

Microarray analysis of transcriptional responses to abscisic acid and osmotic, salt, and drought stress in the moss, *Physcomitrella patens*

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Summary

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Received: 31 May 2007
Accepted: 2 June 2007

- Dehydration tolerance was an adaptive trait necessary for the colonization of land by plants, and remains widespread among bryophytes: the nearest extant relatives of the first land plants. A genome-wide analysis was undertaken of water-stress responses in the model moss *Physcomitrella patens* to identify stress-responsive genes.
- An oligonucleotide microarray was used for transcriptomic analysis of *Physcomitrella* treated with abscisic acid (ABA), or subjected to osmotic, salt and drought stress. Bioinformatic analysis of the *Physcomitrella* genome identified the responsive genes, and a number of putative stress-related *cis*-regulatory elements.
- In protonemal tissue, 130 genes were induced by dehydration, 56 genes by ABA, but only 10 and eight genes, respectively, by osmotic and salt stress. Fifty-one genes were induced by more than one treatment. Seventy-six genes, principally encoding chloroplast proteins, were drought down-regulated. Many ABA- and drought-responsive genes are homologues of angiosperm genes expressed during drought stress and seed development. These ABA- and drought-responsive genes include those encoding a number of late embryogenesis abundant (LEA) proteins, a 'DREB' transcription factor and a Snf-related kinase homologous with the *Arabidopsis* ABA signal transduction component 'OPEN STOMATA 1'.
- Evolutionary capture of conserved stress-regulatory transcription factors by the seed developmental pathway probably accounts for the seed-specificity of desiccation tolerance among angiosperms.

Key words: abscisic acid (ABA), anhydrobiosis, dehydration stress, desiccation tolerance, moss, *Physcomitrella patens*, transcriptional profiling.

New Phytologist (2007) **176**: 275–287

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doi: 10.1111/j.1469-8137.2007.02187.x

Introduction

The green plants constitute one of the major classes of eukaryotes on the planet, comprising approx. 500 000 species. Phylogenetic and palaeobotanic evidence suggests that the present variety of land plants diversified from a single colonization of the land during the mid-Ordovician period (*c.* 480 million yr ago; Kenrick & Crane, 1997). Fundamental to the success of plants

in colonizing a terrestrial habitat is the acquisition of adaptations to an uncertain supply of water. The currently dominant taxa among the land plants – the tracheophytes – display numerous anatomical adaptations to the terrestrial environment, including ramifying root systems to scavenge water from the substratum, extensive vascular tissues for its delivery throughout the plant, and stomates, cuticles and lignin that restrict evaporative loss, whilst facilitating gas

exchange, and providing mechanical strength. By contrast, the first plants to assume a terrestrial lifestyle lacked these adaptations and must, necessarily, have exhibited a variety of biochemical and physiological mechanisms to ensure their survival during times of drought (Oliver *et al.*, 2005).

Most bryophytes retain the property of vegetative desiccation tolerance, in the form of anhydrobiotic survival, and occupy niches characterized by frequent cycles of dehydration and rehydration (Dilks & Proctor, 1974). In the evolution of the vascular plants, this property has been lost, in favour of the adaptations associated with added complexity and increased diversity. True desiccation tolerance, in the form of anhydrobiotic survival, has become restricted to the protection of metabolically quiescent reproductive propagules: spores and pollen (sometimes) and seeds (usually) (Oliver *et al.*, 2000). In only a few remarkable species (termed 'resurrection plants') has vegetative desiccation tolerance re-evolved within the tracheophyte phylogeny (Ingram & Bartels, 1996; Bartels, 2005).

Nevertheless, many processes characteristic of the early stages of embryonic desiccation tolerance may be recognized within water-stressed vegetative tissues. These include the early accumulation of compatible osmolytes, and of potentially stabilizing compounds such as proline, glycine-betaine, polyhydric alcohols and disaccharides (Bianchi *et al.*, 1991; Ishitani *et al.*, 1995; Yoshida *et al.*, 1995; McKue & Hanson, 1996). ABA coordinates these stress responses, mediating physiological processes such as stomatal closure (an immediate response to restrict evaporative water loss), osmolyte accumulation, and also the synthesis of stress-related proteins, including late embryogenesis abundant (LEA) and heat shock proteins (HSPs), as well as compounds associated with the scavenging of reactive oxygen species that are implicated in desiccation-related membrane damage (Leopold *et al.*, 1991; Ingram & Bartels, 1996; Hoekstra *et al.*, 2001).

The molecular responses to dehydration in higher plants have been studied by genome-wide microarray analysis of gene expression processes in the model angiosperm, *A. thaliana*, resulting in the identification of several hundred individual genes whose expression is induced by dehydration stress, salinity stress and cold stress, as well as an additional subset of *c.* 150 genes expressed during the recovery from these stresses. The identification of groups of coregulated genes has enabled the identification of common sequence motifs among the promoters of these genes, and the identification of the transcription factors that regulate their expression (Seki *et al.*, 2002; Oono *et al.*, 2003; Shinozaki *et al.*, 2003).

Since dehydration tolerance is an ancient evolutionary adaptation within the plant kingdom, it is of clear interest to determine the extent to which common genetic mechanisms leading to dehydration stress and desiccation tolerance have been conserved, and to identify the processes by which the phenomenon of desiccation tolerance has become developmentally restricted within the higher plant lineages. For the desiccation-tolerant moss *Tortula ruralis*, the ability to survive

rapid and complete desiccation appears to rely on an ABA-independent, constitutive mechanism supported by the induction of a repair-associated gene set upon rehydration (Oliver, 1991; Oliver *et al.*, 2004, 2005). Such a mechanism is not universal among bryophytes, however, and in the dehydration-resistant, but desiccation-sensitive, moss *Physcomitrella patens*, ABA-related induction of stress-related gene products, clearly homologous with higher-plant stress-related genes, is associated with the response to, and survival of, both dehydration stress (Knight *et al.*, 1995; Machuka *et al.*, 1999; Frank *et al.*, 2005; Kamisugi & Cuming, 2005; Oldenhof *et al.*, 2006), and of freezing tolerance (Minami *et al.*, 2003; Takezawa & Minami, 2004; Oldenhof *et al.*, 2006).

The developing genomic resources available for *Physcomitrella* have been exploited, using an oligonucleotide microarray to identify a larger set of genes expressed in response to ABA and water stress in a near relative of the earliest land plants. A number of genes whose stress-related expression is conserved between moss and angiosperms were identified. Analysis of the putative promoter regions of these genes identifies a number of potential *cis*-acting elements similar to those identified in stress-induced genes of higher plants. A number of GC-rich sequence elements were also identified that appear significantly over-represented in the promoters of the induced gene set, and that represent candidates for further functional characterization.

