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RNA-Seq analysis of the wild barley (*H. spontaneum*) leaf transcriptome under salt stress

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ABSTRACT

Wild salt-tolerant barley (*Hordeum spontaneum*) is the ancestor of cultivated barley (*Hordeum vulgare* or *H. vulgare*). Although the cultivated barley genome is well studied, little is known about genome structure and function of its wild ancestor. In the present study, RNA-Seq analysis was performed on young leaves of wild barley treated with salt (500 mM NaCl) at four different time intervals. Transcriptome sequencing yielded 103 to 115 million reads for all replicates of each treatment, corresponding to over 10 billion nucleotides per sample. Of the total reads, between 74.8 and 80.3% could be mapped and 77.4 to 81.7% of the transcripts were found in the *H. vulgare* unigene database (unigene-mapped). The unmapped wild barley reads for all treatments and replicates were assembled *de novo* and the resulting contigs were used as a new reference genome. This resulted in 94.3 to 95.3% of the unmapped reads mapping to the new reference. The number of differentially expressed transcripts was 9277, 3861 of which were unigene-mapped. The annotated unigene- and *de novo*-mapped transcripts (5100) were utilized to generate expression clusters across time of salt stress treatment. Two-dimensional hierarchical clustering classified differential expression profiles into nine expression clusters, four of which were selected for further analysis. Differentially expressed transcripts were assigned to the main functional categories. The most important groups were "response to external stimulus" and "electron-carrier activity". Highly expressed transcripts are involved in several biological processes, including electron transport and exchanger mechanisms, flavonoid biosynthesis, reactive oxygen species (ROS) scavenging, ethylene production, signaling network and protein refolding. The comparisons demonstrated that mRNA-Seq is an efficient method for the analysis of differentially expressed genes and biological processes under salt stress.

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1. Introduction

Barley is an important cereal crop in terms of productivity and global area of cultivation [1]. Cultivated barley, *Hordeum vulgare* L. ssp. *vulgare* (*H. vulgare*), descended from wild barley, *H. vulgare* L. ssp. *spontaneum* (*H. spontaneum*). Barley is a self-pollinating diploid with 7 pairs of chromosomes and a nuclear genome size of 5.1 Gb [2], and it harbors high levels of genetic variation [3,4] that helps it survive in low input and climatically marginal climates. Although *H. vulgare* is well-studied in terms of genetics, genomics and breeding, little is known about the genetic makeup and genome function of its wild ancestor *H. spontaneum*. Cultivated barley was earlier reported to contain about 40% of *H. spontaneum* alleles [5].

Salt tolerance in wild barley has been reviewed by many researchers because it provides a rich source of genes that can be transferred to other crop plants by genetic transformation as well as to cultivated barley by classical breeding methods [6,7]. For plants to survive under salt stress, they must be able to activate cascades of molecular networks involved in stress perception or sensing [8], signal transduction [9], as well as the induction of specific stress-related genes and their encoded metabolites [10–12]. Some of the signaling pathways are specific, but others may cross talk; e.g., MAPK cascades and the cross talk between ABA signaling and biotic signaling [13]. Previous studies on various plant species demonstrated that cross talk involves complex networks of gene regulation [14,15], some of which are mediated by plant hormones such as abscisic acid [9] and ethylene [16], and influenced through specific transcription factors [17]. Cross talk also results in the expression of diverse functional genes for osmoregulation, cell protection and acclimation, such as dehydrins, aquaporins and chaperones [10,12,18].

A draft genome sequence of cultivated barley has been recently described [2] and transcript profiling for the cataloguing of stress-responsive genes has been reported [19,20]. The present study utilizes mRNA-Seq analysis of leaves of the wild barley (*H. spontaneum*) to examine salt-related genes and biological processes in order to discover novel genes and transcription factors to improve the understanding of the mechanisms underlying the process of salt stress tolerance. The RNA-Seq method has considerable potential to generate high-resolution transcriptome maps sensitive enough to display transcripts with low-levels of expression [21].

2. Materials and methods

2.1. Plant material

Seeds of the self-pollinated wild *H. spontaneum* were collected from a location in Rafah, North Sinai, Egypt (31.313559, 34.205973) near the Mediterranean Sea (~38 g/L), where no specific permission is required. Besides, no endangered or protected plant or animal species are grown in this location. Seeds were germinated in the greenhouse in trays filled with potting mix consisting of vermiculite:perlite (1:1) and grown at 14 h of light per day, 80% humidity and 22 °C for two weeks and watered with half-strength Hoagland solution [22]. Seedlings were then salt stressed (500 mM NaCl or 29.22 g/L in half-strength Hoagland solution) at 0, 2, 12 and 24 h time intervals. Leaves of individual plants in three replicates were harvested at each time point except at time point 0 where only two replicates were gathered. All tissues were flash-frozen in liquid nitrogen and stored at −80 °C.

2.2. RNA isolation

Flash-frozen leaf material from individual plants was crushed into a fine powder in a microcentrifuge tube using a sterilized metal rod. Total RNAs were extracted from similar-sized leaf samples collected from emergent leaves using Trizol (Invitrogen, Life Tech, Grand Island, NY, USA) and treated with RNase-free DNase (Promega Corporation, Madison, WI, USA) in the presence of 1 U/μL of RNasin® Plus RNase Inhibitor (Promega) for 2 h at 37 °C. RNAs were quantified and 30 μg (400 ng/μL) was used for RNA-Seq. To test for the presence of DNA contamination in RNA samples, the *actin* gene was amplified by PCR of the original RNA samples. Purified RNA samples were shipped to Beijing Genomics Institute (BGI), Shenzhen, China in three replicates of each treatment for deep sequencing and generation of datasets (at least 100 million reads per sample).

2.3. Next-generation mRNA sequencing

Filtered reads were aligned with up to two mismatches to the cultivated barley genome as the reference after downloading the *H. vulgare* unigene transcript sequences from the NCBI database (http://www.ncbi.nlm.nih.gov/unigene). This database consisted of 26,941 transcripts including those annotated as complete and partial CDSs. RSEM v1.1.6, an RNA-Seq quantification tool, was used to estimate the relative abundances and expected read counts for the transcripts. By default, RSEM uses the Bowtie aligner (Bowtie v0.12.1) to map the reads against the transcripts. Transcript quantification of the reference-aligned reads was performed with RSEM, which allowed for the assessment of transcript abundances based on the mapping of RNA-Seq reads to the assembled transcriptome.

