Proteolistics: a biolistic method for intracellular delivery of proteins

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Abstract In this work, an intracellular protein delivery methodology termed "proteolistics" is described. This method utilizes a biolistic gun apparatus and involves a simple protein/projectile preparation step. The protein to be delivered is mixed with a gold particle microprojectile suspension and is placed onto a gene gun cartridge, where it is dehydrated using either lyophilization or room-temperature air-drying. Subsequent intracellular protein delivery is achieved in plant and mammalian tissues upon bombardment. Because the method does not require modification of delivery agents or cargo biomolecules and involves a simple physical deposition of the protein onto the microprojectiles, there is no restriction on protein type in terms of molecular weight, isoelectric point or tertiary structure. Because the method delivers protein through the widely used gene gun system, it can be readily applied to any tissue or organism amenable to biolistics. A variety of proteins with molecular weight ranging from 24 to 68 kDa and isoelectric point from 4.8 to 10.1 were tested in this work. It is anticipated that this simple and versatile technique will offer biologists a powerful tool for basic research in areas such as understanding of cell and gene functions and for biotechnological applications such as genome editing.

Keywords Biolistics · Biomolecule delivery · Gene gun · Protein delivery · Proteolistics

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Introduction

In recent years, intracellular delivery of biomolecules other than nucleic acids has been a major focus of study, mostly in biomedical sciences, where different drug delivery systems have been developed for therapeutical purposes (Ravichandran 2009). Intracellular protein delivery is also becoming an important tool for both fundamental and applied research. For instance, study of protein function usually involves generation of a knock-in or knock-out transgenic organism, a process that requires DNA expression constructs, availability of transformation protocols, and characterization of transgenic organisms. Direct intracellular delivery of a protein may be considered a

shortcut because it saves time and cost. Additionally, the protein of interest can be labeled for tracking or can be modified for properties that cannot be achieved by its expression in the host organism. Moreover, multidimensional functional analysis of a protein can be performed if the protein of interest can be co-delivered with other biomolecules or chemicals related to its role in the cell. For biotechnological purposes, delivery of enzymes with a specific function such as stimulating double-strand DNA breakage or recombination could, for instance, improve plant transformation efficiency or facilitate genome editing.

Several methodologies have been developed for delivery of proteins to mammalian cells based on lipid, polymeric or inorganic nanocarriers (reviewed in Gu et al. 2011). Protein transduction is one of the most widely used of these methodologies. In protein transduction, the cell membrane translocation ability of cell-penetrating peptides or protein transduction domains is used to deliver the protein of interest (reviewed in Noguchi et al. 2010). The major challenge for protein delivery in plant tissues is the presence of the plant cell wall in addition to the cell membrane. Available protein delivery methods for plant tissues have been limited mainly to proof-of-concept systems, protein transduction being one of the most frequently used methods (Eggenberger et al. 2009). Other methodologies available involve direct physical methods such as microinjection (Staiger et al. 1994; Wymer et al. 2001) or biolistic delivery of proteins immobilized onto gold projectiles (Wu et al. 2011a, b) or loaded into the pores of mesoporous silica nanoparticles (Martin-Ortigosa et al. 2012a). Even though these methods have been successful for protein delivery in plant cells, they require synthesis and testing of the carrier (Eggenberger et al. 2009; Martin-Ortigosa et al. 2012a), laborious precision work (Staiger et al. 1994; Wymer et al. 2001) or several steps to attach the protein to the carrier (Wu et al. 2011a, b). Therefore, a simpler and more straightforward method for efficient protein delivery to plant cells is needed.

More than a decade ago, the term "diolistics" was used for the first time to refer to a technique in which dyes are dried onto tungsten or gold projectiles and bombarded using a gene gun into nervous system cells for labeling purposes (Gan et al. 2000). This technique has been primarily used to deliver indicator dyes to study different cellular physiological states or to permit visualization of cell architecture (O'Brien and Lummis 2007). Diolistics has also been used in plant and algal cells to monitor changes in cytosolic calcium concentrations (Bothwell et al. 2006). Following the same principle as in diolistics, delivery of RNA to parasitic helminths has also been achieved by the biolistic method, lyophilizing the RNA onto 1.6-μm gold microcarriers (Davis et al. 1999). In the field of vaccination, antigenic agents have been precipitated using ethanol and sodium acetate onto 1.5–2.5-μm gold particles (Chen et al. 2001). These particles were used for delivery by a gene gun for immunization purposes (Chen et al. 2001).

The objective of the work presented here is to test the biolistic method for intracellular delivery of proteins to plant and mammalian tissues. Using a procedure similar to diolistics, proteins were deposited using different drying methods onto microprojectiles for tissue bombardment using a gene gun. The intracellular delivery of intact and active proteins upon bombardment was fast, efficient, and reproducible. The technology presented here has been termed "proteolistics" and is foreseen to be widely applicable by biologists in different fields to answer basic questions about protein roles in the cell and for more applied biotechnological uses.

Materials and methods

Plant materials

Onion epidermis tissue was obtained from scale leaves of white onion bulbs purchased from any grocery store. Rectangular (3×2.5 cm^2) pieces were peeled off immediately before bombardment and placed in the center of a Petri dish containing solid agar medium [0.5 mM 2-(N-morpholino)ethanesulfonic acid (MES), 15 g/l Bacto agar (BD), pH 5.7] with the peeled side upwards. For measurements of green or red fluorescent cells, or cell numbers after fluorescein diacetate staining and X-Gluc histochemical staining, pieces of epidermis of the same scale leaf were distributed among treatments.

Tobacco (Nicotiana tabacum var. Petite Havana) leaves were obtained from 3- to 6-week-old plants grown in vitro on Murashige and Skoog (MS, 1962) medium (2 % sucrose, 2.5 g/l Gelrite, pH 5.7). About 30 min before bombardment, young leaves from 4- to

8-week-old in vitro-grown Petite Havana tobacco plants were placed with the adaxial surface upwards on agar medium. Immature maize embryos of Hi-II genotype were obtained from ears harvested 11–12 days postpollination (provided by the Center for Plant Transformation, Iowa State University). Immature embryos (1–2 mm long) were dissected and cultured as described in Frame et al. (2000).

Projectile/protein coating

Stock solutions for six proteins (three marker proteins and three enzymes) used in this study were prepared as follows: enhanced green fluorescent protein (eGFP, BioVision) was reconstituted in phosphate-buffered saline (pH 7.4) at 1 µg/µl; DsRed fluorescent protein (DsRed, BioVision) was reconstituted in dH$_2$O at 100 ng/µl; tetramethylrhodamine-isothiocyanate-labeled bovine serum albumin (TRITC-BSA, Invitrogen) was reconstituted in Tris–sodium (TS) buffer (250 mM NaCl, 15 mM Tris, pH 8) at 25 µg/µl; β-glucuronidase (GUS, Sigma) and trypsin (Sigma) were both reconstituted in TS buffer at 50 µg/µl; RNAse A (Sigma) was reconstituted in dH$_2$O at 10 µg/µl. Plasmid DNA pLMNC95 (GFP expression, ABRC stock CD3-420; Luke Mankin and Thompson 2001) was used at 500 ng/µl in dH$_2$O. Gold microprojectile suspension (0.6 µm, Bio-Rad, cat. 165-2262) at 30 µg/µl was prepared as described in Frame et al. (2000). The different protein concentrations used in the study were determined by the availability of the materials. In general, where proteins were expensive, the concentrations used in the experiments were decreased accordingly.

