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Symplastic Transport of Carboxyfluorescein in Staminal Hairs of *Setcreasea purpurea* Is Diffusive and Includes Loss to the Vacuole[1]

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ABSTRACT

The kinetics of symplastic transport in staminal hairs of *Setcreasea purpurea* was studied. The tip cell of a staminal hair was microinjected with carboxyfluorescein (CF) and the symplastic transport of this CF was videotaped and the digital data analyzed to produce kinetic curves. Using a finite difference equation for diffusion between cells and for loss of dye into the vacuole, kinetic curves were calculated and fitted to the observed data. These curves were matched with data from actual microinjection experiments by adjusting *K* (the coefficient of intercellular junction diffusion) and *L* (the coefficient of intracellular loss) until a minimum in the least squares difference between the curves was obtained. (a) Symplastic transport of CF was governed by diffusion through intercellular pores (plasmodesmata) and intracellular loss. Diffusion within the cell cytoplasm was never limiting. (b) Each cell and its plasmodesmata must be considered as its own diffusion system. Therefore, a diffusion coefficient cannot be calculated for an entire chain of cells. (c) The movement through plasmodesmata in either direction was the same since the data are fit by a diffusion equation. (d) Diffusion through the intercellular pores was estimated to be slower than diffusion through similar pores filled with water.

Symplastic transport, the intercellular passage of ions and small molecules in the cytoplasm via plasmodesmata, is required for the proper growth and development of plants (5). The kinetics of symplastic transport is of interest to plant physiologists and has been studied by other researchers. It was proposed (14) that: the plasmodesmata constitute the pathway of least resistance for the diffusion of all small solutes; diffusion is the predominant mechanism of transport across the pores for small solutes; solute distribution within the bulk cytoplasm of each cell ought to be by a combination of diffusion and cyclosis; and, there ought to be nearly perfect mixing within the bulk cytoplasm of each cell. Thus, the rate-controlling factor of symplastic transport should be diffusion through plasmodesmata. When fluorescein entered the symplast through a cut in *Tradescantia* staminal hairs (15) and tomato trichomes (1), its symplastic transport was found to be proportional to the square root of time, that is, was accomplished through simple diffusion. Symplastic transport was slow (passing through five *Tradescantia* staminal hair cells after 35 min), suggesting that the plasmodesmata were occluded (15). When CF[2] was microinjected into the cytoplasm of *Setcreasea purpurea* staminal hair cells, symplastic transport was faster (through five cells after 1–2 min) and influenced by additional factors such as the apparent degree of cell-to-cell coupling (13). When cytoplasmic streaming was stopped by metabolic inhibitors (cyanide or azide) or a microfilament disrupting agent (cytochalasin D), the kinetics of symplastic transport were not altered to any extent (12). Thus, diffusion within the staminal hair cells was taken to be adequate for symplastic transport of small, hydrophilic molecules like CF.

To extend our earlier studies on cell-to-cell transport in *S. purpurea* staminal hairs (13), a computer program has now been developed that generates concentration curves and compares them to the actual data of microinjection experiments. We obtain the best fits, defined by minimum least squares differences, when symplastic transport in the model is assumed to depend not only upon a coefficient of intercellular junction diffusion (*K*) but also upon a coefficient of intracellular loss (L). Observations indicate that most of this intracellular loss is movement into the vacuole. A diffusion coefficient of CF (D_{CF}) can then be calculated from the known parameters of the plasmodesmatal pores and it is concluded that the material in the pores impedes diffusion of the dye. In this communication we describe a quantitative approach for studying symplastic transport.

MATERIALS AND METHODS

Plants

Pots of *Setcreasea purpurea* Boom (Commelinaceae) were maintained at room temperature under natural lighting. Immediately prior to the experiment, immature buds (approximately 4 d preanthesis) were removed and kept moist on wetted filter paper. Stamens were plucked from these flower buds, secured to microscope slides with double-stick tape, and covered with a drop of distilled water. Only hairs with cells of

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[2] Abbreviation: CF, carboxyfluorescein.

a uniform size and of the same developmental stage were used.

