RESEARCH PAPER

The V-ATPase from etiolated barley (Hordeum vulgare L.) shoots is activated by blue light and interacts with 14-3-3 proteins

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Abstract

The vacuolar H⁺-ATPase (V-ATPase) is a key enzyme that controls the electrochemical proton potential across endomembranes. Although evidence suggests that V-ATPase is important for photo-morphogenesis, little is known about short-term regulation of V-ATPase upon initiation of the photo-morphogenetic programme by exposure of dark-grown plants to light. In this study, etiolated coleoptiles were given a short blue light treatment and V-ATPase characteristics were determined. The effectiveness of the light treatment was assessed by means of fusicoccin binding to the plasma membrane; this increased 5-fold. The short light treatment also induced a 2-fold to 3-fold increase in the hydrolytic activity of V-ATPase. Members of the 14-3-3 protein family are involved in both blue light perception and the subsequent activation of the P-type ATPase. We provide evidence that 14-3-3 proteins specifically interact with the catalytic A-subunit of the V-ATPase. First, the isolated V₁-part of the V-ATPase co-purifies with 14-3-3 on a gel filtration column. Secondly, in an overlay experiment, 14-3-3 interacts with a 68 kDa band that was identified as the V₁ A-subunit by mass spectrometry. Thirdly, in 14-3-3 affinity chromatography, both A- and B-subunits of the catalytic moiety of the V-ATPase were identified by matrix-assisted laser desorption ionization tandem time of flight mass spectrometry (MALDI TOF/TOF MS) as 14-3-3-interacting proteins. It was shown that the A-subunit can be phosphorylated in vitro by a tonoplast-bound kinase, whose properties are affected by blue light. Taken together, the data show that besides the P- and F-type H⁺-ATPases, the V-type H⁺-ATPase also interacts with 14-3-3 proteins.

Key words: 14-3-3 interaction, blue light, phosphorylation, V-ATPase.

Introduction

The vacuolar H⁺-ATPase (V-ATPase) is a proton-translocating enzyme, present in endomembranes of all eukaryotes. This includes the endoplasmic reticulum, the Golgi apparatus, lysosomes, the tonoplast, and intracellular vesicles that traffic between all these membranes (Dettmer et al., 2006). The V-ATPase transports protons from the cytoplasm into the extraplastic compartments, thereby creating the driving force for a variety of transport events of ions and metabolites. In this way, the pump has a general housekeeping function, but it is also very important for plant growth, development, and adaptation to environmental stress conditions (Schumacher et al., 1999; Ratajczak, 2000; Dietz et al., 2001). In view of its...
role in the regulation of cytoplasmic ion homeostasis and turgor pressure, it is not surprising that the activity of the pump changes in response to salt and drought stress, low and high temperature, acidification of the cytosol, and anoxia and plant hormone treatment (Dietz et al., 2001).

V-ATPase is a tightly regulated proton pump. Several mechanisms at different levels and time spans have been recognized to control its functioning: regulation by gene expression, redistribution of the V-ATPase, modulation of the ATPase activity, and regulation of coupling of ATP hydrolysis to H+ pumping (Finbow and Harrison, 1997; Merzendorfer et al., 1997; Dietz et al., 2001). Adaptations on a long-term basis are achieved through variation of the V-ATPase gene expression, as is often seen in salt-tolerant plants, for example, salt-activated expression of subunits correlating with increased V-ATPase activity (Ratajczak et al., 1994; Barkla et al., 1995; Tsiantis et al., 1996). For short-term variation, the V-ATPase is subjected to biochemical regulation by pH and ions (Cl−, K+, NO3−, ATP/ADP, and Pi, or oxidation/reduction. Relatively little is known thus far about another well-recognized mechanism to control enzyme activity on a short-term basis, i.e. phosphorylation/dephosphorylation. Liu et al. (2004) showed that phosphorylation increased the activity of the V-ATPase in vesicles isolated from maize root. The authors identified the A-subunit as being phosphorylated on a serine residue in a motif conserved in the A-subunit of many species (RLV[S/Q]K). In view of the amino acid sequence of this phosphorylated motif and the fact that the A-subunit of V-ATPases appears to be homologous with the β-subunit of the F-ATPase, it was hypothesized that the V-ATPase might interact with members of the so-called 14-3-3 family of proteins in a phosphorylation-dependent manner, just like the F-ATPase. Recently it was found, that the β-subunit of the plant F-ATPase can bind 14-3-3 proteins (Moorhead et al., 1999; Bunney et al., 2001). Both the mitochondrial and the chloroplast F-ATPase interact with 14-3-3, and the complex is more stable in the presence of phosphatase inhibitors. Binding of the 14-3-3 proteins to the F-ATPases resulted in inhibition of their activity (Bunney et al., 2001). V-ATPases resemble F-ATPases; these pumps probably share common ancestors and show similarity in their complex structure and subunit composition (Nelson and Taiz, 1989; Nelson, 1992).

