Isolation and patch clamp measurements of xylem contact cells for the study of their role in the exchange between apoplast and symplast of leaves

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Abstract

Because of their importance for nutrition, a method was developed to patch xylem contact cells in leaves of Vicia faba and maize. Since the lignification of older cells was a major obstacle for isolating protoplasts which could be patched, only young leaves (fourth fully developed leaf) were used. An important step in the isolation of these cells was the infiltration of the leaves and their exposure to enzymes for several hours, allowing mesophyll cells to be removed whilst having most of the xylem contact cells attached to the xylem. Channel activity in cell-attached mode or in excised patches could only be observed if an internal coating of sialucate was used to block diffusion ions out of the pipette glass. Two different types of K⁺ channels were identified by measuring the reversal potential at different concentrations of KCl. One channel (SC) had a symmetric IV curve with a high probability of remaining open, irrespective of membrane potential; the other channel was an inward rectifier. The symmetrical channel could be blocked weakly by Na⁺ but it was permeable to NH₄⁺.

Introduction

Nutrients and water are transported from the roots to the leaves in xylem vessels. The reentry into the symplast is believed to occur via the plasmalemma of the cells in contact with the xylem. Thus, the transport capacities of these cells are of major interest for the study of nutrition. Wilson et al. (1991) found that the pH in the apoplast close to these cells was more acid than in other areas, which suggests that the activity of proton pumps in the plasmalemma of these xylem contact cells is high in order to energize high transport rates. The proton gradient and the membrane potential provide the driving force for cotransporters and for cations (Sanders et al., 1984).

Patch clamp techniques (Schroeder et al., 1994) are increasingly being used to investigate these transport processes at the molecular level for individual transport proteins (channels). So far, patch clamp experiments on cells of leaves of higher plants have been restricted to guard cells (Schroeder et al., 1994), motor cells (Kim et al., 1993) and, more rarely, to mesophyll cells (Spalding et al., 1992). The first attempt to study cells in contact with the xylem vessels was done using the root. Wegner and Raschke (1994) and Wegner et al. (1995) developed a technique for isolating xylem parenchyma cells of barley roots. Their method took advantage of the Casparian band in order to separate these cells from the mesophyll during digestion by enzymes.

In the leaves, there is no Casparian band, and the lignification of the xylem contact cells renders their isolation difficult. However, the special role of these cells in nutrient uptake has stimulated new attempts to isolate patchable xylem contact cells in the leaves. This paper describes a successful new method for isolating xylem parenchyma cells from leaves of Vicia faba and Zea mays based on a time-dependent enzyme digestion.

Materials and methods

Plant

Maize was grown in an aqueous culture (0.5 mM NH₄NO₃, 0.7 mM K₂SO₄, 0.1 mM KCl, 2 mM Ca(NO₃)₂, 0.5 mM MgSO₄, 0.1 mM KClPO₄, 1 mM H₃BO₃, 0.5 µM MnSO₄, 0.1 µM ZnSO₄, 0.2 µM CuSO₄, 10 mM (NH₄)₂MoO₄, 1 µM FeEDTA) under normal light conditions in a greenhouse (13 hours light). After 4 weeks they had 5 leaves. The fourth fully developed leaf was cut, and about 6 cm of its middle section used for the isolation procedure. It was important to use young cells because increasing lignification (McDougall et al., 1994) rendered patching of older isolated cells impossible. Vicia faba was grown in pots on a window shelf at room temperature.

Patch clamp recording

Electrodes of soft glass (Garner Glass Co., KG 12)
were drawn on a L/M-3P-A Puller (List, Darmstadt, FRG). Patch-clamp currents from excised patches (inside-out) were measured by a Dagan amplifier (Minneapolis, USA). Data acquisition and evaluation was done as described previously (Draber and Hansen, 1994; Hansen et al., 1995; Schultze and Draber, 1993). Briefly, a Dalianco AD converter board inserted into a Pentium computer allowed a sampling rate of 200 kHz with an anti-aliasing filter of 50 kHz. If not otherwise stated the solution was 200 mM KCl, 5 mM CaCl₂ and 20 mM sorbitol in the bath and in the pipette.

**Results**

**Isolation of xylem contact cells**

This was difficult because of the lignification of the cell walls. In the first experiments, incubation of leaves of *Vicia faba* in Rohament PC (Rohm, Darmstadt, FRG) led to a rapid digestion of mesophyll cells and with little effect on xylem contact cells. This lignification made separation easy, but prevented further processing to get patchable protoplasts, so other methods had to be used.

Patchable xylem contact cells were finally obtained by the following procedure: A dissected leaf of *Vicia faba* was put into a 10 ml syringe filled with tap water. By pulling the piston out, negative pressure was applied which made the air come out of the stomata. Upon relaxation of the pressure, the water moved in. This procedure was repeated three times. This method was as effective as infiltration with enzyme solution and was preferred because this method consumed much less of the expensive enzyme solution.

