Suppression subtractive hybridization for studying gene expression during aerial exposure and desiccation in fucoid algae

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Gene expression in *Fucus vesiculosus* L. from the Ria Formosa, Faro, Portugal was investigated by screening a cDNA library generated by suppression subtractive hybridization (SSH) for algae undergoing mild desiccation stress (60–70% tissue water content). The subtractive library was constructed from small amounts (1 μg) of total RNA using PCR-based techniques. Screening by reverse Northern analysis (cDNA Southerns) revealed that between 60% and 70% of clones randomly selected from the library were differentially regulated in desiccated and hydrated algae. Most genes could not be directly identified based on sequence homology with known gene sequences. However, several cDNAs for chloroplast-encoded transcripts were identified and shown to be up-regulated or differentially regulated in desiccated algae relative to hydrated controls. These included partial sequences for ribulose-l,5-bisphosphate carboxylase/oxygenase (Rubisco; *rbc*L/*rbc*S), chloroplast coupling factor ATPase (*atp*H/*atp*I) and a photosystem I P700 chlorophyll *a* binding protein (*psa*A). The usefulness of reverse Northern analysis was confirmed with conventional Northern analysis of transcript abundance for Rubisco, which varied rapidly in response to light and the hydration status of the algae. These results show that SSH is a useful technique for analysing the responses of gene expression to environmental stress, and as a starting point for the subsequent identification of stress-responsive genes in macroalgae.

Key words: cDNA library, chloroplast (plastid) genes, dehydration, desiccation, fucoid algae, *Fucus vesiculosus*, gene expression, stress tolerance, suppression subtractive hybridization, water stress

Introduction

Intertidal macroalgae experience two very different types of environmental conditions on a daily basis, as periods of submergence in seawater during high tide alternate with aerial exposure during low tides. Coping with the physiological conditions imposed by high-frequency (hours) cycles of immersion/emersion is a unique problem for intertidal organisms. Water stress resulting from desiccation, osmotic changes and freezing can be severe, particularly since seaweeds typically lack mechanisms to prevent water loss. The effects of water stresses during emersion cycles may be further compounded by photoinhibitory irradiance and extremes of temperature (see reviews by Chapman, 1995; Davison & Pearson, 1996).

The brown algal genus *Fucus* is a recently diverged genus within the family Fucaceae and comprises a number of very closely related species, based on molecular phylogenetic evidence (Serrão *et al.*, 1999). Several species of *Fucus* occupy distinct, but overlapping, niches on rocky intertidal shores. In common with other fucoid algae, individual species exhibit levels of physiological tolerance to emersion stresses (desiccation, freezing) which broadly correlate with their range of vertical distribution on the shore (Schonbeck & Norton, 1978; Dring & Brown, 1982; Davison *et al.*, 1989; Pearson & Davison, 1993). Although there is a large literature detailing the molecular mechanisms of water stress tolerance in vascular plants (see reviews by Bohnert *et al.*, 1995; Ingram & Bartels, 1996; Shinozaki & Yamaguchi-Shinozaki, 1996, 1997), much less is known about the genetic basis of desiccation tolerance in poikilohydric plants in general, and even less is known for brown algae (but see Li *et al.*, 1998). Fucoid algae are much more tolerant to tissue water loss than vascular plants (Kawamitsu & Boyer, 1999), and are able to recover photosynthetic capacity within only 1–2 h following 90% or greater tissue water loss (e.g. *Fucus vesiculosus* L.: Pearson *et al.*, 2000). Investigation of the underlying mechanisms of desiccation-tolerance in fucoids should yield important insights into this process in a group of photosynthetic organisms that are evolutionarily fairly distantly related to green

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plants. In addition, the co-occurrence of conspecifics with different capacities to recover from desiccation and of ecotypes within *Fucus vesiculosus* varying in desiccation tolerance (Pearson *et al.*, 2000) could be exploited for comparative studies to assess the importance of specific molecular responses to desiccation stress.

As a first step towards identifying genes that play a role in stress tolerance, a common approach is to investigate changes in gene expression during imposition of the stress. Identification of genes that are up-regulated can then provide molecular tools for further investigations of function/regulation. Subtractive cDNA hybridization has been a powerful method for isolating genes expressed during biological processes such as growth, differentiation and responses to environmental variables (Hendrick *et al.*, 1984; Duguid & Dinauer, 1990; Hara *et al.*, 1991). Although these molecular tools are available for macroalgal research, relatively few studies have taken advantage of recent advances to address these questions (e.g. Liu *et al.*, 1994).