14-3-3 proteins are conserved regulatory proteins, ubiquitously present in the eukaryotic kingdom (Aitken, 2006). They are capable of binding to phosphorylated motifs, such as R/K-x-(x)-S/T-x-P, in target proteins (Muslin et al., 1996; Yaffe et al., 1997). In plants, 14-3-3 proteins also bind to the third type of H+-ATPases, the plasma membrane-located P-type ATPase, and form the so-called 'fusicoccin receptor' (Korthout and de Boer, 1994; Marra et al., 1994; Oecking et al., 1994). Binding of 14-3-3 to the P-ATPase plays a role in plant adaptation to abiotic stresses such as low temperature and osmotic stress via activation of the pump (Chelysheva et al., 1999; Babakov et al., 2000). Moreover, in a series of elegant experiments, it was shown that the P-ATPase is activated after a short (seconds) treatment by blue light (Kinoshita and Shimazaki, 1999, 2002; Kinoshita et al., 2003). The activation mechanism involves (i) phosphorylation of the C-terminal domain of the P-ATPase; (ii) binding of 14-3-3 to that domain; and (iii) activation of the enzyme. It is worth noting that the blue light receptor involved in perception of the blue light signal and activation of the pump, phototropin 1 (Phot1), also binds 14-3-3 upon blue light activation (Kinoshita et al., 2003). Intriguingly, the V-ATPase can also be involved in blue light responses. Mutation in one of the V-ATPase subunits in Arabidopsis (det3-mutation) results not only in lack of cell expansion and abolished stomata closure after blue light treatment, but also in a de-etiolated phenotype (Schumacher et al., 1999; Allen et al., 2000).

Thus, keeping in mind that P- and V-ATPases are involved in similar processes in the cell, such as generation of electrochemical potential differences across the plasma membrane and tonoplast, stabilization of cytoplasmic pH, turgor pressure control, and plant cell expansion, and the possibility of analogous regulation of the F- and V-ATPase, the question was addressed of whether 14-3-3 might interact with the V-type ATPase. Since it is well-known that 14-3-3 can act as an 'alarm regulator', changing enzymatic activity at short notice after a given stimulus (Kinoshita and Shimazaki, 1999; Sinnige et al., 2005), blue light was used as a signal to change the activity of the V-ATPase. The P-type ATPase was monitored as a positive control for the action of blue light and regulation by 14-3-3 (Kinoshita and Shimazaki, 2002).

In this study, it is demonstrated that short treatment of etiolated barley coleoptiles with blue light results in activation of specific ATP hydrolysis by the V-ATPase. The results indicate that the V-ATPase hydrolytic part, V1, co-purifies with 14-3-3 in gel filtration experiments and can be pulled-down by 14-3-3 affinity chromatography. The interaction between 14-3-3 and the A-subunit of the V1 part of the ATPase was confirmed in overlay experiments. In an in vitro experiment, it is demonstrated that the A-subunit of the V-ATPase can be phosphorylated and the level of kinase activity increases after the blue light treatment.

Materials and methods

Plant material

Barley (H. vulgare L. cv. Alexis) seeds (300 g) were washed with tap water for 1 h, germinated overnight on wet tissue at 22 °C, then sown in soil and grown in the dark for 4 d at 20 °C. After that, the pots covered in aluminium foil were transferred to a room, illuminated with red light (Kodak GBX-2 safe light filter, intensity of 100 mE m−2 s−1). Coleoptiles (120 g) were cut with a razor.
V-ATPase is activated by blue light and interacts with 14-3-3 proteins

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Microsomal and tonoplast vesicles isolation

