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A Vacuole-Associated Annexin Protein, VCaB42, Correlates with the Expansion of Tobacco Cells[1]

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A Ca-dependent membrane-binding protein of the annexin family, VCaB42, has previously been shown to associate with vacuolar vesicles at physiological levels of Ca. In this study we used suspension-cultured cells of tobacco (*Nicotiana tabacum* BY-2) to show that VCaB42 is enriched 4.5-fold in intact vacuoles, whereas evacuolated protoplasts show a 12-fold reduction in VCaB42. VCaB42 distribution is thus comparable to that of the vacuole-associated H^+-ATPase but is distinct from the endoplasmic reticulum-localized protein calnexin. Because VCaB42 is a vacuole-associated annexin, and given the putative function of annexins in vesicle fusion, we hypothesize a role for this protein in the vacuolation process of expanding cells. Consistent with this hypothesis, we show that VCaB42 levels correlate with age-associated and hormonally induced changes in cell volume in tobacco suspension cultures. The association of VCaB42 with vacuoles and its correlative pattern of expression relative to the expansion of cells is consistent with a possible role for VCaB42 in the early events of vacuole biogenesis.

Plant cell vacuoles carry out diverse functions necessary for cellular homeostasis (for review, see Taiz, 1992; Wink, 1993). Vacuoles are also dynamic organelles capable of large changes in size and morphological appearance, particularly during cell division and during vacuole formation in expanding cells (Raven, 1987; Esau and Gill, 1991). The process of vacuole biogenesis depends in part on the acquisition of molecular components involved in vacuolar function. Targeting of proteins to the vacuole requires either cleavable prosequences at the amino or carboxy terminus or noncleaved sequences in the mature protein that direct the proper sorting of vacuolar proteins into vesicles destined for the vacuole (for review, see Chrispeels and Raikhel, 1992; Neuhaus, 1996). Recent work also suggests that a specific receptor for these signals may be required for proper sorting (Kirsch et al., 1994, 1996). Still, it is not clear where these vesicles (and/or provacuoles) originate and how the large, central vacuole is formed (for review, see Okita and Rogers, 1996).

Marty (1978) provided a detailed analysis of vacuole biogenesis in root meristematic cells of *Euphorbia* sp. In this case small, tubular provacuoles budded from the *trans*-Golgi network and subsequently fused to form larger, autophagic vacuoles. Colocalization of the ER, Golgi, and autophagic provacuoles has also been observed in root tips (Herman et al., 1994) and during the process of vacuole regeneration from evacuolated tobacco protoplasts (Burgess and Lawrence, 1985). In contrast, Hilling and Amelunxen (1985) proposed that after fusion provacuoles dilate, excluding the cytoplasm rather than hydrolyzing its constituents. There has been speculation that provacuoles may be coated with clathrin, since clathrin-coated vesicles may form at the Golgi (Griffing, 1991; Hohl et al., 1996) and both the V-ATPase and the putative vacuole receptor have been localized to clathrin-coated vesicles (Kirsch et al., 1994; Oberbeck et al., 1994). This does not appear to be the case for specialized PSVs located in seed storage tissues. In pea cotyledons PSVs arise directly from the ER/Golgi (Robinson et al., 1995) through a dense vesicle intermediate that is devoid of clathrin but contains characteristic storage proteins and an intrinsic protein, α-TIP, known to specifically associate with PSVs (Hofte et al., 1992; Hohl et al., 1996). Additional mechanisms for PSV formation may also exist in other plants (for reviews, see Okita and Rogers, 1996; Robinson and Hinz, 1996).

Despite vacuole biogenesis being a critical aspect of plant cell development, relatively little is known of factors that may mediate this process. Based on models that suggest that provacuoles fuse during vacuole formation, proteins involved in vesicle fusion may also play a role in vacuole biogenesis. In mammals and yeast a SNARE hypothesis for vesicle fusion has been postulated by which soluble protein factors such as NSF, small GTPases of the Rab/Ypt family, and SNAPs associate with vesicle or target membrane SNAP receptors (v- and t-SNAREs) during intracellular vesicle-targeting events (for review, see Rothman, 1996). Recently, such proteins (Ypt GTPase, NSF, and α-SNAP) have been implicated in homotypic fusion of provacuoles in yeast (Haas et al., 1995; Haas and Wickner, 1996). That similar proteins could also function during vacuole biogenesis in plants is suggested by the recent cloning of both a

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Abbreviations: CFDA, carboxyfluorescein diacetate; NSF, *N*-ethylmaleimide-sensitive fusion protein; PSV, protein storage vacuole; r_{max}, maximal radius; SNAP, soluble NSF attachment protein; SNARE, SNAP receptor; V-ATPase, vacuole-associated H^+-ATPase; V-PPase, vacuole-associated H^+-pyrophosphatase.

t-SNARE homolog and a small Ypt-like GTPase in Arabidopsis, each of which can complement yeast vacuole-targeting mutants (Bednarek et al., 1994; Bassham et al., 1995).