In this paper, we describe the use of polymerase chain reaction (PCR)-based suppression subtractive hybridization (SSH) (Diatchenko *et al.*, 1996; Gurskaya *et al.*, 1996) to produce cDNA libraries enriched for genes up-regulated during desiccation in *Fucus vesiculosus*. We have identified several photosynthetic genes up-regulated during mild tissue water loss (60–70 % tissue water content : TWC), and isolated several other partial cDNA clones for potentially desiccation-responsive genes.

Methods

Experimental tissue and RNA extraction for library construction

Adult plants of *Fucus vesiculosus* were collected from the Ria Formosa, Faro, Southern Portugal (37·12° N, 8·10° W). Apical tips (*c.* 5 cm) that had no visible epiphytes were gently cleaned by rubbing between paper towels before being cut and threaded onto cotton lines with a needle and thread. Threaded tips were then allowed to recover in seawater (SW) overnight before use in experiments. Recovery and experimental treatments were carried out in a temperature-controlled reach-in chamber (Fitoclima 700EDTU, Aralab, Lisbon, Portugal) at 25 °C, 60–70 % relative humidity and a photon flux density (PFD) of 1000 μmol m^{-2} s^{-1}. For desiccation, threaded tips were hung, without blotting surface water, on a metal drying frame. Samples of desiccated tips (*c.* 2 g Fresh Weight (FW) equivalent) were taken after 30 min (60–70 % TWC, data not shown) and immediately frozen in liquid nitrogen (LN_2). An equal amount of control tissue in SW was also sampled and treated the same way. Tissue samples were then stored at −80 °C until used for RNA extractions.

Total RNA was extracted as follows: *c.* 2 g FW equivalent of tissue was ground to a fine powder in LN_2 and resuspended in 10 ml g FW^{-1} extraction buffer (100 mM Tris-Cl buffer, pH 7·5, 1·5 M NaCl, 2 % (w/v) CTAB, 50 mM DTT). The resulting suspension was kept at room temperature for at least 10 min with frequent agitation before extraction with an equal volume of chloroform with agitation for a further 5 min. After centrifugation, the aqueous phase was removed to a clean centrifuge tube and 0·3 volumes of ethanol was slowly added. This step precipitates much of the remaining polysaccharides from the extract. A further chloroform extraction and centrifugation (20 min, 10000 *g*, room temperature) followed, after which the aqueous phase was removed. Total RNA was precipitated with 0·25 volumes of 12 M LiCl at −30 °C overnight in the presence of 1 % (v/v) *β*-mercaptoethanol as antioxidant. The RNA was collected by centrifugation (30 min, 10000 *g*, 4 °C) and redissolved in 1·5 ml TE. After successive phenol (pH 4·5):chloroform:isoamyl alcohol (25:24:1) and chloroform:isoamyl alcohol (24:1) extractions, the purified RNA was precipitated with 2 volumes of ethanol in the presence of 0·3 M NaOAc at −30 °C for 6 h. Following centrifugation (30 min, 10000 *g*, 4 °C) the pellet was washed with cold 70 % ethanol, dried, and stored dry at −80 °C until use.

The RNA was quantified by UV-spectrophotometry (Genequant, Amersham Pharmacia Biotech Europe, Carnaxide, Portugal) using capillary cuvettes (0·5 mm path-length), and RNA integrity was assessed with 2–3 μg on a denaturing agarose gel using standard electrophoretic methods (Sambrook *et al.*, 1989).

Library construction

Prior to construction of the subtractive cDNA library, a PCR-based method was used to generate full-length cDNA using the Switch Mechanism at the 5′ end of RNA Transcripts (SMART) directly from total RNA (1 μg), using a commercially available kit (SMART® PCR cDNA Synthesis Kit, CLONTECH Laboratories, Palo Alto, CA, USA) according to the manufacturer's instructions. This method takes advantage of the terminal transferase activity and template switching ability of reverse transcriptase (RT) to incorporate a specific primer sequence at the 5′ end of fully transcribed cDNAs. The modified oligo-dT primer used for reverse transcription contains the same primer sequence. Subsequent PCR amplification relies on the presence of this primer sequence at both the 3′ and 5′ ends of the cDNA, and thus only full-length transcripts are exponentially amplified. Full-length cDNA was generated from 1 μg of RNA from each of the control and the desiccated samples.

