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TECHNICAL ADVANCE

A generalized method for transfecting root epidermis uncovers endosomal dynamics in Arabidopsis root hairs

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Summary

Progress in analysing the cellular functions of many structural proteins has accelerated through the use of confocal microscopy together with transient gene expression. Several methods for transient expression have been developed in the past few years, but their application has seen limited success beyond a few tractable species and tissues. We have developed a simple and efficient method to visualize fluorescent proteins in Arabidopsis root epidermis using co-cultivation of seedlings with *Agrobacterium rhizogenes*. The method is equally suitable for transient gene expression in other species, including *Thellungiella*, and can be combined with supporting molecular and biochemical analyses. The method promises significant advantages for study of membrane dynamics, cellular development and polar growth in root hairs without interference in the development of the plant. Since the method targets specifically the root epidermis, it also offers a powerful tool to approach issues of root–rhizosphere interactions, such as ion transport and nutrient acquisition. As a proof of principle, we carried out transfections with fluorescent markers for the plasma membrane (NpPMA2–GFP, *Nicotiana plumbaginifolia* L. Plasma Membrane H^+-ATPase 2), the endoplasmic reticulum (YFP–HDEL), and the Golgi apparatus (sialyl transferase–GFP) to trace their distribution in growing Arabidopsis root hairs and epidermis. The results demonstrate that, in Arabidopsis root hairs, movement of the Golgi is faster than previously reported for tobacco leaf epidermal cells, consistent with the high secretory dynamics of the tip growing cell; they show a pattern to the endoplasmic reticulum within the cytoplasm that is more diffuse than found in tobacco leaf epidermis, and they confirm previous findings of a polarized distribution of the endoplasmic reticulum at the tip of growing root hairs.

Keywords: polar tip growth, endoplasmic reticulum, confocal microscopy, membrane transport, cell biology, *Thellungiella*.

Introduction

Generating stably transformed transgenic plants is a lengthy process; selecting homozygous lines can delay key experiments for weeks, if not months; and often the results are not guaranteed. Among other difficulties, analysis of phenotypes and gene function may be frustrated when expressing the gene of interest interferes with the normal development of the plant or of a particular cell type. In many instances, transient gene expression offers a valid alternative or means to a 'first test' for characteristics associated with the gene product. Transient transformation of protoplasts (Pimpl *et al.*, 2006) and biolistic approaches (reviewed by Christou, 1995) are fast and highly efficient methods of visualizing subcellular localization of fluorescent fusion proteins. Nevertheless, such techniques are often highly invasive, disrupting the integrity of the cells and altering the dynamics of subcellular structures and metabolism (Brüdern and Thiel, 1999).

Another approach to introducing exogenous DNA into plants exploits the ability of *Agrobacterium* to transfer T-DNA to plant cells through the intact cell wall. *Agrobacterium tumefaciens*-mediated transient gene expression had become the favourite choice in many functional analyses, taking advantage of the relative ease with which transfection may be made by leaf infiltration (Goodin *et al.*, 2002; Kapila

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et al., 1997; Sutter *et al.*, 2006; Wroblewski *et al.*, 2005; Yang *et al.*, 2000) and fruit injection (Orzaez *et al.*, 2006; Spolaore *et al.*, 2000). Leaf infiltration has been used to trace the distribution of the endoplasmic reticulum (ER) and the movement of Golgi in tobacco leaf epidermal cells (Boevink *et al.*, 1998; daSilva *et al.*, 2004; Nebenführ *et al.*, 1999; Runions *et al.*, 2006) and has provided a fertile testing ground for the roles of structural proteins that function, among others, in secretory membrane traffic (Batoko *et al.*, 2000; Geelen *et al.*, 2002; Sutter *et al.*, 2006). These and similar studies have generally resorted to expression in tobacco, because of difficulties in handling Arabidopsis and its sensitivity to mechanical damage during infiltration.

We were interested in developing protocols for transient transfection of Arabidopsis, and especially of root hairs. Root hairs are lateral projections from the root that function in the uptake of water and minerals from the surrounding environment, and offer an important model for studies of development. These unicellular projections arise from trichoblasts at the root epidermis, and extend by a process called tip growth that depends on a traffic of membrane vesicles from the ER to the plasma membrane at the tip. Fundamental for tip growth are the polarization of cytoplasm and secretion, the generation of internal gradients of Ca^{2+}, and localized transmembrane fluxes of ions (for a review on root hair-tip growth, membrane trafficking and ion gradients see Campanoni and Blatt, 2007). Despite the striking features of these cells, there has been no established method for transient gene expression in root hairs. For this purpose we developed a method for transient transformation of Arabidopsis root epidermal cells based on co-cultivation of Arabidopsis seedlings with *Agrobacterium rhizogenes*. Here we describe the method, equally applicable to other species, such as the halotolerant *Thellungiella halophila*, and provide examples of its application in visualizing fluorescently labelled sub-cellular markers in Arabidopsis.