Tonoplast vesicles were isolated according to the protocol of Maeshima and Yoshida (1989) with minor modifications. In brief, 60 g of coleoptiles were homogenized in 120 ml of ice-cold buffer A [50 mM MES/TRIS pH 7.6, 250 mM sorbitol, 10 mM EDTA, 5 mM K$_2$SO$_4$, 5 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% polyvinylpolypyrrolidone (PVPP), 10 µM cantharidin] with a Waring blender at the highest speed, three times for 30 s each with a 1 min interval. The homogenate was filtered through four layers of Miracloth, centrifuged at 6000 g for 10 min at 4°C, the supernatant was centrifuged at 100 000 g for 35 min at 4°C and the pellet was resuspended in 15 ml of buffer B [10 mM K$_2$HPO$_4$/KOH pH 7.8, 500 mM sucrose, 1 mM EDTA, 2 mM DTT, 10 µM cantharidin]. The resuspended microsomes (5 ml) were layer under 5 ml of buffer C (5 mM MES/TRIS pH 7.3, 250 mM sorbitol, 1 mM DTT) in tubes for a swing-out rotor. Samples were centrifuged at 120 000 g for 35 min at 4°C. The white disc of the interphase was carefully collected, diluted five times in buffer C, and pelleted by centrifugation at 100 000 g for 35 min at 4°C. Samples were stored at –80°C in aliquots.

Fusicoccin binding assay

Binding of [3H]fusicoccin ([3H]FC) to 50 µg of the microsomal membrane fraction was carried out in 100 µl of binding buffer (20 mM MES/TRIS pH 7.0, 5 mM MgCl$_2$, 1 mM CaCl$_2$, 2 mM DTT) at a concentration 60 nM [3H]FC. Microsomal membranes were incubated with [3H]FC for 30 min at room temperature, then samples were filtered through GF-C Millipore filters precharged with 1% polyethyleneimine, and washed three times (5 ml each) with ice-cold 20 mM glycine/KOH (pH 9.5), dried in air and counted on an Rack Beta 1219 LKB counter in a liquid scintillator. Unspecific binding was measured in the presence of 10 µM of unlabelled fusicoccin (FC).

Dissociation of V1-ATPase and gel chromatography

Dissociation of the soluble V1 domain of the V-ATPase was carried out according to the protocol of Muller et al. (1996). Tonoplast vesicles (230 µg) were resuspended in 200 ml of dissociation buffer (10 mM MES/BTP pH 7.6, 20 mM KCl, 1 mM EDTA, 2 mM DTT, 0.1 mM PMSF, 5 mM ATP, 7 mM MgSO$_4$, 500 mM KNO$_3$, and 10 µM cantharidin). Samples were incubated on ice for 1 h and spun down for 15 min at 400 000 g at 4°C. The supernatant was applied on a Superdex-200 chromatography column equilibrated with gel filtration buffer (20 mM HEPES/KOH pH 7.5, 0.5 M NaCl, 1 mM MgCl$_2$, 10 µM cantharidin). Fractions between the void volume and the salt peak were collected and precipitated overnight at 4°C with 10% trichloroacetic acid (TCA), washed twice with cold acetone, and loaded on an SDS–polyacrylamide gel for western blotting of 14-3-3s and the E-subunit of the V-ATPase.

SDS-PAGE and western blotting assay

Tonoplast proteins (5 µg) were separated by 12% SDS–PAGE (Laemmli, 1970) and transferred during 140 min from the gel onto a polyvinylidene fluoride (PVDF) membrane (Bio-Rad) in a semi-dry Transfer Cell (Bio-Rad) at 2 mA cm$^{-2}$ at room temperature (Towbin et al., 1979). Next, the membrane was blocked overnight at room temperature by 5% skimmed milk (‘Marvel’) dissolved in TRIS-buffered saline-Tween (TBS-T; 0.05% Tween-20). Antibodies against barley 14-3-3 isoforms A, B, C, D, and E were applied at a 1:50 000 dilution. As common 14-3-3 antibody, K-19 (Santa Cruz) at a dilution of 1:10 000 was used; antibodies against the E-subunit of the V-ATPase were diluted at 1:5000 (the antibodies were kindly provided by Dr KJ Dietz) in TBS-T buffer with 1% bovine serum albumin (BSA) (Sigma) and incubated for 1 h at room temperature with constant agitation; secondary anti-rabbit horseradish peroxidase (HRP) conjugate (Promega) at 1:3000 dilution was applied in TBS-T. The blot was developed with an ECL kit (Amersham), and ECL film (Amersham) was used for detection of the signal with an exposure time of 1.5 min.

V-ATPase activity assay

Hydrolysis of ATP by V-ATPase was measured according to LeBel et al. (1978) in 1 ml of reaction solution: 50 mM Tricine/KOH pH 7.5, 2 mM MgSO$_4$, 2 mM ATP. For each measurement, 10 µg of tonoplast protein was used. The blank sample contained all components except the tonoplast membrane. Samples were incubated for 10 min at 30°C. The reaction was stopped by adding 1 ml of LeBel stop mixture; the colour developed for 4 min at room temperature and development was stopped by adding 100 µl of 34% Na$_2$-citrate. Samples were centrifuged at 10 000 g for 4 min, and absorption was measured at 750 nm. Baflomycin A (50 nM) was added specifically to inhibit the V-ATPase. All measurements were done in triplicate.

