Ion fluxes, auxin and the induction of elongation growth in *Nicotiana tabacum* cells

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Abstract

Immobilized cultured tobacco cells become polarized upon the addition of naphthalene-1-acetic acid and start to elongate from an initial spherical shape. The question as to how a diffuse-growing cell forms a polar axis is addressed here with approaches successfully applied to the study of tip growth. With two kinds of vibrating probes the electric current flow and proton fluxes were mapped around such elongating cells. No consistent polar pattern of ion fluxes, which is typical for actively tip-growing cells, was detected. Therefore, other signals must provide the positional information needed for polar axis formation. Furthermore, neither a specific pattern of intracellular Ca²⁺ concentration nor a polar distribution of putative ion-channel antagonist-binding sites were found in elongating tobacco cells. Auxin flux, on the other hand, was found to be important as TIBA, an inhibitor of polar auxin transport, clearly inhibited elongation in a concentration-dependent way. Cross-linking of arabinogalactan-proteins with the β-Yariv reagent also resulted in inhibition of elongation. A model is proposed for the induction of polar growth where localized auxin efflux starts a signal cascade that triggers molecules that reorient microtubules. These then guide cellulose deposition in the cell wall, which in turn alters cell wall mechanics and leads to elongation. In this scheme, arabinogalactan-proteins are not causal agents but are probably important regulators of growth and survival of the cell.

Key words: Polar growth, auxin, elongation, vibrating probes, ion fluxes.

Introduction

Plants grow by cell division and differentiation of the newly formed cells. During differentiation most plant cells expand dramatically. Tip-growing cells, such as pollen tubes and root hairs, deposit new membrane and cell wall material exclusively at their growing tip (Miller et al., 1997; Obermeyer and Bentrup, 1996; Shaw et al., 2000). The majority of differentiating cells in a developing plant, however, shows diffuse growth, i.e. the cells extend over the entire surface. Many of these cells exhibit polar growth preferentially along one axis, hereby determining the shape of plant organs, especially of roots and stems (Lyndon, 1990).

In the current hypothesis elongation begins when stress relaxation in the cell wall is mediated by expansins. This would normally result in isotropic expansion. To obtain anisotropic or polar growth of plant cells, cellulose microfibrils are laid down transversally to the axis of elongation forming a spring-like structure that reinforces the cell radially and favours longitudinal expansion (Green, 1980). The direction of expansion is thus controlled by the orientation of cellulose microfibrils, which is thought to be laid down by the orientation of cortical microtubules underneath the plasma membrane. These microtubules are proposed to serve as guides for the cellulose.
synthesizing complexes that can move along the plasma membrane (Giddings and Staehelin, 1991). Most of the studies of cell elongation have been performed by stimulating the growth rate of cells that were already elongating. In coleoptiles, for instance, a controversy developed in such studies as to whether or not the epidermis is a unique target for auxin in elongation growth. Some results are in favour of the epidermis, whereas others such as peeling experiments suggest that the cortical cells are also involved (Cleland, 1991). In growing Arabidopsis roots a complex network of auxin movements has recently been described suggesting that more than one sort of tissue is responsive to the hormone (Gälweiler et al., 1998; Müller et al., 1998).

To circumvent the problems of interpreting results in an organ with a diverse population of cells, it was decided to study the induction of polar growth in individual cells. A single cell culture, based on protoplasts that were isolated from the leaf mesophyll of tobacco, was used. In the intact plant this tissue is composed of cells that undergo no polar elongation. Isolated protoplasts were immobilized on an agarose layer and manipulated in their development by simple hormone signals. When auxins and cytokinins are applied together, the cells enter the cell cycle. Auxin alone induces the cells to elongate in a dose-dependent way (Vissenberg et al., 2000). With tools and methods that have been used to study tip growth, the question as to how a spherical cell forms an axis and starts polarized elongation upon treatment with auxin was addressed.

