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Subunit C of the vacuolar H^+-ATPase of *Hordeum vulgare*

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Abstract The molecular cloning of the first subunit C of the plant vacuolar H^+-ATPase is reported. Tonoplast vesicles were purified from barley leaves by sucrose gradient centrifugation, and the tonoplast polypeptides were separated by two-dimensional (2-D) gel electrophoresis. Using an anti-ATPase holoenzyme antibody, a polypeptide was recognized in the molecular mass range of 40 kDa with an isoelectric point of about 6.0, and tentatively identified as subunit C. The polypeptide spot was excised from about 50 2-D gels and subjected to endo Lys C proteolysis. Two proteolytic peptides were sequenced and the amino acid sequences were used to design degenerated oligonucleotides, followed by PCR amplification with cDNA template and screening of a cDNA library synthesized from *Hordeum vulgare* poly A mRNA of epidermis strips. The full length clone of 1.5 kbp contains an open reading frame of 1062 bp encoding a polypeptide of 354 amino acids with a molecular mass of 39 982 Da and an isoelectric point of 6.04. Amino acid identity with sequences of SUC from animals and fungi is in the range of 36.7 to 38.5%. Expression of the cloned gene was demonstrated by Northern blotting and RT-PCR.

Key words: ATPase; cDNA; Leaf; Subunit C; Root; Vacuole; *Hordeum vulgare*

1. Introduction

The proton concentration of lytic compartments is primarily dependent on the activity of the V-type H^+-ATPases, both in expanding and fully differentiated plant cells. Thus, the V-ATPase is of primary importance for metabolic processes such as cell expansion, cellular ion and pH homeostasis and detoxification processes. The coarse structure of the V-ATPase has been well documented by ultrastructural analysis [1,2], although the precise number of different subunits required for function has yet to be settled firmly for plants; even less is known about the gene products required for assembly of the V-ATPase in plants [3]. For instance recently it has been shown that a 100 kDa polypeptide immunologically related to the yeast VPH1 gene product is associated only with unassembled V_0 complexes but not with fully assembled functional V-ATPase holoenzymes. Li and Sze [4] conclude that in plants the 100 kDa polypeptide might be involved in assembly or sorting.

The head and stalk structure of the ATPase extends into the cytosol and according to present day knowledge contains the structure A_3B_3CDEFG as deduced from stoichiometry measurements by quantitative amino acid analysis of coated vesicle V-ATPase [5]. For plants, cDNAs encoding subunits A, B, D, E and G have been cloned from various sources [6–11]. However, molecular information on the subunits C and F is still missing, but required to initiate work on the relationship of structure and function of V-H^+-ATPase in higher plants for example by reconstitution, studies of protein-protein interaction and investigations on structural changes underlying the catalytic activity.

Therefore, we approached the molecular cloning of subunit C using the established method of protein identification and isolation. Here, we report on the first plant sequence of subunit C of the barley V-ATPase which may now allow to commence the urgently required molecular analysis of structure-function relationship of this important primary pump energizing the vacuolar membrane of plant cells.

2. Materials and methods

2.1. Plant growth and preparation of tonoplast membranes

Barley (*Hordeum vulgare* cv. Gerbel) was grown under controlled conditions (12 h light at 25–27°C, 12 h dark at 15°C, 400 μmol Quanta m^{-2} s^{-1}, 50–70% relative humidity) in soil or hydroponic culture as described [12]. Primary leaves or roots from 7 to 10 days old barley seedlings were harvested and homogenized in buffer containing 0.2 M Tris-HCl (pH 8.0), 1 M sucrose, 32 mM EDTA, 4 mM DTT and 2 mM PMSF (1 ml g^{-1} fw) [13]. The homogenate was passed through nylon gauze (20 μm mesh) and the filtrate was centrifuged at 8000 rpm and 4°C for 20 min. The supernatant was spun at 20 000 rpm and 4°C for 30 min. The pellet containing the crude microsomal fraction was dissolved in 1 ml of a buffer consisting of 0.25 M sucrose, 5 mM PIPES-KOH (pH 7.2) and 2 mM DTT. The tonoplast membranes were enriched by laying the microsomal membranes on a discontinuous sucrose gradient with density steps of 35%, 25% and 16% sucrose (w/w) supplemented with 0.1 M Tris-HCl (pH 7.2), 0.1 M EDTA and 1 mM DTT. Following centrifugation at 100 000 × *g* for 180 min, the tonoplast membranes were recovered from the 25/16% interphase and sedimented in 0.25 M sorbitol, 5 mM PIPES-KOH (pH 7.2) and 2 mM DTT at 20 000 rpm for 30 min.

