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Determination of single-cell gene expression in *Arabidopsis* by capillary electrophoresis with laser induced fluorescence detection

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Abstract

We report a new method for the measurement of gene expression in single cells of *Arabidopsis* using capillary electrophoresis with laser-induced fluorescence (CE–LIF) detection. Initially, the quantitative analysis of *APETALA2* (*AP2*) and *LEAFY* (*LFY*) was performed by CE–LIF method. The detection limits of *AP2* and *LFY* can reach 0.08 and 0.04 ng/ml (signal-to-noise ratio = 3), respectively. This protocol coupling with single-cell reverse transcriptase-polymerase chain reaction (SC-RT-PCR) has been used to monitor *LFY* and *AP2* expression in individual cells from the shoot apical meristem, leaf, root, and stem of *Arabidopsis*, simultaneously. The effect of PCR cycle number on PCR product concentrations has been discussed. The changes of *LFY* expression were determined at single-cell level in different *Arabidopsis* tissues. The relationship between gibberallic acid (GA) and *LFY* expression was also revealed by this method. It was shown that the combination between CE–LIF and SC-RT-PCR could provide a highly sensitive and selective tool for the determination of different gene expression at single-cell level in specific tiny plant tissues.

Keywords: *Arabidopsis*; Gene expression; Single-cell transcriptase–polymerase chain reaction

1. Introduction

Flowering is controlled by both environmental conditions and developmental regulation and an intricate network of signaling pathways creates the complexity of this regulation [1]. The transition from vegetative to reproductive growth is a critical transition in the life cycle of flowering plants. *Arabidopsis* is an excellent model plant in which to approach this complexity of this regulation is created by an intricate network of signal.

The transition to flowering involves a change in the identity of the primordial arising at the flanks of the shoot apical meristem from leaves with associated lateral shoots (paraclades) to bractless flowers. This switch is dependent on the activity of floral meristem identity genes, such as *LEAFY* (*LFY*) and *APETALA1* (*AP1*) [2]. Both genes are expressed in emerging flower primordial, but only *LFY* is expressed in leaf primordial before the transition to flowering is made [3,4]. The level of *LFY* expression in the primordial produced by the shoot apical meristem increases with the age of the plant until it apparently reaches a threshold level. Once this level has been reached, a primordium that would otherwise turn into a leaf/paraclade becomes a bractless flower instead. These additional copies of wild-type *LFY* cause plants to produce fewer leaves before the first flower is formed, indicating that *LFY* expression levels are critical for the fate switch from leaf/paraclade to bractless flower [4,5].

The growth regulator gibberallic acid (GA) promotes flowering of *Arabidopsis* [5]. This was initially demonstrated by applications of exogenous GA, and more evidence was obtained by using mutations that disrupt either GA biosynthesis or signaling. One-way in which GA promotes flowering is by increasing the transcriptional activity of the floral meristem identity gene *LFY*. While expression of *LFY* is reduced in mutants defective in GA biosynthesis, over-expression of *LFY* can restore flowering of mutants defective in GA biosynthesis [5].

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The *LFY* mRNA is extremely rare, and it was not detected on Northern blots. RT-PCR was therefore used to detect the transcript. To understand the transition from vegetative growth to reproductive phase (flowering), the information of *LFY* expression on shoot apical meristem is essential. However, it is a challenging task because of the extremely small size of the shoot apex. Because the expression of *LFY* on only shoot apical meristem determines the flowering time and it is very difficult to get enough meristem cells for common assay of *LFY* expression. Therefore, it is important to develop an effective protocol for detecting *LFY* expression at single-cell level.