It should be noted, however, that some membrane fusion events are not dependent on NSF, Rabs, SNAPs, and SNAREs (Ikonen et al., 1995). Another family of proteins that can play a role in vesicle fusion are the annexins (for review, see Moss, 1997). Annexins are defined by their Ca-dependent membrane binding. They also share a similar primary structure that consists of four or eight repeat domains, each of which contains the conserved endonexin fold implicated in Ca binding and lipid binding. In contrast, the amino terminus varies between different annexins. Although different annexins have been implicated in diverse functions, their ability to fuse ("annex") vesicles during endocytosis and exocytosis has been especially well documented (Creutz, 1992; Burgoyne and Clague, 1994). Vesicle fusion may also be a function shared by plant annexins (for review, see Clark and Roux, 1995). Several plant annexins can bind and aggregate synthetic liposomes or secretory vesicles (Blackbourn and Battey, 1993; Hoshino et al., 1995), whereas others are thought to function in exocytosis because they are localized to secretory cell types (Clark et al., 1992, 1994, 1995) and are expressed during fruit ripening (Wilkinson et al., 1995; Proust et al., 1996). Other properties of annexins such as actin-binding (Calvert et al., 1996), deoxyribonucleotide triphosphatase activity (McClung et al., 1994; Calvert et al., 1996), inhibition of callose synthase (Andrawis et al., 1993), and involvement in oxidative stress responses (Gidrol et al., 1996) suggest a variety of functions for annexin family members.

We have previously identified a Ca-dependent membrane-binding protein in celery that, based on partial amino acid sequencing, is a member of the annexin family of proteins (Seals et al., 1994). This protein, VCaB42, was purified based on its binding to a low-density membrane fraction that was enriched in vacuole membranes. In this study we have used tobacco (*Nicotiana tabacum* BY-2) suspension cultures to confirm vacuole localization and to demonstrate a direct correlation between VCaB42 protein levels and the expansion of cells. The close association between the amount of VCaB42 and the presence of large vacuoles is consistent with a role for this protein in vacuole biogenesis or in the function of mature vacuoles in plant cells.

MATERIALS AND METHODS

Growth of Tobacco Suspension Cultures

Suspension cultures of tobacco (*Nicotiana tabacum* cv Bright Yellow-2) were grown in Murashige and Skoog liquid medium (Murashige and Skoog, 1962) containing 0.2 mg/L (0.9 μM) 2,4-D under continuous, fluorescent light at room temperature with shaking (110 rpm). Other concentrations of 2,4-D were utilized in some experiments (see "Results"). Stationary phase cells were subcultured every 12 to 15 d by placing 3 mL of cells into 30 mL of fresh medium. Packed cell volume was estimated following centrifugation for 1 min at 300*g* at r_{max} of either 0.5- or 1-mL suspensions. [^{3}H]thymidine incorporation into newly synthesized DNA was measured as described previously (Morehead et al., 1995). Both cell number and cell volume were determined following treatment with 15% (w/v) chromic acid for 30 min at 65°C to separate cell clumps (Iraki et al., 1989). Cell number was measured on a hemacytometer. Cell volume was determined by first taking phase-contrast micrographs on a Diaphot 200 microscope (Nikon). Individual cells were analyzed for area (A) and perimeter (P) after imaging the photographs using a scanner (Apple, Cupertino, CA) and the computer image-analysis program NIH Image 1.44. Volume (V) was calculated using the equation for rods and spheres: $V = 8.5A^{2.5}P^{-2}$ (Binzel et al., 1988).

Antibodies

Polyclonal antibodies were obtained from Balb/c mice following intraperitoneal injections of VCaB42 from celery (EGTA supernatant; Seals et al., 1994). VCaB42 antibodies were routinely used at 1:3000 dilution in 5% (w/w) dry milk in 1× PBS as described below. Monoclonal antibody raised against the 70-kD catalytic subunit of the oat V-ATPase was used at 1:200 dilution (Ward et al., 1992), whereas polyclonal antibodies raised against the castor bean ER proteins calnexin and calreticulin were used at 1:2500 dilution (Coughlan et al., 1996).

Western Analysis and Densitometry

All tobacco samples were directly homogenized into an equal volume of boiling 2× SDS-PAGE sample buffer containing 100 mM DTT. Proteins were separated by SDS-PAGE (12% acrylamide) and transferred electrophoretically to nitrocellulose membranes (Towbin et al., 1979). Except where noted, all steps of the western analysis were done in 5% (w/w) dry milk in 1× PBS. Membranes were blocked for a minimum of 1 h with rapid shaking. Primary antibody incubation was done overnight at 4°C, and secondary antibody incubation (goat anti-mouse conjugated to horseradish peroxidase) was done for 30 min at room temperature. Three 10-min washes were carried out after each antibody application, and a second set of three washes in 1× PBS was used before substrate treatment. Membranes were developed using luminol-based chemiluminescence detection according to the manufacturer's recommendations (Renaissance; Dupont NEN). Multiple film exposures were quantitated by densitometry using the NIH Image 1.44 image processing and analysis program in conjunction with the scanner. Care was taken to ensure that quantitation was within the linear range of VCaB42 standards.

Intact Vacuole Isolation

Protoplasts were prepared from 3- or 4-d-old tobacco suspension cultures. Generally, 1 to 2 mL of packed volume cells were incubated for 2 h in 30 mL of Murashige-Skoog medium (substituting 0.4 M mannitol for Suc) containing 1% (w/v) cellulase and 0.1% (w/v) pectinase.

