

TECHNICAL ADVANCE

Near-infrared femtosecond laser pulses as a novel non-invasive means for dye-permeation and 3D imaging of localised dye-coupling in the *Arabidopsis* root meristem[†]

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Summary

We have used near-infrared femtosecond Titanium:Sapphire laser pulses as novel non-invasive means for dye loading into various cell types of the *Arabidopsis* root meristem, and by 3D imaging have assessed the extent of dye coupling between the meristematic cells. The post-embryonic primary root of *Arabidopsis thaliana* has an invariant ontogeny and fixed cellular organisation which makes it an attractive model system to study developmental events involving cell fate determination, cellular differentiation and pattern formation. Local intercellular communication and local transmission of positional signals are likely to play a pivotal role in cell proliferation and regulation of differentiation. We have therefore examined the extent to which the constituent cells in the root meristem are symplastically coupled. Following laser-assisted loading of membrane impermeate fluorescent dye propidium iodide (PI) in single cells, we show by time-lapse and 3D imaging that in the root tip all undifferentiated cells are dye-coupled. When PI is permeated into the central cells, it rapidly moved into the adjacent initials of the columella, cortex, pericycle and stele. Interestingly, when only either of the initials were loaded with the dye, it never moved into any of the central cells. Amongst the epidermal cells, the differentiated hair cells are symplastically isolated. Our data provide evidence (1) for differential dye-coupling behaviour between quiescent centre cells and the neighbouring initials; (2) that cells in the root are coupled during stages at which the cell-lineage pattern is formed and that it becomes progressively secluded as they differentiate and the pattern is fixed. Taken together, our NIR-laser mediated approach is highly efficient and has numerous potential applications for non-invasive permeation of dyes in different cell types.

Introduction

In the last decade, confocal laser scanning microscopy has revolutionised the biologist's ability to visualise microscopic structures within thick living specimens (Berns *et al.*, 1991; Hepler and Gunning, 1998; Terasaki and Dailey, 1989). However, when observing living specimens, particularly at different stages of development, one is confronted with some serious problems with normal confocal fluorescence microscopy. One of these is photobleaching of the fluorescent probe itself mainly due to a large excitation volume compared to the detection volume defined by the confocal aperture. Thus, the fluorescence signal weakens as subsequent scans are made, either to generate a 3D image or to observe a single slice at different timepoints. In addition to photobleaching, phototoxicity is also a problem as excited endogenous and exogenous dye molecules are known to generate toxic free-radicals (König *et al.*, 1996a). Thus, one is obliged to limit the scanning time or the light intensity if one hopes to keep the specimen alive during an extended period of observation.

Both of these major hindrances, namely photobleaching and phototoxicity, during 3D imaging can to a great extent be reduced if the excitation volume is restricted to the detection volume. This has been accomplished by two-photon microscopy (Denk *et al.*, 1990; König *et al.*, 1996b) wherein the chromophore excitation occurs specifically in the sub-femtoliter focal volume through contemporaneous (within femtoseconds) absorption of two low-energy (infrared) photons. The tissue above and below the plane of focus is merely subjected to NIR of lower intensity that causes neither photobleaching nor phototoxicity. The advantages and efficacy of two-photon laser scanning microscopy (two-photon LSM) with a range of living specimens and a variety of chromophores has recently been demonstrated (König *et al.*, 1996b; König, 1997).

On the other hand, the intense light emitted by the NIR laser is potentially capable of causing perturbations to almost any biological material (König *et al.*, 1995; König *et al.*, 1996c; König *et al.*, 1999). Because this NIR laser can be focused through a microscope objective to perturb areas in the submicron range, the multi-photon LSM can be employed as a versatile tool in studies addressing basic cellular processes.

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[†]Annotations to Figure 6 in this paper can be found at: <http://www.blacksci.co.uk/products/journals/suppmat/tpj/tpj603/tpj603sm.htm>.

The primary roots of *Arabidopsis* manifest a simple architecture with the cell number and location being almost invariant (Dolan *et al.*, 1993). Because of its small size and transparent nature, this organ is amenable to experimental manipulation (Scheres *et al.*, 1996). The cells in the root meristem of *Arabidopsis* have predictable fates and is composed of three tiers of cells, which are the upper vascular initials, the four non-dividing central cells (known as the 'quiescent centre') flanked by cells which are contiguous with the files of the endodermis and cortex (the endodermal/cortex initials) and the lower tier of the columella and root cap/epidermal initials.

