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ORIGINAL PAPER

Optimization of conditions for transient *Agrobacterium*-mediated gene expression assays in *Arabidopsis*

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Abstract Transient genetic transformation of plant organs is an indispensable way of studying gene function in plants. This study was aimed to develop an optimized system for transient *Agrobacterium*-mediated transformation of the *Arabidopsis* leaves. The β-glucuronidase (GUS) reporter gene was employed to evaluate growth and biochemical parameters that influence the levels of transient expression. The effects of plant culture conditions, *Agrobacterial* genetic backgrounds, densities of *Agrobacterial* cell suspensions, and of several detergents were analyzed. We found that optimization of plant culture conditions is the most critical factor among the parameters analyzed. Higher levels of transient expression were observed in plants grown under short day conditions (SDs) than in plants grown under long day conditions (LDs). Furthermore, incubation of the plants under SDs at high relative humidity (85–90%) for 24 h after infiltration greatly improved the levels of transient expression. Under the optimized culture conditions, expression of the reporter gene reached the peak 3 days after infiltration and was rapidly decreased after the peak. Among the five *Agrobacterial* strains examined, LAB4404 produced the highest levels of expression. We also examined the effects of detergents, including Triton X-100, Tween-20, and Silwet L-77. Supplementation of the infiltration media either with 0.01% Triton X-100 or 0.01% Tween-20 improved the levels of expression by approximately 1.6-fold. Our observations indicate that transient transformation of the *Arabidopsis* leaves in the infiltration media supplemented with 0.01% Triton X-100 and incubation of the infiltrated plants under SDs at high relative humidity are necessary for maximal levels of expression.

Keywords *Agrobacterium*-mediated transient assay · *Arabidopsis* · GUS · LBA4404 · Detergent · Daylength

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Introduction

Gene expression studies in homologous or heterologous systems constitute a critical component of molecular biological researches in plants. A wide array of methods or techniques has been developed during the past decade to transfer exogenous genes into plants or specific plant organs and to produce transgenic plants through stable or transient transformation. One benefit of the transient genetic transformation is its rapid process compared to the stable genetic transformation, and we can frequently get the results in days.

Most of the transient expression techniques have been developed by employing particle bombardment of epidermal cells and leaves (Sato et al. 1993; Schweizer et al. 1999) or by protoplast transformation and electroporation- and polyethyleneglycol (PEG)-mediated methods (Lindsey and Jones 1987; Sheen 2001). The particle bombardment is an efficient way of gene delivery applicable to virtually all plant species. However, it suffers from several drawbacks: it usually delivers the gene of interest only to a few cells, and the sizes of the genes are limited (Christou 1997; Fisher and Emans 2000). It also requires expensive and

specialized equipments. The PEG-mediated protoplast transformation can be readily carried out using ordinary lab supplies and equipments. However, the experimental procedures are quite complicated and time-consuming (Yang et al. 2000).

Transient *Agrobacterium*-mediated gene expression assays have been successfully used to analyze gene function, gene silencing, and gene-for-gene interactions between host resistance and pathogen avirulence genes (Baulcombe 1999; Frederick et al. 1998; Kapila et al. 1997; Scofield et al. 1996). They have also been employed to characterize production of recombinant antibodies in tobacco and lettuce (Negrouk et al. 2005; Vaquero et al. 1999). Notably, the experimental procedures are relatively simple and do not require expensive supplies and equipments. In particular, this method efficiently works in many plant species, such as tobacco, potato, tomato, lettuce, pea, grapevine, and *Arabidopsis* (Mestre et al. 2000; Santos-Rosa et al. 2008; Van der Hoorn et al. 2000; Wroblewski et al. 2005).

However, current transient transformation systems used in *Arabidopsis* still need to be improved, because optimal plant culture conditions and affordable supplements have not yet been well-defined (Wroblewski et al. 2005).

Genetic backgrounds of the *Agrobacterial* strains considerably influence the efficiency of transient expression. Different *Agrobacterial* strains are defined by their chromosomal backgrounds and the presence of resident Ti plasmids. Most of the widely used *Agrobacterial* strains originate from two representative wild isolates: C58 (Hamilton and Fall 1971) and Ach5 (Hoekema et al. 1983). The AGL1, EHA105, and GV3101 strains have the C58 origin. The LBA4404 strain, one of the most commonly used laboratory strains, originates from the Ach5 chromosomal background.

