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Abscisic acid causes changes in gene expression involved in the induction of the landform of the liverwort *Riccia fluitans* L.

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Abstract. The conversion of the submerged form of *Riccia fluitans* to the landform either by transfer to a moist solid surface or by treatment with abscisic acid (ABA), is accompanied by the formation of a set of new polypeptides and concomitant down-regulation of other polypeptides. Changes in gene expression were analyzed by two-dimensional separations of proteins and differential screening of a cDNA library. One of the landform-specific proteins might depend on the expression of the newly discovered *Ric 1* gene. The deduced amino acid sequence of the isolated *Ric 1* cDNA clone codes for a protein with a molecular mass of 30.1 kDa. This polypeptide possesses two amino acid sequences which are repeated five times each and it is largely hydrophilic with the exception of a hydrophobic carboxyl-terminal region. Under ABA treatment the expression of the *Ric 1* mRNA had already reached its maximum after 1 h of incubation. Transferring submerged thalli onto an agar surface resulted in a slower induction. The *Ric 1* gene product shows homology to an embryo-specific polypeptide of carrot seeds and to the group 3 of late-embryogenesis-abundant (LEA) proteins. Interestingly, ABA treatment improved the desiccation tolerance of the submerged thalli. Additionally, ABA stimulated the synthesis of a protein which is immunologically related to a tonoplast protein. This finding, together with the fact that the ABA-induced landform exhibits an increased activity of several vacuolar enzymes, may indicate a special role of the tonoplast and the vacuole during ABA-induced conversion of the thallus from the submerged to the terrestrial form.

Key words: Abscisic acid – Drought tolerance – Protein synthesis – *Riccia* – Vacuole

Introduction

Abscisic acid (ABA), a stress hormone of higher plants, has been shown to occur in a wide range of species of bryophytes (Hartung et al. 1987; Hartung and Gimmler 1994). Three physiological functions have been found for ABA in bryophytes: (i) ABA can induce closure of stomata in *Anthoceros* and in musci. (ii) ABA induces desiccation tolerance in xerophytic members of the Marchantiales, such as *Exormotheca holstii* (Hellwege et al. 1994) and protonemata of mosses (Werner et al. 1991); (iii) ABA induced landform characteristics of thalli in aquatic species such as *Riccia fluitans* or *Ricciocarpus natans* (Hellwege et al. 1992; Hartung and Gimmler 1994).

The role of ABA in the induction of desiccation tolerance in *Exormotheca holstii* has been studied in more detail by Hellwege et al. (1994). These authors demonstrated that ABA induces the formation of ABA- and desiccation-specific polypeptides which exhibit a significant similarity to dehydrins of higher poikilohydric plants (Bartels et al. 1990; Schneider et al. 1993) and to dehydrins of desiccated corn embryos (Close et al. 1993a, b).

Nothing is known, however, about the action of ABA on the molecular level during induction of landform thalli in *Riccia fluitans*. Therefore, we have used several approaches to answer this question. We (i) studied landform- and ABA-specific polypeptides of *Riccia fluitans*; (ii) established a cDNA library, performed a differential screening and sequenced an ABA- and landform-specific DNA clone; and (iii) carried out some experiments to look for possible functions of the landform-specific proteins.

Materials and methods

Plant material and growth conditions. Riccia fluitans thalli were cultivated under sterile conditions at 20–22 °C and a light-/dark-regime of 14 h/10 h. The nutrient medium for the submerged form contained 1.5 mM KH_2PO_4, 2.0 mM KNO_3, 1.0 mM $CaCl_2$, 1.0 mM $MgSO_4$, 18.0 μM FeNaEDTA, 8.1 μM H_3BO_3, 1.5 μM $MnCl_2$ and glucose (0.2%, w/v). The terrestrial form was grown on agar (1.5% w/v) with the same nutrient composition as the medium

Abbreviations: ABA = abscisic acid; 2-D PAGE = two-dimensional polyacrylamide gel electrophoresis; F_0, F_m = initial and maximal chlorophyll *a* fluorescence; LEA = late embryogenesis abundant; Q_A = primary quinone acceptor of PS II

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for the submerged form, but without glucose. For landform induction in submerged thalli, 10^{-6} M ABA was added to the nutrient solution described above. If not otherwise mentioned, the analysed plants were cultivated for four weeks prior to analysis.

Determination of enzyme activities. The activities of several hydrolytic enzymes were determined by measuring the liberation of *p*-nitrophenol (PNP) from their substrates (shown in parentheses): α-mannosidase (PNP-α-mannopyranoside), β-galactosidase (*o*-nitrophenyl-β-galactopyranoside), phosphatase (PNP-phosphate), phospho-diesterase (bis-PNP-phosphate) and β-N-acetylglucosaminidase (PNP-β-N-acetylglucosaminide). The assay mixture contained 0.1 M citric acid adjusted to pH 4.6 with NaOH, 6.6 mM of the appropriate substrate and 5–80 µl plant extract in a total volume of 400 µl. After incubation at 37 °C for 60 min, 1 ml of 0.2 M Na_2CO_3 was added and the liberated PNP quantified spectrometrically at 405 nm. The activities of the enzymes were calculated from the measured absorption changes using the molar extinction coefficient $\varepsilon_{405} = 18\,300\,l \cdot mol^{-1} \cdot cm^{-1}$.

Measurement of chlorophyll a fluorescence. The quenching of chlorophyll *a* fluorescence was measured as described earlier (Hellwege et al. 1994).

Extraction of proteins. Thalli frozen in liquid nitrogen were homogenised in 100 mM phosphate buffer (pH 7.5) containing 2% β-mercaptoethanol, extracted with phenol, and precipitated from the phenol phase by addition of 0.1 M ammonium acetate in ethanol supplemented with 10 mM dithiothreitol (DTT). For protein determination, an aliquot of the phenol-ethanol extract was centrifuged for 15 min at $12\,000 \cdot g$. The sediment was washed with 70% ethanol, dried and dissolved by boiling in 0.1 M NaOH for 10 min. The protein content of an aliquot was determined using the BCA (bicinchoninic acid) assay of a commercial supplier (Pierce Chemical Comp., Oud-Beijerland, The Netherlands). For separation by polyacrylamide gel electrophoresis, aliquots of the phenol/ethanol phase containing equal protein contents were centrifuged, washed with ethanol containing 10 mM DTT, dried under nitrogen and resuspended in sample or lysis buffer (O'Farrell 1975).

