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Original Paper

A novel autofluorescence-based selection of calli amenable to *Agrobacterium*-mediated transformation in onion (*Allium cepa* L.)

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Abstract Suppression of lachrymatory factor synthase (LFS) activity in onion (*Allium cepa* L.) is expected not only to reduce lachrymatory factor released from the disrupted tissues, but also to increase thiosulfinate and their derivatives associated with the characteristic flavor and various health effects. To test this hypothesis, we used transgenic methods to obtain non-lachrymatory onions in which the *LFS* gene was silenced by RNA interference (RNAi). In transformation experiments, we found that onion calli generated after preconditioning of the suspension-cultured calli on a solid medium were amenable to *Agrobacterium*-mediated transformation. These calli were observed without or with low levels of yellowish autofluorescence under the fluorescence microscope, and were easily distinguished from the recalcitrant calli having a characteristic yellowish autofluorescence. The frequency of transient green fluorescent protein (GFP) expression significantly increased when these amenable calli were infected with *Agrobacterium* harboring the plasmid carrying a GFP expression cassette. Southern blot analysis of adaptor ligation PCR products revealed that 4 independent transgenic lines were obtained. The successful transfer of the RNAi construct was confirmed by using PCR. Analysis of the 4 transgenic lines confirmed that the levels of *LFS* gene transcript, LFS protein, and LFS enzyme activity were reduced to 3.2–11.0%, 0.09–20.7%, and 0.3–10.1%, respectively, of the wild-type control. The thiosulfinate assay suggested that a significant increase in thiosulfinate formation could be expected in the onion bulb extracts in which LFS enzyme activity was suppressed to approximately 1% or less than that of the wild-type control.

Key words: *Agrobacterium tumefaciens*, lachrymatory factor synthase, onion, RNA interference, transformation.

When we cut onion bulbs, tear is shed from our eyes. The lachrymatory sensation is caused by a lachrymatory factor (LF), which has been identified as propanthial *S*-oxide (Brodnitz and Pascale 1971). The LF had long been believed to be spontaneously produced in the disrupted tissues of the onion from 1-propenylsulfenic acid (1-PS), a breakdown product of *trans*-(+)-*S*-1-propenyl-L-cysteine sulfoxide (PRENCSO) by alliinase. The discovery of lachrymatory factor synthase (LFS), an enzyme obligatorily necessary for the conversion of 1-PS to the LF (Imai et al. 2002), raised the possibility of developing tearless onions by suppressing the LFS rather than alliinase. The LFS-suppressed tearless onions are expected to have additional values, because availability of 1-PS as a precursor of thiosulfinates and their derivatives associated with the characteristic flavor and the health effects of onions (Griffiths et al. 2002) is likely to increase in the disrupted tissues (Figure 1). As lack of LF generation and increase in thiosulfinate formation were demonstrated *in vitro* by omission of LFS from a model reaction system (Imai et al. 2002, 2006), to confirm these in the onion bulbs *in vivo*, we developed tearless onions by suppressing the expression of the *LFS* gene using RNA interference (RNAi).

Various protocols for tissue culture, plant regeneration, and transformation of onions have been reported using mature embryos (Zheng et al. 1998, 2001a, 2005), immature embryos (Eady et al. 1998a, 1998b, 2000, 2003), and calli derived from seedling radicles (Ashwath et al. 2006). Among these materials, seedling radicles may be superior to other 2 materials for the high transformation rate and ease of manipulation. However, propagation of the materials is still a labor intensive and technically difficult task, because it requires a considerable amount of aseptic manipulation, such as cutting the seedling radicles and transferring them from one medium to another. Recently, we came across an efficient procedure for callus induction, propagation via a suspension culture, and plant regeneration, in which surface sterilized seeds were

Abbreviations: AL-PCR, Adaptor ligation PCR; GFP, green fluorescent protein; LF, lachrymatory factor; LFS, lachrymatory factor synthase; PRENCSO, *trans*-(+)-*S*-1-propenyl-L-cysteine sulfoxide; 1-PS, 1-propenylsulfenic acid; RNAi, RNA interference.

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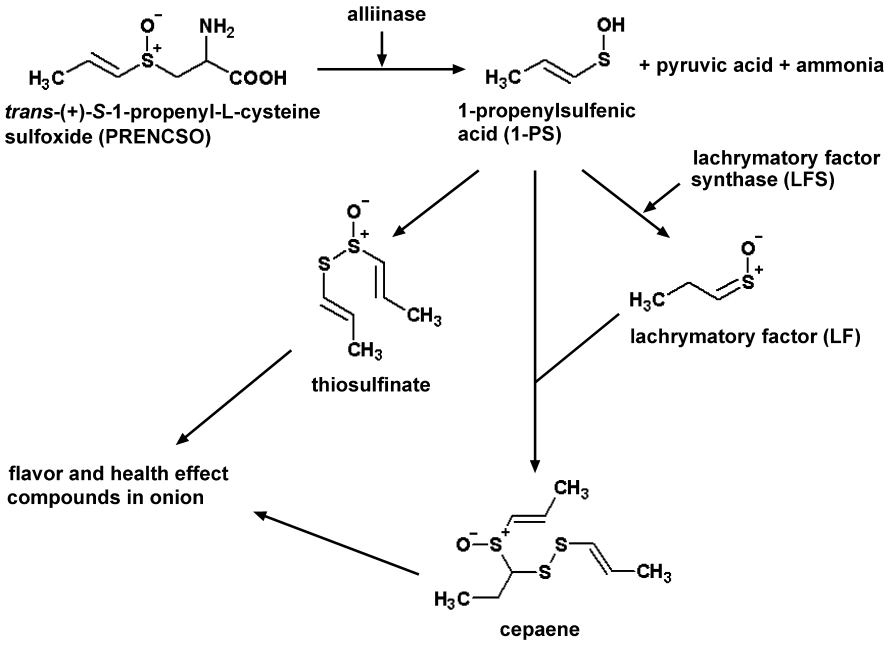

Figure 1. Synthetic pathways of LF, cepaene, and putative di-1-propenyl thiosulfinate from PRENCSO.