PCR-based SSH was used to generate a library enriched for desiccation-responsive transcripts using the PCR-Select® cDNA Subtraction Kit (Clontech Laboratories, Palo Alto, CA). The cDNA from desiccated algae from which specific up-regulated transcripts are to be found is called the 'tester', whereas the cDNA from control algae (fully hydrated) is the reference or 'driver'. The library produced was named D30 (desiccated 30 min). Prior to subtraction, both tester and driver cDNAs were digested with *Rsa*I, a 4-base cutter, to provide a more homogeneous mixture of shorter, blunt-ended molecules. Following the protocol provided with the kit, two different adaptors were ligated to aliquots of the tester cDNA population, and following two rounds of hybridization with the driver (no adaptors) to (i) equalize the representation of rare and common messages and (ii)

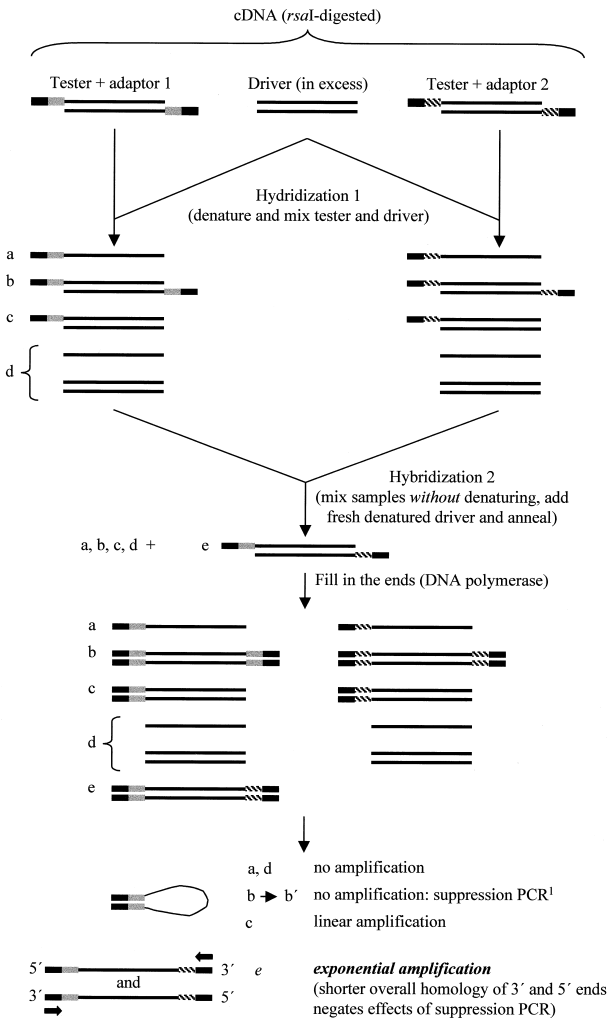

Fig. 1. Scheme outlining the subtractive library construction procedure (modified from the CLONTECH PCR-Select® cDNA Subtraction Kit User Manual). After mixing tester cDNA (from desiccated algae) with driver cDNA (from control hydrated algae) in hybridization 1, molecule types are indicated by the letters a–e. After a second hybridization with fresh denatured driver cDNA, filling in of adaptor ends with DNA polymerase, and PCR with primers complementary to the adaptor sequences, only type e molecules are exponentially amplified. These are sequences over-represented (i.e. differentially expressed) in the tester population relative to the driver. Amplification of type b molecules is effectively suppressed due to the formation of hairpin structures between complementary sequences in the adaptors.

enrich the representation of differentially expressed sequences, two rounds of PCR were performed. The first PCR was to amplify the differentially expressed sequences, and then a second, nested PCR was performed to further reduce background PCR products and enrich the library. A scheme outlining the methodology is shown in Fig. 1.

Library screening by reverse Northern analysis

The subtracted cDNAs, following the second nested PCR (see above) were ligated into a T/A type cloning vector (pGEM-T Easy, Promega Corporation, Madison, WI, USA), and used to transform *E. coli* DH5α competent cells. Transformed cells were plated onto LB-agar containing ampicillin (50 μg ml^{-1}, X-gal (2 mg) and IPTG (5 μl of a 1 N stock), and grown overnight at 37 °C. White colonies were picked at random and the presence of an insert was confirmed by restriction digest (*Eco*RI) of plasmid DNA minipreps, followed by electrophoresis of the products on a 1·2 % agarose gel.

The DNA inserts from individual clones were amplified from the plasmid by PCR, using Ml3 forward and reverse primers, and the resulting PCR reactions were electrophoresed on 1·2 % agarose gels. The PCR product was cut from the gel and purified using the QIAquick PCR Purification Kit (Qiagen, Chatsworth, CA, USA). Approximately 25 ng of purified PCR product was used as a template for generation of random primed ^{32}P-1abelled cDNA probes (Prime-It Kit, Stratagene, La Jolla, CA, USA). To make reverse Northern blots the original full-length SMART cDNA from control and desiccated algae was electrophoresed on agarose gels. cDNAs were transferred to nylon membranes (Hybond-N, Amersham Pharmacia Biotech Europe) using a rapid alkaline transfer procedure (transfer was carried out for at least 4 h in 0·4 M NaOH/0·6 M NaCl, and, after transfer, the membrane was neutralized in 0·5 M Tris-HCl, pH 7·0/1 M NaCl for 10–15 min). Membranes were pre-hybridized for 2 h at 42 °C in 50 % formamide, 5 × SSPE, 5 × Denhardt's reagent, 0·1 % SDS and 200 μg ml^{-1} denatured herring sperm DNA. Hybridization with the denatured probe was carried out overnight at 42 °C under the same conditions, followed by washes in 6 × SSPE/0·1 % SDS (2 × 15 min at room temperature) and 1 × SSPE/0·1 % SDS (2 × 15 min at 37 °C), with a final wash in 1 × SSPE/0·1 % SDS at 65 °C for 1 h. Blots were exposed on Kodak XA film (Kodak, USA).