From the results it is concluded that the specific current patterns found around tip-growing cells are absent and that other mechanisms must be more important in determining the axis of polarity. In this context auxin efflux was found to be important since TIBA, an inhibitor of polar auxin transport, clearly inhibited elongation in a concentration-dependent way.

Materials and methods

Cultures

Nicotiana tabacum L. cv. Petite Havana SR1 plants were grown from seeds under sterile conditions on a Murashige and Skoog medium without hormones (4.7 g l⁻¹; Duchefa, The Netherlands), supplemented with 10 g l⁻¹ sucrose and solidified with 4 g l⁻¹ Gelrite (Duchefa, The Netherlands), pH 5.7. Tobacco protoplasts were isolated (following the method of Stickens et al., 1996) by incubating healthy leaves in 2% cellulase-R10 and 0.2% macerozyme-R10 (Yakult Honsha Co., Ltd., Japan) dissolved in K3A culture medium at pH 5.7 (Potrykus and Shillito, 1986) for 5 h. Living protoplasts were isolated from cell debris by filtration and centrifugation at 60 g and immobilized on the surface of an agarose layer (K3A culture medium solidified with 1.2% agarose) in Petri dishes. The protoplasts were then covered with K3A culture medium supplemented with 5 μM naphthalene-1-acetic acid (NAA, auxin; Sigma). The regenerating cells were kept in culture at 22 °C in a 16 h photoperiod at a light intensity of 24 μmol s⁻¹ m⁻² (Philips tlm 65W/33).

The auxin transport inhibitor 2,3,5-triiodobenzoic acid (TIBA; Sigma) was added to the culture medium at concentrations of 2, 4, 8 or 16 μM. The β- and α-Yariv reagent (Biosupplies, Australia) were supplied at a concentration of 25 or 50 μM.

Measurements of extracellular currents

After 1 h adaptation in the experimental medium (culture medium 10 times diluted with 0.4 M sucrose to decrease the electrical conductivity) elongating cells were transferred to the stage of an inverted microscope and currents were measured with a three-dimensional recording vibrating voltage probe (for technical details see Weisenseel et al., 1992; Meyer and Weisenseel, 1997). Using a custom-made algorithm the x, y and z components of the endogenous currents were converted to current-density vectors and plotted in a system of Cartesian coordinates in which the origin was always the position of measurement (Fig. 1B, C). In this system the x and y axes form the horizontal plane.

Measurements of proton currents

Extracellular proton fluxes were measured around elongating tobacco cells using a proton-specific vibrating electrode (Kühlreiber and Jaffe, 1990; Kochian et al., 1992; Shipley and Feijó, 1999). Details of probe construction are described elsewhere (Feijó et al., 1999). Signals were measured between the probe and a dry reference electrode (World Precision Instruments) inserted into the sample bath and amplified using a purpose-built electrometer (Applicable Electronics, Sandwich, MA, USA). Electrode vibration and positioning was achieved with a stepper-motor-driven three-dimensional positioner. Data acquisition, preliminary processing, control of the 3-D electrode positioner, and stepper-motor controlled fine focusing of the microscope stage were performed with ASET software (Science Wares and Applicable Electronics). The self-referencing vibrating probe oscillated along an excursion of 10 μm. A typical measurement cycle was completed in c. 2.0 s, and included a tunable settling time after each move, two measurement periods (one at each extreme) and the excision time. The measurement close to the membrane was then subtracted by the one further away. This subtraction represents the self-referencing feature of the probe. Proton flux at the surface of the cells was measured by vibrating the electrode tip up to 5 μm of the membrane. Background references were taken at more than 0.5 mm from any cell and the values were subtracted from the μV differential recordings during data processing using Microsoft Excel 4.0. The vibrating electrode system was attached to a Nikon Eclipse TE-300 inverted microscope that was housed inside a copper sheet Faraday cage supported on a vibration-free platform.

