Chapter 4: The whole plant and cell to cell transport

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Abstract
4.1 Introduction
As outlined in Chapter 1 (section 1.1) the characeae plant appears to be similar to land plants or other submerged plants, as consisting of roots, stems and leaves. Upon closer inspection, these structures are made up from large single cells (with corticating cells in some species), separated by multicellular nodes. Instead of flowers and fruit, there are oogonia and antheridia that produce oospores instead of seeds. However, similarly to higher plants, characeae need transport of nutrients from rhizoids to growing apices, as well as transport of photosynthates throughout the plant. For the plant to work as a whole, electrical and hormonal signals must pass from cell to cell and through the nodal complexes.

As characeae do not possess xylem transport driven by transpiration and root pressure or phloem transport powered by Munch mechanism (Raven 2013), another mechanism is needed to move solutes over distances of up to one meter. Diffusive intracellular movement would be very slow (Boot, Libbenga et al. 2012), so the cytoplasmic streaming becomes important. The large characeae cells played an important role in discovering the actin-myosin interaction that powers the streaming (Shimmen and Yokota 1994). The elegant experiments probing the streaming mechanism are described in section 4.2. The other important transport step is crossing of the nodal complexes. The structure and the transport experiments are described in section 4.3.

The rhizoids share gravitropism with roots of land plants and

So, while there are less experiments on characeae in the whole plant context, the large cells still facilitate unique approaches. Perhaps the usefulness of the whole plant experimental model is yet to come.
4.2 Cytoplasmic streaming

4.2.1 Characeae cell started the research into streaming mechanism

The cytoplasmic streaming in characeae follows the orientation of the fixed chloroplast rows, spiralling along helical path around the cell upwards and downwards, streams separated by a chloroplast-free “indifferent zone” (see figure 1). The sight is beautiful and mesmerising: you are drawn into the green depths of the cell! As the chloroplast rows are fixed, the direction of streaming and, indeed, the whole plant morphology are correlated. The oldest lateral cell in each whorl (and axillary new shoots) appears above the ascending stream. In an excised basal node, new rhizoids are produced below the descending stream. The streams are always oriented at “counter-current” on each side of the nodal complex (Hope and Walker 1975). At constant temperature and supply of ATP, the velocity of streaming is comparatively steady. The large characean cells thus became an excellent system to research the streaming mechanism.

(Kamiya and Kuroda 1956) addressed the question whether the motive force for the streaming was generated by the flowing cytoplasm (sol) or by the surrounding static cytoplasmic system (gel). In an intact cell, the narrow ribbon (~10 µm) of viscous cytoplasm appeared to move with the same speed. The movement spread to the vacuolar sap, which exhibited decreasing speed as function of the distance into the cell. The authors were the first to prepare cytoplasm-enriched fragments (see Methods Box 2.3), where the vacuole was removed by centrifugation and ligation. The volume of the flowing cytoplasm was now much greater and similar speed decrease was observed as function of distance from the gel. Kamiya and Kuroda (1956) proposed a “sliding theory”, where the interaction of the gel and the sol surfaces produces the shearing force that moves the sol along. Having established the site of the interaction, the mechanism could be investigated.

Using light microscopy (Kamitsubo 1966) found cable-like structures on the inner surface of the chloroplasts, fixed on the inner surface of the gel. The structures were visualised in greater detail by electron microscopy (Nagai and Rehbin 1966; Kersey and Wessells 1976). Each cable was found to be composed from about 100 microfilaments. (Kamitsubo 1972) proved that the cables are necessary for the
streaming. Strong illumination of a small patch of the cell detached the chloroplasts and cables, strongly inhibiting the streaming. After a few days the cables regenerated, restoring the streaming. (Williamson 1974), (Palevitz, Ash et al. 1974) applied fragment of skeletal muscle myosin, heavy mero-myosin (HMM) sub fragment S1, and obtained arrowhead structure found in animal systems containing actin (see Figure 4.2b). The ability to bind muscle myosin became accepted as a reliable identifier of filaments as actin.

4.2.2 Actin and myosin in characeae
The actin presence was later confirmed by fluorescently labelled phallotoxin (Nothnagel, Barak et al. 1981) or an antibody raised against actin (Williamson and Toh 1979). There was also pharmacological evidence as cytochalasin, that inhibits actin-based motility in animal systems, also stopped cytoplasmic streaming (Williamson 1972; Shimmen and Tazawa 1983).