Methods and Materials

Plant material

Physcomitrella patens ssp. *patens* (Hedwig) ecotype 'Gransden 2004' was propagated in culture as described by Knight *et al.* (2002). Protonemal tissue was subcultured at weekly intervals on cellophane overlays on solid BCD medium containing 5 mM ammonium tartrate. Treatment with abscisic acid and osmotic stressing agents was applied to protonemal (largely chloronemal) tissue, 6 d following subculture: the cellophane overlays were transferred on to filter paper soaked with liquid BCD medium containing 5 mM ammonium tartrate and additional supplements: *cis-trans* ABA was added to a final concentration of 10^{-5} M; mannitol was added at a final concentration of 10% (w/v) and NaCl at a final concentration of 0.3 M. Tissue was incubated for 2 h before harvesting. For dehydration treatments, the cellophane-grown moss was transferred directly to the plastic base of a 9 cm Petri dish by inverting the cellophane and peeling it away from the protonemal tissue. Dishes were placed without lids in desiccators containing saturated NaCl to provide an atmosphere of relative humidity = 75% (Young, 1967). The extent of fresh-weight loss by the tissue was monitored by weighing the dishes at intervals following the onset of treatment. Tissue was harvested and squeezed dry before freezing in liquid nitrogen and storage at -70°C before RNA isolation.

Microarray analysis

Total RNA was isolated from moss tissue by aqueous phenol extraction, as described by Knight *et al.* (2002). Two replicate samples for each treatment were extracted and the quality of RNA samples was monitored in two ways. First, equivalence of stress-induced gene expression between replicate samples was checked by RNA gel blot hybridization using a cDNA probe corresponding to a transcript previously known to be induced by all four stress treatments (*PpLEA-2*: AW497323). Second, integrity of the RNA was determined on an Agilent 2100 bioanalyser using an RNA 6000 LabChip kit. An Agilent-certified microarray service lab (MOgene, LC, St. Louis, MO, USA) was used to perform the microarray experiments. Gene-specific 60-base oligomers were printed on to glass slides by Agilent Technologies Inc. RNA samples (2.5 µg) were labelled using the ULS-Cy 3/5 ULS aRNA Labelling Kit (product no. EA-006, Kretech Biotechnology, San Diego, CA, USA). As a quality control, a dye swap for labelling RNA samples was performed. Once the samples were labelled with reciprocal Cy fluorescent dyes, equal amounts (1 µg) of samples were combined in nuclease-free water and processed using the Gene Expression Hybridization Kit (product no. 5188-5242, Agilent, Palo Alto, CA, USA). The sample was then placed between the Agilent backing slide and the microarray chip, sealed in the hybridization chamber and set to hybridize for 17 h in a 60°C rotating hybridization oven. Upon completion, the slides were washed sequentially in 6 × SSC (0.9 M NaCl, 90 mM Na-acetate, pH 7.0) buffer at room temperature, then 0.1 × SSC (0.15 M NaCl, 15 mM Na-acetate, pH 7.0) on ice. Slides were then dried using nitrogen gas and placed in holders, and scanned using the DNA Microarray Scanner (no. G2565BA, Agilent) with the Agilent Scan Control software. The fluorescent intensities of each feature were extracted using the Feature Extraction Software with default parameters (version 9.1, Agilent). The raw intensity data then were log₂-transformed for normalization before ANOVA (mixed model) analysis. When the raw intensities of both Cy3 and Cy5 channels were below 150 (typical background intensity is 40) and the signal-to-background ratio below 2, the genes were removed from further analysis. Log ratios among different samples and the *P*-values were calculated using the mixed model (Wolfinger *et al.*, 2001). A gene was considered as a significant change in gene expression based on the twofold cutoff with *P*-values < 0.05.

RNA blot hybridization

RNA gel blot hybridization was used to identify individual transcripts using a subset of selected cDNA probes as indicated. Probes were isolated by restriction enzyme digestion of EST clones, for labelling. Gel electrophoresis, blotting, probe synthesis, hybridization and detection were carried out as previously described (Kamisugi & Cuming, 2005).

Gene identification

The genomic sequences corresponding to individual microarray features were retrieved from the *Physcomitrella* genome sequence assembly, version 1 (http://genome.jgi-psf.org/Phypa1_1/Phypa1_1.home.html) using BLAST searches (Altschul *et al.*, 1990). The repeat-masked genome assembly was searched with the sequences of the EST contigs from which the microarray oligonucleotides were designed, using the BLASTN tool, following their retrieval from NIBB Phycobase (<http://moss.nibb.ac.jp>). Gene models were selected, where possible, by alignment with cDNA sequences. Where cDNA sequences were incomplete, the intron and exon sequences determined by the gene prediction software were manually curated following pairwise comparison between the translated *Physcomitrella* genomic assembly and the polypeptide sequence of the closest plant homologues using BLASTX and TBLASTN alignment, and the gene models annotated. *Cis*-acting sequences previously identified in promoters of higher plant genes were identified in 1 kb lengths of *Physcomitrella* DNA sequence located immediately 5′- to the gene coding sequence using the PlantCARE search tool (Lescot *et al.*, 2002: <http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>). Hexameric sequence motifs that were relatively over-represented in the promoters of the ABA- and stress-induced gene set were determined by using the TAIR motif finder software (<http://arabidopsis.org/tools/bulk/motiffinder/index.jsp>).

Gene retrieval

Gene models for all the features up- and down-regulated in this study can be retrieved from the *Physcomitrella* Genome Browser v1.1 using the protein ID number assigned to each chip feature in Tables S1 and S2 (Supplementary material), as follows: (i) go to http://genome.jgi-psf.org/Phypa1_1/Phypa1_1.home.html and select 'Search'; (ii) scroll down to 'Gene models' and select 'Protein id' in the drop-down menu; (iii) enter the ID number and click 'Search models' – results are returned as 'Model search results'; (iv) clicking on 'Model ID' links to the gene model page (gene and transcript sequence, polypeptide sequence, annotation details).

Results

Microarray analysis

The microarray for *Physcomitrella* was designed based on the 22 895 cDNA contigs obtained from Phycobase (<http://moss.nibb.ac.jp>). These represented the total available publicly accessible EST set generated by the Leeds University *Physcomitrella* EST programme and the National Institute for Basic Biology (Nishiyama *et al.*, 2003). The 3′ region in each contig was targeted for computational generation of 60-base oligomers. Among 190 784 oligomers generated, 21 939

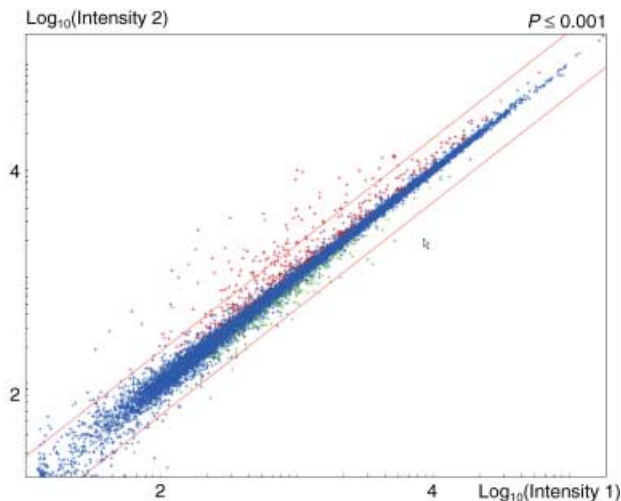


Fig. 1 Microarray analysis of abscisic acid (ABA)-regulated genes. A dot-matrix plot of the signal intensities of each feature on the microarray chip for Cy3-labelled ABA-treated probe (Intensity 2) vs Cy5-labelled control probe (Intensity 1), plotted on a \log_{10} scale. The two red lines indicate the values at which genes are twofold up- or down-regulated. Red data points are indicated as up-regulated by the Agilent software, and green data points as down-regulated. Only those genes indicated as outside the twofold threshold of change in expression were analysed in detail.

