Plant Physiology and Biochemistry 90 (2015) 38–49

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Identification of genes involved in the drought adaptation and recovery in *Portulaca oleracea* by differential display

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ARTICLE INFO

Article history:
Received 20 January 2015
Accepted 28 February 2015
Available online 4 March 2015

Keywords:
Differential display
Drought
Fatty acid desaturase 2
Portulaca oleracea
Rehydration
Ribosomal protein
Zinc finger domain

ABSTRACT

Portulaca oleracea is one of the richest plant sources of ω-3 and ω-6 fatty acids and other compounds potentially valuable for nutrition. It is broadly established in arid, semiarid and well-watered fields, thus making it a promising candidate for research on abiotic stress resistance mechanisms. It is capable of withstanding severe drought and then of recovering upon rehydration. Here, the adaptation to drought and the posterior recovery was evaluated at transcriptomic level by differential display validated by qRT-PCR. Of the 2279 transcript-derived fragments amplified, 202 presented differential expression. Ninety of them were successfully isolated and sequenced. Selected genes were tested against different abiotic stresses in *P. oleracea* and the behavior of their orthologous genes in *Arabidopsis thaliana* was also explored to seek for conserved response mechanisms. In drought adapted and in recovered plants changes in expression of many protein metabolism-, lipid metabolism- and stress-related genes were observed. Many genes with unknown function were detected, which also respond to other abiotic stresses. Some of them are also involved in the seed desiccation/imbibition process and thus would be of great interest for further research. The potential use of candidate genes to engineer drought tolerance improvement and recovery is discussed.

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1. Introduction

Portulaca oleracea is a succulent plant used as food and in traditional medicine (Xiang et al., 2005). It is rich in antioxidant compounds and in other metabolites of importance for human diet (Simopoulos et al., 1992). It is also known for its adaptability to different soils and environments, particularly high temperature, high salt, drought or low nutrient conditions and it is not chilling sensitive (Rinaldi et al., 2010). Regarding drought, *P. oleracea* is generally more tolerant than most crops (Ren et al., 2011). *P. oleracea* employs multiple strategies to cope with drought, from induction of several compounds like flavonoids, betalains, pinitol, free aminoacids and urea; and antioxidant machinery and enzymes (D'Andrea et al., 2014) to the switch in its photosynthetic mode from C_4 to Crassulacean Acid Metabolism (CAM)-like (Lara et al., 2003, 2004). Also, this species is capable of rapid recovery on rehydration, showing restoration of C_4 metabolism and of growth (D'Andrea et al., 2014). This great plasticity enables *P. oleracea* to grow in a wide range of environmental conditions and makes this species a source of candidate genes to improve drought tolerance.

Abiotic stress is the major reason for which crops are lost around the world, causing decreases in more than 50% in average yields for most important crops (Bray et al., 2000). Amongst abiotic stresses, drought is constantly increasing in many regions, being a main constraint to crop productivity (Bartels and Sunkar, 2005; Umezawa et al., 2006). Currently, water accessibility to agriculture is being gradually restricted by degraded soil and water systems, competition with other economic activities, and the need to safeguard aquatic ecosystems (Unesco, 2006).

Abbreviations: DD, differential display; DET, differentially expressed transcript; FA, fatty acid; FAD, fatty acid desaturase; qRT-PCR, quantitative real time RT-PCR; RWC, relative water content; RP, ribosomal protein.

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http://dx.doi.org/10.1016/j.plaphy.2015.02.023
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While studies on the short-term response to drought stress provide much information on stress perception and differential activation of signaling pathways, gene expression changes analysis on plants exposed to a prolonged period of drought provides information regarding adaptations responses to lasting water deficit (Evers et al., 2010). Understanding these mechanisms is vital to identify traits that improve stress tolerance in crops through breeding and transgenic strategies. Microarray technology for transcriptional profiling has provided insights into the multiplicity of plant stress responses (Bohnert et al., 2006; Sreenivasulu et al., 2007); however, commercial arrays are restricted for limited number of species. Recently high-throughput sequencing of cDNA (RNA-seq) has emerged as a powerful alternative (Mortazavi et al., 2008). However, it is still a high-costly technique which is not accessible to all researchers. Differential display (DD) is a powerful tool to obtain sequences that are uniquely expressed in a sample comparing different treatments. While microarray analyses give variation in more than a thousands genes, there are only tens to hundreds of genes that are significantly related to drought resistance (Liang et al., 2011). Thus, although information given by DD is limited it is of great value and provides candidate genes for functional genomic studies. In a previous study we deeply characterized *P. oleracea* plants adapted to drought and recovered after rewatering at metabolic and physiologic level (D'Andrea et al., 2014). Here, to identify genes involved in the drought adaptation and recovery after rewatering of *P. oleracea* we used the DD. The response of selected genes was tested against other abiotic stresses in *P. oleracea* and in *Arabidopsis thaliana*. The behavior of the orthologs genes in *A. thaliana* against abiotic stresses reveals genes which have a similar response versus a different response between these species. Of particular interest are sequences with unknown function, which represent a source of proteins with novel roles in drought adaptation and/or recovery after rewatering. Some of the strategies employed by seeds to withstand water deprivation and rehydration may be commonly also used in leaves.

2. Materials and methods

2.1. Plant material, growth conditions and sampling

P. oleracea L. plants were grown and subjected to drought as in D'Andrea et al. (D'Andrea et al., 2014). Plants watered daily constituted the control group (C). For the stressed (S) group of plants water was withheld during 21–23 days. After that, plants were re-watered during another 21–23 days (re-watered group, R). At least 10 plants were used in each set of experiments. Samples were taken from C, S and R groups.

In other stress experiments, three weeks old *P. oleracea* seedlings were divided in groups containing at least three plants: the control group was kept under well watering conditions (D'Andrea et al., 2014). Other groups were subjected to the following treatments: plants were watered with 300 mM NaCl for 6 days; for cold treatment, plants were kept at 16 °C during the 12 h-photoperiod and at 10 °C during the night for a week and for high light treatment plants were exposed to 800 μmol m^{-2} s^{-1} for 3 h.

A. thaliana plants were grown in soil at 22 °C and 70% relative humidity under a PFD of 120 μmol m^{-2} s^{-1}. Four weeks old seedlings were used for stress treatments: plants were watered with 200 mM NaCl or 300 mM mannitol, samples were taken after 3 h. For drought stress analysis *Arabidopsis* plants were withheld watering for 7 days and then leaves were collected. After that, plants were watered for 2 days and sampled. For each treatment at least 3 plants were analyzed. Stress treatments were conducted at least twice.

In all cases, the leaves sampled after the different treatments were already present and fully expanded before the treatments began. Leaves newly developed during the treatments were not sampled. After collection the leaves were immediately frozen in liquid N_2 and stored at −80 °C for further analyses.

2.2. Plant water status

Relative water content (RWC) was measured using whole fresh leaves (Lara et al., 2003).

2.3. RNA extraction

Total RNA was extracted from 100 mg of *P. oleracea* or *A. thaliana* leaves using the Trizol method (Invitrogen). RNA quality was assessed by agarose electrophoresis and RNA concentration and purity were estimated spectrophotometrically.