Protoplasts (approximately 1.0 mL) were washed free of cell wall-digesting enzymes and filtered through glass wool. To isolate intact vacuoles, protoplasts were osmotically shocked by dilution into 30 mL of 0.2 M KH_2PO_4 (pH 8.0), 1 mM $CaCl_2$, and a protease inhibitor cocktail containing 1 mM PMSF, 0.1 mM benzamidine, 1 μg/mL aprotinin, 1 μg/mL leupeptin, and 1 μM pepstatin. The mixture was gently shaken for 1 h at room temperature and then centrifuged for 10 min at 10*g* at r_{max} in a swing-out rotor (centrifuge model GS-6R, Beckman), and the pellet was discarded. The supernatant was recentrifuged for 10 min at 2600*g* at r_{max}. The pellet, highly enriched in vacuoles, was further purified by flotation through a Percoll cushion. Vacuoles, resuspended in 4 mL of 35% (v/v) Percoll, 0.4 M mannitol, and 1 mM $CaCl_2$, were placed at the bottom of a three-step gradient. The middle layer contained 4 mL of 25% (v/v) Percoll, 0.4 M mannitol, and 1 mM $CaCl_2$, and the top layer contained 2 mL of 0.2 M KH_2PO_4 (pH 8.0), 1 mM $CaCl_2$, and the protease inhibitors described above. Following centrifugation at 4°C for 1 h at 100,000*g* at r_{max} (Ti90 rotor, Beckman), vacuoles were collected at the top of the gradient. Purity was generally >95% vacuoles as estimated following staining with 0.1% (w/v) neutral red (final concentration).

Evacuolation of Protoplasts

A modification of the procedure used by Griesbach and Sink (1983) was developed for protoplast evacuolation. Protoplasts, isolated as described above, were resuspended in 0.4 M mannitol, 20 mM Hepes (pH 7.2), and 1 mM $CaCl_2$ and layered on top of a 17-mL cushion of 0.4 M mannitol and 50 mM $CaCl_2$ in 100% Percoll (pH not adjusted). Evacuolation was carried out by centrifugation at 4°C for 1 h and 45 min at 100,000*g* at r_{max} (SW28 rotor, Beckman). Highly enriched evacuolated protoplasts were recovered just above the Percoll pellet and well below intact protoplasts and vacuoles. Evacuolated protoplasts were washed twice with Murashige-Skoog medium (substituting 0.4 M mannitol for Suc) by centrifugation for 10 min at 900*g* at r_{max}.

CFDA Labeling of Protoplasts

Protoplasts, isolated as described above, were adjusted to 50% total volume in Murashige-Skoog medium (substituting 0.4 M mannitol for Suc) containing 25 μM CFDA and incubated overnight (generally 15 h) at room temperature. Extracellular CFDA was removed using Murashige-Skoog medium (substituting 0.4 M mannitol for Suc), and a further incubation (4–6 h) cleared CFDA from the cytosol. Labeled protoplasts were visualized with a Diaphot 200 phase-contrast microscope and a Chroma filter no. 41001 designed to select for CFDA fluorescence.

RESULTS

VCaB42 Protein Is Present in a Variety of Higher Plants

EGTA extracts of low-density cell membranes were obtained from celery (*Apium graveolens*) petioles to identify Ca-dependent membrane-binding proteins (Seals et al., 1994). One such protein, VCaB42, was purified and polyclonal antibodies against the native protein were obtained. By western analysis, VCaB42 antibodies exhibited a relatively high affinity for a 42-kD polypeptide in purified VCaB42 preparations from celery (1:5000 dilution; data not shown). No reaction was observed for preimmune serum or in samples lacking primary antibody application (data not shown). When the antibodies were tested against membrane proteins from celery, a single band at 42 kD was detected, suggesting that the antibodies were specific for VCaB42 (Fig. 1). VCaB42 antibodies recognized proteins of similar molecular mass in carrot (*Daucus carota*), pea (*Pisum sativum*), soybean (*Glycine max*), tobacco (*Nicotiana tabacum*), and *Arabidopsis thaliana* (Fig. 1). VCaB42-related proteins were also present in cotton (*Gossypium hirsutum*) embryos as well as in seedlings from two monocots: maize (*Zea mays*) and oat (*Avena sativa*; data not shown). Proteins antigenically related to VCaB42 were present in all higher plants tested, although the apparent molecular masses varied from 37.5 kD in pea to 44.5 kD in Arabidopsis.

VCaB42 Is Associated with Vacuoles in Tobacco Cells

The Ca-dependent association of VCaB42 with a low-density membrane fraction co-enriched with V-ATPase and V-PPase activities suggested that VCaB42 was associated with vacuoles (Randall, 1992; Seals et al., 1994). To further test this hypothesis, intact vacuoles were obtained from tobacco protoplasts by osmotic shock and further purified by flotation through Percoll gradients. Vacuoles in intact protoplasts were easily observed by staining with neutral red or CFDA prior to intact vacuole isolation (Fig. 2, A and