Fluorescent stainings

Cellulose was stained using a 1% solution of Congo red (Merck C122120) in culture medium for 10–30 min (Verbelen and Stickens, 1995; Verbelen and Kerstens, 2000). Calcium channel antagonist-binding sites were investigated by incubating protoplasts and cells in DM-Bodipy-dihydropyridine (DHP) and DM-Bodipy-phenylalkylamine (PAA; Sigma) at final concentrations of 1, 2.5, 5 or 10 μM (made from 250 μM stock solutions in DMSO) for 30 min (Vallée et al., 1997).
Observations of the stainings were carried out with a BioRad MRC-600 confocal laser scanning microscope equipped with a Zeiss Axioskop.

Results

Ion fluxes

Knowing that in tip-growing plant cells and organs ionic current, defined as the net movement of cations, always enters the growing parts and leaves the non-growing parts, net ion fluxes around diffuse-growing tobacco mesophyll cells were mapped using a three-dimensional voltage recording vibrating probe. The probe was positioned above both ends and the middle of the cell, resulting in three separate measurements per cell. The endogenous current of a total of 40 elongating tobacco cells was measured and evaluated. Of the cells measured approximately 50% showed current influx all along the upper surface and 20% showed polarity, i.e. there was one side with influxes and the other end with effluxes. The remaining cells displayed a combination of inward and outward current. Figure 1 shows two typical examples. In Fig. 1A the positions of the probe are shown and the boxes indicate the sampling spots along the cell. Figure 1B shows the current vectors at three different sites near the cell wall, indicating a net inward current in all three positions (1, 2 and 3). In Fig. 1C a cell is shown where ionic current entered the cell at positions 1 and 2 and left at position 3. However, no specific and consistent pattern of inward and outward current was detected, in contrast to tip-growing structures that are well characterized in this aspect.

In another set of experiments, proton fluxes were mapped around growing cells using a proton-specific vibrating probe. In Fig. 2A a young cell at the onset of elongation is shown together with its pattern of proton fluxes. Bar = 50 μm.
magnitude of the flux. Fluxes around an older well-elongated cell are drawn in Fig. 2B. Figure 3 is a graphical display of the output of the proton-specific vibrating probe. In this case a cell was measured at six different sites along its surface as indicated by the letters a to f in Fig. 3. After a set of measurements at each position, the probe was repositioned far away from the cell to yield a reference (r) value. As indicated by the negative numbers, protons entered the cell at all six positions, a result that was obtained in all (=40) cells examined. In contrast to the results obtained with the current measuring probe, no proton-efflux was ever detected.

Dihydropyridine and phenylalkylamine binding

With the use of fluorescent dihydropyridine and phenylalkylamine dyes (DM-Bodipy-DHP and DM-Bodipy-PAA, respectively), the presence of antagonist-binding sites of calcium channels was examined in fresh tobacco protoplasts and in elongating cells. With both dyes at 1 or 2.5 μM no polarity-specific binding sites at the plasma membrane were stained. At higher dye concentrations (5 or 10 μM) the complete plasma membrane was uniformly stained (Fig. 4).

Auxin transport

To assess the importance of polar auxin transport for cell elongation, cells were grown in culture medium supplemented with 1 mg l⁻¹ NAA alone or with 1 mg l⁻¹ NAA and 1, 2, 4 or 8 mg l⁻¹ TIBA, a known inhibitor of polar auxin transport. With NAA as the only auxin, about 70% of the cells elongated (i.e. cell length ≥ 2 times cell width). TIBA inhibited elongation substantially (Fig. 5). At equimolar concentration TIBA reduced the elongation by 50% (interpolated value for 5 μM TIBA) which suggests a 1:1 competition between both compounds (NAA and TIBA) at the level of the auxin exporter. The level of inhibition by TIBA exceeded by far the slight inhibition caused by NAA at doses equal to the highest TIBA dose given (see black bar at the right of the graph).

