# Biolistic approach for transient gene expression studies in plants

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**Abstract**

Since its inception in the late 1980s, the delivery of exogenous nucleic acids into living cells via high-velocity micro-projectiles (biolistic, or micro-particle bombardment) has been an invaluable tool for both agricultural and fundamental plant research. Here, we review the technical aspects and the major applications of the biolistic method for studies involving transient gene expression in plant cells. These studies cover multiple areas of plant research, including gene expression, protein subcellular localization and cell-to-cell movement, plant virology, silencing and the more recently developed targeted genome editing via transient expression of customized endonuclease.

**Key words**

Biolistic, micro-particle bombardment, microbombardment, nucleic acid delivery, transient gene expression

## 1 Introduction

The first demonstration of the use of high-velocity micro-projectiles to deliver exogenous nucleic acids into living cells, detected via their transient expression in plant cells, was made in the late 1980s in the laboratory of J.C. Sanford at the Cornell University (*1*). Because this process involves the bombardment of nucleic acid-coated metal micro-particles, it may be considered as biological ballistics and was later termed "biolistic" (*2*); this method is also known as micro-particle bombardment, or simply microbombardment. Since then, the biolistic approach has proven useful for production of transgenic plants via stable transformation followed by selection and regeneration of transformed cells as well as for a wide variety of studies involving transient expression of a gene of interest (*3, 4*). In addition to plants, microbombardment has been used to transfer DNA into many other eukaryotic and prokaryotic organisms, such as bacteria (*5*), algae (*6*), fungi (*7*), and animals (*8, 9*). However, biolistics presents a particular interest for delivery of genes into and their transient expression in intact plant cells, where the cell wall forms an obstacle to other pathways for introduction of transgenes. Indeed, the strong plant cell wall, composed mostly of cellulose, must be either removed (by cell wall digestion enzymes, such as the ones used to generate protoplasts), or physically pierced to allow delivery of foreign nucleic acid. The bombardment of plant cells with high-speed micro-particle coated with nucleic acid molecules allows the penetration of the plant cell wall and delivery of the nucleic acids. In this article, we review the advantages and restrictions of the biolistic methods for transient gene expression studies as well as examples of their major utilizations that demonstrate the importance of this technique in many areas of plant research, from transcriptional regulation of gene expression to targeted genome editing.

## 2 Advantages and limitations of transient expression mediated by biolistics

Three main methods are utilized for delivery of gene constructs and subsequent transient expression of the delivered genes in plant cells: *Agrobacterium*-mediated DNA transfer, for example via infiltration of *Agrobacterium tumefaciens* cell suspension (agroinfiltration) into leaf tissues (*10*) or via inoculation of root segments (*11*), polyethylene glycol (PEG)-mediated transformation of protoplasts (*12*), and biolistics. These three approaches have advantages and constraints that make them more or less suitable for specific applications. The main features of each of these methods are summarized in Table 1. Although a few other methods have been used to transfer nucleic acids into plant cells, such as electroporation, microinjection or silicon carbide fibers, they have not gained popularity, and usually result in significantly lower DNA transfer frequency (*3*).

### 2.1 Range of species and tissue targets

Unlike *Agrobacterium*-mediated and protoplast transformation, which are restricted in their range of host species and tissues, biolistic bombardment can be used with a wider variety of plant species and target cells or tissues. Indeed, using the biolistic approach, nucleic acids are delivered by a mechanical process without the need for compatibility between the host plant and a biological agent or vector, thus presenting virtually no host range limitation and usually no restriction to specific tissues or cell types. In the first report of gene transfer to plant cells via micro-particle bombardment, transient expression of the reporter gene was observed in epidermal tissue of onion scales (*1*); soon after this original study, the technique was used with mature organs or cell cultures of several different species, including such agronomically important crops as rice, wheat and soybean (*13*). In the following years, gene transfer via biolistics has been

successfully employed with species from all plant families and with most types of plant tissues and organs. A 1997 bibliographical review (*14*) noted that more than 80 different plant species were susceptible to biolistics, representing a wide array of families, including monocotyledonous plants and gymnosperms. Moreover, many types of cells have been targeted by biolistics, including callus, suspension cultured cells, reproductive organs (pollen, styles, petals), meristems, seedlings, embryos and mature organs (leaves, stems and roots) (*3, 14, 15*). The range of plant species and tissues that can be used as targets for biolistic transformation studies is still expanding today. For example, this method has been used with cell suspension cultures, leaf sections and somatic embryos of grapevine (*Vitis vinifera*) (*16*), with leaf sections of the aquatic plant *Egeria dens* (*17*), and with petal tissue of Antirrhinum (*Antirhinum majus*) flowers developing in vitro (*18*).

Whereas most plant tissues are amenable to biolistic gene transfer, tissues with strong cuticle, lignified cell wall or hairy surface resist particle penetration (*19*). Another limitation of the biolistic approach is the need to adapt particle bombardment protocols for each type of target tissue, which necessitates the adjustment of several critical variables, e.g., particle diameter, distance from the target material, helium pressure. Generally, these parameters must be configured to avoid the detrimental effect of micro-particle impact on fragile tissues or cultured cells. However, adapting these variables may also help target specific cell types; for example, when an intact plant organ or tissue is used, the transformed cells usually are located in the outer cell layers of the tissue. The cell layer expressing the transgene, therefore, is determined by the penetration power of the micro-particles, which depends on their size, density and velocity.

### 2.2 Target organelles

Biolistics allows transfer and expression of exogenous nucleic acids not only in the nucleus, but also in other organelles, particularly plastids. DNA transfer to the plastid genome was first achieved with the unicellular alga *Chlamydomonas reinhardtii* cells (*20*), and then with tobacco cell suspension (*21*). Subsequently, biolistic plastid transformation has become a widely used technique, mostly for generation of transplastomic plants after selection and regeneration of the cells that have integrated the foreign DNA into their plastid genomes (*22*). However, biolistics also has been used in transient gene expression assays, for example to test the functionality of different promoter constructs in chloroplasts and non-photosynthetic plastids (*23*). DNA transfer to the mitochondrial genome by micro-particle bombardment was also performed in *C. reinhardtii* (*24, 25*), and it is a widely used technology in *Saccharomyces cerevisiae* (*26, 27*). Although this method most probably is applicable to mitochondrial transformation in higher plants, it has not been reported so far.