Results and discussion

Optimizing Agrobacterium and seedling growth for transfection

Co-cultivation of Arabidopsis seedlings with *A. tumefaciens* was used previously in gene silencing based on RNA-mediated interference (Fu and Harberd, 2003), but this application relied on systemic transmission and was not expected to support the large-scale transfection of a tissue that would facilitate work in visualizing fluorescently tagged proteins. Therefore we explored the use of *A. rhizogenes*. This *Agrobacterium* species has been used to infect root cells with a root-inducing (Ri), plasmid-derived T-DNA and to generate stably transformed, so-called hairy-root cultures (Chilton *et al.*, 1982). Hairy-root cultures engender significant changes in root morphology and metabolism, and have been exploited for a number of purposes, including secondary metabolite production and plant–pathogen interaction studies (Christey, 2001; Cleene and Leu, 1981; Diouf *et al.*, 1995; Limpens *et al.*, 2004; Tepfer, 1984; Tepfer *et al.*, 1989; Yibrah *et al.*, 1996). Since we were not interested in generating hairy-root cultures, we omitted to use the Ri plasmids and transformed *A. rhizogenes* strain MSV440 directly with binary vectors carrying several constructs for sub-cellular marker proteins. To determine the efficiency of transformation, both biochemical analysis and confocal laser scanning microscopy were employed. In the latter case, images were quantified by fluorescence yield using standardized settings typical for imaging expression of the same constructs when expressed in tobacco leaves after infiltration. We found that co-cultivation of Arabidopsis seedlings together with *A. rhizogenes* was successful as a simple method for transfecting the root epidermis, and the same approach could be applied to other species, including the close relative of Arabidopsis, *T. halophila* (not shown). The results below summarize the key parameters affecting expression in Arabidopsis.

Bacterial growth conditions had a significant impact on transfection efficiency in every case when quantified by fluorescence yield. Figure 1(a) shows the efficiency of transformation as a function of bacterial growth phase in liquid culture. After harvesting and pre-treatment with acetosyringone, *A. rhizogenes* carrying *YFP–HDEL* and *NpPMA2–GFP* constructs were co-cultivated with 3-day-old Arabidopsis seedlings grown in 0.5× Murashige–Skoog (MS) salts, pH 6.2. The results shown represent optimal final co-cultivation concentrations and detection periods (cf. Figures 1b and 2c). Good expression was obtained with both constructs only when the bacteria were harvested in mid- to late-exponential phase (OD_{600} between 10.0 and 20.0); little or no transformation was detected when the bacteria were taken either early in growth (end lag; OD_{600} between 2.0 and 5.0) or once their growth rate had slowed (saturation; OD_{600} >20.0). By contrast, the concentration of *Agrobacterium* used in co-cultivation had little influence on expression. Again, optimal detection periods were used for the constructs (Figure 2c). When the bacteria were harvested during exponential growth phase, transformation efficiencies for both constructs were maximal with an OD_{600} near 0.5, but for the ER marker showed no obvious trend with bacterial densities between 0.1 and 0.9 OD_{600} (Figure 1b). This difference in expression probably reflects characteristics associated with the two proteins and the ability of the plant cells to tolerate their overexpression, but we cannot rule out effects of the different binary vectors used in this case. Finally, we tested the effect of pH during co-cultivation on expression (Figure 1c) using optimal concentrations of bacteria (OD_{600} 0.1 for *YFP–HDEL*; OD_{600} 0.5 for *NpPMA2–GFP*). Transfection efficiency in this case appeared strongly

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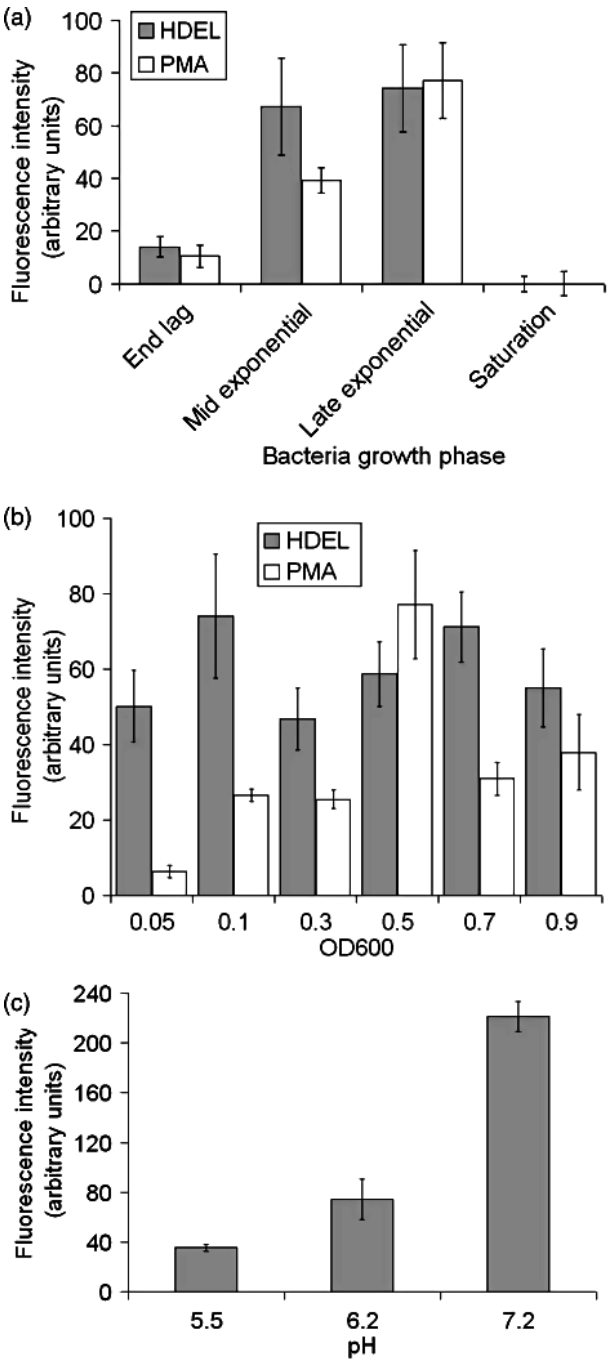

