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First patch, then catch: measuring the activity and the mRNA transcripts of a proton pump in individual *Lilium* pollen protoplasts[1]

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Abstract Combining the patch-clamp method with single-cell reverse transcription polymerase chain reaction (scRT-PCR) a fusicoccin-induced current reflecting the activity of the plasma membrane H^+ ATPase of lily pollen protoplasts was measured and subsequently, the ATPase-encoding mRNAs were collected and amplified. Southern blot signals were observed in all 'patch–catch' experiments and could be detected even in 2560-fold dilutions of the pollen contents. H^+ ATPase mRNAs were detectable only in the vegetative but not in the generative cell of pollen as confirmed by immunolocalisation. In 15% of the scRT-PCR experiments, a random non-reproducibility of the PCR was observed, probably caused by varying amounts of ATPase mRNAs in the protoplasts.

Key words: Patch–catch; Patch-clamp; Plasma membrane H^+ ATPase; Pollen; Single-cell reverse transcription polymerase chain reaction

1. Introduction

All processes and components involved in tip growth of pollen tubes including cell wall synthesis, vesicle transport, exocytosis, cytoskeleton, ion gradients, ion transporters, and turgor pressure must be well-regulated in space and time to produce a regularly shaped, growing pollen tube. All these components interact with each other forming a dynamic and sensitive network [1–5]. Additionally, individual molecules essential for a specific cellular process or for a central function in the regulatory network have been identified and their role in the polar growth process has been studied, e.g. Rho- and Rob-related GTPases [6,7], Ca^{2+}-dependent protein kinases [8,9], protein phosphatases [10], and the H^+-translocating ATPase in the plasma membrane [11].

So far, the majority of investigations used populations of pollen grains, but studies on single pollen grains or tubes, e.g. by imaging techniques, revealed a highly individual activity, probably due to their haploid nature. This individuality is usually reflected by high standard deviations (30–50% of the mean) when cellular parameters were quantified in pollen populations, e.g. growth rates, germination frequencies, enzyme activities. Therefore, to characterise the role of a given molecule, e.g. the plasma membrane (PM) H^+ ATPase, it seems necessary to monitor the expression, activity, and localisation in single, individual cells.

In plants, PCR techniques on the single-cell level have been applied to a few cell types, e.g. maize zygotes [12], pollen grains [13,14], and epidermal as well as guard cells of tomato [15]. In single-cell reverse transcription polymerase chain reaction (scRT-PCR) studies, mRNAs coding for highly abundant proteins in the respective cells, e.g. actin, RubisCO or GAPDH, were transcribed to cDNAs and finally amplified. Additionally, cytoplasm aliquots of single leaf cells of potato or cucumber were collected with a micropipette and the mRNAs of a peroxidase, the RubisCO, and a starch phosphorylase were detected [16].

Compared to these proteins/mRNAs a much lower abundance is expected for the PM H^+ ATPase and its mRNA. Nevertheless, it is known that the activity and expression of ion transporters indeed can be measured in individual neuronal cells [17–19] by the combination of scRT-PCR and patch-clamping. Therefore, we optimised scRT-PCR for the expected low abundance of H^+ ATPase mRNAs, and finally combined it with the patch-clamp technique to measure the activity of the PM H^+ ATPase in the PM of pollen grains of *Lilium longiflorum*.

2. Materials and methods

2.1. Plant material and isolation of protoplasts

Closed anthers from flowers of *L. longiflorum* Thunb. containing the mature pollen grains were collected, surface-sterilised and dehydrated. Anthers were then frozen in liquid nitrogen and stored at −70°C.

Protoplasts were released from pollen grains by enzymatic digestion of the cell wall [20,21]. Single protoplasts were used for patch-clamp experiments or pipetted into PCR tubes containing 1 μl protoplast medium with 20 U RNasin (MBI Fermentas, St. Leon-Rot, Germany), frozen in liquid nitrogen, and subjected to scRT-PCR. Generative cells were released from pollen protoplasts by osmotic shock after adding one volume of distilled water to the protoplast suspension. Individual generative cells were collected.