were selected based on melting temperature (T_m) range, the number of Gs, complexity such as repeats and simple sequences, RNA secondary structure and uniqueness based on BLAST score. The BLAST was performed against the Dec-01-03 version of nucleotide sequences downloaded from NCBI (<http://www.ncbi.nlm.nih.gov>). The E -value cutoff used was $1E^{-06}$ using BLASTN. These oligomers were fabricated on the chip. Because the cDNA contigs from which these oligonucleotides were designed have since undergone several revisions, each feature on the chip has been denoted by a GenBank accession number assigned to a representative cDNA clone. These will be further denoted by a gene model accession number when the genomic sequences are released via GenBank. Initial microarray experiments compared transcripts induced by ABA-treated protonemal tissue with protonemal tissue incubated in hormone-free medium. Tissue was treated with 10^{-5} M ABA for 2 h before harvest. The principal effect of ABA treatment was to cause the appearance of new transcripts (Fig. 1): 60 features indicated significant (twofold or greater) up-regulation, by comparison with only four features whose corresponding transcript abundances fell by the same amount.

Because of the interrelationship between ABA and drought, osmotic and salt stresses, a further series of analyses was undertaken of transcript abundance in protonemal tissue subjected to these treatments. Salt stress resulted from incubation in the presence of 0.3 M NaCl, whilst osmotic stress resulted from incubation in the presence of 10% mannitol. Each of these treatments was applied for 2 h before harvest of

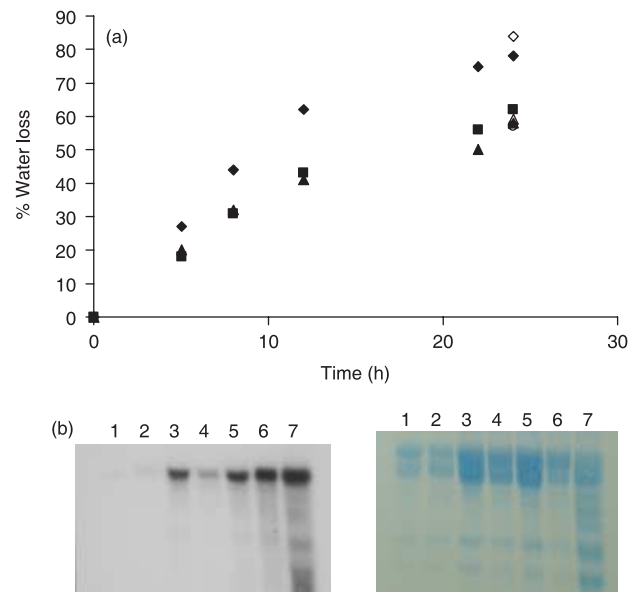


Fig. 2 Dehydration of moss *Physcomitrella* protonemal tissue. (a) Time-course of dehydration of protonemal tissue. Water loss was determined from three replicate plates for each time point except 24 h (six replicate plates). (b) RNA gel blot hybridization of mRNA isolated from tissue dehydrated to different extents: following blot transfer, the blot was hybridized with a cDNA probe corresponding to the group 2 late embryogenesis abundant (LEA) protein gene, *PpLEA-2* (chip feature 15287: EST clone AW497323). The autoradiograph and the corresponding filter stained with methylene blue is shown so that unequal loading of RNA can be taken into account. The region of the filter shown contains the 18SrRNA (topmost band) and fragments derived from 23S and 16S chloroplast rRNA. Tracks contain: 1, control RNA; 2, 8% water loss; 3, 11% water loss; 4, 16% water loss; 5, 30% water loss; 6, 60% water loss; 7, 95% water loss.

tissue. Drought stress was applied by controlled drying of tissue in an atmosphere of 75% relative humidity. Tissue was harvested at intervals (Fig. 2) and monitored for induction of a candidate stress-related transcript (the *PpLea-2* gene product: feature 15287 on the microarray), and for viability following rehydration. Figure 2(a) shows the rate of water loss by individual batches of protonemal tissue over a period of 24 h, and Fig. 2(b) indicates the accumulation of the *PpLea-2* transcript in tissue harvested at progressive stages of dehydration, detected by northern blot hybridization. Tissue dehydrated to 84% water loss was able to resume normal growth following rehydration. Tissue dehydrated to 90% water loss or greater was unviable. There was a general (but inexact) correspondence between the extent of water loss and the accumulation of the *PpLea-2* transcript, and, significantly, RNA extracted from highly dehydrated tissue (95% fresh weight loss) had undergone some degradation (Fig. 2b).

Messenger RNA derived from tissue that had undergone c. 84% fresh weight loss was used to generate the hybridization probe for microarray analysis. The results of this analysis are summarized in Fig. 3, with further detail provided in Tables

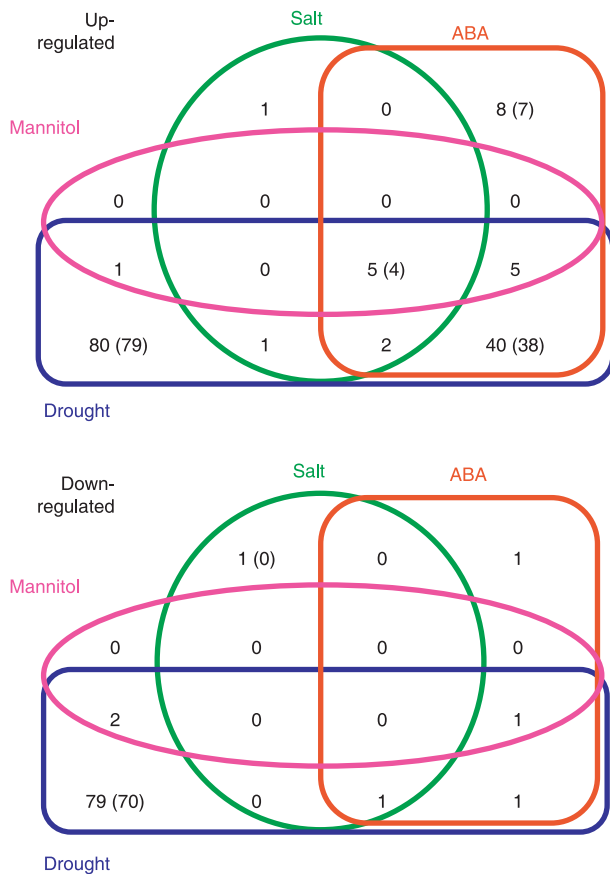


Fig. 3 Genes up- and down-regulated by abscisic acid (ABA) and stress treatments. Transcripts exhibiting significant changes (> twofold) in abundance following 2 h treatment with 10^{-5} M ABA (ABA), 2 h incubation on 10% mannitol (mannitol), 2 h incubation on 0.3 M NaCl (salt) and dehydration to 84% fresh weight loss (drought). Numbers in brackets indicate the numbers of genes, where some chip features correspond to different sequences within the same transcript.

