Plant, Cell and Environment (2010) 33, 211–222 doi: 10.1111/j.1365-3040.2009.02071.x

Comparative analysis of the grain proteome fraction in barley genotypes with contrasting salinity tolerance during germination

KATJA WITZEL[1], ANNETTE WEIDNER[1], GIRIDARA-KUMAR SURABHI[1]*, RAJEEV K. VARSHNEY[2], GOTTHARD KUNZE[1], GERHARD H. BUCK-SORLIN[3], ANDREAS BÖRNER[1] & HANS-PETER MOCK[1]

[1]*Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Corrensstraße 3, 06466 Gatersleben, Germany,* [2]*International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru- 502 324, Greater Hyderabad, India and* [3]*Wageningen University and Research Centre, Crop and Weed Ecology Group, PO Box 430, 6700 AK Wageningen, The Netherlands*

ABSTRACT

In the present paper, we based a search for candidates underlying different levels of salinity tolerance during germination in the Oregon Wolfe Barley mapping population (DOM × REC) by proteomic profiling of the mature grain of lines showing differing levels of salinity tolerance. By contrasting the parents DOM and REC, displaying divergent stress responses, and two tolerant and two sensitive segregants, six protein spots were identified that showed a differential abundance between the tolerant and the sensitive lines. The tolerant lines expressed a higher level of 6-phosphogluconate dehydrogenase and glucose/ribitol dehydrogenase (Glc/RibDH). Both proteins were heterologously over-expressed in an osmo-sensitive yeast strain and over-expression of Glc/RibDH resulted in an enhanced ability of yeast transformants to grow on salt containing media. A quantitative trait locus (QTL) analysis of the population germinating at different salt concentrations led to the identification of two chromosome regions on 5H and one on 7H associated with salt stress response. A dense barley transcript map was employed to map the genomic region of all identified proteins. Two of these, heat-shock protein 70 and Glc/RibDH, co-localized with the identified QTL on chromosome 5H. The putative functional role of the candidates is discussed.

Key-words: 2-D gel electrophoresis; barley grain; mapping population; proteome analysis; QTL analysis; salt tolerance.

Abbreviations: 6PGDH, 6-phosphogluconate dehydrogenase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate; DTT, dithiothreitol; Glc/RibDH, glucose and ribitol dehydrogenase; IPG, immobilized pH gradient; OWB, Oregon Wolfe Barley; QTL, quantitative trait locus.

Correspondence: H.-P. Mock. Fax: +49 39482 5139; e-mail: mock@ipk-gatersleben.de

*Present address: Department of Biology and Program in Molecular Plant Biology, Colorado State University, Fort Collins, CO 80523, USA.

INTRODUCTION

Salinity is one of the most severe abiotic factors threatening agriculture worldwide, whether the occurrence is natural or arose from landscape clearing and irrigation of cultivated land. Natural tolerance to soil salinity is present in some plant species, but despite a growing investment in salinity-related research, much of the mechanisms underlying tolerance remain unclear. Salt tolerance is a multigenic trait, comprising ion partitioning within the plant, osmotic adjustment and morphological changes at both the cellular and subcellular levels (Munns 2005). Mechanisms which allow the maintenance of growth rate under stress conditions tend to limit the entry of salt into the plant and avoid toxic ion concentrations in the cytosol. A number of expression studies have sought to identify both candidate gene and gene products in a range of crop and model plants (Apse & Blumwald 2002; Wang, Vinocur & Altman 2003; Sahi *et al.* 2006; Vij & Tyagi 2007). Among these, genes controlling salt uptake and transport, affecting cellular osmosis or promoting plant growth in saline soil have been prominent (Munns 2005). Over-expression or knockout experiments for some of these genes contributed to the understanding of salt tolerance mechanisms, but, probably due to the complexity of the trait, there is no good example as yet of the directed development of salt-tolerant crops with wild-type performance in the field when the stress is absent (Flowers 2004; Shabala & Cuin 2008).

Barley is considered to be one of the most salinity tolerant of the cereals and cultivars display different levels of tolerance towards salinity (Weidner *et al.* 2006). Previous studies have been devoted to elucidate salt stress-induced transcriptional changes in barley cultivars Golden Promise (Walia *et al.* 2007), Haruna-nijyo (Ueda *et al.* 2006), Morex (Walia *et al.* 2006) and Tokak (Ozturk *et al.* 2002) and resulted in the identification of novel gene candidates associated with salinity stress. A high degree of genetic variation

has been widely documented for mapping populations and, therefore, the employment for gene and gene product identification improving desirable traits has been attempted (Francia, *et al.* 2007; Furukawa *et al.* 2007; Gyenis *et al.* 2007; Marcel *et al.* 2007; Nduulu *et al.* 2007). This variation is even more apparent when both parents show a contrasting phenotype for the respective trait as shown for the Oregon Wolfe Barley (OWB) mapping population. The OWB population was the first one to be established and aimed at mapping morphological traits by crossing dominant and recessive genetic marker stocks (Wolfe & Franckowiak 1991). The parents of the OWB population, DOM and REC, respond differentially in terms of germination under saline conditions (Weidner *et al.* 2006). Based on this observation, we previously carried out a comparative proteome analysis of DOM and REC, and revealed a high level of cultivar-specific protein expression in the grain (Witzel *et al.* 2007).

The mature barley grain is of high importance in feed and malting processes, and proteins present in the grain also determine the plant's performance during germination and affect the final crop productivity. Barley grain proteins fall into three groups according to their function: storage proteins, structural and metabolic proteins as well as protective proteins (Shewry & Halford 2002). The storage proteins are alcohol soluble and account for 30–50% of total grain protein. During germination and seedling growth, they function as source of amino acids and determine the nutritional quality of the grain. Albumins and globulins are soluble in water and dilute saline. Those proteins control metabolic and cellular processes; therefore, they are of high interest when investigating germination under salinity stress. Two-dimensional gel electrophoresis (2-D GE) was applied to establish proteome maps of the temporal and spatial distribution of albumins and globulins in mature grains (Østergaard *et al.* 2002), during maturation (Finnie *et al.* 2002) and germination (Bønsager *et al.* 2007) and provided insights into protein allocation.

The objective of the present study was first to identify differentially expressed proteins in the mature grains of salt-tolerant and salt-sensitive lines of OWB population. The choice of target for the proteomic analysis was governed by the recognition that the quality of a seedling emerging from a saline soil represents an important agronomic trait. For a better biochemical characterization of the complex trait, firstly, we decided to analyse the protein complement of individual lines, rather than to investigate bulked accessions with the same trait for salt stress response as it was done before in a study on boron tolerance (Patterson *et al.* 2007). Some candidates emerging from this analysis were functionally tested by their heterologous expression in yeast. Secondly, we wished to define the genetic basis of the differential salinity tolerance at the germination stage in the OWB mapping population by quantitative trait loci (QTL) analysis. Finally, we have compared the location of the genetic regions associated with salinity tolerance with the location of genes encoding the candidate salinity tolerance-associated proteins.

MATERIAL AND METHODS

Plant material

A mapping population consisting of 94 doubled-haploid (DH) lines was developed from the cross DOM × REC using the *Hordeum bulbosum* method (Costa *et al.* 2001). Characteristics of DOM and REC, which were selected as dominant and recessive morphological marker stocks, are described in Wolfe & Franckowiak (1991).