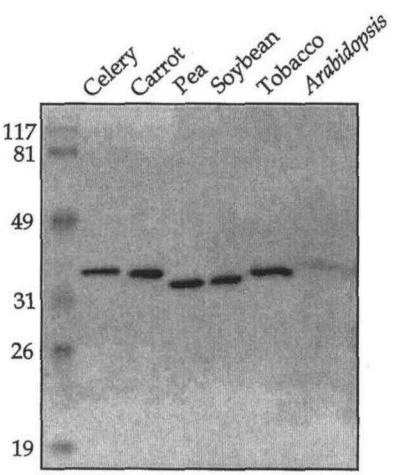


Figure 1. VCaB42-related proteins are present in a variety of plant species. Protein extracts were prepared from low-density membranes of celery (petioles, 2.5 μg), carrot (taproots, 5 μg), and Arabidopsis (whole plants, 80 μg) and from homogenates in pea (seedlings, 20 μg), soybean (seedlings, 16 μg), and tobacco (suspension cultures, 5 μg). Total protein, assayed as described by Bradford (1976) or by using amido black (Kaplan and Pederson, 1985), was resolved by SDS-PAGE, transferred to nitrocellulose, and probed with VCaB42 antibodies. Prestained molecular mass markers in kilodaltons are shown at the left.

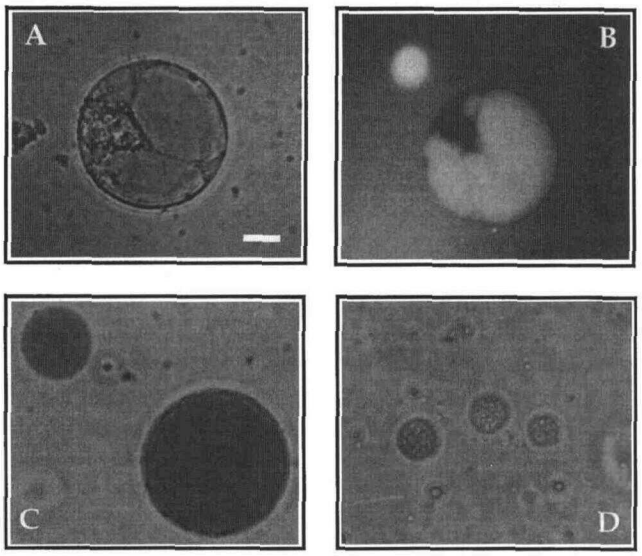


Figure 2. *Protoplasts, vacuoles, and evacuolated protoplasts from tobacco suspension cultures. Protoplasts were stained with 0.1% (final concentration) neutral red (A) or with 25 μM CFDA (B). C, Isolated vacuoles stained with neutral red. D, Evacuolated protoplasts (not stained). All photographs were taken with a Nikon Diaphot 200 phase-contrast microscope at ×244. Scale bar is 10 μm.*

B). These protoplast vacuoles, even in younger tobacco cultures (3 and 4 d), made up a significant proportion (>80%) of the total cell volume. Isolated intact vacuoles also stained with neutral red (Fig. 2C) and CFDA (data not shown) and were of the same approximate size as protoplast vacuoles (Fig. 2, compare A, B, and C). When compared on a protein basis, VCaB42 was significantly enriched (4.5-fold) in intact vacuoles relative to intact protoplasts (Fig. 3A). This enrichment was comparable to that of the V-ATPase, consistent with vacuole localization of VCaB42. The enrichment of VCaB42 in purified vacuoles of tobacco was also similar to that observed for V-ATPase (3.5-fold) and acid phosphatase (4-fold) activities (Saunders, 1979). Other vacuole proteins show similar enrichment in isolated vacuoles (Walker-Simmons and Ryan, 1977; Nishimura and Beevers, 1978; Martinoia et al., 1981). In contrast, calnexin, a marker protein for the ER, showed a reduction in intact vacuoles compared with intact protoplasts, which is indicative of minimal ER contamination of purified vacuoles. Calreticulin, another ER protein (Denecke et al., 1995), showed a lesser reduction than calnexin in intact vacuoles, suggesting that this or a related protein may also be present in vacuoles.

Levels of VCaB42 protein were also analyzed in protoplasts devoid of large vacuoles. We utilized an evacuolation procedure previously developed for leaf mesophyll cells that used differential centrifugation of protoplasts through continuous Percoll gradients (Griesbach and Sink, 1983). Evacuolation was accompanied by a large reduction in vacuole-associated proteins and enzymatic activities (Griesbach and Sink, 1983; Burgess and Lawrence, 1985; Hortensteiner et al., 1992; Hoffmann and Hampp, 1994). When isolated from tobacco suspension cultures, evacuolated tobacco protoplasts had a much reduced volume (approximately 40-fold) due to the loss of large vacuoles (Fig. 2D). Evacuolated protoplasts also failed to stain with neutral red and CFDA (data not shown). The amount of VCaB42 was reduced 12-fold relative to its amount in protoplasts (Fig. 3B). This was comparable to the reduction in V-ATPase levels from protoplasts to evacuolated protoplasts and was similar to the loss of acid hydrolase activity (Hortensteiner et al., 1992; Hoffmann and Hampp, 1994) and V-ATPase levels (Hoffmann and Hampp, 1994) in other protoplast evacuolation studies. In contrast, calnexin showed only a 3-fold reduction. Thus, evacuolation was selective for the removal of large vacuoles, although a portion of other low-density organelles (e.g. ER) were removed as well. Calreticulin showed a moderate reduction (8-fold) from protoplasts to evacuolated protoplasts, supporting the hypothesis that a calreticulin-like protein may be associated with vacuoles. Together, the enrichment of VCaB42 in intact vacuoles and loss of VCaB42 in evacuolated protoplasts suggested that in tobacco, as in celery, VCaB42 was associated with vacuoles.