2.4. Differential display assay

Differential display (DD) experiments were performed following the general protocol reported by Liang and Pardee (1998) with the modifications described in Lauxmann et al. (2012). First-strand cDNA was synthesized using 3 μg of total RNA and 200 U of SuperScript II reverse transcriptase (Invitrogen) following the manufacturer protocol. Four different reverse transcription (RT) reactions were conducted with each cDNA by using 5 μM of one-base anchored oligonucleotide primer. These primers sequences were5′ $T_{(16)}$MN 3', where M was degenerated A, C or G and N was A, C, G or T and designated as AA, AT, AC and AG, respectively. Reactions were conducted at 42 °C for 60 min. A final step of enzyme inactivation consisted of an incubation at 70 °C for 15 min. The cDNAs obtained were diluted 1:10 and aliquots of 1 μl were used for PCR. Reactions were conducted in a final volume of 20 μl and contained 4 μM of corresponding anchored primer; 0.8 μM of one of the RAPD-designed decameric random primers (Table S1), 1X buffer (Promega), 200 μM dNTPs, 2 U of GoTaq DNA polymerase enzyme (Promega). Negative controls were conducted by adding the corresponding dilution of total RNA instead of cDNA so as to verify the absence of genomic DNA in the preparations. Duplicates were performed for each reaction. Cycle parameters were set as follows: an initial step of 3 min at 94 °C, 40 cycles of 20 s at 94 °C, 20 s at 48 °C and 30 s at 72 °C, followed by a final step of 5 min at 72 °C. The obtained PCR products were subjected to electrophoresis on 0.4 mm thick, 5% (w/v) polyacrylamide gels containing 7.5 M urea and 0.5X TBE buffer (44 mM Tris–HCl (pH 8.0), 36 mM boric acid, 50 mM EDTA) sequencing gels (Electrophoresis System. DNA Sequencing System. FB-SEQ-3545, Fisher Scientific). For this, samples were prepared by adding the same volume of denaturing loading buffer (10 mM NaOH; 95% (w/w) formamide; 0.05% (w/v) xylenecyanol and 0.05% (w/v) bromophenol) and heated for 3 min at 95 °C. Seven μl were downloaded in each well. Gels were run at 60 W for 3–4 h. The DNA Silver Staining System procedure from Promega was used to visualize the DNA products. A binary code was used to classify the bands; one (1) was used to designate the expression of a transcript in a sample; that is the presence of a DNA band in the gel, and zero (0) to denote the non-expression of the corresponding transcript (absence of the band) (Table 1).

2.5. Elution, cloning and sequencing of the differentially expressed bands

Selected bands were excised from the gel, eluted and cloned as in Lauxmann et al. (2012) and sequenced by Macrogen Inc. (Korea) facility by using the universal SP6- and T7 promoter sequencing primers.

Table 1
Classification of the differential expressed transcripts in *P. oleracea* after 21–23 days of withholding water and after 21–23 of re-watering. The expression pattern is represented by a binary code in which 1 denotes the occurrence and 0 de absence of the band in control (C), drought stressed (S) and after re-watering (R) plants.

N° of bands (DETs)	Percentage	Expression pattern	Cluster identity
2587	92.7	1.1.1	Not variable
38	1.4	0.1.1	Drought induced
69	2.5	0.1.0	Drought induced
28	1.0	1.0.0	Drought repressed
23	0.8	1.0.1	Drought repressed
22	0.8	0.0.1	Re-watered induced
22	0.8	1.1.0	Re-watered repressed
2789	100		Total

2.6. Sequence analysis and processing

Nucleotide sequences retrieved by Macrogen Inc. (Korea) were edited to remove vector sequences by using VecScreen analysis software (www.ncbi.nlm.nih.gov/VecScreen). BLAST-n and -X (Altschul et al., 1997) were performed using the unigene set as query against the non-redundant protein National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov). The set of unigenes was also subjected to BLAST analysis against local installation of TAIR's *Arabidopsis* protein database using BLASTX program. Sequences longer than 100 nucleotides were annotated as highly similar to the first BLAST hit to plant sequences. Next, over-represented differential expressed transcript sequences were organized as contigs and all the resulting analyzed DNA sequences were presented as unigenes (Table 1). Produced sequences were submitted to GenBank. To understand the functional significance of the sequences, the *Arabidopsis* homologues obtained for each *P. oleracea* sequence were used for gene ontology analysis (GO) (TAIR, www.arabidopsis.org/tools/bulk/go/index.jsp), and the same "biological process" and "molecular function" GO terms were assigned for the *Portulaca* unigene set.

2.7. Differential display validation

Quantitative real time RT-PCR (qRT-PCR) assays on a subset of 10% (9 unigenes) of the identified unigenes by DD were randomly selected for validation as described below.

2.8. Quantification of transcript levels using qRT-PCR

Relative expression was determined by qRT-PCR in an iCycler iQ detection system and the Optical System Software version 3.0a (Bio-Rad, Hercules, CA, USA), using the intercalation dye SYBRGreen I (Invitrogen) as a fluorescent reporter. qRT-PCR assays were prepared in a final volume of 20 μl containing 1X Taq activity buffer (Promega), 200 μM dNTPs, 2.5 mM $MgCl_2$, 0.8 U of GoTaq DNA polymerase enzyme (Promega), 0.5 μM of each primer (Tables S2–S4), 0.5X SYBRGreen I (Invitrogen), and 1 μl of a ten-fold dilution of cDNA each. PCR primers were with the aid of the web based program "primer3" (http://www.frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) (Tables S2 and S3). QRT-PCR controls were performed including dilution of non-reverse-transcribed total RNA in order to ensure RNA preparations were free of DNA contamination. The cycling parameters were as follows: an initial denaturation step at 94 °C for 2 min; 40 cycles of 96 °C for 10 s; 58 °C for 15 s; 72 °C for 1 min, and 78 °C for 1 s to detect fluorescence, and final elongation step at 72 °C for 10 min. The high reading plate temperature of 78 °C was set to avoid the influence of primer dimers to fluorescence detection.

Melting curves for PCR reactions were determined by measuring the decrease of fluorescence with increasing temperature (from 65 °C to 98 °C). The specificity of the PCR reactions was confirmed by melting curve analysis using the software as well as by 2% (w/v) agarose gel electrophoresis of the products. Relative gene expression was calculated using the Comparative $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) and *actin* (GenBank Acc. N° KM349373) and *polyubiquitin 10* (AT4G05320) as reference genes for cDNAs from *P. oleracea* and *A. thaliana*, respectively. Each cDNA sample was run in technical triplicates. At least three independent cDNAs synthesized from mRNAs extracted from leaves of different plants were used. Results were expressed in relation to the values obtained for C plants.

Since in gene databases there were not available sequences of *P. oleracea* encoding for already known references genes, cDNAs encoding Actin I, elongation factor 1α (EF1α) and Tubulin β from this species were amplified and sequenced in this work and submitted to GenBank (Acc. N° KM349373, KM349374 and KM349375, respectively). To obtain them, degenerate primers were designed for each gene (Table S4) and used for RT-PCR using total RNA from *P. oleracea* control leaves extracted as described above. Products were cloned into pGEM-T easy (Promega) and sequenced using

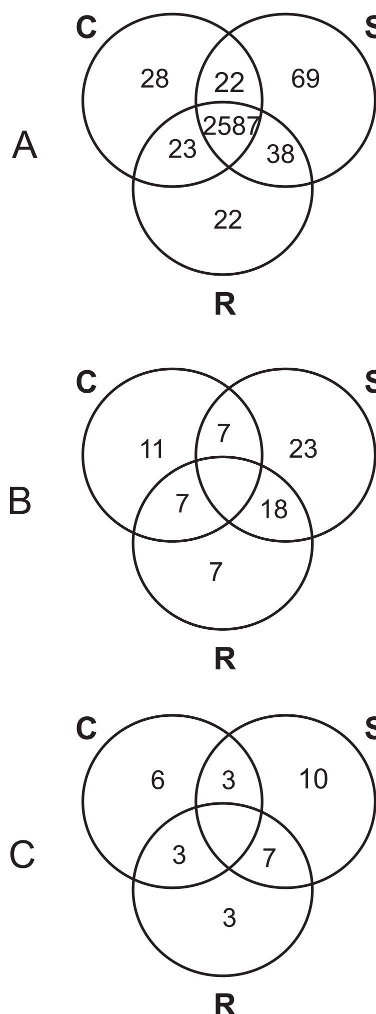

Fig. 1. Venn diagrams representing the sequence distribution obtained by Differential Display. Analysis of total bands identified in the gels (2789) (A). Representation of the sequenced 73 unigenes (B). Distribution of the 32 unigenes that depict homology to sequences published in databases (C). Leaves of well watered plants (C), after 21–23 of withholding water (S) and after 23 days of re-watering (R) were analyzed.