Expected read counts were used as input to differential expression analysis by EdgeR (version 3.0.0, R version 2.1.5). Because we had three biological replicates per time point, the median of these values was used as the common dispersion factor for differential expression (DE) analyses. The remaining unmapped sequences were re-aligned against the contigs collectively assembled *de novo* using the Trinity RNA-Seq Assembly package (r2013-02-25) from total unmapped sequences of all treatments and replicates. Trinity was selected for transcriptome assembly based on recent studies that showed that it performs better than other available methods [23]. DE transcripts were annotated using blast-2-GO software (version 2.3.5, http://www.blast2go.org/). Blastx was performed against the NCBI non-redundant protein database with an E-value cut off of 1 e^{-5}. GO terms were obtained for mapped and unmapped barley transcripts with the default parameters.

To identify clusters with functional enrichment, we determined a significant Pearson correlation through permutation analysis [24]. The resulting clusters were refined by visual inspection and analyzed for GO term enrichment using Blast2GO (http://www.blast2go.org/). We also clustered the RPKM data to provide a representation of absolute abundance of the transcripts.

2.4. Validation of RNA-seq findings by real time PCR

Six transcripts were randomly selected for validating the RNA-Seq data by real time PCR with the *actin* gene as the reference [25]. Primers were designed using Netprimer software (http://www.premierbiosoft.com/netprimer/index.html) with the following criteria: length ~20 bases, GC content ~50%, minimal secondary structures, comparable annealing temperatures (55 °C) of the primer pairs, and PCR products of ~500 bp. Total RNAs were extracted from individual plants salt-stressed for 2, 12 and 24 h and control plants. Extraction was done in three replicates and RNAs from each treatment were then bulked. Expression levels of transcripts were detected by real time PCR using the Agilent Mx3000P qPCR Systems (Agilent technology, USA). First-strand cDNA was synthesized using 1 μg of total RNA, 0.5 μg of reverse primers of each gene (Table S1) and Superscript II reverse transcriptase (Invitrogen). All cDNA-synthesized samples were diluted (1:10) prior to amplification. The reaction (25 μL) components were 12.5 μL Maxima™ SYBR Green/ROX qPCR master mix, 0.2 μM of each gene forward and reverse primers (Table S1), and PCR-grade water was added up to 22.5 μL. Finally, 2.5 μL of diluted cDNA template were added to the reaction mix. Forty PCR cycles for each gene product included denaturation at 94 °C for 15 s, annealing at 55 °C for 30 s, and extension at 72 °C for 45 s. Amplification for each sample was carried out in triplicate along with a no-template control (NTC, PCR-grade water). Data was collected and amplification plots of ΔRn versus cycle number were generated for analysis. Calculations were made to detect the expression level of each gene under a given treatment relative to its expression under control condition.

3. Results

3.1. Analysis of RNA-Seq datasets

Sequencing of cDNA samples yielded between 103 and 115 million reads corresponding to over 10 billion nucleotides per sample (Table 1). The raw sequencing reads were deposited in the Small Read Archive (SRA) at GenBank (accession number SRP032854). Between 74.8 and 80.3% of the wild barley reads could be mapped to the cultivated barley reference genome; the remaining 20–25% matched no sequences (Table 1). The percentage of transcripts of wild barley found in *H. vulgare* unigene database (unigene-mapped) ranged from 77.4 to 81.7%. All mapped sequences were in exonic regions of the genome. The unmapped wild barley reads of all treatments and replicates were assembled *de novo* and the resulting contigs were used as a new reference genome. Then, the unmapped wild barley reads were aligned to this new reference genome and results indicated that between 94.3 and 95.3% of them were *de novo*-mapped to the new reference. The total number of transcripts generated from alignment with both reference genomes ranged from 54,572 to 59,353. The percentage of transcripts generated from alignment with the wild barley genome contigs ranged from 60.9 to 63.9%.

3.2. Clusters of gene expression across time of salt treatment

RNA-Seq data was used to detect the differential expression (DE) of previously annotated barley transcripts, as well as novel transcripts uncovered in this study. To statistically obtain confirmation of the differences in gene expression across treatment time, RPKM-derived read counts were compared using a likelihood ratio test [21]. Statistical analysis was reliable when applied to genes with an RPKM value ≥ 2. To determine DE transcripts, a two-fold (or greater) change in expression and false discovery rate (FDR) of 10^{-3} or less was required. The resulting number of DE transcripts was 9277 (Fig. 1a), 3861 of which represent DE transcripts from the alignment with *H. vulgare* reference genome in the unigene database (Fig. 1b). The remaining DE transcripts (5416) resulted from the alignment with the generated wild barley reference genome (Fig. 1c). The number of unigene-mapped DE transcripts with no blast hits was less than 100, while that of *de novo*-mapped transcripts was over 2300 (Fig. 1b and c, respectively).

Annotated unigene- and *de novo*-mapped transcripts (5100) were utilized in generating the expression clusters across time of salt stress treatment. Expression profiles of the DE transcripts were determined by a cluster analysis based on the *k*-means method using Pearson's correlation distance so that the similarity in relative change for each transcript or among transcripts across time of salt treatment was determined. These data were then subjected to hierarchical clustering using the Pearson correlation as the distance metric. Fig. 2 shows the expression clusters for the DE transcripts mapped on the *H. vulgare* reference genome (Fig. 2a) and those mapped on the *de novo*-assembled wild barley reference genome using the unigene unmapped reads (Fig. 2b). Two-dimensional hierarchical clustering classified DE profiles into nine expression clusters according to the similarity of their expression profiles. Visual inspection of these expression groups suggested diverse and complex patterns of regulation. Four out of the nine expression clusters were selected for further comparisons across the time of salt tolerance treatment (Fig. 3). In general, heterogeneity and redundancy were two significant characteristics for selection. Another criterion for selection was the importance of the expression pattern. We did not have a cluster for genes that were upregulated at 2, 12 and 24 h. All upregulated genes at the 2 h time point returned to the 0 h expression level either at 12 or 24 h time points (Fig. 3a–b). We selected both expression patterns as two clusters of upregulation. The third cluster includes transcripts that were downregulated at 2, 12 and 24 h time points (Fig. 3d), and the fourth represents downregulated transcripts at 2 and 12 h time points then

Table 1
Statistics of RNA-Seq numerical data analysis of *Horduem spotanium*.

