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Strong alkalinization of *Chara* cell surface in the area of cell wall incision as an early event in mechanoperception ☆

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ABSTRACT

Mechanical wounding of cell walls occurring in plants under the impact of pathogens or herbivores can be mimicked by cell wall incision with a glass micropipette. Measurements of pH at the surface of *Chara corallina* internodes following microperforation of cell wall revealed a rapid (10–30 s) localized alkalinization of the apoplast after a lag period of 10–20 s. The pH increase induced by incision could be as large as 3 pH units and relaxed slowly, with a halftime up to 20 min. The axial pH profile around the incision zone was bell-shaped and localized to a small area, extending over a distance of about 100 μm. The pH response was suppressed by lowering cell turgor upon the replacement of artificial pond water (APW) with APW containing 50 mM sorbitol. Stretching of the plasma membrane during its impression into the cell wall defect is likely to activate the Ca^{2+} channels, as evidenced from sensitivity of the incision-induced alkalinization to the external calcium concentration and to the addition of Ca^{2+}-channel blockers, such as La^{3+}, Gd^{3+}, and Zn^{2+}. The maximal pH values attained at the incision site (~10.0) were close to pH in light-dependent alkaline zones of *Chara* cells. The involvement of cytoskeleton in the origin of alkaline patch was documented by observations that the incision-induced pH transients were suppressed by the inhibitors of microtubules (oryzalin and taxol) and, to a lesser extent, by the actin inhibitor (cytochalasin B). The results indicate that the localized increase in apoplastic pH is an early event in mechanoperception and depends on light, cytoskeleton, and intracellular calcium.

1. Introduction

The cell wall (CW) is the first and highly important barrier protecting the cell from mechanical injury and pathogen invasion [1]. The range of plant responses to CW injury is wide, including formation of cell wall appositions [2], ion fluxes [3], cell depolarization, activation of NADPH oxidase (respiratory burst oxidase homologue), and production of reactive oxygen species (ROS) [4]. Apart from biochemical events induced by perception of CW degradation products, direct effects of mechanical stresses and microwounds are now in the focus of interest. Some pathogens employ the force of penetration pegs to perforate the cell wall of a host plant [5]. Natural wounding can be mimicked by impaling the cells with tiny needles or perforating them by flow of accelerated microparticles [6–8]. Stimulation of *Arabidopsis* root cells with a glass micropipette revealed that early stages of mechanoperception comprise the increase in cytosolic Ca^{2+}, ROS generation, cytosol acidification, and apoplast alkalinization [8]. The large rapid shifts in extracellular pH were particularly spectacular and were argued to stabilize the cell wall after local injury by inhibiting wall-loosening proteins, such as expansins, and by activating wall-rigidifying enzymes such as pectin methylesterases. Alkalinization-dependent localized CW hardening at the site of mechanical stimulation presumably enhances cell wall ability to prevent further cell injury.

It is not yet known whether the induction of proton fluxes across the plasma membrane (PM) induced by mechanical injury is specific to cells of dicots or if it is a universal mechanism of defense shared by other organisms that contain carbohydrate-rich cell walls. Land plants and charophycean green algae are believed to have common ancestors (e.g., [9]); the cell wall composition is similar between the two, suggesting an intriguing possibility that they share fundamental cellular mechanisms of mechanostimulus perception and regulation of CW extensibility. Characean internodal cells represent a suitable material for studying mechanoreception [10–13] and other fundamental processes such as pattern formation, excitability, and cytoplasmic streaming. Internodal cells of characean algae produce in the light the alternating regions with high and low pH (reviewed in [14,15]). Interestingly, elongation growth is confined to cell regions with low cell-wall pH [16], suggesting that alkalinization-mediated CW rigidification is an ancient mechanism conserved between higher plants and algae.

Abbreviations: APW, artificial pond water; CW, cell wall; PFD, photon flux density; pH_o, pH in the outer medium near the cell surface; PM, plasma membrane; PSI and PSII, photosystems I and II

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Apart from their role in modifying CW structure, transmembrane proton fluxes play a role in spatial patterning of photosynthesis at rest and after membrane excitation [15] and may directly or indirectly modulate the activity of a number of plasma membrane proteins. The proton fluxes are subject to sensitive regulation by light stimuli, including photoinduced signals transmitted via cyclosis, and by calcium fluxes through gated channels activated by electrical stimuli [17,18]. They might be also involved in cell responses to mechanical stimulation.

We therefore sought to investigate the role of apoplastic pH in early signaling of CW injury in giant internodal *Chara* cells. Large dimensions of characean internodes permit multiple repetitive probing of its cell wall with a microneedle without appreciable damage to the cell. The advantage of this material is that mechanical stimuli can be applied to the same cell under variable experimental conditions. *Chara* takes up mineral nutrients from the aquatic environment via the cell surface and utilizes membrane-permeant CO_2 in photosynthesis. In photosynthesizing cells, the signals generated in the apoplast are tightly linked with the events occurring in the cytosol and chloroplasts [19]. It is thus possible that photosynthetic activity participates in the perception of mechanostimulus.

Considering possible involvement of Ca^{2+} and H^+ fluxes in mechano-stimulated signaling [8], we describe here strong effects of CW microwounding on the surface pH in *Chara corallina* cells and examine possible bases of large rapid pH changes in the apoplast. We show that the microinjury of internodal *Chara* cells is associated with prolonged (up to 40 min) and substantial (up to 3 pH units) increase of the apoplastic pH localized to the stimulation site. It is proposed that the localized increase in apoplastic pH is of complex origin and depends on light, cytoskeleton, and calcium.

2. Materials and methods

2.1. Plant material

Characean alga *C. corallina* Klein ex Willd. was grown in an aquarium at room temperature at daylight illumination (photosynthetic photon fluence rate ~10 μmol m^{-2} s^{-1} during daytime). Individual internodal cells with the length from 3 to 6 cm and 0.8–1 mm in diameter were excised from the strand and kept at least one day in artificial pond water (APW) containing 0.1 mM KCl, 1.0 mM NaCl, and 0.1 mM $CaCl_2$. The pH was adjusted to 7.0 by adding $NaHCO_3$. The isolated cell was placed horizontally into a transparent plexiglas or glass chamber mounted on the stage of an inverted Axiovert 25-CFL microscope (Carl Zeiss, Jena, Germany). The cell in the experimental chamber accommodating 40 ml of solution was illuminated from the microscope upper light source through a blue glass cut-off filter SZS-22 ($\lambda < 580$ nm). The maximal light intensity was 100 μmol m^{-2} s^{-1}; it was attenuated by glass neutral density filters. Experiments were performed under photon flux density (PFD) of 28 μmol m^{-2} s^{-1} unless indicated otherwise.

2.2. Mechanical stimulation and measurements of the cell surface pH

The pH-microelectrodes were fabricated from Pyrex glass capillary tubes (1.1 mm outer diameter) filled with molten antimony [20]. The Sb-filled micropipettes were pulled on a vertical puller using a two-step procedure similar to preparation of patch clamp pipettes. The tip diameter of glass-insulated pH microsensor was about 5 μm. The mechanical stimulus was applied with glass (Pyrex) micropipettes having a tip diameter of less than 1 μm. Three mechanical micromanipulators were placed around the microscope and served to adjust the positions of the pH-microelectrode, the stimulating micropipette, and a fire-polished capillary (1 mm outer diameter) serving as a support for the cell at the point of incision. All microinstruments were slightly inclined to the horizontal plane. The angle between the pH-sensor and the stimulating micropipette was about 45°; the fire-polished capillary tube was positioned on the opposite cell side at approximately equal angles to the pH microsensor and the micropipette. The pH microelectrode was placed at a distance of 5–10 μm from the cell surface. The stimulating micropipette was inserted to a depth of few micrometers from the cell surface and withdrawn in 1–2 s after insertion. In a series of experiments, the micropipette was filled with 1 M KCl solution and the electric potential difference between the pipette and the external Ag/AgCl reference electrode was measured during CW impalements to check the pipette tip position.

In an experiment run, the pH response to CW incision was monitored for 5–8 min, by which time the external pH started to decrease but did not attain the initial level. Before starting the next run, the cell position was shifted to the area where the outer pH (pH_o) remained undisturbed. The mechanical stimulus was applied at a distance of ≥0.2 mm from the previous point of incision. Thus, multiple replicate records of pH_o changes were made on the cell measuring several centimeters in length.

The potential difference between the pH microelectrode and the reference Ag/AgCl electrode was fed into a high-impedance amplifier (input impedance 10^{15} Ω) and displayed on a computer via ADC/DAC interface (National Instruments PCI-6024E, United States) using WinWCP program (Strathclyde Electrophysiology Software).

2.3. Simultaneous measurements of surface pH and chlorophyll fluorescence

These measurements were performed with young fully developed transparent internodes. The procedure for micropipette preparation and insertion was modified slightly compared to that described above. In order to detect chlorophyll fluorescence in the area of mechanical stimulation, the stimulating micropipette was bent under the angle of 45° in the shank region with a heated wire. The region in the center of microscopic cell view was selected, and the micropipette approached the cell surface from the upper side of the internode. Fluorescence was measured on cell areas with a diameter ~50 μm using a ×32/0.4 objective lens. Owing to the depth of field of few micrometers, fluorescence emitted from the upper chloroplast layer was detected. Chlorophyll fluorescence F_m' was determined with the saturation pulse method using Microscopy Pulse-Amplitude-Modulated (PAM) fluorometer (Walz, Effeltrich, Germany) mounted over the Axiovert microscope [21]. Both the surface pH and chlorophyll fluorescence were recorded simultaneously on a computer.