Table 1. List of media used for experiment

Culture medium	Ingredients
SCC	MS salts and vitamins (Murashige and Skoog 1962), 0.1 M sucrose, 1 g l^{-1} casein hydrolysate, 50 μM 4-FPA, 1 μM 2iP, 2 g l^{-1} gellan gum
LCC	MS salts and vitamins, 0.1 M sucrose, 1 g l^{-1} casein hydrolysate, 10 mM MES, 50 μM 4-FPA, 1 μM 2iP
PCO	MS salts and vitamins, 0.1 M sucrose, 20 g l^{-1} glucose, 1 g l^{-1} casein hydrolysate, 10 mM MES, 50 μM 4-FPA, 1 μM 2iP, 2g l^{-1} gellan gum
SCO	MS salts and vitamins, 0.1 M sucrose, 20 g l^{-1} glucose, 1 g l^{-1} casein hydrolysate, 10 mM MES, 50 μM 4-FPA, 1 μM 2iP, 40 mg l^{-1} acetosyringone, 2 g l^{-1} gellan gum
LCO	MS salts and vitamins, 0.1 M sucrose, 1 g l^{-1} casein hydrolysate, 10 mM MES, 50 μM 4-FPA, 1 μM 2iP, 40 mg l^{-1} acetosyringone
SE(-HG)	MS salts and vitamins, 0.1 M sucrose, 1 g l^{-1} casein hydrolysate, 10 mM MES, 50 μM 4-FPA, 1 μM 2iP, 500 mg l^{-1} cefotaxime, 2 g l^{-1} gellan gum
SE	MS salts and vitamins, 0.1 M sucrose, 1 g l^{-1} casein hydrolysate, 10 mM MES, 50 μM 4-FPA, 1 μM 2iP, 500 mg l^{-1} cefotaxime, 50 mg l^{-1} hygromycin B, 2 g l^{-1} gellan gum
RE	MS salts and vitamins, 0.1 M sucrose, 1 g l^{-1} casein hydrolysate, 10 mM MES, 1 μM 2iP, 500 mg l^{-1} cefotaxime, 50 mg l^{-1} hygromycin B, 2 g l^{-1} gellan gum, 5 g l^{-1}agar
RT	MS salts and vitamins, 0.1 M sucrose, 1 g l^{-1} casein hydrolysate, 10 mM MES, 2 g l^{-1} gellan gum

MES, 2-(*N*-morpholino)-ethane sulfonic acid; 4-FPA, 4-fluorophenoxyacetic acid; 2iP, 6-(γ,γ-dimethylallylamino)purine; acetosyringone, 3′,5′-dimethoxy-4′-hydroxyacetophenone

directly sown on a callus induction medium containing 4-fluorophenoxyacetic acid as auxin (Tanikawa et al. 1996). The procedure was successfully applied to 22 cultivars of onion (Tanikawa et al. 1998). However, no successful *Agrobacterium*-mediated transformation was observed, using the calli prepared according to their method. Further studies were needed to develop onion calli amenable to *Agrobacterium*-mediated transformation.

In this paper, we report the development of tearless onion bulbs in which expression of the *LFS* gene was suppressed by RNAi using calli amenable to *Agrobacterium*-mediated transformation. In addition, results of genetic and biochemical analyses of the transgenic bulbs are presented and discussed.

Materials and methods

Plant materials

Cultivar of onion (*Allium cepa* L. cv. Senshu-Chu-Koudakaki) was used in transformation experiments. Callus induction and propagation of light yellow and large (LL) type calli via a suspension culture of onion were performed as described by Tanikawa et al. (1998). Media used for tissue culture are listed in Table 1. The LL type calli were segmented into small pieces (2–3 mm in diameter) and cultured on PCO medium at 25°C in darkness for 1–2 weeks for preconditioning before *Agrobacterium* infection.

Agrobacterium-mediated transformation

Agrobacterium tumefaciens LBA4404 containing the binary vector pLFSRNAi was used for transformation. The T-DNA region of the plasmid is shown in Figure 2. NOS promoter-driven neomycin phosphotransferase II gene (NPTII) was derived originally from pBI121 (Jefferson et al. 1987). The PCR-amplified sense and antisense fragments comprising nucleotides 102–559 of the onion *LFS* gene (GenBank Accession number AB089203) were separated by the first intron fragment of the *FAD2* gene (Smith et al. 2000). This RNAi construct was cloned into the T-DNA region of the plasmid pBI121 by replacing β-glucuronidase (*uidA*) gene. The green fluorescent protein (GFP) expression cassette amplified from the 35Somega-sGFP (S65T) plasmid (Chiu et al. 1996) and the hygromycin resistance cassette in the plant transformation vector pPCV91 (Strizhov et al. 1996) were also inserted in the T-DNA region of the above-mentioned plasmid. The resulting plasmid, pLFSRNAi was introduced into *Agrobacterium tumefaciens* LBA4404 as described by Ditta et al. (1980).

Agrobacterium was grown at 28°C for 3 days on MinA medium (Miller 1972) supplemented with 400 $\mu g\,l^{-1}$ kanamycin. The bacteria were collected with a small spoon and suspended in LCO medium at an optical density of 0.1–0.2 at 600 nm. Approximately 50 clumps of calli were immersed in 40 ml of bacterial suspension for 2 min, followed by paper drying. Twenty-four explants were cultured on SCO medium at 25°C in darkness for 3–6 days. In transformation experiments using the preconditioned calli, the calli were selected on the basis of autofluorescence levels by observation under a fluorescence stereomicroscope. Duplicate transformation experiments (Experiment 1 and 2) were performed using the calli without or low levels of autofluorescence. Selected calli were segmented into small pieces (2–3 mm in diameter), and cocultured as described above. After coculture, the materials were rinsed with 0.5 $g\,l^{-1}$ cefotaxime, placed onto SE(-HG) medium, and incubated for 1 week. The calli showing transient GFP expression were selected and transferred onto SE medium for selection of the transformed calli. The calli were transferred to fresh SE medium 2–3 times at 1- to 2-week intervals, depending on the growth rate of the calli. After 4–6 weeks of selection, the calli showing stable GFP expression were transferred to RE medium for plant regeneration, and cultured at 25°C under a 12 h light (40 $\mu mol\,m^{-2}\,s^{-1}$)/12 h dark. Regenerated plants were transferred to RT medium for root induction, eventually transferred to soil pots, and cultured in a growth chamber under a 14 h light (270 $\mu mol\,m^{-2}\,s^{-1}$ at 25°C)/10 h dark (at 20°C) until bulbs were obtained.