Figure 1. Selection of *Agrobacterium* growth conditions and concentration optimal for transient transformation of Arabidopsis roots.
Arabidopsis seedlings grown in 0.5× MS at pH 6.2 for 3 days were co-cultivated with bacteria carrying the constructs *YFP–HDEL* (HDEL) or *GFP–NpPMA2* (PMA). Efficiency of transformation was determined by confocal imaging at 90 and 40 h after co-cultivation, respectively.
(a) Expression quantified as fluorescence yield as a function of *Agrobacterium* growth phase. Seedlings were co-cultivated with OD_{600} 0.1 of *Agrobacterium* for the construct *YFP–HDEL* and OD_{600} 0.5 for *GFP–NpPMA2*. *Agrobacterium* liquid cultures were harvested at different times along the growth curve.
(b) Expression quantified as fluorescence yield as a function of *Agrobacterium* density in co-cultivation. *Agrobacterium* liquid cultures were harvested at late exponential phase and co-cultivated at different OD_{600} with 3-day-old Arabidopsis seedlings.
(c) Expression quantified as fluorescence yield as a function of co-cultivation pH. *Agrobacterium* liquid cultures were harvested at late exponential phase and co-cultivated at OD_{600} 0.1 for *YFP–HDEL* with 3-day-old Arabidopsis seedlings in 0.5× MS at different pH.

dependent on pH, the highest expression being obtained with a pH of 7.2. The high levels of expression obtained under these conditions were frequently associated with an aberrant distribution pattern of labelling in the ER, which gave way to patches and large sheets (Figure 4g,p). We therefore settled on pH 6.2, as the best compromise between expression efficiency and preservation of subcellular structures and their dynamics.

For ease of transfection and experimental handling, we made use of seedlings germinated in sterile liquid culture. Plants grown on agar up to 0.4% (w/v) were also transfected when co-cultivated with *A. rhizogenes*, but mechanical damage of the root hairs during handling greatly reduced the utility of this approach (data not shown). Arabidopsis seedlings germinated and grown for 3 days in liquid media with full-strength and higher concentrations of MS salts showed little or no expression, but reducing the MS salts to 0.5× and 0.1× gave satisfactory results (Figure 2a) when co-cultivated with *Agrobacterium* that had been harvested in the exponential phase of bacterial growth. Independently, we noted an aberrant growth pattern and swelling of root hairs when Arabidopsis seedlings were grown on full-strength MS salts. The observations suggest that physiological stress associated with higher salt concentrations is an important factor in determining the success of transfections.

Seedling age proved to be equally important for successful expression. When grown in 0.5× MS salts, Arabidopsis seedlings showed a sharp decline in the efficacy of transfection and gene expression over the first week after germination when subsequently harvested for co-cultivation with *Agrobacterium* (Figure 2b). Expression yield roughly followed an exponential decay with a half-time of approximately 3 days after sowing. Little expression of the *YFP–HDEL* construct was detected after 15 days post-sowing. The highest expression levels obtained with 2-day-old seedlings were frequently associated with aberrant and 'patchy' sub-cellular distributions of the fluorescent marker (Figure 4g,p). Thus, again, an optimum of 3 days' growth before co-cultivation was selected as a compromise between expression and preservation of sub-cellular structures and their dynamics.

Finally, each of the constructs exhibited a distinct temporal characteristic of gene expression. Under standardized conditions of seedling germination, *Agrobacterium* growth and co-cultivation, Arabidopsis seedlings showed a narrow window of expression for *NpPMA2–GFP* during the second day of co-cultivation. By contrast, expression of the *YFP–HDEL* marker construct was evident over the period from the

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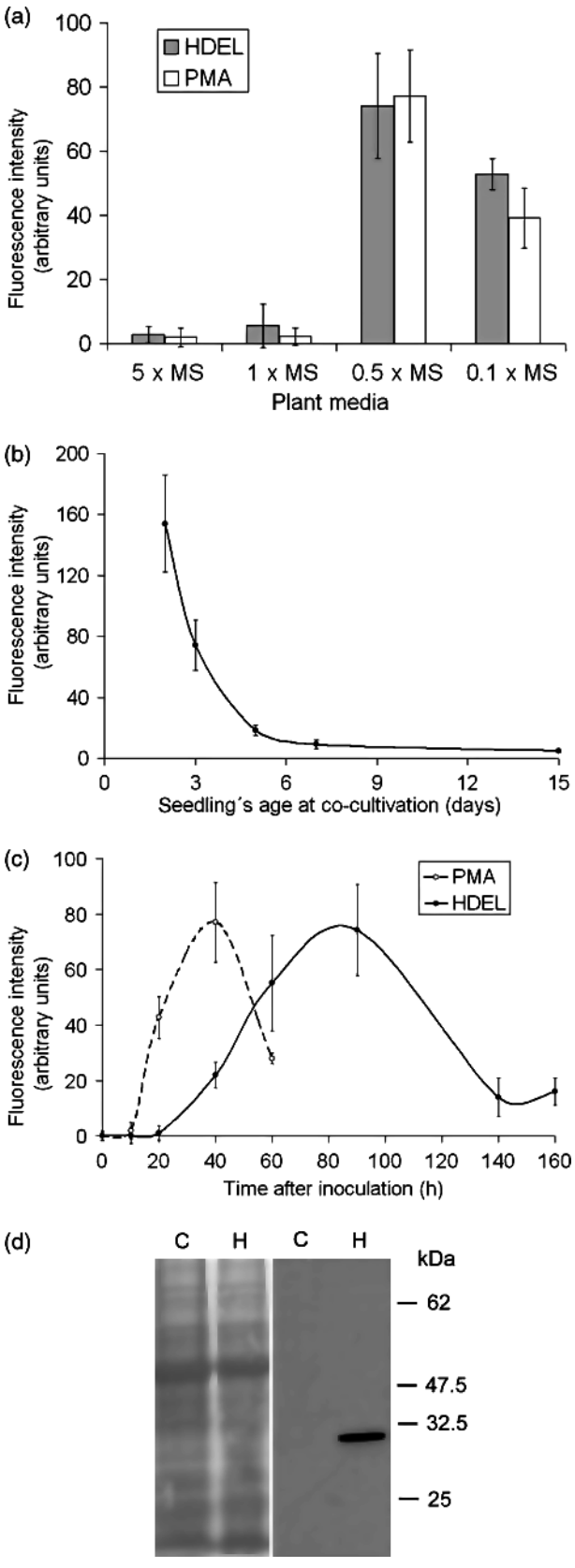