Growth Parameters of Tobacco Suspension Cultures

To characterize the developmental regulation of VCaB42 expression, we analyzed its presence in tobacco suspension cultures as a function of culture age (time after subculture). Several growth-related parameters were first measured to determine the sequence of events leading to cell expansion.

Packed cell volume, the volume of a cell population after low-speed centrifugation of a tobacco suspension aliquot, was an efficient assay for measuring the relative growth of suspension cultures. In tobacco cultures there was a lag

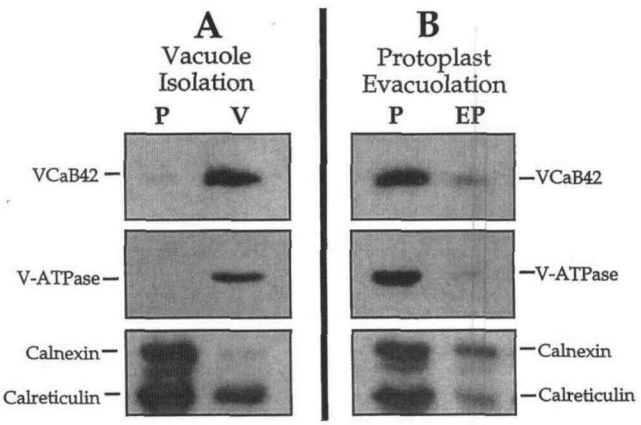
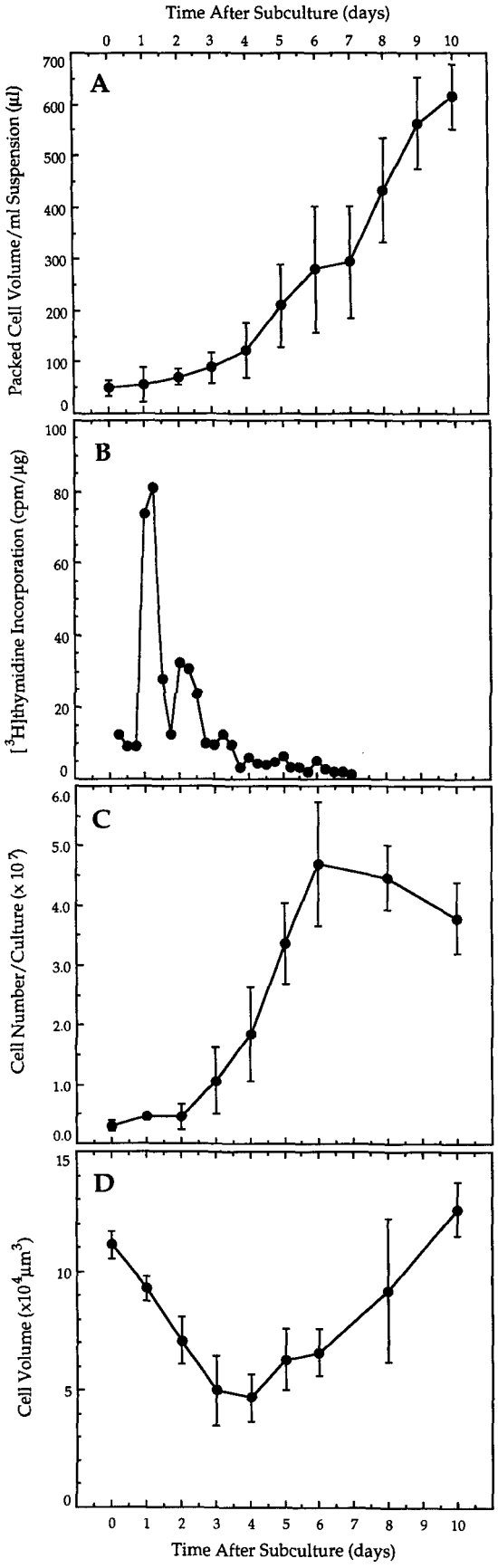


Figure 3. *VCaB42 is associated with vacuoles. Whole-protein extracts were obtained from protoplasts (P), vacuoles (V), and evacuolated protoplasts (EP). Samples were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with either VCaB42 antibodies (top), a monoclonal antibody to the 70-kD catalytic subunit of the V-ATPase (middle), or ER protein antibodies (bottom). Isolation of intact vacuoles (A) and protoplast evacuolation (B) were performed in separate experiments ($n = 3$ and $n = 6$, respectively), as described in "Materials and Methods." Representative data are shown. Note that to more clearly depict the enrichment of VCaB42 and V-ATPase in vacuoles, A was exposed for a much shorter time.*