Sequencing of cDNA clones

Cloned plasmid inserts were sequenced in both directions using the Sanger dideoxy chain termination method for cycle sequencing with dye-labelled terminators (Perkin Elmer) on an ABI 373 automated sequencer (Applied Biosystems, Foster City, CA, USA).

Northern analysis of Rubisco expression

Adult *Fucus vesiculosus* individuals were collected in April 1999 from the Ria Formosa Natural Park, Faro, Portugal. After transportation to the laboratory (*c.* 10 min), apical tips of approximately 5 cm length were cut and placed in SW overnight in outdoor tanks under natural irradiance and temperature conditions. Midday irradiance in tanks under full sunlight was 1000–1200 μmol photons m^{-2} s^{-1}, measured with a LI-COR LI-1000 datalogger and cosine corrected sensor (LI-COR, Lincoln, NE, USA). The following day the algae were exposed to one of six treatments: (1) High irradiance (HL = full sunlight) in SW, (2) HL in air without desiccation, (3) HL in air with desiccation, (4) low irradiance (LL ≈ 50 μmol photons m^{-2} s^{-1}) in SW (5) LL in air without desiccation, (6) LL in air with desiccation. For low-irradiance treatments, tanks were screened with nylon mesh to reduce the irradiance. For treatments in air, algae were placed on mesh screens and either allowed to desiccate, or were sprayed with SW to maintain a hydrated state. Experimental treatments were started at midday, with an air temperature of 27 °C. Samples were

taken after 15, 30, 60 and 180 min, and following 60 min and 24 h recovery in SW at LL. Each sample (*c*. 2 g FW) was frozen rapidly in LN_2 and stored at −80 °C until used for RNA extraction.

Total RNA was extracted as described above, but using a scaled-down extraction procedure in which *c*. 0·2 g fresh weight tissue was ground in LN_2 in 1·5 ml microcentrifuge tubes, before addition of 0·75 ml RNA extraction buffer containing 1·5% *β*-mercaptoethanol as antioxidant in place of DTT. Total RNA (5 μg) was separated by agarose gel electrophoresis and transferred to nylon membranes (Hybond-N, Amersham Pharmacia Biotech Europe) using standard techniques (Sambrook *et al*., 1989). Northern blots were hybridized with a ^{32}P-labelled probe prepared from clone D30 1.3 as described above, containing partial coding sequences of *rbc*L and *rbc*S.

Results

The screening procedure for identifying desiccation-responsive genes of desiccated and control tissue indicated that several photosynthetic genes are up-regulated in response to aerial exposure and/or mild desiccation in *F. vesiculosus*. This was confirmed by Northern blot analysis for ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco).

The subtracted *Fucus vesiculosus* cDNA library was enriched for transcripts up-regulated during mild emersion stress. This was indicated by reverse Northern analysis which showed that 66·7% of the clones tested were differentially expressed (14 of 21). This included two redundant clones for non-mRNAs (16S and 23S chloroplast (cp) rRNAs); there was no evidence that the abundance of cp rRNA differed between desiccated and control tissue (Fig. 2*a*). The exposure time for reverse Northern blots varied between 60 min and 21 days, suggesting that desiccation-responsive cDNAs with different abundances were represented in the subtracted library (Diatchenko *et al*., 1996).

The features of some selected clones are presented in Table 1. Of the clones that have been fully sequenced, size varied between 170 base pairs (bp) and 828 bp.

Sequencing results showed that a total of 9 clones which coded for rRNA sequences were isolated from the library, representing 30% of all clones sequenced. Several of the cDNAs showed little homology to any known genes in the databases, and potentially represent cDNAs from novel desiccation-responsive genes. Six of the clones sequenced were homologous to known gene sequences from chromophyte or rhodophyte algae, or from yeast (see below).

Chloroplast-encoded transcripts

Several cDNAs for chloroplast-encoded genes were identified. Of 6 clones which unambiguously matched sequences with known functions in the database, 5 were chloroplast-encoded genes/operons.