2.2. Protein purification, 2-D gel electrophoresis and Western blotting

Membrane proteins were extracted into water-saturated phenol buffered to pH 6.8 with 100 mM Tris-Cl and supplemented with 10 mM DTT. Proteins were precipitated with ethanol containing 0.1 M ammonium acetate at −20°C, washed with ice-cold ethanol, solubilized in 2-D gel lysis buffer (57% (w/v) urea, 4% (v/v) ampholyte 5-7 (Serva), 1% (v/v) ampholyte 3-10 (Serva), 2% (v/v) Triton X-100 and 20 mM DTT) and separated by 2-D gel electrophoresis ([14] as modified by [15]). Samples were loaded on tube gels of 3.5 mm diameter (first dimension). The proteins were focused for 8000 V h. The gels were equilibrated and loaded on the second SDS-PAGE dimension as described by O'Farrell et al. [14]. Electrophoretically separated polypeptides were visualized by staining the gels with silver nitrate [16] or with Coomassie Brilliant Blue [17]. Immunoblot analysis was performed using an anti-ATPase holoenzyme immune serum [18] as primary antibody at a 1:1500 dilution in TBS containing 1% BSA. The protein spot corresponding to the 40 kDa polypeptide of subunit C of the V-ATPase was excised from about 50 2-D gels following Coomassie blue staining.

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2.3. Peptide sequence determination

In gel digestion with endo Lys C proteinase, fractionation of proteolytic peptides by HPLC and amino acid sequence analysis were performed according to Eckerskorn and Grimm [19].

2.4. Isolation of RNA and cDNA synthesis

Total RNA was isolated from barley roots and leaves. The plant tissues were frozen in liquid nitrogen and RNA was extracted according to Chomczynski and Sacci [20]. First-strand cDNA was synthesized from total RNA using Superscript II RT (Gibco BRL) according to the instructions of the manufacturer.

2.5. Screening of barley epidermis cDNA library

A barley leaf epidermis cDNA library was screened by plaque hybridization [21]. Plaques were transferred to Hybond N-membranes (Amersham-Pharmacia, 0.45 μm). The first membrane was left on the plates for 1 min, the replicate membranes for each 3 min. Following transfer, the membranes were denatured with 0.5 M NaOH/1.5 M NaCl, neutralized with 1.0 M Tris-HCl (pH 7.5)/1.5 M NaCl and washed in 2×SSC. The transferred DNA was cross-linked to the membrane with UV-light. Before hybridization a proteinase K treatment was carried out (2 mg/ml proteinase K in 2×SSC buffer) to digest interfering proteins. Prehybridization and hybridization were preformed at 42°C in 5×SSC, 0.2% SDS, 0.1% *N*-lauroylsarcosin, 2% Blocking Reagent (Boehringer Mannheim) and 50% formamide. The detection probe corresponding to the 348 bp fragment was prepared by PCR with sequence specific primers using digoxigenin-dUTP (Boehringer Mannheim) as a label. The PCR cycle settings were 1 cycle 94°C, 1.30 min; 30 cycles 1 min 94°C, 1 min 57°C, 1 min 72°C; 1 cycle 10 min 72°C. The digoxigenin-labeled DNA was used as probe for hybridization at a concentration of 3 ng per ml hybridization buffer. After hybridization the membranes were washed twice with 2×washing solution (2×SSC, 0.1% SDS) for 15 min at room temperature and twice for 15 min at 42°C in 0.5×SSC. Clones harboring cDNA homologous to the hybridization probe were detected with anti-digoxigenin alkaline phosphatase conjugated Fab fragments (Boehringer Mannheim) and the chemiluminescent substrate CSPD (Boehringer Mannheim). Approximately 20 positive clones were isolated.