File[a] name	Treatment time (h)	Total number of reads[b]	Number of mapped reads (unigene)[c]	%[d] of reads	Number of unmapped reads (unigene)[e]	Number of reads mapped (*de novo*)[f]	%[g] of reads	Number of unmapped[h] reads	Number of transcripts (unigene)[i]	% of transcripts[j]	Number of transcripts (*de novo*)[k]	Number of transcripts (Total)[l]
Bs2c_1.fastq/Bs2c_2.fastq	0	106,495,036	85,560,630	80.3	20,934,406	19,814,424	94.7	1,119,982	21,160	78.5	33,962	55,122
Bs3c_1.fastq/Bs3c_2.fastq	0	115,111,594	91,065,533	79.1	24,046,061	22,922,643	95.3	1,123,418	21,698	80.5	33,830	55,528
Bs1_2_1.fastq/Bs1_2_2.fastq	2	104,137,914	79,466,902	76.3	24,671,012	23,417,657	94.9	1,253,355	21,518	80.0	37,498	59,016
Bs2_2_1.fastq/Bs2_2_2.fastq	2	111,668,026	83,885,570	75.1	27,782,456	26,366,413	94.9	1,416,043	21,439	80.0	37,914	59,353
Bs3_2_1.fastq/Bs3_2_2.fastq	2	106,843,457	81,125,022	75.9	25,718,435	24,274,948	94.4	1,443,487	21,687	80.5	37,319	59,006
Bs1_12_1.fastq/Bs1_12_2.fastq	12	110,659,611	83,224,420	75.2	27,435,191	26,120,724	95.2	1,314,467	20,702	76.8	35,890	56,592
Bs2_12_1.fastq/Bs2_12_2.fastq	12	106,831,568	80,825,061	75.7	26,006,507	24,649,525	94.8	1,356,982	21,418	80.0	36,467	57,885
Bs3_12_1.fastq/Bs3_12_2.fastq	12	103,860,256	77,694,090	74.8	26,166,166	24,841,786	94.9	1,324,380	21,747	80.7	36,572	58,319
Bs1_24_1.fastq/Bs1_24_2.fastq	24	112,611,535	87,575,012	77.8	25,036,523	23,769,264	94.9	1,267,259	20,841	77.4	33,731	54,572
Bs2_24_1.fastq/Bs2_24_2.fastq	24	113,972,947	89,370,676	78.4	24,602,271	23,437,546	95.3	1,164,725	21,346	79.2	34,425	55,771
Bs3_24_1.fastq/Bs3_24_2.fastq	24	112,207,066	89,834,579	80.1	22,372,487	21,098,577	94.3	1,273,910	22,017	81.7	34,277	56,294

[a] Names of RNA-Seq files.
[b] Total number of reads recovered from wild barley RNA-Seq.
[c] Number of wild barley reads aligned with *Hordeum vulgare* reference genome in the unigene database of NCBI (http://www.ncbi.nlm.nih.gov/unigene).
[d] Percentage of wild barley reads aligned with *H. vulgare* reference genome.
[e] Number of reads unaligned with *H. vulgare* reference genome.
[f] Number of reads aligned with the new (*de novo*-assembled) wild barley reference genome generated from contigs generated by *de novo*-assembly from total unaligned wild barley reads of all treatments and replicates with the reference *H. vulgare* genome.
[g] Percentage of reads aligned with the new (*de novo*-assembled) wild barley reference genome.
[h] Number of reads unaligned with the new wild barley reference genome.
[i] Number of wild barely transcripts found in *H. vulgare* unigene database.
[j] Percentage of wild barely transcripts found in *H. vulgare* unigene database.
[k] Number of wild barley transcripts generated from alignment with the new reference genome.
[l] Total number of wild barley transcripts generated from alignment with the two reference genomes.

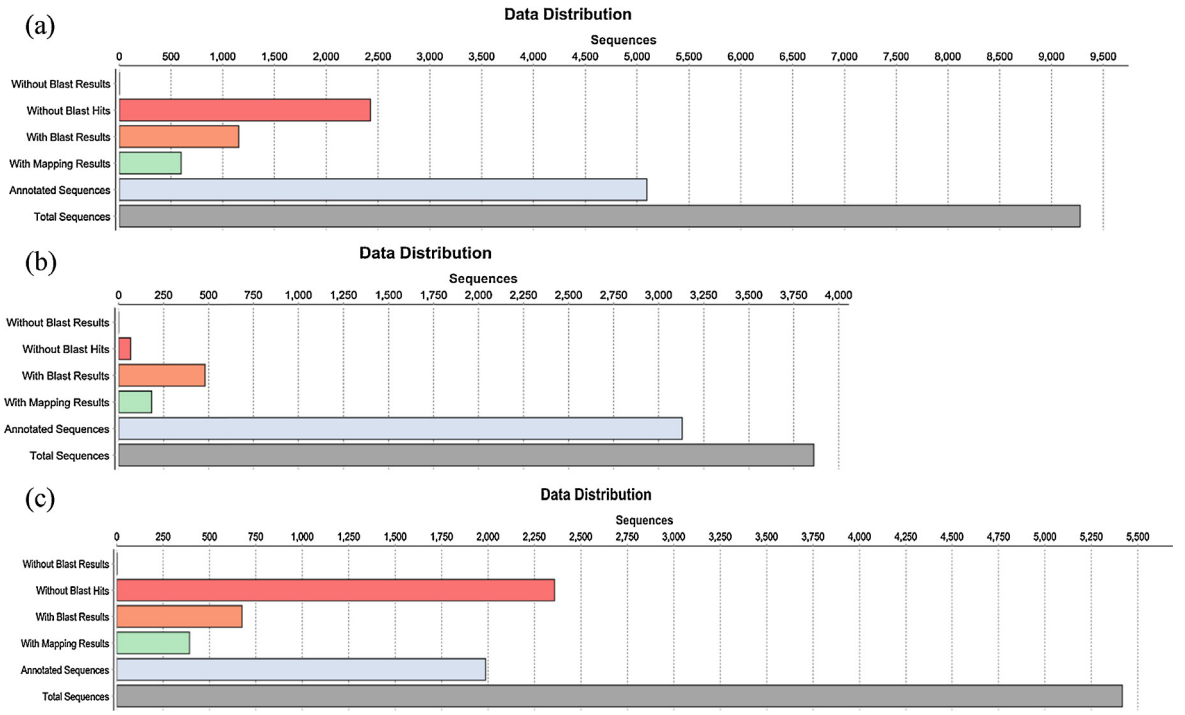

Fig. 1. (Color online.) Total number of differentially expressed (DE) transcripts under salt stress: a: total number of DE transcripts (9277 transcripts) subjected to blast2go resulting from reads either aligned or unaligned with *Hordeum vulgare* reference genome in the unigene database of NCBI (http://www.ncbi.nlm.nih.gov/unigene); b: number of DE transcripts (3861 transcripts) aligned with *H. vulgare* reference genome; c: number of DE transcripts (5416 transcripts) unaligned with *H. vulgare* reference genome.

