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Tissue Culture Note

Protoplast isolation from bamboo leaves

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Abstract Protoplasts with a yield greater than 2.0×10^6 cells per 0.3 g fresh weight were isolated from leaves of four bamboo species: an herbaceous bamboo, *Lithachne pauciflora*, and three woody bamboos, *Phyllostachys meyeri*, *Sasa jotanii*, and *Bambusa vulgaris*. For aseptic protoplast isolation, folded leaves were sterilized in 70% ethanol, and the internal tissues were drawn out from the leaf sheath. The leaf material was incubated for 4 h in a modified White's medium solution containing 0.8 M mannitol (pH 5.8, 32°C) and an enzyme mixture containing 3% Macerozyme R-10, 2.5% Meicelase, and 2% Cellulase Onozuka for woody bamboos, and 2.5%, 2.5%, and 1% of these enzymes, respectively, for *L. pauciflora.* Protoplasts were filtered through a double-layered Miracloth funnel and then washed with 0.6 M to 0.4 M mannitol-containing White's medium (pH 5.8) prior to analysis. Protoplast viability averaged 83% as determined by FDA staining.

Key words: Bamboo protoplasts, *Bambusa vulgaris*, *Lithachne pauciflora*, *Phyllostachys meyeri*, *Sasa jotanii.*

Bamboos provide a sustainable resource for numerous uses, including foods and materials for construction, craft, charcoal, and paper (Higuchi 1981). However, bamboos are known to have a gregarious flowering habit (Janzen 1976) that makes genetic breeding difficult. Although many studies on tissue or organ culture of bamboos have been reported (Chang and Ho 1997; Ogita 2005; Ogita et al. 2008), studies on bamboo protoplasts are scarce. Recently, some tree species with long vegetative growth phase have been genetically modified by introducing flowering genes to shorten the vegetative span, e.g., *FT* homolog in *Citrus* (Endo et al. 2005). Previously, we have already cloned an *FT* homolog from a bamboo, *Phyllostachys meyeri* McClure as *PmFT* (Hisamoto et al. 2009). As a preliminary study on an ectopic *PmFT* induction into any bamboo clones, a direct gene transfer to protoplasts is necessary for transient gene expression analysis. Therefore, we aimed to develop a convenient method to isolate bamboo protoplasts for ectopic gene expression analysis by using an electroporation technique.

Bamboos are classified into two taxonomic groups: herbaceous Olyreae and woody Bambuseae (GPWG 2001). Bambuseae are further divided into three groups, namely, tropical Asiatic bamboos, Andean bamboos, and East Asiatic temperate bamboos (Hisamoto et al. 2008). We examined methods for protoplast isolation from these representative bamboo groups. *Lithachne pauciflora* (Sw.) P.Beauv. ex Poir. (Olyreae) and *Bambusa vulgaris* Schrad. ex J.C.Wendl were maintained in a green house at Utsunomiya University, with the minimum atmospheric temperature in winter at 13°C, while *Phyllostachys meyeri* McClure and *Sasa jotanii* (Ke.Inoue et Tanim.) M. Kobay were cultivated in outdoor gardens at the university, the Fuji Bamboo Garden and the Tateshina Dwarf Bamboo Garden, Japan.

As shown in Figure 1, folded leaves were used for the aseptic preparation of protoplasts from tissues tightly enclosed within the leaf sheaths; the tissues were considered to be free of epiphytic microorganisms. Uppermost branch complements bearing new folded leaves were cut beneath the uppermost node. Unfolded leaf blades were removed, and the twigs were sterilized with 70% ethanol on a clean bench. Folded leaves were carefully drawn from the leaf sheaths to collect the enclosed parts, cutting off the exposed top end. Folded leaves were classified into one of three developmental stages, according to the extent of enclosure within the leaf sheaths: early, more than 80% enclosed; middle, approximately half enclosed; and late, less than one-third was enclosed. The middle-aged leaves were the preferred source for protoplast isolation.