Analyses of the transgenic plants

Genomic DNA was extracted from leaf tissues of wild-type control and putatively transformed onions using the DNeasy Plant Mini Kit according to the manufacturer's protocol (QIAGEN, Germany). The transferred genes in the onions were examined by PCR analysis using the following 3 primer pairs: 35SF (5′-ATCTACCCGAGCAATAATCTCCAGG-3′) and ACEF (5′-TGGAGGGTCCTGAGCACAAG-3′), ACER (5′-CTCTTCGATTTTCTGACCTATCTCAGTAGC-3′) and INTR (5′-GGAGAAATTCACAGAGCAGGAGC-3′), and INTF (5′-GTGGCAATCCCTTTCACAACCTG-3′) and NTR (5′-TGCGGGACTCTAATCATAAAAACCCAT-3′). The annealing positions and orientations of these primers in the T-DNA region are shown in Figure 2. The 25 μl reaction solution comprised 25 ng of template DNA, 0.5 μM of each primer, 0.2 mM of dNTP mixture, 1× PCR Buffer II, 1.5 mM $MgCl_2$, and 0.625 units of AmpliTaq GOLD DNA polymerase (Applied Biosystems, USA). The PCR conditions were as follows: initial denaturation at 94°C for 10 min, followed by 40 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min, and a final extension at 72°C for 10 min.

Adaptor ligation PCR (AL-PCR) was carried out using the LA PCR *in vitro* cloning kit according to the manufacturer's protocol (TaKaRa-Bio, Japan). Briefly, the total DNA extracted from frozen leaf tissue was digested with *Hin*dIII and then ligated with the *Hin*dIII cassette. For amplification of the T-DNA insertion site, the first PCR was carried out with the cassette C1 primer (TaKaRa-Bio) and primer tfsp67f (5′-CATTGCCCAGCTATCTGTCACTTCATCGAAAG-3′) containing the CaMV 35S promoter sequence of the GFP expression cassette. The second (nested) PCR was performed with the cassette C2 primer (TaKaRa-Bio) and inner primer gfp118f (5′-TACGGCAAGCTGACCCTGAAGTTCATCTG-3′). Thermal cycling of these PCRs was performed for 30 cycles of 10 s at 98°C and 15 min at 68°C. The amplified products were separated on a 0.7% agarose gel and transferred to the Hybond-N+ blotting membrane (GE Healthcare, UK). The *sGFP(S65T)* fragment was used as a hybridization probe and was directly digoxigenin-labeled by a PCR process using the PCR DIG Probe Synthesis Kit (Roche, Switzerland). Hybridization, washing, and detection were performed

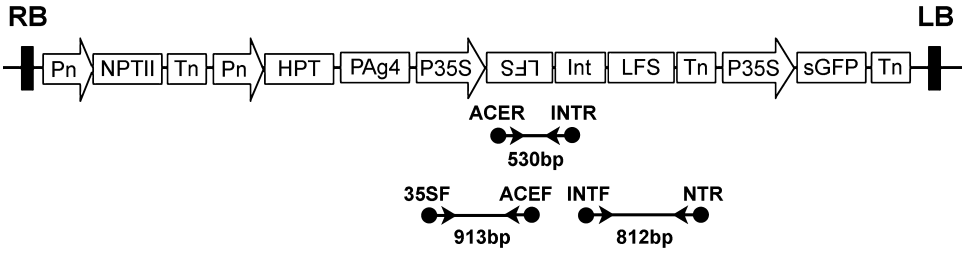

Figure 2. T-DNA region of *LFS* silencing plasmid, pLFSRNAi, constructed from pBI121 binary vector. RB, right border; Pn and Tn, promoter and terminator of nopaline synthase gene; NPTII, neomycin phosphotransferase II gene; HPT, hygromycin phosphotransferase gene; PAg4, polyadenylation signal sequences from gene 4 of T_L-DNA; P35S, cauliflower mosaic virus 35S promoter; LFS, sense and antisense (inverted) fragment of the *LFS* gene; Int, a first intron fragment of *FAD2* gene; sGFP, *sGFP(S65T)* gene; LB, left border. The arrows indicate annealing positions and orientations of the primers used for the PCR analysis.

according to the instruction manual of DIG labeling and detection system with CDP-Star (Roche, Switzerland).

Determinations of LFS enzyme activity and total protein

Frozen onion powder was re-suspended in ice-cold phosphate-buffered saline (PBS) (137 mM NaCl, 8.1 mM $Na_2HPO_4 \cdot 12H_2O$, 2.68 mM KCl and 1.47 mM KH_2PO_4). After centrifugation at 14,000×*g* for 5 min at 4°C, the supernatant was used for determinations of the LFS enzyme activity, total protein, and LFS protein. For the LFS enzyme activity assay, 10 μl of the supernatant and 40 μl of garlic alliinase (50 units ml^{-1}) were mixed in a tightly closed vessel. The assay reaction was initiated by the addition of 20 μl of PRENCSO solution (20 mg ml^{-1}), and the reaction mixture was incubated at room temperature for 3 min. One μl of the reaction mixture was injected into the HPLC system equipped with an ODS column (4.6φ×250 mm, Senshu Scientific Co., Japan) to determine the peak area of the generated LF. The mobile phase was 30% (v/v) acidic methanol, and the HPLC was conducted at a flow rate of 0.6 ml min^{-1} at 35°C, and the eluate was monitored at 254 nm. The total protein in the enzyme extract was determined by the Bradford method (Bradford 1976) using bovine gamma globulin as the standard. Duplicate measurements were made for each sample and the peak area was converted to the amount of the enzyme activity per mg of the total protein in the extract. One unit of the enzyme activity was defined as the amount of enzyme which generated 1 μmole of LF in a 1 min reaction.

