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Simultaneous determination of jasmonic acid epimers as phytohormones by chiral liquid chromatography–quadrupole time-of-flight mass spectrometry and their epimerization study

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ABSTRACT

Jasmonic acid (JA) is an essential plant hormone involved in plant development and defense system. There are four stereoisomeric forms of JA and they act quite differently *in vivo*. In this work, a normal phase liquid chromatography–quadrupole time-of-flight mass spectrometry (NPLC–QTOF-MS) method using cellulose tris (4-methylbenzoate) coated silica gel as the chiral stationary phase was first established for the simultaneous discrimination and direct analysis of all the four JA stereoisomers without need of derivatization. A non-endogenous JA stereoisomer was introduced as the internal standard to ensure the reliability of the developed method. Satisfactory results were obtained in terms of sensitivity (limit of detection, 0.5 ng mL^{-1} or 2.4 fmol), linearity (R^2 = 0.9996) and repeatability (run-to-run RSD of migration time and peak area, 0.37% and 5.9%, respectively, n = 6). Endogenous rise of two natural JA stereoisomers was detected in tobacco leaves and their variations in response to mechanical wounding were monitored. In addition, the configurational stability of JA stereoisomers was investigated using the stereoisomerically pure forms which were not commercially available but easily obtained by our semi-preparative chiral LC method. Experimental evidence indicated that both of the two naturally existing JA stereoisomers were putative signals for wounding response, and the epimerization between them was not a spontaneous process simply promoted by the thermodynamical instability as expected before.

1. Introduction

Jasmonic acid (JA), as well as its precursors and derivatives (jasmonates) is an essential phytohormone for plant development and stress response. JA acts as the regulatory molecule in root elongation, germination, pollen production, fruit ripening and plant senescence [1,2]. It also acts as the signaling compound activating plant defenses against pathogens, herbivory, wounding and abiotic stress [2–4]. The specific bioactivities of JA and other jasmonates are determined by their specific structures whose change will result in hormone inactivation [5]. Distinct structural requirements for JA activity were defined on the basis of various biological responses caused by JA treatment [6–8]. And the key points were summarized in the early stage [2,7,9], for example, an intact pentenyl side chain, a cyclopentanone ring and a keto group at C-6. Requirements like this are within the expectation since they are why these cyclopentanone derivatives have been classified as the hormone family of jasmonates. A comparatively special observation is the stereoselectivity found in JA's bioactivity. As viewed from the chemical structure (Fig. 1), JA possessing two chiral centers at C-3 and C-7 can generate four possible stereoisomeric forms, (−)-(3R,7R)-JA (known as (−)-JA), (+)-(3S,7S)-JA ((+)-JA), (−)-(3S,7R)-JA ((−)-epi-JA) and (+)-(3R,7S)-JA ((+)-epi-JA), among which (−)-JA and (+)-JA are a pair of enantiomers in *trans* form while (−)-epi-JA and (+)-epi-JA are a pair of enantiomers in *cis* form. The epi-JAs are thermodynamically less stable due to higher steric hindrance between the *cis* side chains. So they have a tendency to epimerize at C-7 to their corresponding *trans* forms with the fixed stereochemistry at C-3 [10]. In other words, (−)-JA and (+)-epi-JA are considered as each other's epimers, and so are (+)-JA and (−)-epi-JA. In the plant kingdom, (−)-JA and (+)-epi-JA are believed to be the naturally existing stereoisomers [11]. Extensive studies have been focused on the stereoselective bioactivities of JA family, and different opinions and arguments are inevitable. Previously, (−)-JA and its derivatives were considered as the major naturally existing stereoisomers and more active than (+)-epi-JA and its derivatives [2,7,9,12,13]. Later, kinetic analysis showed some enzymatic reactions had a strong preference for (+)-7-epi-JA as opposed to its epimer (−)-JA [14,15,16]. Meanwhile some other research found that the stereoselective hormone perceptions of JA were different

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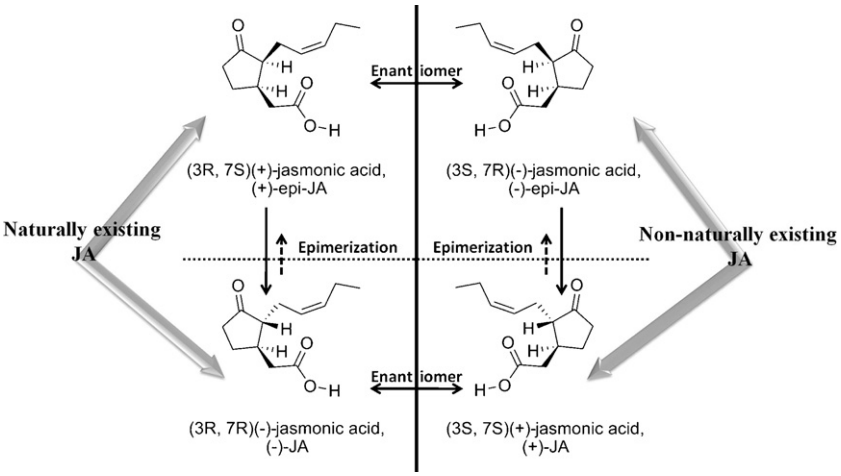

Fig. 1. Chemical structures of four JA stereoisomers.

under different conditions or in different plant species [5,17,18]. Most recently, a hypothesis has been proposed: the epimerization between (+)-epi-JA and (−)-JA might be an *in vivo* mechanism for switching on or off JA activities [19–21].