2.6. Northern blot analysis and RT-PCR of subunit C

RNA was isolated from 7 days old roots and primary leaves of barley. Twenty μg of total RNA from roots and leaves were separated electrophoretically in 1% agarose/formaldehyde gels according to standard procedures [22] and transferred to nylon membrane (Hybond N, Amersham-Pharmacia, 0.45 μm). Prehybridization and washing were carried out as described above for the screening. The membranes were probed with the same digoxigenin-labeled cDNA fragment at a concentration of 10 ng/ml hybridization buffer.

cDNA was synthesized from each 3 μg of total RNA from leaves and roots using Superscript II RT (Gibco BRL). Ten μl of 1:10 dilutions of the cDNA were used as template for PCR amplification in 50 μl standard reactions. A *Hv-vatC* sequence specific forward primer (5′-GAGTTGCTGAGTACGGTA-3′, positions 473 to 490) and a reverse primer (5′-CTAGGACAACAGACAGGA-3′, positions 1008 to 1025) were used for amplification. Actin was amplified with degenerated primers homologous to plant actin sequences (5′-GGNACTGGAATGGTNAAGG-3′ and 5′-GTGATCTCCTTGCTCATACG-3′). The PCR cycle settings were 1 cycle 94°C, 1.30 min; 23, 26 or 29 cycles 1 min 94°C, 1 min 55°C, 2 min 72°C, 1 cycle 10 min 72°C. To verify the amplification of the *Hv-vatC* and the actin fragments, the PCR products were sequenced (sequencing facility of the University of Bielefeld). The PCR products were electrophoresed in 1.5% agarose gels, stained with ethidium bromide, and photographed with a gel documentation system (INTAS, Germany).

3. Results and discussion

3.1. Isolation of subunit C polypeptide

Tonoplast membranes were purified from primary leaves and roots of 5–10 days old barley seedlings by differential sedimentation and gradient centrifugation. The polypeptides of the tonoplast fraction were separated by 2-D gel electrophoresis using ampholytes in the pH range 3 to 10 [23]. More than 50 polypeptide positions were visualized upon Coomassie blue staining (Fig. 1A). In order to identify subunit polypeptides of the vacuolar H^+-ATPase, another 2-D gel was blotted onto nitrocellulose membrane and immunodecorated with antibody directed against ATPase holoenzymes of *Kalanchoe daigremontiana* (Fig. 1B) [18]. In this Western blot, subunits A, B, and E could be identified immediately as prominent signals and are labeled in Fig. 1B accordingly. An additional band was focused at about pH 6 and revealed a relative molecular mass close to 40 kDa. These properties corresponded to the expected characteristics of subunit C. By pattern matching, the subunit C polypeptide position was also identified in silver and Coomassie stained 2-D gels (arrowhead in Fig. 1A).