2.2. ScRT-PCR and hybridisation

Standard protocols for DNA/RNA techniques were used [22]. First-strand cDNA synthesis from a single lily pollen protoplast was performed using Superscript II RNase H^- RTase (Life-Technologies) in a final volume of 10 μl. 9 μl of the RT mix (0.25 mM dNTPs each, 2.5

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[1] This article is dedicated to Prof. F.-W. Bentrup, University of Salzburg, on the occasion of his 66th birthday.

Abbreviations: BTP, Bis-Tris propane; DIG, digoxigenin; HRP, horseradish peroxidase; MES, 2-[morpholino]ethanesulfonic acid; PM, plasma membrane; scRT-PCR, single-cell reverse transcription polymerase chain reaction; PBS, phosphate-buffered saline

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μM 3′-primer ATPase_r2, 20 U RNasin, 1×Superscript first-strand buffer, 10 mM DTT, 40 U RTase) was added to the frozen protoplast, mixed, centrifuged and overlayed with mineral oil. Using a specific primer (ATPase_r2) for the generation of cDNA, the following PCR was more reproducible. After 10 min of primer annealing at 22°C, reverse transcription was carried out in a thermo-cycler (Robocycler, Stratagene) for 75 min at 50°C.

A primer pair flanking an intron was designed (I = inositol): 5′-primer ATPase_f2: 5′-TG(CT)AGTGACAA(AG)ACIGGIACIC-TIAC-3′ and 3′-primer ATPase_r2: 5′-TCATT(AG)AC(AT)C-C(AG)TCICC(AG)GT-3′. In a final volume of 50 μl (0.2 mM dNTPs each, 2.5 mM $MgCl_2$, 1×AmpliTaq buffer, 25 pmol ATPase primer each, 0.5 U AmpliTaq DNA polymerase (Perkin-Elmer), 10 μl RT reaction) the ATPase cDNA was amplified using the following protocol: 1×5 min at 94°C, 35–37 cycles (40 s at 94°C, 40 s at 48°C and 1 min at 72°C), and a final extension step of 7 min at 72°C.

Positive controls were performed for every experiment using single pollen protoplasts or 100 ng genomic DNA for PCR and 100 ng pollen mRNA of *L. longiflorum* for RT-PCR. All pipetting steps were carried out in a clean bench to avoid contaminations with foreign material. Solutions were checked for possible contaminations in parallel to the scRT-PCR experiments.

A digoxigenin (DIG)-labelled probe, specific for the PM H^+ ATPase of lily pollen was generated from the LILHA1 cDNA clone (acc. no. AY029190) using 100 ng plasmid DNA and the described PCR conditions. 15 μl from each scRT-PCR experiment was electrophoresed in a 1% agarose gel and the DNA was transferred onto a membrane. The membrane was prehybridised using standard protocols [22]. Hybridisation with the DIG-labelled, PM H^+ ATPase-specific probe (2 ng ml^{-1}) was done overnight at 65°C in hybridisation solution. The membrane was washed and the DIG-labelled probe was detected by horseradish peroxidase (HRP)-conjugated anti-DIG-Fab fragments (Roche Diagnostics).

2.3. Patch-clamp

Conventional patch-clamp technique was applied in the whole-cell configuration using a patch-clamp amplifier (Axon Instruments, Union City, USA). The glass capillaries were cleaned by incubation in 70% ethanol for 4 h and dried at 80°C. Micropipettes were pulled to an outer diameter of 1.5–2 μm. The pipette medium contained (in mM): 450 mannitol, 10 KCl, 90 K-glutamate, 1 EGTA, 2 Mg-ATP, 0.54 $CaCl_2$ (resulting in 240 nM free Ca^{2+}), 25 2-[morpholino]ethanesulfonic acid (MES) adjusted to pH 7.2 with Bis-Tris propane (BTP). The standard bath medium contained (in mM): 450 mannitol, 10 KCl, 90 K-glutamate, 5 $CaCl_2$, 25 MES/BTP, pH 7.2, ±1 μM fusicoccin (Sigma). A voltage pulse protocol ranging from −200 to 100 mV in 20 mV steps was applied to measure the IV characteristics in the absence and presence of 1 μM fusicoccin. Thereafter, the protoplast was sucked into the patch pipette, transferred into a PCR tube containing 1 μl protoplast medium with RNasin (20 U), and finally frozen in liquid nitrogen.