Yeast strain and cultivation

Over-expression of full-length barley cDNAs was achieved in *Saccharomyces cerevisiae* strain YSH818 (MATa *leu2-3/112 ura3-1 trp1-1 his3-11/15 ade2-1 can1-100 GAL SUC2 hog1*Δ::*LEU2*) (Albertyn *et al.* 1994). Yeast cultures were maintained by growing under selective conditions on synthetic dextrose minimal medium (0.67% bacto-yeast nitrogen base without amino acids, 1.6% bacto-agar) at 30 °C with 2% glucose as a carbon source and the provision of amino acids (20 mg L^{-1} adenine sulphate, 20 mg L^{-1} uracil, 20 mg L^{-1} *L*-tryptophan, 20 mg L^{-1} *L*-histidine HCl, 20 mg L^{-1} *L*-arginine HCl, 20 mg L^{-1} *L*-methionine, 30 mg L^{-1} *L*-tyrosine, 30 mg L^{-1} *L*-leucine, 30 mg L^{-1} *L*-isoleucine, 30 mg L^{-1} *L*-lysine HCl, 50 mg L^{-1} *L*-phenylalanin, 100 mg L^{-1} *L*-glutamic acid, 100 mg L^{-1} *L*-aspartic acid, 150 mg L^{-1} *L*-valine, 200 mg L^{-1} *L*-threonine, 400 mg L^{-1} *L*-serine; Boer *et al.* 2004). Either 2 or 3% NaCl was added to the medium to impose salinity stress.

Germination assay for testing salt tolerance

A total of 92 DH lines from the OWB mapping population and their parents DOM and REC were subjected to salt stress during germination. Two replicates each of 10 grains per genotype were germinated in the presence of 1.5, 2.0, 2.5% NaCl solution or distilled water as control on filter paper in plastic boxes. The chosen concentrations reflect the wide variation in salt tolerance which is expected for *Hordeum* species (Mano, Nakazumi & Takeda 1996; Garthwaite, von Bothmer & Colmer 2005). After 10 days at 20 °C and a 12 h photoperiod, germination was scored in accordance to the scheme of Mano *et al.* (1996).

Protein extraction for 2-D GE

Mature grains of selected lines from the OWB mapping population (OWB21, OWB34, OWB59 and OWB73) including parents DOM and REC were obtained from field-grown plants. Extraction of the albumin and globulin fraction was done following the protocol of Østergaard *et al.* (2002). Approximately 1 g of grain was ground under liquid nitrogen, and 250 mg of the resulting flour was thawed in 1250 μL of 5 mM Tris-HCl, pH 7.5, 1 mM $CaCl_2$, and incubated for 30 min at 4 °C. After a centrifugation for 15 min at 4 °C, the supernatant was mixed with four volumes of ice-cold acetone and incubated at –20 °C for 2 h.

Proteins were pelleted by centrifugation (5 min, 4 °C), dried by vacuum centrifugation and dissolved in 8 M urea, 2% CHAPS, 20 mM DTT, 0.5% IPG buffer by incubation for 1 h at 37 °C. Insoluble material was removed by centrifugation (15 min, room temperature). The protein concentration was determined using the 2-D QUANT KIT (GE Healthcare, http://www.gehealthcare.com) according to the manufacturer's instructions.

2-D GE and protein staining

Protein extracts were subjected to isoelectric focusing and subsequent sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) as described in Schlesier & Mock (2006). A 150 µg sample was loaded by rehydration onto IPG strip of 13 cm length with a pH gradient of 3–10. The separation on an IPGphor II unit (GE Healthcare) was performed with the following parameters: 15 h rehydration, 1 h gradient to 250 V, 1 h gradient to 500 V, 1 h gradient to 4000 V and 5.30 h 4000 V with a total of about 25 kVh. Strips were equilibrated in 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% v/v glycerine, 2% w/v SDS, 20 mM DTT, 0.01% bromphenol blue, and, subsequently, in 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% v/v glycerine, 2% w/v SDS, 135 mM iodoacetamide, 0.01% bromphenol blue for 15 min each at room temperature. The strips were then placed over an 11.25% SDS polyacrylamide gel, covered with 0.5% agarose and electrophoresed using a Hoefer S600 apparatus (GE Healthcare). Before staining, the gels were washed in water for 5 min.

The fluorescent stain was prepared as follows (Rabilloud *et al.* 2001): 0.2 g of potassium pentachloro aquo ruthenate ($K_2Cl_5Ru*H_2O$; Alfa Aesar, http://www.alfa-chemcat.com) was dissolved in 20 mL boiling water under reflux, after which, 0.8 g bathophenanthroline disulfonate was added, and the solution was kept under reflux for additional 20 min. Following this, 5 mL of 500 mM sodium ascorbate solution was added and the mixture was refluxed for further 20 min. The solution was then chilled on ice and the pH was carefully adjusted to 7.0 with NaOH. The volume was adjusted to 26 mL to give a stain concentration of about 20 mM (Rabilloud *et al.* 2001). Aliquots were stored at –20 °C.

With some minor modifications, the staining protocol of Lamanda *et al.* (2004) was used for protein visualization. In short, gels were incubated overnight at 4 °C in 30% EtOH, 10% acetic acid, washed four times for 30 min with 20% EtOH and stained in 1 µM RuBP solution in 20% EtOH for 6 h in the dark. Gels were washed for 10 min with water and destained overnight at 4 °C in 40% EtOH, 10% acetic acid. Prior to scanning, the gels were equilibrated twice in water for 10 min.

Image acquisition and analysis

A Fuji FLA-5100 (Fuji Film, http://www.fujifilm.com) equipped with the Image Reader FLA-5000 v1.0 software (Fuji Film, http://www.fujifilm.com) was used for image acquisition. Scanning parameters were resolution 100 µm, 16 bit picture, excitation wavelength 473 nm, emission filter 580 nm. The Progenesis PG220 v2006 software (Nonlinear Dynamics, http://www.nonlinear.com) was used for the 2-D image analysis with the following parameters: background subtraction method was mode of non-spot with margin 45, spot matching with vector box size 12 and search box size 64, normalization method was total spot volume multiplied by 100 and no spot filtering. Normalized spot volumes were compared for salt-tolerant lines (REC, OWB34, OWB59) and salt-sensitive lines (DOM, OWB21, OWB73), and significant changes were determined using *t*-test.

Mass spectrometry

Selected spots were excised manually from the gel using a standard ultraviolet light box, washed and digested with trypsin as described in Witzel *et al.* (2007). Peptide mass fingerprint data were acquired from a REFLEX III matrix-assisted laser desorption ionization time-of-flight mass spectrometer (Bruker Daltonics, http://www.bdal.com) operating in reflector mode. Spectra were calibrated using external calibration and subsequent internal mass correction. Protein identification was performed with the MASCOT search engine (Matrix Science, http://www.matrixscience.com) searching for Viridiplantae in the National Center for Biotechnology Information (NCBI) non-redundant protein sequence database and for barley expressed sequence tag (EST) Gene Index in the The Institute for Genomic Research (TIGR) database. Search parameters were: mono-isotopic mass accuracy, 100–200 ppm tolerance, one missed cleavage, allowed variable modifications oxidation (Met), propionamide (Cys) and carbamidomethyl (Cys).

When identification was unsuccessful, samples were subjected to nano liquid chromatography electrospray ionization quadrupole time-of-flight tandem mass spectrometry analysis and *de novo* sequencing according to Amme *et al.* (2006). The MS/MS spectra searches were conducted against a protein Viridiplantae index of the non-redundant NCBI database and the barley EST Gene Index in the TIGR database. A 10 ppm peptide, 0.1 Da fragment tolerance, one missed cleavage and variable oxidation (Met) and propionamide (Cys) were used as the search parameters.

Statistical analysis and QTL mapping

The QTL analyses were performed in two steps using QGENE software (Nelson 1997). A preliminary analysis based on a skeletal map developed by Costa *et al.* (2001) was able to define chromosomal location but allowed only a minimal level of intra-chromosomal resolution. This was followed by a second analysis based on a 1000 gene-based marker map (Stein *et al.* 2006), which provided sufficient detail for the localization of the QTL together with the gene function of the detected markers.

