Journal of Bioenergetics and Biomembranes, Vol. 35, No. 4, August 2003 (© 2003)

New Insight Into the Structure and Regulation of the Plant Vacuolar H^+-ATPase

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Plant cells are characterized by a highly active secretory system that includes the large central vacuole found in most differentiated tissues. The plant vacuolar H^+-ATPase plays an essential role in maintaining the ionic and metabolic gradients across endomembranes, in activating transport processes and vesicle dynamics, and, hence, is indispensable for plant growth, development, and adaptation to changing environmental conditions. The review summarizes recent advances in elucidating the structure, subunit composition, localization, and regulation of plant V-ATPase. Emerging knowledge on subunit isogenes from *Arabidopsis* and rice genomic sequences as well as from *Mesembryanthemum* illustrates another level of complexity, the regulation of isogene expression and function of subunit isoforms. To this end, the review attempts to define directions of future research on plant V-ATPase.

KEY WORDS: Gene; plant; regulation; structure; subunit; vacuole; V-ATPase.

INTRODUCTION

The alternating arrangement of plasmatic and extraplasmatic compartments and the connection of secretory spaces by vesicular and/or tubular trafficking are two paradigms of eukaryotic cell organization. Most extraplasmatic compartments are characterized by pH-values below neutrality, positive electrical membrane potential (cytoplasmic side set to 0 as reference), elevated Ca-concentrations, hydrolytic activities with acidic pH optimum, absence of nucleic acids and protein and/or nucleic acid synthesis (Schnepf, 1983). These properties contrast those of the plasmatic compartments with neutral or slightly alkaline pH, low free Ca, presence of nucleic acids, and so on. Generally, all cell membranes separate plasmatic and extraplasmatic cell spaces. Maintenance of the intracellular trans-membrane gradients depends on the activity of primary pumps. Three primary proton pumps are present in plant cells, P-type ATPases, H^+-pumping pyrophosphatases (H^+-PP_iases) and vacuolar-type H^+-pumping ATP hydrolase (H^+-ATPase, VHA). Apparently, each type of primary pumps is indispensable. The specific functional distinction versus "job sharing" between these pumps is only beginning to be unravelled in time, space, and environment, i.e. in development, tissue differentiation and adaptation of plants. The PP_iase is constitutively expressed in all plant cells and suggested to play a particular role in fruit ripening, during adaptation to cold stress and anaerobiosis (Maeshima, 2000). However PP_iase activity may be low in some cases. Roots of *Arabidopsis* Col-0 plants only revealed a "negligible" V-PP_iase activity (Cheng *et al.*, 2003). The V-ATPase is of prime importance for plant cell expansion and stress adaptation. This may partly be attributed to the presence of the large central vacuole as one of the peculiar structures of plant cells that may occupy as much as 99% of the symplastic cell space, for example in parenchymatic tissues of succulent plants. The vacuole functions as intermediate and terminal storage compartment for salts, metabolites,

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0145-479X/03/0800-0377/0

carbohydrates like fructans, amino acids, and conjugates. All these functions are intimately connected to transport processes (Martinoia, 1992). Two examples may be sufficient to illustrate the importance of the V-ATPase in plant development and adaptation at this point.

(1) In a screening approach to identify genes suppressing the etiolation response of plant hypocotyls in the dark, the *det3* mutant of *Arabidopsis thaliana* was identified and the mutated gene shown to represent the *Atvha*-C gene with a single T→A mutation at a putative 3′-splicing site (Schumacher *et al.*, 1999). This mutant reveals a 60% reduction in V-ATPase activity. Apparently, the full V-ATPase activity is a prerequisite for rapid tissue expansion during etiolation in the dark. Suboptimum V-ATPase activity inhibits the etiolation response and allowed to identify the *det3* mutant as apparent etiolation revertant.

(2) Barley root meristematic cells initiated rapid vacuolization upon exposure to salt (NaCl) stress (Mimura *et al.*, 2003). Within 24 h, salt treated root cells were fully vacuolized whereas the salt-free control roots still contained small vacuoles only. The rate of increase was correlated with the external salt concentration. The same process was observed in cell cultures of the Mangrove plant *Bruguiera sexangula*. The salt-induced increase in vacuole volume was coupled to 2.3-fold increase in V-ATPase activity and also V-ATPase protein. A tonoplast Na^+-H^+-antiporter transports Na^+ into the vacuole on the expense of the proton motive force (pmf) that is created by activity of primary H^+-pumps (Garbarino and Dupon, 1988).

The plant vacuolar H^+-ATPase is composed of a head and a central stalk structure (Klink and Lüttge, 1991; Lee Taiz and Taiz, 1991) similar to the F-ATP synthases and the H^+-ATPases of archaea (Nelson and Taiz, 1989). All these pumps are likely to have evolved from a single ancestral prototype structure, characterized by its threefold symmetry in its central parts. The head is composed of a hexamer built from alternating VHA-A and VHA-B subunits. A ring of VHA-c subunits in the membrane extends via VHA-D to the head. Despite these similarities among V-, A-, and F-ATPases, there exist basic differences in the presence of additional subunits, their regulation and sensitivity to inhibitors. Depending on the type of H^+-ATPase, one or several peripheral stalks connect the membrane-associated part V_0 resp. F_0 to the head.

Recently the structure of plant V-ATPase has been refined at a resolution of 2.2 nm on the basis of electron microscopic images (Domgall *et al.*, 2002) and will be discussed below. It is generally believed at present that the V-ATPase functions as molecular motor in analogy to the F-ATP synthase. According to the model, all peripheral stalks and the head form the stator, whereas a ring of six or more VHA-c together with the subunits protruding from the proteolipid into the centre of the head represent the rotor. A conformational torque generated by ATP hydrolysis in VHA-A is converted to a rotation of the membrane-inserted ring. Protons are vectorially translocated from the cytoplasmic side to the lumen of the extraplasmatic compartment. In the following, the present state knowledge on VHA genes and their expression, structure, regulation and ecophysiological significance of plant V-ATPases will be summarised in more detail with emphasis on recent findings.