Sandwich enzyme-linked immunosorbent assay (ELISA) for determination of LFS protein

An antibody against LFS was raised in rabbits by Operon Biotechnologies (Tokyo, Japan) using a recombinant LFS (rLFS) protein prepared according to Imai et al. (2002). Affinity-purified anti-LFS antibody was used as the capture layer in a sandwich ELISA. For the detection antibody, the anti-LFS antibody was biotinylated using Biotin Labeling Kit-NH2 according to the manufacturer's protocol (Dojindo Mol. Technol., USA).

The sandwich ELISA assay was performed as follows: 96-well microtiter ELISA plates (Thermo Fisher Scientific Inc., USA) were coated with the anti-LFS capture antibody in a 50 mM sodium carbonate/bicarbonate buffer (pH 9.6), and blocked with 10 g l^{-1} bovine serum albumin in the same buffer. After the addition of either diluted samples or standards, the wells were incubated with the biotin-labeled anti-LFS detection antibody for 2 h, followed by streptavidin-horseradish peroxidase for 1 h. The wells were washed 3 times with PBS containing 10 g l^{-1} Tween 20 before each step. After development with 3,3′,5,5′-tetramethylbenzidine for 40 min, the color was measured using Precision Microplate Reader Emax (Molecular Devices, USA). The quantification range of the ELISA was 4–4000 ng ml^{-1} of rLFS protein as the standard.

Quantitative reverse transcription (RT)-PCR for determination of LFS mRNA

Total RNA was extracted from the frozen powder of bulb tissue using the RNeasy Plant Mini Kit according to the manufacturer's protocol (QIAGEN, Germany). cDNA was synthesized with the SuperScript III First-Strand Synthesis System for RT-PCR Kit (Invitrogen, USA) and used as a template for quantitative RT-PCR amplification with the following primer pairs: AcLFS01f (5′-GTGGAGGGTCCT-GAGCACAA-3′) and AcLFS01r (5′-TGCTGCAGAATCT-CGGTGAA-3′) for the *LFS* transcript, and AcUbq01f (5′-CCCTTGCTGACTATAACATCCAGAA-3′) and AcUbq01r (5′-AGTCTTTCCCGTCAATGTCTTCA-3′) for the onion ubiquitin-like transcript (GenBank AA451597). The onion ubiquitin-like transcript was used as an internal standard for normalization. Quantitative PCR was performed using the QuantiTect SYBR Green PCR Kit (QIAGEN, Germany) on the ABI Prism 7700 Sequence Detection System (Applied Biosystems, USA). The amplification conditions consisted of 2 min at 50°C, 15 min at 95°C, and 45 cycles of 15 s at 95°C and 1 min at 60°C. Triplicate measurements were made for each sample.

Determination of LF production in macerated onion bulb tissue

Macerated onion samples were obtained through grinding in liquid nitrogen using a mortar and pestle. Mixtures of 0.1 g of macerated onion bulb tissue and 500 μl of water were incubated at room temperature. LF productions in those mixtures were analyzed over time by HPLC, which was conducted similarly for determination of LFS enzyme activity. We calculated the LF production from the maximum LF peak area obtained in a serial measurement.

Colorimetric determination of thiosulfinate

PRENCSO–derived thiosulfinate were determined by using a modified colorimetric assay (Eady et al. 2008). The frozen onion powder (0.3 g) was suspended in 0.6 ml of ice-cold 10 g l^{-1} glycine and incubated at 25°C for 20 min. After centrifugation at 12,000×*g* or 10 min, 0.2 ml of the supernatant was added to 0.02 ml of 0.02% (v/v) formaldehyde and incubated at 40°C for 6 h. The difference in absorbance at 520 nm and 600 nm was measured.

Determination of PRENCSO and alliinase activity

PRENCSO levels were determined as described by Fritsch and Keusgen (2006) with a slight modification. A methanol extract of frozen onion powder was dried and resuspended in distilled water, and an aliquot (1 μl) was subjected to HPLC analysis. Briefly, the ODS column (4.6φ×250 mm, Senshu Scientific Co, Tokyo, Japan) was maintained at 35°C. The mobile phase was 0.1% (v/v) trifluoroacetic acid in water, and the determination was conducted at a flow rate of 0.6 ml min^{-1} and detection wavelength of 230 nm. Alliinase activity levels were determined by the methods of Schwimmer et al. (1963).

Results

Distinction of the calli amenable to Agrobacterium-mediated transformation

Embryogenic calli were induced from the radicles of germinated onion seeds on SCC medium and vigorously growing light yellow and large (LL) type calli were proliferated by suspension cultures of the embryogenic calli in LCC medium as described by Tanikawa et al.

Table 2. Transient expression of GFP in onion calli cocultured with *Agrobacterium*

Calli type	Number of pieces of calli 1–6 days after coculture		
	GFP+[a]	Cocultured	(%)
Embryogenic calli	0	295	0
LL type calli	0	1144	0
Preconditioning-cultured calli	56	312	18
Calli with 0–40% autofluorescence	273	504	54
Calli with 40–60% autofluorescence	94	424	22
Calli with 60–100% autofluorescence	4	144	3

[a] Strongly expressing GFP.

(1998). Embryogenic calli and LL type calli were then cocultured with *Agrobacterium* and transient GFP expression was examined 1–6 days after coculture. There was no expression of GFP in those two types of calli (Table 2), we had to modify the transformation protocol.

