Theor Appl Genet (2013) 126:2335–2351
DOI 10.1007/s00122-013-2139-0

ORIGINAL PAPER

Association mapping of salt tolerance in barley (*Hordeum vulgare* L.)

Nguyen Viet Long · Oene Dolstra · Marcos Malosetti · Benjamin Kilian · Andreas Graner · Richard G. F. Visser · C. Gerard van der Linden

Received: 21 November 2012 / Accepted: 21 May 2013 / Published online: 16 June 2013

Abstract A spring barley collection of 192 genotypes from a wide geographical range was used to identify quantitative trait loci (QTLs) for salt tolerance traits by means of an association mapping approach using a thousand SNP marker set. Linkage disequilibrium (LD) decay was found with marker distances spanning 2–8 cM depending on the methods used to account for population structure and genetic relatedness between genotypes. The association panel showed large variation for traits that were highly heritable under salt stress, including biomass production, chlorophyll content, plant height, tiller number, leaf senescence and shoot Na^+, shoot Cl^- and shoot, root Na^+/K^+ contents. The significant correlations between these traits and salt tolerance (defined as the biomass produced under salt stress relative to the biomass produced under control conditions) indicate that these traits contribute to (components of) salt tolerance. Association mapping was performed using several methods to account for population structure and minimize false-positive associations. This resulted in the identification of a number of genomic regions that strongly influenced salt tolerance and ion homeostasis, with a major QTL controlling salt tolerance on chromosome 6H, and a strong QTL for ion contents on chromosome 4H.

Communicated by P. Hayes.

Electronic supplementary material The online version of this article (doi:10.1007/s00122-013-2139-0) contains supplementary material, which is available to authorized users.

N. V. Long
Graduate School Experimental Plant Sciences, Wageningen University, PO Box 386, 6700 AJ Wageningen, The Netherlands

N. V. Long · O. Dolstra · R. G. F. Visser · C. G. van der Linden (✉)
Wageningen UR Plant Breeding, Wageningen University and Research Centre, PO Box 386, 6700 AJ Wageningen, The Netherlands
e-mail: gerard.vanderlinden@wur.nl

M. Malosetti
Biometris, Department of Plant Sciences, Wageningen University and Research Centre, P.O. Box 100, 6700 AC Wageningen, The Netherlands

B. Kilian · A. Graner
Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Corrensstr. 3, 06466 Gatersleben, Germany

Introduction

Salt stress is a major constraint to agricultural food production because it decreases crop yield and restricts the use of agricultural land. It is estimated that of the 280 million hectares of agricultural land approximately 20 % is salinated (FAO 2008). The problem is increasing annually due to climatic change and poor irrigation management. Most cultivated crops are salt sensitive and therefore salinity is an ever-present threat to agriculture (Flowers and Flowers 2005).

Salt tolerance in crop plants is a genetic and physiological complex trait and is controlled by several quantitative trait loci (Flowers 2004). The plant's response to salinity stress is composed of two phases (Munns and Tester 2008). The first phase concerns the osmotic stress that is perceived immediately upon plant exposure to highly saline conditions. Osmotic stress makes uptake of water by plants difficult and adversely affects shoot and root growth. To facilitate water uptake under such conditions, plants have to accumulate extra solutes to maintain the water balance of the cells. The second phase is

manifested when high concentrations of toxic ions are built up over a longer period of time. As NaCl is a major constituent of saline soil, plants accumulate Na^+ and Cl^- ions up to levels that are toxic, reducing amongst others their photosynthetic capacity. Shoot Na^+ toxicity is associated with a reduction of stomatal conductance while high shoot Cl^- levels affect chlorophyll and inhibit photosystem II (Tavakkoli et al. 2011). Therefore, both shoot Na^+ and Cl^- contents were considered important factors for salt-induced damage (Hasegawa et al. 2000; Munns and Testers 2008; Teakle and Tyerman 2010) even more because the toxicity effects of these ions appear to be cumulative (Tavakkoli et al. 2011). Although the mechanisms conferring salt tolerance and their genetic control in crops are not fully understood, regulation of intracellular content of cations (Na^+, K^+, Mg^{2+} and Ca^{2+}) and anions (Cl^-) and ion transport mechanisms are considered important. Ion homeostasis under salt stress conditions is controlled by several ion channels, pathways of transportation, compartmentalization mechanisms and ion sensing and signalling (Munns and Testers 2008; Zhu 2003).

Barley (*Hordeum vulgare*) is the fourth most important cereal crop worldwide, and it has a long history as a model for genetic studies (Schulte et al. 2009). It is the most salt tolerant cereal (Maas and Hoffman 1977; Munns and Tester 2008). Cultivated barley originates from wild barley (*Hordeum spontaneum*) and was domesticated within the Fertile Crescent, probably multiple times (Kilian et al. 2006). In comparison to other wild cereals, wild barley has a wide natural distribution area to which it is well adapted (Harlan and Zohary 1966; Nevo 2007). Both genetic diversity and the adaptation to a broad spectrum of micro-ecological conditions including water availability, temperature, soil type and altitude have strongly influenced the development of salt tolerance in barley. This resulted in a rich gene pool with a large variation in adaptation to abiotic stresses including drought and salinity (Nevo and Chen 2010; Nevo et al. 2004). Therefore, scientists have advocated barley as a source of favorable alleles to be used in crop salt tolerance improvement by means of conventional and molecular approaches (Colmer et al. 2005; Munns 2005). However, the genetics of the various salt tolerance mechanisms found in the gene pools of barley and wheat are still relatively unknown, which may explain the limited success in exploiting the resources in breeding for salt tolerance. Ellis et al. (2000) and Kilian et al. (2006) pointed out that modern barley cultivars only contain 15–40 % of all alleles present in the barley gene pool. Therefore, it is quite likely that only a part of the barley genetic potential for salt tolerance has been addressed in salt tolerance genetic improvement performed so far.

Traditional QTL mapping or biparental QTL mapping based on a single segregating population derived from two homozygous parental genotypes has been the commonly used approach for genetic dissection of salt tolerance in barley and to identify candidate genes (Mano and Takeda 1997; Xue et al. 2009; Ellis et al. 2002; Witzel et al. 2009). This approach provides valuable information on genomic regions that control quantitative traits but it also has limitations due to poor sampling of the allelic variation present in the barley gene pool for each of the loci affecting salt tolerance, lack of segregation, and poor resolution of this type of the mapping of QTLs. Biparental QTL mapping detects genomic regions with QTLs for a trait with an accuracy ranging on average from a few to several tens of centiMorgans (cM) and such chromosomal regions could harbor a few hundred up to several thousand genes (Ingvarsson et al. 2010). Accurate breeding methods are therefore needed to efficiently exploit the genetic variation for salt tolerance in barley germplasm.

Novel association mapping or linkage disequilibrium approaches have recently been introduced in plant genetic studies (Van Eeuwijk et al. 2004; Mackay and Powell 2007; Zhao et al. 2007; Cockram et al. 2010; Atwell et al. 2010). Association mapping studies in a much broader germplasm are now possible due to fast and affordable genotyping and sequencing technologies (Zhu et al. 2008). Association mapping relies on linkage disequilibrium between markers and QTLs present in collections of diverse germplasm (Pritchard et al. 2000). It exploits the recombination events that have occurred during the long evolutionary history (Nordborg and Tavare 2002) of a crop species, producing shorter linkage blocks than found in biparental QTL mapping studies. QTLs for a salt tolerance trait detected in this way could be more precisely localized than those found through biparental QTL mapping. In addition, association mapping will address major allelic variants of QTLs for salt tolerance when performed with an adequate association mapping panel.

This study aims at the genetic dissection of mechanisms underlying salt tolerance in a worldwide panel of spring barley varieties using association mapping. The collection was chosen to represent a wide range of genetic diversity possible in spring barley (Stracke et al. 2009; Haseneyer et al. 2010) and has already been successfully applied in whole genome association analysis for several agronomical traits (Pasam et al. 2012). The objectives are (1) to evaluate genetic variation for salt tolerance and traits contributing to salt tolerance in a diverse spring barley collection, (2) to estimate genetic properties of the association mapping panel using a different method to account for the confounding of population structure, (3) to establish marker-trait associations for each salt tolerance trait, and (4) to identify major genes/loci affecting salt tolerance in spring barley that can be used for genetic improvement of salt tolerance. Our association mapping revealed a major locus

significantly contributing to salt tolerance, and other major loci determining ion contents and ion homeostasis.

Materials and methods

Barley germplasm collection

The association panel used in this study consisted of 192 spring barley accessions originating from 51 different countries and 4 geographical regions: Europe (EU, $n = 92$), East Asia (EA, $n = 33$), West Asia and North Africa (WANA, $n = 40$), and America (AM, $n = 27$). The set of genotypes comprised breeding materials, traditional and improved cultivars and landraces, including 105 two-rowed and 87 six-rowed varieties. The genotypes were selected among the Barley Core Collection (BCC) (Knüpffer and van Hintum 2003) and the barley collection maintained at the IPK Genebank Gatersleben, Germany (Haseneyer et al. 2010). This worldwide collection was initially investigated by Stracke et al. (2009) using an association mapping approach to map flowering time genes. Haseneyer et al. (2010) studied this collection for several agronomical traits using microsatellite markers. The same population was used in a whole genome scan using SNP markers to identify QTLs associated with agronomical traits (Pasam et al. 2012).

Salt tolerance evaluation

The set of 192 genotypes from the association panel was evaluated at the vegetative stage of plant growth for salt tolerance traits during two consecutive years (2010 and 2011) using a hydroponics system. To this end seeds from the association panel genotypes were germinated in trays with silver sand for one week until the first seedling leaf had fully emerged. Individual seedlings were then transferred to the hydroponics system. The hydroponic growing media was full-strength modified Hoagland's solution which was maintained at pH 5.8. After 7 days on the system, NaCl was gradually added to half of the containers with a 50 mM day^{-1} increment to bring the solution to a final salinity level of 200 mM NaCl. This final concentration was maintained for 3 weeks until the plants were harvested for biomass and ion content measurements.

The hydroponics system used for testing consists of four units of 16 containers with 24 plant positions as described in Nguyen et al. (2013). The experiments in both years had a randomized block design, each with four blocks per treatment. Each plant represented one experimental unit. There were eight replicates per genotype (four per each treatment). Thus, each experiment consisted of eight randomized blocks allocated to two hydroponics units with the control treatment (0 mM NaCl) and to two units with the salt treatment (200 mM NaCl).

