for a variety of traits in barley, such as e.g. grain yield, drought tolerance, and heading date, and QTLs have been identified that previously were not identified with LD mapping (Alqudah *et al*., 2020). Regarding GWAS in Nordic spring barley, Bengtsson *et al*. (2017a) have previously located QTLs for powdery mildew resistance, and Göransson *et al*. (2017) have located an allele combination affecting heat sum to maturity, a trait of importance for expanding the cultivation northwards.

While conducting GWAS, it is important to account for population structure (the differentiation in allele frequencies among populations), to reduce the risk for false positive or negative phenotype-genotype associations (Alqudah *et al*., 2020; Brachi *et al*., 2011; Bergelson & Roux, 2010). Previous population structure studies in barley have divided populations into subpopulations based on growth habit (spring barley or winter barley), row-type (two- or six-rowed), and geographical origins (Bengtsson *et al*., 2017b; Alqudah *et al*., 2020). Various models have been developed to account for population structure for association studies (Alqudah *et al*., 2020). Another important factor to consider while conducting GWAS is linkage disequilibrium (LD), which is defined as the non-independence of alleles at different loci in a population (Waugh *et al*., 2014; Alqudah *et al*., 2020). The rate at which LD declines (the LD decay) with the genetic or physical distance in a given species determines the molecular marker density needed to perform GWAS, and it defines the physical interval on the genome for where to search for candidate genes (Alqudah *et al*., 2020).

1.6 Objectives

1.6.1 Aim

This thesis aims to find out if a low-tech hydroponic method for screening seedling traits, previously developed by Dr. Bertholdsson (Bertholdsson & Kolodinska Brantestam, 2009), can be used for assessing QTLs associated with early root growth traits in seedlings of spring barley. As a way to validate the method, possible identified QTLs/candidate genes will be compared with QTLs/candidate genes detected in similar studies. Another ambition is to investigate if the comprised genotypes differ in early root growth depending on row-types, countries of origin, or genotypes being historic cultivars/landraces or modern breeding lines.

1.6.2 Research questions

- Can QTLs significantly associated with early root growth in spring barley be identified using the hydroponic screening method, described in Bertholdsson & Kolodinska Brantestam (2009)?
- If QTLs/candidate genes are found, have these QTLs/candidate genes been identified in other similar studies?
- Are there differences in early root growth between the two row-types? If so, is it the same or different QTLs associated with early root growth in two-row and six-row spring barley?

- Does the early root growth differ between older material such as landraces and historic cultivars compared to more modern breeding lines?
- Do the breeding lines differ in root growth depending on their country of origin?

1.6.3 Limitations

The thesis comprises a literature survey, a practical experiment in a controlled environment, and statistical analyses regarding the plant material and method described below. It is limited to the two root traits RootW and RootL. The thesis does not include the screening for root traits in the field. No genotyping will be performed since this data already exists. Regarding the localization of QTLs, the thesis is limited to the use of GWAS. While searching for possible candidate genes that might be associated with the traits, the thesis is limited to genes that are reported in similar studies.

2. Material and methods

2.1 Plant material

The plant material comprised 259 genotypes of spring barley, from the Nordic PPP–project (described in chapter 1.1). This material included modern lines from Boreal (Finland), Graminor (Norway), Nordic Seed (Denmark), Sejet Plant Breeding (Denmark), and Lantmännen (Sweden), as well as historic cultivars and landraces from NordGen (Nordiskt Genresurscenter) and 12 historic European cultivars from IPK (Leibniz Institute of Plant Genetics and Crop Plant Research, Germany). The material has previously been genotyped by SciLifeLab, with the Barley 50k iSelect SNP Array (Bayer *et al*., 2017). It has been screened for early vigor traits, such as the digital biomass in a high-throughput phenotyping facility at IPK, and evaluated for yield components under field conditions in Lönnstorp, Sweden, in 2018, and 2019 (unpublished results). In total, the plant material covered 131 two-rowed and 128 six-rowed genotypes pre-dominantly from the Nordic countries. The kernels for the genotypes used in the experiments were collected from plants grown in the same field, in the same year, during a time period of 3-4 weeks, depending on when the kernels of the different genotypes would mature. They were then stored under the same conditions.

2.2 Experimental design

A low-tech hydroponic system, developed by Dr. Bertholdsson, and described in Bertholdsson & Kolodinska Brantestam (2009), was used for screening root traits. This allowed the screening of a high number of genotypes in a small space and under controlled conditions. The seedlings were grown in 25 L containers with a balanced complete nutrient solution for 14 days. The 259 genotypes were arranged in a randomized complete block design with two replicates, each consisting of eight plants. To account for possible edge effects, kernels were sown at the edges of the frames (see Figure 1). Those were not included in the phenotyping. While sowing, kernels considered to deviate too much in size from the mean size for the genotype were discarded to assist a fairly uniform starting point for the genotypes.

The containers were placed in a climate chamber (the Biotron in Alnarp, Sweden) with 16 h artificial light (300 e, in the range of 400-700 nm) at 18/15°C day/night temperature during the growth period. In total, 12 containers were needed to cover the 259 lines with two replicates. After 14 days, the seedlings were phenotyped for root dry weight (RootW) and seminal root length (RootL) (seminal roots, being the roots that develop directly from the embryo radicle (Wahbi & Gregory, 1995)). Seedlings with abnormal growth and seedlings heavily infected by fungi were discarded. The longest seminal root from each seedling was measured with a ruler and registered. Thereafter, the roots were dried for four days (80°C), and the pooled dry weight of the roots of each genotype was measured and registered.

The nutrient solution used for the hydroponic system, based on a balanced complete nutrient solution (Larsson, 1982), has previously been modified regarding nitrogen (N) concentration by Bertholdsson & Koldinska Brantestam (2008). They conducted a series of hydroponic studies with barley, where the nitrogen, as well as the oxygen concentration, was optimized for obtaining a good correlation with field data. Based on their findings no aeration was used in this thesis, together with nutrient-concentrations with minor modifications from their study (see Table 1), as recommended by Dr. Bertholdsson.

Table 1. Content and concentrations used for nutrient stock solution[1] and final working solution.

Compounds	Weight (g) for stock solution	Volume (ml) of stock solution for 25 L nutrient solution	End concentration (mM)
$Na_2HPO_4 \cdot 2H2O$ (Disodium phosphate dihydrate)	39/2 L	200	0.88
KH_2PO_4 (Monopotassium phosphate)	68/2 L	200	2.00
KCL (Potassium chloride)	37/2 L	200	1.99
$MgSO_4 \cdot 7H_2O$ (Magnesium sulfate)	61/2 L	200	0.99
$Ca(NO_3)_2 \cdot 4H_2O$ (Calcium nitrate)	118/2 L	200	2.00
Micronutrients: YaraTera™ REXOLIN® APN (%): B: 0.9%, Cu: 0.25%, Fe: 6%, Mn: 2.4%, Mo: 0.25%, Zn: 1.3%	6.35/0.5 L	25	

[1]2 L of stock solution of macronutrients was enough for 10 x 25 L nutrient solution (10 containers), and 0.5 L of stock solution of micronutrients was enough for 20 x 25 L nutrient solution (20 containers)

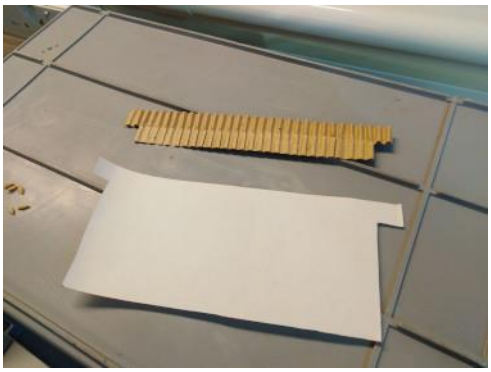
Deionized water was used when preparing nutrient stock solutions, as well as for diluting stock solutions to the final concentrations used in the experiments. The nutrient solution was prepared from the stock solutions just before starting a container. Five days as well as 11 days after starting a container, the nutrient solution was renewed (in total two times during the 14-day growth period). The levels of the nutrient solutions in the containers were checked periodically and when needed all the containers were filled up with deionized water to the start level.

Each container contained two frames with 14 stripes of corrugated paper into where the kernels were sown (see Fig. 1 and Pic. 2). The first and the last stripe were sown to decrease possible edge effects, and these were therefore not included in the subsequent phenotyping and analyses. This left 12 stripes per frame and in total 24 stripes per container. Two genotypes were sown per stripe with eight kernels of the same genotype next to each other. To control for possible container effects, each container contained the control genotype cultivar Tamtam, which is a stable and high yielding Nordic two-row variety. Altogether, one container had room for 47 out of the 259 genotypes. Therefore, to cover all 259 genotypes, six containers were needed for each replicate. Since six containers had room for 282 genotypes (instead of 259), a “fill-up genotype” was used where gaps were created after the position of the 259 genotypes had been distributed in a randomized manner. At the edges of each stripe, as well as for the first and last stripe in each frame, a kernel (same genotype as for filling up gaps) was sown to account for possible edge effects.

