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Plasmodesmata transport of GFP alone or fused to potato virus X TGBp1 is diffusion driven

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Summary. Plasmodesmata (Pd) provide a pathway for exchanging various macromolecules between neighboring plant cells. Researchers routinely characterize the mobility of the green-fluorescent protein (GFP) and GFP fusions through Pd by calculating the proportion of sites in bombarded leaves which show fluorescence in multiple cell clusters (% movement). Here, the Arrhenius equation was used to describe the temperature dependence of GFP and GFP-TGBp1 (potato virus X triple gene block protein1) movement, using % movement values, and to calculate the activation energy for protein transport. The resulting low activation energy indicates GFP and GFP-TGBp1 movement are diffusion driven. Furthermore, GFP movement is inversely proportional to the leaf surface area of expanding leaves. The increase in leaf area results mainly from cell expansion during the sink–source transition. The increasing cell size results in lower Pd density, which decreases the probability that a GFP attains an open Pd by diffusion. The decline in GFP movement as leaf area expands indicates that, in addition to GFP diffusion through Pd, attaining an open Pd by undirected diffusion might be limiting for Pd transport. In summary, this report provides a new quantitative method for studying Pd conductivity.

Keywords: Plasmodesmata transport; Green-fluorescent protein; Viral movement protein; Potato virus X; Diffusion; Intercellular transport.

Abbreviations: Pd plasmodesmata; SEL size exclusion limit.

Introduction

Plasmodesmata (Pd) are cytoplasmic bridges between plant cells that function in the transport of macromolecules, including proteins and nucleic acids. The plasma membrane is continuous between cells through Pd and provides their outer boundary (Robards and Lucas 1990, Ding 1998). The endoplasmic reticulum stretches between cells through Pds, forming the core desmotubule. The cytoplasmic sleeve lies between the plasma membrane and desmotubule and is the primary space for transport of molecules between cells (Robards and Lucas 1990).

Size exclusion limit (SEL) is an important measure of Pd function and conductivity. The SEL describes Pd apertures and is often measured by injecting various fluorescent molecules into cells to identify the sizes (molecular weights) of molecules that can move across Pd or are excluded from moving (Epel 1994, Waigmann and Zambryski 1994). For tobacco leaves, a typical Pd has been described as one having an SEL of <1 kDa. The discovery that viral movement proteins, such as tobacco mosaic virus (TMV) P30 and potato virus X triple gene block protein 1 (PVX TGBp1), could expand Pd SEL to allow movement of fluorescent dextrans up to 20 kDa between cells demonstrated that there is a mechanism triggered by viral movement proteins to increase Pd SEL (Wolf et al. 1991, Waigmann et al. 1994, Angell et al. 1996, Lough et al. 1998, Howard et al. 2004).

The dynamics of movement across Pd has been studied with fluorescent proteins and dyes. Researchers have microinjected high concentrations of fluorescent proteins and dextrans into single leaf epidermal and mesophyll cells and shown that movement of macromolecules across Pd can be quite rapid (Wolf et al. 1991, Derrick et al. 1992, Noueiry et al. 1994, Waigmann et al. 1994, Angell and Baulcombe 1995, Ding et al. 1995, Waigmann and Zambryski 1995, Wymer et al. 2001, Howard et al. 2004). Microinjecting leaf epidermal or mesophyll cells can be technically challenging because of the dimensions of the leaves and the large vacuoles. Therefore, researchers have

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opted to use biolistic bombardment to deliver plasmids expressing green-fluorescent protein (GFP) and GFP fusions to single epidermal cells (Itaya et al. 1997, 1998; Morozov et al. 1997; Oparka et al. 1999; Kotlizky et al. 2000, 2001; Yang et al. 2000; Tamai and Meshi 2001; An et al. 2003; Tamai et al. 2003). Biolistic bombardment produces a large population of single expressing cells in a leaf and at least 10-fold more sites can be analyzed by biolistics than by microinjection in the same time period, making it easier to gather a large population of data that can be analyzed statistically. The data is typically reported as "% Movement", which is a measure of how often a fluorescent marker is seen to move from a transfected cell into neighboring cells. However, using biolistics to deliver plasmids requires longer incubation times for gene expression to occur, and data can only be collected after the protein has moved into neighboring cells. This method has been used successfully to investigate the effects of environmental and developmental changes on Pd conductivity.

