

Identification of desiccation-regulated genes by cDNA-AFLP in *Haberlea rhodopensis*: a resurrection plant

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Abstract The Balkan endemic plant species *Haberlea rhodopensis* belongs to the group of resurrection plants. The members of this small group of angiosperms tolerate extreme dehydration of their vegetative tissues, which are able to recover very rapidly upon rehydration. In this respect, resurrection plants are a unique model intensively studied to reveal the secrets of desiccation tolerance. To date, the knowledge on the molecular biology of *H. rhodopensis* is very limited. Here, we report on the application of a cDNA-AFLP analysis to examine gene expression in leaves of *Haberlea* during dehydration. Twenty transcripts among 33 sequenced cDNA fragments appear to be involved in energy metabolism, transport, cell-wall biogenesis, signal transduction, or are probably transcription regulators according to their putative function. Expression patterns of two up-regulated (*HrhDR8*, *HrhDR35*) and two down-regulated (*HrhDR6*, *HrhDR25*) transcripts were verified by sqRT-PCR analysis at different stages of water stress. The results demonstrated that two up-regulated transcripts *HrhDR8* and

HrhDR35 encoding putative succinate-dehydrogenase and xyloglucan endotransglucosylase/hydrolase (XTH), respectively, were induced during early stage of dehydration, persist in desiccated state, and subsequent rehydration of *Haberlea*. Their possible involvement in drought tolerance is discussed.

Keywords cDNA-AFLP · Dehydration · Gene expression · *Haberlea rhodopensis* · Rehydration · Resurrection plants

Introduction

Plants have developed adaptive strategies to various unfavorable environmental conditions (Ramanjulu and Bartels 2002). Drought tolerance of plants is one of the most exploited areas that focus the efforts of plant biologists around the world. The development of many new technologies to study genes, proteins, and metabolites such as microarrays analysis, mass spectrometry, etc. allows more detailed characterization of the response to drought stress. On the other hand, the critical step in this kind of work is to have a good model to study. In most cases, plants are not able to survive prolonged water deficit or to withstand the drop of their relative water content (RWC) below 20–50% (Toldi et al. 2009). However, a small group of higher plants, called “resurrection” plants, are extremely desiccation-tolerant. Their vegetative organs can reach about 4–13% RWC and to remain dried for several years, upon rehydration, the plants are able to resume normal growth and metabolism within hours (Le and McQueen-Mason 2006). These particular features of resurrection plants make them a unique system to study desiccation tolerance. Studies with resurrection plants highlight various mechanisms involved in cell membranes and organelles

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protection during desiccation, and repair of dehydration-induced damage upon rehydration. The role of key proteins [e.g. late embryogenesis abundant proteins (LEAs) and transcription factors], sugars and compatible solutes in the protection and maintenance of cellular integrity, the changes in cell wall and lipid composition, and the establishment of antioxidant systems have been examined (Le and McQueen-Mason 2006). It was shown that the mechanisms of drought tolerance involve complex changes of gene expression patterns and induction or up- and down-regulation of hundreds of dehydration-regulated genes (Bockel et al. 1998; Collett et al. 2004). A cDNA library constructed from dehydrated microphyll fronds of the resurrection plant *Selaginella lepidophylla* has been used to generate an expressed sequence tag (EST) database (Iturriaga et al. 2006). Small-scale microarray analysis of *Tortura ruralis*, a bryophyte model plant for genomic investigations, showed that only 29% of the desiccation-up-regulated cDNAs had significant similarity to previously identified nucleotide and/or peptide sequences (Oliver et al. 2005). The same approach to *Xerophyta humilis*, an indigenous South African resurrection plant, has revealed that dehydration-up-regulated cDNAs include known stress-responsive genes encoding metallothioneins, galactinol synthases, an aldose reductase and a glyoxalase (Collett et al. 2004). Large number of cDNAs encoding LEAs, dehydrins, and desiccation-related proteins have also been identified, suggesting that proteins providing mechanical and antioxidant protection against water loss are abundant in desiccated *X. humilis* leaf tissue. Several cDNAs representing drought-induced transcripts in *Sporobolus stapfianus* encode proteins related to LEAs, pore-like protein and UDP-glucosyl-transferase (Le et al. 2007). A transcriptomic analysis was performed recently for *Craterostigma plantagineum* using pyrosequencing technology. Identification of 15,000 putative transcripts that mapped to UniProt protein database have been reported (Rodriguez et al. 2010). The study highlighted several pathways such as those required for vitamin K and thiamin biosynthesis as specifically regulated by drought.

Haberlea rhodopensis is a rare endemic and preglacial relict plant species growing on the Balkan Peninsula and a world record-holder in desiccation tolerance (Ganchev 1950). Most of the research so far has been focused on physiological and biochemical mechanisms involved in desiccation tolerance. Ultrastructural changes of chloroplasts (Markovska et al. 1994, 1995), carbohydrate content (Müller et al. 1997), lipid and sterol composition in leaves (Stefanov et al. 1992), photosynthetic and antioxidant activity during dehydration and rehydration have been examined (Peeva and Maslenkova 2004; Georgieva et al. 2005, 2007; Yahubyan et al. 2009; Djilianov et al. 2011).

Information on cDNA sequences of genes involved in the response of *Haberlea* to desiccation, their expression, regulation and molecular characterization is still lacking.

The cDNA-amplified fragment length polymorphism (cDNA-AFLP) is an efficient, sensitive, and reproducible technology for isolation of differentially expressed genes (Bachem et al. 1996; Ditt et al. 2001). It does not require prior sequence information and in contrast to microarrays, often enables identification of novel sequences even in well annotated species. Novel unknown sequences were discovered in *Arabidopsis* by cDNA-AFLP analysis of seed germination (de Diego et al. 2006).

Here, we report application of the cDNA-AFLP technique for isolation of genes, differentially expressed in leaves of *H. rhodopensis* in response to desiccation. Thirty-three transcript-derived fragments (TDFs) corresponding to distinct desiccation-regulated cDNAs were isolated. We do believe that the information provided in the present study will help to better understand the mechanisms of desiccation tolerance in *Haberlea* and will contribute to further genomics and transcriptomics studies of resurrection plants.