Yariv reagent effect

From previous work (Vissenberg et al., 2000) it was concluded that the deposition of a regular cell wall is a prerequisite for elongation in the tobacco cells. In Fig. 6 the effect of different Yariv phenylglycosides, better known as Yariv reagents, is shown. In the presence of the β-D-glucosyl Yariv reagent which selectively binds arabinogalactan-proteins at the plasmalemma (Serpe and Nothnagel, 1996) or cell wall (Li et al., 1992), cells remained spherical, whereas the inactive α-D-galactosyl Yariv reagent did not influence normal cell length or width. From Congo red staining it became clear that addition of the β-Yariv reagent interfered with the deposition of cellulose. Treated cells exhibited no cellulose-based fluorescence (Fig. 7A) whereas both non-treated (Fig. 7B) and α-Yariv reagent-treated cells (Fig. 7C) had a brightly fluorescent cell wall.

Fig. 3. Graphical display of the output of the proton-specific vibrating probe. Proton fluxes are shown at six different places around an elongating tobacco cell (a–f), together with references (r). At all positions protons entered the cell as indicated by the negative values for the fluxes, whereas the background fluxes are more or less zero.

Fig. 4. Localization of calcium channel antagonist-binding sites in freshly isolated protoplasts and growing cells of tobacco with Bodipy-DHP and Bodipy-PAA. Uniform staining of the plasmamembrane with the dye at high concentration. Bar = 25 μm.

Fig. 5. Effect of TIBA on the elongation response of tobacco cells. With only NAA (at 5 μM), about 70% of the cells elongated (0 μM TIBA). Addition of increasing concentrations of TIBA (2, 4, 8 and 16 μM, respectively) inhibited elongation in a concentration-dependent way. Addition of 16 μM NAA (black bar at the right) only slightly inhibited elongation.
Discussion

Many studies on the mechanism of polar growth have been carried out with tip-growing cells in which the deposition of new cell wall material is strictly confined to the growing tip by exocytosis. This is a report on the induction of polar growth in diffuse-growing cells, where new cell wall material is deposited all over the cell surface. Diffuse-growing tobacco cells develop from spherical cells into cells elongating along one axis upon addition of auxin. Elongation starts when randomly organized cortical microtubules rearrange parallel to each other and transversely to the cell’s long axis (Vissenberg et al., 2000). With tools that have been used previously to study tip-growth, the early signs of polarized growth prior to the rearrangement of the cytoskeleton were examined.

In developing fucoid zygotes and growing pollen tubes, ionic current always enters the growing tip (i.e. the rhizoid and pollen tube tip, respectively) and leaves the non-growing part (thallus or pollen grain) (Jaffe, 1966; Nuccitelli and Jaffe, 1976; Weisenseel et al., 1975). With the use of ion-sensitive vibrating probes much of the endogenous current was found to be calcium and proton fluxes which cause specific tip-focused ion-gradients inside the cytoplasm (Feijô et al., 1999, 2001; Malhô et al., 1994; Pierson et al., 1996). In tobacco cells no consistent current pattern was detected, but protons always entered the cells. Around single maize suspension cells, H⁺ effluxes have been reported (Kochian et al., 1992), which were not measured with the ion-sensitive vibrating probe in tobacco cells. Since proton efflux, driven by plasma membrane H⁺-ATPase activity (Oufattole et al., 2000) and respiration has to be present in the cells, the larger influx of protons, overriding the efflux, may be ascribed to H⁺/sucrose or H⁺/monosaccharide co-transport activity (reviewed in Lalonde et al., 1999; Sauer and Stadler, 1993). Another plausible candidate for the generation of a large proton-influx could be the H⁺/auxin co-transporter (Lomax et al., 1995). However, as NAA enters the cells simply by diffusion (Delbarre et al., 1996), the contribution of the auxin-uptake machinery to the H⁺-flux is negligible. Preliminary explorative measurements on calcium currents indicated no specific Ca²⁺ fluxes along the cells (data not shown). Moreover, with the fluorescent dyes dihydropyridine- and phenylalkylamine-analogue, no polarized distribution of putative L-type voltage-gated calcium channels (Knaus et al., 1992; Shaw and Quatrano, 1996; Vallée et al., 1997) was observed at the plasma membrane comparable with the staining in Fucus zygotes (Shaw and Quatrano, 1996). Once inside the cell, these dyes clearly stained the endoplasmic reticulum. As the endoplasmic reticulum is a calcium store, the occurrence of calcium channels in its membrane is expected (Bush et al., 1989) but sequestration of the dye into the ER is another possibility for the heavy staining. So far only H⁺-ions have been found as a part of the endogenous current of tobacco cells.