2.4. Immunolocalisation

Protoplasts were fixed in the respective medium containing 6% (w/v) formaldehyde and 0.1% (w/v) glutaraldehyde for 30 min at 22°C and washed with phosphate-buffered saline (PBS). After blocking with PBS plus 5% (w/v) casein, the cells were incubated with monoclonal anti-PM H^+ ATPase antibodies (AB no. 721, kindly provided by Dr. Serrano) for 45 min, washed in PBS, and incubated with secondary antibody (AlexaFluor 546-conjugated anti-rabbit, Molecular Probes, Eugene, OR, USA). The cells were observed using confocal microscopy (LSM510, Zeiss, Oberkochen, Germany).

3. Results and discussion

A full-length cDNA clone of a PM H^+ ATPase expressed in pollen of *L. longiflorum* was isolated and sequenced (LILHA1, acc. no. AY029190). Alignment of LILHA1 with two well-known PM H^+ ATPases from *Arabidopsis thaliana* (AHA9, acc. no. X73676) and *Nicotiana plumbaginifolia* (PMA4, acc. no. X66737) also expressed in pollen grains [23,24], revealed a high homology between the three ATPases. In the following experiments a DIG-labelled probe generated from the LILHA1 clone was used to identify the scRT-PCR products in Southern blots.

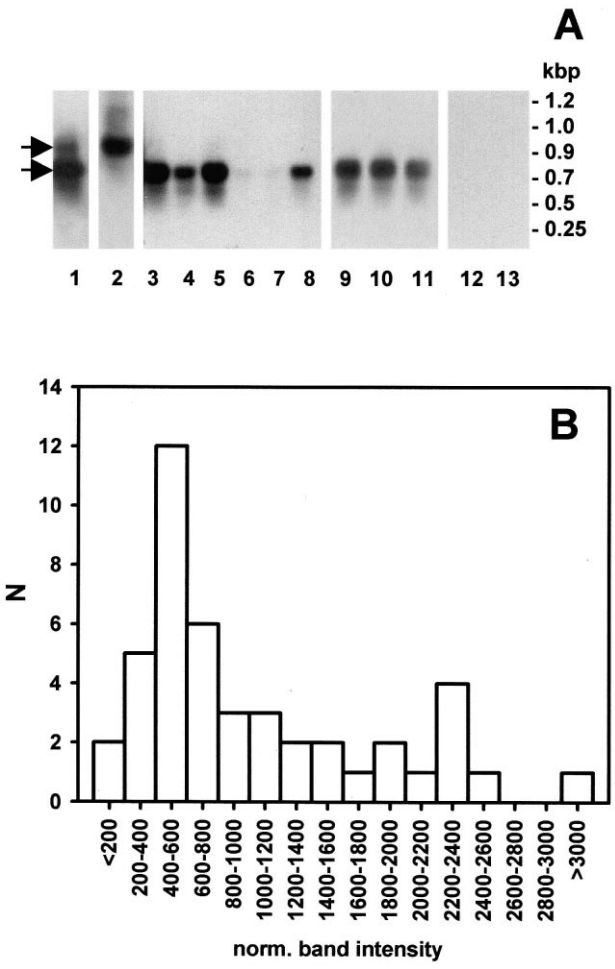

Fig. 1. Typical scRT-PCR experiments. A: scRT-PCR of nine individual protoplasts (lanes 3–11) and the respective controls: RT-PCR using 100 ng lily pollen mRNA (lane 1), genomic PCR using a single pollen protoplast (lane 2), water (lane 12) and protoplast medium (lane 13). The PCR products were hybridised with the DIG-labelled lily pollen H^+ ATPase probe that was detected with a HRP-conjugated anti-DIG antibody. Arrowheads indicate bands at 950 and 800 bp, respectively. B: Histogram of the band intensities of 45 successful scRT-PCR experiments. In 13 experiments no signals were detected. Normalised band intensities were calculated as (intensity×area)/background intensity, and are shown in arbitrary units.

