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Quantitative trait loci diversity for salt tolerance at the early growth stage of barley (*Hordeum vulgare* L.)

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ABSTRACT

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Salt tolerance in crops is multigenic in nature and quantitatively inherited, and therefore controlled by minor genes. To study the diversity of QTLs conferring salt tolerance in barley, four barley mapping populations including L94 × Vada (L × V, 103 RILs), Oregon Wolf Barley (OWB) (Dom × Rec, 94 DHs), SusPtrit × Vada (Su × V, 152 lines), and SusPtrit × Cebada Capa (Su × CC, 113 lines) were tested; L × V and OWB showed unexpected segregation for salt tolerance at an early growth stage and were selected for this study. Two morphological traits (shoot length and total root length) were quantified under different salinity levels (0, 200 and 300 mM of NaCl) at an early growth stage. In total, eight QTLs were mapped in OWB and eight QTLs were mapped in L × V under 200 and 300 mM NaCl; of these, only two QTLs were common to the two populations. In the OWB and L × V populations, two and one QTLs were shared between two traits, respectively. Comparing QTL positions on a consensus map of barley showed that the number and location of the identified salt tolerance QTLs varied depending on the different NaCl concentrations and barley genotypes. Hence, there is a high diversity of QTLs conferring salt tolerance at the early growth stage of barley. In each barley genotype, a set of specific QTLs was responsible for salt tolerance and very few QTLs were common to them.

Keywords: barley, morphological traits, QTL analysis, quantitative inheritance, salt tolerance

INTRODUCTION

Crop plants are exposed to various biotic and abiotic stresses under field conditions that may reduce yield by 50% (Bray *et al.*, 2000; Vij and Tyagi, 2007). Soil salinity is an increasingly serious environmental problem that affects about 6% of all the land in the world, as well as 20% of irrigated agricultural lands, while significantly reducing agricultural production over large areas of the world's farmlands (FAO, 2002; Rengasamy, 2006, 2010).

Due to the constantly growing world population and its food requirements, it is important to find ways of using marginal lands, including saline lands, and of increasing the yield of crop plants that grow in such soils. So far, development of various management approaches such as irrigation and drainage, and domestication of halophytes as new crops to improve the productivity of saline soils have had little success (Colmer *et al.*, 2005; Pitman and Läuchli, 2002). Thus, the development of salt tolerant crops has become an important global priority (Yamaguchi and Blumwald, 2005); an understanding of the genetics of salt tolerance and the application of efficient breeding methods to develop salt tolerant varieties offer a strategy that could make it possible to produce crops in salt-affected areas (Mano and Takeda 1997).

Salt tolerance is defined as the "ability of plants to grow and complete their life cycle on a substrate that contains high concentrations of soluble salt" (Parida and Das, 2005). Many plants develop different mechanisms either to exclude salt from their cells or to tolerate its presence within them. Understanding how plants tolerate this stress is an important step towards developing strategies to improve salt stress tolerance (Colmer *et al.*, 2005; Parida and Das, 2005).

Improving salt tolerance in crops is considered difficult because it is a multigenic and quantitatively inherited trait (Nazar *et al.*, 2011). Crop yield assessment is a useful criterion for evaluating plant responses to environmental stresses such as salinity. However, the basic genetic mechanisms for grain yield are complex due to environmental effects. The complicated inheritance and low heritability of grain

yield and biomass have limited the effectiveness of selection for these traits under salinity stress (Ashraf, 1994).

Various conventional and modern breeding approaches have been used to improve the salt tolerance of crops by introducing genes for salt tolerance into adapted cultivars (reviewed by Munns *et al.*, 2006; Yamaguchi and Blumwald, 2005). The aim of these breeding approaches has been to exploit the variation for salt tolerance present in crops and their progenitors or close relatives, in order to develop new varieties with more tolerance than commercial cultivars. The greatest gains from diversity within a crop species can be achieved by selecting for specific traits from a series of donor parents and then recombining them, as discussed by Yeo and Flowers (1986). This pyramiding approach may allow improving salt tolerance beyond the levels currently available within a specific crop. However, such an objective requires that underlying minor genes be identified in advance.