LL type calli were segmented into small pieces of 2–3 mm in diameter with a scalpel and forceps. The segmented callus pieces were cultured further for 1–2 weeks for preconditioning on PCO medium. The calli that showed substantial growth during this preconditioning period were segmented again into pieces of 2–3 mm in diameter, and were subjected to coculture with *Agrobacterium*. During this coculture period, approximately 18% of the 312 calli exhibited transient GFP expression under the fluorescence stereomicroscope (with the GFP filter block) (Table 2). We further observed that the calli with transient GFP expression had a dark brown appearance (Figure 3A), whereas the calli without transient GFP expression exhibited a yellowish autofluorescence (Figure 3B). We presumed that the calli with the dark brown appearance contained little or no autofluorescence compared to calli exhibiting yellowish autofluorescence. This suggested that the frequency of transient GFP expression was dependent to some degree on the presence of the yellowish autofluorescence. This observation enabled us to further investigate our transformation protocol.

After classifying the callus populations into 3 groups based on the degree of yellowish autofluorescence, we separately treated each group with *Agrobacterium*. In the callus population that contained less than 40% of autofluorescing calli, more than half of the calli (273/504, 54%) showed transient GFP expression (Table 2). However, in the callus populations that contained 40–60% and 60–100% autofluorescing calli, only 22% (94/424) and 3% (4/144) of the calli showed transient GFP expression, respectively. Thus, by selecting populations exhibiting low levels of autofluorescence for coculture, we were able to significantly improve the frequency of transient GFP expression.

Approximately 6–14% of the calli that exhibited strong transient GFP expression exhibited stable GFP expression even after further growth on SE(-HG) and SE

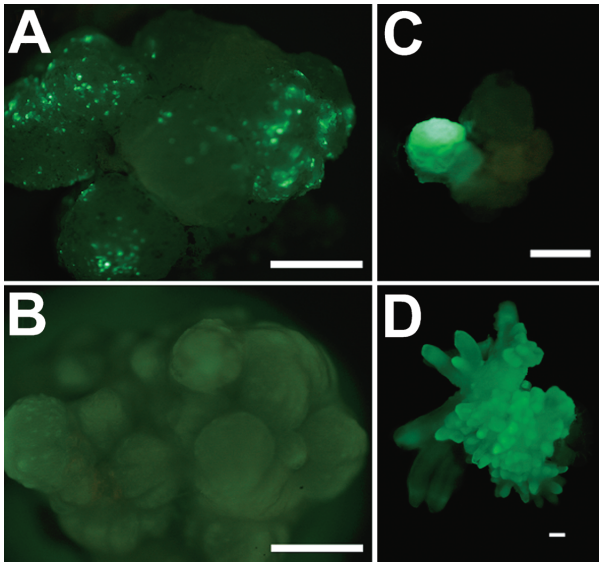

Figure 3. Fluorescence micrographs of onion calli and regenerated shoot clumps transformed with *Agrobacterium*. (A) GFP expressing calli without or with low level of autofluorescence after 6 days of coculture. (B) Yellowish autofluorescing calli without GFP expression after 6 days of coculture. (C) Transgenic calli with stable GFP expression after 4–5 weeks from the coculture. (D) Multiple shoot clumps with GFP developed from embryoids 21 days after transfer to RE medium. (Scale bars indicate 1 mm)

medium (Table 3, Figure 3C). These transgenic calli were transferred onto RE medium 4–5 weeks after coculture. Embryoids became visible about 2–3 weeks after transfer to RE medium. The differentiation of multiple shoots and leaves occurred after the embryoids were transferred several times to fresh RE medium (Figure 3D). Massively regenerating shoots were separated from each other and transferred onto RT medium. In the duplicate transformation experiments using the calli without or with low levels of autofluorescence, approximately 4–5% of the calli cocultured with *Agrobacterium* showed stable GFP expression (Table 3). Of these, 2 calli from Experiment 1 (Exp. 1) and 1 callus from Experiment 2 (Exp. 2) survived the regeneration culture to yield 20 and 10 plants, respectively, that exhibited stable GFP expression. In both experiments, the number of plants with stable

Table 3. Efficiency of onion transformation by *Agrobacterium*

Experiment	Number of pieces of calli				Frequency (B/A, %)
	Inoculated (A)	Transient GFP+[a]	Stable GFP+[a]	Produced GFP+[a] plants (B)	
1	312	110	15	20	6.4
2	168	109	7	10	6.0

[a] Strongly expressing GFP.

GFP expression corresponded to approximately 6% of the calli cocultured with *Agrobacterium*. Approximately 20 weeks after transformation, the rooted plants were transferred to soil pots and cultured in a controlled-environment growth chamber for 7 months until the plants formed bulbs.

Analysis of introduced genes

From the 2 independent transformation experiments, we selected 8 onion bulbs (3 bulbs from Exp. 1 and 5 bulbs from Exp. 2) for molecular genetic analysis. For large genome species like *A. cepa* L., genomic DNA blot hybridization analysis is rather difficult and laborious. As an alternative protocol, AL-PCR method was developed; its patterns of genomic DNA flanking T-DNA borders were specific and reproducible for a given transgenic line (Zheng et al. 2001b). We identified different transgenic lines using the AL-PCR patterns hybridized with a GFP probe according to a slight modification of the method of Akritidis et al. (2008). Southern-blot band patterns of AL-PCR products from these 8 onion plants revealed the presence of 4 transgenic lines (LR11 line from Exp. 1 and LR21, LR22, and LR23 from Exp. 2) (Figure 4A). Southern blot analysis detected more than 1 band in at least 2 transgenic lines (3 bands for LR11 and 2 bands for LR21) revealing that these 2 lines contained multicopies of the *GFP* gene. On the other hand, the remaining 2 lines (LR22 and LR23) contained single-copy inserts. We confirmed the presence of the RNAi construct for the *LFS* gene in these 4 transgenic lines by PCR analysis using 3 transgene-specific primer pairs. As shown in Figure 4B, C, D, all plants yielded amplified bands of expected sizes at the same positions as those amplified from the positive control binary vector, with the exception of the wild-type control. This suggested that at least 1 copy of the RNAi construct for the *LFS* gene was consistently introduced in each transgenic line.