However, the demonstrations of such an assumption and even the previous research on the stereoselective activity of JA have been suffering from methodological problems. Direct distinction of bioactive JA stereoisomers is not possible yet, because no method for simultaneous separation and determination of all the four JA stereoisomers has been developed and no pure JA stereoisomers are commercially available. From the very beginning when the functional analysis of JA was initiated [22], exogenous application of JA to target plants is always the most common approach. But commercially available JA used in biological studies is racemic mixture containing approximately 3–5% each of the (−)-epi-JA and (+)-epi-JA, and 45-47% each of the (−)-JA and (+)-JA [10]. Such a racemic JA sample, in which the proportion of each JA stereoisomers is quite different from that in plant sample, may lead to lots of confusion. For example, the previously proposed active hormone (−)-JA-L-Ile has been recently demonstrated totally inactive, and the misunderstanding of its activity could be attributed to the residual contamination caused by the active epimer (+)-epi-JA-L-Ile [17]. To the best of our knowledge, asymmetric synthesis was developed to obtain pure JA stereoisomers individually but these methods were hardly adopted because of the difficult handling [23]. In separation science, chiral gas chromatography (GC) needs further derivatization of JA into, for example, its methyl ester. However, the direct and specific analysis of endogenous JA isomers could not be achieved [24–27]. The reported chiral high performance liquid chromatography (HPLC) method using the cyclodextrin derivative Nucleodex β-PM as stationary phase could only distinguish (−)-JA and (+)-JA but is powerless for all the four stereoisomers [28]. Thus such a method is not so meaningful for plant samples since the naturally existing isomers are (−)-JA and (+)-epi-JA. Recently, capillary electrophoresis (CE) was used to achieve the direct resolution of both pairs of JA enantiomers [29]. However, improved sensitivity and robustness are still expected, and practical methods to resolve and obtain pure JA stereoisomers are highly desirable

Based on our previous work [29,30], here we report simple but rigorous chiral HPLC methods for JA enantioseparation using cellulose tris (4-methylbenzoate) coated silica gel as the stationary phase. The semi-preparative method was developed to obtain individual JA stereoisomers for exogenous applications. And the method using analytical normal-phase HPLC coupled with quadrupole time-of-flight mass spectrometry (NPLC–QTOF-MS) was developed for the distinction and determination of the endogenous JA stereoisomers. The reliability of this NPLC–QTOF-MS method was guaranteed by the introduction of (+)-JA, a non-endogenous JA stereoisomer as the internal standard. Moreover, endogenous rise of JA stereoisomers in correlation with mechanical wounding was then studied in tobacco leaves.

2. Experimental

2.1. Chemicals and samples

Racemic jasmonic acid and HPLC-grade ammonia solution (28% in water, NH_4OH) were purchased from Sigma–Aldrich (St. Louis, MO, USA). HPLC-grade n-hexane, isopropanol, methanol and acetic acid were purchased from Dikma Technology (Richmond, VA, USA). Diethyl ether was provided by Beijing Chemical Factory (Beijing, China). Purified water was purchased from Hangzhou Wahaha Group Co. (Zhejiang, China).

Stock solution of jasmonic acid (1 $\mu g\,mL^{-1}$) was prepared in methanol. All the sample solutions were filtered through a 0.22 μm membrane filter before use and stored at −20 °C.

2.2. HPLC conditions

An Agilent 1200 series HPLC system (Agilent Technologies, Karlsruhe, Germany), equipped with a binary pump, an online degasser, an auto-sampler, a column oven and a DAD detector, was used. UV detection wavelength was set at 200 nm and 210 nm. Mobile phase was composed of 95/5 (v/v) n-hexane/isopropanol. Semi-preparative isolation and collection was performed on a CHIRALCEL OJ-H (4.6 mm × 250 mm, 5 μm, Daicel, JPN) column with a mobile phase flow rate of 1.0 mL min^{-1}, and LC–MS analysis was performed on a CHIRALCEL OJ-3 (2.1 mm × 150 mm, 3 μm, Daicel, JPN) column with a mobile phase flow rate of 0.25 mL min^{-1}. The column oven was operated at 25 °C.

