Environmental and Experimental Biology (2010) 8: 1–16 *Original Paper*

Transcriptome analysis of the barley *nec3* mutant reveals a potential link with abiotic stress response related signaling pathways

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Abstract

The transcriptome of two fast neutron induced allelic barley mutants, FN362 and FN363, was analyzed with the Affymetrix Barley1 GeneChip microarray in order to characterize the *necrotic leaf spot 3* (*nec3*) gene and its function. Twenty one genes, at least two-fold down-regulated in the mutants compared to the wild-type, were detected, but PCR analyses failed to identify a candidate *Nec3* gene. It is possible that the probe set for the *Nec3* gene is not on the Barley1 GeneChip, or that it is expressed at very low levels or the expression is confined to specific developmental stage or tissue type. Comparison of the genes differentially expressed in FN362 and FN363 mutants with publicly available Affymetrix Barley1 GeneChip expression data sets revealed significant overlap with barley abiotic stress transcriptome. The highest similarity was observed with the transcriptome of barley under drought and freezing stress. These results imply a possible involvement of the wild-type *Nec3* in signaling pathways regulating abiotic stress response in barley.

Key words: Affymetrix GeneChip, barley, lesion mimic mutant, necrotic mutant, *nec3* mutant, transcriptome analysis.
Abbreviations: GO, gene onthology LMM, lesion mimic mutants; nec 3, necrotic leaf spot 3; TBC, transcript based cloning.

Introduction

In order to characterize molecular mechanisms underlying physiological processes, it is essential to establish a link between a phenotype and a corresponding gene. This can be done following either the forward or reverse genetics approach. Reverse genetics requires tools allowing for disruption of a sequence of interest in order to establish a function of a studied gene. Rapid development of genomics, high-throughput sequencing technologies and availability of protocols for routine transformation has facilitated application of reverse genetics approach in *Arabidopsis* (Alonso, Ecker 2006). However, in species where transformation is complicated and targeted mutagenesis is difficult to achieve, forward genetics offers a more promising way to establish a link between gene and a corresponding phenotypic trait (Peters et al. 2003). For economically important species often only those mutants displaying interesting phenotype of altered disease resistance or abiotic stress response draw attention of scientific research. To identify a mutation underlying an interesting mutant phenotype several tools of forward genetics can be applied. One of such techniques is transcript based cloning (TBC) (Zakhrabekova et al. 2002). TBC applies microarray technology to identify mRNAs, which are absent or significantly reduced in mutants, but can still be detected in wild-type (wt) plants. Significant reduction of mRNA abundance in a mutant line can result from either complete or partial deletion of the gene of interest or nonsense mediated decay caused by a premature stop codon (Gadjieva et al. 2004). Alternatively, Bruce et al. (2009) recently reported genomic DNA, instead of mRNA, microarray based gene cloning in rice, thus, avoiding risk of missing the mutated gene due to low expression levels in wt plants.

Availability of microarray platforms encompassing a large proportion of barley genes (Close et al. 2004; Zakhrabekova et al. 2007) and availability of mutant populations has facilitated application of TBC to barley. In barley, transcript based cloning has been tested on several known mutations – *rar1* (Mitra et al. 2004), *rpr1* (Zhang et al. 2006) and magnesium chelatase mutants *xantha* (Zakhrabekova et al. 2002). Recently, TBC was successfully used to identify mutation in gene *HvCAX1* eliciting development of necrotic leaf spots in barley (Zhang et al. 2009) and in *RRP46* regulating rRNA processing and *R* gene-independent cell death in barley-powdery mildew interactions (Xi et al. 2009).

Mutants displaying necrotic phenotype - lesion mimic mutants (LMM) – have been reported in several plant species – maize (Johal 2007), rice (Wu et al. 2008) and *Arabidopsis* (Moeder, Yoshioka 2008). Necrotic phenotype

in LMM is often associated with altered disease resistance (Lorrain et al. 2003). Therefore identification of genes underlying the phenotype can facilitate identification of molecular mechanisms of plant disease resistance. In barley, numerous LMM have been identified in different mutant populations (Lundqvist et al. 1997; Caldwell et al. 2004; Talame et al. 2008).

The aim of the present study was to use Affymetrix Barley1 GeneChip microarray transcriptome analysis for two fast neutron induced *nec3* mutants. The experiment failed to identify a candidate *Nec3* gene, but comparative analysis of the *nec3* transcriptome with wt barley transcriptome under various stress treatments identified a link between missregulated cell death of *nec3* and stress response signaling in barley.

Materials and methods

Plant material and RNA extractions

Barley fast neutron mutants FN362 and FN363 were isolated from a cv. Steptoe seed irradiated with fast neutrons at the IAEA Seibersdorf facility in Austria. Allelism tests with characterized *nec3* mutants GSHO 2065 and GSHO 2066 confirmed that FN362 and FN363 are allelic to *nec3* and they were used for further transcriptome analyses. For transcriptome analyses each biological replicate consisted of a five seedling pool. RNA was isolated from the primary and secondary leaves of 10-day-old cv. Steptoe, FN362 and FN363 plants as described (Zhang et al. 2006). The barley seedlings were grown in a growth chamber with a 16 h light and 8 h dark cycle maintained at 22 °C .

For qRT PCR, total RNA was isolated from leaves of two-week old cv. Steptoe, FN362 and FN363 plants using Trizol-like reagent as described by Caldo et al. (2004). Each RNA sample was extracted from a pool of three plants, and three biological replicates of each barley line (nine plants in total) were used. Integrity of the extracted RNA was monitored using non-denaturing agarose gel electrophoresis. Two μg of the extracted RNA was treated with DNaseI (Fermentas, Vilnius, Lithuania) following manufacturer's instructions and afterwards purified using chloroform-ethanol extraction.

Affymetrix microarray analysis

Two independent biological replicates of cv. Steptoe, and FN362 and FN363 mutants were subjected to Affymetrix Barley1 GeneChip analysis as described (Zhang et al. 2006). The GeneChip data have been submitted to NCBI GEO database under accessions GSE23775. Probeset summary data was obtained using Affymetrix Expression Console 1.1 and the MAS 5.0 processing algorithm (Affymetrix, Santa Clara, CA, USA). The resulting data were exported into Microsoft Excel (Redmond, WA, USA), where all the subsequent analyses were performed. Briefly, a two-tail t-test was used to identify significant ($p < 0.05$) differences in expression for each probeset between the control (cv. Steptoe) and both mutants. Two-fold reduction of expression in the mutant was used as a cut-off for identification of *nec3* candidate genes. For comparison with the publicly available microarray data, all probe sets exhibiting at least two-fold difference in transcript abundance between the control and both mutants were used.

PCR, RT-PCR and quantitative real-time PCR

Gene specific primers (Table 1) were designed by Primer3 software (Rozen, Skaletsky 2000). PCR reactions were carried out in a 20 μL of total volume containing 100 ng genomic DNA, 0.5 μM primers, 1.8 mM $MgCl_2$, 0.2 mM dNTPs and 1 u Hot Start *Taq* DNA polymerase (Fermentas, Vilnius, Lithuania) used with manufacturer-supplied buffers. PCR was carried out as follows: initial denaturing step for 5 min at 95 °C, 35 cycles of 30 s at 95 °C, 30 s at 60 °C, 2 min at 72 °C and final extension of 5 min at 72 °C.

cDNA was synthesized with oligo $(dT)_{18}$ primer in a total volume of 15 μL containing 0.8 μg of total RNA using a RevertAid H Minus First Strand cDNA synthesis kit (Fermentas, Vilnius, Lithuania).

For real-time PCR, aliquots of cDNA were amplified on an ABI Prism 7300 instrument (Applied Biosystems, Foster City, CA, USA) using a QuantiTect SYBR Green PCR kit (Qiagen, Hilden, Germany), in a total volume of 20 μL containing 2 μL of cDNA and 0.3 μM primers. Primers used for real-time PCR are listed in Table 1. Reaction was carried out as follows: initial denaturing step for 15 min at 95 °C followed by 35 cycles of 15 s at 94 °C, 30 s at 60 °C and 30 s at 72 °C (data acquisition step). Standard curves for the quantification of the transcript levels were calculated from serial dilutions of cDNA from cv Steptoe. Transcript levels of analyzed genes were expressed as a percentage of *HvGAPDH* transcript abundance in the same sample.

