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Cell-to-cell transport through plasmodesmata in tree callus cultures

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Summary One factor that contributes to a successful fruit tree grafting is the establishment of symplasmic contacts in the graft interface to facilitate the transfer of compounds between scion and stock. Using novel experimental and theoretical approaches we investigated whether the localized incompatibility, experienced in some *Prunus* grafts, could be related to insufficient plasmodesmal coupling at an early stage of development within one of the partners. Dye-coupling analysis using fluorescent tracers combined with confocal laser scanning microscopy were performed in cultured callus from either the plum rootstock (*Prunus cerasifera* Ehrh. × *Prunus munsoniana* W. Wight et Hedr.) cv. 'Marianna 2624' or from the apricot (*Prunus armeniaca* L.) cv. 'Moniqui' growing in vitro. Fluorescein was loaded into callus cells in a caged form. Following photoactivation of fluorescence within single cells, the uncaged fluorescein could be traced as it was spreading cell-to-cell revealing the existence of functional plasmodesmata. This set of experiments was performed within the 'stock' partner in callus fusions ('callus grafts') as well as in ungrafted callus. The results indicated species-related as well as developmental-related differences in plasmodesmal conductivity. The results further pointed to a novel control factor of connectivity that reaches the graft partner and changes its innate rate of communication: when combining the poorly transporting apricot cultivar with the well-transporting plum cultivar, communication between plum callus cells was much reduced, compared to that in plum homografts. For further support of the hypothesis, we carried out a quantitative analysis in which fluorescein was esterloaded into the callus. Fluorescence redistribution after photobleaching of fluorescein in individual cells gave a measure for the plasmodesmal contact between the cells. We found significant differences between the species with regard to mobile fraction and halftime of redistribution, which confirmed that callus cells are not interconnected to the same extent in Marianna 2624 and Moniqui.

Keywords: callus fusion, grafting, photoactivation, photobleaching, Prunus, size-exclusion limit, symplasmic domains.

Introduction

Vegetative formation by grafting is an essential tool for fruit production. The key to grafting success is the matching of the meristematic tissue of the scion to the cambium layer of the rootstock. Besides environmental factors, the speed of callus formation and vascular differentiation are some of the factors known to affect grafting success (see review by Hartmann et al. 2002). Although most species in the genus *Prunus*, for example, almonds, nectarines, peaches and plums, are easily grafted, there are some inter-specific combinations that show graft incompatibility. This is the case in apricot scions (*Prunus armeniaca* L.), for example, cultivar Moniqui grafted on a plum rootstock (*Prunus cerasifera* × *Prunus munsoniana*) cultivar Marianna 2624. With incompatible apricot/plum grafts, some callus differentiation into cambium and vascular tissues occurs; however, a large portion of the callus never differentiates (Pina and Errea 2005). This lack of differentiation brings about discontinuities in the cambium and the formation of a band of parenchymatous cells interrupting the vascular connections.

The incompatible grafts may grow without external symptoms for several years before breaking at the graft union interface. This type of incompatibility clearly demonstrates that the callus formation and development of functional vascular tissue is not itself an indicator for the graft success (Errea et al. 1994, Hartmann et al. 2002, see review by Pina and Errea 2005). Therefore, knowledge of early structural, biochemical or other events associated with incompatibility would be useful to diagnose the future graft response (Ermel et al. 1999, Errea et al. 2001, Pina and Errea 2008).

One hypothesis on the development of incompatibility is that a late rejection is predetermined already at the initial

step of callus contact. It appears that cell recognition and direct cellular communication between the callus from scion and stock is crucial for grafting success (Jeffree and Yeoman 1983, Kollmann et al. 1985). One of the main communication pathways in plants are plasmodesmata, which are channels in the plant cell wall allowing passage of solutes and macromolecules between the cytoplasm of neighbouring cells. Plasmodesmata are present between callus cells cultured in vitro as well (Turnbull et al. 1981, Jasik et al. 1995, Verdeil et al. 2001). A plasmodesmata consists of a sleeve of plasma membrane with a thin tube of appressed endoplasmic reticulum (ER) running through the centre (Robards 1971, Ding et al. 1992, Overall and Blackmann 1996, Roberts 2005). Plasmodesmata pass through the whole cell wall interconnecting the protoplasts of adjacent stock-scion cells in the callus bridge (Kollmann and Glockmann 1985). The central rod of plasmodesmata is continuous with the cell ER as revealed by fluorescence redistribution after photobleaching (FRAP) studies (Grabski et al. 1993, Martens et al. 2006).

During grafting, plasmodesmata are formed de novo across existing cell walls between opposing surfaces of stock and scion (Kollmann and Glockmann 1991, Yang et al. 1992). The processes leading to the formation of new continuous plasmodesmata involve ER, golgi, plasma membrane and the exact cooperation of both the cell partners (Kollmann et al. 1985, Ehlers and Kollmann 2001, see review by Pina and Errea 2005). Plasmodesmata can transiently be dilated or closed, presumably by changes in the cytoplasmic sleeve and protein–protein interactions, thus changing the quantity of solutes entering or leaving a cell (Schulz 1999).

