Norwegian University of Life Sciences
Faculty of Biosciences
Department of Plant Sciences

Philosophiae Doctor (PhD)
Thesis 2018:50

Phenotypic and genetic studies of waterlogging tolerance in wheat and barley

Fenotypiske og genetiske studier av vannmetningstoleranse i hvete og bygg

Tove Kristina Sundgren

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Moss, June 2018

Tove

SUMMARY

Stress caused by waterlogging may have considerable impact on yields of wheat, barley and other crops. As climate change projections include increased precipitation in parts of the world, one can expect an increasing demand for wheat and barley varieties that are better adapted to temporary waterlogging. Research has shown that genotypes of wheat and barley tolerate waterlogging differently. Still, the progress in developing waterlogging tolerant lines has yet been limited.

This thesis includes three separate studies. The overall aim has been to provide new insights that may contribute to closure of the knowledge gap related to waterlogging tolerance in wheat and barley. In paper I, the waterlogging tolerance of one wheat and one barley population was investigated in field trials. The populations displayed genetic variation for waterlogging tolerance, whereby six wheat and five barley genotypes were identified as more tolerant. Six genotypes, three sensitive and three tolerant, were selected from the wheat population for a subsequent greenhouse study (paper II). By monitoring the root and shoot growth prior to, during and after a waterlogging treatment, we could show that tolerant genotypes were characterized by developing seminal roots faster in the seedling phase and more nodal roots during the treatment. Our results also indicate that a small relative root stele size is beneficial for waterlogging tolerance. In paper III, we identified sixteen QTL on chromosome 1B, 3B, 5BL, 6AL and 7A. *QTL6A.2* was highly significant for foliar chlorosis and was determined to be the most important in the study.

The studies presented in this thesis highlight two main areas that are relevant to investigate further: 1) the potential of early vigor and root stele size as traits that may improve tolerance, and 2) genomic regions, particularly *QTL6A.2*, that are responsive to waterlogging stress. Furthermore, the importance of conducting experiments under conditions that are relevant to the target environment is emphasized and discussed.

SAMMENDRAG

Stress forårsaket av vannmetning kan føre til betydelige avlingstap i hvete, bygg og andre vekster. Det forventes at klimaendringene vil gi økt nedbør i visse områder i verden, og dette kan medføre et økt behov for hvete- og byggsorter som er mer tolerante ovenfor midlertidig vannmetning. Forskning har vist at sorter av hvete og bygg tolererer vannmetning ulikt. Til tross for omfattende forskningsaktivitet har likevel fremgangen ved å utvikle mer tolerante sorter vært begrenset.

Denne avhandlingen består av tre separate studier der den overordnede målsetningen har vært å bidra med økt kunnskap om vannmetningstoleranse i hvete og bygg. I artikkel I ble vannmetningstoleransen hos en hvete- og en byggpopulasjon undersøkt i feltforsøk. Populasjonene viste genetisk variasjon for vannmetningstoleranse, hvorved seks hvete- og fem bygglinjer ble identifisert som mer tolerante. Seks linjer, tre sensitive og tre tolerante, ble valgt ut fra hvetepopulasjonen til et etterfølgende veksthusforsøk (artikkel II). Ved å følge tilveksten av skudd og røtter gjennom periodene før, under og etter vannmetningsbehandlingen, kunne vi vise at tolerante linjer hadde tidlig frøplanteutvikling («early vigor») av frørøtter i etableringsfasen, og de utviklet flere kronrøtter i vannmetningsfasen. Våre resultater indikerer også at det er en sammenheng mellom en smalere sentralsylinder i røttene og økt vannmetningstoleranse. I artikkel III identifiserte vi 16 QTL på kromosomene 1B, 3B, 5BL, 6AL og 7A. *QTL6A.2* var svært signifikant for klorose på bladverket og denne QTL ble fremhevet som det viktigste funnet i denne studien.

Studiene som presenteres i avhandlingen fremhever to hovedområder som er relevante for videre undersøkelser: 1) potensialet for tidlig frøplanteutvikling og størrelsen av røttenes sentralsylinder som egenskaper som kan forbedre toleranse, og 2) genomiske områder, spesielt det for *QTL6A.2*, som responderer på vannmetningsstress. Resultatene viser også at feltforsøk for å screene vannmetningstoleranse må utføres under forhold som er relevante for det miljøet som plantene skal dyrkes i. Dette blir vektlagt og diskutert i avhandlingen.

LIST OF PAPERS

I **Field screening of waterlogging tolerance in spring wheat and spring barley**

Sundgren, T.K., Uhlen, A.K., Waalen, W., Lillemo, M.

Agronomy, 2018, 8(4), 38; DOI: 10.3390/agronomy8040038

II **Rapid seedling establishment and a narrow root stele promotes waterlogging tolerance in spring wheat**

Sundgren, T.K., Uhlen, A.K., Lillemo, M., Briese, C., Wojciechowski, T.

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III **A potential QTL for oxygen sensing detected in wheat subjected to waterlogging stress**

Sundgren, T.K., Belova, T., Uhlen, A.K., Lillemo, M. (Manuscript)

1. INTRODUCTION

The world population is expected to reach almost 10 billion by 2050 (FAO, 2017). To meet the demands for food and biofuels of the growing population, crop yields will need to at least double (Ray et al., 2013). Per annum, Fischer et al. (2014) predicts that yields of wheat, rice and soybean need to increase by 1.2-1.3%. Concurrently, endeavors to reach this target are constantly being challenged by the impacts of global warming and climate change (Mickelbart et al., 2015). When the climate change impact is taken into account, the required yield increase may be closer to 1.7% (Reynolds et al., 2016). This is in contrast to the annual yield increment of 1.0% between 1991 and 2010 (Fischer et al., 2014).

Extreme weather events and abiotic stress caused by drought, heat and waterlogging limit growth and crop yields considerably. Water scarcity and heat stress are the most critical aspects to address in order to prevent large scale yield decline and to meet predicted yield requirements (Bita & Gerats, 2013, Fahad et al., 2017). A paradox of global warming however is the simultaneous increase of precipitation that is expected in certain parts of the world. In Europe, the mean annual precipitation is likely to decrease, but modelling also indicates that severe flooding events are likely to become more frequent (Christensen & Christensen, 2003). Southern parts of Europe will likely suffer more from drought, while precipitation increase is more probable in the Northern region (Trenberth, 2011, IPCC, 2007). As observed, the flooding frequency and total annual precipitation has already increased in many regions over the past century (Rosenzweig et al., 2002, Barua et al., 2014, Parry et al., 2007, Pedersen et al., 2017).

In Norway, a country where precipitation patterns vary considerably between geographical regions, projections indicate an increase of 7-23% in annual precipitation by 2100, relative to the period of 1961 to 1990 (Hanssen-Bauer et al., 2009). This is in addition to the increase that has already been observed (Fig. 1A). Cereal grains are predominantly produced in the South-East of Norway, a region in which spring precipitation (March through May) is more likely to increase and summer precipitation (June through August) to remain relatively constant (Fig. 1B). Additionally, the number of days with heavy rainfall events is expected to rise and flooding patterns to change. Unchanged summer precipitation in combination with a higher temperature suggests that drought events will not be an unlikely scenario.

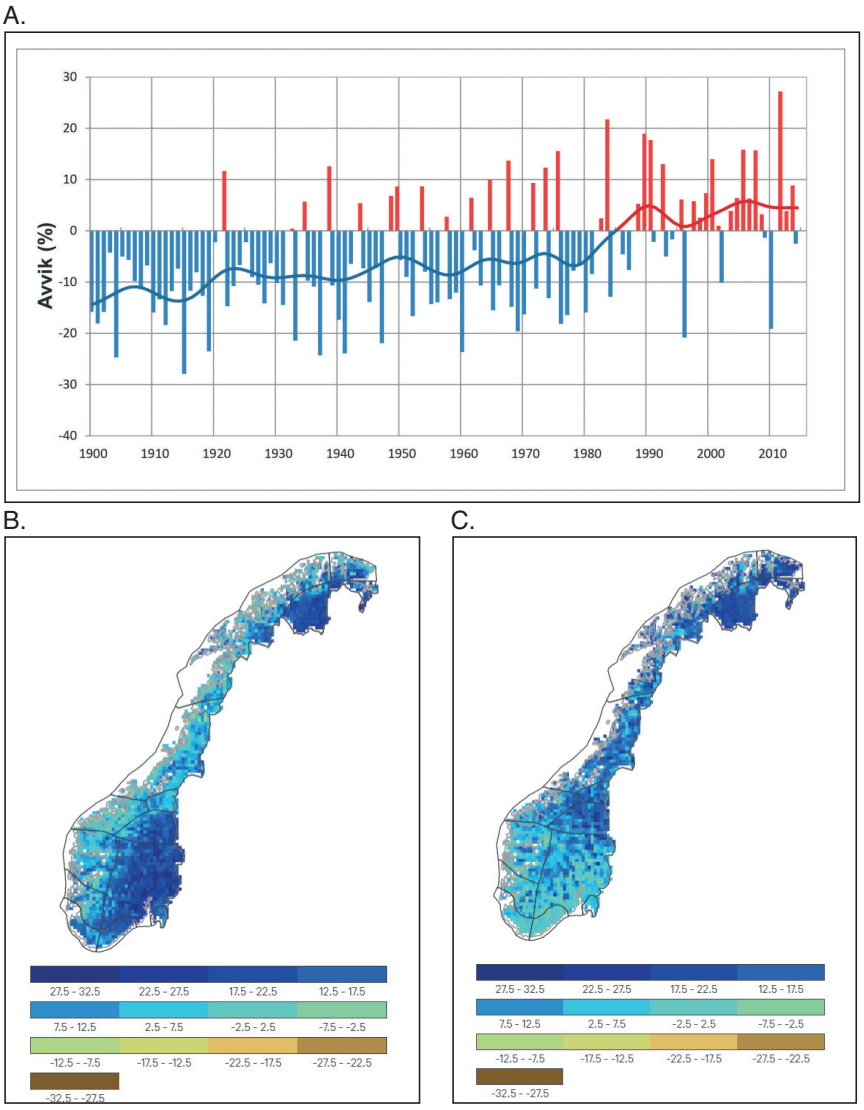

Figure 1. (**A**) The relative deviation of precipitation from 1900 to 2014 compared to the mean precipitation during the normal period of 1971-2000. Relative change (%) in precipitation in Norway between the period of 1971-2000 to 2071-2100 for March through May (**B**) and June through August (**C**) according to the RCP4.5 model. Figure A and B were generated and obtained at the Norwegian Centre for Climate Services (NCCS) and figure C from http://www.miljodirektoratet.no/Documents/publikasjoner/M406/M406.pdf.

1.1 Waterlogging stress

Waterlogging stress occurs in soils when the air in pore spaces are replaced with water. Precipitation, floods and improper irrigation typically initiate waterlogging, whereas the severity of the event depends on several circumstances. Factors that affect the severity can broadly be separated into categories related to:

1. Physical and chemical properties of the soil, particularly soil texture and structure (Saqib et al., 2004), drainage capacity and chemical composition (Ponnamperuma, 1972, Khabaz-Saberi et al., 2006).

2. Crop related factors, such as species, genotype and developmental stage (Setter & Waters, 2003).

3. The waterlogging event itself and concurrent climatic conditions, e.g. duration (Marti et al., 2015), temperature (Trought & Drew, 1982), and whether the crop is submerged or not, either partly or fully (Jackson & Colmer, 2005).

Multiple scenarios can be outlined within each category and combining them creates a very large number of possible growth environments. Waterlogged environments are fundamentally complex and that complicates generalizations of their potential impact on crop yields. When exposed at sensitive developmental stages, wheat and barley may suffer considerably. As in the study by de San Celedonio et al. (2014), waterlogging around anthesis caused yield loss of 79 and 92% compared to drained controls for barley and wheat, respectively. Annual yield losses on a global scale have not been well quantified, but in the mid 90's, it was estimated that 10-15 million hectares of the global wheat growing area were annually subjected to waterlogging (Sayre et al., 1994). As the frequency of floods has increased for every decade on all continents since the 1950's (Pedersen et al., 2017), it is likely that this area has increased since then. With progressed global warming, this area will likely expand further in the years to come (Reynolds et al., 2016).

1.2 Anaerobic soil conditions

Oxygen deprivation is the root cause of waterlogging stress in plants. Restricted oxygen availability does not only affect the plants, but also the microorganisms of the soil and the soil chemistry (Ponnamperuma, 1972). As a soil saturates with water, the bulk of oxygen is physically displaced to the atmosphere, while fractions may be trapped within soil aggregates. Once the

remaining resources are emptied, microorganisms capable of facultative and anaerobic respiration utilize oxygen from other compounds in their respiration (Fiedler et al., 2007). In a preferred order, oxygen in NO_3^-, MnO_2, $Fe(OH)_3$, SO_4^{2-}, and CO_2 serves as electron acceptors in the electron transport chain (Marschner, 2011). Reduction of NO_3^-, commonly referred to as denitrification, diminishes nitrogen resources that would otherwise be available to the plants. Subsequently, reduction of manganese and iron oxides discharges manganese and iron in to plant available forms. Abundance of these minerals may lead to toxic concentrations of plant available Mn^{2+} and Fe^{2+} (Khabaz-Saberi et al., 2010). Besides chemical reduction of these compounds, certain plant nutrients, including NO^{3-}, SO_4^{2-} and K^+ (Alfaro et al., 2004), are predisposed to leaching and may be lost to deeper and inaccessible soil layers or to water streams. In summary, both toxic and deficient conditions may arise, much depending on the waterlogging duration. The slow diffusion rate of gases through water impedes resupply of oxygen to the soil, but also leads to accumulation of gases that are generated therein. One such gas is ethylene, a plant hormone that plays a key role in several plant adaptive responses.

1.3 Plant response under anaerobic conditions

Ethylene is involved in the formation of adventitious roots (Sasidharan & Voesenek, 2015), development of aerenchyma (Yamauchi et al., 2014) and regulation of submergence tolerance as well as internode elongation in rice (Bailey-Serres et al., 2010, Hattori et al., 2009). These traits represent known tolerance mechanisms in cereals that have been studied quite thoroughly in the past. Internal aeration through aerenchymatous nodal roots is often highlighted as one of the most important tolerance traits (Herzog et al., 2016, Setter & Waters, 2003). Aerenchyma forms constitutively in rice and many wetland species (McDonald et al., 2002), while it may be induced by ethylene and reactive oxygen species (Sasidharan & Voesenek, 2015, Yamauchi et al., 2014) in wheat (Xu et al., 2013, Thomson et al., 1990) and barley (Zhang et al., 2016, Pang et al., 2004). Aerenchyma is clearly beneficial for waterlogging tolerance (Thomson et al., 1992, Huang et al., 1994b) and genotypes of barley vary significantly for this trait (Broughton et al., 2015, Zhang et al., 2016). Genetic diversity in wheat is not as extensively documented, but the variation is likely similar to the variation in barley. The majority of previous studies investigating aerenchyma or other tolerance mechanisms have been conducted as greenhouse experiments with seedlings or young plants grown in nutrient solution. Most of the studies were limited to observations during the treatment phase, or immediately after the treatment ended (Striker, 2012). Given the many

factors that influence the outcome of a waterlogging event, these studies confine our current comprehension of waterlogging tolerance in cereals to a quite limited number of circumstances. Seemingly, aerenchyma appears to have limited capacity to form and to convey oxygen. Roots that are longer than 200 mm when waterlogging occurs do not seem to form aerenchyma (Thomson et al., 1990, Huang et al., 1997). Furthermore, in the event that aerenchyma has formed, conveying oxygen to the root apex is limited in roots longer than 100 mm (Thomson et al., 1990). Nodal roots emerge concurrently with tiller formation. In previous studies, waterlogging has often been imposed around this stage. As commonly found, aerenchyma improves waterlogging tolerance around this stage. Considering findings made by Thomson et al. (1990), this is likely due to the fact that nodal roots often emerge during the treatment and have seldom exceeded the length that may be incapable of conveying oxygen. At anthesis, the stage that de San Celedonio et al. (2014) identified as the most vulnerable for yield loss, roots are likely longer than 100 mm. If aerenchyma cannot be formed in roots longer than that, other traits, yet to be specified, may be important contributors to waterlogging tolerance.

Plant tissue in an anaerobic environment convert to anaerobic respiration to sustain ATP production (Ricard et al., 1994). An energy crisis shortly ensues due to the much lower number of ATP produced per unit glucose (Gibbs & Greenway, 2003). The consequences of the energy shortage is manifold. Of major importance is the impairment of the energy requiring H^+-ATPase proton pumps at the plasma membrane (Shabala et al., 2014). Inhibition of H^+-ATPases leads to depolarization of the membrane, which subsequently cause a net influx of H^+ and a net efflux of K^+ from the cytosol (Greenway & Gibbs, 2003, Zeng et al., 2014). Consequently, cytosolic pH declines and uptake of nutrients is reduced (Sze et al., 1999, Sondergaard et al., 2004). Avoiding K^+ loss and cytosolic acidification by maintaining membrane potential is vital to prevent cell damage. Evidently, oxygen supply from shoots to anaerobically exposed roots enables maintenance of membrane potential and K^+ retention in the cytosol, and thereby promoting waterlogging tolerance in barley (Zeng et al., 2014). Concurrent with K^+ efflux, Ca^{2+} spikes and the presence of reactive oxygen species (ROS) increase (Schmidt et al., 2018). The implications of these bio-chemical alterations are not entirely clear but Ca^{2+} and ROS are in addition to ethylene and cytosolic acidification, known to be involved in signaling pathways for induction of low oxygen adaptation (Sasidharan & Voesenek, 2015, Wang et al., 2017, Shabala et al., 2014).

1.4 Breeding for waterlogging tolerance

Low oxygen availability has a comprehensive physiological impact on plants. While roots are directly exposed, the shoots typically reflect the stress that the roots are suffering from. It has been well documented that waterlogging tolerance differs among genotypes of wheat (Gardner & Flood, 1993, McDonald et al., 2006, Musgrave & Ding, 1998, Van Ginkel et al., 1992) and barley (Bertholdsson, 2013, Setter et al., 1999, Pang et al., 2004). A number of wheat lines were identified as having superior waterlogging tolerance in early screening work conducted by the International Maize and Wheat Improvement Center (CIMMYT) (Van Ginkel et al., 1992). Later on, four synthetic hexaploid wheat lines were released by the Wheat Wide Crosses Program of CIMMYT (Villareal et al., 2001). When Khabaz-Saberi et al. (2006) tested a number of lines with reputed waterlogging tolerance, including the waterlogging tolerant CIMMYT line Ducula-4, they found inconsistent ranking of the lines when they were waterlogged in different types of soil. Apparently, Ducula-4 was one of the most sensitive lines when tested in soils in Australia and India (Setter et al., 2009). The authors firmly concluded that waterlogging screening trials need to be conducted in soil from the target environment. Similarly, McDonald et al. (2006) found contrasting waterlogging tolerance of 17 wheat varieties when screened at different locations in Western Australia. The strong genotype x environment interaction that these studies clearly illustrates, adds to the challenge of breeding for waterlogging tolerance in wheat and barley. The set of traits required to tolerate waterlogging in one environment, might be less beneficial in another. As found by Sayre et al. (1994), genotypes of wheat may also perform differently depending on the current growth stage when waterlogging occurs. Clearly, study designs needs to be tailored to the conditions and scenarios that are likely for the target environment.

To date, the progress in breeding for waterlogging tolerant wheat and barley has been limited. To my awareness, no lines with documented tolerance have yet been released for commercial production. This is in contrast to rice, in which the discovery of the submergence tolerance QTL *SUB1 (Xu et al., 2006)* enabled release of lines that can survive complete submergence for up to two weeks (Septiningsih et al., 2008). *SUB1* accounted for 69% of the variation in submergence tolerance and was mapped to rice chromosome 9 by Xu & Mackill (1996). Through marker-assisted back-crossing, *SUB1* was eventually introgressed into modern varieties (Bailey-Serres et al., 2010) that are today grown by 4 million farmers in Asia (Ismail et al., 2013). Indeed, three decades passed in between the discovery of *SUB1* until tolerant lines were made available to

farmers (Bailey-Serres et al., 2010). Still, it proves that persistent work and targeted breeding for abiotic stress is achievable, and is therefore an encouraging example for further advances in wheat, barley and other crops. Given the successes made in rice and the current scientific understanding of waterlogging tolerance in wheat and barley, it appears suitable that scientists direct their efforts to continue the unravelling of tolerance mechanisms and traits, as well as the QTL and genes that control them. This has been the premises and framework of the studies included in this thesis and that are presented hereafter.

2. THE THESIS

2.1 Background, aim and objectives

To date, the objectives in Norwegian cereal breeding have primarily been to improve yield, quality parameters and disease resistance. Waterlogging stress tolerance has not yet been prioritized, but the projected precipitation increase in the coming century suggests a demand for varieties that are better adapted to a more unpredictable and wetter climate.

With this thesis, I aim to provide breeders, the scientific community and farmers with new insights into waterlogging tolerance in wheat and barley, primarily under Norwegian conditions. As outlined below, the thesis includes three separate studies and objectives that relate to phenotyping and genetic diversity (paper I), tolerance traits that confer waterlogging tolerance (paper II) and identification of QTL associated with waterlogging stress in wheat (paper III).

The waterlogging tolerance of genotypes central to Norwegian wheat and barley breeding was completely unknown when the project that this thesis originates from was initiated. An essential first step and objective was therefore to document the waterlogging tolerance of two screening populations, one of wheat and one of barley. To rank the genotypes rightfully, we focused especially on phenotyping and subsequent data analysis. The aim was to identify the most appropriate trait(s) for genotype ranking and the results from this work are presented in paper I. Another objective of the study was to obtain phenotypic data for a subsequent genome-wide association study (GWAS) in wheat (paper III).

Breeding for specific tolerance traits may be particularly effective to quickly gain genetic improvement. Before such breeding efforts can be made, the trait(s) in question need(s) to be defined. A second objective of this thesis was therefore to identify shoot and particularly root traits that may contribute to waterlogging tolerance, by studying sensitive and tolerant genotypes prior to, during and after a controlled waterlogging treatment (paper II).

Crop improvement through modern breeding technologies rely on the identification of significant QTL. Burgos et al. (2001), Ballesteros et al. (2015), Yu & Chen (2013) and Yu et al. (2014) have reported QTL for waterlogging tolerance across the wheat genome. Still, much of the genetics behind the trait is still poorly understood. A third objective of this thesis was therefore to identify significant QTL for waterlogging stress in the field (paper III). The study complements the ones

mentioned above, as it was carried out as a GWAS, as opposed to a linkage mapping study, and with phenotypic data obtained in the field and not in a greenhouse.

3. MATERIALS AND METHODS

3.1 Plant material

The studies in the thesis were conducted with one wheat and one barley population. The populations included varieties, breeding lines, crossing parents, landraces and other genotypes with historic importance in Norwegian wheat and barley breeding. A majority of the lines were of Norwegian or Nordic origin. Several wheat lines originated from CIMMYT, whereas a few of them had known waterlogging tolerance properties. A detailed description of the genotypes can be found in paper I. Six genotypes were selected from the wheat population for the experiments described in paper II.

3.2 Field screening of wheat and barley (paper I)

In 2013 and 2014, we screened the barley and wheat populations in hillplot field experiments (Fig. 2A-D). A controlled waterlogging treatment was imposed at the three-leaf stage. Visual scores of foliar chlorosis and the overall condition around maturation was recorded in both experimental years. In 2014, the genotypes were scored for the ability to recover growth of green biomass. The heading date, number of spikes and plant height of the waterlogged plots were measured and compared with drained controls. To determine the overall waterlogging tolerance of the genotypes, principal component analyses (PCA) were carried out with Best Linear Unbiased Predictors (BLUPs) as input variables. The genotypes were further ranked according to their principal component (PC) 1 scores.

To assess the relationship between phenotypic traits and yield response, we conducted field experiments with larger plots and a subset of wheat genotypes in 2015 and 2016. The subsets included genotypes that were either sensitive or tolerant in the two previous years. In addition to yield measurements, the genotypes were scored for the same traits as in 2013 and 2014. The extent to which phenotypic traits and PC1 scores explained yield response was analyzed in regression models.

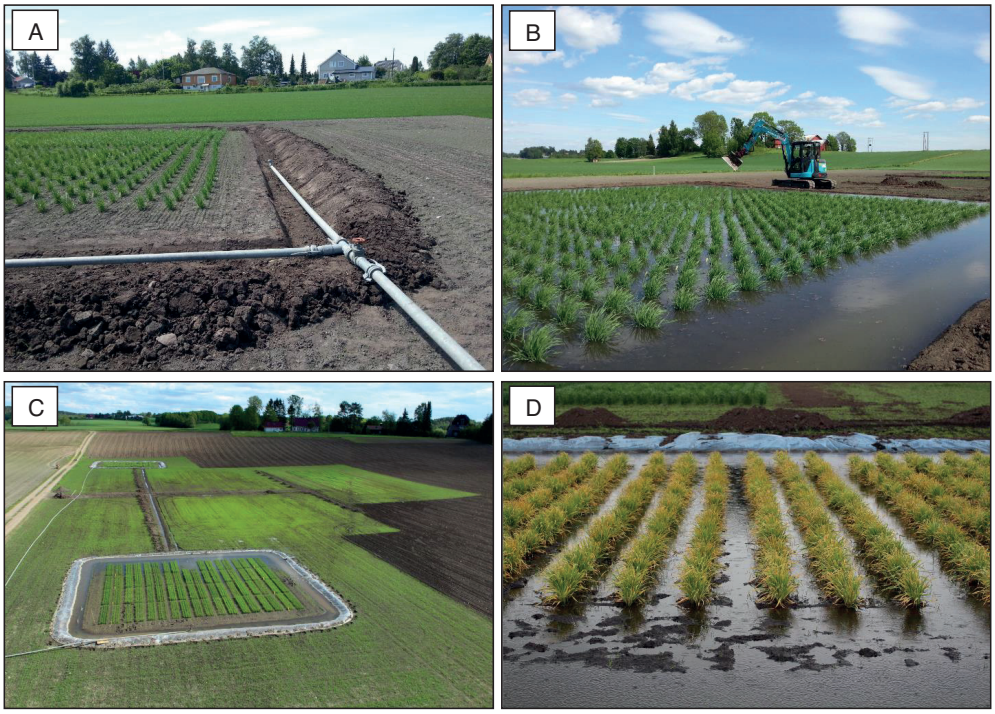

Figure 2. (**A**) The experiments were waterlogged using irrigation pipes, (**B**) with an excavator, trenches and levees were established around the experiments, (**C**) an overview of the experimental site; location N (north) in the forefront and S (south) in the back, (**D**) part of the 2014 hillplot experiment with barley.

3.3 Growth and root anatomy of wheat genotypes in response to waterlogging (paper II)

Six genotypes, three sensitive and three tolerant were selected for two greenhouse experiments (Fig. 3). The experiments were conducted using rhizoboxes (Fig. 4A) and photography for data acquisition (Fig. 4C). In experiment one, root growth of the genotypes was studied during seedling establishment (Fig. 4B) and a subsequent waterlogging treatment, starting at the three-leaf stage and maintained for seven days. In the second experiment, root and shoot growth of previously waterlogged plants was compared between the genotypes during seven days of recovery. At harvest of experiment two (Fig. 4E), root segments were sampled to investigate genotype differences of root cross sectional area, root cortex area, stele area and percentage of aerenchyma. The root anatomical traits were determined by measuring the size of the traits in microscopy images (Fig. 5).

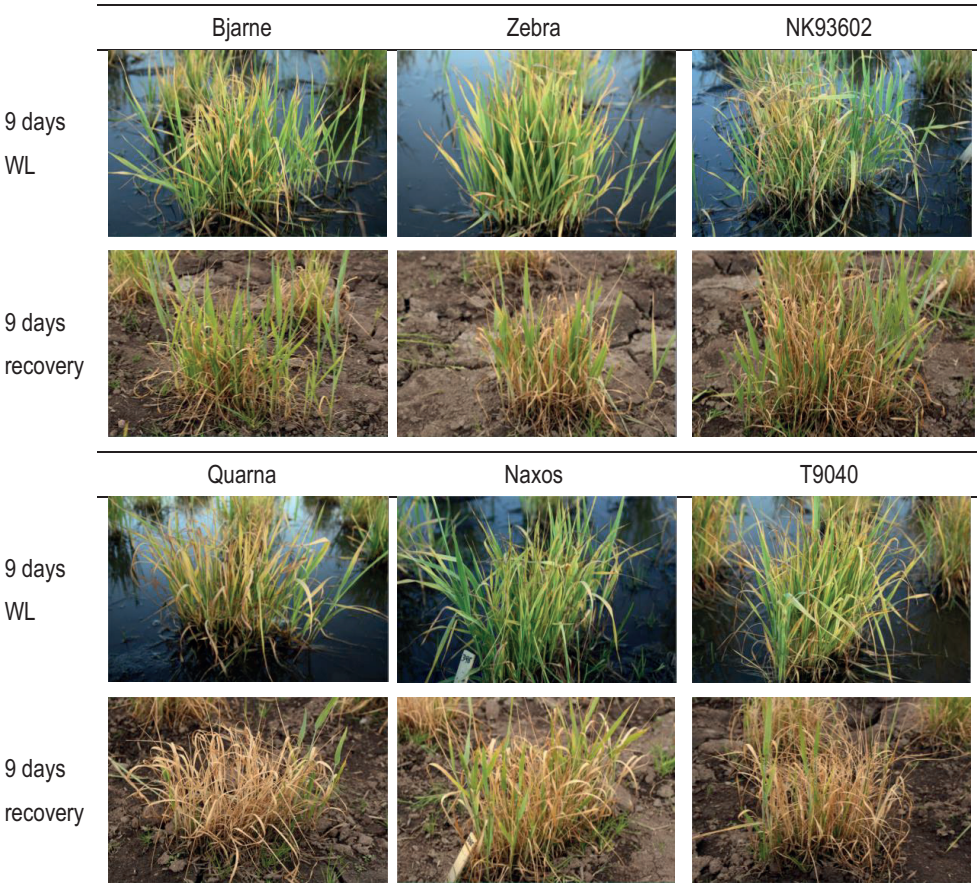

Figure 3. Genotypes selected for the root study presented in paper II. The pictures show the condition of tolerant Bjarne, Zebra and NK93602, and sensitive Quarna, Naxos and T9040 at 9 days of waterlogging (WL) and at 9 days after draining the experiments. Note that plants of sensitive and tolerant genotypes were similarly chlorotic but had contrasting abilities to recover.

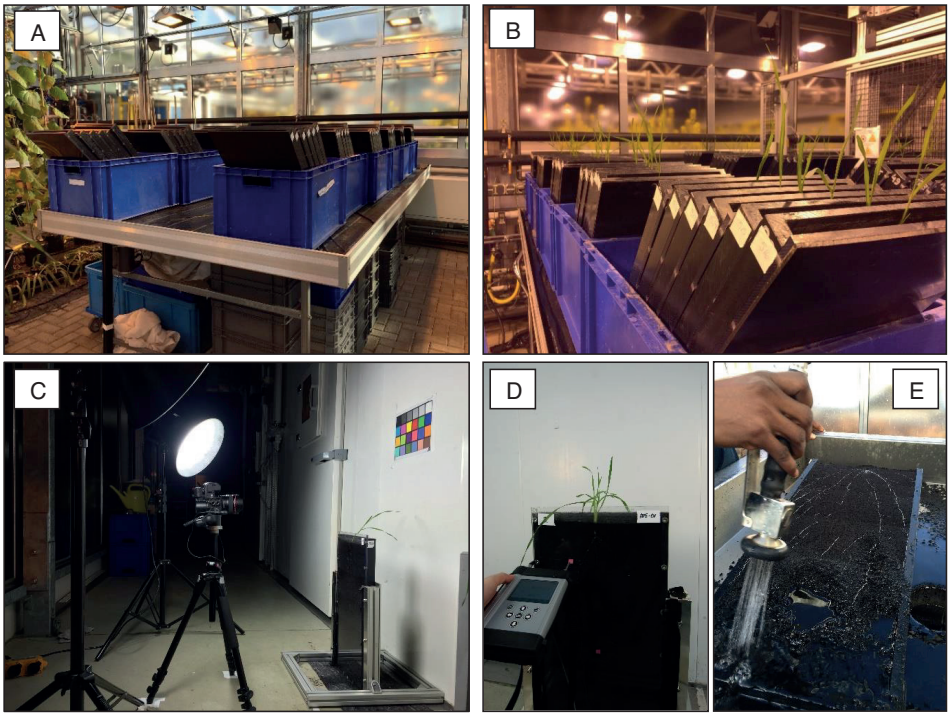

Figure 4. (**A**) the experimental setup of the experiment, (**B**) seedlings growing in the rhizoboxes, (**C**) the photo station setup for photography of the shoots, (**D**) monitoring of oxygen concentration with a fiber optic oxygen transmitter (Fibox 4, PreSens Precisions Sensing, GmbH, Regensburg, Germany), (**E**) cleaning of roots for subsequent sampling.

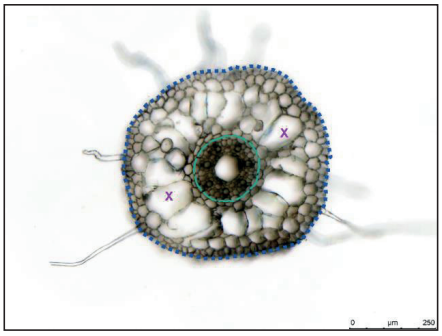

Figure 5. Microscopy image of a transverse section of a NK93602 seminal root. Blue outer dotted line show the circumference of the cross section, green inner dashed line show the circumference of the stele and purple crosses are examples of aerenchyma. The cortex area was calculated as the difference between the area of the whole cross section and the stele area. The percentage of aerenchyma was calculated as the aerenchyma area divided by the cortex area. The image was acquired from a sample at the middle zone of the longest seminal root of this particular plant.

3.4 Genome-wide association study of waterlogging stress in wheat (paper III)

Genetic associations were determined between the phenotypic traits recorded in paper I and SNP markers of the Affymetrix 35K and Illumina iSelect 90K SNP arrays. With Basic Local Alignment Search Tool (BLAST), sequences of significant SNP markers were aligned along the wheat pseudo-chromosome sequences. Assigning the markers with physical positions of the pseudo-chromosomes allowed us to compare markers from the two SNP arrays and to define QTL containing markers from both of them. A QTL was defined when a region in which a minimum of five significant markers were positioned within approximately 10 Mbp distance. A haplotype analysis was performed for one QTL on chromosome 6AL. The QTL included markers with large effects that were determined to be significant in both experimental years. The estimated genotype mean of chlorosis for the haplotypes was calculated and analyzed in simple regression models. The Welch two-sample t-tests were applied to determine the difference in chlorosis between haplotypes.

4. MAIN RESULTS AND DISCUSSION

4.1 Genetic diversity and phenotypic traits

An essential aim and challenge in this study was to identify the most influential and relevant traits for genotype ranking of waterlogging tolerance in the field. As described in paper I, there is no clear consensus as to which traits most accurately describe waterlogging tolerance in cereals. Often, foliar chlorosis or chlorophyll content are used as stress indicators in screenings similar to ours (Van Ginkel et al., 1992). Yield is another common measurement, but has the disadvantage of being confounded by other factors and having low heritability. The hillplot trials in the first two years (2013 and 2014) enabled screening of a large number of genotypes, but the plots were too small to harvest for reliable yield measurements. The waterlogging treatment had a significant effect on all recorded traits and the populations displayed a diversity for them all. To not limit the analyses and genotype ranking to one or a few traits, we applied PCA and ranked the genotypes according to their PC1 score. PCA captures the largest variance from all input variables in the first principal component. Depending on the size of the variance, the input variables contribute to PC1 in a descending order. The phenotypic trait with the largest variance, thereby also representing the greatest phenotypic variation, is the main contributor to PC1. With this statistical approach, we could rank the genotypes based on all affected traits in a weighted manner according to the

variances. When comparing the genotypes' PC1 scores in 2013 and 2014, we found that six wheat and five barley genotypes performed consistently well and were therefore classified as tolerant. Among the tolerant wheat genotypes was CETA/*Ae.tauschii* (895), a CIMMYT line that has been identified as tolerant in previous waterlogging experiments conducted in Mexico. Considering that waterlogging tolerance is strongly affected by the environment, this synthetic line may be a promising candidate for in-depth investigations of genetic and physiologic properties.

The hillplots were exchanged with larger plots but fewer genotypes in 2015 and 2016. Larger plots allowed us to harvest yield and assess the relationship between PC1 scores and individual phenotypic traits with wheat yield response. Our results show that the extent to which PC1 and other phenotypic traits explained yield depended much on the experimental location. The experiments were established on two different locations about 100 meters apart (Fig. 3C). An infiltration test and general observations showed that the infiltration rate of the soil and stress severity differed considerably between the locations. When wheat was tested at the location with low infiltration (in 2013, 2014 and 2015), PC1 scores were strongly predictive of the relative yield (as determined in 2015). Of individual traits, chlorosis percentage explained most of the variation (R^2_{adj}= 0.87 at p<0.001). In contrast, PC1 was a statistically insignificant explanatory variable for relative yield in 2016 when wheat was tested under less severe stress at the location with high infiltration. The overall condition score and percentage of chlorosis were determined as the best yield predictors in this year. Besides PC1 scores, chlorosis and the overall condition score were also considered the most informative of all individual traits in the study. The results from the study also demonstrates the influence of the soil properties, and emphasize the importance of conducting waterlogging experiments under conditions relevant to the target environment.

4.2 QTL for waterlogging stress response

BLUPs of individual traits, in addition to PC1-3 scores obtained from the wheat hillplot experiments in 2013 and 2014 were used in a genome-wide association study (paper III). In the study, we identified significant markers for all recorded traits and PC's. Sixteen QTL were identified on chromosomes 1B, 3B, 5BL, 6AL and 7A. Eight markers: three on 1B, four on 6AL and one on 7A were significant in both experimental years (Table 1). These were significant for principal components, relative plant height and in particular chlorosis and the overall condition

score. The markers on 6AL were positioned within a QTL (*QTL6A.2*) that contained fifteen markers. Fourteen of them were significant for chlorosis in 2013, 2014 or in both years. A few markers within the QTL were of the most significant ones in the whole study. The 35K marker *AX-95092538* (Table 1) was of particular interest. The genomic region of the marker is predicted to be associated with a prolyl 4-hydroxylase (P4H) alpha subunit gene. Intriguingly, P4H is an oxygen-dependent enzyme that plays an important role in oxygen sensing in animals (Jaakkola et al., 2001). In addition to oxygen, it requires Fe^{2+} and ascorbate as co-factors, and 2-oxoglutarate as co-substrate (Kivirikko & Myllyharju, 1998). The role of P4H in plants is not yet fully understood, but studies suggest that it is involved in gene expression related to waterlogging (Asif et al., 2009, Vlad et al., 2007, Zou et al., 2011).

Table 1. SNP markers that were significant in 2013 and 2014.