Two-dimensional gel electrophoresis and autoradiography. Two-dimensional gel electrophoresis was performed as described by O'Farrell (1975) modified by Dietz and Bogorad (1987a). After electrophoresis the gels were either silver-stained according to the method of Blum et al. (1987) or prepared for autoradiography as described by Dietz and Bogorad (1987b).

In-vivo labelling of proteins. After induction in a solution with 10^{-6} M ABA, five tips from thalli branchings were incubated for 2 h in 25 µl nutrient solution containing 10^{-6} M ABA and 85 kBq [^{35}S]methionine. Incorporation was carried out under continuous light at room temperature. After repeated washing of the thalli with 500 µl nutrient solution, proteins were extracted as described above. The incorporated radioactivity was determined after precipitation of an aliquot with trichloroacetic acid as described by Dietz and Bogorad (1987b).

Western blot analysis. Polypeptides were separated on an SDS-polyacrylamide gel (12.5%, w/v; Laemmli 1970) and transferred to nitrocellulose membranes (0.2 µm; Schleicher & Schüll, Dassel, Germany) using a semi-dry blotter (Millipore SDE; Millipore Corporation, Milford, Mass., USA). The immunological detection assay was performed as described by Betz and Dietz (1991).

Construction and screening of a cDNA library. The complementary DNA (cDNA) was synthesised with the Riboclone-cDNA-synthesis-system (C1001; Promega Corporation, Madison, Wis., USA) following the method described by Gubler and Hoffman (1983). Five micrograms poly(A)$^+$mRNA from the ABA-induced landform was used. The double-stranded cDNA was inserted into the *Eco* RI-*Xba* I arms of the lambda GEM™-4 vector. After packaging, the *Escherichia coli* strain LE392 was infected with the phages and the plaques were transferred to nylon filters (Hybond N; Amersham, Braunschweig, Germany). The filters were differentially screened using ^{32}P-labelled first-strand cDNA probes synthesised from poly(A)$^+$mRNA isolated either from the submerged form or the landform of *Riccia fluitans*. Clones hybridising only to the first-strand DNA of the landform were selected and rescreened.

Analysis of DNA sequences. Inserts from the recombinant plasmid were purified by electrophoresis. The cDNA was sequenced from both strand ends with T7, T3 or Sp6 primer. Additionally, the cDNA insert was digested with *Alu* I, *Hae* III, *Dra* I and *Kpn* I and cDNA-fragments were subcloned into *Eco* RV-digested pBSC SK + . These cDNA-fragments were also sequenced. Comparisons of DNA and protein sequences were performed using the program HUSAR (Deutsches Krebsforschungszentrum Heidelberg, Germany) and the SWISS-PROT protein sequence database.

Northern blot analysis. Total RNA (20 µg) denatured in formamide and formaldehyde was size-fractionated in a formaldehyde-containing gel (Sambrook et al. 1989) and blotted to nylon filters (Hybond N; Amersham, Braunschweig, Germany). The filters were baked for 2 h at 80 °C and hybridised with random-primed ^{32}P-labelled cDNA in 4 × SSC (1 × SSC: 0.15 M NaCl, 0.015 M Na citrate-NaOH, pH 7.0), 0.5% (w/v), SDS and 0.1% (w/v) each of Ficoll, polyvinylpyrrolidone and bovine serum albumin at 65 °C. After hybridisation the filters were washed with 0.5% SDS and SSC concentrations decreasing from 2.0 × SSC to 0.5 × SSC at 65 °C. The filters were exposed to X-ray films (Kodak X-OMAT S) for 4–10 d.

Results

Protein patterns of the waterform, landform and ABA-induced landform of Riccia fluitans. In a first approach towards understanding the molecular changes underlying the expression of the landform, polypeptides from submerged, terrestrial and ABA-treated thalli which were grown for four weeks under otherwise identical environmental conditions were extracted and separated by two-dimensional gel electrophoresis. The analysis was based on four independent experiments. Only gels showing changes representative of most or all gels are included in the figure. Although most of the polypeptides were present in all thalli in similar amounts, distinct differences in the protein patterns of the water- and landforms could already be observed in silver-stained gels. Some polypeptides seemed to be specifically associated with the submerged conditions and disappeared after the transformation of the thalli to the landform (Fig. 1A, marked with circles). Simultaneously, 17 new spots appeared exclusively on the gels of the terrestrial thalli (Fig. 1B, marked with squares). Twelve of these landform-specific polypeptides could also be found in the ABA-treated thalli (Fig. 1C).

Effects of ABA on gene expression during the induction of landform structures. In a second approach the time-dependent induction of landform-specific polypeptides by ABA was examined. Polypeptides synthesised in the tips of submerged thalli of *R. fluitans* which were subjected to 10^{-6} M ABA for different time intervals were radioactively labelled with L-[^{35}-S]methionine for 2 h, extracted and separated by two-dimensional (2-D) PAGE. We chose the meristematic tips of the thalli where the induction of