Microinjection

A solution of 5 (6)-CF (mol wt 376; Molecular Probes, Eugene, OR) was made up as a 50 mM solution in 1 M potassium citrate (pH 7.0). Glass micropipettes (1B100F, World Precision Instruments, New Haven, CT) were backfilled with this solution. The tip of the micropipette was carefully placed into the peripheral cytoplasm and away from the interconnecting cell wall of the tip staminal hair cell. Using a Constant Current Generator 260 (World Precision Instruments, New Haven, CT), tracer was microinjected into the cell by iontophoresis using a hyperpolarizing current of 10 nA for 5 to 10 s. The micropipette was then removed. The signal from the fluorescence in the cytoplasm of this microinjected cell, determined by the digitizer, was kept below 3.5 V to ensure its being in the linear region of the response curve. The measured fluorescence intensity (V) was proportional to CF concentration up to 4.0 V and followed the formula $V = 0.033 \times \mu M\ CF + 0.58$.

Carboxyfluorescein was excited by light from a mercury vapor lamp (HBO 50 W) using a Leitz ploemopak filter cube L2 on a Leitz Orthoplan fluorescent microscope (E. Leitz, Rockleigh, NJ). A neutral density filter ($A = 0.30$) was placed between the beam of light and filter cube. A ×25 water immersion lens (Lietz) with a numerical aperture of 0.60 was used.

Video Recording and Analysis

Carboxyfluorescein transport through staminal hairs was videotaped through the fluorescence microscope. The SIT television camera (Dage-MTI, Michigan City, IN) had been adjusted by Mr. J. Jones, electronics technician (MTI, Michigan City) to give a linear response to fluorescence intensity. The camera was set on manual, 1/2 kV and 3/4 gain. Intensity was videotaped on a JVC BR-6400U video recorder. Fluorescence levels in the pool of cytoplasm at the adjoining wall of the microinjected cell (tip cell, cell No. 1) and the two most proximal cells (cells No. 2 and 3) were analyzed with a model 321 video analyzer (Colorado Video Inc, Boulder, CO) and transferred to an IBM Personal Computer (International Business Machine, Corp.; Boca Raton, FL). One hundred thirty-two (132) time points, each point being an average of 100 measurements, were used. Digitized values were stored on a disc.

Calculation and Curve Fitting Program

The following equation was used.

$$\Delta C_i = [K(C_{i-1} - C_i) - K(C_i - C_{i+1}) - L(C_i)](\Delta t) \quad (1)$$

where ΔC_i = change of fluorescence intensity (V), including apparatus scaling factors, in cell i; C_i, C_{i-1}, C_{i+1} = fluorescence intensity (V) at time t for cell i, $i-1$, $i+1$, respectively; K = coefficient of intercellular junction diffusion in s^{-1}; L = coefficient of loss in s^{-1}; Δt = time step of calculation.

A program was written in BASIC to integrate this equation as outlined in the text. The boundary conditions were set with all cells except the first, having zero (0) level of fluorescence at $t = 0$ and the first cell rising linearly to unity of fluorescence in 10 s. Once the fluorescence intensities in each cell were obtained, the resultant data were plotted using the Lotus 1-2-3 program (Lotus Development Corp., Cambridge, MA). The curve-fitting program then minimized the sum of the squares of the differences between calculated and measured cells by varying K and L. This was continued until a minimum in the sum of squares residuals was obtained. The normalized mean square residual was calculated by dividing the mean square for each experiment by the scale value used squared. The model assumed that the loss was dependent upon the concentration of CF in the cytoplasm.

Photodecomposition

A 20 μM solution of CF was placed on a slide containing vacuum grease and covered with a 0.5 mm cover slip. This produced pockets of solution which were similar in size (0.25 μm in diameter) and intensity to the pool of cytoplasm of the adjoining wall. The pockets were videotaped and analyzed as were the microinjected staminal hairs.

Estimate of Diffusion through Plasmodesmata and Vacuole Membrane

A model of a cell-shape with a cylinder as the main body and two halves of a sphere for ends, and containing an identical but smaller inner cell-shape as a vacuole and a sphere as a nucleus, was used to calculate the cytoplasmic volume and surface area of the tonoplast. The dimensions for each cell were taken from the video monitor. Plasmodesmata were taken to be cylinders, 38 nm internal diameter by 290 nm long, containing a central desmotubule of radius 7.5 nm (11). The density of plasmodesmata was taken to be 11 per μm^2 (16) on adjoining walls of neighboring cells of diameter 10 μm (11). The cytoplasm was taken to be a 1.0 μm thick sheet between the cell membrane and tonoplast and to contain a spherical nucleus of diameter 10 μm (11).