Chromosome	SNP array	Marker	Associated traits
1B	90K 90K 35K	BobWhite_c2844_569 BS00039135_51 AX-94413240	Chlorosis and overall condition score (2013, 2014)
6AL	35K 35K 35K 35K	AX-95182345 AX-95153895 AX-94634087 AX-95092538	Chlorosis (2013, 2014),PC 2 (2014), PC 3 (2014)
7A	35K	AX-95629211	Relative plant height (2013), heading delay (2014), PC 1 (2014).

In addition to the sixteen identified QTL, two minor regions associated with heading delay and PC2 scores in 2013 were identified on chromosome 1B and 3B, respectively. These were determined to be in close proximity to two markers previously identified by Ballesteros et al. (2015). BLAST searches of a marker located in the region on 3B showed that this SNP is predicted to encode proteins involved in the biological process of proteolysis and to initiate methionine removal. Interestingly, proteolysis through the N-End rule pathway plays a key role in oxygen signaling in plants and may be an important breeding target (Gibbs et al., 2011, Licausi et al., 2011, Mendiondo et al., 2016). Whether the QTL and markers that were reported in paper III are related to P4H and the N-End rule pathway remains purely speculative at this point. Further investigations are necessary to confirm the significance of the genomic regions and potential candidate genes therein. The proximity of the region on chromosome 3B to the marker reported by Ballesteros et al. (2015), supports a true association with waterlogging stress. To our awareness, the region

covered by *QTL6A.2* has not been identified in previous waterlogging studies. Still, several markers within the QTL were highly significant for chlorosis and the QTL was clearly the most distinct region identified in this study. Several of the markers were solely associated with chlorosis recorded in 2014, likely due to the higher stress intensity compared to 2013. Despite the stress being less severe in 2013, several markers were associated with chlorosis in that year. This indicates that the region is highly receptive, even under less severe waterlogging stress.

The fact that several of the most significant markers in the study were associated with chlorosis supports the conclusion that chlorosis was one of the most important phenotypic traits in the screening trials. In the haplotype analysis conducted in the GWAS, we found that lines of haplotype group 1 had a significantly higher percentage of chlorosis in 2014. Still, the overall condition score indicated that these lines recovered well. This underlines the importance of considering the plants condition during a post-treatment period. This conclusion was also drawn in paper I, and the results from the GWAS supports the conclusion further.

4.3 Tolerance traits

In the greenhouse experiment (paper II), where we investigated the root and shoot properties of three sensitive and three tolerant genotypes, we found that tolerant, in contrast to sensitive genotypes, established faster in the seedling stage. Most notable was the rapid seminal root growth. Total seminal root length increased by 5.9 and 6.9 cm day^{-1} for tolerant Bjarne and NK93602, respectively. For sensitive genotypes, the daily increase was limited to 4.0, 3.8 and 2.6 cm for Naxos, Quarna and T9040, respectively. When the treatment began, Bjarne and NK93602 had developed 3.5 and 4.3 leaves, respectively. The leaf number of Quarna (2.7) and T9040 (2.8) was lower but not significantly different from Bjarne and NK93602. Still, the leaf number seemed to correlate well with the seminal root length, which was in fact significantly different. This finding brought attention to the potential advantage of early vigor for waterlogging tolerance. As tolerance typically increases with higher growth stages (Setter & Waters, 2003), it is possible that genotypes that were determined to be tolerant in the screening trials, also established faster upon sowing. Considering the diversity of the screening populations, it is likely that the developmental stage varied. Monitoring the exact developmental stage is much simpler in a smaller greenhouse experiments compared to a field experiment where hundreds of genotypes are investigated. A rational approach for large field experiments would be to determine the plant size or coverage

using image analysis. Determining the leaf area or soil coverage in 2D images is affordable and straight-forward, but might fail to detect subtle differences. Alternatively, 3D imaging using light detecting and ranging (LIDAR) would enable plant height estimates and measurement of plant architecture (Li et al., 2014). Cheaper alternatives, albeit with lower resolution are ultrasonic sensors or stereo vision (Deery et al., 2014, Li et al., 2014)

Another highlighted result from the greenhouse experiments was the genotypes' variation in relative stele size in nodal and seminal roots. The statistical significance of the stele was stronger than for aerenchyma percentage, a trait that was expected to differ more between sensitive and tolerant genotypes. The tolerant genotypes Bjarne and NK93602 had a significantly narrower stele in seminal roots compared to other genotypes. The relative stele size in nodal roots of Bjarne was also distinctively smaller.

Stele size in wheat roots is a fairly unexplored trait. The potential benefit of a narrower stele for waterlogging tolerance, or if there is a genetic variation of the trait within wheat remains theoretical at this point. While aerenchyma facilitates oxygen diffusion, a narrow stele may contribute to a lower oxygen demand in the root (Armstrong & Beckett, 1987). A few studies have indicated that the stele may decrease in size upon waterlogging (Pang et al., 2004, Huang et al., 1994a, McDonald et al., 2002). In the study by Pang et al. (2004), the decrease was also larger for a tolerant barley variety than for a sensitive variety. A proportionally narrow stele is also a feature of certain wetland species (McDonald et al., 2002). Of anatomical root traits, aerenchyma has often received the most attention in previous waterlogging studies. Results found in paper II suggest that the stele may be an interesting trait to investigate next. The stele might be particularly interesting in seminal roots, as the benefit of aerenchyma is primarily associated with nodal roots. Previous studies indicate that it may be an adaptive trait, and Kondo et al. (2000) found that there is a genetic variation for stele size in rice. Interestingly, Bjarne and NK93602 were both characterized by having a rapid seedling establishment and a narrow seminal root stele. A waterlogging study where the stele size and seminal root elongation are investigated in a larger number of genotypes at early developmental stages could confirm whether there is a correlation between the traits. Such a study should preferably also include a recovery phase and measurements of root respiration rates.

4.4 Challenges in studying waterlogging tolerance in the field

Much of the data presented in this thesis was obtained in the field experiments between 2013 and 2014. This is a strength as the plants are exposed to weather conditions, pathogens and other possible stressors just as they are in a farmer's field. Conducting controlled waterlogging experiments in the field is however demanding. Firstly, the area needs to be as flat as possible to ensure an even water table level within the experiment. Here, we used a levelling instrument to locate the most appropriate areas. Still, even a few centimeters difference in height affects the plants and it is important to conduct the experiment with a suitable design, such as the alpha lattice design. Secondly, we found that the stress needed to be severe in order to differentiate the genotypes using visual scoring methods. Severe stress also leads to major yield losses and may be too large for the data to be reliable. In 2015 and 2016, we conducted experiments with harvestable plots of both wheat and barley but the barley data from 2016 was discarded because the yield loss was indeed too large. The barley data from 2015 was discarded because temporary waterlogging around germination damaged the experiments. Barley is more sensitive to waterlogging than wheat and extra attention needs to be paid to the treatment duration.

4.5 Future perspectives in phenotyping and screening

It has been emphasized in this thesis and by other authors that screening work should be conducted under relevant conditions, in target environments and for full crop cycles. A dilemma with this notion however, is the labor and costs involved in the execution of such experiments. In this context, high-throughput phenotyping and the yet emerging research field of phenomics may potentially aid in gaining not only efficiency in phenotyping but also a wealth of phenotypic data. Considering the comprehensive physiological impact that waterlogging has, it is likely that several imaging techniques, e.g. chlorophyll fluorescence imaging, thermal imaging and hyperspectral reflectance imaging (Chaerle & Van Der Straeten, 2000) could be indicative of waterlogging stress. To date, it appears that few have attempted to employ remote sensing technology in waterlogging field studies, whereas the work by Arguello et al. (2016) seems to be the only published exception. With the spectral reflectance index "Normalized Difference Vegetation Index" (NDVI), Arguello et al. (2016) could predict wheat grain yield (R^2=0.77) and biomass (R^2=0.64) of waterlogged plants, but not of control plants. These measurements were made at the termination of 14 and 28 days long waterlogging treatments. In fact, we also assessed NDVI obtained from hyper- and multispectral images of the wheat experiments in 2015 and 2016. NDVI

measured after three days of stress in 2015 and after five days in 2016 failed to predict yield in our experiments. NDVI is known to be strongly correlated with the leaf area index (LAI, the proportion of an area covered by leaves) and the architecture of plants, e.g. the erectness of leaves (Darvishzadeh et al., 2008). The genotypes in our experiments differed quite much in their architecture as well as their ground coverage and this may have interfered with the measurements.

Another relevant approach is to monitor the photosynthetic activity through chlorophyll fluorescence (Rungrat et al., 2016). Both Bertholdsson (2013) and Pang et al. (2004) used it to characterize the waterlogging tolerance of barley genotypes. However, quantum yield, the main parameter to quantify chlorophyll fluorescence, requires a period of darkness prior to the measurements (Rungrat et al., 2016). Hence, it is primarily suitable for greenhouse experiments and not for the field. Thermal, multi- and hyperspectral cameras are better suited for the field and can also be mounted on unmanned vehicles. In preparatory investigations and measurements of our field experiments, waterlogged as opposed to drained controls, could easily be distinguished in thermal and multispectral images (K Kusnierek, 2018, pers. comm.). The challenge however, lies in differentiating between genotypes.

The ideal phenotypic trait for characterizing genotypes is one which can detect differences in stress already after a few days. That would first of all enable shorter and often more realistic treatment durations. If the trait also predicts recovery or even yield, experiments could be shortened additionally, as a full crop cycle would then be unnecessary. Hypothetically, pots could then replace field experiments and the costs could be cut even more. Phenotypic traits measured in a traditional manner, e.g. chlorosis scoring, are evidently too imprecise to fulfill these requirements. Considering the almost countless number of parameters that can be measured with cameras and sensors, high-throughput phenotyping and new methods of analyzing data, holds at least some promises that a better phenotyping pipeline for waterlogging tolerance can emerge.

5. CONCLUSIONS

Studies included in this thesis have contributed to expand the scientific understanding of waterlogging stress and tolerance in wheat and barley. With these studies, we have documented the genetic diversity in waterlogging tolerance of a wheat and a barley population (paper I), singled out significant QTL for waterlogging stress (paper III), as well as identified traits that may contribute to waterlogging tolerance in wheat (paper II).

With the exception of the genotypes that were included in paper II, it is unknown how and to which extent certain root traits influenced the performance of the genotypes in our field experiments. Genotypes that were less chlorotic might have possessed one set of traits, while other traits might have influenced those that recovered well. Rapid seedling establishment appeared beneficial and I believe that traits related to early vigor and anatomical traits of seminal roots warrant attention in future studies where waterlogging is imposed at vegetative stages. It should be noted that even slight variations in biomass, leaf number or root length might matter for the ability to withstand waterlogging. This is particularly important to consider when the aim is to rank genotypes, or in physiological studies were several genotypes are included. For a complete understanding of waterlogging tolerance, future studies ought to consider not only plant responses during a waterlogging event. Results presented in this thesis clearly show that plant recovery and the traits that are present up to the treatment is imposed, are also crucial aspects to consider. The reappearance of oxygen upon drainage may involve elevated levels of ROS (Blokhina et al., 2003). Sensitivity to, and/or scavenging of such compounds may be interesting aspects to study for understanding plant recovery. For upcoming studies, the CIMMYT line CETA/*Ae.tauschii* (895) is suitable as a tolerant check. For studies relating to Norwegian conditions or for farmers who wish to choose a more waterlogging resistant variety, Bjarne, Zebra and Mirakel are three good alternatives. Farmers are also advised to establish their crops early and properly, independent of which variety they grow. This will likely increase the crops ability to survive a period of transient waterlogging.

Regardless of the tolerance trait a genotype may employ, morphological and/or physiological adaptations rely on the detection of prevailing oxygen conditions in the surrounding environment. The complete picture of how plants sense oxygen is still not entirely clear. Several signaling pathways have been identified and it is currently believed that multiple sensing mechanisms are

involved (Schmidt et al., 2018). For further advances in improving waterlogging tolerance in cereals, it is important to decipher the biochemistry and genetics involved in oxygen sensing. In paper III, we identified QTL that represent genomic regions that may be involved in such processes. In particular, the region of *QTL6A.2* appeared especially significant. The exact role of this QTL is unknown but our results clearly indicate that the region should be investigated further.

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Field Screening of Waterlogging Tolerance in Spring Wheat and Spring Barley

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Abstract: Improved waterlogging tolerance of wheat and barley varieties may alleviate yield constraints caused by heavy or long-lasting precipitation. The waterlogging tolerance of 181 wheat and 210 barley genotypes was investigated in field trials between 2013 and 2014. A subset of wheat genotypes were selected for yield trials in 2015 and 2016. Our aim was to: (1) characterize the waterlogging tolerance of genotypes with importance for Norwegian wheat and barley breeding, and (2) identify which phenotypic traits that most accurately determine the waterlogging tolerance of wheat in our field trials. Waterlogging tolerance was determined by principal component analysis (PCA) where best linear unbiased predictors (BLUPs) of the traits chlorosis, relative plant height, heading delay, relative spike number, relative biomass and an overall condition score were used as input variables. Six wheat and five barley genotypes were identified as consistently more tolerant in 2013 and 2014. This included the waterlogging tolerant CIMMYT line CETA/*Ae. tauschii* (895). Chlorosis and the overall condition score were the traits that best explained the yield response of the genotypes selected for the yield trials. Our results show that early stress symptoms did not necessarily reflect the ability to recover post treatment. Thus, records from full crop cycles appear as fundamental when screening populations with unknown tolerance properties.

Keywords: wheat; barley; waterlogging tolerance; phenotyping

1. Introduction

Heavy precipitation, floods, poorly drained soil or improper irrigation management may cause waterlogging and substantial yield loss of wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*). The worldwide land area subjected to waterlogging has been estimated to 10–15 million hectares per year [1]. Furthermore, climate changes are likely to include a higher risk of floods and increased precipitation in parts of the world [2–4]. Meeting yield requirements of a growing population suggests a demand for wheat and barley varieties better adapted to temporary waterlogging and oxygen deficiency.

During a waterlogging event, water displaces air from the pore spaces in the soil. Soil microorganisms and plant roots respire the remaining oxygen and the reservoirs may be rapidly emptied. Until the soil is drained, the supply will be insufficient for plant root respiration. As the oxygen diffusion rate through water is 10^4-fold slower than through air [5], the absence of oxygen in the soil has immediate impact on plant cell respiration, inhibiting adenosine triphosphate (ATP) synthesis through the oxidative phosphorylation pathway [6]. Plant cells thus convert to anaerobic respiration to sustain vital cell functions. Ethanolic fermentation, the main anaerobic pathway in

plants [7], is far less efficient in synthesizing ATP than oxidative phosphorylation. Hence, the shift from aerobic to anaerobic respiration results in energy shortage [8]. This may inhibit biosynthetic processes such as nutrient uptake [9–11] and photosynthesis [12,13]. Chemical alterations that arise in anaerobic soils may cause additional stress to plants. For instance, the reduction of iron and manganese to plant available Mn^{2+} and Fe^{2+} may reach toxic levels and are especially harmful in acidic soil where they co-occur with aluminum [14,15].

Waterlogging may cause substantial yield loss. Genotypes of both wheat [1,16–18] and barley [19,20] may tolerate waterlogging differently. Setter et al. [20] found that grain yield loss varied from 18 to 81% and from 16 to 49% among sixteen wheat and eight barley genotypes, respectively. Yield loss has been attributed to nearly all yield components: the number of tillers and spikes [21,22], grains per spike [23,24], spikelets per spike and floret formations per spikelet [25], as well as the grain weight [26]. In an outdoor pot study, Marti et al. [27] registered a wheat yield loss equivalent to 175 kg ha^{-1} day^{-1} when waterlogging occurred in the stem elongation period. The yield penalty depends on factors such as the duration of the waterlogging event [27], the developmental stage at the onset [1,28,29] and growth conditions, e.g., temperature [30] and soil properties [21,31]. These environmental factors may cause strong GxE interactions and genotypes may perform inconsistently across different environments [15,17].

Waterlogging may have profound impact on both root and shoot traits. Stress symptoms related to the shoots include ceased leaf extension and/or reduced biomass accumulation [32,33], foliar chlorosis or reduced chlorophyll content, as well as leaf senescence [10,22,24,31]. In addition to these symptoms and the yield components previously mentioned, the plant development, e.g., the date of heading may be delayed [21,24]. A variety of phenotyping approaches have been used to determine waterlogging tolerance in both field and greenhouse experiments. Van Ginkel et al. [18] found that visual scores of foliar chlorosis correlated strongly with yield obtained under waterlogged conditions. Setter et al. [20] on the other hand, reported that actual grain yield was a better selection criterion in Western Australia. In a study by Collaku and Harrison [22], tiller number counted at maturity was the most affected trait. Musgrave and Ding [16] found that the mineral content of roots (sum of Fe, Mn and P) and a visual score of root color correlated with yield under waterlogged conditions. In the assessment by Arguello et al. [34], normalized difference vegetation index (NDVI) was the best yield predictor of both biomass and grain yield. Zhou [35] defined waterlogging tolerance as a combined score of chlorosis and survival to determine the tolerance of 177 double haploid barley lines derived from the cross between the sensitive variety "Franklin" and tolerant "Yerong". Ballesteros et al. [36] calculated an index based on the proportion of shoot or root biomass produced under waterlogged relative to control conditions. Pang et al. [31] recommended chlorophyll fluorescence (Fv/Fm) for tolerance determination of larger populations. However, the dark adaptation requirement for chlorophyll fluorescence measurements makes it less suitable for field screenings. In summary, there appears to be little agreement as to which traits most accurately determine waterlogging tolerance. Although high grain yield is the ultimate goal, as a selection criterion it has low heritability [34,37] and is confounded by other factors. One or a few traits, which clearly differentiate tolerant from sensitive genotypes across different environments, have not been defined. The apparent complexity of the trait, dependence on environmental factors and the comprehensive stress response one may observe are perhaps underlying reasons. This suggests that screening work ought to be concentrated to target environments and under relevant circumstances. It also suggests that the most relevant phenotypic traits may depend on the growth conditions under consideration.

In this paper, we present results from a four-year field screening trial of spring wheat and a two-year field screening trial of spring barley for waterlogging tolerance. The objectives of our research have been to: (1) document waterlogging tolerance of wheat and barley genotypes with unknown tolerance properties, and (2) identify which phenotypic traits most accurately determine the waterlogging tolerance of wheat in our field trials.

2. Materials and Methods

2.1. Experimental Site and Growth Environment

The experiments were conducted at Vollebekk Research Farm (coordinate 59.7° N, 10.8° E) of the Norwegian University of Life Sciences, Aas, Norway. The experimental site was autumn plowed and spring harrowed in all seasons except for 2015 when both plowing and harrowing were done in the spring. Compound fertilizer containing 132 kg N, 18 kg P, 60 kg K ha^{-1} was applied prior to seeding. Sowing occurred in late April or early May or June (Table 1). The weather conditions differed slightly during the years (Table S1).

Table 1. Sowing date, treatment duration and location of the site as well as daily average temperature (°C) during the treatment in the experimental years. Abbreviations: N: northern location; S: southern location.

Year	Sowing Date	Treatment Duration (Days) and Location		Daily Average Air Temperature (°C)
		Barley	Wheat	
2013	06.06.2013	10 N	10 N	14
2014	02.05.2014	13 S	7 N	16
2015	29.04.2015	-	13 N	13
2016	27.04.2016	-	21 S	15

The waterlogging experiments were placed on two different areas of the site (N and S), with north-south orientation, approximately 100 m apart. In 2013, the wheat and barley experiments were located in the northern area of the field (N). In 2014 and 2015, the wheat experiments were also placed in the northern area. The placement of wheat was changed to the south (S) in 2016. The barley experiment was placed in the south in 2014.

2.2. Plant Material

The experiments included 210 genotypes of spring barley and 181 spring wheat genotypes (Tables S2 and S3). The genotype collections included advanced breeding lines, cultivars, landraces and various crossing parents. The majority of wheat genotypes were of Norwegian origin (47%), of Swedish origin (12%), and from CIMMYT (24%). A number of CIMMYT genotypes, previously found to be waterlogging tolerant were included in the experiments [1,38,39]. PRL/SARA, VEE/MYNA, Ducula-1, Ducula-2, Ducula-3, Ducula-4, ALTAR 84/*Ae. tauschii* (221), 68.111/RGB-U//WARD/3/*Ae. tauschii* (454) and CETA/*Ae. tauschii* (895) were included in 2014, while Dulus, BOTNO/*Ae. tauschii* (617) and DVERD_2/*Ae. tauschii* (221) were included in both 2013 and 2014.

The barley collection was also dominated by Norwegian genotypes (58%, Table S2). Other major sources were Sweden (15%), Denmark and Finland (13% in total). The variety Henni was included especially as it performed well in the study by Bertholdsson [19].

The genotypes were screened in hillplots in 2013 and 2014. Based on results from those years, a subset of wheat genotypes (Table 2) were selected for the 2015 and 2016 experiments. Several aspects were considered when selecting genotypes. Our aim was to select genotypes that were either more tolerant or more sensitive. Other factors that influenced our selection was related to their suitability for combine harvest, relatedness (closely related genotypes were avoided) and that good quality seed was available.

Table 2. Wheat genotypes selected for the experiments conducted in 2015 and 2016.

Wheat Genotype	Origin	Expected Tolerance Property	Testing Year
Bjarne	Norway	Tolerant	2015–2016
Chara	Australia	Sensitive	2015
Dulus	CIMMYT	Tolerant	2015–2016
Breeding line 1303	Norway	Sensitive	2016
Breeding line 1327	Sweden	Sensitive	2016
Breeding line 1406	Norway	Tolerant	2016
Breeding line 1416	Norway	Sensitive	2016
Kariega	South Africa	Sensitive	2015–2016
Kukri	Australia	Tolerant	2015–2016
Mirakel	Norway	Tolerant	2015–2016
NK00521	Norway	Sensitive	2015–2016
NK93602	Norway	Tolerant	2015–2016
NK93604	Norway	Tolerant	2016
Quarna	Switzerland	Sensitive	2015–2016
Saar	CIMMYT	Sensitive	2015
T9040	Norway	Sensitive	2015–2016
Zebra	Sweden	Tolerant	2015–2016

2.3. Experimental Design and Treatment Procedure

The experiments were conducted as randomized alpha lattice designs with three and four replicates in 2013–2014 and 2015–2016, respectively. In the first two years, the experiments were carried out in hillplots with 4 gm. of seed in each plot. In the latter two years, the plot size was scaled up to 0.75 × 3 m to allow for yield measurements. Seeding rate was 50 and 70 gm. per plot in 2015 and 2016, respectively. Waterlogged and non-waterlogged control treatments were drill seeded in two separate but adjacent experiments.

An excavator was used to create levees and trenches surrounding the waterlogging experiments. The levees were reinforced with durable plastic. At the 3-leaf stage, water was pumped in to the experiments using irrigation pipes. By maintaining a gentle water flow throughout the treatment period, the water level was kept relatively constant just above the soil surface. The treatment lasted until the plants showed considerable stress symptoms and genotypic differences were easily distinguishable. The treatment duration differed between the years (Table 1).

2.4. Soil Characteristics and Measurements

The soil on the experimental site is classified as a mollic gleysol [40], a soil type that is characterized by being ground-water affected and poorly drained.

To monitor the soil redox potential, platinum electrodes (Ag/AgCl reference (HI 3032) Hanna Instruments, Inc., Woonsocket, RI, USA) connected to a portable pH/millivolt-meter (HI 8424, Hanna Instruments, Inc., Woonsocket, RI, USA) were used. Measurements were made directly in the waterlogged soil at 4–5 cm depth. The redox potential on control plots was not measured, as the electrodes cannot measure in a solid medium. Measurements were taken three and eight days into the waterlogging treatment in 2014, after nine days in 2015 and after three and five days in 2016. The average and standard deviation in millivolt (Eh) was calculated based on a minimum of six measurements.

To quantify the infiltration rate on the waterlogging fields at location N and S, a single ring infiltrometer test with falling head conditions was conducted in the spring of 2017. Plastic rings, 20 cm in length and 9.8 cm in diameter, were hammered down to 5 cm depth directly on the plough pan, approximately 23 cm below the soil surface. The rings were filled with water and the height of the water was measured with 10-min intervals for 30 min in total. Four measurements were made per site. The rings were refilled with water after each reading. Water was also poured in the hole surrounding

the rings to ensure vertical flow within the ring. The infiltration rate in mm h^{-1} was calculated as the difference in water height between 0 and 10 min, divided by the number of minutes.

Soil samples for chemical and physical analysis were taken in the top soil layer in 2014. The soil bulk density and soil porosity (%) in the 4–9 cm layer were determined by using 100 cm^3 steel cylinders. Samples for chemical analysis and soil texture were taken in the 0–15 cm layer. Contents of phosphorus, potassium, calcium and magnesium were determined by ammonium lactate extraction and inductively coupled plasma optical emission spectrometry. A sedimentation analysis with the pipette method was used to determine the soil particle size distribution in the samples. pH was measured in deionized water.

2.5. Measurements and Phenotypic Registrations

Traits clearly affected by the treatment that could be quantified by visual scoring or simple measures were recorded. Not all traits were recorded each year due to slight variations in the expressed stress response. Traits recorded in all years include the percentage foliar chlorosis on plot basis, the date of heading, plant height, and an overall condition score. The percentage of chlorosis was recorded when genotypic variation could be clearly observed. Heading date was determined when 50% of the heads in each plot had fully emerged. The heading delay was calculated as the difference between control and waterlogged plots. The plant height was measured in centimeters from the base of the culm to the first spike node. The number of spikes were counted directly in the hillplots in 2014. In 2015 and 2016, samples from a 50 × 50 cm area (2 rows) were harvested at maturation. These samples were used for the determination of spike number and straw yield. The overall condition score was based on the plants condition around maturation, and included a consideration of spike size, biomass, and the overall vigor. A combined score, on a 1–10 scale with 1 being the poorest, was given. The overall condition score was intended to indicate the yield potential of the genotypes, similar to the agronomic score given by Van Ginkel et al. [18]. In 2013, excessive precipitation caused waterlogging conditions in the control fields. It was therefore necessary to obtain control values for each genotype from a variety trial with similar statistical design, located 100 m from the waterlogging trial.

In 2014, the treatment caused severe stress in wheat and most plants became necrotic. After draining the experiments, the plants recovered growth of existing leaves and/or developed new ones. The ability to recover clearly differed among genotypes and was visually scored on a 1–10 scale, with 1 being the poorest. The percentage of green biomass, relative to senesced biomass, was recorded five and nineteen days after drainage. Wheat grain yield was harvested in 2015 and 2016 and the weights were adjusted to 15% moisture content.

2.6. Statistical Analysis

The R software was used for all statistical analysis [41]. Normally distributed phenotypic results were analyzed in the linear mixed model $y_{ijl} = \mu + \tau_i + \gamma_j + \rho_{i(j)} + \varepsilon_{ijl}$, where τ is the effect of the genotype, γ is the effect of the replicate, ρ is the effect of block within replicate and ε is the error term. Genotype was treated as a random variable and the data was analyzed using the "lme4" package [42]. Results for control and waterlogged plots were analyzed separately. Estimated genotype means from control and waterlogged plots were used to calculate relative values of yield, plant height and number of spikes by dividing the value obtained on waterlogged plots by the control values. In 2013, relative values for plant height and the difference in heading date was calculated prior to the analysis of variance. This was done as the control values were obtained on another variety trial. Chlorosis, green biomass and overall condition scores were given only to waterlogged plants.

Best linear unbiased predictors (BLUPs) constituted the input variables (Tables S2 and S3) for principal component analysis (PCA) on the basis of correlation matrix. The princomp function in the "stats" package was used for the PCA. Scores for principal component 1 (PC 1) were used as an estimate for the genotypes' waterlogging tolerance.

The relationship between PC 1 scores and grain yield data was evaluated in simple linear models with PC 1 as regressor and relative grain yield as response variable. In addition, the individual phenotypic traits and grain yield were analyzed in simple linear regression models.

3. Results

3.1. Soil Measurements

The chemical composition regarding plant available fractions of P, K, Ca, Mg and Mn, pH, total carbon and nitrogen was similar at both locations, in both waterlogged and control experiments (Table S4). The soil texture was determined to silty or medium loams.

Redox potential (Eh) measured at 4–5 cm depth ranged between 181 and 291 from 2014 to 2016 (Table S5). Repeated measurements were made within a few days in 2014 and 2016 but the results did not indicate a difference in the redox potential. The infiltration rate measured in 2017 was vastly different on location S and N. The average infiltration rate in mm h^{-1} $\pm$ the standard deviation at location S was 391 $\pm$ 242, 278 $\pm$ 162 and 211 $\pm$ 126 after 10, 20 and 30 min, respectively. The infiltration at location N was so poor that it was unmeasurable within the mentioned time intervals. Infiltration was still not quantifiable even after approximately 2 h.

3.2. General Stress Responses

Signs of stress were visible three to four days into the treatment, but genotypes were still not easily distinguishable at that time. The first visible symptom was chlorosis of older leaves. Later on, the whole plants became chlorotic and leaves started to senesce. Vegetative growth, i.e., leaf biomass and eventually plant height, was clearly reduced when compared to control plots. For most genotypes, the date of heading was delayed and the size of individual spikes was reduced.

3.3. Screening in Hillplots in 2013 and 2014

PC 1 explained 48.2 and 50.9% of the variance in wheat in 2013 and 2014, respectively (Figures 1 and 2). Variables with arrows pointing in the same direction are positively correlated, while variables with arrows pointing in the opposite direction are negatively correlated. A high or low PC 1 score indicates sensitivity and tolerance, respectively. The variable overall condition had the highest contribution to PC 1 in both years while plant height contributed the least in 2013 and chlorosis in 2014 (Table 3). Chlorosis was correlated with the delay in heading in 2013 (Figure 1; Table S6. In 2014, the overall condition was positively correlated with the green biomass 5 days after drainage (Figure 2). The green biomass recorded 19 days after drainage was positively correlated with the number of heads and negatively correlated with the heading delay. Genotypes that performed well in the experiments generally had a high score for overall condition and their heading date was less delayed.

PC 1 explained 43.6 and 39.3% of the variation in the barley experiments in 2013 and 2014, respectively (Figures 3 and 4). Chlorosis was negatively correlated with the overall condition score in 2013 and with the relative plant height in 2014 (Table S7). The heading delay, overall condition and chlorosis contributed similarly to PC 1 in 2013, while plant height was less important (Table 3). In 2014, the overall condition contributed the least and heading delay the most to PC 1.

Of the 181 wheat genotypes that were screened in 2013 and 2014, 106 were screened in both years. Of the 25% most tolerant genotypes, according to their PC 1 score, six genotypes were consistently more tolerant in both years (Table 4). Of the 25% most sensitive genotypes, twelve of them were consistently more sensitive. Similarly, of the 61 barley genotypes that were screened for both years, five of them were consistently more tolerant and eight were consistently more sensitive (Table 4).

Table 3. Relative contribution of variables to principal component (PC) 1 in wheat and barley.

	Wheat				Barley	
Year	2013	2014	2015	2016	2013	2014
Chlorosis	25.0	1.1	18.2	22.9	29.9	23.8
Plant height	17.8	7.7	17.1	5.5	12.8	28.7
Heading delay	27.4	16.5	14.8	7.5	31.4	30.8
Overall condition	29.8	17.7	17.0	19.0	25.8	2.5
Head number	-	19.9	15.5	23.0	-	14.2
Green biomass 5 days post drainage	-	16.7	-	-	-	-
Green biomass 19 days post drainage	-	20.4	-	-	-	-
Straw yield	-	-	17.5	22.0	-	-

Table 4. Wheat genotypes with consistently poor or good tolerance properties in 2013 and 2014 according to their PC 1 scores.

Wheat Line	Origin	Barley Line	Origin
Consistently more tolerant			
Altar84/*Ae. tauschii* (219)//2 × Seri	CIMMYT	Breeding line 1176	Denmark
CETA/*Ae. tauschii* (895)	CIMMYT	Shirley	Germany
Bjarne	Norway	Frisco	Denmark
Kukri	Australia	Balder	Sweden
Mirakel	Norway	Breeding line 1178	Germany
Zebra	Sweden	-	-
Consistently more sensitive			
Breeding line 1405	Norway	Breeding line 1095	Norway
Breeding line 1327	Sweden	Fredrickson	Japan
T9040 (1995)	Norway	CIho4196	China
BCN*2//CROC_1/*Ae. tauschii* (886)	CIMMYT	Tore	Norway
Breeding line 1303	Norway	Ven	Norway
Sabin	USA	Varde	Norway
512-70	Norway	Herse	Norway
Nobeokabouzu	Japan	Svanhals	Sweden
Kariega	South Africa	-	-
Quarna	Switzerland	-	-
Saar	CIMMYT	-	-
DH 49-18	Norway	-	-

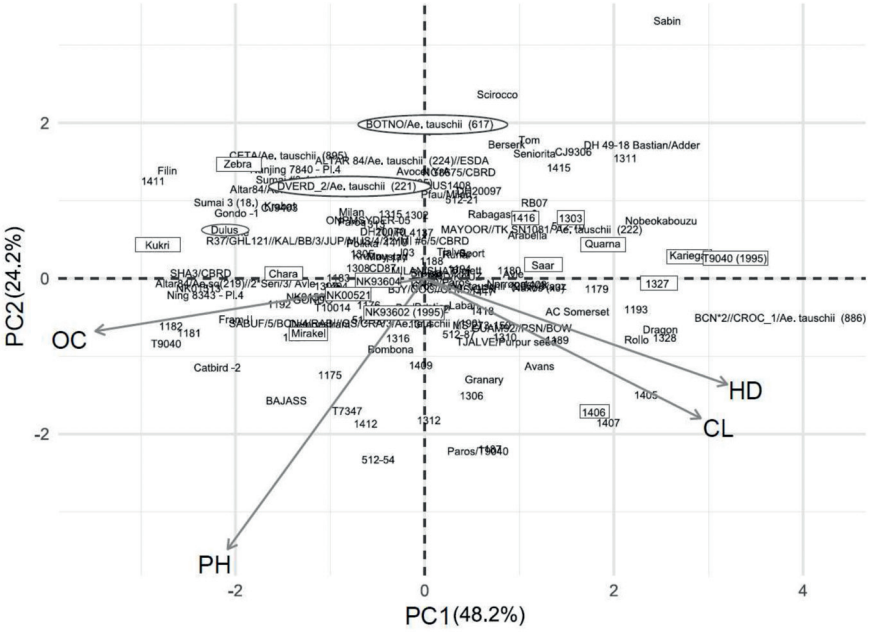

Figure 1. Principal component analysis (PCA) biplot for wheat results in 2013. Circled genotypes have previously been defined as tolerant, while genotypes in boxes were selected for the experiments in 2015 and 2016. Abbreviations: CL, chlorosis; OC, overall condition score; HD, heading delay; PH, relative plant height.

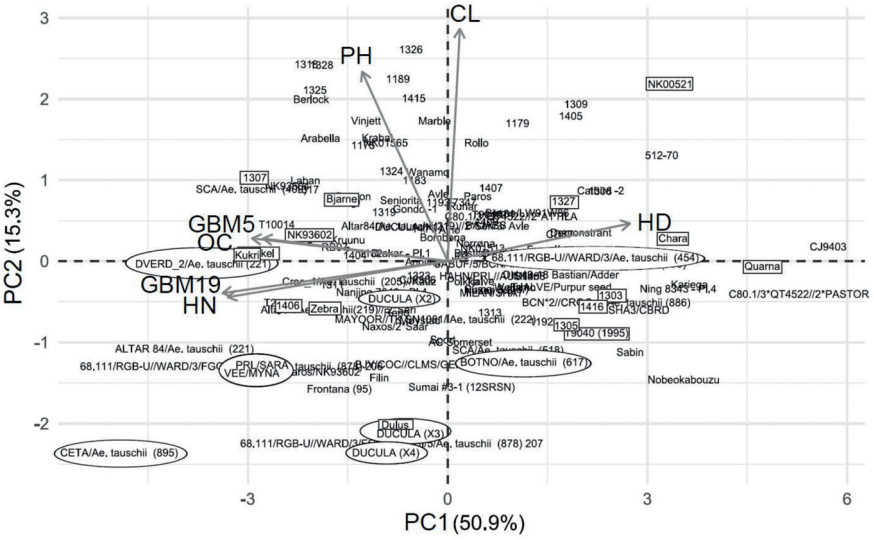

Figure 2. PCA biplot for wheat results in 2014. Circled genotypes have previously been defined as tolerant, while genotypes in boxes were selected for the experiments in 2015 and 2016. Abbreviations: CL, chlorosis; OC, overall condition score; HD, heading delay; PH, relative plant height; HN, relative head number; GBM5, green biomass score 5 days after drainage; GBM19, green biomass score 19 days after drainage.

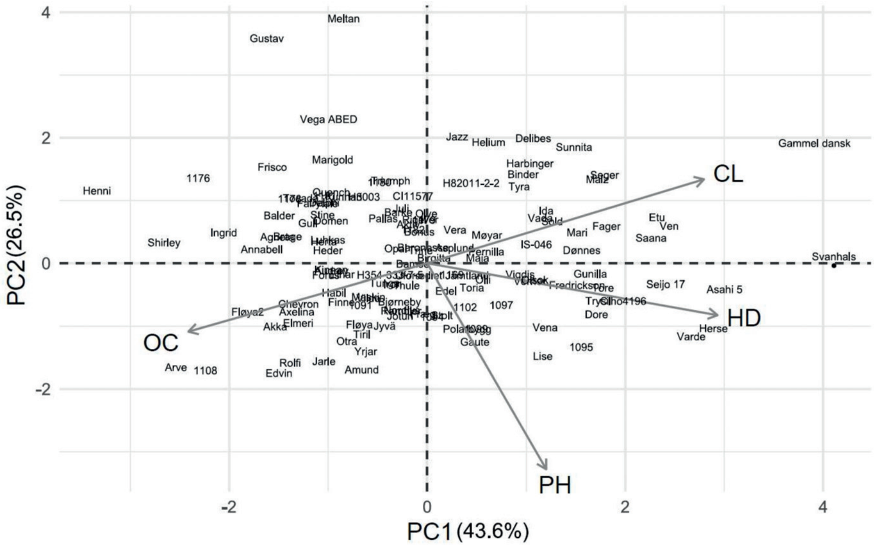

Figure 3. PCA biplot for barley results in 2013. Abbreviations: CL, chlorosis; OC, overall condition score; HD, heading delay; PH, relative plant height.