Further evidence that Pd SEL can be up- or downregulated came from ultrastructural studies showing that Pd architecture changes with development. Detailed analysis of Pd architecture was conducted, comparing tobacco leaves during the sink-to-source transition (Itaya et al. 1998, Oparka et al. 1999, Roberts et al. 2001, Krishnamurthy et al. 2002). Electron microscopic analysis showed that sink tissues contained simple single-channel Pd, while source tissues contained a higher percentage of complex branched Pd. Following delivery of plasmids expressing GFP and GFP fusions into tobacco leaves, GFP movement in young photosynthetic sink leaves was found to be greater than that in mature photosynthetic source leaves (Oparka et al. 1999). Several researchers have suggested that reduced GFP movement could be explained by changes in Pd architecture that may downregulate Pd conductivity as leaves develop from sink to source (Lucas and Wolf 1993, Oparka et al. 1999, Oparka and Cruz 2000, Roberts et al. 2001, Zambryski 2004). The change in Pd architecture correlates with a change in Pd conductivity of GFP and GFP fusions, leading researchers to suggest that Pd structure is a determinant of its function.

Additional factors that downregulate Pd permeability during development or stress have been identified, including calcium, ATP, and plant hormones (Cleland et al. 1994, Ding et al. 1996, Botha and Cross 2000, Baluska et al. 2001). These factors are unrelated to Pd architecture, suggesting that Pd architecture alone does not determine Pd conductivity. Specifically, *ise1* and *ise2* mutants of *Arabidopsis thaliana* show increased Pd SEL with no change in Pd architecture, and the proteins do not localize to Pd (Kim et al. 2002). In fact, *ISE2* encodes an RNA helicase which links Pd conductivity to the RNA silencing machinery (Kobayashi et al. 2007). Additional factors, such as myosin VIII, calreticulin, and actin, have been identified in Pd which may regulate Pd expansion and contraction (Blackman and Overall 1998; Radford and White 1998; Reichelt et al. 1999; Baluska et al. 1999, 2001, 2004).

More recent studies have described two modes of protein movement across Pd, namely, "targeted" or "nontargeted" movement (Crawford and Zambryski 2000). Targeted movement is a term used to describe the movement of non-cell autonomous proteins, including many viral movement proteins, that interact with the Pd apparatus (Crawford and Zambryski 2001). For example, electron micrographs have shown that the TMV movement protein and cucumber mosaic virus (CMV) 3a movement protein accumulate in Pd (Ding et al. 1992, Itaya et al. 1998). Nontargeted movement, also termed passive or diffusive movement, refers to the movement of non-cell autonomous proteins that are sufficiently small to pass between cells without increasing the Pd SEL. These proteins move without interacting with the Pd apparatus and include examples such as GFP, LFY, and SHR (Crawford and Zambryski 2001).

Considering the factors modulating Pd SEL that have been identified and recent descriptions of nontargeted and targeted movement of molecules across Pd led us to investigate methods for studying the energy requirements for protein transport across Pd. While microinjection is more useful than biolistics for viewing and recording the dynamics of protein movement in real time, we demonstrate in this report that data obtained from experiments using biolistics can be employed for studying the activation energy of Pd transport. The activation energy of a process is determined by measuring its temperature dependence. This approach is routinely used for transmembrane ion or metabolite transport, to discriminate between passive and active transport processes (Hille 1992). Passive, diffusion-driven transport processes have a significantly lower activation energy ($\leq$30 kJ/mol) compared with active transport processes ($>$50 kJ/mol) (Obermeyer and Tyerman 2005). The thermodynamic concept of activation energy is widely applicable. The activation energy for RNA transport through nuclear pores has been determined (Clawson and Smuckler 1978), and even the activation energies of the metabolic rates of different organisms have been estimated and compared (Gillooly et al. 2001).

In this study, data was collected by recording the proportion of sites showing GFP movement at different temperatures and in expanding leaves. Experiments were conducted using GFP alone and fused to the PVX TGBp1

protein. PVX TGBp1 is a viral movement protein that is mainly cytosolic and does not target Pd but increases Pd SEL in the absence of virus infection. The temperature dependence of GFP movement was used to calculate the activation energy of GFP and GFP-TGBp1 transport through Pd. The resulting activation energy indicates that GFP movement from cell to cell via Pd is a diffusion-driven process. The dependence of GFP movement on leaf size is discussed in the context of how an increase in cell size is expected to affect diffusion-driven transport through Pd.

Material and methods

Plant material and leaf area measurements

Growth chambers were maintained at 22 °C with 8 h daylight for growing *Nicotiana tabacum* (cv. Petit Havana) seedlings. Plants were grown for 12 days before the first leaves were excised for bombardment. For experiments illustrated in Fig. 3, four leaves were excised from 2 plants, each week for 8 weeks from a total of 16 plants. Leaf area was measured using tracings drawn on graph paper. Squares with 1 cm long sides were drawn and the number of complete squares contained within each trace as counted. Squares bisected by the trace were scored as 0.5 cm squares and added to the total number of complete squares.

