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Research article

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

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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.

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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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.