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Movement of Lucifer Yellow CH in potato tuber storage tissues: A comparison of symplastic and apoplastic transport

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Abstract. The fluorescent dye Lucifer Yellow CH (LYCH) was introduced directly into the symplast of potato (*Solanum tuberosum* L.) tuber storage parenchyma by microinjection and also into the apoplast through cuts made in the stolon cortex. Microinjected LYCH moved away rapidly from a single storage cell and spread radially via the symplast. When the microinjected tissue was subsequently fixed in glutaraldehyde and sectioned the dye was seen clearly to be localised in the cytoplasm but not in the vacuole. In comparison, when LYCH was introduced into cuts made in the stolon cortex the dye entered the tuber by the xylem and subsequently spread apoplastically. No movement of dye was observed in the phloem. In glutaraldehyde-fixed tissues, in which LYCH was introduced to the apoplast, the dye was found within xylem vessels, in the cell walls and in intercellular spaces. Wall regions, possibly associated with plasmodesmata, became stained by the dye as it moved through the apoplast. Three hours after introduction of the dye to the stolon, intense deposits of LYCH were found in the vacuoles of all cells in the tuber, many aligned along the tonoplast. Differentiating vascular parenchyma elements contained large amounts of dye within enlarging vacuoles. However, with the exception of plasmolysed and-or damaged cells, LYCH was absent from the cytoplasm following its introduction to the apoplast. Since LYCH is unable to cross the plasmalemma it is suggested that the most likely pathway from the cell wall to the vacuole was by endocytosis, the dye being transported across the cytoplasm in membrane-bound vesicles. Clathrin-coated vesicles were abundant in the storage cells, providing a possible endocytotic pathway for dye movement. The significance of these observations is discussed in relation to the movement of LYCH in plant tissues and to the movement of solutes within and between storage cells of the tuber.

Key words: Apoplast – Endocytosis – *Solanum* (tuber, transport) – Symplast – Tuber-vacuole

Abbreviation: LYCH = Lucifer Yellow CH

Introduction

The study of symplastic transport in plant tissues has been facilitated greatly in recent years by the advent of microinjection procedures in which fluorescent probes are introduced directly into the cytoplasm of living cells. Fluorescent peptides of different molecular weights have been used to determine the molecular exclusion limit of plasmodesmata (Goodwin 1983) and the highly fluorescent dye, Lucifer Yellow CH (LYCH), which is unable to cross the plasmalemma (Erwee et al. 1985; Terry and Robards 1987; Buckmaster et al. 1987; Sandvig et al. 1987), has proven to be a particularly effective, mobile compound with which to examine symplastic transport in vivo. However, the extent to which the pattern of movement of LYCH parallels that of sucrose remains a matter for conjecture. Madore et al. (1986) demonstrated movement of LYCH between the mesophyll cells and minor veins of *Ipomoea tricolor*, indicating the lack of a symplastic barrier to the diffusion of small molecules throughout the leaf symplast. However, as emphasised by Delrot (1987) there is as yet no evidence that the movement of LYCH, a charged molecule, along its concentration gradient is representative of the movement of sucrose, an uncharged molecule, which frequently moves against a concentration gradient. However, LYCH offers great potential for determining symplastic contin-

uity between plant cells, allowing symplastic 'domains' in plant tissues to be identified (Erwee and Goodwin 1985; Palevitz and Hepler 1985). Recently, Kronestedt and Robards (1987) have also successfully employed LYCH as an apoplastic tracer.

In the potato tuber the storage cells are connected to the internal phloem by numerous plasmodesmata (Oparka 1986) and plasmolysis of storage tissues prevents the transport of [^{14}C] sucrose into solute-collecting wells made in the tuber (Oparka and Prior 1987), indicating that at least part of the transport pathway between the phloem and the storage parenchyma is via the symplast. By contrast, excised storage-parenchyma discs are able to take up exogenous sucrose from the apoplast by active, carrier-mediated transport across the storage-cell plasmalemma (Oparka and Wright 1988a, b). An effective mechanism for sucrose uptake from the apoplast appears at first to be contradictory to the presence of high frequencies of plasmodesmata connecting storage cells, unless the former represents part of a retrieval mechanism for sucrose lost to the apoplast (see Maynard and Lucas 1982) and is not the sole pathway for sucrose movement in vivo.