Production of 14-3-3Cbio and 14-3-3CHis

The N-six-histidine or N-biotin-tagged 14-3-3 barley isoform C (accession number Y14200) was expressed in Escherichia coli. The bacterial cells were induced overnight with 1 mM isopropyl-$p$-thiogalactopyranoside (IPTG). Bacteria were disrupted with a French press in the presence of complete EDTA-free protease inhibitor cocktail (Roche). The lysate was centrifuged at 100 000 g for 35 min at 4°C and the supernatant was filtered through a 0.45 µm filter. 14-3-3CHis was purified on a HiTrap chelating column (Amersham) with a 20–500 mM imidazole gradient according to the manufacturer’s protocol. 14-3-3Cbio was purified on a HiTrap-Q column (Amersham). Proteins were aliquoted and stored at –20°C in the presence of the protease inhibitor cocktail.

14-3-3 affinity chromatography

A 10 µg aliquot of 14-3-3CHis was immobilized on a 5 ml Ni-NTA column. The column was equilibrated in running buffer 20 mM HEPES/KOH with 50 mM imidazole at pH 7.5. A fraction with soluble proteins from 140 g of coleoptiles was obtained according to the protocol of Moorhead et al. (1999). Briefly, the coleoptiles were homogenized in ice-cold buffer (50 mM MES/TRIS pH 7.6, 1 mM EDTA, 1 mM DTT, 0.1 M cantharidin). Fractions between the void volume and the salt peak were collected and precipitated overnight at 4°C with 10% trichloroacetic acid (TCA), washed twice with cold acetone, and loaded on an SDS–polyacrylamide gel for western blotting of 14-3-3s and the E-subunit of the V-ATPase.

blade, gently mixed, split into two samples, and spread out in two Petri dishes. While one Petri dish was covered in aluminium foil, the second sample received a 10 min blue light treatment (Strand Filters Cinelex No 419 Dark Blue Primary H$_2$O$_2$ 450 nm) at a photon intensity of 50 mE m$^{-2}$ s$^{-1}$ superimposed on the background of red light.
bound proteins, the column was washed with 100 ml of running buffer with 100 mM imidazole. Elution was performed in two steps: 100–200 mM and 200–600 mM of imidazole. Proteins from the second elution were collected and precipitated with 10 vols of cold acetone (−20 °C overnight).

14-3-3 overlay assay
Conditions for SDS–PAGE, the amount of protein, and blotting conditions were the same as those described for western blotting (see above). Blocking was performed with 5% BSA (Sigma) overnight at room temperature with constant agitation. 14-3-3-Chi (3 mg ml−1) was incubated in TBS-T with 0.1% BSA (Sigma) with the membrane for 3 h at room temperature with constant agitation. To distinguish between specific and non-specific binding, excess 14-3-3-Chi (3 mg ml−1) was applied together with 14-3-3-Chi. After the membrane was washed three times with TBS-T buffer and avidin-HRP conjugate (Pierce), a concentration of 10 µg ml−1 was incubated for 1 h. After washing three times with TBS-T, the signal was developed with an ECL kit (Amersham) under the same conditions as for western blotting.

\[ {\gamma^{32P}} \text{ATP tonoplast protein labelling} \]
A 20 µg aliquot of tonoplast membranes was incubated in labelling buffer (20 mM TRIS/MES pH 6.5, 250 mM sorbitol, 1 mM DTT, 2 mM Mg2+, 30 µM Ca2+, 15 mM NaF, 0.2 mg ml−1 leupeptin, 10 µM cantharidin, 5 µM microcystin LR) with 0.23 µM \([\gamma^{32P}]\text{ATP for 10, 30, and 60 min at } 3 °C \text{ for the kinetic assay or a 10 min incubation with } 0.23 \text{ µM } [\gamma^{32P}]\text{ATP together with } 0, 0.01, 0.1, 1, 10, 100, \text{ and } 1000 \mu \text{M of unlabelled ATP. The reaction was stopped by boiling for 3 min with SDS sample buffer. Sample proteins were separated by 12% SDS–PAGE. The gel was washed three times for 20 min each with acidic methanol (40% methanol, 10% acetic acid), followed by overnight incubation in TCA-PPi buffer (5% TCA, 1.5% Na-pyrophosphate) with constant agitation, and twice for 20 min each with water. After washing, the gel was stained by colloidal Coomassie, sealed in plastic, placed in a cassette of a phosphorimager, and incubated for 24 h.}