Comparison of nec3 transcriptome with transcriptome changes in barley under biotic and abiotic stress

We compared differentially expressed (at least two-fold up- or down-regulated) probe sets from our experiment with the expression of the same probe sets in a following set of publicly available barley GeneChip experiments from the PlexDB database (Shen et al. 2005; Wise et al. 2008) representing barley transcriptome change in response to abiotic and biotic factors: rar1-BB5 (Mitra et al. 2004), Rpg1_24hpi-BB49 (Zhang et al. 2008), Pseudom.-BB79 (Ueda, Wood 2008), senesc.-BB50 (Parrott et al. 2007), Mla6_8hpi and Mla13_8hpi-BB4 (Caldo et al. 2004), Mercury-BB83, mlo-5-BB7, chilling and freezing – BB81, drought-BB84 (Guo et al. 2009). A cluster dendrogram was designed using the Clique program from the PHYLIP3.66 package (Felsenstein 1989) by analyzing binary data matrix representing data of presence or absence of the gene (probe set) among a differentially regulated gene set from an analyzed experiment. Bootstrap confidence levels

Table 1. Oligonucleotide primers for *nec3* candidate-gene PCR screening and quantitative real time PCR. *, primers used for quantitative real time PCR

Primer	Sequence 5'-3'
ABC3257_L01*	TCAGGAGCTAGCTATCGATGGAGAA
ABC3257_R01*	GAAAGGTCGTTGGCTGGAGGAC
ABC4521_L01*	GCTCGTGGACCACTCCATTGT
ABC4521_R01*	GGTTGTACGACGAGTCCATATCGTG
ABC14229_L01*	GGTCCGACGTACAGTCACTCGTT
ABC14229_R01*	CCAGCGATCAACACATTAAGAAGGA
ABC1954_L01	GCACGTCGCCCTAGAGAAACT
ABC1954_R01	ATAATACTACGCCTGCTCTGCTGTG
ABC2279_L01*	GTCTTCTGCTTGCAAGTTTGACATC
ABC2279_ R01*	CAACGCCTTATTACAGTGAGGTACG
ABC3448_L01	CTACAACAAGAAGATGAAGCCATGC
ABC3448_ R01	GATGCAGAAGCCTCTTTACATTTGA
ABC4024_L01*	ACGGAAATATTGGAGACAAGAGGAG
ABC4024_ R01*	TCAAATGTACACAGAGTTGCAATGG
ABC6708_L01	ATCCTTCAAGGCCTATCTGAATGAC
ABC6708_ R01	GGCAGGAAGAGTTGCAAACTAGAAT
ABC7098_L01	CTATACGTTGTTCGGTTCAATCAGC
ABC7098_ R01	TGGGATACTACGATCATGGACAGTT
ABC7285_L01	CGTGTACCATTCTCCTGTAGGTTCT
ABC7285_ R01	CAAGGTTACACGATACAAGGAAACG
ABC7377_L01*	AGATCATCCTCACCTTCTCCCTTCT
ABC7377_ R01*	ATTTGCTTTCCTAAAAGTCCCAACC
ABC16209_L01*	GAAAACCATGGGAGTAAATGGAAC
ABC16209_ R01*	TACGTATACACCGTACACAGGATGC
ABC18830_L01	CAGGAGCAGGCTCTCAACAAAC
ABC18830_ R01	CGGATCTTATTGTCTCATACGTGTC
ABC19204_L01	CAAGGCCTACCTCAACCGCTAC
ABC19204_ R01	GAAGGCTCCCTCGAAATCAATC
ABC20556_L01	ACGCAAGTGAAAGTGACCAAGAA
ABC20556_ R01	CTCTTCTTCTTCTCGAGCGTCTTTT
ABC21141_L01	GCACCGTGAATATTTGGTTTAATGA
ABC21141_ R01	GCATCTAGTCCTCCTCTAGCCACTC
ABC53072_L01	GCACGATCTTCACAGGTATCACTTT
ABC53072_ R01	CAAAAGATGGGTCTCCTTCCATAAC
ABC29930_L01	GCCCAAGGGACTGTCTAGTG
ABC29930_ R01	TCTAGACTAGGGCTTGCATAAGG
ABC33510_L01	ATGGTGTGTGTGCCTCAGATGT
ABC33510_ R01	CGCTACAAGCTGGTATCATAAGGAG
ABC17652_L01	TCCAGAATTTGCAAGTCATCTTCAT
ABC17652_ R01	TTTGCTGGGATGACAAAAGATGTAT
ABC431_L01	TGTTCACTGGGGAGTGTAAGGAATG
ABC431_ R01	ACAGACTTTATCAAGGGGAGCCTCA
ABC12590_L01	CTGCTAAGCGAGTCCGAGTTCCT
ABC12590_ R01	GTTGAGGTCGAACCGGCAGAT
ABC14129_L01	CTTTACTGGAGAGGCTTTCGCTCAT
ABC14129_ R01	AGGGTCTGACGAAAGCTGGAGTT
ABC12169_L01	GTGTATCAAATGAGCTCGGTGCTG
ABC12169_ R01	CAGGTCATCAAACAAGAATGTGACG

were calculated from 100 iterations using the seqboot programme from the PHYLIP package. A graphical tree representing comparison was visualized using TreeView (Page 1996).

Hierarchical cluster analysis comparing nec3 transcriptome with drought and low temperature treated barley transcriptome data available in the PlexDB database (experiments BB81 and BB84) was performed using the programmes Cluster and Treeview from the EisenSoftware package (Eisen et al. 1998).

Functional categorization of the genes differentially expressed in *nec3* mutant was performed using Gene Ontology service on The Arabidopsis Information Resource website (http://www.arabidopsis.org). Analysis was based on *Arabidopsis* homologues of the *nec3* differentially expressed genes. The GO Term enrichment tool was applied for analysis of representation of different gene groups in nec3 transcriptome in comparison to whole genome data. Analysis was based on *Arabidopsis* and rice homologues of *nec3* differentially expressed genes, using, correspondingly, TAIR and GRAMENE database as background data.

Results

Allelism test

Two fast neutron (FN) irradiated recessive mutants with necrotic spots, FN362 (*nec3l*) and FN363 (*nec3m*), were isolated at the Washington State University (Pullman, WA, USA). The phenotype exhibited was similar to characterized *nec3d* (GSHO 2065) and *nec3e* (GSHO 2066) alleles obtained from Dr. Franckowiak (Franckowiak et al. 1996; Lundqvist et al. 1997). Crosses between FN362 and FN363 and with *nec3d* and *nec3e* alleles all displayed the characteristic *nec3* leaf spot phenotype in F1 confirming that FN362 and FN363 are *nec3* mutants. The recessive nature of the FN362 and FN363 mutants was confirmed in the F2 generation.

Transcript based cloning of nec3 *candidate genes*

Fast neutron irradiation is known to cause large deletions in plant genomes (Li et al. 2001), which may cause complete or partial deletion of one or several genes and, consequently, lack the corresponding mRNA in the plant. Thus, comparison of the transcriptome between mutant and wt plant may identify candidate genes for the mutant phenotype, assuming that the microarray contains the probes for the deleted gene and that the appropriate tissue type and developmental stage, where the gene is expressed, are sampled.

