The 26S proteasome of the resurrection plant *Tortula ruralis*: cloning and characterization of the TrRPT2 subunit

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**Abstract**

*Tortula ruralis* is an important experimental system for the study of plant desiccation tolerance. EST gene discovery efforts utilizing desiccated gametophytes have identified a cDNA *TrRpt2* encoding a predicted polypeptide with significant similarity to the 26S proteosome regulatory subunit IV, TrRPT2, the 446 amino acid deduced polypeptide, has a predicted molecular mass of 49.6 kDa, and a predicted pI of 8.15. Phylogenetic analysis demonstrated that previously characterized RPT2 polypeptide sequences could be reproducibly grouped into 3 major clades and that TrRPT2 forms a discrete evolutionary group. RNA blot hybridizations were used to analyze *TrRpt2* expression in response to: 1) desiccation and rehydration, 2) abscisic acid-treatment, 3) increased NaCl concentration, and 4) NaCl-shock. *TrRpt2* steady-state mRNA transcript levels are unchanged in response to all treatments and the gene is constitutively expressed.

*Additional key words: ABA, bryophytes, desiccation, moss, salinity.*

**Introduction**

In eukaryotic cells, most proteins in the cytosol and nucleus are degraded via the ubiquitin-proteasome pathway (Coux et al. 1996, Voges et al. 1999). The 26S proteasome (EC 3.4.99.46) is a multisubunit, multicatalytic endopeptidase complex that catalyzes the ATP-dependent degradation of ubiquitinated polypeptides (Rechsteiner 1998). In plants, this degradative pathway is involved in pathogen resistance (Becker et al. 1993, Homma et al. 1994), senescence (Homma et al. 1994), hormone response (Ruegger et al. 1998), wound-signalling (Ito et al. 1999) and progress through the cell cycle (Genschik et al. 1994, 1998).

The 26S proteasome (> 2.5 MDa) consists of a barrel-shaped proteolytic core complex (the 20S proteasome), capped at one or both ends by the 19S regulatory complex. The 20S proteasome consists of four stacked rings of seven related α- and seven related β-subunits in a twofold symmetric (α7β7β7α7) configuration (Groll et al. 1997). The 19S regulatory complex is made up of at least 18 subunits and can be dissociated into two subcomplexes, the distal 'lid' and proximal 'base'. The lid complex is essential for the recognition and binding of the ubiquitinated substrate proteins, while the base subcomplex contains six ATPases designated RPT1-to-RPT6 (Glickman et al. 1998, Zwickl et al. 1999). The ATPases are assembled into a ring that forms the interface of the 19S complex with the 20S core particle (Baumeister et al. 1998). The ATPase ring promotes assembly of the 26S complex and directs translocation of substrate protein into the proteolytic core (Voges et al 1999). The RPT ring ATPases belong to the AAA protein super family (ATPases Associated with various cellular Activities) (Dubiel et al. 1992) which direct the binding and utilization of ATP in a wide-range of cellular processes (Patel and Latterich 1998). Rpt2 encoding cDNAs have been identified and characterized from the angiosperm species *Oryza sativa* (Suzuka et al. 1994) and *Arabidopsis thaliana* (Fu et al. 1999) We employ the desiccation-tolerant moss *Tortula ruralis* as a model system for the study of plant vegetative

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**Abbreviations:** ABA - abscisic acid; EST - expressed sequence tag.

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desiccation tolerance and post-transcriptional gene control (Wood and Oliver 1999, Oliver et al. 2000, Wood et al. 2000a,b,c). We postulate that the ubiquitin-proteasome pathway plays a fundamental role in the ability of resurrection plants to survive complete drying of their vegetative structures. In order to pursue our hypothesis at the molecular level, and to further investigate the relationship between salinity-stress and desiccation-stress in _T. rurals_, a cDNA _TrRpt2_ encoding a predicted polypeptide with significant similarity to the AAA protein RPT2 of the human 26S proteasome was isolated and characterized.