Muscle movement involves myosin as well as actin. Thus it seemed likely that myosin also participates in cytoplasmic streaming. (Kato and Tonomura 1977) purified myosin from *Nitella*. (Chen and Kamiya 1975; Chen and Kamiya 1981) performed elegant experiments, where cells were partitioned into two parts and the sol could be moved to one part of the cell by slow centrifugation. In one experiment the part of the cell with no cytoplasm was treated by SH reagent N-ethylmaleimide (NEM) or heat of 47.5 °C. The streaming was not affected, when cytoplasm was returned into that half of the cell. When the same treatments were applied to cytoplasm-containing part of the cell and transferred to the untreated part, the streaming was disrupted. Therefore, similar to muscle, characeae myosin is more sensitive to NEM and heat than actin (for references see (Shimmen and Yokota 1994).

Where is myosin located in the cytoplasm? (Williamson 1975) used tonoplast-free cells (see Methods Box 2.3) to study the effect of ATP depletion. The cytoplasmic organelles became strongly bound to actin cables, but started moving once ATP was re-supplied. Using electron microscopy (Nagai and Hayama 1979) found that endoplasmic organelles had horn-like protuberances with small globular bodies (20 – 30 nm in diameter) arranged in regular arrays (see Figure 4.2c). The authors associated these structures with myosin. (Kachar and Reese 1988) suggested
that myosin is associated with endoplasmic reticulum, which slides along the actin cables.

The velocity of streaming in many plants is in a range of few $\mu$m/sec (close to actin-myosin sliding in skeletal muscle). However, the velocity of streaming in characean cells can reach record 100 $\mu$m/sec! In a series of elegant experiments it was possible to test various combinations of characean, higher plant and skeletal actin and myosin. The sliding of the myosin and actin could be assessed in vitro by coating glass with myosin and observing movement of actin filaments labelled with fluorescence (Kron and Spudich 1986). When the surface of cover glass was coated with an extract of characean cytoplasm (containing myosin), actin filaments from skeletal muscle slid at a velocity close to that seen in the characeae (Shimmen and Yokota 1994). On the other hand, characeae actin could be employed in the tonoplast-free cell and latex beads coated with skeletal muscle myosin were perfused in (Shimmen and Yano 1984). The velocity of this combination was slow. Thus the origin of the myosin determines the speed of the sliding.

By the end of the 90s the procedures for myosin purification from characeae and land plants became more reliable (Shimmen 2007). Genetic characterisation identified land plant myosin and characeae myosin as types of myosin XI. Electron microscopy showed that the molecule has two heads and a tail, which is responsible for binding to organelles (Shimmen 2007). With new molecular and microscopy techniques, the study of various types of myosin has grown into a new field. Recently (Ito, Yamaguchi et al. 2009) discovered that it is the unusual structure of Chara myosin, which contributes to the high speed of streaming. Myosins in many species have positively charged loop 2 with a cluster of lysine residues. The net charge of Chara myosin loop 2 is zero and it lacks the lysine cluster, but there is a positive charge on loop 3. By mutational analysis, the authors demonstrated that the positive charge in loop 3 leads to high ATPase activity, while less charged loop 2 confers higher velocity (see figure 4.2e). They were able to make similar modifications to structure of myosin from Dictyostelium and speed up the sliding.
4.2.3 Some factors affecting streaming