Materials and methods

Plant material, treatments and sampling

Haberlea plants were propagated in vitro as described earlier (Djilianov et al. 2005) and cultured in plant growth chamber at 22°C with a long day (16 h light) photoperiod and 75 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity. Well-developed rooted plants (about 3 months in culture) were taken out of culture vessels and subjected to dehydration and rehydration treatments as previously reported (Djilianov et al. 2011). Leaves of plants sampled at various time points of dehydration and rehydration and untreated control plants were cut, quickly frozen in liquid nitrogen, and stored at -70°C for RNA extraction.

RNA extraction

Total RNA was extracted from the leaves, sampled at indicated time points, using RNeasy Plant Mini Kit (Qiagen, Germany). The RNA quality, integrity, and quantity were determined by running 2 μl of total RNA in a formamide denaturing gel and spectrophotometrically at 260 nm using NanoVue spectrophotometer (GE Healthcare, UK).

cDNA-AFLP analysis

To identify genes responsive to dehydration, cDNA-AFLP analysis was performed on cDNA from leaves of fully

hydrated (control) and dehydrated (treated) intact plants of *Haberlea*. To identify cDNAs that are differentially expressed total RNAs isolated from several time points were pooled together by mixing equal amounts in three “bulks”—(I) early drying, (II) late drying, (III) full desiccation state. The “bulk I” contained pooled RNA from 1, 2, and 3 h of drying representing the RWCs between 73 and 36%, “bulk II”—RNA from 6, 18, and 24 h of drying (RWC of 25–13%) and “bulk III”—RNAs from 72, 168, and 236 h of constant desiccation state (RWC about 12–13%). The cDNA-AFLP patterns of the “bulks” were compared to those of the untreated control RNA (with RWC about 93%). Poly (A)⁺ RNA was purified from the “bulks” of total RNA using the GeneElute kit (Sigma, St Louis, USA) according to the manufacturer’s instructions. The first strand cDNA was synthesized using an oligo (dT)₁₈ primer and M-MuLV Reverse Transcriptase (Fermentas) (1 h at 37°C). The second strand was synthesized at 16°C over night with DNA Polymerase I and DNA ligase in the presence of RNase H (Fermentas). The quantity of double-stranded (ds) cDNA was determined using DyNA Quant 200 fluorometer (Hoefer) fitted with a capillary adaptor kit (Amersham), following the manufacturer’s instructions. Amount of 250 ng ds cDNA was subjected to AFLP template production (Vos et al. 1995) using the restriction enzymes *EcoRI* (Fermentas) and *TruII* (Fermentas). *EcoAd* (5 μM) and *TruIIAd* (50 μM) adaptors were annealed in DEPC water from oligonucleotides *Eco-ad1*, *Eco-ad2* and *TruII-ad1*, *TruII-ad2*, respectively. Adaptors were ligated with T4-DNA ligase (Fermentas) over night at 16°C and 5 μl of ×10 diluted ligase mix was used for preamplification with *Eco-P* (10 μM) and *TruII-P* (10 μM) primers in 50 μl reaction volume. Preamplification reaction was run for 20 cycles at 95°C for 30 s, 56°C for 1 min and 72°C for 1 min and terminated at 72°C for 5 min. The selective AFLP reactions were made with 5 μl of ×10 diluted preamplified solutions, *EcoY* (10 μM) and *TruIIIX* (10 μM) primers. After initial denaturation (94°C for 2 min), 11 cycles were performed with touchdown annealing (94°C for 30 s, 65–56°C in 0.7°C steps for 30 s, 72°C for 1 min) followed by 23 cycles (94°C for 30 s, 56°C for 30 s, 72°C for 1 min). For PCR, 46 combinations of two or three base extensions (denoted as NN or NNN) were used. All oligonucleotides were synthesized by Microsynth (Balgach, Switzerland) and amplifications were performed using Quanta Biotech thermocycler (UK). Oligonucleotide sequences are listed in Table 1. Selective amplification products were separated on a sequencing polyacrylamide gel (6% bisacrylamide, 7 M urea, 1× TBE) using Hoefer electrophoresis unit (Hoefer, Inc., Holliston, MA, USA). DNA fragments were visualized by silver-staining according to Creste et al. (2001).

Isolation and sequencing of TDFs

The differentially expressed TDFs were excised from the gels with a surgical blade, placed in a 1.5 ml microtube containing 50 μl of water and incubated at 60°C for 30 min. Amplifications were performed with the same set of selective primers and conditions used for the selective amplification, except that a 35 cycles and a final 5 min extension were used. The PCR products were resolved in a 2% 1× TAE-agarose gel, and each single band was isolated and purified using illustra GFX PCR DNA and Gel band purification kit (GE Healthcare) and sent for direct sequencing using the selective amplification primers (Table 2). The TDFs were sequenced by Eurofins MWG Operon (Ebersberg, Germany).

Gene function analysis

Database searches were performed using the BLAST Network Service at NCBI, (<http://www.ncbi.nlm.nih.gov/BLAST>). The sequence of each TDF was searched against all sequences in the non-redundant databases using the BLASTX and BLASTN algorithms with the default parameters and cutoff *E* values equal or $<1e^{-5}$. Possible homology of the TDFs to known conserved domains was identified by BlastP against Conserved Domain Database CDD (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) by using translated open reading frame (ORF) as a query. To classify the identified homologues into functional groups, orthologous genes in Arabidopsis were identified by BlastP at TAIR database (<http://www.arabidopsis.org/wublast/index2.jsp>) with a significance threshold of $E \leq 1e^{-5}$ using the protein sequences of the TDF homologues identified in the first step, as template. The functional groups were inferred from gene ontology (GO) annotations of the Arabidopsis orthologs.