The calculated parameters must be then reduced to experimental observables. The amplitude of the fluorescence intensity is normalized to $C_1 = 1$ at $t = 0$ since the data are in a linear range. The time scale is set by the time length of the data sequence, T, and the number of calculated steps, N, *i.e.* $\Delta t = T/N$. The diffusion parameter $K = D_{CF}A/Vl$ where D_{CF} is the diffusion coefficient of CF in the intercellular pores, A is the permeable area of the pores in the plasmodesmata, V is the volume of the cytoplasm, and l is the pore length. Thus measuring K, and given the dimensions of the diffusion elements, one can calculate D_{CF}. The rate constant for the loss is $L/\Delta t$. The loss term is written as a first order rate constant. It may also be diffusive. Diffusive loss has the first order form when the concentration inside the vacuole remains much lower than that in the cytoplasm.

RESULTS

The highest loss of fluorescence due to photodecomposition during five trials was 0.026%/s. This value was subtracted

from L, the loss calculated by the computer to obtain the loss to the vacuole.

No morphological changes in the cytoplasm could be observed during or after iontophoresis or at the termination of the experiment. Cytoplasmic streaming continued vigorously throughout the experiment, and contributed to the success of the assumption that the contents of the cytoplasm of each cell are always well mixed.

The problem of the diffusion of substances through the staminal hair cells can be simplified by considering the cells to be well mixed pools of equal volume connected by diffusion pores, the plasmodesmata. The solution of this problem is the integral of a one-dimensional diffusion equation with the following boundary conditions. At zero time, the concentration of the dye in cell number 1 is C (=1), and that in the remaining cells is zero. For numerical calculations Equation 1 can be used (2). In essence, each plasmodesma is taken as a step in the finite difference representation of the diffusion equation. This states the change in the ith cell, over a time span Δt, given the concentration in it and the neighbor cells. A loss term, with rate proportional to the concentration in the ith cell, is added to the diffusion equation. Although the diffusion equation has a well known analytical solution (2), it is when complications occur, as with the loss term, that the numerical calculation shows its power. Any kind of side reactions of the tracer are easily incorporated. After calculation of each change in C_i (Eq. 1), it is added (algebraically) to the original concentration and another cycle of calculation is begun up to the end of the time points. The truncation error at the last cell is corrected by calculating with five extra cells beyond those measured. This equation adequately fits the data with only two adjustable constants: K and L. The K and L could be different for the different cells, *e.g.* if the cells had markedly different volumes or plasmodesmata, but the present data could be fitted by the above assumptions. In the normalized units, K must be <0.5, so Δt (*i.e.* N) must be so adjusted.

The fit of the data was automated with a program that searched for the minimum in the square of the residual error between calculated and data points (least squares) by varying the parameters systematically (7). The method is slower than the gradient procedures (3), but it can search out a large part of the parameter space by varying the step size and the initial values of the parameter.

A representative curve of experimentally obtained data and computer-generated curve fit is illustrated in Figure 1. The dotted line is the data from cells 1 (the injected cell), 2, and 3 over the 7 min experimental time. In cell 1, the intensity reached 2.3 V in 0.16 min after the microinjection time and then decreased over the remaining 7 min. In cell 2, the intensity reached its maximum of 0.6 V in 0.85 min; while in cell 3, the maximum of 0.25 V was reached in 1.75 min. The solid line is the best fit line generated by the computer, determined by the computer model program. The fit is remarkable, given that only two parameters are used to fit all cells over the complete time. The fit was as good for the other transport kinetic experiments and for further cells in the series which are not shown here. Mismatch can be noted, however, at the beginning and end of the curves.

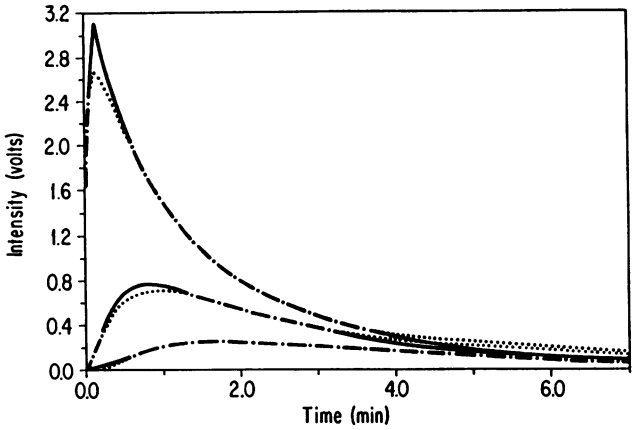

Figure 1. Kinetics of symplastic diffusion. Kinetic curves of the cell-to-cell diffusion of CF in a staminal hair compared to computer generated curves where $K = 0.01319\ s^{-1}$ and $L = 0.04917\ s^{-1}$. The means square residual was 0.86. (·····), Data collected; (——), computer-generated curve.

