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Research article

Enhanced determination of abscisic acid (ABA) and abscisic acid glucose ester (ABA-GE) in *Cistus albidus* plants by liquid chromatography–mass spectrometry in tandem mode

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ABSTRACT

An improved, quick and simple method for the extraction and quantification of the phytohormones (+)-abscisic acid (ABA) and its major glucose conjugate, abscisic acid glucose ester (ABA-GE) in plant samples is described. The method includes the addition of deuterium-labeled internal standards to the leaves at the beginning of the extraction for quantification, a simple extraction/centrifugation process and the injection into the liquid chromatography–electrospray ionization–tandem mass spectrometry (LC–ESI–MS–MS) system in multiple reaction monitoring mode (MRM). Quality parameters of the method (detection limits, repeatability, reproducibility and linearity) have been studied. The objective of this work is to show the applicability of this method for quantifying the endogenous content of both ABA and ABA-GE in *Cistus albidus* plants that have been grown during an annual cycle under Mediterranean field conditions. Leaf samples from winter plants have low levels of ABA which increase in spring and summer showing two peaks that corresponded to April and August. These increases are coincident with the high temperature and solar radiation and the low RWC and RH registered along the year. On the other hand, the endogenous levels of ABA-GE increase until maximum values in July just before the ABA content reaches its highest concentration, decreasing in August and during autumn and winter. Our results suggest that the method is useful for quantifying both compounds in this plant material and represents the advantage of a short-time sample preparation with a high accuracy and viability.

1. Introduction

Acclimation and adaptation of plants to drought have been extensively studied and several mechanisms enabling plants to withstand drought have been described [35]. In the Mediterranean climate area, several species have evolved mechanisms to withstand stress [21,24,26]. One important regulator that coordinates growth and development with responses to the environment is the sesquiterpenoid hormone abscisic acid (ABA) [38]. The plant hormone S-(+)-(ABA) modulates numerous aspects of plant growth and development, including seed dormancy, embryo maturation, stress responses and stomatal aperture [39]. Moreover, plants control their responses to environmental stresses such as drought, cold and high temperatures, or salt stresses by modulating endogenous ABA levels [33,34]. It is also involved in the regulation of many stress-induced gene-expressions, conferring to plants the adaptability toward such environmental stresses [3]. In plant cells ABA is synthesized and degraded continually. Metabolism can occur by several routes involving oxidation, reduction and conjugation [5]. Moreover, a rapid and sudden increase in ABA content in drought-stressed leaves derived from carotenoid precursors has been shown [29]. Metabolism of ABA can occur through a multitude of pathways the nature of which often depends on the species, developmental stage or tissue [9]. The major glucose conjugate of ABA is ABA glucosyl ester (ABA-GE) [1], which exhibits little or no biological activity but appears to be a transported form of ABA [36]; in addition to the glucosyl esters, other conjugates with the hydroxyl groups of ABA and its hydroxylated catabolites (epi-DPA-β-D-glucoside; 8′-hydroxyABA-β-D-glucoside) are conjugated as

Abbreviations: ABA, abscisic acid; d_6-ABA, deuterium-labeled abscisic acid; ABA-GE, abscisic acid glucose ester; d_5-ABA-GE, deuterium-labeled abscisic acid glucose ester; CE, collision energy; DP, declustering potential; ESI, electrospray ionization; LC–ESI–MS/MS, liquid chromatography–electrospray ionization coupled to mass spectrometry in tandem mode; LOD, limit of detection; LOQ, limit of quantification; MRM, multiple reaction monitoring; RWC, relative water content.

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glucosides [14,27]. Continual synthesis, transport and degradation dynamically maintain ABA levels in plant cells [40]. ABA metabolic profiles will help define the relative importance of competing pathways, conversion efficiencies within pathways and the relationship between ABA metabolism, environmental conditions and development [40].

At present, different sensitive and selective methods to quantify individual plant hormones have been developed. The application of liquid chromatography–tandem mass spectrometry (LC–ESI–MS/MS) to the analysis of plant hormones is of particular interest because it offers an opportunity to quantify several compounds simultaneously with high sensitivity and selectivity [4,8,9,11,12]. Triple quadrupole instruments present the advantages of both a qualitative tool (through precursor ion scan, product ion scan or neutral loss scan experiments) and a quantitative tool (through the MRM mode that provides high sensitivity). In this way, LC–MS has been applied to the analysis of ABA in plant extracts after extraction and purification through SPE cartridges [30] and efficient separation of cytokinins from auxins and abscisic acid has been successfully achieved [7]. Recently, an immunoaffinity chromatography (IAC) method has been developed and the successful combination of IAC with sensitive LC–ESI–MS for determining endogenous ABA levels has been demonstrated [16]. Nevertheless, the complexity of any plant matrix makes phytohormone analysis particularly difficult, so an accurate quantification of trace amounts of these compounds requires robust methods. In this work, stable isotope labeled derivatives as internal standard have been used for quantitation of ABA and ABA-GE in MRM mass spectrometry analysis. The MRM technique is an MS/MS acquisition in which the transition of specific parent to fragment ion can be monitorized thus providing a highly specific and sensitive analysis for target compounds. Quantification of ABA in other plant tissues by isotope-dilution LC/MS, single quadrupole, has been reported before [4,8,16,30,36,40,41]. Here we present a short, sensitive and selective analytical method for the specific detection and accurate quantification of both ABA and ABA-GE in *Cistus albidus* samples using liquid chromatography coupled with electrospray ionization–tandem mass spectrometry (LC–ESI–MS/MS) through isotopic dilution method and without the need for purification of the extracts.