The *nec3* mutation causes development of tan and brown necrotic spots on barley leaves (Lundqvist et al. 1997). We used two allelic fast neutron mutants FN362 and FN363 and a parental cv. Steptoe to identify the defective gene responsible for the necrotic phenotype in *nec3*. Out of 22 791 probe sets represented on Affymetrix Barley1

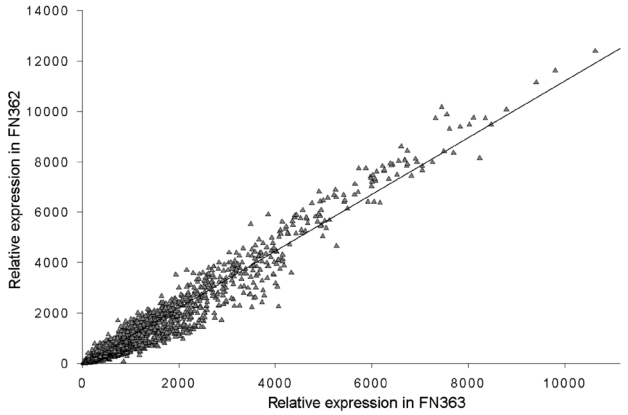

Fig. 1. Correlation between transcriptomes of *nec3* allelic mutants FN362 and FN363. Only genes with transcript abundance above threshold of detection are included.

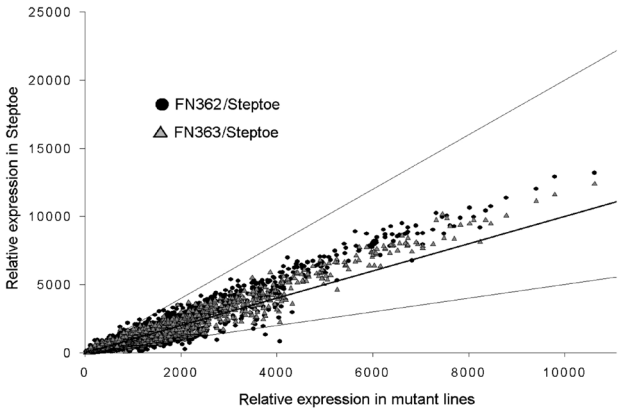

Fig. 2. Transcriptome comparison of allelic *nec3* mutants FN362 and FN363 with parental line Steptoe. Only genes with transcript abundance above treshold of detection are included. Genes outside the trendlines are two-fold up- or down-regulated.

GeneChip, expression of 10507 probe sets could be detected as present and exceeding a threshold signal level of 50 in at least one mutant or parental line. Gene expression values significantly correlated between both mutants $r^2 = 0.959$ (Fig. 1).

Only a small proportion of genes were down-regulated in any of mutants (Fig. 2). To identify the nec3 candidate gene, we tested all 21 probe sets that were at least two-fold down-regulated genes using genomic PCR to identify potential deletions. PCR fragments from all candidate genes of predicted length were present in both mutants and the wild type (Fig. 3). Thus, none of the candidate genes appeared to be deleted to a detectable extent in the FN362 and FN363 mutants.

Characterization of differentially expressed genes in nec3 *mutants*

In total 191 genes were estimated to be differentially expressed (at least two-fold up- or down-regulated) in both mutants (Appendix 1) and only 26 of those were induced more than 10-fold. Microarray data were validated using quantitative real time PCR analysis on selected differentially expressed genes (Fig. 4). Of the seven genes tested, quantitative real time PCR confirmed differential expression of four genes in *nec3*.

We used the HarvEST database (Close et al. 2007) to identify homology-based annotations of the genes differentially expressed in *nec3* and used BLASTX (http://blast.ncbi.nlm.nih.gov/Blast.cgi) homology search for non-annotated barley genes to find the best homologues from *Arabidopsis* and rice. According to annotations, a considerable part of the analyzed genes are known to be differentially regulated upon abiotic stress treatment or pathogen infection (Table 2 and Table 3). Some of these genes could be assigned to general stress-related physiological processes, such as, osmotic regulation and synthesis of storage carbohydrates, whereas others have been shown to be involved in a particular stress response.

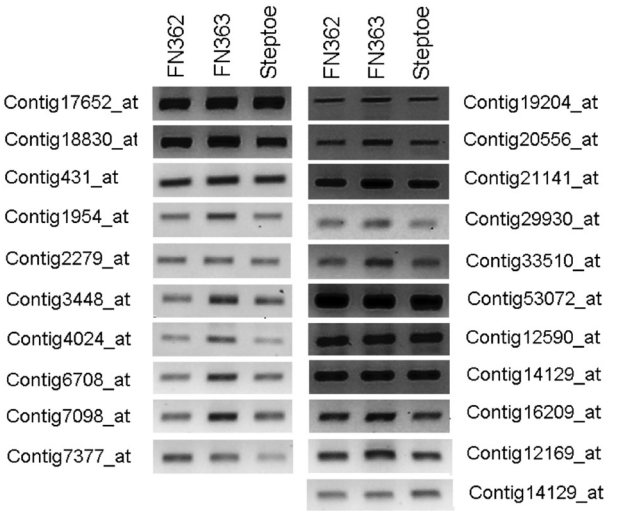

Fig. 3. Genomic PCR analysis of the *nec3* candidate genes in FN362 and FN363 mutants.

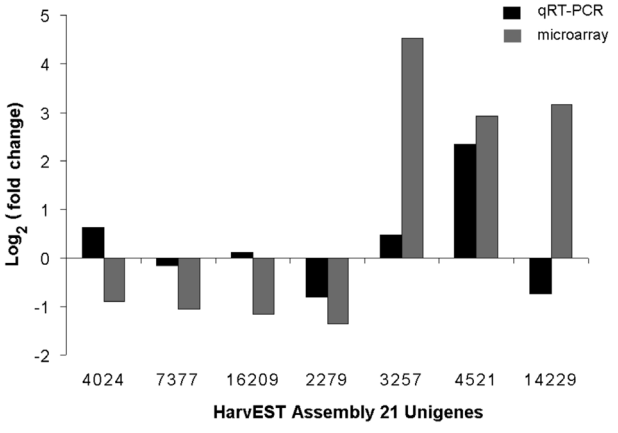

Fig. 4. Validation of microarray data using quantitative real-time PCR analysis of seven differentially expressed genes in *nec3* mutants. Values refer to log2 ratios of fold change in *nec3* (average value of FN362 and FN363) versus parental line Steptoe.

Table 2. List of differentially expressed abiotic stress related genes in the *nec3* mutant

Affymetrix Barley GeneChip probe set	HarvEST Assembly21 Unigene	Best BlastX hit (Uniport Accession)	Function in abiotic stress response	Expression fold-change in FN362; FN363
Contig10558_at	10558	OsRadc1 Q6F4N5	Rice anther peptide down-regulated by chilling, cold acclimation (Yamaguchi et al. 2004)	365; 48
HV11O04r_s_at	39248	Glutamine-dependent asparagine synthetase TaASN1 Q5QFC3	Up regulated by osmotic stress, salt stress and abscisic acid (Wang et al. 2005)	3; 3
HA11P12u_s_at	31829	Sucrose:fructan 6-fructosyltransferase, Hv Q96466	Induced by low temperature treatment (delViso et al. 2009). Abiotic stress related carbohydrate metabolism (Valluru, Van den Ende 2008)	17; 6
Contig19503_at	19503	Fasciclin FLA4 like protein Q06IA2	Fasciclin FLA4 mutation alters salt stress sensitivity of *Arabidopsis* (Shi et al. 2003)	8; 3
Contig7789_at	7789	Fasciclin FLA12 like protein Q06I94		4; 2
Contig7377_s_at	7377	TIP4 aquaporin like protein Q75GA5	Aquaporins are involved in water transport regulation under stress in *Arabidopsis* (Boursiac et al. 2005)	0.4; 0.4
Contig14229_at	14229	NIP1-1 aquaporin like protein A2Y699		14; 6
Contig6156_at	6156	Horcolin Q5U9T2	Proposed to be involved in stress signal perception and transfer (Grunvald et al. 2007)	6; 3
Contig5446_s_at	5446	Cystatin HvCPI8 Q1ENF0	Cystatins are involved in pathogen resistance (Martinez et al. 2003) as well as induced by abiotic stress (Gaddour et al. 2001)	5; 2
Contig4521_s_at	4521	Sucrose-sucrose-1-fructosyltransferase Q70LF5	Induced by drought in *Cichorium intybus* (de Roover et al. 2000), related to freezing tolerance (Li et al. 2007)	14; 4
Contig12073_at	12073	HvRAF (root abundant factor) Q4F8A4	Transcription factor involved in salt tolerance and pathogen resistance related signaling pathways (Jung et al. 2007)	8; 3
Contig6594_at	6594	Phosphatidylinositol 3- and 4-kinase Q5VMR5	Related to salt stress response in *Arabidopsis* (deWald et al. 2001)	2; 2

The *nec3* differentially expressed gene set also comprised a significant number of cell wall modifying enzymes (Table 4).

