Improving Infection Efficiency of *Agrobacterium* to Immature Cotyledon Explants of Japanese Apricot (*Prunus mume*) by Sonication Treatment

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The infection efficiency of *Agrobacterium* to cotyledon explants of Japanese apricot (*Prunus mume*) was markedly improved by sonication treatment. The use of *sGFP(S65T)* reporter gene in this study enabled direct observation of transgene expression, thus allowing the evaluation of *Agrobacterium* infection efficiency. Immature cotyledons of different cultivars and different developmental stages were subjected to sonication treatment of durations ranging from 10 sec to 2 min. When early-stage immature cotyledons of 'Nanko' were subjected to sonication treatment, the transient GFP expression frequency after co-cultivation was nearly 100% and GFP fluorescence was distributed over almost the entire cotyledon surface. In contrast, when the inoculation was carried out according to the standard dipping method, transient GFP expression frequency was less than 10% and GFP fluorescence was observed only in spots. The highest frequency of somatic embryogenesis (SEG) with GFP expression was obtained from 40 sec sonication treatment of May 14 'Nanko' immature cotyledons. However, with younger cotyledons, which have a higher SEG frequency, 20 sec of sonication treatment was found to be sufficient to increase the infection frequency.

Key Words: immature cotyledon, *Prunus*, somatic embryogenesis, sonication treatment.

Introduction

The genus *Prunus*, which contains many fruit tree species, has a relatively small genome size and a short juvenile period. Accordingly, extensive genome studies including linkage map construction and whole genome sequencing have been conducted (Kole and Abbott, 2012). However, the genetic transformation of *Prunus* is difficult because it is recalcitrant to *in vitro* regeneration. Although transformation systems using *Agrobacterium* have been developed in several fruit tree species of *Prunus*, including apricot (*P. armeniaca*) (Laimer et al., 1992; Petri et al., 2004; Pratesi et al., 2004), almond (*P. dulcis*) (Ainsley et al., 2001; Archilletti et al., 1995; Miguel and Oliveira, 1999), sweet cherry (*P. avium*) (Druart et al., 1998; Pratesi et al., 2004), sour cherry (*P. cerasus*) (Dolgov and Firsov, 1999; Song and Sink, 2006), European plum (*P. domestica*) (Mante et al., 1991; Scorza et al., 1994; Scorza et al., 1995a, b; Tian

et al., 2009; Yancheva et al., 2002), peach (*P. persica*) (Hammerschlag and Smigocki, 1998; Pérez-Clemente et al., 2004; Ye et al., 1994), Chinese plum (*P. salicina*) (Urtubia et al., 2008), and Japanese apricot (*P. mume*) (Gao et al., 2010), most of them were transformed with reporter genes and few were successfully transformed with agriculturally useful transgenes. Furthermore, the genetic transformation efficiency reported was usually low in *Prunus*.

One of the *Prunus* fruit tree species, Japanese apricot (*P. mume*), is an important fruit crop in Japan. The total cultivation area of Japanese apricots in 2011 was 16,600 ha, more than that of any other *Prunus* fruit crop, including peach (9,980 ha), Japanese plum (2,970 ha), and cherry (4,440 ha). Its fruits are used to produce a Japanese traditional food called "umeboshi" and a sweet liqueur "umeshu". Owing to its economic importance, there is significant interest in the breeding and improvement of cultivars of *P. mume*. However, conventional cross-breeding for *P. mume*, as of other perennial fruit trees, is a time-consuming and difficult process owing to its long generation time.

Attempts have been made to improve this species by genetic engineering. Previously, we developed a protocol for successful plant regeneration and transformation via

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somatic embryogenesis (SEG) from immature cotyledons of *P. mume* (Gao et al., 2010). However, we obtained only a few transgenic somatic embryo lines from thousands of inoculated immature cotyledon explants. To establish an efficient transformation system for *P. mume*, we need to optimize each step of the *Agrobacterium*-mediated transformation. Here, we attempted to improve the initial step of genetic transformation, the *Agrobacterium* infection step, using a sonication-assisted transformation method. Furthermore, we used an engineered synthetic *GFP* named *sGFP(S65T)* (Chiu et al., 1996; Haas et al., 1996; Niwa et al., 1998), acting as a reporter gene in place of the *gusA*-intron gene that was used in the previous study (Gao et al., 2010) to monitor the initial *Agrobacterium* infection non-destructively.