In recent years, single-cell reverse transcriptase-polymerase chain reaction (SC-RT-PCR) has become an important tool for the determination of specific gene expression in heterogeneous tissues [6]. Most applications of SC-RT-PCR have been described in animal cells [7–10], especially in neuron [11,12]. Only a few SC-RT-PCR protocols were applied to monitor gene expression in single plant cells, which contain mRNA in femtogram amounts [13,14]. Richert et al. [15] promoted a simple method, in which the artificial system of protoplasts was directly used as templates for RT-PCR with specific primers without constructing cDNA libraries. Using this method, Brandt et al. [16] successfully detected gene transcripts from single plant cells in living, undamaged plant tissue. These tested plant cells were extracted by using glass microcapillaries and directly subjected to RT-PCR without any purification. Laval et al. [17] reported a valuable technique for the analysis of cellular locations of the transcripts of the eight isoforms of actins expressed in *Arabidopsis thaliana* (L.) Heynh. In these above methods, the SC-RT-PCR products were determined by slab gel electrophoresis (SGE). Although SGE is the most conventional technique to detect DNA fragment, this technique is time-consuming, low sensitive and non-quantitative. To overcome these shortcomings, capillary electrophoresis (CE) was introduced into the analysis of DNA fragment. Now CE has been proven to have numerous advantages over SGE including fast separation, high efficiency, increased resolution, and the use of noncross-linked polymer solutions [18–20]. Recently, capillary electrophoresis–laser induced fluorescence (CE–LIF) was used to detect SC-RT-PCR products in mammalian cells without quantitation [21,22]. To our knowledge, there are no reports on the determination of gene expression for plant tissue by CE–LIF.

In this paper, we report here a capillary electrophoresis method coupling with SC-RT-PCR, which can be used to monitor *LFY* and *AP2* expression in different tissues of *Arabidopsis*, simultaneously. The *APETALA2* (*AP2*), constantly expressed in all tissues tested [5,23], was used as a control to evaluate the accuracy of SC-RT-PCR. The changes of *LFY* expression were successfully determined at single cell level by this method. The expression of *LFY* in single cell from shoot apical meristem treated by GA was increased about 10–100 times as than pre-treatment of GA.

2. Materials and methods

2.1. Materials

YO-PRO-1 (1 mmol l^{-1} in DMSO) was purchased from Molecular Probes Inc. (Eugene, OR, USA). Hydroxypropylmethylcellulose (HPMC, 3500–5600 c.p., 2% aqueous solution), Polyvinylpyrolidone (PVP, Wt 360000), and Mannitol were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All chemicals were of analytical grade and all solutions were prepared from deionized water purified with a Millipore-Q system (Millipore, Bedford, MA, USA). ϕX174/*HaeIII* for DNA standard containing 11 fragments was prepared by digesting ϕX174 plasmid with *HaeIII* at final concentration of 0.15 μg ml^{-1}.

Gene-specific primers (Primer 1 at concentration of 10 μmol l^{-1}) used to amplify *AP2* cDNA were 5′-CTCAATGCCG-AGTCATCAGG-3′ and 5′-CTCAGCCGCCGGAAACAGTG-3′. The primers (Primer 2 at concentration of 10 μmol l^{-1}) for *LFY* cDNA were 5′-CCCAAGAAG-ATGATTGGACA-3′ and 5′-CGCATTGTTCCGCTCCAAAT-3′. All primers were synthesized by SBS Genetech Co., Ltd (Beijing, China). The standards for *LFY* (731 bp) and *AP2* (267 bp) genes were amplified from our cloned cDNAs, confirmed by sequencing and quantitated by UV spectrometric method.

2.2. Capillary electrophoresis

All CE experiments were performed on an Agilent 3D capillary electrophoresis instrument (Palo Alto, CA, USA) equipped with a ZETALIF laser induced fluorescence detector (Picometrics, Ramonville, France). The excitation wavelength was chosen as 488 nm. Data collection and peak area analysis were performed on a HP Chemstation (Palo Alto, CA, USA). DNA fragments were separated on an uncoated fused-silica capillary of 65 cm (total length) × 50 cm (length to detector window) × 75 μm i.d. × 375 μm o.d. (Yongnian Optic Fiber Inc., He Bei, China).