2.3. HPLC–QTOF-MS

The HPLC system was coupled to an Agilent 6530 Accurate-Mass Quadrupole Time-of-Flight mass spectrometer (QTOF MS) equipped with an Agilent Jet Stream electrospray ionization (ESI) source which operated in negative mode. Instrument parameters were set as follows: drying gas temperature, 325 °C; dry gas flow, 10 L min^{-1}; nebulizer, 60 psi; sheath gas temperature, 350 °C; sheath gas flow, 7.5 L min^{-1}; capillary voltage, 2250 V; fragmentor, 135 V; Skimmer1, 65 V. The MS scan data were collected at a rate of 1.03 spectra/s in the range of m/z 50–1100. The m/z

values of all ions present in the mass spectra were corrected by two reference ions, trifluoroacetic acid anion (m/z 112.985587) and hexakis-trifluoroacetic acid adduct (m/z 1033.988109) (Agilent P/N G1969-85001), which ensured mass error was less than 3 ppm in our experiment. In the targeted MS/MS mode, the MS and the MS/MS data were collected in the same m/z range at the same rate as the MS scan, and collision energy was 16 V. All the MS and MS/MS data were collected with MassHunter Data Acquisition B.02.01 (Agilent), and processed by MassHunter Qualitative Analysis B.03.01 (Agilent). All extracted ion chromatograms (EIC) were obtained with ±5 ppm m/z expansion.

To guarantee an efficient ionization of JA after normal phase LC separation, a make-up solvent composed of 0.1% NH_4OH in isopropanol was added. The post-column mixing of the make-up solvent was carried out by an isocratic pump (Agilent) with a flow rate of 0.25 mL min^{-1} for delivery and a tee junction for mixing.

2.4. Circular dichroism (CD) measurement

Circular dichroism (CD) measurements were performed offline on a JASCO J-810 spectrometer. The temperature was mediated with a PolyScience programmable temperature controller. The light path lengths of the quartz cells used were 0.2–2 mm. The solvent was acetonitrile and the sample concentration was 1.5×10^{-5} mol L^{-1}.

2.5. Tobacco leaves for mechanical wounding

Sterilized tobacco (*Nicotiana tabacum* L. *var*. Winsconsin 38, W 38) seeds were sown in 50 cm × 35 cm × 12 cm plastic dishes which were washed with water and sterilized with 70% ethanol. The soil composed of nutrient soil and vermiculite (1:3, v/v) was sterilized by moist heat under high temperature and pressure. Then the dishes were covered with plastic film and placed in green house. After 8 days, the plastic film was removed. When the tobacco seedlings grown to four-leaf phase, they were moved to growth chamber filled with sterilized soil. The culture condition of day length was light 14 h at 25 °C and dark 10 h at 23 °C.

Fully expanded tobacco leaves were washed with deionized water and then pressed by a hemostat several times across the midvein for mechanical wounding. The damages were performed as consistently as possible for every leaves. Each wounded leave was sampled for six times, that is 0 h (no wounding), 0.5 h, 1 h, 2 h, 3 h and 5 h after wounding. And samples harvested for each time point included leaves from all around the plant (top, middle and bottom). Parallel samples were from three different plants. Undamaged leaves were treated as control samples.

The harvested leaves were immediately ground into fine powder under liquid nitrogen with mortar and pestle. The powder was then divided into several portions, weighted and spiked with known amount of (+)-JA as internal standard. Then methanol/water (80/20, v/v) was added at 10 μL mg^{-1} FW (FW, fresh weight) for extraction, followed by overnight agitation at 4 °C and then centrifugation at 20,000 rpm for 10 min at 4 °C. After centrifugation, the supernatant was collected and evaporated under reduced pressure to remove methanol. The pH of the aqueous residue was adjusted to 2.8 with acetic acid. Then the acidic residue was partitioned twice against an equal volume of diethyl ether. After the aqueous phase was discarded, the organic fraction was vacuum evaporated at room temperature. The solid residue was re-suspended in methanol and filtered through a 0.22 μm membrane filter before analysis.

3. Results and discussion

3.1. Chiral separation and identification of JA stereoisomers

Different types of chiral stationary phases were first investigated and cellulose tris (4-methylbenzoate) coated silica gel was finally demonstrated to be the most effective one for JA enantioseparation. With this stationary phase, the racemic JA sample was easily separated into two groups using n-hexane/isopropanol as mobile phase. The proportion of n-hexane/isopropanol was further optimized for the baseline separation of all the four stereoisomers. Finally, 95/5 (v/v) n-hexane/isopropanol at a flow rate of 1.0 mL min^{-1} for a 4.6 mm × 250 mm OJ column was selected to ensure satisfactory resolution and peak shapes. Although acidic modifiers like trifluoroacetic acid, acetic acid and formic acid were generally believed to be able to enhance the enantioselectivity for acidic analytes, they were avoided in our experiments because no significant improvement on the separation was observed. Every separated peak was collected as a fraction for structure and configuration determination. JA stereoisomers were identified by MS and MS/MS data in combination with CD data. Among the four JA peaks, the first two peaks displayed negative CD signals while the last two peaks displayed positive CD signals. Therefore, the major peaks (Peak 2 and Peak 4) were characterized as (−)-JA and (+)-JA respectively, likewise the minor peaks (Peak 1 and Peak 3) were characterized as (−)-epi-JA and (+)-epi-JA respectively (Fig. S1). To collect the pure JA stereoisomers for later use, the loading capacity was tested to offer high throughput as well as high purity of each fraction. The optimized injection amount for each run was found to be 10 μL racemic JA at a concentration of 20 mg mL^{-1}. All the four fractions were collected within a relatively narrow elution window. Then the individual fractions were concentrated and re-injected into the OJ column to check the enantiopurity, so that pure single JA stereoisomers can be guaranteed (Figs. 3 and S2). To prepare these collections as standard samples, their concentrations were determined as follows: First, each collection was dried and re-suspended in methanol instead of the combined solvent hexane and isopropanol to minimized the concentration alteration caused by solvent volatilization; Second, 0.5 mg mL^{-1} racemic JA sample was analyzed by LC–UV (CHIRALCEL OJ-H column (4.6 mm × 250 mm, 5 μm, Daicel, JPN), UV detection at 200 and 210 nm) using isopropanol at 0.2 mL min^{-1} as the mobile phase. Thus JA was eluted as a single peak (k = 0.1, Fig. S3). The average peak area and the RSD value (RSD1) of peak area were calculated based on twenty consecutive injections. Then a purified and concentrated JA stereoisomer with an estimated concentration of 0.5 mg mL^{-1} was analyzed under the same condition as that for racemic JA sample. Its concentration was adjusted and re-analyzed from time to time until the RSD value (RSD2) of peak area calculated for six rounds of in-turn injections of racemic JA sample and individual JA stereoisomer was no higher than RSD1. Once confirmed to meet with this requirement, these samples were stored at −20 °C and used as the stock solutions of JA stereoisomer standards with a concentration of 0.5 mg mL^{-1} for the following study.