Figure 2. Selection of Arabidopsis growth conditions optimal for transient transformation.
(a) Expression quantified as fluorescence yield with Arabidopsis seedlings grown in different concentrations of MS medium pH 6.2 for 3 days before transfection with *Agrobacterium* at OD_{600} 0.1 for the construct *YFP–HDEL* (HDEL) or OD_{600} 0.5 for *NpPMA2–GFP* (PMA). Efficiency of transformation was detected by confocal laser scanning at 90 and 40 h after co-cultivation for HDEL and PMA, respectively.
(b) Expression quantified as fluorescence yield with Arabidopsis seedlings grown in 0.5× MS medium pH 6.2 for different periods before transfection for 90 h with OD_{600} 0.1 of *Agrobacterium* carrying the construct YFP–HDEL. $t = 0$ indicates the time of sterilization and imbibition.
(c) Time-course for construct expression quantified by fluorescence yield. Arabidopsis seedlings grown for 3 days in 0.5× MS pH 6.2 were transiently transformed with OD_{600} 0.1 for the construct YFP–HDEL (HDEL) or OD_{600} 0.5 for NpPMA2–GFP (PMA). Seedlings were tested for transformation efficiency at different time points after co-cultivation (at $t = 0$).
(d) Western analysis of YFP–HDEL expression. 20 μg total protein extracted from 3-day-old seedlings co-cultivated in 0.5× MS pH 6.2 with non-transformed *Agrobacterium* (c) or with OD_{600} 0.1 of *Agrobacterium* carrying the construct *YFP–HDEL* (h) for 90 h. Left, total protein extract visualized via Ponceau staining; right, Western blot detected with anti-GFP antibodies.

second to the fourth day of co-cultivation (Figure 2c). Western blot analysis confirmed expression of the transgene under these conditions, showing the presence of a single band on SDS polyacrylamide gels that was recognized by polyclonal antibodies to GFP (Figure 2d). For both marker constructs, the expression dynamics approximated a bell curve with a single maximum in fluorescence yield, after which expression levels decayed to values approaching zero. However, a low, but significant level of expression was detected for YFP–HDEL even after 160 h co-cultivation. We suspect that the breadth of variation in each case is a unique property of the expressed protein, and is likely to reflect the ability of the tissue to tolerate its accumulation within the cell as well as feedback mechanisms leading to gene silencing. Nonetheless, differences intrinsic to the binary vector cassette may also contribute to the timing and duration of expression.

Cellular characteristics of transient expression

Figure 3(a–h) shows examples of Arabidopsis seedlings transformed under the optimal conditions identified above, and illustrates several unique features of the method. Most notable was that co-cultivations invariably led to transfection only of the root tissue, resulting in a sharp demarcation of the root–hypocotyl border and absence of expression and fluorescence in the hypocotyl tissues (asterisks in Figure 3a,b). Within the root tissue, transfections were limited to the outer (epidermal) cell layer, as was clearly visible from three-dimensional reconstructions (Figure 3a–c) and planar images (Figure 3d,e). Note that Figure 3(d,e) are tangential sections that pass through the cortex; these images clearly show fluorescence within the outer epidermal cell layer only. No difference in expression was evident between epidermal cell files forming root hairs (trichoblasts) and those that did