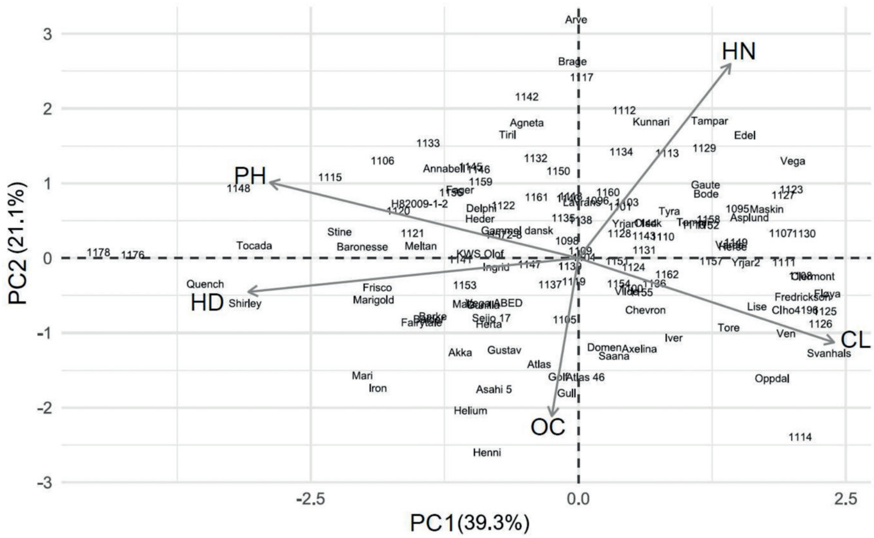

Figure 4. PCA biplot for barley results in 2014. Abbreviations: CL, chlorosis; OC, overall condition score; HD, heading delay; PH, relative plant height; HN, relative head number.

3.4. Yield Registration Experiments in 2015 and 2016

The absolute wheat yields obtained on both waterlogged and control plots were generally lower in 2015 than 2016 (Table 5). Among the ten genotypes that were included in both years, the correlation in yield between 2015 and 2016 was 0.82 ($p = 0.003$) on control plots. Correlations between waterlogged and control plots were 0.82 ($p = 0.003$) in 2015 and 0.80 ($p = 0.005$) in 2016. Relative grain yields obtained by the wheat varieties were of a similar order of magnitude in 2015 and 2016. They ranged from 25% to 37% in 2015 and from 24 to 41% in 2016. Yield obtained on waterlogged plots was non-correlated between 2015 and 2016. Bjarne, which had the highest relative yield in 2015, had a relative yield in the lower range in 2016. Kariega, identified as a consistently more sensitive variety based on the hillplot trials, had 28% relative yield in 2015, but was one of the least affected genotypes in 2016. However, this genotype is significantly lower yielding than most other genotypes.

Table 5. Absolute (kg ha^{-1}) and relative yields obtained by wheat genotypes on waterlogged and control plots in 2015 and 2016. Uppercase letters denote significant differences among genotypes determined by ANOVA and Tukey's honest significance difference test. Controls and waterlogged results were analyzed separately.

	2015			2016		
Variety	**Control**	**Waterlogged**	**Relative Yield (%)**	**Control**	**Waterlogged**	**Relative Yield (%)**
Bjarne	3601 ^{bc}	1299 ^{ab}	37	6482 ^{abc}	2076 ^{bcdef}	32
Dulus	2519 ^{efg}	760 ^{def}	30	4274 ^{ef}	1614 ^{ef}	38
Breeding line 1406	-	-	-	6892 ^{a}	2983 ^{a}	41
Kukri	3433 ^{bcd}	908 ^{cde}	26	6273 ^{abc}	2588 ^{ab}	41
Mirakel	3693 ^{bc}	1230 ^{abc}	34	7156 ^{a}	2167 ^{bcde}	30
NK93602	3668 ^{bc}	1192 ^{bc}	33	6010 ^{abcd}	2380 ^{abcd}	40
NK93604			-	5379 ^{bcde}	1983 ^{cdef}	37
Zebra	2933 ^{def}	1012 ^{bcd}	33	6386 ^{abc}	2193 ^{bcdef}	34
Chara	2179 ^{g}	501 ^{f}	25	-	-	-
Breeding line 1303	-	-	-	5133 ^{cde}	162 ^{def}	32
Breeding line 1416	-	-	-	6460 ^{abc}	2364 ^{abcd}	37
Kariega	2419 ^{fg}	625 ^{ef}	28	3594 ^{f}	1429 ^{f}	40
NK00521	3379 ^{bcd}	977 ^{bcde}	29	5787 ^{abcd}	1641 ^{ef}	28
Saar	-	-	-	4712 ^{def}	1586 ^{ef}	34
Breeding line 1327	-	-	-	6697 ^{ab}	1857 ^{cdef}	24
T9040	3587 ^{bc}	917 ^{cde}	26	7124 ^{a}	2510 ^{abc}	35
Quarna	3108 ^{cde}	801 ^{def}	26	5864 ^{ef}	1607 ^{ef}	27
p-value	<0.001	<0.001		<0.001	<0.001	

Variance explained by PC 1 in wheat was 71.6% in 2015 (Figure 5A). The traits contributed similarly to PC 1 (Table 3). Genotypes expected to be more tolerant showed similar responses (Figure 5A). Yet, Bjarne was differentiated from Zebra, Mirakel, Dulus, Kukri, and NK00521 by having a less reduced plant height and a heading date more similar to the control. Zebra, NK93602, Kukri, Mirakel, and Dulus on the other hand, were more associated with high overall condition scores, many heads per plot, and had a less delayed heading date. The more sensitive genotypes were associated with a high percentage of chlorosis. A fitted regression model with PC 1 as regressor and relative yield as explanatory variable showed that PC 1 explained 74% (R^2_{adj}, $p < 0.001$) of the yield response. Single regression models for individual traits showed that chlorosis alone explained 87% ($p < 0.001$) of the relative yield variation. The straw yield, plant height and overall condition score were also significant explanatory variables for the relative yield response. Correlation analysis showed that these traits were also negatively correlated with chlorosis (-0.86 at $p < 0.001$ for plant height, -0.77 at $p = 0.005$ for straw yield and -0.79 at $p = 0.003$ for the overall condition, Table S8).

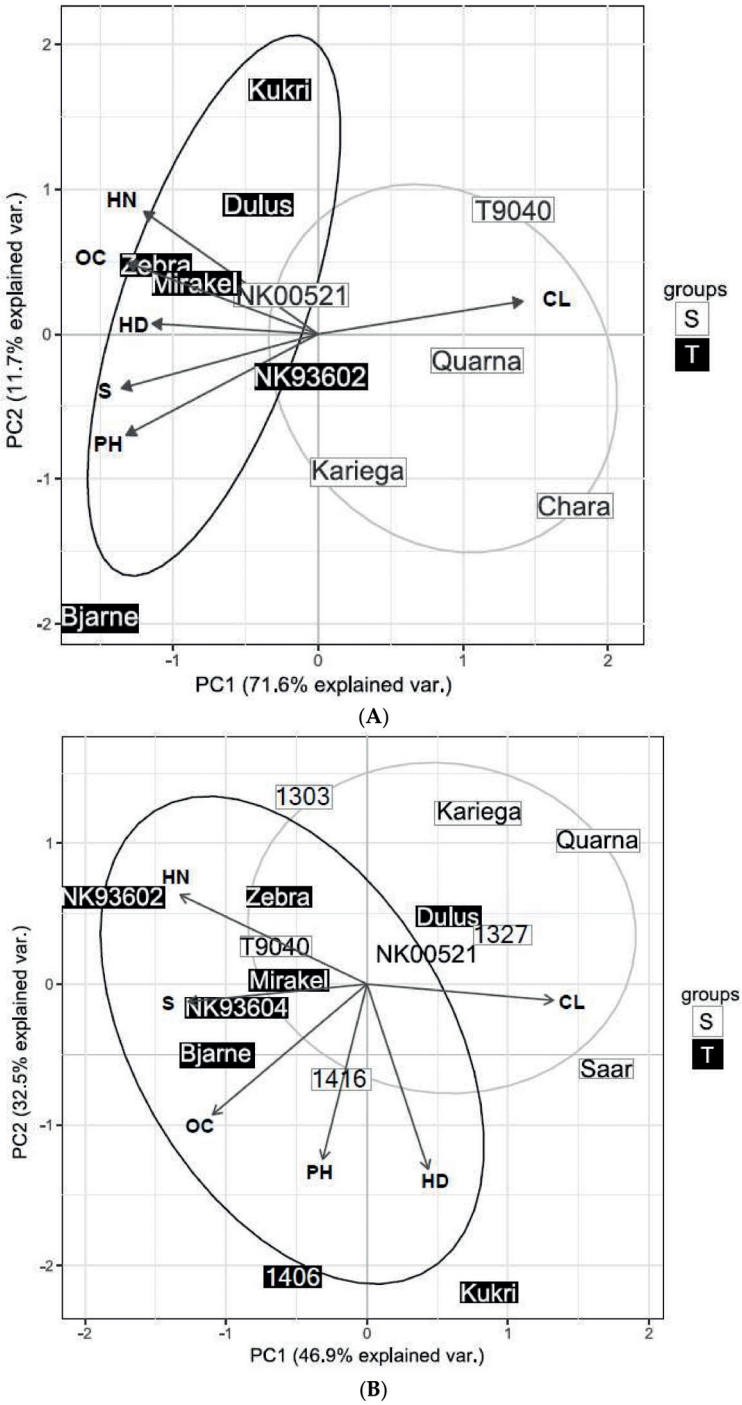

Figure 5. PCA biplot of PC 1 and 2 for selected wheat genotypes expected to be sensitive (S) and tolerant (T) in 2015 (**A**) and 2016 (**B**). Abbreviations: CL, chlorosis; OC, overall condition score; HD, heading delay; PH, relative plant height; S, relative straw yield; HN, relative head number.

PC 1 accounted for 46.9% of the variance in 2016 (Figure 5B). Chlorosis, head number and straw yield were the main contributors to PC1 with 23, 22 and 23%, respectively. Heading delay and plant height had little influence and contributed with only 8 and 5%. The latter two traits were statistically insignificant explanatory variables for yield obtained on waterlogged plots. The overall condition score, straw yield and plant height seemed to be the main differentiator between the genotypes. PC 1 was a non-significant explanatory variable for the relative yield. Chlorosis and the overall condition score were both significant regressors but the R^2_{adj} values were limited to 0.31 ($p = 0.01$) for the overall condition score and to 0.21 ($p = 0.04$) for chlorosis. The other traits were statistically insignificant for the relative yield response. PCA based on data obtained on the waterlogged plots alone showed that yield was strongly associated with PC 1 ($R^2_{adj} = 0.59$, $p < 0.001$) and the overall condition score ($R^2_{adj} = 0.59$, $p < 0.001$) but non-significant for chlorosis.

4. Discussion

In the present study, we have characterized the waterlogging tolerance of wheat and barley varieties based on affected phenotypic traits, found under waterlogged relative to non-waterlogged conditions. PCA was applied to reduce the number of variables into only one (PC 1), which was further used for varietal ranking in 2013 and 2014.

4.1. PCA for Tolerance Determination

Previous research has shown that a multitude of traits may be affected by waterlogging and there is little agreement as to which traits to use as selection criteria. To account for this, our phenotyping approach was to record all affected traits that could be easily quantified and that clearly differentiated the genotypes. To exclude potential noise and maintain only relevant data, PCA was our preferred statistical method. By utilizing PCA for dimensionality and noise reduction, the largest variance of the traits is captured in the principal components. PC 1 contains the largest variance and was therefore used for genotype ranking. Several wheat genotypes (CIMMYT) known to be waterlogging tolerant were included in the 2013, and particularly 2014 experiments [1,38,39]. One of them, the synthetic hexaploid wheat line CETA/*Ae. tauschii* (895), was one of the six genotypes that were consistently tolerant in both screening years (Table 4). This result confirms the previously stated tolerance of this line [38]. Dulus, BOTNO/*Ae. tauschii* (617) and DVERD_2/*Ae. tauschii* (221) were also included in both years. Dulus is a derived line from the waterlogging tolerant CIMMYT line Ducula (pedigree Bobwhite/Neelkant//Ducula/3/Ducula), while the other two are synthetic lines that were determined as waterlogging tolerant in experiments conducted in Mexico [38]. Several more genotypes with known tolerance properties were screened in 2014. Many of them seemed to be associated with low values of PC 1 and PC 2. Dulus, Ducula-3, and Ducula-4 were especially associated with low levels of chlorosis. However, they seemed less able to recover growth post treatment. BOTNO/*Ae. tauschii* (617) and especially 68.111/RGB-U//WARD/3/*Ae. tauschii* (454) appeared as more sensitive. The consistent performance of CETA/*Ae. tauschii* (895) suggests that it may be tolerant in different environments and might be a useful genetic resource in breeding.

Of the genotypes that were selected for the experiments in 2015 and 2016, yields obtained on waterlogged plots were highly correlated with yields on control plots. Hence, genotypes with high yield potential generally performed well also under waterlogged conditions. The varieties Mirakel, Bjarne and Zebra performed consistently well in the hillplot experiments. The relative yield of them were also in the upper range in 2015, when they were tested at the same location as the hillplot experiments in previous years (location N).

4.2. Stress Response as Influenced by Experimental Location

The selected genotypes were clearly more differentiated into groups of sensitive and tolerant in 2015 compared to 2016. This distinction coincides with the locations of the experiments in those years. Wheat was placed on location N in 2015 and location S in 2016. The genotypes included in the

experiments in 2015 and 2016 were selected based on performance in 2013 and 2014. During those years, wheat was evaluated only at location N. When tested on location N, the selected genotypes performed similar as in 2013 and 2014, and PC 1 scores were strongly associated with yield on waterlogged relative to control plots.

Chemical and physical properties of the top soil were similar at the two locations (N and S). The redox potential measured at 4 cm depth was also similar, indicating that the conditions were equally anaerobic in this layer. However, the infiltration rate beneath the plow pan was distinctively different. The water pressure needed to maintain the water level at the soil surface was not quantified, but clearly higher at location S compared to location N. Stress was induced more quickly on location N compared to location S. The difference in imposed stress is also indicated by the yields, which were much lower in 2015 than 2016. The treatment lasted for only 13 days in 2015, while 21 days were necessary to achieve a stress level sufficient to visibly distinguish the genotypes in 2016. It is likely that the total duration of waterlogging was longer at location N due to slower dry-up. The continuous flow of oxygenated water to the basin at location S might have also supplied the plants with oxygen, sufficient to maintain growth and to suppress strongly reducing conditions. At location N, the conditions were clearly stagnant and it is more likely that severe anaerobic conditions arose.

4.3. Evaluation of Phenotypic Results

The agronomic definition of waterlogging tolerance according to Setter and Waters [28], is the ability to maintain yield under waterlogged relative to drained conditions. Another definition relates to the maintenance of high growth rates of physiological traits such as biomass. As discussed by Setter & Waters (2003), the latter definition is relevant since aboveground biomass often is correlated with yield. In the present study, we have considered the influence of waterlogging on both yield and physiological traits. Physiological traits has been emphasized in our study as hillplot experiments are not suitable for yield measurements. However, the smaller size of hillplots allow for screening of a greater number of genotypes. Yield registration in 2015 and 2016 was primarily intended to determine the relevance of phenotypic traits and of PC 1 under the concurrent growth conditions.

The percentage of chlorosis, chlorophyll content or similar traits are commonly used for tolerance determination [18,35,43]. Chlorosis is often a clearly visible stress symptom and it is simple to obtain a differentiation among genotypes if the screening population is diverse and the stress is severe. In our study, chlorosis showed fairly consistent influence on PC 1 over the years. The contribution was however low for wheat in 2014. Instead, chlorosis was strongly associated with PC 2 and PC 3 (data not presented). In 2014, it was apparent that genotypes with similar percentage of chlorosis could have contrasting abilities to recover growth post-treatment. The relationship between percentage of chlorosis and relative yield in 2015 was highly significant. Our results clearly indicate that chlorosis was the superior trait for determining the tolerance properties of the selected genotypes in this year. In contrast, the relationship between chlorosis and relative yield in 2016 was limited. Clearly, chlorosis was a poor predictor of tolerance when the stress was less severe. It was much less distinct between the selected genotypes and thus challenging to score. Even after 21 days of waterlogging in 2016, the wheat plants were in relatively good condition (location S). Setter et al. [20] also reported an inconclusive relationship between chlorosis and relative yield of both wheat and barley varieties in Western Australia. Two of the barley varieties used in their experiment, Franklin and Sterling, had equal scores of chlorosis percentage, but the relative yield of Sterling was three times larger than Franklin. Zhou [35] found that the differentiation among barley genotypes was much stronger after 9 weeks of waterlogging as opposed to only 2 weeks. The correlation of a waterlogging tolerance score (based on leaf chlorosis and survival) between years successively became stronger the longer the treatment lasted.

While the chlorosis percentage was recorded during or in conjunction with the treatment, the overall condition scores were recorded a few weeks later when the plants had been given time to recover. Chlorosis and the overall condition score were significantly correlated in 2013 (-0.42, $p < 0.001$,

Table S6), 2015 (−0.79 at p = 0.003, Table S8) and 2016 (−0.62 at p = 0.01, Table S8), but not in 2014. Largely, the overall condition score reflected the chlorosis percentage that had been previously observed. This was apparent especially for the genotypes selected for the experiments in 2015 and 2016. In the two preceding years when the populations under investigation were much more diverse, the overall condition score was less related to the chlorosis. The green biomass score, recorded for wheat in 2014, also reflected the recovery but at an earlier stage. Genotypes with a high green biomass score were the ones that recovered the fastest. In this particular case, the green biomass score had the most influence on PC 1 and it was strongly correlated with the overall condition. The importance of considering the ability to recover was demonstrated by Setter & Waters [28]. The flooding tolerant wheat genotype Ducula-4 (CIMMYT) and moderately tolerant Chara (Australia) both had 60% relative shoot growth during a 28 day waterlogging event. The relative shoot growth after 21 days of recovery was 45% for Ducula-4 and 7% for Chara. As a comparison, in our experiments, there was a clear difference in the green biomass score between Chara and Ducula-1, a sister line of Ducula-4. Chara had an average green biomass score of 6.6% and Ducula-1 24.7%. The percentage of chlorosis was 40 and 43% for Ducula-1 and Chara, respectively. The plant height, the heading date and the number of spikes were poor predictors of yield on waterlogged plots. Single regression analysis of these traits showed non-significant relationships with yield of wheat in 2016.

Our results, along with cited references, indicate that chlorosis, as a single trait can be unreliable as a single selection criterion for waterlogging tolerance. The fact that there might be a discrepancy between leaf chlorosis and the ability to recover, clearly suggests that a measurement of recovery should always be included in waterlogging tolerance assessments. However, it is noteworthy that yield response might not be correlated to any of these traits. Although maintenance of the yield level is ultimately the most desired trait, it is influenced by many factors, has low heritability and thus may be a delusive selection criterion. Still, yield registrations are important and may reveal possible extreme observations, as observed by Van Ginkel et al. [18], who found certain wheat genotypes to be sterile despite retaining green leaf area.

Differences in the stress expression among genotypes might be a result of underlying tolerance mechanisms and survival strategies. The most favorable survival strategy likely depends upon the given soil conditions, the waterlogging event itself as well as the developmental stage of the plants. For instance, while withstanding elemental toxicity is crucial in soils high in iron and manganese, it might be a redundant trait in soils where these elements are scarce. At the experimental locations used in the present study, it was confirmed by chemical analysis of leaf tissue samples that manganese and iron were not present in toxic levels. Furthermore, long-term, short-term or even reoccurring waterlogging events might require different strategies. Since waterlogging tolerance is a trait that is strongly dependent on the environmental conditions, it is clear that screening and evaluation of tolerance properties in the target environment at relevant growth stages is of utmost importance.

Our analyses have shown that the percentage of chlorosis and the overall condition score were the traits that best determined the yield response of the genotypes we selected for the yield trials. Although these traits were well correlated in 2015 and 2016, they were less so for the larger population screened in 2013 and 2014. Waterlogging tolerance could likely have been determined fairly accurately based on chlorosis alone. However, as the ability to recover might differ, one might discard promising genotypes if such a measurement is neglected. Furthermore, precise scoring of chlorosis in small populations is challenging and severe stress is necessary to achieve differentiation. The relative plant height, the number of heads and the delay in heading did not explain much of the yield response of the selected genotypes in 2015 and 2016. Nonetheless, the traits were much affected by the treatment. Thus, it cannot be excluded that these traits might have been important for certain genotypes in the larger, very diverse populations investigated in 2013 and 2014. As PCA reduces noise and irrelevant information, we consider it as a better tool for tolerance determination of these populations.

5. Conclusions

Our results show that there is a diversity in waterlogging tolerance of the larger populations investigated in 2013 and 2014. A large number of agronomic traits were significantly affected by the treatment. For these genotypes, we consider PCA, where multiple phenotypic traits were considered, better determined tolerance properties instead of single traits. Based on PC 1 scores, six wheat genotypes and five barley genotypes appeared to be consistently more tolerant than others. This includes the CIMMYT line CETA/*Ae.tauschii* (895), which has been determined to be tolerant in previous waterlogging experiments conducted in Mexico. The percentage of chlorosis and the overall condition score given around maturation appeared to be the most important traits for yield response of the genotypes selected for the experiments in 2015 and 2016. The percentage of chlorosis did not necessarily reflect the genotypes' ability to recover growth. Thus, to determine tolerance properties rightfully, records from a full crop cycle, including measurements of the ability to recover growth seem fundamental when screening populations with unknown tolerance properties. The results presented here show that phenotyping waterlogging tolerance based on agronomic traits demands consistency in methodology and testing environment. Precise scoring of chlorosis or other visual traits requires severe stress and diverse populations.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/8/4/38/s1, Table S1: Monthly and accumulated precipitation (mm), temperature (°C) and day degrees from May through August in the experimental years, as compared to the normal period of 1961–1990, Table S2: Barley genotypes, their origin and best linear unbiased predictors (BLUPs) for the percentage of chlorosis, relative plant height, heading delay, overall condition and relative head number, Table S3: Wheat genotypes, their origin and best linear unbiased predictors (BLUPs) for the percentage of chlorosis, relative plant height, heading delay, overall condition, relative head number and green biomass recorded 5 and 19 days post treatment in 2014, Table S4: Mean percentage of clay, silt and sand, bulk density (4–9 cm depth) and porosity, pH, total carbon and nitrogen as well as contents of P, K, Ca, Mg, and Mn at the locations of the experimental site, Table S5: Redox potentials (Eh) ± the standard deviation measured at 4–5 cm depth at location N (north) and S (south) in 2014 to 2016, Table S6: Correlations of traits in barley (2013 and 2014), Table S7: Correlations of traits in wheat (2013 and 2014), Table S8: Correlations of traits in wheat (2015 and 2016).

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Conflicts of Interest: The authors declare that they have no competing interests.

Abbreviations

C	control
WL	waterlogged
PCA	principal component analysis
PC	principal component
N	north
S	south
CL	chlorosis
OC	overall condition score
HD	heading delay
PH	plant height
S	straw
HN	head number
GBM5	green biomass score 5 days after drainage
GBM19	green biomass score 19 days after drainage

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Table S1. Monthly and accumulated precipitation (mm), temperature (°C) and day degrees from May through August in the experimental years, as compared to the normal period of 1961-1990.

Precipitation (mm)					
	2013	**2014**	**2015**	**2016**	**1961-1990**
April	64.6	69.2	13.2	100	39
May	128	44.6	117	50.2	60
June	123.2	28.4	65.2	89.8	68
July	18.2	48.4	158.8	54.8	81
August	71.4	136	140.2	140	83
September	63.2	34.8	220.2	41	90
Sum	468.6	361.4	714.6	475.8	391
Temperature (°C)					
April	3.4	6.7	6.2	5.2	4.1
May	11.8	10.9	8.3	11.6	10.3
June	14	14.7	13.1	15.6	14.8
July	17.1	19.6	15	16.1	16.1
August	15.2	14.8	15.3	14.6	14.9
September	10.8	12	11.5	14.1	10.6
Day degrees May through August					
	1787	1845	1590	1779	1724

Table S2. Barley genotypes, their origin and best linear unbiased predictors (BLUPs) for the percentage of chlorosis, relative plant height, heading delay, overall condition and relative head number.

Year	**Line**	**Chlorosis**	**Plant height**	**Heading date**	**Overall condition**	**Head no.**	**Origin**
2013	1091	-2.20971	-0.01211	1.735772	0.37168	-	Norway
2013	1094	-1.40724	-0.04502	0.235772	-0.09046	-	Norway
2013	1095	-1.68848	-0.05807	7.035772	-0.37231	-	Norway
2013	1097	-2.27268	-0.03674	3.035772	-0.41427	-	Norway
2013	1099	-1.78484	-0.03188	5.435772	0.088206	-	Norway
2013	1102	-0.84998	-0.03775	1.735772	-0.12742	-	Norway
2013	1108	-6.24931	-0.02896	-0.36423	0.879689	-	Norway
2013	1169	1.464034	-0.01506	1.735772	0.135497	-	Sweden
2013	1176	-1.65978	0.063001	-5.36423	0.465105	-	Denmark
2013	1178	0.678418	0.044894	-2.56423	0.470048	-	Germany
2013	1179	0.549801	0.037895	3.735772	0.23002	-	Sweden
2013	1180	0.322653	0.039444	-1.76423	-0.11827	-	Denmark
2013	1181	-0.89379	0.027366	-4.56423	-0.12642	-	Denmark

2013	1182	-0.4276	0.045116	5.535772	0.37419	-	Sweden
2013	Agneta	-7.51054	0.032743	-1.36423	-0.19685	-	Sweden
2013	Akka	-5.89477	0.018528	5.735772	0.9007	-	Sweden
2013	Aktiv	-0.7752	0.016043	-0.16423	-0.16587	-	Czech Republic
2013	Amund	-6.48975	-0.05664	0.935772	-0.02755	-	Norway
2013	Annabell	-5.8278	0.021048	-2.56423	0.027249	-	Germany
2013	Arve	-9.5984	-0.02466	-1.26423	0.582546	-	Norway
2013	Asahi 5	5.729614	-0.05645	7.035772	-0.57713	-	Japan
2013	Asplund	1.936716	0.011159	3.435772	0.248703	-	Sweden
2013	Audrey	-6.8384	0.006018	-0.16423	-0.2576	-	-
2013	Axelina	-2.18485	-0.02106	-1.56423	0.500482	-	Sweden
2013	Balder	-2.66961	0.04168	-1.86423	0.253091	-	Sweden
2013	Bamse	-5.87866	0.001959	1.635772	-0.51849	-	Sweden
2013	Barke	-2.79672	0.022883	-0.76423	-0.42907	-	Germany
2013	Baronesse	1.805879	-0.01963	-3.06423	-0.1515	-	Germany
2013	Binder	1.643378	0.032983	1.935772	-0.56589	-	Denmark
2013	Birgitta	1.700867	0.009082	3.335772	0.372683	-	Sweden
2013	Bjørneby	-1.17671	-0.00913	3.435772	0.38786	-	Norway
2013	Bonus	1.613268	0.007656	-0.26423	0.056793	-	Sweden
2013	Brage	-3.44057	0.02914	-1.46423	0.204793	-	Norway
2013	Chevron	0.63532	-0.01487	-1.16423	0.834356	-	Switzerland
2013	Cl11577	-0.63303	0.040958	1.235772	-0.13411	-	Greece
2013	Clho4196	4.353469	-0.03307	8.235772	0.046728	-	China
2013	Delibes	3.26506	0.058805	2.935772	-0.45276	-	UK
2013	Delphi	-1.15347	0.046226	-0.66423	0.215933	-	Denmark
2013	Domen	-0.38253	0.024819	-2.06423	0.190207	-	Norway
2013	Dore	0.669658	-0.04508	6.435772	-0.34046	-	Sweden
2013	Dønnes	2.201125	-0.00482	5.935772	-0.29462	-	Norway
2013	Edel	0.000704	-0.03338	0.035772	-0.10654	-	Norway
2013	Edvin	-4.77813	-0.05495	-1.56423	0.484485	-	Finland
2013	Einar	-3.60486	-0.01367	-2.56423	-0.15043	-	Finland
2013	Elmeri	-7.06154	-0.01551	0.435772	0.131024	-	Finland
2013	Etu	1.044363	0.037164	12.43577	-0.4177	-	Finland
2013	Fager	3.072516	-0.0062	4.135772	-0.58017	-	Norway

2013	Fairytale	-0.70751	0.036544	-2.46423	0.151552	-	Denmark
2013	Finne	0.653774	-0.01527	0.235772	0.684465	-	Norway
2013	Fløya	0.807799	-0.02177	2.235772	0.832845	-	Norway
2013	Fløya2	-4.93688	0.006748	1.135772	0.731787	-	Norway
2013	Forus	1.028401	-0.0351	-6.56423	0.139246	-	Norway
2013	Fredrickson	6.885807	-0.01814	7.435772	0.510535	-	Japan
2013	Frisco	-1.21936	0.052812	-5.36423	0.013542	-	Denmark
2013	Fræg	-2.29147	-0.03298	1.535772	-0.02806	-	Norway
2013	Gammel dansk	7.396189	0.050879	13.23577	-0.8127	-	Denmark
2013	Gaute	-1.14486	-0.05288	3.235772	0.010056	-	Norway
2013	Gull	-1.88719	0.034859	-1.06423	0.257568	-	Sweden
2013	Gunilla	3.103404	-0.03189	4.235772	-0.32838	-	Sweden
2013	Gustav	-1.91185	0.131667	-5.96423	-0.45495	-	Sweden
2013	H3003	2.134694	0.015669	-4.76423	-0.03676	-	Norway
2013	H354-333-7-5	-2.53803	-0.01056	-0.06423	-0.10271	-	Norway
2013	H82011-2-2	3.854263	0.031164	0.635772	-0.06785	-	Norway
2013	Habil	-0.20869	-0.03284	-4.06423	0.21645	-	Norway
2013	Harbinger	4.54803	0.045261	3.235772	-0.1821	-	Finland
2013	Heder	-2.88745	0.006899	-2.16423	-0.00784	-	Norway
2013	Helium	1.621385	0.067588	3.035772	-0.3417	-	Denmark
2013	Henni	-3.0547	0.072764	-6.36423	0.889647	-	Germany
2013	Herse	4.633767	-0.06818	8.735772	-0.37071	-	Norway
2013	Herta	0.479178	0.024942	-0.06423	0.577029	-	Sweden
2013	Ida	7.084005	0.002909	1.835772	0.023444	-	Sweden
2013	Ingrid	-2.0325	0.029434	-4.26423	0.514037	-	Sweden
2013	Iron	-4.76092	-0.02098	-0.86423	-0.42753	-	Denmark
2013	IS-046	3.144693	-0.0087	2.935772	-0.18106	-	Iceland
2013	Iver	-0.03402	0.019956	0.835772	-0.13609	-	Norway
2013	Jarle	-3.93781	-0.04685	0.335772	0.434652	-	Norway
2013	Jazz	1.231218	0.053047	-1.26423	-0.59065	-	Netherlands
2013	Jotun	-2.04895	-0.028	1.735772	0.168962	-	Norway
2013	Juli	-0.14865	0.029358	0.135772	-0.06439	-	Denmark
2013	Jyvä	-5.6857	-0.01691	3.935772	0.066807	-	Finland
2013	Kinnan	-0.75476	-0.01302	-3.26423	0.177715	-	Sweden

2013	Kunnari	0.008137	0.053414	0.735772	0.338992	-	Finland
2013	Lise	0.59878	-0.07317	4.035772	-0.1043	-	Norway
2013	Luhkas	-6.70353	0.01434	-2.16423	-0.48715	-	France
2013	Maja	1.328223	-0.02862	-1.36423	-0.37045	-	Denmark
2013	Malz	5.999346	0.014139	2.335772	-0.47414	-	Czech Rebublic
2013	Mari	3.902472	-0.00126	4.535772	-0.24199	-	Sweden
2013	Marigold	0.129776	0.047949	-4.56423	-0.16023	-	France
2013	Maskin	-0.68163	-0.01144	1.335772	0.440851	-	Norway
2013	Meltan	2.672833	0.157848	-0.46423	0.032849	-	Sweden
2013	Møyar	1.420866	0.000725	1.535772	-0.22364	-	Norway
2013	Nordlys	-2.10829	-0.02418	1.835772	0.143037	-	Norway
2013	Olli	1.30437	-0.0156	3.335772	0.076286	-	Finland
2013	Olsok	2.751085	-0.02278	4.235772	-0.0004	-	Norway
2013	Olve	3.312376	0.034254	2.635772	0.420783	-	Norway
2013	Opal	-2.30991	-0.00063	-0.96423	-0.26876	-	Denmark
2013	Oppdal	-3.98171	-0.02529	2.735772	0.442613	-	Norway
2013	Otra	1.832329	0.013689	-2.06423	0.112041	-	Finland
2013	Pallas	1.499984	0.006333	4.235772	0.07959	-	Sweden
2013	Pernilla	-2.31695	-0.05864	0.535772	-0.35886	-	Sweden
2013	Polarbygg	-1.00968	0.039867	-2.56423	-0.01257	-	Norway
2013	Quench	-0.73127	-0.04598	-1.76423	-0.01175	-	UK
2013	Rambler	4.297631	0.002234	-2.56423	0.162398	-	-
2013	Rigel	-5.8349	-0.02782	2.335772	0.611296	-	Denmark
2013	Rolfi	6.304897	-0.02108	4.735772	-0.39663	-	Finland
2013	Saana	7.09462	0.027924	4.535772	-0.20631	-	Finland
2013	Seger	4.700991	-0.0436	6.335772	-0.40177	-	Sweden
2013	Seijo 17	-4.20288	0.042725	-3.36423	0.747246	-	Japan
2013	Sold	3.472335	-0.00555	1.635772	-0.42709	-	Norway
2013	Stine	-1.63916	0.038385	-0.76423	0.196253	-	Germany
2013	Stolt	0.652298	-0.04496	0.535772	0.110824	-	Sweden
2013	Sunnita	6.212192	0.043803	3.035772	-0.33109	-	Sweden
2013	Svanhals	10.26399	-0.01911	14.33577	-0.17569	-	Sweden
2013	Thule	0.209874	-0.02494	-0.66423	0.091494	-	Norway
2013	Tiril	-5.98657	-0.03576	0.735772	-0.0877	-	Norway

2013	Tocada	-2.0301	0.041056	-2.96423	0.042997	-	Germany
2013	Tore	6.216657	-0.0825	-2.26423	-0.58832	-	Norway
2013	Toria	1.688528	-0.02874	1.535772	0.053077	-	Norway
2013	Trine	0.242911	-0.01938	-2.86423	-0.2926	-	Norway
2013	Triumph	-0.57233	0.054041	0.935772	-0.05555	-	Germany
2013	Trysil	5.276656	-0.05222	4.035772	-0.06478	-	Norway
2013	Tunga	-2.45807	-0.02313	-1.56423	-0.17114	-	Norway
2013	Tyra	2.442692	0.021866	1.235772	-0.47419	-	Norway
2013	Uforædlet Jämtland	0.348182	-0.02998	-1.16423	-0.19125	-	Sweden
2013	Vada	3.884835	0.006261	2.835772	-0.20503	-	Netherlands
2013	Varde	5.449497	-0.06777	8.835772	-0.10147	-	Norway
2013	Vega ABED	4.666929	0.063851	-6.16423	0.138262	-	Denmark
2013	Ven	4.71192	-0.01615	4.935772	-0.71441	-	Norway
2013	Vena	2.44139	-0.06373	2.735772	-0.10196	-	Norway
2013	Vera	-1.7136	0.013257	1.735772	-0.38618	-	Norway
2013	Verner	-0.75256	-0.0185	4.735772	-0.33145	-	Sweden
2013	Vigdis	1.805101	-0.01166	4.935772	0.028736	-	Norway
2013	Vilde	-1.39307	-0.02912	-1.56423	0.099916	-	Norway
2013	Yrjar	-3.68171	-0.06502	-2.16423	-0.05374	-	Norway
2014	1095	0.00092	0.425981	-1.85943	0.008559	0.768577	Norway
2014	1096	-0.01299	0.484557	-0.97013	-0.00549	0.697414	Norway
2014	1098	0.042367	0.47691	0.115411	-0.01476	0.63333	Norway
2014	1100	0.000428	0.438205	-0.42148	0.022514	0.68474	Norway
2014	1101	-0.02448	0.440568	-0.17841	-0.01493	0.693213	Norway
2014	1103	-0.15787	0.415709	-0.78831	-0.00098	0.716845	Norway
2014	1104	-0.0615	0.452264	-0.35513	0.013096	0.677367	Norway
2014	1105	0.032289	0.471847	-0.2008	0.017809	0.604515	Norway
2014	1106	-0.1277	0.538238	1.76794	0.003734	0.743857	Norway
2014	1107	-0.06827	0.395242	-2.8581	0.017817	0.749583	Norway
2014	1108	0.051243	0.409778	-3.76687	-0.01507	0.590113	Norway
2014	1109	0.003057	0.484179	-0.82903	0.003883	0.65032	Norway
2014	1110	0.006336	0.445308	-1.0211	0.008443	0.716705	Norway
2014	1111	0.098912	0.391055	-1.51628	-0.01022	0.678147	Norway
2014	1112	0.009544	0.512779	-1.85535	-0.03369	0.734824	Norway
2014	1113	0.086647	0.496904	-1.47201	-0.0148	0.76374	Norway
2014	1114	0.056355	0.348486	-2.1258	0.045749	0.579853	Norway
2014	1115	-0.15982	0.556795	2.239322	0.027132	0.772357	Norway
2014	1116	-0.08101	0.411725	-1.57034	0.003728	0.713777	Norway