3.2. Make-up solvent for normal phase LC–MS coupling

ESI is generally considered to be incompatible with normal-phase LC conditions due to the non-polar nature of the mobile phase [31,32], so reversed phase separation using CHIRALCEL OJ-RH (4.6 mm × 150 mm, 5 μm, Daicel, JPN) column was used first in our work. However, the four JA stereoisomers could be only separated into two groups with the retention time longer than 60 min. To highly improve the ionization efficiency of normal phase LC–MS method, in which the four JA stereoisomers were well separated, a post-column addition of 0.1% NH_4OH in isopropanol as

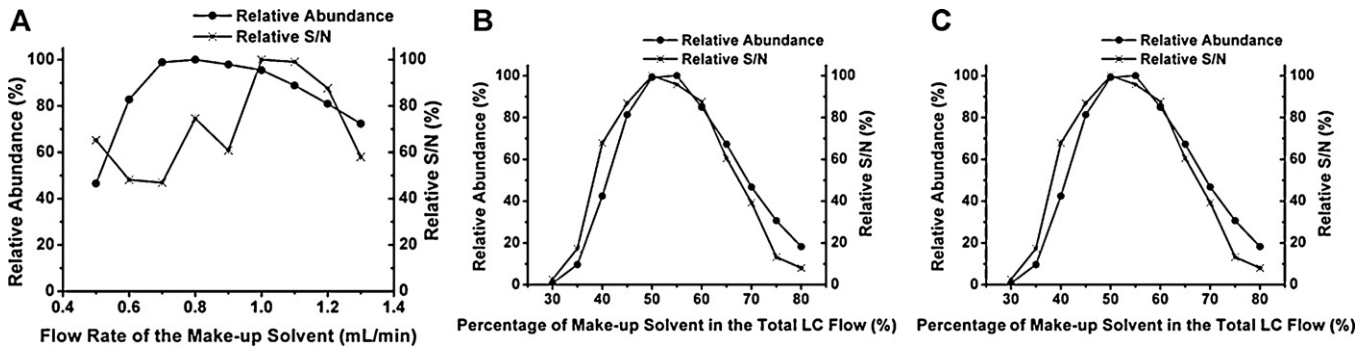

Fig. 2. Optimization of the make-up solvent. (A) Varied flow rate of the make-up solvent with the fixed flow rate of LC mobile phase; (B) varied percentage of make-up solvent with the fixed total flow rate; (C) varied total flow rate with the fixed percentage (50%) of the make-up solvent.

make-up solvent was utilized. One disadvantage of this approach is the post-column dilution of target analytes. Another is the possible insufficient ionization caused by the introduction of too much solvent into MS. Therefore, the percentage of make-up solvent in the total flow, the flow rate of the total flow running into MS and the corresponding MS parameters were systematically optimized.

The ESI source we used features Jet Stream Thermal Focusing technology (Agilent), in which a flow of super heated sheath gas called jet stream is introduced. This jet stream improves the spatial focusing of electrospray droplets and enhances the ion density and desolvation, so that the sensitivity can be improved. What is more important, this feature makes the mass spectrometer compatible with LC flow rate from 0.1 to 2.5 mL min^{-1}. Thus, the OJ column (4.6 mm × 250 mm, typical flow rate 1 mL min^{-1}) could be coupled to MS without splitting the mobile phase. To determine the optimal ratio of make-up solvent to LC mobile phase, the flow rate of the make-up solvent was first optimized with the fixed flow rate of LC mobile phase, and 1:1 was found to be the best ratio considering both MS signal abundance and signal-to-noise ratios (Fig. 2A). Then, the above result was re-checked by varying flow rate of both make-up solvent and LC mobile phase but with the same total flow rate as that in above optimal condition, and 1:1 was still the best ratio (Fig. 2B). So 50% LC mobile phase plus 50% make-up solvent was demonstrated to be a good balance between the ionization efficiency and the dilution effect. Based on this ratio, the total flow rate was further optimized (Fig. 2C). Considering the analysis time and peak shape, the flow rate of LC mobile phase should not be lower than 0.45 mL min^{-1}. For faster analysis and lower solvent consumption, the OJ column (2.1 mm × 150 mm) was also used in our work. The LC conditions were the same as the OJ column (4.6 mm × 250 mm) except the mobile phase flow rate changed to 0.2 mL min^{-1}. Likewise, the make-up solvent and LC mobile phase was carefully adjusted. 0.25 mL min^{-1} LC mobile phase plus 0.25 mL min^{-1} make-up solvent was finally selected and used in the following LC method. Although the resolution was reduced, baseline separation could be achieved within 8 min, shorter than half of that cost on the 4.6 mm × 250 mm column. The influence of the extra post-column volume on the obtained separation resolution must be taken into consideration when the make-up solvent was introduced via a Tee. So the inner diameter of all the PEEK tubes used for post-column connection was 0.1 mm.