Macrogen, Inc. (Korea) facilities. Based on the obtained sequences, primers for qRT-PCR were designed with the aid of the web based program "primer3" (Table S5). To test whether EF1α, Tubulin β and Actin I behave as housekeeping genes in the analyzed samples, the gene expression index was plotted against the sample and linearity and low slope were verified (Brunner et al., 2004). Actin I was the most stable gene and therefore used as endogenous reference gene (not shown).

2.9. Data analysis

Data were tested using one way analysis of variance (ANOVA) and the Bonferroni test was used to calculate the minimum significance differences ($\alpha = 0.05$) with the aid of the Sigma Stat Package.

3. Results

3.1. Clustering analysis of differential transcriptome after drought stress and recovery

In the present work the differential transcriptome of *P. oleracea* leaves was analyzed by comparing three different pools of plants under different watering conditions, as previously described (D'Andrea et al., 2014). Plants from the control group (C) were watered daily and the leaves exhibited a relative water content (RWC) of 76.7 ± 2.8%; after 21–23 days of withholding water, the RWC of leaves decreased an 18% (62.9 ± 1.8%) (S). Thereafter, plants were re-watered during 21–23 days and sampled (R) when the recovered plants displayed the same RWC as controls (75.4 ± 0.4%).

About 2789 bands were detected in the gels. Bands showing a presence-absence pattern were considered for analysis and named as DETs (differentially expressed transcripts). Therefore, in drought adapted and/or recovered plants 202 DETs were detected which showed a 100 to 800 bp size and which represented the 7.2% of the transcriptome amplified by this technique. Representative gels are shown in Supplementary Fig. 1.

A binary code was used to typify the expression patterns of the DETs, with one (1) indicating the appearance of the transcript and zero (0) the absence of it (Table 1). A three-digit sequence identified the expression pattern of each transcript; the left one showing the appearance/non-appearance (1/0) in C, the middle position representing the expression in S and the right in R plants. Thus, a total of six different expression patterns were identified (Table 1).

Of the 202 DETs detected, 107 were induced (0.1.1 and 0.1.0) and 51 transcripts were repressed (1.0.1 and 1.0.0) in drought adapted leaves, accounting for a 5.7% of total transcriptome amplified (Fig. 1A). After re-watering, the pattern of induction and repression was reversed for 41% of the drought stress DETs (0.1.0 + 1.0.1; Fig. 1A). In R plants, 44 bands were exclusively expressed (0.0.1) or repressed (1.1.0; Fig. 1A).

A total of 90 DETs were successfully isolated and sequenced. Over-represented DETs were organized as contigs to eliminate redundancy giving a total of 73 unigenes. Sequences were submitted to GenBank and the corresponding accession numbers are shown in Tables 2–4 and Table S6. These unigenes were further divided into three different groups (Fig. 1B). The drought adapted induced cluster (0.1.0 and 0.1.1) represents the 47% of the total sequenced differential transcriptome and the drought adapted repressed one (1.0.0 or 1.0.1) represents the 36%. The remaining 17% is constituted by unigenes uniquely differentially expressed in re-watered plants (1.1.0 and 0.0.1; Fig. 1B).

3.2. Identity and functional classification of the differential expressed unigenes

Using the BLASTn program and non-redundant databases (NCBI,

Table 2
Unigenes induced in drought adapted *P. oleracea* plants. Functional identity and gene ontology description of the unigenes is shown.

P. oleracea unigene			Blast search			*Arabidopsis* ortholog	
Pattern	Seq n°	GenBank AccN°	NCBI and ESTreeDB functional annotation	NCBI accession n°	e value	TAIR unigene functional annotation	id
0.1.0	Seq041	JZ719118	*Theobrama cacao* BR-signaling kinase1	XM_007017927.1	9e-48	BSK11 – Brassinosteroid signaling kinase 11	AT1G50990
0.1.0	Seq086	JZ719132	*P. oleracea* omega-6 fatty acid desaturase 2	FJ472350.1	1e-49	FAD2 – Fatty acid desaturase 2	AT3G12120
0.1.0	Seq082	JZ719158	*Theobrama cacao* finger protein related mRNA	XM 007051957.1	9e-30	Zinc finger protein-related	AT1G01930
0.1.0	Seq053	JZ719120	*T. cacao* IQ domain32- putative	XM007017680.1	2e-9	Iqd32 – IQ-domain 32	AT1G19870
0.1.0	Seq007	JZ719113	*V. vinifera* ribosomal protein S9	XM_002274110.1	5e-37	Ribosomal protein S5 domain 2	AT3G49080
0.1.0	Seq052	JZ719119	*Silene latifolia* isolate RB16 PSBX gene	KF034820	3e-43	PSBX – Photosystem II subunit X	AT2G06520
0.1.0	Seq121	JZ719142	*P. oleracea voucher Nyffeler* 50S ribosomal L2 protein	HQ621349.1	6e-123	RPL2.2 – Ribosomal protein L2	ATCG01310
0.1.0	Seq015	JZ719115	*R. communis* AP-2 complex subunit α	XM 02527391.1	2e-34	α -ADR – α -Adaptin	AT5G22770
0.1.0	Seq008	JZ719110	Populus EST from severe drought-stressed opposite wood	CU234025.1	4e-68	ATEXPB2 – Expansin B2	AT1G65680
0.1.1	Seq012	JZ719114	*Beta vulgaris* RNA – integrase	EF101866.1	7e-52	Like retrotransposon family	AT4G04145
0.1.1	Seq078	JZ719127	*R. communis* aldose 1-epimerase	XM_002509868.1	5e-70	Galactose mutarotase-like protein	AT3G61610
0.1.1	Seq039	JZ719176	*Pyrus x bretschneideri* 40S ribosomal protein S15a1	XM009340085.1	4e-27	RPS15A – Ribosomal protein S15A	AT1G07770
0.1.1	Seq073	JZ719124	*B.vulgaris* small G protein (clone 1S3)	Z49152.1	2e-29	GTPase AtRAB8 (atrab8)	AT3G53610
0.1.1	Seq004	JZ719112	*Zea mays* PP2Ac-1-Phosphatase 2A isoform 1	EU965808.1	4e-36	PP2A-1 – Protein phosphatase 2A-1	AT1G59830
0.1.1	Seq054	JZ719121	*A. thaliana* 60S ribosomal protein L10-1	NM_101298.2	4e-70	Ribosomal Protein L10 (RPL10)	AT1G14320
0.1.1	Seq056	JZ719122	*Phoenix dactylifera* methyltransferase PMT13	XM 008801303	1e-05	QUA3 – Quasimodo 3	AT4G00740
0.1.0	Seq037	JZ719150	*Prunus mume* nucleolin-like (LOC103324735)	XM 008226830.1	4e-05	Not identified	

Table 3
Unigenes repressed in drought adapted *P. oleracea* plants. Functional identity and gene ontology description of the unigenes is shown.