Since the end of the last century, a novel transformation method has attracted research attention. Sonication-assisted *Agrobacterium*-mediated transformation involves subjecting plant tissue to brief periods of sonication in the presence of *Agrobacterium*. Sonication-assisted *Agrobacterium*-mediated transformation is considered an effective method for transferring foreign genes into recalcitrant target plants (Trick and Finer, 1997). Sonication treatment produces microwounds in tissue and allows the *Agrobacterium* to travel deeper and more completely throughout the tissue than standard dipping inoculation (Liu et al., 2005; Santarém et al., 1998; Tang et al., 2001; Trick and Finer, 1997), thus enhancing bacterial colonization and infection of the tissue.

Sonication treatment has been successfully applied to soybean (*Glycine max*) (Santarém et al., 1998), loblolly pine (*Pinus taede*) (Tang et al., 2001), black locust (*Robinia pseudoacacia*) (Zaragozá et al., 2004), and flax (*Linum usitatissimum*) (Beranová et al., 2008), and attempted in some fruit trees such as citrus (*C. sinensis* and *C. paradisi* × *Poncirus trifoliate*) (de Oliveira et al., 2009) and banana (*Musa* spp.) (Subramanyam et al., 2011). However, sonication can cause cell damage or even rupture. If sonication is used to facilitate the uptake of *Agrobacterium*, it is important to optimize the conditions without causing such damage. Given that tissues differ in their response to sonication treatment, the best treatment method for each tissue must be empirically determined. Treatments that yield high levels of transient gene expression with minimum sonication treatment duration are most desirable. In this study, we investigated the efficacy of sonication treatment on improving the infection efficiency of *Agrobacterium* to cotyledon explants of *P. mume* and identified the most effective sonication duration to obtain the highest expression of the *GFP* reporter gene.

Materials and Methods

Plant materials

Immature fruits were collected on May 15, 2006 from adult trees of two cultivars of *P. mume*, 'Nanko' and 'Ellching', in the orchard of the Horticultural Experiment Center of Wakayama, Japan, and from those of 'Kotsubu Nanko' on May 29, 2006 and 'Nanko' on May 11, 14, and 19, 2007 in the experimental farm of Kyoto University. The fruits were surface sterilized, immature cotyledons were aseptically removed, and explants were prepared as previously described (Gao et al., 2010). The immature cotyledons were used as explants.

Construction of sGFP(S65T) expression vector

A fragment of *sGFP(S65T)* was excised from the plasmid vector pBlue-*sGFP(S65T)*-nos3′ SK (kindly provided by Professor Yasuo Niwa at the University of Shizuoka, Japan; Chiu et al., 1996; Niwa, 1998) with *Pst*I restriction endonuclease, and extracted with QiAquick® Gel Extraction Kit (Qiagen, Venlo, Netherlands). The fragment was blunt-ended with T4 DNA polymerase (Roche Applied Science, Mannheim, Germany). The backbone plasmid pDU92.3103 (Tao et al., 1995), which contains the *β*-glucuronidase gene (*gusA*) and the kanamycin resistance gene *nptII*, respectively, driven by the CaMV 35S promoter, was digested with *Bam*HI, which is located between the CaMV 35S promoter and terminator and blunt end-ligated with *sGFP(S65T)* fragment to generate the plasmid vector of pDU92-*sGFP* (Fig. 1). The plasmid vector was introduced into *Agrobacterium tumefaciens* strain EHA101 using electroporation.

Inoculation methods with Agrobacterium

Freshly excised immature cotyledons of *P. mume* were collected and prepared for sonication treatment. Twenty-five immature cotyledon explants were placed in a 50-mL sterilized polypropylene centrifuge tube containing 20 mL *A. tumefaciens* EHA101 suspension in MS virulence-induction medium, prepared as previously described (Gao et al., 2000), and sonicated for 2 min for the 'Nanko' and 'Ellching' explants of May 15, 2006, and 10, 20, and 30 sec for 'Kotsubu Nanko' of May 29, 2006 with an ultrasonic bath system (Model Ultrasonic Cleaner, SU-27TH; Sibata Scientific Technology Ltd; Tokyo, Japan), operating at 28 KHz frequency and 0.23 W·cm^{-2} intensity. A replicated experiment was
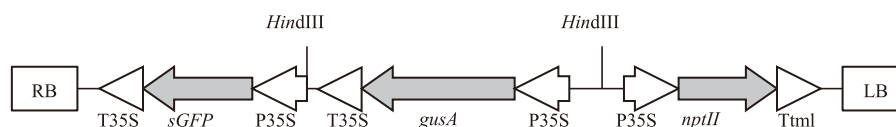

Fig. 1. T-DNA region of pDU92-*sGFP* (19.8 kb). Arrows indicate the direction of transcription. RB: Right border, LB: Left border, P35S: CaMV 35S promoter, T35S: CaMV 35S terminator, Ttml: *Agrobacterium* tml terminator, *nptII*: neomycin phosphotransferase gene, *sGFP*: *sGFP(S65T)* gene, *gusA*: *β*-glucuronidase gene.