In a typical scRT-PCR experiment signals at ca. 800 bp were detected corresponding well to the expected length of the PM H^+ ATPase fragment (795 bp). Positive controls (RT-PCRs from pollen total RNA and genomic PCRs) and negative controls (RT-PCRs with all used solutions) were performed in parallel to the scRT-PCR. In genomic PCR experiments the lily H^+ ATPase-specific probe recognised a longer product (950 bp, Fig. 1A, lane 2) because the two primers flank an intron. The signals varied in intensity probably due to different amounts of ATPase mRNA in the pollen protoplasts. Plotting the normalised band intensities of 45 successful scRT-PCR experiments in a histogram resulted in a Poisson-like distribution with the highest frequency at 400–600 arbitrary units (approximately 500 times higher than the background, Fig. 1B). But in some pollen no signals were detected

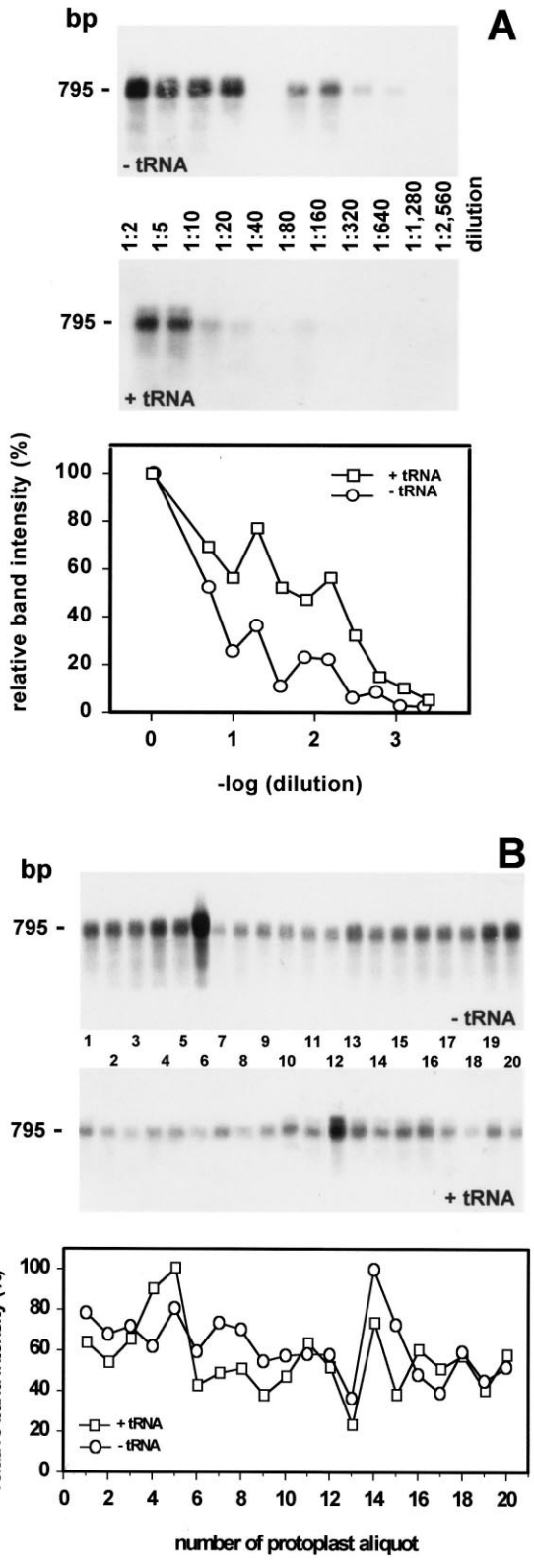

Fig. 2. RT-PCR with diluted cell contents of single protoplasts. A: scRT-PCR of a dilution series of two single protoplasts. Products were detected by Southern blotting. The cell content was diluted as indicated ranging from 1:2 to 1:2560. A protoplast was diluted in the absence or in the presence of 1 μg tRNA. The results of five different dilution series ± tRNA were plotted as the mean of the relative band intensities versus the −log(dilution factor). B: Typical Southern blots of an scRT-PCR with 20 aliquots of two 20-fold diluted pollen protoplasts. The content of one protoplast was diluted in the absence or in the presence of 1 μg tRNA. The mean of the relative band intensities of four experiments was plotted against the aliquot number showing a mean relative band intensity of 62% (−tRNA) and 55% (+tRNA), respectively.