2.4. Inhibitor treatments

The inhibitor of photosynthetic electron transport 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) was dissolved in water and applied at final concentrations of 4 and 8 μM. Cytochalasin B was dissolved in dimethyl sulfoxide (DMSO) and applied at a final concentration of 15 μg/ml; the concentration of DMSO during the treatment was 0.5%. This DMSO concentration had no influence on cell photosynthetic activity, pH band formation, and cytoplasmic streaming (data not shown). Oryzalin was a gift from Prof. Ilse Foissner (Salzburg University, Austria). Stock solution (10 mM) was prepared in DMSO. Prior to experiments it was diluted in APW to 1, 5 and 10 μM. Lanthanum and gadolinium were prepared as water solutions of chloride salts; zinc, as a sulfate salt. All inhibitors were applied as solutions in APW.

2.5. Membrane excitation

The internodal cells were mounted in a two-sectioned chamber and stimulated electrically with external electrodes placed in different water pools [17]. The groove in the partition between two compartments was filled with insulating silicone oil (Baysilone, GE Bayer Silicones). The cells were stimulated with single pulses of

transcellular electric current (~10 μA, 200 ms). The generation of AP was detected from temporal cessation of the cytoplasmic streaming. In the beginning of the experiment, the micropipette was shortly inserted into CW of the resting cell, and the pH response to this insertion was traced. In the next run, 10 min later, the membrane excitation was triggered in advance to mechanical stimulation (20–30 s before CW incision) and the pH response to the brief insertion was recorded. Up to five cycles of alternate measurements at rest and during the post-excitation period were performed with each cell.

2.6. Velocities of cytoplasmic streaming

These were determined from microscopic observations by measuring in triplicates the period during which the moving organelles passed a fixed distance (950 μm).

Experiments on each cell were performed in four to six replicates under standard control conditions, during the treatments (application of chemical agents, changes in osmolarity of the medium, changes in photon flux density), and after return to the initial conditions. Each experiment was repeated at least with four cells. Curves with error bars show averaged traces and standard errors obtained from several recordings on different cells.

3. Results and interpretation

3.1. Basic observations

Fig. 1 shows pH changes near the surface of *C. corallina* internodal cell (pH_o) after a short (for 1–2 s) insertion into CW of a glass micropipette with a tip diameter of ≤1 μm. The initial shift of potential difference between the pH electrode and the reference electrode comprised two jumps of the same polarity occurring upon the insertion and withdrawal of the microneedle (arrows in Fig. 1A). Apart from the short pointed impression of CW, no further experimental manipulations were performed; the insertion had no effect on cytoplasmic streaming (data not shown). As shown in Fig. 1A, after the initial potential shift, there was a lag period of 10–20 s, which was followed by a fast sigmoid pH increase as large as 2–3 units. The slope of this pH increase varied in different cells but it could be as steep as 1 pH unit per 1.2–1.5 s. This pH shift was reversible (Fig. 1B). Unlike the generation of alkaline pH_o, its reversal proceeded slowly and took up to 2500 s (>40 min).

Illuminated characean internodes are known to produce alternating zones of high and low pH along their length. Under bright light the increases in surface pH upon mechanical stimulation of CW were large in the acidic areas and small or negligible in the alkaline zones. The upper limit of incision-induced pH shift never exceeded the high pH values in the alkaline zones. Therefore, at elevated irradiance the pH measurements were performed in the acidic zones. On the other hand, the pH banding was not required for the development of incision-induced alkalinization. Under dim light, used in many experiments, when the cells lost or did not form the pH bands, the localized pH increase upon the CW incision was fully evident. For example, when the alkaline zone decayed after darkening, a large pH increase was observed upon CW incision in the formerly alkaline area (data not shown).

3.2. Identification of the pH response

Upon the replacement of APW with the medium containing 10 mM Mops–KOH buffer (pH 7), the rapid potential shift in response to incision remained evident, though diminished slightly. By contrast, the large electrode signal was suppressed (Fig. 2A). Differential sensitivity of the instant and delayed potential shifts to the presence of buffer indicates that only the signal developing during and after the lag period represents the pH change. The instant signal observed upon perforating CW arises presumably as an extracellular recording of the receptor potential documented by others [12] and may partly result from unspecific leakage and leak sealing.

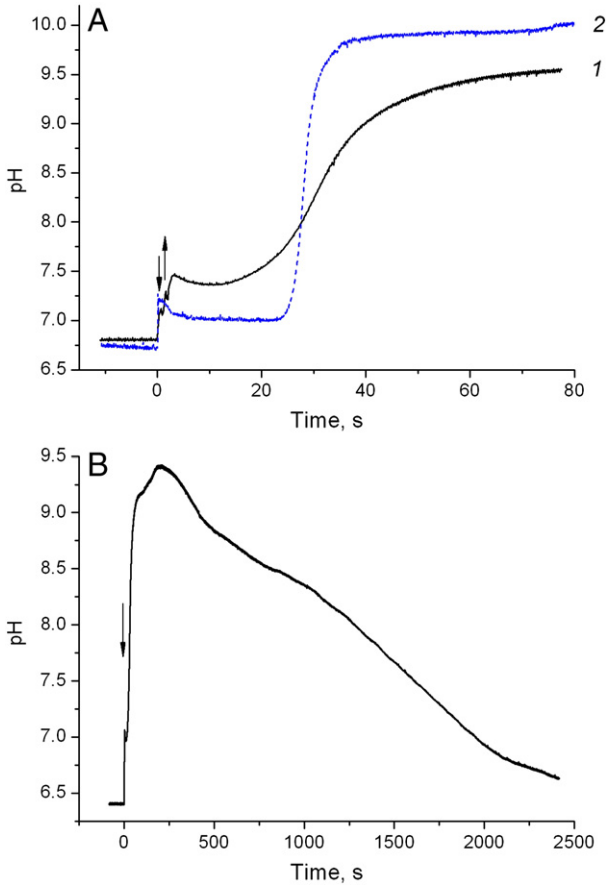

Fig. 1. Localized pH changes on the surface of illuminated *Chara corallina* internode following the microperforation of cell wall (CW) with a glass micropipette. Parts (A) and (B) display rapid and slow stages of the pH-electrode response on different time scales. Traces *1* and *2* represent the pH responses in different cells. Two rapid shifts of the potential difference between the pH microelectrode and reference electrode in trace *1* occurred on the moments of micropipette insertion into CW and its withdrawal from CW (downward and upward arrows, respectively). Zero time ($t = 0$) in this and other figures corresponds to the moment of CW incision.

In a set of experiments, the electric potential difference across the PM was measured during and after CW incision. To this end, the measuring microelectrode was first inserted into the cytoplasm, and then the cell wall was stimulated with another micropipette at a distance of about 20 μm from the first one. The insertion of stimulating micropipette into CW was followed by instant depolarization of 3–5 mV relaxing with a time constant ~6.5 s, which was reminiscent of the mechanically induced receptor potentials [22]. No significant changes were noted in the subsequent period of 200 s after CW incision.

Fig. 2B shows the pH changes induced by microperforation when the micropipette was left inserted for about 250 s before its eventual withdrawal. It is seen that pH changes caused by the micropipette insertion into CW were much smaller than those after the pipette withdrawal. Hence, the key role in alkalinization belongs to the tensile stress produced upon membrane impression into a microscopic cavity or a pore left in the CW after micropipette withdrawal, not to the compression produced by the micropipette during its impact on CW. The local weakening of CW disrupted the balance between turgor pressure and the elastic force, which led to local deformation of CW, the plasmalemma, and underlying cytoskeleton. The plasma membrane impressed under high hydrostatic internal pressure into the CW defect would

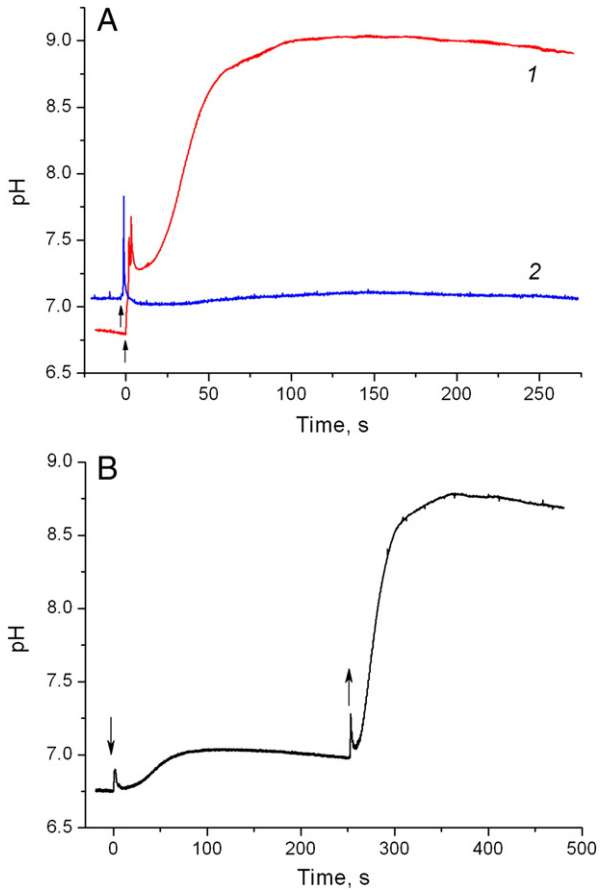

Fig. 2. Separation of the electrode signal components and differential influence of the micropipette insertion and withdrawal. (A) Responses of the pH probe to microperforation of CW in *C. corallina* internode bathed in artificial pond water (APW, curve *1*) and in APW containing 10 mM MOPS–KOH buffer, pH 7.0 (curve *2*). Arrows indicate short periods of micropipette insertion and withdrawal. Note that the presence of buffer selectively eliminated the major part of the electrode response. (B) Different amplitudes of localized pH changes evoked by CW microperforation with the micropipette left inserted (downward arrow) and following the micropipette withdrawal (upward arrow).

experience the increased tension. The tensile stress is expected to activate mechano-percepting systems of the PM. In further experiments only momentary incision was applied for mechanical stimulation.