Intracellular as well as intercellular communication is essential for regulation of growth and development in plants. The precise mechanism by which individual cells in the meristems of higher plants communicate and transmit inductive morphogenetic signals has remained unclear (Tirlapur, 1989; Zhu *et al.*, 1998a; Zhu *et al.*, 1998b). The regulatory inductive signals as well as simple metabolites may move from cell to cell by either of the two possible transport routes. They may move symplastically via plasmodesmata (Lucas *et al.*, 1993; Mezitt and Lucas, 1996; Robards and Lucas, 1990) or they may be transported out of the cell and move to other cells via an apoplastic route which includes intercellular spaces, cell walls and various xylem elements (Epel, 1994).

Plasmodesmata in higher plants are highly specialised gatable cytoplasmic trans-wall microchannels about 2.5 nm in diameter that interconnect contiguous cells and regulate intercellular movement of water, small water-soluble molecules in the range of 850–1000 M_r, nutrients and, in certain cases, macromolecules, including proteins and nucleic acids (Epel, 1994; Ghoshroy *et al.*, 1997; Kragler *et al.*, 1998).

The knowledge of symplastic communication in plants, i.e. the intercellular transport through plasmodesmata between cells, is derived by monitoring the cell-to-cell movement of plasma membrane-impermeable fluorescent dyes of different sizes and characteristics (Oparka, 1991).

To understand the developmental nature and distribution pattern of the plasmodesmata microinjection (Goodwin *et al.*, 1990), phloem loading (Oparka *et al.*, 1994) and other non-invasive techniques such as ester loading (Duckett *et al.*, 1994; Zhu *et al.*, 1998a) have been employed to label cells with low-molecular-weight fluorescent probes such as carboxyfluorescein and Lucifer Yellow.

Dye coupling (the movement of fluorescent dyes) between individual living plant cells has been studied in the epidermis of leaves (Erwee and Goodwin, 1983; Erwee *et al.*, 1985; Palevitz and Hepler, 1985), staminal hairs (Tucker, 1988), roots (Duckett *et al.*, 1994) and

shoot apical meristem (Van der Schoot and Lucas, 1993) using membrane impermeable fluorescent probes in combination with microinjection. Because the single epidermal cells are more amenable for microinjection of fluorescent dyes, our knowledge of symplastic coupling in plants has largely been limited to these cell types.

Furthermore, because of the limitation of the microinjection technique, dye-coupling studies between individual cells or a group of cells that are deeply seated in the meristematic tissue have not been forthcoming thus far.

In this article we report the successful application of near-infrared femtosecond laser beams as a novel non-invasive means for dye permeation/loading and simultaneous excitation of the fluorochrome PI in meristematic cells of a higher plant, *Arabidopsis thaliana* and also monitor local dye-coupling between cells during development by 3D imaging.

Results

Organisation, arrangement and number of cell files in the primary root

Median longitudinal optical sections (obtained with both laser microscopes) of PI labelled primary root of *Arabidopsis thaliana* (Figure 1) revealed that it consists of single cell files of epidermis, cortex, endodermis and pericycle surrounding a centrally located vascular bundle (schematised in Figure 2). These cell files are derived from basally located cells termed as initials. The initials basally produce daughter cells that continue to remain initial-like and proximal daughters that generate cells which differentiate following displacement from the meristem proper. All initials surround a set of four non-dividing central cells known as the quiescent centre. These structural features are consistent with earlier observations (Dolan *et al.*, 1993) and correspond to the closed meristem organisation described by Von Guttenberg (1964).

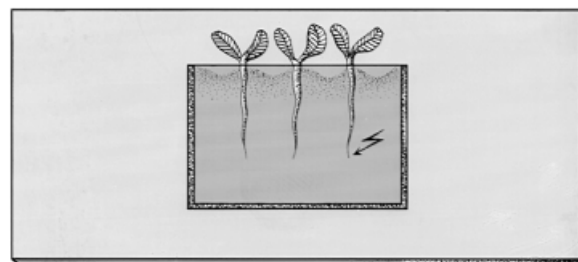


Figure 1. Schematic drawing of the dye permeation chamber consisting of a coverslip sealed on a glass slide. Seedlings were carefully placed in the chamber containing PI and the root tips were examined using an inverted or upright microscope.