S1 and S2. The complete datasets have been deposited in ArrayExpress (Array A-MEXP-646; Experiment E-TABM-225). Drought treatment resulted in the largest number of up-regulated transcripts being detected, with 134 features identified as indicating twofold up-regulation or greater. Osmotic stress (11 features) and salt stress (nine features) were less effective in inducing the accumulation of new transcripts. Drought treatment also resulted in the largest number of down-regulated transcripts (84 features). Because the features on the chip were based on EST sequences clustered in 2003, subsequent genomic analysis has identified that, in some cases, the oligonucleotides correspond to different regions of the same gene. Consequently, the number of genes represented by these features is slightly fewer than the number of chip features.

There is considerable overlap between the sets of genes regulated by the different treatments, with 49 of the 56 ABA-up-regulated genes also showing increased expression in

response to at least one other treatment. Four genes were induced by all four treatments, and seven by three of the treatments. Of the smaller numbers of genes induced in response to salt- and mannitol-induced osmotic stress, all but one were also up-regulated by one or more additional treatment. The single gene expressed only in response to salt treatment (feature 2398) encodes a glutathione-S-transferase most similar to the product of an *Arabidopsis* gene annotated as 'ERD' (early response to dehydration). Of the larger number of ABA- and drought-induced genes, seven were exclusively ABA-induced, while 70 were only induced by drought.

Many of the genes up-regulated by stress and ABA share significant homology with higher plant genes whose expression is ABA- and stress-regulated. The polypeptides encoded by these genes were used in BLAST searches of the plant protein database, and the gene products assigned functional categories following gene ontology analysis. The results of this analysis are summarized in Fig. 4, and the sequences of the polypeptides encoded by both up- and down-regulated genes are provided in Tables S1 and S2. A significant subset of the strongly up-regulated genes ($n = 16$) were found to contain amino acid sequence motifs characteristic of LEA proteins (Table 1). The majority of these have the characteristic signatures of group 3 LEA proteins, while two contain motifs typical of the group 2 ('dehydrin') gene family. Several other sequences have homology with higher plant proteins whose GenBank annotations directly associate their expression with ABA and abiotic stress treatments, including drought, cold and salt stresses (Table 2). Additionally, a number ($n = 14$) encode proteins with membrane association, involved in either signalling or transmembrane transport of solutes: an important feature of osmoregulation in response to water deficit stress in plants (Table 3). Ten genes encoded polypeptides with no significant homologues, and thus correspond to novel moss-specific genes. This category included the most strongly up-regulated gene (feature 5076) and a related transcript (feature 18538) up-regulated 16.8-fold and threefold by drought stress, respectively. The majority of genes down-regulated by drought stress encode chloroplast components, most being photosynthesis-associated (Fig. 4; $n = 53$).

Among the drought up-regulated genes, two encode proteins with possible functions in the ABA and/or drought-stress response. A gene encoding a Snf-related protein kinase similar to the *Arabidopsis* gene 'OPEN STOMATA1' (*OST1*) was induced 3.4-fold (chip features 19662 and 7931; protein ID 228733), and a gene encoding a member of the Apetala-2 'DREB' transcription factor family (chip feature 3326; protein ID 228738) showed 2.4-fold induction. *OST1* is both ABA-induced and required for ABA-mediated stomatal closure in *Arabidopsis* (Mustilli *et al.*, 2002), while DREB transcription factors activate drought-inducible gene expression (Shinozaki *et al.*, 2003).

Microarray analysis provides a powerful and rapid means of identifying genes that are differentially regulated in response

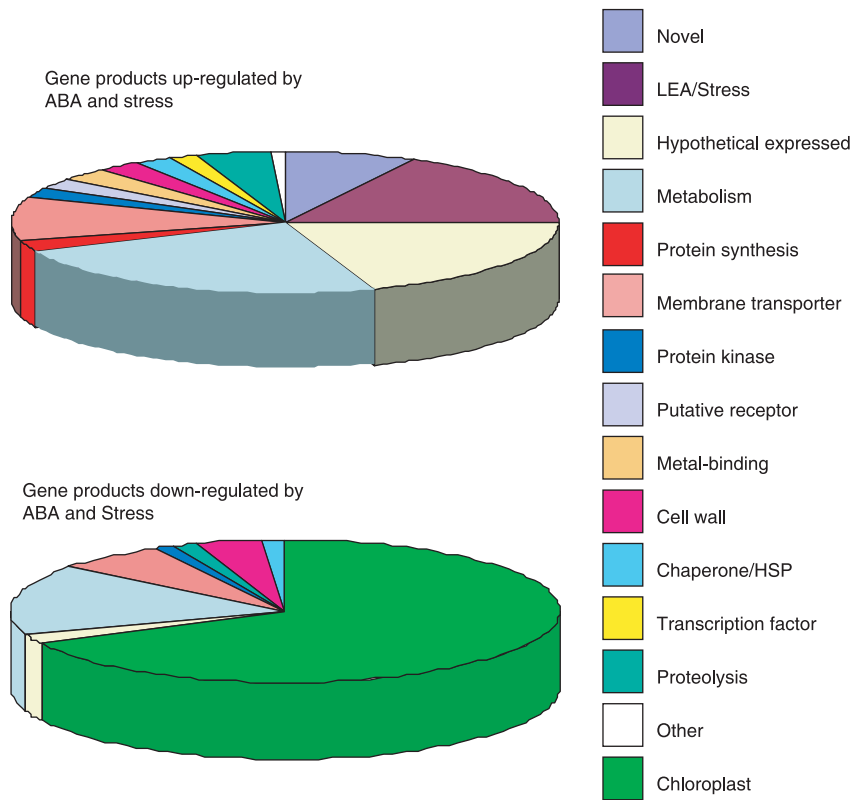


Fig. 4 Functional categorization of up- and down-regulated genes. Conceptual translation of the up- and down-regulated genes was used to determine the polypeptide sequences of their products. BLASTP analysis of the plant protein database was used to identify their nearest homologues and their likely functions were further characterized by GO function searches. ABA, abscisic acid; LEA, late embryogenesis abundant; HSP, heat shock protein.

Table 1 *Physcomitrella* late embryogenesis abundant (LEA) genes up-regulated by abscisic acid (ABA)- and stress treatment

Feature	Protein ID	A	M	S	D	Annotation
15557	166566	6.42	5.61	3.09	7.61	Group 3 LEA
18008	166566	7.65	4.10	3.20	15.95	Duplicate of above
19261 ^b	166566	2.51			3.04	Duplicate of above
11860	228647 ^a	6.99	3.17		4.49	Group 3
10252	211998	6.46	2.51		9.37	Group 3
15287	173331	3.59	3.08	2.44	3.97	Group 2 LEA
8128	228668	3.53			4.77	Group 3
2804	228669	2.84			7.22	Group 3
20869	228673	3.50			3.31	Group 3
13858	228675	3.25			5.88	Group 3
16783	228681	2.69	2.11		3.20	Group 3
15827	166565				4.51	Group 3
5581	166565				2.26	Duplicate of above
12864	223670	2.91			3.65	Group 3
16461	223670	2.44			2.87	Duplicate of above
5350	228668	3.13			3.08	Group 3
15595	228697	2.62			3.26	Group 3
16224	228710	2.12			2.8	Group 3
17721	228735				2.51	Group 2-like
19583	228736				2.45	Group 3

The up-regulation for each gene is indicated: A, ABA; M, 10% (w/v) mannitol; S, 0.3 M NaCl; D, dehydration to 84% fresh weight loss.