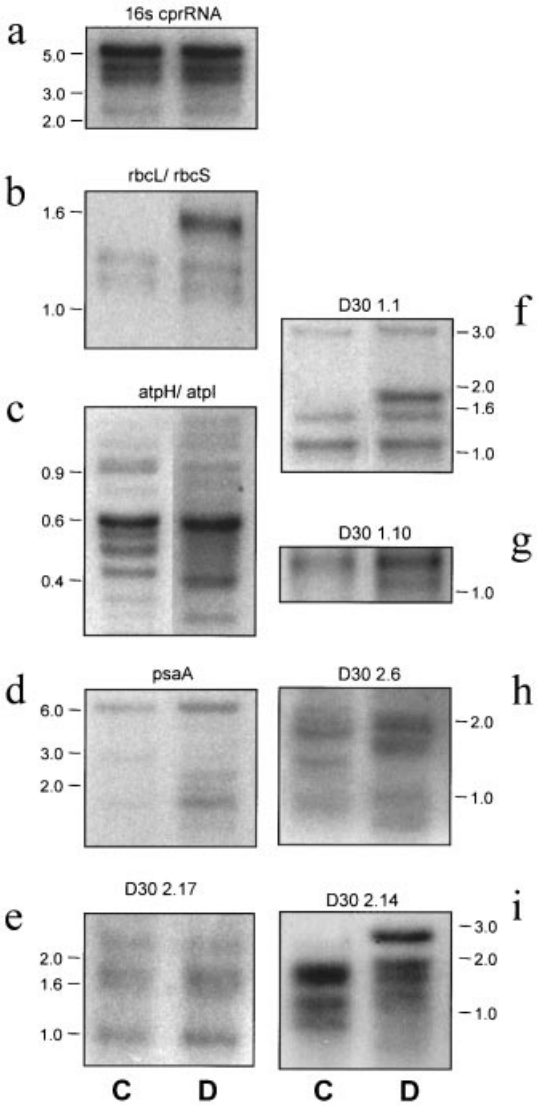

Fig. 2. Reverse Northern blots of cDNA from control hydrated algae (C) and desiccated algae (D), hybridized with the following cDNA clones isolated from the subtracted cDNA library: (*a*) 16S chloroplast ribosomal RNA, (*b*) Rubisco operon containing *rbc*L/*rbc*S partial sequences, (*c*) chloroplast ATPase coupling factor partial sequences for *atp*H/*atp*I, (*d*) partial coding sequence for chloroplast gene *psa*A, encoding subunit 1a of the photosystem I P700-chlorophyll *a* binding protein, (*e*) clone D30 2.17, with similarity to an integrin homologue from the yeast *Saccharomyces cerevisiae*, (*f*) unidentified clone D30 1.1, (*g*) unidentified clone D30 1.10, (*h*) unidentified clone D30 2.6, (*i*) unidentified clone D30 2.14.

Clone D30 1.3 is a 578 bp cDNA encoding part of the Rubisco operon and contains 105 bp of *rbc*L, the intergenic spacer (203 bp), and 270 bp of *rbc*S. Differential expression of Rubisco was indicated by reverse Northern analysis: in particular a band corresponding in size to *rbc*L was abundant after desiccation, but was not detected in controls (Fig. 2*b*).

Clone D30 1.4 encodes part of the CF_0 complex

Table 1. Selected clones from the *Fucus vesiculosus* subtracted cDNA library

Clone	Size (bp)	Expression	Identity	GenBank accession number	Poly-A$^+$
D30 1.1	170	++	Mitochondrial SSU rRNA	AF363451	Yes
D30 1.2	ND	+/−	Unknown	–	ND
D30 1.3	578	++	*rbc*L/*rbc*S Rubisco, large and small subunits	AF346700	No
D30 1.4	547	+	*atp*H/*atp*I	AF346703	No
			ATP synthase CF_0 c and a subunits	AF346702	
D30 1.10	ND	+	Unknown	–	ND
D30 2.1	336	+	*psa*A P700 Chl*a* protein, subunit la	AF346701	No
D30 2.3	637	−	*psb*Y homologue PSII polypeptide Y	AF346705	No
D30 2.4	301	ND	Unknown	BG487395	No
D30 2.6	393	+	Unknown	BG487396	No
D30 2.7	773	+/−	Homologous to vanadium bromoperoxidase 3′ UTR from *F. distichus*	AF362992	Yes
D30 2.8	828	+/−	*rpo*A Chloroplast DNA-directed RNA polymerase, alpha subunit	AF346704	No
D30 2.9	388	ND	Unknown	BG487397	No
D30 2.10	389	+/−	Unknown	BG487398	Yes
D30 2.13	581	−	Unknown	BG487399	Yes
D30 2.14	345	++	Unknown	BG487400	No
D30 2.16	424	ND	Unknown	BG487401	No
D30 2.17	648	+/−	Integrin homologue (*Saccharomyces cerevisiae*)	AF346699	No
D30 2.25	547	ND	Unknown	BG487402	No
D30 2.28	501	+/−	Unknown	BG487403	No