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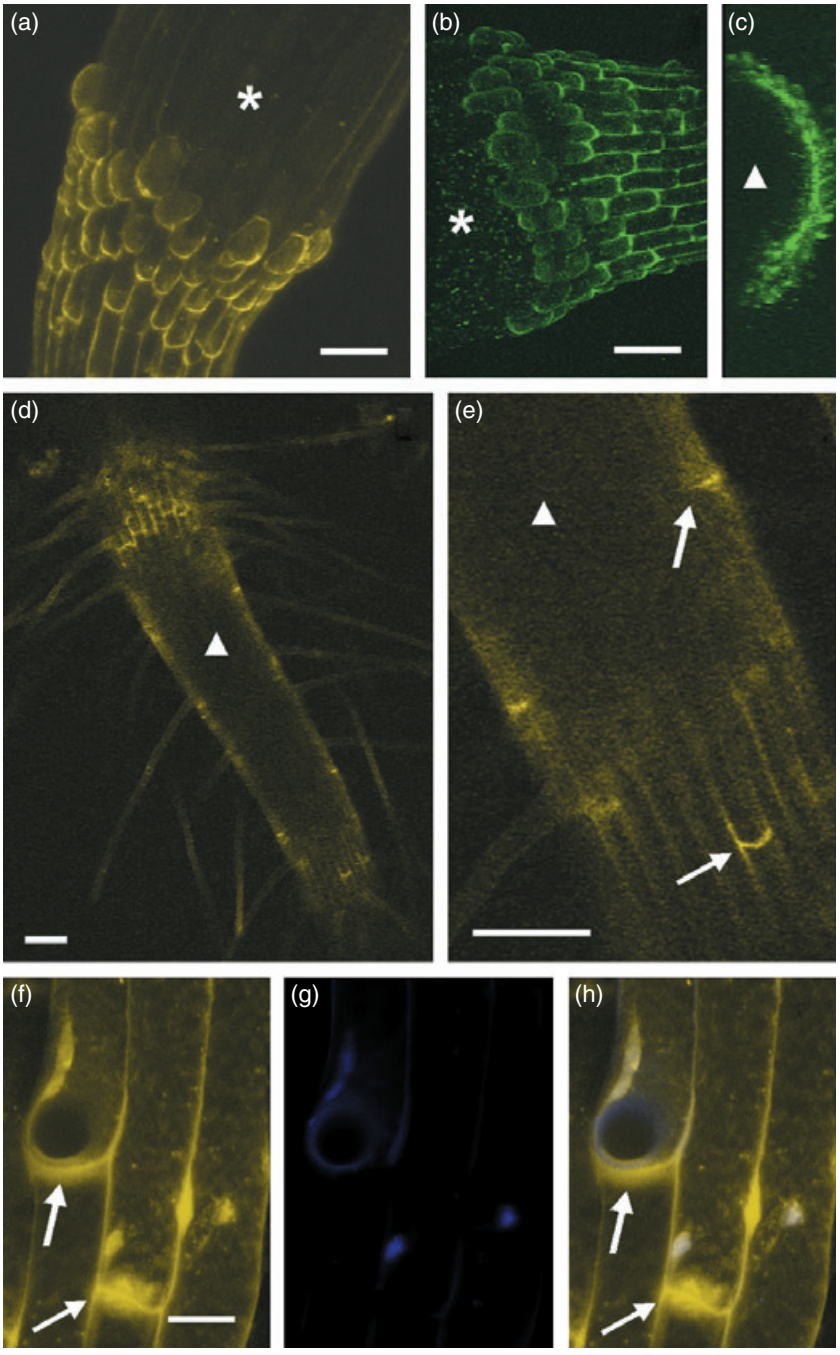

Figure 3. Visualization of transient expressed proteins in root epidermal cells.
Confocal images of roots transformed in optimal conditions with *Agrobacterium* carrying the *YFP–HDEL* or *NpPMA2–GFP* constructs.
(a) Three-dimensional reconstruction of the root–hypocotyl junction in an early developing Arabidopsis seedling transformed with *YFP–HDEL*.
(b, c) Detail of the root–hypocotyl junction in an early developing Arabidopsis seedling transformed with *NpPMA2–GFP*. Images show roughly one-third of the root depth in longitudinal (b) and transverse orthogonal (c) section. Note the expression limited to the epidermis clearly visible in transverse section.
(d) Single longitudinal confocal section of a 7-day-old Arabidopsis root transformed with *YFP–HDEL*, again highlighting the localization of expression to the epidermis.
(e) Detail of YFP–HDEL expression from (d).
(f–h) Three-dimensional reconstruction of ER localization in atrichoblasts and root hair basal regions. The empty space in the reconstruction of the trichoblast at the top-left corner of (f) and (h) corresponds to the vacuole of a root hair in tangential view. (f) YFP–HDEL; (g) DAPI nuclear staining; (h) merge. Asterisks mark position of hypocotyl; triangles indicate root stele. Arrows in (e, f, h) emphasize the polar sub-cellular localization of the expressed construct. Scale bars, 50 μm (a–e) and 15 μm (f–h).

not (atrichoblasts). Furthermore, transfection was observed along the length of the root, including young and growing root hairs that showed active cyclosis and dense cytoplasmic accumulations at the growing tip (see below and Supplementary Material). It appears, therefore, that transfection is restricted to cells exposed directly to the *Agrobacterium* culture during co-cultivation, but that only the rhizodermis is competent for transfection. Equally important, these examples also underscore the advantages of gene expression and confocal image analysis in a tissue with little or no background fluorescence. By contrast, studies using transfections of tobacco leaf epidermis are often plagued by a background of fluorescence from chloroplasts, especially when excited by wavelengths of 514 nm and below (cf. Samalova *et al.*, 2006; Sutter *et al.*, 2006; Vermeer *et al.*, 2006).

We found that the sub-cellular characteristics of the endoplasmic reticulum marker YFP–HDEL (and GFP–HDEL, not shown) differed appreciably from those associated with the ER in tobacco leaf epidermis when labelled with the

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same constructs. An intense fluorescence was associated with the ER surrounding the nucleus in Arabidopsis root epidermal cells (Figure 3f–h), as in tobacco leaf epidermis (Sutter *et al.*, 2006); however, we were unable to identify the lattice-like cortical network that is typical of the ER in the tobacco leaf tissue (Batoko *et al.*, 2000; Boevink *et al.*, 1996; Haseloff *et al.*, 1997; Sutter *et al.*, 2006). Instead, YFP–HDEL distribution yielded a diffuse, peripheral fluorescence in both root hairs and epidermal cells (Figure 4h–k, cf Figure 3f,h). Additionally, the ER marker showed an enhanced density within the first 3–5 μm from the tip of growing root hairs (Figure 4h–k,n–o), and along the lateral walls at the base of epidermal cells (arrows in Figure 3e,f,h), consistent with the patterns of developmental and secretory activity expected of these tissues. By contrast, expression of the plasma membrane marker NpPMA2–GFP showed a uniform