### 2.3 Plant sample preparation

Biolistic bombardment experiments require virtually no preparation of the plant target tissue as opposed, for example, to the cell wall digestion step required for the generation of protoplasts before PEG-mediated transformation. When the objective is to obtain stably transformed transgenic plants, the choice of cell types and growth conditions suitable for the selection and regeneration of the transformed cells is crucial and limits the number of tissues and experimental conditions that can be used. Such restrictions, however, do not exist for transient expression studies. For example, intact leaves and other mature organs from many plant species may be used directly for micro-particle bombardment, allowing high throughput acquisition of data by observing transient gene expression of a large number of constructs or in different plant

material targets. Moreover, the ability to transform directly mature organs or whole plants is useful to assay gene expression in tissues of plants grown under different conditions or subjected to different treatments. However, depending on the device used for the bombardment, the need for plant sample preparation varies. Indeed, whereas the gene gun is semi-portable and can be used directly with various plant organs or even whole plants, with other types of devices, the sample has to be placed in a chamber of limited size and exposed to partial vacuum, which limits the choice of potential targets.

### 2.4 Vector preparation

The vector preparation for biolistics is simple and versatile: coating of micro-particles with nucleic acids is relatively rapid (less than one hour per sample). Most studies employing the biolistic approach employ circular plasmid DNA because purification of such plasmid from a bacterial culture is the simplest way to produce the amount of vector needed for a bombardment experiment. Moreover, several plasmids may be used in different combinations; different plasmids are simply mixed before precipitation onto the micro-particles, which often results in co-expression of these constructs in the transformed cells (*28*). Linear DNA is also a suitable vector for biolistic transformation; for example, a PCR product generated via a system that allows preparation of an expression cassette in a one-step PCR-based amplification has been used for biolistic gene transfer (*29*). Finally, RNA molecules have been used for biolistic delivery as well (*1*). Although, in practice, DNA constructs are generally preferred because DNA vectors are easier to produce and store, biolistic delivery of RNA has the advantage of achieving transient expression while eliminating a potential integration of the vector into the target cell genome. Furthermore, different functional types of RNA molecules can be introduced

biolistically into plant cells, for example, specific siRNA that elicits in targeted gene silencing (*30*).

### 2.5 Invasiveness

Inherently, all available methods for transient expression of transgenes are invasive to a certain extent. The damage caused to plant tissues or interference with host cellular processes may trigger stress responses, which may in turn alter the transgene expression or the activity of the expressed protein and potentially affect the outcome of functional studies.

In the case of agroinfiltration, plant biotic stress responses are activated. Indeed, transcriptional response of plant tissues to *A. tumefaciens* infection has been examined in several studies, all of which demonstrated the activation of a pathogen response shortly after inoculation (*31-36*). For example, a study of the response of *Nicotiana benthamiana* leaves to agroinfiltration showed that the expression of the *PR1* gene, a hallmark of pathogen response, as well as generation small RNAs involved in plant defense increased upon infiltration of the leaves with *A. tumefaciens* suspension (*37*). For PEG-mediated transformation, protoplasts are generated via enzymatic disruption of the cell wall, which results in dedifferentiation and extensive reprogramming of the cell. Indeed, protoplasting of the plant cell triggers important changes in chromatin state (*38, 39*), and the resulting transcriptional changes that resemble stress response (*40, 41*).

It is likely that the wounding of plant cells or tissue occurring as a consequence of micro-particle bombardment also triggers a stress response in the target cell. The response of plant tissues to wounding, which shares common features with pathogen response, is well documented (*42, 43*), but we are not aware of any study about the specific response of plant tissue to micro-

particle bombardment, except for the effect on cell viability described in the next section. However, the impact of biolistics on the target tissue may be less dramatic than of the more protracted exposure of plant tissues to *A. tumefaciens* infection or to enzymatic treatment during protoplast generation.

### 2.6 Early events following micro-particle bombardment

The early events that follow micro-particle bombardment are important for the process of transient expression of the transgene, and they have been investigated in several studies (*19, 44*). In more than 90 % of the cells that transiently expressed a reporter gene, a particle was detected inside the cell nucleus (*19, 45*). Obviously, these studies were performed with constructs designed for expression in the nucleus of target cell, and it is very likely that with constructs designed for expression in plastids or mitochondria, the particle would have been found in the corresponding compartment of the expressing cells. The subcellular compartment to which the vector is delivered may determine for the fate of the transferred DNA. DNA molecules do not diffuse freely within the cell cytoplasm; for example, imaging fluorescently labeled DNA introduced into plant cells revealed that the mobility of DNA molecules larger than 1.5 kb in the cytoplasm and their entry into the nucleus was severely restricted (*46*). The presence of micro-particles in the nucleus of a majority of successfully transformed cells suggests that, with the biolistic approach, the introduced DNA is delivered directly into the nucleus and nuclear import is not required. This represents an important advantage over other techniques for gene delivery, such as *Agrobacterium*-mediated DNA transfer, where nuclear import of DNA represents a critical prerequisite for both transient expression and integration, and it relies on interactions of the transferred DNA with numerous bacterial and cellular factors (*47*).

As a result of the penetration of micro-particles, all cells that show transient expression of the transgene also display a callose plug in their cell wall, which indicates activation of a cellular reaction to wounding that developed within the minutes after bombardment (*19*). Moreover, a large majority of cells that have received a micro-particle in their nucleus died within 48 hours after bombardment, with the percentage of dead cells increasing over this period of time. The events leading to this cell death are not completely understood, but they likely result from the stress triggered by the intrusion of the micro-particle into the major cellular organelle. This process potentially affects the transient expression of the transgene (and the activity of the expressed protein and may also explain the relatively low frequency of cells expressing transgene after bombardment, e.g., 0.1-0.3 % in suspension cell cultures (*44*). However, transient expression likely occurs rapidly after bombardment, and the observation of transgene expression products, usually performed less than 24 hours after bombardment, allows meaningful and reproducible functional studies.