To coat the proteins on the gold particles, different volumes of protein solution were mixed with a fixed amount of gold suspension by pipetting. Specifically, for each shot, 2 µl gold microprojectile suspension was mixed with the following amounts of protein solution: eGFP, 2.5 µl; DsRed, 12 µl; TRITC-BSA, 10 µl; GUS, 5.5 µl; trypsin, 4 µl; and RNAse A, 4 µl.

For the protein–plasmid DNA co-delivery experiment, 4 µl of 37.5 µg/µl TRITC-BSA solution in TS and 2 µl of plasmid pLMNC95 were mixed together. The mix was loaded and evenly distributed over the inner circle (the central part that is struck by the helium blast) of the macrocarrier set (a plastic macrocarrier placed into the macrocarrier holder). The loaded macrocarrier set was frozen in liquid nitrogen for 5–10 min and lyophilized using a lyophilizer (Freezone 2.5 from Labconco) for 1 h. Bombardment was performed immediately after lyophilization.

For air-dried protein delivery, the macrocarrier sets were left in a horizontal flow bench at room temperature (22 °C) for between 2 and 16 h for liquid evaporation. Macrocarrier sets carrying the dried particles and biomolecules were kept in Petri dishes containing Drierite until bombardment.

For delivery of protein alone without projectiles, 12 µl of DsRed protein solution was spread directly onto the macrocarrier set and allowed to air-dry at 22 °C for 16 h. For GUS protein delivery, 6 µl of 12.5 µg/µl solution was spread onto the macrocarrier set and allowed to air-dry at 22 °C for 2–16 h.

For delivery of DNA using gold particles, 2 µl of plasmid pLMNC95 was precipitated on gold microprojectiles using calcium chloride and spermidine as described in Frame et al. (2000).

For delivery of plasmid DNA alone with no projectiles, 300 ng (0.7 µl of 480 ng/µl solution in dH$_2$O) of plasmid pLMNC95 was placed in the center of the macrocarrier set and allowed to air-dry at 22 °C for 16 h or lyophilized for 1 h.

Plant tissue bombardment

A PDS-1000/He gene gun (Bio-Rad) was used according to the general settings described in Frame et al. (2000). For onion epidermis tissue, 1,100 psi rupture discs and 6 cm target distance were used. For tobacco leaf and immature maize embryos, 650 psi and 6 cm target distance bombardment conditions were used.

Mouse ear pinna tissue bombardment

Ear pinnae of 8-week-old CD-1 mice were dissected immediately after the animals were euthanized. The tissues were arranged with the inner part of the ear upwards on an agar medium plate and were immediately bombarded using the PDS-1000/He gene gun at 650 or 1,100 psi, 6 cm target distance, and 28 mmHg vacuum. For bombardment, a mixture of 90 µg of 0.6-µm gold and 200 µg of GUS protein or 1 µg of DsRed per shot was air-dried onto the macrocarrier set for 2 h. Samples bombarded with GUS protein were soaked in X-Gluc solution and incubated at 37 °C overnight.

X-Gluc histochemical staining

GUS histochemical assays on plant and animal tissues were performed based on a procedure previously described in Jefferson (1987). X-Gluc solution containing 50 mM of sodium phosphate buffer (pH 7.0), 5 mM of potassium ferricyanide (pH 7.0), 5 mM of potassium ferrocyanide (pH 7.0), 0.1 % of Triton X-100, 1 % of dimethyl sulfoxide, and 1.5 mM of X-Gluc substrate (Biosynth) was prepared. Immediately after bombardment, bombarded tissues with GUS protein (GUS) were soaked in X-Gluc solution and incubated overnight at 37 °C.

Fluorescein diacetate staining

Fluorescein diacetate staining was used to count the number of dead cells after different treatments and was based on a previous protocol described by Widholm (1972). Onion epidermis tissues were submerged in 10 ml of MS liquid medium to which 100 µl of 5 mg/ml fluorescein diacetate (Alfa Aesar) solution in acetone was added. The samples were incubated for 2–5 min and directly observed under the green channel filter of a fluorescence microscope. For each onion epidermis sample, 12 images were taken with a 5× microscope objective (representing an area of 10.5×8 mm^2) every 0.5 cm (four columns × three rows per sample). The total number of dead cells found in each image (four repeats per treatment) was recorded.

Microscopy

Bright-field and fluorescence images were taken using a Zeiss Axiostar plus microscope. The objectives used were A-Plan 5×/0.12, 10×/0.25, 40×/0.65, and Plan-Apochromat 63×/1.4 oil. For green channel fluorescence images, a GFP BP filter (Chroma Technology Corp.) was used ($\lambda_{ex} = 470$ nm, beam splitter = 495 nm, $\lambda_{em} = 525$ nm). For the red channel, a Texas Red filter (Chroma Technology Corp.) was used ($\lambda_{ex} = 560$ nm, beam splitter = 595 nm, $\lambda_{em} = 645$ nm). Images were taken using ProgRes Capture Pro 2.6 software and a ProgRes C3 digital camera (Jenoptik). Pictures were also taken with an Olympus SZH10 stereo microscope using a SPOT Camera and SPOT version 4.6 software (Diagnostic Instruments, Inc.). For fluorescence images a red channel filter

($\lambda_{ex} = 540$–580 nm, $\lambda_{em} = 600$–660 nm) was used. Red channel images were changed to magenta coloration using Adobe Photoshop software.

For scanning electron microscopy imaging of lyophilized or air-dried samples, 60 µg of 0.6-µm gold (2 µl of 30 µg/µl gold suspension) was subjected to the following treatments: (1) air-drying with 5 µl of 250 mM NaCl, 15 mM Tris pH 8 buffer solution, (2) DNA coating based on CaCl$_2$/spermidine with 1 µg of pLMNC95 plasmid as previously reported (Martin-Ortigosa et al. 2012b), and (3) air-drying or lyophilization with 250 µg of GUS protein (50 µg/µl solution) in 250 mM NaCl, 15 mM Tris pH 8 buffer solution. The macrocarriers holding these different treatments were mounted onto aluminum stubs and lightly sputter-coated using a palladium/gold alloy target in a Denton Desk II sputter coater (Denton Vacuum, LLC). Images were taken using a JEOL (Japan Electron Optics Laboratories) 5800LV scanning electron microscope at 10 kV.

Statistical analysis

Graphs show mean and standard deviation. P values were derived from t tests comparing two independent groups or from one-way analysis of variance (ANOVA). Analyses were done using Microsoft Excel 2010 software.