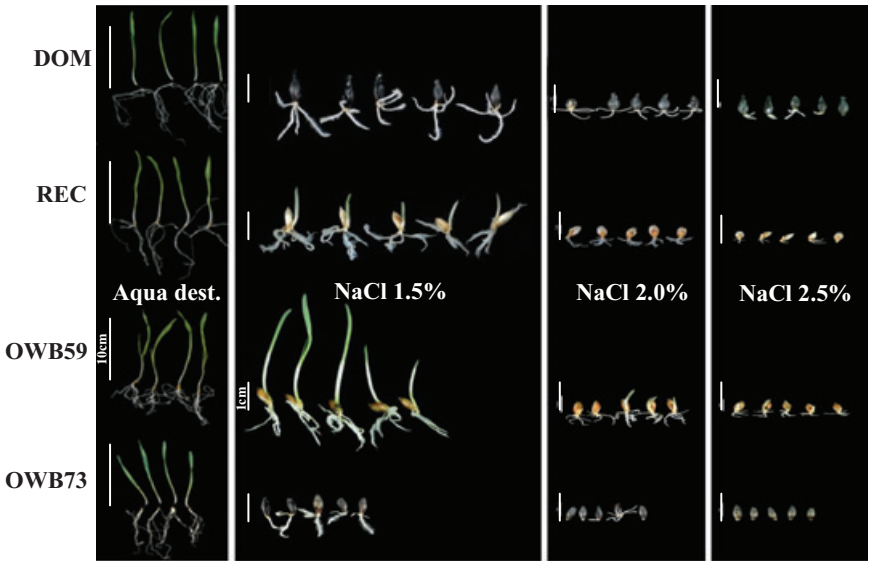

Figure 1. The germination performance of the parents of the Oregon Wolfe Barley (OWB) population, DOM and REC, and two doubled-haploid lines (OWB59 and OWB73) challenged with 0, 1.5, 2 or 2.5% NaCl.

RESULTS

Germination test of the OWB population for salinity tolerance

The effect on germination and seedling growth after 10 days exposure to salinity stress is shown in Fig. 1. Increasing salt concentration decreased the germination rate and subsequent seedling growth. Figure 2 tabulates the mean performance of each line in the form of a cumulative germination score across all the salt concentrations tested. OWB segregants with a high cumulative score under salinity stress were able to develop further than those having a low germination score. All the lines were able to germinate at 1.5% NaCl, while 2.0% NaCl provided the maximum discrimination between lines. At 2.5% NaCl, only the most tolerant lines were able to survive. The REC parent exhibited a better salinity tolerance than the DOM parent. About 20% of the DH lines were more tolerant

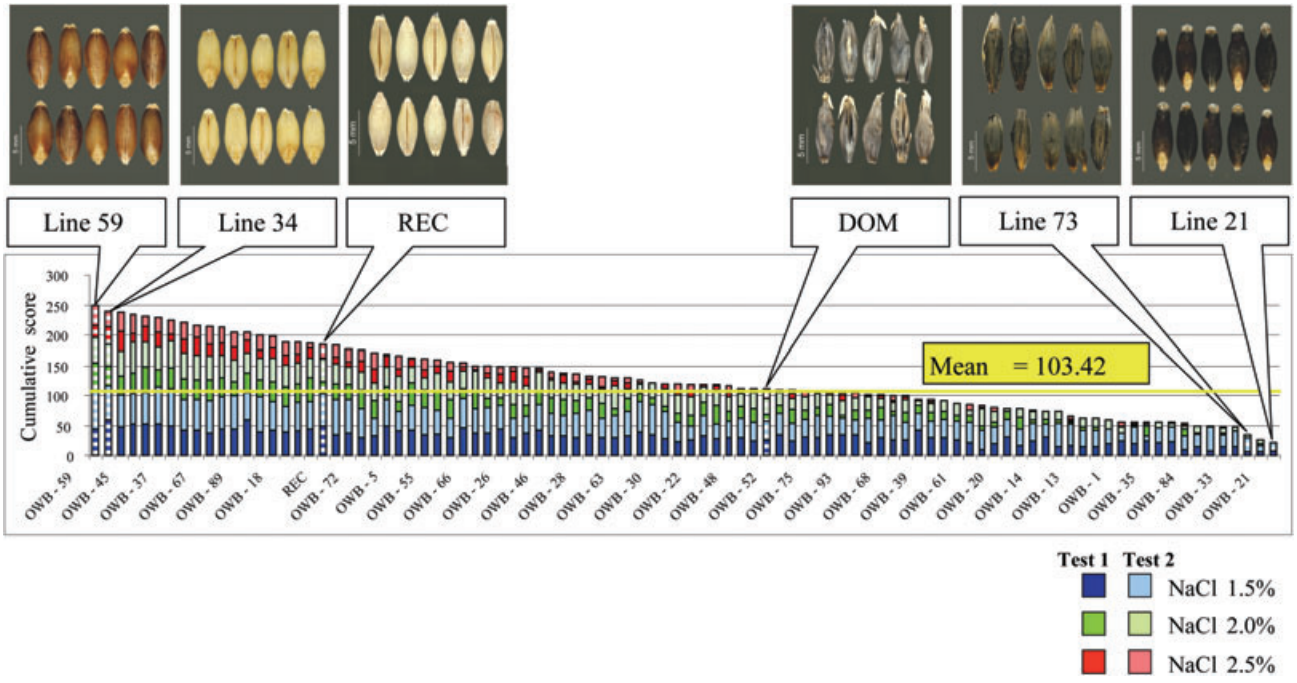

Figure 2. Germination assay for salt tolerance of 92 accessions of the Oregon Wolfe Barley (OWB) mapping population and the parental lines. Three different salt concentrations were used in two independent assays as highlighted in the colour code. The cumulative score for each line and the mean value for the entire population are shown. The grain phenotype of the selected lines for proteome analysis is depicted above.

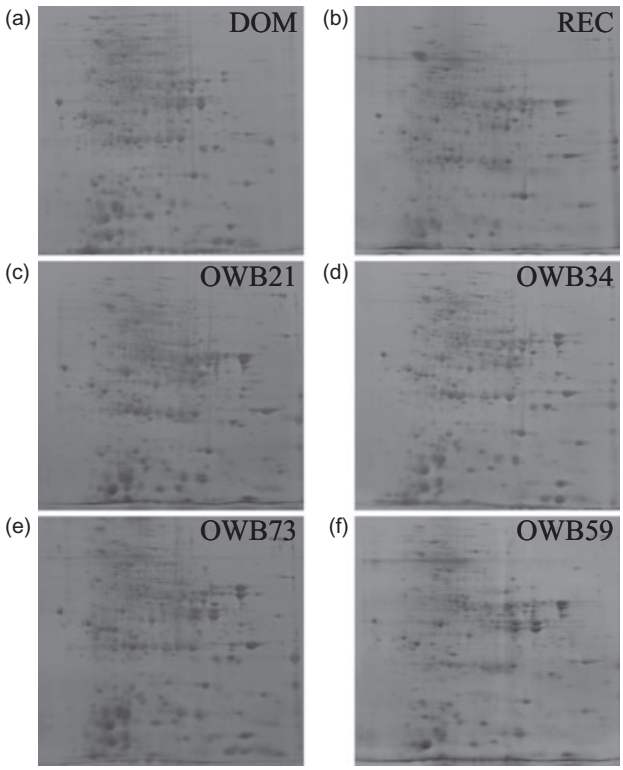

Figure 3. The water-soluble grain proteomes of salinity sensitive (a,c,e) and tolerant barley lines (b,d,f). Sample loading, separation and protein visualization were as described in the Materials and Methods section.

than REC and 44% responded more sensitive than DOM.

Comparative proteome analysis of mature grains

The water-soluble fraction from the mature grain of salt-tolerant (REC, OWB34, OWB59) and salt-sensitive (DOM, OWB21, OWB73) lines was investigated using 2-D GE. On average, about 1000 protein spots could be detected per line and per gel, and representative images from 2-D gels are shown in Fig. 3. A comparison between the parental proteomes revealed that, at a threshold of 1.5-fold, 228 proteins were more abundant in DOM and 108 in REC. The number of differentially expressed proteins decreased considerably when the other four DH lines were included in the analysis (Table 1). When analysing trait-specific protein expression, we found that six and five spots were more abundant in sensitive and tolerant lines, respectively. The example illustrated in Fig. 4 shows the abundance and normalized volumes of spots #8 and #9. The former was only detected in the sensitive lines, while the latter was more highly expressed in the tolerant ones.