2014	1117	-0.13373	0.491685	-0.64115	-0.00534	0.86392	Norway
2014	1118	-0.10847	0.463643	-0.19427	0.013049	0.757555	Norway
2014	1119	-0.07631	0.425805	0.255254	0.003842	0.627707	Norway
2014	1120	-0.14969	0.513537	1.865843	0.027243	0.748354	Norway
2014	1121	-0.17331	0.530907	0.044092	0.02709	0.673124	Norway
2014	1122	-0.11116	0.465612	0.598527	-0.01953	0.642099	Norway
2014	1123	-0.01186	0.37188	-0.99473	0.003662	0.829336	Norway
2014	1124	0.055698	0.450111	-0.09798	0.01329	0.701888	Norway
2014	1125	-0.02865	0.365506	-3.21364	0.027081	0.680149	Norway
2014	1126	0.021808	0.382056	-3.06352	0.036553	0.694754	Norway
2014	1127	0.093727	0.427711	-2.09627	-0.01007	0.756696	Norway
2014	1128	0.071227	0.485006	-0.69698	0.00363	0.700445	Norway
2014	1129	0.132361	0.452758	-0.41505	-0.0384	0.752952	Norway
2014	1130	0.184559	0.419056	-1.93316	-0.02424	0.682929	Norway
2014	1131	0.060349	0.46874	-1.08484	0.003736	0.678066	Norway
2014	1132	-0.12154	0.488133	0.224249	0.017885	0.81845	Norway
2014	1133	-0.13262	0.505758	1.467389	-0.02434	0.702366	Norway
2014	1134	-0.00545	0.478033	-1.25214	-0.03821	0.677797	Norway
2014	1135	-0.01783	0.44732	0.985436	-0.01503	0.681951	Norway
2014	1136	0.000162	0.421612	-0.37168	0.01783	0.690598	Norway
2014	1137	-0.09439	0.425834	1.034053	0.022587	0.684725	Norway
2014	1138	-0.03568	0.454639	0.253322	-0.00098	0.7061	Norway
2014	1139	-0.06167	0.463327	-0.59013	0.013057	0.651856	Norway
2014	1140	-0.04294	0.483508	-0.52969	-0.00563	0.696036	Norway
2014	1141	-0.03743	0.499446	1.06013	0.003649	0.628859	Norway
2014	1142	-0.10414	0.494409	0.069228	-0.03834	0.738793	Norway
2014	1143	-0.01807	0.434061	-1.02316	-0.01945	0.628859	Norway
2014	1144	0.008445	0.428155	-0.23722	-0.01949	0.669638	Norway
2014	1145	-0.17424	0.525946	-0.62313	0.013157	0.736588	Norway
2014	1146	-0.13646	0.48747	0.430008	-0.02409	0.661767	Norway
2014	1147	-0.12841	0.434368	0.729366	0.008603	0.653553	Norway
2014	1148	-0.22807	0.556436	3.488624	0.022314	0.729099	Norway
2014	1149	0.088684	0.425815	-1.31579	-0.00084	0.714517	Norway
2014	1150	0.004382	0.497964	-0.12218	-0.01969	0.704251	Norway
2014	1151	-0.12009	0.430681	-1.10436	0.017808	0.677011	Norway
2014	1152	-0.03232	0.444311	-2.67073	0.003768	0.694153	Norway
2014	1153	-0.06174	0.497369	0.731045	0.022556	0.637851	Norway
2014	1154	0.017831	0.437385	-0.50459	-0.01029	0.592081	Norway
2014	1155	0.014283	0.447936	-1.08017	0.017864	0.65425	Norway
2014	1156	-0.00448	0.536745	1.001259	-0.00558	0.693042	Norway
2014	1157	-0.03448	0.430718	-2.89228	-0.00082	0.626564	Norway
2014	1158	0.01977	0.448589	-2.38756	-0.01022	0.674348	Norway
2014	1159	-0.06927	0.496408	1.18456	-0.0055	0.722672	Sweden

2014	1160	-0.06094	0.462892	-0.77689	-0.00101	0.728694	Norway
2014	1161	-0.10538	0.480388	-0.2907	-0.00084	0.698799	Norway
2014	1162	-0.05756	0.390336	-0.71183	-0.01034	0.616918	Norway
2014	1176	-0.19225	0.572445	4.260954	-0.01503	0.503736	Denmark
2014	1178	-0.38213	0.528897	4.419241	-0.0149	0.483058	Germany
2014	Agneta	-0.06968	0.500183	0.783151	-0.00089	0.829546	Sweden
2014	Akka	0.023526	0.502461	0.675231	0.017737	0.532116	Sweden
2014	Annabell	-0.11998	0.491897	1.410429	-0.02436	0.673491	Germany
2014	Arve	-0.08836	0.49833	0.023983	-0.0365	0.875475	Norway
2014	Asahi 5	-0.03097	0.433895	1.255426	0.01322	0.495479	Japan
2014	Asplund	0.205437	0.42083	-0.207	-0.03348	0.701412	Sweden
2014	Atlas	-0.07791	0.425561	0.222424	0.017831	0.534188	USA
2014	Atlas 46	0.16006	0.44381	0.797941	-0.00551	0.503117	USA
2014	Axelina	0.069034	0.414276	0.303871	0.017805	0.614715	Sweden
2014	Balder	0.036581	0.504616	2.470381	0.031703	0.652011	Sweden
2014	Barke	-0.03742	0.510672	0.436067	0.003735	0.519997	Germany
2014	Baronesse	0.00059	0.51856	3.301815	-0.01022	0.623884	Germany
2014	Bode	-0.01346	0.427554	-1.49348	-0.01019	0.7309	Norway
2014	Brage	-0.09746	0.476623	-0.01853	-0.04898	0.778043	Norway
2014	Chevron	0.103424	0.39603	1.23812	-0.01494	0.606877	Switzerland
2014	Clho4196	0.203969	0.387129	-1.68332	-0.04298	0.526693	China
2014	Clermont	0.326189	0.407992	0.187325	-0.00091	0.759078	France
2014	Delphi	-0.08363	0.533413	-1.71133	-0.03366	0.528387	Denmark
2014	Domen	0.107245	0.443527	-0.10435	-0.01035	0.512719	Norway
2014	Edel	0.056647	0.396322	-0.80422	-0.07385	0.674673	Norway
2014	Fager	0.026655	0.534236	1.015872	-0.01952	0.664154	Norway
2014	Fairytale	-0.06129	0.501318	0.825442	0.008427	0.52803	Denmark
2014	Fløya	0.116151	0.405306	-3.21868	0.003809	0.650016	Norway
2014	Fredrickson	0.162985	0.383698	-1.49533	-0.01507	0.63039	Japan
2014	Frisco	-0.15458	0.48163	1.985318	0.003732	0.567726	Denmark
2014	Gammel dansk	0.044479	0.500904	0.178619	-0.03376	0.572723	Denmark
2014	Gaute	-0.00771	0.45149	-2.17776	-0.0056	0.738344	Norway
2014	Golf	0.041245	0.473703	-0.20722	0.045767	0.600546	UK
2014	Gull	0.110054	0.432278	1.947226	0.041144	0.630844	Sweden
2014	Gunilla	0.0313	0.472686	2.078545	0.013071	0.634148	Sweden
2014	Gustav	-0.00522	0.467423	0.509542	0.013199	0.53703	Sweden
2014	H572-8	-0.12749	0.484977	-0.18498	0.013224	0.672023	Norway
2014	H82009-1-2	-0.16712	0.4556	2.863646	-0.00551	0.701341	Norway
2014	Heder	-0.06169	0.509252	0.858502	0.023258	0.743497	Norway
2014	Helium	0.154011	0.533992	0.322351	0.032068	0.495304	Denmark
2014	Henni	0.078798	0.445967	2.18844	0.042617	0.518806	Germany
2014	Herse	0.059528	0.433405	-1.77624	0.010347	0.724575	Norway

2014	Herta	0.013015	0.49227	0.521406	0.013118	0.567539	Sweden
2014	Ingrid	0.047048	0.451296	2.685078	-0.01934	0.619253	Sweden
2014	Iron	-0.10532	0.493665	1.66997	0.049467	0.550846	Denmark
2014	Iver	0.249846	0.488965	-1.54946	-0.00097	0.556002	Norway
2014	Kunnari	0.11202	0.525096	-1.637	-0.02445	0.770456	Finland
2014	KWS Olof	-0.11016	0.448439	1.450469	-0.01008	0.612921	Germany
2014	Lavrans	-0.09869	0.443779	0.061207	-0.00097	0.722475	Norway
2014	Lise	0.233846	0.410699	0.805297	0.041212	0.81962	Norway
2014	Malz	0.215062	0.524153	2.675031	-0.00081	0.61551	Czech Rebublic
2014	Mari	0.11133	0.501437	3.599878	-0.01025	0.458586	Sweden
2014	Marigold	-0.08515	0.50877	1.866591	0.010365	0.568942	France
2014	Maskin	0.06303	0.416014	-1.36241	0.008373	0.798749	Norway
2014	Meltan	-0.035	0.528001	1.368454	0.013069	0.664239	Sweden
2014	Olsok	0.012298	0.467334	-1.16083	0.013229	0.739314	Norway
2014	Oppdal	0.248944	0.390576	-0.09156	0.022341	0.649962	Norway
2014	Quench	-0.16246	0.566596	2.729241	-0.0055	0.485068	UK
2014	Saana	0.253471	0.499115	-0.15699	0.017762	0.592799	Finland
2014	Seijo 17	-0.00833	0.443557	2.009028	-0.00084	0.576024	Japan
2014	Shirley	-0.27375	0.534585	1.814431	0.022516	0.523901	Germany
2014	Stine	-0.21684	0.488023	2.058047	-0.02432	0.54659	Germany
2014	Svanhals	0.23736	0.402057	-2.11062	0.013506	0.632443	Sweden
2014	Tammi	0.15438	0.469869	-1.46083	-0.02887	0.644273	Finland
2014	Tampar	0.261798	0.525559	-0.98764	-0.0195	0.840422	Faroe Islands
2014	Tiril	-0.13831	0.509144	-0.27195	-0.00254	0.766253	Norway
2014	Tocada	-0.18372	0.504444	4.148857	-0.01485	0.572034	Germany
2014	Tore	0.163486	0.407319	-0.59887	-0.00096	0.61572	Norway
2014	Tyra	0.229609	0.472503	-0.47101	-0.06154	0.587994	Norway
2014	Varde	0.022084	0.436981	-1.61395	0.041305	0.814856	Norway
2014	Vega	0.224105	0.428695	-0.79752	-0.04292	0.759917	Sweden
2014	Vega ABED	0.067287	0.47101	1.949673	-0.00563	0.588316	Denmark
2014	Ven	0.146386	0.42995	-2.80509	0.026936	0.653165	Norway
2014	Vilde	0.141557	0.470079	0.283757	0.022559	0.706977	Norway
2014	Yrjar-1	0.017349	0.440993	0.252229	-0.00565	0.711595	Norway
2014	Yrjar	0.063503	0.408761	-1.19096	0.013232	0.732599	Norway

Table S3. Wheat genotypes, their origin and best linear unbiased predictors (BLUPs) for the percentage of chlorosis, relative plant height, heading delay, overall condition, relative head number and green biomass recorded 5 and 19 days post treatment in 2014.

Year	Line	Chlorosis	Plant height	Heading delay	Overall condition	Head no.	Green biomass19	Green biomass5	Origin
2013	1175	1.035976	0.042612	-0.06559	0.31801	-	-	-	Norway
2013	1176	-1.73488	0.018845	0.337283	0.183835	-	-	-	Norway
2013	1177	-1.18888	0.017157	-0.27236	-0.19575	-	-	-	Norway
2013	1179	1.978852	-0.01485	0.56661	-0.24877	-	-	-	Sweden
2013	1180	1.562213	-0.01138	0.116972	-0.12912	-	-	-	Sweden
2013	1181	-1.19634	0.063052	-0.86757	0.253613	-	-	-	Norway
2013	1182	-3.97635	0.055704	-0.17083	0.460541	-	-	-	Norway
2013	1183	0.355017	0.011664	-0.45512	0.151788	-	-	-	Norway
2013	1186	-0.57142	-0.00024	0.249783	0.046391	-	-	-	Norway
2013	1187	2.557899	0.077631	0.221797	-0.28657	-	-	-	Norway
2013	1188	-1.8192	0.001902	0.332342	-0.04361	-	-	-	Sweden
2013	1189	3.147531	-0.00077	0.517476	-0.03945	-	-	-	Sweden
2013	1192	-1.78863	0.033212	-0.21136	0.248303	-	-	-	Norway
2013	1193	2.084147	-0.00587	0.72398	-0.38062	-	-	-	Sweden
2013	1194	1.788935	0.000665	-0.3494	-0.1694	-	-	-	Norway
2013	1302	-0.40786	-0.02854	-0.00445	0.124482	-	-	-	Norway
2013	1303	0.501229	-0.02796	0.238073	-0.38052	-	-	-	Norway
2013	1305	-0.85821	0.001625	-0.18507	0.106914	-	-	-	Norway
2013	1306	0.449722	0.049856	0.590275	-0.09174	-	-	-	Norway
2013	1307	-2.51381	0.024299	0.057234	0.138514	-	-	-	Norway
2013	1308	-0.25136	0.014044	-0.42289	0.007199	-	-	-	Norway
2013	1310	4.281978	-0.00483	0.138437	0.135501	-	-	-	Norway
2013	1311	-0.35553	-0.06631	0.642852	-0.33827	-	-	-	Norway
2013	1312	1.953446	0.053973	0.3236	0.096409	-	-	-	Norway
2013	1313	0.649982	-0.0123	-0.60722	0.045823	-	-	-	Norway
2013	1314	-1.25986	0.030189	0.372467	-0.04336	-	-	-	Norway
2013	1315	1.145074	-0.03396	-0.35963	0.238692	-	-	-	Norway
2013	1316	-0.46832	0.022121	0.458818	0.217928	-	-	-	Norway
2013	1324	-0.99078	0.037633	-0.04016	0.321685	-	-	-	Sweden
2013	1327	3.23037	-0.01922	0.455596	-0.44686	-	-	-	Sweden
2013	1328	5.469317	-0.03618	0.955973	0.06742	-	-	-	Sweden
2013	1404	-1.85902	0.03293	-0.2517	-0.04308	-	-	-	Norway
2013	1405	3.652469	0.034942	0.598414	-0.52193	-	-	-	Norway
2013	1406	2.972455	0.032005	0.89589	-0.17924	-	-	-	Norway
2013	1407	4.620318	0.025142	0.805231	-0.10365	-	-	-	Norway
2013	1408	-0.05138	0.008574	0.35045	-0.37351	-	-	-	Norway
2013	1409	-0.43607	0.052841	0.219709	-0.14475	-	-	-	Norway
2013	1410	-1.92079	-0.00392	0.141796	0.04128	-	-	-	Norway
2013	Willy	-4.56726	-0.00626	-0.61788	0.533272	-	-	-	Norway

2013	1412	0.680097	0.061417	0.345832	0.245672	-	-	-	Norway
2013	1415	1.217669	-0.06543	0.167876	-0.12511	-	-	-	Sweden
2013	1416	1.409881	-0.03716	0.064531	-0.12114	-	-	-	Norway
2013	1417	1.693409	-0.01154	0.279647	0.092402	-	-	-	Norway
2013	1418	1.322902	0.010657	0.152057	-0.10749	-	-	-	Norway
2013	512-21	-0.49536	-0.03721	0.131483	0.014301	-	-	-	Norway
2013	512-50	-0.89458	0.027027	0.145645	0.130616	-	-	-	Norway
2013	512-54	0.554813	0.091179	0.248677	-0.00831	-	-	-	Norway
2013	512-70	0.015423	-0.03071	0.50591	-0.26615	-	-	-	Norway
2013	512-87	2.399131	0.026607	-0.21485	-0.15568	-	-	-	Norway
2013	AC Somerset	2.968608	-0.01473	0.550857	-0.07249	-	-	-	Canada
2013	Aino	-2.75663	-0.05817	0.067667	0.018634	-	-	-	Finland
2013	ALTAR 84/Ae.tauschii (224)//ESDA	-0.96628	-0.04201	-0.31448	0.039282	-	-	-	CIMMYT
2013	Altar 84/Ae.tauschii (219)//2*Seri	-2.74062	-0.00194	-0.61091	-0.03134	-	-	-	CIMMYT
2013	Altar 84/Ae.tauschii (219)//2*Seri/3/ Avle	-2.48266	0.030143	-0.36274	0.320363	-	-	-	Norway
2013	Arabella	-1.65034	-0.00848	0.453012	-0.36472	-	-	-	Poland
2013	Avans	2.726741	0.046093	-0.07004	-0.51025	-	-	-	Sweden
2013	Avle	1.926826	-0.01064	0.067012	-0.1425	-	-	-	Sweden
2013	Avocet-YrA	1.923919	-0.01815	-1.32273	-0.40455	-	-	-	Australia
2013	BAJASS-5	-2.00126	0.081917	0.066366	0.119476	-	-	-	Norway
2013	Bastian	-0.32348	0.02472	0.072079	-0.13882	-	-	-	Norway
2013	BCN*2//CROC_1/ Ae.tauschii (886)	5.51834	-0.04045	1.247118	-0.51991	-	-	-	CIMMYT
2013	Berlock	0.164553	0.00214	0.374874	0.252659	-	-	-	Sweden
2013	Berserk	-0.19406	-0.04618	-0.31919	-0.36215	-	-	-	Norway
2013	Bjarne	-3.4706	0.023669	-0.63561	0.121851	-	-	-	Norway
2013	BJY/COC//CLMS/ GEN	-1.58007	0.023481	0.212117	-0.22749	-	-	-	CIMMYT
2013	Bombona	1.198509	0.014653	0.321187	0.374722	-	-	-	Sweden
2013	BOTNO/Ae. tauschii (617)	-3.67832	-0.04805	0.096308	-0.1123	-	-	-	CIMMYT
2013	Brakar	0.547226	0.008735	-0.21662	-0.16841	-	-	-	Norway
2013	Catbird	0.368766	0.051485	-0.4989	0.514567	-	-	-	CIMMYT
2013	CBRD/KAUZ	1.442302	-0.00726	-0.02895	0.035086	-	-	-	CIMMYT
2013	CD87	0.065906	0.025369	-0.6654	-0.2515	-	-	-	Australia
2013	CETA/Ae. tauschii (895)	-1.91356	-0.03001	-0.71666	0.216793	-	-	-	CIMMYT
2013	Chara	-3.23368	0.025881	-0.07671	0.191102	-	-	-	Australia
2013	CJ9306	0.119631	-0.05689	0.102897	-0.37095	-	-	-	China
2013	CJ9403	-4.67697	0.011781	-0.27158	0.032667	-	-	-	China
2013	Croc_1/Ae.tausc hii (205)//Kauz	-0.21702	0.003039	0.393089	-0.13088	-	-	-	CIMMYT
2013	Demonstrant	-0.8048	0.03908	-0.21124	0.129296	-	-	-	Norway

2013	DH49-18	1.65101	-0.0757	0.225126	-0.39432	-	-	-	Norway
2013	DH20070	-1.11589	-0.00723	-0.1585	0.039788	-	-	-	Norway
2013	DH20097	-1.06692	-0.03843	0.237006	-0.0612	-	-	-	Norway
2013	Dragon	6.182546	-0.02588	0.4525	-0.14832	-	-	-	Sweden
2013	Dulus	-2.36192	0.004193	-0.56184	0.395966	-	-	-	CIMMYT
2013	DVERD_2/Ae. tauschii (221)	-1.10449	-0.03948	-0.17025	0.346121	-	-	-	CIMMYT
2013	Filin	-3.65156	-0.00648	-0.92888	0.396553	-	-	-	CIMMYT
2013	Fram II	-2.0992	0.044738	-0.30655	0.320009	-	-	-	Norway
2013	Frontana	0.021035	-0.04311	-0.26009	0.176795	-	-	-	Brazil
2013	GONDO	1.253406	0.019289	-0.6299	0.231775	-	-	-	CIMMYT
2013	Gondo	-3.85328	0.00933	-0.47673	0.216543	-	-	-	CIMMYT
2013	Granary	5.702146	0.016792	-0.18456	0.106318	-	-	-	UK
2013	GUAM92//PSN/BOW	4.126548	-0.00709	0.156949	0.062428	-	-	-	CIMMYT
2013	HAHN/PRL//AUS 1408	-1.07295	-0.03537	-0.06746	0.052407	-	-	-	CIMMYT
2013	J03	0.63923	-0.02124	0.027037	0.258957	-	-	-	Norway
2013	Kariega	-1.09064	-0.01629	1.186336	-0.64497	-	-	-	South Africa
2013	Krabat	-4.35022	-0.00093	-0.11016	0.201739	-	-	-	Norway
2013	Kruunu	-2.04226	0.003214	0.120364	0.105835	-	-	-	Finland
2013	Kukri	-2.82564	0.021637	-0.80551	0.46001	-	-	-	Australia
2013	Laban	-1.15626	0.011851	0.612379	-0.03079	-	-	-	Norway
2013	MAYOOR//TKSN1081/Ae.tauschii (222)	1.938625	-0.04052	0.201767	-0.0695	-	-	-	CIMMYT
2013	Milan	-0.20248	-0.02059	-0.44125	0.186654	-	-	-	CIMMYT
2013	MILAN/SHA7	-0.09981	-0.01773	0.415729	0.292119	-	-	-	CIMMYT
2013	Mirakel	-0.88184	0.026817	0.145154	0.410874	-	-	-	Norway
2013	MS273-150	-0.5838	0.026394	0.474032	-0.19437	-	-	-	Norway
2013	Møystad	1.915726	-9.53E-05	-0.72658	-0.01297	-	-	-	Norway
2013	Nanjing 7840	-1.64352	-0.01403	-0.91893	0.033778	-	-	-	China
2013	Naxos	0.973583	-0.00198	0.365045	-0.23743	-	-	-	Germany
2013	NG8675/CBRD	-0.75777	-0.05574	0.227585	0.133871	-	-	-	CIMMYT
2013	Ning 8343	-0.75569	0.034072	-0.80746	0.380573	-	-	-	China
2013	NK00521	-0.32256	0.030733	-0.43729	-0.03897	-	-	-	Norway
2013	NK01513	-4.31243	0.052201	-0.4123	0.191301	-	-	-	Norway
2013	NK01565	0.458688	0.022666	-0.55251	0.200106	-	-	-	Norway
2013	NK93602 (1995)	0.60668	0.013837	0.055001	0.121159	-	-	-	Norway
2013	NK93604	-1.19049	0.015321	0.006084	0.035944	-	-	-	Norway
2013	Nobeokabouzu	2.635364	-0.06379	0.74258	-0.17636	-	-	-	China
2013	Norrøna	2.345309	-0.01551	0.168294	0.004185	-	-	-	Norway
2013	ALTAR 84/Ae.tauschii (224)//2*YACO/3/KAUZ	0.669684	-0.02954	-0.32009	0.29603	-	-	-	CIMMYT
2013	Paros	-1.66658	-0.02065	0.043394	0.293621	-	-	-	Germany

2013	Paros/NK93602	-0.24532	0.016849	0.188069	0.056351	-	-	-	Norway
2013	Paros/T9040	3.879815	0.062393	0.207876	-0.04988	-	-	-	Norway
2013	Pfau/Milan	-0.27853	-0.02997	-0.18345	-0.08575	-	-	-	CIMMYT
2013	Polkka	-2.84249	-0.00193	0.244068	0.150136	-	-	-	Sweden
2013	QUARNA R37/GHL121//KAL/BB/3/JUP/MUS/4/2*YMI	1.259275	-0.0235	0.369975	-0.40914	-	-	-	Switzerland
2013	#6/5/CBRD	-1.84734	0.009569	-0.33805	0.01101	-	-	-	CIMMYT
2013	Rabagast	0.525564	-0.01632	-0.2859	-0.32944	-	-	-	Norway
2013	RB07	1.376376	-0.04078	-0.00793	-0.19776	-	-	-	USA
2013	Rollo	4.870266	-0.00718	0.44877	-0.25009	-	-	-	Norway
2013	Runar	0.111118	-0.02657	0.43678	0.206354	-	-	-	Norway
2013	Saar	-0.00305	-0.00907	0.48274	-0.25895	-	-	-	CIMMYT
2013	Sabin	-0.55905	-0.11083	0.005016	-0.68177	-	-	-	USA
2013	SABUF/5/BCN/4/RABI//GS/CRA/3/Ae.tauschii (190)	-0.90988	0.03252	0.04253	0.11421	-	-	-	CIMMYT
2013	Scirocco	-1.13828	-0.06559	-0.34516	-0.33061	-	-	-	Germany
2013	Seniorita	1.500686	-0.05391	-0.38582	-0.32143	-	-	-	Norway
2013	SHA3/CBRD	0.287379	0.029039	-1.24708	0.299694	-	-	-	CIMMYT
2013	Sport	-0.11912	0.0019	-0.06962	-0.27294	-	-	-	Sweden
2013	Sumai #3 (12SRSN)	-0.88879	-0.02507	-0.70636	0.201945	-	-	-	China
2013	Sumai 3 (18.)	-2.03643	-0.00989	-0.68895	0.418531	-	-	-	China
2013	T10014	-1.34022	0.040498	-0.27854	-0.02392	-	-	-	Norway
2013	T7347	5.405582	0.034893	-0.478	0.482035	-	-	-	Norway
2013	T9040	-2.4628	0.060238	-0.4296	0.475548	-	-	-	Norway
2013	T9040 (1995)	2.682181	-0.04031	0.926114	-0.52395	-	-	-	Norway
2013	T9040/Paros	-1.08675	-1.93E-05	0.486439	0.07197	-	-	-	Norway
2013	Tjalve	-2.33389	0.003266	0.371592	-0.16977	-	-	-	Sweden
2013	TJALVE/Purpur seed	1.679749	0.011972	0.493996	-0.01649	-	-	-	Norway
2013	Tom	-0.93741	-0.04738	-0.10922	-0.41928	-	-	-	USA
2013	TUI/RL4137	0.267167	-0.01644	-0.23241	0.074229	-	-	-	CIMMYT
2013	Vinjett	1.797496	-0.01501	0.012355	0.053557	-	-	-	Sweden
2013	Zebra	-4.18527	-0.01214	-0.56687	0.228532	-	-	-	Sweden
2014	1175	13.41039	0.432855	16.2895	0.169415	0.545176	0.100412	0.310678	Norway
2014	1179	13.66031	0.423901	21.28773	-0.15426	0.3362	-0.23101	0.134417	Sweden
2014	1181	-0.31123	0.446866	17.42848	-0.11656	0.293354	-0.31099	-0.02034	Norway
2014	1182	-10.6001	0.492469	16.31508	0.340926	0.458506	0.035288	0.150846	Norway
2014	1183	8.688025	0.437524	17.034	0.561627	0.464275	0.272576	-0.46803	Norway
2014	1189	19.67172	0.451517	17.15502	-0.01034	0.554876	0.074977	0.024765	Sweden
2014	1192	-0.10721	0.365747	18.8662	-0.65709	0.321915	0.038721	-0.08608	Norway
2014	1193	6.278537	0.430614	17.55464	-0.01108	0.467839	0.064722	-0.10277	Sweden
2014	1303	-0.01405	0.37265	23.03865	-0.52057	0.333961	-0.37365	-0.31327	Norway

2014	1304	-3.73063	0.421511	15.11192	0.097445	0.462934	0.151813	-0.12371	Norway
2014	1305	-3.84855	0.375602	19.39452	-0.15241	0.293097	-0.34106	-0.30063	Norway
2014	1307	-1.73488	0.507571	15.56002	0.445838	0.554601	0.593549	0.591748	Norway
2014	1308	1.307288	0.44262	23.18268	-0.31973	0.303844	-0.58173	-0.27176	Norway
2014	1309	4.463277	0.483833	22.85267	-0.24412	0.244268	-0.68138	0.091295	Norway
2014	1311	1.050651	0.403949	16.92406	0.417797	0.576232	0.52579	0.162614	Norway
2014	1313	-6.84714	0.42171	15.94298	-0.07148	0.348879	-0.10469	-0.33542	Norway
2014	1317	6.641201	0.441813	15.7736	0.367912	0.518271	0.514814	0.471942	Norway
2014	1318	21.17542	0.451261	14.77488	0.137671	0.516816	0.377456	0.683612	Norway
2014	1319	7.796244	0.405856	17.22437	0.316451	0.438238	0.28135	0.306456	Norway
2014	1320	7.677353	0.418739	15.77985	0.057875	0.426259	0.455874	0.620192	Norway
2014	1323	-3.18482	0.432052	18.20851	0.049665	0.506124	0.145858	-0.07102	Sweden
2014	1324	13.34432	0.414856	15.65747	-0.34533	0.44539	0.364526	0.403274	Sweden
2014	1325	17.32927	0.45686	17.87642	0.448314	0.634709	0.345962	0.280611	Sweden
2014	1326	19.5879	0.471581	16.95676	0.007587	0.507777	-0.06026	0.033724	Sweden
2014	1327	10.11768	0.397558	19.62882	-0.43299	0.331015	-0.1543	-0.38926	Sweden
2014	1328	18.93018	0.464517	17.4379	0.823512	0.585713	0.317432	0.048132	Sweden
2014	1404	-4.90413	0.459954	15.851	0.538433	0.554294	-0.02609	0.040414	Norway
2014	1405	14.50395	0.426827	21.9951	-0.26994	0.350942	-0.44789	-0.24098	Norway
2014	1406	-6.50907	0.431139	16.77685	0.653065	0.634723	0.342717	0.396155	Norway
2014	1407	1.183133	0.454315	20.89274	0.144624	0.372911	-0.22462	-0.06205	Norway
2014	1409	-2.96092	0.468897	16.40622	-0.25331	0.365165	-0.26125	-0.1896	Norway
2014	Willy	-4.30447	0.469455	18.95481	-0.09819	0.44417	0.083855	0.172282	Norway
2014	1415	14.45951	0.453074	17.56761	0.526421	0.434134	-0.07269	0.001831	Sweden
2014	1416	-4.89135	0.403843	17.33609	-0.37983	0.296805	-0.65175	-0.50692	Norway
2014	512-70	3.193451	0.448881	25.19476	-0.35969	0.205068	-0.70465	-0.3944	Norway
2014	68.111/RGB-U//WARD/3/Ae. tauschii (454)	5.308094	0.375798	22.82685	-0.49944	0.355903	-0.17893	-0.40655	CIMMYT
2014	68.111/RGB-U//WARD/3/FGO/4/ RABI/5/Ae. tauschii (878) 206	-6.50071	0.402087	11.82948	0.992427	0.629467	0.815327	0.043937	CIMMYT
2014	68.111/RGB-U//WARD/3/FGO/4/ RABI/5/Ae. tauschii (878) 207	-6.99965	0.329002	16.34591	0.294218	0.648333	0.090414	-0.13467	CIMMYT
2014	AC Somerset	-2.77521	0.370026	16.37867	-0.38995	0.393899	0.049362	0.224081	Canada
2014	Aino	-0.97228	0.441702	20.52175	0.22441	0.439334	0.027605	-0.0452	Finland
2014	ALTAR 84/Ae. tauschii (221)	-14.141	0.457312	11.58587	1.52324	0.551612	0.954148	0.136861	CIMMYT
2014	Altar84/Ae. tauschii(219)//2*Seri	-7.183	0.424451	17.98394	0.441298	0.546567	0.286885	0.397049	CIMMYT
2014	Altar84/Ae. tauschii(219)//2*Seri/3/ Avle	-10.3767	0.49962	21.54756	0.504452	0.498088	-0.30264	-0.09005	CIMMYT
2014	Anniina	1.763257	0.399885	19.42171	0.55003	0.429998	0.245587	-0.062	Finland

2014	Arabella	6.469275	0.477229	18.4116	0.664262	0.576808	0.212833	0.319067	Poland
2014	Avle	8.269468	0.42763	15.66959	-0.226	0.416235	0.111515	-0.00506	Sweden
2014	Avocet-YrA	-6.85983	0.456343	19.78724	-0.33297	0.352687	-0.27038	-0.30156	Australia
2014	BAJASS-5	-3.31789	0.467779	17.90652	-0.16284	0.407704	-0.23988	-0.25084	Norway
2014	Bastian	-12.5931	0.490056	20.70099	-0.21876	0.373521	-0.18966	0.207919	Norway
2014	Bau/Milan -2	2.279531	0.409326	17.81223	-0.25114	0.356728	-0.0682	-0.28543	CIMMYT
2014	BCN*2//CROC_1/ Ae. tauschii (886)	-5.17591	0.399497	20.97014	-0.59501	0.239521	-0.34632	-0.24522	
2014	Berlock	16.86117	0.449236	17.08245	0.280005	0.551155	0.496139	0.528535	Sweden
2014	Berserk	-2.52653	0.418985	20.74061	-0.06015	0.316676	-0.33761	-0.12918	Norway
2014	Bjarne	-5.86274	0.511568	16.41096	0.316338	0.543901	0.021274	0.215195	Norway
2014	Bjarne/LW91W8 6	6.027008	0.412542	16.58638	0.1827	0.332029	-0.44597	-0.47591	Norway
2014	BJY/COC//CLMS/ GEN	-9.18706	0.403823	13.35815	-0.45266	0.480286	-0.07821	0.295162	CIMMYT
2014	Bombona	4.573505	0.410479	14.06948	-0.28544	0.367852	-0.08879	0.217347	Sweden
2014	Brakar	-2.05573	0.340821	23.6606	-0.45603	0.486467	0.074278	-0.00598	Norway
2014	Brakar	-3.35421	0.436238	19.46615	0.523895	0.46965	0.024491	0.163199	Norway
2014	C80.1/3*QT4522 //2*ATTILA	0.371593	0.435941	24.60537	-0.07606	0.432964	-0.03483	-0.08804	CIMMYT
2014	C80.1/3*QT4522 //2*PASTOR	2.460703	0.341169	19.07291	-1.60052	0.145743	-1.84708	-0.20912	CIMMYT
2014	Catbird	5.017082	0.432436	18.94853	-0.44983	0.240279	-0.43599	-0.49771	CIMMYT
2014	CD87	-7.80306	0.461173	21.90732	-0.07041	0.280983	-0.62128	-0.05077	Australia
2014	CETA/Ae. tauschii (895)	-19.85	0.411163	13.12248	1.281657	0.759167	1.116992	0.91824	
2014	Chara	0.469419	0.41705	22.25162	-0.84037	0.263641	-0.67789	-0.60474	Australia
2014	CJ9306	-5.1346	0.447772	15.91378	-0.48959	0.481414	0.133476	0.120002	China
2014	CJ9403	-0.56567	0.39643	27.29228	-1.69888	0.216067	-1.41223	-0.52352	China
2014	Croc_1/Ae.tausc hii (205)//Kauz	-5.2825	0.434925	20.48212	0.898682	0.518001	0.561542	-0.13192	CIMMYT
2014	Demonstrant	2.344053	0.422724	18.1907	-0.85703	0.286484	-0.3046	-0.2819	Norway
2014	DH49-18	-8.64071	0.432742	24.96867	-0.03469	0.343421	-0.40585	-0.08149	Norway
2014	Dragon	10.60619	0.419891	17.47514	-0.06466	0.597704	0.526645	0.186909	Sweden
2014	DUCULA (X1)	-2.19118	0.448436	19.2415	-0.2754	0.391924	0.162753	0.697727	CIMMYT
2014	DUCULA (X2)	-4.2358	0.41814	16.75659	0.19967	0.486766	0.096905	0.046696	CIMMYT
2014	DUCULA (X3)	-13.1165	0.381791	14.61013	-0.0132	0.586111	0.045995	-0.29839	CIMMYT
2014	DUCULA (X4)	-11.2189	0.350486	15.90272	-0.31386	0.562551	0.4218	0.208271	CIMMYT
2014	Dulus	-13.2293	0.389194	14.48723	-0.40037	0.504107	0.404637	0.063654	CIMMYT
2014	DVERD_2/Ae. tauschii (221)	-2.31791	0.457081	11.78328	0.791883	0.564048	1.045716	0.348689	CIMMYT
2014	Filin	-6.38313	0.387526	15.59178	-0.21428	0.6406	0.264818	-0.04981	CIMMYT
2014	Fram II	-0.45347	0.407117	20.2535	-0.10782	0.280955	-0.41759	0.042666	Norway
2014	Frontana	-5.71325	0.368253	14.9159	0.5839	0.654839	0.200074	-0.09118	Brazil
2014	GONDO	2.4774	0.410904	14.93053	-0.35941	0.336086	-0.1554	-0.43794	CIMMYT
2014	Gondo	9.978017	0.399074	17.86533	0.422259	0.44784	0.311805	-0.12575	CIMMYT
2014	HAHN/PRL//AUS 1408	-10.4847	0.469229	21.73876	-0.54934	0.508913	-0.24286	-0.02226	
2014	J03	-4.43403	0.437034	19.08014	0.248331	0.393037	-0.24468	0.067958	Norway

2014	Kariega	-3.65484	0.387044	27.60061	-1.31004	0.331857	-0.87225	0.129019	South Africa
2014	Krabat	4.524904	0.500121	16.98053	-0.11439	0.51727	0.18875	0.130807	Norway
2014	Kruunu	-1.52063	0.442694	17.30292	0.457289	0.508236	0.153679	0.395345	Finland
2014	Kukri	-0.54116	0.440393	14.92544	0.870578	0.630188	0.556416	0.364744	Australia
2014	Laban	11.53348	0.422619	15.7394	0.239891	0.584523	0.480673	0.481966	Norway
2014	Marble	21.91319	0.390748	20.53582	0.208872	0.516631	0.181578	0.011932	Finland
2014	MAYOOR//TK SN1081/Ae.tauschii (222)	0.297635	0.383574	17.60159	0.304253	0.533421	0.294476	-0.56171	CIMMYT
2014	Milan	-2.34232	0.418488	20.90892	-0.24873	0.419381	-0.05663	-0.41524	CIMMYT
2014	MILAN/SHA7	-2.03142	0.409959	21.06268	-0.09958	0.456484	0.300372	-0.4884	CIMMYT
2014	Mirakel	9.159597	0.379898	16.54052	0.348902	0.63239	0.864996	0.730523	Norway
2014	Møystad	-1.60314	0.388731	15.00226	-0.24302	0.437289	0.188055	0.14961	Norway
2014	Nanjing 7840	-3.75847	0.432073	16.39348	-0.00218	0.544636	0.319815	-0.04383	China
2014	Naxos	-5.07403	0.40261	25.10146	0.433983	0.490878	-0.25741	-0.00667	Germany
2014	Naxos/2*Saar	-15.6955	0.465103	19.95796	0.488212	0.451083	0.458983	-0.17499	Norway
2014	Ning 8343	4.816247	0.354146	17.45173	-0.85818	0.203103	-0.76196	-0.62231	China
2014	NK00521	4.169316	0.505247	24.03747	-0.36081	0.194926	-0.8632	-0.64826	Norway
2014	NK01513	-0.22441	0.431485	16.7038	-0.41836	0.398491	-0.17788	-0.03435	Norway
2014	NK01565	6.426055	0.481161	14.57198	0.048898	0.418045	0.144641	0.111179	Norway
2014	NK93602 (1995)	-1.16577	0.458169	16.44285	0.772082	0.590599	0.380796	0.02344	Norway
2014	NK93604	-5.09312	0.523274	13.09845	0.66758	0.551923	0.057558	0.211001	Norway
2014	Nobeokabouzu	-2.666	0.319868	21.24768	-1.09271	0.200234	-0.45863	-0.30134	China
2014	Norrøna	1.566618	0.410897	19.60447	-0.12885	0.3967	-0.13689	0.198669	Norway
2014	Paros	0.636782	0.448059	22.75443	0.990679	0.40724	-0.12032	-0.4932	Germany
2014	Paros/NK93602	-6.15553	0.38417	14.74757	0.368588	0.629226	0.308042	0.163568	Norway
2014	Polkka	-1.26855	0.406196	17.67423	-0.19465	0.38876	0.007074	0.105595	Sweden
2014	PRL/SARA	-10.3559	0.411289	14.92258	0.543429	0.581509	0.64814	0.563686	CIMMYT
2014	QUARNA	5.000872	0.349442	25.91667	-0.89297	0.228325	-0.97568	-0.65937	Switzerland
2014	RB07	-2.35875	0.456755	17.67829	0.138707	0.580604	0.480855	0.225345	USA
2014	Reno	-0.08389	0.376545	17.22223	0.07994	0.504626	0.103391	0.350205	Norway
2014	Rollo	3.466275	0.473762	22.18841	0.143666	0.389053	-0.16369	0.078058	Norway
2014	Runar	-5.11727	0.488902	20.14433	-0.24375	0.428114	-0.12199	0.06649	Norway
2014	Saar	-7.11519	0.421117	22.35173	-0.68263	0.290286	-0.41407	-0.31827	CIMMYT
2014	Sabin	-5.44215	0.361696	21.01792	-0.38986	0.296776	-0.58423	-0.51291	USA
2014	SABUF/5/BCN/4/RABI//GS/CRA/3/Ae.tauschii (190)	4.092646	0.387701	19.70231	-0.11774	0.378086	-0.18447	-0.66709	CIMMYT
2014	SCA/Ae. tauschii (409)	-3.14038	0.50883	16.01588	0.056104	0.56218	0.707102	0.819008	CIMMYT
2014	SCA/Ae. tauschii (518)	-7.84778	0.372484	23.30219	0.004301	0.379622	-0.0733	0.1888	CIMMYT
2014	Scirocco	-9.07458	0.377601	21.65705	0.266333	0.407597	-0.22217	-0.18124	Germany
2014	Seniorita	2.333907	0.445969	17.10055	0.332762	0.405366	0.029113	0.243115	Norway
2014	SHA3/CBRD	0.338083	0.372251	19.46271	-0.70548	0.322473	-0.512	-0.73527	CIMMYT
2014	Sport	-4.73624	0.391817	16.17783	-0.13939	0.44918	0.109165	-0.07953	Sweden

2014	Sumai #3 (12SRSN)	-3.33947	0.340258	17.79955	-0.22877	0.428472	0.282755	0.015758	China
2014	Sumai 3 (18.)	3.640165	0.373334	16.95919	-0.1512	0.39978	-0.09688	-0.22023	China
2014	T10014	-3.47651	0.483479	14.42688	0.379002	0.556709	0.460388	0.450038	Norway
2014	T2038	-5.88807	0.442669	13.48887	0.057123	0.652026	0.325516	0.512402	Norway
2014	T7347	6.005738	0.419287	15.59089	0.603892	0.344562	-0.31444	-0.23473	Norway
2014	T9040	-0.22044	0.45235	18.30643	-0.30203	0.368894	-0.15154	-0.00995	Norway
2014	T9040 (1995)	-3.12609	0.354985	23.40824	-0.43346	0.305148	-0.32186	-0.05215	Norway
2014	Tjalve	-3.42192	0.414321	18.5022	0.248772	0.388034	-0.20951	-0.17046	Sweden
2014	TJALVE/Purpur seed	-1.28908	0.391199	23.262	0.036535	0.383331	-0.15834	-0.50095	Norway
2014	VEE/MYNA	-4.65602	0.380417	13.08582	0.393056	0.648131	0.705417	0.470842	CIMMYT
2014	Vinjett	15.67185	0.444908	16.28498	0.003505	0.579029	0.146809	0.197351	Sweden
2014	Wanamo	1.482873	0.487102	15.23102	0.177123	0.39734	-0.02663	-0.25432	Finland
2014	Wellamo	-4.26737	0.423199	18.0594	-0.43978	0.371049	-0.14936	-0.20425	Finland
2014	Zebra	-4.18526	0.417208	15.4505	-0.15074	0.586968	0.284897	0.636788	Sweden

Table S4. Mean percentage of clay, silt and sand, bulk density (4-9 cm depth) and porosity, pH, total carbon and nitrogen as well as contents of P, K, Ca, Mg and Mn at the locations of the experimental site. Abbreviations: c, control; wl, waterlogged. Values for bulk density and soil porosity was determined from six cylinders (two from each replicate) sampled at the borders of the locations in 2014. Soil texture, pH and chemical contents were determined in one sample for each location. Each of these four samples were consisted of sixteen, evenly distributed spot samples across the locations.