To measure growth parameters, all plants from the control and salt stress treatments were weighed at harvest and then separated into shoots and roots. Both plant fractions were dried separately in a forced-air oven at 70 °C until the samples reached stable weight prior to the determination of the dry weight. Salt tolerance (ST) was assessed as the percentage of relative shoot biomass production under saline and non-saline conditions according to the definition of Munns and James (2003).

The shoot and root samples of the plants grown on the same hydroponic unit were pooled per genotype prior to the determination of contents of the cations Na^+, K^+, Ca^{2+} and Mg^{2+}, and one anion (Cl^-). The assessment of the ion contents of each root and shoot sample using Ion Chromatography (IC) system 850 Professional (Metrohm Switzerland) was done similarly to Nguyen et al. (2013).

Data on plant height, root length (cm) and number of tillers were collected for all plants grown under control and saline conditions. Chlorophyll content was measured using a SPAD-502 meter (Minolta, Osaka, Japan) 1 day before the final harvest (3 weeks after final salt concentration was reached). SPAD measurements give an accurate estimation of the total chlorophyll content (James et al. 2002). The SPAD readings were taken near the stem (5 cm from the stem), in the middle and near the tip (5 cm from the end) of the last fully expanded leaf. The leaf was about 15–25 cm long at the time the SPAD reading was taken. The SPAD measurements were done on four plants per genotype per treatment, and three measurements per leaf were averaged per plant. Leaf senescence on each of the three oldest leaves of the main tiller of each plant was scored 1 day before harvesting using a senescence scale from 1 to 9. The average over three lower leaves per plant was used for analysis. The upper shoot leaves did not senesce during the experiment.

Genotyping

The association panel lines used in this study were genotyped with a customized 1536 SNP Illumina GoldenGate Oligonucleotide Pool Assay (OPA) (Close et al. 2009). In total 988 mapped SNPs were polymorphic. The SNPs with rare alleles and poor quality (more than 10 % missing data) were excluded. The final set of 954 good quality SNPs that distributed over the whole barley genome were used to perform LD investigation and association mapping. The average spacing between markers was 1.18 cM. Marker profiling was described in detail in Pasam et al. (2012).

Statistical analysis of phenotypic data

The data of the experiments combined over the 2 years were first inspected trait-by-trait to get insight in the relevance of the genotypic variation within the panel and in genotype-by-environment interactions by means of an overall analysis of variance using Genstat version 14.2. The experimental design with its block structure within both years was taken into consideration. After the preliminary statistical analyses, separate analyses of variance were done using either the salt-stressed or control datasets. The 2-year data of the salt-stressed plants were analyzed to get for each trait estimates of the genotypic variance (σ^2_g), genotype-by-year interactions (σ^2_{gy}), and environmental variances (σ^2_e). These estimates were subsequently used to calculate for each trait the heritability (h^2_m) based on genotypic means over 2-year data by the formula: $h^2_m = \sigma^2_g/(\sigma^2_g + \sigma^2_{gy}/y + \sigma^2_e/ry)$ where y is number of years and r is number of biological replications per year. The dataset of control treatment was analyzed in the same way to get similar population statistics. The relationship between the mean shoot and root ion contents of the lines and their contributions to the variance for salt tolerance was investigated using Pearson correlations. For the salt and control dataset, separate ANOVAs were performed to test the relevance of geographical origin and ear row number type.

Principal component analysis (Eigenanalysis)

Population structure of an association panel is typically assessed using the approach described by Pritchard et al. (2000) implemented in the STRUCTURE software. Haseneyer et al. (2010) and Pasam et al. (2012) used this to assess the structure information of the association panel and revealed subgroups existing within the collections that largely correspond to the row types of the ear and the geographical origins. In this study, we used the Eigenanalysis method proposed by Price et al. (2006) and Patterson et al. (2006) to investigate the population structure. Eigenanalysis was run with help of the QEIGENALYSIS procedure in Genstat 14 (Payne et al. 2011) using the 954 SNP marker set. From a singular value decomposition of the genotype by marker matrix, a set of significant eigenvectors were obtained, which in turn were used as covariables to account for population structure in both the models used to assess LD between markers, and to assess marker-trait associations.

LD decay investigation

The extent of LD within the evaluated barley association panel was studied previously using 45 SSR markers (Haseneyer et al. 2010) and in a candidate gene approach for flowering time using 25 SSR markers (Stracke et al. 2009). In our study, 954 biallelic SNPs were used to study marker–marker associations in relation with genetic distance (LD decay). To study LD between markers, we used a logistic regression model with one marker as response and a nearby marker as regressor. The degree of LD between markers is related to the significance of that regression. The advantage of this approach is that covariables that account for population structure can be added to the model, giving a more accurate estimation of LD than the uncorrected LD measures (simple r^2 between markers are confounded with population structure effects). Geographical origin and ear row type information separately were first used as predetermined subgroups in LD analysis models to reduce the impact of the differences in allele frequencies among subgroups on LD estimation (D'hoop et al. 2010; Pasam et al. 2012). As described before, eigenvectors were used as covariables in the model to assess the LD decay to account for the effects of population structure (Patterson et al. 2006; Price et al. 2006). The Null model (i.e., without covariables), which assumes no population structure and individual relatedness in the association panel, was used as a reference, which is equivalent to the uncorrected r^2 between markers. The LD decay per chromosome was visualized by plotting the $-\log_{10}(P)$ value against the genetic distance between markers in centiMorgan (cM). All analyses and LD graphics were made with procedure QLDDECAY Genstat 14.2 edition (Payne et al. 2011).

Association mapping analysis

In the current study, the phenotypic data of the genotypes from the association mapping panel under saline and control conditions and the marker scores for a set of 954 SNPs were used to perform marker-trait association analysis. To account for effects of the structure of the mapping panel and relatedness among panel members, the three different association analysis models that are available in the procedure QSASSOCIATION in Genstat 14.2 (Payne et al. 2011) were used: (1) Eigenanalysis (Price et al. 2006; Patterson et al. 2006); (2) kinship matrix (Yu et al. 2006; Malosetti et al. 2007; Pasam et al. 2012); and (3) predetermined grouping (Zhao et al. 2007; Pasam et al. 2012). The kinship matrix based on similarities in the SNP scoring patterns between the genotypes in the panel was calculated using a simple matching method present in Genstat. The predetermined grouping approach uses molecular marker information within a Bayesian framework to assign group membership probabilities to the genotypes (Falush et al. 2003; Cockram et al.2010).

We used a marker-trait association model that includes the treatment as an extra factor to study marker and

marker-by-treatment interaction effects. Mean phenotyping data per treatment was used in the single trait-single environment association analyses performed. In this study, we present mainly the results obtained using the Eigen-analysis mixed-model association mapping approach, where eigenvectors are used as covariables in the marker-trait association model (Price et al. 2006; Patterson et al. 2006).

The QTL effects were fitted as fixed effects and tested using the Wald test statistic (Searle et al. 1992; Verbeke and Molenberghs 2000). The Wald statistic is asymptotically distributed as χ^2_r, where r is the number of parameters being estimated. The P-values from the χ^2_r tests are transformed, using a $-\log_{10}(P)$ transformation. The ratio of number of effective tests (total genome size divided to LD decay which was found in the current study at 4 cM) over the significant level ($\alpha = 0.05$) was used to calculate the threshold $= -\log_{10}(0.05/\#\text{tests})$. The threshold level on a $-\log_{10}(P)$ scale at 3.74 was used to claim a significant QTL.

Results

Phenotypic variation and heritability

In the barley association panel grown on hydroponics, a significant reduction of shoot and root growth due to salt stress was observed. There was significant variation ($P < 0.001$) for dry weight shoot and root, and other studied traits (Table 1). Estimates for the heritability (h^2) of growth traits ranged from 0.42 (root length under stress conditions) to 0.86 (leaf chlorophyll content under stress conditions). Heritability estimates for growth traits such as shoot, root biomass, leaf chlorophyll content and leaf senescence under salt stress were generally higher than for the same traits under control conditions. Significant variations in genotype-by-treatment and genotype-by-year interaction were observed for most of the studied traits, except root length and number of leaves on the main culm of barley plants grown under stress conditions (Table 1). As for growth-related traits, significant genotypic variation and clear treatment effects on shoot and root ion contents were observed (Table 2). Heritabilities of shoot Na^+ and K^+ content, and shoot and root Na^+/K^+ under salt stress were high (0.8). The genotypic differences in shoot and root Cl^- contents were highly heritable under both stress and normal growth conditions. Small heritable variation was observed for Ca^{2+} and Mg^{2+} regardless of tissue type and growing conditions.

Salt tolerance and correlation to other traits

Large variation in salt tolerance (ST)—defined as the ratio of dry weight shoot under stress conditions and dry weight shoot under non-stress conditions, expressed as percentage—was

Table 1 Summary of statistics of the association mapping panel describing the genotypic variation for various growth traits determined after 3 weeks of testing with 200 mM NaCl (S) or 0 mM NaCl (C)

Trait	Treatment	Mean	Range		LSD	Variance component			h^2_m
			Max	Min		σ^2_e	σ^2_g	σ^2_{gy}	
Shoot FW (g/plant)	C	23.4	43.9	7.7	0.3	0.09	0.06	0.04	0.66
	S	4.8	10.1	1.4	0.3	0.10	0.14	0.05	0.80
Shoot DW (g/plant)	C	2.3	4.7	0.8	0.3	0.10	0.06	0.04	0.65
	S	0.8	1.7	0.3	0.3	0.09	0.13	0.04	0.80
Root DW (g/plant)	C	0.4	0.8	0.2	0.4	0.17	0.05	0.02	0.61
	S	0.3	0.5	0.1	0.4	0.14	0.05	0.03	0.63
Chlorophyll content (SPAD reading)	C	46.4	54.9	28.2	3.9	15.91	13.45	3.64	0.8
	S	47.3	58.4	23.2	5.2	27.65	40.38	5.97	0.86
Plant height (cm)	C	71.7	90.8	52.2	6.0	37.71	36.90	5.28	0.83
	S	45.0	57.6	30.9	6.3	41.78	27.81	2.06	0.82
Root length (cm)	C	55.6	73.0	38.9	8.9	81.83	21.84	4.90	0.63
	S	29.8	35.5	22.1	4.7	22.51	2.32	0.84	0.42
Number of tillers	C	6.0	10.0	2.6	1.6	2.60	1.47	0.54	0.71
	S	2.9	6.0	1.3	1.0	0.97	0.41	0.13	0.69
Leaf senescence	C	1.5	3.5	1.0	0.7	0.47	0.12	0.06	0.58
	S	5.4	8.9	1.8	1.3	1.88	1.14	0.61	0.68