Results

Proteolistic delivery with lyophilization preparation

Figure 1a shows the scheme of the proteolistic method for intracellular delivery of intact proteins to cells using a gene gun. A mixture of a protein (e.g., eGFP) and microprojectiles routinely used for plant transformation purposes (e.g., 0.6-µm gold particles) is placed on the surface of a macrocarrier set (a plastic macrocarrier film placed in the macrocarrier holder of the PDS-1000/He gene gun) using a pipette. The liquid mixture is then dried by lyophilization (freezing the loaded macrocarrier set in liquid nitrogen for several minutes followed by lyophilization to remove liquid from the suspension), or by air-drying at room temperature (22 °C). As a consequence, the proteins are expected to be deposited on the surfaces of the gold
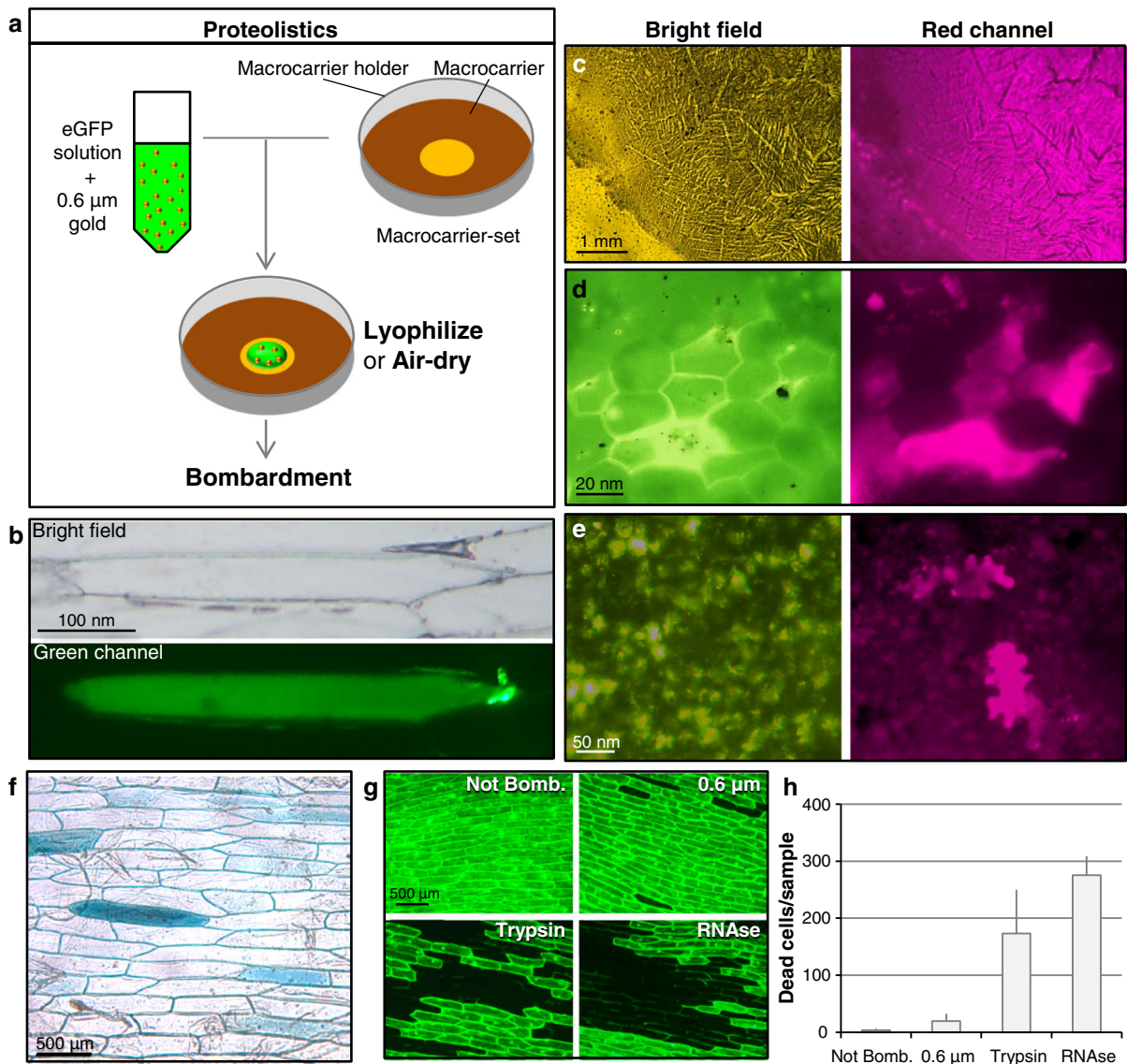
Fig. 1 Scheme of the proteolistics methodology. **a** A solution of a protein (e.g., eGFP) is mixed with a suspension of 0.6-μm gold projectiles. The mixture is placed in the center of the gene gun cartridge, and the macrocarrier set is (1) frozen in liquid nitrogen for a few minutes and lyophilized for 1 h or (2) allowed to air-dry at room temperature. Once dried, a target tissue (e.g., onion epidermis tissue or mouse ear pinnae) is bombarded using a gene gun (Bio-Rad PDS-1000/He). **b** Microscope images of an onion epidermis cell showing green fluorescence after bombardment with eGFP protein lyophilized with 0.6-μm gold particles. **c** Microscope images of TRITC-BSA and 0.6-μm gold projectiles deposited on the macrocarrier plastic film surface after lyophilization. **d** Intracellular TRITC-BSA protein delivery to immature maize embryo scutellum cells after bombardment. In the bright-field image (*left*) *dark dots* corresponding to projectiles can be observed. **e** Microscope images of tobacco leaf cells showing red fluorescence due to TRITC-BSA delivery by the proteolistics method. **f** Bright-field image of an onion epidermis tissue bombarded with lyophilized GUS enzyme/0.6-μm gold projectile mixture. Cells where the active enzyme has been delivered show blue coloration after GUS histochemical staining with X-Gluc solution. **g** Fluorescence microscope images (taken with a ×5 objective) of onion epidermis tissues stained with the vital stain fluorescein diacetate (*dark cells* are dead cells). Samples were not bombarded ("Not Bomb."), bombarded with 0.6-μm gold ("0.6 μm") or with lyophilized trypsin ("Trypsin") or RNAse A ("RNAse") enzyme/0.6-μm gold projectile mixtures. **h** Mean and standard deviation of the number of dead cells found in the different treatments. For each onion epidermis sample, 12 images were taken with the ×5 microscope objective (representing an area of 10.5×8 mm^2) every 0.5 cm (four columns × three rows per sample)

microprojectiles as well as on the plastic film. The macrocarrier sets with protein-coated gold particles are then used for tissue bombardment.

First, lyophilization as a drying method was tested. Because many proteins are labile biomolecules, rapid dehydration of the protein/projectile mixture at low temperature could prevent degradation. As a proof of concept, the fluorescent protein eGFP was chosen. This protein will not fluoresce if it is denatured (Ward and Bokman 1982). As can be seen in Fig. 1b, green fluorescent cells in onion epidermis tissue could be detected 30 min after bombardment with the lyophilized eGFP/gold preparation. The fluorescence was evenly distributed in the entire cell (Fig. 1b), indicating successful intracellular delivery of intact eGFP. To rule out that such results were due to passive diffusion of eGFP through pores of cell wall, eGFP solution was incubated for 30 min on the surface of intact onion epidermis tissues or tissues bombarded with bare 0.6-µm gold projectiles. No intracellular eGFP detection could be observed in these tissues, suggesting that eGFP protein does not passively permeate plant cell wall and membrane.