Image acquisition and dye permeation in meristematic cells with argon-ion and Ti:sapphire NIR laser microbeams

Confocally acquired optical sections using an argon-ion laser (488 nm) reveal that PI clearly outlines all the cells in the root tips of *Arabidopsis* (Figure 3a) The laser power of 107 μ W used (at the specimen plane) during scanning had

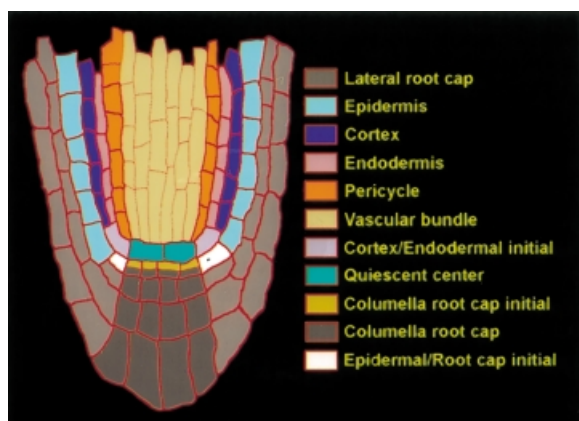


Figure 2. Schematic representation of cell types in the *Arabidopsis thaliana* root meristem. The constituent cell types are depicted in the colour legend.

no deleterious effect on the specimen and there was no dye accumulation in any of the cell types. Nevertheless, when the same beam was parked on the target cell, namely a single central cell located 45–50 μ m deep in the meristematic tissue for 6 sec (for dye permeation), several cells in the optical path besides the target cell accumulated PI (Figure 3b). Thus, the 488 nm laser line from a conventional confocal laser scanning microscope (CLSM) was not suitable for highly specific dye permeation into the target cell.

Therefore, we subsequently used the NIR laser beams for laser-assisted dye permeation. Interestingly we were able to image NIR excited fluorescence of PI in the root tips of *Arabidopsis* with a relatively low mean laser power of 4.6 mW at the same pixel dwell time of 30 μ s. As shown in Figure 3(c) no visible deleterious effect on the cells was registered. Furthermore, when highly focussed NIR laser beam at a mean power of 9 mW was parked on the target cell for a very short duration of 0.047 sec, we were successfully able to permeate PI into the target cell (central cell). This was highly specific and PI fluorescence was restricted only to the target cell while the adjacent cells and those in the layers above the target cell that encountered the beam path did not show any dye accumulation (Figure 3d).

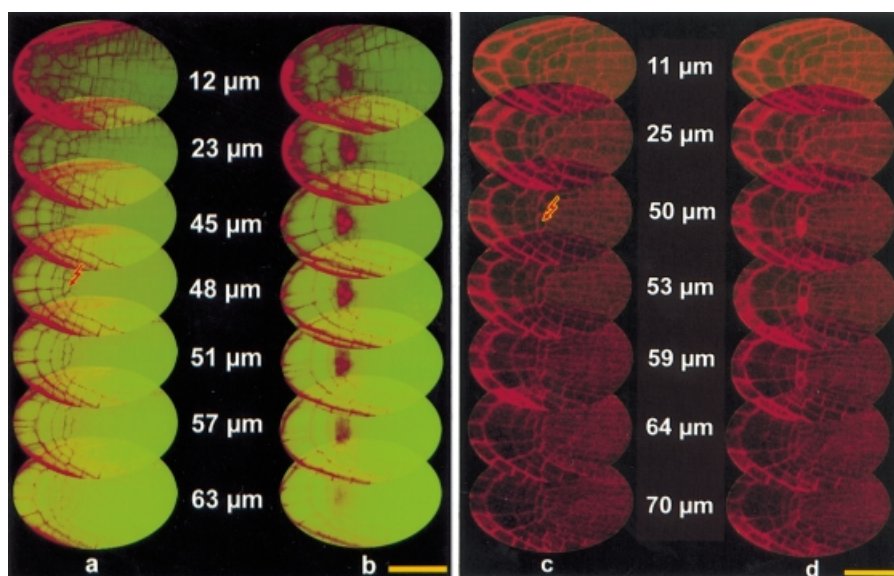


Figure 3. Pseudo-colour coded optical sections of a 3-day-old living root tip labelled with propidium iodide.