FW fresh weight, *DW* dry weight, *LSD* least significant difference at $P < 0.05$; h^2_m heritability of means; σ^2_e environmental variance; σ^2_g genotypic variance; σ^2_{gy} genotype-by-year interactions; *Leaf senescence* (1–9 rating)

Table 2 Summary of statistics of the association mapping panel for various ion content traits collected after 3 weeks of testing under 200 mM NaCl (S) and 0 mM NaCl (C) treatments

Trait	Treatment	Mean	Range		LSD	Variance component			h^2_m
			Max	Min		σ^2_e	σ^2_g	σ^2_{gy}	
Shoot Na^+ (mg/g)	S	38.2	69.6	18.4	7.1	25.74	102.29	37.32	0.80
Shoot K^+ (mg/g)	C	65.2	81.0	48.3	8.0	32.99	28.40	12.86	0.66
	S	25.2	51.6	8.1	5.5	15.82	57.28	19.37	0.81
Shoot Mg^{2+} (mg/g)	C	2.7	4.4	1.8	0.5	0.11	0.10	0.04	0.65
	S	1.7	3.4	0.9	0.6	0.19	0.11	0.06	0.58
Shoot Ca^{2+} (mg/g)	C	8.4	13.0	5.6	1.9	1.79	0.80	0.63	0.51
	S	3.3	7.1	1.3	1.3	0.93	0.40	1.05	0.34
Shoot Cl^- (mg/g)	C	11.6	19.3	7.0	1.5	1.11	3.80	1.61	0.78
	S	45.5	77.9	24.3	8.9	40.75	84.30	18.20	0.81
Shoot Na^+/K^+	S	1.9	5.0	0.6	0.6	0.21	1.13	0.19	0.88
Root Na^+ (mg/g)	S	56.5	65.8	46.5	7.1	25.96	9.45	3.41	0.54
Root K^+ (mg/g)	C	44.8	62.9	31.1	7.5	29.19	21.18	9.56	0.64
	S	11.5	19.5	4.1	3.3	5.69	5.59	1.50	0.72
Root Mg^{2+} (mg/g)	C	3.0	5.5	1.9	1.0	0.52	0.14	0.00	0.52
	S	1.9	3.3	1.2	0.9	0.39	0.03	0.08	0.17
Root Ca^{2+} (mg/g)	C	7.2	12.0	4.6	2.9	4.46	0.12	0.00	0.10
	S	4.4	6.8	1.7	2.4	2.89	0.04	0.44	0.04
Root Cl^- (mg/g)	C	0.7	2.3	0.0	0.5	0.12	0.09	0.01	0.71
	S	54.8	71.6	45.1	7.1	25.86	19.48	4.55	0.69
Root Na^+/K^+	C	0.2	0.4	0.1	0.1	0.00	0.00	0.00	0.08
	S	5.4	14.8	2.9	1.4	1.08	1.99	0.66	0.77

LSD least significant difference at $P < 0.05$, h^2_m heritability of means, σ^2_e environmental variance, σ^2_g genotypic variance, σ^2_{gy} genotype-by-year interactions

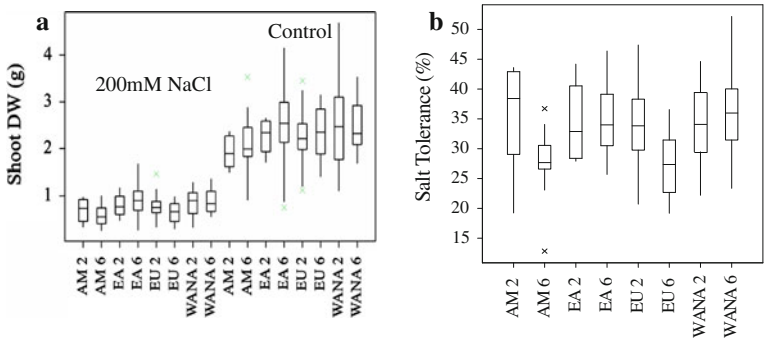


Fig. 1 *Box plots* showing differences in (**a**) shoot DW (g) under saline and control conditions and (**b**) salt tolerance (ST) among barleys from four different geographical origins (*AM*: North America; *EA*: East Asia; *EU*: Erope; *WANA*: West Asia and North Africa) and two ear types (2, two-rowed and 6, six-rowed) subpopulations; *Box edges* show *upper* and *lower* quantile and the median as shown in the middle of the box. Individuals falling outside the rank of *whisker* are shown as crosses

observed within the association panel. The six-rowed East Asia (EA6) and two-rowed West Asia–North Africa (WANA2) genotype groups showed largest variation for shoot dry weight under both control and saline conditions. The six-rowed American (AM6) and European (EU6) groups produced less shoot biomass under saline conditions (Fig. 1a). ST ranged from 12.8 to 52.2 % with a population average of 33 %. Variation in salt tolerance appeared to be at least partly linked to the geographical origin of the germplasm and ear type. The WANA2 and EA6 genotypes had an average ST (34–35 %) that was slightly higher than the population mean while the groups of AM6 and EU6 genotypes generally showed lower ST (Fig. 1b). Taking into account the variation contributed by ear row type within a geographical origin, two-rowed AM and two-rowed EU genotypes showed larger genotypic variation as well as higher means for ST than six-rowed genotypes. Six-rowed EA and two-rowed WANA genotypes displayed the largest variation for shoot dry weight under saline conditions. The best genotype for salt tolerance over the two-year trials was collected from North Africa (ST: 52 %) and a genotype from America was consistently salt sensitive (ST: 12 %).

Shoot dry weight under control and saline conditions was highly positively correlated ($r = 0.83$; $P < 0.001$) (Table S1). Most ion contents under both conditions were negatively correlated with shoot dry weight, except root K^+. Under control conditions, shoot Mg^{2+}, shoot Ca^{2+}, root K^+ and root Na^+/K^+ were negatively correlated to relative shoot dry weight. Under salinity stress, shoot K^+, Mg^{2+}, Ca^{2+} and Cl^- and root K^+, Na^+/K^+, Ca^{2+}, and Cl^- were inversely related to shoot dry weight.

Salt tolerance within the association panel was found to be strongly associated with the amount of biomass produced (both shoot and root dry weight) under stressed condition but no clear relationship was found with control biomass, indicating that the performance of the plants under control conditions was not indicative for ST. Under stress conditions, high concentrations of both shoot and root Cl^- were adversely correlated with ST ($r = -0.33$; $P < 0.001$) and ($r = -0.18$; $P < 0.05$), respectively (Table 3). Under saline conditions, Na^+ contents in shoots compared to roots showed a clearly different relation with ST. Shoot Na^+ ($r = -0.23$; $P < 0.01$) was negatively correlated with ST, in contrast root Na^+ content ($r = 0.19$; $P < 0.01$) was positively correlated with ST. These results suggest that a shoot Na^+ exclusion mechanism may be involved in salt tolerance. Shoot Mg^{2+} was negatively correlated with ST ($r = -0.29$; $P < 0.001$) only under control conditions.

Under saline conditions, leaf chlorophyll content ($r = 0.46$; $P < 0.001$), tiller number ($r = 0.36$; $P < 0.001$), plant height ($r = 0.47$; $P < 0.001$) and shoot DW ($r = 0.63$; $P < 0.001$) were positively correlated with ST (Table S1). Leaf senescence showed a clear negative correlation with ST ($r = -0.40$; $P < 0.001$). Shoot Cl^- was inversely correlated with leaf chlorophyll content ($r = -0.21$; $P < 0.01$) and positively correlated with leaf senescence ($r = 0.32$; $P < 0.001$). This clearly indicates the interdependency between shoot Cl^- content, leaf chlorophyll content, leaf senescence and ST. The effect of shoot Cl^- on plant growth traits is the most obvious for plant height ($r = -0.6$; $P < 0.001$), may even be more harmful than shoot Na^+ which showed smaller correlation with plant height ($r = -0.4$; $P < 0.001$). A higher shoot Na^+ showed no correlation with chlorophyll content of the leaves and the formation of tillers. We observed no clear correlation of growth-related traits with shoot K^+ under stress conditions. There were negative correlations between shoot and root Na^+/K^+ and plant height ($r = -0.22$; $P < 0.01$) and ($r = -0.40$; $P < 0.001$), respectively (Table S1).

Table 3 Coefficients of correlation (r) between various compositional traits of the shoot and root plant fraction and Salt Tolerance (ST) and shoot dry weight of the association mapping panel after 3 weeks of testing on hydroponics with 200 mM NaCl (S) or 0 mM NaCl (C)

Trait	Treatment	ST (%)	Shoot dry weight (g)	
			S	C
Shoot Na^+ (mg/g)	S	−0.23**	NS	NS
	C	NS	NS	NS
Root Na^+ (mg/g)	S	0.19**	NS	NS
	C	NS	NS	NS
Shoot K^+ (mg/g)	S	NS	−0.27***	−0.27***
	C	NS	NS	NS
Root K^+ (mg/g)	S	NS	0.26***	0.24***
	C	NS	0.32***	0.35***
Root Na^+/K^+ (mg/g)	S	−0.17*	−0.30***	−0.30***
	C	NS	−0.28***	−0.23**
Shoot Mg^{2+} (mg/g)	S	−0.44***	−0.58***	−0.46***
	C	−0.29***	−0.40***	−0.34***
Shoot Ca^{2+} (mg/g)	S	NS	−0.20**	−0.19**
	C	NS	−0.15*	−0.18*
Root Ca^{2+} (mg/g)	S	NS	−0.19**	−0.17*
	C	NS	NS	NS
Shoot Cl^- (mg/g)	S	−0.33***	−0.42***	−0.30***
	C	NS	NS	NS
Root Cl^- (mg/g)	S	−0.18*	−0.21**	NS
	C	NS	NS	NS

NS not significant

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Genetic properties of the association panel

The Eigenanalysis resulted in 19 axes (PCs) that describe the relationships between individuals in the association panel (Fig. S1). The PCs 1–6 explained most of the variation and this suggests the presence of major groups in the population. The remaining axes suggest the existence of the more cryptic relationships. The differences in variation explained by 19 axes might reflect differences in relatedness and the population structure within the association panel. The estimates for linkage disequilibrium ($-\log_{10}(P)$) between all possible pairs of SNP markers within each of the seven barley linkage groups were plotted against their genetic distance in cM on the integrated genetic map (Close et al. 2009; Pasam et al. 2012) to determine LD decay. Figure 2 displays the LD decay plots for chromosome 5H with and without correction for population structure.