It is known that some *Agrobacterial* strains are more virulent than others, depending on the target plant species. Such variations are caused by differences in the ability of the bacterial cells to attach to plant cells or by differences in either bacterial-encoded or plant-encoded T-DNA transfer mechanisms (Nam et al. 1997; Yanofsky et al. 1985a, b). However, it is unknown what host compounds affect the recognition process, the transfer of DNA, and the integration of the T-DNA into the host (Ditt et al. 2001). The T-DNA region of the Ti plasmid carries genes encoding tumor-specific low-molecular-weight compounds, opines (Montoya et al. 1977). Opines are metabolized by the bacterial cells as an important source of carbon and energy.

Agrobacterium Ti-plasmids are classified into several types based on the ways of utilizing the opines: octopine (LBA4404 and GV2260), nopaline (C58C1, GV3100, GV3101, GV3850, A136, and EHA 101), agropine (A281 and A543), and succinamopine (EHA105 and AGL1) types (Dessaux et al. 1992). It seems that specific *Agrobacterial* strains work better than other strains in specific plant species. For example, it has been suggested that the C58C1 strain is most suitable for the transient expression assays in lettuce, tobacco, and *Arabidopsis* (Wroblewski et al. 2005).

Detergents are amphiphilic compounds having measurable aqueous solubility both as aggregates and as monomers. They belong to a class of compounds, collectively called surfactants, which reduce the surface tension within the external surface layers of water by absorbing at the liquid–gas interface. They also reduce the interfacial tension between oil and water by absorbing at the liquid–liquid interface. Silwet L-77 is a unique surfactant in that it can improve cuticular penetration of spray mix into plant surfaces. It is compatible with most pesticides and fertilizer solutions. Notably, it has been reported that Silwet L-77 improves the efficiency of the transient *Agrobacterium*-mediated genetic transformation in *Arabidopsis* (Clough and Bent 1998) and wheat (Cheng et al. 1997; Wu et al. 2003).

Tween 20 is a polysorbate surfactant and used as detergent and emulsifier in a number of biological, pharmaceutical, industrial, and food applications. It is distinguished from other Tween members by the lengths of polyoxyethylene chains and fatty acid ester moieties. Triton X-100 is one of the most commonly used, nonionic detergents in various biochemical applications in order to solubilize proteins.

In this work, we developed an optimized system for transient *Agrobacterium*-mediated transformation of the *Arabidopsis* leaves. The parameters explored included plant culture conditions, *Agrobacterial* genetic backgrounds and cell densities, and effects of detergents. We found that optimization of plant culture conditions is the most important factor among the parameters analyzed. The optimized conditions for the transient expression system will be widely applicable to studies on gene function in *Arabidopsis*.

Materials and methods

Plants and growth conditions

Arabidopsis thaliana line used was Columbia (Col-0) ecotype. *Arabidopsis* plants were routinely grown in a controlled culture room set at 22°C either under LDs (16 h light/8 h dark) or SDs (8 h light/16 h dark) with white light illumination (120 μmol photons/m^2 s) provided by fluorescent FLR40D/A tubes (Osram, Seoul, Korea). For transient expression assays, plant pots were covered with clear polyethylene film for 24 h after infiltration to maintain the infiltrated plants at high humidity before further

growing for 1–6 additional days under identical growth conditions. The relative humidity was retained at approximately 85–90% with covering.

Agrobacterial strains and expression vector

We included five *Agrobacterial* strains, as listed in Table 1, and a binary vector pCAMBIA1304 containing the GUS reporter gene was used. The expression vector comprises: the right and left borders of the Ti region, the Cauliflower Mosaic Virus (CaMV) 35S promoter, the Nos-3′ terminator, and the mGFP and GUS reporter genes. It also contains a kanamycin resistance gene for bacterial selection and a hygromycin phosphotransferase gene for selection in plants.

Infiltration of the *Arabidopsis* leaves

The YEP medium (Bacto trypton 10 g/L, yeast extract 10 g/L, NaCl 5 g/L, pH7.5) was used to culture *Agrobacterial* cells at 28°C. The AGL1 cells harboring the pCAMBIA1304 vector were maintained in YEP media supplemented with rifampicin (25 mg/L), kanamycin (50 mg/L), and ampicillin (100 mg/L). Other *Agrobacterial* cells harboring the pCAMBIA1304 vector were maintained in YEP media supplemented with rifampicin (25 mg/L) and kanamycin (50 mg/L). The *Agrobacterial* cells were cultured in 10 mL YEP media overnight at 28°C with vigorous shaking. Small aliquots of the cell cultures were transferred to fresh medium (1:10 ratio, v/v) and grown until the cell density reaches OD_{600} of 1.0. The cell cultures were centrifuged, and the collected cells were resuspended in the infiltration medium (0.5% D-glucose, 10 mM MES, 10 mM $MgCl_2$, 200 μM acetosyringone) at $OD_{600} = 0.6$ prior to infiltration. Acetosyringone is a phenolic compound that can attract *Agrobacterial* cells to wounded plant tissues via chemotaxis and induce the *Vir* genes to initiate T-DNA transfer (Hiei et al. 1994).