Modified White's basal medium (White 1963) was used for enzymatic digestion and protoplast purification and maintenance (Table 1). Yeast extract was excluded to avoid the growth of heterotrophic contaminating

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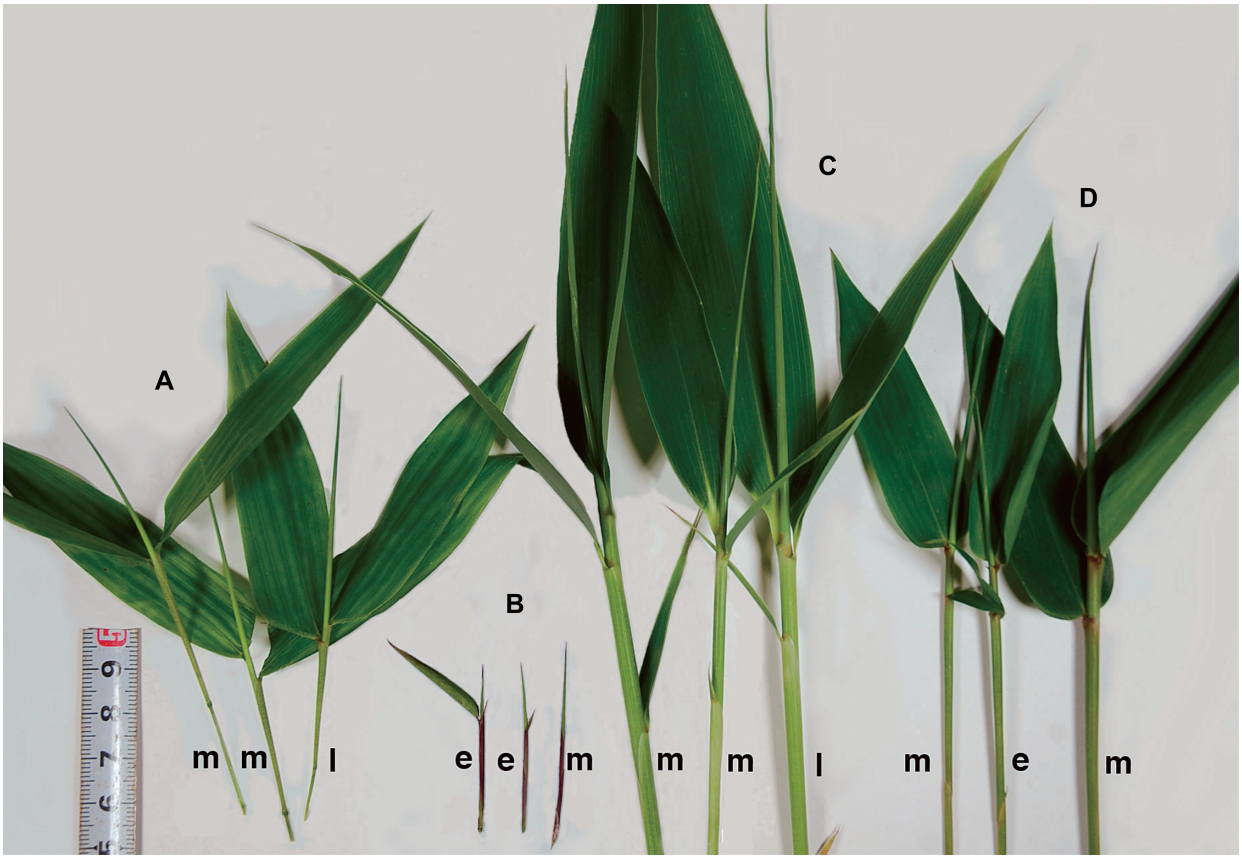

Figure 1. Bamboo branch complements with a folded leaf at each top, in which small letters indicate the folded leaf stage at e, early; m, middle; and l, late. (A) *Lithachne pauciflora*. (B) *Phyllostachys meyeri*. (C) *Sasa jotanii*. (D) *Bambusa vulgaris*.

Table 1. Modified White's medium.

Main inorganic elements (mM)	
KNO_3	0.79
$Ca(NO_3)_2 \cdot 4H_20$	1.64
KCl	0.87
$MgSO_4 \cdot 7H20$	2.92
$NaH_2PO_4 \cdot H_2O$	0.12
Na_2S0_4	1.41
Inorganic microelements (μM)	
$Fe(SO_4)_3$	6
H_3BO_3	24
$MnSO_4 \cdot 4H_2O$	31
$ZnSO_4 \cdot 4H_2O$	10
KI	4.5
Organic microelements (mg l^{-1})	
Glycine	3
$Ca(NO_3)_2 \cdot 4H_20$	1
Thiamine-HCl	0.1
Pyridoxine	0.1
Nicotinic acid	0.5
Buffering organic elements	
Mannitol	0.8/0.4 M
Horse serum	20 ml l^{-1}
pH 5.8	

microorganisms. Horse serum (Ito 1973) was added only when protoplast maintenance over several days was required.