**2.7 Time-frame of biolistic experiments**

The total time of required for transient expression experiments in plant tissues via biolistics is relatively shorter than the agroinfiltration and protoplast transformation methods. Transient expression following biolistic delivery can be observed 24 hours after the vector is ready. In the case of *Agrobacterium* inoculation, a bacterial culture must be started 24 hours before inoculation, and observation of transient expression is performed 2 to 4 days after inoculation, in addition to the 2 days required for the transformation of the *Agrobacterium* cells with the DNA construct.

## 3 Examples of biolistic-mediated transient expression studies

In this section, we will review some of the major uses of biolistics for transient expression studies in plant cells and tissues and highlight the potential for innovative utilization of this method. The most common types of employment of the biolistic approach for transient expression in plants are illustrated in Fig. 1.

### 3.1 Transcriptional regulation and promoter activity

The biolistic delivery of various constructs carrying genes encoding reporter proteins, such as AFP (autofluorescent proteins), GUS (ß-glucuronidase), or luciferase, represents an important tool for rapid evaluation of different genetic elements that control gene expression. Transcriptional activity is affected by different regulatory elements and/or gene sequences (e.g., different promoters, codon usage, presence of introns), by co-expressed effector proteins, or by their cell/tissue specificity. For example, when the activity of different native and mutated promoters was measured in strawberry fruit (*48*), two negative regulatory-elements were identified in the promoter of *ZmHyPRP*, a gene marker of embryo development, via transient expression in immature embryo (*49*). Or, transient expression was used to analyze DNA sequences controlling the expression of the rice *OSCDPK2* gene (*50*) and to define organ-specific elements in the *RBCS2* promoter important for expression in tomato fruit (*51*). The biolistic approach was employed to investigate the functionality of plastid-specific promoters (*23*). The effects of the presence of an intron on transcription levels were also investigated in embryogenic maize and bluegrass cell suspension cultures using transient biolistic transformation (*52*). Also, micro-particle bombardment has been used for investigating the activity of transcription factors, for example, those that affect the induction of anthocyanin

synthesis in maize aleurone and embryogenic callus cells (*53, 54*). Finally, combined hormonal treatments and micro-particle bombardment allowed the study of gene control by hormonal factors in barley (*55*). Overall, the biolistic gene delivery represents a powerful tool for studies of gene expression, and it is also frequently used to validate the expression ability of a transgene construct before using it for stable transformation.

### 3.2 Protein subcellular localization

Tagging of proteins with AFPs, e.g., GFP and its derivatives, has evolved as the method of choice for protein visualization and analyses of subcellular targeting in living cells. In plant research, biolistics represents an efficient method for rapid expression and detection of the AFP-tagged proteins of interest. For example, it has been used as a high throughput method for the study of Arabidopsis glycosyl transferase family proteins (*56*). It was also employed to observe nucleocytoplasmic traffic of proteins in onion leaf cells (*57*), or mitochondria targeting in Arabidopsis (*58*). Expression in onion epidermal cells is often used as a convenient system to study nuclear targeting of proteins from different plants (*59*), and even for localization within different subnuclear compartments (*60*). The versatility of the biolistic approach makes it suitable for use with non-model crop plants, such as *Citrus* species (*61*).

The ability to co-express several constructs by mixing different vectors during micro-particle coating allows the use of biolistics for co-localization of different AFP-tagged proteins. For example, in rice, this method was used to observe co-localization of several proteins with organelles markers (*62*). In another application, protein-protein interactions are visualized by bi-molecular fluorescence complementation (BiFC) experiments, which allows simultaneous detection of interaction and subcellular localization of the interacting proteins in living cells. For

example, interaction was visualized between two transcription factors in Arabidopsis (*63*), or between the Agrobacterium exported F-box protein effector VirF and ASK1, the plant component of the SCF complex E3 ligase, in *N. benthamiana* leaf cells (*64*).

### 3.3 Cell-to-cell protein movement

In most plant tissues, some macromolecules can move symplastically through intercellular connections termed plasmodesmata (*65*). Visualization of this movement in vivo is crucial for functional studies of regulation of intercellular transport of macromolecules in general and of cell-to-cell spread of plant viruses in particular. For example, in one of the first studies using biolistics to investigate protein cell-to-cell movement, published in 1997, the spreading of the cucumber mosaic virus movement protein fused to GFP was demonstrated in tobacco leaf epidermis (*66*). Conceptually, these experiments rely on observing transient expression of AFP-tagged protein of interest. If the labeled protein can move through plasmodesmata, a cluster of AFP-containing cells will be visible after the tagged protein have trafficked to these cells from a single cell, expressing the biolistically delivered construct. Recording the number of cells per cluster at different time points after microbombardment allows quantification of protein movement (*67*). This protocol has been employed in many studies to characterize movement of endogenous as well as viral proteins. For example, the movement protein (MP) of the Rice stripe virus was identified and its role elucidated using this approach (*68*). Besides monitoring movement of viral proteins, the biolistic approach was used to characterize the plasmodesmata themselves, for example, to determine their size exclusion limit using expression of GFP-fusion proteins of different sizes (*69*).

### 3.4 Virus inoculation

Introduction of complete virus genomes or their fragments into plant cells has been achieved using biolistics, with initial studies employing the zucchini yellow mosaic potyvirus and *Cucurbita pepo* (squash) plants (*70, 71*). In this application, particles are coated with cDNA, PCR products, or whole virions from a virus preparation or from sap of infected plants. Biolistics represents a simple approach for delivery of DNA as well as RNA viral genomes into plants, circumventing more laborious techniques, such as insect vectors. Microbombardment has been used, for instance, to assess the infectivity of different virus isolates (*72, 73*) as well as the resistance of different plant varieties or lines. Using biolistic delivery of modified cDNAs of the cucumber mosaic virus, the requirements for the cell-to-cell movement of this virus were investigated (*74*). In fact, biolistic delivery of viral nucleic acids represents a highly efficient system for introduction of infectious viral genomes into plant cells, becoming an essential tool in plant virology experimentation.