Growing evidence indicates a specific role for plasmodesmata in regulating symplasmic communication during development. Injection of fluorescent dyes into the meristem cells has revealed transient symplasmic domains in shoot and root apices (Rinne and Van der Schoot 1998) as well as calluses (Ehlers and van Bel 1999). Whereas symplasmic domains are completely isolated from surrounding tissues by the closure of plasmodesmata, symplasmic fields retain a certain level of communication with surrounding tissues. Such organization suggests coordinated development and signalling events between plant cells by allowing diffusion of information-bearing molecules, for example, proteins and RNA within the cells of a symplasmic field or domain (Gisel et al. 1999, Ding et al. 2003, Complainville and Crespi 2004, Kurata et al. 2005).

The use of tissue culture techniques provides an interesting approach to the studies of graft incompatibility since the physiological and cellular responses of in vitro graft systems mimicked those that occur in vivo (Moore 1984, Richardson et al. 1996, Errea et al. 2001, Espen et al. 2005).

The main purpose of this study was to develop bioimaging tools for cellular communication in in vitro grown callus, which would aid in revealing the localized incompatibility experienced in some *Prunus* grafts. We further wanted to test the hypothesis that symplasmic coupling within one graft partner is influenced by the other graft partner. The work forms the basis for subsequent investigations using in vitro techniques with potential grafting partners of known and unknown compatibility.

Materials and methods

Plant material

Callus tissue was grown from Marianna 2624, a clonally propagated plum rootstock (*P. cerasifera* × *P. munsoniana*) and the apricot cultivar Moniqui (*P. armeniaca*).

Stem fragments of each cultivar were cut into 0.5-cm segments under sterile conditions and placed in vitro with a modified Murashige and Skoog medium (Murashige and Skoog 1962) supplemented with indole-3-butyric acid (0.5 μM), 6-benzylaminopurine (0.5 μM), 3% sucrose and 0.7% Difco-Bacto agar. Cultures were maintained in a growth chamber at 22 ± 2 °C and 16-h photoperiod provided by a cool white fluorescent tube. Calli were subcultured every month. In vitro micropropagated plants used for the dye loading control experiments were obtained from buds, excised from actively growing trees in the field. They were grown in agar medium composed from Murashige and Skoog medium supplemented with 0.7 mg/l benzylaminopurine and in the presence of 0.1 mg/l gibberellic acid. In vitro plants were subcultured every month in the same medium. For microscopy of ungrafted controls, thick hand-sections were mounted on a microscope slide after removing the irregular top layer of surface cells.

Establishment of in vitro callus grafts

Callus tissue from apricot cv. Moniqui and plum rootstock Marianna 2624 – hereafter referred to as MO and MN – were used to establish in vitro callus fusions, termed callus grafts. These were either compatible 'homografts' (MN/MN and MO/MO) or incompatible 'heterografts' (MO/MN). Ungrafted calli served as control. The 'grafts' were established by making two clean-cut callus pieces and placing one on top of the other under sterile conditions in the same culture medium and conditions as used for callus initiation. The graft partners were brought together by gently pressing them down on the agar layer (Figure 2K).

Confocal microscopy

A confocal laser scanning microscope (Leica TCS SP2/MP, Leica Microsystems, Heidelberg, Germany) equipped with argon laser and UV-laser was used in the experiment. Images of 512 × 512 pixels were captured using the 20× water immersion objective.

PI (propidium iodide, Molecular Probes) (5 μg/ml) was used for detection of dead cells. The solution was prepared from a stock solution of 1 mg/ml PI in water. The dye was excited by a 543 nm laser line and emission was recorded in the range 610–700 nm.

For photoactivation experiments fluorescein was administered by CMNB-caged fluorescein [fluorescein bis-(5-carboxymethoxy-2-nitrobenzyl) ether, dipotassium salt, Molecular Probes]. The tissue was incubated for 10 min in 50 μM of the dye kept in darkness and thoroughly rinsed before photoactivation. Photoactivation (uncaging) studies were performed in ungrafted callus tissue after removal of the top layer of irregular cells and in the rootstock partner from callus homografts and heterografts sampled 5 and 10 DAG (days after grafting). The rootstock cells examined were situated 100–200 μm from the graft interface after removing the graft interface that is characterized by interdigitating cells from both partners. Five grafts were examined for each day and combination. The samples were stained with PI before the loading of caged fluorescein. Time-lapse series were done with 488 nm excitation and emission recorded in two channels for fluorescein and PI simultaneously. For orientation, transmission was recorded in a third channel. After a few initial frames, photoactivation was done by a UV-laser (351/364 nm principal lines) that throughout the rest of the series illuminated a region of interest (ROI), which comprised at least one complete cell, denoted donor cell in the following. Confocal images series showing the diffusion of uncaged fluorescein out of the selected ROI were recorded during 300 s with a 20× water immersion objective lens and line average 4. For comparison of different graft combinations (Figure 2M), the fluorescence intensity in acceptor cells, neighbouring the donor cell, was normalized to 100%. Otherwise, images and graphs were corrected for fading only.