SUBUNITS AND GENES OF THE PLANT V-ATPase

VHA-Genes

At present, the plant V-ATPase structure is suggested to be assembled from 12 different subunits and thus mostly similar to yeast V-ATPase (Dietz *et al.*, 2001; Sze *et al.*, 2002). According to the suggested nomenclature by Sze *et al.* (2002), the subunits are denominated VHA-A to VHA-H for hydrophilic subunits composing the V_1-sector protruding into the cytoplasm and VHA-a, -c, -d, and -e for membrane associated subunits. In yeast subunit c is encoded by a small gene family denominated c, c′, and c″. The c-isoforms appear to be present at a strict stoichiometry in the holocomplex, that was proposed to be $A_3B_3CDEFG_2Hac_4c'_1c''_1d$ (Powell *et al.*, 2000). A gene homologous to yeast subunit c′ has been identified in the *Arabidopsis* genome, whereas no gene encoding a homologue to c″ has been found (Dietz *et al.*, 2001; Sze *et al.*, 2002). Although the structural analysis has advanced and the gene identification may be completed, it cannot be excluded that additional subunits or assembly factors will be identified as associated parts of the V-ATPase complexes. In the best case, an ultimate answer to that open question will be obtained from functional reconstitution of the V-ATPase complex in vitro. The very first step of such an analysis is presented in Fig. 1. V-ATPase holocomplex was isolated (Fig. 1(A)), divided into two fractions, one of which was labelled with the fluorescence dye Alexa488, the other one with Alexa546. The dyes have a succinimidyl ester mojety that reacts with primary amines. Following chaotropic disassembly of the labelled complexes probably to inhomogenous subcomplexes, both fractions were mixed and dialyzed. Formation of reassembled partial complexes was

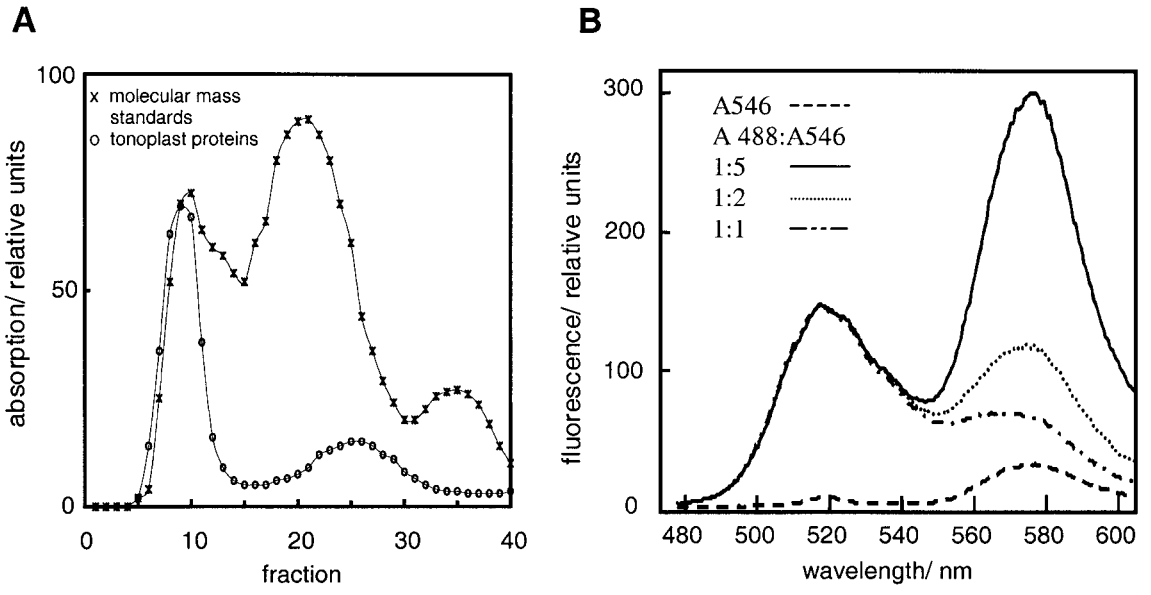

Fig. 1. Förster-Resonance-Energy Transfer (FRET) in V-ATPase subfractions. (A) Tonoplast membranes were isolated from *Mesembryanthemum crystallinum* leaves through density gradient centrifugation. V-ATPase holoenzyme was purified through size exclusion chromatography. The column was calibrated with molecular mass standards. The elution profile as measured at 280 nm was monitored for a mixture of proteins of 669 kDa, 440 kDa, and 66 kDa (x). Superimposed is the separation of a tonoplast preparation (o). The fractions 8–13 were collected and positively tested for the presence of V-ATPase subunits through Western-blotting (not shown). The combined fractions at a protein concentration of 1 mg/mL were divided into two samples each of which was covalently coupled either with the fluorescent dye Alexa488 or Alexa546. After dialysis, the two differentially stained V-ATPase-complexes were chaotropically disassembled with 100 mM KJ. Both fractions were mixed in ratios V-ATPase/Alexa488: V-ATPase/Alexa546 of 1:1, 1:2, and 1:5. After mixing, the ATPase-complexes were dialyzed to remove the KJ. (B) The stained V-ATPase-mixtures and V-ATPase/Alexa546 alone in a twofold concentration as a control were excitated with a laser beam at 440 nm. The emissions were recorded from 480 to 620 nm. The increasing emission at 575 nm is caused by FRET and indicates assembly of V-ATPase subcomplexes. A546: V-ATPase stained with Alexa546, A488: V-ATPase stained with Alexa488.

sensitively monitored using Förster Resonance Energy Transfer (FRET) from Alexa488 as donor to Alexa546 as acceptor dye that only occurs within the range of the Förster radius, i.e. within distance ranges up to 20 nm. The appearance of the emission peak at 575 nm in Fig. 1(B) that increased with the ratio of Alexa546- to Alexa488-labelled fraction indicates interaction of VHA-subunits. This approach will allow to optimize the reassembly conditions and to analyze the generated subcomplexes (Rizzo *et al.*, 2003).

Alternatively, a proteomics approach with isolated functional V-ATPase complex may yield equivalent information, however, with somewhat lower certainty of having picked up all essential subunits. In a recent work, Drobny *et al.* (2002) used the proteomics technique: Following purification of Triton X-100 solubilized V-ATPase complex from tobacco protoplasts, the authors performed immunoprecipitation using an antiserum against VHA-A of V-ATPase from *Mesembryanthemum crystallinum*. Twelve polypeptide positions were identified in SDS PAGE-separations, 11 of which could be assigned to specific subunits by matrix assisted laser-desorption ionization mass spectrometry (MALDI-MS). It was not possible to obtain a suitable mass spectrum for the 12th polypeptide. Its apparent molecular mass was about 50 kDa and could represent VHA-H. In addition to the results from searches in the *Arabidopsis* genome, the MALDI-MS analysis supports the conclusion that 12 subunits compose the functional V-ATPase complex in plants.