For functional categorization of the differentially expressed genes, we applied the TAIR gene onthology tool (Berardini et al. 2004) using the *Arabidopsis* gene set homologous to differentially expressed genes from *nec3*. Based on this classification over 20% of the analyzed genes were considered as stress response related (GO terms GO:0009628, GO:0009607, GO:0006950) (Fig. 5).

Since a large proportion of *nec3* differentially expressed genes might be stress related, we wanted to see, if stress related genes were indeed significantly overrepresented in the analyzed dataset, or if the ratio corresponded to the normal proportion of stress related genes in the genome. The GO term enrichment tool allows for identification of common characteristics of a gene set and also identifies gene groups which are overrepresented in the analyzed dataset compared to the whole genome data (Carbon et al. 2009). Since whole genome data are not yet available for barley, we retrieved *Arabidopsis* and rice gene products homologous to *nec3* differentially expressed genes using the HarvEST database and searched it against TAIR and Gramene databases using the GO term enrichment tool. According to this classification, the analyzed gene set contained a significantly larger number of lipid transport

Table 3. List of differentially expressed disease resistance related genes in the *nec3* mutant

Affymetrix Barley GeneChip probe set	HarvEST Assembly21 Unigene	Best BlastX hit (Uniport Accession)	Function in pathogen resistance	Expression fold-change in FN362; FN363
Contig2773_s_at	2773	Pathogenesis related protein PRP2 Q0IJ88		7; 3
Contig2043_s_at	2043	Type 1 non specific lipid transfer protein Q2PCB9	Involved in plant pathogen defence (Blein et al. 2002)	34; 9
Contig14482_at	14482	Remorin P93788	Delays virus PVX spread in potato and restricts bacterial infections in plants (Raffaele et al. 2009; Lefebvre et al. 2010)	3; 2
Contig2088_s_at	2088	Bowman-Birk type trypsin inhibitor BBBI, P12940	Comprises antifungal activity (Pekkarinen et al. 2007)	3; 3

Table 4. List of differentially expressed cell wall modifying genes in the *nec3* mutant

Affymetrix Barley GeneChip probe set	HarvEST Assembly21 Unigene	Best BlastX hit (Uniport Accession)	Function in cell wall modification	Expression fold-change in FN362; FN363
HZ01K16u_s_at	48563	UDP-glucose dehydrogenase A2YAR2	Cell wall formation, enzyme is regulated by the osmotic state of the cell (Johansson et al. 2002)	7; 5
Contig10778_s_at	10778	Polygalacturonase isoenzyme 1 beta subunit homolog Q6ZA27	Cell wall hydrolytic enzyme involved in fruit softening, plant development and organ senescence (Hadfield, Bennet 1998; Kim et al. 2006)	3; 2
Contig2873_s_at	2873	Expansin EXPB2 Q6QFA2	Cell wall extensibility (Cosgrove et al. 2002)	5; 3
Contig7055_at	7055	Expansin A5 Q6ZGU9		12; 3
Contig4124_s_at	4124	Extensin HvEx1 O49870	Cell wall modifying enzyme (Sturaro et al. 1998)	18; 3
Contig2957_at	2957	Xyloglucan endo-transglycosylase/ hydrolase Q5JZX2	Cell wall polysaccharide modification (Minic, Jouanin 2006)	5; 3

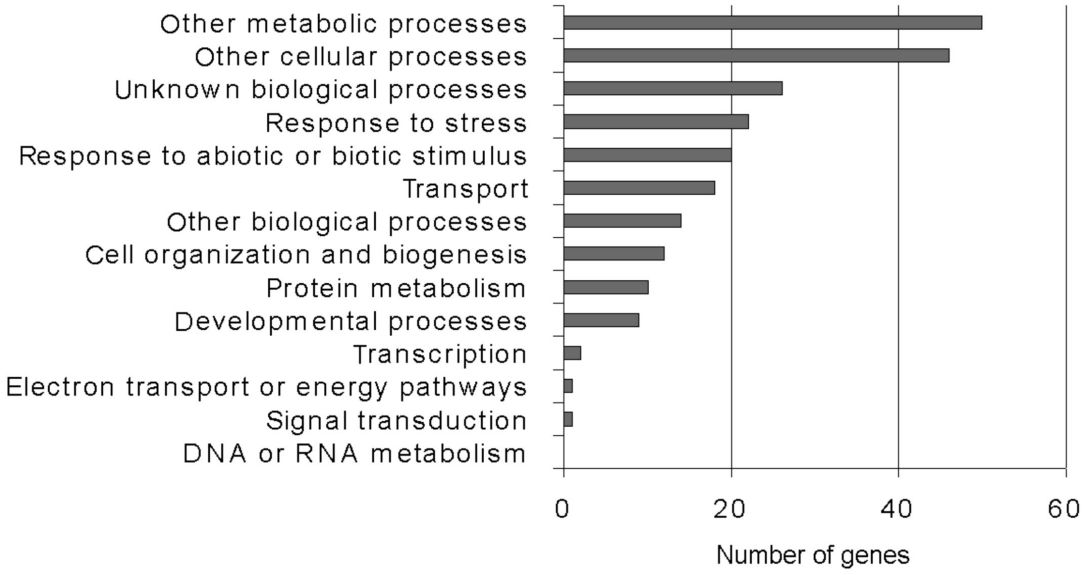

Fig. 5. Functional categorization of *Arabidopsis* homologues of differentially expressed *nec3* genes according to *TAIR* gene onthology (GO) terms. .

related proteins (10% in the analyzed gene set against 2% within the whole genome) and vesicle localized proteins (65% against 30% background frequency).

Comparison of nec3 *transcriptome with barley transcriptome under biotic and abiotic stress*

Whole genome transcript analysis can be used to reveal main signaling pathways activated in response to a particular stress factor or as a result of a mutation. We performed a comparative analysis of differentially expressed genes from *nec3* and publicly available Affymetrix Barley1 GeneChip data on barley transcriptome change under various stress treatments.

We chose a set of barley GeneChip experiments from the PlexDB database (Wise et al. 2008) representing barley transcriptome change in response to four abiotic factors (chilling, freezing temperature, drought, mercury toxicity) and five biotic factors (powdery mildew resistance of specific *Mla* alleles, effect of *mlo-5* and *rar1* mutations, stem rust resistance of transgenic Golden Promise containing *Rpg1* gene and response to *Pseudomonas aeruginosa*). We compared a set of differentially expressed genes from nec3 with the differentially expressed gene sets from the selected microarray experiments. Cluster analysis showed that the set of differentially expressed genes from *nec3* shares at least some common genes with data from all analyzed datasets. However, the highest overlap was established between *nec3* and transcriptome of abiotically stressed barley (Fig. 6). In total, 25% and 22% of differentially expressed genes from *nec3* are also differentially regulated in response to drought or freezing, respectively. Although *nec3* shares some similarity with abiotic stress induced barley transcriptome, the pattern of regulation of the majority of the overlapping genes in *nec3* was reverse to that reported for stress induced genomes (Fig. 7). Only a small subset of fructan biosynthesis related genes was up-regulated in *nec3* and also induced in response to low temperature treatment, whereas the majority of analyzed genes was induced in *nec3* and down-regulated in response to drought and chilling.