The PI-fluorescence was induced either with a 488 nm laser line (a and b) or with an 800 nm NIR laser (c and d).

(a) Images of the root tip at different optical planes. The arrow indicates the target cell (central cell) before the dye was permeated with 488 nm laser pulse.

(b) Corresponding series of images of the same root tip as in (a) but taken immediately after parking the 488 nm laser beam for 6 sec on the target cell (arrow). Note that PI has pervaded into several cell layers along the beam path. In the median optical image, dye accumulation is also seen in cells surrounding the target cell ($n=20$).

(c) NIR laser-induced fluorescence images of a PI labelled root tip at different optical planes. The arrow indicates the target cell (central cell) that is to be dye permeated with laser pulse.

(d) Corresponding images acquired immediately after dye permeation into the target cell (central cell) with NIR 800 nm femtosecond laser microbeam. Note that PI has specifically pervaded only the target cell (arrow) and there is no dye accumulation in the surrounding or cell layers above and below the target cell ($n=27$). Scale bar = 25 μ m.

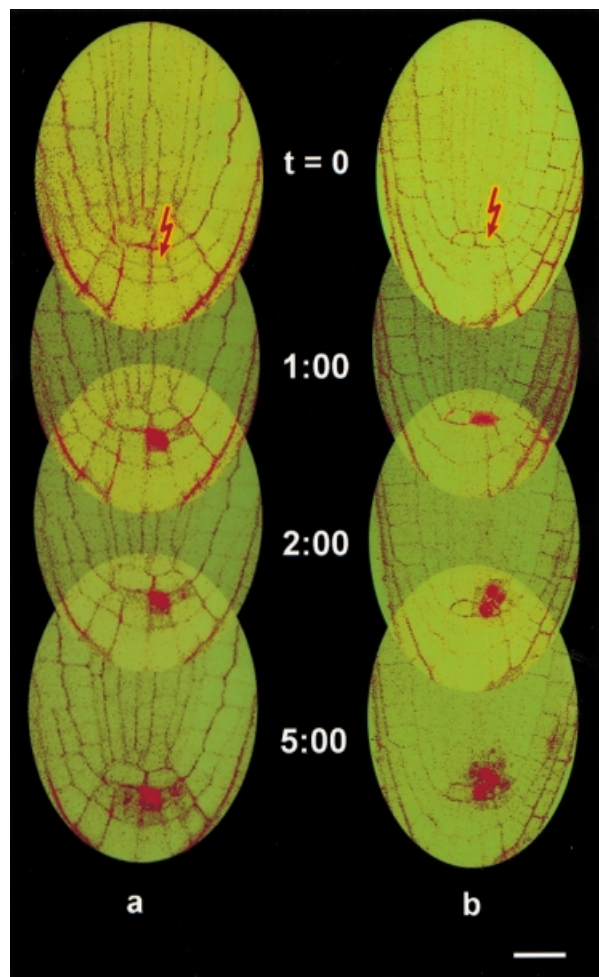


Figure 4. Time lapse imaging of dye-coupling between the cell types in the root meristem.

(a) After dye permeation in one columella initial. Initially, the dye moves into adjacent columella initials and subsequently into the subtending columella cells. Note that there is no visible dye movement into the adjacent central cell ($n=31$).

(b) After dye permeation in one central cell. Note the rapid bi-directional movement of PI into all the initials adjacent to the dye permeated central cell. The nuclei of all dye coupled cells show PI fluorescence ($n=25$). Scale bar = 25 μm .

Time lapse imaging of dye-coupling between the cells following NIR mediated dye permeation into different cell types

With a view to ascertain the pattern of dye coupling we obtained two-photon fluorescence images at different timepoints after dye permeation into specific cell types. When a single columella initial (Figure 4a) was chosen and the laser was parked on it for 0.047 sec and immediately an image was acquired, PI fluorescence was found to be exclusively confined to this cell (data not shown). Furthermore, 60 sec later, the dye was coupled to the adjacent columella initial and 5 min later PI fluorescence was detectable in the subtending columella cells. However, no increase in PI fluorescence was detectable