Semi-quantitative reverse transcription polymerase chain reaction (sqRT-PCR)

Total RNA was prepared using RNeasy Plant Mini Kit (Qiagen) then treated with DNAaseI (Fermentas). Reverse transcription was performed on 1 μg of total RNA at 37°C for 1 h in final reaction mixture (20 μl) containing 0.5 μg oligo (dT)₁₈ primer, 1× M-MuLV reaction buffer, 1 mM dNTPs, 20 units of Ribonuclease inhibitor and 40 units of M-MuLV reverse transcriptase (Fermentas). PCR reactions were performed in 25 μl with 1× PCR buffer (USB), 0.2 mM dNTPs, 0.4 μM of each primer, 1.25 U Taq DNA polymerase (USB), and 1 μl of ×10 diluted cDNA solution. The primers used are listed in Table 3. Primers were designed from the sequences of the cDNA fragments using the Primer3.0 web resource (<http://frodo.wi.mit.edu/cgi-bin/primer3/primer3www.cgi>). Amplifications were carried out

Table 1 Oligonucleotides used in cDNA-AFLP analysis

Adaptors	Preselective primers	Selective primers
Eco-ad1: 5'-CTCGTAGACTGCGTACC-3'	<i>Eco</i> -P: 5'-GACTGCGTACCAATTC-3'	<i>Eco</i> Y: 5'-GACTGCGTACCAATTCNN (or)/NNN-3'
Eco-ad2: 5'-AATTGGTACGCAGTCTAC-3'	<i>Tru</i> II-P: 5'-GATGAGTCCTGAGTAA-3'	<i>Tru</i> IIIX: 5'-GATGAGTCCTGAGTAANN (or)/NNN-3'
<i>Tru</i> II-ad1: 5'-GACGATGAGTCCTGAG-3'		
<i>Tru</i> II-ad2: 5'-TACTCAGGACTCAT-3'		N represents A, T, G, or C

Table 2 Selective primer combinations used for TDFs isolation and sequencing

<i>Eco</i> RI	<i>Tru</i> II	TDF	
AC	CA	<i>Hrh</i> DR19, <i>Hrh</i> DR22	
	GC	<i>Hrh</i> DR2, <i>Hrh</i> DR3, <i>Hrh</i> DR4, <i>Hrh</i> DR5	
	TC	<i>Hrh</i> DR41	
	CAG	<i>Hrh</i> DR8, <i>Hrh</i> DR9, <i>Hrh</i> DR10, <i>Hrh</i> DR11, <i>Hrh</i> DR12, <i>Hrh</i> DR14, <i>Hrh</i> DR15	
	ACC	<i>Hrh</i> DR6, <i>Hrh</i> DR7, <i>Hrh</i> DR13	
	CCA	<i>Hrh</i> DR16, <i>Hrh</i> DR17	
	AAG	CA	<i>Hrh</i> DR26, <i>Hrh</i> DR30
		TA	<i>Hrh</i> DR27, <i>Hrh</i> DR28, <i>Hrh</i> DR38
		GC	<i>Hrh</i> DR37
		CCA	<i>Hrh</i> DR25, <i>Hrh</i> DR35
ACC		<i>Hrh</i> DR23, <i>Hrh</i> DR24, <i>Hrh</i> DR33	
ATA	CAG	<i>Hrh</i> DR34	
	CTA	<i>Hrh</i> DR39	
	CCA	<i>Hrh</i> DR40	

Table 3 Primers used for sqRT-PCR analysis of selected TDFs and an actin gene as internal control

TDF	Forward primer (5'–3')	Reverse primer (5'–3')	T_a (°C)	Size of amplicon (bp)
<i>Hrh</i> DR 25	AGGCTTGTGGAGTCCCCTAC	CTTGCTCAGGCTTCCTTC	57	116
<i>Hrh</i> DR 6	CTTGGCCAGTAATGCTGTCA	TGGGGTCAGTAGTGCCTTTC	55	173
<i>Hrh</i> DR 8	CACGCTTGATCGTTGTAGGA	GGACTAAGCCTAACCGAACG	55	164
<i>Hrh</i> DR 35	TACCCGACAATCAACCGATG	ATGCATTCTTGCCGTTCAA	50	201
<i>Hrh</i> Actin	ACACCCCGTGCTTTAACTG	ACGACCAGCAAGATCCAGAC	53	250

with a Quanta Biotech thermocycler with the following cycling parameters: preliminary denaturation (3 min, 94°C), then denaturation (45 s, 94°C), annealing (45 s, 50–57°C), extension (1 min, 72°C) and final extension (5 min, 72°C). sqRT-PCR was carried out for 32, 35, or 37 cycles to follow amplification kinetics and was repeated twice for the reproducibility of the results. As internal standard for expression we used amplified fragment of *Haberlea* actin gene (*Hrh*Actin, GenBankAccn GT270756).

The same “control” and “treated” RNA bulks from cDNA-AFLP analysis were used as templates for sqRT-PCR with gene specific primers to confirm the expression patterns. For selected differentially expressed genes, more

detailed sqRT-PCR was performed at every time point of desiccation–recovery process.

Results

Identification of desiccation-regulated genes

To identify genes responsive to desiccation, cDNA-AFLP analysis was performed on poly (A)⁺ RNA isolated from leaves of *H. rhodopensis* plants subjected to dehydration by a non-radioactive procedure. A representative example of a cDNA-AFLP expression profile after polyacrylamide gel

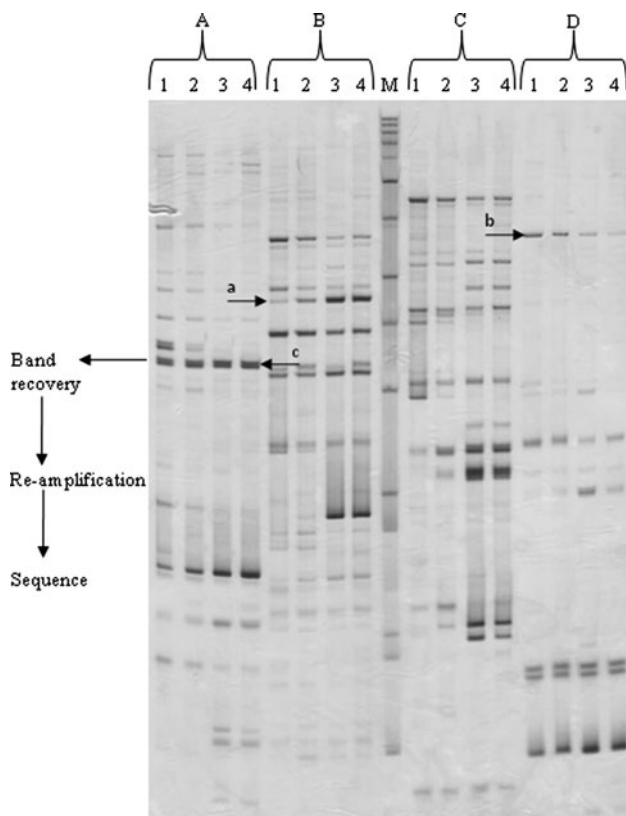


Fig. 1 cDNA-AFLP display of gene expression at different stage of dehydration in *Haberlea rhodopensis*. The horizontal arrows point out on the three main gene expression patterns: up-regulated (a), down-regulated (b) and constitutively expressed (c). Lanes 1 fully hydrated control plants, 2 bulk I: early drying, 3 bulk II: late drying, 4 bulk III: completely desiccated plants; A, B, C and D presents profiles of cDNAs obtained by using of different primer combinations, M marker 50 bp DNA ladder

electrophoresis and silver staining is shown on Fig. 1. Reproducibility of the technique was verified with several primer combinations on independent cDNA synthesis and PCR amplification. More than 257 TDFs were generated, with a size range 100–500 bp and the intensity of all bands were compared among treatments and with untreated control plants. The TDFs were classified according to their expression patterns (Fig. 1; Table 4).