The aim of the present work was to examine further the potential for solute movement through the symplast and apoplast of potato tuber tissues using LYCH as a marker for continuity in both pathways. We compare the movement of LYCH following its microinjection into individual storage cells with its apoplastic spread subsequent to arrival in the xylem and demonstrate that, in both cases, the dye is able to achieve an intracellular location within storage cells, but by quite different transport pathways. The significance of the results are discussed in relation to transport studies utilising LYCH and to the in-vivo transport of solutes within and between plant cells.

Materials and methods

Plant material. Potato (*Solanum tuberosum* L. cv. Record) plants were grown in a heated glasshouse maintained at 15° C, in 20-cm-diameter pots containing compost. The plants were used for experiments when the developing tubers had reached a size of between 0.5–3.0 cm diameter, approx. 70 d after planting.

Symplastic introduction of Lucifer Yellow CH

Tissue preparation. Tubers were removed from their stolons and cut transversely into slices approx. 3 mm thick. The slices were washed thoroughly in distilled water for about 20 s in order to remove the starch and other cell debris from the cut cells at the surface of the tissue slice. The tissue was then immersed briefly in 0.01% (w/v) aqueous cellufluor (Polysciences, Northampton, UK). This allowed the cell walls of the intact storage cells immediately below the surface layer of cut cells to be seen easily and aided in the selection of individual cells for microinjection.

Microinjection. Lucifer Yellow CH (Sigma Chemical Co., Poole, Dorset, UK) was prepared as a 5% (w/v) aqueous solution prior to microinjections. Borosilicate glass capillaries (Narishigi Scientific Instrument Laboratory, Tokyo, Japan) were pulled into microcapillaries of 0.5–1 µm diameter using a Narishigi needle puller (model PB-7). Lucifer Yellow CH was backfilled into the microcapillaries using an Eppendorf centrifuge tube as a dye reservoir. The dye-filled needles were inserted into the arm of a hydraulic micromanipulator type MO-102; Narishigi) bolted to the stage of the microscope and the needles directed using a three-axis joystick (MO-202; Narishigi), hydraulically coupled to the stage micromanipulator. Individual storage cells were impaled with the tip of the micropipette through the base of an empty surface cell.

Microscopy. Microinjections were carried out using a Nikon (Tokyo, Japan) Labophot microscope, with fluorescence attachment, under either a ×10 or ×20 long-working-distance objective. A blue-violet combination filter (Nikon BV-1A, dichroic mirror 455 nm, excitation filter 425–445 nm, barrier filter 460 nm) was used for the initial microinjection. Under this wavelength, cellufluor-induced wall fluorescence appeared blue and the LYCH a bright yellow. Following a successful injection, the needle tip was removed from the cell and the combination filter changed to blue (Nikon B-2E, dichroic mirror 510 nm, excitation filter 450–490 nm, barrier filter 520–560 nm). This successfully removed the cellufluor-induced fluorescence and allowed only the spread of the LYCH to be visualised. Movement of the dye was recorded on Kodak (Hemel Hempsted, Herts., UK) Ektachrome 160 colour transparency film. The microscope and micromanipulator were mounted on a antivibration bench (Photon Control, Cambridge, UK) during microinjections and subsequent photography. Exposure of the tissue to UV radiation was kept to a minimum to prevent damage to the tissue (see Madore et al. 1986). The time taken from cutting the tubers to the observation of dye movement was seldom greater than 2 min.