To further validate these results, different fluorescent protein and plant tissues were tested. The red fluorescently labeled BSA (TRITC-BSA) protein solution was mixed with 0.6-µm gold projectiles. Lyophilized TRITC-BSA protein/gold deposited on the plastic film of the macrocarrier set (Suppl. Fig. 1) showed red fluorescence when observed under a fluorescence scope (Fig. 1c). Intracellular TRITC-BSA protein delivery could be observed in immature maize embryo scutella (Fig. 1d) and tobacco leaves (Fig. 1e) after bombardment with TRITC-BSA protein/gold preparations. Successful fluorescent protein delivery to plant tissues through proteolistics indicated that at least a portion of the bombarded protein retained its integrity and was not degraded.

The next question to be addressed was whether proteolistics could deliver enzymes that showed intracellular activity upon delivery. For this purpose GUS enzyme was chosen. Figure 1f shows an X-Gluc histochemical staining image of onion epidermis tissue bombarded with GUS protein/gold preparations and incubated with X-Gluc substrate for 12–16 h immediately after bombardment. Blue cells could be readily observed as a consequence of successful intracellular delivery of active GUS enzyme. Different

shades of blue were observed, possibly indicating the amount of enzyme delivered (Fig. 1f).

To further confirm active enzyme delivery through proteolistics, trypsin and RNAse A enzymes were used. These enzymes, if active, should cause cell death when delivered in sufficient amounts by disrupting vital cell processes through digestion of protein or RNA molecules. Four different types of treatment along with fluorescein diacetate vital staining to monitor cell death (Fig. 1g, h) were performed: onion epidermis tissues were not bombarded ("Not Bomb."), bombarded with 0.6-µm gold projectiles alone ("0.6 µm"), and bombarded with trypsin ("Trypsin") or RNAse A ("RNAse") lyophilized with 0.6-µm gold projectile suspension. As can be seen in Fig. 1g, the samples not bombarded ("Not Bomb.") showed no damage, as no dark cells (dead cells) could be observed. Onion tissues bombarded with 0.6-µm gold ("0.6 µm") showed some dead cells, likely due to cell damage caused by bombardment. In contrast, samples bombarded with the enzyme/projectile treatments ("Trypsin" and "RNAse") showed vast areas of cell damage (Fig. 1g), suggesting additional damage caused by the intracellular activity of the enzymes. These results are corroborated by the data shown in Fig. 1h, where both enzyme treatments showed significant differences in the number of dead cells ("Trypsin": 173 dead cells/sample, $P = 0.007$; "RNAse": 275 dead cells/sample, $P = 0.000$) compared with the control bombarded with bare projectiles ("0.6 µm": 20 dead cells/sample).

Proteolistic delivery with air-drying preparation

An alternative sample preparation process for the proteolistics technique, i.e., air-drying of the protein/gold mixture in a horizontal flow bench at room temperature (22 °C), was tested. The advantage of this preparation process is that no freezing and lyophilization of the samples is required. Two different proteins, namely the red fluorescent protein DsRed and the GUS enzyme, were used for the experiments. Figure 2a shows the air-dried DsRed and 0.6-µm gold projectiles on a macrocarrier set. Red fluorescence of the dried matter (Fig. 2a, bottom) suggests that some portion of the DsRed protein remains intact and fluorescent after 16 h of air-drying process. Red fluorescent cells were detected in onion epidermis tissues after bombardment, indicating that DsRed
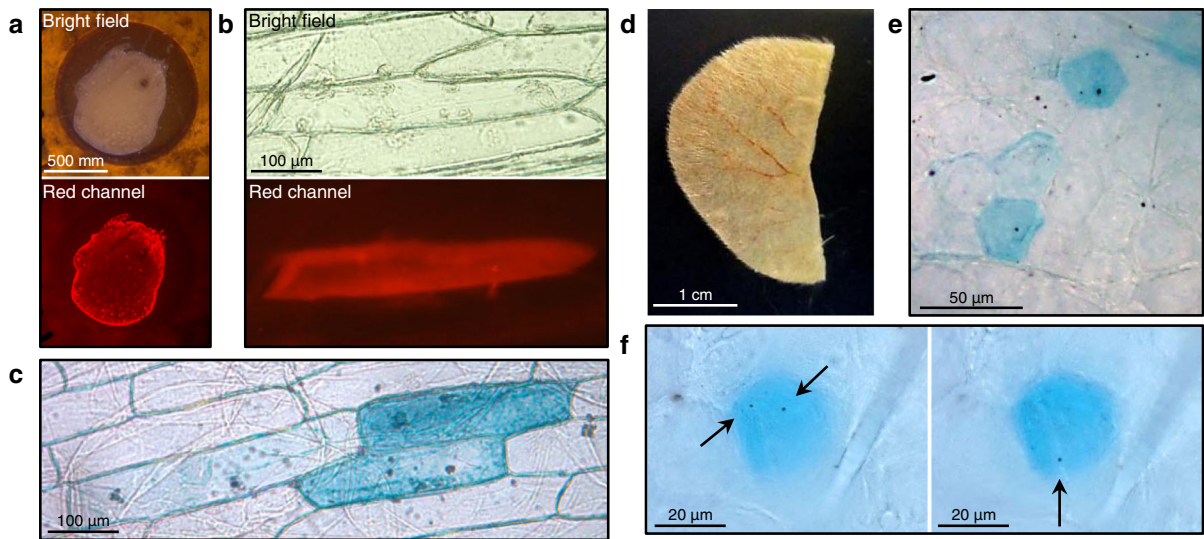
Fig. 2 Proteolistic delivery of air-dried protein/projectile mixtures. **a** Images of a macrocarrier set with a suspension of the red fluorescent protein DsRed/0.6-μm gold projectiles air-dried overnight. **b** Microscope images of an onion epidermis cell showing red fluorescence due to proteolistic delivery of air-dried DsRed/0.6-μm gold mixture. **c** Bright-field microscope image of onion epidermis cells showing *blue* coloration after GUS histochemical staining. The tissue was bombarded with air-dried GUS enzyme/0.6-μm gold projectile mixture. **d** Mouse ear pinna tissue before bombardment. **e** Microscope image of ear pinna tissue cells showing *blue* coloration after X-Gluc solution incubation. Tissues were previously bombarded with air-dried GUS enzyme/0.6-μm gold projectile mixture. **f** Microscope images of a blue ear pinna tissue cell at two different depths (*left* and *right* images). At both depths in the same cell, different 0.6-μm gold particles (*dark dots*) can be detected (indicated by *arrows*)

intracellular protein delivery with air-drying preparation was successful (Fig. 2b).

The same process was performed with the GUS enzyme. Onion epidermis tissues were bombarded, and blue cells were observed after GUS histochemical staining (Fig. 2c), indicating intracellular delivery of active enzyme. Delivery of GUS was also successful using 0.4- and 1.1-μm tungsten projectiles in addition to the aforementioned 0.6-μm gold projectiles (Suppl. Fig. 2).

To evaluate whether proteolistics could work as a protein delivery system for mammalian cells, the technology was tested on mouse ear pinna tissues (Fig. 2d). These tissues were removed immediately after the animals were euthanized and bombarded with GUS protein air-dried onto 0.6-μm gold. As can be seen in Fig. 2e, distinct blue cells were detected after the tissue was incubated in X-Gluc solution. Multiple gold particles could be observed at different depths in one single blue cell (dark dots in Fig. 2f), indicating that GUS protein was successfully delivered and functional in the ear pinna tissues. DsRed fluorescent protein air-dried on gold particles was also tested, but the confirmation of intracellular delivery was not obvious due to the strong red autofluorescence observed in the ear tissue after bombardment (Suppl. Fig. 3).