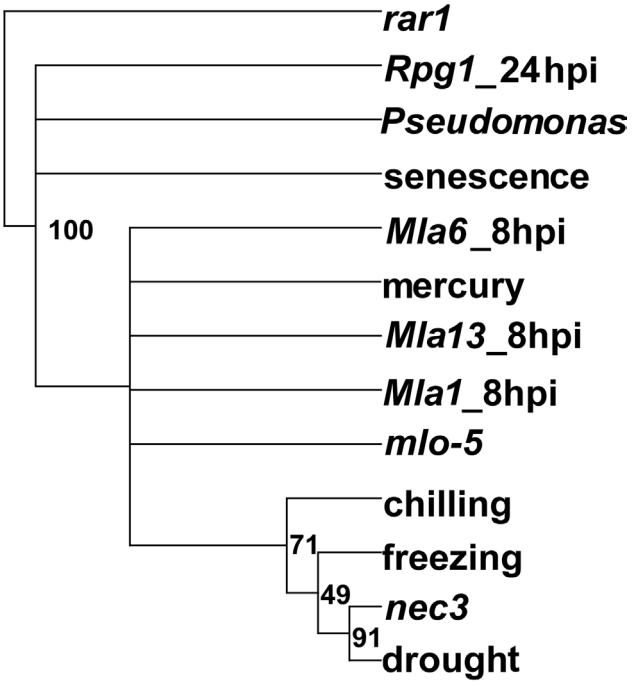

Fig. 6. Hierarchical cluster analysis of differentially expressed genes from nec3 and barley transcriptome change under various stress treatments from data available at PlexDB database. Data from following PlexDB experiments are included in analysis: rar1-BB5, Rpg1_24hpi-BB49, Pseudomonas-BB79, senescence-BB50, Mla6_8hpi and Mla13_8hpi-BB4, mercury-BB83, mlo-5-BB7, chilling and freezing – BB81, drought-BB84. Bootstrap confidence levels (calculated from 100 iterations) higher than 50 are shown..

Discussion

Identification of nec3 *candidate genes using TBC*

Significant reduction of mRNA abundance of a specific gene in a transcriptome of several allelic mutants, in comparison to a parental line, allows identification of a candidate gene for the analyzed mutation (Zakhrabekova et al. 2002). Assuming that the correct tissue at the correct developmental stage is analyzed, transcript based cloning can be more straightforward and a less laborious technique for gene identification than map based cloning. Although SNPs can also affect mRNA abundance of the mutated gene through a mechanism known as nonsense mediated decay (Gadjieva et al. 2004), use of fast neutron mutants containing large deletions encompassing partial or entire gene might be more reliable for gene cloning using microarray hybridization (Bruce et al. 2009). TBC has successfully been applied for identification of candidate genes for several mutations in barley (Zakhrabekova et al. 2002; Mitra et al. 2004; Zhang et al. 2006; Zhang et al. 2009; Xi et al. 2009). We chose two fast neutron mutants FN362 and FN363 displaying tan or light brown necrotic leaf spots, both allelic to *nec3*, for the Affymetrix Barley1 GeneChip experiment to identify the *Nec3* candidate gene. We identified 21 genes down-regulated at least two-fold in at least one of the analyzed mutants, but none of them appeared to be deleted from either of the *nec3* mutants (Fig. 3). Failure to identify a candidate *Nec3* gene may result from several reasons, e.g., (i) the probe sets for *NEC3* gene are not present on the Barley1 GeneChip; (ii) the expression level of wt gene is below the detection threshold of the GeneChip; (iii) the cv. Steptoe allele of the *Nec3* gene is not reliably detected by the GeneChip; (iv) the expression of the wt *Nec3* gene requires either specific environmental conditions or a particular developmental stage. While the Affymetrix Barley1 GeneChip contains 22792 probe sets (Close et al. 2004), it only represents a fraction of the total transcribed portion of the barley genome. As the genomes of *Arabidopsis*, rice and maize contain an estimated number of 25498 (Arabidopsis Genome Initiative 2000), 37544

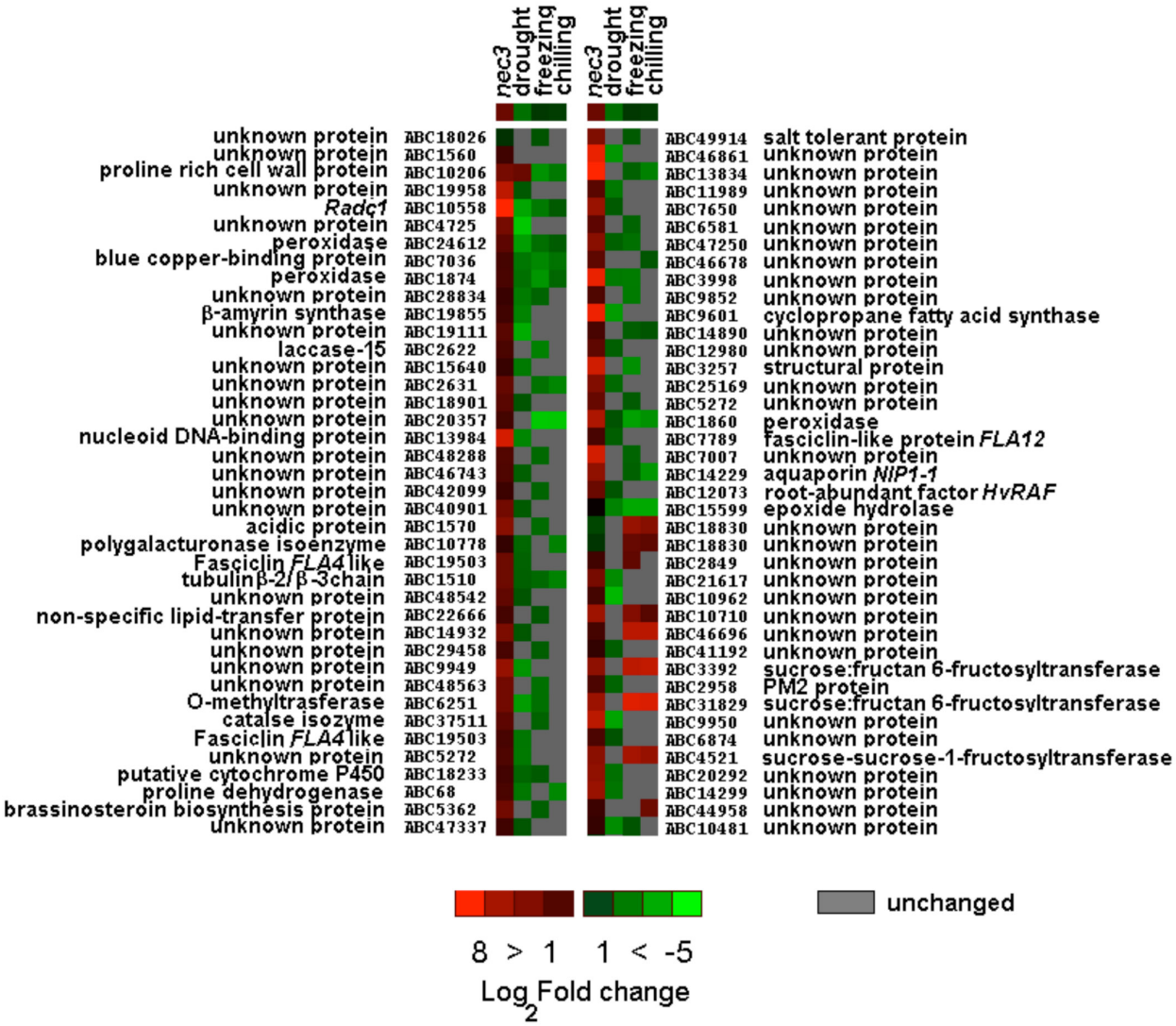

Fig. 7. Expression of overlapping differentially regulated genes from *nec3* and abiotically stressed barley transcriptomes. Barley genes are designated according to the corresponding HarvEST assembly 21 unigene number. Data from following the PlexDB experiments are included in the analysis: chilling and freezing – BB81, drought-BB84. Functional annotations obtained by BLASTX are shown..