carried out with 0-, 20-, 40-, and 60-sec sonication of 'Nanko' immature cotyledon explants from May 11, 14, and 19, 2007. After the sonication treatments, the explants were immersed in the suspension and incubated at room temperature for 10 min. For the control inoculation (dipping method), the explants were immersed in the *A. tumefaciens* suspension for 10 min. The explants were then blotted briefly on a sterilized paper towel and placed on sterilized filter paper on 90 mm × 20 mm Petri dishes containing 35 mL co-cultivation medium. The plates were sealed with Micropore surgical tape (3M; St. Paul, MN, USA) and incubated at 25°C in the dark for 3 days for co-cultivation. The co-cultivation medium consisted of MS medium supplemented with 1 μM 2,4-D, 1 μM BA, 3% sucrose, and 100 μM acetosyringone at pH 5.2, and solidified with 0.8% agar. The acetosyringone was dissolved in dimethylsulfoxide (DMSO) and added aseptically to the medium after autoclaving at 121°C for 15 min.

Elimination of Agrobacterium and induction of somatic embryogenesis

After co-cultivation, the explants were transferred to disinfection medium containing 50 mg·L^{-1} meropen (MEROPEN; Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan), and cultured for 7 days at 25°C in the dark. The explants were then transferred to the primary selection medium containing 50 mg·L^{-1} meropen and 50 mg·L^{-1} kanamycin sulfate (Km) for induction of somatic embryogenesis (SEG). The basic media for disinfection and primary selection were identical to the co-cultivation medium and consisted of MS medium supplemented with 1 μM 2,4-D and 1 μM BA, 3% sucrose, and 0.8% agar, but at pH 5.8, and was described as SEIM-MS medium in our previous report (Gao et al., 2010). The antibiotics were aseptically added to the medium before dispensing to the plates. The plates were sealed with Parafilm (Parafilm M; Chicago, IL, USA) instead of the surgical tape used in co-cultivation. Disinfection and primary selection culture were also performed at 25°C in the dark. After 30-day primary selection culture, the explants with calli and somatic embryos were transferred to somatic embryo propagation medium (SEPM) containing 50 mg·L^{-1} meropen and 50 mg·L^{-1} Km and consisting of MS medium supplemented with 0.1 μM NAA and 5 μM BA and solidified with 0.2% gellan gum (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Transformed somatic embryos showing GFP expression were picked from the somatic embryo cluster and subcultured in fresh medium with a 30-day cycle.

Investigation of GFP expression frequency

The expression of green fluorescent protein (GFP) in the inoculated immature cotyledons was investigated using fluorescence microscopy (OLYMPUS MVX10 MacroView system; Olympus Corp., Tokyo, Japan) after 3 days of co-cultivation, 7 days of disinfection, and 30

and 60 days in selection culture. The GFP expression frequency was defined as the percentage of all inoculated explants that showed any visible green fluorescent spot or area. The frequency of SEG was investigated after 30 and 60 days in selection culture by counting explants that regenerated somatic embryos. The frequency of SEG with GFP expression was defined as the percentage of all inoculated explants that regenerated a GFP fluorescent somatic embryo, whether or not the somatic embryo was chimeric. The somatic embryos (usually forming a cluster) with GFP expression from the same explants were regarded as one line.

Estimation of Agrobacterium infection efficiency

Two-way ANOVA with no replication was performed to analyze the significance of the main effects of sonication treatment and the stage of immature cotyledon on GFP expression and SEG for the experiments in 2007. The percentage data were used after arcsine transformation.

Results

GFP expression in inoculated immature cotyledons

The immature cotyledons of the 'Nanko' and 'Ellching' explants of May 15, 2006 were approximately 5–10 mm in length and filled 50–80% of seed, and the growth stage of 'Ellching' cotyledons was more advanced than that of 'Nanko'. In the sonication treatment experiment using these immature cotyledons sonicated for 2 min, explants showing GFP expression following sonication treatment markedly exceeded those following the control inoculation after 3-day co-cultivation and 7-day disinfection culture, irrespective of the cultivar. The GFP expression frequency for sonication treatment was 49.1% in 'Ellching' and 88.6% in 'Nanko', whereas it was only 4.3% and 2.3%, respectively, for the control inoculation (Table 1). Simultaneously, GFP fluorescence in sonication-treated explants was intense and distributed over the explant surface, whereas GFP expression in non-sonicated control explants was weak and confined to a few green fluorescent spots (Fig. 2). Even after one month of selection culture, GFP expression in sonication-treated explants remained relatively high (13.9% for 'Ellching' and 50.0% for 'Nanko'). However, sonication-treated explants became brown and soft, with many cracks on their surfaces after one month of culture (Fig. 3).