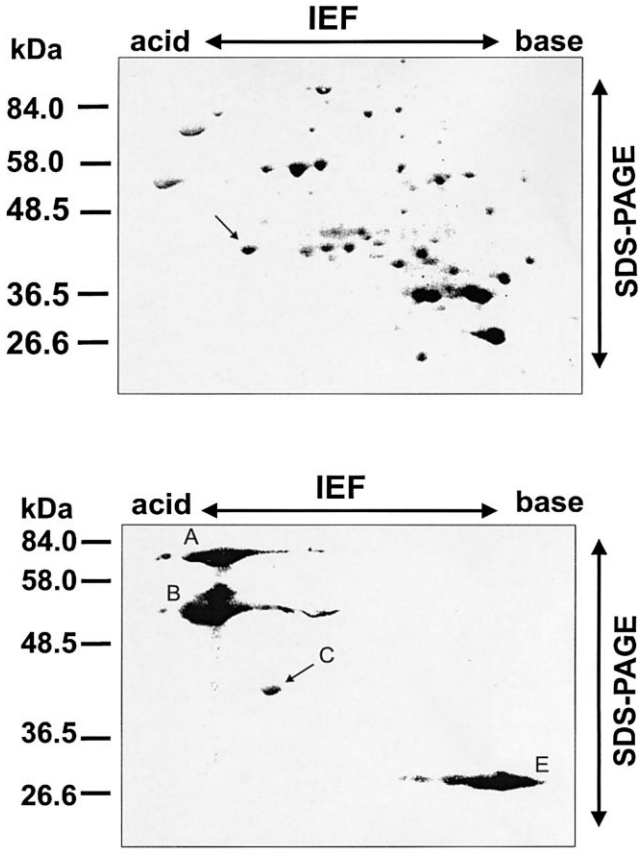

Fig. 1. A: Two-dimensional separation of barley tonoplast polypeptides. Tonoplast vesicles were isolated by discontinuous sucrose gradient centrifugation from 10 days old roots of barley seedlings. The membrane proteins were extracted with phenol followed by precipitation with ammonium acetate in ethanol. The ioelectric focussing (IEF) was performed using a non-linear pH. The gel was stained with Coomassie Brilliant Blue. The area of the gel of a 40 kDa polypeptide tentatively identified as subunit C of the V-ATPase was isolated from approximately 50 2-D gels. B: Western blot analysis of a 2-D gel of barley tonoplast proteins. Following electrophoretic transfer of the polypeptides, the nitrocellulose membrane was incubated with an antibody directed against the tonoplast H^+-ATPase of *Kalanchoe daigremontiana* (1:1500 with TBS containing 1% (w/v) bovine serum albumin). This antibody reacted on the immunoblot with polypeptides of the tonoplast preparation. The arrow indicates the position of the 40 kDa subunit C of V-ATPase.

3.2. Molecular cloning of subunit C

The polypeptide spot was isolated from about 50 2-D gels and subjected to Lys C endoproteinase treatment within the

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1     ACG ACC TCC TCC TCC CCC TCC CCC TCG CCG GCG ACG ACC ACC ATG GCG ACG CGC   54
1                                                             M   A   T   R     4

55    TAC TGG ATC GCC GCC CTG CCC GTC GCC GAC GAC AAC GTC GCC GCC GGC AAG ACC   108
5      Y   W   I   A   A   L   P   V   A   D   D   N   V   A   A   G   K   T    22

109   GCC CTC TGG GCG CGC CTC CAG GAG GCC ATC TCC CGC CAC TCC TTC GAC ACC CCG   162
23     A   L   W   A   R   L   Q   E   A   I   S   R   H   S   F   D   T   P    40

163   CTC TAC CGC TTC ACC GTC CCG GAT CTC CGC CCC GGC ACG CTC GAC TCC CTC CTC   216
41     L   Y   R   F   T   V   P   D   L   R   P   G   T   L   D   S   L   L    58

217   GCC CTC AGC GAC GAC CTC GTA AAG TCC AAC ATC TTC ATC GAG GGC GTC TCG CAC   270
59     A   L   S   D   D   L   V   K   S   N   I   F   I   E   G   V   S   H    76

271   AAG ATC CGC AGG CAG ATC GAG GAC CTG GAG CGC GCC GGA GGG GTC GAG CCC GGC   324
77     K   I   R   R   Q   I   E   D   L   E   R   A   G   G   V   E   P   G    94

325   ACC CTC ACC GTC GAC GGC GTC CCC GTC GAC AGC TAC CTC ACC AGG TTC GTG TGG   378
95     T   L   T   V   D   G   V   P   V   D   S   Y   L   T   R   F   V   W   112