The sieving buffer used for separation has been described previously [24] and contained 0.5% HPMC, 0.5% PVP, and 5% mannitol dissolved in TBE buffer (100 mmol l^{-1} Tris-, 100 mmol l^{-1} boric acid, 2 mmol l^{-1} EDTA, pH 8.3). YO-PRO-1 at the concentrations ranging from 0.25 to 4 μmol l^{-1} was added to the sieving buffer prior to CE.

New capillaries were pre-treated with 1 mmol l^{-1} NaOH for 60 min followed by deionized water for 60 min at room temperature. Prior to use, the capillary was washed with 0.1 mmol l^{-1} and pure water for 5 min at room temperature, respectively, followed by preconditioning with running buffer for 10 min. The above flushing cycle was repeated to ensure the separation reproducibility for each injection. Electrophoresis was carried out in the reversed polarity (negative potential at capillary inlet) using a typical voltage of −15 kV. Samples were electrokinetically injected at −10 kV for 5 s.

2.3. Cell preparation

Arabidopsis thaliana Columbia erecta was grown in a greenhouse (Wuhan University) under LD (16 h light and 8 h dark) conditions. For GA treatment, 0.1 mM GA_3 was sprayed once a week from soon after germination until appearance of the flower bud.

To isolate single cells, *Arabidopsis* tissues were treated with 0.48 mol mannitol (pH 5.7) containing 1.5% pectinase (SABC, Shanghai, China) and 2% cellulose R10 (Yakult, Honsha, Co. Ltd., Tokyo, Japan) for 4–6 h [25]. The protoplasts were isolated under an Olympus BX60 fluorescence microscope (Olympus Optical Co., Tokyo, Japan).

2.4. RT-PCR

To carry out two-step RT-PCR with the isolated single cell in 10 μl reaction mixture, 0.5 μl Oligo $(dT)_{16}$ (500 μg/ml) (GIBCO, USA), 0.5 μl 10 mmol l^{-1} dNTP Mix (10 mmol l^{-1} each dATP, dGTP, dCTP and dTTP at pH 7.0), 5 μl RNase-free water and single protoplast were mixed and incubated at 65 °C for 5 min. After quick chill on ice, 2 μl 5 × first-strand buffer, 1 μl 0.1 mol l^{-1} DTT and 0.1 μl (4 units) RNaseOUT™ recombinant ribonuclease inhibitor (40 units/μl) (Promega, USA) were added and the mixture were incubated at 42 °C for 2 min following by the addition of 8 units SuperScript™ II (200 units/μl) (GIBCO, USA) and continuous incubation at 42 °C for 50 min. After the enzymes in this mixture were inactivated by the incubation at 70 °C for 15 min, the synthesized first-strand cDNAs were used as a template for PCR amplification. To do so, 1 μl cDNA from RT reaction above, 1 μl 10 × PCR buffer (200 mmol l^{-1} Tris-HCl (pH 8.4), 500 mmol l^{-1} KCl), 0.3 μl 50 mmol l^{-1} $MgCl_2$, 0.2 μl 10 mmol l^{-1} dNTP Mix, 0.2 μl specific amplification primer 1, 0.2 μl amplification primer 2, 0.1 μl Taq DNA polymerase (5 U/μl) (MBI Fermentas, Jingmei Biotech. Co. Ltd, China), 7 μl RNase-free water were mixed and incubated at 94 °C for 2 min following by PCR. PCR was performed for 32, 35 and 40 cycles, respectively, under following conditions: 94 °C for 30 s (denaturation), 58 °C for 60 s (annealing), 72 °C for 80 s (extension) in a PTC-100 DNA Engine thermal cycler (MJ Research, Waltham, MA, USA).