Thirty-three randomly selected TDFs with different type of expression were sequenced and the putative gene function for each sequence was predicted from homology obtained by BLAST searches (Tables 4, 5). The clones corresponding to different TDFs were named *HrhDR* (*H. rhodopensis* desiccation-regulated). Most of the homologous proteins were identified by BlastX against non-redundant (nr) protein database, indicating that the respective TDFs are located in the translated part of the transcript (Table 4). Since, many of the homologous proteins, identified by Blast were poorly annotated (Table 4), the putative Arabidopsis orthologs were identified by

BlastP search against the TAIR protein database using each TDF homologous protein as query and the functional category was inferred from the TAIR GO annotation of each ortholog (Table 5). Twenty fragments showed significant homology to function-known genes in the databases. Four other sequences showed homology to DNA or protein sequences with unknown function. Nine of the 33 sequences showed no match to any sequence in the searched databases (Table 4).

The 20 TDFs that showed homology to function-known genes could be grouped into five functional categories - cellular metabolism and energy, cell-wall biogenesis, signal transduction, transport, and expression regulator (Fig. 2). Two out of four function-known TDFs early induced upon dehydration were probably involved in signal transduction (*HrhDR19* and *HrhDR24*) (Table 5). One TDF was related to cellular metabolism and energy (*HrhDR8*). One cDNA fragment was involved in the metabolism of cell wall components, like xyloglucans (*HrhDR35*). Four out of nine late up-regulated TDFs were with possible role in signal transduction (*HrhDR9* and *HrhDR37*) and protein transport (*HrhDR15* and *HrhDR27*). Seven early repressed gene transcript fragments were classified as involved in cellular metabolism (*HrhDR4*, *HrhDR6*, *HrhDR12*, and *HrhDR25*), transport (*HrhDR11* and *HrhDR14*) and *HrhDR26* transcript was encoding a transcriptional regulator. In the late phase of dehydration, down-regulated cDNA fragments related to cellular metabolism as homologues to cytochrome genes (*HrhDR33* and *HrhDR41*) and serine-type peptidase DegP9 (*HrhDR38*) were identified. Two transcript fragments (*HrhDR2* and *HrhDR3*) involved in metabolism were constitutively expressed. Among the TDFs, homologous to unknown or predicted genes two TDFs (*HrhDR34* and *HrhDR39*) were late up-regulated. Other two transcript fragments (*HrhDR28* and *HrhDR40*) were early repressed. Among nine no match TDFs, one transcript was early up-regulated, three transcripts—late up-regulated, three transcripts—early down-regulated and two transcripts were late down-regulated (Table 4).

Verification of expression patterns of selected desiccation-regulated genes using sqRT-PCR

To validate our cDNA-AFLP results two up-regulated—*HrhDR8* and *HrhDR35* and two down-regulated—*HrhDR6* and *HrhDR25* TDFs were randomly selected to perform sqRT-PCR with transcript specific primers. According to the BlastX results *HrhDR8* transcript fragment shared 38 out of 46 encoded amino acid residues (83%) with the succinate dehydrogenase (SDH) protein from *Ricinus communis* (Table 4). Search in the CDD database with the translated *HrhDR8* ORF as query, revealed that the fragment encoded part of the SDH conserved domain (Online

Table 4 Homology analysis of the transcript-derived fragments (TDFs) to gene sequences in the database using BLAST algorithm along with their expression