Fixation and embedding. Lucifer Yellow CH has a free hydrazido group that reacts with aliphatic aldehydes at room temperature (Stewart 1981). Tuber tissue slices, which had been injected with LYCH, were trimmed with a razor blade around the area of dye movement under a ×4 Nikon objective. The excised tissue pieces were immersed immediately in 3% glutaraldehyde/2% paraformaldehyde in 50 mM sodium-cacodylate buffer and fixed for 2 h under slight vacuum. The tissue was then dehydrated through a graded alcohol series and embedded in London Resin (LR White; Agar Scientific, Stanstead, Essex, UK). Sections (1–2 µm) were cut on glass knives on an LKB (Bromma, Sweden) Ultrotome III ultramicrotome, dried on to glass sides and mounted in fluoromount (BDH, Poole, Dorset, UK). Resin sections were viewed under a blue-violet combination filter (Nikon BV-1A) or blue filter (Nikon B-1A, dichroic mirror 510 nm, excitation filter 470–490 nm, barrier filter 520 nm).

Introduction of LYCH to the apoplast

The compost was removed from around the tubers and a series of parallel incisions made into the cortex of the stolon, approx. 3 cm from the tuber, using a sharp needle. A 50 µl Hamilton syringe was loaded with 5% (w/v) aqueous LYCH and the

dye introduced into the cuts from the end of the needle. Approximately 30 tubers were treated in this way. The plants were left for a range of time intervals from 0.5–3 h before harvesting the tubers. At each harvest some of the tubers were sliced transversely and the fresh tissue mounted on a slide to monitor dye movement. The spatial separation of xylem and phloem in the potato tuber (Oparka 1986) allowed the route of entry of the dye into the tuber to be determined. The remaining tubers were sliced transversely or longitudinally into fixative. In the case of very small tubers (<0.5 cm diameter) the entire tuber was immersed in fixative, without cutting, in order to minimise losses of dye from cut surfaces. Subsequent dehydration, embedding and fixation procedures were exactly as described for microinjected tissues.

Electron microscopy

Tissue slices from untreated tubers were fixed as described above and postfixed in 2% osmium tetroxide. Postfixation eliminates LYCH fluorescence (Stewart 1981) and so LYCH-containing tissues were not prepared for electron microscopy. The tissue was dehydrated in a graded alcohol/propylene oxide series and embedded in E-Mix resin (Agar Scientific). Ultrathin sections were collected on formvar-coated copper grids, stained with uranyl acetate and lead citrate, and examined in a JEOL (Tokyo, Japan) JEM 1200 EX electron microscope operating at 80 kV.

Isolation of storage cells

Storage-parenchyma cells were isolated from tissue slices exactly as described previously (Oparka 1986).