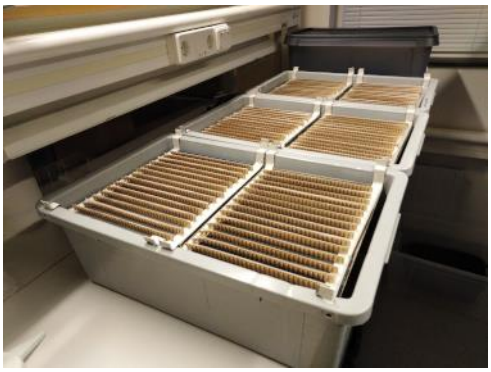
	⓪ ⓪ ⓪ ⓪ ⓪ ⓪ ⓪ ⓪ ⓪	⓪ ⓪ ⓪ ⓪ ⓪ ⓪ ⓪ ⓪ ⓪	
Genotype 84	⓪ ① ② ③ ④ ⑤ ⑥ ⑦ ⑧	① ② ③ ④ ⑤ ⑥ ⑦ ⑧ ⓪	Genotype 13
Genotype 255	⓪ ① ② ③ ④ ⑤ ⑥ ⑦ ⑧	① ② ③ ④ ⑤ ⑥ ⑦ ⑧ ⓪	Genotype 64
Genotype 194	⓪ ① ② ③ ④ ⑤ ⑥ ⑦ ⑧	① ② ③ ④ ⑤ ⑥ ⑦ ⑧ ⓪	Genotype 10
Genotype 207	⓪ ① ② ③ ④ ⑤ ⑥ ⑦ ⑧	① ② ③ ④ ⑤ ⑥ ⑦ ⑧ ⓪	Genotype 118
Genotype 22	⓪ ① ② ③ ④ ⑤ ⑥ ⑦ ⑧	① ② ③ ④ ⑤ ⑥ ⑦ ⑧ ⓪	Control genotype, cv. Tamtam
Genotype 188	⓪ ① ② ③ ④ ⑤ ⑥ ⑦ ⑧	① ② ③ ④ ⑤ ⑥ ⑦ ⑧ ⓪	Genotype 240
Genotype 230	⓪ ① ② ③ ④ ⑤ ⑥ ⑦ ⑧	① ② ③ ④ ⑤ ⑥ ⑦ ⑧ ⓪	Genotype 223
Genotype 133	⓪ ① ② ③ ④ ⑤ ⑥ ⑦ ⑧	① ② ③ ④ ⑤ ⑥ ⑦ ⑧ ⓪	Genotype 187
Genotype 36	⓪ ① ② ③ ④ ⑤ ⑥ ⑦ ⑧	① ② ③ ④ ⑤ ⑥ ⑦ ⑧ ⓪	Genotype 134
Fill-up genotype	⓪ ⓪ ⓪ ⓪ ⓪ ⓪ ⓪ ⓪ ⓪	① ② ③ ④ ⑤ ⑥ ⑦ ⑧ ⓪	Genotype 148
Genotype 9	⓪ ① ② ③ ④ ⑤ ⑥ ⑦ ⑧	① ② ③ ④ ⑤ ⑥ ⑦ ⑧ ⓪	Genotype 184
Genotype 161	⓪ ① ② ③ ④ ⑤ ⑥ ⑦ ⑧	① ② ③ ④ ⑤ ⑥ ⑦ ⑧ ⓪	Genotype 149
	⓪ ⓪ ⓪ ⓪ ⓪ ⓪ ⓪ ⓪ ⓪	⓪ ⓪ ⓪ ⓪ ⓪ ⓪ ⓪ ⓪ ⓪	

Figure 1. Example of the randomized arrangement of the genotypes in one frame consisting of 14 stripes of corrugated paper. Red color: a "fill-up genotype" to account for possible edge effects. Green color: Test genotypes, two genotypes per stripe with eight kernels per genotype. Blue color: cultivar Tamtam used as a control genotype, included to control for possible container effects.

Step-wise description (A-P) of the experimental procedure:

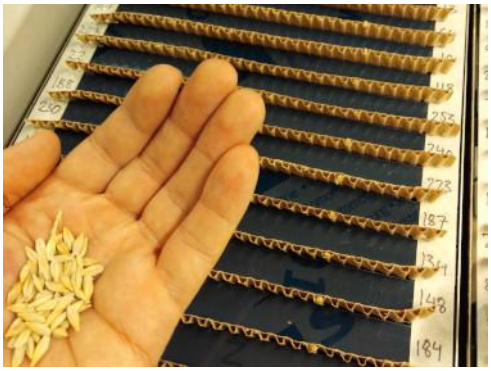


A. Corrugated paper stripes and filter papers were cut to fit the frames. The lower side of the stripes was flattened with an iron, to fit the stripes between the plastic rods in the frame.

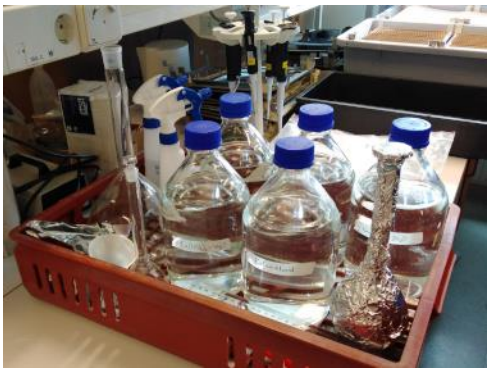


B. The paper stripes and filter paper were placed in between flat movable plastic rods with about 1.5 mm gaps.

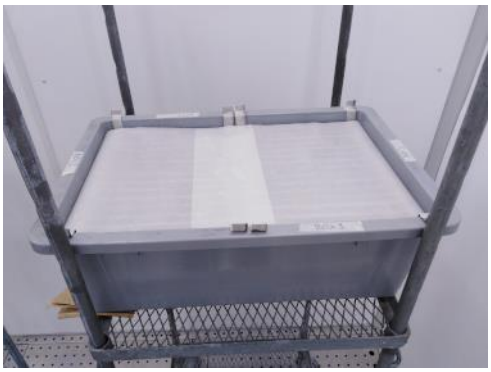
C. Side view showing the position of the corrugated paper stripes and filter papers. The filter paper was used to assure water could reach the seedlings before the roots were long enough to reach the nutrient solution.



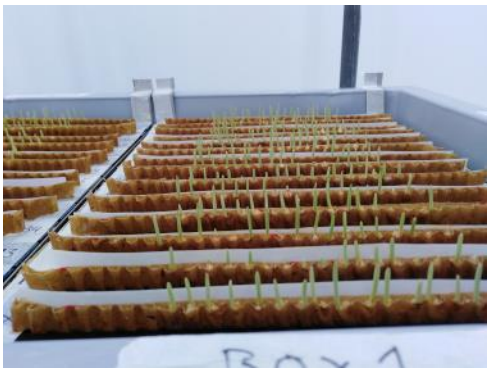
D. Masking tape was used to keep the plastic rods in place. Numbers representing the genotypes were written on both sides since two genotypes were sown in each corrugated paper stripe.



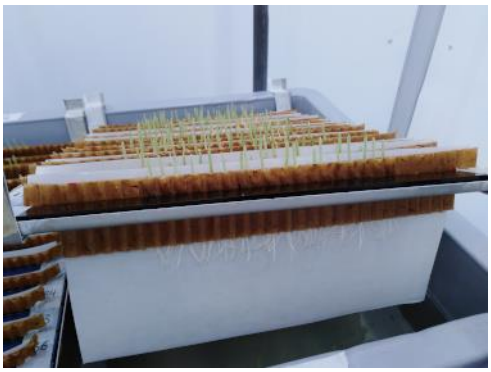
E. Stock solutions with macronutrients were stored in 2 L bottles and the stock solution with micronutrients was stored in an aluminium foil-covered e-piston. For preparing the nutrient solution, stock solutions were diluted with ionized water in a 25 L plastic can before poured into the container.



F. The containers were placed in a climate chamber. A diluted detergent, Tween-20 (0.5% concentration), was sprayed on the corrugated paper stripes to assure that the nutrient solution could penetrate the paper and reach the kernels. The frames were covered with moistened filter paper with both short sides submerged into the nutrient solution.



G. Germinated kernels after the filter paper had been removed, three DAS (days after sowing).

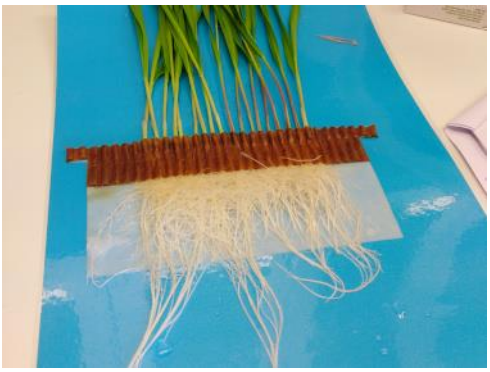


H. Visible roots starting to reach the nutrient solution.

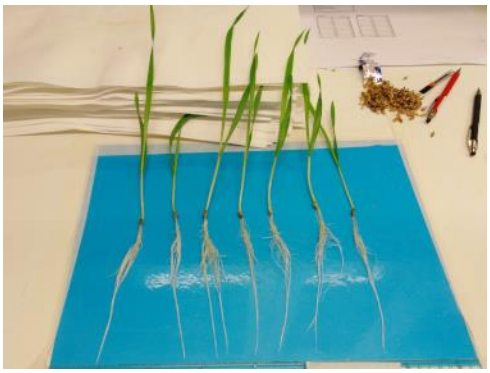
I. The containers were periodically rotated in the climate chamber, to decrease possible climatic variations.

J. Three containers at different growth stages. The container to the right is ready for phenotyping 14 DAS.

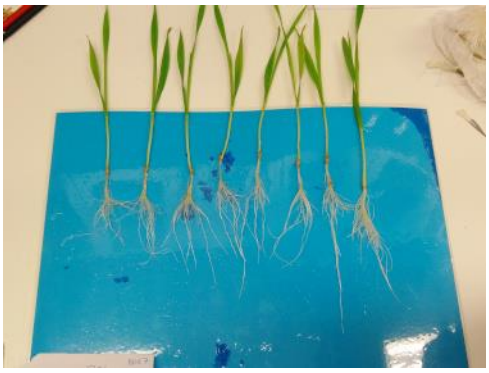
K. Root growth just before phenotyping.