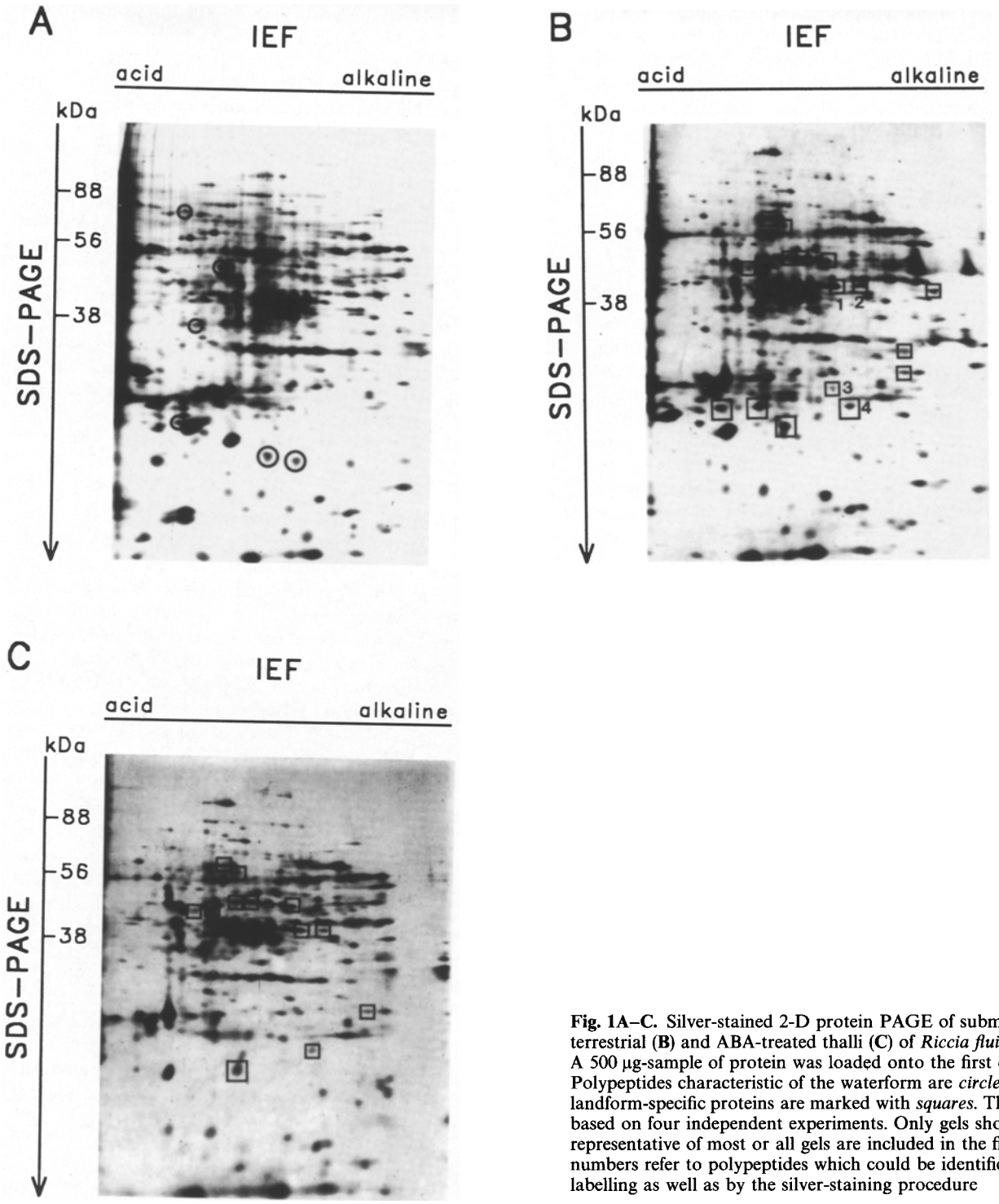


Fig. 1A–C. Silver-stained 2-D protein PAGE of submerged (**A**), terrestrial (**B**) and ABA-treated thalli (**C**) of *Riccia fluitans*. A 500 µg-sample of protein was loaded onto the first dimension. Polypeptides characteristic of the waterform are *circled*, landform-specific proteins are marked with *squares*. The analysis was based on four independent experiments. Only gels showing changes representative of most or all gels are included in the figure. The numbers refer to polypeptides which could be identified by in-vivo labelling as well as by the silver-staining procedure

landform characteristics takes place. Tips of the thalli of the waterform and landform were used as controls.

Similar to the silver-stained gels the majority of the polypeptides in thalli of the submerged and the terrestrial form of *R. fluitans* were identical. However, the synthesis of some polypeptides characteristic of the waterform (Fig. 2A, circled) seemed to be reduced in the landform. In contrast, the polypeptides marked with squares were synthesised in different amounts only under terrestrial conditions (Fig. 2B) and may thus be called landform-specific.

Nearly all radiolabelled landform-specific polypeptides could also be found under ABA treatment. Following the time-dependence of the induction of landform-specific polypeptides by ABA (Fig. 2C–F) we found that the first ABA-inducible landform polypeptides appeared during an early period (3–6 h) of induction (Fig. 2C, marked with squares) and that the incorporated radioactivity into most of these proteins increased up to 24 h. The synthesis

of other landform polypeptides started after 9–15 h (Fig. 2D), while a few proteins could not be detected earlier than after 24 h (Fig. 2E). After 48–72 h almost all polypeptides of the landform were also visible in more or less identical amounts on the ABA gel (Fig. 2F). It should be noted that even after this long time of incubation the amount of newly synthesised thallus was very small and that no effects of wounding could be observed on protein patterns of *R. fluitans* (data not shown).

To determine the abundance of these radioactively labelled polypeptides and to compare them more easily with those of the silver-stained gels the 2-D gels were first subjected to fluorography and then stained also with silver. Using this technique of consecutive detection, some polypeptides could be characterised both in terms of turnover and abundance. Some major landform-specific polypeptides known from the silver-stained gels were also highly radiolabelled (Figs. 1B, 2B, numbered). The polypeptide patterns of waterform and landform were also compared after in-vitro translation of isolated poly(A)$^+$mRNA in a cell-free rabbit reticulocyte lysate system (data not shown). Both approaches showed some polypeptides with identical migration characteristics (Fig. 2B, F, marked with arrows) showing regulation on the level of mRNA abundance.

Isolation of an ABA-inducible landform cDNA. As shown above the two-dimensional separation of polypeptides revealed substantial differences between the waterform and landform of *Riccia fluitans*. Therefore, we established a cDNA library with poly(A)$^+$mRNA from ABA-induced landform thalli of *R. fluitans*. About 40 000 recombinants were obtained in this library which was screened differentially with mRNA of the water- and landforms. One phage plaque, called *Ric 1*, hybridised selectively to the poly(A)$^+$mRNA of the landform. The vector DNA of this clone was isolated and transferred into plasmid DNA. Digestion of this plasmid DNA with *Eco* RI and *Xba* I followed by separation of the products by agarose-gel electrophoresis resulted in an insert of about 1.2 kb.

The nucleotide sequence of the *Ric 1*-cDNA was determined and is shown in Fig. 3, as well as the deduced amino acid sequence. Assuming that initiation of translation occurs at the first methionine, the gene contains an open reading frame of 288 amino acids, which results in a polypeptide with a predicted molecular mass of 30.1 kDa and an isoelectric point of 5.2. Alanine (20,5%), threonine (12,5%) and Lysine (12,2%) are the most abundant amino acids present in the deduced polypeptide. Cysteine is lacking, and hydrophobic amino acids like tryptophan, phenylalanine and isoleucine are rare. The hydropathy plot presented in Fig. 4 indicates that the protein is mainly hydrophilic, with a small hydrophobic region at the carboxy terminus. Within the polypeptide, two sets of repeats of two amino-acid sequences Gly-Ala-Glu-Lys-Thr-Glu-Gln-Ala-Lys (Fig. 3, A1–A5) and Ala-Val-Asp-Lys-Ala-Val-Glu-Gly-Lys-Asp (Fig. 3, B1–B5) are present.