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($n = 13$), probably due to an unsuccessful scRT-PCR; there are several possible reasons, e.g. the protoplast did not break, mRNA adhered to organelles and was not accessible to the RTase, mRNA was digested, loss of mRNA during pipetting steps. Therefore, the scRT-PCR conditions were optimised by addition of RNasin, by the use of sterilised and DEPC-treated media, and by harvesting the closed anthers just before dehydration to avoid contaminations. In comparison to other studies the use of oligo-dT magnetic beads [15] or oligo-dT primer [16] for first-strand synthesis did not improve the scRT-PCR results. In general, using an ATPase-specific primer for the reverse transcription increased the reproducibility of the scRT-PCR experiments. This optimised procedure finally resulted in 84.3% of successful experiments (total $n = 89$).

3.1. Limitations of the scRT-PCR in pollen protoplasts

To estimate the minimum of detectable ATPase mRNA, a dilution series of the cytosol from a single pollen grain protoplast was prepared ranging from 1:2 to 1:2560 (Fig. 2A). The dilution was done in the presence or absence of 1 μg tRNA. The tRNA was added as carrier material for the small amounts of mRNAs from single protoplasts. It was expected that in the presence of tRNA less unpredictable errors like unspecific binding of the pollen mRNA to reaction tubes, pipette tips, may occur resulting in a regular decrease of the Southern blot signal of a dilution series. The addition of tRNA indeed improved the signal quality: less unspecific binding of the probe was observed in the range of shorter PCR products. But the addition of tRNA did not prevent the sometimes observed failure of an scRT-PCR experiment: although clear signals could be observed at almost every dilution step, no signals were observed at the 1:40 dilution step (Fig. 2A, lane 5). In most dilution experiments, no clear detection limit could be determined: a signal was still observed in a 2560-fold dilution of the cell contents. Occasionally, a signal was still detectable in a 10 000-fold dilution (data not shown). Therefore, the copy number of PM H^+ ATPase mRNAs in pollen protoplasts might be estimated as ranging from 2500 to 10 000 molecules.

The randomly occurring non-reproducibility of the complex PCR (Monte-Carlo effect) was described in scRT-PCR experiments on tomato mesophyll and guard cells [15]. In these experiments the Monte-Carlo effect was more likely to occur with low abundant mRNAs/cDNAs. Although this effect was first described when preparing a cDNA library from a single tomato cell, it was also observed in the presented scRT-PCR experiments possibly due to the low copy number of the investigated mRNAs. To further characterise the Monte-Carlo effect in pollen protoplasts, the content of one single protoplast was diluted 20-fold in the presence or absence of tRNA, and the resulting 20 aliquots were subjected to RT-PCR (Fig. 2B). Again, the Southern blot signals were much clearer in the presence of tRNA but a random variation in the signal intensity was still observed. In this specific experiment a Southern blot signal was detected in every diluted aliquot. In similar dilution experiments, signals were observed in 84.4% (total