379   GAC GAG GGC AAG TAC CCC GTC AAC GCC CCG CTC AAG GAG ACC GTC GCC AGC ATC   432
113    D   E   G   K   Y   P   V   N   A   P   L   K   E   T   V   A   S   I   130

433   CAG TCC CAG GTC GCC AAG ATC GAG GAC GAC ATG AAG GTT AGA GTT GCT GAG TAC   486
131    Q   S   Q   V   A   K   I   E   D   D   M   K   V   R   V   A   E   Y   148

487   GGT AAT GTT AAG AGC CAG CTT GGA GCA ATC AAC AGG AAG CAA ACT GGA AGT TTA   540
149    G   N   V   K   S   Q   L   G   A   I   N   R   K   Q   T   G   S   L   166

541   GCA GTT CGT GAC CTT TCC AAC CTC ATA AAA CCA GAG GAT ATG GTC ACT TCG GAA   594
167    A   V   R   D   L   S   N   L   I   K   P   E   D   M   V   T   S   E   184

595   CAT CTA GTG ACA CTC CTT TCT ATT GTG CCA AAG TAT TCC CAA AAA GAC TGG TTA   648
185    H   L   V   T   L   L   S   I   V   P   K   Y   S   Q   K   D   W   L   202

649   GCA AGC TAC GAA TCA CTT GAT ACG TTT GTG GTT CCG AGA TCG TCA AAA AAG CTT   702
203    A   S   Y   E   S   L   D   T   F   V   V   P   R   S   S   K   K   L   220

703   TAC GAG GAC AAT GAG TAT GCT CTC TAC ACT GTA ACA TTA TTT GCC AAG GTT GTT   756
221    Y   E   D   N   E   Y   A   L   Y   T   V   T   L   F   A   K   V   V   238

757   GAC AAC TTT AAG GTC CAT GCT CGT GAG AAA GGC TTC CAA ATT CGC GAT TTC GAA   810
239    D   N   F   K   V   H   A   R   E   K   G   F   Q   I   R   D   F   E   256

811   TAT AGT CCT GAA GCA CAG GAG AGT CGG AAG CAA GAG CTA GAA AAG CTG CTG CAA   864
257    Y   S   P   E   A   Q   E   S   R   K   Q   E   L   E   K   L   L   Q   274

865   GAC CAG GAA GTC ATG AGG ACC TCT CCT ATT GCA ATG GGT GCT ATG CTA GCT ACA   918
275    D   Q   E   V   M   R   T   S   P   I   A   M   G   A   M   L   A   T   292

919   GTG AGG GTA TTT AGC TCC TGG GAT GCA TTT TCC TCT GCT GTT CGT GTC TTC GTA   972
293    V   R   V   F   S   S   W   D   A   F   S   S   A   V   R   V   F   V   310

973   GAG AGT ATT CTG AGA TAT GGG TCT GCC TGC ACG GTT CCT GTC TGT TGT CCT AGC  1026
311    E   S   I   L   R   Y   G   S   A   C   T   V   P   V   C   C   P   S   328

1027  ACC ATC TAC AAA GAG CGA GAA AAA AGT AAG GAA CAT CTT GGA AGG GCT ATG CGG  1080
329    T   I   Y   K   E   R   E   K   S   K   E   H   L   G   R   A   M   R   346

1081  CAA TAC CAA CAG CTA CTG GAG ATC TGA AGA CGA CGT GGG TAT GGC TGC TGG CCT  1134
347    Q   Y   Q   Q   L   L   E   I   *                                     354