Biolistic bombardment of plasmids and microscopy

The pRTL2-GFP and pRTL2-GFP-TGBp1 plasmids were prepared previously (Yang et al. 2000). A mixture of 0.5 mg of gold particles and 5 μg of plasmids was loaded into gene-delivery cartridges and then into the Helios Gene Gun. Tobacco leaves were bombarded at a pressure of 160 kPa, as described previously (Krishnamurthy et al. 2002). For experiments determining the activation energy, bombarded leaves were incubated for 24 h at the required temperatures before the frequency of GFP movement was measured.

GFP and GFP-TGBp1 fluorescence was studied with a Nikon E600 (Nikon Inc., Tokyo, Japan) epifluorescence microscope with a Nikon B2A filter cube. Images were captured with the Optronics Magnafire camera (Intelligent Imaging Innovations Inc., Denver, Colo., U.S.A.) attached to the microscope. The Magnafire cooled charge-coupled-device camera and software are integrated by FireWire technology, which provides live on-screen viewing of fluorescent material and can be used for focusing and framing prior to image capture. The Magnafire dichroic color filters are 12 times more sensitive than cameras using liquid crystal filters. We routinely use the live-imaging mode to view samples under the microscope and adjust zoom, contrast, and brightness, etc., to differentiate artifacts or autofluorescence from GFP expression, as well as to determine whether the GFP fluorescence is in single or multiple neighboring cells. The sensitivity of the camera allows us to identify low levels of GFP or GFP-TGBp1 fluorescence in the nucleus or cytoplasm of neighboring cells. A Leica TCS SP2 system attached to a Leica DMRE microscope was used for confocal imaging.

Quantification of movement

Between 200 and 400 sites containing GFP or GFP-TGBp1 were counted. % movement was calculated by the following equation: % movement = (fluorescent sites consisting of two or more cells)/(fluorescent sites consisting of one cell + sites consisting of two or more cells) × 100. Activation energies were determined using the % movement measured between 12 and 28 °C. Origin (OriginLab Corporation, Northampton, Mass., U.S.A.) was used for data analysis and nonlinear regression analysis.

Results

Determination of % movement

Tobacco leaves were biolistically bombarded with pRTL2-GFP plasmids and fluorescence was seen in transfected epidermal cells following 24 h incubation. Some fluorescent sites were single cells, while others were clusters of neighboring cells (Itaya et al. 1997) (Fig. 1). Sites described as "multiple cell clusters" are those where GFP had moved between neighboring cells through Pd. To measure Pd conductivity, we calculated the % movement of GFP for each leaf. The % movement value represents the proportion of sites containing fluorescent multiple cell clusters relative to the total number of sites viewed (sites containing fluorescence in single cells and multiple cell clusters) on a leaf (Fig. 1). This measure reflects the rate at which GFP is able to move between neighboring cells and has been used in a range of studies to describe the ability of GFP and GFP fusion proteins to move between leaf epidermal cells. In particular, GFP has been fused to several viral movement proteins to study the effects of leaf developmental stage or environmental factors on cell-to-cell transport of viral movement proteins (Itaya et al. 1997, Yang et al. 2000, Tamai and Meshi 2001, Krishnamurthy et al. 2002, Mitra et al. 2003).

Activation energy of transport across Pd

We initially set out to study temperature effects on GFP movement by growing plants at 18 and 25 °C and then bombarding leaves with plasmids. However, we observed that leaves at similar positions on plants grown at 18 and 25 °C did not have the same surface area (data not shown). While it is not surprising that temperature affects leaf expan-

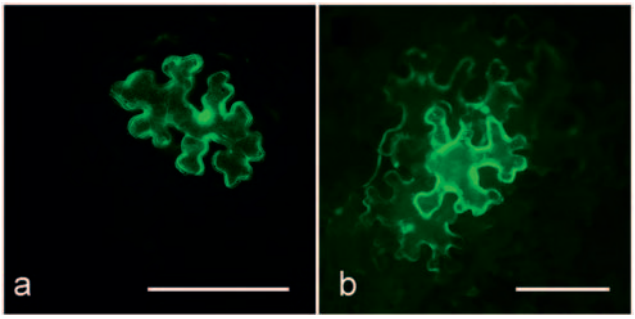

Fig. 1 a, b. Examples of fluorescent sites following biolistic bombardment of plasmids. **a** Single cell expressing GFP. **b** Cluster of neighboring cells expressing GFP-TGBp1. Bars: 100 μm

sion, this could also involve unknown changes in cell size and Pd density and/or architecture. On the basis of these observations, we decided to look at the effects of temperature on protein movement by growing plants at the same temperature, bombarding detached leaves, and then incubating them at different temperatures between 12 and 28 °C.