RT-PCR was also done by one-step RT-PCR in 10 μl reaction containing single protoplast, 1 μl 10 × reaction buffer, 0.3 μl 50 mmol l^{-1} $MgCl_2$, 0.2 μl dNTP mix (10 mmol l^{-1} each), 0.1 μl (4 units) RNaseOUT™ recombinant ribonuclease inhibitor (40 units/μl) (Promega, USA), 0.2 μl amplification primer 1, 0.2 μl specific amplification primer 2, 0.2 μl AMV reverse transcriptase (5 U/μl), DyNAzyme EXT DNA Polymerase 0.4 μl (1 U/μl), 7.5 μl RNase-free water. The mixture was incubated at 48 °C for 40 min followed by incubation at 94 °C for 2 min. The mixture was directly used for PCR amplification for 32, 35 and 40 cycles, respectively, under following conditions: 94 °C for 30 s (denaturation), 58 °C for 60 s (annealing), 72 °C for 80 s (extension) in a PTC-100 DNA Engine thermal cycler (MJ Research, Waltham, MA, USA).

The RT-PCR products from shoot apical meristem treated with GA were diluted five folds with deionized water for CE analysis and the RT-PCR products from other tissues were directly used in CE analysis.

3. Result and discussion

3.1. Effect of dye concentration

The purpose of this work was to detect ultra-low amount products of SC-RT-PCR for monitoring the expression of *LFY* and *AP2* in *Arabidopsis*. High-resolution separation and sensitivity detection of DNA fragments are primary requirement. In our previous work [26], a CE method with non-gel sieving medium, using polyacrylamide-coated capillary, was successfully used to identify the orientation of DNA fragments in recombinant plasmids and characterize cell apoptosis. Unfortunately, the coating method requires in situ synthesis and often met some problems such as capillary fouling, coating inhomogeneity, and limited lifetime [27]. Recently, a low viscosity medium, containing TBE, HPMC, PVP and mannitol, has been developed for the efficient DNA separation [24]. The adding of PVP can reduce the absorption of DNA onto the capillary inner wall [28]. In this work, the sieving medium, contained 0.5% HPMC, 0.5% PVP, and 5% mannitol dissolved in TBE buffer (100 mM tris-100 mM boric acid–2 mM EDTA, pH 8.3), was used in the separation of DNA fragments.

The ratio of dye to DNA is a critical factor in maintaining separation efficiency and detection sensitivity. When the ratio of dye to DNA was too low, it cannot provide enough fluorescent complexes to improve the detection sensitivity. Due to the instability of dye–DNA complexes, it is necessary to use excess dyes to prevent the dissociation of dye–DNA complexes. However, too excess dyes may not only influence the separation efficiency but also lead to the fluorescence quench of fluorescent complexes. It was pointed out that the intercalation of monointercalators induces structural and electrostatic changes in double strand DNA (dsDNA). When the complexes are subjected to electrophoresis, these changes can alter migration dynamics.

In this work, we used ϕX174 marker as model compound to evaluate the effect of increasing fluorescent dye concentrations on fluorescence intensity and migration time in CE–LIF of DNA fragments. When the concentration of ϕX174 marker was chosen as 15 ng/ml, the effect of fluorescent dye on detection and separation was examined by varying the concentration of YO-PRO-1 from 0.25 to 4 μmol l^{-1}.

The migration times of each DNA fragment became slightly larger by the increase of YO-PRO-1 concentration. At YO-PRO-1 concentration lower than 0.5 μmol l^{-1}, the peaks for 271 and 281 bp were partially overlapped. When