Mass spectrometry-based identification of differentially expressed proteins

The 11 differentially expressed proteins taken forward for identification are shown in Fig. 5. The respective spots were collected from gels of all lines under investigation to confirm the spot identity. When spot identification based on peptide mass fingerprinting of tryptic peptides was not successful, *de novo* sequencing was attempted, allowing database searches for homologous sequences from related plant species. Six of the 11 proteins were successfully identified; this rather low proportion reflects in part the limited number of barley protein and EST sequences in the public domain and, in part, the high level of stringency applied by only accepting as valid an identification that was confirmed across all lines. Four of the spots contained unique proteins: cytosolic 6-phosphogluconate dehydrogenase (6PGDH) (#3), elongation factor 1β (#7), heat-shock protein (Hsp) 70 (#8) and a translationally controlled tumour protein homolog (#10) (Table 2); spots #6 and #9 both contained a glucose and ribitol dehydrogenase (Glc/RibDH) homolog. Except for spots #8 and #9, the predicted molecular mass and isoelectric point of these proteins were in agreement with their 2-D GE migration.

Functional analysis of candidate proteins in yeast

A functional analysis of the two proteins which were more abundant in the grain of tolerant lines (6PGDH and Glc/RibDH) was carried out via their heterologous overexpression in *S. cerevisiae*. The full gene sequence for these proteins was obtained from a BLAST search of the

Table 1. The number of differentially expressed (>1.5-fold change in normalized spot volume) proteins in comparisons between the parents and four segregants of the OWB population

	Spots with increased abundance	
	Sensitive lines	Tolerant lines
Comparison of parent lines (DOM versus REC)	228	108
Comparison including additional accessions (DOM + OWB21 + OWB73 versus REC + OWB34 + OWB59)	6	5

Protein spots depicted as increased in one group should be considered as decreased in the other.
OWB, Oregon Wolfe Barley.

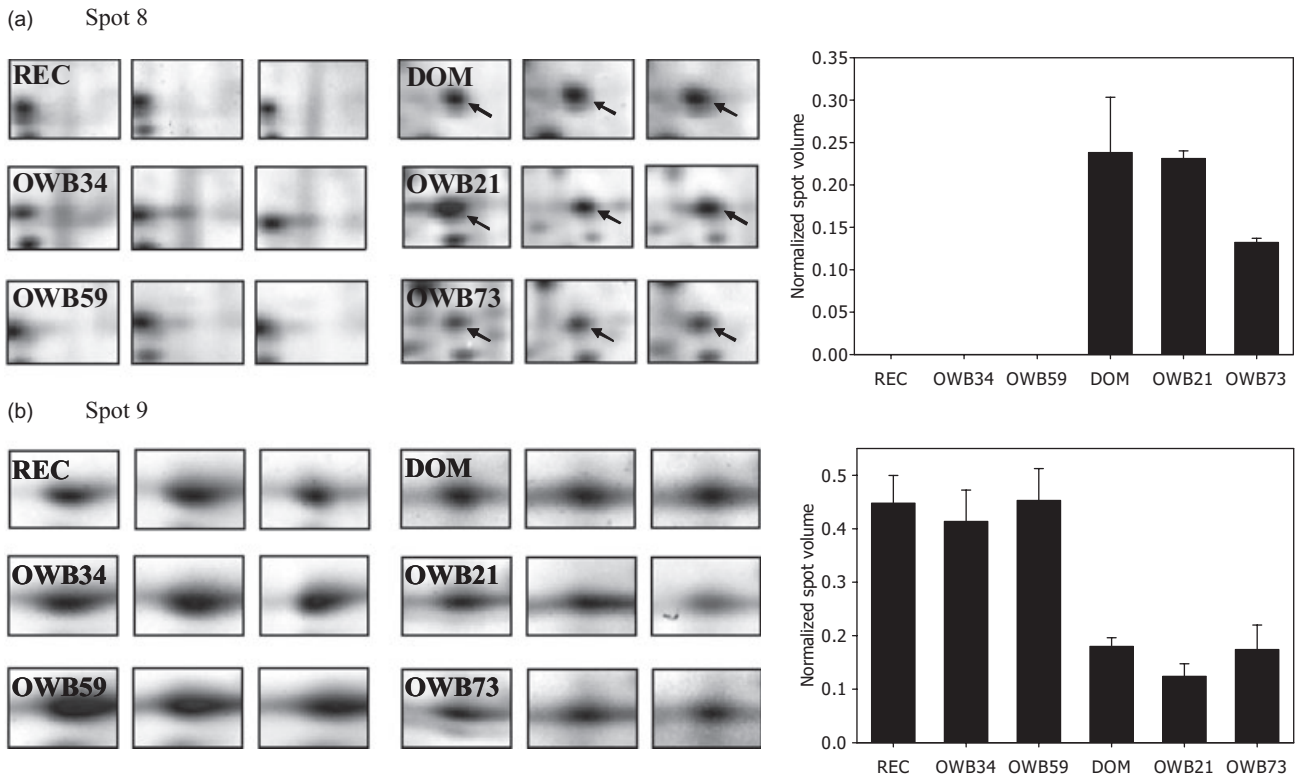

Figure 4. Differentially expressed proteins in the grains of lines showing contrasting salinity tolerance. (a) Spot #8 (arrow), present only in sensitive lines, shown as triplicate gels per sample. The accompanying histogram illustrates normalized spot volumes, with each bar representing the mean of three replicates per line. (b) Spot #9, the abundance of which is enhanced by 1.5-fold in the grain of tolerant lines. OWB, Oregon Wolfe Barley.

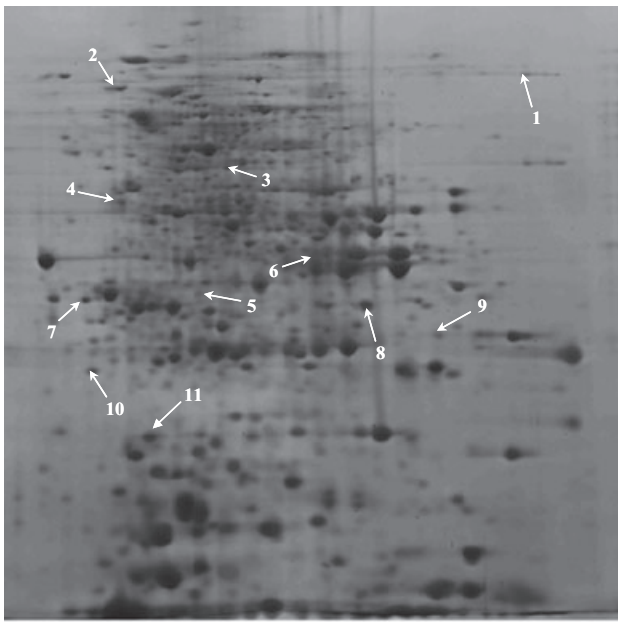

Figure 5. Representative two-dimensional gel electrophoresis separation of the water-soluble protein fraction from mature barley grain. Arrows show the 11 differentially expressed spots selected for protein identification.

CR-EST database (http://pgrc.ipk-gatersleben.de/crest/index.php). Specifically, the nucleotide sequence of EST HS09N23 was 99% identical with that of the derived sequence of Glc/RibDH, while that of HI05J23 was 99.5% identical with that of the derived sequence of 6PGDH. pYES-DEST52 plasmids containing either Glc/RibDH or 6PGDH were introduced into the osmo-sensitive yeast strain YSH818 (Δ*hog1*) (Boer *et al.* 2004). Transformants expressing 6PGDH were not able to grow on agar plates containing 2 and 3% NaCl, whereas the over-expression of Glc/RibDH enhanced the level of salinity tolerance, allowing cell growth even at the presence of 3% NaCl (Fig. 6).