P. oleracea unigene			Blast search			*Arabidopsis* ortholog	
Pattern	Seq n°	GenBank AccN°	NCBI and ESTreeDB functional annotation	NCBI accession n°	e value	TAIR unigene functional annotation	id
1.0.0	Seq079	JZ719128	*Vitis vinifera* hypothetical protein	XM_002280619.1	2e-60	Lysophosphatidic acid acyltransferase	AT4G24160
1.0.0	Seq107	JZ719138	*Bixa orellana* carotenoid cleavage dioxygenase	EF493214.2	6e-9	Carotenoid cleavage dioxygenase 1	AT3G63520
1.0.1	Seq108	JZ719168	*Lotus japonicas cDNA clone*: *LjFL3-021-AA12, HTC*	AK339379.1	4e-27	Not identified	
1.0.0	Seq074	JZ719125	*Vitis vinifera* Histone-lysine N-methyltransferase	XM_002273899.2	3e-14	SDG21 -SUVH8 putative histone methyltransferase	AT2G24740
1.0.0	Seq094	JZ719135	*Talinum paniculatum* ribosomal protein S7	HQ664663.1 2e-70		RPS7 – Chloroplast Ribosomal protein S7	ATCG00900
1.0.0	Seq123	JZ719144	*Nicotiana tomentosiformis* heat shock cognate	XM009595759.1	1e-10	Heat Shock protein 81.4 (Hsp81.4)	AT5G56000
1.0.0	Seq016	JZ719116	*Oryza sativa japónica* clone P0543004	AP004755.3	7e-5	Lysophosphatidylcholine acyltransferase 1	AT1G12640
1.0.1	Seq112	JZ719141	*Malus x domestica* D-lactate dehydrogenase	XM 008388934.1	5e-44	Glycolate dehydrogenase	AT5G06580
1.0.1	Seq077	JZ719126	*S. lycopersicum* alpha glucosidase II (gluII)	NM_001247101.1	5e-5	RSW3 – Radial swelling 3. A subunit of a glucosidase II enzyme	AT5G63840

ESTreeDB and TAIR), the identity of the isolated unigenes was assigned. Of the 73 differentially expressed unigenes, 32 sequences showed homology to already reported gene sequences available in databases while the other 36 sequences did not show homology to any other reported sequence (Table S6). The best BLASTn plants hit is shown in Tables 2–4. Therefore, among the 32 sequences identified, 17 unigenes are induced after withholding water during 21–23 days, 9 unigenes are repressed and 6 only modify their expression upon re-watering of stressed plants (Tables 2–4). As genomic and proteomic sequence information for *P. oleracea* are scarce or null, selected DETs were identified based on homology to nucleotides sequences from other plant species present in databases.

The "Gene Ontology (GO)" function at the TAIR website was used to retrieve the "biological process" and the "molecular function" of each sequence based on the *Arabidopsis* annotations. The histograms of frequency show the distribution of unigenes in each "biological process" (Fig. 2A) or "molecular function" (Fig. 2B) category and they show the number of unigenes with each group that are induced or repressed after prolonged drought and the number of unigenes that are modified only upon re-watering after drought. In addition, the identity of the unigenes belonging to each "biological process" or "molecular function" groups is presented in Tables S7 and S8, respectively.

Based on the "biological process" GO term (Fig. 2A), 19 functional groups were detected; with translation (composed by 4 unigenes up-regulated and one down-regulated by drought) and unknown biological function (composed by 5 unigenes; with 3 unigenes differing their expression only in re-watered plants and one down-regulated and another up-regulated by drought) being the most represented ones. The protein modification category was composed by 3 unigenes and followed by cellular response to water stress, lipid metabolic process, cell wall modification, signal transduction and vesicle-mediated transport categories. Finally, the following categories were composed by only one gene: oxidation-reduction process, defense response, response to abscisic acid stimulus; response to oxidative stress; hyperosmotic salinity response; photosynthesis; epigenetic; galactose metabolic process (Table S7).

Regarding the "molecular function" GO term classification (Fig. 2B) the group encompassing the unigenes with unknown molecular function was the most represented; with 3 unigenes being differentially expressed only when drought stressed plants were re-watered, 3 being drought induced and one being repressed after withholding water. Structural constituent of ribosome was the second most represented category with 5 unigenes. The third most

Table 4
Re-watered unigenes. The functional identity and gene ontology description of unigenes with variable expression exclusively during the rewatering period (repression, 1.1.0 or induction, 0.0.1).

P. oleracea unigene			Blast search			*Arabidopsis* ortholog	
Pattern	Seq n°	GenBank AccN°	NCBI and ESTreeDB functional annotation	NCBI accession n°	e value	TAIR unigene functional annotation	id
1.1.0	Seq002	JZ719111	*Ricinus communis* conserved hypothetical protein	XM002530300.1	1e-07	Unknown protein	AT2G36145
1.1.0	Seq072	JZ719123	*Prunus mume* pentatricopeptide repeat containing protein	XM008240435.1	5e-05	Pentatricopeptide repeat superfamily protein – DYW1	AT1G47580
1.1.0	Seq031	JZ719117	*Nicotiana tomentosiformis* 3-hydroxyacyl-CoA dehyrogenase	XM009610633.1	5e-11	AIM1 – Abnormal inflorescence meristem	AT4G29010
0.0.1	Seq109	JZ719139	Clon SS0ACG19YF03 *Vitis vinifera*	FQ381239.1	4e-57	APX4 – Ascorbate peroxidase 4	AT4G09010
0.0.1	Seq110	JZ719140	*N. tomentosiformis* Pyridoxal kinase	XM009630943.1	1e-25	SOS4 – Salt overly sensitive 4 – pyridoxal kinase	AT5G37850
0.0.1	Seq084	JZ719131	*Glycine max* uncharacterized protein	XM009363364	1e-36	Protein of unknown function (DUF1639)	AT1G55340 AT3G03880

