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Journal of Pharmaceutical Analysis 2014;4(3):190–196

Contents lists available at ScienceDirect

Journal of Pharmaceutical Analysis

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ORIGINAL ARTICLE

Profiling the dynamics of abscisic acid and ABA-glucose ester after using the glucosyltransferase *UGT71C5* to mediate abscisic acid homeostasis in *Arabidopsis thaliana* by HPLC–ESI-MS/MS

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Received 12 July 2013; accepted 21 January 2014
Available online 10 February 2014

KEYWORDS
HPLC–MS/MS;
ABA;
ABA-GE;
AtUGT71C5

Abstract The HPLC–MS/MS method was developed to profile the dynamics of abscisic acid (ABA) and ABA-glucose ester (ABA-GE) after cloning glycosyltransferase enzyme family gene *AtUGT71C5* into *Arabidopsis thaliana*. By constructing over-expression lines (OE) and down-expression lines (DN), we acquired mutant strains to analyze the function of *AtUGT71C5*. The multiple-reaction monitoring (MRM) was used for quantitative determination in negative mode. The transition was m/z 263.1→153.0 for ABA ($[M–H]^+$), m/z 425.1→263.0 for ABA-GE ($[M–H]^+$), and m/z 321.0→152.0 for chloramphenicol. The linear range was 0.8684–217.1 ng/mL for ABA and 0.3920–196.0 ng/mL for ABA-GE. The accuracy was 88.0–109.0% for ABA and 86.6–113.0% for ABA-GE; the inter-day and intra-day precisions were less than 5.4% for ABA and 8.9% for ABA-GE, respectively. This method is simple and sensitive enough for determination of ABA and ABA-GE in *A. thaliana* leaves. All the evidence confirmed the speculation that *AtUGT71C5* can mediate abscisic acid homeostasis.
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Peer review under responsibility of Xi'an Jiaotong University.

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1. Introduction

Traditional Chinese medicine (TCM) is one part of the splendid Chinese cultural heritage. Based on the TCM theory, TCM plays an important role in preventing and treating diseases and in conditioning physical functions [1]. As we all know, high costs, long cycles, and side effects in the research and development of western medicine make the TCM with thousands of years of

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http://dx.doi.org/10.1016/j.jpha.2014.01.004

historical precipitation become one of the sources of the development of new drugs. However, the growth of TCM materials highly depends on the climate and the environment. Especially in recent years, the climate has been becoming increasingly unpredictable, making it difficult to plant TCM materials in large quantities under the complex environment. Therefore, the new method was proposed to solve this problem by introducing gene into plants.

Cruciferous plants contain sinigrin, fatty acids (such as oleic acid, linoleic acid, and linolenic acid), alkaloids, phenolic substances (quercetin and kaempferol), flavonol, rutin, inositol, cardiac glycosides, saponins, bitter substance, isatin and other ingredients, many of which have physiological activities and are also widely used among ordinary people. For example, the seeds of *Erysimum* and *Cheiranthus* with cardiac glycosides are used as a cardiac diuretic; *Brassicas*, *Europe Sinapis alba* and *Descurainia*, which are rich in glucosinolates, can produce hydroxy benzyl isothiocyanate esters or allyl isothiocyanate by hydrolysis. These two ingredients are topical for diminishing inflammation and oral administration can stimulate expectorant. Besides, many *Cruciferous* plants have anti-cancer effects.

In our laboratory, a thorough study of *Thlaspi arvense* Linn, which belongs to *Cruciferae*, *Thlaspi* [2], was carried out. A rapid approach was established to determine the sinigrin, which is the index component in *T. arvense* Linn, by near-infrared absorption spectroscopy (NIR). Based on its near-infrared absorption spectroscopy at 12,500–3600 cm^{-1} and using partial least squares (PLS), a correction model was established to compare with the effects of calibration results after different pretreatments on spectra. This method is suitable for rapid analysis of index ingredients [3]. Additionally, a simple, accurate and reliable (RSD $<$ 1.0%) method has been developed to determine the sinigrin in *T. arvense* Linn by HPLC, which can be used for the quality control of sinigrin in *T. arvense* Linn [4]. Based on these researches, *Arabidopsis thaliana* was chosen for further study of the endogenous substances of *Cruciferous* plants. *A. thaliana*, which is one type of *Cruciferae*, *Arabidopsis*, *A. thaliana* plants because of its good biological characteristics of short-growth cycle, small size, abundance in seeds [5], strong vitality and small genomes, has been used as the model organism in many botany researches. Its role in botany is as important as that of white mice and drosophila in genetics.