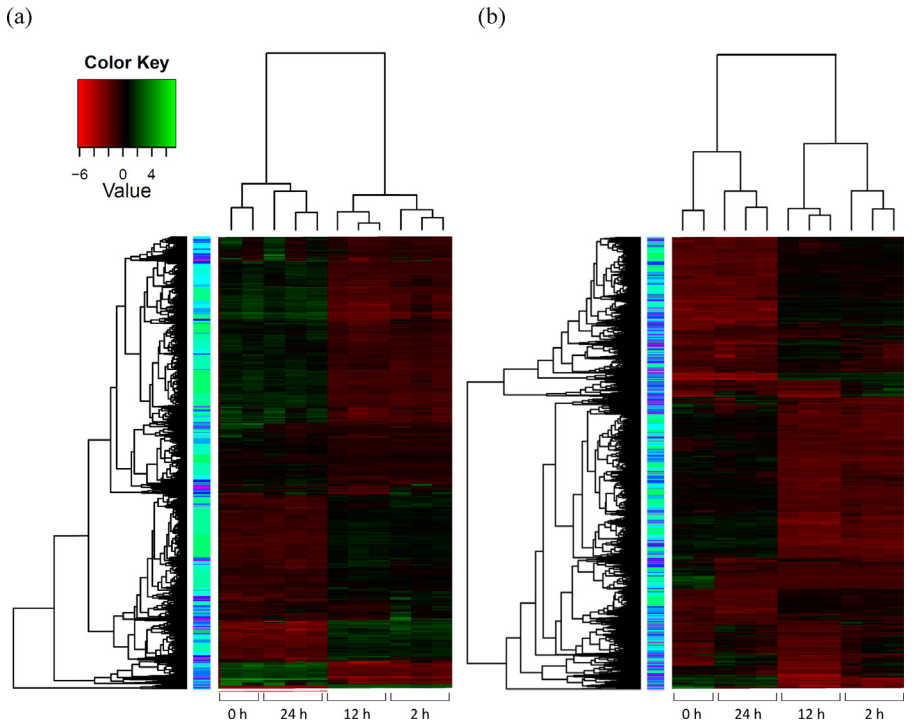

Fig. 2. (Color online.) Hierarchical cluster analysis of gene expression based on log ratio RPKM data for transcripts. Reads were either aligned (a) or unaligned (b) with *H. vulgare* reference genome in the unigene database of NCBI (http://www.ncbi.nlm.nih.gov/unigene).

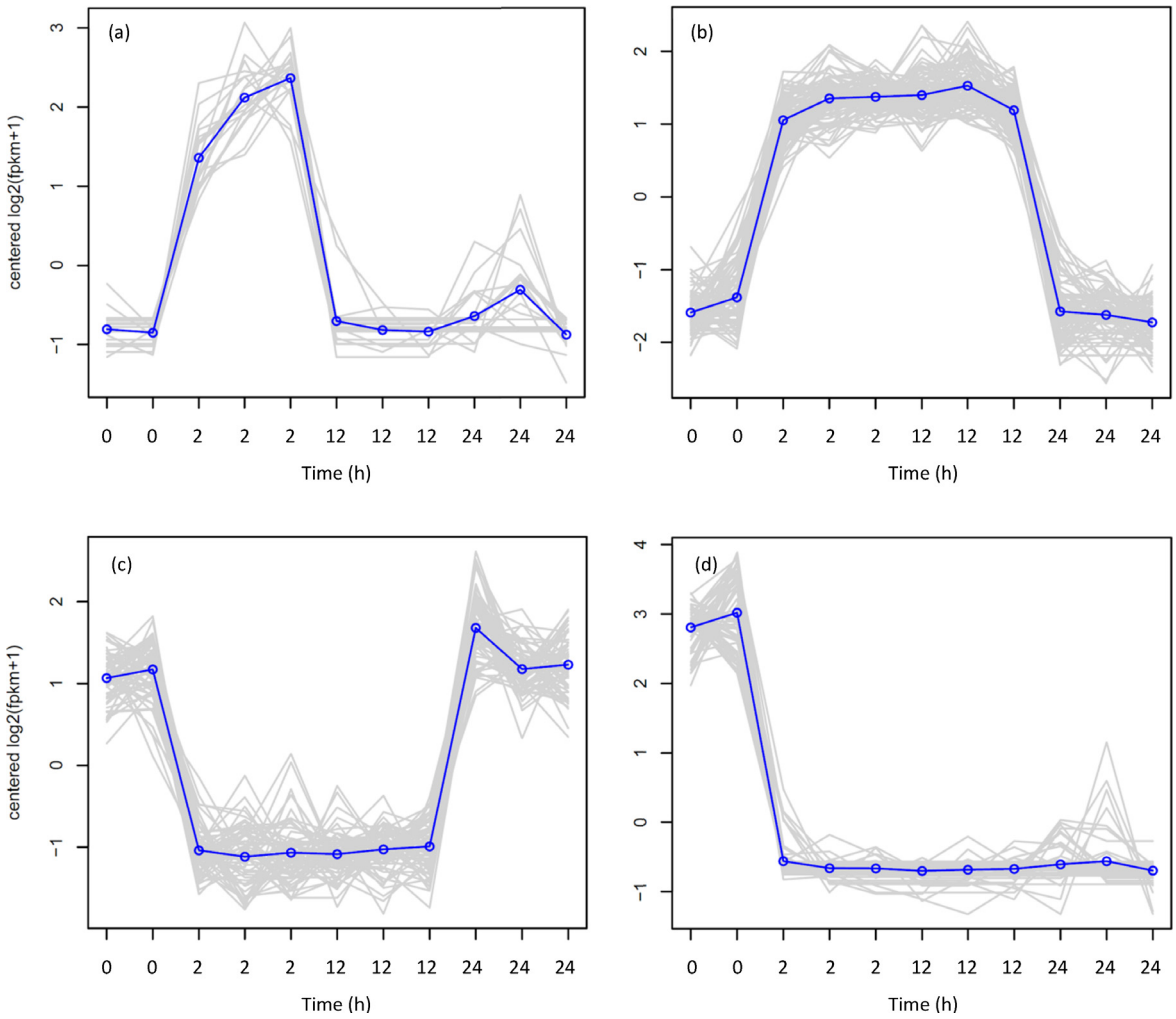

Fig. 3. (Color online.) Four selected clusters of gene expression under salt stress (C1 (a), C2 (b), C3 (c) and C4 (d)). See Table 3 for transcripts generated from reads either aligned or unaligned with *H. vulgare* reference genome in the unigene database of NCBI (http://www.ncbi.nlm.nih.gov/unigene).

returned to the 0 h expression level at 24 h time point (Fig. 3c).