### 3.5 RNA silencing induction

Virus induced gene silencing (VIGS) takes advantage of an antiviral defense mechanism of plants mediated by RNA interference (RNAi). When a plant is infected with a wild-type virus, the viral genome is specifically targeted by this defense mechanism. Using this natural mechanism, a viral vector engineered to carry sequences derived from a host gene can trigger silencing of the endogenous copy of this host gene; this process is termed VIGS. Once such a vector is introduced into a plant cell, the virus will spread systematically throughout the entire plant and the target mRNA will be degraded by the host plant RNA silencing machinery (*75*). The inoculation with the VIGS vector can be done via agroinfiltration (*76*), but biolistics is

frequently used for this purpose with plant species that are not susceptible to Agrobacterium. For example, micro-particle bombardment has been used to inoculate several *Rosaceae* (apple and pear) fruit trees with an apple latent spherical virus-based VIGS vector (*77*), soybean with a bean pod mottle virus-based VIGS vector (*78*), and cassava with an east African cassava mosaic virus-based VIGS vector (*79*). In addition, biolistics was also used to induce gene silencing by transient expression of non-viral constructs carrying an inverted repeat of the target gene (*18*); in that case, the silencing signal spreads locally in the tissue but not systemically, in the whole plant.

### 3.6 Targeted genome editing

Recently, several methods have been developed for targeted genome editing, using customized endonucleases, such as transcription activator-like effector nuclease (TALEN) or zinc finger nuclease, or RNA-guided endonucleases, such as the CRISPR-Cas9 system. Although the ultimate goal of these methods is the production of stably transformed genetically modified organisms, the modification itself is mediated by transient expression of the endonuclease selected to edit the host genome. Because it is generally preferable to obtain genetically modified organisms without any additional, extraneous sequences, a major aspect of these methods is to avoid any integration of the vectors needed to effect the change in the genome. To this end, biolistics was used to transiently express TALEN in tobacco and barley (*80*). Similarly, different elements of the CRISPR-Cas9 system were introduced biolistically into the plant cells to effect precise genome editing. In one genome editing study, a CRISPR-Cas9 construct was introduced as either DNA or RNA and transiently expressed in wheat callus (*81*) whereas another study utilized biolistic delivery of pre-assembled Cas9-gRNA ribonucleo-proteins into the maize

embryo (*82*). In both cases, the mutated plants were subsequently regenerated from the modified cells.

## 4 Discussion and Conclusion

Transient expression of biolistically delivered gene constructs has been employed for a wide array of purposes, representing an important tool in plant research and biotechnology. Besides the classical uses illustrated in this review, several new trends for future applications of biolistics may be of interest. In its broad meaning, transient expression may be interpreted as expression or introduction of biological effectors into the target cell on a temporary basis when long-term persistence of the introduced molecules into the host cell is not required or desired. First, proteins may be introduced in plant cell by microbombardment directly, without the use of their encoding genes. For example, a technique designated "proteolistics" was proposed for biolistic delivery of effector proteins into the host cell (*83, 84*). Different types of RNA-based macromolecules, such as RNA nucleoprotein-complexes, miRNAs or siRNAs can also be introduced in plant cell by particle bombardment. Second, genome editing by transient expression of different editing systems (see section 3.6) will likely be developed. Third, the "next generation" nano-biolistic systems will be developed to employ particles in the nanometer range as opposed to the current methods using particles that range from 0.5 to 2 micrometer in diameter, which likely will result in markedly reduced damage to the target cells (*85, 86*). Indeed, as discussed in section 2.6, mechanical damage caused by entry of a microparticle in the target cell represents a major flaw of the biolistic approach, hampering transient expression efficiency and affecting the results of the functional studies. The use of nanoparticles may

increase the number of cell expressing transgenes and the efficiency of expression, while reducing the effects of the stress caused by bombardment.

Owing to its versatility and applicability to a wide range of target plant species or cell or tissue types, the biolistic approach will remain a major tool for studies involving transient expression in plant cells. Moreover, improvements and novel applications of this technology will certainly continue to be implemented.

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## References

1. Klein, T. M., Wolf, E. D., Wu, R., and Sanford, J. C. (1987) High-velocity microprojectiles for delivering nucleic-acids into living cells. *Nature* **327,** 70-73.
2. Sanford, J. C. (1988) The biolistic process. *Trends Biotechnol.* **6,** 299-302.
3. Taylor, N. J., and Fauquet, C. M. (2002) Microparticle bombardment as a tool in plant science and agricultural biotechnology. *DNA Cell Biol.* **21,** 963-977.
4. Ueki, S., Lacroix, B., Krichevsky, A., Lazarowitz, S. G., and Citovsky, V. (2009) Functional transient genetic transformation of *Arabidopsis* leaves by biolistic bombardment. *Nat. Protoc.* **4,** 71-77.
5. Smith, F. D., Harpending, P. R., and Sanford, J. C. (1992) Biolistic transformation of prokaryotes: factors that affect biolistic transformation of very small cells. *J. Gen. Microbiol.* **138,** 239-248.
6. Mayfield, S. P., and Kindle, K. L. (1990) Stable nuclear transformation of *Chlamydomonas reinhardtii* by using a *C. reinhardtii* gene as the selectable marker. *Proc. Natl. Acad. Sci. USA* **87,** 2087-2091.
7. Armaleo, D., Ye, G. N., Klein, T. M., Shark, K. B., Sanford, J. C., and Johnston, S. A. (1990) Biolistic nuclear transformation of *Saccharomyces cerevisiae* and other fungi. *Curr. Genet.* **17,** 97-103.
8. Cheng, L., Ziegelhoffer, P. R., and Yang, N. S. (1993) *In vivo* promoter activity and transgene expression in mammalian somatic tissues evaluated by using particle bombardment. *Proc. Natl. Acad. Sci. USA* **90,** 4455-4459.
9. Yang, N. S., and Sun, W. H. (1995) Gene gun and other non-viral approaches for cancer gene therapy. *Nat. Med.* **1,** 481-483.