Location	Clay	Silt	Sand	Soil texture type	Bulk density ($g^{-1}cm^3$)	Soil porosity	pH	Tot. C %	Tot. N %	P mg^{-1} kg	K mg^{-1} kg	Ca mg^{-1} kg	Mg mg^{-1} kg	Mn mg^{-1} kg
N (wl)	25	58	18	silty loam	0.95	45	6.1	8.2	0.5	130	130	2500	250	14.2
N (c)	29	55	16	silty loam	0.80	55	5.9	6.3	0.4	160	130	2200	230	6.7
S (wl)	21	53	26	silty loam	0.93	50	5.5	6.5	0.4	140	88	3700	250	19.5
S (c)	14	38	48	medium loam	1.16	38	5.5	3.9	0.3	150	120	1700	100	14.2

Table S5. Redox potentials (Eh) ± the standard deviation measured at 4-5 cm depth at location N (north) and S (south) in 2014 to 2016.

	2014	2015		2016	
Location	N	N	S	S	N
3 days waterlogging	181±34	-	-	291±21	264±36
5 days waterlogging	-	-	-	282±10	290±33
8 days waterlogging	202±41	-	-	-	-
9 days waterlogging	-	213±37	219±33	-	-

Table S7. Correlations of traits in wheat (2013 and 2014). Abbreviations: CL, chlorosis; OC, overall condition score; HD, heading delay; PH, relative plant height; GBM5, green biomass score 5 days after drainage; GBM19, green biomass score 19 days after drainage; HN, relative head number.

2013		CL	PH	HD	OC		
	CL	-	-	-	-		
	PH	-0.27**	-	-	-		
	HD	0.47***	-0.27**	-	-		
	OC	-0.42***	0.52***	-0.51***	-		
2014		**CL**	**GBM19**	**PH**	**GBM5**	**HN**	**HD**
	CL	-	-	-	-	-	-
	GBM19	0.14	-	-	-	-	-
	PH	0.01	0.13	-	-	-	-
	GBM5	0.05	0.64***	0.29**	-	-	-
	HN	0.05	0.85***	0.25**	0.66***	-	-
	HD	-0.10	-0.68***	-0.13	-0.47***	-0.57***	-
	OC	0.08	0.64***	0.40***	0.40***	0.67***	-0.38***

Table S6. Correlations of traits in barley (2013 and 2014). Abbreviations: CL, chlorosis; OC, overall condition score; HD, heading delay; PH, relative plant height.

2013		CL	OC	PH	HD
	CL	-	-	-	-
	OC	-0.34***	-	-	-
	PH	-0.06	-0.07	-	-
	HD	0.43***	-0.26**	-0.37***	-
2014		**CL**	**OC**	**PH**	**HD**
	CL	-	-	-	-
	OC	-0.08	-	-	-
	PH	-0.40***	-0.06	-	-
	HD	-0.37***	0.05	0.55***	-
	HN	0.00	-0.04	-0.11	-0.34***

Table S8. Correlations of traits in wheat (2015 and 2016). Abbreviations: CL, chlorosis; OC, overall condition score; HD, heading delay; PH, relative plant height; S, relative straw yield; HN, relative head number.

2015		**CL**	**OC**	**PH**	**HD**	**S**	**HN**
	CL						
	OC	-0.79**					
	PH	-0.86***	0.57				
	HD	-0.60*	0.50	0.61*			
	S	-0.77**	0.71*	0.85**	0.56		
	HN	-0.61*	0.78**	0.47	0.59	0.58	
2016		**CL**	**OC**	**PH**	**HD**	**S**	**HN**
	CL						
	OC	-0.62*					
	PH	0.00	0.47				
	HD	0.20	0.44	0.42			
	S	-0.50*	0.51*	0.43	-0.34		
	HN	-0.76***	0.43	-0.23	-0.53*	0.61*	

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Rapid seedling establishment and a narrow root stele promotes waterlogging tolerance in spring wheat

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ABSTRACT

Improving the waterlogging tolerance of wheat varieties could alleviate yield constraints caused by excessive rain and poor soil drainage. In this study, we investigated root and shoot growth as well as anatomical traits of six spring wheat genotypes with contrasting waterlogging tolerance properties. Our aim was to identify root traits that differentiate tolerant from sensitive genotypes. Two experiments were conducted using rhizoboxes and photography for data acquisition. In experiment one, root growth of the genotypes was studied during seedling establishment and a subsequent waterlogging treatment, starting at the 3-leaf stage and maintained for seven days. In the second experiment, root and shoot growth of previously waterlogged plants was compared between the genotypes during seven days of recovery. At harvest of experiment two, root segments were sampled to investigate genotype differences of root cross sectional area, root cortex area, stele area and percentage of aerenchyma. The results show that tolerant, in contrast to sensitive genotypes, developed seminal roots faster in the seedling establishment phase and more nodal roots during the waterlogging treatment. NK93602 and Bjarne were the best performing genotypes. Bjarne in particular had a narrower relative stele size of nodal (13.4%) and seminal roots (11.7%) compared to other genotypes (e.g. 16.3% in nodal roots and 13.9% in seminal roots of sensitive Quarna). The results from this study suggests that early vigor is an important trait for waterlogging tolerance in the field. Anatomical root traits, such as a narrow stele and aerenchyma may contribute to improving waterlogging tolerance furthermore.

1. Introduction

Improving the waterlogging tolerance of dryland crops is becoming increasingly important as climate change is projected to increase the precipitation and the frequency of floods and heavy rainfalls in parts of the world (Parry et al., 2007; Barua et al., 2014; Bailey-Serres et al., 2012). Wheat (*Triticum aestivum* L.), one of the world's staple crops, is poorly adapted to waterlogging and substantial yield losses may be a consequence. The severity of the stress and subsequent yield loss depends on factors such as the duration of the event (Marti et al., 2015), the developmental stage at the onset (De San Celedonio et al., 2014), soil and climate conditions (Mcdonald et al., 2006; Watson et al., 1976) as well as the genetic background. Genotypes of wheat are known to tolerate waterlogging stress differently (Mcdonald et al., 2006; Sayre et al., 1994), and the grain yield loss of waterlogged, relative to drained controls may vary from 18 to 81% (Setter et al., 1999). Improving the waterlogging tolerance of wheat has been a longstanding objective. Yet, the advances have been limited, likely due to the complexity of the trait and the dependency on environmental conditions.

Oxygen deficiency in the rhizosphere is the dominant cause of waterlogging stress. It arises as water fills the soil pore space, causing either complete absence (anoxia), or partial absence of oxygen (hypoxia) (Ricard et al., 1994). Until the soil drains, re-supply from the atmosphere will be limited and other gases such as ethylene may accumulate as a result of impeded gas diffusion (Sasidharan and Voesenek, 2015). Plant cells exposed to anaerobic conditions convert to anaerobic respiration and the ethanolic fermentation pathway (Ricard et al., 1994). The conversion curtail ATP production and a subsequent energy shortage can cause cell death or limit energy demanding processes such as nutrient uptake (Colmer and Greenway, 2011), photosynthesis and growth (Malik et al., 2001).

Maintaining oxygenated conditions of the root apices is crucial for stress prevention in the roots. Rice and other plant species native to wetlands may constitutively form intercellular gas spaces in the roots

Abbreviations: C, control; WL, waterlogging; 2dfp-11dfp, 2–11 days from planting; 1dWL-7dWL, 1–7 days of waterlogging; 1dyr-7dyr, 1–7 days of recovery; RCS, root cortical senescence

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known as aerenchyma (Mcdonald et al., 2002). Induced by ethylene and reactive oxygen species (ROS), aerenchyma, sometimes reported as root porosity, is also commonly found in waterlogged roots of wheat (Xu et al., 2013; Yamauchi et al., 2014b) and barley (Broughton et al., 2015; Zhang et al., 2016). Aerenchyma have been associated with stress alleviation (Huang et al., 1994b; Thomson et al., 1992) and maintenance of yield (Setter et al., 1999). However, it is also evident that the mere presence of aerenchyma does not enable cereal crops to persist waterlogging stress. For instance, Zhang et al. (2016) investigated the relationship between a waterlogging tolerance score and a visual aerenchyma score of nodal roots. A significant correlation between scores of aerenchyma and waterlogging tolerance was found among 177 double haploid barley lines, but the correlation coefficient (r) was limited to 0.2 after 7 days of anaerobic stress treatment. In a QTL-mapping study, Broughton et al. (2015) found root porosity of barley nodal roots to be positively correlated with several plant growth parameters including fresh, dry, relative and absolute weight of roots and shoots. Root porosity was significantly correlated with several of these traits (r = 0.25 at the most, for root fresh weight). The authors identified a QTL which was determined to be syntenic with the submergence tolerance gene *Sub1* in rice, and a QTL (*Qaer1.02-3*) associated with aerenchyma in maize. The QTL in question accounted for 39% of the phenotypic variation in root porosity. Similarly, a QTL identified by Zhang et al. (2016) accounted for 44% of the genotypic variation. These results indicate the relevance of aerenchyma for waterlogging tolerance, and that additional traits are likely also involved.

Aerenchyma have been found in both seminal and nodal roots of wheat. However, the extent of it and the ability to act as a diffusion pathway appears to be age and length limited. Thomson et al. (1990) found that neither seminal nor nodal roots that were longer than 200 mm increased in porosity if they had emerged in aerobic nutrient solution and then transferred to anaerobic conditions. Barrett-Lennard et al. (1988) and Trought and Drew (1980) found that nodal roots did not exceed a certain length when emerging under anaerobic conditions. According to their own assumptions, as well as to theory cited by them (Armstrong, 1980), the elongation ceased when the oxygen supply to the root tip was limited by radial oxygen loss (ROL) and internal consumption. Similarly, Huang et al. (1997) found contrasting abilities of two wheat genotypes to increase the root porosity in nodal roots that already existed when a hypoxic treatment was imposed. While seminal roots may cease growth or even senesce under anaerobic conditions (Malik et al., 2002; Thomson et al., 1990), an increased number of nodal roots per tiller, or per unit shoot fresh weight may be found (Malik et al., 2001; Watkin et al., 1998; Thomson et al., 1992). Nodal root emergence is likely triggered by ethylene (Voesenek and Sasidharan, 2013) and is considered as a beneficial trait as they may partly compensate for the loss of functionality of seminal roots (Thomson et al., 1992).

The longitudinal O_2 diffusion and the functionality of aerenchyma could further be improved if coupled with a barrier to oxygen loss (ROL) (Colmer, 2003). Suberization of the hypodermis to prevent ROL is common among wetland species but comparably absent in common wheat. *Hordeum marinum*, a wild relative to wheat and native to wetlands, have a strong ROL barrier (Mcdonald et al., 2001). Through wide hybridization, the trait has been successfully transferred to *H. marinum*-wheat amphiploids (Malik et al., 2011) but further work would be necessary in order to improve the fertility of the offspring (Islam et al., 2007). The prospects of introducing ROL barrier traits from *H. marinum* to wheat was further dismissed by Konnerup et al. (2017), who found that a barrier to ROL was not expressed in disomic addition lines produced from *H. marinum*-wheat amphiploids, nor did they develop more aerenchyma than their wheat parents. Moreover, it is unclear whether improving the barrier to ROL entail tradeoffs such as reduced water and nutrient uptake, or impediments for O_2 entry in the roots once the conditions are oxygenated (Colmer and Greenway, 2011).

Oxygen that is not lost through ROL diffuses in a source-sink manner within roots, where the oxygen concentration may decrease both longitudinally and radially (Armstrong, 1980). The stele tissue is considerably more dense than the surrounding cortex, and stelar anoxia can arise at oxygen concentrations otherwise sufficient for aerobic respiration in cortical cells (Gibbs et al., 1998). The respiration rate of stele tissue may be several times higher than the cortex (Aguilar et al., 2003) and modelling have suggested that a narrower stele may prevent stelar anoxia (Armstrong and Beckett, 1987). Thicker roots and a large cortex area could also be beneficial traits as it would provide more space where aerenchyma can develop (Yamauchi et al., 2014a; Visser et al., 2000).

The majority of previous physiological studies related to anaerobic stress have focused on root and shoot growth under or immediately after the treatment (Striker, 2012). A period of subsequent recovery is less often considered although the ability to recover may differ among genotypes of both wheat (Huang et al., 1994b) and barley (Pang et al., 2004). Contrasting abilities to recover has also been found in field experiments. For instance, Sundgren et al. (2018) found that foliar chlorosis recorded during a waterlogging treatment, did not necessarily correspond with the ability to recover aboveground biomass growth post treatment.

We hypothesize that anatomical root traits of waterlogging sensitive and tolerant wheat genotypes differ and that these traits influence their ability to tolerate waterlogging. We tested this hypothesis by conducting a controlled environment study, investigating the root properties of six spring wheat genotypes with contrasting waterlogging tolerance. Our objective has been to identify root growth and anatomical traits or characteristics that may contribute to the presumed waterlogging tolerance of these genotypes. Two separate greenhouse experiments were conducted to undertake this objective. The first experiment included a seedling establishment phase and a seven days long waterlogging treatment. In the second experiment, the genotypes were compared in their ability to recover from the previous waterlogging treatment.

2. Materials and methods

2.1. Plant material

Six genotypes were used in the experiments, three considered to be tolerant (Bjarne, Zebra and NK93602) and three sensitive (Naxos, Quarna and T9040) to waterlogging. The assumed tolerance properties of the genotypes have been determined in a previous hillplot field screening trial conducted in Norway (Sundgren et al., 2018). Briefly, the tolerance of the genotypes was determined based on measurements of plant height (height under waterlogged conditions relative to drained conditions), the relative number of spikes, the delay in heading date and visual scores of foliar chlorosis as well as a score reflecting their condition at maturity, thus indicative of their ability to recover and to produce yield. Bjarne, NK93602 and T9040 are genotypes with Norwegian origin, while Zebra originates from Sweden, Naxos from Germany and Quarna from Switzerland. Evenly sized seeds were pre-germinated for approximately 65 h in a dark growth cabinet (20 °C) on petri dishes with moist filter paper before transplanting them to the rhizoboxes.

2.2. Growth conditions, preparation of rhizoboxes and experimental setup

The described experiments were conducted in a greenhouse facility at Institute of Bio- and Geosciences (IBG-2 (Plant Sciences); Forschungszentrum Jülich GmbH, Jülich, Germany), at 16 h day length and day/night temperatures of ~20/18 °C. Plants were grown under natural light conditions and supplied with artificial lighting (SON-T AGRO 400, Philips, Amsterdam, The Netherlands) to maintain light intensity > 300 µmol photons $m^{-2} s^{-1}$.

The rhizoboxes used in the experiments were constructed by molded

plastic frames with one side covered by a transparent and removable polycarbonate plate. Each rhizobox (length: 58, depth: 2.1, width: 26.5) was manually filled with 6.2 Liters of peat soil (Graberde; Plantaflor Humus Verkaufs GmbH, Vechta, Germany) containing 150 mg L^{-1} N; 70 mg L^{-1} P_2O_5; 300 mg L^{-1} K_2O. The rhizoboxes were filled with soil in the following manner: 1.2 L of soil were filled and compacted for every 11 cm up to 44 cm. The top 12 cm of the rhizoboxes were filled with soil and compacted four times: two times with 0.5 L and two times with 0.2 L. The height of the soil reached 56 cm. The rhizoboxes were placed in an inclined position (approx. 45°) in plastic boxes to stimulate root growth along the transparent side. 350 mL of water was added to each rhizobox the day before sowing.

In the first experiment, root and shoot growth were observed under waterlogged and control conditions. It included a seedling establishment phase and a seven days long waterlogging treatment. The experiment was conducted as a split-plot with the six genotypes as subplots and treatment (control and waterlogging) as main plots. The waterlogging treatment was imposed at the three leaf stage (15 days old plants). Slight variations in the developmental stage among the genotypes were recorded (Table S1). Waterlogging was simulated by filling the rhizoboxes and the plastic boxes they were placed in with water. As the rhizoboxes were not air-tight, water sieved in from the sides. Water was added from the top of the rhizoboxes regularly to maintain saturation. The rhizoboxes were moved to deeper plastic boxes on the second day of the treatment. This was done in order to ensure anaerobic conditions at all times and to minimize manual watering from the top.

Plants that had been treated with waterlogging were maintained for the second experiment, where the aim was to compare the genotypes' ability to recover from the waterlogging treatment. The second experiment was conducted as a randomized complete block design with the six genotypes and six replicates. Control treatment was excluded and the climatic conditions were equal to the preceding experiment.

2.3. Oxygen measurements

Two extra sets of six rhizoboxes were used for oxygen measurements. Half of them were used to measure oxygen concentration under waterlogged conditions and the other half for control values. Seeds of the six genotypes were pre-germinated and transplanted to the rhizoboxes as described above. At the three leaf stage, the transparent plate of the rhizobox was unscrewed and three 1 × 1 cm oxygen sensitive sensor foils (SF-PSt3, PreSens Precisions Sensing GmbH, Regensburg, Germany), were attached with a thin layer of silicon glue on the side facing the soil. Sensor foils were attached at three positions (8, 29 and 51 cm depth) in each of the twelve rhizoboxes. The sensor foils were calibrated in oxygen-free water and water brought to oxygen equilibrium with atmospheric air (approx. 8.9 mg O_2/L at 23 °C) prior to usage. Oxygen-free water was obtained by dissolving 1 g of sodium sulfite (Na_2SO_3) in 100 mL water. Water which was in oxygen equilibrium with atmospheric air was obtained by bubbling 100 mL of water with air for approximately 20 min and then left for another 10 min.

Measurements were made using a fiber optic oxygen transmitter (Fibox 4, PreSens Precisions Sensing GmbH, Regensburg, Germany) 1 day before the treatment started, on days 1–4 and 6–7 of waterlogging and after 1, 5 and 6 days of recovery. The mean and standard error of the oxygen concentration in percentage was calculated based on 3–4 measurements per sensor foil in each layer of the twelve rhizoboxes. The treatment and growth conditions of these rhizoboxes were identical to the conditions already described.

2.4. Image acquisition and analysis

Images of the root systems were acquired by placing the rhizoboxes in the opening of a closed photo compartment, custom-built for image acquisition of rhizoboxes. Images were taken every second day in the first experiment, beginning from 2 days after planting and until the last day of treatment. In second experiment, roots were photographed after 2, 3, 5 and 7 days of recovery. The camera used was a standard DSLR camera (Canon 70D EF 14 mm f/2.8 with fixed focal length) mounted in a fixed position inside the box. LED-lights installed in the box ensured even illumination. Root length as depictured on the root images was determined by manually tracing the roots using a custom-made software, previously described by Nagel et al. (2012).

In the first experiment, shoot images were acquired of control and waterlogged plants after 1, 3, 5 and 7 days of waterlogging. In the second experiment, previously waterlogged plants were photographed after 2, 3, 5 and 7 days of recovery. The rhizoboxes were placed towards a wall with the sides facing a DSLR camera (Canon 70D EF 14 mm f/20 with fixed focal length) on a tripod placed in a fixed position, 78 cm from the wall.

The image processing pipeline included image conversion, segmentation and mask analysis. Raw images were converted into 3 channel RGB color images. Image segmentation was done pixelwise by a Support Vector Machine (SVM) approach, a previously trained maximum margin classifier using features from HSV color space. The SVM classifier is trained once before starting the image processing pipeline with user marked examples for fore- and background color values.

The binary masks calculated by the segmentation were finally cleaned from small artifacts and holes by using connected-component labeling. Based on these binary masks, projected leaf area and the number of leaf tips were calculated by the software. Images acquired at 1dWL (1 day of waterlogging) was used to determine the number of leaves of each plant (Table S1).

2.5. Harvest, root sampling and determination of anatomical features

On the final day of the second experiment, shoots were separated from the roots at the root-shoot junction to allow for comparison of the shoot biomass recovery of the genotypes. Fresh weight was recorded before the shoots were separated into leaves and tillers and scanned with a leaf area meter (LI-COR Li-3100, LI-COR Inc., Lincoln, NE, USA). Dry weight was recorded after the plants had dried for 3 days at 60 °C.

Roots from plants in the second experiment were carefully washed clean from soil residues. Seminal and nodal roots were separately scanned and analyzed using WinRHIZO (Regent Instruments, Inc., Quebec City, QC, Canada) (gray scale classification method, manual threshold gray value 210, 3 diameter classes, debris removal threshold < 0.01 cm^2). Three samples, 2 cm in length, taken from the longest seminal and the longest nodal root of each plant were preserved in 70% ethanol. To account for varying root lengths, sampling was standardized in a relative manner rather than absolute. That is, three samples were taken at the exact middle of each root, at the exact middle of the basal half of the root and in the exact middle of the distal half of the root. Thus, sampling area was individually determined and adjusted according to the length of each root.

Each root segment was hand cut under a stereo microscope (Leica S8 APO, Leica Microsystems CMS GmbH, Wetzlar, Germany) into thin sections using a double edge razor blade. Images of transverse root sections were acquired on a light microscope at 10× magnification (Leica Microsystems, CMS GmbH, Wetzlar, Germany) and analyzed for anatomical traits with ImageJ (Rasband, 1997). The cross sectional area and stele area was measured by manually tracing the contours in the images. Cortex area was determined by subtracting the stele area from the cross sectional area. Aerenchyma was determined by manually tracing the area of aerenchyma in each image and calculating it as a percentage of the cortex area. The mean of anatomical traits of each root zone was calculated based on two images of six individual plants per genotype of both seminal and nodal roots.

2.6. Statistical analysis

All data were analyzed using R 3.3.3 (R Core Team, 2017). Analysis

of variance (ANOVA) and Tukey's Honest Significant Difference (HSD) were performed using the package "mixlm" (Liland and Sæbø, 2017). Data of root and shoot growth retrieved from the images were analyzed separately for each day images had been acquired. Root and shoot growth data in the first experiment was analyzed with the model $Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \tau k(_i) + \varepsilon_{ijk}$, where μ is the overall mean, αi the effect of *ith* treatment (waterlogged or control), β is the effect of the *jth* genotype, αβ is the interaction effect of genotype and treatment, τk is an error term for the treatment factor and ε is the model error term. Root and shoot growth data acquired in the second experiment was analyzed without the treatment factor, hence the model was simplified to $Y_j = \mu + \beta_j + \varepsilon_j$.

Data of root anatomical traits (aerenchyma, cortex, stele and cross sectional area) was analyzed with a sampling zone factor as well as an interaction term of genotype x sampling zone; $Y_{ij} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \varepsilon_{ij}$. Here, μ is the overall mean, α the effect of *ith* genotype, β is the effect of the *jth* sampling location, αβ is the interaction effect of the *ith* genotype and *jth* sampling zone and ε is the model error term. To determine whether anatomical traits depended on the length of individual roots, a factor of root length was initially included in the model. As root length did not have a statistical effect on the traits, the factor was thus left out from the final model. Root anatomical traits of seminal and nodal roots were analyzed separately.

3. Results

3.1. Oxygen measurements

The oxygen concentration in the upper layer of non-treated rhizoboxes was 19–20%, while the concentration in the middle and lower layer ranged between 17 and 20% (Fig. S1). The oxygen concentration in waterlogged rhizoboxes decreased to 0% in the lower and middle layer on the first and third day, respectively. The oxygen measured in the upper layer decreased to approximately 15% on the first day, to less than 8% on the fourth and sixth day, and to 0% on the seventh day of waterlogging. The upper and middle soil layer returned to 20 and 18% oxygen upon drainage. The lower soil layer remained at 0% oxygen for the rest of the experimental period.

3.2. Seminal root growth

The total root length determined in images at harvest of experiment two was strongly correlated with root length determined by the WinRHIZO system ($R^2 = 0.82$, $p < 0.001$).

Similar total seminal root length increase was observed for all genotypes and both treatments during seedling establishment (2dfp-1dWL, Fig. 1) in experiment one. Zebra was an exception as control plants of this genotype had a smaller total seminal root length increase from 8dfp (8 days from planting) to 1dWL (one day of waterlogging). The increase in mean total seminal root length per day during seedling establishment was significantly ($p < 0.001$) larger for the tolerant genotypes Bjarne (5.9^{ab} cm day^{-1}) and NK93602 (6.9^{a} cm day^{-1}) compared to the sensitive genotypes Naxos (4.0^{cd} cm day^{-1}), Quarna (3.8^{cd} cm day^{-1}) and especially T9040 (2.6^{d} cm day^{-1}). Mean total seminal root increase for tolerant Zebra (5.0^{bc} cm day^{-1}) was significantly larger than for T9040.

The mean total seminal root length one day before the treatment started was significantly larger for Bjarne and NK93602 and the lowest for T9040 (experiment one). Plants of Zebra designated for the waterlogging treatment had an equally large total seminal root length as Bjarne and NK93602. At 3dWL (3 days of waterlogging), seminal root growth stagnated for all genotypes. A significant treatment effect and interaction effect (genotype x treatment) was detected for Bjarne at 3dWL. As the treatment progressed, the interaction effect became significant also for Quarna and NK93602 at 5dWL and 7dWL (5 and 7 days of waterlogging), respectively. Genotypes did not differ in the growth rate during the treatment.

In experiment two, total seminal root length increased for all genotypes after two days of recovery (2dyr, Fig. 2), clearly indicating that all genotypes were able to resume seminal root growth. Total seminal root length continued to increase until the recovery phase ended. The increase in total seminal root length per day was the highest for sensitive Quarna (6.5 cm day^{-1}) and was significantly larger than for sensitive T9040 (2.9 cm day^{-1}). After seven days of recovery, the tolerant genotypes NK93602, Bjarne, Zebra and sensitive Quarna had a significantly larger seminal root length than sensitive Naxos and T9040 (Fig. 2). The length of the longest individual seminal root was shorter for Naxos and T9040 compared to NK93602, Bjarne and Quarna (Table S1). In contrast to Zebra and NK93602, Naxos and T9040 also had a lower number of seminal roots (Table S1).

3.3. Nodal root growth

Nodal roots were detected in experiment one after one (Bjarne, Zebra, Naxos and T9040) or three (NK93602 and Quarna) days of waterlogging (Fig. 3). Zebra, T9040 and Quarna developed nodal roots earlier on waterlogged plants than on corresponding control plants. The increase in total nodal root length per day was significantly ($p < 0.001$) larger for tolerant Bjarne (3.9^{a} cm day^{-1}) and Zebra (4.0^{a} cm day^{-1}), compared to sensitive Quarna (1.7^{c} cm day^{-1}) and T9040 (2.0^{bc} cm day^{-1}). A significant treatment effect was detected at 5dWL, where the mean total nodal root length of waterlogged plants was 10.7 cm, while total nodal root length of control plants was 5.3 cm. There was no significant interaction effect between genotype and treatment. A significant genotype effect (the mean of control and waterlogged for each genotype) was however detected at 3-, 5-, and 7dWL. At 7dWL, tolerant Zebra had a significantly larger total nodal root length than sensitive Quarna and Naxos as well as NK93602 (tolerant).

Total nodal root length did not differ significantly between genotypes during the recovery phase in experiment two (Fig. 4). At harvest, Zebra, NK93602 and Bjarne had a significantly higher number of nodal roots compared to Naxos and Quarna (Table S1). The length of the longest individual nodal root was also shorter for Naxos compared to NK93602.

3.4. Anatomical root traits

A significant genotype and root zone effect was detected for stele area in mm^2, the stele area relative to the cortex and for percentage aerenchyma in nodal and seminal roots (Tables 1 and 2). There was a significant difference among genotypes for cross sectional area and cortex area in mm^2 in nodal roots but not in seminal roots. Bjarne was different from most other genotypes by having a smaller stele in both nodal and seminal roots. A significant interaction effect (root zone x genotype) of waterlogged nodal roots indicated that the relative stele area of Bjarne was especially small in the distal root zone (data not presented). NK93602 was characterized by having thin nodal roots with a small cross sectional area, cortex and stele (mm^2) (Table 1). Similar to Bjarne, the relative stele area was also small. Naxos and Quarna (Fig. 5) were characterized by having thick roots. The cross sectional area and the stele (mm^2) in nodal roots were the largest or in the upper range (Table 1). The relative stele area was especially large in Quarna and the mean percentage aerenchyma was the smallest in nodal roots (Table 1). T9040 had traits similar to Bjarne and NK93602. The seminal roots had a small cross sectional area, cortex and stele. However, the relative stele area in nodal roots was quite large and the percentage of aerenchyma was small in seminal roots (Table 1; Fig. S3).

The size of the cross section, cortex and stele area in roots had a similar distribution pattern along the length of nodal and seminal roots (Table 2). The size of the traits in nodal roots decreased from the basal zone to the middle and distal zone. Seminal roots had an inversed size distribution of the cross sectional area, cortex and stele (mm^2) and

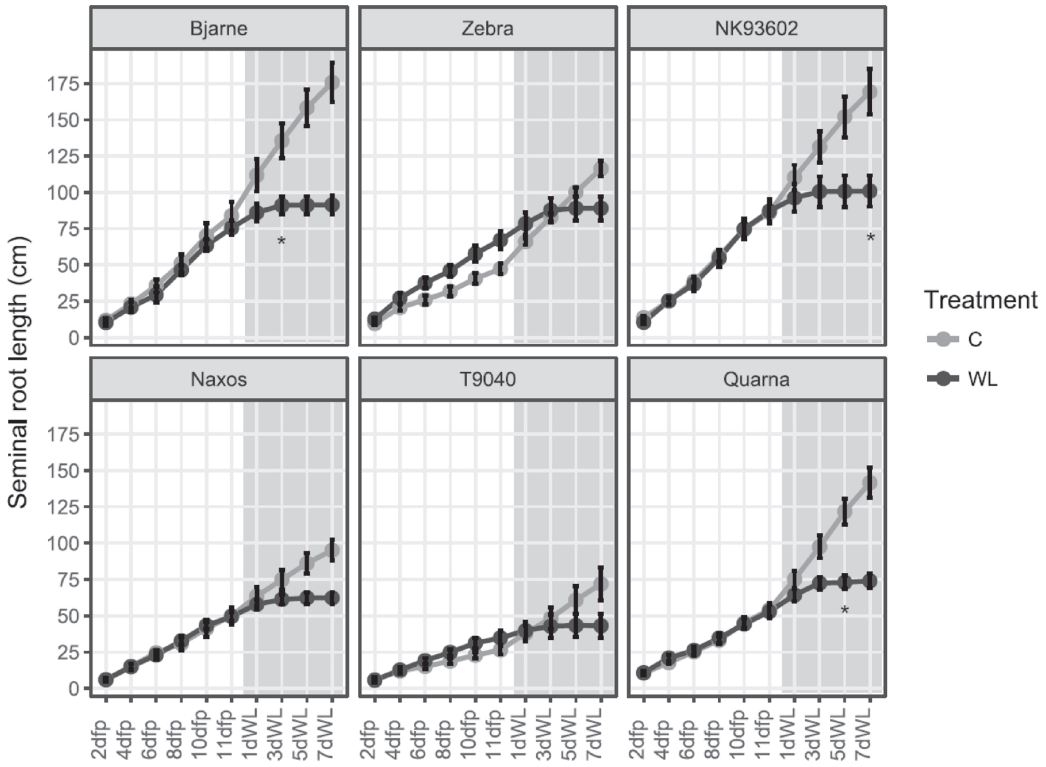

Fig. 1. Mean total seminal root length [cm] in experiment one of the six genotypes during seedling establishment (2dfp-11dfp) and the treatment period (1dWL-7dWL, shaded area) for control (C) and waterlogged (WL) plants. Plant age at 1dWL was 16 days. Error bars are standard error of the mean. Stars denote a significant interaction effect for genotype x treatment. Significant differences for genotype means: 11dfp: Bjarne, NK93602 > all others and T9040 < than all others, Zebra = Naxos = Quarna at $p < 0.001$; 3dWL: T9040 < all others and Zebra = Quarna = Naxos and NK93602, Bjarne > all others at $p < 0.001$; 7dWL: NK93602 = Bjarne > all others and Quarna = Zebra > Naxos, T9040 and Naxos = T9040 at $p < 0.001$.

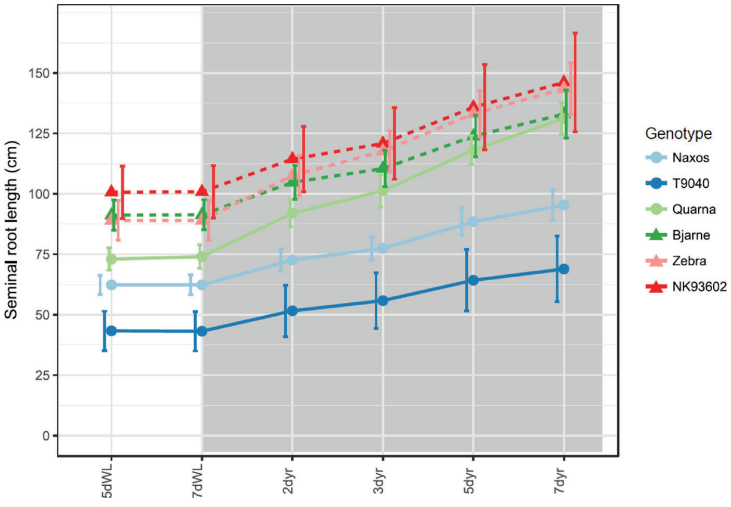

Fig. 2. Mean total nodal root length [cm] in experiment one of the six genotypes during the waterlogging period (1dWL-7dWL) for control (C) and waterlogged (WL) plants. Plant age at 1dWL was 16 days. Error bars are standard error of the mean. Significant differences for genotype means (control and waterlogged roots): 3dWL: Zebra > Quarna at $p = .008$; 7dWL: Zebra > NK93602, Naxos, Quarna and Bjarne > NK93602, Naxos, Quarna at $p < 0.001$.

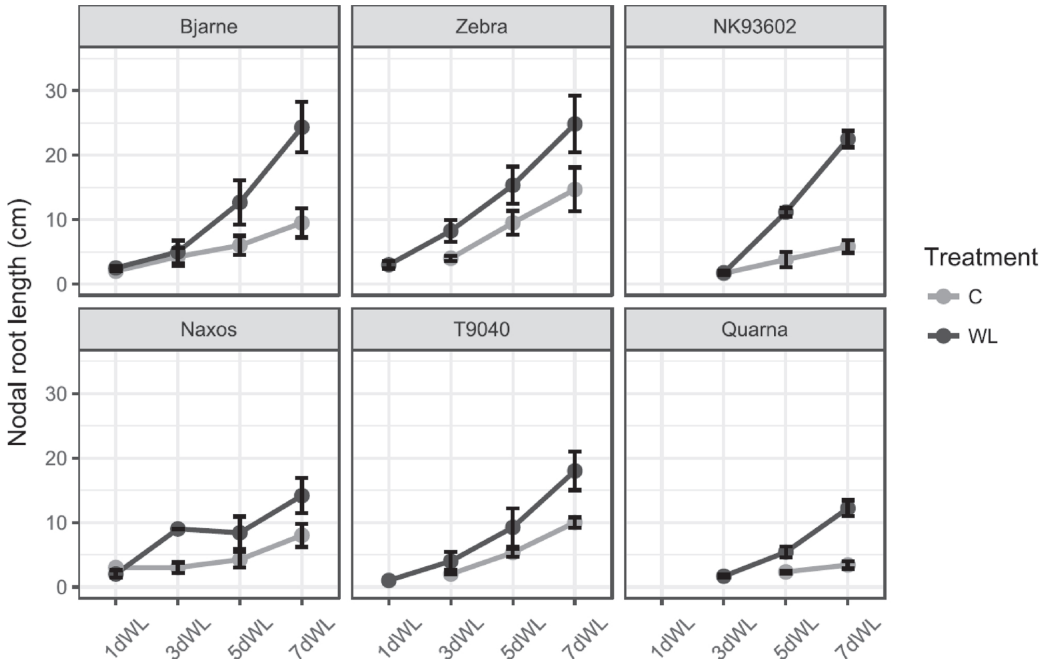

Fig. 3. Mean total seminal root length [cm] in experiment two of the six genotypes in the recovery period (commenced on 7dWL and lasting until 7dyr) of previously waterlogged plants. Shaded area highlights the recovery period. Plant age at 7dyr was 29 days. Error bars are standard error of the mean. Genotypes with triangles are considered tolerant, genotypes with circles are sensitive. Significant differences for genotype means: 3dyr: NK93602 = Zebra = Quarna > Naxos, T9040 at $p < 0.001$; 7dyr: NK93602, Bjarne > Naxos, T9040 at $p < 0.001$.

appeared to increase in size towards the root apex. However, the relative stele area decreased between the basal and distal zone of the seminal roots.