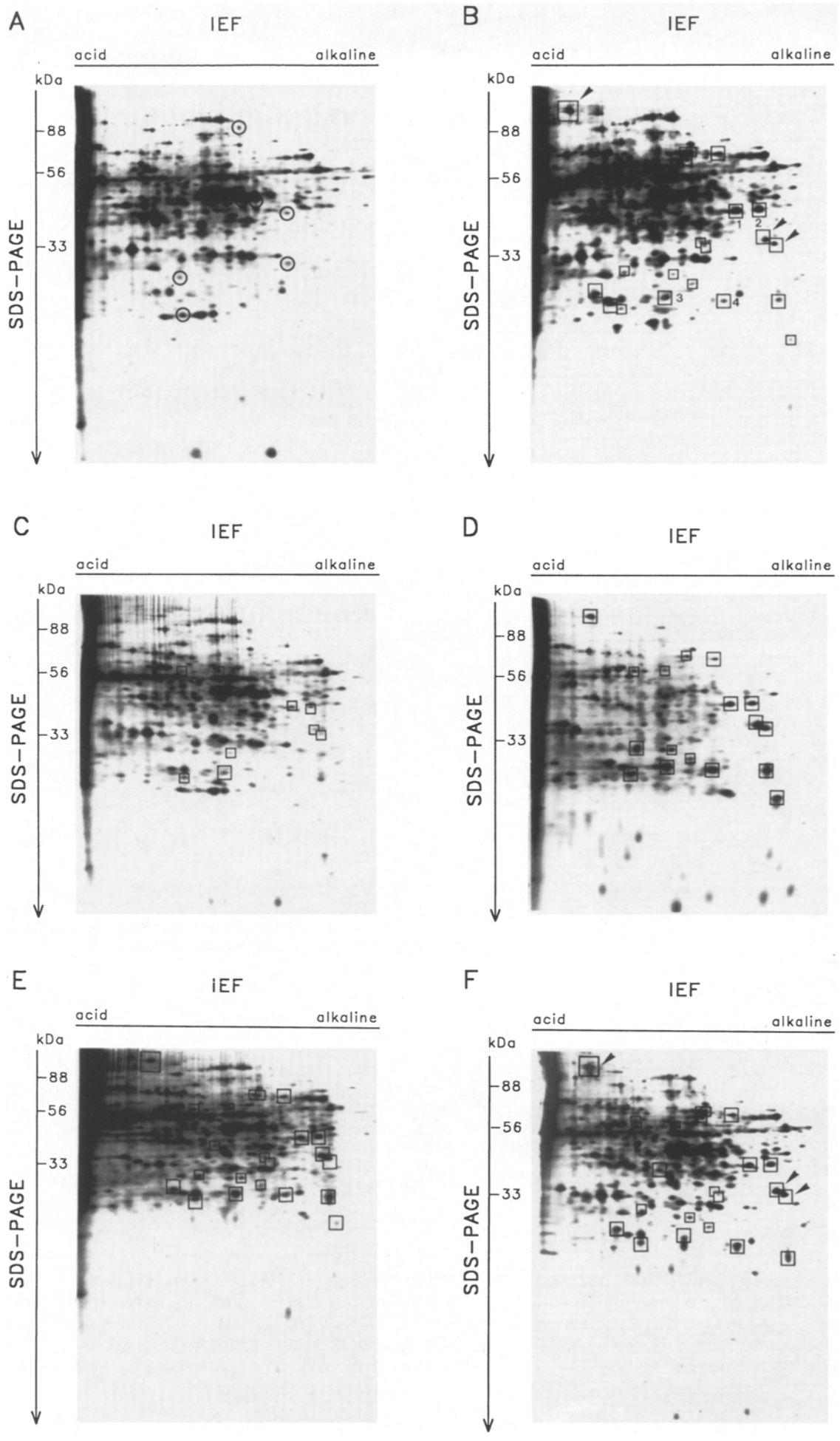
Specificity and induction kinetics of Ric 1. A Northern blot analysis of RNA isolated from submerged, terrestrial and ABA-treated thalli of *R. fluitans* which were probed with the clone *Ric 1* is shown in Fig. 5. The clone hybridised exclusively to a RNA species of the landform and the ABA-induced landform. The size of this RNA species was estimated to be 1.2–1.3 kb, based on the comparison of its mobility to the rRNAs. The induction pattern of the mRNA of the *Ric 1* gene is shown in Fig. 6. The Northern blot analysis indicated undetectable levels of *Ric 1* mRNA in the submerged control thalli. After only 1 h of incubation with 10^{-6} M ABA the *Ric 1* message reached a high level of expression (Fig. 6A). The message remained at this level for the next 24 h of ABA incubation and seemed to show a gradual decline during the following 7 d.

Transferring submerged thalli onto an agar surface resulted in a longer period of induction (Fig. 6B). Only small amounts of the mRNA could be detected during the first 3 h; maximal gene expression was reached between 8 and 9 h. These observations indicated that ABA synthesis and accumulation must take place first (Hellwege et al. 1992), before maximal gene expression is reached.

Comparison of Ric 1 with published sequences. The amino acid sequence deduced from the cDNA nucleotide sequence was compared with published protein sequence data. The Ric 1 polypeptide possesses a 28% homology (Fig. 7) to an embryo-specific protein of carrot seeds (Franz et al. 1989), whose expression is controlled by ABA (Hatzopoulos et al. 1990). Additionally, it shares similarities with polypeptides of the D7 family of the LEA proteins (Baker et al. 1988; Hong et al. 1988; Harada et al. 1989; Seffens et al. 1990; Curry et al. 1991). Like these proteins, the Ric 1 gene product is ABA-inducible, primarily hydrophilic and contains a large proportion of the amino acids alanine, lysine and threonine.