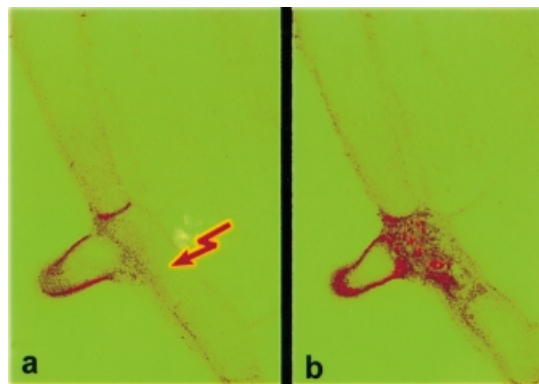


Figure 5. NIR laser-induced fluorescence images of epidermis in the differentiation region of the root labelled with PI.

(a) A root hair cell before dye loading with NIR laser pulse.

(b) The same root hair 8 min after dye loading. Note that the dye does not move out of the cell into adjacent cells ($n=19$).

in any of the adjoining central cells up to 30 min following dye loading into the columella initial.

Similarly, when a single central cell (Figure 4b) was chosen and the laser was parked on this target cell (for 0.047 sec) and subsequently imaged, the dye was found to be confined to the cell for about 60 sec. Interestingly, in the subsequent 60 sec the dye rapidly moved into the adjacent cortex/endodermal initial, pericycle initial and cells of the stele. By 5 min a significant increase of PI fluorescence was also detected in the columella and epidermal initials.

Time lapse imaging of dye uncoupling in root hair cells

When the epidermal cells in the root tip were dye-permeated there was rapid movement of the dye into the neighbouring epidermal cells (data not shown). However, when a single root hair cell (Figure 5a) in the differentiation zone of the root was loaded with PI, the dye invariably remained in that cell and no PI fluorescence was detected in the adjacent epidermal cells even after 8 min (Figure 5b).

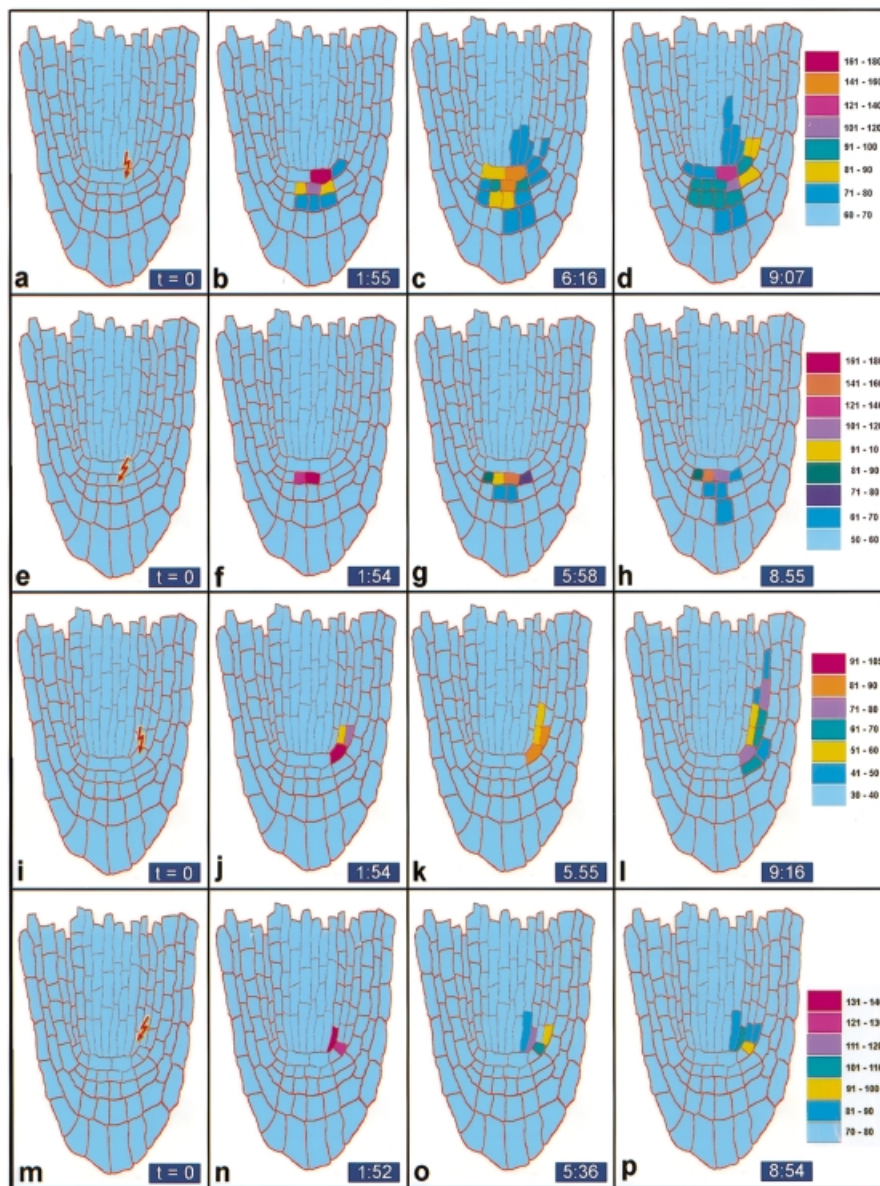
Relative fluorescence intensity changes during dye coupling in the root meristem

