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Lab Note

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Isolation of nuclei from transient transfection plants under abiotic stress

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The isolation of nuclei is often the first step to study the processes such as protein–DNA regulation and interactions. Therefore, rapidly obtaining nuclei from woody plants with relatively high purity and minimal contamination is highly desirable. Many methods have been mainly employed in animal tissues because the plant cell wall represents an obstacle for the extraction of nuclei. The knowledge of nuclei of plant is less advanced than that of animals. Nevertheless, with the currently available techniques for nuclear isolation from whole plants, satisfactory yield of intact nuclei cannot be achieved because nuclei and protoplasts are more fragile during liquid nitrogen grinding and they have delicate plasma membranes that are susceptible to bursting when subject to mechanical shock. Therefore, it is very important to develop new methods that have reduced damage to the nuclei.

Here, we describe an efficient scheme for the preparation of pure and intact nuclei using protoplasts as a system. The basic technique applies to a variety of protoplast types and is expected to facilitate the experiments in many plants. It is based on co-cultivation of total *Betula platyphylla*. Target gene is taken into *B. platyphylla* using *Agrobacterium* vector system. Transient gene expression system offers several advantages over stable expression. For instance, transient gene expression is simple and easy to perform by virtue of its time and labor efficiency [1]. The purpose of this protocol is not only to complete the stress but also to obtain high-quality nuclei and nuclear DNA yields.

The isolation and purification of nuclei from transient protoplasts, in principle, involves three steps: (i) transient transformation using whole plants, (ii) disruption of protoplasts in an appropriate buffer, and iii) separation of released nuclei from other organelles and fragmentation of nuclear DNA.

(1) Wild-type *B. platyphylla* were grown under standard environmental conditions with a photoperiod of 16 h light and 8 h dark and a mean temperature of 25°C. For all assays, 3- to 4-week-old plants were used in the experiments. Binary vectors, carrying the gene of interest, containing the green fluorescent protein (GFP) and the *nptII* genes within the T-DNA borders (e.g. *CDF3*) were cloned into pBI121. *Agrobacterium tumefaciens* strain EHA105 was used in all experiments. The whole *B. platyphylla* was infected with *A. tumefaciens* EHA105. The transient plants were co-cultivated on MS medium (PhytoTechnology Laboratories, New York, USA) with 200 mM salt for 3 days, and GFP expression was monitored daily with an imaging system (Fig. 1A).

(2) The *B. platyphylla* leaf tissues were cut into pieces (~0.1 cm × 0.5 cm). In a petri dish, ~1 g of fresh leaves was mixed with 10 ml enzyme solution [Cellulase onozuka R-10 2% (w/v), Macerozyme R-10 0.5% (w/v), $CaCl_2$ (10 mM), KCl (20 mM), Mannitol (0.6 M)]. After 2 h of incubation, the protoplast enzyme mixture was filtered through miracloth to remove cell debris. The protoplast was washed by re-suspensing and centrifuging in 2 ml of washing buffer [2 mM MES (pH 5.7), 154 mM NaCl, 5 mM KCl, and 125 mM $CaCl_2$] and then centrifuged and resuspended in modified MMg solution [4 mM MES (pH 5.7), 0.4 M Mannitol, and 15 mM $MgCl_2$]. Protoplast yield was evaluated by cell counting with a hemocytometer under an inverted light microscope, and their viability was assessed by Food and Drug Administration. The protoplasts were kept on ice for 30–60 min after which they settled at the bottom of the tube by gravity, and then other components were removed without touching the protoplast.

(3) A total of 2 µl of 20 mM Tris-HCl (pH 7.5), containing 25% Glycerol, 2.5 mM $MgCl_2$, and 0.2% (v/v) Triton X-100 was added to the protoplast, and the nuclei was re-suspended by pipetting. The mixture was incubated for 10 min on ice to lyse the chloroplasts before centrifugation at 3500 *g* for 10 min at 4°C. This procedure was repeated twice. The supernatant was discarded, and 2 ml of 20 mM Tris-HCl (pH 7.5), containing 25% Glycerol and 2.5 mM $MgCl_2$, was added to the protoplast. The nuclei were re-suspended by pipetting. The samples were centrifuged at 3500 *g* at 4°C for 10 min [2]. The purpose of this