Plant growth and development are regulated by internal signals and external environmental conditions [6]. Plant hormones are low-molecular-weight natural products that act at micromolar (or even lower) concentrations to regulate essentially all physiological and developmental processes during a plant's life cycle [7]. One important regulator that coordinates growth and development with responses to the environment is abscisic acid (ABA) [8], which is the sesquiterpenoid hormone with many functions in plants, including roles in seed development, dormancy and germination, root and shoot growth, as well as maintenance of water relations and stress tolerance [9]. The level of this hormone is regulated by the relative rates of biosynthesis, catabolism, conjugation and redistribution throughout the plant's life [10]. Control of these processes enables plants to increase or decrease the level of ABA in different cells at specific time during growth and development, as well as in response to biotic and abiotic stresses. When plants are under abiotic stress (drought, salinity, cold acclimation, flooding, etc.), it will activate the appropriate response mechanisms. When plants are in dry status, the concentration of ABA in them will instantly rise, leading to stomatal closure by reducing the water loss rate to cope with drought stress. After the dry status disappears, the concentration of ABA will decrease to the normal level.

ABA-glucose ester (ABA-GE), the most important storage form of ABA and the most widespread ABA conjugate, has been thought to be a physiologically inactive end-product of ABA metabolism. There is no evidence that endogenous ABA-GE is cleaved in plants to release free ABA. Furthermore, it has been shown that the bulk of ABA-GE is found in vacuoles and that levels of the conjugate do not decrease in response to stress [11]. These observations suggest that ABA-GE is an inactive end product of ABA metabolism. However, it has been proposed recently that ABA-GE may act as a root to shoot stress signaling of ABA, since ABA-GE has been detected in the xylem sap of several plant species. The concentration of ABA-GE in the xylem of these plants increases under stress conditions [12]. It is known that ABA-GE can only play a role as a hormonal signal when ABA is released from the physiologically inactive conjugate [13].

Glycosyltransferase family (GTs) is widespread in animals, plants, and fungi. This family gene has a function of substrate glycosylation. It is reported that more than one member of the family can catalyze ABA generating ABA-GE *in vivo*. In 2006 it was reported that *AtUGT71B6* was the only candidate, which can identify the natural ABA conformation.

In summary, introducing gene into plants would be an inevitable trend of study on plant hormones, so effective detection is necessary. Liquid chromatography–tandem mass spectrometry (LC–MS/MS) is an alternative method that is essentially free of the limitations described above. Abram's work reported on the development of a highly sensitive and selective method for the simultaneous profiling and quantification of a wide variety of plant hormone groups and their metabolites using high-performance liquid chromatography (HPLC) coupled with electrospray ionization-tandem mass spectrometry (ESI-MS/MS) [7]. However, in their researches, isotope internal standard had to be used and the method reproducibility was not validated. In this research, we cloned *Arabidopsis* gene *AtUGT71C5*, constructed and identified its over-expression (OE) and down-expression (DN) strains, and the mutant strain of this gene from the ABRC that was obtained. Through the germination rate, drought stress, water loss rate, and stomatal aperture, lines' phenotypes were analyzed. Finally, a sufficiently simple, rapid and sensitive method was established to detect endogenous ABA and ABA-GE of these lines by HPLC–MS/MS. In this method, chloramphenicol was used as the internal standard, and this method can be widely used to profile the dynamics of endogenous hormone in plants. Besides, all these proofs further confirmed the speculation that *AtUGT71C5* can mediate abscisic acid homeostasis.

2. Experimental

2.1. Materials and reagents

Standard compounds of ABA (Sigma Chemical Co., Mississauga, Ontario, Canada, molecular weight 264.3) and ABA-GE (chemical purity $>$98.0%, molecular weight 426.4) were kindly provided by College of Life Science, Sichuan University. Chloroamphenicol (internal standard, IS, molecular weight 323.1) was obtained from Shanghai Taishi Biological Technology Co., Ltd. (TASH). HPLC-grade methanol and acetic acid were purchased from Sigma (St. Louis, MO, USA). Distilled water was purified “in-house” using a milli-Q20 system Millipore (MA, USA). An Oasis® HLB cartridge (200 mg, 3 mL, 50 pkg) was purchased from Waters Corp. (Bristol, UCT, USA).