(International Rice Genome Sequencing Project 2005) and 32000 (Schnable et al. 2009) genes, respectively, and the number of genes in barley genome is likely to be similar to rice and maize, it is possible that the Affymetrix Barley1 GeneChip does not contain probe sets for the *Nec3* gene. In addition, because the Barley1 GeneChip was designed primarily from the EST data, it only contains probesets for genes that were expressed in the tissue and at the developmental stages sampled during EST sequencing. The EST based unigenes that were used for Barley1 GeneChip design were designed from multiple barley accessions; however, the cv. Steptoe was not sampled. Natural allelic diversity, in cases of sequence mismatch between cRNA target and oligonucleotide probe, may result in an artificially lower mRNA hybridization signal, a phenomenon that has been used for single feature polymorphism discovery in barley (Rostoks et al. 2005) and yeast (Ronald et al. 2005) transcriptomes. Thus, it is possible that the cv. Steptoe allele of the *Nec3* gene may not be detected using the Barley1 GeneChip, even though the probe sets for *Nec3* are present on the microarray, resulting in a undistinguishable expression level between cv. Steptoe and the mutants. There is also the possibility that the fast neutron induced mutation is a small deletion or SNP that was not detected by our analyses. Such fast neutron mutations are rare, but not unknown, as exemplified by *nec1* mutations we previously analyzed (Rostoks et al. 2006)

The main disadvantage of TBC might be a requirement for an above-threshold expression of the target gene in the parental line. Since many genes require specific conditions to be induced, this significantly restricts the range of genes that can be identified using TBC (Bruce et al. 2009). The typical *nec3* lesions normally appear on leaves of three to four weeks old plants, while in our experiment we analyzed

transcriptome of two weeks old seedlings. If *Nec3* expression is developmentally or environmentally controlled, the observed failure to identify *Nec3* gene may be caused by the lack of its expression under our experimental setup.

Transcriptome analysis of the barley nec3 *mutants FN362 and FN363*

Studies of mutants displaying specific phenotype or altered response to abiotic or biotic stimulus help in identification of genes critical for plant adaptation to adverse conditions (Svensson et al. 2006). Whole genome transcript analysis can be used to reveal the main signaling pathways activated in response to a particular stress factor or as a result of a mutation (Hoth et al. 2002; Ozturk et al. 2002). In general, different stressors elicit stress specific signaling pathways with only a minor part of induced genes overlapping between various treatments (Kreps et al. 2002). The analysis of mutants with altered stress response can often help in unraveling molecular mechanisms of stress response, since mutations disrupting a certain signaling pathway can mimic the effect of stress treatment (Bohnert et al. 2006). Analysis of nec3 transcriptome aids in a better understanding of molecular mechanisms underlying missregulation of cell death in barley and the probable link between regulation of cell death and other physiological processes. Due to the phenotypic similarity, it is tempting to associate missregulated cell death of lesion mimic mutants with hypersensitive response and disease resistance. Numerous lesion mimic mutants display enhanced resistance to certain pathogens (Lorrain et al. 2003; Mur et al. 2008; Wu et al. 2008; Zhang et al. 2009). However, several studies demonstrate enhanced or impaired abiotic stress resistance of lesion mimic mutants (Jambunathan et al. 2001; Mateo et al. 2004; Muhlenbock et al. 2007; Yamanouchi et al. 2002), suggesting that the necrotic phenotype of lesion mimic mutants does not necessarilly result from alterations of disease resistance pathways, but can also be linked to abiotic stress response. Comparison of differentially expressed genes from *nec3* with data from publicly available barley GeneChip experiments revealed a common gene set between *nec3* and abiotically stressed barley transcriptomes (Fig. 6). The fact that the same genes were up- or down-regulated in *nec3* as those that were induced or repressed by drought, freezing or chilling suggests that *nec3* mutation might interfere with signaling pathways required for abiotic stress response in barley. Examination of homology-based annotations of *nec3* differentially expressed genes highlighted a set of genes involved in general abiotic stress response, such as cell membrane stabilization and synthesis of storage carbohydrates, as well as a set of genes specifically involved in a particular stress response (Table 2, 3, 4). The *nec3* mutants analyzed significantly over-expressed fructan synthesis related genes (Table 2). Grasses synthesize and accumulate fructans as short-term storage carbohydrates (Vijn, Smeekens 1999), but fructans also serve for cold and drought acclimation through membrane stabilization (Hincha et al. 2000; Hincha et al. 2002; Valluru, van den Ende 2008). Genes participating in fructan biosynthesis have been shown to enhance freezing tolerance when over-expressed in transgenic plants (Li et al. 2007). We also detected significant induction of several putative aquaporins in the mutants analyzed, supporting the link between *nec3* and abiotically stressed barley. Although the physiological function of the specific aquaporin-like genes detected in the *nec3* transcriptome has not yet been characterized, aquaporins, in general, are known to be involved in drought, cold and salt resistance (Boursiac et al. 2005). Induction of fructan biosynthesis and over-expression of aquaporins in *nec3* might render the mutant more resistant to drought or subzero temperatures. The role of *nec3* mutation in abiotic stress related signaling pathways is also supported by GO term enrichment tool analysis, confirming overrepresentation of membrane synthesis related genes such as lipid transport related and vesicle localized proteins in the *nec3* transcriptome. Changes in lipid membrane composition and induction of genes involved in lipid biosynthesis are known to occur upon abiotic stress treatment (Blein et al. 2002; Gigon et al. 2004; Svensson et al. 2006).

Although transcriptomes of *nec3* and abiotically stressed barley share significant overlap, the majority of *nec3,* differentially expressed genes are inversely regulated in *nec3* compared to abiotically stressed barley. An opposite pattern of regulation might cause a competition between the *nec3* and signaling pathways required for abiotic stress response.

Together these results suggest that nec3 mutation affects expression of a significant number of genes involved in abiotic stress response. However, physiological experiments are required to determine if *nec3* affects actual abiotic stress resistance in barley.

Acknowledgements

The study was supported by a Latvian Council of Science grant 09.1095 to NR and USDA NRI grant 2007-35301-18205 to AK. Seed of the barley *nec3* mutants GSHO 2065 and GSHO 2066 was obtained from Dr. J. Francowiack.

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Received 30 August 2010; received in revised form 8 September 2010; accepted 10 September 2010

Appendix 1. Affymetrix Barley GeneChip probesets having altered transcript abundance in *nec3*. Os, *Oryza sativa*; Hv, *Hordeum vulgare*; Ta, *Triticum aestivum*; Zm, *Zea mays*