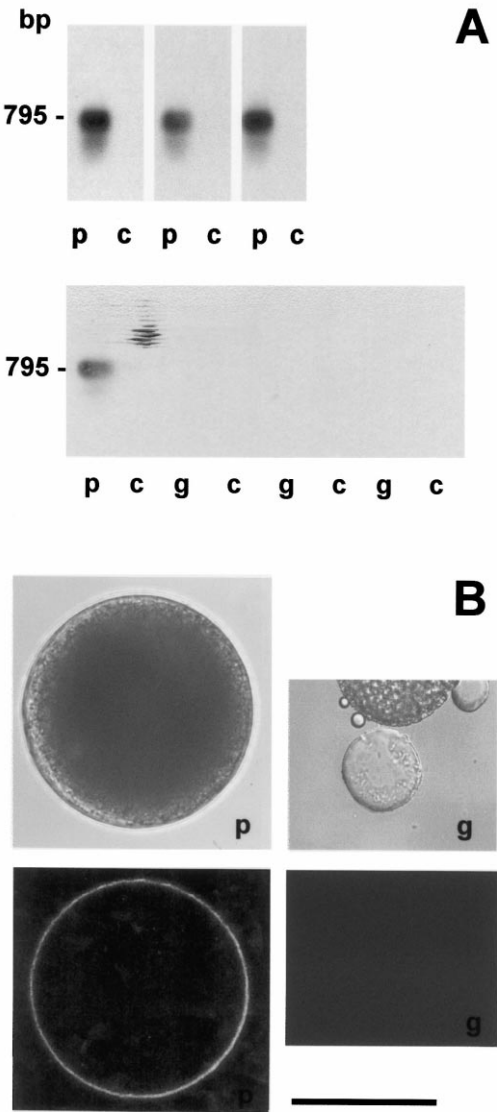

Fig. 3. Localisation of the ATPase mRNA. A: scRT-PCR experiments were performed with lily pollen protoplasts (p), isolated generative cells (g), and the respective control experiments (c = 1 μl medium). Southern blot signals were only detected with entire protoplasts. B: Bright field and corresponding fluorescence images of a lily pollen protoplast and a generative cell localising the H^+ ATPase in the PM of the vegetative cell but not in generative cells. The fluorescence images were taken with the same settings of the confocal laser scanning microscope (pin hole: 1.3 arbitrary units = 0.9 μm optical slice thickness, detector gain: 700 V, 52% laser intensity). Bar = 50 μm.

$n = 32$); the same success rate observed in the series of experiments with undiluted single protoplasts. Additionally, an all-or-nothing principle was observed meaning that all 20 dilution aliquots of a single protoplast showed a positive response or all RT-PCRs of the 20 aliquots failed. Therefore, one may conclude that the Monte-Carlo effect in pollen protoplasts results mainly from the variability of the amounts of a specific mRNA in individual cells rather than from errors of the scRT-PCR method itself, e.g. loss of mRNA during the several pipetting steps.

3.2. Localisation of the H^+ ATPase and its mRNA

Lily pollen grains consist of two cells, a generative cell embedded in the cytoplasm of the vegetative cell that grows the pollen tube. Both cells may express a PM H^+ ATPase but no ATPase mRNA was detected in isolated generative cells (Fig. 3A) whereas in all pollen protoplasts (that is, vegetative and generative cells) a Southern blot signal was observed. Therefore, no PM H^+ ATPase is expressed in generative cells, although they possess translatable mRNAs and protein synthesis has been detected [25]. Immunolocalisation was used to verify the absence of the H^+ ATPase protein in the PM of the generative cell (Fig. 3B). In pollen grain protoplasts the H^+ ATPase was detectable in the PM of the vegetative cell but neither in the generative cell inside the protoplast nor in isolated generative cells. Therefore, any detectable, PM H^+ ATPase-specific signal from an scRT-PCR experiment using pollen grain protoplasts is caused by the mRNAs of the vegetative cell. The absence of the H^+ ATPase from the PM of the generative cell may be explained by the unique arrangement of vegetative and generative cells in pollen grains because a H^+ ATPase localised in the PM of the generative cell would acidify the cytoplasm of the vegetative cell.

3.3. Patch–catch experiments

The two single-cell techniques were now combined by measuring the activity of the PM H^+ ATPase in a patch-clamp experiment before subjecting the same protoplast to the optimised scRT-PCR (Fig. 4). A fusicoccin (FC)-induced current was measured in a pollen grain protoplast (Fig. 4A). The difference of the IV curves before and after FC addition gives the voltage dependence of the FC-induced current (Fig. 4B) showing typical IV characteristics of ion pumps [26,27]. Then, the protoplast was sucked into the tip of the patch pipette and ATPase mRNAs were amplified by scRT-PCR (Fig. 4C, lane 7). Combined ('patch–catch') experiments were performed for other protoplasts and variability in the Southern blot signals probably corresponding to the various amounts of ATPase mRNAs can be noticed (Fig. 4C). A synopsis of all 'patch–catch' experiments performed so far reveals no correlation between the FC-induced current and the mRNA transcripts (Fig. 4D) indicating that the well-documented, post-translational regulation of the PM H^+ ATPase [28,29] plays an important role in lily pollen, too [11]. Note that all transcription levels in this study were given in arbitrary units because no absolute quantification seemed plausible in single, haploid cells by using RT-PCR quantification protocols. Therefore, any correlation between activity and transcription level may