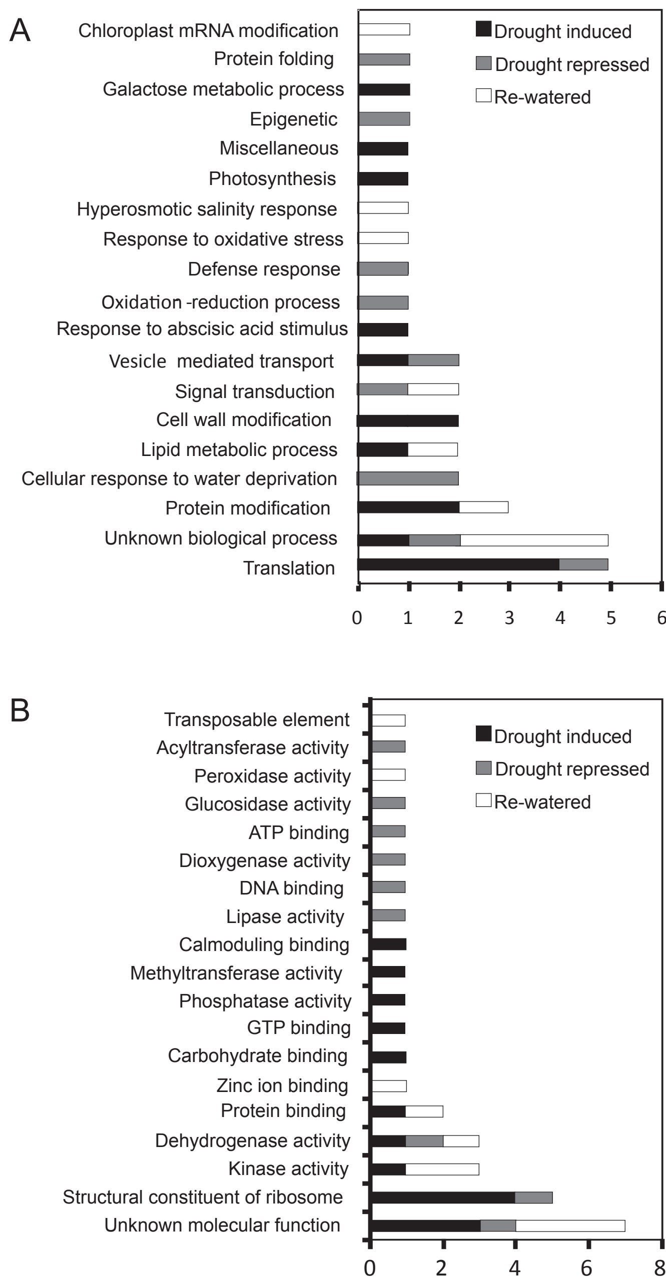

Fig. 2. GO-term functional classification of the differentially expressed unigenes. *Arabidopsis thaliana* orthologous to the identified unigenes were used for GO terms assignation. Histograms showing distribution based on biological process (A) and GO molecular function (B) are presented.

represented molecular function groups (3 unigenes) were kinase and dehydrogenase activity (Table S8).

3.3. Validation of expression patterns using qRT-PCR

The transcriptional profiles obtained by DD were verified by quantifying the expression of 9 randomly chosen unigenes with different patterns of expression (Fig. 3; Seq012, Seq041, Seq008, Seq086, Seq053, Seq005, Seq007, Seq052 and Seq001). Statistical analysis confirmed the significant differences in the relative level of expression of the unigenes chosen and validated the DD experiments (Fig. 3).

3.4. Analysis of the response of selected sequences to other abiotic stresses

The response of four sequences induced in drought adapted plants (Seq039, Seq041, Seq053 and Seq86) was tested in *P. oleracea* against other abiotic stresses such as high light (HL), cold or NaCl treatment. While sequences 086 and 039 were also induced by NaCl treatment, sequences 041 and 053 were not increased in any of the stress conditions tested except for drought (Fig. 4).

Seqs 074 and 104 repressed in drought exposed plants, were also decreased by cold and HL treatments in both cases. While Seq074 was not decreased by NaCl exposure, it decreased under salt treatment (Fig. 4).

3.5. Study of the behavior of some candidate genes in Arabidopsis subjected to drought and other abiotic stresses

Arabidopsis is widely used as a model plant to study stress and adaptation responses. Therefore the behavior of the orthologous genes to some of those identified by DD in *P. oleracea* was explored in *A. thaliana* under different stress conditions.

In *A. thaliana* AT3G12120 (the orthologous of Seq086, Table 2) encodes for Fatty Acid Desaturase 2 (FAD2) which is induced 7-fold after NaCl treatment (Fig. 5). Similarly, AT1G07770 (the orthologous of Seq039, Table 2), encodes a Ribosomal Protein S15A (RPS15A) which expression increased 12- and 20-fold in leaves of *Arabidopsis* NaCl treated and drought exposed plants, respectively (Fig. 5). In addition, the levels of the transcript of AT1G19870 (orthologous to Seq053, Table 2) encoding a IQ-domain 32 protein, increased 2.5-fold in leaves of mannitol treated plants with respect to C (Fig. 5). While the expression of AT1G19870 was not induced in drought stressed leaves (Fig. 5), its relative expression was induced 7-times in roots of drought treated plants (results not shown). AT1G01930 encodes a protein with a zinc finger domain. As its orthologous sequence in *P. oleracea* (Seq082), the levels of AT1G01930 were induced 4-fold by mannitol and 15-fold by drought and decreased to control levels after re-watering (Fig. 5).

Among DETs repressed by drought in *P. oleracea*, the expression of the gene orthologous to sequences 074 (Table 3) was explored in *A. thaliana*. AT2G24740 encodes a putative histone methyltransferase (SDG21 -SUVH8) which expression was significantly decreased in drought-stressed plants (Fig. 5), as in the case of *P. oleracea* plants (Seq074, Table 3). In addition, AT2G24740 was also significantly decreased in *A. thaliana* plants treated with NaCl (Fig. 5).

To identify genes regulated after the re-watering period, the relative expression of the orthologous genes of three sequences with differential expression in drought-recovered *P. oleracea* plants (Seq084, 0.0.1; and Seq002, 1.1.0) was investigated in *Arabidopsis*. In *A. thaliana* AT1G55340 and AT3G03880 encode proteins of unknown function (DUF1639), both sequences are homologous to Seq084 and were increased two- and three-fold, respectively in leaves of *A. thaliana* re-watered plants after drought stress with respect to controls, as it is the case of *P. oleracea*. On the other hand, AT2G36145 is the orthologs sequence of Seq002 and also encodes a protein of unknown function. QRT-PCR showed that AT2G36145 transcripts decreased after drought stressed plants were re-watered (Fig. 5).

4. Discussion

In order to identify genes involved in the drought adaptation and in the recovery after re-watering we used the DD technique. This method has been demonstrated as a successful means for the isolation of DETs for species where sequence data is not available