To test whether there was any loss of enzymatic activity during the air-drying sample preparation process, the activity of GUS enzyme subjected to different drying methods was measured using a spectrophotometric assay. As shown in Suppl. Fig. 4, the differences in the activity of freshly prepared, 1-h lyophilized or overnight air-dried GUS were not statistically significant ($P = 0.464$). This suggests that, under the conditions tested, the activity of GUS enzyme is not affected by the air-drying process.

Scanning electron microscopy investigation of the drying process

For better understanding of the protein coating of projectiles by the lyophilization and air-drying processes, scanning electron microscopy images of macrocarrier plastic film with 60 μg of 0.6-μm gold projectiles subjected to different treatments were
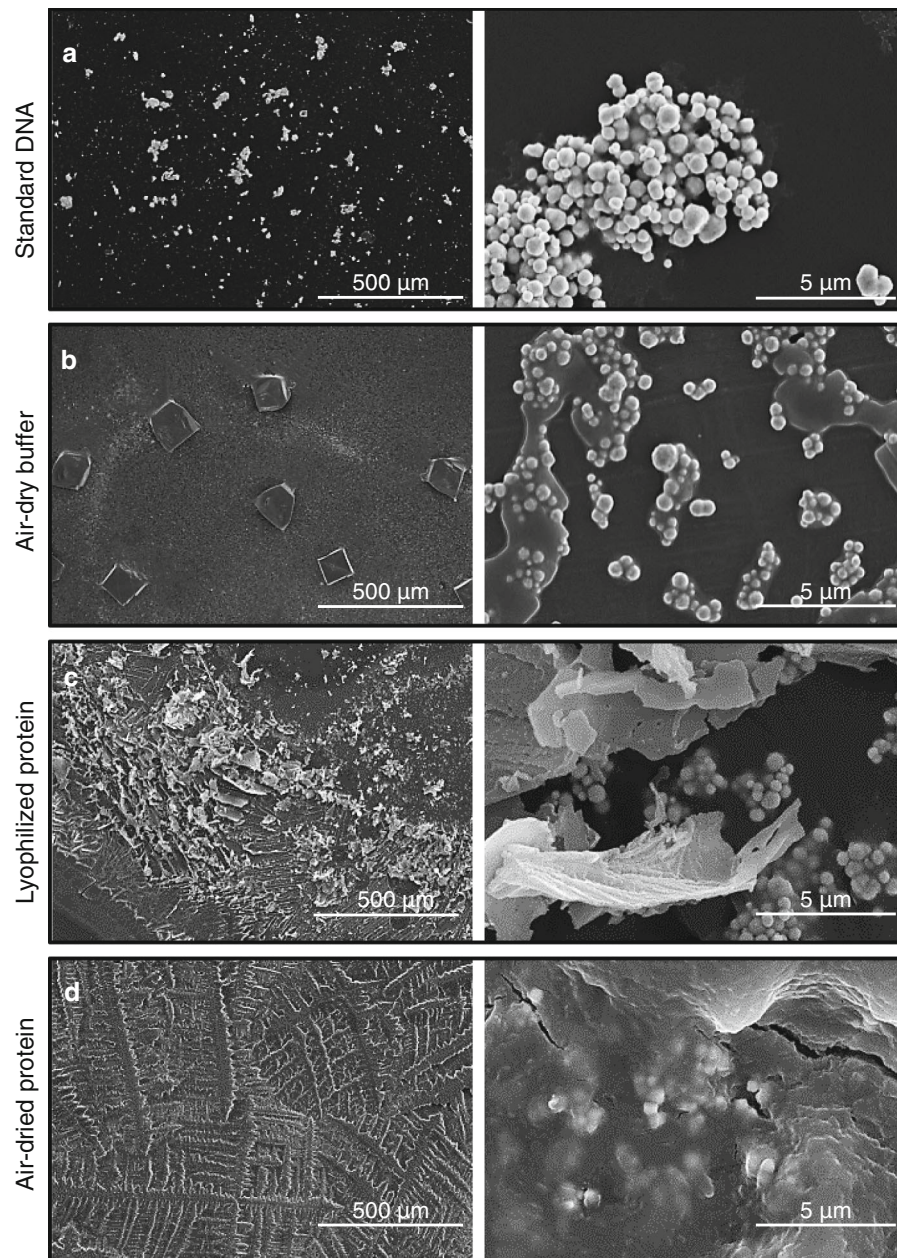
Fig. 3 Scanning electron microscopy images of the layout of 0.6-µm gold particles on the macrocarrier (*left* wide-field view; *right* close-up view) after the following treatments: **a** standard DNA: standard DNA coating with CaCl$_2$/spermidine; **b** air-dry buffer: TS buffer only, no protein; **c** lyophilized protein: lyophilized GUS protein solution; **d** air-dry protein: air-dried GUS protein solution

obtained (Fig. 3). The treatments were the following: "Standard DNA" for CaCl$_2$/spermidine-based plasmid DNA coating (Fig. 3a); "Air-dry buffer" in which projectiles were mixed with TS buffer without protein (Fig. 3b); "Lyophilize protein" (Fig. 3c) and "Air-

dry protein" (Fig. 3d) in which 250 µg of GUS enzyme was lyophilized or air-dried along with the projectiles, respectively. All treatments resulted in different layouts of the particles on the macrocarrier (Fig. 3). The DNA-coated particles were found in

clusters of different sizes scattered throughout the macrocarrier (Fig. 3a). In the control "Air-dry buffer," particles were found dispersed and embedded in a solid matrix formed by the crystallized saline buffer (Fig. 3b). When the gold particles were coated with 250 µg of GUS protein through lyophilization, a laminated pattern of protein/saline buffer was found (Fig. 3c). When the enzyme was air-dried along with the projectiles, a fern-like solid matrix was formed, completely embedding the particles in a two-dimensional solid structure (Fig. 3d). All the patterns of the protein/gold preparations after the dehydration processes described above appeared to be different and heterogeneous.

Proteolistic co-delivery of protein and DNA

While the main purpose of the proteolistics technique is intracellular delivery of proteins, the utility of the methodology was also investigated for delivery of other chemicals and biomolecules that can be dried onto the projectiles. Firstly, chemicals such as bromophenol blue (BPB) were mixed with the gold particles and lyophilized onto the macrocarrier set. BPB is a color marker commonly used to monitor the process of gel electrophoresis and is not permeable to plant cells (Suppl. Fig. 5a). Intracellular delivery of BPB to onion cells was achieved after bombardment with BPB lyophilized onto gold microprojectiles (Suppl. Fig. 5b). Additionally, circular or linear plasmid DNAs lyophilized or air-dried along with 0.6-µm gold were also successfully delivered into different plant tissues (Suppl. Fig. 6). In transient experiments using DNA, lyophilized plasmid DNA/gold preparations resulted in less transient expression, but air-dried plasmid DNA/gold preparations were comparable to standard CaCl$_2$/spermidine-based precipitation preparations (Suppl. Fig. 6d).