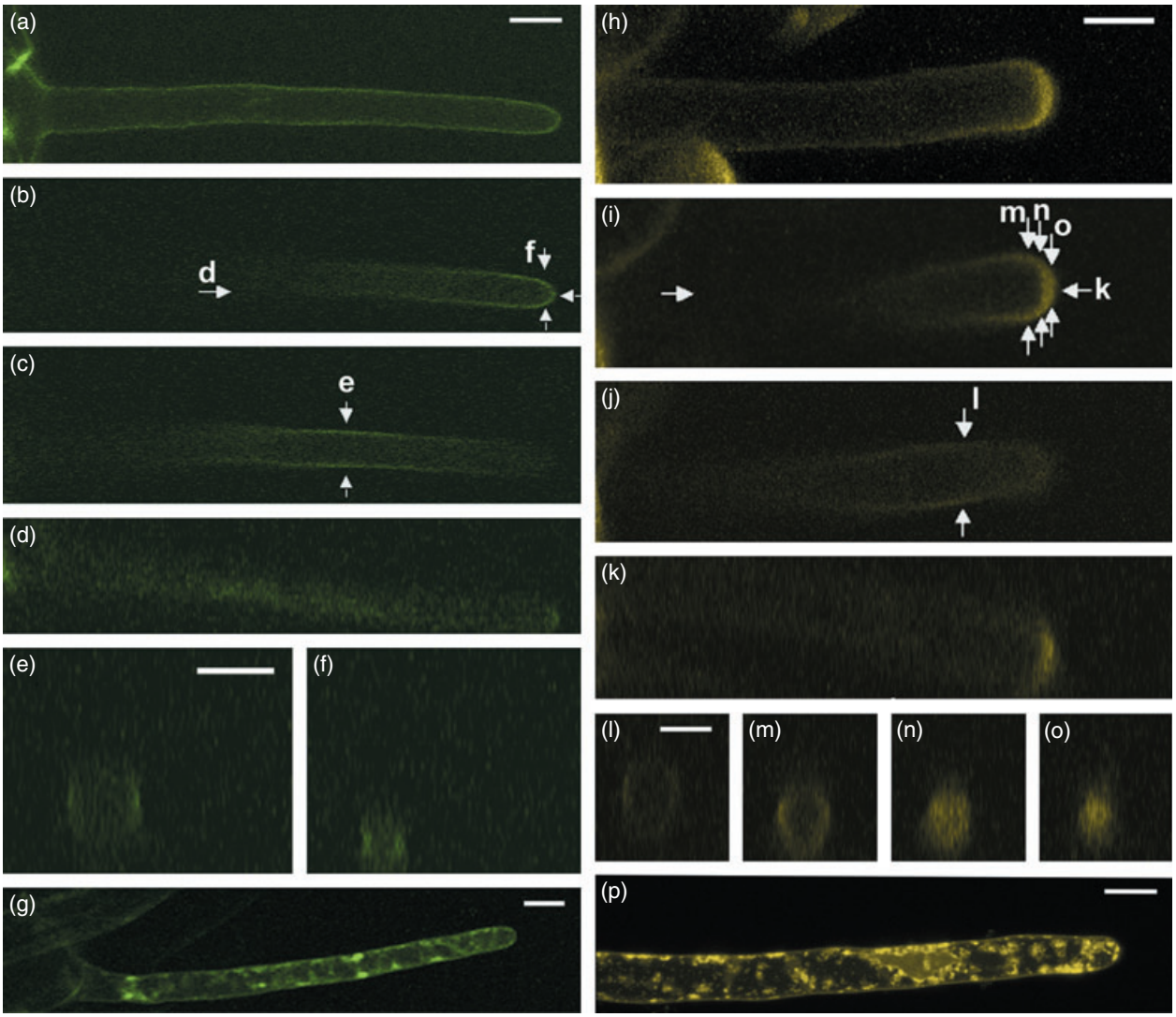

Figure 4. Visualization of the transiently expressed proteins in root hairs. Examples of root hairs transformed with OD_{600} 0.5 of bacteria carrying the construct for NpPMA2–GFP (a–g) or OD_{600} 0.1 of bacteria carrying the construct for YFP–HDEL (h–p).
(a–f, h–o) Seedlings were transiently transformed under optimal conditions as described in the text.
(a, h) Reconstructed three-dimensional confocal images of root hairs transformed in optimal conditions. (b–c, i–j) Single planar images of the root hairs shown in (a) and (h).
(d–f, k–o) Orthogonal projections of the single planar images in (b, c) and (i, j): (d, k) are longitudinal vertical projections; (e, f and l–o) are transverse projections. The axes of transverse and vertical projections are indicated as arrows and in lower case on corresponding planar sections. Due to physical limitations of the single photon confocal microscope, the fluorescence in (e, f) and (l–o) appears as two dense areas, which results from the scattering of the fluorescence into diffraction cones below and above the focal points.
(g, p). Reconstructed three-dimensional confocal images of root hairs from seedlings transformed 2 days post-germination with *NpPMA2–GFP* (g) and *YFP–HDEL* (p) to give super-optimal expression. Note the sheet- and inclusion-like distribution of constructs when strongly overexpressed. Images collected as described in the text, using standardized settings. Scale bars, 10 μm.