Gene Families of VHA-Subunits in *Arabidopsis thaliana* and *Mesembryanthemum crystallinum*

The availability of sequence information of the *Arabidopsis* genome allowed to identify gene families for VHA subunits (The *Arabidopsis* Genome Initiative 2000; Dietz *et al.*, 2001; Sze *et al.*, 2002). Recently, the major part of the rice genomic DNA-sequence has become available (TIGR database). In addition a tentative estimation of the number of subunit isoforms was made from EST entries with distinct nucleotide sequences for *Mesembryanthemum crystallinum* in the dbEST data bank (NCBI, USA) (Kluge *et al.*, 2003). Table I summarizes the information on VHA subunits for *A. thaliana*, *M. crystallinum*,

Table I. Number of Isogenes Encoding VHA Subunits in Plant Genomes

	Isogene numbers		
VHA-subunit	*A. thaliana*	*M. crystallinum*	*O. sativa*
A	1	2	2
B	3	3	2
C	1	4	1
D	1	1	1
E	3	3	2
F	1	2	1
G	3	1	3
H	1	1	2
a	3	3	3
c/c′	5	Unknown	4
d	2	2	1
e	2	1	1

Note. The number of isogenes was determined for *A. thaliana* through a FASTA search in the MATDB database (www. mips.gsf.de), and estimated for *M. crystallinum* through a BLAST search in the database dbEST (www. ncbi.nlm.nih.gov/dbEST) and for *O. sativa* through a BLAST search in the TIGR database (www.tigr.org).

and *Oryza sativa*. VHA-D, is encoded by a single gene in all three species. Slightly contrasting copy numbers in the plants are predicted for VHA-A (A.th./M.c./O.s. = 1/2/2), VHA-G (3/1/3), VHA-d (2/2/1), and VHA-e (2/1/1). An identical multiple copy number of 3 each was revealed for VHA-B, VHA-E, and VHA-a in *A. thaliana* and *M. crystallinum*, but only two isogenes of VHA-B and VHA-E have been idenfied in *O. sativa* up to now. VHA-E is also present in at least three gene copies in mungbean (Kawamura *et al.*, 2001). A highly variable copy number is deduced for VHA-C (1/4/1). Prediction of isoform number for VHA-c was not possible in *M. crystallinum* due to the high degree of sequence conservation among the isogenes. In fact the same limitation may account for the low gene number encoding small subunits VHA-G and e. From these data, it has to be concluded, that the gene copy numbers is not related to the location in either the V_1- or V_0-sector as previously suggested (Sze *et al.*, 2002). In a converse manner, it is tempting to speculate that the conserved three-(two?)fold diversity of VHA-B, VHA-E, VHA-a, and possibly VHA-G has a functional significance. As will be discussed below, these three subunits are known to interact and have been suggested in recent models to form peripheral stalks connecting the head and the membrane sector (cf. Domgall *et al.*, 2002; Sze *et al.*, 2002; Fig. 2). On the basis of these models, the existence of three VHA-G-isogenes as known from the *A. thaliana* and *O. sativa* genome may also be predicted for other plants. Supekova *et al.* (1996) cloned the first cDNA of a G-subunit from bovine chromaffin granules and described the similarity of the deduced amino acids

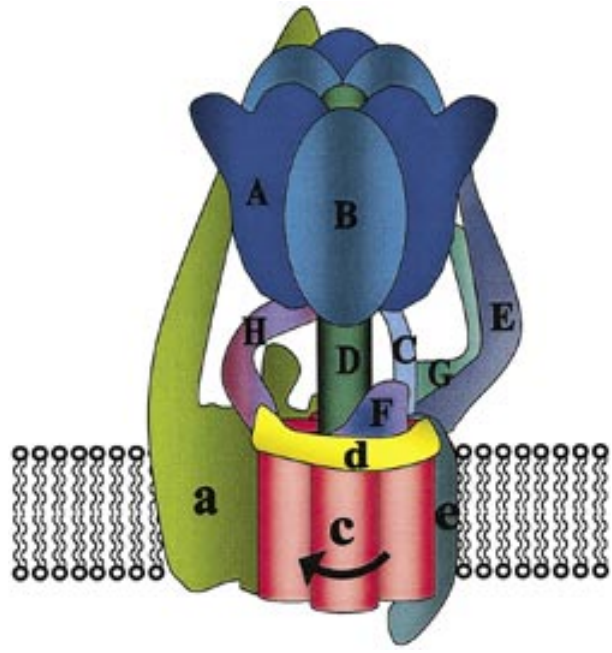

Fig. 2. Model of the plant V-ATPase. The arrangement and interaction of VHA-subunits is based on results from cross-linking studies, the 3D map of V-ATPase of *Kalanchoe daigremontiana* (Domgall *et al.*, 2002) and model suggestions for V-ATPase of *Manduca sexta* (Radermacher *et al.*, 2001) and *Bos bovis* (Wilkens *et al.*, 1999; Wilkens and Forgac, 2001). Subunits of the V_1-part are labelled with capital letters, subunits of the V_0 are labelled with lower case letters.

sequence with subunit b of the F-ATPase. A dimer of subunit b constitutes the single peripheral connector between the V_0 and the V_1 sectors of F-ATP synthases (Junge *et al.*, 1997). Figure 3 illustrates the amino acid (aa) sequence similarity of the three VHA-G subunits present in the *A. thaliana* genome and the VHA-G cloned from *M. crystallinum* with the carboxyterminus of the b-subunit of *A. thaliana* chloroplast F-ATPase. Out of 106 aa residues, 10 aa positions are conserved and 25 aa are similar among all five sequences. This observation indicates a common evolutionary origin of VHA-G and subunit b of F-ATPase with implications for a similar structural function, i.e. as peripheral connector between V_1 and V_0 (Supekova *et al.*, 1996).

