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Characterization of the *Egeria densa* Leaf Symplast: Response to Plasmolysis, Deplasmolysis and to Aromatic Amino Acids

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Summary

The fluorescent dyes 6-carboxyfluorescein and fluorescein glutamylglutamic acid, which move freely in the *Egeria densa* leaf symplast, fail to move from cells subjected to plasmolysis, demonstrating that plasmolysis disrupts symplastic continuity. Dye movements begins again within 10 minutes of removal of the osmoticum and becomes more extensive with increasing recovery time. The re-established symplastic links show a number of distinctive features compared to untreated leaves: dyes of up to 1678 dalton can pass, compared to the normal limit of 665 dalton; and Ca^{2+} ions, which completely inhibit dye movement in untreated cells, only reduce the extent of dye movement. Aromatic amino acids and their fluorescein conjugates prevent intercellular movement in untreated cells. In deplasmolysed cells the aromatic conjugates move freely. The increased symplast permeability persists for at least 20 hours. Thus, after plasmolysis followed by deplasmolysis, the symplast shows a marked increase in permeability associated with an increased molecular exclusion limit, indicating an increase in pore size, and symplast permeability becomes relatively insensitive to Ca^{2+} ions or to the aromatic conjugates.

Keywords: Aromatic amino acids; *Egeria densa*; Fluorescent probes; Intracellular communication; Plasmolysis.

1. Introduction

Plasmolyzed plant cells have the plasmalemma pulled away from the cell wall, and it is thought that plasmodesmatal connections between cells are severed and hence symplastic continuity is broken. This technique has repeatedly been used to study the importance of symplastic continuity; for example to determine the mode of auxin transport (CANDE and RAY 1976, DRAKE and CARR 1978), of ion transport in roots (JARVIS and HOUSE 1970, VAN IREN and BOERS-VAN DER SLUIJS 1980), amino acid transport in *Vallisneria leaves* (BRÄUTIGAM and MÜLLER 1975), and of phloem loading (GEIGER *et al.* 1974); to study electrical coupling between cells (DRAKE *et al.* 1978), virus-host interactions (COUTTS 1978, GULYÁS and FARKAS 1978) and for developmental studies (reviewed by CARR 1976). However no definitive evidence is available to show that symplastic continuity is lost when cells are plasmolyzed, or regained once cells are deplasmolyzed. Hechtian strands are found between protoplasts of oat coleoptile even though cells are severely plasmolyzed (DRAKE *et al.* 1978). We have determined directly what effect plasmolysis and deplasmolysis have on symplastic continuity by injecting into cells dyes which do not pass the plasmalemma.

2. Materials and Methods

Egeria densa (Planch.) was collected from a pond in the Royal Botanical Gardens, Sydney. Healthy young leaves $^2/_3$ fully expanded (approximately 10 mm in length) were removed from the shoot apex and washed in bathing medium (0.1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.1 mM KCl, 0.5 mM NaCl, 0.05 mM $NaSO_4$ and 1.0 mM 3-[-N-morphalenolpropanesulfonic acid) pH 7.0 prior to plasmolysis. Leaves were plasmolyzed by soaking the leaves for 2 hours in a 0.7 M mannitol solution, after which time the protoplasts had drawn away from the cell wall and rounded up. Leaves were deplasmolyzed either rapidly, by immediate transfer to bathing medium, or slowly, by stepwise transfer through 0.5, 0.35, 0.25, and 0.175 M mannitol, 30 minutes each, before transfer to bathing medium.