TDF	Length (bp)	GenBank accn	Homolog accn	Blast	Identity	E value	Organism and putative homolog annotation
Early up-regulated							
<i>Hr</i> hDR19	140	GT270774	AAB47968.1	blastx	36/39 (92%)	3e-17	<i>Arabidopsis thaliana</i> protein kinase (ADK1)
<i>Hr</i> hDR8	232	GT270764	XP_002534311.1	blastx	38/46 (83%)	4e-19	<i>Ricinus communis</i> succinate dehydrogenase, putative
<i>Hr</i> hDR35	231	GT270785	ACQ85271.1	blastx	40/50 (80%)	5e-24	<i>Musa acuminata</i> xyloglucan endotransglucosylase/hydrolase
<i>Hr</i> hDR24	183	GT270777	ADG96403.1	blastx	34/57 (60%)	4e-17	<i>Olea europaea</i> S-locus glycoprotein
<i>Hr</i> hDR5	142	GT270757	No match				
Late up-regulated							
<i>Hr</i> hDR27	327	GT270780	CAN62174.1	blastx	57/102 (56%)	4e-11	<i>V. vinifera</i> hypothetical protein,
<i>Hr</i> hDR37	214	GT270786	XP_002514582.1	blastx	44/64 (69%)	4e-19	<i>R. communis</i> inositol or phosphatidylinositol kinase, putative
<i>Hr</i> hDR39	140	GT270788	ACQ63637.1	blastx	46/46 (100%)	2e-23	<i>Streptomyces</i> sp. sugar O-methyltransferase
<i>Hr</i> hDR34	174	GT270784	XP_002297764.1	blastx	32/49 (65%)	5e-15	<i>Populus trichocarpa</i> predicted protein
<i>Hr</i> hDR15	101	GT270771	AK323365.1	blastm	78/86 (91%)	1e-06	<i>Solanum lycopersicum</i> , cDNA, clone: LEFL1055BC03
<i>Hr</i> hDR9	157	GT270765	DQ792736	blastm	59/72 (82%)	5e-08	<i>Glycine max</i> transcription factor bZIP104-like (bZIP104) mRNA
<i>Hr</i> hDR22	138	GT270761	No match				
<i>Hr</i> hDR23	118	GT270776	No match				
<i>Hr</i> hDR30	178	GT270782	No match				
Early down-regulated							
<i>Hr</i> hDR11	137	GT270767	XP_002284552.1	blastx	30/43 (70%)	8e-11	<i>V. vinifera</i> Ca ²⁺ -transporting ATPase, hypothetical
<i>Hr</i> hDR28	312	GT270781	XP_002313437.1	blastx	68/102 (67%)	8e-42	<i>P. trichocarpa</i> predicted protein
<i>Hr</i> hDR26	150	GT270779	XP_002271519.1	blastx	42/49 (86%)	7e-22	<i>V. vinifera</i> hypothetical protein isoform 2
<i>Hr</i> hDR6	222	GT270762	XP_002327232.1	blastx	61/66 (92%)	2e-22	<i>P. trichocarpa</i> predicted protein
<i>Hr</i> hDR25	145	GT270778	XP_002521339.1	blastx	33/45 (73%)	2e-13	<i>R. communis</i> conserved hypothetical protein
<i>Hr</i> hDR12	166	GT270768	XP_002528637.1	blastx	19/32 (59%)	7e-06	<i>R. communis</i> ATPP2-A13, putative
<i>Hr</i> hDR40	178	GT270787	CAJ33098.1	blastx	51/58 (88%)	2e-23	<i>Lactobacillus mali</i> phenylalanyl-tRNA synthase alpha subunit
<i>Hr</i> hDR4	149	GT270760	AM411650	blastm	46/52 (88%)	1e-08	<i>Petunia hybrida</i> mRNA for obtusifolior-14-demethylase (cyp51 gene)
<i>Hr</i> hDR14	206	GT270770	AB026909	blastm	52/64 (81%)	1e-05	<i>Ipomoea batatas</i> FI-delta-1 gene for mitochondrial FI-ATP synthase delta subunit
<i>Hr</i> hDR7	196	GT270763	No match				
<i>Hr</i> hDR13	131	GT270769	No match				
<i>Hr</i> hDR17	190	GT270773	No match				
Late down-regulated							
<i>Hr</i> hDR33	283	GT270783	XP_002275806.1	blastx	75/85 (88%)	1e-43	<i>V. vinifera</i> hypothetical protein
<i>Hr</i> hDR41	130	GT270789	XP_002334134.1	blastx	25/28 (89%)	1e-09	<i>P. trichocarpa</i> cytochrome P450
<i>Hr</i> hDR38	142	GT270775	CB115070.3	blastx	36/39 (92%)	2e-16	<i>V. vinifera</i> unnamed protein product
<i>Hr</i> hDR10	245	GT270766	No match				
<i>Hr</i> hDR16	126	GT270772	No match				

Table 4 continued

TDF	Length (bp)	GenBank accn	Homolog accn	Blast	Identity	E value	Organism and putative homolog annotation
Constitutively expressed							
<i>HrhDR2</i>	275	GT270758	CBI30854.3	blastx	46/62 (74%)	1e-21	<i>V. vinifera</i> unnamed protein product
<i>HrhDR3</i>	231	GT270759	XM_002275247	blastn	60/75 (80%)	4e-06	<i>V. vinifera</i> hypothetical protein

Transcripts induced up to 3 h of dehydration were classified as early up-regulated, after 6 h late up-regulated, TDFs repressed up to 3 h early down-regulated, after 6 h late down-regulated, TDFs with constitutive expression constitutively expressed

Resource 2). The translated *HrhDR35* TDF showed 80% identity to xyloglucan endotransglucosylase/hydrolase (XTH) protein from *Musa acuminata*. The CDD database search revealed that the homology of the translated *HrhDR35* ORF was located in the conserved domain of glycosyl hydrolase 16 superfamily including xyloglucan endotransglucosylases (Online Resource 3). The translated *HrhDR6* transcript fragment was 92% identical to *Populus trichocarpa* predicted protein which shared 75% identity with the Arabidopsis ortholog AT1G34430 (Tables 4, 5). Blast analysis of the translated *HrhDR6* ORF against CDD database showed homology to the conserved catalytic domain of 2-oxoacid dehydrogenase family (Online Resource 1). The translated ORF of the *HrhDR25* cDNA fragment was 73% identical (33 out of 45 amino acids) to *R. communis* hypothetical protein that shared 57% identity (69% similarity) with Arabidopsis ortholog AT1G72640 (Tables 4, 5). The expression profiles obtained by sqRT-PCR confirmed the results for the respective TDFs obtained by the cDNA-AFLP method (Fig. 3).

Expression of selected desiccation-regulated transcripts during dehydration/rehydration cycle

Expression patterns of the above mentioned TDFs were followed at various time points of desiccation/rehydration cycle by sqRT-PCR (Fig. 4). The presence of 164 bp mRNA transcript encoded by *HrhDR8* gene was detected at low levels in control and 1 h dehydrated leaf tissue (Fig. 4a). The level of expression of *HrhDR8* gene increased at 3 h drought stress. Transcripts were abundant during severe stress and after rehydration. The expression of 201 bp *HrhDR35* mRNA transcript was induced after 1 h of dehydration and reached its maximum at 6 h of water stress (Fig. 4b). *HrhDR35* transcript abundance declined slightly after 24 h of recovery.

Other two transcripts 173 bp *HrhDR6* and 116 bp *HrhDR25* showed gradual reduction of their expression during the time course of dehydration (Fig. 4c, d). After 6 and 24 h of re-watering the expression increased rapidly for both transcripts and reached the levels in control plants.