Because the explants showed serious damage after treatment with 2 min of sonication, shorter sonication times of 10, 20, and 30 sec were tested using immature cotyledons from 'Kotsubu Nanko' of May 29, 2006. The cotyledons of 'Kotsubu Nanko' at this stage were somewhat hard and filled 80–100% with seed. GFP expression frequencies after 7-day disinfection increased with sonication time and were 22.4%, 54.6%, and 71.0% for 10, 20, and 30 sec, respectively (Table 2). Although the GFP expression frequency remained high after one

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Table 1. GFP expression and SEG from inoculated 'Nanko' and 'Ellching' immature cotyledons of May 15, 2006.

Inoculation method		GFP expression after disinfection		GFP expression after one-month selection		SEG after one-month selection		SEG with GFP expression after one month	
			%		%		%		%
Ellching	Non-sonicated control	6/141[z]	4.3	5/141	3.5	6/141[y]	4.3	0/141[x]	0.0
	2 min sonication treatment	107/218	49.1	30/216	13.9	7/216	3.2	0/216	0.0
Nanko	Non-sonicated control	3/131	2.3	2/122	1.6	28/122	23.0	0/122	0.0
	2 min sonication treatment	140/158	88.6	79/158	50.0	20/158	12.7	7/158	4.4

[z] Explants of GFP expression/total cultured explants.
[y] Explants of SEG/total cultured explants.
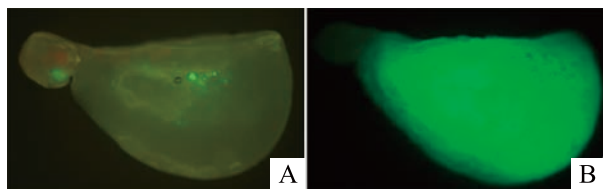[x] Explants of GFP expression SEG/total cultured explants.

Fig. 2. GFP expression after 7-day disinfection culture in the control inoculation of standard "dipping" (A) and sonication-treated (B) 'Nanko' immature cotyledon explants. Note that the color version of this figure is published only online.
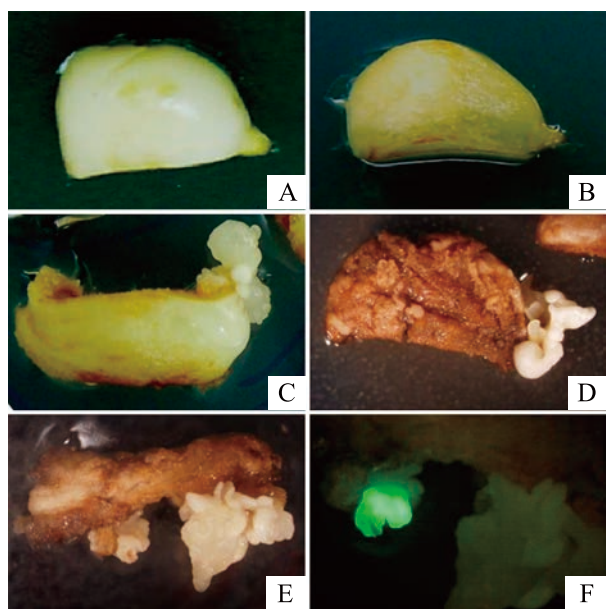

Fig. 3. Appearance and SEG from 'Nanko' immature cotyledon explants of May 15, 2006 with non-sonicated control inoculation (A, C) and 2 minutes of sonication treatment (B, D, E, F). A and B: after 7-day disinfection culture, C and D: after one-month selection culture, showing primary SEG, E and F: after 3 months selection culture, illustrating GFP expression of somatic embryos (E: bright field image, F: fluorescence microscope image showing an embryo with GFP expression). Note that the color version of this figure is published only online.

month of selection culture, the intensity of GFP fluorescence weakened greatly.