RECENTLY IDENTIFIED ADDITIONAL SUBUNITS IN THE COMPLEX: VHA-H,−a,−d,−e

Many plant VHA-subunits have been identified on the basis of sequence similarity with subunits from other organisms, mainly yeast *Saccharomyces cerevisiae*, *Bos*

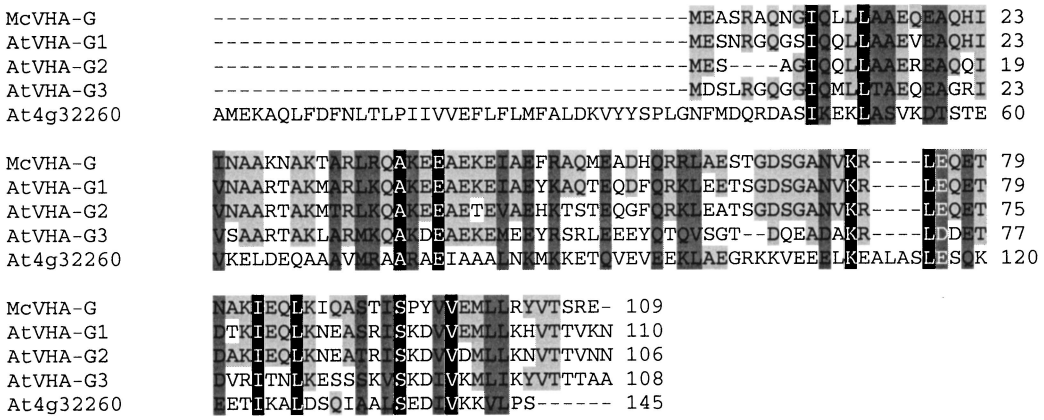

Fig. 3. Amino acid sequence alignment of plant VHA-G and subunit b of chloroplast F-ATP synthase. The VHA-G aa sequences are from *M. crystallinum* (AccNr. AJ438591) and *A. thaliana* (MIPS protein entry code At4g23710, At3g01390, and At4g25950).

taurus, and *Manduca sexta*. Four of these VHA subunits have been identified in plants only recently and are discussed in slightly more detail in the following. From yeast it is known that VHA-H is dispensable for assembly but required for catalytic function (Ho *et al.*, 1993). Recently VHA-H from *S. cerevisiae* was crystallized and structural predictions were derived (Sagermann *et al.*, 2001). In plant VHA-H, two highly conserved regions are also likely to be separated by a variable intervening sequence suggesting structural flexibility (Kluge *et al.*, 2003). The characteristic element of the 3D structure of VHA-H are two armadillo repeat motifs that form superhelices from three α-helices. These elements are also present in the plant proteins and mediate protein–protein interaction (Hirschl *et al.*, 1996). VHA-H is suggested to link V_1 and V_0 and activate V-ATPase. Cross-linking studies with mammalian clathrin coated vesicle and yeast V-ATPase have revealed interaction of VHA-H with VHA-E, VHA-F, and VHA-a (Landolt-Martincorena *et al.*, 2000; Xu *et al.*, 1999).

VHA-a has a molecular mass of about 90 kDa and is the largest subunit of V-ATPases. VHA-a has a chimeric structure with a less conserved hydrophilic aminoterminal part (aa 1-400) and a more conserved hydrophobic C-terminal part (aa 401-816). The C-terminal part has 6 to 9 predicted transmembrane domains and contains amino acid residues involved in the vectorial H^+-translocation across the membrane. These amino acid residues are conserved throughout all published VHA-a sequences, i.e. K743, H743, Q789, R799 (Kluge *et al.*, in press; Leng *et al.*, 1996) indicating a catalytic function. However, the presence of a complete VHA-a subunit in the functional V-ATPase complex of plants is still discussed controversially (Ratajczak, 2000). Li and Sze (1999) could not identify VHA-a in catalytically active V-ATPase and suggested that VHA-a is required for assembly, but absent in the functional complex. In the proteomics approach, VHA-a could not be analyzed since in the immunoprecipitate too many polypeptides exhibited a molecular mass of about 100 kDa (Drobny *et al.*, 2002). This apparent contradiction awaits to be solved.

VHA-d was identified as subunit tightly associated with the V_0 sector although it is devoid of transmembrane domains (Wang *et al.*, 1988). VHA-d (VMA6 in yeast) could be dissolved from the yeast V_0 sector by treatment with chaotropic salts (Baeuerle, 1993). An antibody directed against VMA6 reacted with a 46 kDa polypeptide that separated upon treatment with chaotropic reagents from tonoplast membranes isolated from red beet (Baeuerle *et al.*, 1998). The VHA-d sequences are highly conserved among species. In VHA-d from *M. crystallinum*, 41.5% of all aa positions are identical with VMA6, the degree of conservation increases to 67% in the mid part of the aa sequence (aa 97–194).

The 8-kDa VHA-e is the smallest subunit of the V-ATPase and was first identified in chromaffin granules of *Bos taurus* (Ludwig *et al.*, 1998) and cloned from the insect *Manduca sexta* V-ATPase (Merzendorfer *et al.*, 1999). Interestingly, in yeast, no polypeptide has been found with a high similarity to VHA-e in size and function. It is discussed that the corresponding function in assembly might be realized by the yeast chaperon VMA21p (Ludwig *et al.*, 1998). Despite the low degree of aa identity between VHA-e of all species, VHA-e is characterized by two highly conserved putative membrane-spanning helices. The discrepancy between the theoretical molecular mass of the gene product predicted from

the cDNA-sequence and the molecular mass of VHA-e in V-ATPase separations could be attributed to glycosylation at positions N68-T71 and N84-G87 (Merzendorfer *et al.*, 1999). A putative glycosylation motif is also present in the *M. crystallinum* VHA-e sequence, but absent in the VHA-e sequences encoded in the *A. thaliana* genome (Kluge *et al.*, 2003). Apart from the discussion of their sequences, VHA-d and VHA-e have not been analyzed in plants yet.