The Agilent 1100 liquid chromatography system used was equipped with a G1311A binary high-pressure gradient pump,

ultimate online degasser, a G1316A thermostated column oven and a G1313A autosampler (Agilent, USA). Mass spectrometric detection was performed on a triple quadrupole instrument (AB Sciex, API3000, USA). The Agilent1.4 software packages were used to control the HPLC–MS/MS system as well as for data acquisition and processing.

2.2. *Recombinant UGT71C5 catalyzes ABA glucosylation in vitro and construction of transgenic line*

UGT71C5 was expressed, and fused with His-tagged polypeptides in *Escherichia coli* and purified by affinity chromatography. When purified recombinant *UGT71C5* was added to the reaction mixture, ABA-GE was detected as control because it was not observed in the reaction mixture containing denatured *UGT71C5*.

UGT71C5 was overexpressed in *A. thalia*na plants (ecotype RLD) under control of the Cauliflower mosaic virus (CaMV) 35S promoter from binary vector pJR1Ri. 71C5-DN is a transposon-insertion line obtained from the Sainsbury Laboratory Arabidopsis Transposants (SLAT) collection. The 71B6-KO line was complemented by transformation with pART27 vector carrying the genomic fragment containing the *UGT71B6* gene with its own promoter (440 bp) and terminator (1000 bp) to give rise to the 71B6-RE lines. NCED3-OX was kindly provided by Kazuo Shinozaki; ABA3-2 was previously described by Leon-Kloosterziel et al. Plants were grown on Levington's seed and modular compost in a controlled environment of 16:8 h light–dark cycles (22 °C during the day, 18 °C at night). Salt treatment was performed as follows: 4-week-old plants, grown hydroponically in 0.5 MS medium, were incubated with their roots submerged in media supplemented with 150 mM NaCl for 24 h. For post-germinative growth and glucose-sensitivity experiments, seeds of the same age were surface sterilized with chlorine gas and plated on 1 MS medium containing 0.8% (w/v) agar and 0.5 μM (þ)-ABA, PBI-514, PBI-413 or 6% (w/v) glucose. After stratification at 4 °C in the dark for 4 days, the seeds were transferred to a controlled environment of 16:8 h light–dark cycles (22 °C during the day and 18 °C at night). Experiments were performed with three replicates of at least 150 seeds.

2.3. *Preparation of stock solution, standards and quality control samples*

Stock solutions of ABA (1.085 mg/mL), ABA-GE (196.0 μg/mL) and chloramphenical (142.5 μg/mL) were prepared by dissolving accurately weighed standard compounds in methanol–water (50:50, v/v), stored in a refrigerator at −20 °C, and protected from light until use. Working solutions of ABA (ranging from 0.8684 to 217.1 ng/mL), ABA-GE (ranging from 0.3920 to 196.0 ng/mL) and IS (14.25 ng/mL) were prepared by serial dilution of the stock solution in mobile phase solution [methanol:water:acetic acid (45:54:1, v/v/v)].

Calibration standard samples were prepared by adding 100 μL ABA, 200 μL ABA-GE and 40 μL IS of the appropriate standard working solutions into 1 mL methanol:water:acetic acid (10:89:1, v/v/v), to yield calibration concentrations of 0.8684, 1.737, 5.427, 21.71, 54.27, 108.6 and 217.0 ng/mL for ABA, and 0.3920, 0.7840, 1.960, 7.840, 19.60, 78.40 and 196.0 ng/mL for ABA-GE.

Quality control (QC) samples were similarly prepared at concentrations of 1.737 (low-) for ABA and 0.7840 (low-) for ABA-GE; 21.71 (medium-) for ABA and 19.60 (medium-) for ABA-GE; and 108.6 (high-) for ABA and 98.00 (high-) ng/mL for ABA-GE. All stock standard solutions and spiking/working standard solutions were stored in a refrigerator at −20 °C and thawed at room temperature before use.