For photobleaching experiments, fluorescein was ester-loaded into the callus cells with 0.001% CFDA [5-(and-6)-carboxyfluorescein diacetate, Molecular Probes, Leiden, The Netherlands]. A stock solution was made by dissolving 10 mg CFDA in < 10 μl acetone and making up to 10 mg/ml solution with distilled water. Thick hand-sections of ungrafted callus tissue were incubated for 30 min in CFDA at room temperature and thoroughly rinsed in distilled water for the removal of extracellular fluorochrome before being mounted in water under a cover slip. The probe was excited with 488 nm laser and emission was recorded at 505–525 nm. The mobility of fluorescein molecules was quantified using FRAP in ungrafted callus tissue. The cells were loaded with CFDA, which is split by endogenous esterases in living cells to release fluorescein, which labels the dense cytoplasm with a bright green fluorescence. Living cells did not show any fluorescent signal in the vacuoles. The fluorochrome remained in the cytoplasm during observation and the vacuoles remained unstained. The FRAP was performed within ROI using rapid switching between low-intensity imaging and a high-intensity bleach mode, both at 488 nm, in 10 replicates. Cell-to-cell diffusion of dye was measured as follows. A pre-bleach image was collected using a 20× water immersion objective lens (unidirectional scan, 25% laser intensity, zoom 4×, resolution 512 × 512, line average 2) in a focal plane in which the

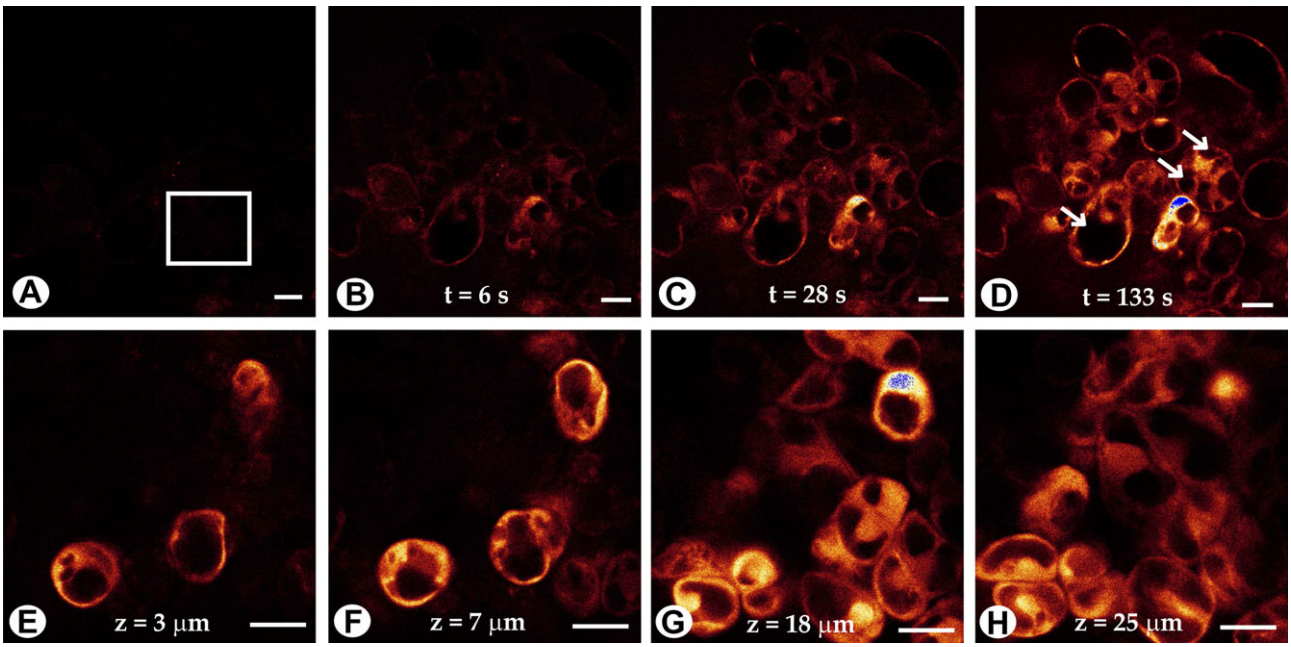

Figure 1. The spread and accumulation of photoactivated fluorescein in *Prunus* callus grown in vitro. Aggregates of undifferentiated in vitro grown callus labelled with CMNB-caged fluorescein; before photoactivation (A) and after 6 s exposure to UV-laser within the region outlined (B), 28 s (C) and 133 s (D). The focus plane is about 20 μm from the surface. See also Supplementary Movie1 for the spread of dye. Note that the released fluorescein accumulates in the dense cytoplasm and does not label the vacuoles (arrows). After photoactivation, a 3D-recording (E–H) through the uppermost cell layers reveals several layers of labelled cells, see also Supplementary Movie2. The cell viability seems unaffected by the treatment as seen in the time-lapse Supplementary Movie3. Bar = 20 μm.