The assumption that the micropipette produced either a cavity or a pore in the CW was tested. According to early studies, the electric potential of CW in characean cells can be measured with capillary microelectrodes [23]. Using electric potential measurements as a suitable indicator of micropipette position, we checked whether the impalement of CW alone suffices for the development of external alkalinization. To this end, the micropipette used for CW incision was filled with 1 M KCl, and the electric potential difference between the micropipette and an external Ag/AgCl reference electrode was recorded during the pipette insertion into CW; the surface pH was also monitored in each measurement. The experiments made on four cells, with 3–4 replicates per cell, have shown that the alkalinization response can be induced by shallow puncturing, without piercing the plasma membrane. The potential shifts recorded with microcapillary electrodes upon CW incisions were -32 ± 8 mV (mean $\pm$ SD, $n = 16$); such incisions sufficed to cause the usual alkalinization of the cell surface. Hence, the indentation of CW without penetration through the PM is sufficient for the alkalinization response.

In continuation of the same series of experiments, we inserted the capillary microelectrode into the cytoplasm and found out that these impalements also evoked the localized increase in the surface pH. The membrane potentials observed in these trials ($E_m = -137 \pm 7$ mV, mean $\pm$ SD, $n = 11$) were close to the equilibrium potential for H^+ distribution between the cytoplasm and external alkaline zones and fall within the range of previously reported E_m values across the plasma membrane in illuminated *C. corallina* cells [24,25]. Altogether, these results indicate that the localized injury of CW is the only essential factor for external alkalinization and that the pH response can be induced by both indentation and perforation of CW.

3.3. The pH response is confined to a narrow area

The mechanically induced increase in the surface pH could be detected with a pH-indicating dye phenol red. When the thickness of dye solution in the area of measurements was reduced to about the internode diameter, we observed localized absorbance changes following the CW incision. A red colored area developed around the place of incision, which indicated the localized alkalinization of the medium. The high pH region was confined to an area of about 100 μm in diameter (data not shown). Unlike phenol red, whose absorbance change is saturated at pH ≥ 8.2, pH microelectrodes exhibit a linear electrode response as a function of pH.

Fig. 3 illustrates the localized distribution of high pH around the point of CW incision. These measurements were made at the stage when the pH was settled at a quasi-stationary level after CW incision. Only stable pH profiles obtained during repeated displacements of the pH-microelectrode were considered. The axial profiles of the pH shifts for individual cells were normalized to summarize data obtained with four cells. It is seen that the shifts in position of pH microelectrode along the cell by 50 μm induced a considerable drop in surface pH. The pH drop upon the electrode displacement from the point of incision was not due to mixing in the boundary layers, because the return of pH microprobe to the initial position was accompanied by the increase in pH. The distribution of pH around the point of incision was asymmetric, and this asymmetry was related to the direction of cytoplasmic streaming. When the electrode was shifted by 50 μm upstream of the incision point (left branch of the distribution profile in Fig. 3), the pH drop was larger than after the equidistant downstream shift of the electrode position (right part of the profile). Apparently, some regulating factor promoting elevation of surface pH was carried from the incision zone to a short distance by

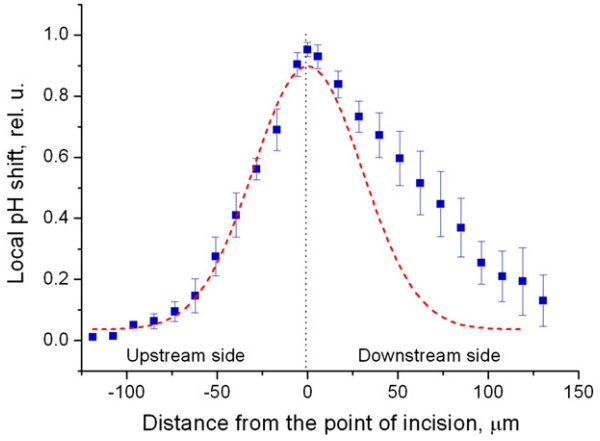

Fig. 3. Localized pattern of alkaline pH around the point of CW incision. Data points show the distribution of pH around the point of CW incision; the line is a predicted approximation of experimental data with a Gaussian curve, provided the pH shifts on the downstream side would be symmetrical to the pH shifts on the upstream side. The left part of the distribution corresponds to the upstream side with respect to the cytoplasmic flow. Symbols are mean values and standard errors obtained with four cells.

streaming cytoplasm. It should be noted that the pH microelectrode was displaced along the axial coordinate, whereas the streaming direction is tilted with respect to the cell axis. Thus, the asymmetry of the alkaline patch is likely to be higher than for the averaged profile shown in Fig. 3.

Although, the width of alkaline spots induced by mechanical stimulation (~100 μm) was at least tenfold lower than that of alkaline bands produced upon dark–light transition, the maximal pH values established after CW incision were similar to those in the light-dependent alkaline bands. This similarity raises the question of whether the incision-induced pH changes are also light-dependent and related to photosynthesis.

3.4. Relation to photosynthesis

Fig. 4A shows the pH responses to the CW incision at various light intensities (the period of adaptation after the change in irradiance was 10–20 min). It is seen that impaling CW at lowered irradiance produced smaller pH_o shifts with a faster relaxation to the initial level. It is worth noting that the mechano-induced pH response was observed even at very low light intensities (10^4-fold attenuation of maximal irradiance with a neutral density filter). Photosynthesis was not needed directly: the incision-induced pH changes with an amplitude 1.0–1.5 unit could be observed after keeping the cells for 45 min in darkness (Fig. 4B, curve *1*). These pH signals were strongly inhibited only after keeping the cells for 60 to 90 min in darkness (curves *2*–*3*). The transfer of the cell to light after long incubation in darkness resulted in a rapid recovery of mechano-induced pH changes (Fig. 4B, curve *4*). When the pH_o increased slowly, as in curve *4*, the biphasic rise could be noted. Thus, the mechano-induced pH response was light-dependent, although its disappearance in darkness proceeded very slowly. The above results imply that the pH response may have a complex origin and comprise stages with different light requirements.

In order to exclude regulatory effects of light unrelated to photosynthesis, we tested the influence of DCMU, an inhibitor of photosynthetic electron transport. Fig. 4C shows that a 5-min incubation in the presence of 4 μM DCMU suppressed the mechanically stimulated pH changes (curve *2*); the signals were almost completely flattened after 20 min incubation (curve *3*). The inhibitory effect of DCMU was reversible: it was relieved after washing the cell with a fresh medium (curve *4*). Clearly, the incision-induced pH response depended strongly on oxidoreduction state of photosynthetic electron transport chain. The intersystem electron carriers are expected to be completely oxidized in DCMU-treated illuminated cells owing to electron drainage by photosystem I (PSI), whereas these carriers can be partly reduced in darkness by chlororespiratory electron flow [26]. Thus, the light-dependent and incision-induced alkalinization of the cell surface have a common property of being dependent on the redox state of photosynthetic electron transport chain.

3.5. Changes in chlorophyll fluorescence

Seeking for possible similarity between alkaline spots arising upon mechanostimulation and illumination, we wondered whether chloroplasts in the area of CW excision exhibit stronger non-photochemical quenching, like the chloroplasts residing under alkaline bands, compared to other cell regions [21]. Fig. 5 shows that the local pH increase upon mechanical stimulation with a microneedle was accompanied by the decrease in chlorophyll fluorescence F_m' induced by saturating light pulses. The decrease in F_m', concurrent with the increase in external pH, designates the enhancement of non-photochemical quenching. The fluorescence F_m' recovered gradually in parallel with restoration of the initial pH. Thus, similar relations were found between the H^+ fluxes and photosynthetic activity in alkaline cell domains arising from different causes. These data suggest that the alkalinization of the apoplast results from the transmembrane H^+ transport and is accompanied by acidification of the cytoplasm, since non-photochemical quenching is known to increase with lowering the cytoplasmic pH [27].