QTLs for phenotypic parameters associated with salt tolerance in different plant species have been identified (see Mano and Takeda, 1996, 1997; Foolad *et al.*, 1999; Xue *et al.*, 2009; Shavrukov et al., 2011; Rivandi *et al.*, 2011; Zhou *et al.*, 2012; Eleuch *et al.*, 2008; Genc *et al.*, 2010; Xu *et al.*, 2012). In many plant species (including tomato, rice, barley, citrus, and arabidopsis), QTLs conferring salt tolerance have been mapped on different chromosomal locations at various growth stages (Mano and Takeda, 1996, 1997; Foolad *et al.*, 1999; Foolad and Lin, 1997; Quesada *et al.*, 2002; Flowers and Flowers, 2005; Yano and Sasaki, 1997) and under different salinity levels (Monforte *et al.*, 1997b; Foolad *et al.*, 1999).

Another feature of QTLs associated with salt tolerance may be the diversity of QTLs that control the trait; also, the location of QTLs may differ from one genotype to another of the same crop species, but there is not enough information to address this aspect of salt tolerance. To address this question, in this study we tested six barley accessions, the parents of four mapping populations. All four barley mapping populations had been used for mapping QTLs for traits other than salt tolerance. Surprisingly, we found segregation for salt tolerance at an early growth stage in the commonly used Oregon Wolfe Barley (OWB) reference mapping population (Costa *et al.*, 2001). We also found clear segregation for salt tolerance in L94 × Vada, a mapping population that was developed at Wageningen University for mapping QTLs for partial resistance to *Puccinia hordei* (Qi *et al.*, 1998). The availability of two populations segregating for salt tolerance allowed us to map QTLs for salt tolerance and address the question of whether barley accessions share the same salt tolerance genes or whether different genes control salt tolerance in different barley accessions. This will lead to an understanding of the pattern of inheritance of salt tolerance in barley and would explain the diversity of salt tolerance genes.

MATERIALS AND METHODS

Plant materials

Four mapping populations of barley were used in this study, including three recombinant inbred line (RIL) populations developed at Wageningen University (The Netherlands) and a doubled haploid (DH) population developed in North America. The RIL populations consisted of lines derived from crosses between L94 and Vada (L × V, 103 lines; Qi *et al.*, 1998), between SusPtrit and Vada (Su × V, 152 lines; Jafary *et al.*, 2006), and between SusPtrit and Cebada Capa (Su × CC, 113 lines; Jafary *et al.*, 2008). The DH population, consisting of lines derived from crosses between Dom and Rec (OWB, 94 lines; Costa *et al.*, 2001), is a reference mapping population that has been subjected to extensive genotyping and phenotyping.

Available linkage mapping data

The available data sets of the three RIL populations consisted predominantly of AFLP markers. The marker segregation data for L × V were obtained from Qi *et al.* (1998), whereas for Su × V and Su × CC, marker segregation data were obtained from Jafary *et al.* (2006; 2008). The OWB segregation data sets were downloaded from the Oregon State University (USA) Barley Project web site (http://www.barleyworld.org/).

Locus genotype files that include marker information were downloaded from the above sources. To prepare the skeleton map and the data files as the map file for QTL mapping, the more reliable markers distributed throughout the genome that had been used for constructing the saturated map were selected to construct a map file with suitable marker spacing (about 5-10 cM). From 769 markers in the locus genotype file, a subset of 133 markers was used as a skeleton map of OWB. The base maps of other mapping populations were constructed based on the same considerations.