To ascertain the relative changes and the pattern of dye coupling, we measured the PI fluorescence intensity in different cell types before and at different timepoints up to 10 min after dye permeation into specific cell types of the root meristem.

The rate of dye coupling between the cells of the root meristem was found to be relatively different. For instance, when a single central cell (Figure 6a) was selected and the dye was permeated into it, the dye remained in the cell for the initial 60 sec and subsequently within the next 60 sec moved laterally into the cortex/endodermal initial, colu-

figure 6. Colour-coded representations of the patterns of dye-coupling and relative fluorescence intensity changes during symplastic communication in *Arabidopsis* root meristem.

Schematic representation of PI fluorescence before (a,e,i,m) and at different timepoints following permeation of the dye into a single central cell (b–d), columella initial (f–h), cortex/endodermal (j–l) and pericycle initial (n–p). The relative changes in the PI fluorescence intensities in the cells are depicted in the respective colour legends on the right. When dye is loaded into a single central cell (a) there is rapid bi-directional movement of PI into all the neighbouring cells (d). Note the lack of dye movement into the central cells when the dye is loaded into either columella (f–h), cortex/endodermal (j–l) and pericycle initial (n–p).



mella initials, and the columella cells but not into the adjacent central cell, pericycle initials or those of the stele (Figure 6b). Five to six min later the dye was coupled to almost all the surrounding initials including the adjacent central cell (Figure 6c). The fluorescence intensity in the target cell immediately after dye permeation was between 160 and 180 units, which gradually decreased to values between 141 and 160 units. This was accompanied by an increase in the fluorescence intensity of the adjacent central cell and the columella initial. On the whole, the dye movement into the columella initials and the subtending columella cells was relatively more pronounced as compared to the movement into the stelar cells. Between 9 and 10 min high PI fluorescence was seen in all the initials and the respective derivatives (Figure 6d), and the cells

furthest away from the central cell had high PI fluorescence.

Following dye permeation into a single columella initial (Figure 6e), the fluorescence intensity was in the range of 161–180 units. Subsequently, the dye moved laterally into the adjacent columella initial within 1 min (Figure 6f). In the next 5–6 min the dye was coupled to all the columella initials (Figure 6g) and a few columella cells. The central cells invariably maintained the basal fluorescence intensity values (50–60 units). Even after 8 min the dye did not move into the adjacent central cells (Figure 6h).

When the dye was loaded into a single cortex/endodermal initial (Figure 6i), dye coupling was more obvious between the cells of the endodermis and cortex (Figure 6j,k). Nine minutes later the endodermal and cortical cell

files had a high level of fluorescence and manifested extensive dye coupling while the adjacent central cell, the pericycle and stelar initials were uncoupled (Figure 6l).

After dye permeation into a single pericycle initial, the fluorescence intensity was in the range of 131–140 units (Figure 6m). This value gradually decreased as the dye moved initially into the adjoining cortex/endodermal initial and that of the stele (Figure 6n). Subsequently, the derivatives of the endodermis and the cortex manifested dye coupling (Figure 6o) while the central cells remained uncoupled even after 8 min (Figure 6p).