2. Results

2.1. LC–MS/MS optimization

Mass spectra for ABA in MS and MS/MS modes had been previously studied [22]. For optimization of MS and MS/MS parameters of d_6-ABA, ABA-GE and d_5-ABA-GE, infusion of individual standard solutions of each one as described in Section 4 into the mass spectrometer. The spectrum generated for d_6-ABA in full scan mode in negative ion mode gave the deprotonated molecule $[M-H]^-$ (m/z 269). The product ion scan spectrum of the m/z 269 ion gave the ion m/z 159 ion that corresponds to the loss of the side chain, as occurred for ABA. ABA-GE showed (m/z 425) ion which corresponds to the deprotonated molecule $[M-H]^-$ and (m/z 263) that corresponds to the loss of the glucose unit $[M\text{-}H\text{-}Gluc]^-$. The same behavior was observed for d_5-ABA-GE showing m/z 430 and m/z 268.In Table 1, there is a summary of MRM transitions and potentials (DP, declustering potential and CE, collision energy) obtained as optimum after the optimization process.

Fig. 1 shows the chromatograms of *C. albidus* crude extracts analyzed (A and B, January plants and August plants, respectively). Positive identification of a compound was done when a peak at the same retention time as this of the standard appears in the same MRM trace chromatogram. As can be seen, no interferences are present in these chromatograms, even taking into account that a fast and simple extraction protocol has been applied without clean-up. That is very useful in order to facilitate the processing of a high number of samples.

Table 1
MRM transitions and potentials for ABA and ABA-GE.

Compound	MRM transition	DP (V)	CE (V)
ABA	263/153	−30	−30
d_6-ABA	269/159	−30	−30
ABA-GE	425/263	−50	−20
d_5-ABA-GE	430/268	−30	−15

2.2. Performance characteristics of the LC–ESI–MS/MS method

To study the run-to-run and day-to-day precision of the LC–MS/MS method, three standard solutions containing 2, 100 and 400 ng ml^{-1} of each ABA and ABA-GE (0.01 ng, 0.5 ng and 2 ng injected), and 200 ng ml^{-1} (1 ng) of the same deuterium-labeled compounds were quantified ($n = 10$) on three different days using calibration curve (from 0.01 ng to 2 ng) prepared daily. Fig. 2 shows the calibration curves for ABA (A) and ABA-GE (B); as can be observed, good correlation coefficients ($r > 0.998$) were obtained in the concentration range studied. The analysis of residuals [17] for this range of concentrations (expressed as mean ± SD) was 102.4 ± 1.7%. The results for reproducibility were a relative standard deviation (RSD %) of 1.4% for run-to-run precision and 8.8% for day-to-day precision on concentration.

Detection limits for standard solutions were calculated on the basis of the concentration that gives a signal-to-noise ratio of 3:1. The method presented good sensitivity for ABA with an LOD of 0.07 ng ml^{-1} (0.35 pg injected) and a relatively high LOD for ABA-GE of 0.63 ng ml^{-1} (3.1 pg injected) probably due to the broad peak of this compound (5 μL injected). For samples, the LODs were 1.4 ng g^{-1} f.w. and 7.8 ng g^{-1} for ABA and ABA-GE, respectively. LOQ based on a signal-to-noise ratio of 10:1 were 4.8 ng g^{-1} for ABA and 26 ng g^{-1} for ABA-GE.

In the last months, several interesting papers related to plant hormones analysis using LC–MS/MS in different species have been published [10,15,23,28]. The sensitivity of an analytical method depends strongly on the technology employed but also on the sample treatment (weight of sample, dilution injected volume, etc.). In this section we present both the detection limit for standard solutions and the detection limits in plant samples to show a real situation of the analysis proposed. The linearity range studied in this work is also described (from 0.01 ng to 2 ng per injection). Moreover, the weighting ($1/x^2$) for the curve has been optimized in order to give the best accuracy for the analytical range studied.

The MS and MS/MS parameters such as ionspray voltage, declustering potential, collision energy and cell exit potential have been optimized through infusion experiments, in order to generate the highest signal intensities. The standard solutions were introduced into the mass spectrometer a 5 μl/min via a syringe pump and the software (using the quantitative optimization feature) gave as a result the best parameters for analyte analyses. Other important parameters such as gas flow-rates (nebulising gas, curtain gas and auxiliary gas) and temperature were optimized in FIA (flow-injection analysis) mode: a standard solution of 1 ng ml^{-1} of each compound was injected (5 μl) and the LC run was applied. The final conditions chosen were those that gave the best signal-to-noise ratio; moreover, some different extraction conditions have been tested: number of replicates of extraction, volume of extractant (the minimum one to avoid long evaporation times) and composition of extractant.