Materials and methods

Plants: _Gametophytes of Tortula rurals_ (Hedw.) Gaertn., Meyer & Scherb._ (= Syntrichia rurals_ F. Weber & D. Mohr) were prepared for experimentation as described previously (Wood et al. 1999). Hydrated moss was obtained after a 24 h rehydration period. Desiccated moss was obtained by placing cut hydrated gametophytes on a filter paper disc over activated silica gel (rapid-dried, RD) or a stirred saturated solution of sodium nitrate at 20 °C (RH 66 %) for 24 h (slow-dried, SD). The air-dried dry mass is achieved in 1 h for rapid-dried and 6 h for slow-dried gametophytes. Desiccated moss was obtained by the addition of de-ionized H_{2}O to desiccated moss. Abscisic acid (ABA) treated moss was obtained by incubation with 50 to 500 μM ABA solutions (cis, trans, Sigma, St. Louis, USA). Salt-treated moss was obtained by the stepwise addition of NaCl (50 mM 2 h, 50 mM + 50 mM 2 h, and 100 mM + 50 mM 2 h; 6 h total application) or by incubation in 50, 100 or 150 mM NaCl for 6 h.

cDNA isolation: The EST clone RNP129 (A1305024) was previously isolated from a _T. rurals_ cDNA expression library cDNA derived from the polysomal, mRNP fraction of desiccated gametophytes (Wood et al. 1999). Polymorphic chain reaction (PCR) amplification of the cDNA library was performed using the T3 primer and an antisense primer designed from RNP 129 (primer A, 5'-GAAGCCAAAAGCCATAGAA-3'). The full-length cDNA clone was obtained by 5' random amplification of cDNA ends (5'-RACE) using the FirstChoice RLM-RACE Kit (Ambion, Austin, USA) as described by the manufacturer utilizing the gene specific primers primer B (5'-GAAGCCAAAAGCCATAGAA-3') and primer C (5'-GATCAGCTCAATCCAAAC-3'). Reverse transcription was done at 50 °C. First and second rounds of PCR amplification was adjusted to 65 and 67 °C, respectively, utilizing HotStarTaq DNA polymerase (Qiagen, Valencia, USA).

DNA sequence analysis: DNA sequence was determined by the Plant Biotechnology and Genome Center (Southern Illinois University) using an automated sequencer (ABI model 377; Applied Biosystems, Foster City, USA). Vector NTI suite (InforMax, North Bethesda, USA) was used for sequence assembly, analysis and homology searches. Similarity of the _T. rurals_ sequences to nucleotide sequences in GenBank, EMBL, DDBJ, and PDB databases were determined using the FASTA and BLASTN server as described by Wood et al. (1999). Molecular mass and pl prediction, and alignment of deduced amino acid sequences were performed using software available on the ExPASy molecular biology server (www.expasy.ch/). Multiple alignments of the deduced amino acid sequence for _TrRpt2_ and related _Rpt2_ were created using Clustal-X and phylogenetic trees were constructed using the Neighbor-Joining algorithm. The amount of support for each node of the resultant trees was examined with 1000 bootstrap replicates. The data set consisted of the following predicted amino acid sequences: _Arabidopsis thaliana_ (CAB43918), _Homo sapiens_ (Q03527), _Saccharomyces cerevisiae_ (546613), _Drosophila melanogaster_ (AAF56205), _Neurospora crassa_ (CAB88559), _Oryza sativa_ (P46466), and _Brassica napus_ (C14432).

RNA isolation and RNA blot hybridizations: Total RNA was isolated using the RNeasy kit (Qiagen) as described by the manufacturer. RNA (approximately 10 μg) was separated by electrophoresis in a formaldehyde-agarose gel and transferred to nitrocellulose under standard conditions (Duff et al. 1999). The DNA probe (i.e. the 897 bp _EcoRI_ fragment derived from the 5'-RACE product) was labeled with [α^32P]-dCTP (PerkinElmer Life Sciences Inc., Boston, USA) using the random prime labeling kit (Decaprime H Kit, Ambion, Austin, USA). Membranes were prehybridized and hybridized at 42 °C using ULTRAhybTM (Ambion) as described by the manufacturer. Membranes were washed at 42 °C in 2× standard saline citrate (SSC), 0.1 % sodium dodecyl sulphate (SDS) for 2 × 5 min, followed by washing in 0.1×SSC, 0.1 % SDS for 2 × 15 min. Blots were stripped and reprobed with rRNA-DNA to demonstrate equal loading.
Results and discussion