STRUCTURE AND SUBUNIT COMPOSITION OF PLANT V-ATPase

The concept of rotational catalysis as basis for V-ATPase-dependent H^+-translocation into endomembranes was adopted from F-ATP synthase (Noji *et al.*, 1997). According to that model, ATP-driven rotation requires the existence of two functionally distinct units within the V-ATPase complex, a stator and a rotor. The head structure has to be fixed to eccentrically located membrane integrated subunits by peripheral connectors. The rotor consists of a ring of VHA-c subunits and VHA-D which extends into the head. According to this assumption, a molecular mass of the rotor may be calculated with about 140 kDa, or slightly higher in the case that additional subunits might be associated. During the last decade, research in the group of R. Ratajczak and U. Lüttge (TU Darmstadt, D) has consequently advanced our knowledge on plant V-ATPase structure. Recently, the image analysis of Domgall *et al.* (2002) provided a detailed set of data on the 3D organization of the plant V-ATPase in the absence or presence of the ATP-analogue AMP-PNP (adenosine[5′-β, γ-imido]triphosphate). This compound mimics nucleotide concentrations present in the cytosol. During electron microscopic analysis, the samples were tilted by 20 to 30° for calculation of the 3D structure. In the side view of the AMP-PNP structure, the V_0 section appeared bean shaped. In a section parallel to the plane of the membrane V_0 showed a dense outer ring and a diffuse centre (Domgall *et al.*, 2002). A spike was located on top of the V_1-head in an asymmetrical position and was suggested to represent the N-terminal part of VHA-a (Fig. 2). Three knob-like structures were detected in the periphery of the head, perpendicular to the central symmetry axis, and assigned to the N-termini of VHA-A. Three peripheral stalks connected the V_1-head to the V_0-sector and were denominated prominent (4.9 nm), intermediate (3.6 nm) and faint (2.4 nm) stalk, respectively. The thin central stalk had a diameter of 3.6 nm. Omission of AMP-PNP strongly altered the 3D map of V-ATPase. A peculiar response to nucleotide depletion was that the V_0-sector was tilted by 30° as related to the axis formed by the head and stalk, and both the prominent and intermediate stalks were detected but much less defined (Domgall *et al.*, 2002).

Increasing evidence confirms the function of VHA-D as central part of the putative rotor that extends to the head and converts conformational energy in the V_1-sector to rotational movement and H^+-translocation in the V_0 sector. VHA-F is in close vicinity of VHA-D. Both subunits can be cross-linked chemically in V-ATPase complexes of clathrin coated vesicles (Xu *et al.*, 1999). VHA-E and VHA-G interact and are suggested to form the faint peripheral stalk (Domgall *et al.*, 2002; Tomashek *et al.*, 1997). Crystal structure analysis of VHA-H has revealed the modular composition (Sagermann *et al.*, 2001) that matches the appearance of the intermediate stalk and could well be fitted into the 3D map of plant V-ATPase (Domgall *et al.*, 2002). The prominent stalk may be composed of the hydrophilic part of VHA-a and VHA-C.

CELLULAR LOCALIZATION OF VHA-SUBUNITS AND PLANT V-ATPase HOLOCOMPLEX

Knowledge about the distribution of V-ATPase subunits on endomembrane compartments in plants is still fragmentary. One particular drawback is the lack of suitable antibodies against specific plant V-ATPase-subunits. Early localization studies on isolated purified membrane fractions from plants have been accomplished with antibodies directed against VHA-A, -G, and against the purified V-ATPase holocomplex. These studies gave evidence on a localization of VHA-subunits on the endoplasmatic reticulum (ER), the Golgi-apparatus (GA), the plasma-membrane (PM), and the tonoplast of plant cells (Herman *et al.*, 1994; Matsuoka *et al.*, 1997; Robinson *et al.*, 1996a,b; Rouquie *et al.*, 1998). Confocal microscopy techniques allow to examine the distribution of vacuolar ATPase subunits, for example in maize root cells (Kluge, Bolte, and Dietz, unpublished data; Fig. 3). Antisera against VHA-A and -E from the cytoplasmic orientated V_1-sector showed similar staining patterns: They marked the tonoplast and some smaller endomembrane-surrounded compartments in a strong manner (Fig. 4(A) and (B): arrow heads). In contrast to this staining, the antiserum anti-VHA-a_{nterm} that was raised against the N-terminal section of the 100 kDa subunit VHA-a of the V_0-sector labelled endomembranes surrounding small compartments, but not the tonoplast (Fig. 4(C): arrow heads).

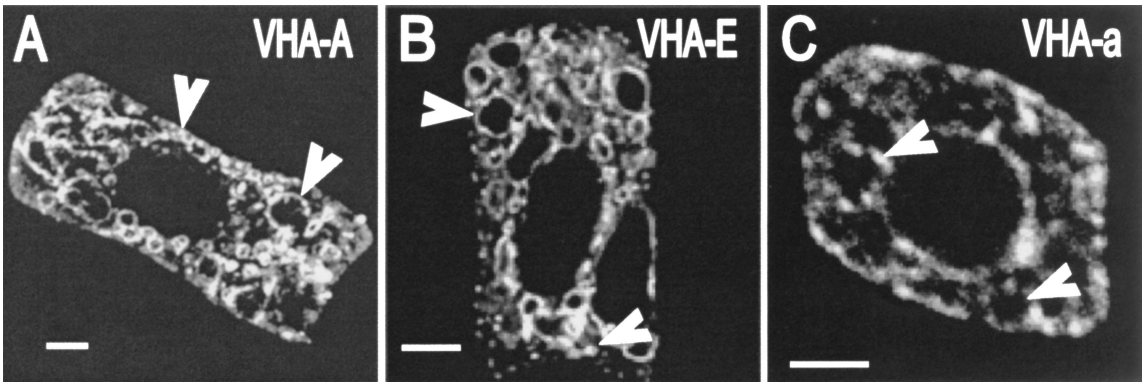

Fig. 4. Immunolocalization of VHA-subunits in maize root cells. Fixed maize root cells from squashed roots were incubated with sera against specific VHA-subunits. (A) Maize root cell incubated with antiserum against VHA-A (Fischer-Schliebs *et al.*, 1997) at a 1:1000-dilution. Bound antibodies were detected with an anti rabbit IgG-antibody coupled with the fluorescent dye CY3 (Sigma, Germany) (1:800). (B) Maize root cell immunodecorated with anti-VHA-E serum (Betz and Dietz, 1991) (1:1000), the antigen–antibody complexes were detected with an anti-rabbit-IgG-Cy3 antibody (1:800). (C) Maize root cell incubated with the anti-VHA-a_{nterm} serum (Kluge and Dietz, unpublished data) (1:1000). The bound antigen–antibody complexes were detected with anti-guinea pig antibody coupled to CY5-fluorescent dye. All images were obtained with a Leica confocal microscope system using 40× magnification. The scalebar is equivalent to 8 μm.