A total of 0.3 g of aseptically collected folded leaf tips were dipped in 0.8 M mannitol-containing White's medium in a glass dish, cut into strips of approximately 5 mm in length, and then torn with forceps into several fibrous pieces for enzymatic digestion. The torn pieces were divided into five equal portions, which were incubated in 5 ml of enzyme solution in a 20-ml Erlenmeyer flask at 32°C with shaking for 2–12 h at 100 rotations per minute. After enzymatic digestion, the flasks were stored at 4°C for 30 min to stabilize the protoplasts (Nagao 2002) in order to prevent protoplast rupture and a consequent reduction in protoplast yield. The digested aliquots were filtered with a funnel made of Miracloth (Calbiochem. Lot. No. 106791, La Jolla, USA) inside a 50-ml centrifuge tube (Figure 2). A 12-ml centrifuge tube with a double-layer Miracloth funnel enabled us to aseptically separate the digested protoplasts from the degraded leaf tissues by centrifugation at 300 rpm for 5 min. The filtered portion in the 12-ml tube receiver was transferred to a new 15-ml centrifuge tube, and the protoplasts were collected by centrifugation at 1,200 rpm for 20 min. Each protoplast pellet was washed with 0.8 M, 0.6 M, and 0.4 M mannitol-containing White's media under the same centrifugation conditions. An electroporation technique

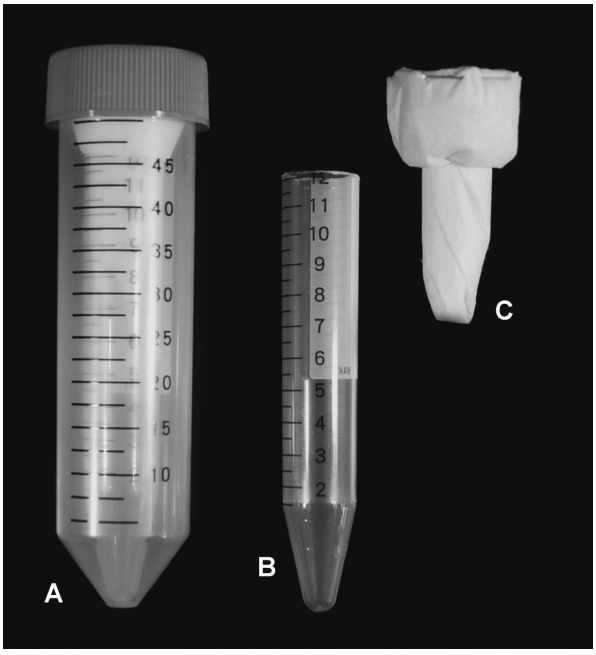

Figure 2. A tube equipped with a Miracloth funnel for protoplast purification. (A) A 50-ml centrifuge tube with a Miracloth funnel. (B) A 12-ml tube receiver. (C) Miracloth funnel.

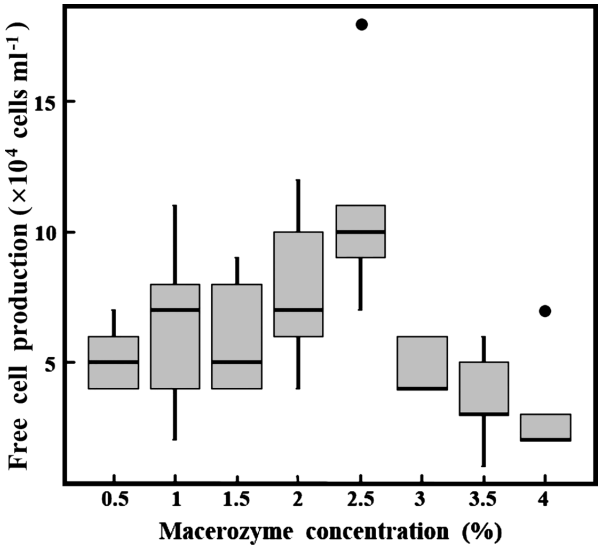

Figure 3. Effect of Macerozyme R-10 concentration on free cell production from *Lithachne pauciflora* leaves. Horizontal bars, vertical lines, and dots show medians, maximum and minimum values, and outliers, respectively.