3.3. Validation of transcript profiles by real time PCR

Real time PCR of six randomly selected DE transcripts from the mRNA-Seq data resulted in successful amplification of the bands of expected sizes (Fig. S1). Quantification of the band intensities in relation to the *actin* control supported the direction of change of expression as detected by mRNA-Seq for these transcripts in which expression pattern of four of them fit within cluster 2, while those of the other two fit within cluster 3.

3.4. Analysis of differentially expressed genes

Differentially expressed genes were assigned to functional categories using blast2GO (http://www.blast2go.org/), which provided a valuable resource for detecting specific processes, functions, and pathways during salt stress in wild barley. The results indicated that 3184, 4189 and 4106 transcripts were assigned to the three main categories; "biological process", "cellular component" and "molecular function", respectively (Tables 2–4). Most transcripts were assigned to the cluster 3 pattern of gene expression for downregulated transcripts across time of treatment followed by cluster 2 for upregulated transcripts at 2 and 12 h time points (Fig. 3). The number of transcripts recovered by gene ontology analysis for the three main categories was higher than the total number of DE transcripts, which likely indicates that some transcripts were assigned to more than one category. The same criterion was observed for the number of subgroups in a functional group. The numbers of functional groups for the three main categories were 21, 8 and 12 transcripts, respectively (Tables 2–4). The numbers of subgroups within groups of the three categories were 71, 12 and 49 transcripts, respectively. In the three categories, numbers of groups for cluster(s) with more than 1000 transcripts were 2, 3 and 2, respectively. These groups were "cellular process" and "metabolic process" for the "biological process" category, "membrane", "organelle" and "cell" for the "cellular component" category, and "binding" and

Table 2
GO functional categorization of DE barley transcripts based on biological process. C1, C2, C3 and C4 represent four clusters of gene expression patterns under salt stress.

Level	GO ID	GO Term	*C1	C2	C3	C4
1	GO:0008150	Biological Process	80	505	2422	177
2	GO:0008283	Cell proliferation		4	5	
2	GO:0002376	Immune system process		8	66	
3	GO:0002252	Immune effector process	2	2	8	
2	GO:0015976	Carbon utilization	2		6	
2	GO:0040007	Growth	1	15	46	2
3	GO:0016049	Cell growth	1	15	37	1
3	GO:0048589	Developmental growth	1	10	33	
2	GO:0043473	Pigmentation	1		7	
3	GO:0043476	Pigment accumulation	1		7	
2	GO:0040011	Locomotion			5	
3	GO:0042330	Taxis			4	
2	GO:0022610	Biological adhesion			4	
3	GO:0007155	Cell adhesion			4	
2	GO:0065007	Biological regulation	16	102	438	28
3	GO:0065008	Regulation of biological quality	6	23	101	5
3	GO:0065009	Regulation of molecular function		15	53	
3	GO:0050789	Regulation of biological process	13	89	394	23
2	GO:0051179	Localization	10	69	429	30
3	GO:0033036	Macromolecule localization	3	12	112	
3	GO:0051234	Establishment of localization	10	66	417	30
2	GO:0000003	Reproduction	6	28	170	8
3	GO:0019953	Sexual reproduction		2	17	
3	GO:0022414	Reproductive process	6	28	164	4
2	GO:0023052	Signaling	1	35	95	22
3	GO:0007267	Cell-cell signaling		1		
2	GO:0071840	Cellular component organization or biogenesis	9	54	430	13
3	GO:0016043	Cellular component organization	9	51	385	13
3	GO:0044085	Cellular component biogenesis		12	242	
3	GO:0071554	Cell wall organization or biogenesis		8	55	
3	GO:0071841	Cellular component organization or biogenesis at cellular level	6	42	392	
2	GO:0009987	Cellular process	60	380	1899	126
3	GO:0006928	Cellular component movement		5	10	
3	GO:0016044	Cellular membrane organization		3	89	
3	GO:0019725	Cellular homeostasis	3	3	48	3
3	GO:0007017	Microtubule-based process		5	17	
3	GO:0007049	Cell cycle	5	19	31	1
3	GO:0007059	Chromosome segregation	2	1	6	
3	GO:0007154	Cell communication	2	43	131	23
3	GO:0010118	Stomatal movement	1	2	13	
3	GO:0019725	Cellular homeostasis		11		5
3	GO:0030029	Actin filament-based process		3	24	
3	GO:0051641	Cellular localization	2	17	137	
3	GO:0048869	Cellular developmental process	3	20	102	3
3	GO:0051301	Cell division	1	11	32	
2	GO:0016265	Death		3	39	3
3	GO:0008219	Cell death			39	3
2	GO:0032502	Developmental process	7	69	339	14
3	GO:0007568	Aging	1	8	5	
3	GO:0021700	Developmental maturation	1	8	19	
3	GO:0022611	Dormancy process	1		7	
3	GO:0048856	Anatomical structure development	4	55	269	6
2	GO:0008152	Metabolic process	64	408	2022	135
3	GO:0006807	Nitrogen compound metabolic process	25	120	701	19
3	GO:0044238	Primary metabolic process		307	1453	91
3	GO:0009056	Catabolic process	6	81	307	29
3	GO:0009058	Biosynthetic process	13	108	988	38
3	GO:0019637	Organophosphate metabolic process	1	13	140	
3	GO:0019748	Secondary metabolic process	1	5	83	4
3	GO:0032259	Methylation		5	112	
3	GO:0042440	Pigment metabolic process	3	3	128	
3	GO:0043170	Macromolecule metabolic process	38	182	1004	55
3	GO:0044281	Small molecule metabolic process	8	90	560	
3	GO:0055114	Oxidation-reduction process	6	69	476	
3	GO:0070988	Demethylation		1		
3	GO:0071704	Organic substance metabolic process	4	41	254	
3	GO:0042445	Hormone metabolic process	1	2	20	

Table 2 (*Continued*)