L. Two genotypes (8 x 2 seedlings) were grown beside each other. The genotypes could easily be separated from each other, the corrugated paper stripes and the filter paper. The edge seedlings (one per edge) were removed before phenotyping.



M. One of the genotypes, where the roots of the seedlings with little effort had been separated from each other.



N. Another genotype for comparison with M, with a less uniform root length

O. Roots were separated from the shoots with a razor blade. The longest seminal root for all seedlings was measured with a ruler. Shoot lengths were measured as well, although not included in this thesis due to time restrictions.

P. The roots (and shoots, although not included in this thesis) were stacked on filter papers, one paper per genotype, and dried for four days (80°C) and thereafter weighed.

Figure 2. Step-wise description (A-P) for the experimental procedure, as described in 2.2.

2.3 Correlations, descriptive statistics & Analysis of Variance (ANOVA)

The statistical analyses included data for the two continuous traits RootL and RootW. Since row-type previously has been shown to account for strong population structure, the analyses were performed on the whole panel (comprising all the genotypes) as well as the two-row and six-row panel separately (Bengtsson *et al*., 2017b). Before conducting statistical analyses, outliers were removed using the boxplot.stats function in R (R Core Team, 2020). Observations outside 1.5 * the 'Inter Quartile Range' were removed. The mean value for each genotype was calculated, and the values were divided with the mean value for the control genotype in each container set as 1 to account for environmental effects.

Spearman's rank correlation was calculated in R using the function "cormat" included in the package *corrplot* version 0.84 (Taiyun Wei & Viliam Simko, 2017). Since a few genotypes were removed for RootW (outliers), the correlations were performed with 248 genotypes (instead of 259) for the whole panel, 122 (instead of 131) for the two-row panel and 126 (instead of 128) for the six-row panel. This was due to the package *corrplot* not being able to handle missing values (same number of genotypes were needed for both traits for creating combined plots). Descriptive statistics, including the number of observations (n), mean, standard deviation (sd), median, absolute deviation (mad), minimum (min), maximum (max), and standard error (se), were retrieved with the *psych* package version 2.0.9 (Revelle, 2020). The same package was used for plotting frequency distributions.

To evaluate the relative contributions of genotype and experiment regarding each trait, analysis of variance (ANOVA model III with Satterthwaite's method) was analyzed using the "lmer" function in the *lme4* R package (Bates *et al*., 2015). The model assumed the genotype and replicate effect to be fixed and the effect of the containers and genotypes nested within the containers to be random.

2.4 GWAS

GWAS was conducted with R and the package *GAPIT* version 3.0 (Lipka *et al*., 2012). Before the analysis, the markers were filtered to exclude monomorphic markers, markers with a call rate below 95%, and markers with a minor allele frequency (MAF) below 5%. This left 33 230 SNP markers for the whole panel (n=259), 27 832 SNP markers for the two-rowed panel (n=131), and 27 678 SNP markers for the six-rowed panel (n=128). To retrieve the physical position of the SNP markers based on the barley reference genome, version 1.0 (Bayer *et al*., 2017; Mascher *et al*., 2017), the online tool BARLEYMAP (http://floresta.eead.csic.es/barleymap) was utilized (Cantalapiedra *et al*., 2015).

To avoid the inflation of false positive or negative associations, several models were tested to find the optimal one for each trait. Population structure was accounted for using kinship-matrix with the VanRaden method (VanRaden, 2008) as well as using PCA (Principle components analysis); both included in *GAPIT*. The software TASSEL v. 5.2.59 (Bradbury *et al*., 2007) was used to calculate the percent variation explained by the first two principal components. Bayesian information criterion (BIC) was retrieved with GAPIT for determining the optimum number of PCs (principal components) to include for each trait. The tested models were general linear model (GLM), mixed linear model (MLM) (Zhang *et al*., 2010), multiple loci mixed linear model (MLMM) (Segura *et al*., 2012), and fixed and random model circulating probability unification (FarmCPU) (Liu *et al.*, 2016). To compare the performance of the models, quantile-quantile plots (Q-Q plots) were created with R and the package

CMplot (Yin *et al*., 2020). The model with the smallest deviation from the expected distribution for each trait was chosen for the subsequent GWAS.

The Genome-Wide Association Studies were conducted for each trait and panel, and the package *CMplot* was used to create combined Manhattan-plots. Bonferroni thresholds were set to determine the significance level. The thresholds were calculated as 0.05/number of effective markers for each panel, where the number of effective markers for the panels was calculated as described in Gao *et al*. (2008). This gave a LOD (logarithm of the odds) score of 3.79 as the threshold for the whole panel, 3.83 for the two-row panel, and 3.82 for the six-row panel.

2.5 QTL assignment & candidate genes

TASSEL v. 5.2.59 (Bradbury *et al*., 2007) was used to evaluate intra-chromosomal LD between all the significant markers, to determine if the significant markers could be regarded as the same or distinct QTLs. The significant SNP markers for all panels and traits were subjected to search for possible candidate genes, with the range of +/- 1.5 Mbp using the online tool BARLEYMAP (Cantalapiedra *et al*., 2015). The range was decided based on the LD decay, 3 Mbp, for the whole panel (unpublished results).

3. Results

3.1 Phenotypic data & ANOVA

A significant and high correlation (r ≥0.75, p≥0.01) for RootL between the two replicates for all panels was observed (whole panel, two-row panel, and six-row panel) (Fig. 3). The correlations for RootW between the replicates were lower compared to RootL, although still significant for the whole panel and the two-row panel. In the six-row panel, no significant correlation between the replicates for RootW was observed. There were significant correlations between the root-traits (RootW and RootL) in both replicates and across the replicates in the whole panel. In the two-row panel, no significant correlations were obtained between the traits, whereas significant correlations were obtained in the six-row panel between RootW in both replicates with RootL in replicate two.

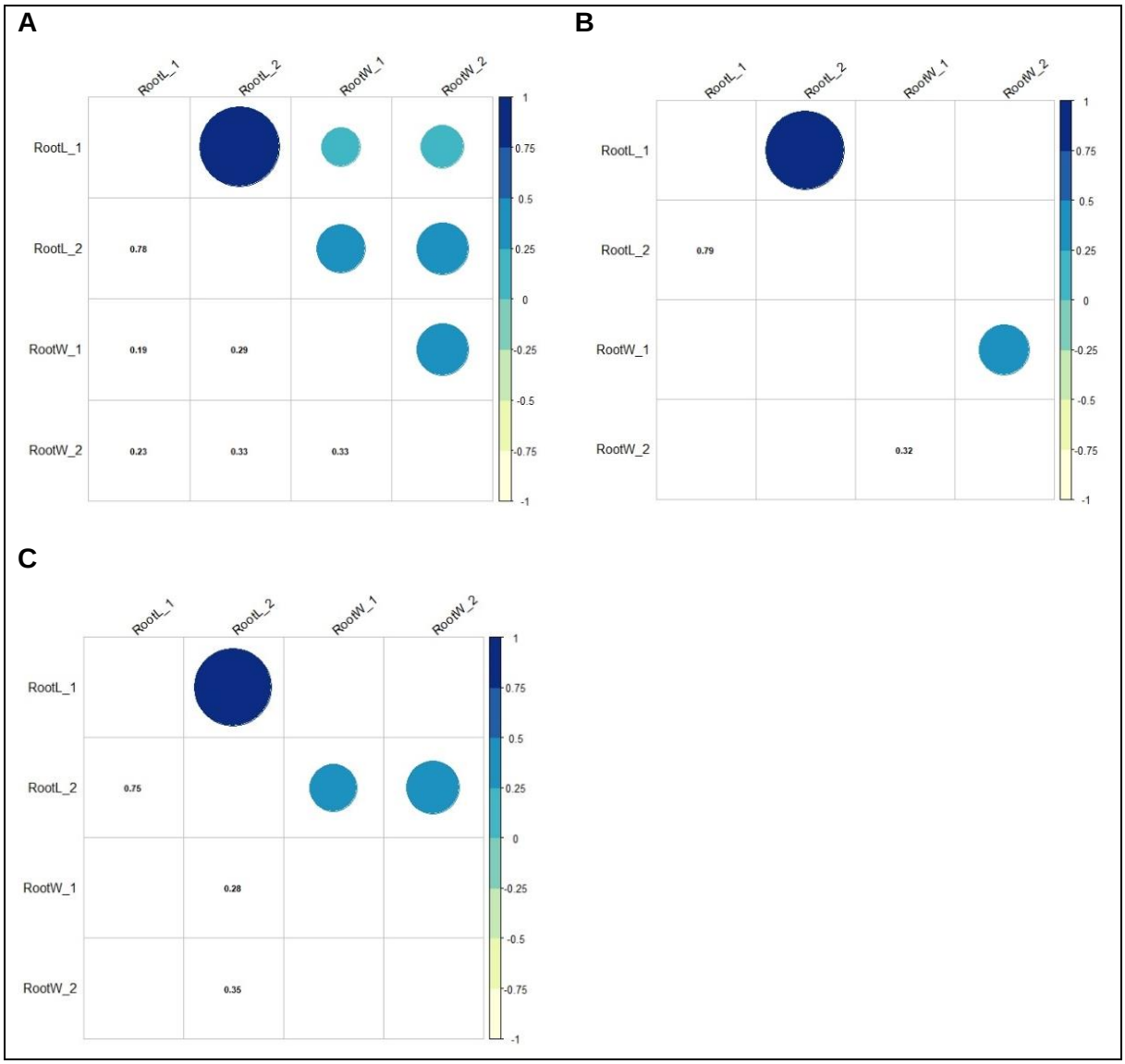


Figure 3. Pearson's correlations between the traits (RootW and RootL) and replicates (one and two, marked as numbers after the trait names) for the whole panel (A), the two-row panel (B), and the six-row panel (C). The color bar to the right of the plots shows the correlation coefficient, and the correlations are plotted as circles with the corresponding color and adjusted sizes (larger circles meaning larger correlations) on the upper-right half of the plots. On the lower-left half of the plots, the correlation coefficients are written. Correlations not significant at p≥0.01 are left blank. The graphs were generated with the Corrplot package in R.