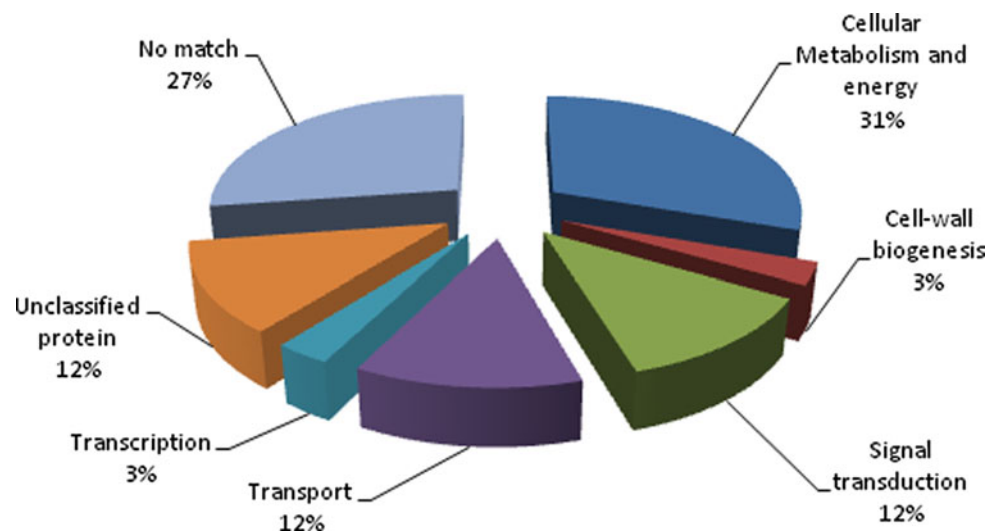
Discussion

The present study is focused on the elucidation of genes regulated by desiccation in resurrection plant *H. rhodopensis*. We applied a cDNA-AFLP technique, which have already been proven as a successful method for isolation of differentially expressed genes in many plant species like rice (Mao et al. 2004), tobacco (Bove et al. 2005), pine (Dubos and Plomion 2003), and aspen (van Raemdonck et al. 2005). Our investigation could be regarded as a first

Table 5 Functional classification of the identified in *Haberlea* TDF homologs based on the GO annotation in *Arabidopsis*

Functional group	TDF	Homolog Accn	TAIR putative ortholog	Identity	E value	Putative Arabidopsis ortholog annotation
Early up-regulated						
Signal transduction	<i>HrhDR19</i>	AAB47968.1	AT1G03930.1	301/309 (97%)	1.4e-164	ADK1, CKL9ALPHA, CKL9BETA dual specificity kinase 1
	<i>HrhDR24</i>	ADG96403.1	AT4G27290.1	197/409 (48%)	1.1e-111	S-locus lectin protein kinase family protein
Metabolism	<i>HrhDR8</i>	XP_002534311.1	AT5G65165.1	203/277 (73%)	1.2e-117	SDH2-3, succinate dehydrogenase 2-3
Cell-wall biogenesis	<i>HrhDR35</i>	ACQ85271.1	AT4G25810.1	134/182 (73%)	4.9e-75	XTR6, XTH23, xyloglucan endotransglycosylase 6
Late up-regulated						
Signal transduction	<i>HrhDR9</i>	DQ792736	AT5G28770.1	70/154 (45%)	3.8e-29	BZO2H3, AtbZIP63, bZIP transcription factor family protein
Protein transport	<i>HrhDR37</i>	XP_002514582.1	AT2G03890.1	455/661 (68%)	9.4e-239	ATPI4K GAMMA 7, UBDK GAMMA 7, P14 K GAMMA 7, phosphoinositide 4-kinase gamma 7
	<i>HrhDR15</i>	AK323365.1	AT4G24920.1	52/69 (75%)	3.2e-23	secE/sec61-gamma protein transport protein
	<i>HrhDR27</i>	CAN62174.1	AT1G09070.1	51/137 (37%)	6.2e-20	SRC2, (AT)SRC2 soybean gene regulated by cold-2
Unclassified	<i>HrhDR34</i>	XP_002297764.1	No match			
	<i>HrhDR39</i>	ACQ63637.1	No match			
Early down-regulated						
Transcriptional regulators	<i>HrhDR26</i>	XP_002271519.1	AT1G71080.1	146/288 (50%)	3.7e-61	RNA polymerase II transcription elongation factor
Metabolism	<i>HrhDR4</i>	AM411650	AT1G11680.1	375/488 (76%)	1.3e-207	CYP51G1, EMB1738, CYP51A2, CYP51, CYTOCHROME P450 51G1
	<i>HrhDR6</i>	XP_002327232.1	AT1G34430.1	220/292 (75%)	2.6e-142	EMB3003 2-oxoacid dehydrogenases acyltransferase family protein
	<i>HrhDR12</i>	XP_002528637.1	AT3G53000.1	231/299 (77%)	1.6e-131	AtPP2-A15, PP2-A15 phloem protein 2-A15
	<i>HrhDR25</i>	XP_002521339.1	AT1G72640.1	178/307 (57%)	1.8e-86	NAD(P)-binding Rossmann-fold superfamily protein
Ion transport	<i>HrhDR11</i>	XP_002284552.1	AT1G07810.1	872/1063 (82%)	0.00	ECA1, ATECA1, ACA3, ER-type Ca ²⁺ -ATPase 1
Electron transport	<i>HrhDR14</i>	AB026909	AT5G13450.1	135/242 (55%)	8.1e-66	ATP5 delta subunit of Mt ATP synthase
Unclassified	<i>HrhDR28</i>	XP_002313437.1	AT3G46540.1	157/295 (53%)	1.7e-72	ENTH/VHS family protein
	<i>HrhDR40</i>	CAJ33098.1	No match			
Late down-regulated						
Metabolism	<i>HrhDR33</i>	XP_002275806.1	AT2G45970.1	395/534 (73%)	2.1e-218	CYP86A8, LCR cytochrome P450, family 86, subfamily A, polypeptide 8
	<i>HrhDR38</i>	CB115070.3	AT5G40200.1	395/464 (85%)	7.0e-218	DegP9, DegP protease 9
	<i>HrhDR41</i>	XP_002334134.1	AT5G42650.1	87/147 (59%)	2.4e-43	AOS, CYP74A, DDE2, allene oxide synthase
Constitutively expressed						
Metabolism	<i>HrhDR2</i>	CB130854.3	AT4G18260.1	145/229 (63%)	2.5e-71	Cytochrome b561/ferric reductase transmembrane
	<i>HrhDR3</i>	XP_002275283.1	AT1G07080.1	127/191 (66%)	6.5e-73	Thioredoxin superfamily protein

Fig. 2 Functional distribution of the TDFs isolated by cDNA-AFLP from leaves of *H. rhodopensis* based on gene ontology. Unclassified protein denotes sequence that is homologous to unknown or predicted genes. No match indicates low similarity to existing nucleotide or protein sequences below the cutoff *E* value



preliminary attempt to apply this method in resurrection plants, using *H. rhodopensis* as a model. The approach enabled us to identify genes, not reported previously for this species. Of 33 isolated by cDNA-AFLP and sequenced TDFs, 20 were assigned to a known function by search similarities to public databases. Among them 18 were found to be differentially regulated by desiccation. The knowledge on these cDNAs can help us to describe the response of *Haberlea* to desiccation, as well to identify candidate genes for molecular breeding programs. More detailed studies of four randomly selected differentially expressed TDFs were performed by sqRT-PCR. The homology analysis and expression data presented here suggest that two of the four transcript fragments, *HrhDR8* and *HrhDR35* were most probably related to desiccation tolerance.