Including structure information in the analysis models helped to reduce noise and LD decay more rapidly (Fig. 2). Without correcting for population structure, the average LD decay was typically 10 cM (Null model). The predetermined models that included geographical origin and ear row type information had similar effects with the mean marker distance from 7–8 cM. The LD estimates obtained with the Eigenanalysis showed a clear decay in each linkage group between markers spaced up to about 4 cM on the integrated map. A similar extent of LD was found

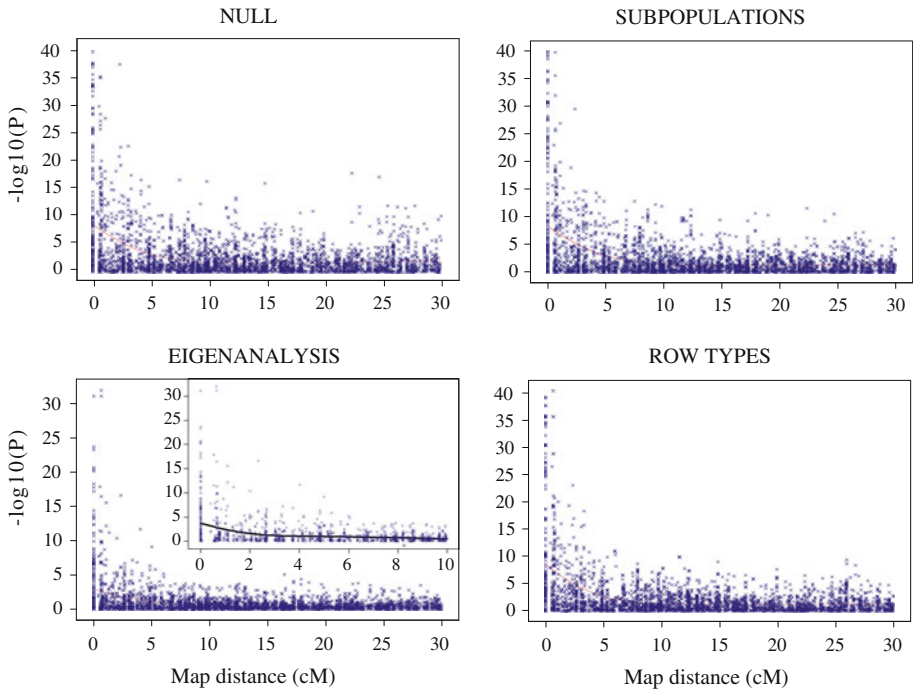


Fig. 2 LD $-\log_{10}(P)$ decay plot of marker pairs as a function of genetic distance on chromosome 5H. The curve illustrates LD decay trend line based on the nonlinear regression of $-\log_{10}(P)$ on genetic distance. The title of each plot shows the model used to account for population structure while investigating LD decay. The inset in the Eigenanalysis model provides an enhanced view of LD decay for markers located <10 cM apart. Similar LD decay was found on other chromosomes, see Fig. S2

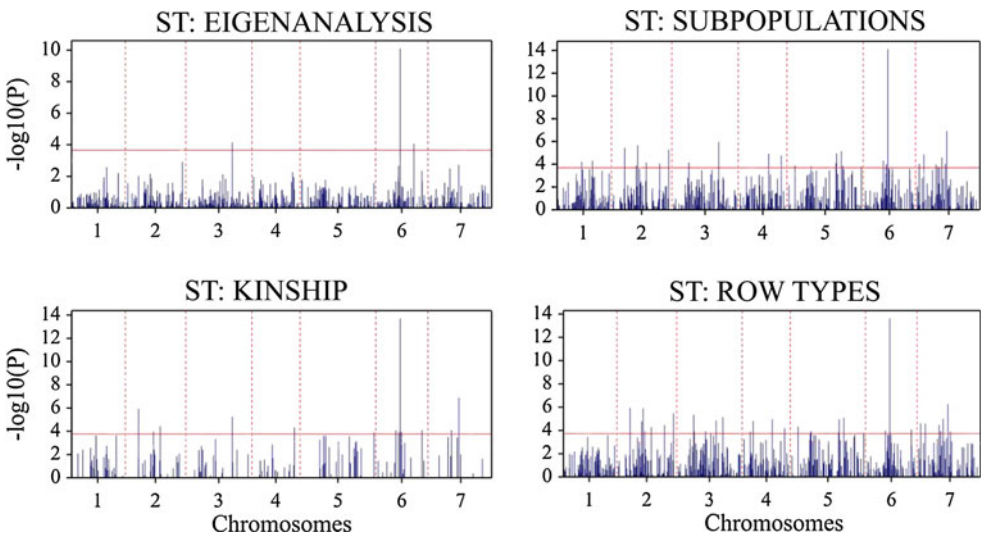


Fig. 3 Association profiles showing outputs of salt tolerance (ST) association mapping analysis using different models to prevent the confounding of population structure. The title of each plot shows the model used to account for population structure or relatedness in association mapping analysis. *Horizontal axis* presents seven chromosomes (1–7H) of barley genome. *Vertical axis* is the $-\log_{10}(P)$ values of QTLs according to the Wald test. The *horizontal red line* indicates the $-\log_{10}(P)$ threshold (3.74) (color figure online)

across the whole barley genome with LD rapidly decaying with map distance between markers (Fig. S2). These results indicate that correction for relatedness is essential and that Eigenanalysis may give less but more likely significant marker-trait associations, reducing the number of false-positive associations. The LD decay at 4 cM (found in Eigenanalysis model) was used to calculate the threshold for marker-trait associations in the next section as described in "Materials and methods".

Association mapping

Three models accounting for population structure were used in the association mapping analysis and all three reduced background and effects of population structure over the Null model. The number of significant QTLs identified ($-\log_{10}(P)$ threshold > 3) differed from model to model. As expected from LD decay analyses, the association mapping approach using the Eigenanalysis model found less QTLs than the models that incorporated either the kinship matrix or predetermined group (subpopulation or ear row type) information (Fig. 3). The Eigenanalysis association mapping procedure identified markers associated with most of the studied traits, which were scattered over the whole barley genome. An overview of significant trait-marker associations identified under Eigenanalysis model with genome positions, $-\log_{10}(P)$ scores and allele effects of the gene-specific

Table 4 Significant marker-traits associations (>3.74) or close to significance (>3.0) and their position (cM) on the barley chromosome (Chr.) identified under saline condition using Eigenanalysis association mapping with $-\log_{10}(P)$ score, allele frequency (Fq), allele effects and standard error (S.E)

Salt tolerance/growth related trait	Marker	Chr.	cM	$-\log_{10}(P)$	Allele Fq	Allele effects	SE
Salt tolerance	SNP405	3	126.3	4.15	0.27	2.207	0.556
	SNP779	6	60.2	10.09	0.26	−11.505	0.528
Shoot FW (g)	SNP518	4	79.6	3.67	0.46	−0.506	0.137
	SNP779	6	60.2	10.84	0.26	−3.095	0.134
Shoot DW (g)	SNP779	6	60.2	11.32	0.26	−0.485	0.021
Root DW (g)	SNP395	3	111.4	3.56	0.39	−0.02	0.005
	SNP518	4	79.6	3.02	0.46	−0.019	0.006
	SNP696	5	161.6	3.20	0.09	0.031	0.009
Leaf chlorophyll content (SPAD reading)	SNP14	1	31.1	3.44	0.25	−1.985	0.557
	SNP548	5	6.4	3.03	0.26	−1.824	0.551
	SNP742	6	45.4	3.39	0.30	2.98	0.843
	SNP779	6	60.2	14.19	0.26	−17.995	0.484
	SNP840	7	4.9	3.33	0.10	−5.896	0.911
Plant height (cm)	SNP164	2	59.2	3.27	0.23	−2.284	0.660
	SNP779	6	60.2	6.09	0.26	−4.261	0.471
	SNP840	7	4.9	4.81	0.10	−3.49	0.807
	SNP871	7	61.3	3.32	0.24	1.999	0.572
Root length (cm)	SNP643	5	110.3	3.08	0.12	−1.013	0.303
	SNP860	7	46.2	3.23	0.23	0.737	0.215
Tiller number	SNP518	4	79.6	3.50	0.46	−0.226	0.063
	SNP777	6	60.2	6.54	0.35	−1.266	0.056
	SNP864	7	54.4	3.08	0.47	0.205	0.061
Leaf number	SNP436	3	170.1	3.80	0.09	0.257	0.068
	SNP543	4	123.3	3.61	0.25	−0.189	0.051
	SNP639	5	108.2	4.52	0.38	−0.174	0.042
Leaf senescence (rating 1–9)	SNP97	1	114.8	3.25	0.25	−0.456	0.132
	SNP160	2	59.2	3.54	0.20	−0.853	0.129
	SNP236	2	113.5	3.24	0.46	1.023	0.120
	SNP779	6	60.2	5.80	0.26	0.999	0.111
Ion content trait							
Shoot Na^+	SNP535	4	103.1	3.37	0.22	−7.005	1.103
	SNP541	4	119.1	16.53	0.39	6.838	0.809
	SNP906	7	83.4	3.24	0.19	4.022	1.169
Shoot K^+	SNP541	4	119.1	21.67	0.39	−5.665	0.582
	SNP776	6	60.2	3.13	0.40	5.123	0.830
	SNP873	7	63.7	3.68	0.38	6.624	0.713
Shoot Mg^{2+}	SNP779	6	60.2	7.77	0.26	1.259	0.035
Shoot Ca^{2+}	SNP89	1	99.2	3.37	0.29	0.328	0.093
	SNP779	6	60.2	4.28	0.26	0.735	0.095
	SNP949	7	149	3.14	0.11	0.458	0.136
Shoot Cl^-	SNP23	1	47.5	3.13	0.44	3.613	1.071
	SNP541	4	119.1	4.14	0.39	3.198	0.806
	SNP840	7	4.9	3.52	0.10	5.215	1.444
Shoot Na^+/K^+	SNP541	4	119.1	28.42	0.39	1.23	0.074
	SNP874	7	64.8	3.52	0.06	0.728	0.202
Root Na^+	SNP852	7	34.8	3.21	0.42	−1.206	0.352

Table 4 continued

Salt tolerance/growth related trait	Marker	Chr.	cM	$-\log_{10}(P)$	Allele Fq	Allele effects	SE
Root K^+	SNP61	1	66	4.68	0.35	−0.914	0.215
	SNP541	4	119.1	4.54	0.39	1.011	0.242
	SNP647	5	129.4	3.13	0.22	0.923	0.274
	SNP779	6	60.2	4.32	0.26	−1.008	0.248
	SNP855	7	38.3	3.52	0.39	−0.88	0.243
Root Mg^{2+}	SNP164	2	59.2	4.20	0.23	0.141	0.035
Root Ca^{2+}	SNP215	2	86.6	3.13	0.31	−0.835	0.070
	SNP422	3	148.9	3.10	0.34	−0.229	0.068
	SNP871	7	61.3	4.35	0.24	−0.554	0.074
Root Cl^-	SNP200	2	74.4	3.71	0.25	9.403	0.537
	SNP921	7	104.8	3.02	0.36	−1.404	0.425
Root Na^+/K^+	SNP61	1	66	4.89	0.35	0.513	0.118
	SNP489	4	55.6	4.97	0.48	0.472	0.107
	SNP770	6	55.9	3.13	0.29	−0.419	0.124
	SNP855	7	38.3	3.46	0.39	0.462	0.129

FW fresh weight, *DW* dry weight

markers for QTLs detected under saline conditions are given in Table 4, respectively.