Infiltration of the *Agrobacterial* cell suspensions into the *Arabidopsis* leaves was carried out as described previously (Schob et al. 1997). *Arabidopsis* plants were grown in soil pots at 22°C for 4 weeks under LDs or for 6 weeks under SDs, and the 4th–5th leaves were used for infiltration. Equal volumes, each containing approximately 0.3 mL, of the *Agrobacterial* cell suspension were injected to the abaxial side of leaf with a 1 mL syringe lacking needle. Six injections, three injections on each side of the leaf vein, were carried out for individual leaves. After infiltration, the LD-grown or SD-grown plants were incubated at 22°C for 24 h either under LDs or under SDs, respectively, with or without polyethylene film covering. The film cover was then removed, and the plants were further incubated under identical light regime for 1–6 additional days before histochemical GUS staining and fluorometric measurements of GUS activities. Various detergents, such as Triton X-100 (Roche Diagnostics GmbH, Mannheim, Germany), Tween-20 (Sigma-Aldrich, St. Louis, MO, USA), and Silwet L-77 (Lehle Seeds, Round Rock, TX, USA), were added to the infiltration media at a series of varying concentrations.

β-Glucuronidase (GUS) activity assays

Histochemical GUS staining and fluorometric measurements of the GUS activity were conducted as described previously (Jefferson et al. 1987). The infiltrated leaves were cut into pieces and soaked in the GUS staining buffer (100 mM sodium phosphate, 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 10 mM Na_2EDTA, pH 7.0). They were incubated for 16 h at 37°C. The stained leaf tissues were rinsed with 70% ethanol until pigments, such as chlorophylls, were completely cleared and photographed.

Fluorometric measurements of the GUS activity were carried out using 4-methyl-umbelliferyl-β-D-glucuronide (4-MUG, Sigma-Aldrich) as a substrate. The infiltrated leaf samples were homogenized in the extraction buffer (50 mM sodium phosphate, 10 mM Na_2EDTA, 0.1% Triton X-100, 0.1% sarcosyl, 10 mM β-2-mercaptoethanol, pH 7.0). The homogenates were centrifuged for 15 min at 13,000 rpm, and aliquots of the supernatants, containing

Table 1 *Agrobacterium tumefaciens* strains used in this study

Strains	Chromosomal backgrounds	Marker genes[a]	Ti plasmids	Marker genes[a]	Opines[b]
LBA4404	TiAch5	rif	pAL4404	spec and strep	Octopine
C58C1	C58	rif	Cured	–	Nopaline
GV3101	C58	rif	Cured	–	Nopaline
EHA105	C58	rif	pEHA105 (pTiBo542DT-DNA)	–	Succinamopine
AGL-1	C58, RecA	rif and carb	pEHA105 (pTiBo542DT-DNA)	–	Succinamopine

[a] Antibiotic resistance genes used to select for chromosomal backgrounds or Ti plasmids. rif, Rifampicin resistance; carb, carbenicillin and ampicillin resistance; spec and strep, spectinomycin and streptomycin resistance; –, no marker gene

[b] The opines were grouped according to the opine catabolism of the original progenitor wild-type strain and/or non-disarmed parental Ti plasmid. Note that the classification of *Agrobacterium* strains does not necessarily imply that their disarmed counterparts still make opines

approximately 25 μg proteins, were incubated for 1 h in the extraction buffer containing 1 mM 4-MUG at 37°C. The reaction was terminated by adding 0.2 M Na_2CO_3. Fluorescence measurements were carried out using a fluorescence spectrophotometer (Multiple Plate Reader Victor3, Perkin Elmer, Waltham, MA, USA). The standard was calibrated using 4-methylumbelliferone (4MU, Sigma-Aldrich). Protein concentrations were determined using BSA as standard (Bradford 1976).

Results

Determination of optimal *Agrobacterial* cell density

With an aim of optimizing the conditions for transient *Agrobacterium*-mediated expression assays in *Arabidopsis*, we extensively analyzed the effects of plant culture conditions and different *Agrobacterial* strains as well as those of detergents that are frequently used in plant laboratories. The *Agrobacterial* strains we examined included AGL1, EHA105, C58C1, GV3101, and LBA4404. Whereas the AGL1 strain is resistant to rifampicin and carbenicillin, other strains have an intrinsic resistance to rifampicin. The pCAMBIA1304 vector containing the GUS reporter gene was for quantitation of the expression levels.