Screening parents of the mapping populations for salt tolerance

We screened parents of available mapping populations under different salinity levels to identify those showing different reactions to the levels of

salinity. For this purpose, parents were tested in 0, 100, 200 and 300 mM NaCl using a randomized complete block design with three technical and two biological replications. Fifteen seeds of each parent were rolled between two layers of filter paper and placed vertically in individual plastic containers. About 100 mL of just one of the above mentioned NaCl solutions were added to each container; the containers were then placed in an aquarium containing double-distilled water. The aquarium was covered with nylon and placed in an incubator in darkness at 21±1 ºC.

After 10 days of incubation, morphological traits including root length (total, maximum and mean root length), number of roots, and length of coleoptiles and shoots were measured. A t-test was performed on each pair of parents to identify statistical differences between parents of each population. We then selected two mapping populations (OWB-DH and L × V-RIL) whose parents showed significant polymorphism for the evaluated traits.

Testing populations for salt tolerance

Based on the parents' tests, all available individuals of the two selected populations (OWB-DH and L × V-RIL) were examined in 0, 200 and 300 mM NaCl using a randomized complete block design with three technical and two biological replicates. The treatment and assessment methods were the same as those used on the parents, except that in these experiments, we used 20 seeds per line.

QTL analyses

QTL analysis in both RIL and DH populations was performed using Map QTL5 software (Van Ooijen, 2004). To map QTLs for salt tolerance, interval mapping was performed for each measured trait in two replications and the average separately. The QTL mapping procedure was followed by automatic co-factor selection, multiple QTL mapping (MQM) and restricted MQM mapping. Significant LOD thresholds at the 5% probability level were obtained for each trait by running a permutation test on the data sets. The QTLs detected in two replications and in the average of two replications at a salinity level were considered as accepted QTLs.

Comparing QTL positions on the barley map

To compare the distribution of QTLs for salt tolerance and find potentially overlapping QTLs mapped in the two different populations, an integrated consensus map of barley constructed by Marcel *et al.* (2007) was used. This high-density map was constructed based on six mapping populations, of which two (OWB-DHs and L × V-RILs) were used in the present study. The consensus map contains 3,258 markers spanning 1,081 cM with an average distance between two adjacent loci of 0.33 cM, and has been divided into 210 BINs of about 5 cM each. MapChart software (Voorrips, 2002) was used to show QTL locations on the map.

RESULTS

Reaction of parents of four segregating populations to salt stress

Data analyses showed that the parents (L94 and Vada) of the L × V population had different reactions to salinity (0, 200 and 300 mM NaCl; data not shown) in two evaluated traits (total root length and shoot length); this population was therefore used to map QTLs involved in salt tolerance. Similarly, the parents of the OWB mapping population (Dom × Rec) showed different reactions to different levels of salinity. Therefore, the OWB mapping population was used to determine whether QTLs conferring salt tolerance in the L × V mapping population were the same as those mapped in the OWB mapping population.

Correlation between traits and replications

The estimated correlation between two measured traits was r = 0.54 in 0 mM NaCl in the L × V population, and it increased to 0.61 and 0.69 in 200 and 300 mM NaCl, respectively. The correlation between two traits was mainly high (e.g., r = 0.88 for 300 mM NaCl in OWB). The estimated correlation coefficient between replications in OWB for 300 mM NaCl for both root and shoot length was r = 0.77. These values for 300 mM NaCl in the L × V population were r = 0.56 and r = 0.45, and for 200 mM NaCl r = 0.44 and r = 0.42 for root and shoot length, respectively. The high correlation between replications implies the repeatability of the experiment. As the salinity level increased, the correlation between traits and between replications also increased. Frequency distribution graphs for both traits at different levels of salinity were continuous in the two mapping populations, as expected for quantitative and polygenic traits (due to the high number of graphs, they are not shown here).

QTLs mapped in the OWB population

In this study, eight QTLs were mapped for root and shoot length; of these, two QTLs on chromosomes 1(7H) and 2(2H) were common for both traits (Tables 1 and 2; Fig. 1). The most effective QTL was located between *Bmac0303a-Nud* markers on chromosome 1(7H), which explains 47%

Table 1. QTLs for total root length detected in the L × V and OWB populations under different salinity levels.