\[ \text{In-gel digestion and MALDI TOF analysis of peptides} \]
Protein bands were cut out from the colloidal Coomassie-stained gel. Trypsin in-gel digestion and peptide extraction were performed according to the protocol of Li et al. (2004). Samples of extracted peptides (0.5 µl) were mixed with 0.5 µl of 3-cyano-4-hydroxycinnamic acid (5 mg ml−1) and spotted on a target plate of an Applied Biosystems 4700 Proteomics Analyzer. A database search was performed using GPS Explorer V.3.0 against the Swiss-Prot green plant database with 100 ppm peptide mass tolerance, and with tandem mass spectrometry (MS/MS) tolerance of 0.2 Da. Common contaminants such as human keratin and trypsin peaks were excluded from the analysis.

Results

Blue light affects the P-ATPase
To examine the effectiveness of the blue light treatment, an investigation was carried out to determine whether blue light activates the plasma membrane (PM) P-type ATPase in etiolated coleoptiles as it does in guard cells (Goh et al., 1995; Kinoshita and Shimazaki, 1999). As a marker for ATPase activation, the specific binding of FC to membranes isolated from blue light-treated coleoptiles was measured. As high affinity FC binding sites have been widely used as a marker for plant PMs (Babakov et al., 2000), it was decided to measure the binding directly on the microsomal fraction without further PM purification. Figure 1 shows a typical response of the plasma membrane FC receptor to blue light treatment (10 min 50 µE m−2 s−1) in the background of red light (100 µE m−2 s−1).

After this treatment, the specific binding of FC increased about five times compared with coleoptiles treated only with red light. The increase in FC binding indicates that the number of 14-3-3–P-ATPase complexes increased and indirectly shows the activation of the P-ATPase in this experiment.

The V-ATPase activates ATP hydrolysis under blue light
To check whether the blue light treatment activates V-ATPase as it does with P-ATPase, tonoplast vesicles were purified from coleoptiles. Figure 2 presents the data of the V-ATP hydrolysis in the tonoplast fraction isolated from coleoptiles treated with blue light. The total tonoplast ATPase activity from control (red light as a background) coleoptiles was 360 nmol Pi mg−1 protein min−1. The specific V-ATPase activity was calculated by subtracting background hydrolysis (the same measurement in the presence 50 nM bafilomycin A1, a specific V-ATPase inhibitor) from the total ATPase activity (Fig. 2). This shows that the specific activity in the tonoplast was 300 nmol Pi mg−1 protein min−1, i.e. 85% of the total activity.

At the same time, blue light-treated plants showed a 2.5-fold to 3-fold increase in bafilomycin A1-sensitive ATPase activity, 950 nmol Pi mg−1 protein min−1. The background
activity in the presence of bafilomycin remained unchanged after the blue light treatment. This indicates that blue light activates only the hydrolytic activity of the tonoplast V-ATPase.

The amount of 14-3-3 bound to tonoplast vesicles increases after blue light treatment

In stomatal guard cells, blue light induces an increase in binding of 14-3-3 to the plasma membrane (Kinoshita and Shimazaki, 1999). To determine whether 14-3-3 proteins are involved in the blue light response of the plant vacuole as well, a western analysis of the tonoplast fraction isolated from coleoptiles was performed using 14-3-3 common and isoform-specific antibodies. The total amount of 14-3-3 on the tonoplast vesicles was estimated with K-19 antibodies (Santa Cruz), which can detect all five barley 14-3-3 isoforms (data not shown). A semi-quantitative western analysis of the tonoplast fraction from coleoptiles was performed using 14-3-3 common and isoform-specific antibodies. The total amount of 14-3-3 on the tonoplast vesicles was estimated with K-19 antibodies (data not shown). Figure 3 shows representative results of a semi-quantitative western. First, quantification of the band intensity of three independent protein isolations shows that 14-3-3 is not a minor protein in the tonoplast fraction; the amount of 14-3-3 is \(1.9 \pm 0.2\) µg mg\(^{-1}\) of the total tonoplast protein (average ±SE). Secondly, after the blue light treatment, the total amount of 14-3-3 associated with tonoplasts increases 1.8 times (3.4 ± 0.3 µg mg\(^{-1}\) of total tonoplast protein). The increase in the amount correlates with the increase in ATP hydrolysis by V-ATPase (Fig. 2).