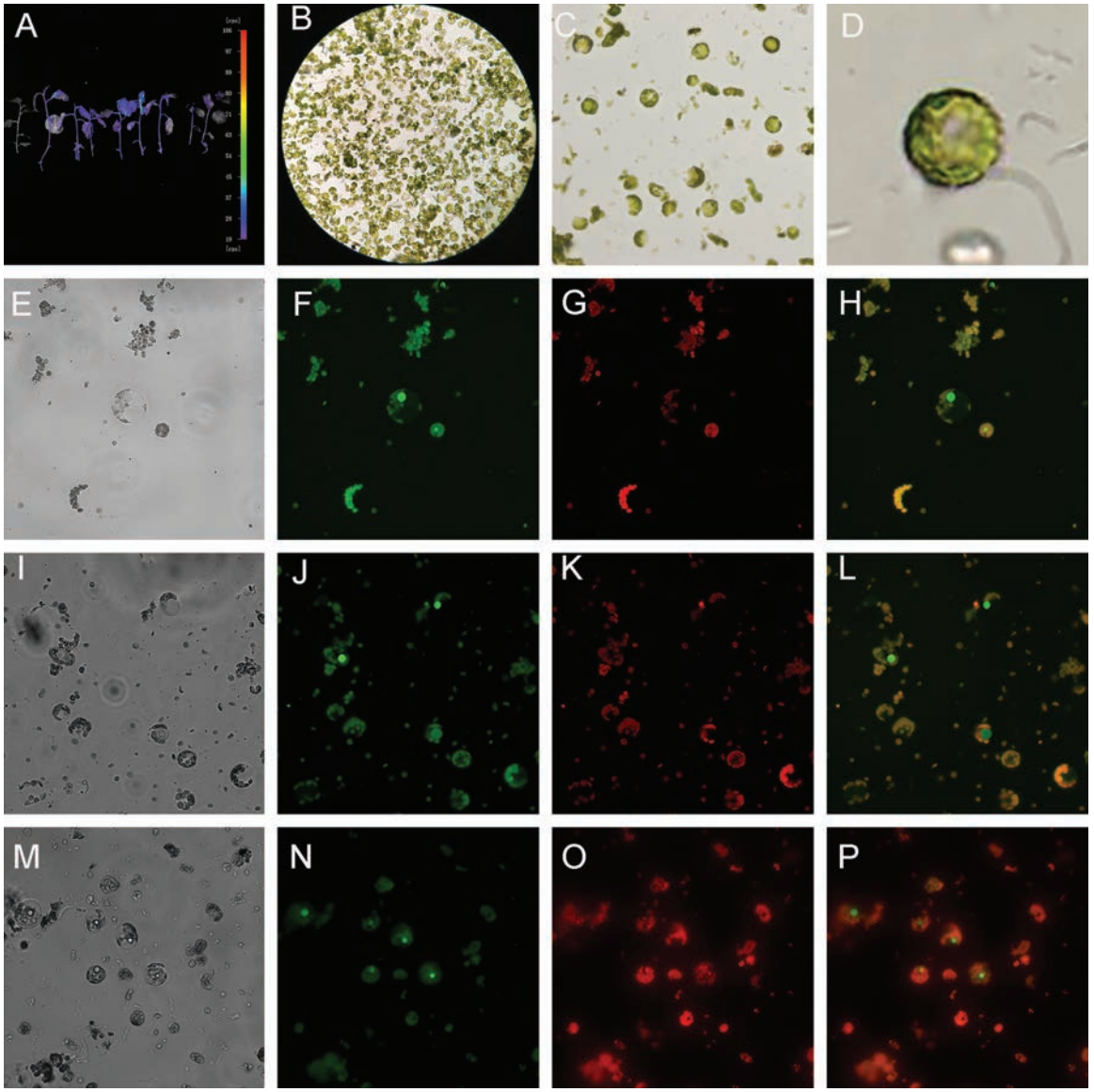

Figure 1. Transient gene expression and the location of protoplasts leaf tissue by the enzymatic method after infection with *A. tumefaciens* (A) Transgenic *B. platyphylla* lines expression GFP with different localizations were tested for their emission of fluorescence using the NightSHADE LB985 imaging system. (B) Microscopic view of mesophyll protoplasts at the end of digestion of leaf tissues after 2 h at 25°C. (C,D) After incubation in the enzyme solution, all mesophyll cells became protoplasts. (E–P) Subcellular localization of *BpHSFA4A, BpGTE8,* and *BpCDF3 in B. platyphylla* (E), (I), and (M) were bright-field. (F), (J), and (N) were GFP. (G), (K), and (O) were chlorophyll autofluorescence (Auto). (H), (L), and (P) were merged images. The 35::GFP fusion was used as the positive protein control and was detected in the nuclei of *B. platyphylla*. GFP:*HSFA4A*, GFP:*GTE8*, and GFP:*CDF3* fusion constructs were each transiently expressed into *B. platyphylla*. Green fluorescence signals were detected by microscopy after 48 h. (B–P) Scale bar = 20 μm.