^aThree identical tandemly arrayed genes recognize chip feature 11860: see Table 5.

^bThis chip feature was designed from an incompletely spliced transcript and thus has reduced identity with the mature mRNA.

to abiotic stresses, but it is a technique that is subject to many sources of experimental variability, and it is prudent to verify the results obtained using an alternative means of estimating steady-state transcript abundance. A number of genes were therefore selected whose sequence analysis indicated that they encoded LEA proteins, and their transcript abundance was independently determined by northern blot hybridization. The results obtained (Fig. 5) generally confirmed the accuracy of the microarray analysis for these sequences. For each of the LEA genes analysed, the highest transcript abundances were observed following ABA and drought treatment, with significantly lower increases in transcript abundance occurring in response to treatments with 10% mannitol and 0.3 M NaCl. Notably, although these two treatments were much less effective in inducing transcript accumulation than ABA and drought treatment, an increase in steady-state transcript abundance was detectable by blot hybridization in relation to the control samples for these treatments. This is in agreement with several studies that have noted that microarray analysis, whilst an excellent tool for large-scale identification of up-regulated sequences, can significantly underestimate the true extent of transcript accumulation (Yuen *et al.*, 2002; Barczak *et al.*, 2003). Some genes are represented by duplicate chip features. Generally, the amounts of transcript induction detected by these features correspond well, although it is well attested that different oligonucleotide designs can result in markedly different signal intensities corresponding to the

Table 2 Other *Physcomitrella* genes homologous with flowering plant abscisic acid (ABA)- and stress-induced genes

Feature	Protein ID	A	M	S	D	Flowering plant annotation
490	228645	3.57			4.23	Unknown dehydration-associated (<i>Xerophyta humilis</i>)
6027	228654	3.72	3.36		3.78	At5g01300 cold-regulated phosphatidylethanolamine binding protein
9277	228674	2.60			3.86	AT3g05500 stress-related rubber elongation factor
13762	228678	2.41	2.40	2.26	5.56	WCOR413 cold-stress related
2398	228689			2.17		Glutathione-S-transferase 'ERD'
7195	110135	2.33			3.07	At3g50830.1 cold-acclimation protein
18914	181142	2.05			4.51	At3g50830.1 cold-acclimation protein
16491	177939				2.76	ERD-1 ATP-binding chaperone (chloroplast?)
6933	205434 ^a				2.22	Small heat-shock protein.
	70357 ^a					
4257	171674				2.13	Ethylene-responsive rice protein: contains 'universal stress protein' domain
13165	228750				2.09	HSP40 type heat-shock protein (DNAJ domain)
7842	215149				3.03	ALDH21 family aldehyde dehydrogenase

The up-regulation for each gene is indicated: A, ABA; M, 10% (w/v) mannitol; S, 0.3 M NaCl; D, dehydration to 84% fresh weight loss.

^aChip feature 6933 identifies two near-identical genes encoding Protein ID205434, and Protein ID 70357.

Table 3 Membrane-associated abscisic acid (ABA)- and stress up-regulated genes

Feature	Protein ID	A	M	S	D	Annotation
17684	166082	4.85			4.29	AWPM-19 membrane channel protein
20213	228663	2.78			2.34	At2g47770 benzodiazepene receptor like
16234	228676	2.98			4.73	AWPM-19 membrane channel protein
13762	228678	2.41	2.40	2.26	5.56	WCOR413 cold-acclimation protein
15531	228695	2.21			2.13	At1g22710 sucrose transporter
7195	110135	2.33			3.07	WCOR413 cold-acclimation protein
18914	181142	2.05			4.51	WCOR413 cold-acclimation protein
6967	228698	2.26				AWPM-19 membrane channel protein
14509	164391	2.35			2.43	Chloroplast envelope protein
5415	109889				2.64	Voltage-dependent ion channel
17738	191107	2.29			2.96	Tonoplast intrinsic protein (aquaporin)
3290	228745				2.19	Predicted integral membrane protein
4457	228747				2.16	Calcium exchange protein
5484	189127				2.00	Sugar transporter

The up-regulation for each gene is indicated: A, 10⁻⁵ M ABA; M, 10% Mannitol; S, 0.3 M NaCl; D, dehydration to 84% fresh weight loss.

same transcript (Barczak *et al.*, 2003). In one case (the gene encoding group 3 LEA protein 166533), one chip feature (19261) was designed from an incompletely spliced EST contig, and so has only partial homology with the mature transcript (Table 1).

The transcriptional response to ABA is very rapid. The significant transcript accumulation that occurred in response to ABA treatment did so within 2 h of the application of the growth regulator. To elucidate the rate of the response in more detail, the accumulation of selected transcripts was monitored at very much shorter intervals during this two-hour period. Northern blot analysis demonstrates that, for representative *LEA* transcripts, accumulation of transcript can be observed during the first 15 min of application, with transcript abundance showing a steady increase throughout the 2 h period (Fig. 6).

Promoter analysis of ABA- and stress-regulated genes

The coordinate regulation of gene expression is most frequently mediated by transcriptional induction. Bioinformatic analysis represents a powerful tool with which stress-responsive genes may be recognized through their common promoter elements (Zhang *et al.*, 2005). The sequences 5'-proximal to the mRNA coding sequences (the putative promoters) were therefore examined to identify the presence of conserved sequence motifs that might correspond to binding sites for *trans*-acting transcription factors, and represent targets for future experimental investigation. Two approaches were taken: a scan for sequences known to be implicated in ABA- and abiotic stress-mediated transcription in higher plants, and an approach not based on *a priori* expectations, in which all possible hexameric sequences were evaluated for whether they