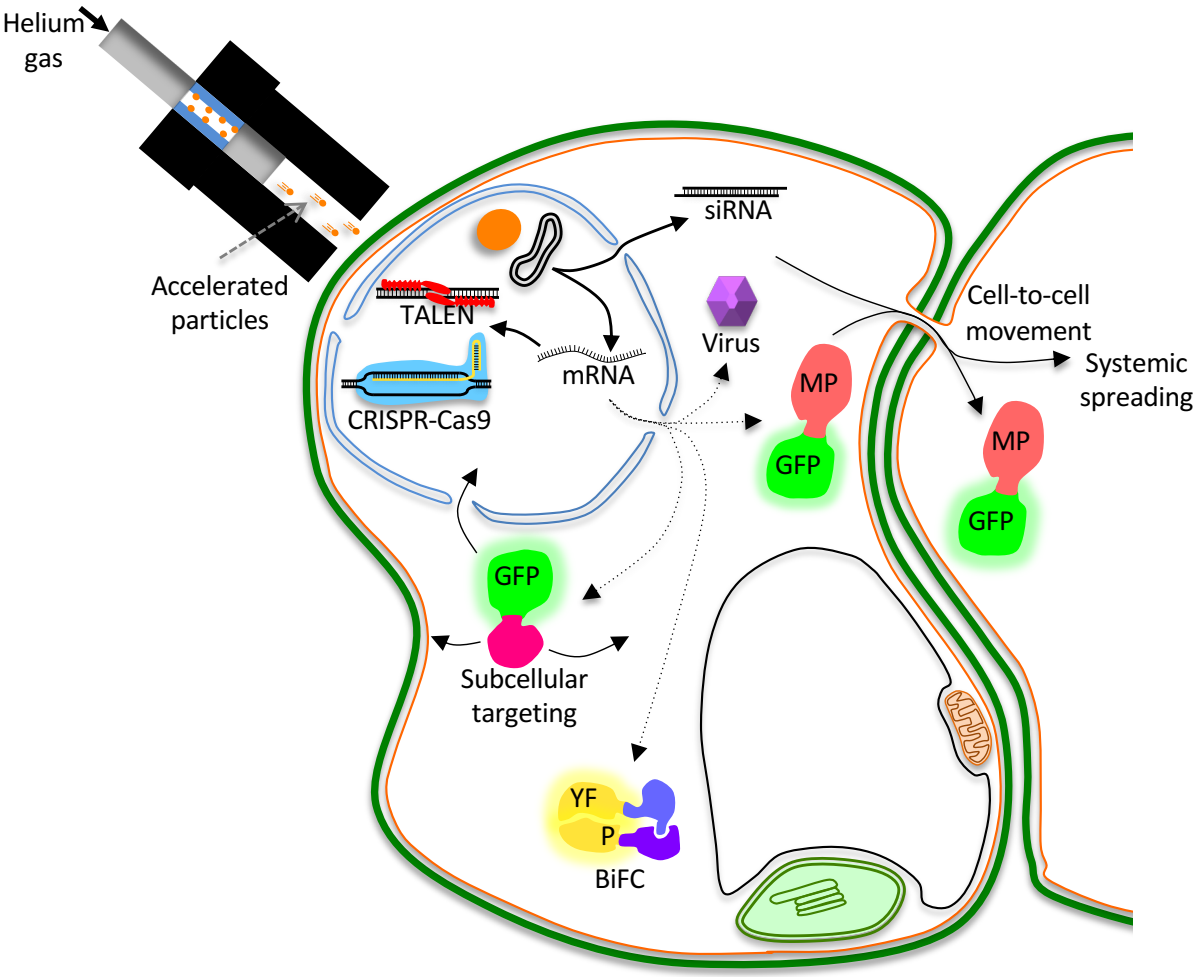
10. Kapila, J., De Rycke, R., Van Montagu, M., and Angenon, G. (1997) An *Agrobacterium*-mediated transient gene expression system for intact leaves. *Plant Sci.* **122,** 101-108.
11. Nam, J., Matthysse, A. G., and Gelvin, S. B. (1997) Differences in susceptibility of *Arabidopsis* ecotypes to crown gall disease may result from a deficiency in T-DNA integration. *Plant Cell* **9,** 317-333.
12. Junker, B., Zimny, J., Luhrs, R., and Lorz, H. (1987) Transient expression of chimaeric genes in dividing and non-dividing cereal protoplasts after PEG-induced DNA uptake. *Plant Cell Rep.* **6,** 329-332.
13. Wang, Y. C., Klein, T. M., Fromm, M., Cao, J., Sanford, J. C., and Wu, R. (1988) Transient expression of foreign genes in rice, wheat and soybean cells following particle bombardment. *Plant Mol. Biol.* **11,** 433-439.
14. Luthra, R., Varsha, Dubey, R. K., Srivastava, A. K., and Kumar, S. (1997) Microprojectile mediated plant transformation: a bibliographic search. *Euphytica* **95,** 269-294.
15. Finer, J. J., Finer, K. R., and Ponappa, T. (1999) Particle bombardment mediated transformation. *Curr. Top. Microbiol. Immunol.* **240,** 59-80.
16. Jelly, N. S., Valat, L., Walter, B., and Maillot, P. (2014) Transient expression assays in grapevine: a step towards genetic improvement. *Plant Biotechnol. J.* **12,** 1231-1245.
17. Osaki, Y., and Kodama, Y. (2017) Particle bombardment and subcellular protein localization analysis in the aquatic plant *Egeria densa*. *PeerJ* **5,** e3779.
18. Shang, Y., Schwinn, K. E., Bennett, M. J., Hunter, D. A., Waugh, T. L., Pathirana, N. N., Brummell, D. A., Jameson, P. E., and Davies, K. M. (2007) Methods for transient assay of gene function in floral tissues. *Plant Methods* **3,** 1.
19. Hunold, R., Bronner, R., and Hahne, G. (1994) Early events in microprojectile bombardment: cell viability and particle location. *Plant J.* **5,** 593-604.
20. Boynton, J. E., Gillham, N. W., Harris, E. H., Hosler, J. P., Johnson, A. M., Jones, A. R., Randolphanderson, B. L., Robertson, D., Klein, T. M., Shark, K. B., and Sanford, J. C. (1988) Chloroplast transformation in *Chlamydomonas* with high-velocity microprojectiles. *Science* **240,** 1534-1538.
21. Daniell, H., Vivekananda, J., Nielsen, B. L., Ye, G. N., Tewari, K. K., and Sanford, J. C. (1990) Transient foreign gene expression in chloroplasts of cultured tobacco cells after biolistic delivery of chloroplast vectors. *Proc. Natl. Acad. Sci. USA* **87,** 88-92.
22. Maliga, P. (2004) Plastid transformation in higher plants. *Annu. Rev. Plant Biol.* **55,** 289-313.
23. Hibberd, J. M., Linley, P. J., Khan, M. S., and Gray, J. C. (1998) Transient expression of green fluorescent protein in various plastid types following microprojectile bombardment. *Plant J.* **16,** 627-632.
24. Randolph-Anderson, B. L., Boynton, J. E., Gillham, N. W., Harris, E. H., Johnson, A. M., Dorthu, M. P., and Matagne, R. F. (1993) Further characterization of the respiratory deficient *dum-1* mutation of *Chlamydomonas reinhardtii* and its use as a recipient for mitochondrial transformation. *Mol. Gen. Genet.* **236,** 235-244.
25. Larosa, V., Coosemans, N., Motte, P., Bonnefoy, N., and Remacle, C. (2012) Reconstruction of a human mitochondrial complex I mutation in the unicellular green alga *Chlamydomonas*. *Plant J.* **70,** 759-768.