Discussion

Imaging and dye-permeation with visible (488 nm) and NIR (800 nm) lasers

Visual comparisons of serial optical sections following dye-permeation with the argon-ion laser from a conventional confocal microscope unequivocally demonstrate that several cells of the upper surface layers of the root meristem in the beam path and also the cells surrounding the target cell accumulate PI and hence are substantially affected by the 488 nm laser line. This is not unexpected because in conventional confocal microscopes the excitation volume is larger than the detection volume and therefore several cells all along the excitation cone experience photo and thermal stress resulting in dye accumulation. Hence there is no highly localised permeation of the dye with the 488 nm laser microbeam.

In contrast to the nature of dye-permeation observed using the argon-ion laser, the NIR laser-mediated permeation of the dye is significantly superior and is substantially specific to the target cells. Furthermore, none of the cells in the beam path are affected as is discernible by the absence of PI in these cells. This is a great advantage because NIR excitation occurs in the sub-femtoliter focal volume through simultaneous absorption of two photons with one-half of the energy required for conventional one-photon excitation.

It has been demonstrated previously (Kitzes *et al.*, 1977) that the NIR laser can induce transient membrane alterations in myocardial cells. We believe that the permeation of PI with the NIR laser into the target cell as observed in the present study possibly occurs due to transient local changes in the plasma membrane.

Movement of PI into adjacent cells is not due to thermal effects

It can be argued that dye entry into the neighbouring cells from the dye permeated target cell could be due to thermal effect (localised cell heating) caused by the NIR laser beam. This is quite unlikely because when PI is permeated

into either the columella or cortex/endodermal initials no dye accumulation is discernible in the contiguous central cell. Similarly, there is no dye accumulation in the neighbouring epidermal cells when the dye was permeated into a single root hair cell. Moreover, heating by NIR microbeams of a mean power of less than 100 mW (laser tweezers and multi-photon microscopes) was found to be of the order of less than 2°C (Liu *et al.*, 1995; Schönle and Hell, 1998). Hence, it is conceivable that the dye from the permeated cell moves symplastically through plasmodesmata.

Evidence for directional dye-coupling between meristematic cells

Cell-cell communication in the primary root apical meristem of *Arabidopsis* has been investigated by visualising the coupling pattern of fluorescent dye loaded into cells using NIR femtosecond laser pulses. With this novel technique it is possible to non-invasively load a fluorescent probe in any single cell deeply seated in the meristematic tissue of the root and almost immediately monitor symplastic exchange of dye molecules between contiguous cells. We demonstrate that the meristematic initial cells and their respective derivatives in the *Arabidopsis* root tip are symplastically coupled, allowing the movement of PI, a membrane impermeate dye with a molecular mass of 668.4 Da, to pass from cell to cell. This intercellular symplastic coupling is likely to enable cells to exchange position-dependent information molecules during pattern formation in the root meristem.

It is noteworthy that we have not detected movement of the dye into the central cells (quiescent centre) when a single cell in the adjacent two tier of initials was loaded with the dye. However, on the other hand when a single central cell is loaded with the PI, the dye rapidly moved into the contiguous initials of the columella, stele, cortex, endodermis and epidermis. This unidirectional movement of the fluorochrome from the central cells into the immediately adjacent initials of the columella, cortex, pericycle and stele is perhaps due to uni-directional gating of the plasmodesmata.

Interestingly, when the PI is loaded into a single cortex/endodermal initial, the dye to a greater extent preferentially moved into the derivative cell files of the cortex and the endodermis rather than to the lateral pericycle and stelar initials. This finding of preferential dye movement within the cortex and the endodermal cell files is quite consistent with the recent detailed ultrastructural studies (Zhu *et al.*, 1998a) which have indicated higher frequencies of primary plasmodesmata in the transverse walls than in the lateral walls.

Root hair cells are uncoupled from the surrounding cells

When dye is non-invasively permeated into a single epidermal cell in the tip zone, PI rapidly moves into the surrounding cells. This movement of the dye occurs in 1–2 min. However, when a single hair cell in the differentiation zone of the root is loaded with the dye, the dye never moves out of the cell. This suggests that the hair cells are symplastically isolated from other epidermal and subepidermal cortex. These results are highly consistent with those of Duckett *et al.* (1994) who found that when *Arabidopsis* hair cells were microinjected with carboxy-fluorescein that the dye did not move out of the cell. Similarly, in *Allium* and *Commelina* the guard cells and the surrounding epidermal cells are dye coupled during early stages of development but at maturity the guard cells are isolated (Palevitz and Hepler, 1985). It is therefore conceivable that with the maturation of the epidermal cells symplastic communication is significantly curtailed.