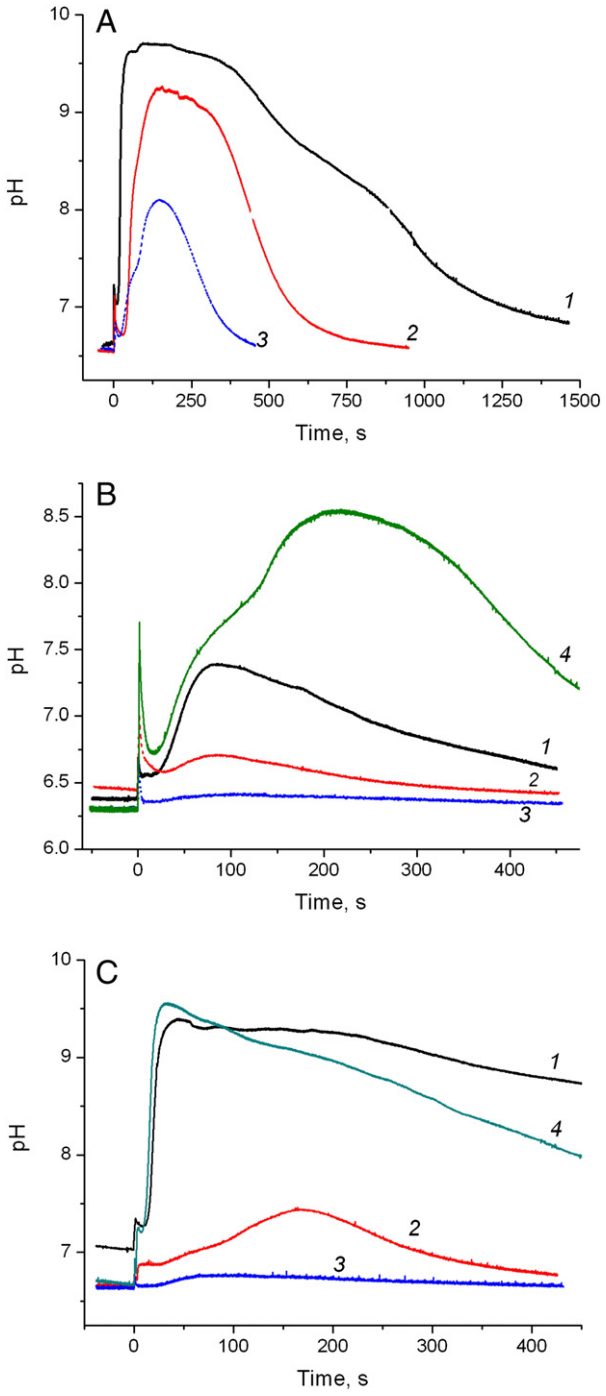

Fig. 4. Dependency of the pH changes induced by CW incision on light intensity and photosynthesis. (A) Kinetics of pH changes arising upon pointed perforation of CW with a micropipette at various photon flux densities: *1* – 100, *2* – 1.0, and *3* – 0.01 μmol m^{-2} s^{-1}. Records were made after 10–20 min incubation at the light intensity indicated. (B) Gradual disappearance of the incision-induced pH response during dark adaptation of various lengths: *1* – 45 min, *2* – 60 min, *3* – 90 min. Curve *4* shows the response recovery in 2 min after transferring the cell to light (photon flux density 100 μmol m^{-2} s^{-1}). (C) Reversible inhibition of the pH response to CW incision in the presence of 4 μM DCMU, an inhibitor of photosynthetic electron transport: *1* – in the absence of inhibitor; *2,3* – after 5-min and 20-min incubation in the presence of 4 μM DCMU; *4* – after 20-min washing the cell with APW. Photon flux density: 28 μmol m^{-2} s^{-1}. A representative experiment for $n = 4$ is shown.

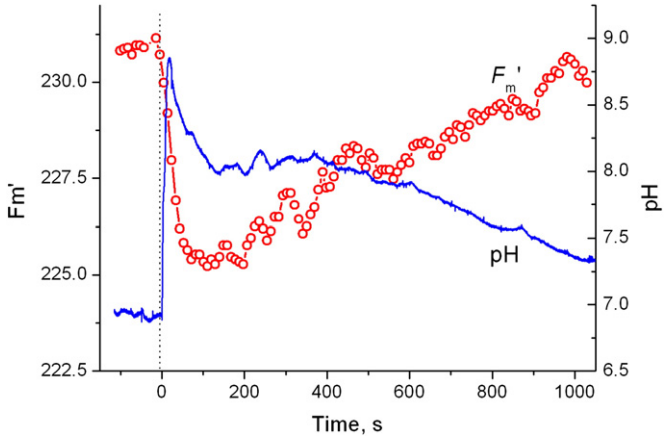

Fig. 5. Simultaneous measurements of maximal chlorophyll fluorescence F_m' (circles) and localized pH changes (solid line) induced by CW incision with a glass micropipette. The mechanical stimulus was applied at time $t = 0$ marked with a dotted line. Representative of $n = 3$.

3.6. Stretching the plasma membrane as an early step of mechanoperception

The penetration of a micropipette into CW should disturb the native arrangement of the cellulose fibrils, moving them apart and creating free space devoid of a rigid support for the membrane. The intracellular hydrostatic pressure would press the membrane into this defect, thus activating stretch sensors. This assumption was verified by decreasing the membrane tension through the increase in osmotic pressure of external medium and, accordingly, the decrease in intracellular hydrostatic pressure. Fig. 6 shows the pH_o changes that were triggered by CW incision in cells bathed with normal APW, in the same cells after the addition of 50 mM sorbitol to the medium, and following the replacement of sorbitol solution with the standard medium (curves *1–3*, respectively). The increase in osmolarity of the external solution resulted in slowing down of mechanically triggered pH changes and in lowering of their amplitude (curves *1, 2*). These modifications were reversible and developed without a noticeable delay. After washing the cell with normal APW, the amplitude and the slope of ascending front of pH transients increased significantly (curve *3*). These observations suggest that the pH increase is the consequence of membrane tension, arising in response to local disturbance of CW

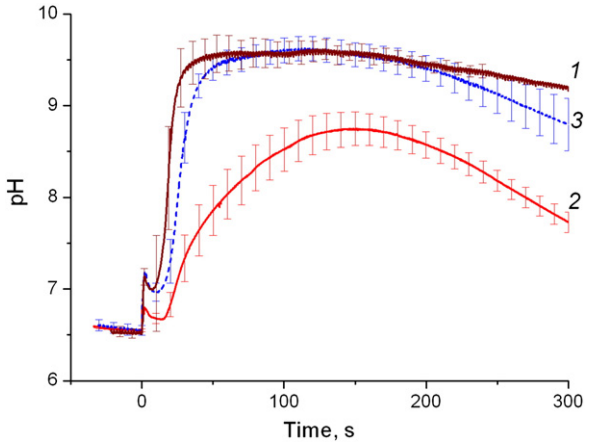

Fig. 6. Effect of osmolarity of external medium on the pH increase induced by CW incision. During the experiment artificial pond water (APW) was replaced with APW containing 50 mM sorbitol and then again with a standard APW. *1* – pH response of the cell in APW; *2* – in the presence of 50 mM sorbitol; *3* – after washing the cell. Photon flux density: 28 μmol m^{-2} s^{-1}. Data are averaged curves and standard errors for $n = 4$ (curves *1, 3*) and $n = 8$ (curve *2*).

structure. Previous studies on *Chara* cells and *Arabidopsis* roots provided evidence that the tensile stress activates stretch-sensitive Ca^{2+} channels, which initiates the Ca^{2+} influx and elevates the cytoplasmic Ca^{2+} level [8,10–13]. Therefore, we tested the suggestion that Ca^{2+} channels are involved in the response of *Chara* internodal cell to mechanical stimulation.

3.7. Involvement of Ca^{2+} influx in mechanoperception

We checked the possible role of Ca^{2+} fluxes in mechanoperception by examining the influence of Ca^{2+} channel blockers on the mechano-stimulated pH changes. Lanthanum is known to inhibit the Ca^{2+} influx through the gated Ca^{2+} channels. As can be seen in Fig. 7A, the addition of 10 μM La^{3+} to the external medium resulted in strong attenuation of the pH response. A stronger inhibition was caused by 100 μM La^{3+}.

A similar suppression of the incision-induced pH increase was observed after a 30-min incubation of the cell in the presence of 10 μM gadolinium (Gd^{3+}), an inhibitor of stretch-activated channels [10]. The inhibitory effect of gadolinium was removed upon washing the cell with APW (Fig. 7B).

Zinc ions are assumed to inhibit the activation of mechanosensitive Ca^{2+} channel [28] and act as a blocker of H^+ channels [29] in *Chara* cells. The addition of Zn^{2+} to the medium at concentrations of 20 or 100 μM resulted in strong inhibition of incision-induced pH rise (Fig. 7C). The rapid deflection was not inhibited, which is in line with the reported insensitivity of the receptor potential to Zn^{2+} [28]. The inhibition of the pH changes by Zn^{2+} was irreversible.

In addition to prevention of Ca^{2+} influx and cytosolic Ca^{2+} rise, we examined the influence of enhanced Ca^{2+} influx and elevation of $[Ca^{2+}]_c$ on the mechano-stimulated pH response. This was accomplished by triggering the action potential (AP) with a short pulse of electric current in advance (~20 s) to mechanical stimulation. During the AP generation the intracellular Ca^{2+} level increases from ~200 nM to 10–40 μM (discussed in [30,31]), which is accompanied by cessation of cytoplasmic streaming. The streaming starts recovering in about 30 s after AP, presumably in parallel with the decrease in cytoplasmic Ca^{2+} level. It should be noted that the AP generation elevates the cytoplasmic Ca^{2+} level over the whole cell, whereas the supposed Ca^{2+} increase in the region of stretch-activated channels is a strictly localized phenomenon. In Fig. 8A the mechano-stimulated local pH changes are compared for resting cells (curve *1*) and for the same cells when the AP was triggered by electric stimulus 20 s prior to mechanical stimulation (curve *2*). It is seen that preliminary PM excitation accelerated the development and elevated the magnitude of mechano-stimulated pH changes. It is possible that the AP-induced inhibition of the proton pump [17,32] facilitated the pH elevation in the incision-induced alkaline patch, because the pH increase was not counteracted by H^+ extrusion in the circumferential plasma membrane regions.