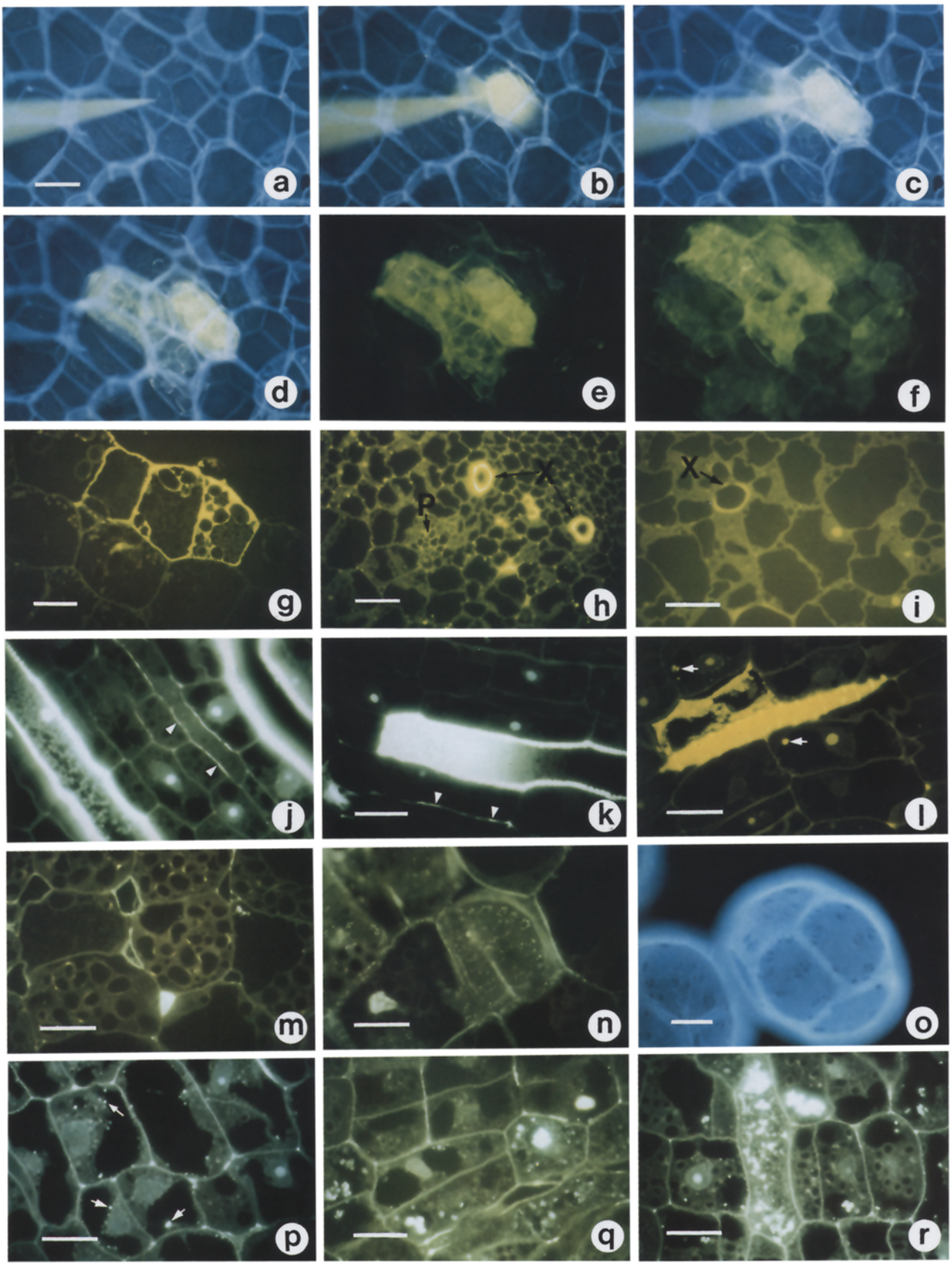
Results

Symplastic movement of LYCH. Figure 1 (a–f) shows the time course of movement of LYCH through potato tuber storage-parenchyma cells following its injection into a single cell. Within approx. 30 s the dye had spread into adjoining cells, as the original cell continued to be injected. Injections into the vacuole resulted in the dye remaining in the original cell without movement. However, the relatively large ratio of cytoplasm to vacuole in immature storage tissues facilitated the frequency of successful cytoplasmic injections (see also Terry and Robards 1987). As the dye moved it was frequently observed to spread around the amyloplasts making them appear silhouetted against the intense yellow cytoplasm. However, in cells which became ruptured or damaged during impalement the starch grains became intensely fluorescent, presumably as a consequence of penetration of the dye across the damaged amyloplast membrane. The rapid staining of starch with LYCH provided a convenient means for detecting when cellular damage had occurred during microinjection and such tissue was discarded. With all cytoplasmic injections the dye showed a rapid and radial spread away from the injected cell. Tissue which had been fixed in glutaraldehyde and sectioned showed that the LYCH was confined to the cytoplasm and did not penetrate the tonoplast or amyloplast membranes (Fig. 1 g).