Characterization of TrRPT2: We have employed EST analysis to discover a number of genes associated with desiccation stress including Aldh21A1 (Chen et al. 2002a), Aldh1B6 (Chen et al. 2002b), Vca7 (Chen et al. 2002c), Elip and Elipb (Zeng et al. 2002), and the ribosomal proteins Rp15, Rp51, Rp516 and Rp123 (Wood et al. 2000a, Zeng and Wood 2000). In this paper, we describe the isolation and characterization of an EST-derived T. ruralis cDNA encoding a predicted polypeptide with significant similarity to the 26S proteasome regulatory subunit IV. The full-length cDNA was obtained using 5'-RACE and designated TrRpt2 in accordance with the proposed unified nomenclature for the proteasome (Finley et al. 1998). The composite TrRpt2 cDNA was 1845 bp in length and contained a single, continuous open reading frame from nucleotide 187 to 1524 flanked by a 186 bp 5'UTR, and a 321 bp 3'UTR (data not shown). The initiation and termination sequences conformed to the known consensus sequences in plants (Lutcke et al. 1987), and the cDNA contained putative polyadenylation signal sequences (Wood et al. 2000b) (data not shown). The ORF encodes a polypeptide of 446 amino acids with a predicted molecular mass of 49.6 kDa and predicted pl of 8.15. The deduced T. ruralis polypeptide TrRPT2 is 84% identical to 26S proteasome RPT2 homologues from Oryza sativa and Arabidopsis thaliana (data not shown). TrRPT2 contains the AAA cassette (residues 227 - 414, Walker et al. 1982) that incorporates the P-loop ATP/GTP-binding site (GEPGKT, residues 232-239) and the AAA-protein family signature sequence (VKVILATNRIESLDPLL, residues 331-349) (Swafield and Purugganan 1997).

![Fig. 1. Alignment of the deduced polypeptide sequence of TrRPT2 from Tortula ruralis (AF432345), Arabidopsis (CAB43918) and yeast (S46613). The sequences are numbered from the presumed translation initiation methionine (M) and are aligned using ClustalV to give maximal alignment.](image)

Phylogenetic analysis of TrRPT2: To examine the structural relationship between the predicted polypeptide TrRPT2 and similar RPT2 subunits, the deduced amino acid sequences were analyzed by the Neighbor-Joining method. The gene tree was assembled from the pairwise alignment of these deduced polypeptide sequences (Fig. 2). To our knowledge, TrRPT2 is the first full-length RPT2 homologue to be characterized in bryophytes and this analysis demonstrates its evolutionary relationship to other RPT2 sequences. The RPT2 sequences could be reproducibly grouped into 2 major clades, one for plants and animals, and one for fungi. TrRPT2 is distinct from animals and tracheophytes, and forms a discrete evolutionary group.

Expression of TrRpt2 in T. ruralis gametophytes: The steady-state mRNA accumulation of T. ruralis TrRpt2 transcripts were analyzed by RNA blot hybridization in
response to a desiccation-rehydration cycle, ABA-treatment or NaCl treatment (Fig. 3). Total RNA was isolated from hydrated, slow-dried, slow-dried rehydrated, rapid-dried, rapid-dried rehydrated, ABA-treated or salinized gametophytic tissues as described (see Materials and methods). To enable normalization of the hybridization signals to account for loading anomalies, the membrane was re-probed after the initial analysis using a plant 18S nuclear rRNA probe. *T. ruralis TrRpt2* hybridized to a single mRNA species of approximately 1850 bp (Fig. 3). TrRpt2 steady-state mRNA transcript levels are unchanged in response to desiccation or rehydration cycle (Fig. 3A), ABA-treatment (Fig. 3B), or stepwise application of NaCl and NaCl shock (Fig. 3C).