Pop.	Salinity level	Chromosome	Position (cM)*	LOD	Flanking markers		Additive**
OWB	**300**	**1(7H)**	**57.7-112.5**	**10.51**	**Bmac0303a**	**Nud**	**-21.7**
OWB	**300**	**2(2H)**	**52.7-68.5**	**3.24**	**ABG356**	**Bmac0144f**	**12.6**
OWB	**300**	**2(2H)**	**92.7-123.1**	**3.82**	**Bmac0144b**	**ABG072**	**14.4**
OWB	**300**	**7(5H)**	**154.9-214.5**	**4.45**	**ABG496**	**ABG391**	**-16.8**
LV	**300**	**4(4H)**	**3.0-26.3**	**6.94**	**GBM1252**	**E37M33-191**	**22.4**
LV	**200**	**2(2H)**	**161.0-173.6**	**4.20**	**E40M32-590**	**E42M40-644**	**-24.8**
LV	**200**	**3(3H)**	**7.1-35.2**	**3.33**	**E42M32-116**	**E41M40-358**	**-21.4**
LV	**200**	**4(4H)**	**19.3-36.1**	**5.10**	**GBM1252**	**E37M33-191**	**32.0**
LV	**0**	**5(1H)**	**104.2-117.4**	**4.10**	**P16M50-378**	**E37M32-260**	**-7.0**
LV	**0**	**6(6H)**	**43.9-59.6**	**3.90**	**E35M61-269**	**E35M55-216**	**-5.0**

*** Positions of QTLs ± 2 cM as confidence interval.**
**** Additive effect of QTLs.**
Rows in boldface indicate common QTLs.

Table 2. QTLs for shoot length detected in the L × V and OWB populations under different salinity levels.

Pop.	Salinity level	Chromosome	Position (cM)*	LOD	Flanking markers		Additive**
OWB	**300**	**1(7H)**	**58.7-118.2**	**8.17**	**Bmac0303a**	**Nud**	**-6.90**
OWB	**300**	**2(2H)**	**85.0-115.0**	**3.37**	**Bmac0144b**	**ABG072**	**5.00**
OWB	**300**	**2(2H)**	**115.0-140.0**	**3.73**	**ABG072**	**cnx1**	**4.50**
OWB	**300**	**2(2H)**	**14.0-170.0**	**3.24**	**Zeo**	**wst**	**4.10**
LV	**300**	**2(2H)**	**65.6-75.6**	**5.20**	**E42M32-324**	**E33M54-187**	**-5.50**
LV	**300**	**4(4H)**	**66.0-84.4**	**4.90**	**EBmac0701**	**E33M58-504**	**5.00**
LV	**200**	**5(1H)**	**42.4-59.7**	**6.00**	**E33M54-263**	**P15M53-163**	**-5.10**
LV	**200**	**4(4H)**	**1.0-20.3**	**3.10**	**P14M54-435**	**E42M40-94**	**4.00**
LV	**0**	**(6H)**	**115.3-129.9**	**6.00**	**P15M53-525**	**MWG897**	**-0.07**

*** Positions of QTLs ± 2 cM as confidence interval.**
**** Additive effect of QTLs.**
Rows in boldface indicate common QTLs.