Table 2. Two-way ANOVA on Macerozyme R-10 concentration and temperature for maceration of *Lithachne pauciflora* leaves.

Macerozyme (%)	Temperature (°C)	25	35
	Leaf stage	$\times 10^4$ cells ml^{-1}	
2	Early	0,1,0,2,1	2,1,7,1,2
	Middle	0,2,1,3,1	1,2,6,3,6
	Late	2,7,7,4,6	3,7,3,2,3
2.5	Early	2,0,2,2,1	2,7,4,1,5
	Middle	1,2,2,2,3	4,1,4,6,4
	Late	4,2,6,4,4	4,7,6,5,6
Sample repeat	All stage	All leaves	
Concentration	**		
Temperature	**	*	
Conc.×Temp.			
Leaf stage	Early	Middle	Late
Concentration			
Temperature	*	*	
Conc.×Temp.			.

Signif. Codes: '***'=0.001, '**'=0.01, '*'=0.05, '·'=0.1.

for transient gene expression analysis is usually performed at a tonicity level of 0.4 M mannitol solution (Okada et al 1986, Chen et al. 2006), making the accommodative treatment from 0.8 M to 0.4 M necessary.

We first optimized the concentration of Macerozyme R-10 (Yakult, Japan) for free cell preparation by varying the enzyme concentration from 0.5% to 4% in 0.8 M mannitol-containing White's medium at pH 5.8. Data were analyzed by one-way ANOVA with the R program package and displayed as a box and whisker plot (Figure 3), in which the F value was 4.4268 (Pr=0.001553) at a 1% significance level. The optimum concentration was found between 2% and 2.5%. In a similar fashion, we optimized enzyme concentrations for Cellulase Onozuka (Yakult) and Meicelase (Cellulase conc. Lot No. GCEF61920, Meiji Seika Co. Ltd., Japan). Cellulase and Meicelase were tested at 0.5%, 1%, 1.5%, 2%, and 2.5% in a 2% Macerozyme R-10 and 0.8 M mannitol-containing White's medium using five pairs of folded *Lithachne pauciflora* leaves. We determined that the optimal mixture was a combination of 0.5% Cellulase Onozuka and 2.5% Meicelase (data not shown). Next, we examined the effects of 2% and 2.5% Macerozyme at temperatures of 25°C and 35°C, using three different-aged leaves of *L. pauciflora* (early, middle, or late) by two-way ANOVA (Table 2). Early, middle, and late leaves were tested with each treatment in replicates of five; both concentration and temperature were found to have significant effects (p=0.01). Temperature affected all leaves, regardless of age (n=15, p=0.05), while a slight interaction between concentration and temperature was observed only in late leaves (p=0.1). Gamborg (1976) reviewed general conditions for plant protoplast isolation, with temperatures ranging from 23°C to 32°C.

On the basis of these results and those reported by Gamborg (1976), we selected the following conditions for protoplast isolation from *L. pauciflora*: 2.5% Macerozyme R-10, 2.5% Meicelase, and 1% Cellulase Onozuka with 0.8 M mannitol containing White's medium with pH 5.8 at 32°C. We identified different optimal enzymatic conditions for protoplast isolation

from woody bamboos. As shown in Figure 4, we examined *Phyllostachys meyeri* with three combinations of enzyme concentrations as Macerozyme R-10 : Meicelase : Cellulase Onozuka at (a) 3% : 2.5% : 2% (open circle), (b) 3% : 2% : 2.5% (closed circle), and (c) 3.5% : 2.5% : 2.5% (dotted circle), respectively.