The % movement values for GFP were measured and used to estimate the activation energy of Pd transport. As expected, increasing temperatures caused the frequencies of GFP movement to increase (Fig. 2). Data were compiled from two or more data sets obtained from different bombardments and show the reproducibility of this experimental approach. An Arrhenius plot (Fig. 2b) showed a linear relationship between the inverse of temperature ($1/T$) and the logarithm of % movement, indicating that the concept of activation energy is applicable. To determine the activation energy for GFP transport across Pd, the data in Fig. 2a were fitted to the Arrhenius equation, % movement $= A \exp(-E_a/RT)$, where A is the preexponential factor, E_a is the activation energy, R is the gas constant (8.31447 J/mol·K), and T is the absolute temperature in kelvins. This fit resulted in $E_a = 38.0 \pm 2.0$ kJ/mol for GFP transport. This rather low activation energy is comparable to that of temperature-dependent transport through an open ion channel and is significantly lower than the activation energy values calculated for carrier-mediated transport or enzyme-catalyzed reactions (Obermeyer and Tyerman 2005).

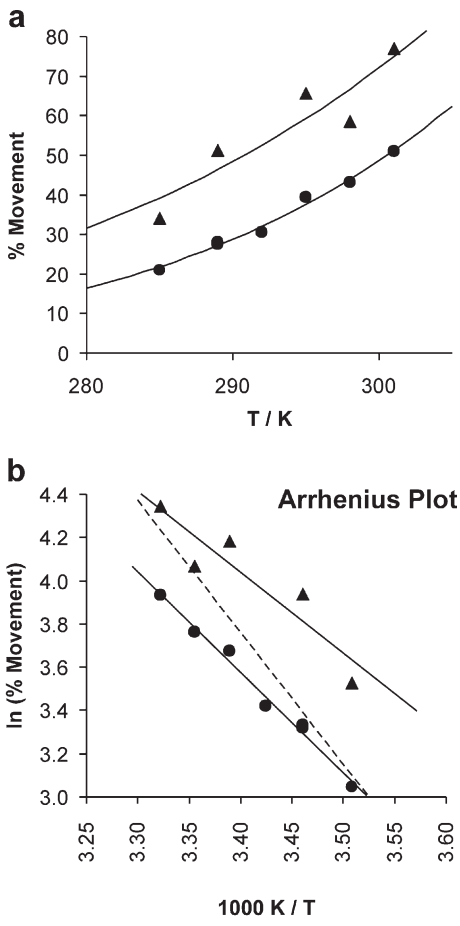

Fig. 2 a, b. % movement as a function of absolute temperature, T (in kelvins). Movement of GFP (circles) is compared to movement of GFP-TGBp1 (triangles). **a** Direct plot of data (% movement $= f(T)$). Both data sets were fitted to an Arrhenius equation (% movement $= A \exp(-E_a/RT)$) resulting in $A = 2.04 \times 10^8 \pm 1.62 \times 10^8$, $E_a/R = 4575 \pm 244$ K for GFP (circles) and $A = 7.49 \times 10^6 \pm 2.44 \times 10^7$, $E_a/R = 3465 \pm 993$ K for GFP-TGBp1 (triangles). Data for GFP were compiled from two data sets originating from different bombardments (transfections). The smaller sample size of the GFP-TGBp1 data set results in larger scattering. **b** For comparison a classical Arrhenius plot of the same data is shown, where ln (% movement) is plotted against $1/T$ (in 1/K × 1000). Linear regression results in ln (% movement) $= -4652.7/T + 19.4$, $R^2 = 0.987$ for GFP (circles) and ln (% movement) $= -3740.6/T + 16.8$, $R^2 = 0.854$ for GFP-TGBp1 (triangles). A dashed line was added to illustrate a process with $Q_{10} = 2$ ($A = 2.6 \times 10^{10}$, $E_a/R = 5989$ K) for comparison

The PVX-encoded TGBp1 movement protein is known to gate Pd, increasing their SEL (Angell et al. 1996; Lough et al. 1998, 2000; Howard et al. 2004). To test the effect of TGBp1 on GFP movement, the temperature dependence of GFP-TGBp1 movement was examined (Fig. 2). The resulting data could be described by the Arrhenius equation, resulting in $E_a = 28.8 \pm 8.3$ kJ/mol (Fig. 2). The E_a values for GFP and GFP-TGBp1 were not significantly different (with overlapping error ranges). Only the preexponential factor, A, differed. This means conductivity of Pd was higher for GFP-TGBp1 compared with GFP, but transport of both proteins was diffusion driven.

Compared to the 29 to 38 kJ/mol estimated here for the activation energy of diffusion-driven protein transport through Pd, values of 30 kJ/mol can be calculated for the temperature dependence of protein diffusion through the cytosol (Papadopoulos et al. 2001). The activation energy for GFP and GFP-TGBp1 transport estimated here is close to that for protein diffusion in the cytosol, indicating that transport of GFP and GFP-TGBp1 across Pd is diffusion driven and does not require an interaction of these proteins with Pd. There are no significant energy barriers for GFP or GFP-TGBp1 to overcome when crossing Pd.