Affymetrix Barley GeneChip probe set	HarvEST Assembly21 Unigene	Best BlastX hit	Fold-change in FN362	Fold-change in FN363
Contig10558_at	10558	Radc1 Os Os03g0186900	365	48
Contig13834_at	13834	OSIGBa0142C11.7 Os protein	186	22
Contig3998_at	3998	Putative uncharacterized protein Os A2XB03	79	5
Contig9601_s_at	9601	Cyclopropane fatty acid synthase, putative, expressed Os Os12g0267200	74	13
HVSMEn0018H10r2_at	48425		51	32
Contig7007_s_at	7007	Os06g0517700 protein Os	49	6
Contig13984_at	13984	Nucleoid DNA-binding-like protein Os Os07g0658600	44	13
Contig3257_at	3257	Structural protein Hv Q43493	41	13
Contig5710_at	5710	Putative CBS domain containing protein Os Os08g0313200	40	69
Contig9093_at	9093	Putative uncharacterized protein Os A2YX88	39	12
Contig2046_at	2046	Type 1 non specific lipid transfer protein Ta Q2PCB9	35	9
Contig17985_at	17985	Putative uncharacterized protein Os A2XAT9	33	7
Contig19958_at	19958	Os01g0728100 protein Os	30	9
Contig9950_s_at	9950	Os01g0216500 protein Os	30	9
Contig11308_at	11308	AMP-binding enzyme family protein, expressed Os Os11g0558300	23	8
Contig1860_s_at	1860	Peroxidase Os A7J0U4	21	10
Contig19747_at	19747	Os09g0374900 protein	19	10
Contig3256_s_at	3256	Structural protein Hv Q43493	19	4
Contig10710_at	10710	Putative uncharacterized protein Os A2WM98	18	6
Contig4124_s_at	4124	Extensin Hv O49870	18	3
HA11P12u_s_at	38223	Sucrose:fructan 6-fructosyltransferase Q96466	18	6
Contig7650_at	7650	Putative uncharacterized protein Os A3BL87	17	6
Contig9949_at	9949	Os01g0216500 protein Os	16	5
Contig12272_s_at	12272	Putative nucleic acid binding protein Os Q69ME2	15	4
Contig20292_at	20292	Putative uncharacterized protein Os A2X2Y2	15	5
Contig3392_at	3392	Sucrose:fructan 6-fructosyltransferase Hv Q96466	15	4
Contig14229_at	14229	Aquaporin NIP1-1 Os	14	6
Contig14299_at	14299	Putative uncharacterized protein Os A2Y699	14	8
Contig4521_s_at	4521	Sucrose-sucrose-1-fructosyltransferase Hv Q70LF5	14	4
HW01K06u_s_at	48542		14	3
Contig1689_at	1689	Type 1 non specific lipid transfer protein Q2PCD1	13	6
Contig5794_s_at	5794	Putative uncharacterized protein Os A2Z3Y4	13	4
Contig10838_at	10838	Putative threonine synthase Os Os05g0549700	12	2
Contig25169_at	25169	Putative uncharacterized protein Os A2Y6K4	12	4
Contig7055_at	7055	Expansin-A5	12	3
Contig7977_at	7977		12	4
rbags19n19_s_at	32923	Salt tolerant protein Ta Q0IJ88	12	3
Contig10206_s_at	10206	Proline-rich protein Zm Q9ZNY1	11	2
Contig14932_at	14932	Putative uncharacterized protein Os Os08g0405700	11	3
Contig1570_s_at	1570	Acidic protein Hv THN3	11	6
Contig6804_at	6804	Expressed protein Os Os12g0563600	11	3
HY07I12u_s_at	49846		11	5
Contig5362_at	5362	Brassinosteroid biosynthesis-like protein Zm Q5YFA2	10	3
rbags12n24_s_at	32039		10	5
Contig1510_s_at	1510	Tubulin beta-2/beta-3 chain	9	2
Contig16460_at	16460	Putative uncharacterized protein Os Os09g0542000	9	3
Contig19503_at	19503	Fasciclin-like protein FLA4 Ta Q06IA2	9	3
Contig19638_at	19638	PREDICTED: hypothetical protein UPI0000DB6CCB	9	4
Contig21617_at	21617	Os11g0210100 protein Os	9	4
Contig4725_s_at	4725	Proline-rich protein Zm Q9SBX4	9	4
Contig6251_at	6251	O-methyltransferase 3 Ta A5HB57	9	3
Contig12073_at	12073	Root abundant factor Hv Q4F8A4	8	3
Contig5363_at	5363	Os06g0326400 protein Os	8	3
Contig6642_at	6642	Putative 3-oxoacyl-[acyl-carrier-protein] synthase I, chloroplast Os Os06g0196600	8	2

Appendix 1. *continued*

Affymetrix Barley GeneChip probe set	HarvEST Assembly21 Unigene	Best BlastX hit	Fold-change in FN362	Fold-change in FN363
Contig6950_s_at	6950	Putative uncharacterized protein Os A3C0D8	8	3
HVSMEb0003M21r2_x	42805		8	4
Contig15396_at	15396	Putative transcription activator RF2a Os Os01g0756200	7	2
Contig2043_s_at	2043	Type 1 non specific lipid transfer protein Ta Q2PCB9	7	4
Contig2773_s_at	2773	Salt tolerant protein Ta Q0IJ88	7	3
Contig3756_at	3756	Nucleolar protein Nop56, Os Os03g0352300	7	3
Contig4400_s_at	4400	Putative uncharacterized protein Os A2ZK04	7	3
Contig5272_at	5272	Putative uncharacterized protein Os A2YMW1	7	3
Contig7275_at	7275	Os01g0266400 protein Os	7	5
Contig9290_at	9290	Coatomer alpha subunit Hv Q6RYF4	7	3
HU08O12u_s_at	40951	Putative uncharacterized protein Os A2YS16	7	2
HY10H19u_s_at	49914		7	2
HZ01K16u_s_at	50075	Putative uncharacterized protein Os A2YAR2	7	5
Contig11003_at	11003		6	3
Contig11989_at	11989	Putative uncharacterized protein Os A2YVX5	6	3
Contig12980_at	12980	Os05g0373400 protein Os	6	3
Contig18901_at	18901	Tetratricopeptide repeat protein-like Os Os01g0218200	6	2
Contig19111_at	19111		6	2
Contig22198_at	22198	Putative uncharacterized protein Os A2XZC7	6	3
Contig23598_at	23598	Putative uncharacterized protein Os A3BVT2	6	3
Contig3165_at	3165	Os09g0327100 protein	6	2
Contig5833_at	5833	3-hydroxy-3-methylglutaryl-CoA reductase Os Q4246	6	4
Contig6157_s_at	6157	Horcolin Hv Q5U9T2	6	3
Contig6581_at	6581	Putative uncharacterized protein Os A2YQF1	6	3
Contig725_s_at	725	Protein disulfide-isomerase Ta	6	2
Contig7790_at	7790	Putative uncharacterized protein Os A2YER6	6	2
HT06F11u_s_at	39312	Catalase isozyme 2	6	3
HV12N24u_s_at	42099	H/ACA ribonucleoprotein complex subunit 1-like protein 1, Os Os11g0579800	6	2
HVSMEh0094M14f_s_at	46861		6	2
HVSMEn0013N19f_s_at	47337		6	3
HW06A08u_s_at	48937	UDP-glucose dehydrogenase Populus Q6S4U9	6	3
Contig12191_at	12191	Putative uncharacterized protein Os A2X2T0	5	3
Contig1633_at	1633	Putative reversibly glycosylated polypeptide Os Os07g0604800	5	2
Contig18244_at	18244	Putative uncharacterized protein Zm Q9XHF3	5	2
Contig2243_s_at	2243	Putative uncharacterized protein wrsi5-1 Ta Q6QAX7	5	3
Contig24612_at	24612	Peroxidase Os A7J0U4	5	4
Contig2631_at	2631	Putative uncharacterized protein Os A3B722	5	5
Contig2873_s_at	2873	Expansin EXPB2 Ta Q6QFA2	5	3
Contig2957_at	2957	Xyloglucan endo-transglycosylase/hydrolase Zm Q5JZX2	5	3
Contig2958_at	2958	PM2 protein Hv P93669	5	2
Contig4887_s_at	4887	Cysteine protease Ta Q76CZ3	5	5
Contig5272_s_at	5272	Putative uncharacterized protein Os A2YMW	5	2
Contig5446_s_at	5446	Cystatin Hv-CPI8 Hv Q1ENF0	5	2
Contig5663_at	5663	Putative uncharacterized protein A2YEP6	5	2
Contig6156_at	6156	Horcolin Hv Q5U9T2	5	3
Contig6690_at	6690	H/ACA ribonucleoprotein complex subunit 1-like protein 1 Os Os11g0579800	5	2
Contig6874_at	6874	Os07g0645000 protein Os	5	2
Contig71_s_at	71	Endoplasmin homolog Hv	5	2
Contig8646_at	8646	Putative uncharacterized protein Os A2Y0I5	5	2
Contig9113_s_at	9113	Dor1-like family protein, expressed Os Os12g0538300	5	3
Contig9693_at	9693	Putative uncharacterized protein Os A2WYW1	5	2
HA24C19r_s_at	38360		5	3
HVSMEn0015P15r2_at	48288	Putative uncharacterized protein Os A2YLQ9	5	3
HY03N19u_s_at	49657		5	3
HZ51D22r_s_at	31632		5	2