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labelling at the periphery both of root hairs and of epidermal cells (Figure 4a–d; cf Figure 3b). At the extreme tip of growing root hairs, NpPMA2–GFP fluorescence marked a narrow cortical layer that surrounded a non-fluorescent cytoplasm (Figure 4a,b) consistent with its localization to the plasma membrane. Only with supra-optimal expression did both YFP–HDEL and NpPMA2–GFP markers show additional accumulations within discrete sub-cellular domains, either in sheets or in irregular and oblong endosomal structures (Figure 4g,p).

These observations underscore another important advantage of our co-cultivation method. A comparison with the effects of overexpressing the *YFP–HDEL* construct in tobacco (Runions *et al.*, 2006) leads us to conclude that these sub-cellular accumulations probably reflect a 'backlog' of tagged protein within the secretory pathway, and do not represent the normal distributions of these markers in the cells. This assessment is also supported by a comparison with stable expression patterns of GFP–KDEL already reported in the literature (cf. Figures 1A and 2A,C,D in Flückiger *et al.*, 2003) which indicates that the diffuse, peripheral distribution of the ER marker does not interfere with the normal development of the plant. Nonetheless, these examples highlight the difficulties associated with interpreting protein distributions when expressed under the control of constitutive promoters. In many instances, the distributions of tagged proteins align closely with that of the native proteins (cf. Sutter *et al.*, 2006; Tian *et al.*, 2004; Uemura *et al.*, 2004), and only with extreme overexpression are these proteins mistargeted or accumulate in other cellular compartments. Validation in these instances can benefit from the facility to control the level of transgene expression by manipulating the co-cultivation conditions (Figures 1 and 2).

As a further test of the transient expression method and its facility for marking the secretory pathway, we carried out co-cultivations with *Agrobacterium* carrying the rat sialyl transferase–GFP (*ST–GFP*) marker that was previously used to tag and track the Golgi apparatus in tobacco (Batoko *et al.*, 2000; Boevink *et al.*, 1998; daSilva *et al.*, 2004; Geelen *et al.*, 2002; Saint-Jore *et al.*, 2002). As in these previous studies, the ST–GFP marker was found to accumulate in discrete, punctate structures with diameters of approximately 0.5–1 μm (Figure 5a–d), and time-lapse studies showed a rapid cyclosis of these structures, especially around the periphery of the root hairs. In tobacco leaf epidermal cells, individual Golgi move at rates of approximately 0.25 μm sec^{-1} (Boevink *et al.*, 1998; Runions *et al.*, 2006). Runions *et al.* (2006) reported the fastest Golgi to move at 0.47 ± 0.12 μm sec^{-1} and Boevink *et al.* (1998) quoted rates of 2.2 μm sec^{-1} for movement along well defined 'tracks' of the ER. We observed individual Golgi to move along similarly defined spatial pathways within root hairs, both towards the tip and away from it (cf. scheme in Figure 5f), with mean speeds of 1.30 ± 0.10 μm sec^{-1} and maximum speeds up to 3.45 ± 0.05 μm sec^{-1} between frequent saltatory (stop-and-go) movements. This higher rate of movement is not entirely surprising. By contrast with epidermal cells of expanded leaves, which might be expected to show a relatively low level of secretory activity, root hairs exhibit highly polarized and active secretion at the growing tip. This behaviour is entirely consistent with the dynamics of the endoplasmic reticulum and Golgi apparatus that we observed.

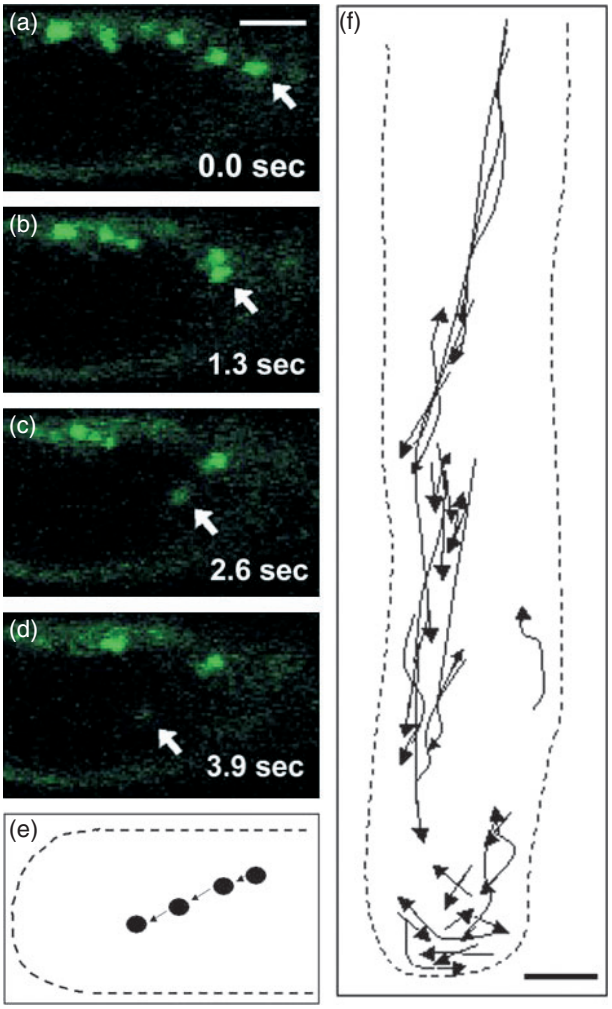

Figure 5. An application of the method: tracking Golgi bodies in root hairs using ST–GFP as cellular marker.
(a–e) Time-lapse series on a section at the cortical region of a root hair transformed in optimal conditions with OD_{600} 0.1 of bacteria carrying the construct *ST–GFP* (a–d). Sample times are indicated on each image. White arrows trace the movement of a single Golgi apparatus. Scheme in (e) resumes the total movement. Sample times are indicated in each image.
(f) Schematic tracking of individual Golgi in Arabidopsis root hairs. Every line represents the movement of a single Golgi body. Small black arrows indicate the direction of movement. Scale bars, 2 μm.