Three strong QTLs for ST were detected. These associations were consistently detected independent of the model that was used to account for the population structure. The strongest QTL for ST on chromosome 6H ($-\log_{10}(P)$ 10) was consistently found in all models (Fig. 3). This QTL co-localizes with QTLs for other growth-related traits such as shoot dry weight, chlorophyll content, tiller number, plant height and leaf senescence under stress conditions—located at around 60 cM on chromosome 6H, with $-\log_{10}(P)$ scores ranging from 6 to 14 (Fig. 4). Another important region was found at 119 cM on chromosome 4H with highly significant QTL $-\log_{10}(P)$ scores (4–28) for the ion homeostasis-related traits: shoot Na^+, K^+, Na^+/K^+ ratio and Cl^-, and root Na^+/K^+ (Fig. 4). The QTL for ion contents were mainly detected in salt-stressed plants. QTLs affecting shoot growth and related traits were found under both stress and non-stress conditions. However, we observed significantly higher $-\log_{10}(P)$ scores and effects for QTL(s) determining growth traits under stress conditions than under control conditions.

Discussion

Unravelling the mechanisms underlying salt tolerance in higher plants is challenging, due to the complexity of the adaptive mechanisms of the plants in response to salt stress. In this study, we present the genetic dissection of the naturally occurring genetic variation of salt tolerance in a worldwide collection of spring barley genotypes, linking traits that contribute to salt tolerance to specific regions in the barley genome. A number of genomic regions with genes affecting salt tolerance and related physiological and ion homeostasis traits were identified. In particular, QTLs for salt tolerance, biomass production, chlorophyll content, leaf senescence, tiller number and plant height accumulated on a prominent genomic region located on chromosome 6H. QTLs for Na^+, K^+ and Cl^- content were found on chromosome 4H (Fig. 4 and Table 4). Association mapping has proven to be a powerful approach to dissect the complexity of quantitative traits in plants (Flint-Garcia et al. 2003; Nordborg and Tavare 2002; Mackay and Powell 2007); the current study has shown that this also holds true for salt stress tolerance in barley.

Genetic variation for salt tolerance in barley

Association mapping is becoming an important tool for identifying alleles and loci responsible for traits of agronomical importance. Kraakman et al. (2004) demonstrated the clear potential of association mapping to dissect highly complex traits such as yield and yield stability in barley. Its success, however, depends on the species under study, the trait, the association panel and how the peculiarities of the available panel are tackled (Zhu et al. 2008). A first critical step before initiating an association mapping study for target traits is to consider the species and its available germplasm. In the current study, we have chosen a worldwide barley collection to map loci controlling salt tolerance and related growth and ion homeostasis traits with the ultimate aim to discover useful alleles of candidate genes for crop salt tolerance improvement. The collection consisted of 192 genotypes originating from a wide range of ecological habitats. The population size should be

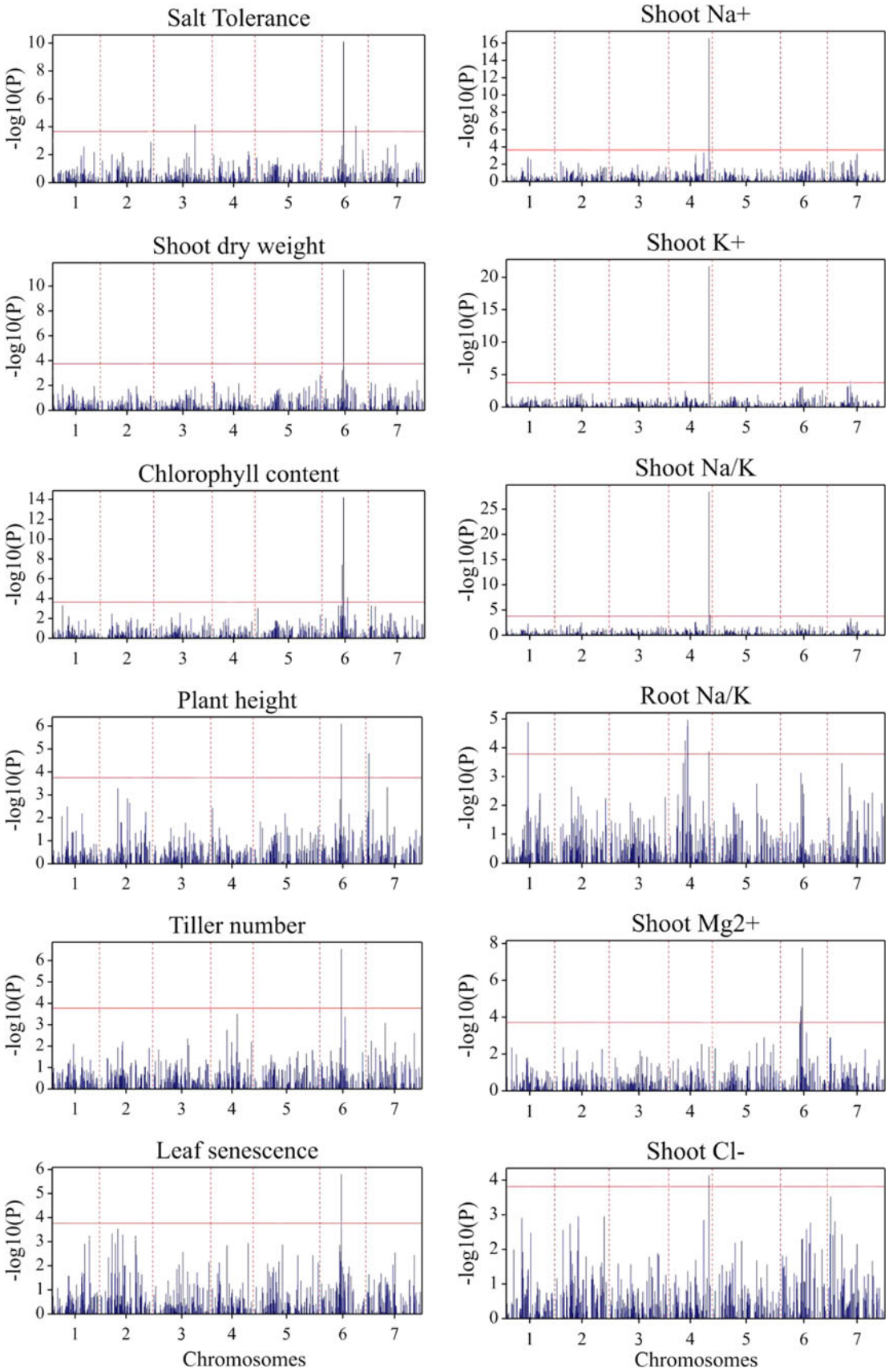
sufficient to detect relevant genetic factors determining variation present in the barley gene pool for the trait of interest (Zhu et al. 2008; Haseneyer et al. 2010). Nevertheless, some genetic factors may be missed due to high allelic diversity that may not be linked to the biallelic SNP markers used in this study. Some SNPs may lump several alleles with different effects which diffuse the association with the functional trait, and decrease the statistical power to detect QTLs in this diverse population. On the other hand, the narrowing of genetic diversity due to plant breeding activities implies that elite barley germplasm is not necessarily the most promising source material for genetic improvement of tolerance to abiotic stress in crops (Roy et al. 2011). The barley lines from Europe and America in our association panel were mainly selected to perform well under relatively favorable conditions and may hardly have alleles that confer resistance to salt stress. The large variation in salt tolerance found in the association panel may be attributed to the fact that it includes barley materials that were domesticated in environments where salt and drought stresses often occur which will increase the genetic diversity for salt tolerance (Nevo and Chen 2010; Munns et al. 2006). It is likely that in areas where barley is under selection in arid and semi-arid conditions the frequency of favorable alleles for drought and salt tolerance is higher. Nevertheless, the alleles contributing to the two important QTLs for salt tolerance related traits detected in this study were also present in the European and American cultivars.

Population structure and LD

An association mapping panel assembled on the basis of different geographical origins, location of adaptation and a long evolution history usually is not fully random (Pritchard et al. 2000). Genotypes originating from the same area may be more closely related than the ones from different areas. This may result in spurious marker-trait associations (Zhao et al. 2007). Malysheva-Otto et al. (2006) reported that a global population of cultivated barley consisting of 953 accessions was highly structured due to geographical origins and row types. We compared LD decay information obtained with the Eigenanalysis with other methods. The LD values observed within the population between markers decayed within 4 cM (Eigenanalysis) and 7–8 cM (subpopulations). Our LD decay result using subpopulation methods is similar to the finding of Pasam et al. (2012) where LD decay was found from 5 to 10 cM in the same population and the same marker dataset. The LD decay (2–4 cM) found using the Eigenanalysis model in our study is also consistent with other studies in barley. Comadran et al. (2011) reported LD decay within a distance of 5 cM between markers for a panel with 190 elite cultivated barley varieties and a large set of markers (2132 SNPs). However, the estimates for LD decay from our study differed strikingly from those of Haseneyer et al. (2010) who showed weak intra and interchromosomal LD, using the same association panel as in our study but with only a few markers (45 SSRs). The difference with our study is likely due to the low number of markers that were relatively widely spaced. One further reason might be the marker type. Malysheva-Otto et al. (2006) showed for a large worldwide barley collection and only 48 SSR markers that LD can extend over a marker distance of up to 50 cM, which strongly depends on population structure. It demonstrates that assessment of LD decay with multiallelic nature of SSRs makes them highly susceptible to pick up LD arising from population structure. None of the studies that use SSR markers have enough genome coverage to reach valid conclusions about genome-wide LD. With a set of 549 DArT markers and a restricted diversity in a Tibetan barley collection, the decay of LD was reported to be 8.9 cM on a single chromosome (5H) (Wu et al. 2011). Using 134 AFLP markers, Kraakman et al. (2006) showed LD decay beyond 10 cM in a 146 spring barley collection with restriction in European genotypes. The relatively fast LD decay (4 cM) observed in the current study is probably due to (1) a fairly large and genetically diverse population, (2) dense marker coverage and (3) the confounding effect of population structure that has been accounted for using Eigenanalysis. This more rapid LD decay in barley than expected in a selfing species is consistent with studies in various barley populations (Rodriguez et al. 2012; Zhang et al. 2009; Comadran et al. 2009). This suggests that barley might have benefited from artificial outcrossing forced by breeding which breaks LD. The higher allele frequency per locus, the high heritability of the salt tolerance traits together with the LD decay of up to 4 cM in our association panel facilitate association studies with a medium marker density (approximately 1000 evenly distributed markers) (Rostoks et al. 2005; Comadran et al. 2009).