After infiltration, the lower epidermis was removed by a pair of forceps. An area of about 3 cm² was cut out and transferred to a Petri dish with the lower side upwards. This was immersed in an enzyme solution made up as a replacement of Rohament PC which was no longer available: 1.5% cellulase, 0.5% macerozyme (Yakult Honsha), 2.0% pectinase (Sigma), 0.5% BSA (Sigma), 5 mM CaCl₂, 0.5 M sorbitol, pH 6.5 (buffered with Tris / Mes).

After three hours of incubation, the upper epidermis could be pulled off with forceps. Exposure to the enzyme solution for a further 30 min made the leaf disintegrate. The mesophyll cells were then washed out using a stream of enzyme solution from a fine pipette. Many xylem contact cells were also removed from the xylem stringe a the same time, but enough remained attached. Their cell walls were so strongly weakened that the patch electrode could penetrate and seal to the plasmalemma.

The infiltration of maize xylem contact cells worked better with positive pressure. After infiltration, the leaf was dissected longitudinally and was exposed to the enzyme solution for 6 h at room temperature or for 3 h at 40°C. The leaf was then stretched using needles to loosen the tissue between the veins, and left in the solution for an additional 30 min.

After that time, the cell wall was very loose. The patch electrode could seal to blebs (protoplasts protruding out of the cell wall debris) or the remaining cell wall could be pushed away by the patch electrode. Figure 1 shows xylem vessels with attached xylem contact cells with one being patched.

Sealing in *Vicia faba* was different from that observed for other tissues. For instance, in the case of a *Chlamydomonas* droplet, sealing occurred immediately or within a few seconds. In the case of xylem contact cells, sealing was only found to occur after a much longer time. A typical sealing procedure for *Vicia faba* was as follows: The bottom of the petri dish was coated with Poly-L-lysine in order to fix the protoplast. When pressing the pipette against the protoplast, a resistance of about 30 MOhm was obtained immediately, but when the positive pressure in the pipette was removed, the resistance increased to 70 MOhm. Repetitive applications of negative pressure made the resistance go up in steps until a stable value of 1 to 2 GOhm was reached after 15 to 60 min, possibly because the fibrils had to be pushed away from the mouth of the pipette.

In the case of maize, the final seal was obtained earlier, often after only a few seconds, but the procedure could also last for 30 min.

**Blocking ions**

Sensitivity to blocking ions from the pipette glass

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*Fig. 1. Xylem vessels of maize with adhering isolated xylem contact cells with one being patched.*
was high in xylem contact cells. Initially the cells scaled, but no activity was obtained, or activity ceased soon after scaling. According to Copello et al. (1991) ions diffusing out of the glass of the pipette can block the ion channels. This was avoided by coating the capillaries internally with sigma/ate (Sigma) before pulling the micropipettes. Internal coating by sigma/ate was also a powerful means of reducing the noise of the pipette current. Unfortunately boro-silicate glass which produced less noise (Fairley and Walker, 1989) did not seal.

Time course of patch clamp current from a xylem contact cell of *Vicia faba* and of *Zea mays* (Fig. 2) in excised mode (inside out) were recorded with a sampling rate of 200 kHz and an anti-aliasing filter of 50 kHz. In both cases, the currents at 80 mV were not very high (2 to 3 pA). This was much smaller than the case of the dominant K⁺ channel in *Chara* (Hansen et al., 1997), and thus the signal-to-noise ratios were poor in recordings from xylem contact cells. In *Zea mays*, the number of channels in a patch was usually very high (about 10), and these channels showed very fast gating. This did not allow strong filtering of the data, and thus the evaluation suffered from poor signal-to-noise ratios.

The dominant K⁺ channel is identified by the results in Figure 3 showing the dependence of the reversal potential on the potassium concentrations on either side of the membrane. The straight line gives the theoretical Nernst potential Eₜ. At high concentrations on either side ([K⁺]/[K⁺] = 1), reversal potential was close to Eₜ, but deviated at lower concentrations. The usual explanation is that the channel is also permeable to other ions, Ca²⁺ or Cl⁻ in this case. However, it is also possible that where [K⁺]/[K⁺] > 1 high concentrations of KCl from the bath diffused into the pipette, thus decreasing the concentration gradient.

![Figure 2](image)

**Fig. 2.** Time series (section of 5.2 s, 2.3 pA) of patch clamp current in an excised patch of xylem contact cells of maize measured at +100 mV. The smooth line is the "original time course" of the channel current as reconstructed by a Hinkle detector (Schütze and Dräber, 1993).

![Figure 3](image)

**Fig. 3.** Reversal potential measured at different [K⁺]/[K⁺] activity ratios. At ratios higher than 1, the lumen (pipette) side was constant at 200 mM KCl, and the concentration of KCl was varied (and supplemented by sorbitol). At ratios smaller than 1, the cytosolic (bath) side was constant at 200 mM KCl and different solutions were used in the pipette.