Level	GO ID	GO Term	*C1	C2	C3	C4
3	GO:0044237	Cellular metabolic process	50	299	1617	77
2	GO:0032501	Multicellular organismal process	7	62	317	14
3	GO:0007585	Respiratory gaseous exchange	1	1		
3	GO:0008037	Cell recognition		1	3	
3	GO:0007275	Multicellular organismal development	6	58	310	14
3	GO:0043480	Pigment accumulation in tissues	1		7	
3	GO:0032504	Multicellular organism reproduction	1	1	20	
3	GO:0009606	Tropism		3	3	
2	GO:0051704	Multi-organism process	8	25	185	2
3	GO:0044419	Interspecies interaction between organisms		3	4	
3	GO:0035821	Modification of morphology or physiology of other organism		1	3	
3	GO:0051707	Response to other organism	7	22	171	
2	GO:0048511	Rhythmic process		3	13	
3	GO:0007623	Circadian rhythm		3	13	
2	GO:0016032	Viral reproduction		2	3	
3	GO:0022415	Viral reproductive process		2	3	
2	GO:0050896	Response to stimulus	26	159	52	55
3	GO:0051716	Cellular response to stimulus	6	55	99	22
3	GO:0009628	Response to abiotic stimulus	9	998	388	14
3	GO:0006955	Immune response	2	7	61	
3	GO:0006950	Response to stress	19	92	435	26
3	GO:0009605	Response to external stimulus	2	11	54	2
3	GO:0009607	Response to biotic stimulus	7	23	172	12
3	GO:0009719	Response to endogenous stimulus	6	32	111	11
3	GO:0042221	Response to chemical stimulus	11	77	387	
3	GO:0051606	Detection of stimulus		4	12	

*Time	0	2 h	12 h	24 h
C1 (cluster 1):	Control level	Up	Down	Down
C2 (cluster 2):	Control level	Up	Up	Down
C3 (cluster 3):	Control level	Down	Down	Up
C4 (cluster 4):	Control level	Down	Down	Down

"catalytic activity" for the "molecular function" category. The numbers of subgroups for cluster(s) with more than 1000 transcripts were 3, 3 and 0, respectively. These subgroups were "primary metabolic process", "macromolecule metabolic process" and "cellular metabolic process" for the "biological process category", and "membrane-bounded organelle", "organelle part" and "cell part" for the "cellular component" category (Tables 2–4). There were some groups and subgroups of the three main categories in which DE transcripts were either upregulated (clusters 1 and/or 2) or downregulated (clusters 3 and/or 4). The most important components were "cell–cell signaling" (cluster 2) and "cell death" (clusters 3 and 4) subgroups of the "biological process" category and the "electron carrier activity", "superoxide dismutase activity", "electron transporter transferring electrons within the cyclic and noncyclic electron transport pathways of photosynthesis activity", "electron transporter transferring electrons within cytochrome b6/f complex of photosystem II activity" and "structural constituent of cytoskeleton" (clusters 1 and 2) subgroups of the "molecular function" category. There were six subgroups in the "response to stimulus" group that demonstrated both upregulation and downregulation of many different transcripts under salt stress. These subgroups were "cellular response to stimulus", "response to abiotic stimulus", "response to stress", "response to external stimulus", "response to endogenous stimulus", and "response to chemical stimulus".

Annotated unigene-mapped and *de novo*-mapped transcripts under subgroups of "electron carrier activity" and "response to abiotic stimulus" that were upregulated after 12 h of salt stress exposure with fold change (FC) of $\geq$4 (Table S2) fit within the expression pattern cluster 2. These subgroups were selected because they included the most important highly upregulated transcripts related to salt stress. There were thousands of GO hits whose expression was altered $\geq$4 fold under salt stress (Table S2). The number of GO hits under the selected "electron carrier activity" subgroup was 44, 13 of which were unigene-mapped and 31 were *de novo*-mapped. These GO hits represented nine genes/gene families, one unigene-mapped and eight *de novo*-mapped. The number of GO hits under "response to abiotic stimulus" subgroup was 866, 432 unigene-mapped, 99 *de novo*-mapped and 335 unigene/*de novo*-mapped. These GO hits represented 88 genes/gene families, 55 unigene-mapped, 15 *de novo*-mapped and 18 unigene/*de novo*-mapped.

4. Discussion

Organisms that survive in saline water possess mechanisms to maintain their osmotic balance [26]. It is evident that the number and percentage of transcripts generated from the RNA-Seq data of different replicates grown under salt stress at 2 or 12 h time points are slightly higher than at 0 or 24 h time points (Table 1). This increase may be due

Table 3
GO functional categorization of DE barley transcripts based on cellular component. C1, C2, C3 and C4 represent four clusters of gene expression patterns under salt stress.

Level	GO ID	GO term	*C1	C2	C3	C4
1	GO:0005575	Cellular component	79	1302	2636	172
2	GO:0016020	Membrane	30	449	1110	62
3	GO:0044425	Membrane part	6	57	416	
2	GO:0043226	Organelle	66	837	2348	106
3	GO:0043227	Membrane-bounded organelle	63	822	2304	104
3	GO:0044422	Organelle part	12	76	1082	9
3	GO:0043228	Non-membrane-bounded organelle	4	43	399	8
3	GO:0031982	Vesicle	25	91	335	
3	GO:0019867	Outer membrane		2	9	
2	GO:0005623	Cell	72	1189	2523	159
3	GO:0044464	Cell part	72	1184	2523	157
2	GO:0032991	Macromolecular complex	2	49	532	3
3	GO:0043234	Protein complex	2	28	226	
3	GO:0032993	Protein-DNA complex			15	
2	GO:0005576	Extracellular region	5	35	159	9
3	GO:0048046	Apoplast	4	7	143	
3	GO:0044421	Extracellular region part		2		
2	GO:0055044	Symplast	5	9	48	
2	GO:0031974	Membrane-enclosed lumen		30	179	9
2	GO:0030054	Cell junction	5	9	48	
3	GO:0005911	Cell-cell junction	5	9	48	

* See Table 2.

to the high level of salt-related gene expression at time points 2 and 12 h, followed by recovery of gene expression after 24 h exposure to salt stress to similar levels as the control plants. The number of DE transcripts (5416) from the alignment with the *de novo*-assembled wild barley reference genome indicates that many of these transcripts likely represent more than one contig of single genes (Fig. 1c). This conclusion is supported by the fact that the number of annotated DE transcripts mapped on the *H. vulgare* unigene reference genome is over 3000 out of 3861 (Fig. 1b), whereas transcripts mapped to the *de novo*-assembled wild barley reference genome number less than 2000 out of 5416 (Fig. 1c).