1135  GGG AGG CGA GAC AGA GTC CCA CCC ATA CGT GTC CTT CAC CAT AAA CTT TGT CTG  1188
1189  AGG GTG TTT GAT GTA TAT TAT TTT GTG ACA CCA ATT CTT TCA TGG TAT GTC GGT  1242
1243  TTC GGT TCA TAC TTC TGT AGT AGA TGA TCC GCG CCG ATC AAT AAT ATA GTG TTT  1296
1297  CGG AGA ACC TCG AAC GTA TCC TGG CAC AAA GTG GGA CTT ATC TTT TCT TGT TCT  1350
1351  GCT ACG GTG TTG CAA CTG TTT ACT TTT TGT GCA GTG TTG TAT TAC TGG GTA CAT  1404
1405  TAT TGT AAT TTT GAG TAA CCA GGT TCC ATA ATA ATA AAA TCA GGG TGA ATA AGC  1458
1459  TGG GTA ATA TAA ACA AAA AAA AAA AAA AA                                 1487
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Fig. 2. cDNA and deduced amino acid sequences of the barley V-ATPase subunit C. Nucleotide sequence and deduced amino acid sequence of cDNA clone *Hv-vatC*. The amino acid sequences of proteolytic peptides #5 and #10 from the microsequence analysis are underlined. The numbers of nucleotides and amino acids are shown at left and right.

gel matrix followed by peptide separation using reverse phase chromatography and amino acid sequencing [19]. Lys C peptide #5 had the sequence VRVAEYGNV and Lys C peptide #10 the sequence GFQIRDFEYSPEAQE. Degenerated oligonucleotides were designed from both peptides for both the sense and antisense strand: #5 sense GTI MGI GTI GCI GAR TAY GGI AAY G; #5 antisense CRT TIC CRT AYT CIG CIA CIC KIA C; #10 sense GGI TTY CAR ATH MGI GAY TTY GAR TAY TC; #10 antisense GAR TAY TCR AAR TCI CKD ATY TGR AAI CC. PCR was performed with cDNA synthesized from barley primary leaf RNA using the two possible primer combinations, i.e. #5s↔#10as and #10s↔#5as. The primer combination #5s↔#10as allowed the amplification of a 348 bp fragment which was sequenced and used to screen a cDNA library constructed in λGEM 4-vector from poly A mRNA of leaf

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                    *                                   *  *    * *  *      *
H.v. : ------MATRYWIAALPVADDNVAAGKTALWARLQEAISRHSFDTPLYRFTVPDLRPGTLDSLLALSDDL :  64
B.t. : -------MTEFWLISAPGE-----KTCQQTWEKLHAATTKNNNLAVSSKFNIPDLKVGTLDVLVGLSDEL :  58
H.s. : -------MTEFWLISAPGE-----KTCQQTWEKLHAATSKNNNLAVTSKFNIPDLKVGTLDVLVGLSDEL :  58
D.d. : ----MSETQEFWLISAPN------LPGADIFDQVNQKTAKENSLSENKKFNTPALRVGTLNSLITLNDEL :  60
S.c. : MATALYTANDFILISLPQNAQPVTAPGSKTDSWFNETLIGGR--AFVSDFKIPEFKIGSLDTLIVESEEL :  68

         *          *                      *          *   * *   *             *
H.v. : VKSNIFIEGVSHKIRRQIEDLER--AGGVEPGTLTVDGVPVDSYLTRFVWDEGKYPVNAPLKETVASIQS : 132
B.t. : AKLDAFVEGVVKKVAQYMADVLEDSKDKVQE-NLLANGVDLVTYITRFQWDMAKYPIKQSLKNISEIIAK : 127
H.s. : AKLDAFVEGVVKKVAQYMADVLEDSKDKVQE-NLLANGVDLVTYITRFQWDMAKYPIKQSLKNISEIIAK : 127
D.d. : QKIDTIVESTTKKIARQLVDLVG-TKPGKDK-SLSINGHTIPQYLQQFAWDDAKYNLKLSLQEIVEKISS : 128
S.c. : SKVDNQIGASIGKIIEILQGLNE-TSTNAYR-TLPINNMPVPEYLENFQWQTRKFKLDKSIKDLITLISN : 136