QTL analysis of the OWB population for salinity tolerance and the assignment of the chromosomal location of genes encoding candidate proteins for salinity tolerance

An initial QTL analysis based on the classical map (Costa *et al.* 2001) revealed one major salinity tolerance locus on chromosome 5H and a second on chromosome 7H. The definition of both QTL was clearest under the highest level of stress (Fig. 7). However, the extent of the trait/marker correlation was insufficient to allow marker-assisted selection, because of the size of the genetic intervals in the key regions. The use of a more densely populated gene-based

Table 2. Identification of differentially expressed proteins in the mature grain of salinity tolerant and sensitive barley lines, as obtained via either MALDI-TOF MS or nanoLC-ESI-Q-TOF MS

Spot number	Expression in salt tolerant lines	Fold change	*P*-value	Protein name	Accession number	Theoretical pI/MW (kDa)
3	↑	4.4	0.026	Cytosolic 6-phosphogluconate dehydrogenase, *Oryza sativa*	TC146849	5.85/52.7
6	↑	3.2	2.65E-04	Glucose and ribitol dehydrogenase homolog, *Hordeum vulgare*	gi:7431022	6.54/31.6
7	↓	4.4	5.29E-03	Putative elongation factor 1β, *Hordeum vulgare*	gi:7711024	4.52/24.5
8	*	–	9.34E-06	Hsp 70, *Triticum aestivum*	gi:2827002	5.17/70.9
9	↑	1.5	2.18E-09	Glucose and ribitol dehydrogenase homolog, *Hordeum vulgare*	gi:7431022	6.54/31.6
10	↓	3.1	0.013	Translationally controlled tumour protein homolog, *Hordeum vulgare*	gi:20140865	4.53/18.8

Column 1: spot numbers; column 2: (↑), (↓) indicate, respectively, an increased and decreased abundance of the protein in the tolerant lines. (*) present only in the sensitive lines; column 3: fold change of the spot, based on normalized spot volumes; column 4: *P*-value as determined by *t*-test, column 5: spot identity based on MS analysis and the source species of the orthologue; column 6: TIGR EST and NCBI non-redundant protein database accession number; column 7: predicted isoelectric point (pI) and molecular weight (MW) as calculated using ExPASy tools (http://www.expasy.ch).
Hsp, heat-shock protein; LC-ESI-Q-TOF MS, liquid chromatography electrospray ionization quadrupole time-of-flight mass spectrometry; MALDI-TOF-MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry.

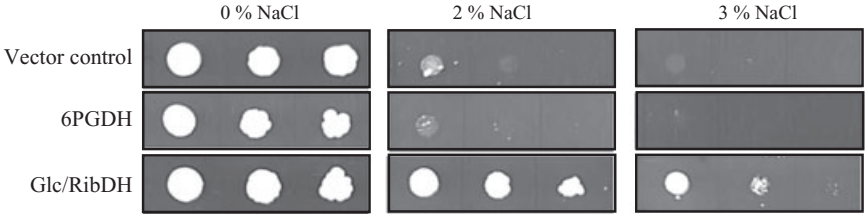

Figure 6. Complementation of an osmo-sensitive phenotype of the *Saccharomyces cerevisiae* Δ*hog1* mutant (YSH818) by over-expression of 6-phosphogluconate dehydrogenase (6PGDH) and glucose and ribitol dehydrogenase (Glc/RibDH). Yeast cultures (3×10^6 cells mL^{-1}) were spotted in serial 10-fold dilutions onto agar plates containing 0, 2 and 3% NaCl, and incubated for 4 days at 30 °C before photographs were taken. As vector control served the pYES vector without insertion.

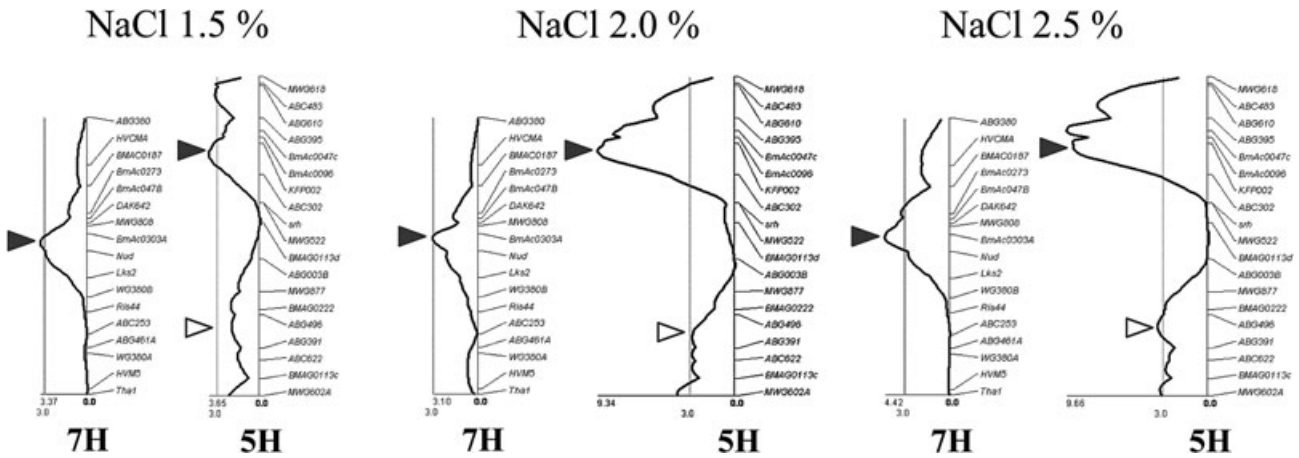

Figure 7. Major salinity tolerance quantitative trait locus at three NaCl concentrations of the Oregon Wolfe Barley mapping population at germination stage based on a skeletal genetic map. Filled arrowheads indicate the locations on chromosome 5H and 7H where the logarithm of the odds score reached a peak.