Abscisic acid increases drought tolerance in Riccia fluitans. The homology of the Ric 1 gene product to desiccation-related proteins posed the question of whether the landform transformation of *R. fluitans* also includes a drought-dependent component and how ABA could play a role in this process. To analyse this, submerged thalli of *R. fluitans*, which had been pretreated for 3 d either with 10^{-6} M ABA in nutrient solution or with control media, were dried to 30% of their initial fresh weight, arrested in this stage for 2 h and transferred again to nutrient solution. Furthermore, a terrestrial thallus was also exposed

Fig. 2A–F. Fluorography of in-vivo-labelled proteins separated by 2-D PAGE. Thalli of *R. fluitans* were labelled for 2 h with [^{35}S]methionine. Equal amounts of radioactivity were loaded onto the gels. Polypeptides were extracted from thalli of the waterform (**A**), the landform (**B**) and from waterform thalli which had been incubated with 10^{-6} M ABA for 6 h (**C**), 15 h (**D**), 24 h (**E**) or 48 h (**F**). Polypeptides characteristic of the terrestrial thalli are indicated with *squares* (□), those specific for the submerged thalli are *circled* (○). The analysis is based on three independent experiments. Only gels showing changes representative of most or all gels are included in the figure. The numbers refer to polypeptides which could be identified by in-vivo labelling as well as by the silver-staining procedure. The *arrows* mark proteins which showed similar migration characteristics on the two-dimensional gels of in-vivo-labelled and in-vitro-translated proteins.


A
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   1    C CAA ACT GAG CTC TCT CAT CTC TAT TTC AGC ATT CAT AAA GGT TAG      46

  47  AAA TAT TCG ATT CTT CTA TCA GCG CTC AAC AGT TTC AGC TGG TAG TCG      94

   1                 Met Ala Ser Lys Gln Tyr Glu Gln Ser Tyr Asp Thr      12
  95  TTA GTT GCA ACG ATG GCG TCG AAG CAG TAC GAA CAG AGT TAC GAC ACC     142

  13  Thr Ala Gln Lys Thr Glu Glu Ala Lys Asn Ala Ala Ala Gly Thr Ala      28
 143  ACT GCC CAG AAG ACG GAG GAG GCG AAG AAC GCA GCC GCC GGT ACC GCC     190

  29  Glu Gln Thr Lys Gln Ser Ala Ala Glu Thr Ser Asp Lys Thr Lys Gln      44
 191  GAG CAG ACC AAA CAA TCT GCC GCC GAG ACA TCC GAT AAA ACC AAG CAA     238
                          A1
  45  Leu Gly Ala Glu Lys Thr Glu Gln Ala Asn Arg Ala Ala Ser Glu Ala      60
 239  CTC GGG GCG GAG AAA ACC GAA CAG GCT AAT CGC GCG GCA TCG GAG GCG     286

  61  Ala Gly Thr Val Gln Glu Lys Ala Gln Val Ala Arg Asp Thr Gly Ala      76
 287  GCC GGA ACG GTC CAG GAG AAG GCG CAA GTC GCC AGG GAT ACT GGA GCC     334
            A2                                        A3
  77  Glu Lys Ala Glu Gln Ala Lys Asn Tyr Gly Ala Glu Lys Thr Glu Gln      92
 335  GAG AAG GCG GAG CAA GCG AAG AAT TAC GGC GCG GAG AAA ACC GAG CAA     382

  93  Ala Lys Ser Ala Ala Ser Asp Met Ala Gly Thr Leu Gln Glu Lys Ala     108
 383  GCC AAG AGC GCG GCA TCC GAT ATG GCC GGT ACG CTC CAA GAG AAG GCC     430
                                        A4
 109  Gln Ala Thr Arg Asp Val Gly Ala Glu Lys Thr Glu Gln Ala Lys Gln     124
 431  CAA GCC ACC AGA GAC GTT GGA GCT GAG AAG ACC GAA CAG GCG AAG CAG     478
                          A5
 125  Tyr Gly Ala Glu Lys Thr Glu Gln Ala Lys Gly Ala Ala Ser Glu Thr     140
 479  TAC GGA GCC GAG AAA ACG GAG CAG GCC AAA GGC GCG GCT TCG GAA ACC     526

 141  Ala Gly Thr Ala Gln Asp Lys Ala Gln Thr Val Trp Glu Gln Ala Lys     156
 527  GCC GGT ACC GCG CAG GAC AAG GCG CAG ACT GTT TGG GAG CAG GCG AAG     574

 157  Gln Thr Ala Ala Asp Ala Thr Gln Tyr Val Gln Glu Lys Ala Thr Gln     172
 575  CAA ACA GCC GCG GAT GCC ACT CAA TAT GTG CAA GAG AAA GCT ACG CAA     622

 173  Ala Trp Glu Ser Thr Lys Gln Thr Thr Ser Asp Thr Thr Thr Thr Ala     188
 623  GCC TGG GAA TCC ACC AAG CAA ACC ACG TCG GAT ACC ACC ACA ACT GCT     670
                  B1                                              B2
 189  Gly Asn Lys Ala Val Glu Ala Lys Asp Tyr Ala Val Asp Thr Ala Val     204
 671  GGA AAC AAG GCT GTA GAG GCG AAA GAC TAC GCC GTG GAC ACG GCA GTC     718
                                        B3
 205  Gln Gly Lys Asp Phe Ala Val Asp Lys Ala Val Gln Gly Lys Asp Tyr     220
 719  CAG GGC AAG GAC TTC GCG GTG GAC AAG GCT GTG CAA GGC AAG GAC TAC     766
                     B4
 221  Ala Val Asp Lys Ala Leu Gln Gly Lys Asp Tyr Thr Gly Glu Lys Ala     236
 767  GCG GTG GAC AAG GCT TTG CAA GGC AAG GAC TAT ACC GGT GAG AAG GCA     814
                                           B5
 237  Gly Gln Ala Gly Gln Tyr Thr Thr Asp Arg Ala Val Gln Gly Lys Asp     252
 815  GGA CAA GCC GGA CAG TAC ACC ACG GAC AGA GCA GTG CAG GGG AAA GAT     862

 253  Phe Thr Val Ala Lys Thr Ala Glu Ala Val Lys Gly Val Val Leu Gly     268
 863  TTC ACG GTA GCA AAG ACG GCA GAA GCT GTT AAA GGA GTC GTC CTC GGT     910