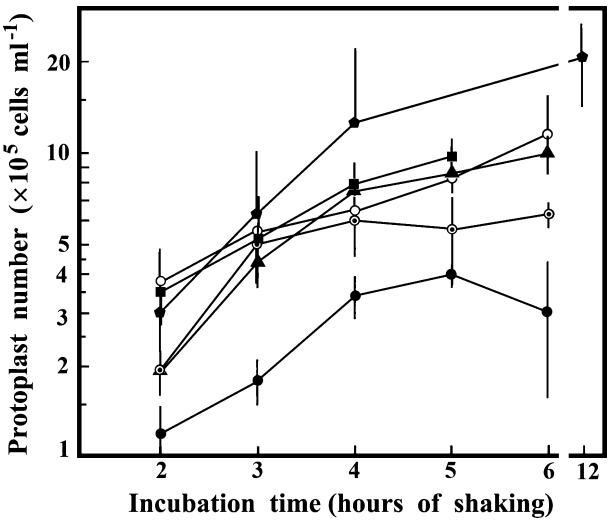

Figure 4. Effect of mixed enzyme solution on protoplast production from leaves of *Phyllostachys meyeri* with 3% : 2% : 2.5% (closed circle), 3.5% : 2.5% : 2.5% (dotted circle), or 3% : 2.5% : 2% (open circle) of Macerozyme R-10 : Meicelase : Cellulase Onozuka. *Sasa jotanii* (pentagon) and *Bambusa vulgaris* (square) were treated with the same enzyme mixtures, while *Lithachne pauciflora* (triangle) was treated with 2.5% : 2.5% : 1%. Each circle and vertical bar shows the mean value and standard deviation for five sampling repeats.

Combination (a) showed the most stable and highest protoplast yield, at approximately 2.0×10^6 cells per 0.3 g fresh weight. Figure 4 also shows the protoplast yield for *Bambusa vulgaris* (square) and *Sasa jotanii* (pentagon) under the same enzymatic conditions as *P. meyeri*, while *L. pauciflora* (triangle) was distinct. In summary, after 2 h of enzymatic digestion, the protoplast yield was approximately $3–4\times10^5$ cells ml^{-1} and more than 2.0×10^6 cells per 0.3 g fresh weight after 4 h of incubation. As shown in Figure 1, folded leaf size was the smallest in *P. meyeri* than in other woody bamboos, that the other two taxa were more easily to attain the amount of protoplast yield per fresh weight.

Protoplasts were prepared for electroporation by washing in 0.4 M mannitol- containing White's medium with 5 mM 2-morpholinoethanesulfonic acid (MES, pH 5.8), or cultured for a few days with 2% horse serum (Ito 1973). Protoplast pellets in 100 μl aliquots were used for observation with an OLYMPUS BH2 light microscope equipped with Nomarski optics on a Thoma hemocytometer.

As shown in Figure 5, after 4 h of enzymatic digestion, larger protoplasts with ca. 25 μm in diameter were produced (Figure 5A, B, D for *L. pauciflora*, *P. meyeri*, *B. vulgaris*, respectively), among which extremely large protoplasts with 50 μm in diameter appeared in *L. pauciflora* (Figure 5A). However, after 12 h of treatment, the majority of protoplasts were small (ca. 10 μm in diameter), and the large protoplasts had disappeared for all bamboo species (Figure 5C, e.g., *S. jotanii*). Many