The composition of make-up solvent and MS parameters were next studied for sensitivity improvement. Addition of NH_4OH into make-up solvent significantly enhanced the MS response of JA in negative detection mode, but more than 0.1% (v/v) NH_4OH would raise the background signal and show suppression effect. Meanwhile, MS parameters including capillary voltage, fragmentor voltage, drying gas temperature, dry gas flow rate, nebulizer pressure, sheath gas temperature as well as sheath gas flow rate was systematically optimized, and the first two of these parameters were demonstrated to be the key factors for sensitivity enhancement. MS spectra generated in negative ion detection mode gave the deprotonated molecular ion $[M-H]^-$ at m/z 209.1183 for all the four JA stereoisomers. The fragmentation of m/z 209.1183 in targeted MS/MS mode resulted in two predominant product ions, $[M-COOH]^-$ at m/z 165.1285 and $[CH_3COO]^-$ at m/z 59.0139. Modulation of the collision energy gave different ratios of $[M-COOH]^-$ to $[CH_3COO]^-$. Interestingly, the $[M-H]^-/[CH_3COO]^-$ transition gave a signal 50 times greater than $[M-H]^-/[M-COOH]^-$ under their respective optimum conditions. But when compared with the EIC obtained in MS mode, EIC obtained in MS/MS mode did not show much better detection sensitivity as expected, instead, the results were comparable. This may due to the relatively narrow m/z expansion settled for chromatogram extraction in MS mode, which minimized the background noise and offered higher sensitivity. Therefore, $[M-H]^-/[CH_3COO]^-$ transition was only used for identification while the EIC of $[M-H]^-$ was used for quantification.

3.3. Internal standard selection and method validation

The reliability and robustness of this method were highly improved by selecting (+)-JA as the internal standard. Except for chromatographic behavior, this stereoisomer internal standard appears to be almost identical with the target analyte either during the whole procedure of sample preparation or in the process of ionization and fragmentation for MS detection (Table S1). Moreover, as (+)-JA is a non-endogenous stereoisomer, plant samples can be treated as the "blank sample" for such an internal standard. Of course, (−)-epi-JA is also a good candidate considering its stereoisomeric structure and its absence in plants, but finally it was not adopted due to its relatively poor thermodynamic stability.

The proposed chiral LC–MS method enabled the separation of all the four JA stereoisomers as shown in Fig. 3A, and it was then validated under the optimum conditions discussed above. To study the linearity of this procedure, calibration curve was obtained by using solutions containing increasing amounts of (−)-JA and (+)-epi-JA and fixed amounts of (+)-JA. The amount of (+)-JA as internal standard was identical to that added into plant sample for spiking. Prepared from a plot of concentration ratio against the response ratio of standard samples to the internal standard, the calibration curve indicated a linear behavior (R^2 = 0.9996) in the concentration range from 2 to 500 ng mL^{-1}. The quantification limit (S/N = 10) and the detection limit (S/N = 3) for single JA stereoisomer were 1 ng mL^{-1} (4.7 fmol) and 0.5 ng mL^{-1} (2.4 fmol), respectively. The repeatability of this NPLC–MS method was evaluated at three concentrations, 2, 20 and 200 ng mL^{-1}. The RSD values calculated by migration time are 0.10%, 0.37% and 0.37% for run-to-run precision (n = 6) respectively, and 0.14%, 0.52% and 0.58% for day-to-day precision (n = 5) respectively. The RSD values calculated by peak area are 6.1%, 5.9% and 2.4% for run-to-run precision (n = 6) respectively,