The percentage of aerenchyma was distinctly different in the three zones. Aerenchyma percentage generally increased towards the root apex of seminal roots. Seminal roots had 6% aerenchyma in the basal zone, while the percentage was determined to 18 and 27% in the middle (Fig. S3) and distal zone, respectively. Aerenchyma in nodal roots had an inversed distribution with the highest percentage in the basal zone and the lowest percentage in the zone closest to the apex.

3.5. Shoot growth

Linear regression analysis showed that leaf area measurements

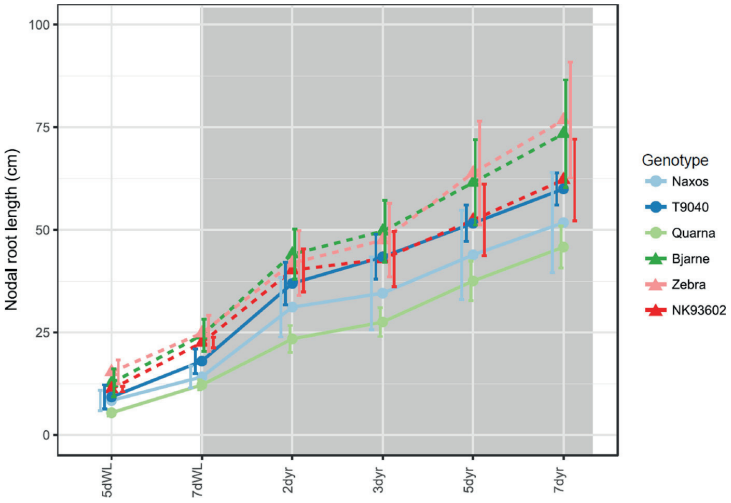

Fig. 4. Mean total nodal root length [cm] in experiment two of the six genotypes in the recovery period (commenced on 7dWL and lasting until 7dyr) of previously waterlogged plants. Shaded area highlights the recovery period. Plant age at 7dyr was 29 days. Error bars are standard error of the mean. Genotypes with triangles are considered tolerant, genotypes with circles are sensitive.

Table 1
Mean and standard error of the mean of anatomical traits of the six genotypes found in the seminal and nodal roots of previously waterlogged plants (sampled after 7 days of recovery (7dyr), experiment two). Uppercase letters denote significant differences determined by Tukey's HSD among genotypes within the specific root class.

		Seminal	Nodal
Cross sectional area [mm^2]	Bjarne	0.18 ± 0.011^{a}	0.37 ± 0.015^{a}
	NK93602	0.21 ± 0.009^{a}	0.32 ± 0.011^{b}
	Zebra	0.19 ± 0.082^{a}	0.34 ± 0.014^{ab}
	Naxos	0.19 ± 0.008^{a}	0.37 ± 0.021^{a}
	Quarna	0.20 ± 0.010^{a}	0.37 ± 0.017^{a}
	T9040	0.18 ± 0.077^{a}	0.34 ± 0.020^{ab}
	p-value	NS	< 0.001
Cortex [mm^2]	Bjarne	0.16 ± 0.01^{a}	0.33 ± 0.013^{a}
	NK93602	0.18 ± 0.08^{a}	0.27 ± 0.009^{b}
	Zebra	0.17 ± 0.07^{a}	0.29 ± 0.012^{ab}
	Naxos	0.17 ± 0.07^{a}	0.31 ± 0.016^{ab}
	Quarna	0.17 ± 0.09^{a}	0.31 ± 0.013^{ab}
	T9040	0.16 ± 0.07^{a}	0.29 ± 0.017^{ab}
	p-value	NS	0.005
Stele [mm^2]	Bjarne	0.021 ± 0.001^{c}	0.050 ± 0.0025^{c}
	NK93602	0.025 ± 0.0097^{abc}	0.047 ± 0.0018^{c}
	Zebra	0.026 ± 0.0009^{ab}	0.053 ± 0.0028^{bc}
	Naxos	0.026 ± 0.0013^{ab}	0.060 ± 0.0044^{ab}
	Quarna	0.027 ± 0.0012^{a}	0.061 ± 0.0043^{a}
	T9040	0.022 ± 0.0009^{bc}	0.050 ± 0.0032^{c}
	p-value	< 0.001	< 0.001
Stele [%]	Bjarne	11.7 ± 0.24^{c}	13.4 ± 0.35^{c}
	NK93602	12.2 ± 0.26^{c}	14.7 ± 0.33^{b}
	Zebra	13.8 ± 0.30^{a}	15.4 ± 0.30a^{b}
	Naxos	13.5 ± 0.25^{ab}	15.8 ± 0.39^{ab}
	Quarna	13.9 ± 0.32^{a}	16.3 ± 0.58^{a}
	T9040	12.6 ± 0.26^{bc}	15.7 ± 0.31^{b}
	p-value	< 0.001	< 0.001
Aerenchyma [%]	Bjarne	17 ± 3.2^{ab}	28 ± 8.3^{a}
	NK93602	13 ± 2.9^{b}	25 ± 10.6^{ab}
	Zebra	22 ± 2.7^{a}	26 ± 11.1^{ab}
	Naxos	19 ± 2.5^{ab}	25 ± 11.3^{ab}
	Quarna	18 ± 2.7^{ab}	19 ± 6.7^{b}
	T9040	12 ± 3.0^{b}	21 ± 9.6^{ab}
	p-value	0.003	0.03

Table 2
Mean and standard error of the mean of anatomical traits in the basal, middle and distal half of seminal and nodal roots of waterlogged plants (sampled after 7 days of recovery (7dyr), experiment two). Uppercase letters denote significant differences determined by Tukey's HSD within traits at three locations of each root class.

	Root zone	Seminal roots	Nodal roots
Cross **sectional area [mm^2]**	Basal	0.178 ± 0.0062^{b}	0.416 ± 0.010^{a}
	Middle	0.180 ± 0.0004^{b}	0.343 ± 0.008^{b}
	Distal	0.216 ± 0.0067^{a}	0.305 ± 0.009^{c}
	p-value	< 0.001	< 0.001
Cortex [mm^2]	Basal	0.154 ± 0.004^{b}	0.350 ± 0.008^{a}
	Middle	0.157 ± 0.004^{b}	0.289 ± 0.007^{b}
	Distal	0.189 ± 0.006^{a}	0.263 ± 0.008^{c}
	p-value	< 0.001	< 0.001
Stele [mm^2]	Basal	0.024 ± 0.00086^{b}	0.066 ± 0.0024^{a}
	Middle	0.023 ± 0.00064^{b}	0.053 ± 0.0016^{b}
	Distal	0.027 ± 0.00088^{a}	0.042 ± 0.0015^{c}
	p-value	< 0.001	0.001
Stele [%]	Basal	13.4 ± 0.2^{a}	15.8 ± 0.3^{a}
	Middle	12.9 ± 0.2^{ab}	15.6 ± 0.3^{a}
	Distal	12.5 ± 0.2^{b}	13.8 ± 0.2^{b}
	p-value	0.002	< 0.001
Aerenchyma [%]	Basal	6 ± 1.1^{c}	30 ± 1.6^{c}
	Middle	18 ± 1.5^{b}	24 ± 1.3^{b}
	Distal	27 ± 1.6^{a}	18 ± 1.3^{a}
	p-value	< 0.001	< 0.001

determined by the leaf area meter at harvest of plants in the second experiment was positively correlated with leaf area measured as cm^2 leaf area in RGB images ($R^2 = 0.55$). The relationship between dry weights (Table S1) and leaf area measured in the images was similar ($R^2 = 0.56$).

The accumulation of leaf area in cm^2 for the six genotypes during the waterlogging treatment is shown in Fig. 6. The treatment did not have a significant effect on the leaf area expansion. On average for both control and waterlogged plants, T9040 had a significantly lower leaf area than all other genotypes at 3dWL, 5dWL and 7dWL. Leaf area of Quarna, NK93602 and Bjarne appeared to be unaffected by the treatment. T9040 had a significantly smaller leaf area throughout the recovery period (Fig. S2) and a smaller dry and fresh weight (Table S1).

4. Discussion

The objective of this study was to identify root traits that may contribute to waterlogging tolerance in the selected genotypes. The experiments included the tolerant genotypes Bjarne, NK93602, Zebra, Naxos, Quarna and T9040. The three former ones have previously been determined to be more tolerant than the three latter in a field screening trial (Sundgren et al., 2018). Our results show that tolerant NK93602, Bjarne and Zebra were associated with developing a larger seminal root system in the seedling phase and to quickly develop nodal root system during the waterlogging treatment. Seminal roots of all plants resumed growth in the recovery phase. Thus, the ability to recover seminal root growth did not clearly differentiate the genotypes in our study.

4.1. Aerenchyma

Aerenchyma is generally perceived as the most important trait for waterlogging tolerance. Besides constructing a pathway for oxygen diffusion, aerenchyma may also contribute to a lower oxygen demand in the root (Huang and Johnson, 1995; Colmer, 2003; Armstrong, 1980). In this experiment, we found aerenchyma in both seminal and nodal roots of all genotypes. A significant genotype effect was detected in seminal roots. However, the development of seminal root systems did not seem to correspond with the presence of aerenchyma. The aerenchyma percentage in nodal roots of tolerant Bjarne (28%) and sensitive Quarna (19%) was distinctly different (Fig. S3). As aerenchyma may both lower the oxygen demand and increase the oxygen diffusion capability, the more advanced aerenchyma in roots of Bjarne may have contributed to the larger nodal root system that was found in this genotype (Fig. 3; Fig. 4). Bjarne, in addition to tolerant Zebra and NK93602 developed a higher number of nodal roots compared to sensitive Naxos and Quarna. These were presumably aerated and supported the plants with nutrient uptake and maintenance of shoot biomass.

The highest percentage of aerenchyma in seminal roots was found in the distal root zone, approximately 10–12 cm from the seminal root apices in all genotypes. The presence in the basal root zone was limited to 6% (Table 2). The aerenchyma distribution along the seminal roots is similar to what Haque et al. (2012) found in roots of 5 days old seedlings that had been waterlogged for 7 days. Haque et al. (2012) concluded that aerenchyma formation in the investigated genotypes could not explain the otherwise varying ability to tolerate waterlogging. The results found here suggests a similar interpretation. To construct a low-resistance pathway, continuity is an obvious requirement. Compared to aerenchyma in nodal roots, seminal root aerenchyma is considered negligible for waterlogging tolerance. As empirically proven by Thomson et al. (1990) and mathematically described by Armstrong (1980), oxygen diffusion to the root apex in roots > 100 mm is limited. In our study, the roots had exceeded this length considerably at sampling (43–50 cm, Table S1). Still, it is possible that aerenchyma may have contributed to a lower oxygen demand in seminal roots. It should be noted that the method of determining aerenchyma in microscopy

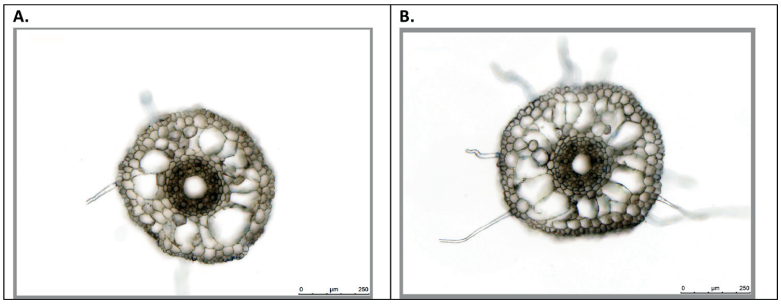

Fig. 5. Examples of cross section images of waterlogged seminal roots sampled at 7dyr (experiment two). **A.** Quarna (sensitive), distal root zone (relative stele size: 13.8%, aerenchyma: 30%). **B.** NK93602 (tolerant), distal root zone (relative stele size: 11.1%, aerenchyma: 36%). Note that the absolute stele size [mm^2] was similar in these particular samples. Additional root images may be found in the supplementary file.

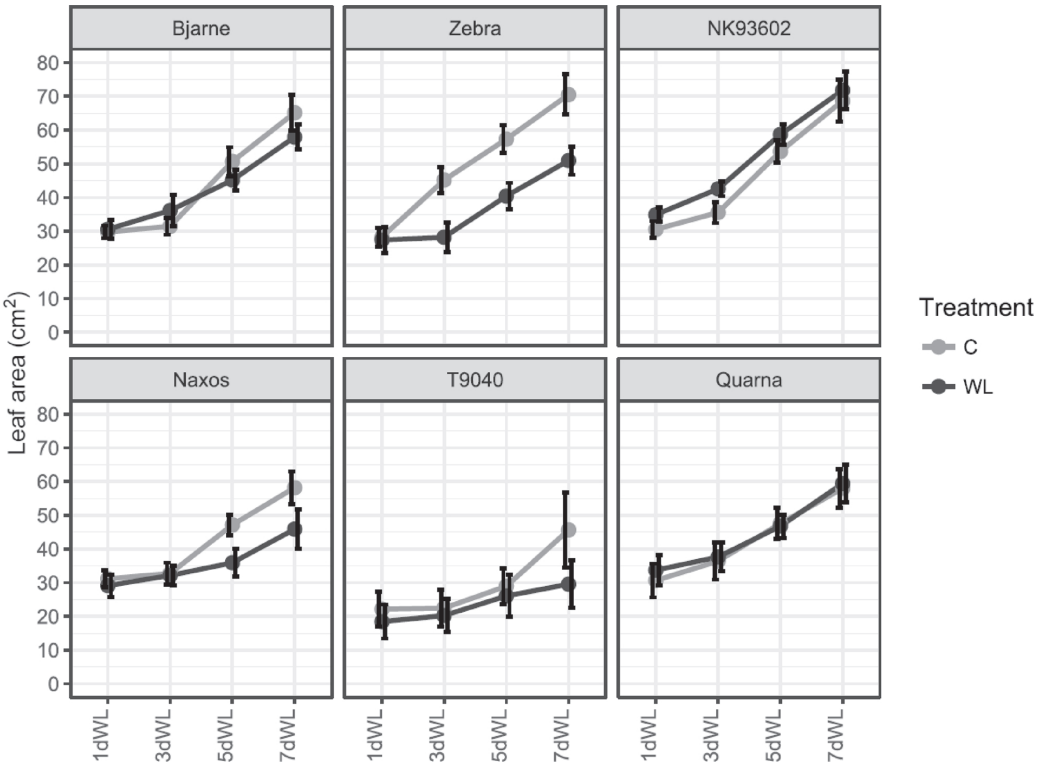

Fig. 6. Mean total leaf area [cm^2] in experiment one of the six genotypes during the waterlogging period (1dWL-7dWL) of control (C) and waterlogged (WL) plants. Significant differences for genotype means: 1dWL: NK93602, Quarna > T9040 at p = .006; 5dWL: T9040 < all others and NK93602 > Naxos at p < 0.001; 7dWL: 7dWL: NK93602 > Naxos, T9040 at p < 0.001.

images is disposed to subjectivity and involves procedures of handling and cutting the roots which may disrupt the samples. Supplementing measurements of root porosity would be necessary to firmly conclude whether aerenchyma is of importance for the waterlogging tolerance in these genotypes.

Axial development pattern of aerenchyma in wheat nodal roots have not been well-defined. Available literature suggests that it starts to form in the apical part (1 cm behind the root tip) and increase in size as the root matures (Huang et al., 1994a; Yamauchi et al., 2013). Aerenchyma in nodal roots of two barley genotypes studied by Pang et al. (2004), followed the same pattern but clearly decreased in size near the root-shoot junction. Our results show that aerenchyma percentage in nodal roots increased basipetally and was continuous in the root zones that we sampled. However, the basal root zone was located 6.5–10 cm from the root shoot junction for all roots. Thus, the presence of aerenchyma in this important area is unknown, and the study by Pang et al. (2004) showed that it can differ between genotypes.

4.2. Aerenchyma in contrast to root cortical senescence

Research has shown that aerenchyma clearly is a beneficial trait for waterlogging tolerance in wheat. Still, to which extent is less evident, especially at the genotype level. A process that resembles, but has been suggested to be distinct from aerenchyma (Deacon et al., 1986), is root

cortical senescence (RCS, synonymous to root cortical death). RCS is known to form in cereal crops by programmed cell death and is considered a natural aging process of the roots. As RCS progresses, the cells are replaced with air spaces and the physical appearance is much alike aerenchyma. RCS and aerenchyma both include cell lysis of cortical cells and may be enhanced by nutrient deficiencies (Saengwilai et al., 2014; Fan et al., 2003; Schneider et al., 2017; Lascaris and Deacon, 1991). The resemblances between aerenchyma and RCS raises the question whether RCS at times may be mistaken for aerenchyma. As nitrogen deficiency is a consequence of stress in waterlogged wheat, we speculate whether what is assumed to be aerenchyma may actually be RCS in an enhanced state. The loss of cortex cells in samples in this study was assumed to be aerenchyma as it is the common assumption in waterlogging studies as the present. However, we cannot exclude that the loss of cortex was a result of RCS. Better understanding of these processes in relation to each other is clearly needed.

4.3. The stele

The difference between genotypes in relative stele size was highly significant in both nodal and seminal roots. Bjarne and partly NK93602 (Fig. 5) had a significantly smaller stele size compared to Quarna (Fig. 5), Zebra and Naxos. The stele diameter is known to influence the oxygen demand and some wetland species have a considerably smaller stele than dryland species (Mcdonald et al., 2002). In the study by Mcdonald et al. (2002), the stele area of oat nodal roots decreased from 19.7% under aerated conditions to 15.1% when grown in stagnant nutrient solution for 36 days. Similarly, Pang et al. (2004) found that waterlogging caused a decrease of the stele diameter and xylem vessel area of two barley genotypes with contrasting waterlogging tolerance. The decrease in stele diameter of TX9425 (tolerant) was much larger than for the waterlogging sensitive variety Naso Nijo. TX9425 also had a high fraction of aerenchyma in waterlogged roots and a relatively smaller stele diameter in the control. A smaller area occupied by xylem vessels was also found in waterlogged nodal roots. This is similar to a reduced meta-xylem vessel size found in wheat that had been waterlogged for 17 days (Huang et al., 1994a). Apart from the reduced stele and xylem area, the diameter of nodal roots have also been found to increase when roots emerge in anaerobic conditions (Yamauchi et al., 2014a). The ability to increase root thickness and the cortex may be beneficial as it creates more space where aerenchyma can form.

In the current study, the relative stele size in waterlogged nodal roots ranged from 13.4% in Bjarne to 16.3% in Quarna. Bjarne and Quarna had the smallest and largest relative stele size in both seminal and nodal roots. The properties of the stele and its response to waterlogging is fairly unexplored for wheat, especially in larger populations. In rice, Kondo et al. (2000) found a clear genotypic variation in stele diameter. Upland varieties, as opposed to lowland varieties, appeared to have a larger stele and meta-xylem vessels, and the QTL controlling these traits are likely unrelated to traits associated with root thickness (Uga et al., 2008). The narrower stele of lowland rice varieties suggests that it might be a trait which have been unintentionally selected for when the actual target have been high yield potential and waterlogging tolerance.

An inherently narrower stele may be especially beneficial as it is already present if anaerobic conditions occur. On the contrary, aerenchyma takes time to develop in already emerged roots. Depending on the stress intensity, it may take up to 72 h (Haque et al., 2010; Xu et al., 2013), and as previously mentioned, roots > 100 mm in length, might even be incapable of conveying oxygen (Thomson et al., 1990). Since the stress impact may be instantaneous, an inherent trait or a mechanism which acts equally fast to alleviate the stress has more potential to improve the tolerance. For young wheat plants, which rely on seminal root functionality, a narrow stele might be an especially advantageous trait at early developmental stages. In combination with well-developed aerenchyma, the longitudinal oxygen diffusion could be enhanced. A narrow stele also has the potential to improve the drought tolerance in wheat (Schoppach et al., 2014). As our climate is expected to become more unpredictable and extreme, traits that are beneficial for a variety of abiotic stresses are especially attractive.

4.4. Developmental stage and stress response

Similar to previous reports (Malik et al., 2001; Barrett-Lennard et al., 1988) shoot growth was less affected than root growth. Signs of foliar chlorosis were nearly absent in the current study. Plants used in the waterlogging treatment differed slightly in the number of leaves they had developed when the treatment started. Bjarne and NK93602 had a higher leaf number (3.5 and 4.3 respectively) compared to Quarna and T9040 (2.7 and 2.8 respectively). The difference was too small to have a statistical effect on the total leaf area, but we suspect it to be an important factor for the subsequent stress response. Growth stages prior to tillering and the reproductive phase have been identified as the stages where waterlogging tolerance among genotypes of wheat and barley vary the most (Setter and Waters, 2003). Growth rate of seedlings may vary even under normal conditions (Rebetzke et al., 2004) and evidence suggests that early vigor may be associated with a higher nitrogen uptake (Liao et al., 2004). This is particularly interesting since reduced nutrient uptake is a major consequence of waterlogging stress (Drew and Sisworo, 1977). Robertson et al. (2009) found no effect of N applied prior to waterlogging on the tolerance of wheat at the tillering stage. In contrast, when young wheat seedlings were pretreated with nitrate, Trought and Drew (1981) found that the plants were less stressed and accumulated more fresh and dry weight in the shoots as well as root dry weight during two weeks of anaerobic stress. A high N status in the plants may improve the plants' ability to withstand the stress. Provided that the seminal roots maintain their functionality, deeper roots will also be in a better position to retrieve nitrogen resources that have been leached to deeper soil layers (Foulkes et al., 2009). Whether Bjarne and NK93602 had a higher N uptake, or to which extent their rapid seedling establishment benefited them during the treatment phase is unknown. Considering the dry weight, rather than the growth stage concurrently with the treatment being imposed could be an interesting approach. As growth rate is genotype dependent, it could be especially relevant where multiple genotypes are investigated. Slightly more advanced genotypes might be in an advantageous position since tolerance typically increase with higher growth stages. Meanwhile, variations in the growth stage, even as moderate as this, could possibly explain the large genetic variation that have been found in stages prior to tillering (Setter and Waters, 2003).

5. Conclusion

Results found in the present study indicate that tolerant genotypes were associated with rapid seedling establishment. This suggests that early vigor may be an important trait for waterlogging tolerance in the field. It also suggests that proper establishment in commercial production is highly recommended, independent of the genotype. Furthermore, tolerant genotypes appeared to be capable of developing a large nodal root system. Our study also indicates that anatomical root traits, such as a narrower stele and aerenchyma may contribute to improving waterlogging tolerance. Further studies, in controlled environment and in the field, as well as computer simulations are needed to determine the importance of these single traits, or in combination of traits for waterlogging tolerance in genotypes of wheat.

Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jplph.2018.04.010.

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Table S1. Mean and standard error of the mean of number of leaves determined at 1dWL (one day of waterlogging), fresh and dry weight at harvest (experiment two), number of seminal and nodal roots as well as the mean length of the longest seminal and nodal root of each genotype after 7 days of recovery (experiment two). Uppercase letters denote significant differences determined by Tukey's HSD test.

	Leaf no.	Fresh weight [g.]	Dry weight [g.]	Seminal root no.	Nodal root no.	Longest seminal root [cm]	Longest nodal root [cm]
Bjarne	3.5±0.3^{ab}	3.3±0.23^{a}	0.35±0.03^{a}	5.2±0.30^{ab}	7.5±0.76^{a}	48.6±1.8^{a}	39.0±1.8^{ab}
NK93602	4.3 ±0.2^{a}	2.8±0.17^{a}	0.29±0.02^{a}	6.3±0.42^{a}	6.8±0.31^{a}	49.7±2.3^{a}	40.9±2.0^{a}
Zebra	3.0 ±0.4^{abc}	3.0±0.15^{a}	0.31±0.02^{a}	6.0±0.26^{a}	7.8±0.65^{a}	46.9 ±1.8^{ab}	38.3±2.0^{ab}
Naxos	3.2 ±0.3^{abc}	2.4±0.24^{ab}	0.26±0.03^{ab}	4.3±0.61^{b}	4.7±0.49^{b}	43.5±1.6^{b}	35.2±2.6^{b}
Quarna	2.7 ±0.2^{c}	2.9±0.32^{a}	0.29±0.03^{a}	5.0±0^{ab}	4.7±0.33^{b}	48.5 ±3.2^{a}	39.3±3.6^{ab}
T9040	2.8 ±0.3^{bc}	1.5±0.36^{b}	0.18±0.04^{b}	3.7±0.2^{b}	6.0±0.57^{ab}	43 ±1.4^{b}	37.5±1.6^{ab}
p-value	0.005	0.004	<0.001	<0.001	<0.001	<0.001	0.01

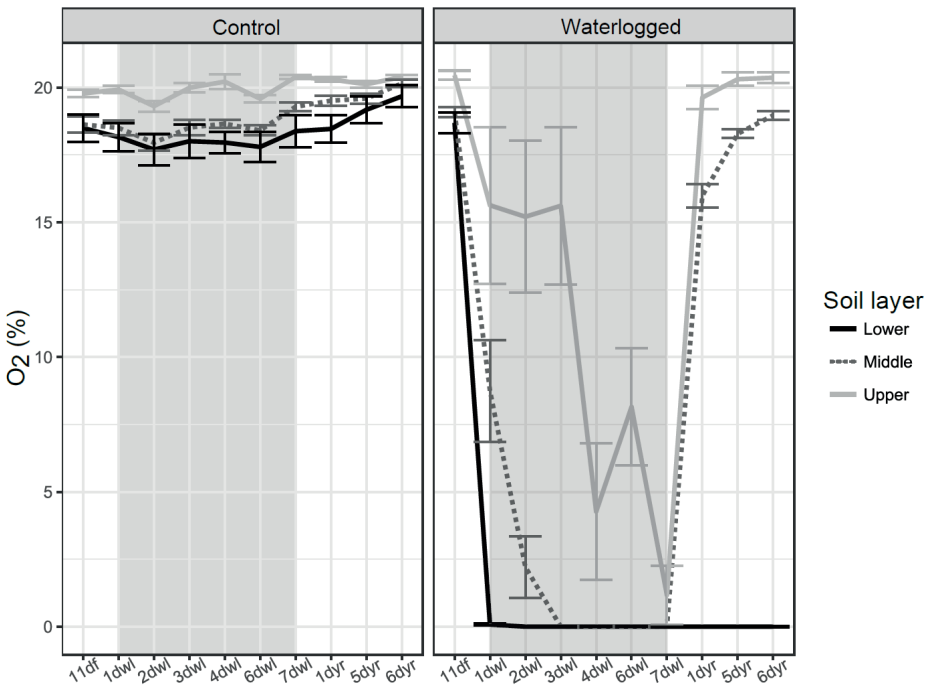

Fig. S1. Oxygen concentration [%] measured at three depths; upper (8 cm deep), middle (29 cm deep) and lower (51 cm deep) in rhizotrons (length: 58, depth: 2.1, width: 26.5) under waterlogged and normal soil moisture conditions. Shaded area highlights the waterlogging time period. Abbreviations: 11df, 11 days from planting/1 day before the treatment started; 1-7dwl, 1-7 days of waterlogging treatment; 1-, 5- and 6dyr, 1, 5 and 6 days of recovery.

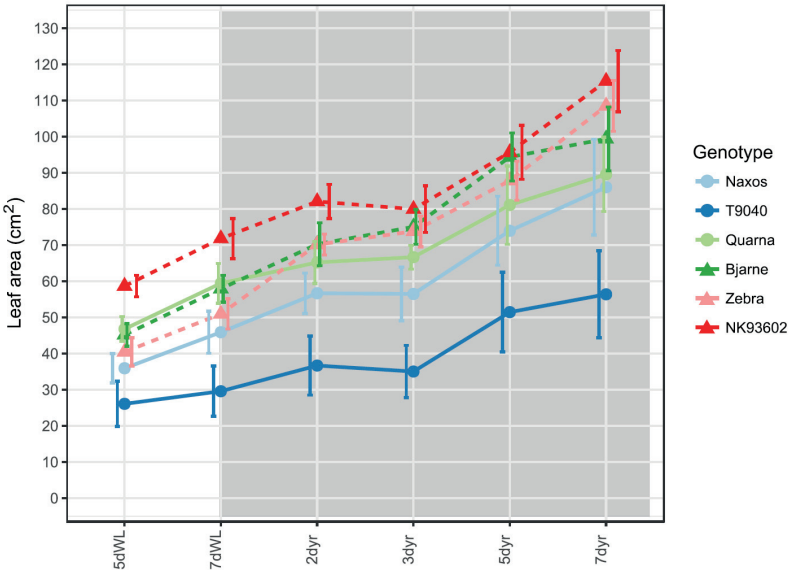

Figure S2. Mean leaf area [cm^2] of the six genotypes during the recovery period (experiment two). Significant differences for genotype means: 3dyr: T9040<NK93602, Bjarne, Zebra, Quarna at $p<0.001$; 7dyr: NK93602=Zebra=Bjarne>T9040 at $p<0.001$.

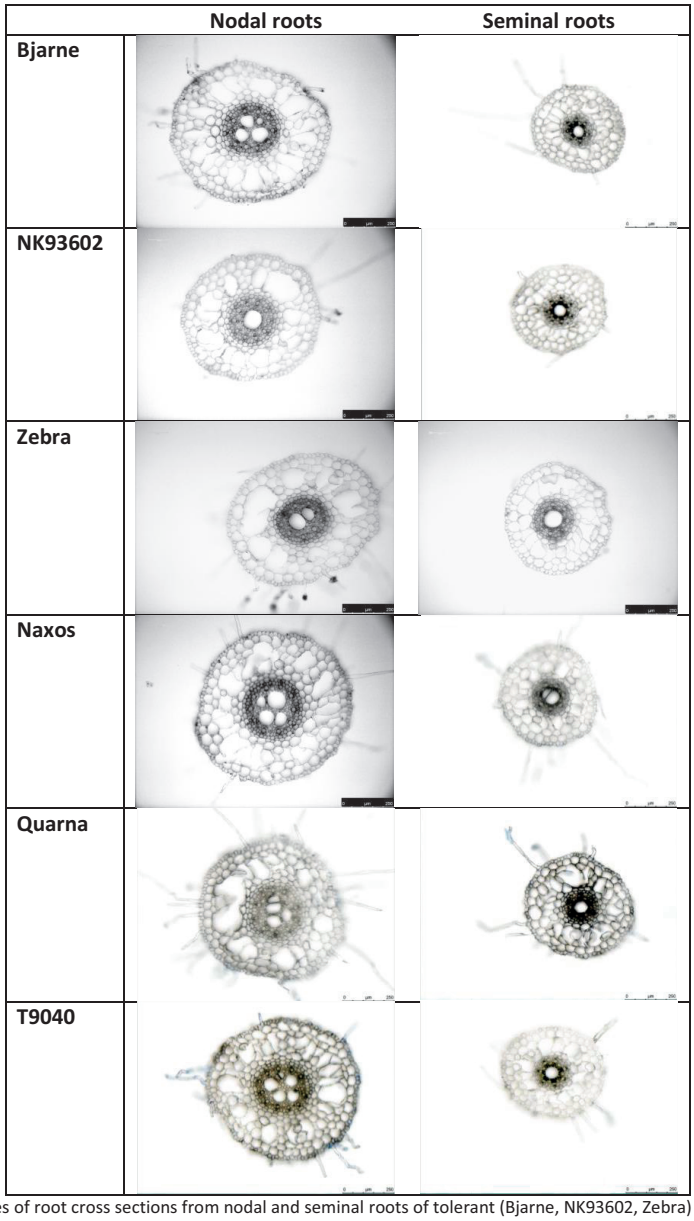

Figure S3. Examples of root cross sections from nodal and seminal roots of tolerant (Bjarne, NK93602, Zebra) and sensitive (Naxos, Quarna, T9040) genotypes. The root samples were acquired from root segments (2 cm in length) from the exact middle of the longest nodal and seminal root of each plant.

Paper III

A potential QTL for oxygen sensing detected in wheat subjected to waterlogging stress

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ABSTRACT

Waterlogging causes major yield losses in wheat worldwide. Breeding for improved tolerance to waterlogging has the potential to reduce the impact, but the genetics of the trait is still poorly understood. In this genome-wide association study (GWAS), we aimed at detecting quantitative trait loci (QTL) for waterlogging stress in the field. A panel of 181 lines, and a core group of 100 lines were phenotyped for the following traits: foliar chlorosis, relative plant height, heading delay, number of spikes, an overall condition score and principal component (PC) scores obtained from PC analyses. Genetic associations were determined between the phenotypic traits and SNP markers of the Affymetrix 35K and Illumina iSelect 90K SNP arrays. Significant SNP markers of both arrays were assigned a physical position by comparing their sequences with the wheat pseudo-chromosome sequences in BLAST. Sixteen QTL were defined on chromosomes 1B, 3B, 5BL, 6AL and 7A. Eight markers: three on 1B, four on 6AL and one on 7A were significant in both experimental years. Markers of *QTL6A.2* were highly significant for foliar chlorosis. One marker within *QTL6A.2* was predicted to be associated with prolyl-4-hydroxylase, a catalyzer of the transcription factor HIF (Hypoxia Induced Factor), a key regulator of oxygen homeostasis in mammals and possibly in plants. A haplotype analysis of *QTL6A.2* showed that lines of one haplotype group were significantly more chlorotic than others. The results from this study provide new insights into promising genomic regions in wheat that are highly relevant for further investigations.

ABBREVIATIONS

PCA, principal component analysis; PC, principal component; CL, chlorosis; OC, overall condition score; HD, heading delay; PH, plant height; HN, head number; GBM5, green biomass score 5 days after drainage; GBM19, green biomass score 19 days after drainage; QTL, quantitative trait loci.

KEYWORDS

Waterlogging tolerance, QTL, wheat, prolyl-4-hydroxylase, N-End Rule Pathway

1. INTRODUCTION

Flooded or waterlogged soil may rapidly become oxygen depleted and cause severe stress reactions of wheat and other dryland crops. Yield loss, the ultimate consequence of waterlogging is typically preceded by stress symptoms including chlorosis (Van Ginkel et al., 1992), reduced shoot and root growth (Pang et al., 2004), as well as delayed development (Amri et al., 2014). Waterlogged soil may be a particularly hostile environment if minerals containing manganese and iron are abundant and pH is at suboptimal levels (Ponnamperuma, 1972, Khabaz-Saberi et al., 2006). The longer a waterlogging event lasts, the more intense and detrimental the stress becomes (McDonald et al., 2006). For young wheat plants, three days of stress may be sufficient to cause considerable and long-term effects (Malik et al., 2002). Genotypes of wheat tolerates waterlogging differently (Gardner & Flood, 1993, Sayre et al., 1994, McDonald et al., 2006) and the grain yield loss may vary considerably (Setter et al., 1999). Maintenance of grain yield is the most desired trait in a breeding scheme, but the heritability is low and yield is typically confounded by many factors (Collaku & Harrison, 2005). Obtaining reliable yield data from waterlogging field experiments is laborious and researchers are often prompted to substitute yield data with measurements of other stress indicators. Foliar chlorosis (Van Ginkel et al., 1992), survival rate (Li et al., 2008) or biomass measurements (Yu et al., 2014) are commonly used as indicators of tolerance properties of cereal crops. Such approaches are considered acceptable as they are often correlated with yield response (Setter & Waters, 2003, Van Ginkel et al., 1992, Sundgren et al., 2018a).

Cereal crops may adapt to anaerobic conditions by various tolerance mechanisms (Herzog et al., 2016). Particular interest has been subjected to the development of intercellular gas spaces known as aerenchyma. Evidently, aerenchyma formation improves oxygen diffusion to the root apex and has been associated with less stress impact and maintenance of wheat grain yield (Setter et al., 1999). The apparent benefit of aerenchyma formation has compelled scientists to understand the genetic basis of this tolerance trait. Recent advances include the identification of a new allele for aerenchyma formation in a wild barley accession (TAM407227, *H. spontaneum*) (Zhang et al., 2017). The phenotypic variation in aerenchyma formation explained by the QTL was 76.8% in TAM407227, which was also clearly correlated with the overall waterlogging tolerance. The QTL was also identified in domesticated barley (Broughton et al., 2015, Zhang et al., 2016). The correlation between waterlogging tolerance and the presence of aerenchyma in *H. vulgare* was

however much lower, suggesting that also other traits are involved in waterlogging tolerance of barley.

Previous genetic studies in wheat under waterlogged conditions have primarily considered the overall performance in terms of biomass and other stress indicators. Less is known of specific tolerance traits such as aerenchyma, although there appear to be a genetic variation also in wheat (Huang et al., 1994b). A large number of putative waterlogging tolerance QTL has been reported from QTL mapping studies using recombinant inbred lines (RIL) of wheat. Collectedly, Burgos et al. (2001), Yu & Chen (2013), Yu et al. (2014) and Ballesteros et al. (2015) have reported QTL on all wheat chromosomes. While the two former studies considered germination and seedling stages, Ballesteros et al. (2015) studied adaptive traits at the vegetative stage. Measured traits included lengths and weights of roots and shoots, chlorophyll content and various indices of growth and survival.

Improving wheat through marker-assisted selection (MAS) relies on the detection of genetic markers that are closely linked to the trait of interest. QTL mapping with pre-designed populations, as in the aforementioned linkage studies, is highly useful to identify genomic regions which holds significant QTL. The precision of targeting these QTL may however be low as such populations have typically undergone a limited number of recombination events. Thus, the intra-chromosomal linkage disequilibrium (LD) may be high and the mapping resolution therefore low (Flint-Garcia et al., 2003). The genetic variation within the population is also relatively confined to the variations introduced by the parents (Ingvarsson & Street, 2011). This requires that the two parents and their subsequent offspring segregate for trait of interest. Selecting appropriate parents can be challenging especially when dealing with complex traits such as waterlogging tolerance. In contrast, genome-wide association studies (GWAS) exploit the countless recombination events that has occurred over time in natural populations (Korte & Farlow, 2013). Diverse populations naturally introduces a higher allelic diversity and thereby increases the mapping resolution and precision (Zhu et al., 2008). Although GWAS overcomes limitations typical for linkage mapping, it comes with other disadvantages that needs to be addressed, e.g. multiple testing problems (Gupta et al., 2014) and impacts of population structure (Breseghello & Sorrells, 2006). If these drawbacks are considered appropriately, GWAS has great potential to efficiently provide insight to the genetics behind a trait, aid in the discovery of genes and serve as a guide for choice of parents for

development of subsequent mapping populations (Hall et al., 2010, Korte & Farlow, 2013). In this paper, we present results and putative QTL for waterlogging stress in spring wheat. The identified QTL are highly relevant for further investigations and potential targets for improving waterlogging tolerance. To our awareness, this is the first GWAS of waterlogging tolerance in wheat under field conditions.