From this staining pattern with anti-VHA-a_{nterm} it may be hypothesized that VHA-a is part of the V-ATPase assembly complex located on the membranes of the ER (Frey and Randall, 1998) as it was proposed by Li and Sze (1999). Another possibility is an isoform-depended localization of VHA-a on distinct endomembranes were it forms functional V-ATPase complexes with compartment specific properties. The latter hypothesis is favored at present since VHA-a has been shown to be an essential component participating in proton translocation (Kawasaki-Nishi *et al.*, 2001; Leng *et al.*, 1996). The availability of sequence information for all VHA-a isogenes from *A. thaliana* and other plants allowed a comparative analysis that revealed the presence of all catalytically essential charged aa also in the plant VHA-a polypeptides (see above). Furthermore the antibody VHA-a_{nterm} was raised against the aminoterminal part of the protein, that exhibits the highest sequence variation among all known isoforms from plant-V-ATPase subunits (Kluge *et al.*, 2003). Thus the antibody may have detected the VHA-a isoform specifically located on nonvacuolar endomembranes. It is unknown at present whether the detection of the VHA-a subunit is correlated with a fully assembled, active V-ATPase or whether the detected subunits are elements of partial assembly complexes of the V-ATPase generated on endomembranes. Functional V-ATPase is required on all endomembranes that surround secretory compartments in order to correctly allocate at least some vacuole-destined soluble proteins (Matsuoka *et al.*, 1997). The binding kinetics of the vacuolar sorting receptor BP-80 represents a good example for a strict pH dependent process at endomembranes (Kirsch *et al.*, 1994).

GENETIC REGULATION OF V-ATPase

In plants, transcriptional regulation is a prominent mechanism to adjust V-ATPase activity to the prevailing growth condition and to the developmental requirements. In response to salinity, changes in transcript levels of single VHA-subunits have been analyzed in a variety of plants since Narasimhan *et al.* (1991) reported upregulation of *Vha*-A-mRNA levels in salt stressed cell cultures of tobacco. In most investigations, salt treatment increased expression of VHA-genes (for summary see Ratajczak, 2000). High amounts of *Vha*-mRNAs are also correlated with growth. When analyzing *Vha*-transcripts in dependence of tissue age, highest amounts of *Vha*-E were found in the young growing part of barley leaves (Dietz *et al.*, 1995). Development and ripening of grape berries represent another recently described example of developmental control of V-ATPase accumulation. During the 15 weeks from pollination to harvest, the berries undergo distinct phases of development. At the time point of véraison, i.e. 7–8 weeks after pollination, the berries soften and start to accumulate sugars (Terrier *et al.*, 2001). The transition was accompanied by a dramatic increase in V-ATPase protein and activity.

The most detailed analysis of transcript levels and tissue distribution is available for *M. crystallinum*, a

facultative CAM (Crassulacean acid metabolism) plant, that switches from normal C3-photosynthesis to nocturnal accumulation of organic acids under drought, salinity and upon addition of the plant growth hormone abscisic acid. In the following light phase, decarboxylation of stored organic acids malate and citrate provide CO_2 for photosynthesis. Stomata may remain closed during the day when the water saturation deficit is high. CAM enables the plants to optimize water use efficiency. Organic acids are stored in the vacuole. Accordingly, an essential component of development of CAM is the activation of V-ATPase (Ratajczak *et al.*, 1994). Recently, salt induced changes in gene expression were compared in juvenile and adult plants (Golldack and Dietz, 2001). Non-salt-tolerant juvenile plants were unable to respond to salinity with up-regulation of *Vha*-E transcripts. In a converse manner, salt-tolerant plants of 5 week age exposed to salinity increased *Vha*-E transcript levels in leaves, but not in roots. These results are in line with the strategy of *M. crystallinum* to export salt from the roots to the shoots where it is stored in the vacuoles mainly of mesophyll and bladder cells. In situ hybridization and immunolocalization studies assigned a special role to V-ATPase during salt adaptation in the tissue surrounding the vasculature. Transcript and protein amounts were down-regulated in root cortex cells and in the cells of the vascular cylinder of the roots and preferentially up-regulated in cells surrounding the vasculature in leaves (Golldack and Dietz, 2001). Apparently, vacuolar deposition of Na^+ is suppressed in the root cells and stimulated in the leaves. The contrasting regulation is particularly pronounced in the cells adjacent to the long distance transport systems.

In all studies, transcript analyzes were restricted to single or few VHA-subunits. Recently, transcript levels of all 12 different VHA-subunits were studied in parallel by macroarray hybridization during short term stress treatment of *M. crystallinum* (Kluge *et al.*, in press). For most *Vha*-isogenes, the changes in transcript abundance were coordinate, for example up-regulation in leaves upon salt stress, no change in heat-stressed leaves and osmotically stressed roots, and down-regulation in salt-stressed roots, cold-treated leaves and roots, heat-stressed roots and leaves from osmotically treated plants (Fig. 5). Also indicated in the figure are those VHA-subunits whose mRNA amounts changed differently from the majority. *Vha*-B transcripts revealed the most pronounced responses to the stress treatments five times, *Vha*-A and *Vha*-c three times, *Vha*-a and -C two times (Kluge *et al.*, in press). By comparison with the established or proposed isogene numbers (Table I) it can be deduced that subunits encoded by several isogenes have a higher propensity to strong changes in transcript amounts upon stress than subunits encoded by