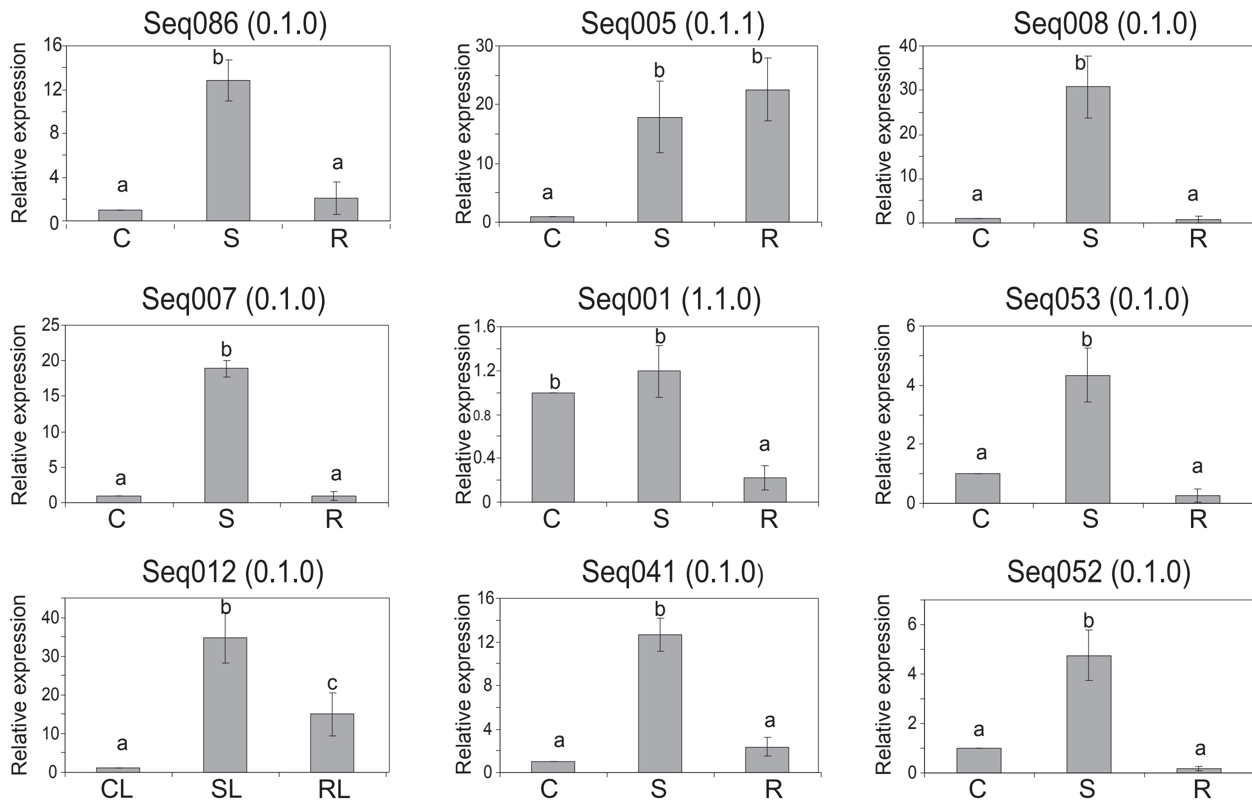

Fig. 3. Validation of differentially expressed unigenes by qRT-PCR. The relative-to-control level of accumulation of the DETs was determined in the different samples studied. Relative expression level indicated by black bars emphasized the presence-absent pattern of expression observed in the DD experiments. Gene expression levels were normalized against *P. oleracea* actin 1 gene. Values represent the mean ± SD. Bars with at least one equal letter mean no statistically significant difference ($p < 0.05$). Samples were taken as described in Fig. 1.

(Acevedo et al., 2013; Pessino et al., 2001; Bovie et al., 2004). About 7.2% of the transcriptome amplified exhibited changes (appearance/disappearance of a band) during drought and/or after recovery. This value is similar to those reported in other transcriptomic works studying the plant drought stress-responses. For example, about 10% of genes were differentially regulated in barley and about 4% in *Arabidopsis* under drought stress (Talame et al., 2007; Seki et al., 2002). Even though hundreds of genes that respond transcriptionally to drought have been identified by microarray analysis or other techniques in species like *Arabidopsis*, maize, rice and sorghum (Oono et al., 2003; Seki et al., 2007; Nakashima et al., 2009), the search for new genes involved in stress resistance or tolerance is still of great need. Moreover, the identification of genes contributing to the recovery after drought is of importance as well.

4.1. Many sequences with unknown identity were obtained

About half of the DETs rendered no homology to sequences published in different databases (Table S6). This is in agreement with the DD employed in this work which uses one-base anchored oligonucleotide primer to get cDNA transcripts and therefore, isolated sequences contain the full 3′UTR which is highly variable among species and in some cases only include a small part of the 3'coding region. Similarly, in the resurrection plant *Haberlea rhodopnesis*, 30% of the identified sequences by cDNA-AFPL during dehydration showed no match to any sequence in the searched databases (Georgieva et al., 2012). On the other hand, a study on *A. thaliana* under stress revealed many of the sequences identified by tilling array may not encode proteins and would function as non-protein-coding RNA molecules (Matsui et al., 2008).

4.2. Protein biosynthesis and regulation are main process affected by prolonged drought and re-watering

Translation is the biological function largest represented among the identified DETs, with four transcripts encoding ribosomal proteins (RPs) being induced (Seqs 007, 039, 054 and 121) and one repressed after drought (Seq 094; Fig. 2A). The composition of the ribosome determines which genes will be translated, and thus plays a key role in regulating gene expression during both stress response and adaptation. Changes in the expression of genes involved in protein biosynthesis has been related to stress tolerance in plants (Sormani et al., 2011), as up-regulation of transcripts encoding RPs has been shown after drought (Kawaguchi et al., 2004; Huang et al., 2007; Zheng et al., 2004; Gulyani and Khurana, 2011), cold (Machida et al., 2008) and salt stress exposure (Baldwin and Dombrowski, 2006; Wong et al., 2007). Here, the expression of transcripts encoding RPs S5 and L2 is modified only during drought and then reversed after re-watering. In contrast, that of S15A, L16p/L10e and S7 is kept in recovered plants. While the first genes could be involved in the translation of proteins implicated in the drought response and/or in CAM induction, the second group could constitute a type of cellular memory that maintains the expression of certain proteins that, in the case of a new period of stress, would allow the plant to respond quickly (Ding et al., 2013).

Transcripts encoding RPS15A were also significantly induced in response to NaCl in both *P. oleracea* (Seq039, Fig. 4) and in *A. thaliana* (AT1G07770, Fig. 5) constituting a strong candidate towards understanding the translational control in drought and salty conditions.

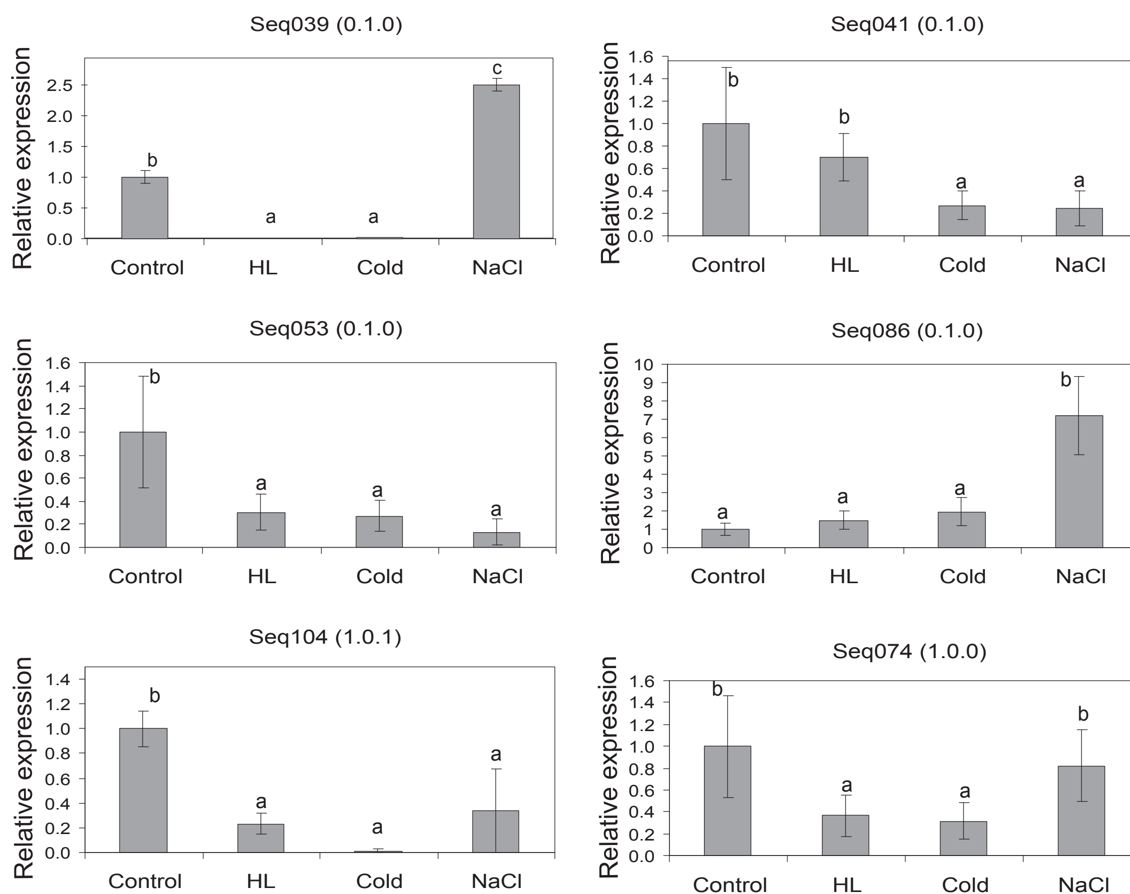

Fig. 4. Stress response analysis of some drought-responsive transcripts (DETs) in *P. oleracea*. The relative expression of some candidate genes was evaluated in cold-, high light (HL)- or salt-treated plants by qRT-PCR and expressed in relation to control plants. Gene expression levels were normalized against *P. oleracea* actin 1 gene. Values represent the mean ± SD. Bars with at least one equal letter mean no statistically significant difference ($p < 0.05$).