4.1. Salt stress tolerance via electron transport and exchanger mechanisms

Many cytochrome p450 proteins detected in salt-stressed wild barley are likely involved in electron transfer chains as a mechanism of salt tolerance regulation. p450 usually acts as a terminal oxidase in electron-transfer chains under salt stress with a number of fundamental redox domains, e.g., FAD-containing flavoproteins, ferredoxins [27]. Besides cytochrome p450, we detected a number of salt-regulated transcripts encoding electron transfer flavoproteins and ferredoxins. Flavoproteins function in detoxifying salts in the plant cell [28,29], while ferridoxins were recently reported for their role in abiotic stress signaling [29]. Other detected transcripts encoding vacuolar cation as well as cation proton exchanger in wild barley may act as key factors in the sequestration of sodium (Na^+) into vacuoles to avert ion toxicity in the cytosol of plants under salinity stress. Upon influx of N+ into the cell, different ATPases (PM-ATPases, V-ATPases, and V/H-ATPases) are activated [30]. This results in Na+ efflux into the outer rhizosphere by PM Na^+/H^+ antiporters and/or influx into vacuoles by tonoplast Na^+/H^+ leading to cellular ion homeostasis, consequently salt tolerance [31]. The cytoplasmic domain of the vacuolar H+-ATPase (V-ATPase), whose transcripts were also salt-regulated in wild barley, were reported to present in a SOS (salt overly sensitive)-containing protein complex [32] with a key role in regulating ion transport under salt stress. Regulation of V-ATPase activity represents an additional key function of SOS_2 in the coordination of ion transport changes during salt stress, thus promoting salt tolerance [32]. Upregulation of transcripts encoding plasma membrane (PM)-ATPase in wild barley complements those of other ATPases, as it supported the occurrence of an electrochemical gradient generated under salt stress in the intertidal C_4 grass *Spartina patens* [33]. Although wild barley plants in the present study were stressed by NaCl, an additional transcript encoding calcium-transporting ATPase was upregulated. This enzyme transport protein in the plasma membrane serves to remove calcium (Ca^{2+}) from the cell [34].

4.2. Salt stress tolerance via flavonoid biosynthesis

Flavonoid biosynthesis seems to be one of the biological processes regulating salt tolerance in wild barley. One upregulated transcript, major latex protein gene (*MLP*) (Table S2), was previously studied in cotton (*Gossypium hirsutum*) and expression in roots was induced by salt stress [35]. The *Gh-MLP* promoter contains potential *cis*-acting elements for response to salt stress and fungal elicitors. Results of RT-PCR showed that expression of *Gh-MLP* in *Arabidopsis* is rapidly induced by NaCl, and induction was maintained over 72 h [35]. In our case, expression of this gene dropped back to the control level after 24 h of salt stress exposure. *Gh-MLP*-transgenic *A. thaliana* plants showed enhanced salt stress tolerance

Table 4
GO functional categorization of DE barley transcripts based on molecular function. C1, C2, C3 and C4 represent four clusters of gene expression patterns under salt stress.

Level	GO ID	GO term	*C1	C2	C3	C4
1	GO:0003674	Molecular function	97	1459	2368	182
2	GO:0060089	Molecular transducer activity	1	27	27	
2	GO:0000988	Protein binding transcription factor activity		2		
3	GO:0000989	Transcription factor binding transcription factor activity		2		
2	GO:0045735	Nutrient reservoir activity		1	4	
2	GO:0016209	Antioxidant activity		2	33	
3	GO:0004784	Superoxide dismutase activity	3	2		
3	GO:0004601	Peroxidase activity		2	22	
2	GO:0005488	Binding	66	982	1414	134
3	GO:0001871	Pattern binding	4	6	9	
3	GO:0003676	Nucleic acid binding	19	265	375	23
3	GO:0003682	Chromatin binding		5		
3	GO:0005515	Protein binding	6	141	160	16
3	GO:0008144	Drug binding			9	
3	GO:0008289	Lipid binding	1	23	13	4
3	GO:0008430	Selenium binding		5		
3	GO:0019825	Oxygen binding		1	4	
3	GO:0030246	Carbohydrate binding	4	19	24	7
3	GO:0031406	Carboxylic acid binding	1	3	14	
3	GO:0036094	Small molecule binding	32	377	557	67
3	GO:0042277	Peptide binding	1	1	4	
3	GO:0042562	Hormone binding		1		
3	GO:0043021	Ribonucleoprotein complex binding		1		
3	GO:0043167	Ion binding	21	143	541	
3	GO:0046906	Tetrapyrrole binding	1	8	112	
3	GO:0048037	Cofactor binding	4	38	136	
3	GO:0051540	Metal cluster binding		6	48	
2	GO:0003824	Catalytic activity	65	949	1519	125
3	GO:0004133	Glycogen debranching enzyme activity			3	
3	GO:0008641	Small protein activating enzyme activity		1		
3	GO:0008907	Integrase activity		1		
3	GO:0016491	Oxidoreductase activity	10	79	418	
3	GO:0016740	Transferase activity	31	404	511	57
3	GO:0016787	Hydrolase activity	19	271	450	28
3	GO:0016829	Lyase activity	5	18	99	
3	GO:0016853	Isomerase activity	2	12	83	
3	GO:0016874	Ligase activity	2	24	62	
3	GO:0070283	Radical SAM enzyme activity			3	
2	GO:0009055	Electron carrier activity	7	179		
3	GO:0045156	Electron transporter, transferring electrons within the cyclic electron transport pathway of photosynthesis activity	6	2		
3	GO:0045157	Electron transporter, transferring electrons within the noncyclic electron transport pathway of photosynthesis activity	3	1		
3	GO:0045158	Electron transporter, transferring electrons within cytochrome b6/f complex of photosystem II activity	3			
2	GO:0030234	Enzyme regulator activity	1	30	17	
3	GO:0004857	Enzyme inhibitor activity		8	6	
3	GO:0008047	Enzyme activator activity		5	4	
3	GO:0019207	Kinase regulator activity		1		
3	GO:0060589	Nucleoside-triphosphatase regulator activity	1	5	3	
3	GO:0061134	Peptidase regulator activity		7	3	
3	GO:0004871	Signal transducer activity	1	27	27	
2	GO:0001071	Nucleic acid binding transcription factor activity	4	52	37	
3	GO:0003700	Sequence-specific DNA binding transcription factor activity	4	52	37	4
2	GO:0004872	Receptor activity	4	32	32	12
3	GO:0038023	Signaling receptor activity	1	1	8	
2	GO:0005198	Structural molecule activity		5	248	2
3	GO:0003735	Structural constituent of ribosome		2	223	

Table 4 (*Continued*)

Level	GO ID	GO term	*C1	C2	C3	C4
3	GO:0005199	Structural constituent of cell wall		1		
3	GO:0005200	Structural constituent of cytoskeleton			5	
2	GO:0005215	Transporter activity	3	93	229	
3	GO:0022857	Transmembrane transporter activity	3	31	173	
3	GO:0022892	Substrate-specific transporter activity	3	23	153	
3	GO:0042910	Xenobiotic transporter activity		3	3	
3	GO:0051184	Cofactor transporter activity			6	