Clone size is in base pairs (bp). Expression pattern: differential expression during desiccation is indicated by + or ++; +/− indicates that some evidence of differential expression was detected but that further analysis is required to verify expression patterns; − indicates no differential expression; ND, not determined. The identity of genes from which the clones originate is based on homology to known sequences in the database (if any). It is also indicated whether a poly-A$^+$ tail was found in the cloned sequence.

of the chloroplast ATP synthase operon and contains parts of *atp*I (296 bp) and *atp*H (180 bp), separated by a spacer region (70 bp). In both Heterokonta and Rhodophyta, all the subunits of the CF_0CF_1 ATP synthase complex are chloroplast encoded with the exception of the *Atp*G subunit of CF_1 (Pancic *et al.*, 1992; Pancic & Strotmann, 1993; Reith & Munholland, 1993). When PCR-amplified cDNA was probed with this clone in a reverse Northern analysis, a complex banding pattern was observed, probably a result of the probe including parts of two co-transcribed genes. However, the hybridization pattern was different between control and desiccated tissues (Fig. 2*c*), with evidence for increased expression and differential splicing and/or editing.

A third clone, D30 2.1 (336 bp), was found to be a partial cDNA for *psa*A, encoding subunit 1a of the P700 chlorophyll *a* binding protein of PSI. Relative to cDNA from hydrated controls, after 30 min desiccation this cDNA hybridized to several bands on a reverse Northern blot, including a prominent band at approximately 6 kb (Fig. 2*d*). The *psa*A gene product is *c*. 2·2–2·3 kb (Raven & Falkowski, 1997), but this band could represent a *psa*A/*psa*B primary transcript. Other bands with increased intensity were apparent at *c*. 2·5 kb and < 2 kb.

Clone D30 2.3, which was not differentially expressed between hydrated and desiccated tissues (results not shown), has strong homology with chloroplast open reading frames for Photosystem II polypeptide Y. The clone D30 2.8 was identified as having sequence homology with the chloroplast RNA polymerase alpha subunit (*rpo*A). Hybridization of this probe to reverse Northerns was weak and did not provide conclusive support for an increased expression of this transcript in response to desiccation (results not shown).

Overall, the results indicated that chloroplast-encoded transcripts are common in the library, and in the majority of cases, were not present as background (i.e. false positives), but represented true, differentially expressed genes in *F. vesiculosus* undergoing desiccation.

Nuclear and/or unidentified clones

The expression patterns of other isolated clones are shown in Fig. 2*e–i*. Most of these are not identified, as they did not show unambiguous homology to other known gene sequences. However, comparison of the sequence of clone D30 2.7 to sequences in the database (GenBank, BLAST algorithm) revealed strong homology to the 3′-UTR of a vanadate bromoperoxidase from *Fucus distichus*. Clone D30 2.17 was similar to a sequence from baker's yeast, *Saccharomyces cerevisiae*, reported to be an integrin homologue (Hostetter *et al.*, l995). However, reverse Northern analysis did not unambiguously confirm