2.4. *Extraction of ABA and ABA-GE*

For turgid tissues, 4-week-old rosettes were harvested and frozen immediately in liquid N_2. The wilting treatment was carried out according to the method of Leon-Kloosterziel et al. Detached rosettes were dehydrated in dry air at 20 °C for 3 h. The stressed material was kept in polythene bags at 22 °C for 6 h and then frozen in liquid N_2. The frozen material was freeze-dried and the dry weight was determined. ABA and ABA-GE were extracted for analysis as described. Freeze-dried tissue (50–100 mg) was ground to powder; 1 mL extraction solvent [isopropanol:water:glacial acetic acid (80:19:1, v/v/v)] was added. Samples were mixed by vortexing and then incubated with shaking, overnight at 20 °C. After centrifugation (16,000*g* for 2 min, 20 °C), the supernatant was collected and pellets were rinsed with a further 0.5 mL extraction solvent. The supernatants were pooled in a fresh tube and dried by centrifugation under vacuum. Following resuspension in 1 mL isopropanol:acetic acid (99:1, v/v) by vortexing and sonication, the samples were centrifuged (16,000*g* for 2 min, 20 °C); the supernatant was transferred to a fresh tube and then dried again. The samples were dissolved in 50 μL methanol:acetic acid (99:1, v/v) and further 450 μL water:acetic acid (99:1, v/v) was added [9]. Oils in the samples were removed by partitioning using 1 mL hexane and the remaining aqueous extracts (bottom layer) were again removed into a fresh tube and dried by centrifugation under vacuum and then stored in a refrigerator with temperature maintained at −20 °C.

2.5. *Sample preparation*

Extracts were thawed to room temperature before dissolved in 100 μL of methanol:glacial acetic acid (99:1, v/v) and topped up to 1 mL with water:glacial acetic acid (99:1, v/v) [9]. The mixture was vigorously vortexed for 1 min and then a 40 μL aliquot of the IS solution (14.25 ng/mL), 100 μL, then 200 μL of methanol: water:acetic acid (10:89:1, v/v/v) was added and vortexed for 1 min again. Oasis® HLB cartridge (200 mg, 3 mL, 50 pkg, Waters Corp., Bristol, UCT, USA) was conditioned with 2 mL of 100% methanol and equilibrated with 2 mL of methanol:water: glacial acetic acid (10:89:1, v/v/v). After loading the samples, the cartridge was washed with 2 mL of methanol:water:glacial acetic acid (10:89:1, v/v/v); then ABA, ABA-GE and IS were eluted with 2 mL of methanol:water:glacial acetic acid (80:19:1, v/v/v). The eluate was evaporated to dryness at 40 °C under a gentle stream of nitrogen, and the residue was reconstituted by addition of 200 μL methanol:water:acetic acid (45:54:1, v/v/v), vigorously vortexed for 1 min and then centrifuged at 12,000*g* for 5 min. The supernatant was filtered with 0.22 μm aperture microporous membrane before transferring to an autosampler vial for HPLC insert, and the autosampler vials were placed in the autosampler for analysis.

2.6. *Detection method*

Chromatographic separation was achieved on an Inertsil ODS-3 column (2.1 mm × 100 mm, 5 μm) protected by a phenomenex C18 guard column (4 mm × 3.0 mm i.d.; Torrance, CA, USA).

The column was maintained at 25 °C and eluted with methanol: water:acetic acid (45:54:1, v/v/v) as mobile phase at a flow rate of 0.30 mL/min. For analysis, 10 μL aliquots of sample were loaded. The column elution was analyzed by MS/MS spectrometry. The retention times were about 6.4 min for ABA, 2.3 min for ABA-GE, and 3.8 min for chloroamphenicol.