Because 'Nanko' immature cotyledons had the highest GFP expression frequency in the three cultivars used in 2006, replicated experiments were designed using 'Nanko' in 2007. Factorial combinations of the age of cotyledons from May 11, 14, and 19 and the duration of sonication of 0, 20, 40, and 60 sec were made to test the GFP expression after *Agrobacterium* infection. The cotyledons from May 11 were approximately 2–3 mm long and their tissues were soft, those of May 14 were 4–5 mm long with somewhat harder tissues, and those of May 19 were over 5 mm long and filled more than half with seed, and with still harder tissues. After 3-day co-cultivation, transient GFP expressions were observed in almost all sonicated explants, including those of 20-, 40-, and 60-sec in the three tests. The intensity of GFP fluorescence was very strong and distributed over almost the entire surfaces of explants, with no distinct difference among the 20, 40, and 60 sec sonication-treated explants. However, in the control inoculation treatments (0 sec), GFP expression was observed in only a few explants, and the intensities were weak and confined to spots or limited areas as described above. For the May 11 and 14 explants, the GFP expression frequencies were over 90% after 7-day disinfection, but they were lower for the May 19 explants (54% for 20 sec, 76.6% for 40 sec, and 78.7% for 60 sec) (Table 3). After one month of selection culture, stable GFP expression was observed both in the explants and calli or somatic embryos derived from the explants. Two-way ANOVA indicated that the main effects of sonication treatment on the percentages of the explants that expressed GFP immediately after disinfection and one month after selection were significant (Table 3). The main effect of the explant stage on the GFP expression frequency immediately after disinfection was also significant, while that after one month of selection was nonsignificant.

Somatic embryogenesis from inoculated immature cotyledons

In the sonication treatment experiment using 'Ellching' and 'Nanko' immature cotyledons of May 15, 2006, sonication-treated explants showed some transparency after 2 min of sonication, as though the *Agrobacterium* solution had infiltrated the explant tissue (Fig. 3). Somatic embryogenesis (SEG) was observed in the inoculated explants after two weeks of primary selection culture. The somatic embryos formed directly on the surface of the cotyledons and were white, smooth spheres,

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Table 2. GFP expression and SEG from 'Kotsubu Nanko' immature cotyledons of May 29, 2006.

	Duration of sonication treatment	Total no. explants	GFP expression after disinfection		GFP expression after one-month selection		SEG after one-month selection		SEG with GFP expression after two months	
			no. explants	%	no. explants	%	no. explants	%	no. explants	%
Control (non-inoculation)	0 sec[z]	80	—	—	—	—	21	26.3	—	—
	30 sec[y]	80	—	—	—	—	7	9.1	—	—
Inoculated with *Agrobacterium*	10 sec	200	45	22.4	71	35.5	72	36.0	1	0.5
	20 sec	194	106	54.6	88	45.4	83	42.8	8	4.1
	30 sec	200	142	71.0	119	59.5	76	38.0	2	1.0

[z] Control culture (CC).
[y] Control sonicated culture (CSC).

Table 3. GFP expression and SEG for sonication treatment using 'Nanko' immature cotyledons of 2007.

Explants stage		Duration of sonication treatment	Total no. explants	GFP expression after disinfection		GFP expression after one-month selection		SEG after one-month selection		SEG with GFP expression after one month	
				no. explants	%	no. explants	%	no. explants	%	no. explants	%
11-May	Control culture		82					32 (49)[z]	39.0 (59.8)		
	Inoculation	0 sec	122	12	9.8	4	3.3	66	54.1	2	1.6
		20 sec	108	104	96.3	51	47.2	52	48.1	7	6.5
		40 sec	106	103	97.2	37	34.9	17	16.0	1	0.9
		60 sec	105	101	96.2	23	21.9	13	12.4	1	1.0
14-May	Control culture		100					18 (29)	18.0 (29.0)		
	Inoculation	0 sec	116	4	3.4	1	0.9	29	25.0	0	0.0
		20 sec	128	117	91.4	42	32.8	61	47.7	9	7.0
		40 sec	111	110	99.1	46	41.4	33	29.7	8	7.2
		60 sec	120	117	97.5	57	47.5	33	27.5	7	5.8
19-May	Control culture		100					12 (18)	12.0 (18.0)		
	Inoculation	0 sec	107	5	4.7	5	4.7	15	14.0	0	0.0
		20 sec	107	58	54.2	39	36.4	21	19.6	1	0.9
		40 sec	111	85	76.6	41	36.9	10	9.0	0	0.0
		60 sec	108	85	78.7	33	30.6	20	18.5	2	1.9
ANOVA[y]											
Sonication treatment					**		**		NS		NS
Explant stage					*		NS		NS		NS

[z] The data in parentheses were collected after two-months selection culture.
[y] Two-way analysis of variance with no replication was conducted for the main effects of sonication treatment and the explant stage.
 * Indicates statistical significance at the 5% level.
** Indicates statistical significance at the 1% level.
[NS] Not significant.

as described in our previous report (Gao et al., 2010). Although sonication-treated explants showed extensive damage and browning after one month of selection culture, SEGs were observed on the edges or surfaces of some explants (Fig. 3). The SEG frequencies in sonication-treated explants, which were 3.2% for 'Ellching' and 12.7% for 'Nanko', were lower than those of non-sonicated control explants, 4.3% for 'Ellching' and 23% for 'Nanko' (Table 1). However, somatic embryos showing GFP expression were observed only in sonication-treated explants of 'Nanko' (Table 1; Fig. 3).