Based on the semiquantitative analysis of fluorescence intensity profiles on per cell basis it can be concluded that undifferentiated cells in the root meristem are indeed dye coupled and are symplastically connected. Furthermore, the cells become symplastically isolated as they differentiate.

Taken together, our findings and those of Duckett *et al.* (1994) provide analogy to gap junction-mediated communication between animal cells which is pivotal for growth and development (Warner, 1992). Recent immunogold labelling and immunological studies using antibodies raised against two gap junction proteins connexin32 and connexin43 have shown that the plasmodesmata of maize mesocotyl contain two different proteins that crossreact with the two gap junction antibodies (Yahalom *et al.*, 1991).

The molecular nature of the position-dependent signal or morphogen involved in root apical development is still not known. However, recent ultrastructural studies (Zhu *et al.*, 1998a) on the distribution of plasmodesmata in the root tips of *Arabidopsis* have demonstrated primary plasmodesmatal connections between cells of the same tissue layers. Although more rigorous experimentation is essential to draw firm conclusions, the ultrastructural and functional analyses (Zhu *et al.*, 1998a; Zhu *et al.*, 1998b) taken together with the dye coupling data presented in this study provide indirect evidence for cytoplasmic transmission of the positional information during the growth and development of the *Arabidopsis* root.

Experimental procedures

Seeds of the *Arabidopsis thaliana* ecotype 'Columbia' were imbibed for 2 days at 4°C in the dark in water. Seedlings were then grown in microtiter plates at 22°C, 80% humidity and a 15 h light/9 h dark cycle.

Laser systems and technique for permeation/loading of propidium iodide

Seedlings were placed in a small chamber (Figure 1) consisting of a single cover slip (18 × 18 mm) sealed on three sides on a slide (76 × 26 mm) containing 5 µg ml⁻¹ propidium iodide (Molecular Probes Inc.). The seedlings were placed into these chambers so that the cotyledons were not submerged in the PI solution. Individual chambers could contain 4–6 plants. Within a few minutes of incubation propidium iodide (PI) outlines all individual cells of the root tip. PI labelled root tips were imaged with a conventional upright confocal laser scanning microscope (Zeiss LSM 310) equipped with an external 10 mW Argon-ion laser (488 nm). In addition, we used a compact mode-locked Ti:sapphire NIR (800 nm) femtosecond laser (Vitesse, Coherent: 80-MHz pulse repetition frequency, ≈1 W power, ≈90 fs pulse length out-put) coupled to inverted Zeiss CLSM Model 410 (König *et al.*, 1999). In both cases the laser beam was focussed through a high numerical aperture (N.A., 1.3) 40 × Zeiss Neofluar objective.

Laser powers of both continuous wave Argon-ion (488 nm) as well as that of the NIR in pulsed mode (800 nm) following transmission through the microscope objectives were routinely measured (at the object plane) in air using the FieldMaster FM power meter (Coherent, Santa Clara, USA) before and after the completion of the experiments. According to the manufacturer's, the meter accuracy is <2% whilst the absolute accuracy including that of the detector head and the instrument is ± 5%.

The pulse duration at the sample was determined to be 180 fs. For dye permeation the point exposure mode or irradiation of a small (1 µm²) region of interest (ROI) were used. The area of exposure was chosen in the median optical image where dye permeation was to occur.

For 488 nm laser line mediated permeation of PI the laser power at the specimen plane was 107.0 µW and the beam was parked on the cell for 6 sec. Lower laser powers (4 µW, 2 µW and 300 nW) obtained through the standard built-in neutral density filters of the microscope and durations between 1 and 6 sec were ineffective in permeating the dye into the target cell.

With the NIR laser a mean power of 4.6 mW was held constant for image acquisition, and for dye permeation a mean power of 9 mW was used and the laser beam was parked on the centre of the target cell as a defined ROI of 1 × 1 µm² for 0.047 sec. All images were acquired at zoom setting 2 and scan speed of 8 sec/frame (512 × 512 pixels consisting of a 160 µm × 160 µm sample area) which corresponds to a pixel dwell time of about 30 µsec. Wherever required, Z series (in the axial plane) images were collected at every 1 µm space steps.

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