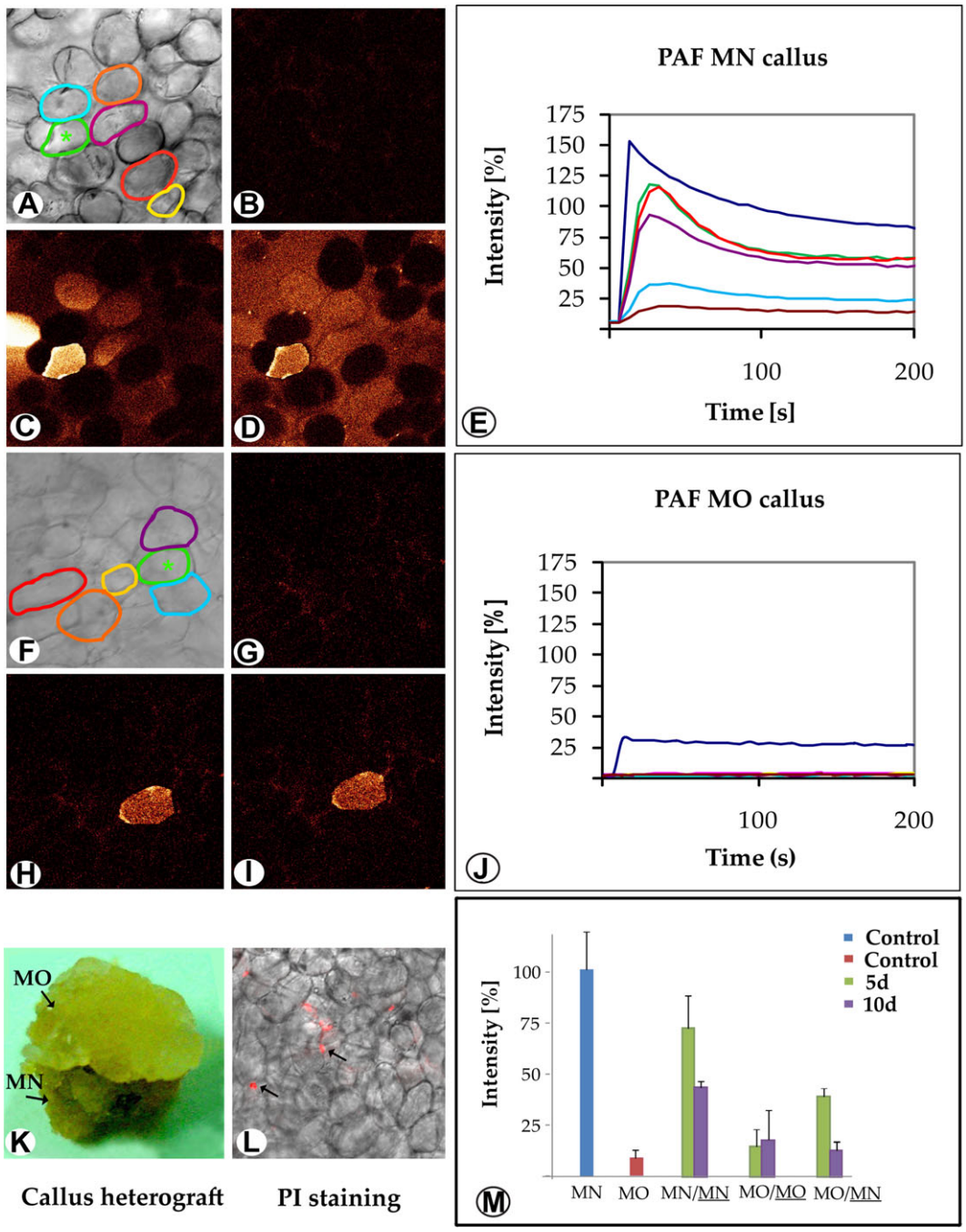

Figure 2. The PAF in *Prunus* ungrafted callus tissue and in in vitro callus unions. The PAF in single callus cells from MN (A–E) and MO (F–J). Transmission image (A and F) showing the donor cell (star) and its neighbouring cells in a population. Before photoactivation (B and G). Low resolution images from the time course, 13 and 144 s after photoactivation (C–D and H–I). Changes in the fluorescence intensity over time in six regions (E and J). The plot for MN callus indicates that the initial respond is limited to the cell exposed to UV, while the neighbouring cells lag behind and the more remote regions hardly respond at all (E). In MO, only minor amounts of photoactivated fluorescein leave the donor cell (J). Heterograft callus union showing MO grafted onto MN (K). Dead cells revealed by PI staining (L). The spread of uncaged fluorescein out of the donor cell in the stock partner of in vitro callus unions 5 and 10 DAG (M). The data are represented as mean values of uncaged fluorescein transfer into three cells adjacent to the selected ROI. Mean values ($n = 5$) $\pm$ *SD* from independent experiments are indicated.

cytoplasm of several neighbouring cells was in focus. A whole cell was selected, and its fluorescence was photobleached during about 20 s (20 bidirectional scans, 100% laser intensity, zoom 16×, resolution 512 × 512, line average 2). The settings were changed back to low-intensity imaging (duration of handlings before post-bleach recording was altogether 40 s) and a series of post-bleaching scans were performed with the same settings as the pre-bleaching

scans. The post-bleach fluorescence was sampled every 30 s for 300 s followed by sampling every 60 s for 500 s. Provided that the bleached cell is connected to its adjacent unbleached neighbours by permeable plasmodesmata, an increase in fluorescence intensity (excited by weak laser intensity) is observed and recorded in the bleached cell. The FRAP curve provides two independent parameters: the mobile fraction M (the degree in percent to which the final fluorescence approaches the pre-bleach value) and halftime ($T½$, time at which fluorescence recovers by 50% of the start value).