The presence of succinate dehydrogenase conserved domain (SDH; EC 1.3.5.1) was identified after ORF analysis of amino acid (aa) sequence corresponded to *HrhDR8* transcript (Online Resource 2). The expression of *HrhDR8* transcript increased in the early phase of dehydration and high abundance in late dehydrated and re-hydrated samples was observed (Fig. 4a). Our results correlated with previously reported microarray data showing that a cDNA clone corresponding to SDH gene was up-regulated in leaves of dehydrated *X. humilis* plants (Collett et al. 2004). It was shown that desiccation tolerance in resurrection plants involves many genes implicated in seed desiccation tolerance (Rascio and La Rocca 2005). In agreement, it was found that SDH 2–3 transcripts appear during maturation of *Arabidopsis* seeds and are abundant through desiccation in dry stage but markedly decline during germination (Elorza et al. 2006). We presume that high abundance of *HrhDR8* transcript at recovery in *Haberlea* could be an evidence for different types of gene regulation in germination of seeds and recovery of vegetative tissues in resurrection plants.

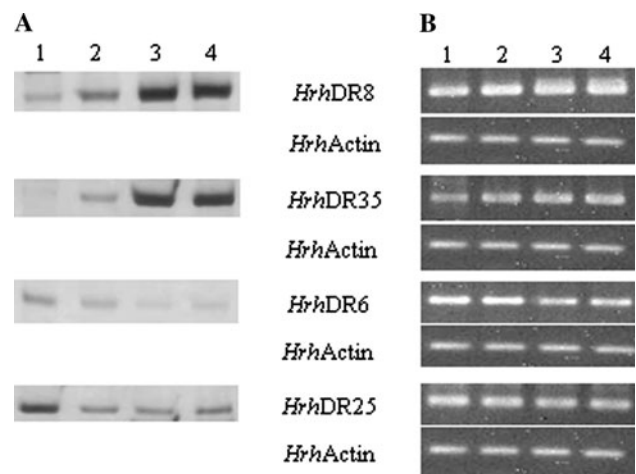


Fig. 3 Confirmation of the expression patterns of four TDFs by sqRT-PCR. **a** Expression profiles of *HrhDR8*, *HrhDR35*, *HrhDR6*, and *HrhDR25* obtained by cDNA-AFLP. **b** Expression profiles of the same TDFs by sqRT-PCR with transcript specific primers. *HrhActin* was co-amplified as a house-keeping control. RNA was isolated from control hydrated *H. rhodopensis* intact plants (Lanes 1) and at different rate of dehydration: Lanes 2 bulked RNA from 1, 2, and 3 h of dehydration (bulk I), Lanes 3 bulked RNA from 6, 18, and 24 h of dehydration (bulk II) and Lanes 4 bulked RNA from fully desiccated plants at 72, 168, and 236 h of dehydration (bulk III). See Materials and methods for additional details

The translated *HrhDR35* transcript fragment showed high amino acid sequence similarity to *M. acuminata* xyloglucan endotransglucosylase/hydrolase enzyme (XTH; EC 2.4.1.207). In addition, the homology of the translated *HrhDR35* ORF to the conserved domain of glycosyl hydrolase 16 superfamily that includes xyloglucan endotransglucosylases (Online Resource 3) supports the idea that *HrhDR35* derived from a transcript encoding protein with similar function. XTHs are a class of wall-modifying enzymes involved in xyloglucan metabolism (Liu et al. 2007). Vicré et al. (1999) reported that cell wall folding in

dehydrated leaves of *C. wilmsii* was accompanied by a marked increase of XG epitopes, which declined during rehydration. Further analysis proposed that remodeling of cell wall during desiccation is probably a result of the activity of xyloglucan-modifying enzymes that altered the number of XG epitopes (Vicré et al. 2004). Interestingly, recent transcriptomic studies of *C. plantagineum* revealed that transcripts involved in cell wall remodeling are

abundant in fully hydrated plants (Rodriguez et al. 2010). The authors associated this with the maintenance of the cell wall plasticity required to prevent cells from the mechanical stress that appears in result of water loss and uptake during dehydration/rehydration process. Our results demonstrated a rapid induction of *HrhDR35* transcript upon dehydration and after 24 h of rehydration the level of expression decline to the level in untreated control plants (Fig. 4b). These facts, prompted us to assume that *HrhDR35*, encoding a putative XTH enzyme is involved in the XG modification of *H. rhodopensis* cell wall and is drought-regulated. Changes in XG have previously been reported to play a role in several plant species in response to abiotic stress (Zwiasek 1991; Kubacka-Zebalska and Kacperska 1999). Constitutive expression of abiotic stress-inducible hot pepper *CaXTH3* improves drought and salt tolerance in transgenic *Arabidopsis* plants (Cho et al. 2006). Further studies including isolation of full cDNA sequence of XTH gene from *Haberlea* and study on expression and function of respective protein will be a good contribution to our knowledge on cell wall of resurrection plants and could provide important information for engineering stress tolerant crops.

Our cDNA-AFLP analysis showed that four out of eight function-known up-regulated (early and late) transcripts were involved in stress signaling in *Haberlea* (Table 5). Drought-induced transcription factors have been isolated and showed differential expression and regulation of response to desiccation and to ABA in different tissues of *C. plantagineum*—the myeloblastosis (MYB) family (Iturriaga et al. 1996), homeodomain-leucine zipper (HD-Zip) family (Deng et al. 2002, 2006), basic leucine zipper domain (bZIP) family (Ditzler and Bartels 2006), novel zinc finger (Hilbricht et al. 2002), and NAC transcription factors (Rodriguez et al. 2010).