The stimulatory effect of AP on the localized alkalinization is at variance with the AP-induced decline of high pH in the light-dependent alkaline zones [17,33]. Such clear difference indicates that properties of membrane transporters operating in light-activated and incision-induced alkaline areas differ substantially. One may suppose either (i) operation of different transport systems or (ii) regulation of one system by internal cytoplasmic factors (e.g., pH) distributed unevenly under stationary alkaline (light-dependent) zones and stationary acidic zones (a prospective alkaline spot prior to CW incision). Furthermore, the pH response to CW incision might have a complex origin and comprise Ca^{2+}-stimulated and Ca^{2+}-inhibited stages that dominate at early and late stages of the pH transients, respectively.

The supposed involvement of Ca^{2+} as a mediator of the pH increase upon microwounding of CW was additionally tested by elevating the Ca^{2+} concentration in APW from 0.1 to 1.0 mM. Such a replacement of the bath solution was immediately followed by the

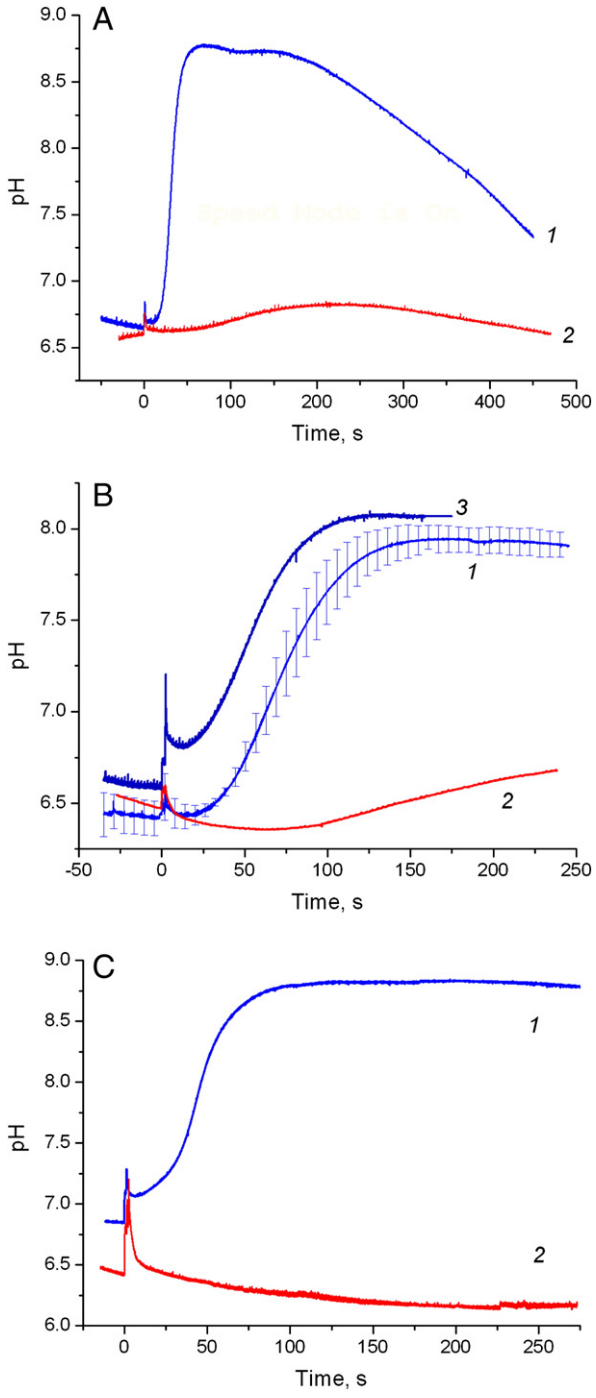

Fig. 7. Inhibition of the mechanically stimulated pH increase by Ca^{2+} channel blockers. (A) Effect of 10 μM La^{3+}: *1* – in the absence of inhibitor, *2* – in the presence of 10 μM $LaCl_3$. (B) Reversible inhibitory action of 10 μM Gd^{3+}: *1* – in the absence of inhibitor, *2* – in the presence of 10 μM Gd^{3+}, *3* – after washing the cell with APW. (C) Inhibition of the pH changes by Zn^{2+}: *1* – untreated cell, *2* – in the presence of 20 μM $ZnSO_4$. Photon flux density: 28 μmol m^{-2} s^{-1}. Bars on curve *1* in (B) represent standard errors of measurements for untreated cells ($n = 3$). Other traces show results of representative measurements.

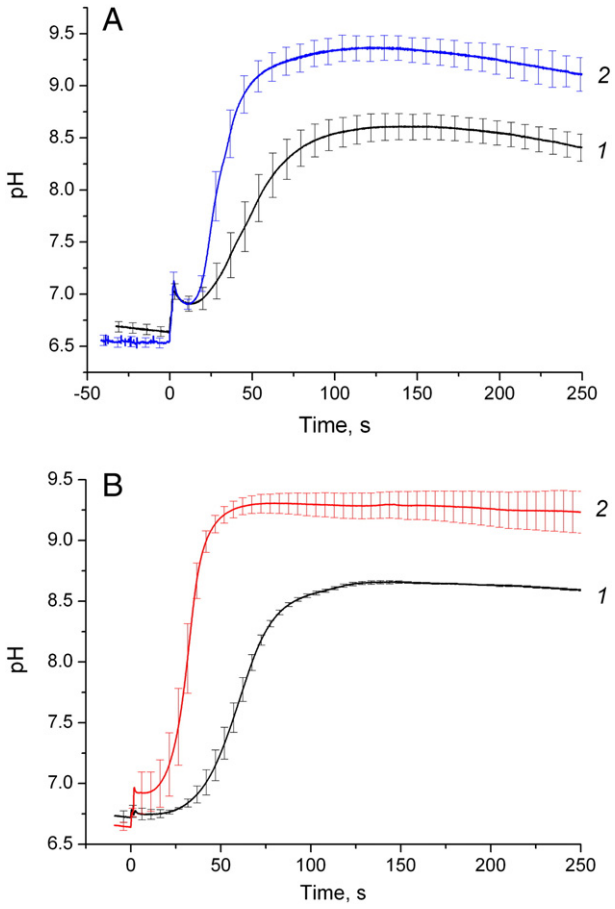

Fig. 8. Stimulatory effect of plasma membrane excitation and elevated Ca^{2+} concentration on the pH changes induced by CW incision. (A) Enhancement of the pH response by the action potential (AP) elicited in advance to CW incision. *1* – pH response of the resting cell ($n = 12$); *2* – response to a similar incision applied in 20–30 s after AP generation ($n = 10$). (B) Stimulation of the pH response after replacing standard APW (0.1 mM $CaCl_2$) with a similar medium containing 1 mM $CaCl_2$. *1* – 0.1 mM Ca^{2+} ($n = 3$); *2* –1.0 mM Ca^{2+} ($n = 4$).

increase in both amplitude and the maximum rate of the incision-induced pH changes (Fig. 8B).

3.8. Role of cytoskeleton

Cytoskeleton is significant for mechanoreception [34–38]. Physical stress may directly induce changes in actin polymerization, thus modulating stress perception. Cytoskeleton is closely related to the operation of ion channels, including mechanosensitive (stretch-activated) channels, since ionic channels are often tethered to actin filaments [39]. It is also known that the spatial alignment of cellulose microfibrils is determined by orientation of microtubules. We examined the influence of oryzalin, which depolymerizes microtubules and disturbs orientation of cellulose microfibrils, and the influence of cytochalasin B, which disturbs the cortical actin fibers and inhibits the cytoplasmic streaming.

Oryzalin at concentrations of 5 and 10 μM strongly inhibited mechanically induced alkalinization without having significant effect on cytoplasmic streaming. However, oryzalin at concentrations ≥2 μM was reported to affect also the morphology of the endoplasmic reticulum [40]. According to immunofluorescence study with *C. corallina* internodal cells [41], oryzalin concentration as low as 0.5 mg/L (~1.45 μM) causes complete depolymerization of microtubules, whereas a lower concentration (0.1 mg/L) was partly effective. Therefore, the effect of 1 μM oryzalin on the pH response was tested. The inhibition was clearly evident after the cells were incubated for 60–120 min in the presence of this agent (Fig. 9A). The inhibitory effect of oryzalin on stress-stimulated pH transients was reversible. A substantial recovery of the pH response was already noted after washing the cells for 30–40 min.