Fig. 4 shows the frequency distribution-plots for all panels, where the plots for RootL for the whole panel (Fig. 4B) and RootL for the two-row panel (Fig. 4D) are slightly right-skewed. The summary statistics, shown in Table 2, illustrates general differences in mean-values, as well as the distribution for the traits, between the two-row and six-row panel. The mean values for the two-row panel are 1.07 for RootW (min: 0.56; max: 1.61), and 0.97 for RootL (min: 0.63; max: 1.23), compared to the six-row panel where the mean values are 0.91 for RootW (min: 0.50; max: 1.56) and 0.90 for RootL (min: 0.59; max: 1.20). The ANOVA (Table 3) confirmed a significant genotype effect for RootW as well as for RootL ($p < 0.0001$). No significant replicate effect was obtained for RootL, whereas a significant replicate effect was obtained for RootW ($p < 0.0001$).

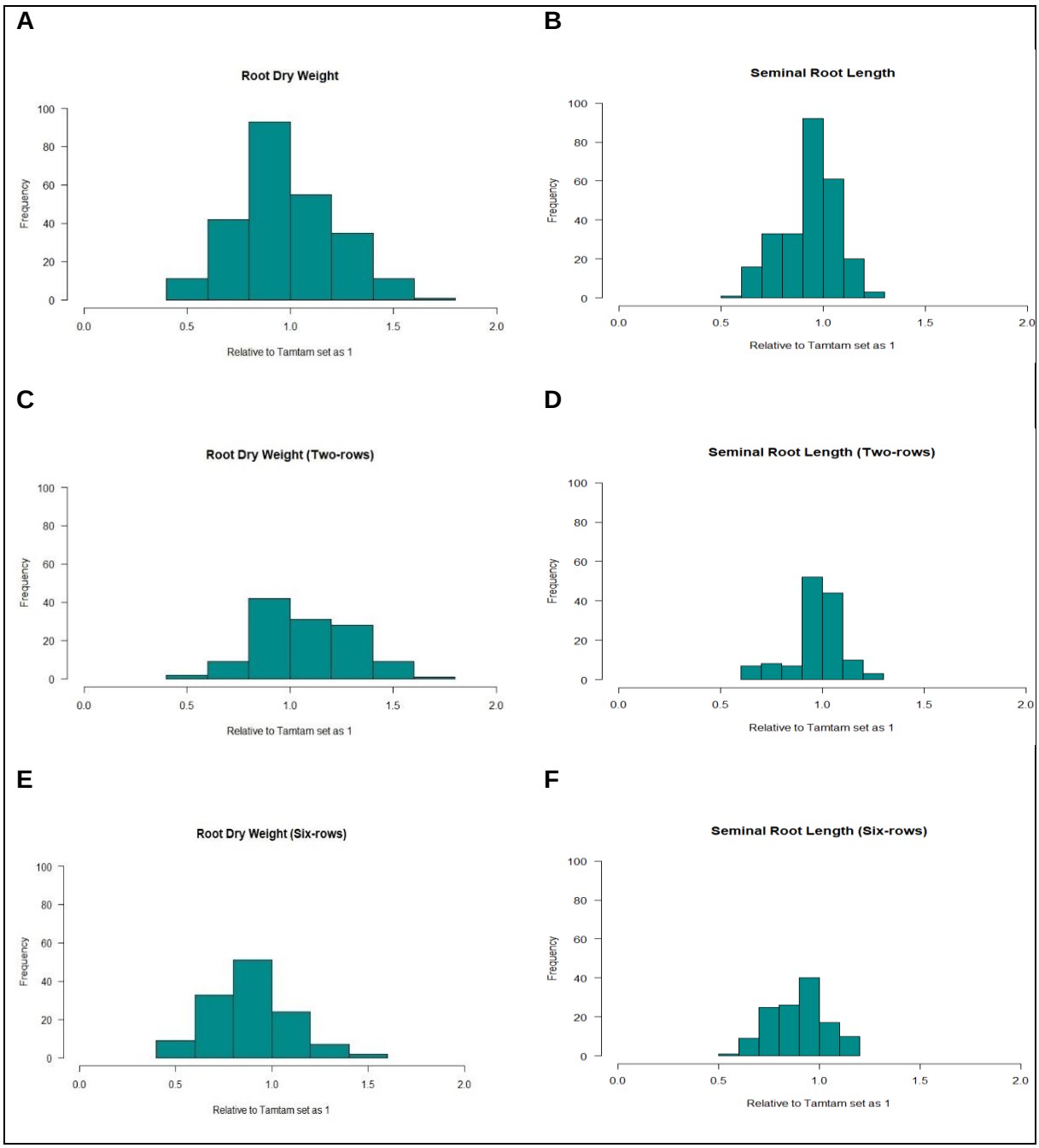


Figure 4. Frequency distribution-plots for all panels of RootW and RootL, where the x-axis represents the registered values of the traits 14 DAS (divided with the values of a control genotype, set as 1) and the y-axis shows the frequency. RootW, whole panel (A), RootL, whole panel (B), RootW, two-row panel (C), RootL, two-row panel (D), RootW six-row panel (E), RootL, six-row panel (F).

Table 2. Summary statistics for RootW and RootL for all panels, both replicates (rep. 1 and rep. 2), and the mean values of the two replicates. n=number of genotypes, sd=standard deviation, mad= median absolute deviation, se=standard error.

Panel/replicate	n	mean	sd	median	mad	min	max	se
Whole panel								
RootW rep. 1	248	1.06	0.29	1.00	0.27	0.50	2.11	0.02
RootW rep. 2	248	0.92	0.27	0.91	0.27	0.33	1.64	0.02
Mean	248	0.99	0.23	0.96	0.24	0.50	1.61	0.01
RootL rep. 1	259	0.92	0.15	0.94	0.12	0.55	1.30	0.01
RootL rep. 2	259	0.95	0.15	0.97	0.12	0.60	1.27	0.01
Mean	259	0.94	0.14	0.96	0.12	0.59	1.23	0.01
Two-row								
RootW rep. 1	122	1.14	0.27	1.11	0.27	0.62	2.00	0.02
RootW rep. 2	122	1.01	0.27	0.97	0.27	0.38	1.64	0.02
Mean	122	1.07	0.22	1.04	0.23	0.56	1.61	0.02
RootL rep. 1	131	0.96	0.14	0.98	0.10	0.59	1.30	0.01
RootL rep. 2	131	0.99	0.13	1.00	0.10	0.60	1.27	0.01
Mean	131	0.97	0.13	0.99	0.09	0.63	1.23	0.01
Six-row								
RootW rep. 1	126	0.98	0.29	0.95	0.23	0.50	2.11	0.03
RootW rep. 2	126	0.83	0.24	0.82	0.27	0.33	1.55	0.02
Mean	126	0.91	0.21	0.90	0.23	0.50	1.56	0.02
RootL rep. 1	128	0.89	0.14	0.89	0.15	0.55	1.25	0.01
RootL rep. 2	128	0.91	0.15	0.94	0.17	0.61	1.23	0.01
Mean	128	0.90	0.14	0.91	0.13	0.59	1.20	0.01

Table 3. Type III Analysis of Variance (ANOVA) with Satterthwaite's method, showing the relative contributions of genotype and replicate for RootL and RootW, respectively. The genotype and replicate effect was considered to be fixed, and the effect of the containers and genotypes nested within the containers to be random.

	Sum	Sq Mean	Sq NumDF	DenDF	F value	P-value[1]
RootL						
Genotype	5495.80	21.22	259	262.07	12.13	<2.00e-16***
Replicate	2.00	2.02	1	9.42	1.15	3.09e-1
RootW						
Genotype	10007.60	38.64	259	261.39	3.36	<2.20e-16***
Replicate	400.20	400.16	1	9.04	34.79	2.26e-4***

[1]Significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

3.2 GWAS

3.2.1 Population structure

The population structure analyses of the panels, including PCA and Kinship-matrix, showed a clear division between the row-types of the whole panel (Fig. 5A & 5B), with 32.9% (29.5%+3.4%) of the variance explained by the first two principal components. The variance explained by the first two principal components was 18.3% (13.2%+5.1%) in the two-row panel (Fig. 5C & 5D), with no obvious explanation due to the characteristics of the comprised genotypes. In the six-row panel, the historic cultivars and landraces were separated from the breeding lines (highlighted with a circle in Fig. 5E and red line in Fig. 5F), and the variance explained by the first two principal components was 21.1% (12.8%+8.3%).