In some experiments, a related technique fluorescence loss in photobleaching (FLIP) was applied. In these cases, the ROI was continuously bleached for 3 min, then the cell population was imaged in a time series to study symplasmic domains.

Control experiments

In photoactivation experiments, the presence of any uncaged tracer in the intercellular space due to insufficient washing was tested by UV-radiation of ROI between cells. Thereby, the contribution of apoplastic spread and uptake of the tracer in donor cells could be excluded. In FRAP experiments, guard cells from *Prunus* plants were used as control to test that fluorescein was not leaking out of cells and into the medium.

Quantification of image data

The $T½$ redistribution curves were corrected for general fading caused by monitoring. Data were normalized to 100% before quantification using Leica confocal software and Microsoft Excel. The images shown were kept in their original fluorescence level. The $T½$ values were calculated from the normalized redistribution curves.

Results and discussion

Prunus callus tissue

The formation of a successful graft is a complex biochemical and structural process that includes an immediate wound response, callus formation, interdigitable growth and establishment of new vascular connections across the graft interface (for review see Kollmann and Glockmann 1990, Hartmann et al. 2002, Pina and Errea 2005). Incompatibility reactions may appear at the cellular level in these highly differentiated tissues, but are known to take place in (undifferentiated) cultured callus tissues as well (Jonard et al. 1990). We studied in vitro grown callus from the plum rootstock (*P. cerasifera* × *P. Munsoniana*) cv. Marianna 2624 (MN) and from the apricot (*P. armeniaca*) cv. Moniqui (MO) using bioimaging methods under the assumption that in vitro callus resembles grafting callus at the cellular level.

The callus consisted of an undifferentiated amorphous mass of loosely arranged, thin-walled parenchyma cells arising from the proliferating cells of the stem segment. There seemed to be no predictable organization pattern. In contrast to most other plant cell types, in which the vacuole forms a large compartment, a highly fragmented vacuole is present in most callus cells.

Features such as the ability to form shared plasmodesmata and the size-exclusion limit (SEL) of plasmodesmata in the callus could affect the compatibility of graft partners. In this work, methods were developed for studying symplasmic transport within callus tissue. The methods were applied to the ungrafted callus as well as the bottom partner of callus homografts (MN/MN and MO/MO) and the bottom partner of callus heterografts (MO/MN).

We first established our approach by performing PAF (photoactivation of fluorescence) to track the diffusion of the released fluorescein (see below) through plasmodesmata. This method has been used to measure diffusion coefficients and protein turnover in intact animal cells (Politz 1999), but has also been applied to plants (Martens et al. 2004). Both PAF and callus grafting are novel techniques. The FRAP, which is a well-established method to quantify molecular movements, was applied for comparison.

PAF in in vitro callus grafts

Intercellular communication within callus cells was studied by confocal microscopy using PAF. Conversion of caged compounds involves the local photolysis of caged molecules based on the ability to chemically 'cage' the molecule of interest and activate it by breaking the caging bonds with UV light, causing a local and rapid increase in the concentration of the activated molecule (Blancaflor and Gilroy 2000, see review by Fricker et al. 2006). By PAF, a fluorophore can be released in single cells and its movement, if any, can be traced in time and space. Figure 1, and the Supplemental movies Movie1, Movie2 and Movie3, illustrate the principle of photoactivation in MN callus. The fluorophore, continuously released within the marked area, spreads to other cells and clearly accumulates in the cytoplasm in these cells (Figure 1A–D, Movie1). Loading of the caged dye is not restricted to the outermost cells but reaches several cell layers down as seen in Figure 1(E–H, Movie2), allowing for analysis within unwounded cells. This is of great importance since it is well-known that plasmodesmata alter their permeability to cope with physiological traumas that arise as a result of stress or wounding (Roberts and Oparka 2003). The cytoplasm within the callus cells showed cytoplasmic streaming (Movie3) and accordingly seemed unaffected by the treatment.

The PAF produced a fast rise in fluorescence in the donor cell (green outline) and a delayed response in the surrounding callus cells from MN as exemplified in Figure 2A–E. The delayed response in the neighbours resulted from diffusion of uncaged fluorescein out of the donor cell via plasmodesmata (Figure 2E). The UV-laser was constantly illuminating the donor cell and uncaging both those caged