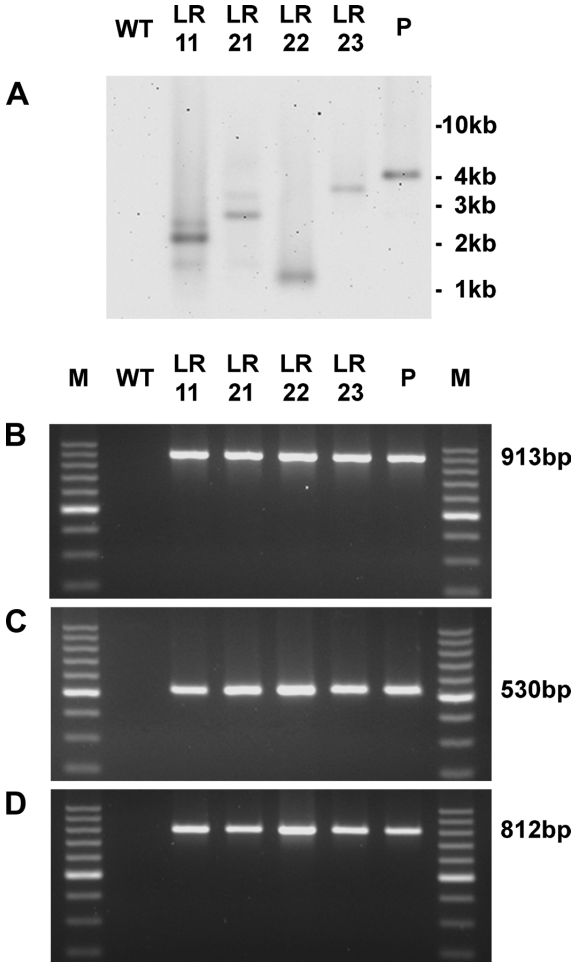

Figure 4. Molecular analysis of transgenics. (A) Southern hybridization analysis of AL-PCR products from transgenic plants. *Lanes*: WT wild-type onion plant, LR11, LR21, LR22, and LR23 transgenic onion plants, P the 35Somega-sGFP(S65T) plasmid. (B, C, D) PCR analyses of transgenics and wild-type onion for the delivered gene amplified with 3 primer pairs shown in Figure 2. Amplicons shown are the fragments encompassing, CaMV 35S promoter and *LFS* antisense fragment (B), *LFS* antisense fragment and *FAD2* 1st intron (C), *FAD2* 1st intron, *LFS* sense fragment, and nopaline synthase terminator (D). *Lanes*: WT wild-type onion plant, LR11, LR21, LR22, and LR23 transgenic onion plants, P pLFSRNAi vector, M 100bp ladder marker.

Measurements of LFS suppression levels in the transgenic bulbs

LFS enzyme activities in the 4 transgenic bulbs were suppressed to 0.3–10.1% of the average activity in the wild-type controls (n=5) (Figure 5A). To confirm whether these reductions in enzyme activity in the transgenic bulbs were due to RNAi-mediated suppression of *LFS* gene expression, we directly measured the level of LFS protein using the sandwich ELISA and the relative level of *LFS* mRNA using quantitative RT-PCR in each transgenic line.

On average, LFS protein levels in the transgenic plants were 0.09–20.7% of the wild-type control levels (Figure 5B), and *LFS* mRNA levels in the transgenic bulbs were 3.2–11.0% of the wild-type control levels (Figure 5C). These results indicated that the lower LFS enzyme activities observed in the transgenic bulbs were due to reduced LFS protein levels and suppressed *LFS* mRNA levels during the transcription process.

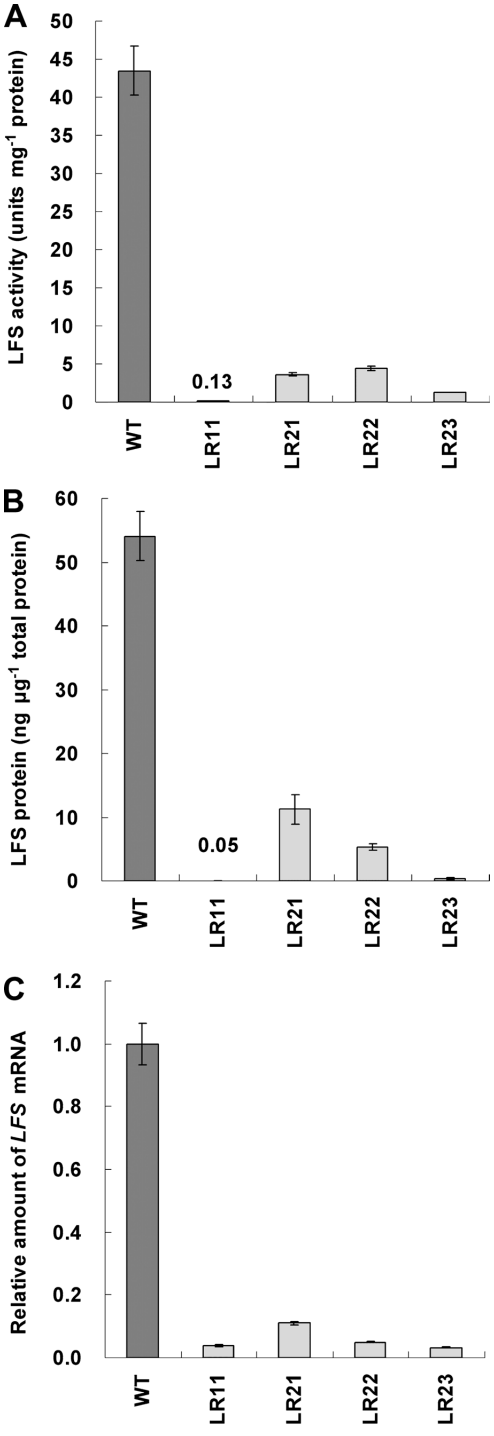

Figure 5. Levels of LFS enzyme activity (A), LFS protein (B) and *LFS* transcript (C) of wild-type and transgenic bulbs. WT wild-type onion, LR11, LR21, LR22, and LR23 transgenic onion plants. Data for WT are the average of 5 wild-type bulbs. The measured values, which are too small to be seen in the graphs, are shown as numeric values.