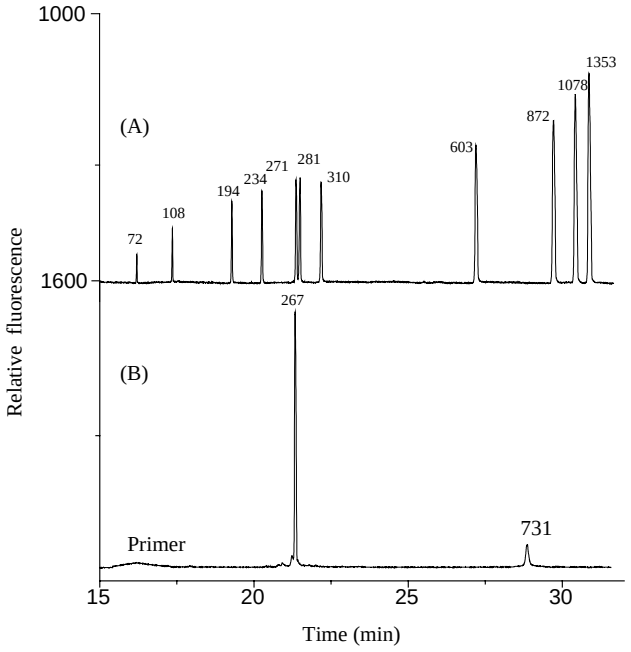

Fig. 1. Electropherogram of (A) the ϕX174 marker with YO-PRO-1 and (B) standards of *AP2* and *LFY* gene fragments. Conditions: 100 mmol l^{-1} Tris-100 mmol l^{-1} boric acid–2 mM EDTA, pH 8.3; 0.5% HPMC; 0.5% PVP; 5% mannitol; 2 μmol l^{-1}; voltage: −15 kV; the total concentration of DNA fragments: 15 ng/ml; the effective length of column: 50 cm; the total length of column: 65 cm; electrokinetic injection time: 5 s at −10 kV. 267 bp is for *AP2* gene and 731 bp for *LFY*.

the concentration of YO-PRO-1 was higher than 4 μmol l^{-1}, It caused a delay in migration with peak broadening.

The fluorescent intensity for DNA fragments can be significantly changed by the variable of YO-PRO-1 concentration. When the concentration of YO-PRO-1 was varied in the range of 0.25–4 μmol l^{-1}, higher concentration of YO-PRO-1 can lead to the increase of fluorescent intensity for DNA fragments. Furthermore, the peak areas of each DNA fragments can reach maximum when the concentration of YO-PRO-1 was 2 μmol l^{-1}. As it mentioned, too high concentration of fluorescent dyes may decrease the fluorescent intensity of DNA-fragments. It was observed that the fluorescent intensities of each DNA fragment were decreased with the increase of YO-PRO-1 concentration when the YO-PRO-1 concentration was beyond 2 μmol l^{-1}. Thus, a 2 μmol l^{-1} of YO-PRO-1 was used in the analysis of DNA fragments.

Under the optimum CE separation medium procedure, the eleven DNA fragments of ϕX174 marker were well separated (Fig. 1A). Fig. 1B shows the separation of the standards of *AP2* and *LFY* gene fragments with 267 and 731 bp, respectively.

3.2. Quantitation of RT-PCR products

Several groups [29,30] have described quantitation of nucleic acids by capillary electrophoresis recently. Butler et al. has reported the use of CE with LIF for quantitation of PCR products. Using internal standards of known size and concentration, peak migration time was blow 0.1% relative standard deviation (R.S.D.) with a peak area precision of 3% R.S.D. Compared to quantiation by hybridization, (i.e. slot blot) and spectrometric analysis, this method shows distinct due to its ability to separate un-incorporated primers and PCR byproducts from targeted PCR products. Fasco et al. [30] also employed CE–LIF to detect YOYO-1 complexes of PCR products. RT-PCR mixtures can be analyzed either directly (without primer and protein removal) or by electrokinetic injection following desalting.

It has been found that the electrokinetic injection yields a more efficient separation than hydrodynamic injection. Although hydrodynamic injection allows direct injection of DNA sample without prior sample cleanup, the co-existing primers and salts causes decrease of detection sensitivity and apparent peak broadening [29]. In comparison to hydrodynamic injection, electrokinetic injection can produces better peak shape and detection sensitivity. In our experiment, we choose electrokinetic injection as injection mode.