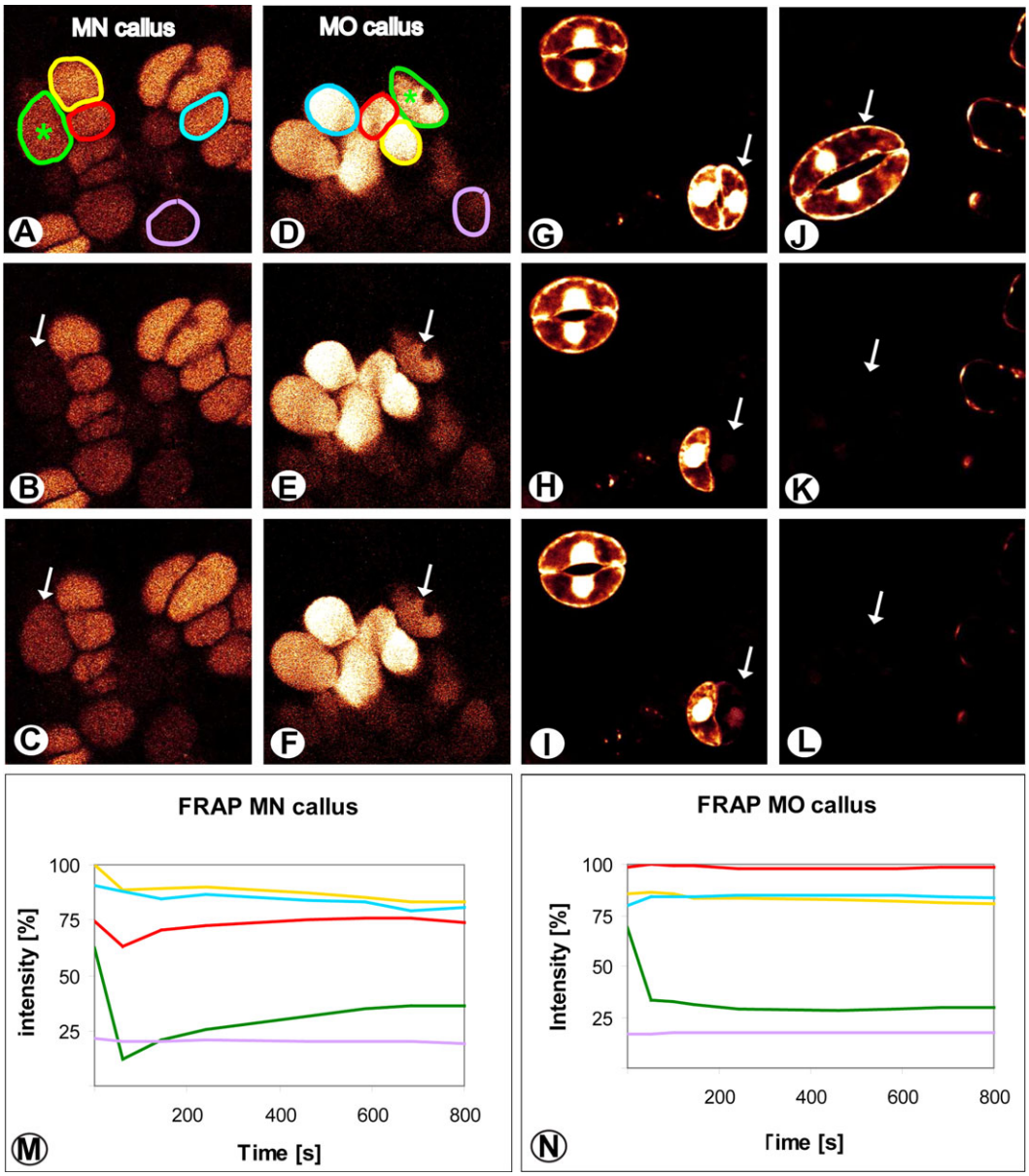

Figure 3. The FRAP in *Prunus* callus grown in vitro. The FRAP of single callus cells from MN (A–C and M) and MO (D–F and N) labelled with CFDA. Before bleaching, showing the donor cell (star) and neighbouring cells within a population (A and D). Immediately after bleaching of donor cell (B and E). Following 700 s of low-radiation monitoring (C and F). Redistribution curve showing intensity plotted versus time for bleached cell (green) and intensity changes in other ROI (M and N). The redistribution into single bleached guard cell (G–I) and the lack of dye movement into bleached guard cell complexes (J–L) are used as control experiments for symplasmic movement of the dye. Note that guard cells are raised above the surrounding epidermis cells, these are therefore out of focus in the images.

molecules existing in the donor cell at the start of the experiment and those entering it from the neighbour cells during the experiment. As long as the caged, non-fluorescing fluorescein (826 Da) can pass plasmodesmata, it can be assumed that the pool of fluorescing molecules within the donor cells is constantly being replenished by new caged molecules from the surrounding cells. Photoactivated (uncaged) fluorescein (376 Da) labelled the callus cells similarly to callus stained with CFDA.

In MO callus, however, the transport of uncaged fluorescein out of the donor cell was very limited (Figure 2F–J) indicating low cell-to-cell transport between the MO callus cells, possibly due to a small number of plasmodesmata or to a small SEL. Statistically, the difference in symplasmic communication is highly significant as seen when comparing fluorescence export in MN and MO callus (Figure 2M).

PI, which only enters dead cells and emits a red fluorescence, was used as a cell viability indicator (Figure 2L).

PI was suitable as a nucleic acid binding fluorescent dye and greatly facilitated the visualization of the cells that had lost their membrane integrity. In control experiments, using uncaging in dead cells or apoplasmic spaces, the fluorescence intensity curve over time was different from that of symplasmic transport between living cells.