It has been known that the cell density of bacterial suspension cultures is important for high transient expression in *Arabidopsis*. Whereas cell suspensions with the cell density of less than $OD_{600} = 0.1$ often result in low transient expression, those with the cell density with above $OD_{600} = 1.0$ result in tissue yellowing or wilting (Wroblewski et al. 2005). To determine the cell density that gives rise to maximal transient expression, we tested several different cell densities at $OD_{600} = 0.3, 0.6, 0.9$, or 1.2. While severe wilting and yellowing symptoms were observed when cell suspensions with $OD_{600} = 1.2$ were used, lower transient expression was obtained using cell suspensions at $OD_{600} = 0.3$ or lower. We obtained the highest transient gene expression with cell suspensions with $OD_{600} = 0.6$–0.9 (Table 2). Notably, the LBA4404 and GV3101 strains showed relatively higher expression at $OD_{600} = 0.6$. In contrast, the EHA105 strain showed the highest transient expression at $OD_{600} = 0.3$. Transient expression using the C58C1 strain was not detectably affected by variations in cell densities, indicating that the optimal cell densities of individual *Agrobacterial* strains are different from each other.

Effects of daylengths and humidity

Environmental factors, such as daylengths and humidity, significantly influence plant growth and physiology. We therefore examined the effects of different daylengths and relative humidity on the efficiency of transient expression. The LBA4404 cells containing the GUS reporter gene was used at $OD_{600} = 0.6$ for infiltration.

Table 2 Effects of different cell densities on the transient expression system

Strain OD	0.3	0.6	0.9	1.2
LBA4404	++	++++	+++	++
C58C1	++	++	++	++
GV3101	+	+++	++	++
EHA105	+++	++	++	+
AGL-1	+	+	++	+

The 4th–5th leaves of *Arabidopsis* plants grown in soil pots under SDs for 6 weeks were infiltrated with *Agrobacterial* cell suspensions. Five different *Agrobacterial* strains at different cell densities were analyzed. Histochemical GUS staining was carried out 3 days after infiltration as described previously (Wroblewski et al. 2005). The numbers of (+) represent the levels of histochemical GUS staining

OD optical density at 600 nm

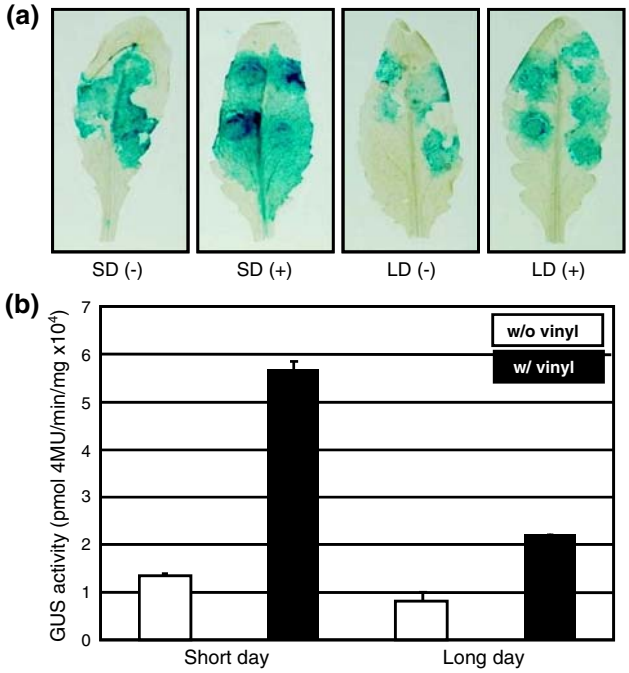

Fig. 1 Effects of different culture conditions on the transient GUS expression. The 4th–5th leaves of the *Arabidopsis* plants grown in soil pots either under LDs for 4 weeks or under SDs for 6 weeks were infiltrated. *Agrobacterial* strain LBA4404 cells containing the GUS reporter gene were infiltrated to the abaxial surfaces of the *Arabidopsis* leaves. **a** Histochemical GUS staining. The infiltrated plants were incubated at 22°C for 24 h under LDs with (+) or without (−) polyethylene film covering. The infiltrated plants were further incubated under identical conditions but without polyethylene film covering. Histochemical GUS staining was carried out 3 days after infiltration. **b** Fluorometric measurements of GUS activities. The measurements were carried out using the leaves shown in Fig. 1a. Three measurements, each consisting of five different leaves, were averaged. *Bars* indicate standard error of the mean