In the biological literature, the Q_{10} value is frequently used instead of activation energy. Q_{10} and activation energy, E_a, at a certain temperature, T, can be interconverted by

$$Q_{10} = \frac{\exp\left(\frac{E_a}{R(T-5)}\right)}{\exp\left(\frac{E_a}{R(T+5)}\right)} \Leftrightarrow E_a = \frac{R \ln Q_{10}}{\frac{1}{T-5} - \frac{1}{T+5}}.$$

Since Q_{10} values for enzyme-catalyzed reactions are usually in the range of 2.0 or higher (Raven and Geider 1988, Atkin and Tjoelker 2003), we introduced a dashed line in Fig. 2b depicting a process with a Q_{10} of 2 (E_a = 49.5 kJ/mol).

GFP diffusion into neighboring cells continuously declines during leaf expansion

Prior investigations showed that young sink leaves show greater movement of GFP than mature, fully expanded source leaves. As we noticed in preliminary experiments that plants grown at different temperatures have different surface areas, experiments were conducted to determine if changes in leaf surface area may coincide with changes in GFP cell-to-cell movement.

To investigate changes in Pd conductivity during leaf growth, % movement values were determined for leaves of different age and size. A set of 16 plants were grown simultaneously and leaves from two plants were excised weekly for 8 weeks and labeled L1 through L4, where L1 is the leaf closest to the soil and L4 is the fourth leaf above L1. Data were compiled from two data sets obtained from different bombardments and show the repro-