26. Johnston, S. A., Anziano, P. Q., Shark, K., Sanford, J. C., and Butow, R. A. (1988) Mitochondrial transformation in yeast by bombardment with microprojectiles. *Science* **240,** 1538-1541.
27. Montanari, A., Francisci, S., Fazzi D'Orsi, M., and Bianchi, M. M. (2014) Strain-specific nuclear genetic background differentially affects mitochondria-related phenotypes in *Saccharomyces cerevisiae*. *MicrobiologyOpen* **3,** 288-298.
28. Hadi, M. Z., McMullen, M. D., and Finer, J. J. (1996) Transformation of 12 different plasmids into soybean *via* particle bombardment. *Plant Cell Rep.* **15,** 500-505.
29. Lu, Y., Chen, X., Wu, Y., Wang, Y., He, Y., and Wu, Y. (2013) Directly transforming PCR-amplified DNA fragments into plant cells is a versatile system that facilitates the transient expression assay. *PLOS ONE* **8,** e57171.
30. Dalakouras, A., Wassenegger, M., McMillan, J. N., Cardoza, V., Maegele, I., Dadami, E., Runne, M., Krczal, G., and Wassenegger, M. (2016) Induction of silencing in plants by high-pressure spraying of *in vitro*-synthesized small RNAs. *Front. Plant Sci.* **7,** 1327.
31. Ditt, R. F., Nester, E. W., and Comai, L. (2001) Plant gene expression response to *Agrobacterium tumefaciens*. *Proc. Natl. Acad. Sci. USA* **98,** 10954-10959.
32. Ditt, R. F., Nester, E., and Comai, L. (2005) The plant cell defense and *Agrobacterium tumefaciens*. *FEMS Microbiol. Lett.* **247,** 207-213.
33. Ditt, R. F., Kerr, K. F., de Figueiredo, P., Delrow, J., Comai, L., and Nester, E. W. (2006) The *Arabidopsis thaliana* transcriptome in response to *Agrobacterium tumefaciens*. *Mol. Plant-Microbe Interact.* **19,** 665-681.
34. Lee, C. W., Efetova, M., Engelmann, J. C., Kramell, R., Wasternack, C., Ludwig-Müller, J., Hedrich, R., and Deeken, R. (2009) *Agrobacterium tumefaciens* promotes tumor induction by modulating pathogen defense in *Arabidopsis thaliana*. *Plant Cell* **21,** 2948-2962.
35. Gohlke, J., and Deeken, R. (2014) Plant responses to *Agrobacterium tumefaciens* and crown gall development. *Front. Plant Sci.* **5,** 155.
36. Duan, K., Willig, C., de Tar, J. R., Spollen, W. G., and Zhang, Z. (2018) Transcriptomic analysis of *Arabidopsis* seedlings in response to *Agrobacterium*-mediated transformation process. *Mol. Plant-Microbe Interact.* **31,** 445-459.
37. Pruss, G. J., Nester, E. W., and Vance, V. (2008) Infiltration with *Agrobacterium tumefaciens* induces host defense and development-dependent responses in the infiltrated zone. *Mol. Plant-Microbe Interact.* **21,** 1528-1538.
38. Zhao, J., Morozova, N., Williams, L., Libs, L., Avivi, Y., and Grafi, G. (2001) Two phases of chromatin decondensation during dedifferentiation of plant cells: distinction between competence for cell fate switch and a commitment for S phase. *J. Biol. Chem.* **276,** 22772-22778.
39. Tessadori, F., Chupeau, M. C., Chupeau, Y., Knip, M., Germann, S., van Driel, R., Fransz, P., and Gaudin, V. (2007) Large-scale dissociation and sequential reassembly of pericentric heterochromatin in dedifferentiated *Arabidopsis* cells. *J. Cell Sci.* **120,** 1200-1208.
40. Grafi, G., Chalifa-Caspi, V., Nagar, T., Plaschkes, I., Barak, S., and Ransbotyn, V. (2011) Plant response to stress meets dedifferentiation. *Planta* **233,** 433-438.
41. Chupeau, M. C., Granier, F., Pichon, O., Renou, J. P., Gaudin, V., and Chupeau, Y. (2013) Characterization of the early events leading to totipotency in an *Arabidopsis* protoplast liquid culture by temporal transcript profiling. *Plant Cell* **25,** 2444-2463.