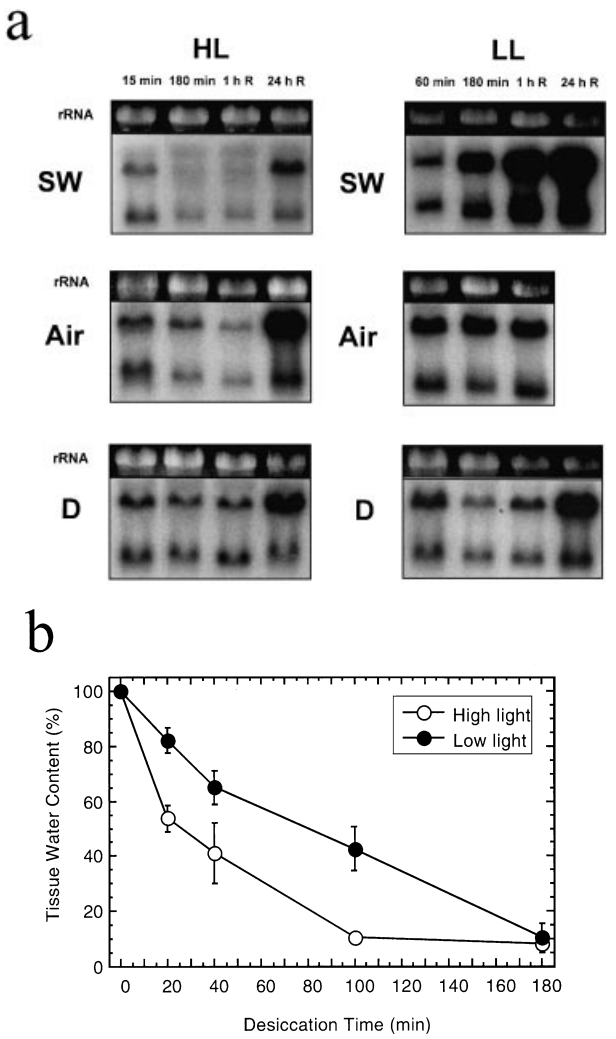

Fig. 3. (*a*) Northern blots probed with cDNA containing *rbc*L/*rbc*S partial coding sequences (see Fig. 2*b*) and showing abundance of Rubisco mRNA isolated from *Fucus vesiculosus* in full sunlight (HL: i.e. in conditions similar to those used during library construction) and in reduced light (LL). HL was approximately 1000–1200 μmol photons m^{-2} s^{-1} and LL was approximately 50 μmol photons m^{-2} s^{-1}. Results shown are for algae in seawater (SW), hydrated in air (Air) and desiccated in air (D). The first two lanes show mRNA abundance under mild (60–70% thallus water content, TWC) and severe (*c.* 10% TWC) desiccation respectively (see below), and the third and fourth lanes are after 1 h and 24 h recovery following a return to low irradiance (*c.* 50 μmol m^{-2} s^{-1}). (*b*) Desiccation rates of algae in HL and LL experimental conditions used for Northern analysis. Values are means ± SE ($n = 5$). Note that tissue water contents in HL after 15 min desiccation were comparable to those in LL after 60 min.

that these transcripts were more abundant following desiccation than in controls (Fig. 2*e* for D30 2.17).

Various patterns of transcript abundance for other differentially expressed clones are illustrated in Fig. 2*f–i*: Clone D30 1.1 hybridized to three common bands from control algae, but to a fourth band from desiccated algae at *c.* 1·9 kb (Fig. 2*f*). A simple increase in transcript abundance was seen for clone D30 1.10, which hybridized to bands at *c.* 1·0 and 1·2 kb (Fig. 2*g*). In contrast, clone D30 2·6 showed complex differences in hybridization pattern between control and desiccated algae (Fig. 2*h*). Finally, clone D30 2.14 hybridized to a band at *c.* 2·5 kb from desiccated, but not from control algae. In addition there were other more subtle changes in expression at *c.* 1·6 kb (Fig. 2*i*).

Northern analysis of Rubisco expression

Standard Nothern blotting was used to verify the usefulness of reverse Northerns (cDNA Southern blots) for detecting differential gene expression. Results for Rubisco expression are presented in Fig. 3*a*. At high irradiance Northern analysis provided qualitatively similar information to the reverse Northern (Fig. 2*b*), with transcript abundance higher in desiccated than in SW control algae (Fig. 3*a*). While this result validates the use of reverse Northerns for identification of differentially expressed genes, the complexity of the situation for Rubisco is indicated in the more detailed experiment in Fig. 3. These results showed that Rubisco transcript abundance declined in SW during the HL treatment, but remained relatively stable during desiccation in air. When algae remained hydrated in air, there was a decline in Rubisco abundance after 3 h in air that continued during the first hour of recovery (at LL), but the decline was much lower than that observed in SW, and final transcript levels were higher after 24 h recovery (Fig. 3*a*).

In contrast to the situation in HL, after transfer to LL conditions, Rubisco transcript abundance increased in SW, and remained high after 1 h and 24 h 'recovery' (17:00 and 16:00 hours local time, respectively). These observations suggest that a strong circadian pattern of transcript abundance might occur under HL, which was disrupted by transfer to LL. Hydrated and desiccated tissue showed fairly stable levels of Rubisco expression during the treatment and after 1 h recovery in SW (Fig. 3*a*).

Discussion

The PCR-based subtractive hybridization technique is a useful method for obtaining cDNA clones of genes whose expression is up-regulated during emersion and desiccation in fucoid algae. Nearly 70% of clones randomly picked from the library were shown to be more abundant in desiccated tissues than in hydrated (immersed) controls. Significantly, all the (non-rRNA) cDNAs sequenced (21 clones) have been unique, suggesting that the library was both equalized for rare and abundant sequences, and well-represented for different gene products. We used total RNA as the starting material for

cDNA synthesis, although it is likely that the percentage of redundant clones for rRNA (*c.* 30 %) in the library could be reduced by using purified poly-A^+ mRNA. The SMART cDNA synthesis methodology used to construct the library is designed to select only full-length mRNAs possessing a poly-A^+ tail for exponential amplification by PCR following reverse transcription. The reason for the presence of cDNA clones for rRNA in the library is not known. It is likely that the relative abundance of rRNA (most clones were for chloroplast rRNAs) in the original total RNA used for cDNA synthesis was so great that a certain amount of mis-priming and reverse transcription was inevitable.