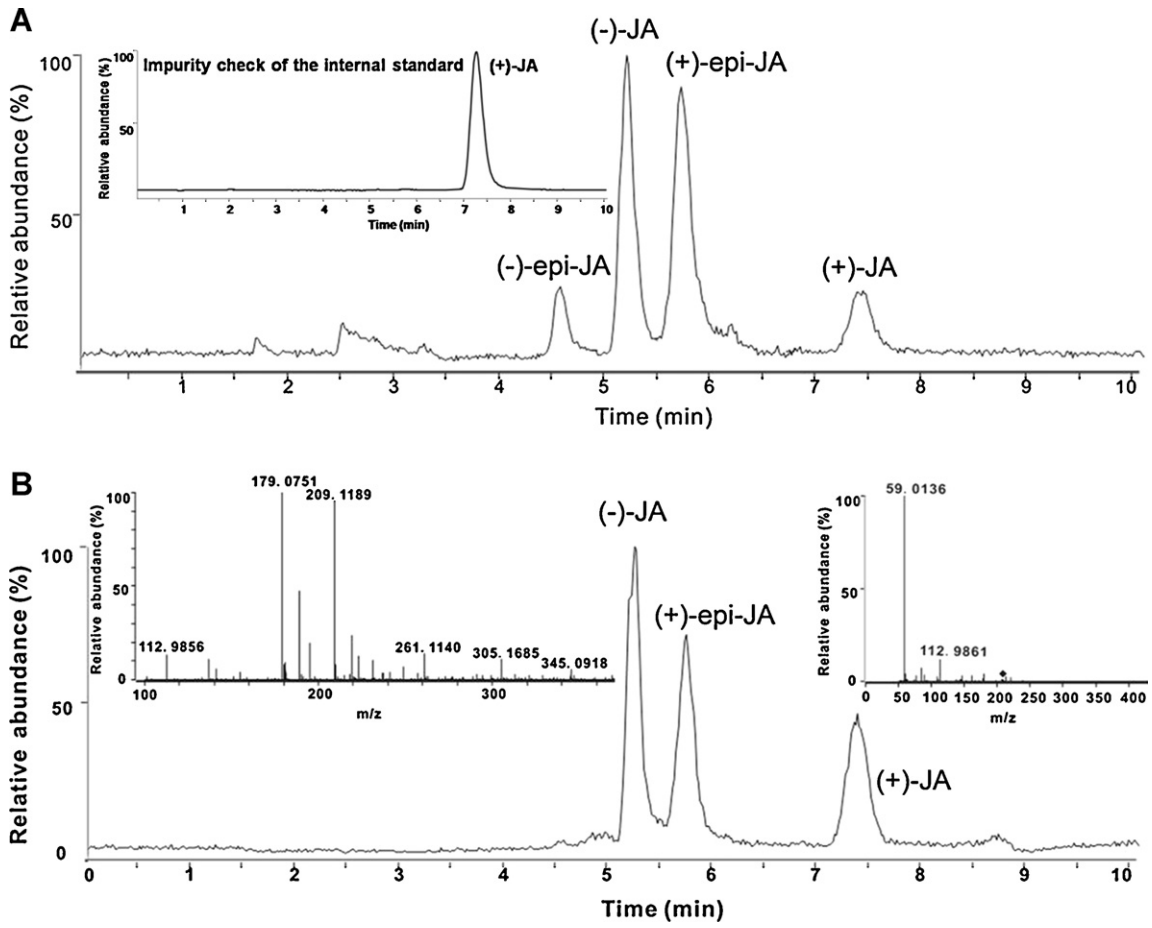

Fig. 3. Extracted ion chromatogram and the MS, MS/MS spectrum of the separated JA stereoisomers. (A) Extracted ion chromatogram of the JA standard prepared by stereoisomerically pure forms of JA collected though the semi-preparative chiral LC method. The inserted extracted ion chromatogram demonstrated the isomeric purity of collected (+)-JA which was used as the internal standard; (B) extracted ion chromatogram of the extraction of wounded tobacco leaves with (+)-JA added as the internal standard. The inserted MS and MS/MS spectrum is the same for all JA peaks (see Section 2 for LC–MS conditions).

and 7.1%, 7.3% and 5.3% for day-to-day precision ($n = 5$) respectively. The recovery and accuracy were evaluated at three spiking concentrations, 5, 75 and 150 ng g^{-1} FW, at which the recovery was calculated as 101%, 97.6% and 98.2%, respectively, and the accuracy was calculated as 106%, 98.5% and 99.0%, respectively. These results proved the feasibility of the proposed method for JA stereoisomer determination.

3.4. Determination of endogenous JA epimers in tobacco leaves

This established method for direct identification of JA stereoisomers allows new experimental strategy to study their stereoselective bioactivities. One of the best studied signal-transduction pathways of JA is wounding response [2]. So in our work, endogenous rise of (−)-JA and (+)-epi-JA was monitored at different time points after mechanical wounding. The whole sampling process was performed as rigorously as possible to ensure a reliable data collection (see Section 2.5). Before the construction of calibration curve, trial analysis of an early, middle and late time point selected from the wounding status was carried out to confirm that (+)-JA never naturally occurred in tobacco samples and to know roughly that how much (−)-JA or (+)-epi-JA was in the samples, so that appropriate amount of (+)-JA as internal standard could be added. Among all the peaks detected from plant samples, (−)-JA and (+)-epi-JA were identified by retention time and distinct precursor-to-product ion transitions (see Fig. 3B). The amounts of endogenous (−)-JA and (+)-epi-JA were calculated in relation to the internal standard that provided correction for hormone loss during sample preparation and chromatography.