Appendix 1. *continued*

Affymetrix Barley GeneChip probe set	HarvEST Assembly21 Unigene	Best BlastX hit	Fold-change in FN362	Fold-change in FN363
Contig10962_at	10962	Putative uncharacterized protein Os A2Y1M8	4	2
Contig12799_at	12799	Putative glycosyltransferase protein Os Os03g0413400	4	2
Contig13262_at	13262	Putative uncharacterized protein Os A2Y424	4	2
Contig1391_at	1391	Actin-11	4	2
Contig14890_at	14890	Putative uncharacterized protein Os A2YGX0	4	2
Contig1560_at	1560	OSJNBb0012E08.10 protein Os	4	2
Contig1615_s_at	1615	Luminal-binding protein 2 Zm BIP2	4	2
Contig17107_at	17107	Non-specific lipid-transfer protein Os A2YIN7	4	3
Contig17136_at	17136	Isoform 2 of Q8GU87 Os	4	2
Contig18233_at	18233	Putative cytochrome P450 Q9ATV2	4	2
Contig1860_x	1860	Peroxidase Os A7J0U4	4	3
Contig1874_at	1874	Peroxidase Os A7J0U4	4	2
Contig19855	19855	Beta-amyrin synthase Q6IW97	4	2
Contig22092_at	22092	Proline-rich protein Ta Q01979	4	3
Contig2622_at	2622	Laccase-15 Os	4	2
Contig4656_at	4656	H0313F03.20 protein Os	4	2
Contig5494_at	5494	H0212B02.14 protein Os	4	2
Contig5933_at	5933	Eukaryotic translation initiation factor Ta IF4E2	4	2
Contig6682_at	6682	Universal stress protein / early nodulin ENOD18-like Os Os02g0773200	4	2
Contig68_at	68	Proline dehydrogenase family protein, Os Os10g0550900	4	3
Contig7036_at	7036	Blue copper-binding protein-like Os Os07g0112700	4	2
Contig7789_at	7789	Fasciclin-like protein FLA12 Ta Q06I94	4	2
Contig840_s_at	840	Pyrophosphate-energized vacuolar membrane proton pump Hv	4	2
Contig8891_at	8891	Viral A-type inclusion protein repeat containing protein, Os Q10RF6	4	2
Contig9135_at	9135	Putative uncharacterized protein Os A3ADI5	4	2
Contig9852_at	9852	Putative uncharacterized protein Os A2YAR2	4	3
EBed01Q002_G15_s_at	28834, 19503	Fasciclin-like protein FLA4 Ta Q06IA2	4	2
EBro02Q007_A14_at	30500		4	3
HA03F12u_s_at	37511		4	2
HF11O19r_at	39248	Glycosyl transferase protein A-like Os Q67WK4	4	3
HVSMEb0005C06r2_at	43600	Putative uncharacterized protein Os A2X8R7	4	3
HVSMEn0014H06r2_s_at	47512		4	2
HW02O23u_s_at	48563		4	2
Contig10481_at	10481	Putative uncharacterized protein Os A2WWB0	3	2
Contig10518_at	10518	Putative uncharacterized protein Os Os07g0202900	3	2
Contig10778_s_at	10778	Putative polygalacturonase isoenzyme 1 beta subunit homolog Os Os08g0380100	3	2
Contig14482_at	14482	Remorin	3	2
Contig14613_at	14613	Putative uncharacterized protein At Q9XIL9	3	2
Contig15231_at	15231	WD-40 repeat family protein-like Os Os01g0653800	3	2
Contig15599_at	15599	Putative epoxide hydrolase Os Q8W3F2	3	0.2
Contig15640_at	15640	Putative uncharacterized protein Os A2Y699	3	2
Contig17957_at	17957	Uclacyanin 3-like protein Os Q949E8	3	3
Contig18035_at	18035	Flavonol-sulfotransferase Hv A9UKM5	3	2
Contig19504_at	19504	Os07g0175500 protein Os	3	3
Contig20357_at	20357	Putative uncharacterized protein Os A2YPR0	3	3
Contig20393_at	20393	Putative gamma-adaptin 1 Os Q948F4	3	2
Contig2088_s_at	2088	Bowman-Birk type trypsin inhibitor Hv	3	3
Contig22666_at	22666	Non-specific lipid-transfer protein Os A2YIN7	3	3
Contig2499_s_at	2499		3	3
Contig25307_at	25307	Protein kinase domain containing protein Os Q2QVC2	3	2
Contig2849_at	2849	Putative uncharacterized protein Os A2WK87	3	3
Contig553_s_at	553	Protein TolA B1DS62	3	2
Contig6734_at	6734	OSIGBa0159F11.8 protein Os	3	2
Contig6843_at	6843	Putative leucine-rich repeat transmembrane protein kinase Os Os02g0190500	3	2
Contig6931_at	6931	Putative uncharacterized protein Os A3B8E2	3	2

Appendix 1. *continued*

Affymetrix Barley GeneChip probe set	HarvEST Assembly21 Unigene	Best BlastX hit	Fold-change in FN362	Fold-change in FN363
Contig7450_at	7450	Putative uncharacterized protein OS A2Z1K2	3	2
Contig8226_at	8226	Plant integral membrane protein TIGR01569 2containing protein, Os	3	2
Contig8936_at	8936	Nucleoside diphosphate kinase 1 Os	3	2
EBed01Q002_G15_at	28834		3	2
EBem10Q002_L14_s_at	29458		3	3
EBro08Q012_G23_at	31559	Expressed protein (With alternative splicing) (Protease inhibitor/seed storage/LTP family protein, expressed) Os Q75GY5	3	3
HV11O04r_s_at	41630	Glutamine-dependent asparagine synthetase Ta Q5QFC3	3	3
HVSMEf0003C10r2_at	46696		3	3
HVSMEf0022D18r2_s_at	46743	Putative uncharacterized protein Os A3BH34	3	3
HVSMEl0010A03r2_s_at	47250		3	3
rbags36i03_s_at	34191		3	2
Contig19813_at	19813		2	2
Contig19815_at	19815	Transferase family protein, Os Os11g0507200	2	2
Contig20832_s_at	20832	Hydrolase-like Os Os01g0636400	2	2
Contig6594_at	6594	Phosphatidylinositol 3-and 4-kinase family-like Os Os06g0283400	2	2
Contig7707_at	7707	Putative mevalonate disphosphate decarboxylase Os Q6ETS8	2	2
HU05P03u_at	40901		2	3
HV_CEa0002I05r2_at	41192	Putative uncharacterized protein Os A2YL56	2	3
HVSMEb0011L02r2_x	44958		2	2
HVSMEc0006O07r2_at	46678		2	2
Contig6708_at	6708	Ureide permease 2, Os Q2QQ91	0.5	0.5
Contig7377_s_at	7377	Probable aquaporin TIP4-1 Os	0.5	0.5
Contig18830_at	18830	MtN19 like protein	0.4	0.4
Contig1675_s_at	1675	23 kDa jasmonate-induced protein JI23	0.3	8
rbaal17b01_s_at	31829		0.3	13
Contig18830_s_at	18830_s	MtN19 like protein	0.2	0.2
Contig1954_at	1954	Serine-glyoxylate aminotransferase	0.4	0.6
Contig2279_at	2279	LOL3	0.2	0.6
Contig2279_s_at	2279	LOL3	0.3	0.6
Contig3348_s_at	3348		0.4	0.7
Contig4024_at	4024	Protein kinase domain containing proteine	0.5	0.6
Contig7098_at	7098	Secretory acid phosphatase	0.4	0.6
Contig16209_at	16209		0.4	0.5
Contig17652_at	17652		0.3	0.5
Contig19204_at	19204	Heading date 5 Os	0.4	0.6
Contig20556_at	20556		0.6	0.4
Contig21141_at	21141	Flavin containing monooxygenase 3-like Os	0.4	0.6
HVSMEI0012A13f_at	53072		0.6	0.5
ContigEBpi01_SG004_C23_at	29930		0.4	0.6
ContigHD05F08r_at	33510	Putative anthocyanin 5-O-glucosyltransferase	0.5	0.7