To assess the co-delivery of two biomolecules, GFP expression plasmid pLMNC95 and TRITC-BSA protein solutions were mixed with 0.6-µm gold particle suspension and lyophilized onto the macrocarrier set. Onion epidermis tissues were bombarded. One day after bombardment, cells showing fluorescence in both green (due to plasmid DNA expression) and red (due to TRITC-BSA protein delivery) could be observed as a consequence of biomolecule co-delivery (Fig. 4a). To evaluate whether protein/plasmid DNA co-delivery would affect DNA delivery, measurement of green
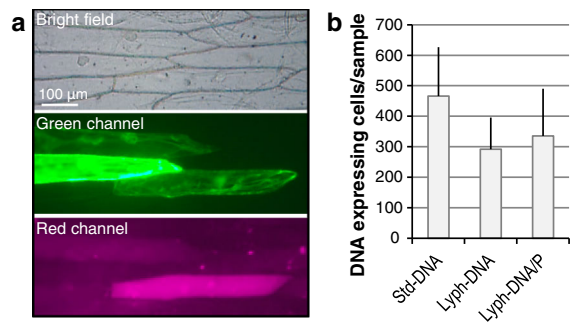

Fig. 4 Co-delivery of protein and plasmid DNA to plant cells. **a** Microscope images of onion epidermis cells after bombardment with lyophilized protein (red fluorescent protein TRITC-BSA), DNA (*gfp* gene carrying plasmid pLMNC95), and 0.6-µm gold mixture. Cells simultaneously fluoresced green due to GFP expression from the plasmid pLMNC95 and red due to TRITC-BSA protein delivery. **b** Mean and standard deviation of the number of onion epidermis cells transiently expressing the plasmid for GFP expression pLMNC95 1 day after bombardment. Samples (four per treatment) were bombarded with plasmid DNA coated on the projectiles following a standard CaCl$_2$/spermidine precipitation protocol ("Std-DNA"), lyophilized plasmid DNA ("Lyph-DNA"), and lyophilized DNA and TRITC-BSA protein mixture ("Lyph-DNA/P")

fluorescent cells was performed. The number of GFP-expressing cells was compared in onion tissue samples bombarded with pLMNC95 plasmid DNA using a standard biolistic gun method (CaCl$_2$/spermidine + 0.6-µm gold, "Std-DNA") versus DNA only (0.6-µm gold, "Lyph-DNA") and DNA/protein (0.6-µm gold, "Lyph-DNA/P"), both using the lyophilization procedure described in this work. Figure 4b shows the transient GFP expression results 1 day after bombardment. The standard coating procedure resulted in a higher DNA delivery frequency ("Std-DNA", 466 fluorescent cells/sample) when compared with samples bombarded with the lyophilization procedure ("Lyph-DNA", 291 fluorescent cells/sample), as expected. However, when using the lyophilization preparation procedure, the DNA delivery frequencies in samples bombarded with DNA only ("Lyph-DNA", 291 fluorescent cells/sample) were comparable to the samples bombarded with DNA and TRITC-BSA protein ("Lyph-DNA/P", 335 fluorescent cells/sample). In fact, the differences between the three treatments were not significant ($P = 0.674$). This result indicates that, under the conditions tested, DNA delivery is not affected by TRITC-BSA protein co-delivery.
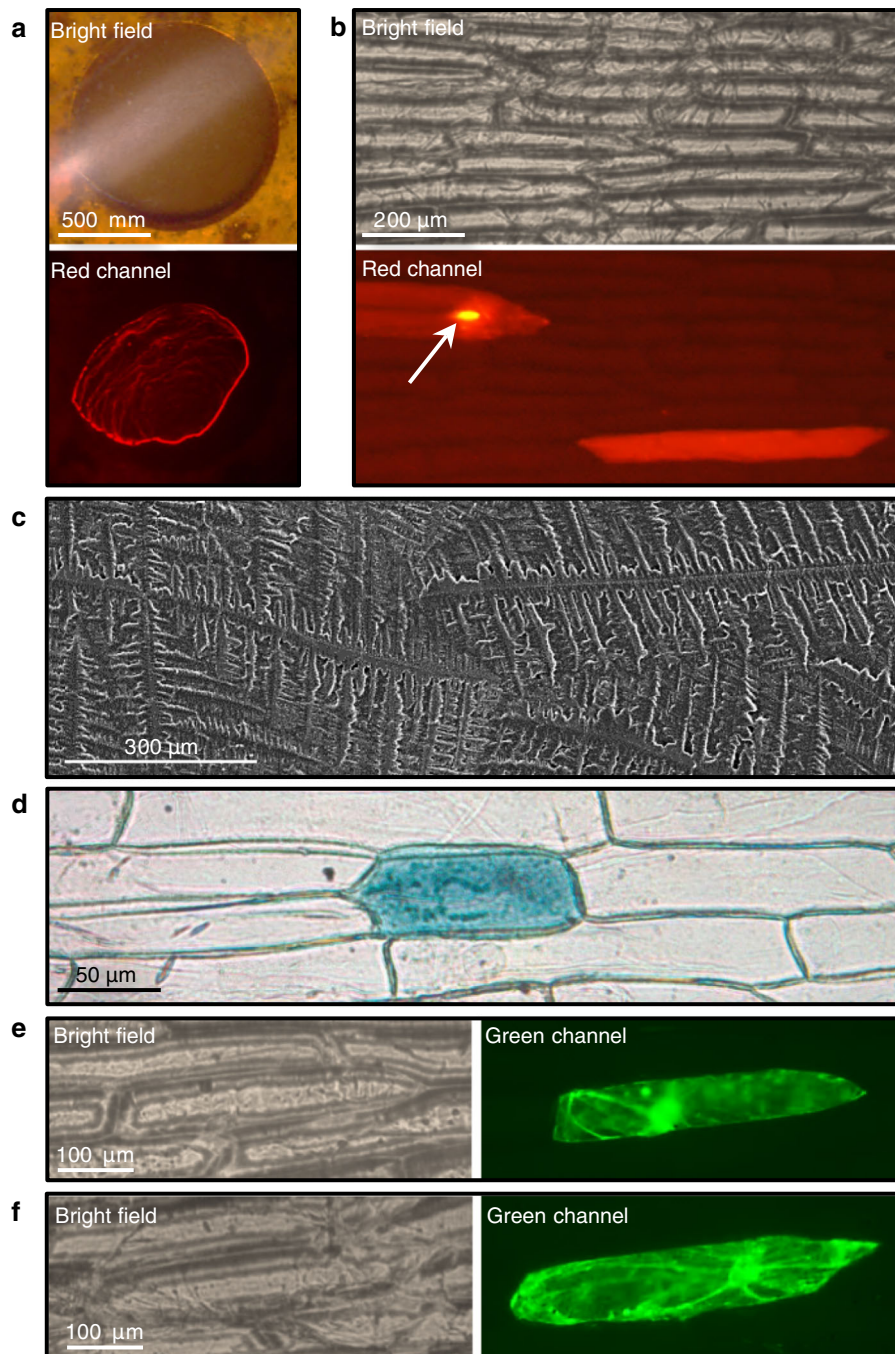
Fig. 5 Protein and DNA delivery to plant tissues without gold particles. **a** Images of a macrocarrier set with a solution of 1.2 μg of DsRed protein air-dried overnight. **b** Microscope images of onion epidermis tissues bombarded with air-dried DsRed protein; cells showing red fluorescence can be observed as a consequence of the red fluorescent protein delivery. Bright red fluorescent clumps of DsRed protein can be seen (*white arrow*) on the surfaces. **c** Scanning electron microscopy image of a macrocar- rier plastic film on which 250 μg of GUS protein was air-dried. **d** Microscope image of a blue onion epidermis cell after GUS staining. The tissue was bombarded using a macrocarrier on which 75 μg of GUS protein was air-dried overnight. **e** Onion cell showing GFP expression after bombardment with air-dried DNA solution of plasmid pLMNC95. **f** Onion cell showing GFP expression after bombardment with lyophilized plasmid pLMNC95 DNA solution