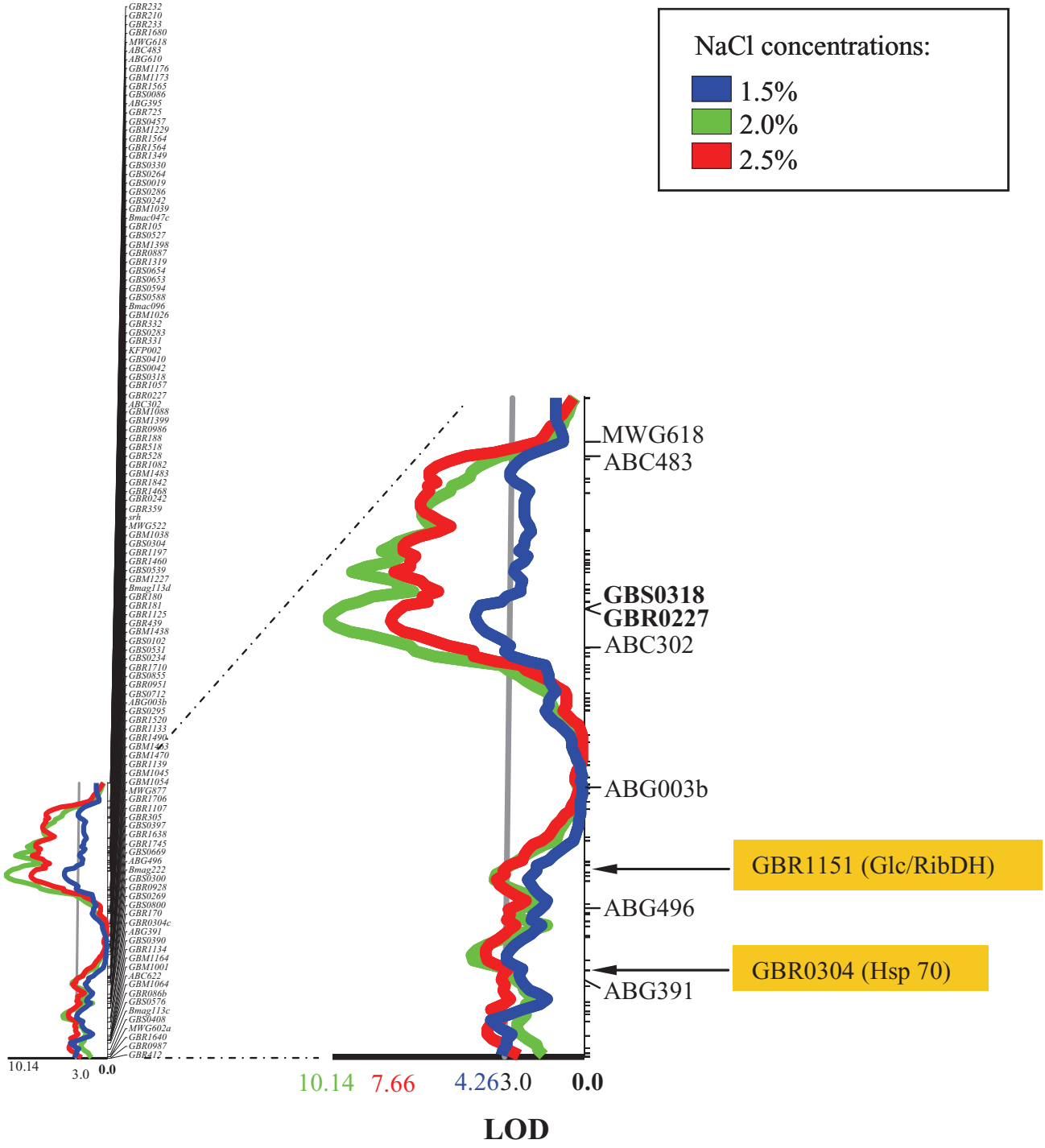

Figure 8. Quantitative trait locus (QTL) localization on chromosome 5H of barley based on a dense gene-based marker map. Each colour represents a different NaCl concentration. Loci marked in bold are genetically linked to the QTL, and those highlighted in yellow were identified from the proteomic analysis. LOD, logarithm of the odds.

map (Stein *et al.* 2006) confirmed the importance of chromosomes 5H and 7H. Two separate QTL were detected on the former chromosome, along with a minor QTL on each of chromosomes 7H and 2H. One of the chromosomes 5H QTL was responsible for 42% of the phenotypic variation, and was consistently identifiable across all NaCl concentrations. The closest markers to this locus were GBS0318 (EST: AL511699) and GBR0227 (EST: AL502175) (Fig. 8). The functions of these two ESTs were, respectively, 'hypersensitive-induced reaction protein 3' and 'mitogen-activated protein kinase kinase kinase 11' (MAP3K11). Previously reported markers for grain yield

under salinity stress on chromosome 2H were not identified (Eleuch *et al.* 2008).

Using the fine-scale barley genetic map (Stein *et al.* 2006), both encoding loci for Hsp 70 and Glc/RibDH were assigned a firm genetic location. The inferred nucleotide sequence of Hsp 70 had a BLAST score of 154 and an *e*-value of 9.00E-46 when compared to that of EST 500877 (marker GBR0304); similarly, the Glc/RibDH sequence is highly homologous to EST 502447 (marker GBR1151) at high BLAST score (192) and high *e*-value (5.00E-48). GBR1151 and GBR0304 both map to chromosome 5H at, respectively, positions 153.7 and 176.6 cM, which is the same genomic region where a major salinity tolerance QTL was mapped (Fig. 8).

DISCUSSION

Proteomic analysis of the mature grain reveals candidates for salinity stress

The mature barley grain is of high importance in feed, food and malting industries, and proteins present in the grain contribute to the ability to germinate even under unfavourable environmental conditions. The comparative analysis of proteins in the water-soluble fraction of grains from salinity tolerant and sensitive lines led to the identification of differentially expressed proteins. One of the proteins present in enhanced abundance in the salinity tolerant entries was a cytosolic 6PGDH, an enzyme involved in the pentose phosphate pathway. The function of this pathway is to provide reduced nicotinamide adenine dinucleotide phosphate (NADPH) used as electron donor in reductive biosynthetic reactions like synthesis of lipids, aromatic amino acids and coenzymes. It is also essential for the functioning of the ascorbate–glutathione cycle, an important component of the plant antioxidant protection system (Corpas *et al.* 1998). The expression of NADPH-dependent dehydrogenases is enhanced by both oxidative and salinity stress, as, for example, in olive, where salinity stress increases the activity of glucose-6-phosphate dehydrogenase, isocitrate dehydrogenase and malic enzyme (Valderrama *et al.* 2006; Fu-Yun *et al.* 2007). Similarly, salinity treatment of rice rapidly induces 6PGDH transcription (Huang *et al.* 2003; Fu-Yun *et al.* 2007; Hou *et al.* 2007). Thus, the association of higher levels of 6PGDH expression in the mature grain of salinity tolerant barleys, as observed in the present experiments, may indicate that the tolerant lines possess a more active enhanced pentose phosphate pathway.

The second protein with higher abundance was orthologous to a bacterial Glc/RibDH, a class of proteins belonging to short alcohol dehydrogenases (Jornvall *et al.* 1984). Both Glc/RibDH transcripts and protein were found in developing barley embryos, and the level of transcript was observed to decrease during germination (Alexander *et al.* 1994). Glc/RibDH catalyses the oxidation of D-glucose (but not sugar phosphates) using NAD as co-substrate, and the specificity was determined by enzymatic assays. This substrate specificity suggests the presence of a novel metabolic pathway in barley, and that a specific carbohydrate metabolism may be important for early embryo development. The up-regulation of Glc/RibDH in the grain of tolerant genotypes is indicative of an enhanced carbohydrate metabolism, where sugars with osmo-protective function are synthesized. Glc/RibDH was present in two separate protein spots (#6 and #9), the gel migration position for one of which is consistent with the protein's properties deduced from the database sequence, whereas the other appears to have a lower molecular weight and a higher predicted isoelectric point. Neither peptide mass fingerprinting nor *de novo* sequencing were able to reveal any further insights into the structural integrity of the proteins present in these two spots, so it remains unresolved as yet whether they represent post-translational modification products or whether the protein is translated as multiple isomers.

Expression of Glc/RibDH complements an osmo-sensitive yeast phenotype

The yeast expression system is appropriate for the analysis of plant protein function, because of global commonality in signal transduction pathways and stress tolerance mechanisms that exist between these organisms (Serrano, Culianz-Macia & Moreno 1999; Hohmann 2002; Quintero *et al.* 2002; Liu *et al.* 2007). Functional complementation of an osmo-sensitive yeast strain was tested both for 6PGDH and Glc/RibDH. While yeast cells transformed with the former could not grow on NaCl-containing agar plates, over-expression of the latter increased the growth rate considerably. The enzyme 6PGDH is involved in the NADPH-dependent detoxification of reactive oxygen species (ROS). However, the negative effect of salinity stress on yeast involves osmotic maladjustment rather than ion toxicity and ROS generation, as the cell is well equipped with ion transporters which ensure the efficient transport of major cations (Serrano 2004). Although the transgene failed to complement an osmo-sensitive yeast strain, its role in ion-specific stress reactions is worth to be investigated *in planta*. The over-expression of Glc/RibDH enhanced the growth of salinity-stressed yeast, which suggests a role in the accumulation of sugars with osmo-protective function. Osmotic shock is known to stimulate the glycolytic pathway, in response to the greater demand for energy needed for continued proliferation and biomass production under stressed conditions (Hohmann 2002). Complementation of the osmo-sensitive effect in *S. cerevisiae* by Glc/RibDH provides evidence for its role in augmenting salinity tolerance in barley.