The linear range, calibration graph and detection limit (S/N = 3) of two gene fragments, *AP2* and *LFY*, calculated from integrated peak areas are listed in Table 1. When the concentration of *AP2* was from 10 down to 2 ng/ml, the peak area was changed slightly. The same result can be also obtained in the determination of *LFY* (Fig. 2). These results indicate that electrokinetic injection is independent of concentration when the sample concentration is variable in a given range.

3.3. Single cell RT-PCR

The quantity of mRNA that can be harvested from a single cell is in the order of 1 pg at best [6]. Therefore, to obtain valuable gene-expression data in single cell, well-optimized or specialized amplification protocols must be applied. SC-RT-PCR provides a valuable tool for molecular characterization using a limited amount of starting materials.

Table 1
Quantitation data

Gene	Linear range (ng/mL)	Cablibration graph	Correlation coefficient	%R.S.D. for migration time ($n = 6$)	%R.S.D. for peak area ($n = 6$)	Detection limit (ng/ml)[a]
AP2	0.1–2	$y = 1075x + 41.3$	0.9952	0.28	4.3	0.08
LFY	0.17–3.4	$y = 990x + 40.1$	0.9938	0.53	6.5	0.04

[a] S/N = 3.

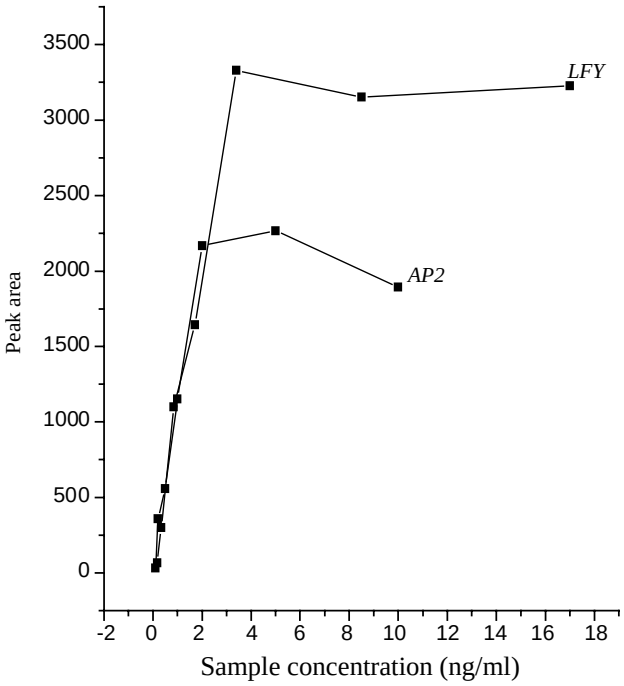

Fig. 2. Effect of DNA fragments concentrations on peak area. Conditions for CE–LIF were described in Fig. 1.