phase of growth (based on packed cell volume) that lasted until d 2 to 3; thereafter there was a rapid increase up to d 10 (Fig. 4A), which is consistent with previous data (Morehead et al., 1995). Little change occurred in packed cell volume after d 10 (data not shown), indicating that the cells had reached stationary phase. It was previously reported that most of the DNA replication in this culture system occurs by d 2 to d 3 of growth (Morehead et al., 1995). Measurement of [^{3}H]thymidine uptake into cells indicated a peak of incorporation at d 1, followed by a second peak at d 2 (Fig. 4B). It was likely that several rounds of DNA replication occurred prior to cell division, since the number of cells per culture did not increase significantly until after d 2 (Fig. 4C), and some multinucleated cells were observed in 3- and 4-d-old cultures (data not shown). Multinucleated cells have also been observed in other tobacco cultures (Ros and Wernicke, 1991). Cell number measurements showed an increase from d 2 to d 6 (Fig. 4C). The maximum number of cells per 33 mL of culture occurred at d 6 (4.7×10^7), but this was followed by a decline late in culture growth (after d 6) that could be attributed to cell death. Cell volume measurements varied from 5×10^4 μm^3 to as large as 12×10^4 μm^3 during the 10-d growth period (Fig. 4D). Cell volume declined early in culture from d 0 to d 3, remained at minimal levels until d 4, and then increased up to d 10.

VCaB42 Levels Directly Correlate with Cell Volume

Measurement of the amount of VCaB42 protein in total cell extracts indicated a dramatic decline in the 1st d after subculture (Fig. 5A), a time that was coincident with DNA replication (Fig. 4B), but which preceded the observed decrease in cell volume (Fig. 4D). By d 5, levels of VCaB42 started to increase and continued to increase up to d 10. This increase in VCaB42 did not correlate with the onset of cell division (between d 2 and d 6) but, rather, when normalized by protein or by cell number, correlated well with increases in cell volume (after d 4). This direct correlation between the amount of VCaB42 and cell volume extended from d 3 to d 10, as shown by their linearity when plotted against each other (Fig. 5B).

2,4-D Alters VCaB42 Levels and Cell Volume

The growth properties of suspension-cultured plant cells can be altered by changing the concentration of the growth regulator 2,4-D. Previous studies indicated that lower 2,4-D concentrations cause cells to greatly enlarge, whereas higher 2,4-D concentrations result in reduced cell size (Lloyd et al., 1980; Sakai et al., 1996). To test the expression

Figure 4. Growth properties of tobacco suspension cultures. A, Packed cell volume measurements derived from low-speed centrifugation of suspension aliquots (n = 7). B, Incorporation of [^{3}H]thymidine into newly synthesized DNA. Measurements were taken every 6 h for the first 7 d of tobacco culture growth and normalized by protein (Bradford, 1976). Data are those of Morehead et al. (1995). C, Cell number per 33-mL culture (n = 3). D, Average individual cell volumes (n = 3). The total number of cells counted for each treatment varied from 82 to 145.

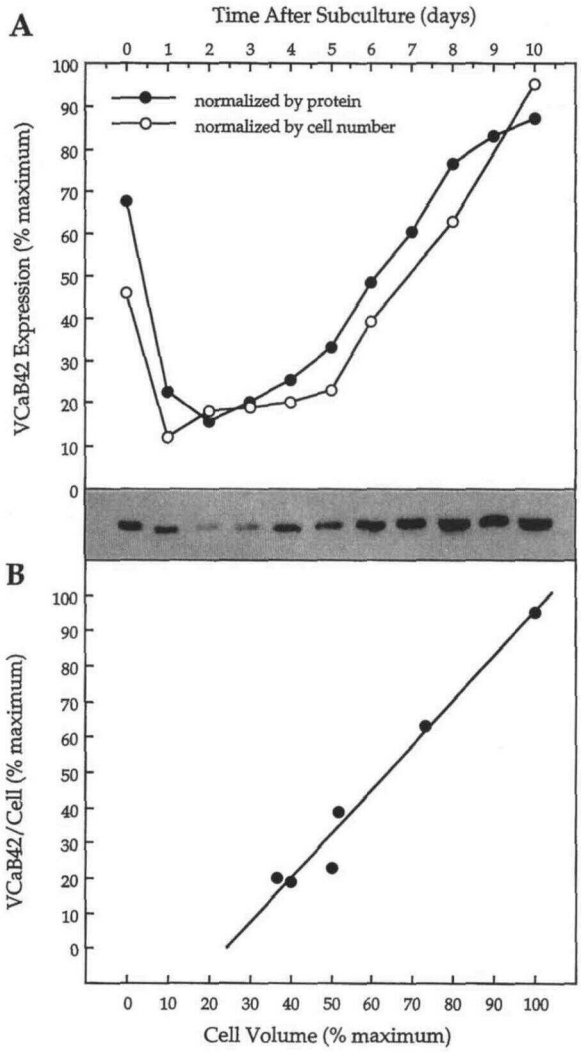


Figure 5. VCaB42 levels directly correlate with cell volume during culture growth. A, Homogenates were obtained from 100 μL of packed cells and 5 μg of protein was resolved by SDS-PAGE, transferred to nitrocellulose, and probed with VCaB42 antibodies. A representative blot is shown. Blot quantitation (n = 4) was expressed as the percentage of maximum levels either as a function of total protein or by cell number (from Fig. 4C). B, Percentage of maximum VCaB42 levels (as a function of cell number) were plotted against percentage of maximum cell volume (from Fig. 4D). Only the data from d 3 to d 10 were used in the correlative analysis.

of VCaB42 in cells of the same age but of different volumes, we treated cultures with Murashige-Skoog medium containing different concentrations of 2,4-D and analyzed cells at d 6, the mid-point of growth under normal conditions (0.2 mg/L 2,4-D; see Fig. 4A).