Delivery of proteins or DNA without gold projectiles as carriers

When protein/gold particle suspensions were dried, irregular aggregations were observed on the macrocarrier plastic film (Fig. 2a; Suppl. Fig. 1). Because the concentrations of protein were high, it was likely that some of these aggregates were protein powder with no microprojectiles. This was further corroborated by scanning electron microscopy images (Fig. 3c, d) in which dehydrated protein aggregates containing no microprojectiles were observed in samples prepared with gold microprojectiles. To test whether these types of dehydrated protein clumps or aggregates could be delivered directly through a gene gun to plant tissues, DsRed or GUS protein solutions (without gold microprojectiles) were air-dried onto macrocarrier sets overnight (Fig. 5a). Red fluorescent onion epidermis cells were observed upon bombardment with these macrocarrier sets (Fig. 5b), indicating successful DsRed protein delivery. In some cases, bright clumps of proteins could be detected in the surface of the tissue (white arrow in Fig. 5b). The same procedure was performed for GUS protein delivery. Scanning electron microscopy imaging of the protein solution dried onto the plastic macrocarrier film showed fern-like patterns (Fig. 5c) similar to those obtained when using 0.6-µm gold particles (Fig. 3d). Blue-colored onion epidermis cells were observed after X-Gluc staining of the tissues bombarded using these macrocarrier sets (Fig. 5d).

Both results suggest that it is possible to deliver functional dehydrated proteins to plant tissues from the macrocarrier set using the biolistic method without using gold microprojectiles as carriers. Additionally, DNA molecules were also successfully delivered to plant tissues without using microprojectiles as carriers following the same principle. This was indicated by the presence of fluorescent cells containing GFP-expressing plasmid DNA 1 day after bombardment (Fig. 5e, f). Although there may be some utility to this approach for experiments where metal projectiles cannot be used, the delivery frequencies for both proteins and DNA were considerably lower compared with bombardment using gold microprojectiles. For DNA delivery, the number of fluorescent cells per sample ranged from 0 to 2 cells for lyophilization and from 5 to 20 cells for the air-dry method, whereas the standard $CaCl_2$/spermidine-based 0.6-µm gold microprojectile DNA coating method resulted in over 800 fluorescent cells per sample.

Discussion

Biolistic guns, also known as gene guns, have been used widely for delivery of DNA molecules into various tissues in many organisms. Here we report a simple procedure for delivering proteins into plant and animal tissues using the biolistic or gene gun method. "Proteolistics" is the technology involved in dehydrating protein with a microprojectile suspension directly onto the cartridge of the gene gun followed by intracellular delivery of the protein/microprojectiles into the target tissues and cells via bombardment. We demonstrated that fluorescent proteins such as eGFP, DsRed, and TRITC-BSA, as well as enzymes such as GUS, trypsin, and RNAse A, can be successfully delivered to plant and mammalian tissues. The delivery of functional proteins to cells measured by cell fluorescence, GUS histochemical staining, or fluorescein diacetate staining was reproducible, but variable.

Protein delivery through the biolistic method has been previously reported by Wu et al. (2011a) and Martin-Ortigosa et al. (2012a). Wu et al. delivered a Tn5 transposase into rice for plant transformation purposes (Wu et al. 2011a, b). One major hurdle for this method is that the transposase needs to be immobilized onto the gold particles via covalent binding of 6xCys-tag sulfhydryl groups to the gold surface. Our group has previously shown that fluorescent marker proteins can be delivered into plant cells using gold-functionalized mesoporous silica nanoparticles (Martin-Ortigosa et al. 2012a). The method described in this study offers a simple procedure for preparing and delivering proteins to tissues.

In the proteolistics method, biomolecules such as proteins are dehydrated with a microprojectile suspension directly onto a biolistic gun macrocarrier set prior to bombardment. Two different dehydration methods, namely lyophilization and air-drying, were evaluated and proven to be adequate for preparing the proteins tested. Lyophilization (−45 °C, 1 h) can be used to prevent degradation of labile protein molecules (Tang and Pikal 2004). Air-drying (22 °C, 2–16 h depending on starting volume) can be used for stable proteins and requires no special equipment.

A small volume of protein/microprojectile mixture, around 10 μl, on the macrocarrier set can be dried at room temperature (22 °C) in a few hours by evaporation. The two drying systems involve different processes for water removal (sublimation or evaporation) that imprint different protein/microprojectile landscapes onto the macrocarrier plastic film (Fig. 3). Therefore, variability in the protein delivery results was expected.

Various parameters such as the amount and type of protein, the nature of the protein buffer, the type of projectile, the protein-to-microprojectile ratio, the final volume to be dried, the way the mixture is placed onto the plastic macrocarrier film, the area covered, and the drying process of choice need to be optimized for each experiment; For example, equal amounts of GUS protein in different volumes could lead to different amounts of blue cells per sample in transient X-Gluc assay (Suppl. Fig. 7). Quantification of protein delivery by visual detection was difficult due to the limitation of the eye in detecting fine differences in blue coloration between cells when GUS enzyme and X-Gluc assays were used. When only distinguishable blue cells were counted, the range of cells varied from 0 to over 300 cells per sample depending on the amount of protein used (5 or 250 μg of protein per shot, respectively; Suppl. Fig. 7). In general, intracellular protein delivery was detected in most of the experiments performed and was reproducible. Only in the cases where the amount of protein was low (Suppl. Fig. 7a) or when no gold microprojectiles were used (Fig. 5) was protein delivery more difficult to detect.

It has been found that it can be difficult to spread an even layer of liquid depending on the interaction of the protein/microprojectile suspension with the plastic surface of the macrocarrier. In this case, the protein/microprojectile suspension can be pipetted in a "dotted" fashion in approximately 1–3-μl drops over the surface of the macrocarrier (Suppl. Fig. 1b). The drying of the protein/microparticle suspension can lead to formation of irregular agglomerates or matrices on the plastic film of the macrocarrier. It is expected that these structures will break down during the blast caused by the bombardment, releasing the crystallized protein/particle mix into the cell. In our study, these structures did not cause notable additional damage to plant cells compared with samples bombarded with microprojectiles alone (Suppl. Fig. 8).