Characteristics of the transgenic onions

Wild-type control bulbs generated considerable amounts of LF. On the other hand, no lachrymatory sensation was observed when the transgenic bulbs were cut with a knife. HPLC analysis of LF produced in macerated onion bulb material demonstrated that the LF levels in LR11 transgenic onion (180 μg g^{-1} fresh mass) were reduced by half compared with those of wild-type controls (350 μg g^{-1} fresh mass). A similar extent of LF reduction were previously reported in the LFS silencing onions (Eady et al. 2008). The reduced levels of lachrymatory sensation were consistent with the LFS suppression levels in the transgenic bulbs. In contrast, there were significant differences in the amount of pink color formation in the bulb extracts (Figure 6A). Pink color formation in the bulb extract from the LR11 onion was markedly higher than those in the bulb extracts from LR21, LR22, and LR23, as well as those in the wild-type onions. Addition of purified rLFS protein in an amount

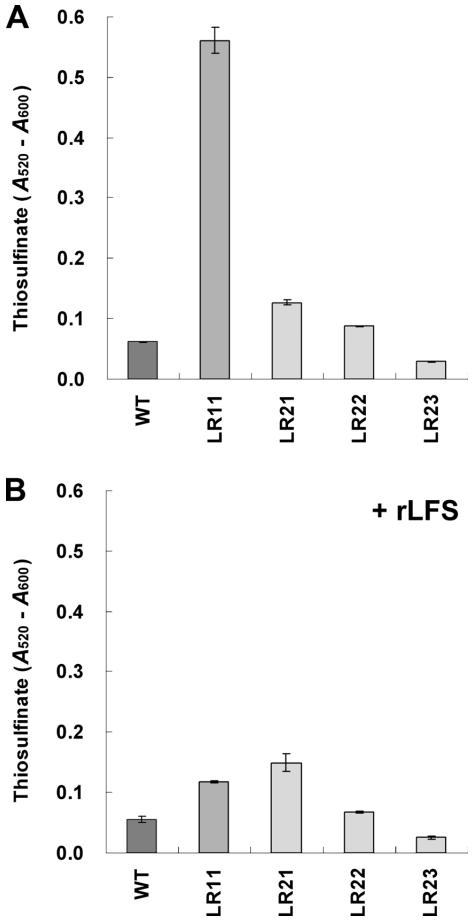

Figure 6. Pink pigment formations as a measure of thiosulfinate levels. (A) without external rLFS, (B) with external rLFS (ca. 76 units). WT wild-type onion, LR11, LR21, LR22, and LR23 transgenic onion plants.

Table 4. Summary of biochemical factors[a] concerning generations of LF and thiosulfinate

Onion plants	PRENCSO mg g^{-1} fresh mass	Alliinase activity units mg^{-1} protein	LFS activity units mg^{-1} protein	Thiosulfinate A_{520}–A_{600}
Wild-type	0.98 ± 0.07	1.02 ± 0.02	43.446 ± 3.196	0.0617 ± 0.0004
LR11	3.14 ± 0.15	0.82 ± 0.06	0.134 ± 0.002	0.5606 ± 0.0154
LR21	0.76 ± 0.01	1.51 ± 0.05	3.637 ± 0.159	0.1268 ± 0.0040
LR22	0.94 ± 0.02	0.92 ± 0.15	4.367 ± 0.282	0.0874 ± 0.0001
LR23	1.18 ± 0.01	1.32 ± 0.10	1.316 ± 0.017	0.0288 ± 0.0005

[a] Values are means ± SD of duplicate measurement.

comparable to the level in wild-type onions (ca. 76 units) resulted in decreased pink color formation in the LR11 bulb (Figure 6B). These results suggested that the high amount of PRENCSO-derived thiosulfinate depended on the low level of LFS enzyme activity in the transgenic bulb from the LR11 line. On the other hand, the levels of color formation in bulb extracts with moderately reduced LFS enzyme activity, such as LR21, LR22, and LR23, were similar to those in the wild-type controls. Biochemical analyses of factors related to the generation of the LF and thiosulfinate are shown in Table 4. PRENCSO content and alliinase activity associated with generation of LF in the transgenic and wild-type plants were within the range reported for onion (Fritsch and Keusgen 2006; Kitamura et al. 1997; Kopsell and Randle 1999; Nock and Mazelis 1987). These biochemical analyses revealed that the differences in color formation among the onions analyzed depended on the differences in the reduction of LFS enzyme activity.

Discussion

The successful production of transgenic plants requires efficient protocols for transformation and subsequent plant regeneration. We preliminarily examined the frequency of plant regeneration from both radicle-derived embryogenic calli and proliferated LL type calli in suspension cultures prepared by the methods of Tanikawa et al. (1998). The percentage of calli that regenerated shoots among the calli transferred onto RE medium was 34% for the embryogenic calli and 58% for the LL type calli. These regeneration frequencies were comparable with a previous report by Tanikawa et al. (1998), who tested 22 onion cultivars and obtained an average regeneration frequency of 43.6% for the embryogenic calli and 60.5% for the LL type calli. Thus, we considered that these calli were suitable for transformation experiments.