To find out which of the 14-3-3 isoforms is involved in the blue light response, a western analysis was performed using isoform-specific antibodies raised against a synthetic peptide derived from the C-terminal part of each protein (Van den Wijngaard \textit{et al.}, 2005). Figure 4 shows the results of a representative western blot. From three independent experiments, the optical density of the 14-3-3 bands was scanned and averaged, and the values of the blue light-treated samples were normalized to those of the respective controls (red light). These ratios are shown below the bands (Fig. 4) and only the 14-3-3A isoform shows a significant increase in density (3.9 times). In the same set of experiments and in the same fashion, the amount of the E-subunit of the V-ATPase was also measured with an antibody raised against the E-subunit (kind gift of Dr. KJ Dietz; Golldack and Dietz, 2001). As shown in Fig. 4, no significant changes in the amount of E-subunit were observed. Sometimes, an extra band with an apparent molecular mass of 35 kDa was detected in the blue light treatment by the E-subunit antibodies; this may be due to a post-translational modification since it is known that phosphorylated proteins will migrate more slowly in an SDS–polyacrylamide gel (Lu \textit{et al.}, 1994).

14-3-3 co-purifies with the V1-ATPase on the gel filtration column

Previously, it was shown that the structural and functional analogue of the V-ATPase, the F-ATPase, isolated from barley chloroplasts or mitochondria, can be co-purified with 14-3-3 (Bunney \textit{et al.}, 2001). To check whether 14-3-3 interacts and co-migrates with the V-ATPase holoenzyme, the V1 head structure was dissociated from the whole enzyme and run on a size exclusion column. As shown in Fig. 5, in fractions collected between the void column volume and salt peak, both 14-3-3 and the E-subunit of the V-ATPase were detected on western blot. The molecular mass of the V1-domain of the V-ATPase is 440 kDa, and column calibration showed that this corresponds to fraction number 4.

The A-subunit of the V-ATPase specifically interacts with 14-3-3 in an overlay assay

Next, the question of which subunit of the V-ATPase multisubunit complex interacts with 14-3-3 proteins was addressed. To do this, a so-called far-western or overlay assay with 14-3-3 was performed. In the assay, the tonoplast fraction and one of the 14-3-3 isoforms labelled with biotin (14-3-3Cbio) were used to detect 14-3-3-interacting partners. Bands were visualized with avidin–HRP conjugate.
using enhanced chemiluminescence (ECL). To show the specificity of the interaction, a control experiment was done where the 14-3-3Cbio overlay buffer was supplemented with a 1000-fold excess of unlabelled 14-3-3C (histidine-tagged, but this tag is not detected with avidin–HRP). Figure 6B shows that 14-3-3Cbio specifically recognizes one band at 68 kDa of the tonoplast membrane fraction. This band is not seen in the control experiment with excess 14-3-3His (Fig. 6C). This band was excised from the gel, digested with trypsin, and the peptide mass fingerprint of the protein was determined by matrix-assisted laser desorption ionization tandem time of flight mass spectrometry (MALDI TOF/TOF-MS; model 4700 ABI). Figure 7 shows that this proved to be the A-subunit of the V-ATPase. No statistically significant difference between the 14-3-3 signal intensity in the control (red light) and the blue light-treated sample of the tonoplast fractions (Fig. 6) was seen, which may be due to the fact that the overlay with the 14-3-3C is not quantitative enough.

**The V1-ATPase interacts with 14-3-3 on a 14-3-3 affinity column**

To identify possible targets for 14-3-3 interaction after blue light treatment of etiolated barley shoots, 14-3-3 affinity chromatography of the 70% ammonium sulphate-precipitated protein fraction was performed. The protein fraction was applied to a 14-3-3 affinity column; non-specifically bound proteins interacting with the gel matrix were washed away with an imidazole gradient from 100 mM to 200 mM, and finally 14-3-3 protein together with interacting proteins were stripped from the column in a 200–600 mM imidazole gradient. The eluted proteins were identified by MALDI TOF/TOF MS (Fig. 8) and compiled into the list of proteins presented in Table 1. Besides a number of other interesting proteins, the core subunits of the V1-ATPase catalytic moiety were clearly identified, i.e. both the A- and the B-subunit. This corroborates the outcome of the gel filtration and overlay experiment and provides further evidence for the conclusion that the tonoplast V-ATPase in coleoptiles interacts with members of the 14-3-3 family.