The mass spectrometer was operated in the negative ion mode with electrospray ionization-tandem mass spectrometry (ESI-MS/MS) [14]. Nitrogen was used as the nebulizer, auxillary, collision and curtain gas. The main working source/gas parameters of mass spectrometer were optimized and maintained as follows: collision activated dissociation (CAD) gas, 6; curtain gas, 8; nebulizer gas, 8; turbo ion spray (IS) voltage, −4.0 kV; and source temperature, −450 °C. The compound parameters like declustering potential (DP), entrance potential (EP), focusing potential (FP), collision energy (CE), and collision cell exit potential (CXP) were optimized and set at −40, −5, −200, −15 and −25 for ABA; −40, −5, −100, −15 and −10 for ABA-GE; and −40, −5, −100, −25 and −25 for IS, respectively. Analytes were detected by tandem mass spectrometry using multiple reaction monitoring (MRM) of the most intensive precursor→fragment transitions with 200 ms dwell time, at m/z 263.1→153.0 for ABA, m/z 425.1→263.0 for ABA-GE, and m/z 321.0→152.0 for chloramphenical (IS) (Fig. 1). In this assay, the mass spectrometer was operated at low mass resolution (peak width at half-height set at 0.7 Da) for Q1 and unit mass resolution for Q3. For each injection, the total data acquisition time was 10 min.

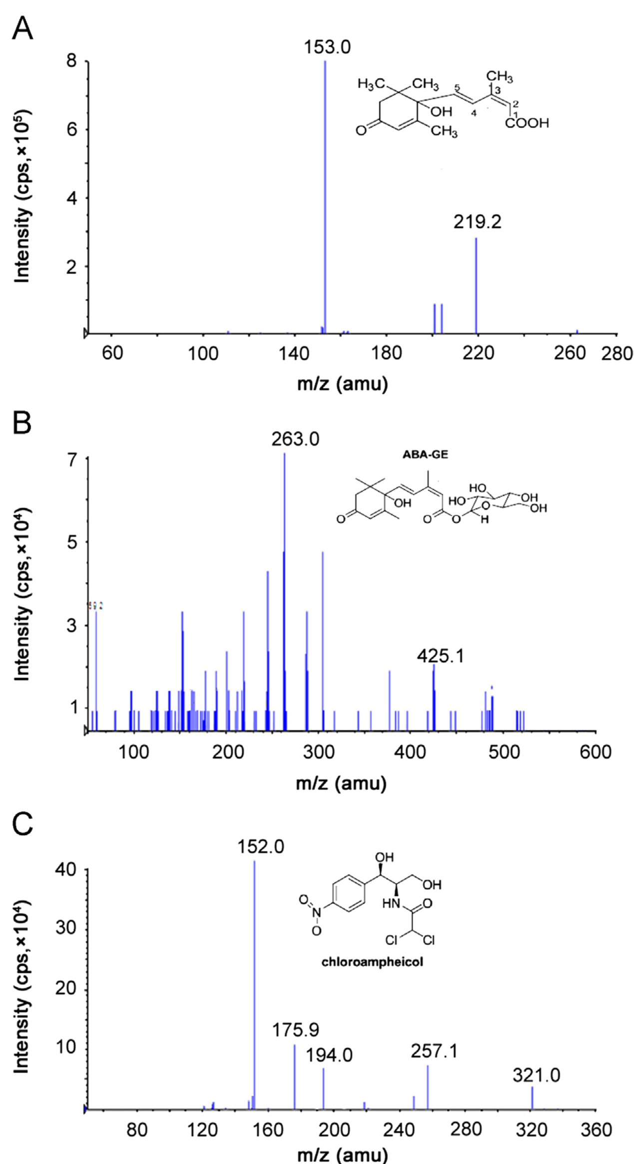

Fig. 1 MS/MS spectra of ABA (A), ABA-GE (B) and IS (C).

3. Results and discussion

3.1. Optimization of internal standard

Due to the high price and difficulty to obtain, the isotope internal standard (d4-abscisic acid) is hard to be used as internal standard. However, chloroamphenicol is easily available and cheap, and most importantly, chloroamphenicol and ABA can both be detected in the negative ion mode with ESI-MS/MS. In our study, we investigated the retention time (t_R) and sensitivity of chloroamphenicol, ABA and ABA-GE under the same chromatographic conditions. The t_R of chloroamphenicol was about 3.80 min and that of ABA and ABA-GE was about 6.40 min and 2.33 min, respectively, indicating that chloroamphenicol can be used as an internal standard.

In addition, because of the great chemical structural differences between chloroamphenicol and ABA, and the complex pretreatment that made it hard to predict and guarantee the recovery of chloroamphenicol during the extraction of ABA and ABA-GE process, finally, IS was added before solid phase extraction (SPE). In addition, ABA and ABA-GE are endogenous plant hormones and blank matrix was unavailable, so mobile phase was chosen in place of plant blank sample.