Association mapping of salt tolerance

Biparental QTL mapping for salt tolerance has resulted in the detection of several genomic regions with candidate genes controlling salt tolerance-related traits (Mano and Takeda 1997; Xue et al. 2009; Nguyen et al. 2013). However, the QTLs found with biparental mapping strategies often have not lead to the identification of candidate genes for crop improvement, mainly because of the low resolution of QTL mapping due to genetic linkage blocks as a consequence of the small number of recombination events between the two parental genomes (Bernardo 2008). Our previous biparental QTL mapping using the


Salt Tolerance
Shoot Na+
Shoot dry weight
Shoot K+
Chlorophyll content
Shoot Na/K
Plant height
Root Na/K
Tiller number
Shoot Mg2+
Leaf senescence
Shoot Cl-
-log10(P)
Chromosomes

◀ **Fig. 4** Association profiles showing significant markers associated with salt tolerance and related growth traits (*left*) and ion contents in shoot and root under saline conditions (*right*) using Eigenanalysis association mapping method. *Horizontal axis* presents seven chromosomes (1–7H) of barley genome. *Vertical axis* is the $-\log_{10}(P)$ values of QTLs according to Wald test. The *horizontal red line* indicates $-\log_{10}(P)$ threshold (3.74). Associations of markers and other traits as well as the allele effects for QTLs can be found in Table 4 (color figure online)

Steptoe × Morex DH population with a similar hydroponics experimental setup resulted in several QTL regions—some of which are the same locations with QTLs found in the current study such as the 6H QTL—controlling growth, ST, and ion homeostasis, but the QTL regions were 15–30 cM in size (Nguyen et al. 2013).

In this study, we have identified strong QTLs affecting ST and related traits using an association mapping approach while correcting for population structure and relatedness (Fig. 4). We found a number of trait-marker associations in different regions of the barley genome controlling salt tolerance and related traits (Table 4). Some of these genes/factors may not be specific for stressed conditions as they were identified under both control (data not shown) and stress conditions which might relate to developmental traits or growth vigor. However, the relatively high heritability values for growth and related traits as well as the higher QTL $-\log_{10}(P)$ scores and effects under stress conditions compared to control conditions indicate that traits phenotyped under saline conditions are the strongest indicators for salt tolerance selection. The genomic region on chromosome 6H identified in the current study strongly influenced ST as well as chlorophyll content, plant height, tiller number and leaf senescence under salinity stress. This suggests that these related traits may be controlled by a single or only a few gene(s). We previously detected a QTL controlling biomass produced under saline conditions in a similar region on chromosome 6H using the Steptoe × Morex DH population (Nguyen et al. 2013). These QTL had a large confidence interval (30 cM). Xue et al. (2009) used a DH population derived from CM72 × Gairdner and mapped QTLs for Na^+/K^+ ratio and plant height on chromosome 6H close to our QTL region. In the current study, the QTLs affecting ST and growth-related traits were found in a small interval of 2 cM on chromosome 6H. The 6H QTL region is close to the centromere and the high gene density may complicate the identification of candidate genes. The accumulation of QTLs for ST and traits such as leaf senescence, chlorophyll content and tiller number as well as the absence of strong QTLs for ion contents in this region suggests that it may contribute to osmotic stress tolerance. Osmotic stress is the first stress that plants encounter in saline soil and it has an immediate influence on the growth of plants under salinity stress (Munns and Tester 2008). High concentrations of salts in root growing media cause osmotic pressure and reduced water uptake or loss of water in roots (Horie et al. 2011). The molecular response of barley to the osmotic phase has been targeted in several transcriptome studies (Ueda et al. 2006; Walia et al. 2007; Walia et al. 2006) and resulted in the discovery of early response genes controlling osmoprotection under salinity stress. Recent studies on osmotic stress tolerance in barley revealed that under saline conditions, aquaporins—channel proteins that mediate transport of water and small neutral molecules across cellular membrane—relate to salt tolerance (Katsuhara et al. 2002; Katsuhara et al. 2003; Katsuhara et al. 2011). The syntenic region to this interval in rice includes, amongst others, two aquaporin genes as well as two dehydrin genes, and one *CBF* gene. Walia et al. (2006) showed that the expression of dehydrin, aquaporin and *CBF* genes was associated with the response of barley plants to osmotic stress induced by high salt concentration in hydroponics. In plants, the *CBF* gene family is critical in an osmotic stress signalling pathway caused by drought and salinity. The *CBF3* gene was associated with drought and cold stress in barley (Choi and Close 2000). In addition, three *CBF* genes partly explained the variation in salt tolerance in Tibetan barley (Wu et al. 2011). Dehydrins were found to play an important protective role during cellular dehydration, improving enzyme functioning under the conditions of low water availability. Du et al. (2011) showed that two dehydrin genes might contribute to improved drought and salt tolerance of Tibetan and wild barley. Hv-WRKY38 is a barley gene coding for a WRKY protein, whose expression is involved in cold and drought stress response which was mapped close to the QTL region (Mare et al. 2004). Hv-WRKY38 was early and transiently expressed during exposure to low non-freezing temperature, in ABA-independent manner. Furthermore, it showed a continuous induction during dehydration and freezing treatments. The aquaporin, dehydrin, CBF genes and Hv-WRKY38 may be putative candidate genes that underlie the QTL effect on ST.

Association mapping of ion content traits

The QTLs for Na^+, K^+ and Na^+/K^+ ratio identified on chromosome 4H with high $-\log_{10}(P)$ scores were only detected under saline conditions. Forster et al. (2000) showed that chromosome 4H in barley harbors several loci involved abiotic stress tolerance including salt and drought, but no QTL for ion content on chromosome 4H of barley has been detected before. QTLs for other salt tolerance or yield-related traits found in the same region or close to our 4H QTL include QTLs for spike numbers per plant and tiller numbers under saline conditions (Xue et al. 2009) and a major QTL for yield under normal field conditions (Ellis

et al. 2002). Previous studies showed that a decrease in growth under saline conditions could be mainly attributed to ionic effects caused by toxic levels of Na^+ in the leaves (Mano and Takeda 1997; Shabala et al. 2010; Storey and Jones 1978). Several genes which are differentially expressed under saline conditions may underlie the ion exclusion mechanism regulated by the QTL region on chromosome 4H under salt stress conditions. Candidate genes may include ion transporter(s), a proton pump(s) or ion channel(s) that control ion exclusion in roots and shoots and the gene might mediate constitutively shoot K^+ over Na^+ discrimination under saline conditions. Dubcovsky et al. (1996) mapped the *Knal* gene on chromosome 4D of wheat which partly explains the better Na^+ exclusion or K^+/Na^+ discrimination of bread wheat over durum wheat. In durum wheat *Nax2* was proposed by Byrt et al. (2007) to have the same function as the *Kna1* gene in bread wheat. *HKT1;5* is a strong candidate for *Nax2*, a gene mapped on the long arm of chromosome 4H as well (Huang et al. 2008). Our results suggest that the QTL on the long arm of 4H in barley might relate to both *Knal* and *Nax2* which may explain why barley is more salt tolerant than both bread and durum wheat (Munns and Tester 2008). *HKT1;5* therefore is a strong candidate gene for our 4H QTL. The *HKT1;5* gene from ancestral wheat was used recently to produce salt-tolerant durum wheat which showed increased salt tolerance with yield increases of 25 % on saline soil (Munns et al. 2012). Another association mapping QTL for Na^+, K^+ and Na^+/K^+ ratio locates near the center of chromosome 7H might be related to the *HvNax3* locus by Shavrukov et al. (2010). However, this locus in our association mapping study has a small effect compared to the QTL on 4H ($-Log_{10}(P)$ 3 compared to 20).

Previous studies mainly focused on Na^+ and K^+ homeostasis in their salt tolerance studies. Niu et al. (1995), Zhu (2003) and Munns and Testers (2008) suggested to consider the interrelationship between other ions such as Ca^{2+} and Mg^{2+} in relation to ion homeostasis under saline conditions. Tavakkoli et al. (2010, 2011) provided evidences to consider the important role of Cl^- content in shoot—as higher contents of this ion in shoot is highly toxic to many plants (White and Broadley 2001) including barley (Teakle and Tyerman 2010). Measuring the five most important ions that are major constituents of saline soil (Tavakkoli et al. 2010) we were able to assess the role of ion homeostasis in plants under salt stress condition in a wider context. In addition to Na^+ and K^+, a QTL for Cl^- was found in the same region which supports the recently made suggestion by others to consider the role of Cl^- in relation to ion homeostasis and salt tolerance in barley (Tavakkoli et al. 2010; Nguyen et al. 2013). Our results suggested that accumulation of Cl^- in both roots and shoots might be toxic for barley plants. In addition, shoot Cl^- content consistently showed a stronger negative correlation with salt tolerance than shoot Na^+ in our Steptoe × Morex biparental mapping study (Nguyen et al. 2013). In contrast to Na^+, there is little known about mechanisms or genes that control Cl^- transport/uptake and no QTL have been detected for Cl^- in cereals. In our study, the QTL on chromosome 4H was found controlling homeostasis of Na^+/K^+ and Cl^- as well, which may suggest that the gene that controls Cl^- loading/uploading at the xylem/symplast boundary (*CCC*) could be a target for further investigation. Recently, the *Arabidopsis thaliana AtCCC* gene encoding a cation-chloride co-transporter was cloned and shown to control both shoot and root Cl^- homeostasis under saline conditions (Colmenero-Flores et al. 2007). In rice, the *OsCCC1* gene was shown to play a significant role in ion homeostasis and rice development under saline conditions (Kong et al. 2011). The genes underlying the QTLs for Cl^- content may also include transporters having either direct or indirect effects on Cl^- exclusion or control of a Chloride channel. The CLC gene family was found to control Cl^-/H^+ antiporters and Cl^- (Lv et al. 2009). The CLC subclass I family was found to be located on the tonoplast membrane in *Arabidopsis* and was suggested to be involved in sequestering Cl^- in the vacuole under salinity stress (Li et al. 2006; Teakle and Tyerman 2010).