Intracellular protein delivery can facilitate cellular and biochemical studies and provide a tool for DNA-free genome editing. Protein delivery systems have been developed predominantly in the medical field (Ravichandran 2009). The challenge for protein delivery in the plant science field is the presence of cell walls in plant cells. Because the biolistic method for DNA delivery is a technology extensively used in the plant science field, the same apparatus is readily adoptable for delivery of protein molecules in plants. The biolistic method can also be used for delivering proteins to any tissue (such as intact animal tissue as demonstrated in this work) or organisms amenable to the biolistic method. In the present work, most of the experiments were performed using onion epidermis cells as a model tissue to test this proof-of-concept methodology. However, other tissues such as immature maize embryo (Fig. 1d), maize embryogenic callus (Suppl. Fig. 6c), and tobacco leaves (Fig. 1e; Suppl. Fig. 6b) were also used for protein or DNA delivery after lyophilization or air-drying. Because these tissues are amenable to in vitro culture and regeneration, we anticipate that whole plants may be regenerated from cells after proteolistic delivery of an enzyme to modify, for example, the genome of the plant.

Compared with existing protein delivery methodologies such as protein transduction or nanoparticle-mediated delivery (Chugh et al. 2009; Ravichandran 2009; Lu et al. 2010; Martin-Ortigosa et al. 2012a), proteolistics is a simple and versatile method. It does not require any modification of the delivery agent or of the cargo biomolecules to promote a covalent or electrostatic bond between the two prior to delivery (Wu et al. 2011a, b). Because it is a procedure that involves simple physical deposition of protein on microprojectiles, the method does not pose restrictions on protein type in terms of molecular weight, isoelectric point or tertiary structure. In this work, proteins ranging from 24 to 68 kDa in size and from 4.8 to 10.1 in isoelectric point were delivered (Suppl. Table 1). The protein delivery is immediate, and the delivered protein is likely dispersed in the intracellular space of the cell without having to be released from the delivery agents. However, there is no control over the timing or the amount of protein released using the proteolistic method described here. It is worth mentioning that the simple biomolecule/microprojectile preparation procedure can also be used to deliver DNA

molecules (Suppl. Fig. 6) and chemicals (Suppl. Fig. 5). Moreover, protein or DNA can also be dehydrated onto the gene gun cartridge and delivered into cells without using any microprojectiles (Fig. 5). Delivery of this type of "bioprojectile" is not efficient under the conditions tested, but could be useful in experiments where the presence of metal particles is not desired.

The advantage of the proteolistics method is its simplicity and versatility. It utilizes the widely used biolistic gun apparatus to deliver proteins to walled cells and tissues. A variety of proteins and enzymes were tested and delivered successfully into plant and mammalian tissues. It is likely that the delivery frequency will differ depending on different protein types as well as the target tissue and cell types. Future work should include further optimization of both sample preparation and bombardment procedures. It is anticipated that this methodology will form a part of the toolbox of biologists for basic biological research as well as agricultural and biomedical applications.

References

Bothwell JHF, Brownlee C, Hetherington AM, Ng CKY, Wheeler GL, McAinsh MR (2006) Biolistic delivery of Ca^{2+} dyes into plant and algal cells. Plant J 46:327–335

Chen D, Weis KF, Chu Q, Erickson C, Endres R, Lively CR, Osorio J, Payne LG (2001) Epidermal powder immunization induces both cytotoxic T-lymphocyte and antibody responses to protein antigens of influenza and hepatitis B viruses. J Virol 75:11630–11640

Chugh A, Amundsen E, Eudes F (2009) Translocation of cell-penetrating peptides and delivery of their cargoes in triticale microspores. Plant Cell Rep 28:801–810

Davis RE, Parra A, LoVerde PT, Ribeiro E, Glorioso G, Hodgson S (1999) Transient expression of DNA and RNA in parasitic helminths by using particle bombardment. Proc Natl Acad Sci USA 96:8687–8692

Eggenberger K, Birtalan E, Schröder T, Bräse S, Nick P (2009) Passage of Trojan peptoids into plant cells. ChemBioChem 10:2504–2512

Frame BR, Zhang HY, Cocciolone SM, Sidorenko LV, Dietrich CR, Pegg SE, Zhen SF, Schnable PS, Wang K (2000) Production of transgenic maize from bombarded type II callus: effect of gold particle size and callus morphology on transformation efficiency. In Vitro Cell Dev Biol Plant 36:21–29

Gan W-B, Grutzendler J, Wong WT, Wong ROL, Lichtman JW (2000) Multicolor "Diolistic" labeling of the nervous system using lipophilic dye combinations. Neuron 27:219–225

Gu Z, Biswas A, Zhao M, Tang Y (2011) Tailoring nanocarriers for intracellular protein delivery. Chem Soc Rev 40:3638–3655

Jefferson R (1987) Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol Biol Rep 5:387–405

Lu S-W, Hu J-W, Liu BR, Lee C-Y, Li J-F, Chou J-C, Lee H-J (2010) Arginine-rich intracellular delivery peptides synchronously deliver covalently and noncovalently linked proteins into plant cells. J Agric Food Chem 58:2288–2294

Luke Mankin S, Thompson WF (2001) New green fluorescent protein genes for plant transformation: intron-containing, ER-localized, and soluble-modified. Plant Mol Biol Rep 19:13–26

Martin-Ortigosa S, Valenstein JS, Lin VSY, Trewyn BG, Wang K (2012a) Gold functionalized mesoporous silica nanoparticle mediated protein and DNA codelivery to plant cells via the biolistic method. Adv Funct Mater 22:3576–3582

Martin-Ortigosa S, Valenstein JS, Sun W, Moeller L, Fang N, Trewyn BG, Lin VSY, Wang K (2012b) Parameters affecting the efficient delivery of mesoporous silica nanoparticle materials and gold nanorods into plant tissues by the biolistic method. Small 8:413–422

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473–497

Noguchi H, Matsushita M, Kobayashi N, Levy MF, Matsumoto S (2010) Recent advances in protein transduction technology. Cell Transplant 19:649–654

O'Brien JA, Lummis SC (2007) Diolistics: incorporating fluorescent dyes into biological samples using a gene gun. Trends Biotechnol 25:530–534

Ravichandran R (2009) Nanotechnology-based drug delivery systems. NanoBioTechnology 5:17–33

Staiger CJ, Yuan M, Valenta R, Shaw PJ, Warn RM, Lloyd CW (1994) Microinjected profilin affects cytoplasmic streaming in plant cells by rapidly depolymerizing actin microfilaments. Curr Biol 4:215–219

Tang X, Pikal M (2004) Design of freeze-drying processes for pharmaceuticals: practical advice. Pharm Res 21:191–200

Ward WW, Bokman SH (1982) Reversible denaturation of *Aequorea* green-fluorescent protein: physical separation and characterization of the renatured protein. Biochemistry 21:4535–4540

Widholm JM (1972) The use of fluorescein diacetate and phenosafranine for determining viability of cultured plant cells. Biotech Histochem 47:189–194

Wu J, Du H, Liao X, Zhao Y, Li L, Yang L (2011a) An improved particle bombardment for the generation of transgenic plants by direct immobilization of relleasable Tn5 transposases onto gold particles. Plant Mol Biol 77:117–127

Wu J, Du H, Liao X, Zhao Y, Li L, Yang L (2011b) Tn5 transposase-assisted transformation of indica rice. Plant J 68:186–200

Wymer CL, Fernández-Ábalos JM, Doonan JH (2001) Microinjection reveals cell-to-cell movement of green fluorescent protein in cells of maize coleoptiles. Planta 212:692–695