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Fluorescein isothiocyanate isomer I[F] and lissamine rhodamine B [LRB] conjugates with L-glutamylglutamic acid [$(Glu)_2$], L-hexaglycine [$(Gly)_6$], insulin A chain, L-leucyl-diglutamylleucine [LGGL], phenylalanine [Phe], the pentamer (propyl-propylglycine)$_5$ [$(PPG)_5$], tyrosine [Tyr] and tryptophan [Trp] were synthesized as by GOODWIN (1983). Electrophysiological techniques and iontophoretic injection of dye were carried out as previously described (ERWEE and GOODWIN 1983). Five minutes after injection the leaf was observed under an epifluorescence microscope. F conjugates were observed with a blue excitation filter K 490 with a TK 510 dichroic beam splitter mirror and a K 515 suppression filter. LRB conjugates were observed with green excitation filters KP 560 and BG 36, with a TK 580 dichroic beam splitter and a K 580 suppression filter. Photographs were taken on Ektachrome 400 daylight film rated at 1,600 ASA. Injections where the dye moved into the injected cell only, or where the injected and adjacent cells were equally heavily loaded with dye, but there was no dye in other cells, were recorded as non-movement of the dye. Data were analyzed using an analysis of variance on log transformed data and χ^2-test.

3. Results

3.1. Plasmolysis

After 2 hours of plasmolysis in 0.7 M mannitol the protoplasm had pulled away from the cell wall and rounded up (Fig. 1). Plasmolysis stopped cytoplasmic streaming and suppressed chlorophyll fluorescence. Injections into protoplasts were very difficult to achieve, but in those few cases where $F(Glu)_2$ was successfully injected, the dye remained within the protoplast. Plasmolysis of cells after injection with 6-carboxyfluorescein resulted in numerous protoplasts containing the dye, while no dye was seen in the cellular space outside the protoplasts (Figs. 2 and 3). Again no dye movement was seen after plasmolysis, showing that symplastic continuity had been disrupted. Mannitol at 0.25 M neither caused plasmolysis nor interrupted dye movement. Thus the inhibition of cell-to-cell communication is due to plasmolysis, rather than to a specific response to mannitol.