**The A-subunit can be phosphorylated in vitro**

Although there are exceptions to the rule, the paradigm of 14-3-3 interaction states that a 14-3-3 target protein interacts with 14-3-3 via its conserved (C) domain, which is phosphorylated in vivo. To test if the A-subunit of the V-ATPase can be phosphorylated by 14-3-3, the A-subunit was incubated with 14-3-3Cbio and 14-3-3His. The A-subunit was incubated with 14-3-3Cbio and 14-3-3His, and the resulting proteins were separated by SDS-PAGE and detected by Western blotting (Fig. 9). The results showed that the A-subunit can be phosphorylated by 14-3-3, which provides further evidence for the interaction of the 14-3-3 family with the V-ATPase.
contains a conserved 14-3-3 binding motif, that will be phosphorylated on a serine or threonine residue for high-affinity interaction (Van Heusden, 2005). The A-subunit of the barley V-ATPase potentially can bind 14-3-3 because it has putative 14-3-3 binding motifs that are conserved in many homologous proteins (RGVS83VP and RLVS544QK; numbering according to alignment with the full-length A-subunit from *Hordeum*; accession no. Q40002). The latter peptide was shown to be phosphorylated in the maize homologue (Liu et al., 2004). To address the question of...
whether the barley V-ATPase can be phosphorylated, in vitro labelling with $[^{32}\text{P}]\text{ATP}$ was carried out. Integral protein kinase activity, both Ca$^{2+}$ and calmodulin independent, is expected to be present in purified tonoplast membranes, as shown by Chen et al. (2002). The tonoplast fraction was mixed with Mg-$[^{32}\text{P}]\text{ATP}$ in the presence of phosphatase inhibitors and 10 $\mu$M free Ca$^{2+}$ (Liu et al., 2004). Figure 9 shows several phosphorylated bands, with the strongest labelling shown by a 69 kDa and a 55 kDa band. The peptides of these bands were identified with MALDI TOF/TOF MS as the A- and the B-subunit of the V-ATPase, respectively (data not shown). Phosphorylation occurs very rapidly: already after 10 min the bands are visible, and further incubation with $[^{32}\text{P}]\text{ATP}$ did not increase the intensity. At the same time, no significant difference between control and blue light-treated samples was seen. However, it is possible that in vitro assay light-induced phosphorylation of the subunit is rapidly saturated.

A competition assay was performed to reveal possible differences in the kinase activity after blue light treatment. The same amounts of tonoplast vesicles were incubated with a fixed amount of $[^{32}\text{P}]\text{ATP}$ and increasing amounts of unlabelled ATP. If the blue light affects the affinity of the kinase for ATP as compared with the control treatment, differences in the competition curve are to be expected. Phosphorylation of the 69 kDa band was quantified with a phosphoimager and plotted against the amount of unlabelled ATP (Fig. 10). Blue light treatment shifted the curve about two orders of magnitude, which means that 100-fold more unlabelled ATP was necessary to reduce labelling by 50% as compared with the control (red light). Therefore, blue light treatment can stimulate phosphorylation of the A-subunit affecting the activity of the kinases.

### Table 1. List of proteins eluted from the 14-3-3 affinity column and identified with the Swiss-Prot green plant database

The search of the proteins was performed with 100 ppm precursor peptides mass tolerance and 0.2 Da MS/MS tolerance.

<table>
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<th>Protein name</th>
<th>Accession no.</th>
<th>Protein mol. wt, Da</th>
<th>Protein pl</th>
<th>Peptide count</th>
<th>Total ion score</th>
<th>Total ion score CI %</th>
<th>14-3-3 binding motif</th>
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<td>5.9</td>
<td>2</td>
<td>37</td>
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<td>Putative disease resistance protein</td>
<td>DRL38_ARATH</td>
<td>101 219</td>
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<td>2</td>
<td>27</td>
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<td>Exopolypolygalacturonase clone GBGE184 precursor</td>
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Fig. 9. In vitro labelling of tonoplast proteins with $[^{32}\text{P}]\text{ATP}$. Tonoplast vesicles (20 $\mu$g per lane) from the control (red light, RL) and the blue light (BL)-treated coleoptiles were mixed with 0.23 $\mu$M $[^{32}\text{P}]\text{ATP}$, incubated for 10, 30, and 60 min, boiled, and run on an SDS–polyacrylamide gel. The gel was exposed on a screen of a phosphoimager for 24 h. The band labelled with an asterisk was identified by mass fingerprinting as the A-subunit of the V-ATPase.