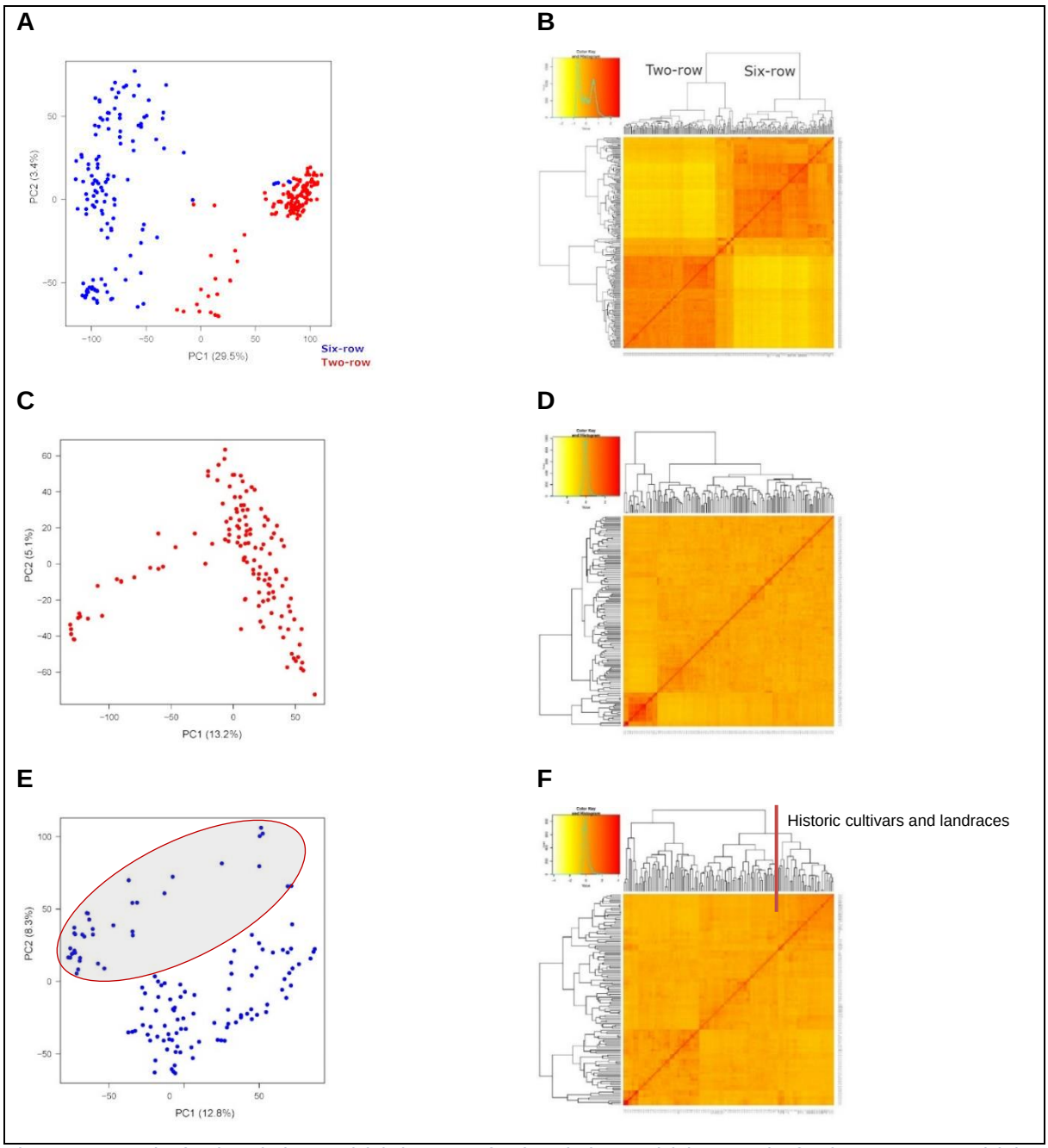


Figure 5. PCA-plot for the whole panel (A), heatmap for the whole panel (B), PCA-plot for the two-row panel (C), heatmap for the two-row panel (D), PCA-plot for the six-row panel (historic cultivars and landraces marked with a red oval) (E), heatmap for the two-row panel (historic cultivars and landraces on the right side of the red line) (F).

3.2.2 Model selection

The Bayesian Information Criterion (BIC)-table in GAPIT indicated that it was optimal to exclude PCs for both traits for the analyses, for not risking an overcompensation for population structure in the subsequent analyses (Table 4).

Table 4. BIC (Bayesian information criterion) table for RootL and RootW, obtained with GAPIT. The largest BIC number corresponds to the optimal number of PCs/covariates to include for the GWAS models.

Nr of PCs	BIC (larger is better) - Schwarz 1978	log Likelihood Function Value
RootW		
0	28.199	36.469
1	26.785	37.812
2	26.399	40.183
3	23.295	39.835
4	20.346	39.643
5	17.323	39.376
6	14.504	39.315
7	11.482	39.050
8	8.487	38.811
9	6.084	39.165
10	4.337	40.175
RootL		
0	184.751	193.086
1	182.188	193.302
2	179.736	193.628
3	176.949	193.619
4	174.159	193.608
5	171.412	193.639
6	170.551	195.556
7	167.742	195.526
8	165.292	195.855
9	163.253	196.594
10	160.460	196.579

The Q-Q plots, consisting of the models GLM, MLM+K (Kinship-matrix), MLMM+K, and FarmCPU+K, showed that the models with the least deviation from the expected distribution were FarmCPU+K in the case of RootW and MLMM+K in the case of RootL (Fig. 6). Thus, these models were chosen for the respective trait for conducting the association studies.

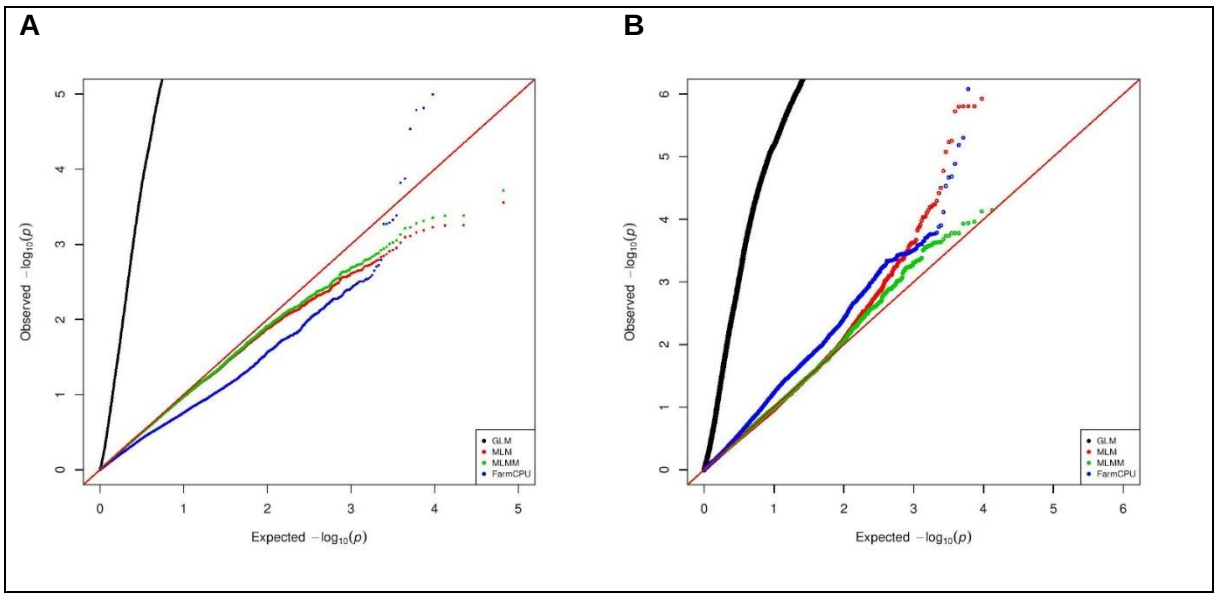


Figure 6. Q-Q (quantile-quantile) plot for RootW (A) and RootL (B) for the whole panel. The Y-axis represents the observed negative base 10 logarithms of the P-values, and the X-axis represents the expected observed negative base 0 logarithms of the P-values, with the assumption that the P-values follow a uniform distribution.

3.2.3 GWAS results, QTL assignment & candidate genes

In total, 36 significant markers with a known physical position on the barley reference genome were obtained, considering both traits and all panels (see Appendix, Table 5). For RootW, nine significant markers were obtained for the whole panel, zero for the two-row panel, and six for the six-row panel. Regarding RootL, seven significant markers were obtained for the whole panel, two for the two-row panel, and 12 for the six-row panel. The markers could be grouped into 23 QTLs: 14 QTLs for RootW and 10 for RootL, where RootW and RootL shared one of the QTLs. For RootW, nine QTLs were obtained for the whole panel, zero for the two-row panel, and six for the six-row panel. Regarding RootL, four QTLs were obtained for the whole panel, two for the two-row panel, and five for the six-row panel. Manhattan plots for each panel, including QTLs, are shown in Fig. 7.

The search for gene candidates for the 36 significant markers, using a range of +- 1.5 Mbp from each of the markers, resulted in a total of 2130 gene candidates. Genes located at the same position as the significant markers are included in Table 5 in Appendix.