step was to remove the Triton X-100. Mild detergents such as Triton X-100 can be used for the isolation of nuclei as they can disrupt the plasma membrane while having little effect on the nuclear membrane if used under optimized concentrations and incubation time. The chloroplasts are dispersed by the neutral detergent Triton X-100 that is based on the selective dissolution of the cytoplasmic membrane (not the nuclear membrane) [3]. One potential drawback is that higher concentrations of detergents or longer incubation time can lead to nuclear membrane damage [4]. So 0.2% (v/v) Triton X-100 and an incubation time of 10 min were used. Triton X-100 was stored as a 30% percent w/v stock at 15°C and diluted with isolation buffer.

The results showed that a fluorescent reporter gene was easily introduced into *B. platyphylla* leaves and that most of the plants showed fluorescence when NightSHADE imaging system was used. The transient transformation was monitored and assayed by the expression within a range between 340 nm and 488 nm. GFP reached its maximum within 3–4 days following infection and decreased slowly afterwards. By contrast, untransformed controls did not show any GFP signal (Fig. 1A).

Freshly isolated nuclei were translucent, spherical to oval in shape, and had an average diameter of about 10 μm. Integrity of the isolated nuclei was analyzed by staining with DAPI and observation with a light and fluorescence microscope. The nuclei were uniform.