The response to particular stress including drought leads to a complex regulation of the genes by induction or repression of their expression. In resurrection plants down-regulated genes were also investigated in order to describe the mechanisms of drought tolerance in depth. In *C. plantagineum*, studies have revealed that transcripts encoding proteins relevant to photosynthesis (e.g. small subunit of Rubisco enzyme) are down-regulated and have been estimated to represent 36% of the total number of genes altered during dehydration process (Ramanjulu and Bartels 2002). In monocotyledonous resurrection plant *X. humilis*, the genes associated with photosynthesis (e.g. chlorophyll *a/b* binding protein, *psbY* and *psbO* components of PSII) and metabolism were identified exclusively among the dehydration down-regulated genes (Collett et al. 2004). The sqRT-PCR analysis confirmed that *HrhDR6* and *HrhDR25* transcripts, with role in cellular metabolism, were down-regulated at early stages of dehydration

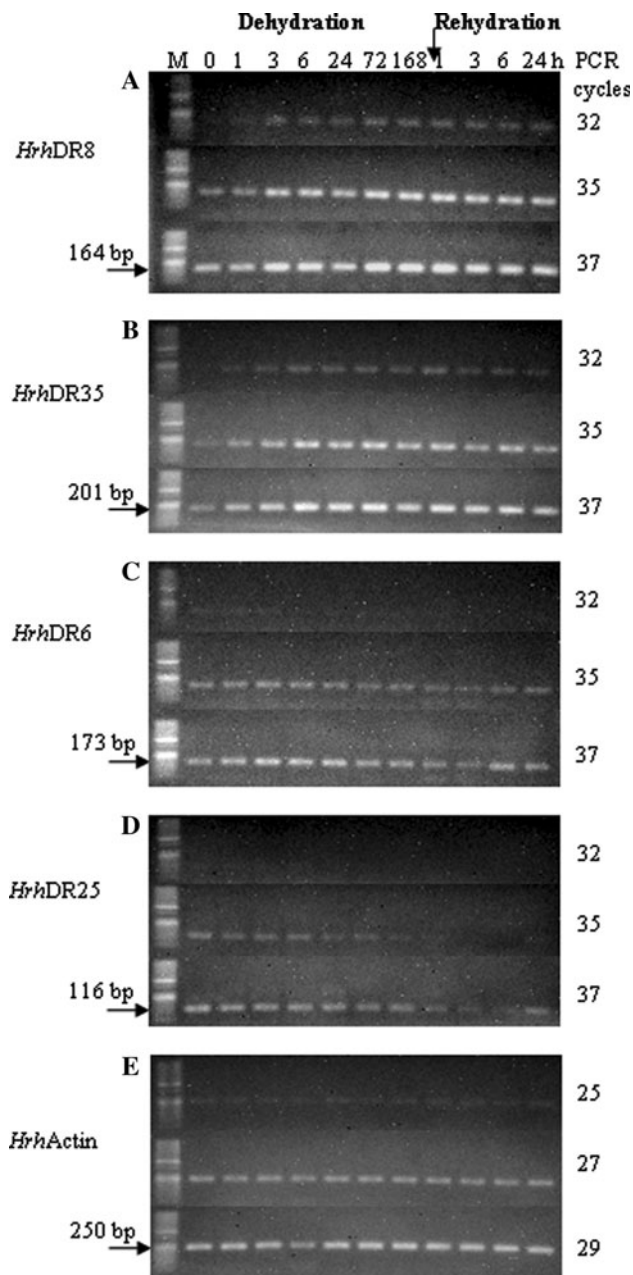


Fig. 4 sqRT-PCR analysis of *HrhDR8* (a), *HrhDR35* (b), *HrhDR6* (c) and *HrhDR25* (d) cDNAs in leaves of *Haberlea* at different time points of dehydration and subsequent rehydration. PCR was carried out for 32, 35, and 37 cycles to follow amplification kinetics. *HrhActin* (e) was co-amplified for 25, 27, and 29 PCR cycles as a house-keeping control. *M* Marker 50 bp DNA ladder

(Table 4; Fig. 4c, d). The design of our experiment allowed us to follow the changes in their expression also in state of rehydration. The results showed that this inhibition is reversible for both transcripts after 6 and 24 h of rehydration, respectively. This is in agreement with the main feature of resurrection plants to restore their metabolism after severe water stress. Also, several physiological studies demonstrated fully recovery of photosynthetic activity in *Haberlea* after rehydration (Georgieva et al. 2005, 2007).

About 30% of the identified *Haberlea* sequences show no significant similarity to any sequence in the searched databases and were differentially regulated. The analysis of respective ORFs to no match TDFs revealed a coding capacity for peptide sequences with approximately 40 aa for some of them. Previously Furini et al. (1997) reported the isolation of a novel gene (CDT-1) in *C. plantagineum* involved in dehydration tolerance of callus tissue. The authors suggested that the CDT-1 transcript carried ORF that could be translated into a short peptide (22 aa), which did not share homology to any known protein. Furthermore a cDNA-AFLP analysis of seed germination in *Arabidopsis* identified a set of sequences that showed no homology to any sequences in the public databases (de Diego et al. 2006). The study revealed that some may encode transposons that may help in the annotation of new genome sequences and identification of regulatory mechanisms. In *Haberlea* further analysis are needed to identify the no match sequences.

Our results on gene regulation of *H. rhodopensis* in response to desiccation could be considered as promising first attempt in the area. More detailed studies on gene and protein level are needed for better characterization of the extreme desiccation tolerance of this plant species.

Conclusion

In summary, cDNA-AFLP technique was used for the first time in resurrection plants to identify genes that are differentially expressed during dehydration in leaf tissues of *H. rhodopensis*. Thirty-three genes newly reported for *Haberlea* were found to be regulated by dehydration. Two of the genes with function in plant mitochondria (*HrhDR8*) and cell wall re-modeling (*HrhDR35*) were shown to be strongly up-regulated during dehydration and could be suggested as involved in the desiccation tolerance mechanisms. Further experiments including isolation and characterization of full-length cDNA sequences, with subsequent transformation of the species and functional analysis are underway and could help us to identify the complex mechanisms specific for desiccation tolerance of *Haberlea*.

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Conflict of interest All benefits in any form from a commercial party related directly or indirectly to the subject of this manuscript or any of the authors must be acknowledged. For each source of funds, both the research funder and the grant number should be given.

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