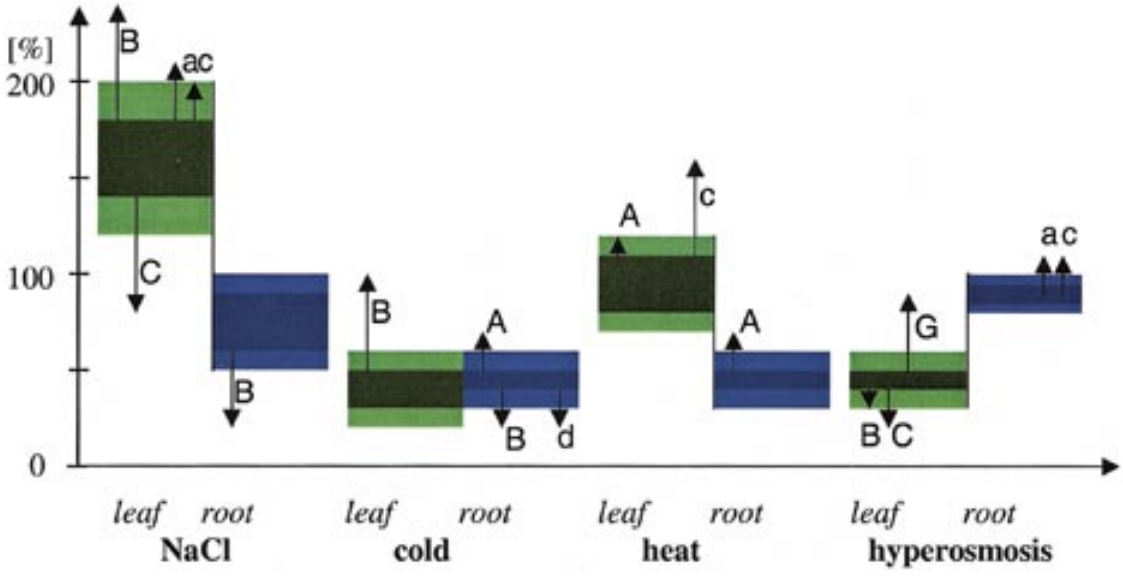

Fig. 5. Coordinate versus distinct regulation of V-ATPase subunits upon short-term stress treatment. A macroarray with cDNA-fragments of all subunits of V-ATPase of *M. crystallinum* was probed with labelled cDNA synthesized from leaf or root tissue subjected to a 6-h stress regime, i.e. salt (400 mM NaCl in the hydroponic medium), hyperosmosis (700 mannitol in the hydroponic medium), cold (4°C), and heat (42°C). The boxes indicate the range of mean change ± SD of all subunit transcripts in percent of control, the dark areas the range of most subunits. Arrows indicate particularly large changes observed for specific VHA-transcripts (letters close to arrow heads). Data taken from Kluge *et al.* (in press).

single genes. On the basis of this relation, it may be hypothesized that the isogenes either encode subunit forms with distinct features necessary or favorable under certain stress conditions or that they are under control of stress-specific promoters in order to allow for appropriate response to the specific stress. The next step of research will have to be the detailed transcript analysis with gene-specific probes to distinguish between the response of individual genes encoding VHA-isoforms.

In addition to transcriptional regulation of V-ATPase synthesis, Chen *et al.* (2002) obtained evidence for post-transcriptional control under salt stress. The moss *Tortula ruralis* is being used as model system to study desiccation tolerance of plants. *Vha*-c transcript levels increased in *T. ruralis*-tissue in response to salinity. Simultaneously, *Vha*-c mRNA was preferentially recovered in the polyribosomal fraction. It has been hypothesized that differential recruitment of mRNAs to the polysomal fraction is related to the maintenance of the constitutive protection system (Wood *et al.*, 2000). The V-ATPase may be part of this protection system under conditions of desiccation and salinity.

Many environmental parameters affect V-ATPase expression and activity. For that reason, Lüttge *et al.* (2001) denominated the H^+-V-ATPase of higher plants "eco-enzyme." In addition to its function as house-keeping enzyme, V-ATPase undergoes specific modifications in structure and activity that are crucial for environmental adaptation. Certainly, V-ATPase function needs further attention in this context. The range of investigated species and growth conditions will have to be broadened. *Suaeda salsa*, an important halophyte from northern China, was suggested to rely on upregulation of V-ATPase amount as main strategy for establishing salt tolerance (Wang *et al.*, 2001). In wheat, a 51-kDa polypeptide cosegregating with the aluminium-resistance phenotype was identified as VHA-B isoform. The *det3* mutant described in the Introduction section exhibited a complex phenotype in addition to its lack of the etiolation response (Schumacher *et al.*, 1999). *det3* has defects in activity of the shoot apical meristem, hypocotyl expansion, brassinosteroid response and stomata regulation. Stomata closure in *det3* was still induced by the plant hormone abscisic acid and cold, but external application of Ca^{2+} and H_2O_2 was ineffective (Allen *et al.*, 2000). The results indicate a function of V-ATPase in specific intracellular signalling events.

BIOCHEMICAL REGULATION OF V-ATPase

The activity of V-ATPase is subjected to posttranslational regulation. Recently, Ratajczak (2000) and Dietz *et al.* (2001) have summarized regulatory and biochemical properties of the plant V-ATPase. Among the parameters affecting ATP hydrolysis and H^+-translocation rates are the slightly alkaline pH optimum, stimulation by chloride, nitrate-sensitivity, moderate affinity to ATP, inhibition by ADP and inorganic phosphate and phosphorylation of certain subunits in dependence of a lysophospholipid-activated kinase. In the latter set of experiments, Martiny-Baron *et al.* (1992) correlated increased H^+-activity with increased phosphorylation of VHA-B and other tonoplast polypeptides. Four elements of biochemical regulation were studied in slightly more detail recently, i.e. redox, adenylate binding, organic acids, and glycolipids.

(i) Oxidation sensitivity of V-ATPase has been known for almost 20 years when Hager and Biber (1984) described the inhibitory effect of SH-group modifying reagents on nitrate-sensitive ATP hydrolysis of tonoplast vesicles. In V-ATPase from clathrin coated vesicles, C-252 and C-532 were identified in VHA-A as targets of redox sensitivity of V-ATPase (Feng and Forgac, 1994). Recently oxidative inactivation of V-ATPase was further investigated in barley leaf preparations (Tavakoli *et al.*, 2001). H_2O_2 inactivated ATP hydrolysis and vectorial H^+-transport in a dose-dependent manner. Reduced glutathione reactivated both H_2O_2-inhibited ATPase activities with a half effective concentration of 1 mM. The transfer from oxidative to reductive conditions was accompanied by electrophoretic shifts of both VHA-A and VHA-E in nonreducing, denaturing SDS-PAGE. Barley VHA-E subunit possesses two cysteine residues at positions 134 and 186, that are absent in nonplant V-ATPases. The authors hypothesized on the existence of a redox-regulatory adjustment of V-ATPase activity that might specifically have evolved in plants. Redox regulation and homeostasis is known to be of high importance in all organisms, but particularly so in sessile plants with oxygenic photosynthesis (Dietz, in press).