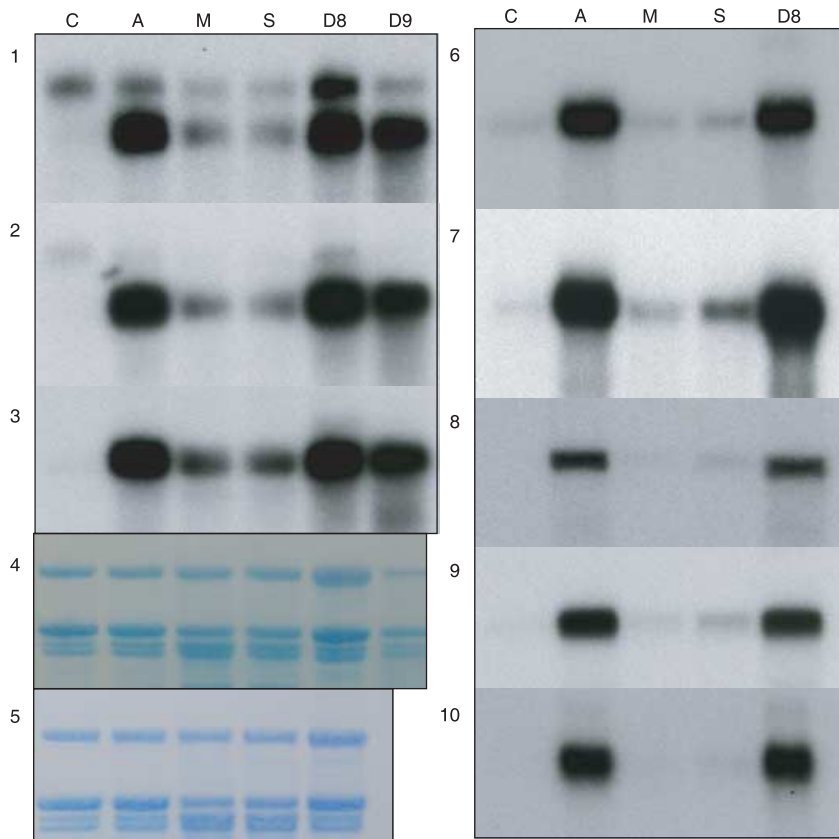


Fig. 5 RNA gel blot hybridization with selected cDNA probes. RNA used for the various chip hybridizations was analysed by RNA gel blot hybridization with selected cDNA probes. C, control treatment; A, 10^{-5} M abscisic acid (ABA); M, 10% (w/v) mannitol; S, 0.3 M NaCl; D8, dehydration to 84% water loss; D9, dehydration to 95% water loss. Probes used were as follows: panel 1, feature 15557/18008 (group 3 LEA: EST clone BQ827691); 2, feature 17684 (AWPM-19 membrane channel protein: EST clone BQ041987); 3, feature 6027 (cold-regulated phosphatidylethanolamine-binding protein: EST clone BQ826761); 6, feature 17209 (protein of unknown function: EST clone AW476845); 7, feature 15287 (*PpLEA-2*: EST clone AW497323); 8, feature 8128 (group 3 LEA protein: EST clone BQ826612); 9, feature 13858 (group 3 LEA protein: EST clone BQ827490); 10, feature 15827/5581 (group 3 LEA protein: EST clone BQ827063). Equivalence of sample loading is shown in panels 4 and 5; methylene blue-stained RNA transferred to the nylon membrane on each of two filters that were successively probed, stripped and re-probed. Panel 4 corresponds to hybridizations 1–3 and panel 5 to hybridizations 6–10. The region of the stained filters shown contains 26S rRNA (topmost band) and 18S rRNA, and fragments derived from the chloroplast 23S and 16S rRNA (these fragments derive from denaturation of the rRNA molecules which contain 'hidden breaks').

were overrepresented in the promoter regions, relative to the entire sequence of each up-regulated gene. Highly conserved stress-related transcription factors that have been identified in flowering plants include members of the basic domain-leucine zipper (bZip) family that associate with 'G-box' elements (motifs containing an ACGT core sequence) in ABA-response elements (ABREs). Such a motif has been previously identified as instrumental in the ABA-induction of the gene *PpLea-1*, encoding a *Physcomitrella* group 1 LEA protein (Kamisugi & Cuming, 2005). Additionally, 'DREB' transcription factors of the ethylene response element binding factor/*APETALA-2* (EREB/At2) class interact with dehydration response elements (DREs) (also known as C-repeat (CRTs), or 'coupling elements' when found in tandem with ABREs; Shen & Ho, 1995) to mediate expression of genes in response to drought and low-temperature stress. In flowering plants, the DRE/CRT motif contains a conserved A/GCCGAC sequence that is the binding site for these factors. Other transcription factors implicated in similar abiotic stress responses include the basic helix-loop-helix-zipper domain (bHLHZip) MYC factors (binding site CANNTG) and helix-turn-helix MYB factors (binding site YAAGTG) (Abe *et al.*, 1997, 2003). Hexanucleotides corresponding to these consensus sequences were found with relatively high

frequency in the presumptive promoters, being especially concentrated within the 400 bp region immediately adjacent to the TATA box. The relative distribution of these and other motifs in the promoters of the 25 most strongly up-regulated genes is summarized in Table 4 and Fig. 7.

The search for hexameric sequences identified a number of GC-rich motifs as being significantly over-represented in the promoters of the *Physcomitrella* ABA- and stress-induced gene set (Table 5). Significantly, the promoters of the *Physcomitrella* genes are generally less GC-rich overall (41% GC) than the coding sequences (46% GC), implicating these sequences as candidates for *cis*-acting regulatory elements. Notably, this approach also identified the canonical ACGT-containing elements as being significantly over-represented among the promoters of the induced gene set.

Discussion

During dehydration, substantial accumulation of novel transcripts was observed, many of which are also induced by the growth regulator ABA, and which encode conserved members of gene families associated with the acquisition of desiccation tolerance. A significant number of these encode LEA proteins: a class of protein originally identified in seeds

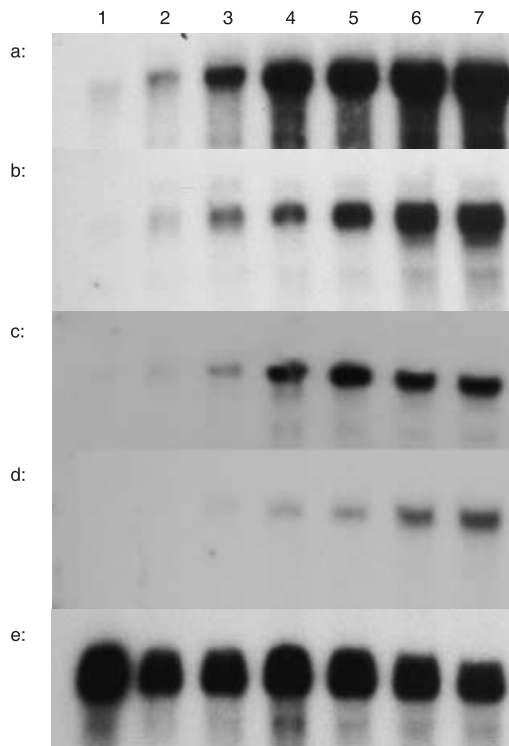


Fig. 6 Time-course of late embryogenesis abundant (LEA) mRNA accumulation. RNA was harvested at intervals following the application of 10^{-5} M abscisic acid (ABA) and analysed by RNA gel blot hybridization with a selection of cDNA probes encoding group 3 LEA proteins. Track 1, control (zero time); 2, 15 min; 3, 30 min; 4, 45 min; 5, 60min; 6, 90min; 7, 2 h. Probes used were as follows: panel a, AW497323 (*PpLea-2*: chip feature 15287; protein ID 173331); b, BU052152 (group 3 LEA: chip features 15557/18008; protein ID 166566); c, AJ225549 (group 3 LEA: chip feature 12864; protein ID 223670); d, BQ827572 (group 3 LEA: chip feature 11860; protein IDs 228647, 228650, 228651); e, AW509984 (plastocyanin: chip feature 17161; protein ID 170637) to monitor RNA loading.

during the later stages of embryogenesis, and since found to be highly conserved in other anhydrobiotic taxa, including green algae (Honjoh *et al.*, 1995), nematodes (Browne *et al.*, 2001) and rotifers (Tunnacliffe *et al.*, 2005). LEA proteins are believed to relieve the consequences of desiccation through sequestration of water or ions (Cuming, 1999; Hoekstra *et al.*, 2001), acting as chaperone-like 'molecular shields' (Goyal *et al.*, 2005) or through structurally reinforcing the cell following desiccation-induced structural changes (Goyal *et al.*, 2003; Wise & Tunnacliffe, 2004). Thus, there appears to be a close correspondence between the responses of ABA- and dehydration-stressed *Physcomitrella* protonemal tissue and the preparations made by the seeds of higher plants for the desiccation that is both an inherent part of their development and a requirement for the longevity that ensures their dispersal in time as well as in space.