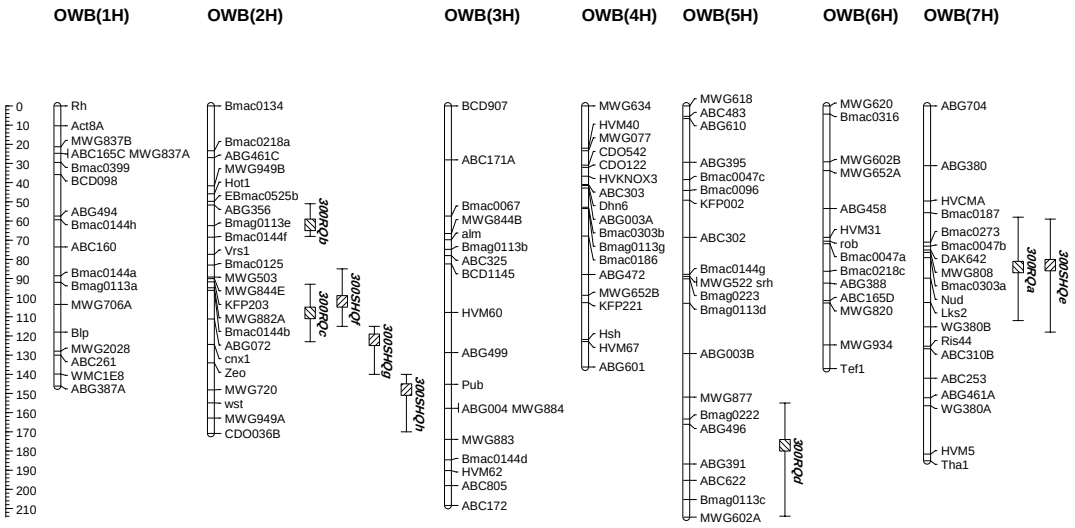

Fig. 1. Position of QTLs mapped for total root length and shoot length under different salinity levels in the OWB mapping population.

Note: Bars show the positions of QTLs ± 2 cM as confidence interval; Detected QTLs for root length; Detected QTLs for shoot length. Numbers on the bars indicate the level of salinity, R indicates QTL detected for total root length, and S indicates QTL detected for shoot length.

and 30% of root and shoot length variance, respectively. One of the QTLs detected on chromosome 2(2H) (between *E38M54-134* and *E38M54-176*) for root length was the same as the QTL in the L × V population under 200 mM NaCl, and one QTL on chromosome 2(2H) (between *E33M55-109* and *ABG356*) was the same as the QTL in the L × V population under 300 mM NaCl for root length. However, the OWB population did not have any QTLs in common with the L × V population under control conditions (Fig. 3).

QTLs mapped in the L × V population

Under 300 mM NaCl, three QTLs (one for root length and two for shoot length, with no QTLs in common) were detected (Fig. 2). One QTL under 300 mM NaCl was the same as a QTL under 200 mM NaCl mapped on chromosome 4(4H) (between