To investigate the effect of sonication on SEG frequency of 'Kotsubu Nanko' immature cotyledons, 80 cotyledon explants were cultured on SEIM-MS medium for 30 days to induce primary SEG and then transferred to SEPM for propagation of the somatic embryos, following the method described in our previous report (Gao et al., 2010) (expressed below as "control culture" and abbreviated as CC). Simultaneously, another 80 cotyledon explants were sonicated for 30 sec in 20 ml MS virulence-induction medium without *Agrobacterium* and then cultured as described above (expressed below as "control-sonicated culture" and abbreviated as CSC). As a result, the SEG frequency after one-month culture was 26.3% for CC and 9.1% for CSC, respectively (Table 2). The SEG frequency for CSC explants was lower than that for CC explants.

In the sonicated inoculation treatment experiments

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using the same stage of 'Kotsubu Nanko' immature cotyledons, the SEG frequency for any sonication-treated explants following 10, 20, or 30 sec of sonication was higher than those of either CC or CSC (Table 2). The highest SEG frequency was found in 20-sec sonication-treated explants (42.8%), followed by 30-sec (38%) and 10-sec (36%)-treated explants.

The SEG ability of 'Nanko' immature cotyledons of May 11, 14, and 19, 2007 was investigated by culturing the explants in SEIM-MS for the first month and in SEPM for the later subculture (expressed below as "control culture"). The SEG frequency of May 11 explants was markedly higher than those of the May 14 and 19 explants, being 39.0% for May 11 explants, 18.0% for May 14 explants, and 12.0% for May 19 explants after one-month culture. After two months of subculture, the SEG frequency was 59.8% for May 11 explants, 29.0% for May 14 explants, and 18.0% for May 19 explants (Table 3). In sonicated inoculation treatment experiments using the same stages of immature cotyledons, the highest SEG frequency was obtained from non-sonicated (0 sec) explants of May 11 (54.1%) (Table 3). Similarly to the control, SEG frequency decreased with the growth of immature cotyledons, being highest for May 11, followed by May 14 and May 19 explants in 0-sec and 20-sec sonication treatments, respectively. The SEG frequency of May 11 explants treated with 20-sec sonication was somewhat lower than those of non-sonicated (0 sec) explants, and explants treated with 40- and 60-sec sonication treatment had still lower SEG frequencies. In contrast, SEG frequencies of May 14 and 19 explants treated with 20-sec sonication were higher than those of non-sonicated (0 sec) explants. However, ANOVA analyses of the SEG frequency indicated that there were no significant main effects of sonication treatment and the explant stage, possibly because there

seemed to be an interaction between the two factors.

Somatic embryogenesis with GFP expression

In the sonication treatment experiment of May 15, 2006, the SEG with GFP expression was observed only in sonication-treated 'Nanko' explants, although the GFP expression in somatic embryos showed mosaic patterns (Fig. 3). The frequency of SEG with GFP expression after one-month selection culture was 4.4% (Table 1).

SEG was also observed in 'Kotsubu Nanko' explants from May 29, 2006 that were treated with 10-, 20-, and 30-sec sonication (Table 2). After 2 months selection culture, somatic embryo clusters with GFP expression were obtained from 8 explants with 20-sec sonication treatment, markedly more than that from the 10- or
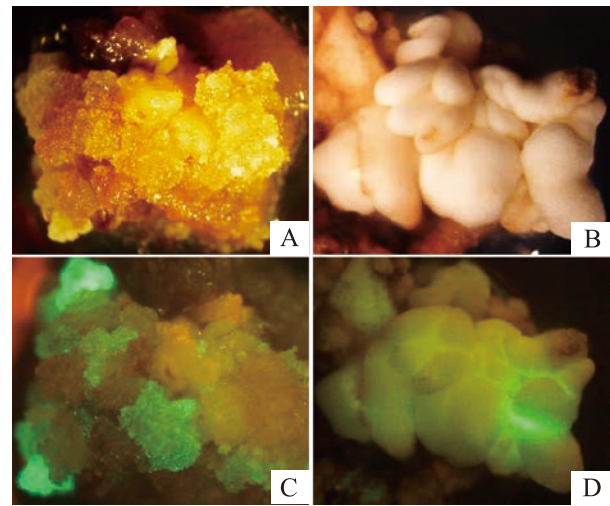