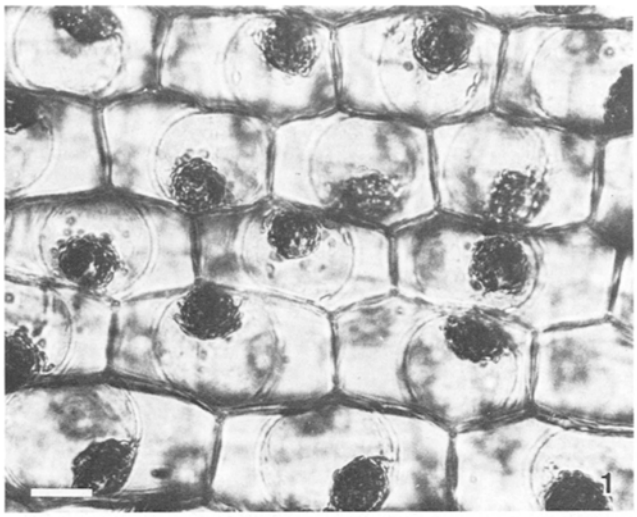
3.2. Deplasmolysis

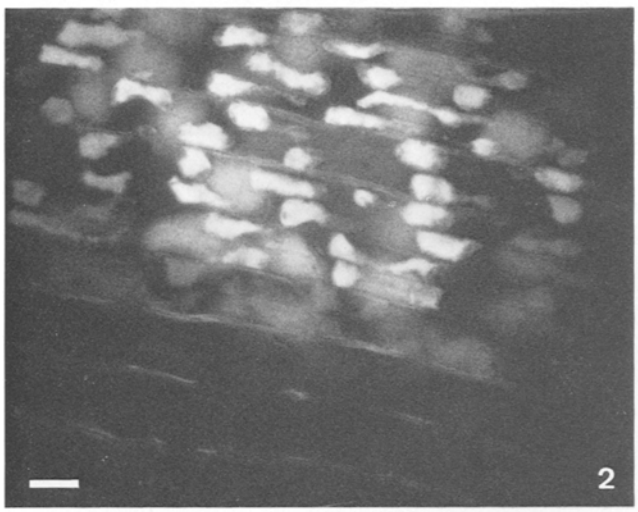
The time course of recovery of movement of $F(Glu)_2$ after rapid deplasmolysis is summarized in Tab. 1. Dye movement occurred from only 31% of cells which had been deplasmolyzed for 5 minutes, even though by this time the plasmalemma was resting against the cell wall (Figs. 6 and 7). Dye movement from cell-to-cell was common within 10 minutes after deplasmolysis (Fig. 8), though the extent of dye movement was limited. The extent of dye movement increased as the recovery time increased, so that after 120 minutes dye movement was to 15.2 cells on average as compared to 9.1 cells in the control leaves (Fig. 9). This suggests that plasmolysis and subsequent deplasmolysis increases symplast permeability. This could be due to a substantial increase in the number of functional plasmodesmata, or to the newly reestablished plasmodesmata being more permeable. The latter was the case (Tab. 2) since $F(Gly)_6$ (749 dalton) which does not move in control leaves moves freely in treated leaves. Movement of other large molecules which never move in untreated leaves was noted. The smallest non-mobile dye was LRB insulin A chain (4,158 dalton; Figs. 10 and 11). This is a substantial increase in symplast permeability. The increase in symplast permeability is not due to injury caused by the speed of deplasmolysis, as even with slow deplasmolysis, $F(PPG)_5$ moved in 87% of injections one hour after transfer to bathing buffer. The increased symplastic permeability lasted for at least 20 hours, $F(PPG)_5$ still moving from cell-to-cell after this time period. Leaves exposed only to low (non-plasmolysing) concentrations of mannitol before transfer to bathing buffer showed the same symplastic characteristics as control leaves.

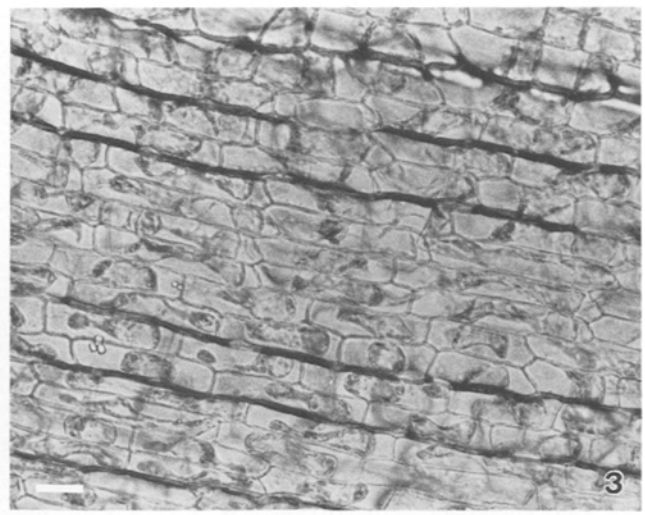
Plasmolysis and deplasmolysis may alter plasmodesmatal physiology and hence alter symplast permeability. To test this, cells were injected with Ca^{2+} ($CaCl_2$ 10^{-2} M in the electrode tip) prior to $F(Glu)_2$ injection. Ca^{2+} has been shown to inhibit movement of this dye in untreated leaf cells (ERWEE and GOODWIN 1983). In treated cells Ca^{2+} did not significantly inhibit the frequency of dye movement but did reduce the extent of dye movement, dye moving to an average of 7.2 cells as compared to 15.2 cells without Ca^{2+} injection. The difference is statistically significant. The molecular exclusion limit was apparently unaltered by