Apoplastic movement of LYCH. When LYCH was introduced into cuts in the stolon cortex the dye appeared rapidly within the growing tuber. An examination of free-hand sections taken from about 20 tubers revealed that in all cases the dye entered the tuber via the xylem ring. No dye movement was observed in the internal phloem, which in the potato tuber is spatially separated from the xylem (Oparka and Prior 1987). Sections taken from tissue which had been fixed in glutaraldehyde showed the secondary thickenings of the xylem vessels to be intensely fluorescent (Fig. 1 h). Control tissue which was untreated with LYCH showed only a pale yellow-green background autofluorescence and lacked the intense yellow fluorescence associated with LYCH (Fig. 1 i). In many tubers, intense LYCH fluorescence was seen within the vessel lumen, presumably where LYCH had reacted with the glutaraldehyde in situ (Fig. 1 j, k, l). In such instances the LYCH frequently had a coagulated appearance (Fig. 1 j, k). Some of the parenchyma elements bordering xylem vessels which contained LYCH were plasmolysed and the dye was found within the cytoplasm of these cells. However, no symplastic spread of dye was observed into adjoining unplasmolysed cells (Fig. 1 l). Following its arrival in the xylem, LYCH was detected in the cell walls and intercellular spaces of both vascular parenchyma (Fig. 1 j, k) and storage parenchyma (Fig. 1 m, p).

Glancing sections of the walls of vascular and storage-parenchyma cells demonstrated numerous small and intensely fluorescent spots (Fig. 1 n). Such areas were not visible in control tissues lacking LYCH. It was not possible to determine whether these areas represented localised accumulations of the dye or whether the dye had stained specific wall areas as it moved through the apoplast. Many of the fluorescent wall spots were arranged in discrete areas resembling plasmodesmatal pit fields, as seen in isolated storage cells stained with cellufluor (Fig. 1 o) and also aniline blue (data not shown).

Intracellular deposition of LYCH following apoplastic uptake. Growing tubers in which LYCH had entered via the apoplast also contained numerous, intensely fluorescent deposits of dye within their cell vacuoles. In storage cells, large accumulations of dye were found within the central vacuole and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numerous small fluorescent deposits were aligned along the tonoplast (Fig. 1p). The most concentrated accumulations of dye were seen within the enlarging vacuoles of differentiating vascular parenchyma elements (Fig. 1q, r). Despite an extensive search, dye deposits were not seen in the cytoplasm of tubers in which LYCH had entered apoplastically. In the electron microscope, several types of vesicle were observed in the cytoplasm of the storage cells (Fig. 2). Vesicles derived from the Golgi apparatus were abundant, several containing fibrillar material which appeared to be destined for the cell wall. Clathrin-coated vesicles were also abundant in the cytoplasm, some in the vicinity of the wall (arrows, Fig. 2). However, since LYCH possesses no inherent electron density (Stewart 1981) the precise subcellular location of the dye could not be determined.

Fig. 1. a–f Time course of movement of LYCH between storage-parenchyma cells of potato tubers. The dye was injected into the cytoplasm through the base of an empty cell at the cut surface. The tissue was surface-stained with cellufluor to aid the visualisation of individual cell walls and viewed under blue-violet excitation (**a–d**; Nikon BV-1A filter). Following a successful injection the wavelength was changed to blue (**e**, **f**; Nikon B-2E filter). This removed the wall fluorescence and allowed the spread of dye to be followed. The photographs were taken at 30-s intervals except for **f** which was taken 3 min after **e**. ×80; bars = 100 μm.

g Resin section (2 μm) of potato tuber storage tissue which had been microinjected with LYCH and then immersed in fixative. LYCH can be seen clearly in the cytoplasm of three cells immediately below the cut surface. The vacuoles and the amyloplasts are unstained. Blue excitation (Nikon B-1A); ×80; bar = 100 μm.

h Resin section showing intense fluorescence in the secondary walls of xylem vessels (*X*) in the potato tuber following the introduction of LYCH to the stolon apoplast. A neighbouring internal phloem group (*P*) does not contain LYCH. Blue excitation (Nikon B-1A); ×40; bar = 200 μm.

i Control section of tuber in which LYCH was omitted. The cells show a pale green-yellow background autofluorescence. Nucleoli autofluoresce a bright yellow. *X* = xylem. Blue excitation (Nikon B-1A); ×400; bar = 25 μm.