### Discussion

**Tonoplast-associated 14-3-3 proteins**

Although 14-3-3 proteins have previously been shown to down-regulate the activity of the slow vacuolar channels located on the tonoplast (Van den Wijngaard et al., 2001), they have not been reported to be co-isolated with the tonoplast before. In the present work, the tonoplast was...
isolated in the presence of phosphatase inhibitors which increases the stability of the 14-3-3–target complexes because phosphorylation increases the affinity. Quantification of western blotting data shows that 14-3-3 is not a minor protein in the tonoplast fraction (Fig. 3). An estimation shows that 0.2% of tonoplast-associated 14-3-3s (60 kDa as a dimer) is enough to regulate up to 20% of the V-ATPase (600 kDa the holoenzyme) in the case of formation of a complex between 14-3-3 and the V-ATPase. After blue light treatment, the amount of 14-3-3 on the tonoplast vesicles increased almost 2-fold. It should be noted that not all 14-3-3 isoforms react with their targets in a similar fashion. Only one isoform, 14-3-3A, responded to blue light treatment with a significant increase in the amount of tonoplast bound (>3.5-fold). A similar increase in 14-3-3 protein binding to the plasma membrane after a blue light impulse has been shown for a plasma membrane fraction isolated from guard cells (Kinoshita and Shimazaki, 1999). Interestingly, in the case of the effect of blue light on guard cells of *Vicia faba*, isoform-specific binding of vF14-3-3a (81% homology with 14-3-3A on the protein level) to the plasma membrane has also been observed (Emi et al., 2001).

**14-3-3 interaction with the V-ATPase**

The V-ATPase as a key enzyme has to be tightly regulated in several different ways involving different mechanisms of regulation. In its multimeric structure, the V-ATPase contains regulatory subunits that influence enzyme activity. However, there are indications that regulation can be carried out by proteins that are not conventionally included in the holoenzyme. In mammals, two proteins were discovered that activate the V-ATPase: a 35 kDa cytosolic activator from bovine kidney (Zhang et al., 1992) and a 6 kDa membrane-bound activator from bovine brain (Xie et al., 1993). Thus, the V-ATPase can be regulated not only autonomously, but ‘external’ regulatory subunits might be involved.

In our view, 14-3-3 proteins are good candidates to have such an activator function after blue light treatment of barley etiolated coleoptiles. 14-3-3s have already been reported to be important regulators of both the P-ATPase (Korthout and de Boer, 1994; Marra et al., 1994; Oecking et al., 1994) and the F-ATPases (Bunney et al., 2001). Moreover, 14-3-3 proteins bind very rapidly to the P-ATPase in response to a blue light pulse (Kinoshita and Shimazaki, 1999). Evidence has been provided for direct interaction of 14-3-3 proteins with the V1-holoenzyme complex and specific interaction with one of the subunits, the A-subunit of the V-ATPase, in the gel filtration assay (Fig. 5) and the 14-3-3 overlay assay (Fig. 6). Affinity chromatography of soluble coleoptile proteins shows that both the V1-ATPase core subunits A and B can be retrieved with 14-3-3 after blue light treatment (Table 1). Phosphorylation of 14-3-3 target proteins, needed for interaction of 14-3-3s with their partners, occurs in the case of the V-ATPase on the catalytic A-subunit of the V-ATPase (Liu et al., 2004; Fig. 9). Blue light also increases the level of kinase activity, which results in higher concentrations of ATP to compete with the 68 kDa band phosphorylation (Fig. 9B).

**Co-ordinated regulation of the P- and V-ATPases under blue light**

What might be happening at very early stages of blue light treatment at the cellular level in etiolated coleoptiles? Hager (1996) reported that in maize coleoptiles, blue light activates a photoreceptor kinase localized in the plasma membrane, which then phosphorylates the P-ATPase. This receptor kinase is probably encoded by *phot1*, since it was recently shown that *Phot1* mediates the rapid inhibition of *Arabidopsis* stem growth by blue light (Folta and Spalding, 2001). Upon blue light activation, *Phot1* itself interacts with 14-3-3 proteins (Kinoshita et al., 2003), and subsequently rapid phosphorylation of the C-terminal autoinhibitory domain of the P-ATPase results in binding of 14-3-3 and activation. Activation of the H*-ATPase will hyperpolarize the membrane potential, which can result in the activation of hyperpolarization-activated K*+* channels (Fuchs et al., 2003), activation of chloride channels (Noh and Spalding, 1998), and activation of voltage-dependent and Ca**2**+-permeable channels (Stoelzl et al., 2003). Therefore, blue light strongly affects the machinery responsible for ion homeostasis and in this light it is not surprising that the vacuolar H*-ATPase is
also involved in blue light regulation. For example, blue light-induced Ca"^{2+} influx in the cytoplasm (Stoelzle et al., 2003) can activate a kinase located on the tonoplast (Chen et al., 2002), phosphorylate the A-subunit of the V-ATPase, followed by 14-3-3 binding and activation of the whole holoenzyme. The increase in the electrochemical gradient across the tonoplast can then drive secondary active transport of other ions and solutes across the tonoplast, necessary for the proper photomorphogenic response.

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References