Based on the observation that MN and MO show different rates of symplasmic transport in callus culture it was obvious to test the mutual influence of symplasmic communication in grafts. We tested this in 5- and 10-day-old in vitro callus unions (callus grafts) using the combinations MN/MN, MO/MO and MO/MN (Figure 2K). We observed callus cell proliferation from both partners at 5 and 10 DAG. Cells of opposing partners made contact and showed interdigitated growth. The two partners were detached and photoactivation experiments were performed within cells from the stock partner. The examined stock cells were situated 100–200 μm from the graft interface, thus avoiding the zone of interdigitation in which the individual partners can hardly be recognized. After UV-activation of fluorescein, the diffusion of uncaged fluorescein out of a donor callus cell was evaluated. We found significant differences between plasmodesmal conductivity within the stock partner of callus homografts (MN/MN and MO/MO) throughout graft development, indicating species-related differences, as also recognized in ungrafted callus (Figure 2M, blue and red bar).

There seemed to be a negative influence of MO on MN in heterografts (MO/MN), since MN showed a strong reduction in fluorescein export when grafted onto MO, compared to the MN/MN homograft. This indicates that already after 5 days of co-existence, MO as graft partner induces a change in the rate of symplasmic transport in MN, possibly through an hitherto unknown signalling pathway. It has been postulated that the critical event leading to compatible and incompatible grafts may occur already when the callus cells first touch due to cell-to-cell communication (Hartmann et al. 2002, Pina and Errea 2005). This communication would comprise a signal, crossing the graft interface either via an apoplastic step or through inter-specific plasmodesmata (Kollmann and Glockmann 1985), and thereafter causing a change of connectivity in the graft partner. Meristematic cells of root and shoot apices are known to exchange signalling molecules to coordinate the development of the organs, and plasmodesmata seem to play an important role in the regulation of the symplasmic communication (Jackson 2001). Cultured plant protoplasts showed outer cell wall plasmodesmata already after 3.5 days, seeking to develop contact to neighbouring cells (Ehlers and Kollmann 1996). Therefore, a transport across the graft interface through secondary plasmodesmata after 5 days is conceivable.

In MN/MN, there was a clear decrease in transport with time (5 DAG versus 10 DAG). This difference might be explained by cells getting increasingly specialized. Local intercellular communication plays an important role in the early establishment of the cell pattern in, that is, root and shoot apexes, enabling cells to register their position and fate (Duckett et al. 1994, Gisel et al. 1999). The undifferentiated cells in the root apex of *Arabidopsis thaliana* (L.) Heynh., that is, cells in the meristem and the elongation zone, are symplasmically well-connected, but gradually become isolated as they differentiate (Duckett et al. 1994). It is likely that also newly formed callus tissues are divided into symplasmic domains (see Figure 4, Rinne and Van der Schoot 1998, 2003, Ehlers and van Bel 1999).

Measuring molecular mobility

The PAF experiments reported above could be biased by the fact that caged and uncaged fluorescein have different molecular sizes (826 versus 376 Da). If the plasmodesmata of a graft partner have a SEL in this range, reduced export of uncaged fluorescein and reduced replenishing of the pool of uncaged fluorescein in the donor cells could not be discriminated. At the same time, there is no method established for quantifying PAF experiments. Therefore, we used as a complementary approach the FRAP with the fluorescent dye CFDA. The FRAP is also a non-invasive

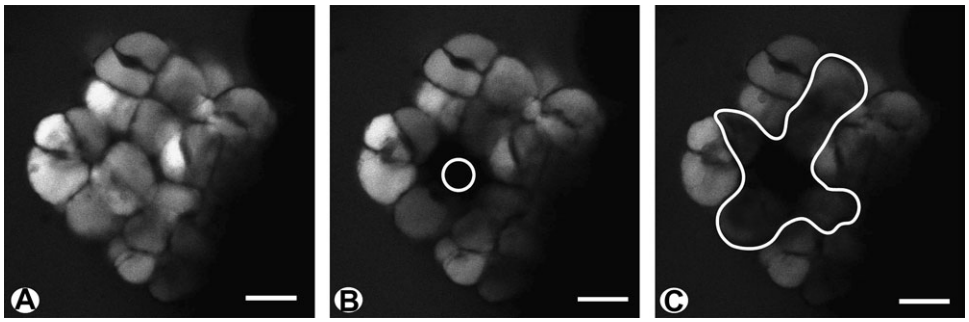

Figure 4. The FLIP in *Prunus* callus grown in vitro. Small populations of newly divided cells were found to be strongly coupled suggesting that symplasmical connectivity in the callus tissue is subdivided in many smaller domains, possibly indicating a first switch of a coordinated development. The CFDA-labelled callus cells before bleaching (A). The middle image represents the first image after repetitive photobleaching of the circular ROI for 1 min (B). The situation after 3 min of bleaching outlining a symplasmic domain (C). Prolonged bleaching had no further effect on the fluorescence intensity. Bar = 20 μm.

technique that enables quantitative measurements of the movement of fluorescently tagged molecules or structures within living cells (Hush et al. 1994, Ward and Brandizzi 2004, Weiss 2004).

In the FRAP experiments, a significant fraction of the fluorescent molecules within one cell is irreversibly photobleached. If functional plasmodesmata are present, the fluorescence recovers, giving evidence for redistribution of fluorescent molecules from the surrounding, non-bleached cells. The degree of plasmodesmal connectivity between cells can be quantified by comparing fluorescence intensity in the cells before and after bleaching. By determining the percentage of the mobile fraction and the $T½$ of redistribution ($T½$ of the accumulation of fluorescence in the bleached area), quantitative data are available to compare plasmodesmal connectivity in different tissues and species.