2. MATERIALS AND METHOD

Plant material

The genotype collection in this study included advanced breeding lines, cultivars, landraces and various crossing parents. In total, 181 genotypes were tested in our field experiments during 2013 and 2014, whereas 100 of them were tested in both years. The majority of genotypes were of Norwegian origin (47%), of Swedish origin (12%) and from CIMMYT (24%). Details of the lines can be found in Sundgren et al. (2018a).

Field experiments and phenotypic data

Phenotypic data was collected in controlled field trials conducted in Ås in southeast Norway in 2013 and 2014. The field experiments, previously described by Sundgren et al. (2018a), were conducted using hillplots arranged in alpha lattice designs with three replicates. The waterlogging treatment began at the 3-leaf stage and lasted for 10 and 13 days in 2013 and 2014, respectively. Foliar chlorosis was the first visible stress symptom that appeared. The percentage of chlorosis on plot basis was determined once a clear difference among genotypes could be observed. The heading delay was calculated as the difference between the number of days to heading in control and waterlogged plots. The relative plant height and the number of spikes were calculated as the relative difference between waterlogged and control plots. The overall conditions score was recorded around maturation and was intended to indicate the vigor and yield potential of the genotypes. A combined score on a 1-10 scale with 1 being the lowest was given.

The phenotypic traits were analyzed in linear mixed models using R (R Core Team, 2017) and the "lme4" package (Bates et al., 2015). Best linear unbiased predictors (BLUPs) of the traits were further used in principal component analysis (PCA). BLUPs and PCA scores (PC1-PC3) of the

lines constituted the phenotypic data for the association analyses. BLUPs used in the analyses can be found in the supplementary file of Sundgren et al. (2018a).

Genotyping

DNA extracted from leaf tissue was genotyped with the Illumina iSelect 90K SNP array (Wang et al., 2014), the Affymetrix 35K SNP array (Allen et al., 2017) or both. Ninety-one lines were genotyped with both SNP chips. The genotyping procedures were previously described by Jansen (2015) and Windju (2017) for the 90K and the 35K SNP array, respectively. Prior to the association analyses, the genotypic data was filtered in order to exclude minor allele frequencies (MAF) of ≤0.05. The final genotype datasets included 22 031 markers in the 90K SNP dataset and 14 136 markers in the 35K SNP dataset.

Population structure

Population structures in the 90K SNP and 35K SNP datasets were determined and described by (Jansen, 2015) and Windju (2017), respectively. In brief, the calculations were made in the software STRUCTURE v. 2.3.4, using subsets of markers evenly distributed across the chromosomes. For the 90K SNP dataset, 338 SNP markers with 5 cM intervals were selected, while 938 markers were used for the 35K dataset. Structure analysis by Windju (2017) was carried out with 299 spring wheat lines, while Jansen (2015) included 123 lines. Parameters were set to 5000 burnin length and 50 000 reps over three iterations and K=10. According to the analyses, the population was constructed by three subpopulations with the 35K genotypic data and five subpopulations with the 90K genotypic data. These population structures were further included in the association analyses carried out in the present study.

Linkage disequilibrium

Pairwise linkage disequilibrium (LD) between markers and the estimated squared allele frequency correlations (r^2) were determined in the software TASSEL v. 5.2 (Bradbury et al., 2007). Markers with a minor allele frequency of less than 5% were excluded from the analysis. Corresponding *p*-values were obtained with the two-sided Fisher's Exact test and markers with *p*-values ≤0.001 were considered significant. The half-decay distance was calculated based on the maximum

estimated value of LD. The estimated value and the critical value for LD significance was obtained by fitting the r^2-values in a non-linear model as described by Marroni et al. (2011).

Association analysis

The association analysis was performed using TASSEL v. 5.2. The analyses were carried out as mixed linear models in the format of $y=X\beta+Qv+u+e$. Here, y is a vector of the phenotypic observations, X is the vector of SNP marker genotypes, β is the vector of marker effects, Q is the population structure, v is a vector of fixed effects according to the population structure, u is the vector of random effects and e is the error term. Phenotypic observations from the two experimental years were analyzed separately with the 35K and 90K SNP datasets and the corresponding population structure matrices. Markers with a *p*-value in the lower 0.1 percentile of the distribution were considered significant.

To identify promising genomic regions, we investigated all significant markers for commonalities. Markers that were significant in both experimental years, or markers that were significant for more than one trait were used as indicators. Subsequently, we saturated the identified regions with additional significant markers. With Basic Local Alignment Search Tool (BLAST), we aligned sequences of significant SNP markers of the 90K and 35K arrays along the wheat pseudo-chromosome sequences (IWGSC, submitted). Here, we required hits with identity over 99% and marker coverage over 99% along the pseudo-chromosomes. Assigning the markers with pseudo-chromosome physical positions allowed us to compare markers from the two SNP arrays and to define putative QTL containing markers from both arrays. A QTL was defined when a region in which minimum five significant markers were positioned within approximately 10 Mbp distance.

Haplotypes

Haplotype analysis was performed for one of the selected QTL on chromosome 6AL. The QTL included markers that were determined to be significant in both experimental years. The QTL was associated with the percentage of chlorosis and was mapped to the distal end of chromosome 6AL. The QTL contained significant markers from both SNP arrays. Haplotypes were therefore constructed using both types of markers (Table 1). The estimated genotype means of chlorosis for the haplotypes were calculated and analyzed in simple regression models using R. Welch two-sample t-tests were carried out to analyze the difference in chlorosis between haplotype groups.

3. RESULTS AND DISCUSSION

Phenotypic results

Phenotypic traits recorded in both experimental years include the percentage of chlorosis, relative plant height, delay in heading and the overall condition score. Results from the PCA, based on BLUPs of the individual traits, have been described by Sundgren et al. (2018a). Here, PCA was used to rank the genotypes' overall performance, but foliar chlorosis and the overall condition score were considered the most important traits.

Except for the overall condition score, the traits were positively correlated between the years (Table 2). The overall condition score was non-correlated between 2013 and 2014, and the correlations among the other traits were limited to approximately 0.3. Environmental conditions are known to strongly influence waterlogging tolerance (McDonald et al., 2006, Setter et al., 2009). It is likely that contrasting weather conditions in the experimental years, with lower temperatures and higher precipitation in 2013 than in 2014, affected the plants differently. Additionally, the treatment duration was prolonged in 2014 to impose a higher stress level and to obtain a more distinct genotype differentiation. This delayed the plant development (determined by heading date) considerably in 2014. The overall condition score, which was recorded around maturation was also clearly different in 2014, compared to 2013.

Association analysis and linkage disequilibrium

LD of the 90K and 35K markers which displayed alleles frequencies of >0.05, decayed at 1 and 3 cM for the 35K and 90K SNP arrays, respectively (Fig. S1). The estimated r^2 value for half decay was 0.24 for both arrays and is within a comparable range with the CIMMYT wheat association mapping initiative population (Lopes et al., 2015).

A total of 729 35K and 90K markers were identified as significant across the genome. No significant markers were identified on chromosomes 2D or 4D, and only a few ones were located on chromosomes 1D, 4B, 5A and 7D (data not presented). Regions on chromosomes 1BL, 3B, 5B, 6AL and 7A were identified as particularly interesting. These included markers that were either significant in both years, or that they were significant for several traits from one of the years (Table 3; Table S1).

Chromosome 1B

The largest number of significant markers were located on chromosome 1B (Table 3). Altogether, these markers were distributed across the whole chromosome and covered 5.3% of the total pseudo-chromosome sequence length. The markers were further divided into five QTL (Table 3). *QTL1B.5* was highly saturated with markers that explained 10 to 20% of the phenotypic variation (Table S1). Three markers, one 35K marker (*AX-94413240*) and two 90K markers (*BS00039135_5, BobWhite_c2844_569*) were considered significant for traits in both 2013 and 2014. While the markers were associated with the overall condition score in 2013, they were related to chlorosis in 2014. BLAST searches in the National Center for Biotechnology Information (NCBI) showed that the markers are predicted to be associated with electron carrier activity (GO:0009055) and iron-sulphur cluster binding (GO:0051536). Chlorosis percentage and the overall condition scores recorded in 2013 were negatively correlated (Table 2), indicating that the overall condition score given around maturation reflected the chlorosis percentage recorded earlier in the growing season (Sundgren et al., 2018a). Of the twenty-one significant markers located on *QTL1BL.5*, nineteen of them were associated with chlorosis or the overall condition score. Approximately 45 Mbp apart from *QTL1B.5*, we identified a minor region including three closely linked markers (*RAC875_c102886_73, IAAV1732 and BobWhite_rep_c62955_567*) associated with heading delay. These were determined to be in close proximity with two markers identified by Ballesteros et al. (2015). The markers identified in our study were positioned approximately 2.7 and 10 Mbp's from *wmc728* and *gwm259* (Ballesteros et al., 2015), respectively. The QTL that contained the markers reported by Ballesteros et al. (2015) were associated with fresh and dry biomass of roots and shoots after 28 days of waterlogging. The marker *wmc728* explained 22 and 27% of the phenotypic variation in root and shoot fresh weight, respectively. The other four QTL that we identified on chromosome 1B were associated with relative plant height or PC2 in 2013. Plant height was the trait that contributed the most to PC2 in 2013 (Table S2). This strongly suggests that *QTL1B.1-QTL1B.4* were all highly associated with the relative plant height. The phenotypic variations explained by the markers (R^2) were low and did not exceed 0.12. The semi-dwarfing genes *Rht-B1* and *Rht-D1*, which largely regulate plant height in modern wheat, are located on chromosomes 4B and 4D (Ellis et al., 2002). This suggests that the markers we identified here may be related to biomass rather than the actual plant height.

Chromosome 3B

On chromosome 3B, we identified fifty-five significant markers covering 2.6% of the pseudo-chromosome sequence (Table 3). Two closely located QTL were defined, including fifteen (*QTL3B.1*) and twenty-two (*QTL3B.2*) significant markers, anchored at the start of the pseudo-chromosome sequence. The markers within *QTL3B.1* were associated with the overall condition score, relative plant height and heading delay. These traits are indicative of biomass maintenance and recovery from the waterlogging treatment. Similarly, traits associated with *QTL3B.2* also describes the ability to recover and the overall stress tolerance, as the QTL was primarily associated with the overall condition and PC1 scores. In addition, we detected a minor QTL, including four 90K markers (*Kukri_rep_c92293_249, GENE-4458_691, Kukri_c7860_911, RAC875_c66953_100*). According to their physical position on the pseudo-chromosome sequence, these markers were located approximately 8 and 12 Mbp from two markers (*wmc632, gwm340*) reported by Ballesteros et al. (2015). *wmc632,* was related to shoot dry biomass and a flooding tolerance index in their field experiment. *gwm340,* was identified under both control (greenhouse experiment) and waterlogged conditions (field experiment) for chlorophyll concentration, and for shoot dry biomass of controls in the greenhouse experiment. The markers identified in our study were exclusively associated with PC2 scores in 2013. The relative contribution of traits to PC2 (Table S2) in this experimental year was dominated by relative plant height (48% relative contribution) and secondly by chlorosis score (25% relative contribution). BLAST searches showed that the genomic region of *GENE-4458_691* is predicted to encode proteins involved in the biological process of proteolysis and initiating methionine removal. Interestingly, proteolysis through the N-End rule pathway has been revealed to play a key role in regulating low oxygen signaling in both *Arabidopsis* (Gibbs et al., 2011, Licausi et al., 2011) and barley (Mendiondo et al., 2016). Protein degradation by the N-End rule pathway, activated by group VII Ethylene Responsive Factors (ERF-VII), occurs in a specific sequence in certain proteins, whereby an initial step includes cleaving of methionine by methionine aminopeptidase (Voesenek & Sasidharan, 2013, Gibbs et al., 2011). Mendiondo et al. (2016) found that transgenic RNAi barley plants, which had reduced expression of the N-recognin E3 ligase *HvPRT6* gene, were less affected by waterlogging in terms of maintaining biomass accumulation and chlorophyll content. The N-End rule pathway was concluded to be a promising breeding target for improved waterlogging tolerance. However, Giuntoli et al. (2017) recently reported that the responses controlled by the

N-End Rule pathway seem to be age dependent in *Arabidopsis*. While the ERF-VIIs activated hypoxia-responsive genes in young seedlings, the transcription factor appeared to have lost its activation function in older plants. To date, the role of the N-End Rule Pathway for oxygen sensing in plants is unclear and it has been suggested that a multitude of signaling functions are involved in low oxygen sensing in plants (Schmidt et al., 2018). This includes Ca^{2+} signaling, reactive oxygen species (ROS) signaling and cytosolic pH (Wang et al., 2017). Further investigations would be necessary to determine whether the QTL reported here is related to the N-End pathway. The proximity to the QTL reported by Ballesteros et al. (2015) supports that the region is likely involved in waterlogging stress response.

Chromosome 5B

The second largest number of significant markers was identified on chromosome 5BL, whereas a majority of them were only significant for traits in 2014. The markers were further divided into four QTL. Traits associated with the QTL were green biomass recorded five days after drainage (*QTL5B.1*), heading delay (*QTL5B.1, QTL5B2, QTL5B.3, QTL5B.4*) and PC3 in 2014 (*QTL5B.4*). Plant height and chlorosis were the main contributors to PC3 in 2014 (Table S3). In addition, we detected a minor region, including three 90K markers (*Excalibur_c3165_730, Kukri_c59540_137, RAC875_c43383_483*) and one 35K marker (*AX-94516158*) that were associated with heading delay and PC2 in 2014 (plant height and chlorosis as main contributors). This region, and particularly the marker *RAC875_c43383_483* was determined to be in close proximity to the *Vrn-B1* gene when comparing pseudo-chromosome physical distances of markers reported by Voss-Fels et al. (2018). *Vrn-1,* which is best known for its regulatory role in vernalization-induced flowering in cereals (Deng et al., 2015), have recently been shown to affect the root architecture both spatially and temporally (Voss-Fels et al., 2018). Near isogenic lines (NILs) carrying the winter allele displayed a narrower root angle and a reduced root length at seedling stages but longer roots at anthesis. In contrast, NILs with the spring allele were predisposed to produce more roots at the 60-80 cm depth. At anthesis, these lines had a lower root:shoot ratio compared to lines with the winter allele. Under anaerobic conditions, seminal root growth often succumb while adventitious root growth is initiated; resulting in a higher number of adventitious roots per tiller or per unit biomass (Malik et al., 2001, Watkin et al., 1998). Whether the root architecture modulated by *Vrn-B1* has implications for waterlogging tolerance is unclear and may depend on the developmental stage when waterlogging occurs. Sundgren et al. (2018b) found that genotypes that

rapidly developed seminal roots in the seedling phase appeared to be more tolerant than genotypes with slower development. That suggests a preference for the spring allele. There is a scarcity in studies investigating genetic variation in root traits leading up to a waterlogging event. The ability to form aerenchymatous nodal roots is clearly advantageous and varies among wheat genotypes (Huang et al., 1994b, Huang et al., 1994a). Nevertheless, aerenchyma formation is not a constitutive trait in wheat and the development is a slow process (Haque et al., 2010, Xu et al., 2013) considering the instantaneous impact that low oxygen concentration may have. The importance of other, inherent traits that may be beneficial for waterlogging tolerance would be interesting topics for future research.

Chromosome 6AL

A small region on chromosome 6A, covering 2% of the chromosome sequence, was identified to hold two distinct QTL (Table 3). Both were located on the long arm although distant apart. *QTL6A.1* appeared to be associated with the green biomass score 19 days after drainage (GBM19). The second QTL (*QTL6A.2*) appeared to be a highly significant and important region in our study. It included markers from both SNP arrays, whereby four of them were significant in both experimental years (Table S1). Seven of the markers were associated with more than one trait (chlorosis, PC2 and PC3 scores). Three markers were among the four markers with the lowest *p*-value, and two of them (BS00099401_51 and Tdurum_contig29607_413) had the highest R^2 values of the whole study, each explaining 24% of the phenotypic variation (Table S1). Few waterlogging stress QTL have been reported on chromosome 6A before. Yu & Chen (2013) identified two, whereas one of them was located on the short arm, hence distant from the ones reported here. The other QTL was positioned on the long arm, although a few hundred Mbp from *QTL6A.2.* Burgos et al. (2001) also reported a QTL on 6A. Unfortunately, due to the lack of marker information in that study we were unable to compare marker positions. Burgos et al. (2001) found that the QTL was significant for a shoot growth index and the percentage of plants with the first root longer than 1 mm after imbibing seeds for 24 hours.

BLAST searches of the 90K markers in *QTL6A.2* indicated a connection to zinc ion binding (*Tdurum_contig29607_413*), ATP binding protein (*Jagger_c5046_63*), as well as defense response to fungus (*BS00099401_51*). BLAST searches of the 35K marker *AX-95092538* (*QTL6A.2*) revealed that the marker is predicted to be associated with a prolyl 4-hydroxylase (P4H)

alpha subunit gene. According to BLAST, molecular functions related to the marker includes iron ion binding (GO:0005506), oxidoreductase activity (GO:0016491), L-ascorbic acid binding (GO:0031418) and the biological process of oxidation-reduction (GO:0055114). In animals, the transcription factor Hypoxia Inducible Factor 1 (HIF-1), is known to be the principal regulator of O_2 homeostasis, as well as activating signaling cascades under low oxygen conditions (Semenza, 2001, Schofield & Ratcliffe, 2004). HIF-1 protein exists in two subunits, HIF-1α and HIF-1β (Semenza, 2001). Under normoxic conditions, HIF-1α is continuously being degraded, but accumulates under hypoxia (Guzy & Schumacker, 2006) due to inactivity of P4H, an enzyme which requires oxygen as a co-substrate (D'Angelo et al., 2003, Jaakkola et al., 2001). HIF-1α then binds to specific sequences of the DNA and activates hypoxia-responsive genes (Guzy & Schumacker, 2006, Bruick & McKnight, 2001). P4H belong to the large family of oxidoreductases (2-oxoglutarate-dependent dioxygenases) that requires Fe^{2+} as cofactor, ascorbate, as well as 2-oxoglutarate and O_2 for the reaction to take place (Kivirikko & Myllyharju, 1998, Berra et al., 2006). P4Hs have not been well characterized in plants but studies with *Arabidopsis* (Asif et al., 2009, Vlad et al., 2007) and maize (Zou et al., 2011) suggests that they are involved in gene expression related to waterlogging, mechanical wounding (Vlad et al., 2007) and in the regulation of root hair development in *Arabidopsis* (Velasquez et al., 2015). A phylogenetic analysis by Wang et al. (2017) showed that P4H in *Arabidopsis* was closely related to the orthologous protein in the fungus *Taphrina dephormans.* Moreover, when comparing the *Arabidopsis* genome with sequences of known oxygen-sensing domains in animals, Wang et al. (2017) found that the prolyl hydroxylase domain (PHD) likely has similar oxygen sensing mechanism in plants as in mammals. Results found here supports a link between P4Hs and waterlogging stress response under field conditions. The genomic region was clearly significant in both 2013 and 2014, despite the slightly differing experimental conditions.

Chromosome 7A

Three QTL were defined on chromosome 7A, one on the short arm (*QTL7A.1*) and two on the long arm (*QTL7A.2, QTL7A.3*). *QTL7A.1* consisted of three 90K markers and four 35K markers, all of them mapped to the same position according to the consensus maps (Wang et al., 2014, Allen et al., 2017). This QTL was significant for the relative number of heads in 2014 and relative plant height in 2013. The two QTL on the long arm were fairly closely located (approximately 9 Mbps apart) and were significant for traits in 2014; the green biomass score recorded 19 days after

drainage (*QTL7A.2*), PC 1 (*QTL7A.2, QTL7A.3*), PC 2 (*QTL7A.2*) and PC 3 in 2014 (*QTL7A.3*) and the relative head number (*QTL7A.3*). One 35K marker (*AX-95629211*) was significant for relative plant height in 2013 and for heading delay and PC 1 scores in 2014. Flanking markers were too distant for the marker to be included in a near-by QTL. *QTL7A.2* was the closest one and the marker was mapped approximately 19 Mbp apart from this. Despite being distant from other markers, it may represent a promising region as it was identified in both experimental years.

Haplotypes

To further assess the assumed importance of *QTL6A.2*, we constructed haplotypes of this locus using 90K and 35K markers (Table 1) in two separate analyses. The selected 90K markers were anchored to the pseudo-chromosome within a region of 124 Kbp. The marker correlations (r^2) indicate high LD, particularly for the two most closely located markers, *Tdurum_contig29607_413* and *Tdurum_contig413_220* (Table 4). The 90K markers were significant for chlorosis in 2014, whereby *Tdurum_contig29607_413* was highly significant for this trait and explained 24% of the phenotypic variation (Table S1). Similarly, selected markers of the 35K array were in complete or high LD (Table 1). They were mapped to the same genetic position (Table S1) and covered a region within *QTL6A.2* of approximately 670 Kbp. All three markers were identified as significant for chlorosis in 2013 and in particular 2014 (Table 1; Fig. 1). The phenotypic variation explained by the markers ranged from 21 to nearly 26% in 2014.

The genotypes were divided into three haplotypes by both marker sets (Table 5). Of the 90K markers, haplotype 1 included 16 genotypes, haplotype 2 was the smallest group with only four genotypes, and haplotype 3 was the largest with 90 genotypes. Similarly, haplotypes constructed by 35K markers also divided the genotypes into three groups: haplotype 1 included 30 genotypes, haplotype 2 had 3 genotypes, and haplotype 3 was assigned to 125 genotypes. A larger number of genotypes had been genotyped with the 35K than with the 90K array. Except for two lines, the assigned haplotype group was equal for all other lines with both 35k and 90K marker sets. Simple regression analyses showed that there was a significant relationship between the estimated genotype mean of chlorosis and haplotypes in 2013 and 2014 (Fig. 2; Fig. 3; Table 5). According to a Welch two-sample t-test, the estimated genotype mean of chlorosis was significantly different between haplotype group 1 and 3 in both 2013 (*p*-value for 35K markers=6.88×10^{-4}; for 90K markers=5.024×10^{-3}) and 2014 (*p*-value for 35K markers=1.57×10^{-12}; for 90K markers=$6.08 \times 10^{-}$

[8]). Interestingly, although genotypes of haplotype 1 were clearly more chlorotic, they seemed to recover quite well from the treatment. The overall condition score recorded in 2014 was not correlated with chlorosis, indicating that chlorosis percentage did not necessarily describe the condition of the plants later in the season. PCA biplots of BLUPs show that these genotypes share similar properties, as they appeared to cluster in the same region (Fig. 4; Fig. 5). Several of the lines also shared the cultivar Vinjett as a parent in their pedigrees (Table S4). Lines of haplotype 1 seemed to be associated with a delay in heading date in 2013 and with relative plant height in 2014. However, haplotype groups were not statistically different for these traits.

As previously described, *QTL6A.2*, which differentiate the haplotypes may be related to P4H and possibly oxygen sensing. Recognizing that oxygen is at a suboptimal level is a crucial step for subsequent acclimation. With a few exceptions, haplotype 1 lines had the highest chlorosis percentage scores in 2014. A high chlorosis percentage, as of these lines, equals a higher stress level. It is therefore tempting to speculate whether they were more stressed because of a poor ability to detect the low oxygen status. However, judging by their overall condition score, it is clear that these lines were still able to adapt and to recover. Ethylene is involved in the regulation of several traits that confer adaptation to waterlogging; adventitious root formation, aerenchyma formation, shoot elongation and submergence tolerance in rice (*Oryza sativa* L.), hyponasty and possibly post-hypoxic stress tolerance (Sasidharan & Voesenek, 2015). Post-hypoxic stress relates to the reappearance of oxygen and the elevated levels of ROS that may arise. Without efficient scavenging systems, ROS may damage membranes and the viability of cells (VanToai & Bolles, 1991, Biemelt et al., 1998). The physiology behind the recovery of haplotype 1 lines remains unknown but it is likely that they possessed one or several of the outlined traits. Similar to the scarcity in studies investigating root traits prior to a stress event, there is also a knowledge gap in post-stress responses and recovery. The physiology and genetics related to these traits are important aspects to investigate for further advances in waterlogging tolerance breeding.

4. CONCLUSION

In this GWAS, we have identified SNP markers and QTL for waterlogging stress using phenotypic data obtained in field experiments. Sixteen QTL were defined on chromosomes 1B, 3B, 5BL, 6AL and 7A. The QTL were significant for principal components, plant height, chlorosis, overall condition score, heading delay, green biomass and head number. In addition, we identified two minor regions on chromosomes 1B and 3B which were in close proximity to markers reported in a previous QTL mapping study for waterlogging tolerance (Ballesteros et al., 2015). The *QTL6A.2*, significant for chlorosis in 2013 and 2014, was distinctively the most important QTL in our study. It contained highly significant markers of both SNP arrays. A haplotype analysis showed that one group of lines were more chlorotic than others. This QTL, as well as other regions that were identified in both experimental years are highly relevant for further investigations.

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Table 1. The marker ID of 35K and 90K SNPs used to construct haplotypes, their corresponding physical position on the pseudo-chromosome sequence and statistical results in 2013 (top) and 2014 (bottom). *Significant also for PC2 and PC3 in 2014 and PC2 in 2013.

SNP marker	Marker ID	Position (start-stop)	Marker R^2	*p*-value	Year and trait
35K	AX-95182345	611661167-611661237	0.070 0.257	0.00227 $1.47x10^{-7}$	CL 2013-2014
	AX-94634087	612329194-612329124	0.074 0.214	0.0017 $1.1x10^{-6}$	CL 2013-2014
	AX-95153895	612302284-612302354	0.083 0.217	0.0010 $1.11x10^{-6}$	CL 2013-2014*
90K	BS00099401_51	613256472-613256572	0.244	$1.18x10^{-4}$	CL 2014
	Tdurum_contig29607_413	609379961-609380061	0.244	$1.13x10^{-5}$	CL 2014
	Tdurum_contig413_220	609380763-609380664	0.135	$8.2x10^{-4}$	CL 2014

Table 2. Correlations of phenotypic traits recorded in 2013 and 2014. Abbreviations: chlorosis; OC, overall condition score; HD, heading delay; PH, plant height; S, straw; HN, head number; GBM5, green biomass score 5 days after drainage; GBM19, green biomass score 19 days after drainage.

	CL13	PH13	HD13	OC13	CL14	GBM19	PH14	GBM5	HN14	HD14	OC14
CL13											
PH13	-0.27**										
HD13	0.47***	-0.27**									
OC13	-0.42***	0.52***	-0.51***								
CL14	0.34***	0.07	0.22*	0.07							
GBM19	-0.02	-0.01	0.00	0.26**	0.14						
PH14	-0.13	0.28**	-0.07	0.12	0.01	0.13					
GBM5	-0.10	0.07	0.12	0.09	0.05	0.64***	0.29**				
HN14	-0.11	-0.01	0.02	0.19	0.05	0.85***	0.25**	0.66***			
HD14	0.09	-0.24*	0.29**	-0.41***	-0.10	-0.68***	-0.13	-0.47***	-0.57***		
OC14	0.04	0.01	0.00	0.16	0.08	0.64***	0.40***	0.40***	0.67***	-0.38***	

Table 3. List of chromosomes in which QTL were identified, the number of significant markers and the markers' coverage of the pseudo-chromosme sequences. Below are the QTL listed, with the start and end of the pseudo-chromosome sequence, the Mbp span of the QTL, the number of markers, their associated traits and the years in which the QTL were significant.

Chromosome	Pseudo-chromosome coverage	Marker number	QTL number			
1B	5.3%	86	5			
3B	2.6%	55	2			
5BL	6.5%	74	4			
6AL	2%	23	2			
7A	3.9%	30	3			
QTL	**Start**	**End**	**Size (Mbp)**	**Marker number**	**Associated traits**	**Year**
1B.1	367439538	374340321	6.9	24	PC2	2013
1B.2	426683381	433432212	6.7	5	PH, PC2	2013
1B.3	480841563	486543032	5.7	16	PH	2013
1B.4	568964639	571061423	2.1	5	PC2	2013
1B.5	630063204	637622522	7.6	21	CL, OC, PH, PC3	2013-2014
3B.1	3240179	9092678	5.9	15	OC, PH, HD	2013-2014
3B.2	15521996	21385848	5.9	22	PC1, OC, CL	2013-2014
5B.1	473114456	478756057	5.6	28	HD, GBM5 , GBM15,	2014
5B.2	506789406	519742356	13.0	10	HD, PC2	2013-2014
5B.3	600187536	606021096	5.8	7	HN, HD, PC1	2014
5B.4	707131602	712860420	5.7	9	PC3, HD	2013-2014
6AL.1	9326153	14609435	5.3	6	OC, GBM19	2014
6AL.2	607056087	613761987	6.7	15	CL, PH, PC2, PC3	2013-2014
7AS.1	78430481	80861530	2.4	8	HN, PH	2013-2014
7AL.2	689949417	692345021	2.4	5	GBM15, PC1, PC2	2014
7AL.3	701293964	712417060	11.1	9	HN, PC3	2014

Table 4. Marker ID, R^2 and *p*-value of the 90K and 35K markers used for construction of haplotypes.

SNP chip	Marker ID	R^2	p-value
90K	Tdurum_contig413_220 BS00099401_51	0.84	$2.1x10^{-20}$
	Tdurum_contig413_220 BS00099401_51	0.58	$7.9x10^{-13}$
	Tdurum_contig29607_413 BS00099401_51	0.66	$5.3x10^{-14}$
35K	AX-95153895 AX-94634087	1.0	$2.2x10^{-16}$
	AX-95182345 AX-94634087	0.94	$2.2x10^{-16}$
	AX-95182345 AX-95153895	0.95	$2.2x10^{-16}$

Table 5. Mean chlorosis for the haplotypes on 6AL based on the estimated genotype mean for genotypes within each haplotype. *p*-value for 35K marker haplotypes was <0.001 in 2013 and 2014. *p*-value for 90K marker haplotypes in 2013=0.005 and <0.001 in 2014.

	Haplotype	N	2013	2014
35K	1 (ATT)	30	2.00	12.68
	2 (ATA)	3	1.56	0.22
	3 (TAA)	125	-0.25	-1.95
90K	1 (AAT)	16	1.79	13.08
	2 (TAT)	4	-0.02	-1.10
	3 (TTA)	90	-0.38	-2.00

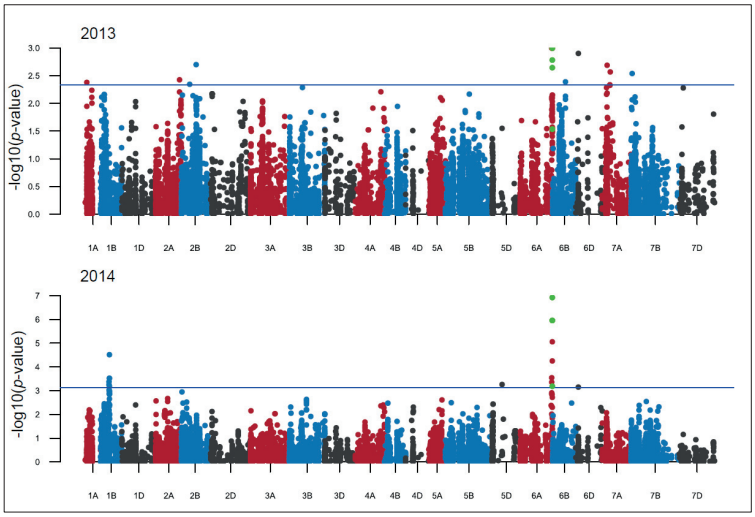

Figure 1. Manhattan plots of 35K SNP markers in association with chlorosis percentage recorded in 2013 (upper panel) and 2014 (lower panel). The markers highlighted in green (chromosome 6A) were used to define haplotypes. Significance threshold (blue horizontal line) was set to the lower 0.01 percentile of the *p*-value distribution. Markers plotted above the significance threshold were considered significant.

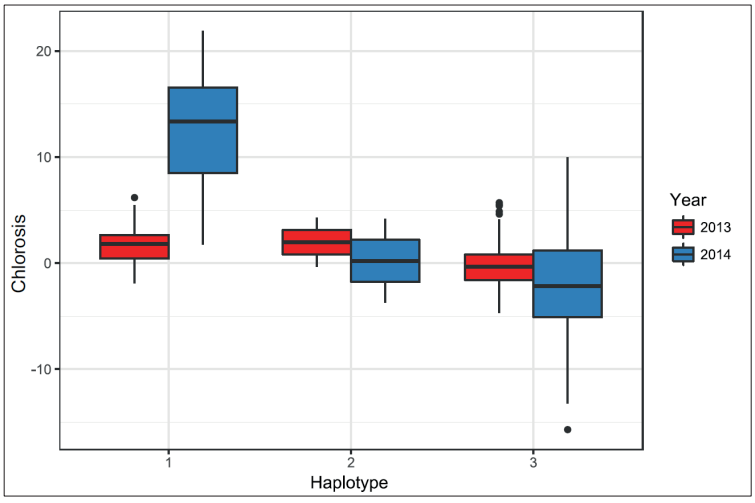

Figure 2. Mean percentage of chlorosis in 2013 and 2014 of three haplotypes on chromosome 6AL constructed by the 35K SNP markers *AX-94634087, AX-95182345* and *AX-95153895.*

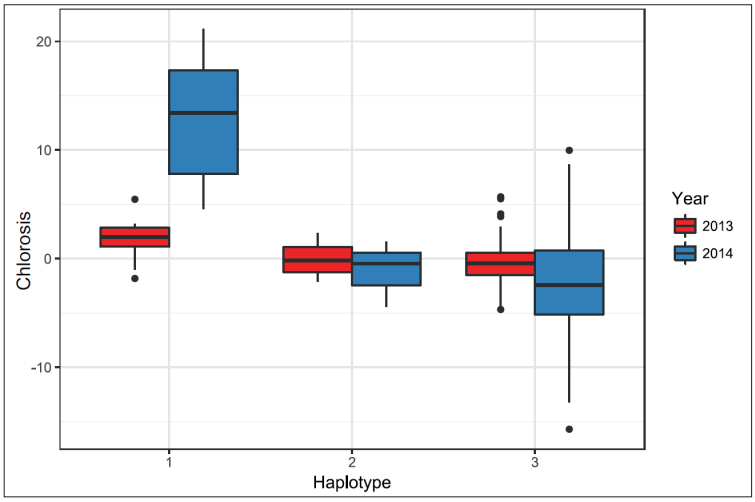

Figure 3. Mean percentage of chlorosis (BLUP) in 2013 and 2014 of three haplotypes on chromosome 6AL constructed by the 90K SNP markers BS00099401_51, Tdurum_contig29607_413 and Tdurum_contig413_220.

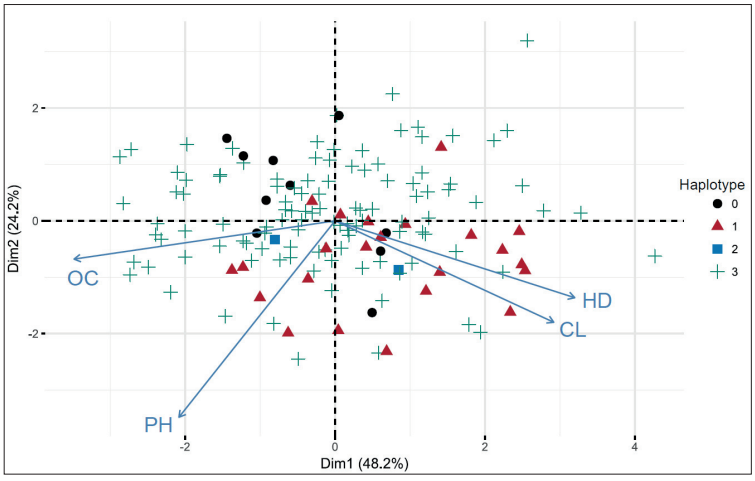

Figure 4. PCA biplot of 143 wheat genotypes and the phenotypic traits recorded in 2013. Scores in the biplot represent genotypes, whereby symbols denote their corresponding haplotype group (triangles are haplotype group 1, squares are haplotype group 2, crosses are haplotype group 3 and circles are genotypes which were not genotyped).

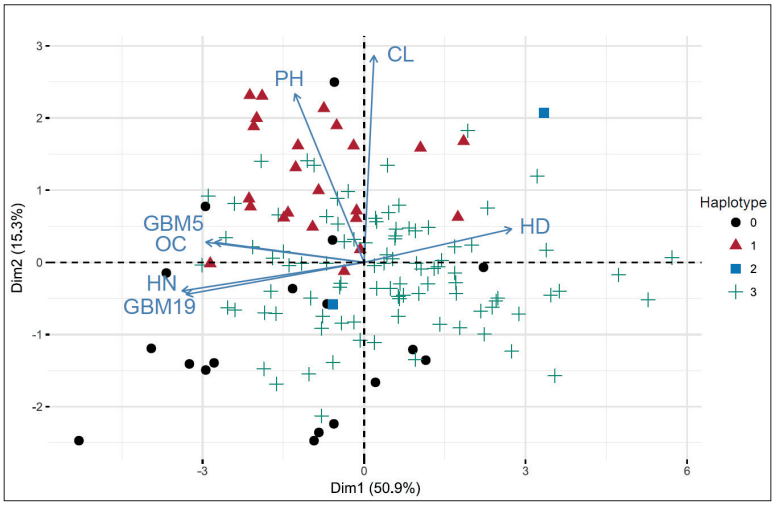

Figure 5. PCA biplot of 123 wheat genotypes and the phenotypic traits recorded in 2014. Scores in the biplot represent genotypes, whereby symbols denote their corresponding haplotype group (triangles are haplotype group 1, squares are haplotype group 2, crosses are haplotype group 3 and circles are genotypes which were not genotyped).

Table S1. List of QTL and nearby markers that were identified by assigning the 35K and 90K SNP markers with a physical position according to the pseudo-chromosome sequences. The QTL and markers were singled out by using markers that were significant in both experimental years or were significant for several traits within one year as indicators. The table shows the marker ID of 35K and 90K markers, their start and end position (Mbp) according to the pseudo-chromosome sequence, the corresponding chromosome, as well as the marker R^2, *p*-value, associated trait(s) and which year the markers were significant in.