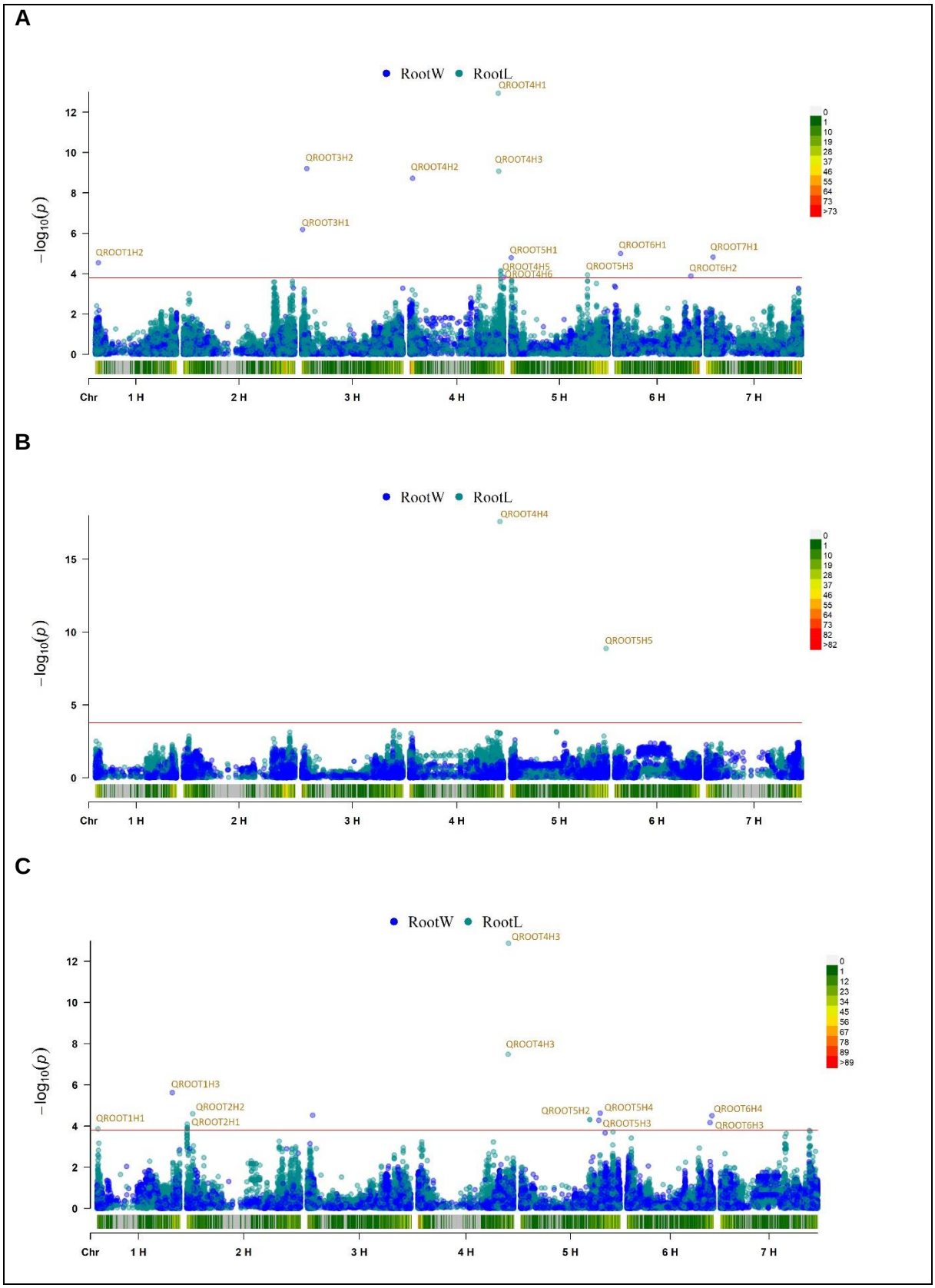


Figure 7. Manhattan plots showing significant associations between trait and marker (Bonferroni threshold in red) for both continuous traits, RootW and RootL, for the whole panel (two-row and six-row) (A) the two-row panel (B), and the six-row panel (C). The x-axis shows the physical distance over the seven barley chromosomes. The bar under the x-axis shows the SNP distribution on each chromosome, where 0 ->89 depicts SNP density. QTL names are presented in the figure.

4. Discussion

4.1 Utilization of hydroponics for screening root traits

The root traits of the 259 genotypes were successfully phenotyped using the hydroponic screening method, described in Bertholdsson & Kolodinska (2009). This allowed the assessed data to be utilized for GWAS and for identifying candidate genes (see 4.2). Correlations were high for RootL between the two replicates in all three panels. The ANOVA for RootL confirmed a significant genotype effect together with non-significant replicate effects. For RootW, the correlation was significant between the replicates for the whole-panel and the two-row panel (although lower compared to RootL). No significant correlation was obtained for RootW for the six-row panel. In contrast with the ANOVA results for RootL, significant replicate effects were obtained. Since six containers were needed to cover all genotypes of one replicate, where seedlings were grown for two weeks before phenotyping, the significant replicate effects might have been reflecting possible environmental effects. The phenotype data for RootW and RootL were both used for GWAS. Although, the GWAS results for RootL are given more strength, considering the outcome of the statistical analyses. The more variable statistical results for RootW in this thesis are discussed below.

Since the study comprised a high number of genotypes and the time for this thesis was limited, time-consuming steps for the practical part that were speculated not to be critical for generating useful results were excluded. Therefore, TKW (thousand kernel weight) of the genotypes was not considered while sowing. Although, kernels that visually deviated from the "normal size" for the genotype were discarded. The relationship between TKW and RootW, as well as RootL, has previously been investigated. Bertholdsson & Kolodinska (2009) studied the effects of TKW on the traits using three different genotypes of spring barley. They concluded that root dry weight and seminal root length reflected differences in TKW, but the phenotype of the genotypes was still expressed if seeds of the same weight were used. In a genome-wide association study comprising the two traits, by Jia *et al*. (2019), TKW was included as a co-factor for the published results. Although, they stated that the same QTLs were obtained without considering TKW, and that seed weight might have little impact on identifying QTLs of the comprised seedling characteristics.

In other studies on root traits of barley, kernels have been sterilized before sowing (Wang *et al*., 2017). Kernel-sterilization was not performed in this thesis. There was to some extent fungal growth during the growth period on the paper stripes that contained the seedlings. Moreover, more fungal growth was observed during the second replicate compared to the first one. Seedlings with visually abnormal growth were discarded, and in some cases, it could be suspected that the fungi had affected the seedlings. One might speculate if RootW to a higher extent can be affected by fungi compared to RootL, taking the differences in mean-values between replicate one and two into account for the traits (Table 2), as well as the significant replicate effect for RootW in contrast to RootL (Table 3). However, no studies confirming or dismissing this speculation have been found. Another consideration has been if the weighing process for the dry root weight could have been too imprecise, but since measures were taken to proceed in a uniform and precise manner with a sensitive scaler, it is unlikely to be the case. To confirm that the method of weighing was accurate enough, several genotypes were re-weighed to assure that the same values were obtained again. It might as well be that data from more than two replicates would have generated higher correlations and lower environmental effects.

Several other root characteristics have previously been phenotyped in studies on barley seedlings (Jia *et al*., 2019; Wang *et al*., 2017; Abdel-Ghani *et al*., 2019). While traits related to the spatial arrangement of roots, such as root spreading angle cannot be assessed using hydroponics, seminal root numbers could have been included by manually counting if time had been allowed. A previous study on spring barley, by Robinson *et al*. (2018), has shown a genetic relationship between seminal root number and yield, and in a study by Liu *et al*. (2013), seminal root number correlated with yield in wheat. If scanning and image processing techniques are utilized, as described in Jia *et al*. (2019), the hydroponic method used in this thesis could have been included screening for total seminal root length.

4.2 Phenotypic differences between genotypes

The summary statistics (Table 2) show higher mean values for both root traits (RootW and RootL) for the two-row panel compared to the mean values for the six-row panel. Notably, the number of QTLs identified in the two-row and six-row panel differed to a high extent, with two QTLs obtained in the two-row panel compared to 11 in the six-row panel (Table 5 in Appendix). The differences between the row-types in this thesis, considering early root growth as well as the number of detected QTLs, might be a result of the composition of the panels. While the two-row panel is mainly composed of modern breeding lines, the six-row panel includes several genotypes of historic cultivars and landraces as well. A trend was observed where the mean values of the traits generally were higher for the modern breeding lines compared to the mean values for the historic cultivars and landraces (data not shown). In contrast, no obvious trend could be detected while comparing the mean values when the genotypes were grouped after the country of origin. A theory considering the higher mean values identified for the modern lines compared to historic cultivars/landraces might be that it could be due to an indirect selection of the comprised early root growth traits in the Nordic breeding programs during recent years. Bertholdsson & Kolodinska Brantestam (2009) studied how seedling growth traits had changed in breeding material during one hundred years of barley breeding in Sweden and Denmark. They found a general declining trend for early root growth, where the decline in one of the studied traits, seminal root growth, coincided with a period of increases in artificial fertilizers usage. Although, this trend was reversed in Denmark during the later years of the studied period, which coincided with policies on restricted fertilizer inputs in agriculture. Early root growth traits, such as seminal root length and root dry weight have previously been shown to correlate with nitrogen uptake in barley (Bertholdsson & Kolodinska Brantestam, 2009; Karunarathne *et al*. 2015) It is known that a well-adapted root system is crucial for crops to maintain a high yield under nitrogen-limited conditions (Jia *et al*., 2019; Garnett *et al*., 2009).

4.3 The use of GWAS for QTL and candidate gene assessment

In total 23 QTLs associated with the two traits RootW and RootL were identified with the genome-wide association study. QTLs were identified on all seven chromosomes, with three of the QTLs on 1H, two on 2H, two on 3H, six on 4H, five on 5H, four on 6H, and one on 7H. In general, separate QTLs responded for the two traits, except QROOT1H1 and QROOT5H3 that were associated with both traits (see Table 5 in Appendix). The GWAS results showed some similarities with earlier reported root growth-related QTLs and candidate genes in barley located in the same region as QTLs obtained in this study.