j–l Localisation of LYCH within the lumina of xylem vessels in the potato tuber following the introduction of the dye to the stolon apoplast. Note that the dye has a coagulated appearance in some vessels (**j**, **k**). The cell walls of xylem-parenchyma elements also contain LYCH (*darts*, **j**, **k**). In **l** the vessel lumen is filled with LYCH and an adjoining plasmolysed cell contains dye in the cytoplasm. Vacuolar deposits (*arrows*) are evident in some of the neighbouring cells but the dye is absent from the cytoplasm of these cells. **j**, **k**: blue-violet excitation (Nikon BV-1A); ×400; bar = 25 μm. **l**: blue excitation (Nikon B-1A); bar = 25 μm.

m Localisation of LYCH in intercellular spaces between storage-parenchyma cells following introduction of the dye to the apoplast. Blue-violet excitation (Nikon BV-1A); ×400; bar = 25 μm.

n Glancing section of the wall of a storage-parenchyma cell. Intense fluorescent spots of LYCH are evident, some arranged in discrete whorls. Blue-violet excitation (Nikon BV-1A); ×400; bar = 25 μm.

o Isolated storage cells stained with cellufluor. Pit fields, revealed as dark patches on the wall, are clearly visible. Blue-violet excitation (Nikon BV-1A); ×160; bar = 50 μm.

p–r Vacuolar deposition of LYCH following introduction of the dye to the apoplast. **p** Storage-parenchyma cells contain intense fluorescent deposits within their vacuoles, many close to the tonoplast (*arrow*). **q**, **r** Intensely fluorescent deposits of LYCH are present in the enlarging vacuoles of differentiating xylem-parenchyma elements. The cell walls and intercellular spaces also contain dye. Blue-violet excitation (BV-1A); ×400; bar = 25 μm

Discussion

Lucifer Yellow continues to be used extensively to study intercellular communication in plants. The dye is highly mobile in the symplast, intensely fluorescent and of low toxicity to cells in which it is moving (Stewart 1981). Stewart (1981) has shown that LYCH reacts with aldehyde-containing fixatives, possibly through the free hydrazido group of the LYCH molecule, so that its distribution within cells can be studied in semi-thin resin sections at relatively high resolution. Robards (1984) has shown that LYCH can also be fixed in plant tissues with glutaraldehyde. In the present study, LYCH remained intensely fluorescent following its fixation in microinjected tissues and its distribution in resin sections was as predicted from its observed movement in fresh tissue slices (compare Fig. 1d and Fig. 1g). When LYCH was introduced into the apoplast of the stolon it quickly entered the tuber via the xylem. In this case, also, the observed arrival of the dye in the xylem, as seen in fresh tissue, was substantiated by the presence of intense concentrations of LYCH in the apoplast of fixed tissues. Furthermore, the presence of large accumulations of the dye within the lumina of xylem vessels indicates that much of the dye had reacted with the penetrating glutaraldehyde in situ. It is conceivable that some dislocation of LYCH occurred during the fixation of the tuber tissues and this was not assessed quantitatively. However, the reaction of LYCH with fixative clearly removes several of the problems normally associated with the localisation of water-soluble dyes in plant tissues (see Canny and McCully 1986).

When LYCH was microinjected into a single potato storage cell it spread radially into surrounding tissues, demonstrating symplastic continuity between storage-parenchyma cells. Such cells are connected to each other by numerous plasmodesmata arranged in discrete pit fields (e.g. Fig. 1o). The storage cells are in turn connected to either