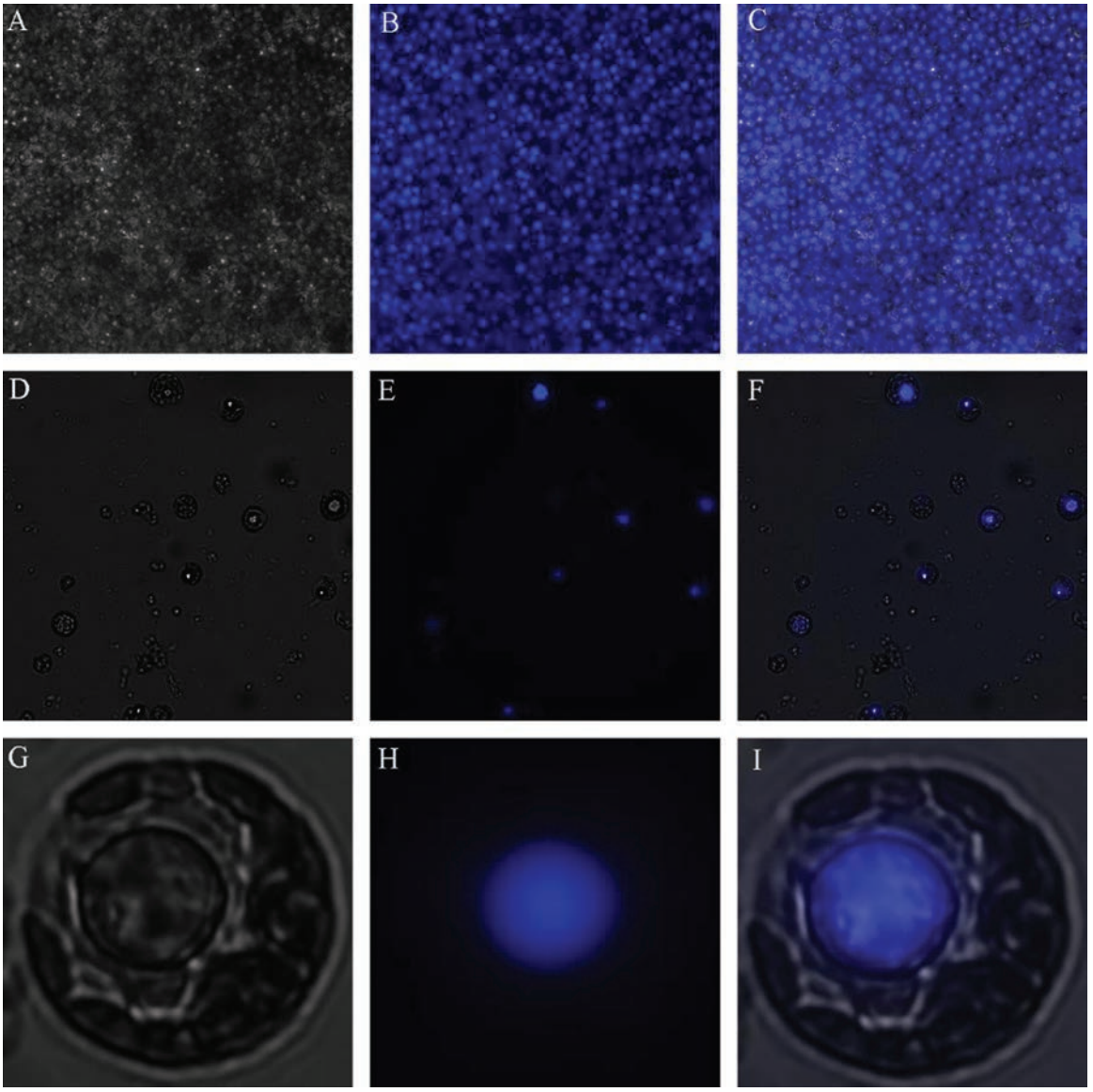

Figure 2. Nuclei microscopy in woody plants and the nuclear morphological observation in DAPI channel DAPI-stained nuclei (shown in blue) were observed with a fluorescence microscope and DAPI served as a positive marker for nuclear localization. (A–I) Scale *bar* = 50 μm. (A–C) Digested *B. platyphylla* leaf tissues after 1.5 h at 25°C. (D–F) Digested *B. platyphylla* leaf tissues after 3 h at 25°C. (G–I) Subcellular localization of *BpHSFA4A*.

DAPI staining results showed that the nuclei appeared intact and were easily recognized. About 80% of the nuclei present could be recovered in an undamaged state (Fig. 2).

In this protocol, cell-wall digesting enzymes are introduced into the cells without apparent damage to the nuclei. The chloroplasts are dispersed by the neutral detergent Triton X-100. Using this protocol, the localization of three transcriptional factors was visualized in the nuclei. This protocol has the following benefits: (i) has little or no visible coloration, (ii) can extract DNA fragments with a length between 200 bp and 600 bp (data not shown), (iii) can be used to isolate DNA from adult plant leaves as well as younger tissues and work best if the tissue is fresh, and (iv) can be successfully validated in a wide variety of plant tissues.

In summary, using the procedure described above, we can obtain nuclei from *B. platyphylla* much more rapidly, with high nuclei yields, which is uncontaminated by other cell organelles. This method combined with ChIP-Seq represents a new tool for testing the DNA integrity of leaf tissues in higher plants under stress conditions. This protocol was optimized for the preparation of the whole ChIP DNA from woody plant followed by high-throughput sequencing to identify the targets of TFs that regulate stress response. The protocol is also an easy, efficient, and rapid method described for the isolation, purification of nuclei, and fragmentation of nuclear DNA from plant protoplasts. The isolated nuclei can be used for a variety of downstream analyses such as ChIP-Seq, experimental procedures like further isolation and purification of nuclear protein complexes, nuclear protein detections, nuclear protein immunoprecipitation, and other chromatin-bound proteins and should be suitable for many other molecular biology applications. Most importantly, it can be used to isolate nuclear DNA from a wide variety of plants.

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