VCaB42 levels were inversely related to 2,4-D concentration (Fig. 6, A and inset). VCaB42 levels were highest at 0.02 mg/L 2,4-D (3.5-fold higher than control levels) and were lowest at 5.02 mg/L 2,4-D (2-fold lower than control levels). Cell volume was also inversely related to 2,4-D concentration (Fig. 6B). At low levels of 2,4-D (0.02 mg/L) the average cell volume was 16×10^4 μm^3, an approximate 2.5-fold increase over control levels. These cell volumes were greater than those ever attained by control cells (compare with Fig. 4D). Higher concentrations of 2,4-D (5.02 mg/L) showed an average cell volume of 3×10^4 μm^3, a 2-fold reduction relative to controls. Similarly, these cells were smaller than control cells of any age (compare with Fig. 4D). These changes in individual cell volumes (5-fold total difference) were similar to the effect of 2,4-D concentration on VCaB42 levels (7-fold total difference) and were linear over the 2,4-D concentrations tested (Fig. 6B, inset).

DISCUSSION

To better understand the role of the vacuole in intracellular Ca regulation, our laboratory has focused on the

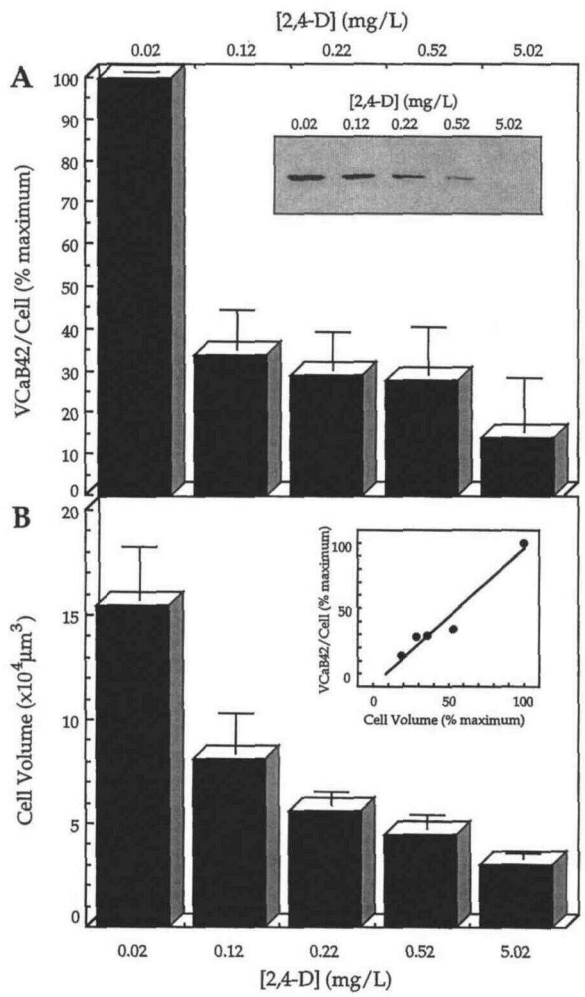


Figure 6. Variations in 2,4-D concentration alter VCaB42 levels and cell volume. A, One-hundred microliters of packed cells was obtained from 6-d-old tobacco suspension cultures treated with various 2,4-D concentrations. Homogenates (5 μg of protein) were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with VCaB42 antibodies. Inset shows a representative blot. Blot quantitation (n = 4) was expressed as the percentage of maximum VCaB42 levels (as a function of cell number) at 0.02 mg/L 2,4-D. B, Average individual cell volumes from various 2,4-D treatments. The total number of cells counted for each treatment varied from 129 to 184. Inset shows percentage of maximum VCaB42 levels (as a function of cell number) plotted against percentage of maximum cell volume (from Fig. 4D).

identification and characterization of vacuole-associated Ca-binding proteins. One such protein, VCaB42, is a member of the annexin family of Ca-dependent membrane-binding proteins, which previously have been shown to mediate the aggregation and fusion of vesicles (Moss, 1997). Here we have confirmed that VCaB42 is associated with vacuoles in tobacco cells. We have also shown that VCaB42 correlates with the presence of large vacuoles in tobacco suspension-cultured cells both as a function of culture age and upon manipulation of culture growth with hormones. Based on these results, we hypothesize that as a vacuole-associated annexin, VCaB42 could play a role in the early events of vacuole biogenesis in plant cells.

Polyclonal antibodies raised against a partially purified preparation of VCaB42 recognized unique proteins at or near 42 kD in extracts from a wide variety of plants (Fig. 1). It is likely that these proteins are distinct, vacuole-associated annexins, since the antibodies are highly specific for VCaB42. No cross-reactivity of antibodies has been observed between VCaB42 and human annexins (I, II, IV, and VI from Biodesigns International [Kennebunk, ME] and VIII, see Hauptmann et al. [1989]; data not shown) or with the pea annexin, p35 (Clark et al., 1992; data not shown), despite the existence of a VCaB42-related protein in pea extracts of 37.5 kD (Fig. 1).