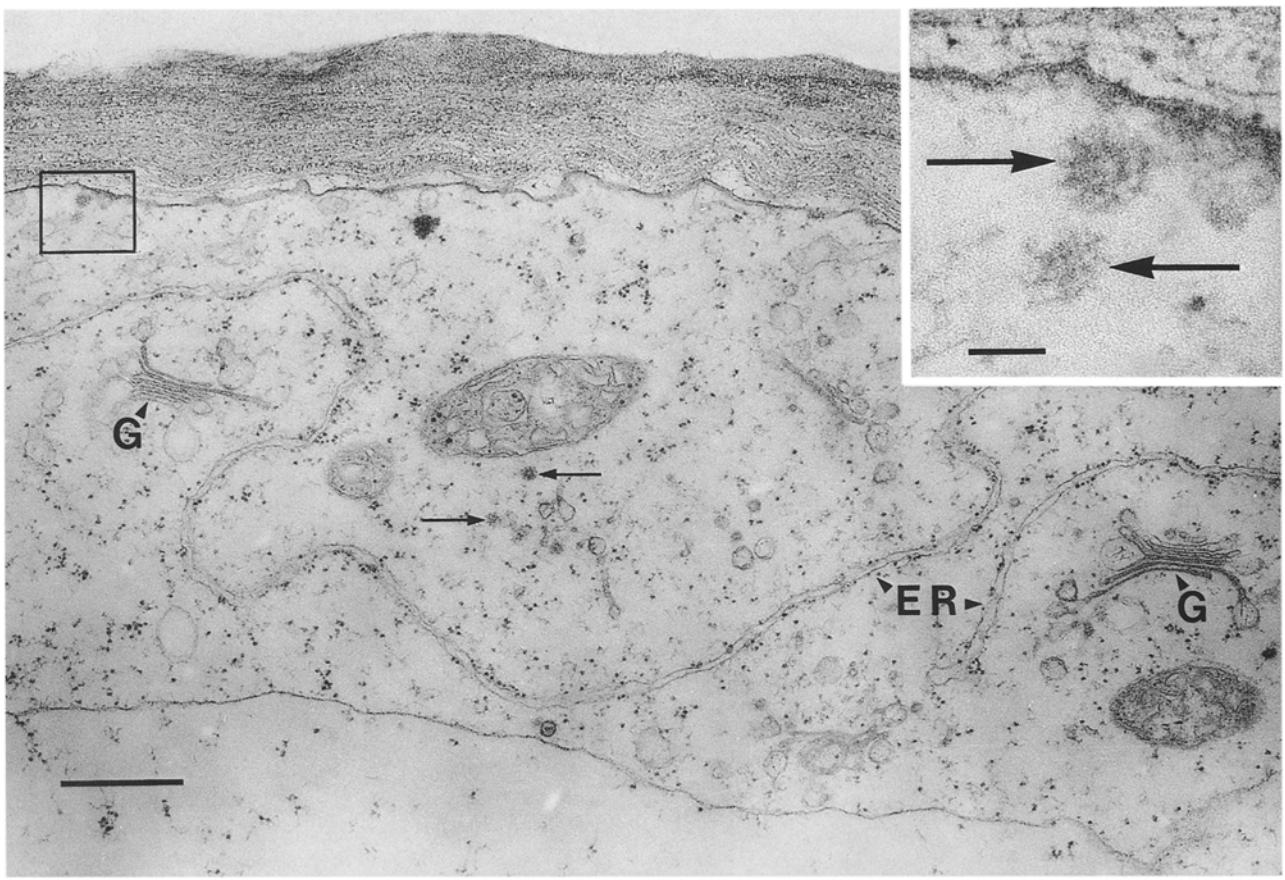

Fig. 2. Thin section through the cytoplasm of a potato tuber storage-parenchyma cell. Golgi-derived vesicles are abundant. Clathrin-coated vesicles appear in the cytoplasm and in the vicinity of the wall (*arrows*). *ER*, endoplasmic reticulum; *G*, Golgi apparatus. × 32000; bar = 500 nm. *Insert*: Enlargement of enclosed region, showing clathrin-coated vesicles (*arrows*). × 205000; bar = 50 nm

vascular parenchyma elements or directly to sieve elements (Oparka 1986). From a structural viewpoint, alone, unloading of sucrose from the phloem could occur by an entirely symplastic pathway, provided that sucrose (MW 342) is able to move as freely through the storage-cell symplast as LYCH (MW 457). If the hydrodynamic radius of the solute molecule is the key determinant for its passage through plasmodesmata (Terry and Robards 1987) then there appears to be no reason why sucrose should not move freely in the symplast of potato tubers. However, this is not to say that this is the sole pathway of sucrose movement in the tuber (see Oparka and Wright, 1988a, b). There is no evidence that LYCH can exchange between apoplast and symplast, and so direct comparisons between the mobility of sucrose and LYCH are likely to be of limited value unless both are moving in the symplast.