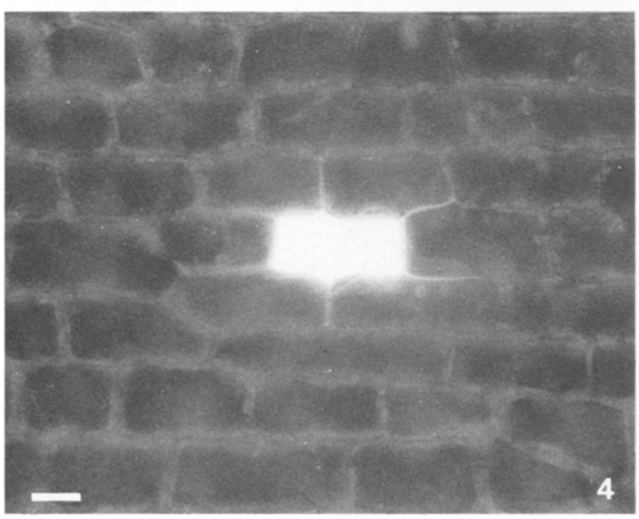
Table 1. *Recovery of movement of $F(Glu)_2$ in the leaf of Egeria densa with time, following 120 minutes of plasmolysis (0.7 M mannitol).* R is the fraction of injections where dye movement occurred. The numbers in ( ) are the total number of injections per treatment. The average cell number is the average number of cells containing dye, including only those injections where movement occurred. [1] Values of R significantly different from the control at the 1% level; for average cell number, means with the same superscript are not significantly different from one another

Deplasmolysis time (minutes)	R	Average cell number
Control	0.93 (15)	9.1[b]
5	0.31 (16)[1]	5.4[b]
10	0.75 (16)	6.4[b]
30	0.93 (15)	11.6[a]
120	1.00 (15)	15.2[a]









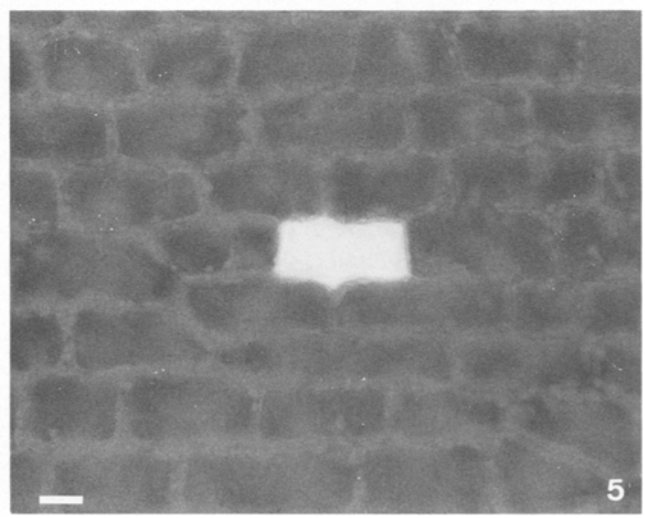


Fig. 1. *Egeria densa* cells after 2 hours of plasmolysis in 0.7 M mannitol. The protoplasts have drawn away from the cell wall and rounded up. Bar is 25 μm

Fig. 2. 6-carboxyfluorescein was injected prior to plasmolysis with 1.0 M mannitol. The dye is located in the protoplasts of the abaxial cell layer. The fluorescence in the background is due to protoplasts in the adaxial cell layer. Photographed after 15 minutes of plasmolysis. Bar is 50 μm

Fig. 3. A light photograph matching Fig. 2 showing the extent of plasmolysis. Bar is 50 μm

Fig. 4. F(Tyr) co-injected with LRB into leaf cells of *Egeria densa*, using F settings of filters to show location of F(Tyr). Dyes move across the tonoplast but not intercellularly. Bar is 50 μm

Fig. 5. A photograph matching Fig. 4 showing LRB location. It has not left the injected cell. LRB injected by itself moves freely from cell-to-cell. Bar is 50 μm