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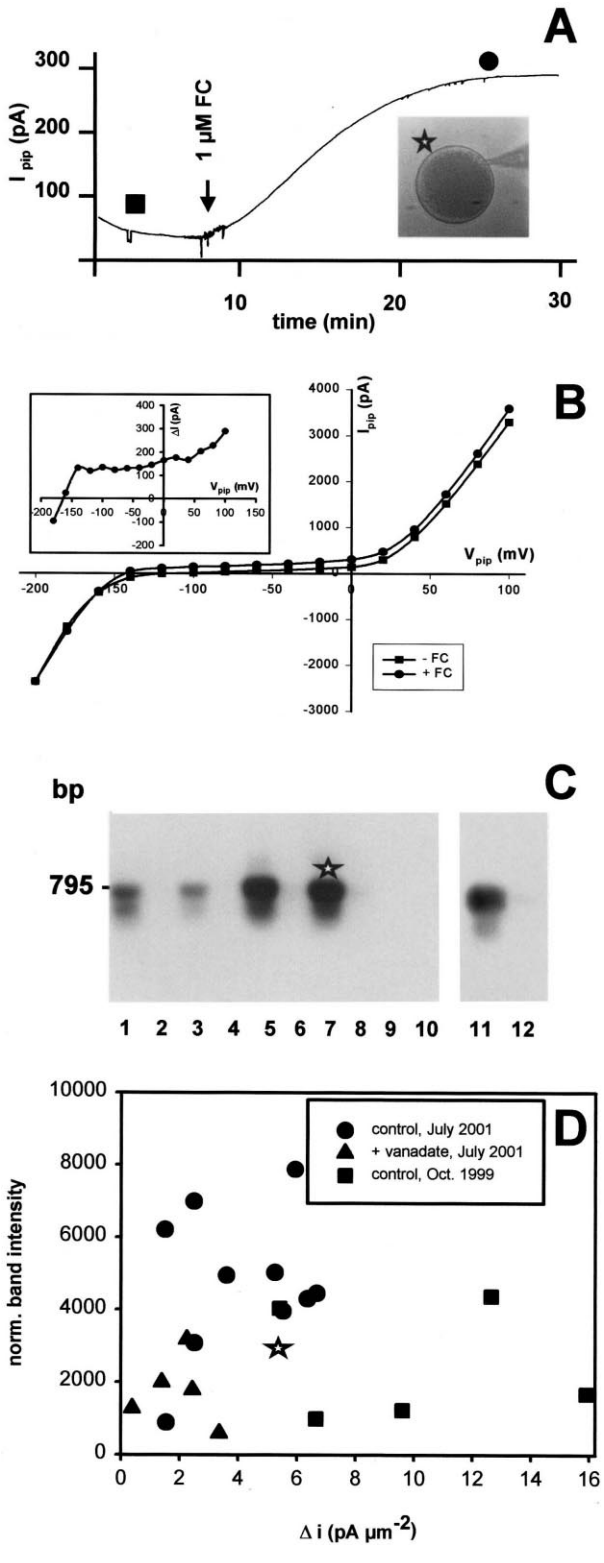
Fig. 4. 'Patch–catch' experiment. A: Recording of the FC-induced current from a single protoplast (34 μm radius) and B: IV characteristics of the same protoplast before (square) and after (circle) FC addition. Insert: The difference of both IV curves giving the voltage dependence of the FC-induced current. C: After the patch experiments the protoplast was sucked into the patch pipette and its contents subjected to scRT-PCR. The Southern blot signal marked by a star represents the mRNA from the protoplast shown in A. Lanes 2, 4, 6, 8–10, and 12 are controls, lanes 1, 3, 5, 7, and 11 are obtained from single protoplasts in which an FC-induced current was measured before. D: The current density of FC-induced currents (Δi) of all 'patch–catch' experiments was plotted against the Southern blot signals for each protoplast (normalised band intensity). 20 experiments performed in July 2001 (●) and October 1999 (■) are shown. Additionally, the PM H^+ ATPase was inhibited by 250 μM vanadate (▲). The star marks the parameters (square) obtained from the protoplast shown in A.



only be observed if the specific mRNA can be quantified possibly by real-time scRT-PCR.

In conclusion, by combination of the patch-clamp with the scRT-PCR technique it was possible to measure the activity of the PM H^+ ATPase and detect its mRNA in a single plant cell. The optimised scRT-PCR protocol included (a) the use of a specific primer in the RT reaction, (b) an intron-flanking primer pair to identify any product amplified from DNA impurities during the PCR, and (c) addition of tRNA to the scRT-PCR to reduce the generation of unspecific products. With this protocol even mRNA from fractions of individual protoplasts could be detected. The presented results look promising for further investigations to detect other ion transporter mRNAs and to quantify specific mRNAs in single plant cells.

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