Fig. 4. Callus regeneration (A and C) and SEG (B and D) from sonicated 'Nanko' immature cotyledon explants illustrated chimeric GFP expression. A and B: bright field image, C and D: fluorescence microscope image.
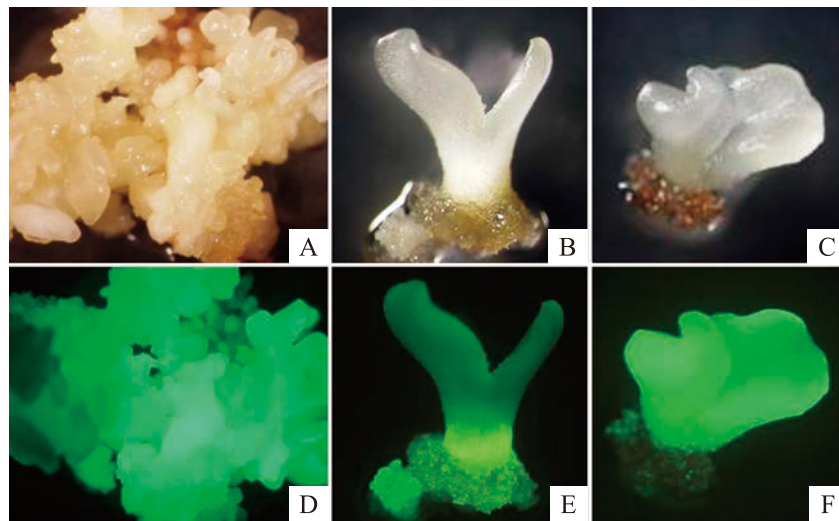

Fig. 5. Somatic embryo cluster with solid GFP expression from 'Nanko' explants treated by sonication. A and D: somatic embryo cluster with GFP expression, B and E: somatic embryo developed into cotyledonary stage, C and F: cotyledon-like structures illustrated irregular development of somatic embryo. A–C: bright field image, D–F: fluorescence microscope image.

30-sec sonication-treated explants.

More somatic embryos with GFP expression were obtained from sonication-treated 'Nanko' immature cotyledons in 2007, especially from May 11 and 14 explants, and more were obtained from May 14 than from May 11 explants, although there was no significant main effects of the explant stage and sonication treatment (Table 3). The highest frequency of SEG with GFP expression was obtained from 40-sec sonication-treated explants of May 14, followed by 20-sec sonication-treated explants of May 14 and 20-sec sonication-treated explants of May 11. Most of the somatic embryos and calli showed chimeric GFP expression (Fig. 4). As mentioned in our previous report (Gao et al., 2010), the somatic embryos could be easily distinguished from calli, which were usually loose and yellow or brown in color, and were usually clustered as a somatic embryo mass and easily detached from the surrounding tissue. When somatic embryos showing GFP expression were isolated from the chimeric cluster and subcultured, some of them could be proliferated and purified to a non-chimeric somatic embryo mass with solid GFP expression (Fig. 5A, D). Occasionally, the somatic embryos developed heart-shaped, torpedo-shaped, and cotyledon stages (Fig. 5B, E), but usually atypical transgenic somatic embryos such as cotyledon-like and leafy structures were observed (Fig. 5C, F).

Discussion

We developed a successful regeneration and genetic transformation system for *P. mume* via SEG from immature cotyledons in our previous study (Gao et al., 2010). However, the transformation efficiency was low. To develop an efficient transformation system for *P. mume*, we need to optimize every step of the transformation from *Agrobacterium* infection to plant regeneration. This study was conducted to improve the first step of transformation, the infection of *Agrobacterium* to cotyledon explants of *P. mume*, using sonication treatment. We identified the most effective sonication duration to obtain the highest expression of the *GFP* reporter gene, named *sGFP(S65T)*. The engineered *sGFP(S65T)* sequence with codons optimal for high expression of eukaryotes, modified by replacement of serine with threonine at position 65, provided up to 100-fold brighter fluorescent signals than the original jellyfish *GFP* sequence in plant cells (Chiu et al., 1996; Haas et al., 1996; Niwa, 1998). The use of the *sGFP(S65T)* reporter gene provides a tool for monitoring in real time the infection of *Agrobacterium* and the regeneration of transgenic tissues. *sGFP(S65T)* driven by the 35S promoter used in this study seemed to be nonfunctional or at most weakly functional in *Agrobacterium*, given that we observed no green fluorescence in the *Agrobacterium* suspension or colony using our detection system. This result is reliable evidence that the fluorescence observed originated from transformed plant cells and not from *Agrobacterium*

cells. Niwa (2003) reported that *sGFP(S65T)* driven by the CaMV35S promoter in pTH2 plasmid was, for unknown reasons, not functional in *Agrobacterium*, so there appears to be no need to introduce an intron into *GFP* to distinguish the fluorescence of transformed plants from that of *Agrobacterium*.