The reversal potential $E_{rev}$ in Figure 3 was determined from current-voltage relationships. Figure 4 shows two typical examples. The voltage across the excised patch was imposed by the patch clamp amplifier. The determination of the single-channel current was difficult because of two reasons. The current is small and shows fast flickering. Attempts to reduce the noise by filtering (averaging) resulted in a decrease of the apparent single-channel current (Hansen et al., 1997), and thus could not be applied. In addition the amplitude histograms were very broad. Investigations showed that the most accurate way of determining single-channel currents was to use our evaluation programme to adjust horizontal lines in the original time series to those which an experienced researcher regarded as discrete current levels. This determination of the single-channel current was compared with the value obtained from fitting the amplitude histogram with a sum of gaussians. Because of the low signal to noise ratio and the fast flickering in xylem parenchyma cells, the data points at low currents are mostly missing.

Two different types of K⁺ channels could be distinguished by their apparent IV-curves (Fig. 4) and their gating behaviour (Fig. 5). In most records, a symmetric channel is observed in symmetric solutions. It leads to the symmetric current voltage relationship labelled "SC" in figure 4. Very rarely, a second channel, an inward rectifier "IR", was found for which few openings at positive potentials were observed, as indicated by the points on the abscissa of Figure 4. The currents of both channels were very
Fig. 4. Current-voltage relationships for two different types of K⁺ channels found in excised maize patches at 100 mM KCl + 5 mM CaCl₂ on either side. SC = symmetric channel, IR = inward rectifier.

Similar at negative potentials.

The difference in gating behavior becomes more obvious when the probability of opening is considered. Figure 5 shows the probability of exactly x % of the resident n channels being simultaneously open. This probability was determined by reconstructing the “noise-free” time-series from the original data by means of a 4-th order Hinkley detector (smooth line in Fig. 2; Hansen et al., 1995; Schultze and Draber, 1993) and adding the detected dwell times in each level.

Figure 5 shows the dependence of the open probabilities on membrane potential for the two different types of channels. It is clear that the probability distributions for the symmetric channel being open were similar at positive (SC+) and at negative potential (SC-). However, probabilities for the inward rectifier were different. IR- at negative potentials coincides with the SC curves. At positive potentials the open probability is strongly reduced. It may be suggested that the SC channel is the superposition of the IR channel and a simultaneously resident OR (outward rectifying) channel. However, this would imply that the number of IR channels is always equal to the number of OR channels in order to give the coincidence of the SC and SC+ curves in Figure 5. This would be in contradiction to the finding that records with only IR channels but never records with only the putative OR channel were found.

The influence of Na⁺ on the symmetric K⁺ channel in maize was investigated by replacing 200 mM KCl on the cytosolic side by 100 mM KCl and 100 mM NaCl. This resulted in current-voltage curves with unchanged inward currents, but outward currents reduced by 20 to 40%. This could have been caused by a reduction in the concentration of permeant ions (if only K⁺ and not Na⁺ is transported) or to a blocking effect from the Na⁺ ion being swept into the pore with the current. In order to find out which effect occurs the open probabilities similar to those in Figure 5 were investigated. Figure 6 shows that there is a clear reduction in open probability. In pure Na⁺ solutions (200 mM NaCl plus 5 mM CaCl₂, data not shown) the channels remained very active. This indicates that blocking of the symmetric channel in maize is weaker than expected from findings for example in Chara (Hansen et al., 1997), where fast flickering occurs because of an apparent reduction of single channel conductivity due to the averaging effect of the anti-aliasing filter.

Permeability to NH₄⁺ is of the same magnitude as the permeability to K⁺. Replacing KCl by NH₄Cl on the cytosolic sides of the membrane did not lead to a change in the current-voltage relationship of the symmetric channel (Fig. 7). One record showed a shift in reversal potential of the inward rectifier in asymmetric solutions, but this is unlikely to be statistically significant.
advantage that the cells stay in place.

The results suggest that the inward rectifier (Figs. 4 and 5) imports $K^+$ into the symplast when the membrane potential is hyperpolarized by the $H^+$ pump as has been demonstrated for guard cells (Schroeder et al., 1987). Enhanced activity of the pump in the plasmalemma of xylem contact cells has also been indicated by acidification near xylem elements as observed by Wilson et al. (1991).

These studies are just the beginning. In addition to the two potassium channels described in Figures 4 and 5, other channels are expected to be identified. Studies of these will not be without difficulties, as illustrated by the low single-channel currents, and the fast flickering which precluded averaging by filtering. In addition the high number of channels in a patch renders the analysis of gating difficult, even though multi-channel analysis is possible (Albertsen and Hansen, 1994).

However, for many questions related to plant nutrition, whole cell recordings (Hedrich and Schroeder, 1989) may be more useful. Unfortunately, investigations in this area were not successful. If the pipettes were narrow, the mouth closed soon after breakthrough. If the pipettes were wide, they did not seal. Obviously, wide pipettes as necessary for whole-cell recordings cannot push away the cell wall debris.

Figures 6 and 7 give examples of the questions which now can be investigated on the molecular level of a single transport protein. Further investigation of the blocking effect of $Na^+$ (Fig. 6) will contribute to understanding of the relationship between transport and salt stress (Matsumoto and Chung, 1988). Jeschke et al. (1992) have shown that $Na^+$ reaches the apoplast via the xylem. The ability of the $K^+$ channel to transport $NH_4^+$ is important if aerial uptake of ammonia by leaves is studied near fields fertilized with liquid manure.

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