![Gene tree](image)

**Fig. 2.** Gene tree derived from a data set of deduced polypeptides for the *Tortula ruralis* TrRpt2 and related deduced polypeptides. The unrooted Neighbor-Joining tree was constructed using *ClustalV*. See Materials and methods for species name and accession numbers; the scale bar indicates the number of changes per unit length. Numbers below the lines represent bootstrap percentages (based on 1000 replicates).

The proposed mechanisms of desiccation tolerance differ between bryophytes and tracheophytes. *T. ruralis* employs a constitutive protection system and an active rehydration induced recovery mechanism (Oliver et al. 2000). This is in contrast to the strategy proposed for the angiosperm *Craterostigma plantagineum* that utilizes a dried irrigation of abscisic acid to trigger the accumulation of gene products that mediate the establishment of a cellular protection system prior to desiccation (Ingram and Bartels 1996, Bartels and Salamini 2001). Unlike many plant stress responses, the alteration in gene expression within *T. ruralis* gametophytes elicited by desiccation stress is primarily regulated at the post-transcriptional level as a result of differential selection and/or recruitment of rehydrin mRNAs from a qualitatively constant mRNA pool (Oliver 1991). We have hypothesized that genes essential to recovery and cellular repair are preferentially expressed upon rehydration of desiccated gametophytes, i.e. the rehydrins (Oliver and Bewley 1997, Duff et al. 1999, Wood and Oliver 1999). We have also postulated that constitutive transcripts that are maintained in the slow dried state consist of both important house keeping genes and those genes that establish the constitutive desiccation tolerance protection system(s). TrRpt2 must play a role in the maintenance of 26S proteasome integrity and/or function during drying, in the desiccated state, and upon rehydration.

![RNA blot analysis](image)

**Fig. 3.** RNA blot analysis of TrRpt2 using the total RNA fraction of desiccated (A), ABA-treated (B) or salt-treated (C) *Tortula ruralis* gametophytes. A: C - control, SD - slow-dried, Re - rehydrated, R - rapid-dried, and RRe - rapid-dried rehydrated. B: gametophytes incubated in solutions of 50, 100, 200 or 500 μM ABA. C: C - control, 50 - 50 mM, 100 - 100 mM or 150 - 150 mM NaCl for 6 h or the step-wise addition of NaCl 50+ - 50 mM 2 h, 100+ - 50 mM + 50 mM 2 h, and 150+ - 100 mM + 50 mM 2 h. RNA was extracted as described in Materials and methods. RNA (approximately 10 μg) was separated by electrophoresis in a formaldehyde-agarose gel and transferred to nitrocellulose under standard conditions. The resulting RNA blot was hybridized with 32P-labeled cDNA probe for *TrRPT2* and blots were re-probed with rRNA-DNA to demonstrate equal loading.

Efficient targeted gene disruption (i.e. homologous recombination) is a well-established tool in the moss *Physcomitrella patens* (Schaefer and Zryd 1997). This capability is at present unique amongst all plants and represents an extremely powerful technique for the functional analysis of plant genes (Reski 1999, Cove 2000, Wood et al. 2000b, Schaefer and Zryd 2001). Girod et al. (1999) have characterized the 26S proteasome subunit MCB 1 (RPN 10) using homologous recombination in *P. patens*. MCB 1 was found to have high affinity for multiubiquitin chains and was postulated to play a key role in hormone-triggered developmental processes. In addition, the *P. patens* EST Programme
(PEP) (www.moss.leeds.ac.uk) has identified several 26S proteasome associated ESTs including the RPT2 subunit (AWS99382). Mosses such as *T. rurals* and *P. patens* will be key experimental systems for the study of 26S proteasome function *in vivo*, and will provide greater insight to the role which the ubiquitin-proteasome pathway plays in adaptation to elevated NaCl concentration, desiccation tolerance, and to stress-inducible post-transcriptional gene control.

References