In a study by Jia *et al*. (2019), 221 accessions (including cultivars, landraces, and breeding lines) of spring barley seedlings were grown in rhizoboxes under controlled greenhouse conditions, screened for various root system architectural traits, and assessed with GWAS. The two RootW-QTLs QROOT1H3 and QROOT4H2 in this thesis were found to be located less than 5 Mbp from QTLs in Jia *et al*. (2019) associated with traits such as "root system depth", "total seminal root length", and "root spreading angle" (Table 6 in Appendix). The RootL-QTL QROOT1H1 was found to be located 3.4 Mbp from a QTL associated with the trait "root spreading angle" (qRSA2) (Table 6 in Appendix). Abdel-Ghani *et al*. (2019) evaluated 233 spring barley genotypes of worldwide origin for root and shoot architecture traits. Seedlings were grown in paper rolls in a growth chamber, then phenotyped and assessed with GWAS. Three RootW-QTLs in this thesis, QROOT1H3, QROOT3H2, and QROOT4H2 were found to be located less than 1 Mbp from QTLs associated with several root traits in the study by Abdel-Ghani *et al*. (2019), such as "maximum root length", "total root volume", and "average root thickness" (Table 6 in Appendix). Two QTLs associated with RootL, QROOT4H3, and QROOT4H5 identified in the current study, were found to be located less than 0.5 Mbp from two QTLs (QTL-4H-7 and QTL-4H-8), associated with the traits "maximum root length" and "total root length" in Abdel-Ghani *et al*. (2019) (Table 6 in Appendix).

It was noted that several genes coding for proteins involved in various aspects of auxin, gibberellin, and ethylene signaling were listed when assessing candidate genes located +/- 1.5 Mbp from the significant markers obtained with GWAS. It is known that these phytohormones are involved in many aspects of plant development, including the regulation of root growth (Tanimoto *et al*., 2005; Muday *et al*., 2012). Two genes coding for gibberellin-regulated proteins, specified as "Gibberellin-regulated family protein" and "Gibberellin-regulated protein 1" in BARLEYMAP, were found in the vicinity of the RootW-QTLs QROOT1H3 and QROOT2H1, respectively (Table 7 in Appendix). The genes *ARF16* and *ARF2*, coding for auxin response factor proteins were found in the vicinity of the two RootL-QTLs QROOT4H1 and QROOT4H5, respectively (Table 7 in Appendix). Although, a more thorough analysis would be required to elucidate the possible roles of these genes for the phenotypic variation in root-growth obtained in this thesis.

In the study by Abel-Ghani *et al*. (2019), the gene HORVU6Hr1G076110 (with the alternative name *PIN7*), with the description "Auxin efflux carrier family protein" in BARLEYMAP, is highlighted as a promising candidate. This gene is located 0.46 Mbp from the RootW-QTL QROOT6H2 in this thesis and is co-located with a QTL in Abdel-Gani *et al*. (2019) associated with the traits "root dry weight" and "average root thickness" (Table 7 in Appendix). *PIN7* has been shown to encode auxin transporters that control radial root growth in *Arabidopsis* (Rosquete *et al*., 2018). Moreover, the gene *LBD14* is mentioned as a candidate gene for the trait "maximum root length" in Abdel-Ghani *et al*. (2019). This gene is located at 4.1 Mbp from the RootL-QTL QROOT4H3 in this thesis (Table 7 in Appendix). Abdel-Ghani *et al*. (2019) found that that *LBD14* showed high sequence similarity to genes known to affect root formation by regulating polar auxin transport, such as *ARL1* and *CRL1* in rice (Liu *et al*., 2005; Inukai *et al*., 2005), and *RTCS* in maize (Taramino *et al*., 2007). In a GWAS study by Karunarathne *et al*. (2020), who screened seedlings of 282 barley accessions, focusing on improved nitrogen use efficiency, the gene HORVU5Hr1G119650 with the description "Ethylene receptor" in BARLEYMAP is listed as a potential candidate gene. In their study, this gene is associated with the traits "relative root dry weight" as well as

“relative shoot dry weight”. It is located 0.12 Mbp from the RootL-QTL QROOT5H5 in this thesis (Table 7 in Appendix).

Overall, several QTLs and candidate genes are located close to QTLs for root growth in related GWAS studies. This might indicate that the hydroponic method used in this thesis can work well for screening seedling traits to elucidate the underlying genetics for early root growth in spring barley.

4.4 Conclusions

The root traits were successfully phenotyped using the low-tech hydroponic screening method, and in total 23 significant QTLs associated with the two traits (RootW and RootL) were identified in the GWAS. When comparing the result with other association studies comprising root traits in spring barley, several of the QTLs in this thesis were located close to QTLs located in these studies. Some of the proposed candidate genes in related studies, associated with RootW and RootL, were found to be located in the vicinity of obtained QTLs in this thesis. It was noted that several genes coding for proteins involved in various aspects of phytohormone signaling were listed when assessing candidate genes in BARLEYMAP. However, a more thorough analysis would be required to elucidate the possible roles of these genes for the phenotypic variation in root growth obtained in this thesis.

The hydroponic screening method proved to be useful for screening RootL of many genotypes in a short time, which is a trait that previously has been shown to correlate with nitrogen uptake and yield. This by itself could motivate its usefulness. Moreover, the simplicity of the method compared to other more high-tech solutions might make it useful in situations with a restricted budget, when the screening comprises many genotypes, and/or where modern screening systems are out of reach. Although, the statistical analyses for RootW regarding correlations between the replicates and the Analysis of Variance (ANOVA) were low, and a significant replicate effect was shown. This might well have been due to fungi growth or the low number of replicates, but the source of the variable results for RootW should nevertheless be attributed for the method to be useful for the trait. Another early root growth trait that could have been included with this method is “seminal root number”, which previously has been shown to correlate with yield. The number of roots could have been counted manually, but this would most likely be more time consuming than the two screened traits in this thesis, and hence not possible considering time restriction. The use of scanning and image processing techniques might be a more convenient alternative.

No obvious differences in early root growth were observed considering the country of origin of the genotypes. While comparing early root growth of historic cultivars/landraces and modern breeding lines, a trend was observed where the modern lines generally had higher values for RootW and RootL. These traits have been shown to correlate with nitrogen uptake. Sufficient nitrogen supply in turn is necessary to maintain high yields. In the light of policies restricting fertilizer use in the past years, it might be speculated if the breeding efforts in recent years have indirectly led to breeding for early root growth.

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Appendix

Table 5. Significant markers and QTL associated with RootW and RootL for the three panels (whole panel, two-row panel, and six-row panel). Blue color= RootW, Green color= RootL, Chr= Chromosome, MAF= minor allele frequency. Candidate genes within the same physical positions as the significant markers and descriptions for these have been obtained with the online tool BARLEYMAP, http://floresta.eead.csic.es/barleymap (Cantalapiedra et al., 2015).

Trait	SNP	Chr	Position	QTL	LOD	P.value	maf	Effect	Gene	Description
RootL_sixrow	JHI_Hv50k_2016_7035	1H	5768430	QROOT1H1	3.86	0.000	0.06	NA	HORVU1Hr1G002830	Chaperone protein DnaJ
RootW_wholepanel	JHI_Hv50k_2016_15705	1H	20485873	QROOT1H2	4.54	0.000	0.27	0.047	HORVU1Hr1G009240; HORVU1Hr1G009250	unknown function; receptor kinase 2
RootW_sixrow	JHI_Hv50k_2016_40077	1H	507587740	QROOT1H3	5.61	0.000	0.37	-0.067	HORVU1Hr1G074290	undescribed protein
RootL_sixrow	JHI_Hv50k_2016_60468	2H	2900843	QROOT2H1	3.94	0.000	0.13	NA	HORVU2Hr1G001370	unknown function
RootL_sixrow	JHI_Hv50k_2016_60479	2H	2956832	QROOT2H1	3.94	0.000	0.13	NA	HORVU2Hr1G001410; HORVU2Hr1G001420	Disease resistance protein RGA2; undescribed protein
RootL_sixrow	JHI_Hv50k_2016_60538	2H	2960326	QROOT2H1	4.09	0.000	0.11	NA	HORVU2Hr1G001410; HORVU2Hr1G001420	Disease resistance protein RGA2; undescribed protein
RootL_sixrow	JHI_Hv50k_2016_60539	2H	2960449	QROOT2H1	3.94	0.000	0.13	NA	HORVU2Hr1G001410; HORVU2Hr1G001420	Disease resistance protein RGA2; undescribed protein
RootL_sixrow	JHI_Hv50k_2016_60597	2H	3097234	QROOT2H1	3.85	0.000	0.11	NA	HORVU2Hr1G001450	Lipid A export ATP-binding/permease protein MsbA
RootL_sixrow	JHI_Hv50k_2016_60646	2H	3116063	QROOT2H1	3.85	0.000	0.11	NA	HORVU2Hr1G001480	undescribed protein
RootL_sixrow	JHI_Hv50k_2016_76569	2H	39982873	QROOT2H2	4.59	0.000	0.17	NA	HORVU2Hr1G017240	myb-like transcription factor family protein
RootW_wholepanel	JHI_Hv50k_2016_152480	3H	7210838	QROOT3H1	6.18	0.000	0.20	0.076	HORVU3Hr1G002780	fructose-bisphosphate aldolase 2
RootW_sixrow	JHI_Hv50k_2016_162361	3H	34943605	QROOT3H2	4.52	0.000	0.46	-0.052	HORVU3Hr1G015010; HORVU3Hr1G015020	Pentatricopeptide repeat-containing protein; undescribed protein
RootW_wholepanel	JHI_Hv50k_2016_162361	3H	34943605	QROOT3H2	9.20	0.000	0.26	-0.086	HORVU3Hr1G015010; HORVU3Hr1G015020	Pentatricopeptide repeat-containing protein; undescribed protein
RootL_wholepanel	JHI_Hv50k_2016_262784	4H	607801267	QROOT4H1	12.9	0	0.49	NA	HORVU4Hr1G078530	undescribed protein
RootW_wholepanel	JHI_Hv50k_2016_231199	4H	19086956	QROOT4H2	8.72	0.000	0.36	0.092	HORVU4Hr1G007260	unknown function
RootL_sixrow	JHI_Hv50k_2016_262883	4H	608057877	QROOT4H3	7.48	0.000	0.19	NA	HORVU4Hr1G078620	Calmodulin-binding transcription activator 2
RootL_sixrow	JHI_Hv50k_2016_263152	4H	610308780	QROOT4H3	12.86	0.000	0.41	NA	HORVU4Hr1G079030	Xyloglucan galactosyltransferase KATAMARI1 homolog
RootL_wholepanel	JHI_Hv50k_2016_263152	4H	610308780	QROOT4H3	9.07	0.000	0.27	NA	HORVU4Hr1G079030	Xyloglucan galactosyltransferase KATAMARI1 homolog
RootL_tworow	JHI_Hv50k_2016_265284	4H	619703876	QROOT4H4	17.57	0.000	0.07	NA	HORVU4Hr1G081570	zinc induced facilitator-like 1