The *Arabidopsis* plants grown either under LDs for 4 weeks or under SDs for 6 weeks were used for infiltration. The infiltrated plants were incubated at 22°C for 24 h either under LDs or under SDs, respectively. The infiltrated plants in soil pots were covered with clear polyethylene vinyl film to maintain a high relative humidity. By doing this, whereas the relative humidity was approximately 50% in the growth chamber, it was maintained at approximately 85–90% with covering. The vinyl film was then removed, and the infiltrated plants were further incubated for 2 more days under identical light regime.

To access the efficiency of transient expression, histochemical GUS staining and fluorometric measurement of GUS activity were carried out. As illustrated in Fig. 1, the level of reporter gene expression was approximately 2 times higher when plants grown under SDs were used for infiltration than those grown under LDs, as determined by fluorometric GUS activity measurements. Notably, the GUS activity was increased significantly when the plants in soil pots were covered with vinyl film both under LDs and SDs. At high relative humidity, the GUS activities were elevated by 4.3-fold and 2.7-fold in the SD-grown and LD-grown plants, respectively (Fig. 1). These observations indicate that use of SD-grown plants for infiltration and maintenance of the infiltrated plants under SDs at high relative humidity for 24 h after infiltration are necessary for higher levels of transient expression in *Arabidopsis*. We therefore used these conditions for further studies.

Effects of incubation times

Expression of the foreign gene continues only for a short time period in the transient system. It is therefore critical to know when the gene expression reaches the peak and thus when the plants should be harvested after infiltration to obtain maximal transient expression.

To determine the optimal incubation time, the infiltrated, LD-grown or SD-grown plants were incubated at 22°C either under LDs or under SDs, respectively, with vinyl film covering for 24 h, the cover was then removed, and the infiltrated plants were further incubated for various time periods upto 7 days under identical light regime. The GUS activities were gradually elevated until 3 days after infiltration. After the peak, they were gradually decreased (Fig. 2), indicating that the highest expression can be obtained in the plants 3 days after incubation.