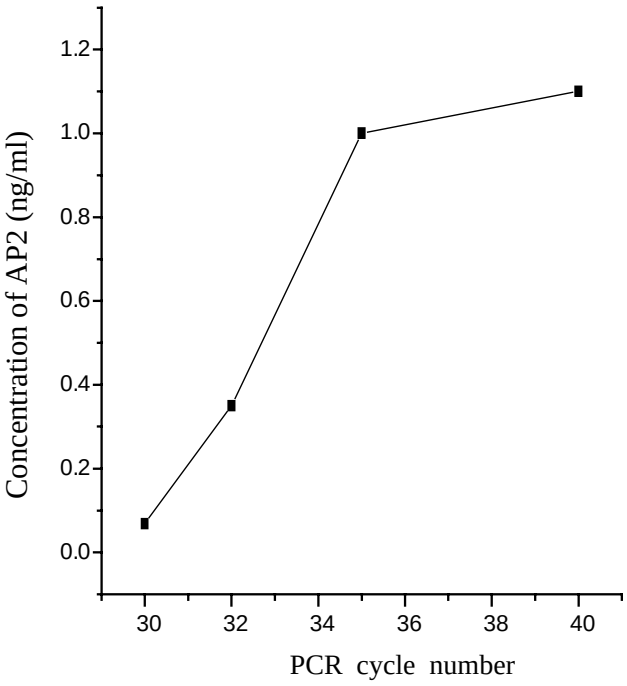

Fig. 3. Effect of PCR cycle number on the PCR amplification efficiencies. A 267-bp segment of the *AP2* was amplified by SC-RT-PCR as described under Section 2. Other separation conditions are described in Fig. 1.

Both RT and PCR efficiency are all important in SC-RT-PCR. The common RT-PCR methods can be divided into one-step RT-PCR and two-step RT-PCR. In this paper, the efficiencies of two kinds of RT-PCR methods were investigated by CE–LIF.

The amount of *AP2* by two-step RT-PCR and one-step RT-PCR from single cell were 1.01 ng/ml and 0.32 ng/ml, respectively. Although one-step RT-PCR kit can provide more facile and simple operation, the efficiency of one-step RT-PCR method is lower than two-step RT-PCR probably due to reaction buffer, which is not adapt to improve the efficiency for reverse transcription and PCR simultaneously. The decrease of PCR efficiency is frequently problem in one-step compared to two-step RT-PCR.

The relationship of the amplification efficiencies of *Ap2* and PCR cycle number was investigated (Fig. 3). In this work, the PCR was performed for 30, 32, 35, and 40 cycles. The amplified fragment cannot be detected by CE–LIF when the PCR cycle number was below 30. PCR amplification was close to exponentially increase in the range of 30–35 PCR cycles. When the PCR cycle number was 40, the amplified fragment was only slightly more intense than 35 cycles. In our experiment, the PCR cycle number was chosen as 35, which can provide better stability and reproducibility.

Single cells, used in the study of SC-RT-PCR, were isolated from the shoot apical meristem, leaf, root, and stem of *Arabidopsis*. Typical single-cell electropherograms are shown in Fig. 4. The amounts of some SC-RT-PCR products were described in Table 2. It can be seen that the amounts of *AP2* by SC-RT-PCR in each cell from different tissues only slightly change. However, there are significant differences of *LFY* expression between cells from different tissues. The *LFY* expression can be detected in single cells on the shoot apical meristem (Fig. 4B). It can be not detected the *LFY* expression in single cells on other tissues of *Arabidopsis* by CE–LIF methods (Fig. 4A).

The growth regulator gibberallic acid (GA) promotes flowering of *Arabidopsis* [5,31]. This was initially demonstrated by applications of exogenous GA [32], and more evidence was obtained by using mutations that disrupt either GA biosynthesis or signaling. One-way in which GA promotes flowering is by increasing the transcriptional activity of the floral meristem identity gene *LFY*. While expression of *LFY* is reduced in mutants defective in GA biosynthesis, over-expression of *LFY* can restore flowering of mutants defective in GA biosynthesis [5]. As a preliminary application, the relationship of GA and *LFY* was investigated by this CE–LIF method. Fig. 4C shows the representative electropherograms of RT-PCR product from shoot apical

Table 2
The amounts of SC-RT-PCR products

Gene[a]	Shoot apical meristem			Shoot apical meristem[c]			Root[d]	Stem[d]	Leaf[d]
	1	2	3	1	2	3			
AP2	1.27	0.71	0.83	0.63	0.84	0.96	1.33	1.14	1.01
LFY	0.38	0.30	0.32	4.3	15.2	13.4	ND[b]	ND	ND

[a] All results are listed in ng/ml.
[b] ND: not determined
[c] GA treatment
[d] Average ($n = 3$)