Interestingly, Seqs 094 and 121 would encode for RPs S7 and L2 located in the chloroplasts. Their plastidic occurrence reveals that changes in the translational machinery take place not only in the cytosol but also in the organelles, modifying the content of protein encoded in the plastids. During the salt stress induction of CAM in *Mesembryanthemum crystallinum*, changes in the plastidic genes encoding subunits of photosystem I (psaA and psaB) and II (psbA and psbD), and the large subunit of Rubisco (rbcL) were reported (Niewiadomska et al., 2010). In *P. oleracea* during the transition from C_4 to CAM due to drought, decreases in the plastidic encoded RbcL protein have been detected (Lara et al., 2004), confirming that changes in the proteins synthetized in the chloroplasts indeed take place.

Genes involved in protein modification are also represented in differential transcriptome (Table S1). Like the induction in drought-adapted and recovered plants (Table 2) of Seq004, which would encode the regulatory subunit of a serine/threonine phosphatase (PP2A) (Janssens and Goris, 2001). PP2A participates in diverse cellular functions including signaling. Regarding stress, changes in PP2A were observed in *A. thaliana* subjected to oxidative stress (Blakeslee et al., 2008), in *Oryza sativa* exposed to drought (Yu et al., 2003) and in ABA treated *Medicago sativa* (Tóth et al., 2000). Moreover, an enhanced drought tolerance was found in tobacco expressing a wheat PP2A catalytic subunit (Xu et al., 2007). Most of these studies were conducted after few hours or days following the stress stimulus. Here, the response was evaluated three weeks after withholding water, revealing that changes in the expression of PP2A are involved not only in the short but also in the long term response.

Another sequence involved in protein modification induced by drought in *P. oleracea* is Seq041 which encodes a kinase (BSK) that mediates the signal transduction from the brassinosteroids (BR) receptor kinase (Tang et al., 2008). Moreover, the action of PP2A, also induced by drought in *P. oleracea*, activates transcriptions factors BES1/BZR1 involved in the BR response (Hao et al., 2013). These data, together with the fact that the application of BR to plants has been used to increase tolerance to diverse abiotic stresses (Peleg and Blumwald, 2011), indicate a crosstalk between drought and BR signaling.

4.3. Genes involved lipid metabolism are also affected by drought and recovery

Lipid metabolism was also modified upon treatment. The induction of the sequence encoding a Ω-6-fatty acid desaturase 2 (FAD2; Seq086, GenBank Acc n° FJ472350, 0.1.0) was observed in both drought adapted (Table 2) and NaCl stressed plants (Fig. 4). Increases in different FADs were reported in *P. oleracea* in response to wounding, salt or cold, which correlated with increases up to two-fold in the levels of total fatty acid (FA) and in the relative amounts of unsaturated FA (Teixeira et al., 2010). This is not a minor detail, since *P. oleracea* is one of the plant presenting the highest levels of polyunsaturated FAs (PUFAs), particularly Ω-3 and -6 FA (Ezekwe et al., 1999). In planta, FADs fulfill different roles from modulation of membrane fluidity by changing the degree of FA unsaturations to the synthesis of trienoic acids synthetized that act

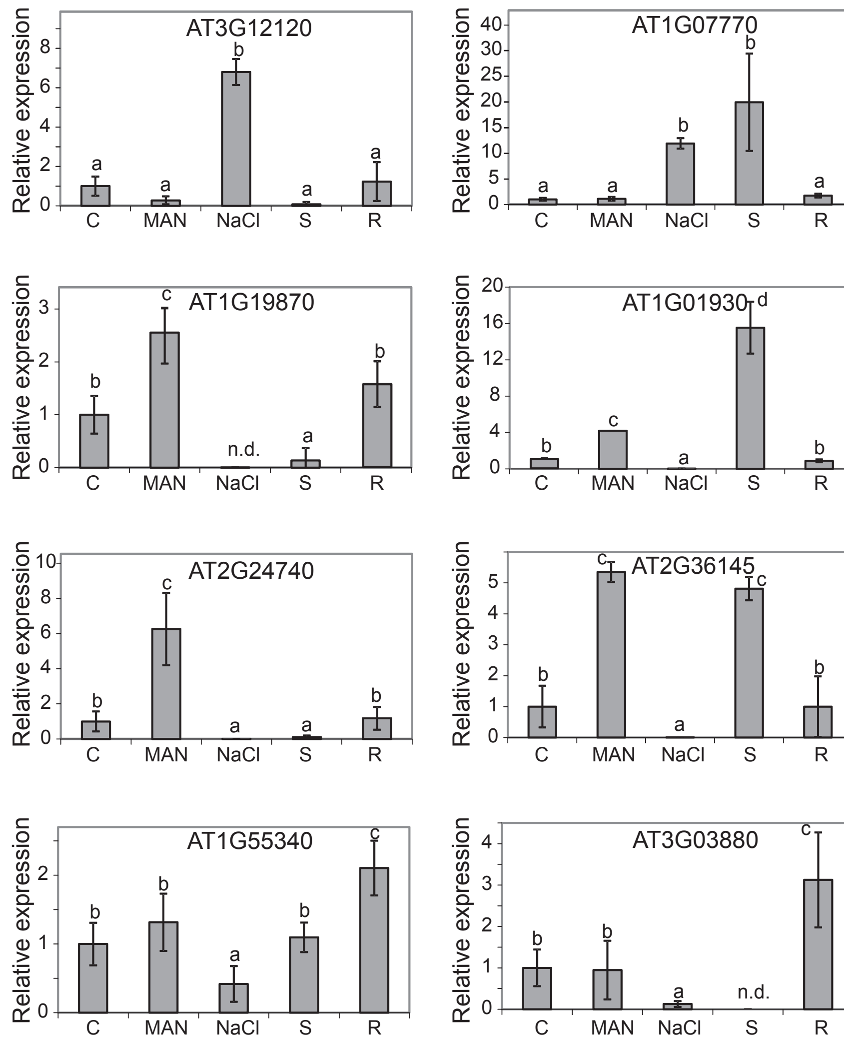

Fig. 5. Response to drought and other abiotic stresses of the orthologous genes of selected DETs in *A. thaliana*. The relative expression of *A. thaliana* orthologous genes of some candidate DETs was evaluated in *A. thaliana* subjected to different abiotic stress treatments (300 mM mannitol = MAN; 200 mM NaCl; drought stress by withholding water during 7 days = S, following rewatering during 2 days = R) and expressed in relation to control plants (C). The relative amount of the transcripts was assessed by qRT-PCR and normalized against *polyubiquitin 10* from *A. thaliana*. Values represent the mean ± SD. Bars with the same letters are not statically different ($p < 0.05$). n.d. = not detected.

as precursors for signaling molecules, such as traumatic acid and jasmonic acid (Turner et al., 2002). In *Arabidopsis*, FAD2 is essential for salt tolerance (Zhang et al., 2012), and its expression was also induced here by salt but not under drought (Fig. 5). Moreover, overexpression of FAD3 or FAD8 in tobacco resulted in increased tolerance to drought and osmotic stress (Zhang et al., 2005). Differences in the response of the gene to drought (as it is also the case of AT1G19870) may explain, at least in part, differences in the drought tolerance of both species.