QTL analysis of the OWB population for salt tolerance at the germination stage and candidate genes co-localizing with a QTL region

The Triticeae group 5 chromosomes have frequently been associated with QTL concerned with plant adaptation to

the environment (Cattivelli *et al.* 2002). Here, we have shown that chromosome 5H carries one QTL for germination under salinity stress conditions in its centromeric region, and a second one on its long arm. In the 'Steptoe–Morex' barley mapping population, a major salinity tolerance QTL was located near the centromere of 5H (Mano & Takeda 1997), which is in line with the present conclusions from the OWB population. QTL important for the determination of osmotic stress resistance (Lohwasser & Börner 2007) and control of pre-harvest sprouting (Hori, Sato & Takeda 2007) in the OWB population have previously been identified in the same region. This indicates a more general stress response in the early phase of plant development. The marker GBS0318 with the function 'hypersensitive-induced reaction protein 3' would support this fact. The overexpression of such a protein in transgenic *Arabidopsis* seedlings resulted in slower germination and a growth retardation in the lateral roots in response to both salinity and osmotic stress (Jung *et al.* 2008). The function of the second closely linked 'MAP3K11' marker GBR0227 is involved with the perception of extracellular stimuli and signal transduction. MAPK genes inducible by salinity stress have been identified on rice chromosomes 3, 5 and 6 (Rohila & Yang 2007). According to our present understanding of orthology between rice and wheat chromosomes (Moore *et al.* 1995) and the synteny to other cereals, these regions are represented in barley by chromosomes 5H and 7H.

The availability of the transcript map of barley provided the opportunity to locate the position of candidate genes for salinity tolerance on the barley genetic map. Both this analysis and the present re-analysis have placed the key QTL in the same genomic region where the genes encoding Hsp 70 and Glc/RibDH also map. Validation of these candidate genes requires either their expression profiling across the full mapping population and/or transgenic approach where each of the genes is over-expressed separately and together in a salinity-sensitive cultivar. Neither of these experiments represents a trivial investment in time and resources. For the moment, we suggest that there is sufficient evidence for these proteins to have a role in enhancing salinity tolerance. As both the genes are marked by well-characterized assays (GBR0304 and GBR1151), a direct outcome of the study would be to use these as a means of applying marker-assisted breeding for salinity tolerance in barley (Varshney, Graner & Sorrells 2005).

Salinity tolerance is a multigenic trait controlled by a range of gene products expressed at various developmental stages of the plant. To detect proteins which contribute to salinity tolerance at the seedling stage, we will extend the analysis from the mature to the salinity-stressed germinating grain.

ACKNOWLEDGMENTS

This work was supported by the BMBF (GABISEED II; FKZ 0313115 to H.-P. M.), COST short-term scientific mission fellowship (FA0603-03178 to KW), DFG (Mo 479/4-1 and 4-2) and by DAAD/Leibniz post-doc scholarship (A/03/27383 to G.-K. S.). We thank Dagmar Böhmert, Elis Fraust and Annegret Wolf for technical assistance. The valuable comments of three anonymous reviewers are gratefully acknowledged.

REFERENCES

Albertyn J., Hohmann S., Thevelein J.M. & Prior B.A. (1994) Gpd1, which encodes glycerol-3-phosphate dehydrogenase, is essential for growth under osmotic-stress in *Saccharomyces cerevisiae*, and its expression is regulated by the high-osmolarity glycerol response pathway. *Molecular and Cellular Biology* **14,** 4135–4144.

Alexander R., Alamilla J.M., Salamini F. & Bartels D. (1994) A novel embryo-specific barley cDNA clone encodes a protein with homologies to bacterial glucose and ribitol dehydrogenase. *Planta* **192,** 519–525.

Amme S., Matros A., Schlesier B. & Mock H.-P. (2006) Proteome analysis of cold stress response in *Arabidopsis thaliana* using DIGE-technology. *Journal of Experimental Botany* **57,** 1537–1546.

Apse M.P. & Blumwald E. (2002) Engineering salt tolerance in plants. *Current Opinion in Biotechnology* **13,** 146–150.

Boer E., Wartmann T., Dlubatz K., Gellissen G. & Kunze G. (2004) Characterization of the *Arxula adeninivorans AHOG1* gene and the encoded mitogen-activated protein kinase. *Current Genetics* **46,** 269–276.

Bønsager B.C., Finnie C., Roepstorff P. & Svensson B. (2007) Spatio-temporal changes in germination and radicle elongation of barley seeds tracked by proteome analysis of dissected embryo, aleurone layer, and endosperm tissues. *Proteomics* **7,** 4528–4540.

Cattivelli L., Baldi P., Crosatti C., Di Fonzo N., Faccioli P., Grossi M., Mastrangelo A.M., Pecchioni N. & Stanca A.M. (2002) Chromosome regions and stress-related sequences involved in resistance to abiotic stress in Triticeae. *Plant Molecular Biology* **48,** 649–665.

Corpas F.J., Barroso J.B., Sandalio L.M., Distefano S., Palma J.M., Lupiáñez J.A. & Del Río L.A. (1998) A dehydrogenase-mediated recycling system of NADPH in plant peroxisomes. *The Biochemical Journal* **330** (Pt 2), 777–784.

Costa J.M., Corey A., Hayes P.M., *et al.* (2001) Molecular mapping of the Oregon Wolfe Barleys: a phenotypically polymorphic doubled-haploid population. *Theoretical and Applied Genetics* **103,** 415–424.

Eleuch L., Jilal A., Grando S., Ceccarelli S., Schmising M.V., Tsujimoto H., Hajer A., Daaloul A. & Baum M. (2008) Genetic diversity and association analysis for salinity tolerance, heading date and plant height of barley germplasm using simple sequence repeat markers. *Journal of Integrative Plant Biology* **50,** 1004–1014.

Finnie C., Melchior S., Roepstorff P. & Svensson B. (2002) Proteome analysis of grain filling and seed maturation in barley. *Plant Physiology* **129,** 1308–1319.

Flowers T.J. (2004) Improving crop salt tolerance. *Journal of Experimental Botany* **55,** 307–319.

Francia E., Barabaschi D., Tondelli A., *et al.* (2007) Fine mapping of a Hv CBF gene cluster at the frost resistance locus Fr-H2 in barley. *Theoretical and Applied Genetics* **115,** 1083–1091.

Furukawa J., Yamaji N., Wang H., Mitani N., Murata Y., Sato K., Katsuhara M., Takeda K. & Ma J.F. (2007) An aluminum-activated citrate transporter in barley. *Plant & Cell Physiology* **48,** 1081–1091.

Fu-Yun H., Ji H., Shan-Lin Y. & Hong-Sheng Z. (2007) The 6-phosphogluconate dehydrogenase genes are responsive to

abiotic stresses in rice. *Journal of Integrative Plant Biology* **49,** 655–663.

Garthwaite A.J., von Bothmer R. & Colmer T.D. (2005) Salt tolerance in wild *Hordeum* species is associated with restricted entry of Na+ and Cl– into the shoots. *Journal of Experimental Botany* **56,** 2365–2378.

Gyenis L., Yun S.J., Smith K.P., Steffenson B.J., Bossolini E., Sanguineti M.C. & Muehlbauer G.J. (2007) Genetic architecture of quantitative trait loci associated with morphological and agronomic trait differences in a wild by cultivated barley cross. *Genome* **50,** 714–723.

Hohmann S. (2002) Osmotic stress signaling and osmoadaptation in yeasts. *Microbiology and Molecular Biology Reviews* **66,** 300–372.

Hori K., Sato K. & Takeda K. (2007) Detection of seed dormancy QTL in multiple mapping populations derived from crosses involving novel barley germplasm. *Theoretical and Applied Genetics* **115,** 869–876.

Hou F.Y., Huang J., Yu S.L. & Zhang H.S. (2007) The 6-phosphogluconate dehydrogenase genes are responsive to abiotic stresses in rice. *Journal of Integrative Plant Biology* **49,** 655–663.