Fixation of microinjected tissues showed that LYCH was clearly located in the cytoplasm, but not in the vacuole, of storage-parenchyma cells and LYCH which was microinjected into the vacuole failed to enter the cytoplasm and be transported. These observations are in agreement with other studies using LYCH (Goodwin 1983; Palevitz and Hepler 1985; Terry and Robards 1987) which demonstrate that LYCH is unable to cross the tonoplast from either the cytoplasm or the vacuole. In marked contrast to these observations, LYCH readily entered the vauoles of storage cells when the dye was introduced to the apoplast. However, despite the presence of intense concentrations of the dye in both the cell wall and the vacuole, LYCH was not detected 'in transit' across the cytoplasm. The dye was only found to enter the cytoplasm from the apoplast when the cells were damaged and-or severely plasmolysed, and it did not spread from these to adjoining unplasmolysed cells, an observation in agreement with that of Erwee and Goodwin (1984) that plasmolysis prevents the symplastic movement of LYCH.

Since LYCH is a membrane-impermeant dye (Buckmaster et al. 1987; Sandvig et al. 1987) and is unable to cross the plasmalemma of plant cells (Erwee et al. 1985; Terry and Robards 1987) then the most likely transport mechanism of dye from the apoplast to the vacuole was by endocytosis, the dye being transported across the cytoplasm in membrane-bound vesicles formed at the plasmalemma. There is now considerable evidence for the endocytosis of macromolecules into plant cells (Baker and Hall 1973; Steer 1988 and references therein) although the uptake of low-molecular-weight solutes by this mechanism has seldom been reported. However, endocytosis of LYCH has recently been demonstrated to occur in a range of animal cells (Bernardini et al. 1987; Sandvig et al. 1987) and also in yeast cells (Reizman 1985) and the dye appears to be a particularly effective marker for fluid-phase endocytosis (Swanson et al. 1987; Sandvig et al. 1987). Uptake of LYCH by endocytosis would involve the packaging of the dye at the cell surface into clathrin-coated vesicles prior to its transit across the cytoplasm (Steer 1988 and references therein). Clathrin-coated vesicles were abundant in the cytoplasm of the storage cells. However, fluorescent points indicating the presence of such vesicles were not seen in the cytoplasm, possibly because such small vesicles (50 nm) contained insufficiently high concentrations of dye to distinguish them from the general autofluorescent background of the cytoplasm. Since LYCH possesses no inherent electron density, and therefore cannot be seen in the electron microscope (Stewart 1981), the exact mechanism of transport across the cytoplasm remains a matter for conjecture at the present time.

Studies of solute transport in plants frequently regard the apoplast and symplast as mutually exclusive transport compartments (see Delrot 1987 and references therein) with a tendency for a particular solute molecule to move freely in either one or the other. Some solutes, such as sucrose, are able to cross the plasmalemma by active, carrier-mediated transport, usually at discrete cellular locations within the plant. However, little is known of the ability of low-molecular-weight xenobiotics to exchange between different plant compartments. Recently, Canny (1987) has shown that the fluorescent dye sulphorhodamine G, normally confined to the apoplast when introduced via the xylem, is able to enter the cytoplasm of the paraveinal mesophyll in soybean leaves. This movement into the cytoplasm was interpreted as evidence for the presence of active proton-extrusion pumps at the plasmalemma of these cells, the low localised wall pH causing the dye to become undissociated and thus cross the plasmalemma. This mechanism appears to be quite distinct from the movement of dye from apoplast to vacuole reported here but, nonetheless, emphasises that fluorescent probes, when introduced to the apoplast, may gain access to cells by a number of potential pathways and mechanisms.

The high concentration of LYCH found in vascular parenchyma cells in the present study indicates that some cell types may be specialised for solute uptake by endocytosis. We suggest that introduction of LYCH to the apoplast of different plant organs, followed by the fixation of specific tissues, may aid in the identification of cell types active in solute uptake by this mechanism.

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