           *       *           **  * *  * *                          **
H.v. : QVAKIEDDMKVRVAEYGNVKSQLGAINRKQTGSLAVRDLSNLIKPED-MVTSEHLVTLLSIVPKYSQKDW : 201
B.t. : GVTQIDNDLKSRASAYNNLKGNLQNLERKNAGSLLTRSLAEIVKKDDFVLDSEYLVTLLVVVPKLNHNDW : 197
H.s. : GVTQIDNDLKSRASAYNNLKGNLQNLERKNAGSLLTRSLAEIVKKDDFVLDSEYLVTLLVVVPKLNHNDW : 197
D.d. : AVSKIDDDLKIKSSEYSTLSSVASEERKASGNLQVRTLNDLITADN-IVQTDYFTTAFVVIPKQSEKEF : 197
S.c. : ESSQLDADVRATYANYNSAKTNLAAAERKKTGDLSVRSLHDIVKPEDFVLNSEHLTTVLVAVPKSLKSDF : 206

          **     *   *      *    *  * ** *    *     *     * *          *
H.v. : LASYESLDTFVVPRSSKKLYEDNEYALYTVTLFAKVVDNFKVHAREKGFQIRDFEYSPEAQESRKQELEK : 271
B.t. : IKQYETLAEMVVPRSSNVLSEDQDSYLCNVTLFRKAVDDFRHKARENKFIVRDFQYNEEEMKADKEEMNR : 267
H.s. : IKQYETLAEMVVPRSSNVLSEDQDSYLCNVTLFRKAVDDFRHKARENKFIVRDFQYNEEEMKADKEEMNR : 267
D.d. : LACYETISDFVLGRSAKRVAQDNDYFLYSVILFKKFYENFKTKIIEKKWVVRDFKLEDN--KP-TQERSK : 264
S.c. : EKSYETLSKNVVPASASVIAEDAEYVLFNVHLFKKNVQEFTTAAREKKFIPREFNYSEELIDQLKKEHDS : 276

                                 *  *        ** *** ** *     *
H.v. : LLQDQEVMRTSPIAMGAMLATVRVFSSWDAFSSAVRVFVESILRYGSACTVPVCCPSTIYKEREKSKEHL : 341
B.t. : LSTDKKKQFG-PLVRWLKVNFSEAFIAWIHVK-ALRVFVESVLRYG----LPVNFQAMLLQPNKKTMKKL : 331
H.s. : LSTDKKKQFG-PLVRWLKVNFSEAFIAWIHVK-ALRVFVESVLRYG----LPVNFQAMLLQPNKKTLKKL : 331
D.d. : LTEDKKNCRT-SLIRWCRLNFPEAFMAWVHLK-VRVFVESVLRFG----IPFNFQAILMKPQKGADKKV : 328
S.c. : AASLEQSLRV-QLVRLAKTAYVDVFINWFHIK-ALRVYVESVLRYG----LPPHFNIKIIAVPPKNLSKC : 340

                  *
H.v. : GRAMRQYQQLLEI---------------------------------------------- : 354
B.t. : REVLYELYKHLDSSAAAIIDAPMDIPG--------LNLSQQEYYPYVYYKIDCNLLEFK : 382
H.s. : REVLHELYKHLDSSAAAIIDAPMDIPG--------LNLSQQEYYPYVYYKIDCNLLEFK : 382
D.d. : RDILFDQFKYLGS--AHIS-GKNETD----------DS--EKFYPYISVSVNWEN---- : 368
S.c. : KSELIDAFGFLGGN-AFMKDKKGKINKQDTSLHQYASLVDTEYEPFVMYIINL------ : 392
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Fig. 3. Amino acid sequence alignment between *Hv-vatC* (barley) and subunit C of various sources. Calculated homology between barley *Hv-vatC* and that of *Bos taurus* was 37.46% (accession number P21282), *Homo sapiens* 37.76% (accession number P21283), *Distiostelium discoideum* 38.54% (accession number P54648, unpublished) and *Saccharomyces cerevisiae* 36.72% (accession number P31412). Amino acids being identical in all selected specie are marked by asterisks.

epidermis strips [21]. Among about 50 λ-phase clones identified in three screening approaches by hybridization with the PCR probe, *Hv-vatC* was identified as clone containing the longest insert of 1487 bp (Fig. 2). An open reading frame of 1062 bp extends from base 43 to base 1104, encoding a polypeptide of 354 amino acids with calculated molecular mass of 39 982 Da and an isoelectric point of 6.04. The proteolytic peptides #5 and #10 from the microsequence analysis were present at positions aa 143–151 and aa 249–259. The deduced aa sequence is hydrophilic with an average hydrophobicity index of −0.27 according to Kyte and Doolittle [24] and contains no particular hydrophobic domains.