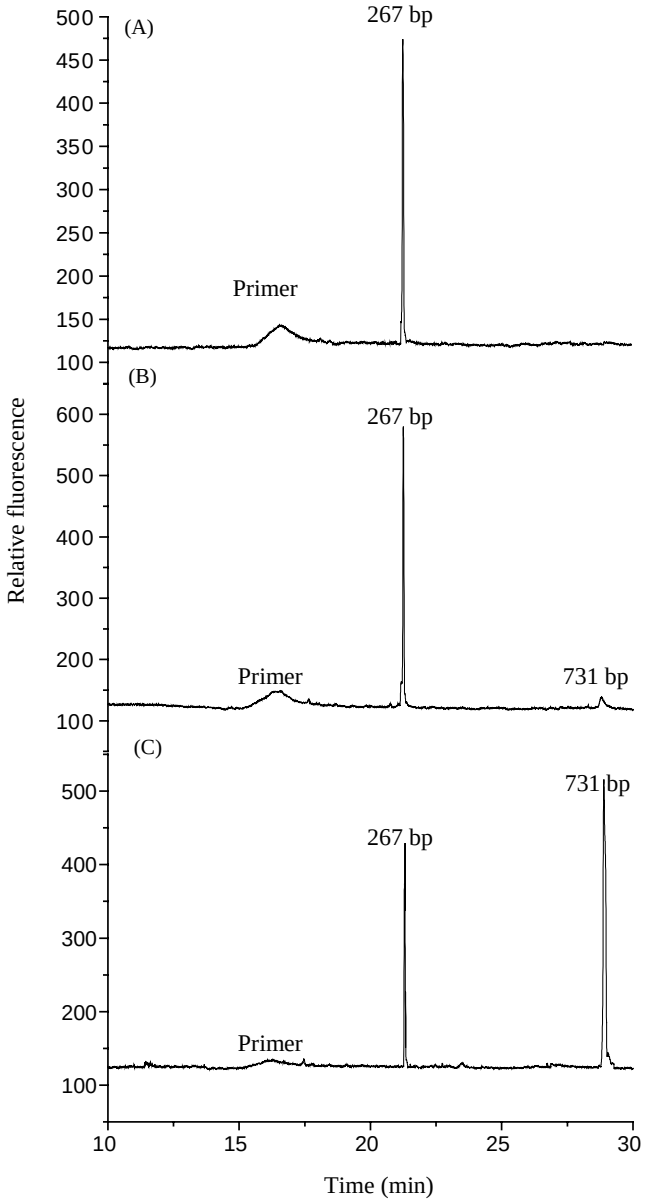

Fig. 4. Typical electropherogram of RT-PCR Products of single *Arabidopsis* cells: (A) single cell on other tissues (root, leaf or stem) except shoot apical meristem; (B) single cell on shoot apical meristem; (C) single cell on shoot apical meristem by GA treatment. Other separation conditions are described in Fig. 1.

meristem by GA treatment. In presence of GA, the amounts of the PCR product of LFY in single cell increase about 10-100 times as than pre-GA treatment. The results indicated that GA stimulates expression of *LFY* in *Arabidopsis* as demonstrated by genetic analyses [32].

4. Conclusion

In this paper, we demonstrated that CE–LIF coupling with SC-RT-PCR was firstly used to monitor the *AP2* and *LFY* expression simultaneously at single cell level in *Arabidopsis*. The amounts of SC-RT-PCR products, which cannot be detected by common methods, were quantified by CE–LIF and used to evaluate the efficiency of SC-RT-PCR. The discriminations of *LFY* expression for different single cells from various *Arabidopsis* tissues were investigated. In addition, the relationship between GA and *LFY* expression was revealed by this method. The proposed method can provide a rapid and effective protocol used for the determination of specific gene expression in tiny plant tissues. Further work will focus on the application of this method to monitoring related genes expression in *Arabidopsis* at single cell level and studying the relationship of these genes in detail.

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