(ii) Nucleotides, preferentially ATP, bind to the catalytic subunit VHA-A and the regulatory subunit VHA-B in the head of V_0. Binding of the nonhydrolyzable ATP analogue AMP-PNP to VHA-A induced formation of a disulfide bridge in VHA-E (Kawamura *et al.*, 2001). Apparently, nucleotide binding to VHA-A causes major conformation changes that affect the peripheral stalk constructed from VHA-E. Interestingly,

addition of ATP did not result in oxidation of VHA-E. The authors suggested that the transition state required for oxidation of the dithiols in VHA-E is short-lived because of rapid hydrolysis of bound ATP even at 0°C. In a converse manner, AMP-PNP may arrest the initial state of ATP-binding allowing oxygen to oxidize the target thiols. In that case, AMP-PNP-induced oxidation of VHA-E is an indicator of major structural rearrangements in the complex following nucleotide binding and does not describe a regulatory process with physiological relevance. These data are in line with a change of shape and size of V_1-ATPase from *Manduca sexta* observed upon oxidation (Gruber *et al.*, 2000). A massive nucleotide-induced change of conformation was also observed in the 3D projection map of plant V-ATPase analyzed in the AMP-PNP-bound and -free state (Domgall *et al.*, 2002). The prominent and intermediate stalks partly disappeared in the absence of AMP-PNP and the V_0 sector was tilted with respect to V_1. The destabilised form mimics ATP deprivation and could be the first step in a controlled disassembly under conditions of starvation.

(iii) The plant V-ATPase catalyzes acidification of endomembrane-surrounded compartments including the vacuole. On the basis of the threefold symmetry of the head with three alternating VHA-A and VHA-B, and $\geq$6 VHA-c subunits in the rotor ring with probably one H^+-binding site per subunit, a stoichiometry of 2 H^+ transported per 1 ATP hydrolyzed can be calculated. Under the assumption of two binding sites per VHA-c, the coupling stoichiometry could increase to 4H^+/ATP. H^+/ATP ratios as high as 3.28 and 3.5 have been reported from patch clamp studies with red beet and yeast vacuole (Davies *et al.*, 1994; Yabe *et al.*, 1999). The ratio may decrease by a slip mechanism. The coupling ratio may also change by modulating the size of the rotor by increasing the number of VHA-c units in each V-ATPase (Ratajczak, 2000). A stoichiometry of 2:1 will allow to generate a pH gradient of 4.6 units (Davies *et al.*, 1993) which is sufficient to explain the level of acidification measured in extraplasmatic compartments of most plant cells including fruit vacuoles. Thus, theoretical maximum acidification is not observed suggesting kinetic regulation of V-ATPase activity in most species. However, in some species such as *Begonia lucerna*, vacuolar pH may reach values below 1 (Taiz and Zeiger, 1998) and in lemon pH 2.2 (Müller *et al.*, 1996). In such tissues, H^+/ATP-coupling ratios should be about 1. Interestingly, lemon juice sac V-ATPase was shown to be marginally inhibited by the inhibitor bafilomycin, partially sensitive to vanadate and less sensitive to oxidative inactivation than V-ATPase from epicotyls (Müller *et al.*, 1999). The authors suggested the existence of two interchangeable forms of V-ATPase with a different H^+/ATPase coupling ratio. Insensitivity to inhibitors as revealed for the juice sac-form may indicate the absence of kinetic regulation allowing the V-ATPase to approach thermodynamic equilibrium. Recent results indicate that the epicotyl form also can switch from an H^+/ATP-ratio of more than two to about 1.1 (Müller and Taiz, 2002). Furtheron, luminally accumulated acid anions affected efficiency of H^+-pumping and acidification of vesicles. The order of effectiveness was citrate>malate>sulfate>chloride. Organic acids that accumulate in acidic plant vacuoles reduced the slip at low luminal pH (Müller and Taiz, 2002). The regulatory variability of plant V-ATPase is likely to be reflected in structural features that are still unexplored.

(iv) Delipidated and purified V-ATPase looses most of its activity. ATPase activity is restored by addition of certain phospholipids (Yamanishi and Kasamo, 1993). In a recent study, Yamaguchi and Kasamo (2001) used lipid fractions extracted from rice tonoplasts and observed contrasting effects of phospholipids and glycolipids. Soybean asolecithin and rice tonoplast phospholipids activated delipidated V-ATPase in contrast to glycolipids and neutral lipids. Interestingly, phospholipid-activated V-ATPase was partially inhibited by addition of glycolipids, particularly acyl steryl glucoside. The ratio of acyl steryl glucoside to phospholipids increased in tonoplast during chilling stress and is suggested to modulate tonoplast V-ATPase (Kasamo *et al.*, 2000; Yamaguchi and Kasamo, 2001).

CONCLUSION

Studies with plants have revealed an extraordinary dynamics of structure and regulation of V-ATPase that has not been described for other organisms. The potential for fast growth, the need for rapid secretion into the

vacuole for detoxification, the crucial dependence on immediate adaptation to changing environmental conditions, and the plasticity of plant metabolism in different cells and species are characteristics of plants that may also explain the dynamics of plant V-ATPase. Despite the significant progress made in elucidating the structure of the *Kalanchoe* V-ATPase (Domgall *et al.*, 2002), identification of regulatory mechanisms and cloning subunit genes, there remain many open questions to be addressed in the near future: (1) The complete understanding of the process of V-ATPase assembly, including the differentiation of self-assembly processes and catalyzed reactions, (2) the role of subunit isoforms in the structural, enzymatic and regulatory context of V-ATPase function, (3) the regulatory modulation of V-ATPase structure for optimum balance between H^+-pumping activity and demand for pmf, (4) the relevance of redox regulation of plant V-ATPase in vivo, (5) the mechanism of slip, (6) the expressional regulation of isogenes in distinct tissues, developmental stages and under stress, and (7) the demonstration that indeed a rotor mechanism couples ATP hydrolysis and H^+-translocation. Some approaches towards answering these questions have been addressed in this review.

ACKNOWLEDGMENTS

Support and fruitful discussions by Dr Béatrice Satiat-Jeunemaître (CNRS, Gif Sur Yvette, France) is gratefully acknowledged. The own work presented here was performed within the framework of the Sonderforschungsbereich 613 of the University of Bielefeld.

NOTE

Three recent reports have proven the rotor-stator structure and the rotational mechanism of V-ATPase in yeast (Imamura, H., Nakano, M., Noji, H., Muneyuki, E., Ohkumia, S., Yoshida, M., Yokoyama, K. (2003). Proc. Natl. Acad. Sci. USA 100, 2312–2315; Hirata, T., Iwamoto-Kihara, A., Sun-Wada, G. H., Okajima, T., Wada, Y., Futai, M. (2003). J. Biol. Chem. published on line April, 1, M 3027 56200; Yokoyama, K., Nakano, M., Imamura, H., Yoshida, M., Tamakoshi, M. (2003). J. Biol. Chem. published online April 21, M 303104200).

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