Coefficients of intercellular junction diffusion (K), coefficient of intracellular loss (L), the average cytoplasmic volume of cells analyzed (V), diffusion coefficient for CF in the intercellular pores (D_{CF}), and rate of loss (K_1) are listed in Table I for the experiment in Figure 1 (trial 4) and for four separate experiments. The average K value was 12×10^{-3}/s, while the average L value was 50×10^{-3}/s. The L values were remarkably similar to each other (6% standard deviation). The K values were somewhat more variable (30% standard deviation) which may reflect differing cell volumes and plasmodesmata characteristics. The average D_{CF} was calculated to be $5.37 \times 10^{-8}\ cm^2/s$. The average rate of movement or diffusion across the vacuolar membrane was calculated to be $9.44 \times 10^{-7}/\mu m^2/s$. The mean square residual (MS) calculated for each curve was small, confirming that the experimental and computer generated curves were very similar.

DISCUSSION

The pulse-microinjection and computer-assisted quantification reported in this communication is an improvement over our previously published method (13). The small quantity of CF microinjected into the peripheral cytoplasm (*i.e.* away from the plasmodesmata-containing adjoining-wall) is presumed to cause minimal damage to the cell. Thus, the physiology of the cell was not altered by this technique. Results were found to be very reproducible.

The data obtained by microinjection of CF clearly fitted the curves generated from the computer model program. Thus, we conclude that:

1. Symplastic transport is governed by diffusion through plasmodesmata and, at least for CF, by intracellular loss.

2. Being purely diffusive, the rate of transport in either direction through plasmodesmata is the same.

3. The calculated diffusion coefficient of CF through the pores is smaller than the diffusion of fluorescein (F) through similar pores containing water, [D_{CF} (plasmodesmata) = $5.4 \times 10^{-8} \pm 1.5\ cm^2\ s^{-1}$, D_F (water) = $4.5 \times 10^{-6}\ cm^2\ s^{-1}$) (15)].

4. The rate of diffusion between the adjacent cells along

Table I. *Symplastic Diffusion and Intracellular Loss Coefficients for CF Microinjected into Staminal Hairs of S. purpurea*

K, coefficient of intercellular junction diffusion; *L*, coefficient of intracellular loss; average V, average volume of cytoplasm being analyzed; D_{CF}, diffusion coefficient of CF in the intercellular pores; k_1, loss across vacuole membrane; *MS*, normalized mean square residual.

Trial	$K \times 10^{-3}$	$L \times 10^{-3}$	Average V	$D_{CF} \times 10^{-8}$	$k_1 \times 10^{-7}$	*MS*
	s^{-1}		μm^3	$cm^2\ s^{-1}$	$\mu m^{-2}\ s^{-1}$	
1	9.5	50	7190	5.37	6.86	1.30
2	18.4	50	5210	7.37	9.63	0.73
3	11.4	58	6060	5.42	9.45	0.35
4	13.2	49	5130	5.31	9.68	0.39
5	7.5	44	5460	3.22	11.59	2.84
Average	12.0 ± 4.2	50 ± 3	5810 ± 850	5.37 ± 1.52	9.44 ± 1.69	

the staminal hair through plasmodesmata is approximately the same, 9.4 ± 1.7 $\mu m^2/s$, although differences are predicted for cells of very differing volumes or plasmodesmata characteristics.

5. The rate of loss in adjacent cells is the same.

These results support the hypothesis that the rate controlling factor of symplastic transport is diffusion through plasmodesmata (9, 14). We add that for some molecules, loss is also an important factor in determining the rate of their symplastic transport. We believe that most of this loss is due to movement into the vacuole since, when unpigmented cells (no pigment in vacuole) are used, fluorescence is clearly seen in the vacuole. In these experiments, the fluorescence in the vacuole increased with the concomitent decrease of fluorescence in the cytoplasm. In fact, with extended time (20–30 min) the cytoplasm became completely clear of fluorescence while the vacuole fluoresced brightly. The fluorescence did not leave the vacuole after 5 h in cells that appeared morphologically healthy. Thus, it appears that CF is bound in the vacuole. When pigmented cells (pigment in vacuole) were used in other experiments, fluorescence was not noted in the vacuole. However, we are of the opinion that CF accumulated in the vacuole of pigmented cells as it did in unpigmented cells, but it could not be seen. This may explain why some researchers noted fluorescence in the vacuole (4, 6) while others did not (15). An explanation for how or why *S. purpurea* cells remove CF from their cytoplasm and transport it into the vacuole has not been put forth.