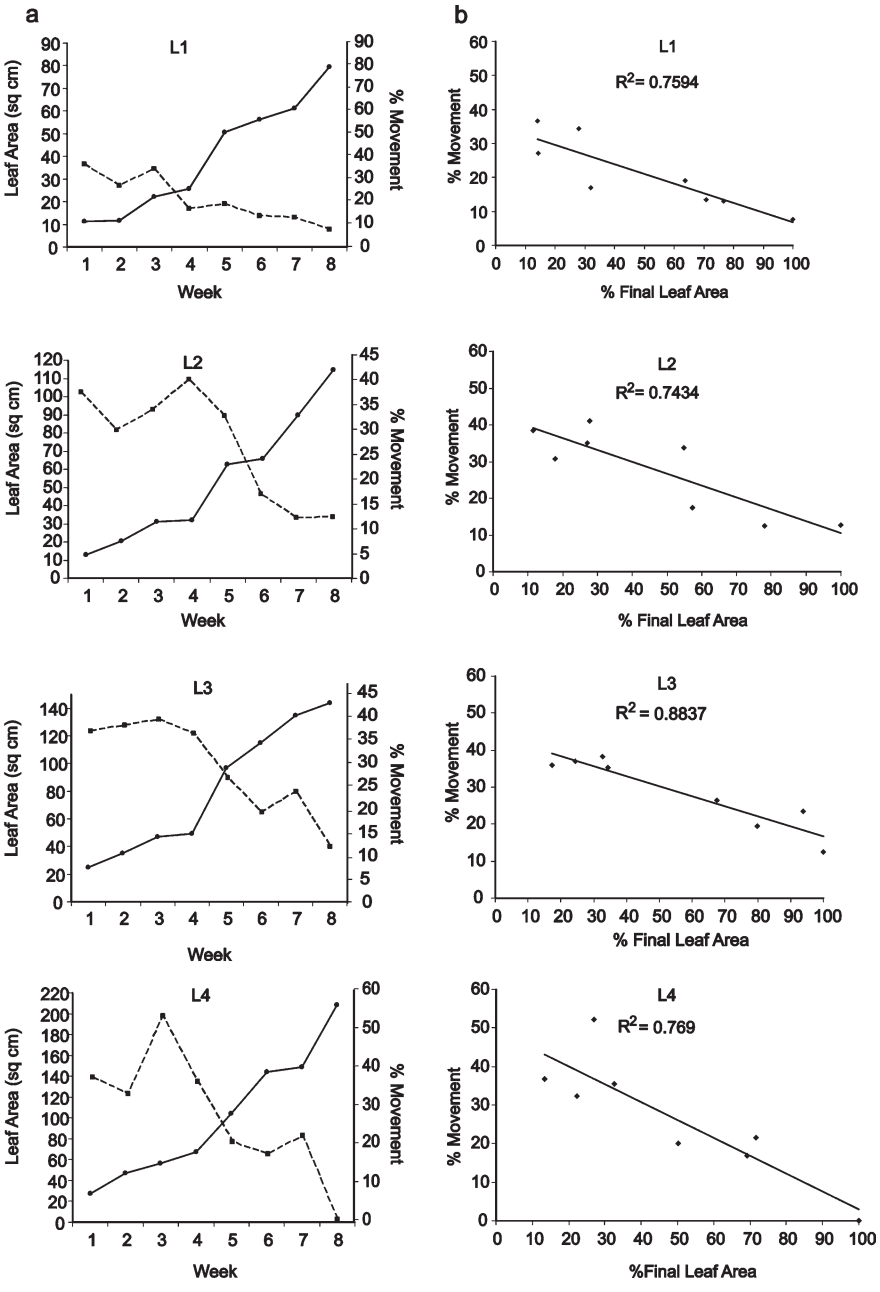

Fig. 3 a, b. Leaf area and % movement of GFP measured each week for 8 weeks. Data from each L1 to L4 leaf are represented in separate panels. **a** Graphs show that the average leaf area (solid line) for each leaf increased over the 8 weeks. For each leaf, there is at least a twofold expansion in leaf area after week 4. The % movement values (broken line) indicate the percentage of sites permitting GFP to move into neighboring cells. The first significant drop in % movement of GFP occurs at week 3 or 4, concomitant with the beginning of major leaf expansion. **b** Average leaf area was calculated as a percent of the final area determined at 8 weeks. % movement values are plotted against relative leaf area. The relationship appears linear. As leaf area expands there is less movement of GFP into neighboring cells

ducibility of this experimental approach. Although more leaves emerged above L4 during the 8 weeks in each experiment, we restricted our analysis to only the first four leaves. At 24 h post bombardment, leaves were scored for the proportion of sites showing GFP expression in multiple cell clusters. In addition, the surface area for each L1 to L4 leaf was measured when it was excised and the average leaf area was determined from the compiled data sets.

The % movement of GFP and the average leaf areas determined during the 8 weeks displayed opposing trends (Fig. 3a). During the first three weeks when leaves were still small, 35–45% of sites showed GFP moving into neighboring cells, regardless of the leaf position. The % movement values decreased during the following weeks as leaves expanded, and by 8 weeks only 0–13% of sites showed GFP in multiple cell clusters in all leaves. Thus, GFP movement was greatest in smaller leaves and declined in all leaves between 4 and 8 weeks when they displayed rapid expansion growth. The average leaf surface area for each L1 to L4 was plotted against time (Fig. 3a). The dimensions of L1 and L2, which were closest to the soil surface, reached a maximum area of between 80 and 115 cm^2, while L3 and L4 continued to expand to 140 and 210 cm^2, respectively. The surface area of each leaf nearly doubled between weeks 4 and 5. The average area for L1 changed from 25.5 to 50 cm^2, L2 changed from 31.9 to 62.8 cm^2, L3 changed from 49.4 to 97 cm^2, and L4 changed from 67.7 to 104.5 cm^2. Thus, the beginning of the downward trend in GFP diffusion into neighboring cells corresponds to a time when the leaf surface area changes most significantly (Fig. 3a).

To directly compare the relation of leaf area to % movement, leaf areas were recalculated as a percent of the final leaf area measured at 8 weeks, and % movement values were plotted against relative leaf size (Fig. 3b). In these plots, the relationship between GFP movement and leaf area appears to be linear. For each leaf, regardless of its position on the plant, as leaf area expanded the % movement of GFP declined.

Discussion

% movement

In this study we employed the % movement measure, which is an established method for measuring Pd conductivity of GFP in experiments involving biolistic bombardment of plasmids into tobacco leaves. This method has been used in a range of studies to quantify GFP movement between epidermal cells in leaves at different developmental stages and under different environmental conditions (Itaya et al. 1997, Yang et al. 2000, Tamai and Meshi 2001, Krishnamurthy et al. 2002, Mitra et al. 2003). Is the % movement measure really a reliable quantitative indicator for Pd conductivity? We think it is, and as a relative measure, it is inherently independent of changes in absolute concentrations. To understand why % movement is a reliable and stable measure for Pd conductivity, it might help to have a look at measurements made at lower GFP concentrations. When GFP concentrations are decreased, there will be more cells containing so little GFP that they are below the detection limit. Yet both more transfected and more neighboring cells drop below the threshold, while the ratio remains the same. The ratio between the number of all transfected cells (above GFP detection threshold) and transfected cells displaying (detectable GFP) movement into neighboring cells only depends on the connectivity or conductance between transfected cells and neighboring cells. The % movement value reflects this ratio and thus is a quantitative measure of Pd conductance. The higher the conductance between cells, the higher the % movement value. The lower the conductance between cells, the more neighboring cells are below the GFP detection threshold, and the lower the % movement value. As expected, with increasing Pd conductivity, both the % movement value and the number of cells per cell cluster increase (data not shown; see Crawford and Zambryski [2000]).