3.3. Sequence comparison of subunits C

Presently six full length cDNA sequences encoding subunit C of the vacuolar ATPase are known from animals and fungi. The length of the encoded polypeptides varies within only narrow limits. Fig. 3 aligns the sequence of *H. vulgare* with those of *Homo sapiens* [25], *Bos taurus* [26], *Dictyostelium discoideum* and *Saccharomyces cerevisiae* [27]. Identity between the various subunits ranged from 36.7 to 38.54%, similarity from 52 to 57%. Stretches of high homology are present at aa positions 44 to 66, 199 to 238 and 324 to 336.

3.4. Expression of Hv-vatC in leaves and roots

Expression of the gene in roots and leaves was investigated by RT-PCR and Northern blotting (Fig. 4). To quantitate

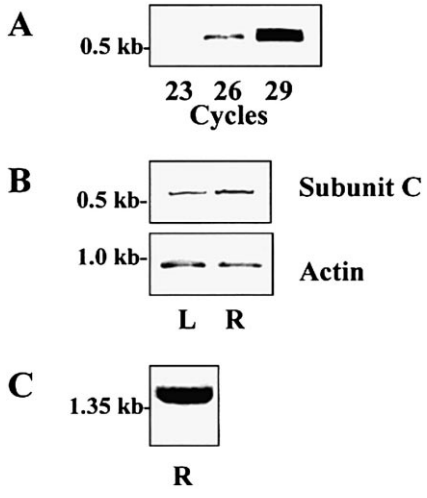

Fig. 4. Analysis of the expression of barley V-ATPase subunit. A: Cycle dependendy of RT-PCR amplification of a 553 bp fragment of *Hv-vatC*. B: Amplification of *Hv-vatC* from leaf and root tissue of 10 days old barley seedlings using actin as an amplification control. L, leaf; R, root. The cDNA was amplified for 26 cycles. C: Northern blot analysis for transcript abundance of subunit C of V-ATPase in barley roots. Total RNA (20 μg) isolated from roots of 7 days old barley was hybridized with a 348 bp DIG-labeled cDNA fragment. A mRNA band with a length of about 1.5 kb was obtained.

expression of the V-ATPase subunit C, a 553 bp fragment from the coding region of the sequence was amplified with gene specific primers by RT-PCR. Linearity of amplification was tested in up to 29 cycles as shown in Fig. 4A. In the linear range of amplification, subunit C signals could be obtained from leaf and root tissue with slightly stronger expression in roots than in leaves (Fig. 4B). Amplification of actin served as a loading control.

In Northern blot experiments, an mRNA band with a length of about 1.5 kb was obtained (Fig. 4C). Expression was seen both in roots and leaves, where expression in roots was lightly stronger when comparing the expressional level of subunit C with that of an actin control (not shown).

4. Conclusions

Here the first nucleotide and amino acid sequence information for subunit C of the vacuolar H^+-ATPase is reported for a plant. Homology of *Hv-vatC* with known sequences from animals and fungi is about 36.7–38.5% and, therefore, in the range of homology reported for other subunits. Since the cloning experiment was started from the protein and expression was also demonstrated on the mRNA level, the proof for cloning the functional gene encoding subunit C is completed. Based on this information and the recent data on subunits D and G [8,10] it will now be possible to initiate the structure-function analysis of plant V-type H^+-ATPase in more detail for instance in respect to subunit interaction.

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