4.2.3.1 Energy source: adenylates and Mg$^{2+}$

The tonoplast-free cell system was convenient for controlling the amounts of ATP, ADP, Pi and Mg$^{2+}$. From initial studies it was clear that ATP provides energy for the myosin head to let go (Williamson 1975; Tazawa, Kikuyama et al. 1976). In absence of ATP, cytoplasmic streaming stops and the organelles are connected to actin cables by rigor cross-bridges (Nagai and Hayama 1979). (Shimmen 1978) found that maximum streaming velocity was reached at ATP concentrations above 200 µM. The normal concentration of ATP in the cytoplasm is 0.5 – 3.4 mM, well above the saturation level for fastest streaming speed (see section 2.2.2). (Reid and Walker 1983) found linear relationship between streaming and ATP concentration (see Fig. 4.2a). Consequently, inhibitors that affect ATP concentration also affect streaming (see Table 12.1 in Hope and Walker 1875). (Reid and Walker 1983; Shimmen 1988) perfused tonoplast-free cells with cytoplasm-like medium containing in mM: 1.6 ATP, 0.6 ADP, 0.8 AMP, 14.7 Pi and 2 pyrophosphate and observed streaming speed very close to that in intact cells. At higher concentrations ADP, Pi and pyrophosphate inhibited streaming in a competitive manner with ATP. If the cells were perfused with medium containing ADP and no ATP, streaming was observed after a delay. A hypothesis that ADP was converted to ATP by adenylate kinase bound to chloroplasts was supported by addition of adenylate kinase inhibitor to ADP medium, which abolished streaming recovery.

In muscle contraction Mg$^{2+}$ is necessary for the ATPase reaction of myosin to transduce chemical energy into sliding force. Shimmen (1978, 1988) found that streaming was severely inhibited, when Mg$^{2+}$ concentration was lower than that of ATP. Mg$^{2+}$ chelator in the perfusion medium caused irreversible inhibition of streaming in the tonoplast-free cells and permeabilized cells (Shimmen and Tazawa 1983). Thus Mg$^{2+}$ is necessary not only for myosin ATPase reaction, but also for maintenance of the streaming system. If Mg$^{2+}$ was added as SO$_4^{2-}$, streaming was also inhibited. SO$_4^{2-}$ seemed to compete with ATP.

4.2.3.2 Temperature, pH and light

The sensitivity of cytoplasmic streaming to temperature was observed for more than a century (see Shimmen and Yokota 1994 for references). A recent detailed study was
done by (Shimmen and Yoshida 1994). In the tonoplast-free cells, where the medium pH and calcium concentrations were well buffered, the streaming velocity increased linearly as temperature increased from 0.5 – 25 °C. Some intact cells did show similar temperature dependence, while others exhibited steeper drop in streaming speed as temperature decreased from 15 to 10 °C (see figure 4.3b). The authors suggested that the decline might result from changes in cytoplasmic pH and [Ca$^{2+}$]. Their homeostasis depends on active transport, which may be affected at some critical temperature. On the other hand (Ding and Tazawa 1989) reported that ATP concentration in the cytoplasm is temperature independent.

The tonoplast-free system was also convenient for exploring the effect of pH (Fujii, Shimmen et al. 1979; Tazawa and Shimmen 1982). As actin and myosin are proteins, their dependence on pH was expected. The streaming velocity is, indeed, highest at neutral pH and decreases at both low and high pH. The ability to manipulate the internal pH in the tonoplast-free cells enabled (Shimmen and Tazawa 1985) to understand the effect of myrmicacin (carboxylic acid secreted by leaf-cutting ant). This fatty acid strongly inhibits cytoplasmic streaming, but only if it is administered in low pH medium (pH 4.5). The undissociated form of the carboxylic acid is abundant at low pH and can permeate the plasma membrane. At near neutral pH in the cytoplasm (see section 2.2.2), the acid dissociates and cytoplasm is acidified. Similar effect can be observed with other carboxylic acids.

(Barr and Broyer 1964) reported higher velocity of streaming upon illumination. This effect was abolished by application of photosynthesis inhibitor DCMU (Plieth and Hansen 1992). Possible explanation was provided by (Miller and Sanders 1987), who measured a decrease in cytoplasmic calcium concentration upon illumination. The authors proposed that there is a Ca$^{2+}$ uptake by the photosynthesising chloroplasts. The effect of cytoplasmic Ca$^{2+}$ on streaming is discussed in the next section.

### 4.2.4 Calcium concentration and action potential

(Hayama and Tazawa 1980) isolated cytoplasmic drops from internodal cells of *Chara* by fast centrifugation. The drops contained rotating chloroplasts. The authors assumed that same actin-myosin mechanism was involved as in cytoplasmic
streaming. They injected either Ca\(^{2+}\) or K\(^+\) iontophoretically by inserting two electrodes: one filled by KCl, the other with CaCl\(_2\) and applied voltage between them. The rotation stopped or slowed only when Ca\(^{2+}\) was injected. After some minutes the rotation spontaneously recovered. Different combinations of ions were tried: K\(^+\) and Mg\(^{2+}\) had no effect; Sr\(^{2+}\) had same effect as Ca\(^{2+}\); Mn\(^{2+}\) and Cd\(^{2+}\) induced slow irreversible decline in rotation; Ba\(^{2+}\) sometimes behaved similarly to Ca\(^{2+}\), sometimes also produced slow irreversible decline in rotation.