Although transfected cells without visible GFP fluorescence in neighboring cells are frequently labeled as displaying “no movement”, it should probably be “too little GFP movement to be detected”. If one accepts that there is a detection limit for GFP (every technique has its detection limit), and that a certain number of transfected cells and neighboring cells contain GFP concentrations below the detection limit, then % movement gives an accurate and stable measure for Pd conductance. On the basis of the % movement measure, the estimated activation energy for GFP transport indicates diffusion-driven transport. This is in line with earlier publications claiming that GFP transport through Pd is diffusion driven (Crawford and Zambryski 2000, Zambryski and Crawford 2000, Liarzi and Epel 2005) and corroborates the assumption that % movement values correctly reflect Pd conductivity.

How does the % movement measure compare to methods that try to quantify GFP concentrations in transformed cells and neighboring cells (Liarzi and Epel 2005)? Should a more quantitative approach not give a better value for Pd conductivity and thus activation energy? Not necessarily.

The % movement approach uses the detection limit for GFP as a cutoff to determine Pd conductivity and includes all transformed cells (with a detectable GFP concentration). Approaches that quantify GFP fluorescence in transformed and neighboring cells can calculate Pd conductivity for each separate cell cluster, but single transformed cells without detectable GFP fluorescence in neighboring cells cannot be analyzed. Depending on the growth conditions, these are 10 to 20% of the transformed cells (Liarzi and Epel 2005). While the % movement values give average values for Pd conductivity for all transformed cells (with a detectable GFP concentration), the quantification of GFP can be used to measure Pd conductance in each separate cell cluster with detectable GFP in neighboring cells.

Activation energy for GFP transport

The % movement values determined after incubating leaves at various temperatures showed that the % movement of GFP increased with increasing temperatures, and the relation between temperature and % movement could be adequately described by the Arrhenius equation (Fig. 2). The concept of activation energy has been applied to characterize transport through ion channels or carriers (Obermeyer and Tyerman 2005) and nuclear pores (Clawson and Smuckler 1978). Here, it is shown that this concept is also applicable to protein movement through Pd. Since several different processes (GFP expression, GFP movement towards Pd, and GFP transport across Pd) are added in our experimental approach, it is not self-evident that the simple concept of a definite activation energy is applicable. Yet, the linear correlation between the logarithm of % movement and $1/T$ (see Arrhenius plot in Fig. 2b) in the temperature range from 12 to 28 °C demonstrates that this approach makes sense. The rather low values for activation energy estimated for GFP movement through Pd (E_a = 38 kJ/mol) are indicative of transport by passive diffusion. This is in line with prior reports assuming nontargeted GFP movement through Pd is driven by passive diffusion (Crawford and Zambryski 2000, Zambryski and Crawford 2000, Liarzi and Epel 2005). To our knowledge this is the first study to determine E_a values for protein Pd transport, thereby indicating a diffusion-driven process. Incubating leaves at different temperatures for 24 h after bombardment affects GFP expression as well as intracellular and intercellular GFP transport. The rather low activation energy determined for the complete process – expression and transport – indicates that Pd transport is a diffusion-driven process. It also shows that GFP expression is most unlikely to be a limiting factor in the studied system.

Activation energy for GFP-TGBp1 transport

Comparing GFP with GFP-TGBp1 movement, the larger fusion protein had a higher % movement (Fig. 2), while the Arrhenius plot showed a comparable activation energy (E_a = 28 kJ/mol), demonstrating that movement of both proteins is diffusion driven. PVX TGBp1 has been shown in several studies to increase Pd SEL. In two such studies, fluorescent dextrans of various sizes were injected into either TGBp1-expressing transgenic or nontransgenic plants along with purified TGBp1. In both cases, TGBp1 enabled the transfer of 10 kDa F-dextrans between cells, which were otherwise restricted to single cells in the absence of TGBp1 (Lough et al. 1998, Howard et al. 2004). Related biolistic bombardment studies also showed that the % movement of GFP-TGBp1 is higher than that of GFP in four different plant species (Howard et al. 2004). In line with these earlier findings, the data presented here (Fig. 2) show that TGBp1 increases Pd conductivity. The larger fusion protein displays a higher % movement, which means Pd conductivity is higher.

PVX TGBp1 is a multifunctional protein acting as a Pd-gating factor and silencing suppressor, and promotes virus translation (Angell et al. 1996, Atabekov et al. 2000, Kiselyova et al. 2003, Howard et al. 2004, Bayne et al. 2005). In spite of its association with many cellular processes, GFP-TGBp1 is mainly a cytosolic protein, similar to GFP, and its mobility across Pd is characterized by comparable temperature dependence. The activation energies of 38 kJ/mol for GFP and 29 kJ/mol for GFP-TGBp1 transport are close to that of 30 kJ/mol for protein diffusion in the cytosol (Papadopoulos et al. 2001), while a value of >50 kJ/mol reflects a threshold for enzymatic processes. An increase in % movement, on the one hand, indicating TGBp1 gates Pd, and a low activation energy, on the other, indicating a diffusion-driven process, may seem irreconcilable. Gating Pd certainly indicates specific interactions, while the low E_a values imply that there is no significant specific interaction or binding. Yet, gating Pd between epidermal cells probably requires only a small fraction of the GFP-TGBp1 proteins synthesized. A tobacco leaf epidermis cell has less than 1000 Pd (<1 Pd per μm and <1000 μm of anticlinal cell wall) (Roberts et al. 2001), while 100,000 copies of wild-type GFP per typical cell are needed for the cell to yield twice the background fluorescence (Tsien 1998). Therefore, it is reasonable to assume that a relatively small portion of TGBp1