The involvement of microtubules in sensing the microwounding was additionally tested by treating *C. corallina* cells with a microtubule-stabilizing drug, taxol. Taxol binds specifically and reversibly to polymerized tubulin, thus preventing the microtubules from disassembly. Fig. 9B

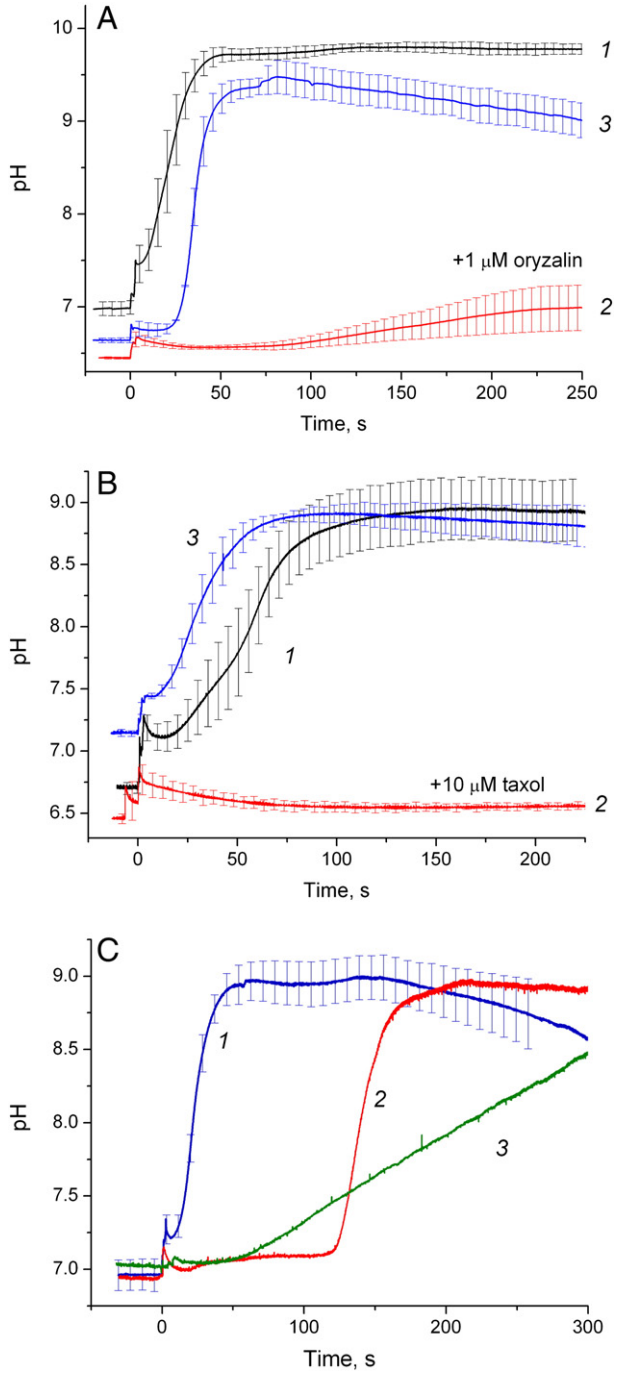

Fig. 9. Dependence of pH changes induced by CW incision on the cytoskeletal structures. (A) Reversible elimination of the pH response to CW incision by 1.0 μM oryzalin. *1* – pH transients in untreated cells; *2* – after 40 to 120-min incubation in the presence of oryzalin; *3* – after 20- to 40-min washing the cell from the inhibitor. Data are average curves ± SE for $n = 4$, 6, and 2 for untreated, oryzalin-treated, and washed cell, respectively. (B) Effect of a microtubule-stabilizing drug, taxol (10 μM) on the alkalinization response induced by CW incision. *1* – untreated cell; *2* – after 20- to 60-min incubation in the presence of taxol; *3* – after 30- to 90-min washing the cell from the inhibitor. Data are average curves ± SE for $n = 4$. (C) Effect of cytochalasin B on the pH changes induced by CW incision. *1* – untreated internodal cells (velocity of cytoplasmic streaming 75–85 μm/s), data are averaged traces and standard errors calculated for $n = 7$; *2, 3* – examples of different pH responses in the presence of 15 μg/ml cytochalasin B (streaming velocities <25 μm/s).

shows strong inhibition of the alkalinization induced by CW incision in the presence of 10 μM taxol. The inhibition was relieved upon washing the cell with a fresh medium. Thus, microtubules appear to be important elements in the transduction of CW mechanical stress to activation of the proton fluxes.

Incubation of the cell in the presence of cytochalasin B (20 μg/ml) resulted in gradual retardation, until complete cessation, of cytoplasmic streaming and modified the pH response to the CW incision. The averaged curves demonstrated the retarded development of pH transients, and their lower amplitude compared to the pH changes in untreated cells. Inspection of individual traces revealed that the shapes of mechano-induced pH responses in CB-treated cells varied to a large extent (Fig. 9B, curves *2, 3*). Some traces displayed a long lag period (100–150 s) followed by an abrupt increase in pH by more than 2 pH units (curve *2*), whereas others showed very slow, almost linear increase in pH after CW microperforation (curve *3*). This variability of pH changes induced by mechanical impact might be related to the appearance of the dispersed pH pattern in CB-treated illuminated cells [42]. If heterogeneity of the cytoplasmic composition increases in the absence of streaming as a stirring factor, the cell responses to mechanical stress can exhibit large variability too, because all physicochemical stages subsequent to tension of the plasma membrane would proceed at unequal conditions in different cell parts.

4. Discussion

Large photosynthesizing cells of characean algae are particularly suitable for studying the origin and significance of pH changes in perception and transduction of mechanical stimuli. The measurements of pH transients can be done on a single cell under variable experimental conditions within the period of several hours. The photosynthesizing capacity of characean cells confers additional traits to the mechanically induced pH responses, which cannot be observed with heterotrophic tissues, such as roots. Previous reports on sensitivity of characean cells to mechanical stresses employed the methods of cell stimulation with a dropping rod [12,13] or with an electro-mechanical compression device [10] or by changes in osmolarity of the medium [11]. These studies revealed the existence of the receptor potential and the possible role of mechano-sensitive Ca^{2+} channels in the transduction of mechanical stress signal. However, the involvement of protons escaped from notice.

We show here that mechanical stimulation of *C. corallina* internodal cell induces extensive alkalinization of the apoplast in the vicinity of the stimulation site (Figs. 1, 3). The influence of buffer on these pH transients (Fig. 2) indicates clearly that the initial jump of the electrode potential represents the extracellular recording of the receptor potential documented by others [10,12,13,28]. The fast deflection is a convenient mark of the stimulation moment, but only the subsequent pH transients represent meaningful pH changes. The localized response of *C. corallina* internodal cells to mechanical stimulation comprises a comparatively short (100–200 s) upturn in pH and prolonged slow reversal phase that lasts up to 40 min. The mechanically induced proton fluxes are localized to the area with the diameter of about 100 μm around the point of incision. The axial profile of the pH shift exhibits asymmetry related to the direction of cytoplasmic streaming (Fig. 3), indicating that some cytoplasmic factor carried by cyclosis affects the transmembrane ion fluxes and that the alkaline patch is elongated along the streaming path. Similar pointed mechanical stimuli applied to epidermal cells of *Arabidopsis* root triggered fairly symmetrical extracellular pH elevation in the area with diameter about 10 μm that lasted about 60 s and did not show prolonged reversal phase [8].

Our results advocate that mechanically induced inward proton fluxes is an ancient response that precedes evolution of land plants. It is interesting to investigate if this response is shared by other photosynthetic organisms with carbohydrate-based cell walls, such as red and brown algae.

The physiological role of the apoplastic pH elevation is apparently related to strengthening of the CW structure. Rapid changes in surface pH affect the cell properties either directly, by modulating activity of CW proteins, such as expansins [43] and pectin methylesterases [44]

or indirectly by affecting the rate of insolubilization of structural wall proteins, such as extensins. Extracellular alkalinization induced by CW incision is significantly more extensive in *C. corallina* internodal cells than in *Arabidopsis* root epidermal cell. In terms of cell dimension the *Chara* internode is substantially larger than the root epidermal cells. Consequently, *Chara* cell wall is much thicker and its rigidification is likely to require longer exposure to alkaline pH. The experiments were mostly conducted with non-growing cells. Nevertheless, young growing cells also responded to CW incision by large increase in the surface pH. There was a trend to faster recovery of low pH after CW incision in young growing cells than in mature nongrowing cells.

The localized incision of CW might release small fragments of cell wall material that can trigger defense responses associated with extensive alkalinization of the external media [45,46]. In order to determine if the observed increase in apoplastic pH was dependent on localized stretching of the membrane or on the release of wall material at the site of mechanical stimulation, we studied dependence of the pH response on osmolarity of the external medium. The decreased hydrostatic pressure would decrease the membrane tension but would not affect the incision-mediated release of CW material. The increase in osmolality of the medium induced reversible inhibition of the pH shift (Fig. 6). Dependency of the *Chara* pH response on osmolarity of the external medium and, accordingly, on the internal hydrostatic pressure acting on the plasma membrane indicates that the CW incision activates membrane tension-dependent molecular mechanisms that initiate signaling cascade responsible for extracellular alkalinization.

The membrane tension is known to activate the Ca^{2+} influx through the tension-sensitive Ca^{2+} channels [47]. Inhibition of the pH increase by La^{3+}, Gd^{3+}, and Zn^{2+} ions is in accord with the supposed mediation of the pH response by Ca^{2+} influx and the respective increase in cytosolic Ca^{2+} level (Fig. 7). The generation of AP in *Chara* internodal cell is associated with a substantial increase in cytoplasmic Ca^{2+} concentration [30,48]. Therefore we tested the effect of AP on proton fluxes in *Chara* internodal cell. The action potential did not trigger immediate H^+ influx, indicating that the increase in cytosolic Ca^{2+} level alone is not sufficient for the induction of the transmembrane H^+ influx. On the other hand, when the AP was triggered in advance to CW incision to elevate the background level of $[Ca^{2+}]_c$, the rate and extent of the pH increase were enhanced, which supports the idea of Ca^{2+} participation in the induction of H^+ influx after CW impalement. Calcium influx through the tension-sensitive Ca^{2+} channels depends on the concentration difference between the cytoplasm and the external media. We modulated the CW incision-mediated calcium influx by changing external Ca^{2+} concentration. When Ca^{2+} concentration in the medium was elevated from 0.1 mM to 1 mM, the pH changes induced by mechanical impact were stimulated, suggesting that calcium plays a pivotal role in CW incision-mediated proton fluxes.

Unfortunately, toxic effects of calcium ionophore A23187 on *C. corallina* internodes [49] prevented us from using it to test direct effect of increase in cytoplasmic Ca^{2+} concentration on proton fluxes.