Another significant group of *Physcomitrella* genes induced both by ABA and by dehydration encode proteins with a probable function in osmoregulation. These include membrane

Table 4 The 25 genes most strongly up-regulated by abscisic acid (ABA)

Gene	Feature	Protein ID	A	Identity
1	18008	166566	7.65	Group 3 LEA
2	5076	228642 228647 ^a	7.41	No significant homologue Group 3 LEA: three tandemly arrayed genes recognize the same chip feature
3	11860	228650 ^a 228651 ^a	6.99	
4	10252	211998	6.46	Group 3 LEA
5	17684	166082	6.42	AWPM-19 membrane channel
6	18538	166706	4.85	Similar to gene 2
7	8165	228658	4.84	Protein of unknown function (At5g01750)
8	10956	228655	4.22	Protein kinase C
9	6027	228654	4.00	Cold-regulated PE-binding protein
10	14636	228643	3.72	Protein of unknown function (At4g31830)
11	13200	228672	3.68	'Little protein 1' (<i>O. sativa</i>)
12	15287	173331	3.67	Group 2 LEA
13	490	228645	3.59	Unknown dehydration- associated
14	3795	228670	3.57	Phosphoglycerate kinase
15	8128	228668	3.55	Group 3 LEA
16	20869	228673	3.53	Group 3 LEA
17	13858	228675	3.50	Group 3 LEA
18	8316	228662	3.25	Alpha-amylase type B isozyme
19	5350	228668	3.16	Group 3 LEA
20	22055	228680	3.13	Possible LEA protein
21	7860	228683	3.08	Translation elongation factor 1a
22	11817	228692	3.02	Unknown – possible protein kinase
23	16234	228676	2.98	AWPM-19 membrane channel
24	12864	223670	2.98	Group 3 LEA
25	2804	228669	2.91	Group 3 LEA

The up-regulation by ABA is indicated in A.

^aThis chip feature identifies three identical tandemly repeated genes.

transport functions such as aquaporins and sugar transporters (Table 3), but also metabolic enzymes such as a stress-associated aldehyde dehydrogenase of the moss-specific ALDH21 sub-family (Chen *et al.*, 2002; Kirch *et al.*, 2004) (Table 2).

There are more genes whose transcripts accumulate in response to dehydration, than in response to other treatments, and also a significant number of genes that are down-regulated by dehydration but not by the other treatments. This may simply reflect the longer period over which dehydration stress was applied, relative to other treatments (24 vs 2 h). Alternatively, it may indicate the existence of ABA-dependent and ABA-independent response pathways. In flowering plants, ABA-specific induction of stress-related gene expression typically operates via the binding of bZip transcription factors to ABREs, a mechanism that also occurs in *Physcomitrella* (Knight *et al.*, 1995; Kamisugi & Cuming, 2005). The

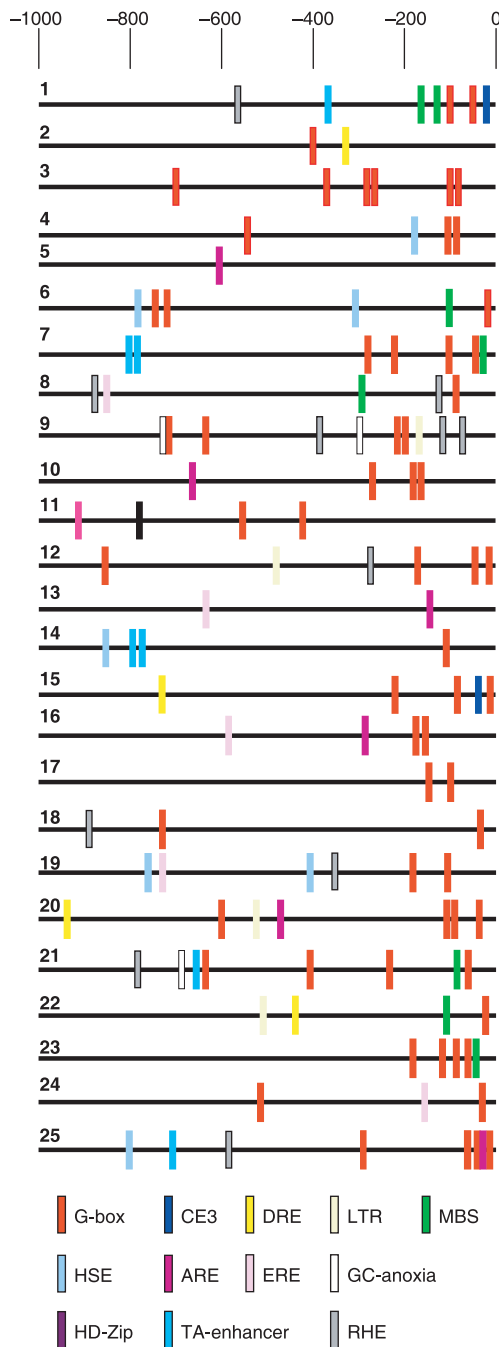


Fig. 7 Distribution of putative *cis*-acting sequences. The first 1000 bp immediately 5'- to the presumptive TATA box in the 25 genes most strongly up-regulated by abscisic acid (ABA; Table 5) were scanned for sequences corresponding to known higher plant *cis*-acting sequences using the PlantCARE search tool. G-box, ACGT-core motifs; CE3, 'coupling element' associated with *VP1/ABI3* mediated gene expression; DRE, drought responsive element; LTR, low-temperature responsive element; MBS, *MYB*-binding site; HSE, heat-shock element; ARE, anaerobic response element; GC-anoxia, GC-rich anoxia response element; HD-Zip, homeodomain-basic leucine zipper binding site; TA-enhancer, TA-rich sequence required for high expression; RHE, rehydration responsive element. Candidate MYC-binding elements are not shown; owing to their degenerate nature, these were too numerous for clear reproduction.