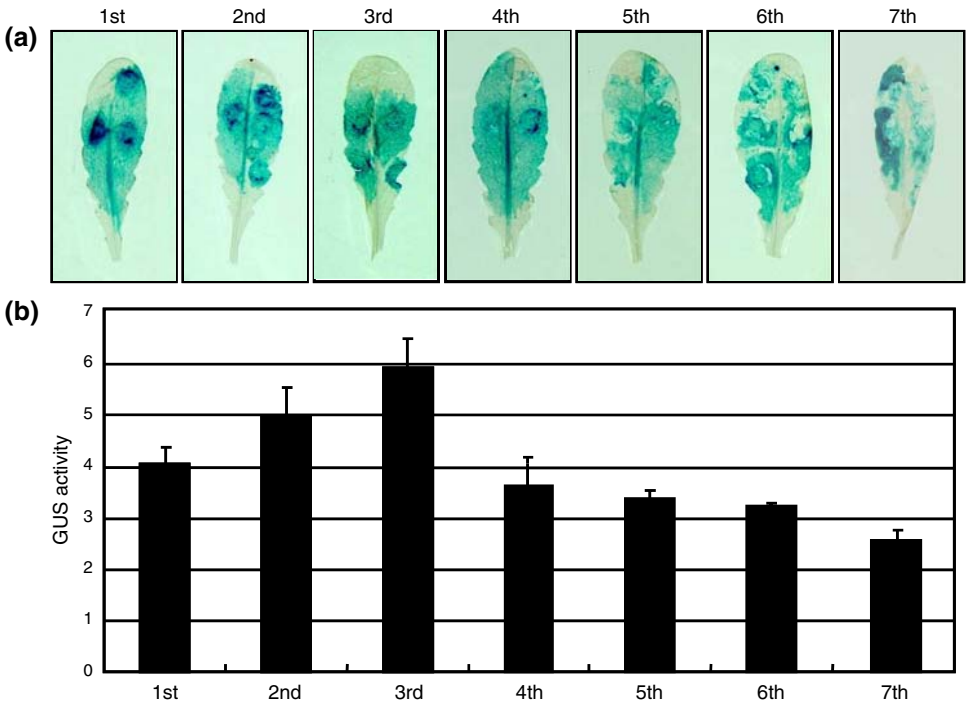

Fig. 2 Levels of transient GUS expression at different time points after incubation. The 4th–5th leaves of the *Arabidopsis* plants grown in soil under SDs for 6 weeks were infiltrated. *Agrobacterial* strain LBA4404 cells containing the *GUS* reporter gene were infiltrated to the abaxial surfaces of the *Arabidopsis* leaves. The infiltrated plants were incubated at 22°C for 24 h under SDs with polyethylene film covering. The infiltrated plants were further incubated under identical conditions but without polyethylene film covering. The leaves were harvested at the indicated time points after infiltration and subject to histochemical GUS staining and fluorometric measurements of GUS activities. **a** Histochemical GUS staining. **b** Fluorometric measurements of GUS activities. Measurements of the GUS activities were carried out as described in Fig. 1b. *Bars* indicate standard error of the mean

Determination of most effective *Agrobacterial* strains

Our data indicated that the highest reporter gene expression occurs when the infiltrated SD-grown plants were incubated at 22°C under SDs at high relative humidity for 3 days after incubation. A question was whether the optimized conditions established with the LBA4404 strain are also valid for other *Agrobacterial* strains.

We examined the levels of transient expression under the optimized conditions but using different *Agrobacterial* strains. Notably, the levels of transient expression, as determined by histochemical GUS staining and fluorometric GUS activity measurements, were highly variable, depending on the strains used. The LBA4404 strain exhibited the highest expression among the strains examined (Fig. 3). The levels of transient expression in the plants infiltrated with the LBA4404 cells were higher by approximately1.6-fold than those obtained with the nopaline strains (C58C1 and GV3101) and by higher than 3.5-fold those achieved with the succinamopine strains (EHA105 and AGL1). These observations revealed that the LBA strain is a choice of *Agrobacterial* strains for transient expression in *Arabidopsis*.

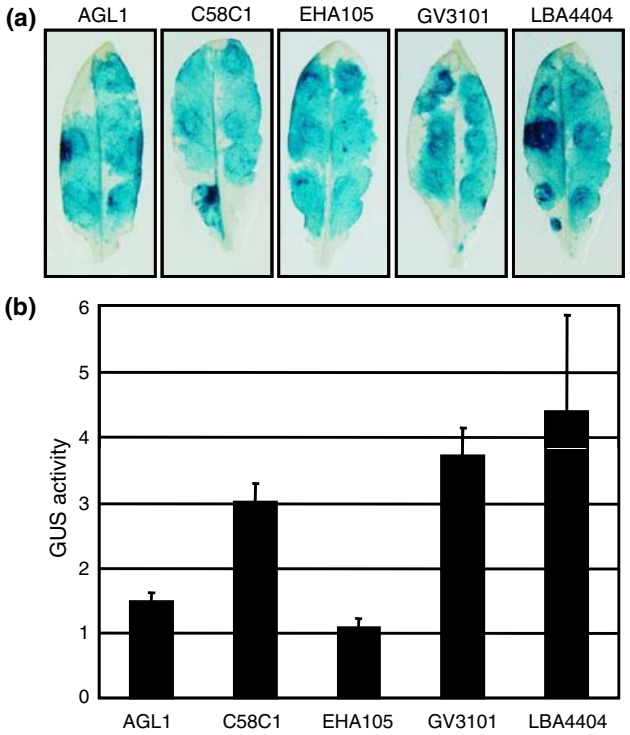

Fig. 3 Effects of different *Agrobacterial* strains on the transient GUS expression. Incubation conditions of the plants were as described in Fig. 2. Five different *Agrobacterial* strains, including AGL1, EHA105, C58C1, GV3101, and LBA4404, containing the GUS reporter gene were used for infiltration assays. Histochemical GUS staining and fluorometric measurements of GUS activities were carried out 3 days after infiltration. **a** Histochemical GUS staining. **b** Fluorometric measurements of GUS activities. Measurements of the GUS activities were carried out as described in Fig. 1b. *Bars* indicate standard error of the mean

Effects of detergents

Some chemicals, such as acetosyringone, L-cysteine, L-glutamine, and azaserine, have been reported to influence transformation efficiency and transient gene expression in many plant species (Olhoft and Somers 2001; Rogowsky et al. 1987; Roberts et al. 2003; Stachel et al. 1986). It was also envisioned that detergents would also affect both the stable and transient expression systems.