The plasma membranes of plant cells contain several Ca^{2+}-regulated transport systems. The increase in $[Ca^{2+}]_c$ is known to inactivate the PM H^+-ATPase [50–52], which may cause alkalinization of the external medium [53]. However, upon the inhibition of the PM H^+-ATPase in *Chara* by AP generation, the external pH shifted usually by less than 0.4 unit, approaching the pH ~7 of the bulk medium [32]. Thus, inhibition of PM H^+-ATPase cannot account for the large-scale alkalinization observed upon the mechanical impact.

The PM of *Chara* was proposed to contain Ca^{2+}-sensitive H^+-conducting channels [24] that are responsible for very high PM conductance in the area of alkaline zones. The highest external pH values attained at the point of CW incision (up to pH 10) are close to the pH in the light-dependent alkaline zones. Furthermore, both the light-dependent alkaline bands and the incision-induced alkaline patches were abolished by Zn^{2+} ions ([29] and Fig. 7C). However, the H^+-transporting system at the incision site was not blocked by electrical excitation of the plasma membrane, unlike that of alkaline bands [24]. Moreover, the generation of AP enhanced the initial pH increase (Fig. 8A). This distinction may be either due to operation of different H^+-transporting systems in incision-stimulated and light-activated alkaline regions or to strong modulation of a single H^+-transporting system by cytoplasmic composition distributed heterogeneously under the alkaline and acidic bands. Variations of cytoplasmic composition in these cell regions are evidenced by different photosynthetic activity of chloroplasts residing under alkaline and acidic bands [21,33].

Manifestations of structural heterogeneity in *Chara* cells comprise uneven distribution of microtubules (they are more abundant under the acid areas than under alkaline bands) [41] and specific location of charasomes in the areas of proton extrusion [54,55]. Different amplitudes of the incision-induced pH responses in the alkaline and acid regions are hardly related to different densities of charasomes in these regions, because charasomes are very stable structures remaining for several days after darkening, whereas large mechanically stimulated pH responses in the formerly alkaline area were restored after comparatively short darkening (~15 min).

In plants mechanical stimulation is often associated with ROS production. ROS have a profound effect on cellular signaling; they can affect the activity of calcium and potassium channels, aquaporins and other membrane proteins. We therefore tested if ROS are a part of cellular machinery that triggers mechanically induced H^+ fluxes. The addition of ascorbate at a concentration of 0.5 mM to scavenge superoxide and hydrogen peroxide had no effect on the pH response of internodal cells to CW incision (not shown). Our data indicate that ROS-dependent cellular mechanisms are not part of the signaling cascade that triggers mechanically induced CW alkalinization.

The results of this study reveal the dependence of the incision-induced pH rise on the cytoskeleton. The disturbance of microtubules by oryzalin and taxol had a strong and reversible action on the pH response. Fast disappearance of the mechanically induced response (within 2 h of incubation in the presence of 1 μM oryzalin and within 1-h incubation with 10 μM taxol) suggests that the pH response to CW incision is related to early stages of oryzalin and taxol action on microtubules. The effect of microtubule-disrupting drugs on mechanically induced H^+ fluxes suggests that dynamic instability of microtubule-based cytoskeleton plays a pivotal role in *Chara* mechanosensing. The role of microtubule cytoskeleton in cell mechanosensing is now in the center of attention. In human endothelial cells local changes in MT dynamics are the essential part of cell mechanosensing machinery. It was shown recently that morphogenesis at the *Arabidopsis* shoot apex depends on the microtubule cytoskeleton, which in turn is regulated by mechanical stress [34]. Mechanoresponses of *Arabidopsis* root are regulated by Calmodulin-like 24 gene (CML24) that is thought to act by controlling cortical microtubule orientation [37]. In plants microtubules respond to continuous directional mechanical stress, and this response is inhibited by Gd^{3+}, suggesting the involvement of stretch-activated calcium channels in slow mechanoresponse [35]. Our data indicate that microtubule-based cytoskeleton is an essential part of *Chara* mechanosensing signaling.

The inhibitory action of oryzalin and taxol on the pH changes might also be related to the parallel alignment of cellulose microfibrils and microtubules in *Chara*. Specifically, impairment of microtubules by microtubule-disrupting drugs might disturb the alignment of microfibrils and modulate the mechanical properties of the cell wall. Furthermore, if tension-activated channels are tethered to microtubules [39], the disordering of microtubules may interfere with the function of ionic channels.

Relation of the pH response to the actin function was less clear than in the case of microtubules. In the presence of cytochalasin B, the kinetics of mechanically induced pH responses exhibited large variability in terms of the lag period (variations from 30 to 150 s), the amplitude and the maximal slope of pH changes. This might result

from the increased intracellular heterogeneity upon suppression of cyclosis-mediated intracellular communications.

Previous study showed the effect of CW incision on heterotrophic cells of *Arabidopsis* root. *Chara* internodal cells contain chloroplasts and photosynthesize. We therefore investigated the role of photosynthesis on CW incision-mediated pH response. Photosynthetic electron transport is not needed directly for the origin of pH changes. Indeed, the pH response, although comparatively small, was retained for almost 1 h in darkness (Fig. 4). Nevertheless, photosynthetic electron transport seems essential for the increase of pH_o to the upper limit (pH ~10). A surprising finding was that the inhibition of photosynthesis by DCMU led to a faster inhibition of stress-induced pH changes than cell darkening. After 20-min incubation in the presence of DCMU the pH response was strongly suppressed, whereas the response remained quite large after similar incubation in darkness in the absence of the inhibitor. One obvious difference between these two treatments is that illumination in the presence of DCMU strongly oxidizes the pool of intersystem electron carriers between PSII and PSI in plant leaves [56], whereas this pool remains partly reduced in darkness. Variations in the reduction state of intersystem electron carriers may affect chloroplast kinases having multiple regulatory functions.

Interestingly, the generation of high pH region proceeded much faster than the reversal stage that lasted up to 40 min at high irradiance and was shortened at lower irradiances (Fig. 4). The latter observation might suggest the presence of both light-dependent and light-independent stages in mechanical stress perception.

The pH changes of comparatively small magnitude, like those shown in Fig. 2B, would occur on standard microelectrode insertions into the cytoplasm or vacuole of characean cells. Nevertheless, they remained unnoticed so far because the membrane conductance changes, confined to a small cell area (~10^{-4} cm^2), are not accompanied by gross changes in membrane conductance of cell parts whose surface area is larger by several orders of magnitude.