RootL_wholepanel	SCRI_RS_148773	4H	625044073	QROOT4H5	4.13	0.000	0.19	NA	HORVU4Hr1G083340	Diacylglycerol kinase family protein
RootL_wholepanel	JHI_Hv50k_2016_267614	4H	625046820	QROOT4H5	3.96	0.000	0.29	NA	HORVU4Hr1G083340	Diacylglycerol kinase family protein
RootL_wholepanel	JHI_Hv50k_2016_267629	4H	625144615	QROOT4H5	3.93	0.000	0.18	NA	HORVU4Hr1G083360	PIN2/TERF1-interacting telomerase inhibitor 1
RootL_wholepanel	JHI_Hv50k_2016_267631	4H	625144885	QROOT4H5	4.15	0.000	0.20	NA	HORVU4Hr1G083360	PIN2/TERF1-interacting telomerase inhibitor 1
RootW_wholepanel	JHI_Hv50k_2016_275256	4H	643470107	QROOT4H6	3.82	0.000	0.22	-0.052	HORVU4Hr1G089740	unknown function
RootW_wholepanel	JHI_Hv50k_2016_278615	5H	4898519	QROOT5H1	4.79	0.000	0.33	-0.054	HORVU5Hr1G001730	Leucine-rich receptor-like protein kinase family protein
RootL_sixrow	SCRI_RS_161711	5H	466198616	QROOT5H2	4.30	0.000	0.09	NA	HORVU5Hr1G059690; HORVU5Hr1G059700	N-terminal protein myristoylation; undescribed protein
RootL_sixrow	JHI_Hv50k_2016_306021	5H	466198631	QROOT5H2	4.30	0.000	0.09	NA	HORVU5Hr1G059690; HORVU5Hr1G059700	N-terminal protein myristoylation; undescribed protein
RootL_wholepanel	BOPA2_12_10674	5H	529427076	QROOT5H3	3.94	0.000	0.05	NA	HORVU5Hr1G070560	unknown protein; Has 361 Blast hits to 333 proteins in 92 species: Archae - 2; Bacteria - 55; Metazoa - 145; Fungi - 36; Plants - 42; Viruses - 19; Other Eukaryotes - 62 (source: NCBI BLink)
RootW_sixrow	JHI_Hv50k_2016_311632	5H	526808592	QROOT5H3	4.28	0.000	0.24	-0.054	HORVU5Hr1G070050	Blue copper protein
RootW_sixrow	JHI_Hv50k_2016_313451	5H	535197408	QROOT5H4	4.62	0	0.30	-0.055	HORVU5Hr1G072260	Vacuolar protein sorting-associated protein 52 A
RootL_tworow	JHI_Hv50k_2016_360315	5H	656514953	QROOT5H5	8.86	0.000	0.10	NA	HORVU5Hr1G119700	prohibitin 1
RootW_wholepanel	JHI_Hv50k_2016_384496	6H	40418298	QROOT6H1	5.00	0.000	0.50	-0.054	HORVU6Hr1G017220	P-loop containing nucleoside triphosphate hydrolases superfamily protein
RootW_wholepanel	SCRI_RS_4653	6H	524529284	QROOT6H2	3.88	0.000	0.19	-0.041	HORVU6Hr1G076190	oxoprolinase 1
RootW_sixrow	JHI_Hv50k_2016_423202	6H	559840153	QROOT6H3	4.17	0.000	0.23	0.071	HORVU6Hr1G085720	Protein kinase superfamily protein
RootW_sixrow	JHI_Hv50k_2016_428046	6H	572429583	QROOT6H4	4.50	0.000	0.07	0.088	HORVU6Hr1G087780	Methyltransferase WBSCR22
RootW_wholepanel	BOPA1_2585_2901	7H	47582498	QROOT7H1	4.81	0	0.47	0.053	-	-

Table 6. QTLs associated with RootW (blue) and RootL (green) in this thesis within a range of +- 5 Mbp of early root growth-related QTLs in related studies. The physical positions of significant markers have been obtained with the online tool BARLEYMAP, http://floresta.eead.csic.es/barleymap (Cantalapiedra et al., 2015).

QTL in thesis	Chr	Position	SNP in thesis	Mentioned in	SNP in study	QTL in study	Distance (bp)	Associated with
QROOT1H1	1H	5768430	JHI_Hv50k_2016_7035	Jia et al. (2019)	BOPA2_12_30950	qRSA2	3 392 774	"root spreading angle"
QROOT1H3	1H	507587740	JHI_Hv50k_2016_40077	Jia et al. (2019) Abdel-Ghani et al. (2019)	SCRI_RS_213675 BOPA1_2935-1634 SCRI_RS_197910	qTSRL1 qASRL1 QTL-1H-6	4 138 592 4 336 433 978 025	"total seminal root length" "average seminal root length" "average root thickness"
QROOT3H2	3H	34943605	JHI_Hv50k_2016_162361	Abdel-Ghani et al. (2019)	BOPA2_12_10968	QTL-3H-1	16 127	"total root volume"
QROOT4H2	4H	19086956	JHI_Hv50k_2016_231199	Jia et al. (2019) Abdel-Ghani et al. (2019)	BOPA1_4616-503 BOPA1_3687-271 BOPA1_4616-503	qRSD7 qTSRL6 qASRL4 QTL-4H-3	843 133 1 170 419 1 170 419 843 133	"root system depth" "total seminal root length" "average seminal root length" "maximum root length" & "total root volume"
QROOT4H3	4H	608057877- 610308780	JHI_Hv50k_2016_262883 JHI_Hv50k_2016_263152	Abdel-Ghani et al. (2019)	SCRI_RS_168399 SCRI_RS_25685	QTL-4H-7 QTL-4H-7	374 131 3 560 190	"maximum root length" "total root length" (under stress cond.) & "maximum root length" (under stress cond.)
QROOT4H5	4H	625044073	SCRI_RS_148773	Abdel-Ghani et al. (2019)	SCRI_RS_160461	QTL-4H-8	479 058	"maximum root length"

Table 7. Phytohormone-related genes within a range of +- 1.5 Mbp of QTLs associated with RootW (blue) and RootL (green), as well as candidate genes mentioned in similar studies within a range of +- 5 Mbp of QTLs associated with the traits. The physical positions of significant markers, candidate genes, and descriptions for these have been obtained with the online tool BARLEYMAP, http://floresta.eead.csic.es/barleymap (Cantalapiedra et al., 2015).

QTL	Chr	Position	Gene	Description	Closest SNP	Distance (bp)	Mentioned in
QROOT1H3	1H	507587740	HORVU1Hr1G074530 HORVU1Hr1G074580	gibberellin-regulated family protein gibberellin-regulated family protein	JHI_Hv50k_2016_40077 JHI_Hv50k_2016_40077	695 443 800 167	- -
QROOT2H1	2H	2900843-3116063	HORVU2Hr1G001540	gibberellin-regulated protein 1	JHI_Hv50k_2016_60646	122 182	-
QROOT4H1	4H	607801267	HORVU4Hr1G078490 (*ARF16)*	*auxin response factor 16*	JHI_Hv50k_2016_262784	99 508	-
QROOT4H3	4H	608057877-610308780	HORVU4Hr1G080160 (*LBD14*)	LOB domain-containing protein 14	JHI_Hv50k_2016_263152	4 101 608	Abdel-Ghani et al. (2019), associated with “maximum root length”
QROOT4H5	4H	625044073-625144885	HORVU4Hr1G083690 (*ARF2)*	*auxin response factor 2*	JHI_Hv50k_2016_267631	1 254 129	-
QROOT5H5	5H	656514953	HORVU5Hr1G119650	ethylene receptor	JHI_Hv50k_2016_360315	121 217	Karunarathne et al. (2020), associated with “relative root dry weight” and “relative shoot dry weight”
QROOT6H2	6H	524529284	HORVU6Hr1G076110 (*PIN7)*	auxin efflux carrier family protein	SCRI_RS_4653	445 447	Abdel-Ghani et al. (2019), associated with “root dry weight” and “average root thickness.