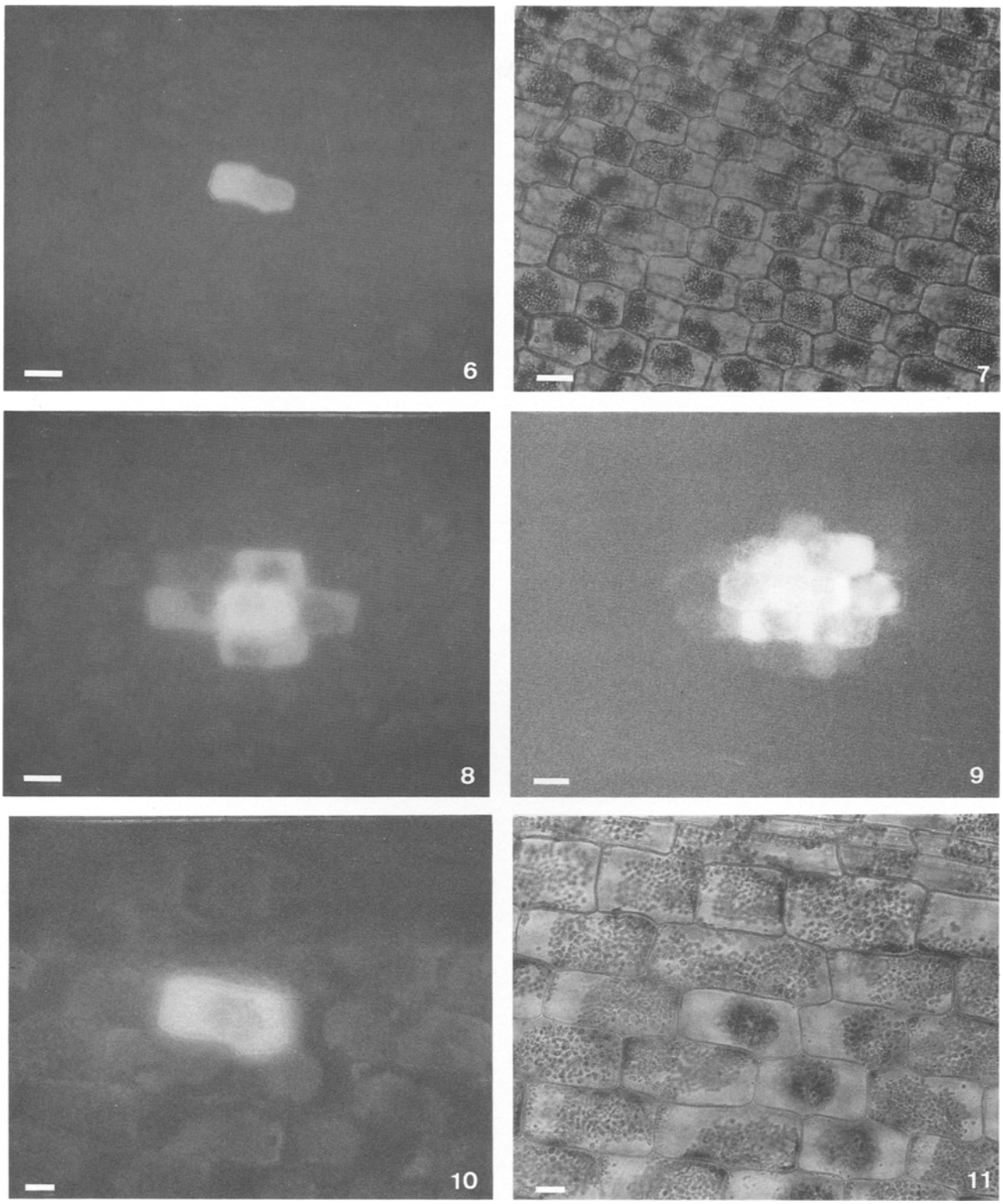


Fig. 6. Non-movement of $F(Glu)_2$ in deplasmolyzed leaf cells of *Egeria densa*. Cells have been plasmolyzed for 2 hours in 0.7 M mannitol and then removed from the osmoticum for 5 minutes. Bar is 50 µm