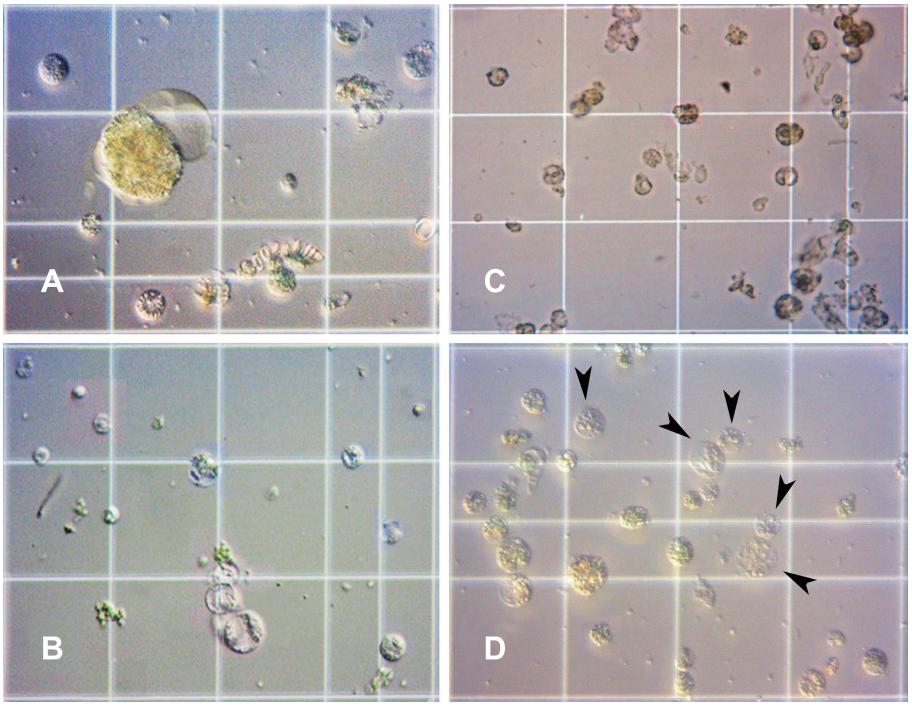

Figure 5. Light micrographs of protoplasts on a Thoma hemocytometer with 50×50 μm squares equipped with Nomarski optics except for (C). (A) *Lithachne pauciflora,* (B) *Phyllostachys meyeri*, (C) *Sasa jotanii,* and (D) *Bambusa vulgaris* in which arrowheads indicate flattened protoplasts.

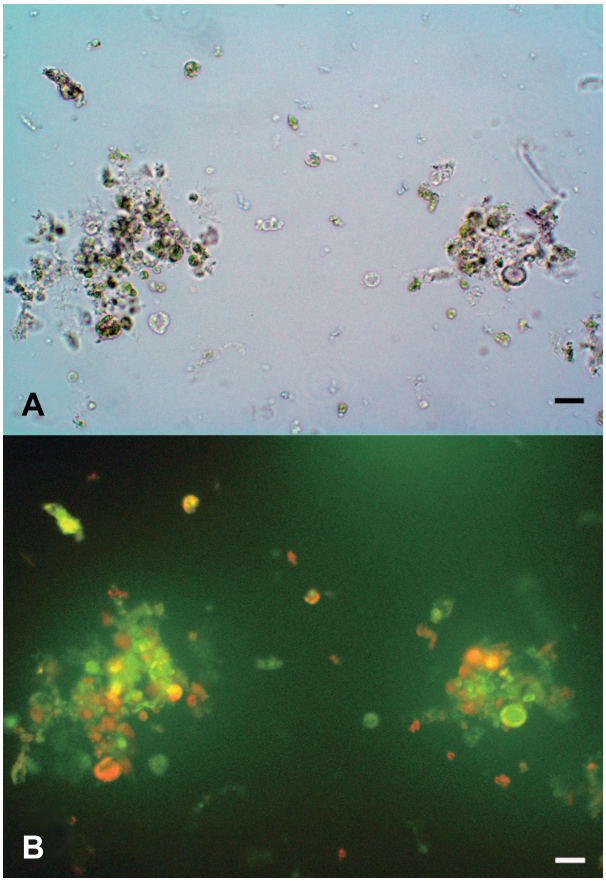

Figure 6. Light micrographs of FDA-stained protoplasts of *Lithachne pauciflora* taken under a Leica DM5000B light microscope; each bar shows 10 μm. (A) Bright-field micrograph taken with Nomarski optics. (B) Epifluorescent micrograph with a 510-nm excitation wavelength filter for GFP *Plus*.

flattened protoplasts in *B. vulgaris* (Figure 5D; arrowheads) suggested that the species was more osmotic labile even under the 0.8 M mannitol concentration.

To assess viability of collected protoplasts, an acetone stock solution (5 mg ml^{-1}) of fluorescein diacetate (FDA, Invitrogen) was added to protoplast suspensions of *L. pauciflora* (Figure 6) or *B. vulgaris* to a final concentration of 0.01%. After incubation for 20 min at room temperature, the pellets were observed and photographed under a Leica DM5000B light microscope combined with a CCD camera CTR5000 (Hamamatsu, Japan) in a bright field equipped with Nomarski optics (Figure 6A) and an epifluorescent microscope equipped with a GFP *Plus* IF filter for 510 nm wavelength excitation (Figure 6B). Mesophyll protoplasts were considered viable if they showed yellow/green fluorescence, and were considered nonviable if they did not show fluorescence or if only red fluorescence (due to chloroplasts) was observed. Protoplast viability was estimated to 83.4% or 83.2% for *L. pauciflora* or *B. vulgaris*, respectively.

High efficiency protoplast isolation is essential to investigate transient ectopic gene expression (Chen et al. 2006). We have already cloned an *FT* homolog, *PmFT*, from *P. meyeri* (Hisamoto et al. 2008) and are currently developing a method for transient *PmFT* gene expression analysis using an electroporation technique, the results of which will be published elsewhere.

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