3.2. Optimization of column

In order to improve the sensitivity and obtain good peak shape, ultimate XB-C18 (2.1 mm × 50 mm, 5 μm) column and Inertsil ODS-3 (2.1 mm × 100 mm, 5 μm) column were chosen for investigation. Using ultimate XB-C18 (2.1 mm × 50 mm, 5 μm) column, the peak of ABA was frontier and shoulder peak. After comparison, Inertsil ODS-3 column (2.1 mm × 100 mm, 5 μm) was selected.

3.3. Selectivity

Selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. The selectivity of the method was evaluated by analyzing a blank sample, a blank sample spiked with the lower limit of quantitation (LLOQ, 0.8684 ng/mL for ABA, 0.3920 ng/mL for ABA-GE) and an *E. coli* sample, which grew under normal conditions without over-expression (OE) or down-expression of recombinant proteins *UGT71C5*. All samples were prepared as 2.5 sample preparation. As can be seen in Fig. 2, the selectivity of this method was good.

3.4. Linearity of calibration curve and lower limit of quantification

The linear regression of the peak-area ratios versus concentrations was fitted over the concentration range of 0.8684–217.1 ng/mL for

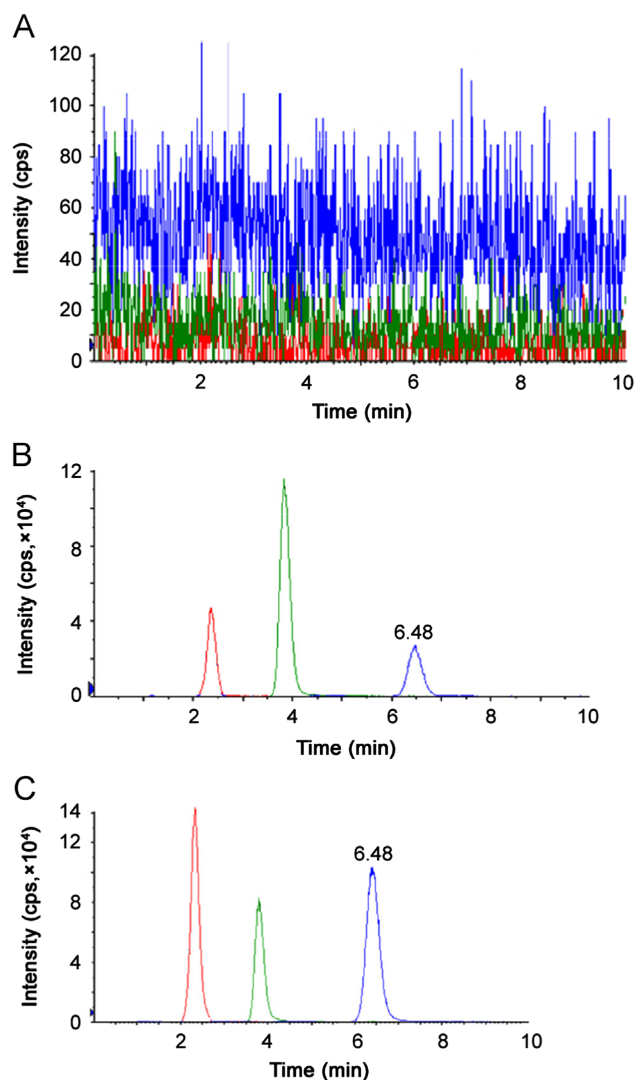

Fig. 2 Specific chromatograms of ABA and ABA-GE with MS/MS detection. (A) Blank sample; (B) a blank sample spiked with LLOQ (0.8684 ng/mL for ABA, 0.3920 ng/mL for ABA-GE and 14.25 ng/mL for IS); (C) an *E. coli* sample.

ABA and 0.3920–196.0 ng/mL for ABA-GE. The standard curves were calculated by a weighted (1/*x*) least squares method through the measurement of the peak-area ratio of the analytes to IS. A typical regression equation was $y=0.0152x+0.00265$ with a correlation coefficient (*r*) of 0.9992 for ABA and $y=0.0158x+0.00829$ with a correlation coefficient (*r*) of 0.9988 for ABA-GE, where *y* represents the peak-area ratio of analytes to IS and *x* represents the concentration of analytes.