Huang J., Zhang H., Wang J. & Yang J. (2003) Molecular cloning and characterization of rice 6-phosphogluconate dehydrogenase gene that is up-regulated by salt stress. *Molecular Biology Reports* **30,** 223–227.

Jornvall H., von Bahr-Lindstrom H., Jany K.-D., Ulmer W. & Froschle M. (1984) Extended superfamily of short alcohol-polyol-sugar dehydrogenases: structural similarities between glucose and ribitol dehydrogenases. *FEBS Letters* **165,** 190–196.

Jung H.W., Lim C.W., Lee S.C., Choi H.W., Hwang C.H. & Hwang B.K. (2008) Distinct roles of the pepper hypersensitive induced reaction protein gene *CaHIR1* in disease and osmotic stress, as determined by comparative transcriptome and proteome analyses. *Planta* **227,** 409–425.

Lamanda A., Zahn A., Röder D. & Langen H. (2004) Improved Ruthenium II tris (bathophenantroline disulfonate) staining and destaining protocol for a better signal-to-background ratio and improved baseline resolution. *Proteomics* **4,** 599–608.

Liu N., Chen A.-P., Zhong N.-Q., Wang F., Wang H.-Y. & Xia G.-X. (2007) Functional screening of salt stress-related genes from *Thellungiella halophila* using fission yeast system. *Physiologia Plantarum* **129,** 671–678.

Lohwasser U. & Börner A. (2007) *Detection of loci controlling pre-harvest sprouting and dormancy in the Triticeae* . Proceedings of the 14th International EWAC Conference, Istanbul, Turkey.

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.

Mano Y., Nakazumi H. & Takeda K. (1996) Varietal variation in and effects of some major genes on salt tolerance at the germination stage in barley. *Breeding Science* **46,** 227–233.

Marcel T.C., Varshney R.K., Barbieri M., Jafary H., de Kock M.J., Graner A. & Niks R.E. (2007) A high-density consensus map of barley to compare the distribution of QTLs for partial resistance to *Puccinia hordei* and of defence gene homologues. *Theoretical and Applied Genetics* **114,** 487–500.

Moore G., Devos K.M., Wang Z. & Gale M.D. (1995) Cereal genome evolution – grasses, line up and form a circle. *Current Biology* **5,** 737–739.

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

Nduulu L., Mesfin A., Muehlbauer G. & Smith K. (2007) Analysis of the chromosome 2(2H) region of barley associated with the correlated traits *Fusarium* head blight resistance and heading date. *Theoretical and Applied Genetics* **115,** 561–570.

Nelson J.C. (1997) QGENE: soft ware for mapping-based genomic analysis and breeding. *Molecular Breeding* **3,** 239–245.

Østergaard O., Melchior S., Roepstorff P. & Svensson B. (2002) Initial proteome analysis of mature barley seeds and malt. *Proteomics* **2,** 733–739.

Ozturk Z.N., Talame V., Deyholos M., Michalowski C.B., Galbraith D.W., Gozukirmizi N., Tuberosa R. & Bohnert H.J. (2002) Monitoring large-scale changes in transcript abundance in drought- and salt-stressed barley. *Plant Molecular Biology* **48,** 551–573.

Patterson J., Ford K., Cassin A., Natera S. & Bacic A. (2007) Increased abundance of proteins involved in phytosiderophore production in boron-tolerant barley. *Plant Physiology* **144,** 1612–1631.

Quintero F.J., Ohta M., Shi H.Z., Zhu J.K. & Pardo J.M. (2002) Reconstitution in yeast of the *Arabidopsis* SOS signaling pathway for Na^+ homeostasis. *Proceedings of the National Academy of Sciences of the United States of America* **99,** 9061–9066.

Rabilloud T., Strub J.-M., Luche S., Dorsselaer A.v. & Lunardi J. (2001) A comparison between Sypro Ruby and ruthenium II tris (bathophenanthroline disulfonate) as fluorescent stains for protein detection in gels. *Proteomics* **1,** 699–704.

Rohila J.S. & Yang Y.N. (2007) Rice mitogen-activated protein kinase gene family and its role in biotic and abiotic stress response. *Journal of Integrative Plant Biology* **49,** 751–759.

Sahi C., Singh A., Kumar K., Blumwald E. & Grover A. (2006) Salt stress response in rice: genetics, molecular biology, and comparative genomics. *Functional and Integrative Genomics* **6,** 263–284.

Schlesier B. & Mock H.-P. (2006) Protein isolation and 2-D electrophoretic separation. In *Arabidopsis Protocols* (eds J. Sanchez-Serrano & J. Salinas) pp. 381–391. Humana Press, Totowa, NJ, USA.

Serrano R. (2004) Halotolerance genes in yeast. In *Salinity: Environment – Plants – Molecules* (A. Leuchli & U. Luttge) pp. 491–504. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Serrano R., Culianz-Macia F.A. & Moreno V. (1999) Genetic engineering of salt and drought tolerance with yeast regulatory genes. *Scientia Horticulturae* **78,** 261–269.

Shabala S. & Cuin T.A. (2008) Potassium transport and plant salt tolerance. *Physiologia Plantarum* **133,** 651–669.

Shewry P.R. & Halford N.G. (2002) Cereal seed storage proteins: structures, properties and role in grain utilization. *Journal of Experimental Botany* **53,** 947–958.

Stein N., Prasad M., Scholz U., *et al.* (2006) A 1000 loci transcript map of the barley genome – new anchoring points for integrative grass genomics. *Theoretical and Applied Genetics* **114,** 823–839.

Ueda A., Kathiresan A., Bennett J. & Takabe T. (2006) Comparative transcriptome analyses of barley and rice under salt stress. *Theoretical and Applied Genetics* **112,** 1286–1294.

Valderrama R., Corpas F.J., Carreras A., Gomez-Rodriguez M.V., Chaki M., Pedrajas J.R., Fernandez-Ocana A., Del Rio L.A. & Barroso J.B. (2006) The dehydrogenase-mediated recycling of NADPH is a key antioxidant system against salt-induced oxidative stress in olive plants. *Plant, Cell & Environment* **29,** 1449–1459.

Varshney R.K., Graner A. & Sorrells M.E. (2005) Genomics-assisted breeding for crop improvement. *Trends in Plant Science 10th Anniversary Issue – Feeding the World: Plant Biotechnology Milestones* **10,** 621–630.

Vij S. & Tyagi A.K. (2007) Emerging trends in the functional genomics of the abiotic stress response in crop plants. *Plant Biotechnology Journal* **5,** 361–380.

Walia H., Wilson C., Wahid A., Condamine P., Cui X. & Close T. (2006) Expression analysis of barley (*Hordeum vulgare* L.) during salinity stress. *Functional and Integrative Genomics* **6,** 143–156.

Walia H., Wilson C., Condamine P., Liu X., Ismail A.M. & Close T.J. (2007) Large-scale expression profiling and physiological characterization of jasmonic acid-mediated adaptation of barley to salinity stress. *Plant, Cell & Environment* **30,** 410–421.

Wang W., Vinocur B. & Altman A. (2003) Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. *Planta* **218,** 1–14.

Weidner A., Varshney R.K., Buck-Sorlin G.H., Stein N., Graner A. & Börner A. (2006) *QTLs for salt tolerance: comparison of barley mapping populations*. Proceedings of 57th Annual Meeting of the Society of the Austrian Plant Breeders, Seed Producers and Seed Offerers, Gumpenstein, Austria 2006.

Witzel K., Surabhi G.-K., Jyothsnakumari G., Sudhakar C., Matros A. & Mock H.-P. (2007) Quantitative proteome analysis of barley seeds using ruthenium(II)-tris-(bathophenanthroline-disulphonate) staining. *Journal of Proteome Research* **6,** 1325–1333.

Wolfe R. & Franckowiak J. (1991) Multiple dominant and recessive genetic marker stocks in spring barley. *Barley Genetics Newsletter* **20,** 117–121.

Received 6 July 2009; received in revised form 12 October 2009; accepted for publication 13 October 2009