Conclusions

Our study showed extensive genetic variation for salt tolerance that can be exploited for barley improvement. Results obtained by Eigenanalysis that is incorporated in the association mapping approach defined the linkage disequilibrium of the barley collection decaying within 4 cM. The current study showed that the medium density genetic map with a thousand markers is sufficient for an association study on barley. Association mapping identified QTLs for salt tolerance, growth-related traits and ion homeostasis-related traits. We presented numerous significant maker-traits associations over the whole barley genome; among them, 66 QTLs were detected under control and 58 QTLs were found under saline conditions. We showed a strong QTL on 6H independently from population structure controlling salt tolerance that co-localized with QTL for other traits such as biomass growth, chlorophyll content and leaf senescence. Another strong QTL was identified on 4H controlling contents of various ion including Na^+, K^+ Na^+/K^+ and Cl^-. The genomic regions that harbor QTLs for salt tolerance and ion contents on chromosome 4H and 6H in our study can be used for targeting candidate gene(s) for salt tolerance and uptake/transportation of both Na^+ and Cl^-, which are important factors for salt tolerance improvement of barley.

Acknowledgments We thank Rients Niks and Björn D'Hoop for their valuable discussions on this work. We would like to acknowledge Raj K. Pasam, Rajiv Sharma and Kerstin Neumann for discussions and critical reading of the manuscript. This work was financially supported by the Ministry of EL&I, The Netherlands and the Vietnamese Ministry of Education. M.M. acknowledges the support of the Generation Challenge Program—Integrated Breeding Platform project 2.2.1. and 3.2.4.

References

Atwell S, Huang YS, Vilhjalmsson BJ, Willems G, Horton M, Li Y, Meng D, Platt A, Tarone AM, Hu TT, Jiang R, Muliyati NW, Zhang X, Amer MA, Baxter I, Brachi B, Chory J, Dean C, Debieu M, de Meaux J, Ecker JR, Faure N, Kniskern JM, Jones JD, Michael T, Nemri A, Roux F, Salt DE, Tang C, Todesco M, Traw MB, Weigel D, Marjoram P, Borevitz JO, Bergelson J, Nordborg M (2010) Genome-wide association study of 107 phenotypes in *Arabidopsis thaliana* inbred lines. Nature 465:627–631

Bernardo R (2008) Molecular markers and selection for complex traits in plants: learning from the last 20 years. Crop Sci 48:1649–1664

Byrt CS, Platten JD, Spielmeyer W, James RA, Lagudah ES, Dennis ES, Tester M, Munns R (2007) HKT1;5-like cation transporters linked to Na+ exclusion loci in wheat, Nax2 and Kna1. Plant Physiol 143:1918–1928

Choi DW, Close TJ (2000) A newly identified barley gene, Dhn12 encoding a YSK2 DHN, is located on chromosome 6H and has embryo-specific expression. Theor Appl Genet 100:1274–1278

Close TJ, Bhat PR, Lonardi S, Wu YH, Rostoks N, Ramsay L, Druka A, Stein N, Svensson JT, Wanamaker S, Bozdag S, Roose ML, Moscou MJ, Chao SAM, Varshney RK, Szucs P, Sato K, Hayes PM, Matthews DE, Kleinhofs A, Muehlbauer GJ, DeYoung J, Marshall DF, Madishetty K, Fenton RD, Condamine P, Graner A, Waugh R (2009) Development and implementation of high-throughput SNP genotyping in barley. BMC Genomics 10:582–595

Cockram J, White J, Zuluaga DL, Smith D, Comadran J, Macaulay M, Luo ZW, Kearsey MJ, Werner P, Harrap D, Tapsell C, Liu H, Hedley PE, Stein N, Schulte D, Steuernagel B, Marshall DF, Thomas WTB, Ramsay L, Mackay I, Balding DJ, Waugh R, O'Sullivan DM, Consortium A (2010) Genome-wide association mapping to candidate polymorphism resolution in the unsequenced barley genome. Proc Natl Acad Sci USA 107:21611–21616

Colmenero-Flores JM, Martinez G, Gamba G, Vazquez N, Iglesias DJ, Brumos J, Talon M (2007) Identification and functional characterization of cation-chloride cotransporters in plants. Plant J 50:278–292

Colmer TD, Munns R, Flowers TJ (2005) Improving salt tolerance of wheat and barley: future prospects. Aust J Exp Agric 45:1425–1443

Comadran J, Thomas WT, van Eeuwijk FA, Ceccarelli S, Grando S, Stanca AM, Pecchioni N, Akar T, Al-Yassin A, Benbelkacem A, Ouabbou H, Bort J, Romagosa I, Hackett CA, Russell JR (2009) Patterns of genetic diversity and linkage disequilibrium in a highly structured *Hordeum vulgare* association-mapping population for the Mediterranean basin. Theor Appl Genet 119:175–187

Comadran J, Ramsay L, MacKenzie K, Hayes P, Close T, Muehlbauer G, Stein N, Waugh R (2011) Patterns of polymorphism and linkage disequilibrium in cultivated barley. Theor Appl Genet 122:523–531

D'hoop BB, Paulo MJ, Kowitwanich K, Sengers M, Visser RGF, van Eck HJ, van Eeuwijk FA (2010) Population structure and linkage disequilibrium unravelled in tetraploid potato. Theor Appl Genet 121:1151–1170

Du JB, Yuan S, Chen YE, Sun X, Zhang ZW, Xu F, Yuan M, Shang J, Lin HH (2011) Comparative expression analysis of dehydrins between two barley varieties, wild barley and Tibetan hulless barley associated with different stress resistance. Acta Physiol Plant 33:567–574

Dubcovsky J, Maria GS, Epstein E, Luo MC, Dvorak J (1996) Mapping of the K+/Na+ discrimination locus Kna1 in wheat. Theor Appl Genet 92:448–454

Ellis RP, Forster BP, Robinson D, Handley LL, Gordon DC, Russell JR, Powell W (2000) Wild barley: a source of genes for crop improvement in the 21st century? J Exp Bot 51:9–17

Ellis RP, Forster BP, Gordon DC, Handley LL, Keith RP, Lawrence P, Meyer R, Powell W, Robinson D, Scrimgeour CM, Young G, Thomas WT (2002) Phenotype/genotype associations for yield and salt tolerance in a barley mapping population segregating for two dwarfing genes. J Exp Bot 53:1163–1176

Falush D, Stephens M, Pritchard JK (2003) Inference of population structure using multilocus genotype data: linked loci and correlated allele frequencies. Genetics 164:1567–1587

FAO (2008) FAO Land and plant Nutrition Management Service. http://wwwfaoorg/ag/agl/agll/spush

Flint-Garcia SA, Thornsberry JM, Buckler ESt (2003) Structure of linkage disequilibrium in plants. Annu Rev Plant Biol 54:357–374

Flowers TJ (2004) Improving crop salt tolerance. J Exp Bot 55:307–319

Flowers TJ, Flowers SA (2005) Why does salinity pose such a difficult problem for plant breeders? Agric Water Manag 78:15–24

Forster BP, Ellis RP, Thomas WTB, Newton AC, Tuberosa R, This D, El-Enein RA, Bahri MH, Ben Salem M (2000) The development and application of molecular markers for abiotic stress tolerance in barley. J Exp Bot 51:19–27

Harlan JR, Zohary D (1966) Distribution of Wild Wheats and Barley. Science 153:1074–1080

Hasegawa PM, Bressan RA, Zhu JK, Bohnert HJ (2000) Plant cellular and molecular responses to high salinity. Annu Rev Plant Phys 51:463–499

Haseneyer G, Stracke S, Paul C, Einfeldt C, Broda A, Piepho HP, Graner A, Geiger HH (2010) Population structure and phenotypic variation of a spring barley world collection set up for association studies. Plant Breeding 129:271–279

Horie T, Kaneko T, Sugimoto G, Sasano S, Panda SK, Shibasaka M, Katsuhara M (2011) Mechanisms of water transport mediated by PIP aquaporins and their regulation via phosphorylation events under salinity stress in barley roots. Plant Cell Physiol 52:663–675

Huang S, Spielmeyer W, Lagudah ES, Munns R (2008) Comparative mapping of HKT genes in wheat, barley, and rice, key determinants of Na+ transport, and salt tolerance. J Exp Bot 59:927–937

Ingvarsson PK, Hall D, Tegstrom C (2010) Using association mapping to dissect the genetic basis of complex traits in plants. Brief Funct Genomics 9:157–165

James RA, Rivelli AR, Munns R, von Caemmerer S (2002) Factors affecting CO2 assimilation, leaf injury and growth in salt-stressed durum wheat. Funct Plant Biol 29:1393–1403

Katsuhara M, Akiyama Y, Koshio K, Shibasaka M, Kasamo K (2002) Functional analysis of water channels in barley roots. Plant Cell Physiol 43:885–893

Katsuhara M, Koshio K, Shibasaka M, Hayashi Y, Hayakawa T, Kasamo K (2003) Over-expression of a barley aquaporin

increased the shoot/root ratio and raised salt sensitivity in transgenic rice plants. Plant Cell Physiol 44:1378–1383

Katsuhara M, Rhee JY, Sugimoto G, Chung GC (2011) Early response in water relations influenced by NaCl reflects tolerance or sensitivity of barley plants to salinity stress via aquaporins. Soil Sci Plant Nutr 57:50–60

Kilian B, Ozkan H, Kohl J, von Haeseler A, Barale F, Deusch O, Brandolini A, Yucel C, Martin W, Salamini F (2006) Haplotype structure at seven barley genes: relevance to gene pool bottlenecks, phylogeny of ear type and site of barley domestication. Mol Genet Genomics 276:230–241

Knüpffer H, van Hintum T (2003) Chapter 13 summarised diversity—the barley core collection. In: von Bothmer R, van Hintum T, Knüpffer H, Kazuhiro S (eds) Developments in plant genetics and breeding. Elsevier, pp 259–267