 269  Val Lys Asp Ala Ile Val Gly Glu Thr Gly Ala Asn Lys Pro Gly Pro     284
 911  GTC AAG GAT GCT ATC GTG GGT GAG ACT GGA GCC AAC AAG CCA GGT CCT     958

 285  Thr Ser Lys His                                                  288
 959  ACA AGC AAA CAT TAG ATG CTT AAA TCG GAG CAG ATT AGG AGA TTC TAG    1006

1007  CCT CTT GTG GAT TCA TTT AAA AAG CAT ATC TTG TTG ATG TAG GAC TGT    1054

1055  GAG GTG TGT ATG TAA ATA TAC ACA ACA CCT TGG TAC TAG CTA CGG CGG    1102

1103  GAG GGG TTT TAC CCC CAC TGT GCC CTG AAC CTT ATT TGT ACA GGT TTC    1150

1151  ATT TAA TCG ACA CTT CGG ATC TTT CAA AAA AAA                        1183
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Fig. 3. Nucleotide sequence and the deduced amino acid sequence of the *Ric 1* clone. The marked sections *A1–A5* and *B1–B5* indicate the repeats of the two repetitive amino acid sequences

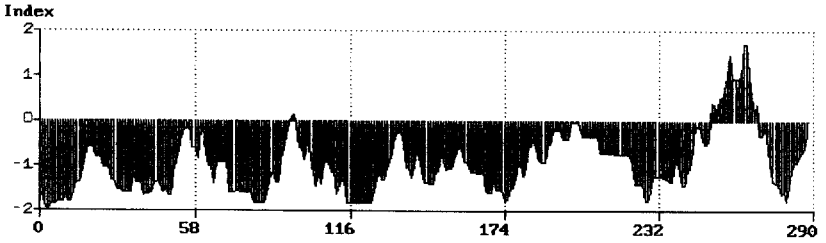

Fig. 4. Hydropathy plot of the polypeptide encoded by the cDNA clone *Ric 1*. The span of amino acids used is 11 and the hydropathy values of the amino acid residues are those of Kyte and Doolittle (1982). *Ordinate*: hydropathic index; *abscissa*: amino acid position

to the same desiccation process and recultivated afterwards on an agar surface. During the desiccation/rehydration cycle, chlorophyll *a* fluorescence quenching was employed as an independent indicator of the metabolic status (Fig. 8) and viability (Hellwege et al. 1994).

At the beginning of the experiment the thalli showed similar mean ratios of maximal to basal fluorescence F_m/F_0 (4.2, 4.6, 4.8; Fig. 8A) and oxidation states of the primary quinone acceptor of PSII Q_A (Fig. 8B; 16%, 20%, 21%). During the following 2 d the F_m/F_0 ratio varied

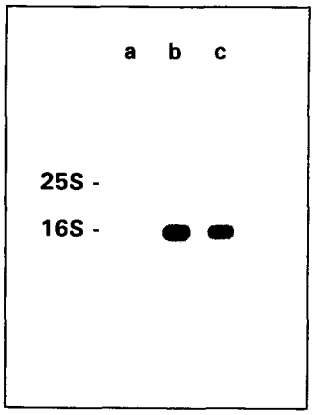

Fig. 5. Northern hybridisation of the ABA-induced landform cDNA clone *Ric 1* to total RNA (20 μg) isolated from thalli of the waterform (*a*), ABA-treated thalli (*b*) or the landform (*c*) of *Riccia fluitans*. 16 S and 25 S indicate the positions of the respective rRNAs on the gel which were visualized by ethidiumbromide fluorescence under UV light. The size estimation of the *Ric 1* mRNA was based on the comparison of its mobility relative to these two rRNAs

within narrow limits. The oxidation state of Q_A of the terrestrial and ABA thalli increased slightly in comparison to the submerged thalli. On day 3 the desiccation process was started by transferring the thalli to open Petri dishes. At the end of the desiccation process, F_m/F_0 of the waterform had decreased to values of "1" and the oxidation state of Q_A dropped to "O", indicating complete inhibition of photosynthetic metabolism. The submerged thalli showed no recovery after transfer to the nutrient solution. Treatment with ABA enabled the submerged thalli to survive the water deficiency. The oxidation state of Q_A of the ABA-treated and the terrestrial thalli was not affected by the water loss although the values oscillated more than before starting the drying process. However, the photosynthetic apparatus of these thalli seemed to be also partially damaged or inhibited, because F_m/F_0 of the thalli decreased to values of 2–3 during the drying process and showed no recovery within 24 h. In contrast to the waterform, which had totally lost its chlorophyll after 2 d, these thalli remained green and kept growing as the controls.

Adaptation of the vacuole to terrestrial conditions. During occupation of the land the role of vacuoles should change. In terrestrial thalli, vacuoles take up, sequester and store products which can be released to the surrounding medium by the submerged living thalli. Tonoplast-specific polypeptides could be very important in this context. We therefore probed *R. fluitans* proteins in a Western blot analysis with an antibody against a tonoplast-specific 31-kDa polypeptide (Betz and Dietz 1991). Figure 9 shows that a related protein could also be detected in extracts of submerged, terrestrial and ABA-incubated thalli. Under ABA treatment the concentration of the recognised polypeptide seemed to be increased. In the terrestrial thalli only a small number of meristematic cells was exposed to increased ABA concentrations for a short period (Hellwege et al. 1992) whereas ABA treatment of submerged thalli affected all the cells of the thallus for four weeks. This may explain the weak response in the terrestrial thallus.