Wounding is an integral step in *Agrobacterium*-mediated transformation, as it allows the bacterium to infect the target tissue. In addition, wounded tissue often produces inducers of the T-DNA transfer process (Stachel et al., 1985). The earliest attempt to use sonication to wound the target tissue to enhance *Agrobacterium* infection was described by Trick and Finer (1997). They found that sonication treatment could increase the transient expression of *GUS* by 100- to 1400-fold in several different plant species. Subsequently, they reported another study on the dynamics and optimization of *Agrobacterium* colonization and infection in the transformation of soybean cotyledons using sonication treatment and a *GFP* reporter (Finer and Finer, 2000). They described that GFP provided a quick, non-destructive method to evaluate, in real time, *Agrobacterium* colonization of cotyledon surfaces as well as infection of internal cells, and that sonication produced extensive microwounds on the surface of plant tissue, allowing increased access by the bacterium to plant cells. In our present study, we evaluated the efficacy of sonication treatment on the infection of *Agrobacterium* to *P. mume* immature cotyledons using *sGFP(S65T)* reporter gene.

This study showed that both transient and stable GFP expression frequencies in sonication-treated explants, irrespective of sonication time from 10 sec to 2 min, were significantly higher than that in standard dipping-treated explants. The intensity and area of GFP fluorescence in sonication-treated explants was also stronger and bigger than in control-inoculated explants. Although the transient GFP expression frequency of younger immature cotyledons (e.g., those of 'Nanko' from May 11 and 14) was not affected by sonication time from 20 to 60 sec, the stable GFP expression frequency decreased with increasing sonication time. This was perhaps because younger immature cotyledons were too soft and weak to bear sonication stress. Liu et al. (2006) reported that treatments that yielded high levels of GUS expression with a minimum sonication treatment duration were most desirable. We concluded that 20 sec of sonication was sufficient to increase foreign gene delivery efficiency into immature cotyledons of early developmental stages, whereas the sonication time can be prolonged when immature cotyledons of later developmental stages are used.

Several researchers reported that sonication treatment significantly decreased the morphogenic potential or shoot proliferation of explants (de Oliveira et al., 2009; Meurer et al., 1998). In this study, we investigated the influence of sonication treatment on SEG from immature cotyledons of *P. mume*. Our results indicated that the SEG was inhibited by sonication treatment

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(Tables 1 and 2), probably owing to damage from sonication wounding. However, the SEG frequencies of sonicated and inoculated explants were increased irrespective of sonication duration as compared with control non-inoculated explants of 'Kotsubu Nanko' from May 29 (Table 2). Considering that the SEG frequencies of inoculated and non-sonicated explants were all higher than those of non-inoculated and non-sonicated control explants in 'Nanko' (Table 3), inoculation with *Agrobacterium* infection seemed to have stimulation effects on SEG, as reported previously (Gao et al., 2010). As higher SEG frequency was often observed with inoculated and sonicated explants of later developmental stages than with inoculated and non-sonicated explants (Tables 2 and 3), the enhanced SEG frequency may be explained by increased *Agrobacterium* penetration into plant tissues by sonication. For younger explants, however, *Agrobacterium* may have readily penetrated tissues even without sonication treatment and the damage caused by sonication had adverse effects on SEG, resulting in reduced SEG frequency, as observed with May 11 'Nanko' explants with 40 sec and 60 sec of sonication (Table 3). These results agreed with our previous study, reporting that enhanced SEG by *Agrobacterium* inoculation was observed with relatively larger cotyledon explants, but that smaller explants were not strong enough to withstand inoculation by *Agrobacterium* (Gao et al., 2010).

We previously reported that 25 mg·L^{-1} Km was not sufficient to inhibit non-transgenic SEG from immature cotyledons of *P. mume*, and accordingly, in this study the Km concentration was increased to 50 mg·L^{-1} in selection media. However, the selection efficiency appeared not to be improved. Although many somatic embryo lines were obtained, only a few showed GFP expression. Most of the somatic embryo lines with GFP expression were obtained from sonication-treated explants, especially from younger immature cotyledons with 20 and 40 sec sonication treatments.

In conclusion, we have described an effective method for improving the infection frequency by *Agrobacterium*, which is one of the key factors to improve the transformation efficiency of *P. mume*. A sonication duration of 20–40 sec was the most suitable for immature cotyledons of appropriate developmental stages. Further studies on plant regeneration from somatic embryos are required to develop an efficient transformation system for *P. mume*.

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