Another question that arises from this library screening is whether chloroplast mRNA from *F. vesiculosus* is polyadenylated, given the fairly high proportion of chloroplast cDNAs identified. None of the cloned (partial) cDNAs for chloroplast encoded genes were polyadenylated, although only 5 of the 18 (restriction-digested) cDNA sequences presented in Table 1 included a poly-A^+ tail. There is evidence that chloroplast mRNA may be polyadenylated as part of a targeting mechanism for degradation (reviewed by Schuster *et al.*, 1999). This raises the possibility that subtracted chloroplast cDNAs may in fact represent transcripts targeted for degradation via polyadenylation, and amplified by the SMART RT-PCR from which cDNA for reverse Northerns was produced. In a previous study, reverse Northern (i.e. cDNA Southern) analysis using SMART cDNA was shown to be a reliable method yielding quantitatively similar results for gene expression to standard Northern blotting techniques (Endege *et al.*, 1999). Northern blotting results of this study also confirmed the corresponding reverse Northern data in showing greater Rubisco transcript abundance during mild desiccation than in SW at high irradiance (Fig. 3). Therefore, our data suggest that the presence of cDNAs for chloroplast transcripts in the library is unlikely to be a result of a polyadenylation/degradation pathway. A more likely explanation for the presence of chloroplast sequences is mis-priming during reverse transcription, perhaps due to the AT-rich nature of chloroplast DNA, and subsequent PCR amplification.

The results from Northern analysis illustrate that care must be taken before assigning a 'stress-responsive' role to any particular gene solely from comparison of hydrated and stressed tissues. For example, Rubisco abundance did not increase in response to desiccation *per se*, but rather *decreased* during the high-irradiance treatments in SW, but not (to the same extent) when hydrated in air, or during desiccation. In contrast, an increase in transcript accumulation was observed under low irradiance in SW, but with little change in hydrated or desiccated algae. Thus, depending on whether high- or low-irradiance conditions are used to treat algae prior to RNA extraction and library construction, Rubisco gene expression might appear to be up-regulated or down-regulated.

Both the molecular mechanisms and the environmental signals underlying the large and rapid (within 15 min) changes in Rubisco mRNA abundance that were observed require further study. Light is clearly an environmental signal, but other interacting factors are involved in the differential responses under immersed versus emersed conditions. In particular, changes in inorganic carbon availability and the operation of carbon-concentrating mechanisms may play a role (Johnson & Raven, 1986; Surif & Raven, 1989; Schmid, 1998). In higher plants, changes in (nuclear encoded) *rbc*S transcript abundance have been observed in response to reductions in external CO_2 concentration (Majeau & Coleman, 1996). In the experiment reported here endogenous rhythms of plastid photosynthetic mRNA abundance may also have been superimposed on responses to other factors, since quite large shifts in Rubisco abundance were observed under constant HL conditions. Chloroplast gene expression is mainly controlled at the post-transcriptional level, either by regulation of transcript stability via 5′ UTR control regions (Shiina *et al.*, 1998; Salvador & Klein, 1999) or via light-dependent translational control (reviewed by Bruick & Mayfield, 1999). However, for *rbc*L, transcription rate is light-regulated, but transcript stability increases in the dark (Shiina *et al.*, 1998). It remains to be seen which processes mediate the rapid decrease in Rubisco mRNA levels at high irradiance in SW versus air in *F. vesiculosus*.

This study is the first attempt to investigate changes in gene expression during desiccation in fucoid algae, despite a long history of interest in these highly desiccation-tolerant intertidal algae (Kawamitsu & Boyer, 1999; Pearson *et al.*, 2000; reviewed by Chapman, 1995; Davison & Pearson, 1996). A disadvantage of the approach used here is that no information about the identity of the genes responding to the variables under investigation is guaranteed and therefore further analysis (e.g. sequencing of full-length cDNAs) is often required. One of the main advantages of PCR-based methods is the efficiency of subtractive hybridization (Diatchenko *et al.*, 1996; Gurskaya *et al.*, 1996), with each PCR cycle representing a round of subtraction; another is the equalization of rare and abundant transcripts in the subtracted library. However, in order to achieve efficient subtraction, the lengths of the starting cDNA molecules are made more equal by digestion with restriction enzymes. This results in cDNA fragments that are

rather short, reducing the amount of sequence information subsequently available, and therefore reducing the likelihood of identifying genes directly from clones. However, with additional molecular tools (Rapid Amplification of cDNA Ends (RACE)-PCR and/or full-length cDNA libraries), these limitations can be overcome and will allow identification of many stress-responsive genes in macroalgae.

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