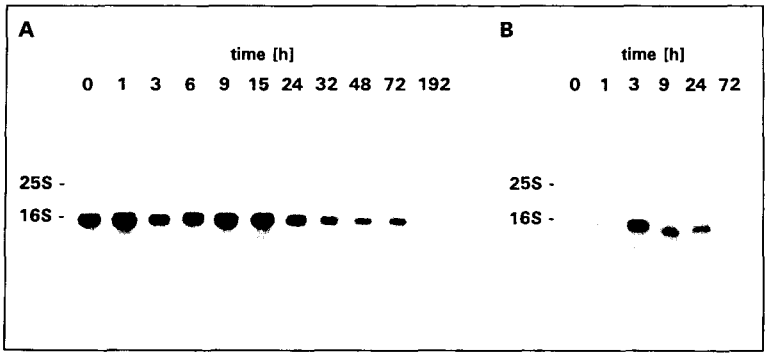

Fig. 6. Northern blots of the ABA-induced landform clone *Ric 1* of *Riccia fluitans* hybridized to total RNA (20 μg) of submerged thalli which were subjected to 10^{-6} M ABA for different time intervals (**A**) or transferred to terrestrial conditions for the same time (**B**) 16 S and 25 S, see Fig. 5

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DC 8  102 QKAEEAKEKAKMAKDTTMGKAGEYKDYTAQKAEEAKEKAAQKAEETKEKAGEYKNYTAQK 161
Ric 1   8 QSYDTTAQKTEEAKNAAAGTAEQTKQSAAETSDKTKQLGAEKTEQANRAASEAAGTVQEK  67

          *  *.**   **  .  *.*.*.*.  .** .....*.  .**.   .*  .*.*.  .**  .*
DC 8  162 AGEAKDTTLGKAGEYKDYAAQKAAEAKDTTAQKAAEAKEKTGEYKDYAAQKAAEAKVLAA 221
Ric 1  68 AQVARDTGAEKAEQAKNYGAEKTEQAKSAASDMAGTLQEKAQATRDVGAEKTEQAKQYGA 127

          .*. .** ... .       * .**     . ..* ...  .*  .*.... ..*... .  .
DC 8  222 QKAAEAKDTTGKDGEYKDYAAQKAAEAKDATMQKTGEYKDYAAQKTAETKDATMEKAKEY 281
Ric 1  128 EKTEQAKGAAS-ETA--GTAQDKAQTVWEQAKQTAADATQYVQEKATQAWESTKQTTSDT 184

             *..** **** ... . . **.. .** . ** ...*. . ****..**... . *
DC 8  282 KEYAAQKAAEAKDATMQKTGEYKDYSAQKAAETKDATMEKTKEYKDYTAQKAAETKDATM 341
Ric 1  185 TTTAGNKAVEAKDYAVDTAVQGKDFAVDKAVQGKDYAVDKALQGKDYTGEKAGQAGQYTT 244