Fig. 7. A light photograph matching Fig. 4 showing cells have returned to full turgor with the plasmalemma lying against the cell wall. Bar is 50 µm

Fig. 8. Movement of $F(Glu)_2$ 10 minutes after removal of the osmoticum. Leaves were subjected to 120 minutes of plasmolysis in 0.7 M mannitol and then placed in bathing medium. Bar is 50 µm

Fig. 9. Movement of $F(Glu)_2$ 120 minutes after removal of the osmoticum. Bar is 50 µm

Fig. 10. Non-movement of LRB-Insulin A chain in leaves subjected to plasmolysis and allowed to recover for 120 minutes. Bar is 25 µm

Fig. 11. A light photograph matching Fig. 8. Bar is 25 µm

Table 2. *Movement of dyes in leaves of Egeria densa which have been subjected to plasmolysis (120 minutes in 0.7 M mannitol) and deplasmolysis (120 minutes in bathing solution).* R, ( ), average cell number as in Tab. 1

Dye	Molecular weight (dalton)	R		Average cell number (deplasmolyzed only)
		Control	Deplasmolyzed	
$F(Glu)_2$	665	0.93 (15)	1.00 (15)	15.2
$F(Gly)_6$	749	0.00 (15)	1.00 (15)	10.9
F(LGGL)	874	0.00 (15)	0.50 (20)	5.0
$F(PPG)_5$	1,678	0.00 (14)	0.75 (16)	3.7
LRB-insulin A chain	4,158	0.00 (10)	0.00 (14)	0.0

Table 3. *Movement of amino acid-dye conjugates in Egeria densa leaves.* R, ( ), as in Tab. 1

Control			Deplasmolyzed	
Dye	R	Average cell number	R	Average cell number
F(Tyr)	0.13 (15)	5.0	1.0 (8)	16.3
F(Phe)	0.07 (15)	2.5	1.0 (8)	22.8
F(Trp)	0.13 (16)	3.5	1.0 (8)	20.0

Table 4. *Movement of F(Glu) in Egeria densa after intracellular injection of aromatic amino acids and glutamic acid (10 mM in the electrode tip).* R, ( ), [1] as in Tab. 1

	R	Average cell number
Control	1.0 (15)	10.5
Glutamic acid	0.73 (15)	15.4
Tyrosine	0.14 (14) [1]	7.0
Tryptophan	0.33 (15) [1]	7.4
Phenylalanine	0.21 (14) [1]	7.0

Ca^{2+}, $F(PPG)_5$ moving in 71% of injections after Ca^{2+} injection.

Dye conjugates with aromatic amino acids rarely move from the injected cell in *Egeria* (Tab. 3). Lissamine rhodamine and F(Glu) normally moved freely from cell-to-cell. However, when co-injected with the aromatic dye conjugates LRB fails to move (Figs. 4 and 5), indicating that the aromatic amino acid conjugates fail to move because, like group II ions, they reduce symplast permeability. Similarly coinjection of the non-conjugated aromatic amino acids with F(Glu) inhibits the movement of the dye (Tab. 4). Coinjected glutamic acid has no significant effect. The site of aromatic amino acid control of symplast permeability is not directly accessible from the external solution, since soaking leaves for 90–120 minutes in a 10 mM solution of phenylalanine has no effect on F(Glu) movement. After plasmolysis followed by deplasmolysis the aromatic dye conjugates move freely from cell-to-cell (Tab. 3).

4. Discussion

Plasmolysis usually results in a number of plasmodesmata being ruptured, the number depending on the severity of protoplasmic shrinkage (BURGESS 1971). Symplastic continuity in *E. densa*, as detected by dye movement from cell-to-cell, is broken when cells are plasmolyzed. No dye was seen to move between protoplasts even when protoplasts were in mutual contact with a cell wall. Membrane integrity remained intact since no dye was seen to enter or leave the protoplasts. Concentrations of mannitol insufficient to cause plasmolysis had no effect on symplastic continuity.