The lower limit of quantification (LLOQ), taken as the lowest concentration (0.8684 ng/mL for ABA and 0.3920 ng/mL for ABA-GE) on the calibration curve that could be measured with acceptable accuracy and precision, was determined in six replicates on one consecutive validation day. The precision was expressed as relative standard deviation (RSD). The RSD(%) of the precision of ABA was $\leq 7.2\%$, ABA-GE was $\leq 4.5\%$, and the accuracy was 86.4–101.0% for ABA and 88.6–90.0% for ABA-GE. The present LLOQ was sensitive enough to investigate the concentration of ABA and ABA-GE in *A. thaliana.*

3.5. Extraction recovery

The recovery of analytes was estimated at three concentration levels by comparing the peak-area ratios of the analyte to IS. Samples that were spiked with the analytes prior to SPE were compared with samples to which the analyte was added after extraction. The IS was added to the two sets of samples after extraction. The extraction recovery of the IS was determined in a similar way, using the QC samples at medium concentration as a reference.

The recoveries of ABA extracted from leaves of *A. thaliana* were $82.6\pm2.2\%$, $74.7\pm8.7\%$ and $69.3\pm6.6\%$ at concentrations of 1.737, 21.71 and 108.6 ng/mL, respectively ($n=6$). Those of ABA-GE were $91.9\pm7.0\%$, $71.5\pm5.7\%$ and $64.8\pm3.7\%$ at concentrations of 0.7840, 19.60 and 98.00 ng/mL, respectively ($n=6$). Mean recovery for the IS was $65.3\pm6.5\%$ ($n=6$). The results indicated that the extraction efficiency for ABA and ABA-GE using SPE was satisfactory.

3.6. Precision and accuracy

Precision and accuracy of the method were assessed by the determination of QC samples at three concentration levels (1.737, 21.71 and 108.6 ng/mL for ABA; 0.7840, 19.60 and 98.00 ng/mL for ABA-GE) in six replicates on 3 validation days. The intra- and inter-day precisions were required to be below 15%, and accuracy was required to be between 85% and 115% of QC concentrations for both within- and between-assay [15].

The method showed good precision and accuracy. Table 1 summarizes the intra- and inter-day precision and accuracy values for QC samples. In this assay, the intra- and inter-assay precisions were measured to be below 5.5% and 5.3% for ABA and 8.4% and 8.8% for ABA-GE, respectively. Intra- and inter-day accuracies were less than $\pm6\%$. These values were within the acceptable range; therefore, the method was judged to be suitably accurate and precise.

3.7. Stability

The stability experiments aimed at testing all possible conditions that the samples might experience during the shipping and handling such as a short storage at room temperature (bench-top) and during analysis such as extracted samples sitting in sample tray or refrigerator. All stability results are summarized in Table 2. It shows that 6 h room temperature storage for QC samples had no substantial effect on the results. Keeping extracts at room temperature for approximately 12 h prior to injection did not affect the quantitative determination of ABA and ABA-GE in samples. The 7-day storage stability of QC samples was also tested. The results showed that the QC samples were stable for at least 7 days if stored frozen at approximately -20 °C (Table 2).

3.8. Methodology application

In this study, we characterized *AtUGT71C5* in ABA metabolism through sequence alignment with *AtUGT71B6*. Expression of recombinant proteins *UGT71C5* in *E. coli* confirmed its capacity to glucosylation of ABA *in vitro*. This study described the impact of excess or absence of *UGT71C5* in *Arabidopsis*. De-regulated *UGT71C5* exhibited significantly ABA over-accumulated phenotypes, such as delayed germination, closed stomata, decreased water

Table 1 Intra-day and inter-day precision and accuracy of QC samples.