In conclusion, our method for transient transformation offers a powerful tool to investigate endosomal organization, localization and dynamics in growing root hairs. The fact that this method of transfection targets specifically the

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root epidermis of intact seedlings, with virtually no alteration in the general physiology of the plant, opens the door to a wide range of applications including, for example, analysis *in planta* of root–rhizosphere interactions, ion transport, nutrient acquisition and interactions with symbionts. Because the transfection method is fast and simple, and because growth of seedlings requires little space, it may also find applications in high-throughoutput screens for identification and testing of candidate genes of interest.

Experimental procedures

Bacteria

Agrobacterium rhizogenes cells strain MSV440 were transformed with the binary vector pBi101 carrying the fusion protein NpPMA2–GFP (Lefebvre *et al.*, 2004), or pVKH carrying the fusion proteins YFP–HDEL, a chromatic variance of the construct *GFP–HDEL* (Batoko *et al.*, 2000), or ST–GFP (Saint-Jore *et al.*, 2002), all under the control of a 35S CaMV promoters. After selection on solid 2YT medium (16 g l^{-1} bacto-tryptone, 10 g l^{-1} yeast extract, 5 g l^{-1} NaCl and 1% agar, pH 7.2), positive clones were spread on solid YEB medium (5 g l^{-1} beef extract, 1 g l^{-1} yeast extract, 5 g l^{-1} peptone, 5 g l^{-1} sucrose, 0.5 g l^{-1} $MgCl_2$ and 1% agar) and grown at 28°C overnight. A volume of 100–200 μl solid culture was inoculated into 4 ml liquid YEB (OD_{600} between 1.0 and 2.0) and incubated at 28°C and 250 rpm for different periods according to the different experimental conditions. Before transfection, bacteria were harvested and acetosyringone activated for 1 h in 10 mM $MgCl_2$ according to Sutter *et al.* (2006).

Plant material and co-cultivation

Arabidopsis thaliana L. (ecotype Colombia) seeds were vernalized for at least 5 days at 4°C in the dark, and sterilized for 2 min in ethanol absolute and 8 min in 2% sodium hypochloride and 0.01% Triton X100, before incubation in different concentrations of Murashige–Skoog (MS) basal salt medium with minimal organics (Sigma-Aldrich, http://www.sigmaaldrich.com) at pH 6.2 (when not differently stated). Arabidopsis seeds were germinated in a cycle of 16 h 65 μmol sec^{-1} m^{-2} light and 22°C, 8 h dark and 18°C. At different stages after sowing, six seedlings per sample were transferred into 2 ml fresh medium plus 100 μM acetosyringone (3,5-dimethoxy-acetophenone; Fluka, Steinheim, CH; http://www.sigmaaldrich.com), and co-incubated with activated *Agrobacterium* in darkness (to avoid transpiration and therefore changes in medium salt concentration) under the same temperature at which they were grown before inoculation.

Confocal microscopy and quantification

Expression was tested by confocal laser imaging, using a Carl Zeiss CLSM510-UV confocal laser scanning microscope with an argon ion laser, using 488 nm excitation, a NTF545 chromatic beam splitter and a 505–530-nm bandpass filter for GFP, 514 nm excitation, a NTF515 chromatic beam splitter and a 535–590-nm bandpass filter for YFP. Laser power, detector gain and offset were kept fixed for all the different specimens, to allow comparison between the different samples. Transformation, distributions and fluorescence tracking were quantified by using the Carl Zeiss LSM 510 software. For each value reported, a minimum of three independent experiments (at least 18 seedlings) was used. Background fluorescence and noise measured in controls co-cultivated with wild-type *Agrobacterium* are subtracted from all data reported. For nuclear staining, seedlings were incubated for 5 min in 50 μg ml^{-1} DAPI (Sigma-Aldrich) and 0.05% Triton X100, and washed for 10 min in 0.5× MS before detection at the confocal laser scanning microscope using an Enterprise II UV laser at 351 and 364 nm excitation, a NTF545 chromatic beam splitter and a 385-nm longpass filter.

Total protein extraction and Western analysis

For molecular analysis, 100–150 seeds were sterilized and incubated with the bacteria as described above, in a convenient volume of medium. After testing the transfection rate at the confocal laser scanning microscope, samples were harvested, flash-frozen and ground under liquid nitrogen. Total proteins were extracted in denaturing extraction buffer (50 mM Tris–HCl pH 6.8, 8 M urea, 2% sodium dodecylsulfate, 5% beta-mercaptoethanol, 30% glycerol, 0.05% bromophenol blue) with the addition of Complete protease inhibitors (Roche, http://www.roche.com) and 1 mM PMSF (BDH, http://www.biolabgroup.com). SDS–PAGE and Western analyses were performed according to Sutter *et al.* (2006). Polyclonal rabbit anti-GFP primary antibody (Abcam, http://www.abcam.com) was diluted 1:5000, and bound antibodies were detected using ECL-Plus chemi-luminescent substrates (Amersham Biosciences, http://www.amersham.com).

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Supplementary materials

The following supplementary material is available for this article online:

Figure S1. Young root hairs transformed with ER or Golgi Marker.

Movie S1. Tracking Golgi bodies in root hairs using ST-GFP as cellular marker.

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