As shown in Fig. 4A, mechanical wounding induced remarkable accumulations of both natural JA stereoisomers within 1 h, that was more than 800-fold increase for (−)-JA and more than 700-fold increase for (+)-epi-JA. Then the amounts of them decreased significantly as the wounding time passed. Although (−)-JA and (+)-epi-JA kept the same trend for wound response, they differed in their alteration rates during this process. This can be evaluated by the ratio of (−)-JA to (+)-epi-JA. As suggested in Fig. 4B, the (−)-JA/(+)-epi-JA ratio gradually shifted at an increasing rising rate. This phenomenon could be easily explained by the unstable nature of (+)-epi-JA which is generally believed to rapidly epimerize to (−)-JA *in vivo* [2,5,6,33]. However, a logical consequence of above assumption should be that (−)-JA is the major JA epimer in natural samples, which is not consistent with our results. Based on our data, although the absolute amount of (−)-JA was more than that of (+)-epi-JA, the endogenously detected (−)-JA and (+)-epi-JA were at the same concentration level at different intervals before and after wounding. Likewise, although the (−)-JA/(+)-epi-JA ratio shifted, it shifted slightly. All of these are worth further discussion rather than simply explained by the spontaneous epimerization from (+)-epi-JA to (−)-JA. First, the sharp increase of (−)-JA and (+)-epi-JA demonstrates that both (−)-JA and (+)-epi-JA are highly effective indicators for wounding response. Second, the pronounced decrease of (−)-JA and (+)-epi-JA in wounded leaves suggests two possible downstream transitions. One is the transportation of them within the plant for long-distance signaling events. But according to the

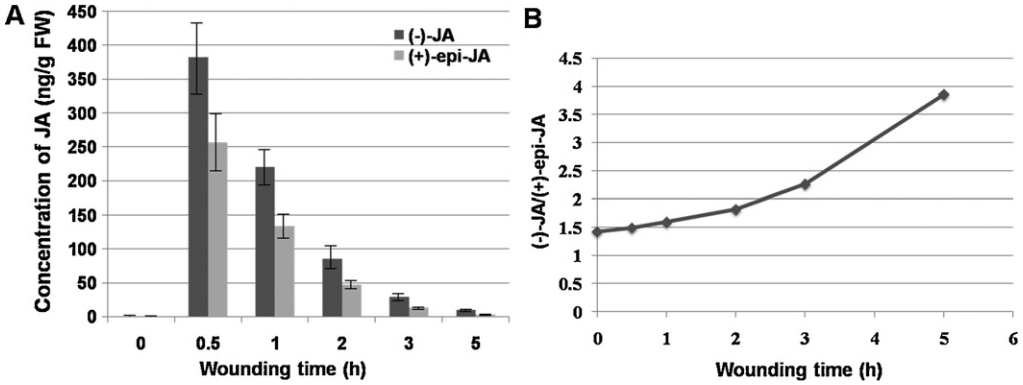

Fig. 4. (A) Endogenous amounts of (−)-JA and (+)-epi-JA detected at different intervals before and after the mechanical wounding; (B) calculated ratios of (−)-JA to (+)-epi-JA responding to the mechanical wounding.

previously reported results [34], the transported amount of JA is only the minor portion of the total amount. So the other possible transition may be more persuasive, that is the conversion of (−)-JA and (+)-epi-JA to their respective metabolites. As proposed by Wasternack [2], JA may act as a systemic signal, leading to systemic expression of genes encoding proteinase inhibitors (PINs) and other foliar compounds with negative effects on wounding attack. And this presumption can also explain the third phenomenon that no significant but only a slight trend of epimerization is observed from (+)-epi-JA toward (−)-JA, because signal attenuation may be accomplished by either derivatization or epimerization of the initially biosynthesized (+)-epi-JA [19], and there is no convincible data obtained by our experiments to demonstrate epimerization as a key contributor to JA deactivation for wounding response. Rigorous testing of these ideas is highly needed. Although this is an arduous task requiring identification of the whole network among the stereoselective JA family and understanding of the related metabolism involving enzymes, we hope our method could at least provide a simple and direct way to monitor JA stereoisomers for better understanding of their stereoselectivity related behavior.

3.5. Configurational stability of JA epimers

Another open question is to what extend the detected (−)-JA and (+)-epi-JA could reflect the real equilibrium status of JA epimers *in vivo*. If epimerization is inevitable during the post treatment process like extraction, it must be identified how much interference should be got rid of for data processing. This confirmation could be hardly carried out before because of lack of individual JA stereoisomer. Therefore, the configurational stability of JA epimers was investigated from two aspects. First, the extent of epimerization was studied throughout the whole procedure of plant sample preparation. Tobacco leave powder was divided into several portions, spiked with either (+)-epi-JA or (−)-JA, and followed by the treatment as described in Section 2.5. Relatively large amount was spiked, so that even the recovery was considered, 0.4%