QC sample	QC concentration (ng/mL)	Intra-day ($n=6$)			Inter-day ($n=18$)		
		Average ± SD (ng/mL)	RSD (%)	Accuracy ± SD (%)	Average ± SD (ng/mL)	RSD (%)	Accuracy ± SD (%)
ABA	1.737	1.630 ± 0.07	4.43	94.02 ± 4.25	1.600 ± 0.05	3.34	92.22 ± 3.14
	21.71	20.92 ± 0.51	2.45	96.40 ± 2.34	20.96 ± 1.08	5.16	96.51 ± 4.93
	108.6	111.0 ± 5.93	5.35	102.2 ± 5.57	107.3 ± 5.70	5.32	98.84 ± 5.23
ABA-GE	0.784	0.740 ± 0.04	5.88	94.95 ± 5.45	0.730 ± 0.04	4.91	93.24 ± 4.52
	19.60	19.45 ± 1.65	8.50	99.28 ± 8.36	20.32 ± 1.80	8.84	103.7 ± 9.17
	98.00	93.43 ± 4.95	5.30	95.30 ± 5.05	100.8 ± 6.40	6.35	102.8 ± 6.50

Table 2 Stability data for ABA and ABA-GE under various conditions ($n=6$).

Condition	QC sample	Concentration (ng/mL)	Average ± SD (ng/mL)	RSD (%)	Rate of change (%)
Room temperature stability (6 h)	ABA	1.737	1.710 ± 0.07	3.81	6.31
		21.71	21.48 ± 1.55	7.23	4.97
		108.6	107.1 ± 7.20	6.72	−1.56
	ABA-GE	0.780	0.746 ± 0.03	3.38	0.09
		19.60	19.77 ± 0.13	0.66	1.63
		98.00	103.7 ± 1.75	1.69	−1.58
In injector stability (12 h)	ABA	1.737	1.680 ± 0.15	8.76	4.45
		21.71	20.75 ± 1.11	5.34	1.38
		108.6	102.6 ± 5.77	5.62	−5.73
	ABA-GE	0.780	0.802 ± 0.01	1.25	7.61
		19.60	19.63 ± 0.54	2.75	0.93
		98.00	95.43 ± 2.67	2.80	2.14
Long-term stability (7 days)	ABA	1.737	1.570 ± 0.08	4.82	−2.38
		21.71	19.40 ± 0.56	2.91	−5.29
		108.6	102.4 ± 3.34	3.27	−6.27
	ABA-GE	0.780	0.752 ± 0.03	4.00	0.96
		19.60	18.94 ± 1.03	5.43	−2.62
		98.00	96.27 ± 1.84	1.91	3.04

Table 3 Results of determination of content of endogenous ABA/ABA-GE in AtUGT71C5 transgenic lines.

Transgenic lines	Normal ambient		After 4 h drought treatment	
	ABA (pmol/g)	ABA-GE (pmol/g)	ABA (pmol/g)	ABA-GE (pmol/g)
DN-11	554.58	76.09	5540.74	72.60
DN-52	720.55	36.89	3709.47	70.94
DN-73	438.34	68.43	4112.27	59.15
RLD	358.15	51.76	2683.77	102.62
OE-11	243.88	101.04	2111.77	233.29
OE-41	313.68	51.66	2062.27	122.34
OE-51	234.30	73.66	2425.03	113.05
Col	324.68	41.97	2767.00	70.83
ugt71c5	586.41	21.07	4161.62	39.48
ugt71b6	334.96	23.74	3853.35	41.48

loss rate and drought stress resistance. However, up-regulated *UGT71C5* displayed obviously ABA deficient phenotypes compared with de-regulated *UGT71C5*. Using the established HPLC–MS/MS method to profile accounts of ABA and ABA-GE in *UGT71C5* sense, anti-sense lines and loss-of-function *UGT71C5* mutant, it revealed that the levels of ABA were significantly higher in anti-sense lines and loss-of-function mutant than in sense lines. Correspondingly, the contents of ABA-GE in anti-sense lines and loss-of-function mutant were lower than those of sense lines. Finally, the transcription levels of ABA related-genes analysis by

qRT-PCR revealed that ABA-related genes were un- or down-regulated in down- or up-expressed *AtUGT71C5* transgenic plants, respectively. Complementary *in vitro* and *in vivo* evidence confirmed our conclusion that *UGT71C5* plays a primary role in ABA glucosylation for homeostasis mediation. The main results are shown in Table 3.

4. Conclusions

The validated HPLC–MS/MS method is simple, sensitive and accurate. It is suitable for profiling the dynamics of ABA and ABA-GE in *A. thaliana* leaves. Determination of the sample used in this study showed satisfactory results. All the evidence confirmed the speculation that *AtUGT71C5* can mediate abscisic acid homeostasis.

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