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References

[1] T.S. Nühse, Cell wall integrity signaling and innate immunity in plants, Front. Plant Sci. 3 (December 11 2012), http://dx.doi.org/10.3389/fpls.2012.0 0280.
[2] D.B. Collinge, Cell wall appositions: the first line of defence, J. Exp. Bot. 60 (2009) 351–352.
[3] G.B. Monshausen, S. Gilroy, Feeling green: mechanosensing in plants, Trends Cell Biol. 19 (2009) 228–235.
[4] M.E. Maffei, A. Mithofer, W. Boland, Before gene expression: early events in plant–insect interaction, Trends Plant Sci. 12 (2007) 311–316.
[5] R.J. Howard, M.A. Ferrari, D.H. Roach, N.P. Money, Penetration of hard substrates by a fungus employing enormous turgor pressures, Proc. Natl. Acad. Sci. U.S.A. 88 (1991) 11281–11284.
[6] I. Foissner, The relationship of echinate inclusions and coated vesicles on wound healing in *Nitella flexilis* (Characeae), Protoplasma 142 (1988) 164–175.
[7] Y. Kobayashi, I. Kobayashi, Microwounding is a pivotal factor for the induction of actin-dependent penetration resistance against fungal attack, Planta 237 (2013) 1187–1198.
[8] G.B. Monshausen, T.N. Bibikova, M.H. Weisenseel, S. Gilroy, Ca^{2+} regulates reactive oxygen species production and pH during mechanosensing in *Arabidopsis* roots, Plant Cell 21 (2009) 2341–2356.
[9] M. Turmel, P. J.-F., P. Charlebois, C. Otis, C. Lemieux, The green algal ancestry of land plants as revealed by the chloroplast genome, Int. J. Plant Sci. 168 (2007) 679–689.
[10] T. Kaneko, C. Saito, T. Shimmen, M. Kikuyama, Possible involvement of mechanosensitive Ca^{2+} channels of plasma membrane in mechanoperception in *Chara*, Plant Cell Physiol. 46 (2005) 130–135.
[11] T. Kaneko, N. Takahashi, M. Kikuyama, Membrane stretching triggers mechanosensitive Ca^{2+} channel activation in *Chara*, J. Membr. Biol. 228 (2009) 33–42.
[12] V.A. Shepherd, M.J. Beilby, S.A.S. Al Khazaali, T. Shimmen, Mechano-perception in *Chara* cells: the influence of salinity and calcium on touch-activated receptor potentials, action potentials and ion transport, Plant Cell Environ. 31 (2008) 1575–1591.
[13] V.A. Shepherd, M.J. Beilby, T. Shimmen, Mechanosensory ion channels in charophyte cells: the response to touch and salinity stress, Eur. Biophys. J. 31 (2002) 341–355.
[14] M.J. Beilby, M.A. Bisson, pH banding in charophyte algae, in: A.G. Volkov (Ed.), Plant Electrophysiology: Methods and Cell Electrophysiology, Springer, Berlin, 2012, pp. 247–271.
[15] A.A. Bulychev, Membrane excitation and cytoplasmic streaming as modulators of photosynthesis and proton flows in Characean cells, in: A.G. Volkov (Ed.), Plant Electrophysiology: Methods and Cell Electrophysiology, Springer, Berlin, 2012, pp. 273–300.
[16] J.P. Metraux, P.A. Richmond, L. Taiz, Control of cell elongation in *Nitella* by endogeneous cell wall pH gradients. Multiaxial extensibility and growth studies, Plant Physiol. 65 (1980) 204–210.
[17] A.A. Bulychev, N.A. Kamzolkina, J. Luengviriya, A.B. Rubin, S.C. Mueller, Effect of a single excitation stimulus on photosynthetic activity and light-dependent pH banding in *Chara* cells, J. Membr. Biol. 202 (2004) 11–19.
[18] S.O. Dodonova, A.A. Bulychev, Cyclosis-related asymmetry of chloroplast–plasma membrane interactions at the margins of illuminated area in *Chara corallina* cells, Protoplasma 248 (2011) 737–749.
[19] A. Shapiguzov, J.P. Vainonen, M. Wrzaczek, J. Kangasjärvi, ROS-talk – how the apoplast, the chloroplast, and the nucleus get the message through, Front. Plant Sci. 3 (2012).
[20] Y.N. Antonenko, A.A. Bulychev, Measurements of local pH changes near bilayer lipid membrane by means of a pH microelectrode and a protonophore-dependent membrane potential. Comparison of the methods, Biochim. Biophys. Acta 1070 (1991) 279–282.
[21] N.A. Krupenina, A.A. Bulychev, U. Schreiber, Chlorophyll fluorescence images demonstrate variable pathways in the effects of plasma membrane excitation on electron flow in chloroplasts of *Chara* cells, Protoplasma 248 (2011) 513–522.
[22] T. Shimmen, Studies on mechano-perception in characean cells: development of a monitoring apparatus, Plant Cell Physiol. 37 (1996) 591–597.
[23] L.N. Vorobiev, Potassium ion activity in the cytoplasm and the vacuole of cells of *Chara* and *Griffithsia*, Nature 216 (1967) 1325–1327.
[24] A.A. Bulychev, N.A. Krupenina, Transient removal of alkaline zones after excitation of *Chara* cells is associated with inactivation of high conductance in the plasmalemma, Plant Signal. Behav. 4 (2009) 727–734.
[25] A.A. Bulychev, N.A. Kamzolkina, Differential effects of plasma membrane electric excitation on H^+ fluxes and photosynthesis in characean cells, Bioelectrochemistry 69 (2006) 209–215.
[26] P.J. Nixon, Chlororespiration, Philos. Trans. R. Soc. B 355 (2000) 1541–1547.
[27] A.A. Bulychev, A.V. Alova, A.B. Rubin, Fluorescence transients in chloroplasts of *Chara corallina* cells during transmission of photoinduced signal with the streaming cytoplasm, Russ. J. Plant Physiol. 60 (2013) 33–40.
[28] K. Iwabuchi, T. Kaneko, M. Kikuyama, Mechanosensitive ion channels in *Chara*: Influence of water channel inhibitors, $HgCl_2$ and $ZnCl_2$, on generation of receptor potential, J. Membr. Biol. 221 (2008) 27–37.
[29] S. Al Khazaaly, M.J. Beilby, Zinc ions block H^+/OH^- channels in *Chara australis*, Plant Cell Environ. 35 (2012) 1380–1392.
[30] G.N. Berestovsky, A.A. Kataev, Voltage-gated calcium and Ca^{2+}-activated chloride channels and Ca^{2+} transients: voltage-clamp studies of perfused and intact cells of *Chara*, Eur. Biophys. J. 34 (2005) 973–986.
[31] P.K. Hepler, Calcium: a central regulator of plant growth and development, Plant Cell 17 (2005) 2142–2155.
[32] S.O. Dodonova, N.A. Krupenina, A.A. Bulychev, Suppression of the plasma membrane H^+-conductance on the background of high H^+-pump activity in dithiothreitol-treated *Chara* cells, Biochem. (Mosc.) Suppl. Ser. A Membr. Cell Biol. 4 (2010) 389–396.
[33] N.A. Krupenina, A.A. Bulychev, Action potential in a plant cell lowers the light requirement for non-photochemical energy-dependent quenching of chlorophyll fluorescence, Biochim. Biophys. Acta 1767 (2007) 781–788.
[34] O. Hamant, M.G. Heisler, H. Jönsson, P. Krupinski, M. Uyttewaal, P. Bokov, F. Corson, P. Sahlin, A. Boudaoud, E.M. Meyerowitz, Y. Couder, J. Traas, Developmental patterning by mechanical signals in *Arabidopsis*, Science 322 (2008) 1650–1655.
[35] T. Ikushima, T. Shimmen, Mechano-sensitive orientation of cortical microtubules during gravitropism in azuki bean epicotyls, J. Plant Res. 118 (2005) 19–26.
[36] K.A. Myers, K.T. Applegate, G. Danuser, R.S. Fischer, C.M. Waterman, Distinct ECM mechanosensing pathways regulate microtubule dynamics to control endothelial cell branching morphogenesis, J. Cell Biol. 192 (2011) 321–334.
[37] Y. Wang, B. Wang, S. Gilroy, E. Wassim Chehab, J. Braam, CML24 is involved in root mechanoresponses and cortical microtubule orientation in *Arabidopsis*, J. Plant Growth Regul. 30 (2011) 467–479.
[38] J. Zhou, B. Wang, Y. Li, Y. Wang, L. Zhu, Responses of chrysanthemum cells to mechanical stimulation require intact microtubules and plasma membrane–cell wall adhesion, J. Plant Growth Regul. 26 (2007) 55–68.
[39] P. Nick, Mechanics of the cytoskeleton, in: P. Wojtaszek (Ed.), Mechanical Integration of Plant Cells and Plants, Springer, Berlin, Heidelberg, 2011, pp. 53–90.
[40] M. Langhans, S. Niemes, P. Pimpl, D.G. Robinson, Oryzalin bodies: in addition to its anti-microtubule properties, the dinitroaniline herbicide oryzalin causes nodulation of the endoplasmic reticulum, Protoplasma 236 (2009) 73–84.
[41] G.O. Wasteneys, R.E. Williamson, Microtubule organization differs between acid and alkaline bands in internodal cells of *Chara* but bands can develop in the absence of microtubules, Planta 188 (1992) 99–105.
[42] W.J. Lucas, J. Dainty, Spatial distribution of functional OH^- carriers along a Characean internodal cell: determined by the effect of cytochalasin B on $H^{14}CO_3^-$ assimilation, J. Membr. Biol. 32 (1977) 75–92.
[43] D.J. Cosgrove, L.C. Li, H.-T. Cho, S. Hoffmann-Benning, R.C. Moore, D. Blecker, The growing world of expansins, Plant Cell Physiol. 43 (2002) 1436–1444.

[44] F. Micheli, Pectin methylesterases: cell wall enzymes with important roles in plant physiology, Trends Plant Sci. 6 (2001) 414–419.
[45] G. Felix, M. Regenass, T. Boller, Specific perception of subnanomolar concentrations of chitin fragments by tomato cells: induction of extracellular alkalinization, changes in protein phosphorylation, and establishment of a refractory state, Plant J. 4 (1993) 307–316.
[46] M.D. Spiro, J.F. Bowers, D.J. Cosgrove, A comparison of oligogalacturonide- and auxin-induced extracellular alkalinization and growth responses in roots of intact cucumber seedlings, Plant Physiol. 130 (2002) 895–903.
[47] R. Dutta, K.R. Robinson, Identification and characterization of stretch-activated ion channels in pollen protoplasts, Plant Physiol. 135 (2004) 1398–1406.
[48] M.J. Beilby, Action potential in charophytes, Int. Rev. Cytol. 257 (2007) 43–82.
[49] R.J. Reid, F.A. Smith, Toxic effects of the ionophore A23187 on *Chara*, Plant Sci. 91 (1993) 7–13.
[50] P. De Nisi, M. Dell'Orto, L. Pirovano, G. Zocchi, Calcium-dependent phosphorylation regulates the plasma-membrane H^+-ATPase activity of maize (*Zea mays* L.) roots, Planta 209 (1999) 187–194.
[51] T. Kinoshita, M. Nishimura, K. Shimazaki, Cytosolic concentration of Ca^{2+} regulates the plasma membrane H^+-ATPase in guard cells of fava bean, Plant Cell 7 (1995) 1333–1342.
[52] B. Lino, V. Baizabal-Aguirre, L.E. Gonzalez de la Vara, The plasma-membrane H^+-ATPase from beet root is inhibited by a calcium-dependent phosphorylation, Planta 204 (1998) 352–359.
[53] A. Schaller, C. Oecking, Modulation of plasma membrane H^+-ATPase activity differentially activates wound and pathogen defense responses in tomato plants, Plant Cell 11 (1999) 263–272.
[54] P.M. Schmolzer, M. Hoftberger, I. Foissner, Plasma membrane domains participate in pH banding of *Chara* internodal cells, Plant Cell Physiol. 52 (2011) 1274–1288.
[55] V.R. Franceschi, W.J. Lucas, Structure and possible function(s) of charasomes: complex plasmalemma-cell wall elaborations present in some characean species, Protoplasma 104 (1980) 253–271.
[56] A.A. Bulychev, W.J. Vredenberg, Induction kinetics of photosystem I-activated P700 oxidation in plant leaves and their dependence on pre-energization, Russ. J. Plant Physiol. 57 (2010) 599–608.