We investigated the effects of several detergents on the transient *Agrobacterium*-mediated expression in the *Arabidopsis* leaves. We examined different concentrations of Triton X-100 (Fig. 4a), Tween-20 (Fig. 4b), or Silwet L-77 (Fig. 4c). The detergents were added to the infiltration media at different concentrations. Overall, the effects of the detergents were somewhat variable. However, it is evident that higher concentrations of the detergents, such as 0.5% Triton X-100 and 0.05% Silwet L-77, drastically reduced the reporter gene expression, apparently by causing severe wilting and necrosis symptoms (Fig. 4). The detergents exhibited some degree of positive effects on the reporter gene expression when they were used at lower concentrations. In all the detergents tested, the levels of transient expression were highest at a concentration of 0.01%.

Comparison of the GUS activities between plant samples treated with different detergents revealed that treatments with 0.01% Triton X-100 and 0.01% Tween-20 showed the most promotive effects. However, the effects of Silwet-L-77 were relatively lower than those of Triton X-100 and Tween-20. Together, these observations indicate that detergents, such as Triton X-100 and Tween-20, are also a factor of consideration for the transient *Agrobacterium*-mediated expression assays in *Arabidopsis*, particularly when highest expression is required.

Discussion

Arabidopsis thaliana is an annual herb in which floral induction is regulated by multiple flowering genetic pathways that respond to diverse environmental signals, such as changes in daylengths and vernalization (exposure to cold temperature for a long time period). *Arabidopsis thaliana* belongs to a long day plants: its flowering is promoted under LDs. In other words, it is drastically delayed and vegetative growth is extended under SDs (Greb et al. 2003; Melzer et al. 2008). The molecular mechanisms underlying this discrepancy are currently unclear. Considering that the leaf cells of plants undergoing reproductive growth are developmentally older than