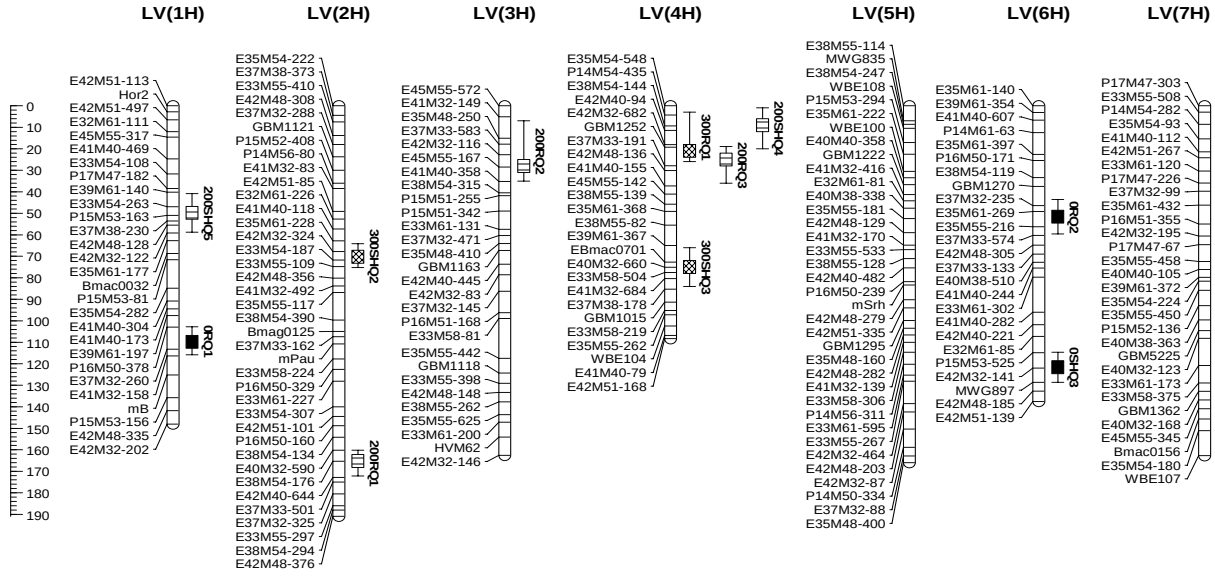

Fig 2. Position of QTLs mapped for total root length and shoot length under different salinity levels in the L × V mapping population.

Note: Bars show the positions of QTLs ± 2 cM as confidence interval; Detected QTLs in 0 mM NaCl; Detected QTLs in 200 mM NaCl; Detected QTLs in 300 mM NaCl. Numbers on the bars indicate the salinity level, R indicates QTL detected for total root length, and S indicates QTL detected for shoot length.

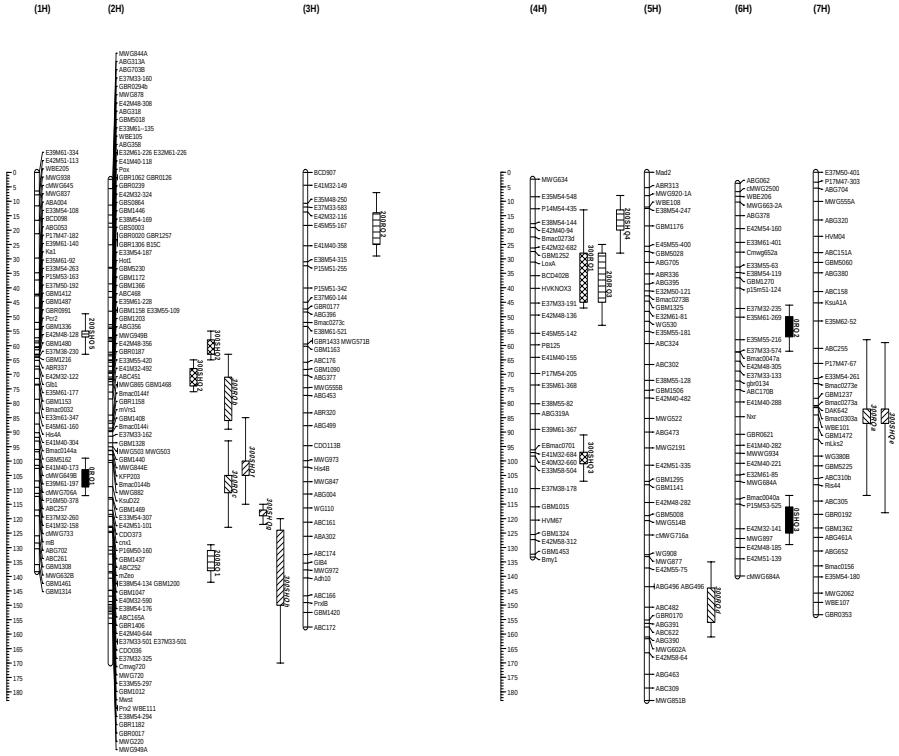

Fig. 3. Comparison of the positions of QTLs mapped for total root length and shoot length under different salinity levels in the L × V and OWB populations on a consensus map of barley (Marcel *et al.*, 2007).