Kong XQ, Gao XH, Sun W, An J, Zhao YX, Zhang H (2011) Cloning and functional characterization of a cation-chloride cotransporter gene OsCCC1. Plant Mol Biol 75:567–578

Kraakman ATW, Niks RE, Van den Berg PMMM, Stam P, Van Eeuwijk FA (2004) Linkage disequilibrium mapping of yield and yield stability in modern spring barley cultivars. Genetics 168:435–446

Kraakman ATW, Martinez F, Mussiraliev B, van Eeuwijk FA, Niks RE (2006) Linkage disequilibrium mapping of morphological, resistance, and other agronomically relevant traits in modern spring barley cultivars. Mol Breeding 17:41–58

Li WYF, Wong FL, Tsai SN, Phang TH, Shao GH, Lam HM (2006) Tonoplast-located GmCLC1 and GmNHX1 from soybean enhance NaCl tolerance in transgenic bright yellow (BY)-2 cells. Plant Cell Environ 29:1122–1137

Lv QD, Tang RJ, Liu H, Gao XS, Li YZ, Zheng HQ, Zhang HX (2009) Cloning and molecular analyses of the *Arabidopsis thaliana* chloride channel gene family. Plant Sci 176:650–661

Maas EV, Hoffman GJ (1977) Crop salt tolerance-current assessment. J Irrig Drain 103(IR2):115–134

Mackay I, Powell W (2007) Methods for linkage disequilibrium mapping in crops. Trends Plant Sci 12:57–63

Malosetti M, van der Linden CG, Vosman B, van Eeuwijk FA (2007) A mixed-model approach to association mapping using pedigree information with an illustration of resistance to Phytophthora infestans in potato. Genetics 175:879–889

Malysheva-Otto LV, Ganal MW, Roder MS (2006) Analysis of molecular diversity, population structure and linkage disequilibrium in a worldwide survey of cultivated barley germplasm (*Hordeum vulgare L.*). BMC Genet 7:6

Mano Y, Takeda K (1997) Mapping quantitative trait loci for salt tolerance at germination and the seedling stage in barley (*Hordeum vulgare L.*). Euphytica 94:263–272

Mare C, Mazzucotelli E, Crosatti C, Francia E, Stanca AM, Cattivelli L (2004) Hv-WRKY38: a new transcription factor involved in cold- and drought-response in barley. Plant Mol Biol 55(3):399–416. doi:10.1007/s11103-004-0906-7

Munns R (2005) Genes and salt tolerance: bringing them together. New Phytol 167:645–663

Munns R, James RA (2003) Screening methods for salinity tolerance: a case study with tetraploid wheat. Plant Soil 253:201–218

Munns R, Tester M (2008) Mechanisms of salinity tolerance. Annu Rev Plant Biol 59:651–681

Munns R, James RA, Lauchli A (2006) Approaches to increasing the salt tolerance of wheat and other cereals. J Exp Bot 57:1025–1043

Munns R, James RA, Xu B, Athman A, Conn SJ, Jordans C, Byrt CS, Hare RA, Tyerman SD, Tester M, Plett D, Gilliham M (2012) Wheat grain yield on saline soils is improved by an ancestral Na(+) transporter gene. Nat Biotechnol 30:360–364

Nevo E (2007) Evolution of wild wheat and barley and crop improvement: studies at the Institute of Evolution. Israel J Plant Sci 55:251–262

Nevo E, Chen GX (2010) Drought and salt tolerances in wild relatives for wheat and barley improvement. Plant Cell Environ 33:670–685

Nevo E, Chen GX, Suprunova T, Krugman T, Fahima T (2004) Ecogeographic and genetic determinants of kernel weight and colour of wild barley (Hordeum spontaneum) populations in Israel. Seed Sci Res 14:137–146

Nguyen V, Ribot S, Dolstra O, Niks R, Visser RF, Linden CG (2013) Identification of quantitative trait loci for ion homeostasis and salt tolerance in barley (*Hordeum vulgare L.*). Mol Breed 31(1):137–152. doi:10.1007/s11032-012-9777-9

Niu XM, Bressan RA, Hasegawa PM, Pardo JM (1995) Ion Homeostasis in NaCl stress environments. Plant Physiol 109:735–742

Nordborg M, Tavare S (2002) Linkage disequilibrium: what history has to tell us. Trends Genet 18:83–90

Pasam RK, Sharma R, Malosetti M, van Eeuwijk FA, Haseneyer G, Kilian B, Graner A (2012) Genome-wide association studies for agronomical traits in a worldwide spring barley collection. BMC Plant Biol 12:16

Patterson N, Price AL, Reich D (2006) Population structure and eigenanalysis. PLoS Genet 2:2074–2093

Payne RW, Harding SA, Murray DA, Soutar DM, Baird DB, Glaser AI, Welham SJ, Gilmour AR, Thompson R, Webster R (2011) GenStat Release 14 Reference Manual, Part 3 Procedure Library PL20. VSN International, Hemel Hempstead

Price AL, Patterson NJ, Plenge RM, Weinblatt ME, Shadick NA, Reich D (2006) Principal components analysis corrects for stratification in genome-wide association studies. Nat Genet 38:904–909

Pritchard JK, Stephens M, Rosenberg NA, Donnelly P (2000) Association mapping in structured populations. Am J Hum Genet 67:170–181

Rostoks N, Mudie S, Cardle L, Russell J, Ramsay L, Booth A, Svensson JT, Wanamaker SI, Walia H, Rodriguez EM, Hedley PE, Liu H, Morris J, Close TJ, Marshall DF, Waugh R (2005) Genome-wide SNP discovery and linkage analysis in barley based on genes responsive to abiotic stress. Mol Genet Genomics 274:515–527

Roy SJ, Tucker EJ, Tester M (2011) Genetic analysis of abiotic stress tolerance in crops. Curr Opin Plant Biol 14:232–239

Schulte D, Close TJ, Graner A, Langride P, Matsumoto T, Muehlbauer G, Sato K, Schulman AH, Waugh R, Wise RP, Stein N (2009) The International Barley Sequencing Consortium—at the threshold of efficient access to the Barley Genome. Plant Physiol 149:142–147

Searle SR, Casella G, McCulloch CE (1992) Variance components. Wiley, New York

Shabala S, Cuin TA, Pang J, Percey W, Chen Z, Conn S, Eing C, Wegner LH (2010) Xylem ionic relations and salinity tolerance in barley. Plant J 61:839–853

Shavrukov Y, Gupta NK, Miyazaki J, Baho MN, Chalmers KJ, Tester M, Langridge P, Collins NC (2010) HvNax3—a locus controlling shoot sodium exclusion derived from wild barley (*Hordeum vulgare* ssp. spontaneum). Funct Integr Genomics 10:277–291

Storey R, Jones RGW (1978) Salt stress and comparative physiology in gramineae. I-Ion relations of two salt and water stressed barley cultivars, California Mariout and Arimar. Aust J Plant Physiol 5:801–816

Stracke S, Haseneyer G, Veyrieras JB, Geiger HH, Sauer S, Graner A, Piepho HP (2009) Association mapping reveals gene action and interactions in the determination of flowering time in barley. Theor Appl Genet 118:259–273

Tavakkoli E, Rengasamy P, McDonald GK (2010) The response of barley to salinity stress differs between hydroponic and soil systems. Funct Plant Biol 37:621–633

Tavakkoli E, Fatehi F, Coventry S, Rengasamy P, McDonald GK (2011) Additive effects of Na^+ and Cl^- ions on barley growth under salinity stress. J Exp Bot 62:2189–2203

Teakle NL, Tyerman SD (2010) Mechanisms of Cl^- transport contributing to salt tolerance. Plant Cell Environ 33:566–589

Ueda A, Kathiresan A, Bennett J, Takabe T (2006) Comparative transcriptome analyses of barley and rice under salt stress. Theor Appl Genet 112:1286–1294

Van Eeuwijk FA, Kraakman ATW, Niks RE, Van den Berg PMMM, Stam P (2004) Linkage disequilibrium mapping of yield and yield stability in modern spring barley cultivars. Genetics 168:435–446

Verbeke G, Molenberghs G (2000) Linear mixed models for longitudinal data. Springer Verlag Berlin

Walia H, Wilson C, Wahid A, Condamine P, Cui X, Close TJ (2006) Expression analysis of barley (Hordeum vulgare L.) during salinity stress. Funct Integr Genomics 6:143–156

Walia H, Wilson C, Condamine P, Ismail AM, Xu J, Cui XP, Close TJ (2007) Array-based genotyping and expression analysis of barley cv Maythorpe and golden promise. BMC Genomics 8:87

White PJ, Broadley MR (2001) Chloride in soils and its uptake and movement within the plant: a review. Ann Bot 88:967–988

Witzel K, Weidner A, Surabhi GK, Varshney RK, Kunze G, Buck-Sorlin GH, Borner A, Mock HP (2009) Comparative analysis of the grain proteome fraction in barley genotypes with contrasting salinity tolerance during germination. Plant Cell Environ 33:211–222

Wu DZ, Qiu L, Xu LL, Ye LZ, Chen MX, Sun DF, Chen ZH, Zhang HT, Jin XL, Dai F, Zhang GP (2011) Genetic Variation of HvCBF Genes and their association with salinity tolerance in tibetan annual wild barley. PLoS One 6(6):e22938

Xue DW, Huang YZ, Zhang XQ, Wei K, Westcott S, Li CD, Chen MC, Zhang GP, Lance R (2009) Identification of QTLs associated with salinity tolerance at late growth stage in barley. Euphytica 169:187–196

Yu JM, Pressoir G, Briggs WH, Bi IV, Yamasaki M, Doebley JF, McMullen MD, Gaut BS, Nielsen DM, Holland JB, Kresovich S, Buckler ES (2006) A unified mixed-model method for association mapping that accounts for multiple levels of relatedness. Nat Genet 38:203–208

Zhang LY, Marchand S, Tinker NA, Belzile F (2009) Population structure and linkage disequilibrium in barley assessed by DArT markers. Theor Appl Genet 119:43–52

Zhao J, Paulo MJ, Jamar D, Lou P, van Eeuwijk F, Bonnema G, Vreugdenhil D, Koornneef M (2007) Association mapping of leaf traits, flowering time, and phytate content in *Brassica rapa*. Genome 50:963–973

Zhu JK (2003) Regulation of ion homeostasis under salt stress. Curr Opin Plant Biol 6:441–445

Zhu C, Gore M, Buckler ES, Yu J (2008) Status and prospects of association mapping in plants. 103835/plantgenome2008020089 1:5–20