Figure 3A–C, M shows an example of FRAP analysis in a callus section from ungrafted MN callus. The target cell (asterisk) is nearly completely bleached directly after high-intensity illumination (Figure 3B) and imports nearly 50% of the original fluorescence from the neighbouring cells within 10 min (Figure 3M). In 10 independent experiments, the redistribution of fluorescent dye in the bleached callus cells amounted to a mobile fraction of 11.3 ± 5.5% and a $T½$ recovery of 120 ± 2 s. We observed no or little changes in the background signal, that is, no uptake of fluorescein from the apoplast. The photobleaching results indicated that plasmodesmata were present and functional in the unwounded callus cells situated at minimum one cell layer below the cut surface. Compared to the plum cultivar MN, the redistribution of fluorescence from the neighbouring cells was much lower in the apricot cultivar MO. When the mobile fraction is compared the difference is clearly highly significant since it is only a fifth part of the MN. According to the $T½$ values, redistribution is three times longer ($M = 2.3 \pm 2.1\%$, $T½ = 389 \pm 3$ s, $N = 10$) (Figure 3D–F, N). Thus, cells from the MN callus tissue revealed a higher degree of coupling than the MO callus. The FRAP experiments confirm the results of a poor rate of symplasmic communication in the apricot MO cultivar as achieved with the PAF experiments. The significant differences in mobile fraction and $T½$ might affect the passage of nutrients and macromolecules at early stages of development between these two genotypes that constitute an incompatible heterograft in the field when MO is grafted onto MN (Errea and Felipe 1993).

As control we used guard cell pairs of *Prunus* leaves that are interconnected but symplasmically isolated from the neighbouring epidermal cells. Photobleaching of fluorescein within one guard cell of a stomatal complex leads to recovery of the dye (Figure 3G–I). The redistribution from one guard cell into the other guard cell is slow, but significant, as seen within this 6-min time series. Photobleaching of the guard cell pairs, on the other hand, resulted in no recovery (Figure 3J–L). The guard cells are raised above the surrounding epidermis cells, therefore, their fluorescence cannot be visualized within the same focus plane as the epidermis.

Some groups of CFDA-labelled callus cells did not take part in the dye diffusion, as seen with continuous bleaching of a defined area, a technique termed FLIP. The FLIP analysis in MN callus indicated the presence of – possibly transiently – symplasmic domains (Figure 4). Two adjacent cells were repeatedly bleached for 3 min using full laser power inactivating all fluorophores entering the area. Cells neighbouring the bleached cells showed loss in fluorescence, as seen after 1 min (Figure 4B), indicating that they were connected by functional plasmodesmata with the bleached cells. Following 3 min of bleaching, a time series revealed that nearly all fluorescence had been depleted from these cells and from other cells within same domain, whereas cell fluorescence outside this region was largely unaffected (Figure 4C). Prolonged bleaching had no further effect on the measurements.

Conclusions

We have shown here that communication via plasmodesmata in in vitro grown callus can be evaluated using both techniques, FRAP and PAF. With the technique FLIP, the existence of symplasmic domains can furthermore be demonstrated in the callus.

The problem of incompatibility in grafted *Prunus* trees may be related to low intercellular transport capacity – low SEL or a low number of functional plasmodesmata – in either rootstock or scion callus or between the two. In this work, we have given evidence of lower plasmodesmal connectivity within the apricot cultivar MO than within the plum rootstock MN. It can only be speculated whether a strong difference in the rate of symplasmic transport per se leads to an incompatibility that goes undetected in the early stages of graft development. There is, however, a finding in this study that points to a novel control factor of connectivity that reaches the graft partner and changes its innate rate of communication: when combining the poorly transporting apricot cultivar with the well-transporting plum cultivar, communication between plum callus cells was much reduced, compared to that in plum homografts. Future experiments shall elucidate the nature of the signal that obviously is crossing the graft interface and characterize the pathway of this signal, which could be symplasmic via the newly formed plasmodesmata or apoplastic.

In fact, before the scion can become dependent on the rootstock in grafts, cellular contact must be established to enable the formation of a symplasmic and apoplasmic transport system between graft partners. Thus, the question whether callus cells in compatible and incompatible grafts differ in their ability to establish symplasmic connections may well be answered by bioimaging techniques as described here. Bioimaging allows evaluation of the overall transport capacity between living cells using FRAP (see also Martens et al. 2006). With classical transmission electron

microscopy, the existence and number of plasmodesmata can be determined. However, it is not possible to assess the functionality of plasmodesmata with this technique.

The different patterns of plasmodesmal transport between stock cells from MN/MN homografts and MO/MN heterografts may be related to the compatible and incompatible behaviour of these combinations. Hence, the differences in the symplasmic coupling, found at early phases of graft development, at the stock level between MN/MN homografts and MO/MN heterografts could potentially affect the fate of grafts, either to the successful formation of a functional and permanent graft or an unsuccessful graft.

Supplementary Data

Supplementary data for this article are available at *Tree Physiology* Online.

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