In addition to VCaB42 being immunologically unique, VCaB42 may also be a functionally distinct plant annexin, because in two different plant families, celery (Seals et al., 1994) and tobacco (Fig. 3), a single protein of 42 kD is shown to associate with vacuoles. Although no previous annexin has been shown to localize to plant vacuoles, several mammalian annexins (I, II, and VI) have been shown to have a role in vesicle trafficking between endosome and lysosome compartments (Emans et al., 1993; Futter et al., 1993; Jackle et al., 1994; Seemann et al., 1996). In plants several annexins have been detected in tissues specialized for secretion (pollen tubes, root cap, plumules, and rhizoids) and on vesicular compartments within the secretory system (Golgi and plasma membrane; Blackbourn et al., 1992; Clark et al., 1992, 1994, 1995). VCaB42 is found primarily on the vacuole; however, the secretory system can be viewed as a continuum, and consequently, VCaB42's distribution may not be limited to this organelle.

The conclusion that VCaB42 was associated with vacuoles suggested a more detailed characterization of this protein's expression during the vacuolation and expansion of suspension-cultured cells. Two processes contributed to the growth of cultures and could be distinguished temporally: cell division (measured as an increase in cell number between d 2 and d 6; Fig. 4C) and cell expansion (measured as an increase in cell volume after d 4; Fig. 4D). The amount of VCaB42 closely correlates with changes in cell volume (Fig. 5B). It is noteworthy that the decrease in VCaB42 levels following subculture slightly precedes the decrease in cell volume, whereas increases in the amount of VCaB42 (d 3 or after) slightly precede increases in cell volume (after d 4). Such changes in VCaB42 levels may reflect changes that occur in vacuole morphology. In rapidly dividing cells, vacuoles may be partitioned to allow the proper formation of cell plates, but following cell division, vacuoles may fuse back together (Esau and Gill, 1991). We further determined whether VCaB42 would correlate with hormonally induced changes in cell volume. Our work supports previous conclusions that higher 2,4-D concentrations stimulate continued cell division, thereby reducing the net volume of individual cells; whereas in cells treated with lower 2,4-D concentrations, the mitogenic signal is below a threshold level, and cells increase their volume rather than divide (Lloyd et al., 1980; Sakai et al., 1996). Consistent with the correlation between VCaB42 and cell volume as a function of culture age, VCaB42 and cell volume were also similarly affected by 2,4-D concentrations (Fig. 6).

It is possible that other plant annexins from maize and cotton have a similar role in cell expansion. Maize annexins are heavily transcribed in the cell elongation region of root tips (Battey et al., 1996), whereas the expression of cotton fiber annexins coincides with the elongation phase of fiber development (Andrawis et al., 1993; Potikha and Delmer, 1997). The relationship of these annexins to VCaB42 is presently unknown.

It is likely that vacuole volume is an important contributing factor to cell volume. The observation that a decrease in cell volume occurs in plants expressing antisense V-ATPase transcripts (Gogarten et al., 1992) and that an increase in cell volume occurs when evacuolated protoplasts regenerate vacuoles (Griesbach and Sink, 1983; Burgess and Lawrence, 1985; Hortensteiner et al., 1992; Davies et al., 1996) supports this hypothesis. The relative volume of protoplasts, vacuoles, and evacuolated protoplasts described here is also consistent with vacuoles making up a vast majority of the volume of most cells (Fig. 2). Although we have not made comprehensive measurements of vacuole volume, it is noteworthy that the contribution of the vacuole to protoplast volume is >80% (estimated from protoplasts in Fig. 2). We have also shown that the physical removal of vacuoles from protoplasts not only results in a 12-fold loss of VCaB42 (Fig. 3) but also greatly diminishes (40-fold) cell volume (Fig. 2). Hence, it is likely that VCaB42 levels and vacuole volume are directly correlated.

If VCaB42 expression directly correlates with the vacuolation of expanding cells, then one must distinguish between the possibilities that VCaB42 expression increases because vacuoles are present (implying a role for VCaB42 in mature vacuoles) or whether VCaB42 is directly involved (perhaps required) for vacuole formation and cell expansion. To further address the function of VCaB42, the effect of sense and antisense VCaB42 transcripts on the well-defined growth properties of tobacco suspension cultures described here can be tested. Clearly, a number of factors affect vacuolation. Since larger, mature vacuoles are acidic, the H^+-pumping activities of the V-ATPase and V-PPase may play a role in vacuole biogenesis (Gogarten et al., 1992). Tonoplast intrinsic proteins, which have water channel activity, may also be involved in osmotic-induced changes in vacuole volume (Ludevid et al., 1992; Maurel et al., 1993), especially since osmolarity can affect vacuolation (Hortensteiner et al., 1994; Davies et al., 1996). The cloning of plant homologs to SNAP/SNARE-mediated vesicle fu-

sion proteins, as well as the demonstration of their ability to complement yeast vacuole-targeting mutants, opens up other possibilities with regard to vacuole formation in plants (Bednarek et al., 1994; Bassham et al., 1995). Based on this report, the role of annexins in general and of VCaB42 in particular must also be considered in the process of vacuole biogenesis in plant cells.

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