42. León, J., Rojo, E., and Sánchez-Serrano, J. J. (2001) Wound signaling in plants. *J. Exp. Bot.* **52,** 1-9.
43. Reymond, P., Weber, H., Damond, M., and Farmer, E. E. (2000) Differential gene expression in response to mechanical wounding and insect feeding in *Arabidopsis*. *Plant Cell* **12,** 707-720.
44. Iida, A., Yamashita, T., Yamada, Y., and Morikawa, H. (1991) Efficiency of particle-bombardment-mediated transformation is influenced by cell-cycle stage in synchronized cultured-cells of tobacco. *Plant Physiol.* **97,** 1585-1587.
45. Yamashita, T., Iida, A., and Morikawa, H. (1991) Evidence that more than 90-percent of beta-glucuronidase-expressing cells after particle bombardment directly receive the foreign gene in their nucleus. *Plant Physiol.* **97,** 829-831.
46. Gisel, A., Rothen, B., Iglesias, V. A., Potrykus, I., and Sautter, C. (1998) *In situ* monitoring of DNA: the plant nuclear envelope allows passage of short DNA fragments. *Plant J.* **16,** 621-626.
47. Lacroix, B., and Citovsky, V. (2013) The roles of bacterial and host plant factors in *Agrobacterium*-mediated genetic transformation. *Int. J. Dev. Biol.* **57,** 467-481.
48. Agius, F., Amaya, I., Botella, M. A., and Valpuesta, V. (2005) Functional analysis of homologous and heterologous promoters in strawberry fruits using transient expression. *J. Exp. Bot.* **56,** 37-46.
49. Jose-Estanyol, M., and Puigdomènech, P. (2012) Cellular localization of the embryo-specific hybrid PRP from *Zea mays*, and characterization of promoter regulatory elements of its gene. *Plant Mol. Biol.* **80,** 325-335.
50. Morello, L., Bardini, M., Cricri, M., Sala, F., and Breviario, D. (2006) Functional analysis of DNA sequences controlling the expression of the rice *OsCDPK2* gene. *Planta* **223,** 479-491.
51. Baum, K., Groning, B., and Meier, I. (1997) Improved ballistic transient transformation conditions for tomato fruit allow identification of organ-specific contributions of I-box and G-box to the *RBCS2* promoter activity. *Plant J.* **12,** 463-469.
52. Vain, P., Finer, K. R., Engler, D. E., Pratt, R. C., and Finer, J. J. (1996) Intron-mediated enhancement of gene expression in maize (*Zea mays* L.) and bluegrass (*Poa pratensis* L.). *Plant Cell Rep.* **15,** 489-494.
53. Goff, S. A., Klein, T. M., Roth, B. A., Fromm, M. E., Cone, K. C., Radicella, J. P., and Chandler, V. L. (1990) Transactivation of anthocyanin biosynthetic genes following transfer of *B* regulatory genes into maize tissues. *EMBO J.* **9,** 2517-2522.
54. Sainz, M. B., Goff, S. A., and Chandler, V. L. (1997) Extensive mutagenesis of a transcriptional activation domain identifies single hydrophobic and acidic amino acids important for activation *in vivo*. *Mol. Cell Biol.* **17,** 115-122.
55. Xie, Z., Zhang, Z. L., Hanzlik, S., Cook, E., and Shen, Q. J. (2007) Salicylic acid inhibits gibberellin-induced alpha-amylase expression and seed germination *via* a pathway involving an abscisic-acid-inducible *WRKY* gene. *Plant Mol. Biol.* **64,** 293-303.
56. Lao, J., Oikawa, A., Bromley, J. R., McInerney, P., Suttangkakul, A., Smith-Moritz, A. M., Plahar, H., Chiu, T. Y., González Fernández-Niño, S. M., Ebert, B., Yang, F., Christiansen, K. M., Hansen, S. F., Stonebloom, S., Adams, P. D., Ronald, P. C., Hillson, N. J., Hadi, M. Z., Vega-Sánchez, M. E., Loqué, D., Scheller, H. V., and Heazlewood, J. L. (2014) The plant glycosyltransferase clone collection for functional genomics. *Plant J.* **79,** 517-529.

57. Alinsug, M. V., Chen, F. F., Luo, M., Tai, R., Jiang, L. W., and Wu, K. Q. (2012) Subcellular localization of class II HDAs in *Arabidopsis thaliana*: nucleocytoplasmic shuttling of HDA15 is driven by light. *PLOS ONE* **7,** e30846.
58. Edmondson, A. C., Song, D. Q., Alvarez, L. A., Wall, M. K., Almond, D., McClellan, D. A., Maxwell, A., and Nielsen, B. L. (2005) Characterization of a mitochondrially targeted single-stranded DNA-binding protein in *Arabidopsis thaliana*. *Mol. Genet. Genomics* **273,** 115-122.
59. Kuijt, S. J., Lamers, G. E., Rueb, S., Scarpella, E., Ouwerkerk, P. B., Spaink, H. P., and Meijer, A. H. (2004) Different subcellular localization and trafficking properties of KNOX class 1 homeodomain proteins from rice. *Plant Mol. Biol.* **55,** 781-796.
60. Moriguchi, K., Suzuki, T., Ito, Y., Yamazaki, Y., Niwa, Y., and Kurata, N. (2005) Functional isolation of novel nuclear proteins showing a variety of subnuclear localizations. *Plant Cell* **17,** 389-403.
61. Levy, A., El-Mochtar, C., Wang, C., Goodin, M., and Orbovic, V. (2018) A new toolset for protein expression and subcellular localization studies in citrus and its application to *Citrus tristeza virus* proteins. *Plant Methods* **14,** 2.
62. Dangol, S., Singh, R., Chen, Y., and Jwa, N. S. (2017) Visualization of multicolored *in vivo* organelle markers for co-localization studies in *Oryza sativa*. *Mol. Cells* **40,** 828-836.
63. Hollender, C. A., and Liu, Z. (2010) Bimolecular fluorescence complementation (BiFC) assay for protein-protein interaction in onion cells using the Helios gene gun. *J. Vis. Exp.* **40,** e1963.
64. Lacroix, B., and Citovsky, V. (2015) Nopaline-type Ti plasmid of *Agrobacterium* encodes a VirF-like functional F-box protein. *Sci. Rep.* **5,** 16610.
65. Ueki, S., and Citovsky, V. (2011) To gate, or not to gate: regulatory mechanisms for intercellular protein transport and virus movement in plants. *Mol. Plant* **4,** 782-793.
66. Itaya, A., Hickman, H., Bao, Y., Nelson, R. S., and Ding, B. (1997) Cell-to-cell trafficking of cucumber mosaic virus movement protein:green fluorescent protein fusion produced by biolistic gene bombardment in tobacco. *Plant J.* **12,** 1223-1230.
67. Ueki, S., Meyers, B. L., Yasmin, F., and Citovsky, V. (2010) A cell-to-cell macromolecular transport assay *in planta* utilizing biolistic bombardment. *J. Vis. Exp.* **42,** e2208.
68. Xiong, R., Wu, J., Zhou, Y., and Zhou, X. (2008) Identification of a movement protein of the *Tenuivirus* rice stripe virus. *J. Virol.* **82,** 12304-12311.
69. Oparka, K. J., Roberts, A. G., Boevink, P., Santa Cruz, S., Roberts, I., Pradel, K. S., Imlau, A., Kotlizky, G., Sauer, N., and Epel, B. (1999) Simple, but not branched, plasmodesmata allow the nonspecific trafficking of proteins in developing tobacco leaves. *Cell* **97,** 743-754.
70. Gal-On, A., Meiri, E., Huet, H., Hua, W. J., Raccah, B., and Gaba, V. (1995) Particle bombardment drastically increases the infectivity of cloned DNA of zucchini yellow mosaic potyvirus. *J. Gen. Virol.* **76,** 3223-3227.
71. Gal-On, A., Meiri, E., Elman, C., Gray, D. J., and Gaba, V. (1997) Simple hand-held devices for the efficient infection of plants with viral-encoding constructs by particle bombardment. *J. Virol. Methods* **64,** 103-110.