However, the absence of transient expression of GFP after coculture with *Agrobacterium* revealed that both the embryogenic calli and the LL type calli were recalcitrant to transformation. Successful transformation required further improvement of both the procedure and the conditions for culturing calli. By selecting the calli without or with low levels of autofluorescence from the preconditioned culture for subsequent coculture with *Agrobacterium,* we observed improved gene-transfer frequency as indicated by transient GFP expression. The calli without or with low levels of autofluorescence were obtainable by segmenting the LL type calli into small pieces (2–3 mm in diameter), and pre-culturing them for 1–2 weeks on a solid PCO medium. Those calli which exhibited robust growth tended to have many non-autofluorescing regions. The yellowish autofluorescence observed in the calli recalcitrant to transformation may be attributable to the presence of phenolic substances and/or lignin (Franceschi et al. 1998; Hassan et al. 2004; Rech et al. 2003). Lignin, a well-known phenolic compound, is the most common polymer in the secondary cell wall that develops at the inner region of the primary cell walls of the cells that have stopped growth and their wall no longer needs to be extensible (Alberts et al. 2002). Lignin is also known for its roles in the cellular defense mechanism in vascular plants (Campbell and Sederoff 1996). Thus, calli exhibiting strong autofluorescence are likely to be more recalcitrant to *Agrobacterium*-mediated transformation, and for efficient gene transfer, it is important to select the calli without or with low levels of yellowish autofluorescence under the blue excitation light.

Preculturing the LL type calli on a solid PCO medium before coculturing with *Agrobacterium* may have had a positive effect on transformation frequency. Our initial attempts to directly transform the embryogenic calli induced on the solid medium or the LL type calli cultured subsequently in a liquid medium, were not quite successful. Hiei et al. (1997) reported that preculturing suspension-cultured cells on a solid medium for several days was efficient for high-frequency transfer in rice (*Oryza sativa* L.). Moreover, segmenting calli into smaller pieces before preconditioning on the solid medium may have promoted cell division and vigorous growth of the calli, resulting in reduced accumulation of phenolic compounds.

Southern blot analysis of AL-PCR products from transgenic plants indicated that more than 1 copy of the T-DNA was integrated in the LR11 and LR21 transgenic lines. However, no significant difference was observed in the *LFS* gene transcript levels between single-copy transgenic lines and multicopy lines. Kerschen et al.

(2004) also reported that multicopy RNAi lines did not demonstrate greater reduction of target transcript levels compared to single-copy RNAi lines. Thus, the differences in both the LFS enzyme activity and the LFS protein among the 4 transgenics may be due to variabilities in RNAi-mediated silencing. The expression levels of transgenes in multicopy lines are more variable and more unstable over generations than those in single-copy lines; therefore, further investigation of the phenotypic behavior and biochemical analysis would be necessary for their progenies.

The transgenic bulbs obtained in this study did not cause any lachrymatory sensation when cut with a knife, consistent with the measured LFS enzyme activity. As a consequence of reduced LFS enzyme activity, more of the 1-PS produced from PRENCSO breakdown is expected to be diverted to thiosulfinate formation. The thiosulfinates and their derivatives have been reported to have antiasthmatic, antithrombotic, and anticancer effects (Block et al. 1997; Dorsch et al. 1989; Guyonnet et al. 1999). They are also associated with the characteristic flavor of onion (Randle 1997). Therefore, the increase in thiosulfinate formation can be seen as an added value of the LFS suppressed non-lachrymatory onions.

Since di-1-propenyl thiosulfinate, the putative condensation product of 2 molecules of 1-PS, had never been isolated, the increase in thiosulfinate levels was assessed by making use of the discoloration phenomenon known as "pinking of onion", in which pink pigments are reportedly formed from 1-propenyl-containing thiosulfinate reacting with an amino acid such as glycine, and an aldehyde such as formaldehyde (Imai et al. 2006, Kubec et al. 2004, Lee and Parkin 1998).

Despite the absence of lachrymatory sensation and the considerably large reduction in LFS enzyme activity found in the bulbs from LR21, LR22, and LR23 lines (3–10% of the wild-type control), the amount of pinking was not significantly different from that observed in the wild-type bulbs. These results indicate that a significant increase in thiosulfinate levels would require a reduction in LFS enzyme activity to approximately 1% or less than that of the wild-type control, as found in the bulbs from LR11 line. Eady et al. (2008) reported a large accumulation of thiosulfinate in onion plants in which LFS enzyme activity was reduced by between 500- and 1500-fold compared to nontransgenic controls. Because the isomerization reaction catalyzed by LFS is presumably much faster than the spontaneous reactions forming thiosulfinate and pink pigments, the residual LFS enzyme activity found in the transgenic bulbs from LR21, LR22, and LR23 lines could have been high enough to limit the availability of 1-PS for thiosulfinate formation, but low enough to limit the rate of LF release below the threshold level for lachrymatory stimulation. Recently, Musah et al. (2009) reported a comparable result that the formation of thiosulfinate depended upon the relative concentrations of the LFS and the alliinase.

Although we mainly focused on LF and thiosulfinate as the 1-PS derived products, it is possible that the unexpected sulfur-containing products derived from 1-PS accumulated in the transgenic bulbs from LR21, LR22, and LR23 lines. The complex chemistry of 1-PS, LF, and their derivatives in the disrupted tissues of onions, and the pathways of their formation, were described by Block (2010). For example, cepaenes, which have been reported to have antiasthmatic effects (Dorsch 1996), could be derived from both 1-PS and the LF (Figure 1). In this instance, the degree of LFS enzyme activity reduction may play a key role in cepaenes accumulation. Thus, different levels of reduction in LFS enzyme activity may lead to the production of new onion varieties having different types and/or levels of sulfur-containing compounds. To regulate the reduction rates in LFS enzyme activity, it would be necessary to apply moderate gene-silencing techniques, such as an antisense method, or conversion to a weaker promoter for driving RNAi constructs. This study demonstrated that *LFS* gene expression, which plays an essential role in generating the LF, can be effectively suppressed. Thus, the tearless onions may serve as model plants for elucidating the complex pathways of sulfur-containing compounds that occur in the disrupted onion tissues.

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