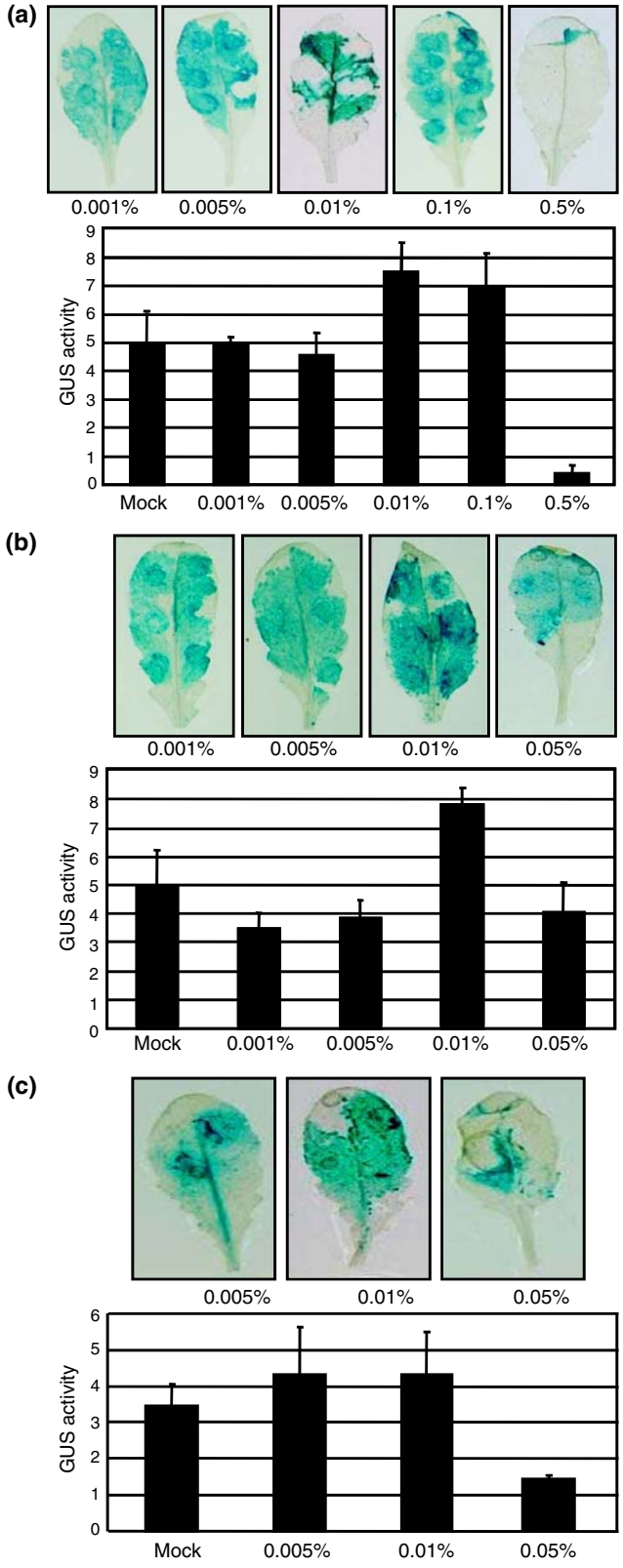

Fig. 4 Effect of different detergents on the transient GUS expression. The plant materials used were as described in Fig. 3. The infiltration media were supplemented with varying concentrations of Triton X-100 (**a**), Tween-20 (**b**), or with Silwet-L77 (**c**). *Agrobacterial* strain LBA4404 cells containing the GUS reporter gene were used. Histochemical GUS staining and fluorometric measurements of the GUS activities were carried out as described in Fig. 1. Bars indicate standard error of the mean. **a** Effects of Triton X-100. It was included in the infiltration media at the final concentrations of 0, 0.001%, 0.005, 0.01, 0.1, or 0.5%. **b** Effects of Tween-20. It was included in the infiltration media at the final concentrations of 0, 0.001, 0.005, 0.01, or 0.05%. **c** Effects of Silwet-L77. It was included in the infiltration media at the final concentrations of 0, 0.005, 0.01, or 0.05

those in their vegetative growth, it is envisioned that developmental age of the leaves is critical for higher levels of transient expression.

Humidity in the air surrounding the plants is an important environmental factor that influences diverse aspects of plant growth and development. The most direct effect of humidity is exerted at the rate of transpiration or evaporation of water from the leaves. Both environmental and intrinsic factors significantly affect the rate of transpiration. Environmental factors influencing transpiration rate include relative humidity, air movements, ambient temperature, water supply from the roots, and light intensity. Endogenous regulatory factors include leaf surface area, thickness of epidermis and cuticle layer, stomatal frequency and size, and distribution of stomata (Ares and Fownes 1999; Satisha et al. 2006). Dry conditions are certainly stressful to plants, and physiological activities would be reduced in the leaf cells under these conditions. This view entails that the cell viability is also important for high transient expression.

Under SDs with high relative humidity, vegetative growth is sustained without flowering, but the vegetative-to-reproductive transition is delayed. Transpiration rate would be lower under SDs than under LDs, perhaps due to shorter daylength, supporting high cell viability. It is therefore anticipated that individual leaf cells may be in a physiologically more active state when plants are grown under SDs than under LDs. Under SDs with high relative humidity, the physiological activities of the cells are higher, and thus gene expression would be promoted. Alternatively, the cells undergoing active vegetative growth would be more susceptible to *Agrobacterial* infections (Gelvin 2003).

Our data indicate that levels of transient expression were higher in plants grown under SDs than in plants grown under LDs. Furthermore, incubation of the plants at high relative humidities (85–90%) for 24 h after infiltration greatly improved the levels of transient expression.

Surfactants are known to function either as enhancers of cuticle penetration of spray mix by making the plant cuticular membrane susceptible to solute transfer or by acting as cosolvents, thereby enhancing the movement of herbicidal active ingredients into the plant cells (Madhou et al. 2006). There are several types of surfactants,

depending on their charges: anionic, cationic, amphoteric, and nonionic surfactants. The nonionic surfactants are most commonly used in the horticulture, because they have less toxic to plant growth.

The herbicidal chemicals are used to suppress the growth of unwanted plants. Application of surfactants together with the herbicide solution is often an effective method of improving the performance of herbicides. In *Arabidopsis*, it has been found that plants treated with 0.02% NUL1026, a nonionic surfactant, undergoes premature leaf senescence, since the chemical activates genes involved in jasmonic acid (JA) biosynthesis, including *LYPOXIGENASE 3* (*LOX3*, At1g17420), *ALLENE OXIDE SYNTHASE* (*AOS*, At5g42650), *ALLENE OXIDE CYCLASE 2* (*AOC2*, At3g25780) (Madhou et al. 2006). As a result, JA biosynthesis is induced, and it acts an activator of leaf senescence, signifying that although surfactants improve the efficiency of transient expression by facilitating infection of the plant tissues by *Agrobacterial* cells, its doses should be tightly controlled.

We also obtained similar results on the effects of a few detergents, such as Triton X-100, Tween 20, and Silwet L-77 on the levels of transient expression. When they were applied at a concentration of 0.01% or lower, the level of transient expression was markedly elevated. However, concentrations of higher than 0.05% caused leaf wilting and yellowing, resulting in drastic reduction of gene expression. It is interesting that while Silwet L-77 is most effective in the *Arabidopsis* floral transformation system (Chung et al. 2000; Clough and Bent 1998), it is least effective in the transient expression assays in the leaves.

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