          ..* ..** ** **.*
DC 8  342 EKAKEAKDTTVQKTGE 357
Ric 1  245 DRAVQGKDFTVAKTAE 260
```

Fig. 7. Amino acid sequence homology between the Ric 1 gene product of *Riccia fluitans* and the ABA-regulated embryo-specific DC 8 protein of carrot seeds (Franz et al. 1989). Identical amino acid residues are indicated with *asterisks*, similar ones by *points*

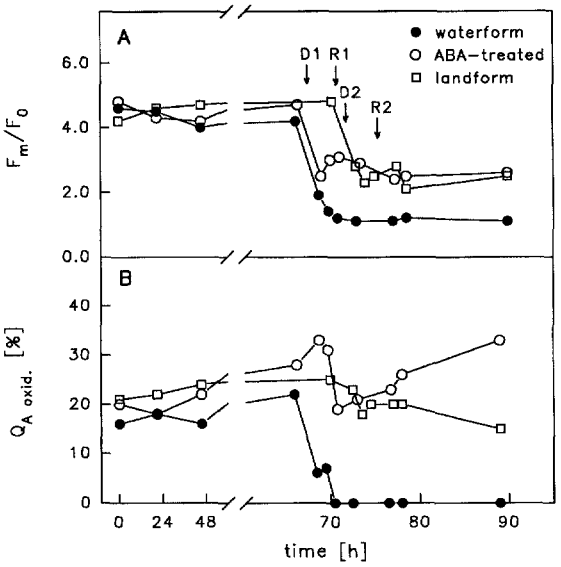

Fig. 8 A,B. Functional characterisation of the photosynthetic apparatus during the time course of a dehydration/rehydration cycle. Chlorophyll *a* fluorescence transients were recorded and used to calculate the F_m/F_0 ratios (**A**) and the redox state of the primary acceptor of photosystem II (**B**) (cf. Hellwege et al. 1994). Waterform thalli of *Riccia fluitans* were either pretreated with ABA (●) or with nutrient solution (○), dried to 30% of their fresh weight, arrested in this stage for 2 h and transferred again to nutrient solution. Additionally, landform thalli were exposed to the same desiccation process and recultivated on agar (□). The start points of desiccation (*D1*) and rehydration (*R1*) of the submerged and ABA-treated thalli are indicated by *arrows* as well as the starting of desiccation and rehydration of the landform (*D2*, *R2*)

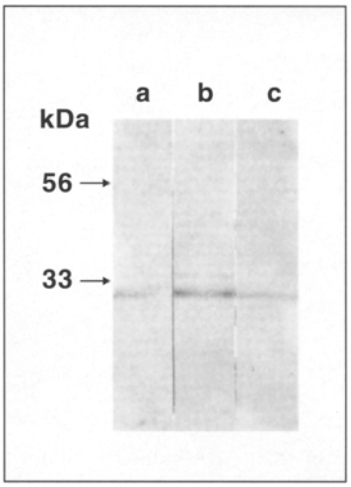

Fig. 9. Western blot analysis of protein extracts (120 µg) of the submerged (*a*), ABA-induced (*b*) and terrestrial thalli (*c*) of *Riccia fluitans* with an antibody against a 31-kDa tonoplast protein of barley leaves (Betz and Dietz 1991). The figure shows one of a number of similar Western blots in all of which the ABA-treated thalli contained the highest amount of antigen

Since a variety of hydrolases seems to be specifically or at least predominantly compartmentalised in the vacuoles (Boller and Kende 1979) we investigated some of them in the different extracts of *R. fluitans*. All measured hydrolytic enzymes exhibited higher activities in the terrestrial and ABA-induced thalli than in the submerged thalli (Table 1). However, there were significant differences in their increase. Most impressive was the effect of the changing environments on the α-mannosidase. Its activity increased up to eightfold under terrestrial conditions and by a factor of 4–5 under ABA treatment. The activities of the phosphatase, phosphodiesterase and the N-acetylglucosaminidase rose only by a factor of 1.5–2.5 and were lower in ABA-induced thalli than in those of the landform. Both conditions still increased the activity of the β-galactosidase up to threefold.

Discussion

The role of abscisic acid in liverworts was obscure until Hellwege et al. (1992, 1994) demonstrated that ABA seems to control both developmental processes in aquatic liverworts as well as the induction of desiccation tolerance in xerophilic species. It has been shown that ABA contents increase transiently by a factor of up to 30 when submerged thalli of *Riccia fluitans* are transferred to a solid surface (agar or moist filter paper) and that treatment of submerged thalli with ABA induces formation of landform characteristics. The role of ABA as an inducer of desiccation tolerance in bryophytes has been demonstrated (Werner et al. 1991; Hellwege et al. 1994), but nothing is known about the molecular basis of this process.

Our data show that in *R. fluitans* a set of polypeptides is formed during the conversion of submerged to terrestrial thalli. Additionally, a group of proteins which seems to be specific for the submerged thalli disappears during landform formation. Most of the landform-specific polypeptides could also be induced by ABA. Some of these proteins are already formed 3–6 h after the start of an ABA treatment. A few landform polypeptides were expressed in such high amounts that they could be visualised in silver-stained gels, others were highly labelled with

Table 1. Differences in the activity of hydrolases in the submerged, terrestrial and ABA-treated thalli of *Riccia fluitans*. Whole thalli of four week-old plants were extracted and analysed

	Activity [nkat·(g FW)$^{-1}$]		
	Submerged	Terrestrial	Submerged + ABA
Acid phosphatase	18.40 ± 5.10	35.90 ± 10.20	28.60 ± 8.40
Phosphodiesterase	6.90 ± 1.60	17.30 ± 3.70	11.40 ± 3.40
N-Acetylglucosaminidase	1.29 ± 0.16	1.91 ± 0.37	1.79 ± 0.20
β-Galactosidase	0.13 ± 0.02	0.43 ± 0.13	0.37 ± 0.06
α-Mannosidase	0.15 ± 0.04	1.18 ± 0.64	0.67 ± 0.24

[^{35}S]methionine but hardly detectable by the staining procedure. As a first simple interpretation one can assume that structural proteins occur in higher amounts in the cells, whereas polypeptides involved in regulation of enzymatic functions often have a high turnover. Therefore, these proteins are highly labelled, but are present only in small amounts (Bray 1990).

To elucidate the physiological importance of a few of these polypeptides a cDNA library of the ABA-induced landform has been established and screened with the mRNA of the waterform and the landform. We isolated an ABA-inducible clone (*Ric 1*) whose gene product exhibits a homology with desiccation-related proteins in carrot seed (DC 8; Franz et al. 1989; Hatzopoulos et al. 1990) and in barley aleurone layers (pHV A1; Hong et al. 1988, 1992). Northern blot studies demonstrate that mRNA formation of the *Ric 1* gene can already be observed 1 h after the start of ABA treatment. After transferring thalli to a solid agar surface, it takes some hours before the mRNA is expressed at its maximum. This might indicate that first ABA synthesis and accumulation must take place before maximal gene transcription is induced. The kinetics of induction of *Ric 1*-mRNA abundance suggests that Ric 1 protein is expected to be among the rapidly appearing proteins during a transition from water- to landform.

The Ric 1-homologous pHV A1 protein (Hong et al. 1988) belongs to the D 7-family of the LEA proteins (Dure 1993) and the DC 8 protein (Franz et al. 1989) shows similarities to it. Members of this family have been found in cotton (Baker et al. 1988; Dure et al. 1989), *Brassica napus* (Harada et al. 1989), carrot (Seffens et al. 1990) and wheat (Curry et al. 1991). The Ric 1 gene product resembles these polypeptides in its hydrophilicity as well as in its high content of the amino acids alanine, lysine, threonine and glutamic acid. An interesting sequence element of these molecules is the repeated amino acid polymers which are predicted to form amphiphilic α-helices. From computer-driven molecular modelling, Dure (1993) concluded that the D 7 proteins could bind phosphate ions thereby preventing their precipitation in desiccating tissue. The Ric 1 protein shows its greatest homology with these polypeptides in the region of the repeating sequences; however, it does not share these elements. The Ric 1 gene product contains two other imperfect repeats, Gly-Ala-Glu-Lys-Thr-Glu-Gln-Ala-Lys and Ala-Val-Asp-Lys-Ala-Val-Glu-Gly-Lys-Asp. Further investigations must show whether these sequences may also form amphiphilic α-helices and how they are involved in the function of the protein. Besides the vacuole and the cytoplasm (protein bodies, ER) the DC 8 protein could also be detected in the cell wall (Franz et al. 1989). Thus, another possible function of these polypeptides might be the stabilization of cell structures.

Since some ABA-inducible genes of seed embryos and leaf tissues are related to desiccation tolerance (Bartels et al. 1988, 1990; Goday et al. 1988; Close et al. 1989; Dure et al. 1989; Skriver and Mundy 1990; Curry et al. 1991; Anderberg and Walker-Simmons 1992; Schneider et al. 1993) we investigated whether ABA could play a role in the induction of drought tolerance of *R. fluitans*. Indeed, ABA could establish a drought tolerance of thalli which had lost 30% of their water, a treatment which was lethal to the non-ABA-treated controls. This indicated that Ric 1 protein might also be related to drought tolerance of the hygrophytic *Riccia* thalli. It should be noted, however, that ABA-dependent proteins of seed embryos and aleurone layers are formed under severe stress conditions whereas *Riccia* thalli already respond to a very mild stress treatment (Hellwege et al. 1992).

During the occupation of land the role of the vacuole must have changed. While the submerged thalli of *R. fluitans* can release by-products or toxic compounds of their metabolism to the surrounding medium the terrestrial thalli have to sequester, to store and to metabolise them. Investigating the occurrence of a tonoplast-specific protein (Betz and Dietz 1991) and the activities of several vacuolar marker enzymes (Boller and Kende 1979) we found that the hydrolytic enzymes were activated during landform induction. The content of the tonoplast protein seemed to be increased under ABA treatment. So, these findings may indicate a special role of the tonoplast and the vacuole during ABA-induced conversion from the submerged to the terrestrial thallus form. However, further investigation is needed to confirm this hypothesis.

With the development of landform characteristics in *Riccia fluitans* only primitive, i.e. phylogenetically early, mechanisms to survive outside a water environment may be investigated. Nevertheless, this plant seems to be a perfect model system. It may answer certain questions in the context of the evolutionary important transition from an aqueous to a terrestrial habitat. Such questions deal with the role of ABA in the transition from an aquatic to a terrestrial environment, the necessity to acquire (a low level of) stress resistance, the function of the vacuole and the regulation of gene expression.

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