The slight difference between the experimental and computer-generated graphs may be due to incorrect or incomplete assumptions about intracellular loss. For example, the loss term may be dependent upon concentration of CF in the vacuole as well as the concentration of CF in the cytoplasm. However, the fact that CF microinjected into a vacuole has never been observed to move into the cytoplasm would argue against this assumption. We believe a more plausible reason for curve mismatch is related to the manner of CF microinjection. CF could not be microinjected in the perfectly smooth manner assumed by the program. Thus, the graph of the experimental data has some variation in the early time range which challenge the curve-fitting program to find values that satisfy all points in the graph. Possible binding of CF in the cytoplasm can account for the tailing of fluorescence noted in the late time range. Further refinement of the curve-fitting program may resolve this question.

The use of Equation 1 assumes that diffusion across the cell junction was the time-limiting factor. The time required to diffuse across a cell is considered insignificant. Therefore, we treat each cell as its own diffusion system. The diffusion coefficient cannot be calculated from the system as a whole as has been done previously (1, 15), that is, it is inconsistent with the observation of rapid mixing in the cytoplasm to divide the square of the total distance by the total time to find the diffusion coefficient.

In earlier and unpublished experiments we found that CF microinjected midway along the hair, traveled at an equal rate in both directions. These results show that symplastic transport for small molecules is not polarized in either direction. In the present communication, the assumption that the movement was purely diffusive through the junction, relies on this observation. The agreement of the data with the computer-generated curves confirms this view. That symplastic transport of CF is not polar has also been reported for *Tradescantia* staminal hairs (15), tomato trichomes (1), and *Elodea* leaf cells (4).

The average diffusion coefficient of CF through the annular pore of plasmodesmata was calculated to be $5.4 \pm 1.5 \times 10^{-8}$ $cm^2\ s^{-1}$ (Table I), which is approximately 120 times less than diffusion through water, *i.e.* 4.5×10^{-6} $cm^2\ s^{-1}$ (15). This factor compares to that of 330 calculated for the slowing of diffusion of small ions through plasmodesmata of *Nitella translucens* (10) and to the factor of 50, calculated for the rate of CF diffusion through beetle larvae gap junctions, 2.7×10^{-7} $cm^2\ s^{-1}$ (8). Since, neglecting wall effects, the time to diffuse a given distance is equal to that distance squared divided by twice the diffusion coefficient, we estimate that if the pore were filled with pure water it would take approximately 1×10^{-4} s to diffuse through it. This is much faster than actually observed. The same calculation with the computed diffusion coefficient gives the time to cross a cell junction to be 3×10^{-2} s. These calculations support other researchers' findings that plasmodesmata are occluded (1, 9, 10, 15). Also, the electron micrographs of *S. purpurea* staminal hair cells show occluded plasmodesmata (11).

Equation 1 assumes that the *K* and *L* values are the same for each cell along the chain. Because the computer-generated curves fit the experimental curves so closely, we conclude that, at least for the chosen staminal hair cells, the rates of diffusion between cells is similar and the rates of loss within each cell is similar. These results do not contradict our pre-

vious reported finding that some staminal hair cells are more coupled than others (13). In the experiments presented here, great care was taken to use only staminal hairs composed of cells which were the same size, shape, and stage of development, whereas these parameters varied in the earlier studies. The present calculations are readily extended to the case of differing *K*s and *L*s for each cell. However, the fit is less elegant because of the numerous parameters. It is worth pointing out that although the model proposed here is very simple, the method of calculation is very powerful. No approximations to the diffusion equation are made. For example, the usual assumptions of steady state diffusion or of limiting rate decreasing as $t^{-1/2}$ are not used and in fact are inapplicable to the problem: the steady state occurs only for a vanishing moment at the peak of cellular content of CF in a cell, and this time varies for each cell. By contrast this method fits the complete time course of the diffusion, and in any number of sequential cells. Thus, it has the advantage of a global analysis where a large amount of data is fit with a minimum number of parameters.

The computer generated curve-fit method described in this paper may be useful to plant physiologists who study cell-to-cell communication in plants. A method using the integrated diffusion equation has been used in studies of cell-to-cell communication in animals (8, 17). For plants, even slight changes in diffusion either through plasmodesmata or across the vacuole membrane will be reflected in changes of coefficients of diffusion and loss, respectively. The method presented in this paper allows direct measurement of these coefficients.

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