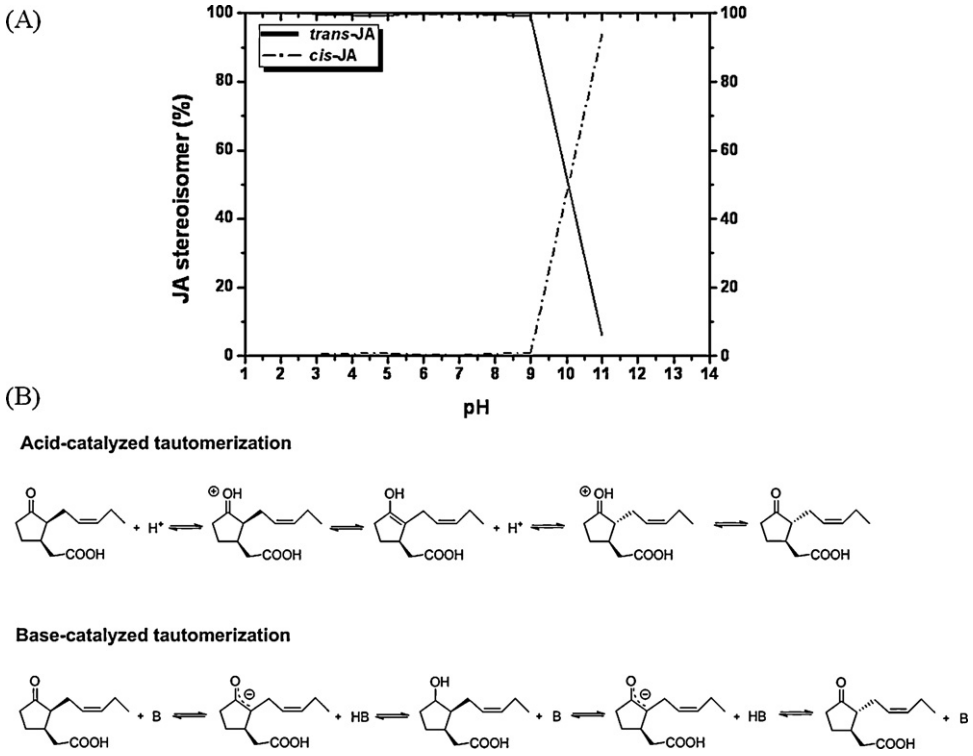

Fig. 5. (A) Epimerization of (+)-epi-JA in aqueous solutions at different pH values; (B) schematic illustration of acid and base catalyzed epimerization from (+)-epi-JA to (−)-JA.

epimerization could be monitored with a signal-to-noise ratio of at least 10. Analysis was performed using our NPLC–MS method after every step, and no conversion was observed. Second, the stability of all the four JA stereoisomers under different pH conditions was evaluated. Pure (−)-JA, (+)-epi-JA, (+)-JA and (−)-epi-JA were treated with acetic acid or ammonia solutions with varied pH. As shown in Fig. 5A, conversion ratio was calculated in the pH range from 2 to 11 after 1 h treatment. Pronounced epimerization was only observed under alkaline condition for either (+)-epi-JA to (−)-JA or (−)-epi-JA to (+)-JA. This facile epimerization at C-7 to the *trans* arrangement can be easily explained by the keto-enol tautomerization as described in Fig. 5B. However, the relevant pH values for us are 2.8, 5.3, 7.0 and 7.4. The pH value of 2.8, well below JA's pK_a, was used for the efficient extraction of JA from aqueous phase to organic phase (see Section 2.5). 5.3 was the pH value of the treated tobacco leaves from different batches before the pH adjustment and extraction. The pH value of 7.0 and pH 7.4 were the mimic condition for *in vivo* cellular environment. Under such unique pH values, all the four JA stereoisomers remained stable when analyzed either one hour or one day later. Therefore, despite of the assumed instability and the possible epimerization during extraction [6,17,33,35], our results provide the direct experimental evidence that little epimerization of (+)-epi-JA could happen once the tobacco leaves are harvested, which also confirms that detection of (−)-JA and (+)-epi-JA using our method could describe the actual equilibrium of endogenous JA epimers. Moreover, the demonstrated stability of (+)-epi-JA at cellular pH indicates a regulated rather than spontaneous process of *in vivo* epimerization, which is consistent with the discussion in Section 3.4. But whether this assumed process is subjected to a certain enzymatic control or is just a simple consequence of local pH changes mediated by other mechanism still requires further investigation.

4. Conclusions

In this work, both pairs of JA enantiomers can be simultaneously discriminated and sensitively detected without any derivatization using our newly developed NPLC–QTOF-MS method. And every single JA stereoisomer was first obtained individually in a most practical way using our semi-preparative LC method. Endogenous rise of (−)-JA and (+)-epi-JA was detected in tobacco leaves and the changes of their amounts and ratios at different intervals after mechanical wounding were monitored. The role of JA as a wounding reporter was re-defined, but this time both naturally existing JA epimers, (−)-JA and (+)-epi-JA, were first clearly demonstrated effective for wounding response. The reliability of all the analysis was guaranteed by the introduction of (+)-epi-JA, a non-endogenous stereoisomer, as the internal standard. No pronounced difference of JA stereoisomers was observed in the study of wounding response related biological activity, and experimental evidence suggests that the epimerization between (+)-epi-JA and (−)-JA is not a spontaneous process simply promoted by the instability of (+)-epi-JA as expected before. However, whether and how it is enzymatically driven and whether this epimerization is a symbol for signal attenuation or activation for some specific bioactivities of JA still require further exploration.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2012.02.062.

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