Table 5 Hexameric sequences significantly over-represented in the promoters of abscisic acid (ABA)- and stress-induced genes

Promoter	Entire gene		
Motif	Occurrences	Occurrences	% in promoter
CCCCCC	188	263	71.48
GGGGGG	188	263	71.48
GGGCCC	68	104	65.38
GCGCGC	44	68	64.71
CGCGCG	50	82	60.98
CACGTG	126	224	56.25
ACGTGG	156	280	55.71
CCACGT	156	280	55.71
GGCCCC	61	119	51.26
GGGGCC	61	119	51.26
GGGGGA	100	202	49.50
TCCCCC	100	202	49.50
CCCCCA	102	217	47.00
TGGGGG	102	217	47.00
CCCCCG	52	111	46.85
CGGGGG	52	111	46.85
CGCGCC	37	80	46.25
GGCGCG	37	80	46.25
CGTGGC	127	277	45.85
GCCACG	127	277	45.85
CCC CGC	33	73	45.21
CGCGGG	33	73	45.21

All possible hexameric sequences were identified in the induced gene set, and scored for their occurrence in the promoter and protein-coding sequences. The number of occurrences in each region and the percentage of these in the promoter region are shown. The promoter regions of the entire sequence set represented 45% of the total quantity of sequence analysed, and only those hexamers that occur in promoters with a frequency > 45% are shown. Hexamers that correspond to all or part of an ACTG-containing 'G-box' element are in bold type.

angiosperm ABA-independent dehydration-specific response is mediated by the 'DREB' class of *Apetala2*-type transcription factors binding to a GC-rich DRE (Shinozaki & Yamaguchi-Shinozaki, 1997; Dubouzet *et al.*, 2003; Shinozaki *et al.*, 2003). The existence of a comparable pathway in mosses has yet to be experimentally demonstrated. However, our observation that a DREB-like transcription factor is up-regulated during dehydration stress, together with the identification of DRE-like elements within the 5'-flanking sequences of some dehydration-induced genes, suggests that this mechanism may also be conserved between bryophytes and angiosperms.

Transcripts exhibiting a substantial reduction in abundance included a preponderance of chloroplast-specific gene products. This was also observed in *Arabidopsis* (Seki *et al.*, 2002) and likely accounts for the generally inhibitory effect of drought stress on photosynthetic activity. However, mosses typically exhibit morphogenetic changes following prolonged exposure to dehydration stress, or to ABA, resulting in the formation of

brachyocytes, or 'brood cells' (Bopp & Werner, 1993): these are typically thick-walled, lipid-rich vegetative spores (Schnepp & Reinhard, 1997), with substantially altered chloroplast morphology. Thus, widespread changes in chloroplast gene products may reflect the early stages of brachyocyte differentiation. Similarly, proteomic studies of *Physcomitrella* brachyocyte formation have identified specific changes in the extracellular protein spectrum in response to ABA (S. Tintelnot, pers. comm.), including changes in several gene products identified in this study (pectin methylesterase, proline-rich wall proteins, LRR-containing proteins, a plant-specific fasciclin and germin-like proteins) with probable wall-modifying functions.

Desiccation tolerance is widespread among bryophytes, yet the underlying mechanism of tolerance and its relationship to the mechanisms operating in the desiccation-tolerant stages of angiosperm development remain unclear. In the desiccation-tolerant moss *Tortula ruralis*, desiccation-associated changes in gene expression occur principally during the rehydration phase, rather than in the period during which water loss is occurring, leading to the suggestion that such species are constitutively prepared for desiccation, and that novel gene expression is required for the rapid repair of desiccation-induced cellular damage (Oliver, 1991; Oliver *et al.*, 2004, 2005). It is clear that *Physcomitrella* differs in this respect, and interesting that many of the genes induced before dehydration encode proteins similar to those identified in the rehydration transcriptome of *Tortula* (Oliver *et al.*, 2004, 2005).

It is noteworthy that the genes up-regulated by ABA and stress treatment of *Physcomitrella* are generally fewer in number than those identified in similar experiments undertaken with *Arabidopsis thaliana*. *Physcomitrella* is intermediate in its degree of dehydration stress tolerance in comparison with *Tortula* and *Arabidopsis*; although protonemal tissue does not survive desiccation, it is nevertheless highly tolerant of dehydration (Frank *et al.*, 2005). Moreover, plants comprising both protonemata and gametophores will tolerate complete desiccation following slow drying, if first pretreated with ABA, a process that is associated with a substantial increase in intracellular concentrations of sucrose (Oldenhof *et al.*, 2006), as is cold-acclimation leading to freezing tolerance (Nagao *et al.*, 2005). This is similar to the ABA-mediated induction of desiccation tolerance in cultured tissue of the resurrection plant, *Craterostigma plantagineum*, and to the ABA-mediated acquisition of desiccation tolerance during angiosperm embryogenesis (Bartels *et al.*, 1988, 1990; Bianchi *et al.*, 1991; Ooms *et al.*, 1993; Bartels, 2005; Smith-Espinoza *et al.*, 2005). It may be that *Physcomitrella* retains a residual population of constitutively expressed genes with a stress-protective function, whereas the corresponding genes in angiosperms have undergone 'evolutionary capture' by an inducible mechanism.

It is clear that genes that confer desiccation tolerance have not been lost during the evolution of the land plants. Instead, their expression has become developmentally sequestered

within the reproductive stages of the life cycle (typically during seed development). Moreover, the frequency with which vegetative desiccation tolerance has independently re-evolved throughout the land plant phylogeny (Oliver *et al.*, 2005) implies that mutations in a relatively small number of regulatory genes may account for the this developmental sequestration.

Within the angiosperms, a subset of the genes expressed during late embryogenesis is restricted to this stage of development owing to a requirement for seed-specific transcription factors. Principal among these is the ABI3 class of transcriptional activator that mediates ABA-induced gene expression specifically in developing seeds through an interaction with the ABI5-type bZip factors (Ezcurra *et al.*, 2000; Nakamura *et al.*, 2001). In *Physcomitrella* protonemata, a similar mechanism activates the ABA-mediated expression of the group 1 *LEA* genes that in angiosperms are seed-specific (Knight *et al.*, 1995; Kamisugi & Cuming, 2005; Marella *et al.*, 2006). Interestingly, whereas all fully characterized angiosperm genomes contain only a single *ABI3* gene, the *Physcomitrella* genome contains at least three such genes (Marella *et al.*, 2006). If this multiplicity of the ABI3 family within the *Physcomitrella* genome reflects the situation more widely among the bryophytes, then it could be hypothesized that the evolutionary loss of the additional copies during the tracheophyte divergences, coupled with the developmental 'capture' of this transcription factor by the embryogenic developmental programme, would have resulted in the wholesale capture of its subordinate genes to this stage of the life cycle.

Acknowledgements

ACC thanks the UK Biotechnology and Biological Sciences Research Council for their support of the *Physcomitrella* EST programme (PEP). SHC was the recipient of a fellowship from the Korea Research Foundation (MOEHRD, Basic Research Promotion Fund, M01-2004-000-10317-0). The support of the *Physcomitrella* Genome Consortium by the US Department of Energy's Community Sequencing Program at the Joint Genome Institute is gratefully acknowledged.

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Supplementary Material

The following supplementary material is available for this article online:

Text S1 Supplementary information

Tables S1 and S2 Relative fold-induction or down-regulation

Figs S1 and S2 Products of up- and down-regulated genes

This material is available as part of the online article from: <http://www.blackwell-synergy.com/doi/abs/10.1111/j.1469-8137.2007.02187.x>
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