* See Table 2.

and transgenic plants allowed seeds to germinate normally after treatment with 75 mM NaCl. Total flavonoid was also enhanced in transgenic *Arabidopsis* as compared to the control, suggesting that *Gh-MLP* might also be involved in altering flavonoid content under salt stress [35]. Two wild barley transcripts coding for chalcone synthase (CHS) and two others coding for chalcone isomerase (CHI) were reported to interact under salt stress [36]. CHS, which belongs to a family of polyketide synthase enzymes (PKS), is ubiquitous in higher plants and serves as the initial step for flavonoid biosynthesis [36]. CHI was recently reported to enhance salt tolerance in salt-sensitive yeasts [37]. In the salt-tolerant *Millettia pinnata*, the level of transcripts involved in flavonoid biosynthesis showed the most remarkable change under salt stress [37]. A transcript for isoflavone reductase-like enzyme was also upregulated in the present study. The enzyme is a key in the isoflavonoid phytoalexin biosynthesis pathway [38] and over expression in transgenic rice (*Oryza sativa*) reportedly conferred resistance to reactive oxygen species (ROS) stress [39]. Two additional wild barley transcripts encoding transcription factors of the basic helix-loop-helix (bHLH) superfamily are involved in a wide range of growth and developmental signaling pathways, including abscisic acid signaling [40], flavonoid biosynthesis [41] and abiotic stress [42].

4.3. Salt stress tolerance via reactive oxygen species scavenging

It is well-known that cytosolic superoxide dismutase enzymes, either cytSODs (Cu,ZnSOD) or mitSODs (MnSOD), act as antioxidants and protect cellular components during salt stress from being oxidized by reactive oxygen species (ROS) [43–45]. Our results suggest that this mechanism may also be playing a role in salt stress tolerance of wild barley as three transcripts for superoxide dismutase (SOD) activity were highly expressed, while two were less expressed ($FC \leq 4$) under salt stress. Other wild barley transcripts encoding electron transporter iron ion binding proteins function in iron-sulfur cluster binding. In several studies, iron ion binding proteins function in detoxification of ROS under salt stress [46–48].

A highly expressed dehydrin (DHN) transcript (FC = 8.44) was also detected. DHNs, or group 2 late embryogenesis abundant (LEA) proteins, have several roles in protecting the plant cell from dehydration stress. One of them exhibits ROS scavenging [49] mediated by the interactions between their amino acid residue and ROS species (e.g., superoxide anion radical O_2^-; singlet oxygen $^1O_2$; hydroxyl radical HO^-; Hydrogen peroxide H_2O_2). DHNs also function as antioxidants [50], ion sequestrants [51], and metal ion transporters in plant phloem sap [52]. Under reduced hydration, the K-segments of DHNs adopt α-helical conformation [53]. The amphipathic α-helices can interact with partly dehydrated surfaces of various other proteins protecting them from further loss of the water envelope. DHNs also act as "space-fillers" in which they participate in keeping the original, non-harmful distances among different intracellular complexes that helps maintain the original cell volume, thus preventing cellular collapse [49].

4.4. Salt stress tolerance via ethylene production

Ethylene production is suggested to be a mediator of the stress responses in wild barley. A detected transcript, encoding the ethylene-forming enzyme (e.g., 1-aminocyclopropane-1-carboxylate [ACC] oxidase or ACO), was shown earlier to be indirectly induced under salt stress [54] as it relies on the expression of another set of genes, ethylene receptor genes (such as NTHK1 in tobacco, see Cao et al. [55]). Recent studies have demonstrated that salt tolerance was induced by exogenous 1-aminocyclopropane-1-carboxylate in *Arabidopsis* [56,57]. More recently, Li et al. [58] indicated that ethylene production and activities of ACO in cucumber (*Cucumis sativus*) seedlings were increased significantly under salt stress (75 mM). Another detected transcript encoding ethylene-responsive element binding factor (ERF) was considered crucial in earlier studies of cotton under stress [59]. Over expression of sugarcane and soybean ERFs in tobacco conferred tolerance to dehydration [60] and high salinity [61] (200 mM NaCl) stresses.

4.5. Salt stress tolerance via a signaling network

Transcripts encoding two serine threonine-protein kinases (STKs), SpkG and SkpC, were salt-regulated in wild barley. These STKs were previously reported to regulate various cellular functions including stress responses [62,63]. Nonetheless, Liang et al. [64] indicated that growth characteristics of a *spkC* mutant of the unicellular cyanobacterium *Synechocystis* were not affected under high salt stress conditions, while, growth of a *spkG* mutant was completely impaired. The *spkG* gene plays an essential role in sensing high salt signal directly, rather than mediating signals among other kinases [64,65]. Two transcripts encoding mitogen-activated protein kinases (MAPK) and MAP2K seem to complement the effect of SpkG in wild barley as the MAKP superfamily

is part of the serine/threonine kinases. This superfamily is a key player in some of the most essential roles in plant signaling networks, and is tolerant to a variety of stresses including drought and salinity [66].

4.6. Salt stress tolerance via a protein refolding mechanism

Salt stress, like other stresses, results in aggregation of cytoplasmic proteins. The present study is the first to indicate the possible role of a transcript encoding ATP-dependent Clp protease adaptor protein (ClpS) in plants with FC of 9.4. This *de novo*-mapped transcript was previously recovered in rice (NCBI database, http://www.uniprot.org/uniprot/Q0JNQ7) but information on its response to salt stress was not reported. Expression of ClpS in a metagenomic clone recovered [26] was shown to be involved in increased recognition of aggregated protein for refolding or degradation by the ClpA protease (ClpAP) complex resulting in salt stress tolerance. The Clp family has been shown to act as a molecular chaperone in bacteria [12]. These proteins can reverse protein aggregation resulting from salt stress and also play a role in ATP-dependent degradation of polypeptide chains under salt stress [67].

We can conclude that mRNA-Seq is an efficient high-throughput method for analyzing the wide diversity of genes expressed under salt stress. This technology is a very valuable tool to enhance our understanding of the genetics underlying salt stress tolerance mechanisms in plants. In the present study, a valuable dataset of thousands of DE transcripts was detected, some of which are involved in novel biological processes regulating salt tolerance. Some of the most likely candidates involved in salt stress in wild barley are genes involved in electron transport and exchanger mechanisms, flavonoid biosynthesis, superoxide dismutase (SOD) activity, ethylene production, signaling network, and protein refolding. The results of these comparisons can be utilized to improve salt stress tolerance in cultivated barley as well as other important cereal crops.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.crvi.2015.03.010.

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