Note: Bars show the positions of QTLs ± 2 cM as confidence interval; Detected QTLs in L×V population with 0 mM NaCl (R= total root length and S= shoot length); Detected QTLs in L×V population with 200 mM NaCl (R= total root length and S= shoot length); Detected QTLs in L×V population with 300 mM NaCl (R= total root length and S= shoot length); Detected QTLs for total root length in the OWB population under 300 mM NaCl; S Detected QTLs for shoot length in the OWB population with 300 mM NaCl.

E42M32-682 and *E37M33-191*) (Tables 1 and 2; Fig. 2). Three QTLs for root length and two QTLs for shoot length were detected under 200 mM NaCl (Fig. 2). No common QTL was detected for these two traits at this level of salinity (Tables 1 and 2). Two QTLs (one for root length and one for shoot length) that were detected at the top of chromosome 4(4H) were close to each other (Fig. 2). Two QTLs for root length and one QTL for shoot length were mapped on chromosomes 5(1H) and 6(6H) under 0 mM NaCl (Fig. 2). There was no QTL in common for these two traits in the two populations.

DISCUSSION

Abundance of QTLs for salt tolerance in barley

In this study, shoot and root length, two important growth characteristics that can be severely affected by salt stress at the early growth stages of barley were selected. These traits were quantified in all individuals of two barley mapping populations at different salinity levels; as well as in control those data were used for QTL mapping. Our data showed that a total of 8 QTLs were involved in salt tolerance in the L × V population under 200 and 300 mM of NaCl; none of these QTLs appeared under control conditions and three of them were detected for both traits and were located close to each other on chromosome 4(4H). A QTL was reported by Mano and Takeda (1997) in the S × M population for salt tolerance at the seedling stage and for germination response to ABA; it is located on chromosome 4(4H), about 4 cM from the position that we mapped in this study. Xu *et al.* (2012) detected one QTL in this region on chromosome 4(4H) that is associated with waterlogging tolerance at the vegetative growth stage. On other arm of chromosome 4(4H) a QTL for shoot length was mapped only in 300 mM Nacl. No QTL was mapped in this region under the other salinity levels as well as under control conditions. In earlier studies, one QTL for number of tillers plant^{-1} in both salinity and control conditions was mapped in this region (Xue *et al.*, 2009; Ellis *et al.*, 2002; Huang *et al.*, 2008).

Studies on the subgroup Triticeae (i.e., wheat, barley, rye, and related species) have revealed some QTLs on chromosome 4 under salinity stress (see Genc *et al.*, 2010; Huang *et al.*, 2008; Dubcovsky *et al.*, 1996). Forster *et al.* (1990) and Forster (1992) reported that genes for abiotic stress tolerance in the Triticeae were located primarily on chromosomes 4 and 5. In this study, chromosome 4(4H) in the L × V mapping population was one of the major chromosomes that effectively control salt tolerance, a finding that is in agreement with earlier studies. However, no QTL for salt tolerance was mapped on chromosome 4(4H) of the OWB mapping population, where chromosome 2(2H) was the most important chromosome for salt tolerance. This indicates that in different mapping populations different chromosomes may host QTLs for abiotic stress, including salinity.

Another QTL for shoot length located on chromosome 2(2H) of the L × V population was identified in our experiment under 300 mM NaCl. Previous studies detected QTLs in this region for different traits related to salt tolerance (Ellis *et al.*, 2002; Zhou *et al.*, 2012; Xu *et al.*, 2012). In the present study, other regions detected under 200 mM NaCl on chromosomes 2(2H) and 5(1H) of the L × V population were not found in the control or under 300mM NaCl. Other researchers have detected QTLs near this region under different levels of salinity (Ellis *et al.*, 2002; Siahsar and Narouei, 2010; Nguyen *et al.*, 2012; Zhou *et al.*, 2012) and under control conditions (Xue *et al.*, 2009) for other traits.