Removal of the osmoticum resulted in rapid cell-turgor recovery. There is some confusion in the literature as to whether symplastic continuity is restored once cells have been plasmolyzed and then deplasmolyzed. STRASBURGER (1901) thought that no plasmodesmata were reformed after disruption by plasmolysis. DRAKE *et al.* (1978) found that electrical coupling between cells which had undergone plasmolysis and deplasmolysis was non-existent within 60 minutes of deplasmolysis,

but they could detect some coupling 6 hours after recovery. On the basis of viral transmission symptoms COUTTS (1978) doubts that symplastic continuity is restored after plasmolysis, at least within 48 hours after treatment. In *E. densa* symplastic continuity was not fully re-established 5 minutes after removing the leaves from the osmoticum, even though cell turgor had recovered. However, within 10 minutes frequent dye movement occurred, and dye movement become more extensive with increasing recovery time. Within 30 minutes dye movement exceeded that in untreated leaves. It appears that plasmolysis followed by deplasmolysis causes plasmodesmata to show increased permeability, associated with a larger molecular exclusion limit, probably reflecting a larger pore size. This state of increased symplast permeability persists in cells for at least 20 hours after treatment.

Whether these re-established plasmodesmata are structurally identical to normal plasmodesmata is unknown. However, they do show differing physiological characteristics. In untreated cells dye conjugates of aromatic amino acids do not pass out of the injected cell. In cells which have undergone plasmolysis and deplasmolysis, the aromatic conjugates move freely from cell-to-cell. They no longer reduce symplast permeability. Similarly Ca^{2+} has been shown to be effective in reducing symplast permeability, so that even low molecular weight dyes such as 6-carboxyfluorescein (376 dalton) do not move out of the injected cell. In deplasmolyzed leaves a Ca^{2+} injection prior to $F(Glu)_2$ injection reduces the average cell number containing dye from 15.2 to 7.2, but the frequency of movement of this and of larger dyes is unaffected. Thus the reformed symplast links in deplasmolyzed leaves appear to be relatively insensitive to regulation.

Failure of conjugates of fluorescein and aromatic amino acids to move from cell to cell was reported by TUCKER (1982), working with staminal hairs of *Setcreasea purpurea*. In *Egeria* the aromatic conjugates move through both the vacuole and cytoplasm of the injected cell, so the tonoplast is not the barrier. Since co-injected LRB or F(Glu) do not move, it appears that the aromatic amino acid conjugates restrict plasmodesmatal permeability in some manner. Since co-injected unconjugated aromatic amino acids inhibit F(Glu) movement, it appears that certain aromatic molecules are able to reduce intercellular communication whether or not they are conjugated to fluorescein. Bathing the tissue with phenylalanine had no effect on dye movement, indicating that these compounds act within the cell, presumably at the plasmodesmata. The aromatic amino acids and related compounds which reduce intercellular permeability are not likely to be able to move in the symplast.

The increase in plasmodesmatal permeability after deplasmolysis suggests that studies on tissues which have been deplasmolyzed need to be reassessed. For example the abnormal branching of filamentous fern (*Pteris vittata*) gametophytes following plasmolysis is proposed to be due to a disruption of cell-to-cell communication (NAKAZAWA 1963). However, the phenomenon may be due to an increase in symplastic permeability, which allows for exchange between cells of chemical stimuli which otherwise would not be able to move, this causing abnormal development.

Plasmolysis appears to be a valid method to study cellular isolation and symplastic transport phenomena, at least in *E. densa*, since we have shown that no dye movement occurs between plasmolyzed protoplasts. Plasmolysis followed by deplasmolysis offers an addition to techniques for studying the symplast: a simple method to increase symplast permeability. Should this prove to be a general phenomenon, it may be a valuable addition to methods for studying the role of symplast continuity and cell isolation in transport and differentiation in plants.

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