It thus seems that in diffuse-growing tobacco cells not polarized ion fluxes but other mechanisms are responsible for defining the axis of cell growth. From the literature it is known that inside plants auxin moves in a polar way (Lomax et al., 1995). As the lipophilic auxin NAA enters the cell in a non-polarized way, i.e. simply by diffusion (Delbarre et al., 1996), one of the probable mechanisms for polarization is the polar efflux of auxin. Addition of the inhibitor of polar auxin efflux, TIBA, indeed interfered with elongation in a concentration-dependent way. Support for this assumption comes from the results of Goldsworthy and Mina who found that, in the presence of IAA alone, weak electric currents stimulated callus growth, whereas the addition of TIBA abolished this stimulation (Goldsworthy and Mina, 1985). To date it is not known whether the proteins responsible for auxin efflux are polarly localized in the elongating tobacco cells because in tobacco no such proteins have yet been characterized. In the Arabidopsis root, however, parts of the efflux carrier were recently identified and shown to be cytolocalized in a polar way (Gälweiler et al., 1998; Müller et al., 1998). These researchers proposed a scheme
with specific auxin movement in the elongation zone of the root. The experimental data reported above support the importance of polar auxin transport for the induction of cell elongation.

After the development of a polar axis, the next step is the rearrangement of the cytoskeleton and the cell wall constituents. It has already been shown that the cortical microtubules and the cellulose microfibrils rearrange at the onset of polar growth (Vissenberg et al., 2000). In the present study it was also found that the arabinogalactan-proteins that function in cell adhesion and cell signalling, are involved in the elongation process of tobacco cells. From the β-Yariv reagent it is known that it interferes with cell division in suspension-cultured Rosaceae cells (Serpe and Nothnagel, 1994) and with elongation in suspension-cultured carrot cells that were induced to elongate and in the Arabidopsis root (Willats and Knox, 1996). As discussed by Du et al. it is suggested that arabinogalactan-proteins (AGPs) could associate specifically with charged pectic galacturonans and galacturonorhamnans, or that they could act in a ligand-receptor association (Du et al., 1996). Binding of the β-Yariv reagent to AGPs leads to the aggregation of AGPs which disrupts the normal interactions with other cell surface components or triggers the programmed cell death pathway (Gao and Showalter, 1999).

In conclusion, it can be stated that one of the first steps during the induction of polar growth in tobacco cells is the development of polarized sites of auxin efflux in the cells. This may set an axis in the cells and trigger a still unknown signal transduction pathway that transmits information to molecules that orient the cortical microtubules into parallel arrays (Chan et al., 1999). The concomitant parallel deposition of cellulose microfibrils in the cell wall favours longitudinal expansion. Arabinogalactan-proteins are most likely an important factor controlling continued plant cell growth.

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References


Delbarre A, Muller P, Imhoff V, Guern J. 1996. Comparison of mechanisms controlling uptake and accumulation of 1,4-dichlorophenoxy acetic acid, naphthalene-1-acetic acid and indole-3-acetic acid in suspension cultured tobacco cells. Planta 198, 532–541.


Jaffe LF. 1966. Electrical currents through the developing Fucus egg. Proceedings of the National Academy of Sciences, USA 56, 1102–1109.


Polarized growth of tobacco cells