On the other hand, drought repressed Seq016 (1.0.0, Table 3) which encodes a lysophosphatidylcholine acyltransferase 1 (classified in cellular response to water deprivation, Supplementary Table 7) (Lager et al., 2013). *Arabidopsis* mutants in this enzyme exhibited increased of very-long-chain FAs and modifications in the PUFAs in triacylglicerides (Bates et al., 2012). Rewatering *P. oleracea* drought adapted plants repressed Seq031 (1.1.0, Table 4); its orthologous in *Arabidopsis* is AT4G29010 and encodes an enoyl-CoA hydratase involved in FA β-oxidation. Collectively, these results indicate that lipid metabolism is significantly modified in *P. oleracea* subjected to drought and that FADs are major targets for manipulation to enhance tolerance to drought and salt.

4.4. Genes with unknown function is one of the largest biological process category represented which constitutes a source of potential candidates for stress response engineering and for research

Among DETs, some genes with poor annotation were identified and others, although annotated, do not possess function assigned yet. In fact, "unknown molecular function" was the most dominant category (Fig. 2). This is not surprising given that in species like *A. thaliana* which genome has been completed sequenced, about the 30–34% of its genome is composed by 'unknown' genes, based on different GO aspects (Lamesch et al., 2012). In addition, comparisons of the functional categories with the most differentially expressed genes in other extremophiles subjected to water deprivation; revealed that a large proportion of the *Thellungiella salsuginea (Eutrema salsugineum)* (48.9%; Lee et al., 2013) and *Populus euphratica* (20.8%; Tang et al., 2013) unigenes corresponded to genes without a clear classification or unknown genes. Similarly, in the *Physcomitrella patens*, a moss that highly tolerates desiccation, a 29.6% of the differentially proteins during dehydration were classified under the unclear on unknown function category (Wang et al., 2009). Moreover, in the case of the halophyte *M.*

crystallinum, when the CAM metabolism is stress-induced a 13.6% and 34.6% of the decreased and increased transcripts, respectively, felt under the categories of unclassified proteins or unknown gene products (Cushman et al., 2008). As pointed out before, it seems that genes up-regulated by stress conditions are not as prone to be investigated as genes regularly expressed under normal conditions (Kore-eda et al., 2004). Thus, there is a large number of genes contributing to water deprivation tolerance whose functions remain to be elucidated and deserve attention.

Particularly, regarding the DETs unified in this work, Seq082 was induced by drought (Table 3). Its orthologous in *Arabidopsis* (AT1G01930) encodes a protein containing a zinc finger domain and an ankyrin repeat-containing domain (IPR020683, http://www.ebi.ac.uk/interpro/entry/IPR020683;jsessionid=B308CD01ED30B7866E564712B21B47AC), one of the most common protein–protein interaction motifs in nature. The putative protein is predicted to be targeted to the nucleus, where it could regulate gene expression in drought adapted plants. In *Arabidopsis*, AT1G01930 transcripts increased fifteen-fold in drought stressed leaves and decreased after re-watering (Fig. 5), denoting the conserved function of this gene in both species. According to Nakabayashi et al. (2005), AT1G01930 is highly expressed in dry seeds and decreases after 6 h of seed imbibition.

Within sequences with unknown function modified only after re-watering, Seq084 was induced in leaves of *P. oleracea* (Table 4). Similarly in *A. thaliana*, transcripts of AT1G55340 (the orthologous to Seq084) were induced in leaves after rehydration (Fig. 5) and were detected in seeds only after 12 and 24 h after imbibition (Nakabayashi et al., 2005). AT1G55340 encodes a putative protein of unknown function of 23.5 kDa with a pI of 10.7 which would be targeted to the nucleus. The protein (DUF1639) (http://pfam.xfam.org/family/PF07797) has an approximately 50 residue region, with a highly basic 5 amino-acid stretch towards its center, which is found in a number of sequences derived from hypothetical plant proteins. Blast searches (not shown) reveal that the putative protein is limited to the plant kingdom. Thus, Seq084 and Seq082 from *P. oleracea* and their orthologous AT1G01930 and AT1G55340 are involved in the response to hydration, in both embedded seeds and leaves of re-watered plants. Further studies are needed to establish the roles of the putative proteins that could be involved in the regulation of the gene expression by interacting with other proteins. Comparable mechanisms to those involved in seed desiccation/imbibition would be involved in the adaptation of leaves to water deficit and water supply after drought stress. In this respect, transcriptome analysis of *Craterostigma plantagineum* (resurrection plant) reveals that this vascular plant engages desiccation mechanisms usually used in seeds and pollen (Suarez Rodriguez et al., 2010).

Another sequence encoding a putative protein of unknown function is Seq002 which is expressed in control and drought adapted *P. oleracea* leaves (1.1.0). It's orthologous sequence in Arabidopsis is AT2G36145, which is induced by drought (Fig. 5). Again, differences in the expression pattern of the orthologous genes could account for differences in the drought tolerance of both species. The expression of Seq002 in control plants could be part of a priming strategy to cope with drought of *P. oleracea* plants.

Finally, it is important to note that sequences encoding enzymes involved in carbon metabolism were not identified during the CAM induction followed by reversion to C_4 studied here by DD, a technique which is based in the analysis transcripts that are completely induced or repressed. Enzymes participating in CAM metabolism (stressed plants) involved either in carbon fixation (i.e. NAD- and NADP malic enzymes, RuBisCO, Phosphoenolpyruvate carboxykinase) or in further metabolization (i.e. Phosphofructokinases, aldolase) are already present in leaves performing C_4 photosynthesis (control plants). Previous works at protein level have shown that they change their relative amount and/or regulatory properties (i.e. Phosphoenolpyruvate carboxylase is differentially phosphorylated) during the C_4-CAM transition induced by drought, but they are not *de novo* synthesized (Lara et al., 2003, 2004).

4.5. Concluding remarks

The available data confirm that many of the identified genes involved in the drought response of *P. oleracea*, like *RPS15A*, *RPL2*, and *FAD2* also participate in the stress response in other species, ratifying that they are strong candidates for engineering. In addition, unknown yet gene candidates identified here (such as AT1G01930) might also be involved the drought adaptation. Even though the function of many of the DETs identified in this work remains to be elucidated, it is evident that they also play a role in the response of plants to other abiotic stresses (due to some shared consequences, e.g. altered relative water content, osmotic stress). Results regarding stress behavior of some genes (Figs. 4 and 5) indicate that some of them show a conserved expression status in *A. thaliana* and in *P. oleracea*, while for others the response differs between these species. The knowledge gained in this study on differential expressed genes under drought and after recovery adds to our understanding of stress and recovery responses and provides some genes as potential candidates for engineering drought tolerance in cultivated plants and for further research. We are currently conducting the characterization of the products of some of the candidates genes identified in this work.

Author contributions

C.S.A. and M.V. L. conceived the project. R.M.D, A.T. and M.I.C. performed the experiments. R.M.D, C.S.A. and M.V.L. analyzed the data. M.V.L. wrote the article.

Acknowledgments

This study was supported by Agencia Nacional de Promoción Científica y Tecnológica to MVL (PICT N° 2010–0187 and N° 2012–1376). CSA and MVL are members of the Researcher Career of Argentine National Research Council (CONICET) and RMD was a fellow of the same institution.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.plaphy.2015.02.023.

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