72. Briddon, R. W., Liu, S., Pinner, M. S., and Markham, P. G. (1998) Infectivity of *African cassava mosaic virus* clones to cassava by biolistic inoculation. *Arch. Virol.* **143,** 2487-2492.
73. Brown, J. K., Ostrow, K. M., Idris, A. M., and Stenger, D. C. (1999) Biotic, molecular, and phylogenetic characterization of *Bean calico mosaic virus*, a distinct begomovirus species with affiliation in the squash leaf curl virus cluster. *Phytopathology* **89,** 273-280.
74. Canto, T., Prior, D. A., Hellwald, K. H., Oparka, K. J., and Palukaitis, P. (1997) Characterization of cucumber mosaic virus. IV. Movement protein and coat protein are both essential for cell-to-cell movement of cucumber mosaic virus. *Virology* **237,** 237-248.
75. Lange, M., Yellina, A. L., Orashakova, S., and Becker, A. (2013) Virus-induced gene silencing (VIGS) in plants: an overview of target species and the virus-derived vector systems. *Methods Mol. Biol.* **975,** 1-14.
76. Velásquez, A. C., Chakravarthy, S., and Martin, G. B. (2009) Virus-induced gene silencing (VIGS) in *Nicotiana benthamiana* and tomato. *J. Vis. Exp.* **28,** 1292.
77. Sasaki, S., Yamagishi, N., and Yoshikawa, N. (2011) Efficient virus-induced gene silencing in apple, pear and Japanese pear using *Apple latent spherical virus* vectors. *Plant Methods* **7,** 15.
78. Zhang, C., Yang, C., Whitham, S. A., and Hill, J. H. (2009) Development and use of an efficient DNA-based viral gene silencing vector for soybean. *Mol. Plant-Microbe Interact.* **22,** 123-131.
79. Beyene, G., Chauhan, R. D., and Taylor, N. J. (2017) A rapid virus-induced gene silencing (VIGS) method for assessing resistance and susceptibility to cassava mosaic disease. *Virol. J.* **14,** 47.
80. Budhagatapalli, N., Schedel, S., Gurushidze, M., Pencs, S., Hiekel, S., Rutten, T., Kusch, S., Morbitzer, R., Lahaye, T., Panstruga, R., Kumlehn, J., and Hensel, G. (2016) A simple test for the cleavage activity of customized endonucleases in plants. *Plant Methods* **12,** 18.
81. Zhang, Y., Liang, Z., Zong, Y., Wang, Y., Liu, J., Chen, K., Qiu, J. L., and Gao, C. (2016) Efficient and transgene-free genome editing in wheat through transient expression of CRISPR/Cas9 DNA or RNA. *Nat. Commun.* **7,** 12617.
82. Svitashev, S., Schwartz, C., Lenderts, B., Young, J. K., and Cigan, A. M. (2016) Genome editing in maize directed by CRISPR-Cas9 ribonucleoprotein complexes. *Nat. Commun.* **7,** 13274.
83. Martin-Ortigosa, S., Peterson, D. J., Valenstein, J. S., Lin, V. S., Trewyn, B. G., Lyznik, L. A., and Wang, K. (2014) Mesoporous silica nanoparticle-mediated intracellular cre protein delivery for maize genome editing *via loxP* site excision. *Plant Physiol.* **164,** 537-547.
84. Martin-Ortigosa, S., and Wang, K. (2014) Proteolistics: a biolistic method for intracellular delivery of proteins. *Transgenic Res.* **23,** 743-756.
85. O'Brien, J. A., and Lummis, S. C. (2011) Nano-biolistics: a method of biolistic transfection of cells and tissues using a gene gun with novel nanometer-sized projectiles. *BMC Biotechnol.* **11,** 66.
86. Joldersma, D., and Liu, Z. (2018) Plant genetics enters the nano age? *J. Integr. Plant Biol.*, in press.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Subcellular
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YF
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## Legends to Figures

**Figure 1**: Illustration of the main applications of biolistics for transient expression in plant cells (see text, section 3, for details).

**Table 1**: Comparison of the three major methods for transient expression of transgenes in plant cells (see text, section 2, for details).

| | **Biolistic bombardment** | **Agrobacterium infiltration (agroinfiltration)** | **PEG-mediated transformation (protoplast)** |
|---|---|---|---|
| Target species | Wide range | Limited | Limited |
| Target tissue | Versatile | Mostly mature leaves, roots | Tissue suitable for protoplast preparation |
| Target organelle | Nucleus, plastids | Nucleus | Nucleus, plastids |
| Plant sample preparation | Minimal | Minimal | Cell wall digestion (protoplasting) |
| Vector preparation | Coating of micro-particles with nucleic acid | Agrobacterium transformation with binary plasmid | None required (DNA is used directly) |
| Invasiveness | Plant tissue mostly intact; impact of micro-particles may trigger mechanical damage stress response | Plant tissue mostly intact; agroinfiltration triggers mechanical and biotic stress responses | Tissue disrupted, protoplasts devoid of cell wall. Protoplasts undergo extensive transcriptional and chromatin changes (dedifferentiation). |
| Detection timing | Observation 24 hours after the vector DNA is ready | Observation 6 days after the binary vector DNA is ready | Observation 24 to 48 hours after the vector DNA is ready |