QTL	Marker ID	SNP chip	Start pseudo-chromosome sequence (Mbp)	End pseudo-chromosome sequence (Mbp)	Chromosome	Marker R^2	*p*-value	-log10(*p*-value)	Associated trait	Significant in year:
	AX-94564319	35K	1430941	1430880	1B	0,09	1,5E-03	2,83	PC2	2014
	AX-94990491	35K	16478917	16478987	1B	0,09	1,3E-03	2,88	HN	2013
	AX-94736092	35K	18419670	18419600	1B	0,07	2,3E-03	2,64	HN	2013
	AX-94569385	35K	18421387	18421317	1B	0,08	1,3E-03	2,89	HN	2013
QTL1B.1	AX-94571756	35K	367439538	367439608	1B	0,08	8,7E-04	3,06	PC2	2013
QTL1B.1	Ex_c34713_501	90K	367439623	367439523	1B	0,11	7,4E-04	3,13	PC2	2013
QTL1B.1	GENE-0193_197	90K	367440899	367440999	1B	0,11	7,4E-04	3,13	PC2	2013
QTL1B.1	wsnp_Ku_c66585_65967792	90K	368543850	368544050	1B	0,11	7,4E-04	3,13	PC2	2013
QTL1B.1	Ra_c33845_794	90K	368559068	368559168	1B	0,11	7,4E-04	3,13	PC2	2013
QTL1B.1	Tdurum_contig65023_587	90K	368675725	368675625	1B	0,11	7,4E-04	3,13	PC2	2013
QTL1B.1	AX-94398036	35K	368680051	368679981	1B	0,08	1,0E-03	2,99	PC2	2013
QTL1B.1	wsnp_Ex_rep_c66802_65172754	90K	369590693	369590606	1B	0,11	7,4E-04	3,13	PC2	2013
QTL1B.1	wsnp_Ex_rep_c66802_65172754	90K	369601374	369601258	1B	0,11	7,4E-04	3,13	PC2	2013
QTL1B.1	wsnp_Ex_c10233_16784994	90K	369602204	369602404	1B	0,11	7,4E-04	3,13	PC2	2013
QTL1B.1	wsnp_Ku_c9014_15193623	90K	370035179	370035379	1B	0,11	7,3E-04	3,14	PC2	2013
QTL1B.1	Ku_c42700_2798	90K	371455438	371455538	1B	0,11	7,4E-04	3,13	PC2	2013
QTL1B.1	IAAV178	90K	372149372	372149274	1B	0,11	7,4E-04	3,13	PC2	2013
QTL1B.1	JD_c10376_670	90K	373143452	373143352	1B	0,11	7,4E-04	3,13	PC2	2013
QTL1B.1	AX-94462077	35K	373143935	373144005	1B	0,08	1,1E-03	2,94	PC2	2013
QTL1B.1	Kukri_c37856_123	90K	373144020	373143920	1B	0,11	7,4E-04	3,13	PC2	2013
QTL1B.1	RAC875_c24109_600	90K	374020281	374020381	1B	0,11	7,4E-04	3,13	PC2	2013
QTL1B.1	Kukri_c76762_166	90K	374319295	374319395	1B	0,11	7,4E-04	3,13	PC2	2013
QTL1B.1	CAP7_rep_c10850_139	90K	374319462	374319362	1B	0,11	7,4E-04	3,13	PC2	2013
QTL1B.1	Excalibur_c3140_697	90K	374329075	374329175	1B	0,11	7,4E-04	3,13	PC2	2013

QTL1B.1	AX-94837039	35K	374329121	374329051	1B	0,08	1,0E-03	2,99	PC2	2013
QTL1B.1	AX-95181560	35K	374329259	374329189	1B	0,08	1,1E-03	2,94	PC2	2013
QTL1B.1	Kukri_c2297_181	90K	374329469	374329369	1B	0,11	7,4E-04	3,13	PC2	2013
QTL1B.1	Kukri_rep_c95031_104	90K	374340231	374340321	1B	0,11	7,4E-04	3,13	PC2	2013
	CAP11_c1969_268	90K	384298858	384298758	1B	0,11	7,4E-04	3,13	PC2	2013
QTL1B.2	Excalibur_c1841_368	90K	426683381	426683281	1B	0,13	2,7E-04	3,57	PH,PC2	2013,2013
QTL1B.2	AX-94820546	35K	430617279	430617349	1B	0,08	7,4E-04	3,13	PC2	2013
QTL1B.2	wsnp_Ex_c8865_14815305	90K	433431611	433431811	1B	0,14	2,1E-04	3,68	PH,PC2	2013,2013
QTL1B.2	Kukri_rep_c73393_848	90K	433431761	433431661	1B	0,14	2,1E-04	3,68	PH,PC2	2013,2013
QTL1B.2	Ku_c10106_313	90K	433432112	433432212	1B	0,14	2,1E-04	3,68	PH,PC2	2013,2013
	Ra_c34433_417	90K	453878458	453878558	1B	0,12	5,7E-04	3,25	PC2	2013
QTL1B.3	wsnp_Ex_c9960_16397347	90K	480841563	480841745	1B	0,08	3,5E-03	2,46	PH	2013
QTL1B.3	Ra_c68984_1882	90K	480974251	480974351	1B	0,08	3,5E-03	2,46	PH	2013
QTL1B.3	IAAV9039	90K	481709261	481709139	1B	0,09	2,0E-03	2,69	PH	2013
QTL1B.3	AX-94982191	35K	482181976	482181906	1B	0,08	1,1E-03	2,97	PH	2013
QTL1B.3	wsnp_Ku_c19618_29134473	90K	482713559	482713652	1B	0,09	2,0E-03	2,69	PH	2013
QTL1B.3	IAAV7936	90K	483115329	483115129	1B	0,09	3,2E-03	2,50	PH	2013
QTL1B.3	AX-94642545	35K	484147517	484147587	1B	0,10	4,0E-04	3,40	PH	2013
QTL1B.3	AX-94642545	35K	484214504	484214574	1B	0,10	4,0E-04	3,40	PH	2013
QTL1B.3	BS00083072_51	90K	484376891	484376991	1B	0,09	2,0E-03	2,69	PH	2013
QTL1B.3	AX-95021596	35K	484463139	484463069	1B	0,09	9,2E-04	3,04	PH	2013
QTL1B.3	wsnp_Ex_rep_c66643_64952627	90K	484887642	484887510	1B	0,09	2,0E-03	2,69	PH	2013
QTL1B.3	AX-94508976	35K	484887645	484887575	1B	0,07	1,5E-03	2,82	PH	2013
QTL1B.3	Excalibur_rep_c67541_1585	90K	484888093	484888016	1B	0,10	1,8E-03	2,74	PH	2013
QTL1B.3	AX-94553348	35K	485291102	485291033	1B	0,07	1,5E-03	2,82	PH	2013
QTL1B.3	RAC875_c92464_53	90K	485606493	485606593	1B	0,09	2,0E-03	2,69	PH	2013
QTL1B.3	AX-94439461	35K	486543102	486543032	1B	0,08	1,4E-03	2,84	PH	2013
	wsnp_Ex_c23992_33235984	90K	532167827	532167668	1B	0,14	2,6E-04	3,59	PC2	2013
	wsnp_Ex_c23992_33235984	90K	532167963	532167920	1B	0,14	2,6E-04	3,59	PC2	2013
	Ra_c1652_588	90K	532173878	532173978	1B	0,13	3,4E-04	3,47	PC2	2013

	IAAV4702	90K	537694595	537694795	1B	0,13	3,4E-04	3,47	PC2	2013
	RAC875_rep_c108853_108	90K	545801338	545801238	1B	0,12	5,9E-04	3,23	PC2	2013
QTL1B.4	Excalibur_c10496_651	90K	568964639	568964598	1B	0,11	7,0E-04	3,16	PC2	2013
QTL1B.4	Excalibur_c10496_651	90K	568965237	568965175	1B	0,11	7,0E-04	3,16	PC2	2013
QTL1B.4	AX-94981461	35K	569977032	569977102	1B	0,09	5,4E-04	3,26	PC2	2013
QTL1B.4	wsnp_CAP11_c287_244799	90K	570023870	570024069	1B	0,12	4,4E-04	3,36	PC2	2013
QTL1B.4	wsnp_Ku_c13043_20902807	90K	571061552	571061423	1B	0,12	5,4E-04	3,27	PC2	2013
	AX-94613350	35K	589134055	589133987	1B	0,10	6,3E-04	3,20	OC	2014
QTL1B.5	RAC875_rep_c114694_99	90K	630063204	630063124	1B	0,14	5,4E-04	3,27	PH,PC3	2014,2014
QTL1B.5	AX-94553813	35K	630568103	630568173	1B	0,10	5,6E-04	3,25	CL	2014
QTL1B.5	AX-95212058	35K	630742246	630742188	1B	0,10	7,3E-04	3,14	PC3	2014
QTL1B.5	IACX4411	90K	630831775	630831656	1B	0,15	4,2E-04	3,38	CL,PC3	2014,2014
QTL1B.5	wsnp_JD_c20621_18304110	90K	630832418	630832367	1B	0,14	6,0E-04	3,22	CL,PC3	2014,2014
QTL1B.5	BobWhite_c4375_347	90K	630926640	630926739	1B	0,14	5,9E-04	3,23	CL,PC3	2014,2014
QTL1B.5	BobWhite_rep_c54139_273	90K	630926795	630926695	1B	0,16	4,2E-04	3,38	CL	2014
QTL1B.5	BobWhite_c4375_347	90K	630931963	630932062	1B	0,14	5,9E-04	3,23	CL,PC3	2014,2014
QTL1B.5	Ra_c2772_4514	90K	632479204	632479104	1B	0,13	9,5E-04	3,02	CL	2014
QTL1B.5	AX-95076244	35K	632479809	632479741	1B	0,10	6,5E-04	3,19	CL	2014
QTL1B.5	Kukri_c18109_331	90K	636263256	636263356	1B	0,17	1,8E-04	3,74	CL	2014
QTL1B.5	AX-95251106	35K	636264039	636264109	1B	0,15	3,1E-05	4,51	CL	2014
QTL1B.5	Kukri_c18109_649	90K	636264147	636264247	1B	0,18	1,2E-04	3,94	CL	2014
QTL1B.5	Kukri_c18109_682	90K	636264180	636264280	1B	0,18	1,2E-04	3,94	CL	2014
QTL1B.5	RAC875_c7357_1107	90K	636264240	636264340	1B	0,20	6,1E-05	4,21	CL	2014
QTL1B.5	BS00110435_51	90K	636725957	636725857	1B	0,17	1,8E-04	3,74	CL	2014
QTL1B.5	RAC875_c13258_955	90K	636738190	636738090	1B	0,14	6,4E-04	3,20	CL	2014
QTL1B.5	BobWhite_c2844_569	90K	636753153	636753054	1B	0,13	7,8E-04	3,11	CL,OC	2014,2013
QTL1B.5	BS00039135_51	90K	636753153	636753053	1B	0,13	7,8E-04	3,11	CL,OC	2014,2013
QTL1B.5	AX-94413240	35K	636753169	636753099	1B	0,11	3,0E-04	3,52	OC,CL	2013,2014
QTL1B.5	Excalibur_rep_c70674_83	90K	637622422	637622522	1B	0,11	8,2E-04	3,09	OC	2013
	RAC875_c102886_73	90K	682469968	682469868	1B	0,13	4,7E-04	3,33	HD,PC1	2013,2013

	IAAV1732	90K	682472813	682472658	1B	0,15	1,4E-04	3,86	HD,PC1	2013,2013
	BobWhite_rep_c62955_567	90K	682485509	682485409	1B	0,15	1,5E-04	3,83	HD,PC1	2013,2013
QTL3B.1	AX-94855154	35K	3240179	3240249	3B	0,07	1,9E-03	2,72	PH	2013
QTL3B.1	Tdurum_contig50954_1264	90K	3240264	3240167	3B	0,08	3,4E-03	2,46	PH	2013
QTL3B.1	AX-94877000	35K	3240474	3240544	3B	0,07	1,8E-03	2,75	PH	2013
QTL3B.1	RAC875_c62483_112	90K	3240993	3240907	3B	0,09	2,7E-03	2,57	PH	2013
QTL3B.1	AX-95632197	35K	3241476	3241546	3B	0,07	2,0E-03	2,70	PH	2013
QTL3B.1	Tdurum_contig50954_394	90K	3241550	3241461	3B	0,09	2,9E-03	2,54	PH	2013
QTL3B.1	RFL_Contig3008_1370	90K	4144621	4144521	3B	0,10	1,3E-03	2,89	OC	2013
QTL3B.1	AX-95081626	35K	6392749	6392817	3B	0,08	1,5E-03	2,82	OC	2013
QTL3B.1	BobWhite_c7454_363	90K	6754538	6754438	3B	0,14	1,2E-03	2,92	HD	2014
QTL3B.1	RFL_Contig5299_1905	90K	7077068	7077168	3B	0,13	1,6E-03	2,79	HD	2014
QTL3B.1	wsnp_Ex_c8386_14128029	90K	7187840	7187640	3B	0,11	2,3E-03	2,64	HD	2014
QTL3B.1	AX-94705969	35K	7189564	7189494	3B	0,09	1,0E-03	2,99	OC	2014
QTL3B.1	BS00098868_51	90K	8133877	8133777	3B	0,10	3,0E-03	2,53	HD	2014
QTL3B.1	wsnp_Ex_rep_c66766_65123941	90K	8528873	8529073	3B	0,11	2,7E-03	2,57	HD	2014
QTL3B.1	BS00105741_51	90K	9092578	9092678	3B	0,11	2,7E-03	2,57	HD	2014
QTL3B.2	BS00064956_51	90K	15521996	15521896	3B	0,12	6,3E-04	3,20	PC1	2013
QTL3B.2	wsnp_BE497169B_Ta_2_2	90K	16038488	16038608	3B	0,11	1,1E-03	2,96	PC1	2013
QTL3B.2	GENE-1856_1005	90K	16442613	16442704	3B	0,13	4,4E-04	3,35	OC,PC1	2013,2013
QTL3B.2	Excalibur_c9206_836	90K	16443167	16443264	3B	0,11	2,4E-03	2,62	PC1	2014
QTL3B.2	IAAV6442	90K	16443566	16443370	3B	0,10	1,8E-03	2,74	CL	2013
QTL3B.2	Excalibur_rep_c104498_194	90K	16444601	16444503	3B	0,11	1,8E-03	2,74	HD,PC1	2014,2014
QTL3B.2	Excalibur_rep_c104498_168	90K	16444627	16444527	3B	0,13	5,7E-04	3,24	OC,PC1	2013,2013
QTL3B.2	Kukri_rep_c101341_425	90K	16451329	16451427	3B	0,11	1,3E-03	2,87	OC	2013
QTL3B.2	Kukri_rep_c107316_393	90K	16453313	16453215	3B	0,11	1,3E-03	2,87	OC	2013
QTL3B.2	Excalibur_rep_c67448_528	90K	16453571	16453670	3B	0,11	1,3E-03	2,87	OC	2013
QTL3B.2	Excalibur_rep_c104532_80	90K	16453641	16453741	3B	0,11	1,3E-03	2,87	OC	2013
QTL3B.2	TA003677-1077	90K	16454659	16454606	3B	0,11	1,3E-03	2,87	OC	2013
QTL3B.2	IACX8266	90K	16455197	16455400	3B	0,11	1,3E-03	2,87	OC	2013

QTL3B.2	RAC875_c8885_74	90K	16455213	16455313	3B	0,11	1,3E-03	2,87	OC	2013
QTL3B.2	BS00018764_51	90K	16455466	16455366	3B	0,11	1,3E-03	2,87	OC	2013
QTL3B.2	RAC875_c36432_172	90K	16461773	16461873	3B	0,13	4,6E-04	3,34	OC,PC1	2013,2013
QTL3B.2	RAC875_c36432_197	90K	16461798	16461898	3B	0,12	9,2E-04	3,04	OC,PC1	2013,2013
QTL3B.2	BS00023188_51	90K	18156407	18156507	3B	0,11	3,1E-03	2,51	PC1	2014
QTL3B.2	AX-94837831	35K	21343742	21343812	3B	0,08	8,3E-04	3,08	OC,PC1	2013,2013
QTL3B.2	AX-94593149	35K	21385021	21384951	3B	0,07	1,5E-03	2,83	PC1	2013
QTL3B.2	AX-94446828	35K	21385872	21385802	3B	0,07	1,5E-03	2,83	PC1	2013
QTL3B.2	AX-95199184	35K	21385918	21385848	3B	0,07	1,5E-03	2,83	PC1	2013
	Kukri_c14140_347	90K	53471387	53471287	3B	0,13	1,1E-03	2,97	CL	2014
	BS00045330_51	90K	474028991	474028891	3B	0,13	8,4E-04	3,07	GBM19	2014
	RAC875_c43893_213	90K	482004601	482004501	3B	0,13	1,0E-03	2,99	GBM19	2014
	AX-94905063	35K	506307604	506307534	3B	0,12	2,8E-04	3,55	GBM19	2014
	BS00062734_51	90K	545068980	545069048	3B	0,11	2,1E-03	2,68	HN	2014
	AX-94486440	35K	561232538	561232468	3B	0,10	5,8E-04	3,24	OC	2014
	AX-94467360	35K	561569290	561569220	3B	0,18	6,9E-06	5,16	OC	2014
	BS00066357_51	90K	561702018	561701918	3B	0,23	1,7E-05	4,76	OC,PC1	2014,2014
	Excalibur_c57658_54	90K	586142991	586142891	3B	0,14	6,7E-04	3,17	PC3	2014
	Excalibur_c57658_54	90K	586143106	586143067	3B	0,14	6,7E-04	3,17	PC3	2014
	AX-94423155	35K	740546844	740546776	3B	0,09	1,5E-03	2,81	PC2	2014
	BS00040742_51	90K	741304069	741304165	3B	0,10	4,2E-03	2,37	PC2	2014
	AX-95111761	35K	741304084	741304154	3B	0,09	1,3E-03	2,89	PC2	2014
	AX-94481286	35K	785677214	785677144	3B	0,07	2,0E-03	2,70	PH	2013
	Kukri_rep_c92293_249	90K	814607853	814607753	3B	0,12	4,7E-04	3,33	PC2	2013
	GENE-4458_691	90K	814940717	814940813	3B	0,13	3,9E-04	3,41	PC2	2013
	Kukri_c7860_911	90K	815046531	815046431	3B	0,13	4,1E-04	3,39	PC2	2013
	RAC875_c66953_100	90K	815048807	815048907	3B	0,13	4,1E-04	3,39	PC2	2013
	wsnp_Ku_c17875_27051169	90K	457343223	457343257	5B	0,14	7,0E-04	3,16	GBM5	2014
	Ex_c1583_1688	90K	457395612	457395514	5B	0,14	1,2E-03	2,94	GBM5	2014
	Ex_c1583_1138	90K	457396663	457396563	5B	0,14	7,0E-04	3,16	GBM5	2014

	wsnp_Ex_rep_c66903_65319487	90K	458150611	458150811	5B	0,14	7,0E-04	3,16	GBM5	2014
QTL5B.1	IAAV8847	90K	473114456	473114256	5B	0,13	9,1E-04	3,04	GBM19	2014
QTL5B.1	AX-94674310	35K	473114724	473114654	5B	0,00	1,1E-03	2,97	HD	2014
QTL5B.1	wsnp_Ku_c5472_9710435	90K	473114789	473114589	5B	0,13	9,1E-04	3,04	GBM19	2014
QTL5B.1	RAC875_rep_c116598_137	90K	473115622	473115722	5B	0,13	9,1E-04	3,04	GBM19	2014
QTL5B.1	RAC875_rep_c106337_414	90K	473116179	473116079	5B	0,13	9,1E-04	3,04	GBM19	2014
QTL5B.1	TA005387-0575	90K	473642382	473642329	5B	0,17	2,4E-04	3,62	OC,GBM19	2014,2014
QTL5B.1	RFL_Contig773_790	90K	473740462	473740362	5B	0,13	1,3E-03	2,90	GBM19	2014
QTL5B.1	Ra_c8939_653	90K	476797852	476797752	5B	0,14	7,0E-04	3,16	GBM5	2014
QTL5B.1	wsnp_Ex_c50413_54706626	90K	476798611	476798411	5B	0,14	7,0E-04	3,16	GBM5	2014
QTL5B.1	wsnp_Ex_rep_c66667_64981526	90K	476801449	476801643	5B	0,14	7,0E-04	3,16	GBM5	2014
QTL5B.1	Kukri_c11815_706	90K	476804888	476804857	5B	0,14	7,0E-04	3,16	GBM5	2014
QTL5B.1	RAC875_c38511_91	90K	476805518	476805600	5B	0,14	7,0E-04	3,16	GBM5	2014
QTL5B.1	AX-95245374	35K	476910461	476910528	5B	0,09	1,7E-03	2,76	GBM5	2014
QTL5B.1	Tdurum_contig68343_339	90K	477498956	477498860	5B	0,14	6,2E-04	3,21	GBM5	2014
QTL5B.1	wsnp_Ku_c12562_20256747	90K	477667547	477667518	5B	0,15	4,4E-04	3,36	GBM5	2014
QTL5B.1	wsnp_Ku_c12562_20256747	90K	477667808	477667634	5B	0,15	4,4E-04	3,36	GBM5	2014
QTL5B.1	wsnp_Ex_c10644_17356566	90K	477669253	477669155	5B	0,14	6,6E-04	3,18	GBM5	2014
QTL5B.1	Kukri_c12562_453	90K	477670491	477670391	5B	0,15	4,4E-04	3,36	GBM5	2014
QTL5B.1	RFL_Contig570_515	90K	477675504	477675418	5B	0,15	4,4E-04	3,36	GBM5	2014
QTL5B.1	GENE-2550_181	90K	478126565	478126665	5B	0,15	3,8E-04	3,42	GBM5	2014
QTL5B.1	Ku_c1083_375	90K	478128017	478128098	5B	0,15	3,8E-04	3,42	GBM5	2014
QTL5B.1	Ku_c1083_1314	90K	478130055	478130155	5B	0,12	2,2E-03	2,66	HD	2014
QTL5B.1	wsnp_Ex_c19724_28721128	90K	478305718	478305518	5B	0,15	3,8E-04	3,42	GBM5	2014
QTL5B.1	wsnp_Ex_c19724_28720939	90K	478305741	478305707	5B	0,15	3,8E-04	3,42	GBM5	2014
QTL5B.1	AX-95223960	35K	478306242	478306172	5B	0,09	1,2E-03	2,92	GBM5,HD	2014,2014
QTL5B.1	AX-95007595	35K	478491077	478491147	5B	0,10	6,1E-04	3,21	GBM5,HD	2014,2014
QTL5B.1	AX-94434561	35K	478613281	478613349	5B	0,10	6,3E-04	3,20	HD	2014
QTL5B.1	AX-95227398	35K	478756123	478756057	5B	0,09	1,3E-03	2,89	HD	2014
	wsnp_Ex_rep_c105478_898916 34	90K	485905616	485905660	5B	0,13	1,0E-03	2,98	PC2	2014

	Kukri_c62247_248	90K	485997860	485997960	5B	0,13	1,0E-03	2,98	PC2	2014
	AX-95651903	35K	490759761	490759831	5B	0,07	2,0E-03	2,69	PC3	2013
	AX-95258852	35K	499120629	499120559	5B	0,09	4,1E-04	3,39	HD	2013
QTL5B.2	AX-94621773	35K	506789406	506789336	5B	0,09	4,3E-04	3,37	HD	2013
QTL5B.2	AX-94783438	35K	506951431	506951501	5B	0,09	5,2E-04	3,28	HD	2013
QTL5B.2	AX-95187492	35K	507030763	507030833	5B	0,13	5,3E-05	4,27	HD,PC1	2013,2013
QTL5B.2	AX-94713727	35K	507041113	507041147	5B	0,14	2,2E-05	4,65	HD,PC1	2013,2013
QTL5B.2	AX-94871419	35K	510883971	510884041	5B	0,09	5,1E-04	3,29	HD	2013
QTL5B.2	AX-94383375	35K	511606979	511607049	5B	0,11	1,4E-04	3,84	HD	2013
QTL5B.2	AX-94699913	35K	511697302	511697235	5B	0,10	3,1E-04	3,51	HD	2013
QTL5B.2	BS00068200_51	90K	512244926	512244966	5B	0,14	2,0E-04	3,70	HD,PC1	2013,2013
QTL5B.2	AX-95685162	35K	517776500	517776432	5B	0,11	2,7E-04	3,57	HD	2013
QTL5B.2	RFL_Contig487_1286	90K	519742256	519742356	5B	0,11	3,1E-03	2,51	PC2	2014
	Tdurum_contig41907_1014	90K	557951782	557951682	5B	0,15	3,8E-04	3,42	GBM5	2014
	Excalibur_c3165_730	90K	584853802	584853902	5B	0,10	3,2E-03	2,50	HD	2014
	AX-94516158	35K	584886618	584886548	5B	0,09	1,1E-03	2,96	HD	2014
	Kukri_c59540_137	90K	586352515	586352418	5B	0,14	6,8E-04	3,17	PC2	2014
	RAC875_c43383_483	90K	586354398	586354298	5B	0,10	3,0E-03	2,52	HD,PC2	2014,2014
QTL.5B.3	Tdurum_contig47071_1322	90K	600187536	600187636	5B	0,12	2,1E-03	2,68	HN	2014
QTL.5B.3	Tdurum_contig47833_484	90K	604068254	604068154	5B	0,12	1,5E-03	2,82	HD	2014
QTL.5B.3	Tdurum_contig70554_1006	90K	604140815	604140715	5B	0,13	1,1E-03	2,98	HN	2014
QTL.5B.3	Tdurum_contig70554_1004	90K	604140817	604140717	5B	0,13	1,1E-03	2,98	HN	2014
QTL.5B.3	RFL_Contig2772_1693	90K	605480992	605480892	5B	0,14	7,9E-04	3,10	HD,GBM19,PC1	2014,2014, 2014
QTL.5B.3	RAC875_rep_c112818_870	90K	605788615	605788699	5B	0,12	1,5E-03	2,81	PC1	2014
QTL.5B.3	BS00003655_51	90K	606020996	606021096	5B	0,12	1,5E-03	2,84	HD,PC1	2014,201
	AX-94438836	35K	663223861	663223931	5B	0,09	1,4E-03	2,86	PC1	2014
	BS00064853_51	90K	668825400	668825500	5B	0,13	7,8E-04	3,11	PH	2014
	BS00039874_51	90K	672953348	672953248	5B	0,16	2,8E-04	3,55	PC3	2014
	RFL_Contig5337_1453	90K	682839878	682839806	5B	0,15	1,6E-04	3,80	HN	2013
	AX-95218919	35K	683517335	683517405	5B	0,09	1,9E-03	2,72	PC2	2014

	AX-94530393	35K	684766124	684766054	5B	0,08	2,6E-03	2,58	GBM5	2014
	AX-94522259	35K	686049796	686049728	5B	0,11	3,4E-04	3,47	OC	2014
QTL5B.4	Tdurum_contig28552_88	90K	707131602	707131702	5B	0,18	1,4E-04	3,86	PC3	2014
QTL5B.4	Tdurum_contig28552_211	90K	707131725	707131825	5B	0,17	1,5E-04	3,83	PC3	2014
QTL5B.4	AX-94932343	35K	707131810	707131740	5B	0,12	2,0E-04	3,70	PC3	2014
QTL5B.4	RAC875_rep_c106589_784	90K	707131825	707131725	5B	0,17	1,5E-04	3,83	PC3	2014
QTL5B.4	RAC875_rep_c106589_184	90K	707133151	707133091	5B	0,13	7,3E-04	3,14	PC3	2014
QTL5B.4	RAC875_rep_c106589_184	90K	707133513	707133470	5B	0,13	7,3E-04	3,14	PC3	2014
QTL5B.4	IACX2594	90K	707134230	707134125	5B	0,13	7,3E-04	3,14	PC3	2014
QTL5B.4	Tdurum_contig92922_58	90K	707134618	707134531	5B	0,17	1,5E-04	3,83	PC3	2014
QTL5B.4	AX-94476475	35K	712860353	712860420	5B	0,10	4,2E-04	3,38	HD	2013
QTL.6A.1	Ex_c21841_1883	90K	9326153	9326117	6A	0,12	1,2E-03	2,91	GBM19	2014
QTL.6A.1	Excalibur_rep_c110867_88	90K	9327835	9327749	6A	0,12	1,2E-03	2,90	GBM19	2014
QTL.6A.1	Kukri_c52953_562	90K	9327835	9327735	6A	0,13	9,8E-04	3,01	GBM19	2014
QTL.6A.1	BobWhite_c22086_444	90K	9328036	9327936	6A	0,13	1,1E-03	2,94	GBM19	2014
QTL.6A.1	Kukri_rep_c70457_607	90K	11274035	11274135	6A	0,12	1,2E-03	2,91	GBM19	2014
QTL.6A.1	AX-95103885	35K	14609505	14609435	6A	0,09	1,2E-03	2,92	OC	2014
	tplb0032i10_420	90K	103760106	103760206	6A	0,10	1,5E-03	2,83	PC1	2013
	BS00034886_51	90K	103958669	103958769	6A	0,10	1,5E-03	2,83	PC1	2013
QTL6A.2	AX-94774725	35K	607056087	607056019	6AL	0,12	1,9E-04	3,72	PC2	2014
QTL6A.2	AX-94540705	35K	608294687	608294757	6AL	0,11	5,7E-04	3,25	PH	2014
QTL6A.2	Jagger_c5046_63	90K	609256632	609256721	6AL	0,14	6,8E-04	3,17	CL	2014
QTL6A.2	Tdurum_contig29607_413	90K	609379961	609380061	6AL	0,24	1,1E-05	4,95	CL,PC2	2014,2014
QTL6A.2	Tdurum_contig413_220	90K	609380763	609380664	6AL	0,14	8,2E-04	3,09	CL	2014
QTL6A.2	AX-94411794	35K	609453850	609453920	6AL	0,12	2,9E-04	3,55	CL	2014
QTL6A.2	AX-94926681	35K	610406777	610406707	6AL	0,08	1,0E-03	3,00	CL	2013
QTL6A.2	AX-94963438	35K	611563809	611563739	6AL	0,15	5,6E-05	4,25	CL,PC3	2014,2014
QTL6A.2	AX-94549612	35K	611566257	611566327	6AL	0,08	1,0E-03	2,99	CL	2013
QTL6A.2	AX-95182345	35K	611661167	611661237	6AL	0,07	2,3E-03	2,64	CL,CL,PC3,PC2	2013,2014, 2014,2014
QTL6A.2	AX-95153895	35K	612302284	612302354	6AL	0,08	1,0E-03	3,00	CL,PC2,CL,PC3 ,PC2	2013,2013, 2014,2014, 2014

QTL6A.2	AX-94844120	35K	612327838	612327768	6AL	0,18	8,7E-06	5,06	CL	2014
QTL6A.2	AX-94634087	35K	612329194	612329124	6AL	0,07	1,7E-03	2,78	CL,PC2,CL,PC3,PC2	2013,2013, 2014,2014, 2014
QTL6A.2	BS00099401_51	90K	613256472	613256572	6AL	0,24	1,2E-05	4,93	CL,PC2	2014,201
QTL6A.2	AX-95092538	35K	613761917	613761987	6AL	0,10	6,5E-04	3,18	PC2,CL,PC2	2013,2014, 2014
	AX-94781123	35K	10254830	10254760	7A	0,10	8,4E-04	3,08	PC3	2014
	Kukri_c74599_85	90K	14190518	14190418	7A	0,11	1,9E-03	2,71	HD	2014
	AX-94826773	35K	20768325	20768375	7A	0,09	4,9E-04	3,31	PC2	2013
	wsnp_CAP12_c3056_1439567	90K	48056206	48056082	7A	0,11	1,1E-03	2,97	OC	2013
QTL.7A.1	wsnp_Ku_c57198_60433631	90K	78430481	78430404	7A	0,11	2,2E-03	2,66	HN	2014
QTL.7A.1	wsnp_Ku_c57198_60433631	90K	78433357	78433318	7A	0,11	2,2E-03	2,66	HN	2014
QTL.7A.1	Ra_c9427_300	90K	78434149	78434249	7A	0,11	2,2E-03	2,66	HN	2014
QTL.7A.1	wsnp_Ex_c41150_48040078	90K	78434401	78434601	7A	0,11	2,2E-03	2,66	HN	2014
QTL.7A.1	AX-94877599	35K	79648887	79648957	7A	0,09	5,5E-04	3,26	PH	2013
QTL.7A.1	AX-94805980	35K	80067005	80067075	7A	0,07	1,8E-03	2,74	PH	2013
QTL.7A.1	AX-94452237	35K	80861397	80861467	7A	0,07	1,9E-03	2,72	PH	2013
QTL.7A.1	AX-94486561	35K	80861460	80861530	7A	0,08	1,5E-03	2,83	PH	2013
	AX-94492491	35K	581848865	581848935	7A	0,08	3,0E-03	2,52	GBM5	2014
	AX-94894168	35K	642736925	642736855	7A	0,06	4,6E-03	2,34	CL	2013
	AX-95652966	35K	645092433	645092503	7A	0,07	2,7E-03	2,57	CL	2013
	AX-95629211	35K	671416510	671416572	7A	0,09	5,4E-04	3,27	PH,HD,PC1	2013,2014, 2014
QTL7A.2	AX-94538262	35K	689949417	689949347	7A	0,14	9,0E-05	4,05	GBM19,PC2,PC1	2014,2014, 2014
QTL7A.2	BS00022237_51	90K	689949432	689949332	7A	0,15	3,8E-04	3,42	PC2	2014
QTL7A.2	wsnp_Ex_c53442_56678505	90K	692344121	692344192	7A	0,13	9,1E-04	3,04	PC2	2014
QTL7A.2	wsnp_Ex_c53442_56678505	90K	692344761	692344829	7A	0,13	9,1E-04	3,04	PC2	2014
QTL7A.2	AX-94424070	35K	692345091	692345021	7A	0,09	1,4E-03	2,84	GBM19	2014
QTL7A.3	Excalibur_rep_c102327_102	90K	701293964	701294043	7A	0,19	7,0E-05	4,15	HN,PC1	2014,2014
QTL7A.3	Tdurum_contig17062_221	90K	701529556	701529456	7A	0,16	2,8E-04	3,55	HN	2014
QTL7A.3	wsnp_RFL_Contig2805_2579582	90K	701532526	701532426	7A	0,13	9,3E-04	3,03	HN	2014
QTL7A.3	Kukri_c57593_79	90K	701629525	701629622	7A	0,13	9,4E-04	3,03	HN	2014

QTL7A.3	Kukri_rep_c98227_230	90K	701638136	701638236	7A	0,16	2,8E-04	3,55	HN	2014
QTL7A.3	Kukri_rep_c98227_390	90K	701638296	701638396	7A	0,18	1,4E-04	3,84	HN,PC1	2014,2014
QTL7A.3	Excalibur_rep_c66918_307	90K	701638820	701638720	7A	0,16	2,8E-04	3,55	HN	2014
QTL7A.3	tplb0032g12_1241	90K	701683460	701683560	7A	0,16	2,7E-04	3,56	HN,PC1	2014,2014
QTL7A.3	AX-95179928	35K	712417130	712417060	7A	0,10	8,7E-04	3,06	PC3	2014

Table S2. Relative contribution of traits recorded in 2013 to principal components.

	Comp.1	Comp.2	Comp.3	Comp.4
CL	0.249	0.245	0.427	0.037
OC	0.298	0.092	0.127	0.440
PH	0.178	0.475	0.117	0.222
HD	0.273	0.186	0.327	0.300

Table S3. Relative contribution of traits recorded in 2014 to principal components.

	Comp.1	Comp.2	Comp.3	Comp.4	Comp.5	Comp.6	Comp.7
CL	0.011	0.405	0.314	0.065	0.018	0.005	0.010
OC	0.176	0.039	0.096	0.328	0.098	0.261	0.122
GBM19	0.203	0.055	0.067	0.056	0.060	0.058	0.436
GBM5	0.167	0.039	0.028	0.399	0.166	0.178	0.070
PH	0.077	0.330	0.334	0.062	0.108	0.106	0.049
HN	0.198	0.062	0.049	0.046	0.113	0.330	0.241
HD	0.164	0.065	0.108	0.040	0.433	0.059	0.069

Table S4. Lines of haplotype group 1. Genotypes denoted with a star share the cultivar Vinjett as a parent.

Name	Origin
Avle	NO
Vinjett	SE
Breeding line 1175	NO
Laban	NO
Breeding line 1179	SE
Breeding line 1187	NO
Breeding line 1188*	SE
Breeding line 1189	SE
Bombona	SE
Breeding line 1193	SE
Breeding line 1312	NO
Breeding line 1317	NO
Breeding line 1318	NO
Breeding line 1319	NO
Breeding line 1320	NO
Breeding line 1324	SE
Breeding line 1325*	SE
Breeding line 1327*	SE
Breeding line 1328	SE

Mirakel	NO
Breeding line 1405	NO
Breeding line 1410	NO
Breeding line 1412*	NO
Breeding line 1413*	SE
Breeding line 1415	SE
Breeding line 1417	NO
Avans	SE
Anniina	FI
Marble*	FI
Dragon	SE

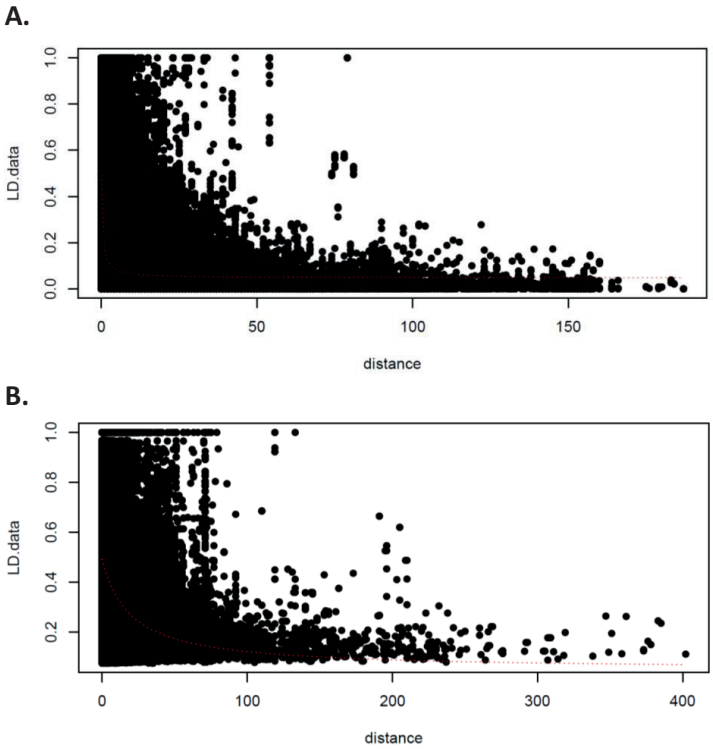

Figure S1. Genome-wide linkage disequilibrium (LD) decay of 35K (A) and 90K (B) SNPs. LD decayed at 1 and 3 cM for the 35K and 90K SNP array, respectively. The estimated r^2 value for half decay was 0.24 for both arrays.

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