proteins (which do not significantly contribute to E_a) is sufficient to gate Pd, while the majority of the GFP-TGBp1 proteins move through Pd by simple diffusion, resulting in the low E_a observed. Considering the results of this and prior investigations, the data suggest that GFP-TGBp1 reaches open Pd by diffusion. A small fraction of TGBp1 specifically increases Pd SEL, which promotes the diffusional movement of the remaining fraction of TGBp1 through Pd into neighboring cells. It seems very unlikely that each TGBp1 molecule transported binds factors inside Pd to propel itself forward.

Viral movement proteins, such as TMV P30 and CMV 3a, are able to increase Pd and accumulate inside them, which is the basis for the term "targeted movement" (Crawford and Zambryski 2001). Accumulation of proteins inside Pd has often caused researchers to query whether viral proteins must interact with factors residing inside Pd in order to provide the force needed to exit the other side of the pore. A recent investigation showed that GFP-TGBp1 accumulates along Pd when expressed from the PVX genome but not when expressed from the cauliflower mosaic virus 35S promoter (Samuels et al. 2007). Since the PVX coat protein accumulates inside Pd (but does not dilate them), it was suggested that TGBp1 interactions with the coat protein might cause it to accumulate in the same place (Santa Cruz et al. 1996, Samuels et al. 2007). Combining the results of this investigation with evidence that TGBp1 increases Pd SEL in the absence of virus infection (Krishnamurthy et al. 2002, Howard et al. 2004), it is reasonable to assume that PVX TGBp1 may require dilated Pd to move from cell to cell and does participate in Pd dilation.

GFP transport and leaf expansion

Several studies have reported that Pd conductivity of nontargeted proteins is affected by developmental transitions (Itaya et al. 1998, Oparka et al. 1999, Zambryski and Crawford 2000, Roberts et al. 2001, Kim et al. 2002). There is greater movement of GFP in newly emerging, young leaves than in fully expanded, mature leaves (Itaya et al. 1998). This has been mainly explained by differences in Pd architecture and density. In young leaves, Pd are single-channeled structures with a high SEL, while mature leaves have Pd that are branched and have a lower SEL (Oparka et al. 1999, Roberts et al. 2001).

The data presented in Fig. 3 show that GFP movement declines as leaf areas expand and the relationship between these two parameters appears to be linear. This linear relationship between % movement and leaf area points to a possible explanation for the observed decrease in Pd conductivity during leaf maturation. The increase in leaf area reported here is caused primarily by cell expansion, since cell division stops relative early during tobacco leaf development, especially in the epidermis (Avery 1933). Cell expansion results in increasing distances between primary Pd formed during cell division. Even though secondary Pd are formed during leaf development, the density of Pd between epidermal cells decreases during cell expansion (Oparka et al. 1999, Oparka and Roberts 2001, Roberts et al. 2001). Accepting that GFP transport through Pd is diffusion driven, two factors will affect the measured Pd conductivity (Paine et al. 1975, Heinlein and Epel 2004): (i) the probability that GFP reaches the open pore of a Pd by diffusion (Ferry 1936) and (ii) the resistance to diffusion through the cytoplasmic sleeve of the Pd. The decrease in Pd density caused by cell expansion does decrease the probability that GFP attains the opening of a Pd. The correlation between leaf size (i.e., cell size) and GFP movement indicates that the decrease in Pd density caused by cell expansion may significantly contribute to the decrease in GFP movement observed during leaf growth. Lower Pd density means longer diffusion times for GFP and longer diffusion times at fixed observation intervals (24 h here) result in lower values for % movement to neighboring cells.

Previous reports comparing the movement of GFP in young photosynthetic sink leaves and mature photosynthetic source leaves suggested that Pd conductivity was downregulated during the transition from sink to source by changes in Pd architecture. Obviously, longer or narrower Pd will increase the resistance to diffusion within the pore and thus decrease Pd conductivity. Accepting that nontargeted GFP movement is a diffusion-driven process (Fig. 2), a decrease in Pd density due to cell expansion causes increased diffusion times resulting in a decrease in % movement with increasing leaf area (Fig. 3). In addition to developmental changes in Pd architecture, which slow down diffusion through Pd, lower Pd density reduces Pd conductivity by decreasing the probability that GFP attains open Pd by diffusional movement inside the cytoplasm.

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