Three QTLs on chromosomes 5(1H) and 6(6H) were identified for morphological traits (root and shoot length) in the L × V population under controlled conditions, which were not detected under salt stress levels (in either the OWB or the L × V population) and may consist of genes that control seedling growth but are not related to salt tolerance. Xue *et al.* (2009) detected several QTLs on these chromosomes for plant height and Na^+ : K^+ ratio under control conditions, as well as several for spikes line^{-1} and grain yield in 200 mM Nacl at the vegetative growth stage. Other studies (Xu *et al.*, 2012; Zhou *et al.*, 2012; Xue *et al.*, 2009; Mano and Takeda, 1997) have detected a few QTLs for salt tolerance on these chromosomes but none of them was close to the chromosomal regions where we found salt tolerance QTLs.

Our data for the OWB population were similar, to some extent, to the results of Witzel *et al.* (2010), who performed QTL analysis at the germination stage under different salt concentrations using the OWB mapping population (Dom × Rec) and identified two chromosome regions on 5H and one on 7H that are associated with salt stress response.

Specific QTLs for different salinity levels and different traits

Comparison of the locations of QTLs mapped in the L × V population under 200 and 300 mM NaCl indicated that shoot and root length are controlled by a set of different QTLs with minor overlapping specificities. If we consider seedling shoot and root length as indices that reflect salt tolerance, it can be postulated that in a specific barley genotype the location of QTLs depends mainly on the salinity

level. Therefore, when the salinity level increases, the position of QTLs may change. Our finding in this case is in agreement with previous studies. Foolad *et al.* (1999) and Monforte *et al.* (1997b) reported that the QTLs for salt tolerance that they identified were treatment sensitive. If we consider that in barley each QTL includes at least one minor gene for salt tolerance, one may conclude that at different salinity levels, different mechanisms may be involved. This implies that tolerant cultivars that have been screened under low salinity conditions may behave differently when exposed to higher levels of salt stress and vice versa.

In the present study, different sets of QTLs were detected for different evaluated traits. Very few of these QTLs were shared between the two traits. Effectiveness of a QTL for both shoot and root length under salt stress can be due to the gene cluster or pleiotropic effects. Salinity can cause several morphological and physiological imbalances in growing plants. Quantifying the more representative trait is crucial for QTL mapping. Our data indicate that it would make sense to use aboveground or underground growth indices in barley for QTL mapping under salinity stress.

Diversity of QTLs for salt tolerance in different barley populations

Of the eight QTLs that were mapped in the OWB-DH population under salinity stress, five were located on chromosome 2(2H) and two were in the same position as QTLs mapped in L × V. Comparing the results of the L × V and OWB populations under 200 and 300 mM NaCl levels indicates that, in these two barley populations, shoot and root length under different salinity levels are controlled by sets of different QTLs with minor overlapping specificities. This suggests there is great diversity in QTLs conferring salt tolerance, and that the positions of these QTLs vary from one barley genotype to another.

Mano and Takeda (1997) showed that different QTLs control salt tolerance in Harrington/TR 306 and Steptoe/Morex under 250 and 300 mM NaCl. Monforte *et al.* (1997a) showed that the QTLs for salt tolerance they detected are population specific and that their detection in closely related populations may be low or even zero. In both these studies, the positions of QTLs were not compared on a consensus map. Comparing the positions of QTLs mapped in two different populations on a consensus map of barley (Fig. 3) allowed us to show that each barley line has a specific set of QTLs that control plant growth under salt stress conditions. This finding, however, reveals that pyramiding minor genes for salt tolerance is not an easy task and may explain, to some extent, why progress on improving salt tolerance has had little success. Other reasons for the slow progress on salt tolerance are:

1) There is a lack of reliable and efficient selection methods (Xu *et al.*, 2012).

2) Direct selection of salt-tolerant genotypes is significantly influenced by environmental factors (Richards, 1996).

3) Salt tolerance of plants at one stage of development is not always associated with tolerance at other stages (see Foolad, 2004).

4) Introgression of salt tolerance genes into adapted cultivars is influenced mainly by the trait's quantitative inheritance.

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