(Kikuyama and Tazawa 1982) injected CaCl\(_2\) directly into cytoplasm of intact *Nitella* cells and also produced transient inhibition of streaming. (Williamson 1975; Hayama, Shimmen et al. 1979) studied the effect of Ca\(^{2+}\) concentration on streaming in tonoplast-free cells. The streaming was inhibited at high Ca\(^{2+}\) concentrations of ~ 1 mM and recovered only partially after Ca\(^{2+}\) removal. (Tominaga and Tazawa 1981) found that streaming in tonoplast-free cells became more sensitive to Ca\(^{2+}\) as a function of time after perfusion.

The connection between an increase in Ca\(^{2+}\) and “freezing” of the cytoplasm also emerged from study of the characeae action potential (AP) (see section 3.2.2). (Williamson and Ashley 1982) used aequorin to measure the Ca\(^{2+}\) concentration in the cytoplasm of intact characean cells at the time of an AP (see Figure 4.3). The peak concentration was 43 µM in *Nitella* and only 6.7 µM in *Chara*. The streaming stopped completely and restarted slowly after some minutes (see Figure 4.3b). So, the data from the tonoplast-free system were misleading: the Ca\(^{2+}\) concentration necessary to stop the streaming was much too high. The problem was confirmed by employing the permeabilized system, where 1 – 10 µM Ca\(^{2+}\) stopped the streaming (Shimmen and Tazawa 1983) in agreement with the aequorin results (Williamson and Ashley 1982). The main difference between the tonoplast-free and permeabilized systems is that in the former the native cytoplasm is washed away or severely disrupted, while in the latter the cytoplasm remains in place.

Which component of the streaming is Ca\(^{2+}\) sensitive? The answer was once again approached by substitution from animal systems. Myosin in skeletal muscle has no Ca\(^{2+}\) sensitivity. (Shimmen and Yano 1986) substituted skeletal muscle myosin coated beads and sliding occurred irrespective of calcium concentration. The skeletal
muscle has troponin-tropomyosin incorporated into the actin filaments and calcium is needed to activate sliding. If troponin-tropomyosin complex was incorporated into characean actin, then higher calcium was also needed to start streaming. Thus in the characeae the calcium sensitivity is associated with myosin, explaining why the loss of native cytoplasm in the tonoplast-free system changed the calcium sensitivity.

(Tominaga, Wayne et al. 1987) performed elegant experiments with both tonoplast-free cells, which were perfused slowly to retain calcium sensitivity, and permeabilized system. In various animal and mould systems myosin produces sliding either in phosphorylated or de-phosphorylated state. Introducing protein phosphatase-1 into tonoplast-free cells abolished the streaming stoppage at high Ca^{2+} concentrations. The inhibitors of phosphatase-1, on the other hand, totally inhibited streaming. The authors hypothesised that characean myosin only promotes streaming in de-phosphorylated state. ATP-γ-S irreversibly inhibited the recovery of streaming, which was stopped by high Ca^{2+} concentration (thio-phosphorylated proteins are not de-phosphorylated with phosphatases). The phosphorylation therefore requires Ca^{2+}, protein kinase and ATP. Tominaga et al. (1987) then applied their model to the streaming stoppage at the time of AP (see Figure 4.5). The streaming stops within 1 second of AP onset. Thus the kinase-mediated phosphorylation is a fast process. Once the Ca^{2+} concentration declines, the kinase activity also diminishes. The recovery is much slower, taking minutes after the Ca^{2+} concentration diminished. The authors suggest that the phosphatase is activated indirectly through Ca^{2+} binding to calmodulin. This hypothesis was supported by (Tominaga, Muto et al. 1985), who found that calmodulin inhibitors only abolish streaming recovery after high Ca^{2+} concentration, rather than steady state streaming.
4.3 Cell to cell transport

4.3.1 Structure of plasmodesmata
References


