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Determination of abscisic acid and its glucosyl ester in embryogenic callus cultures of *Vitis vinifera* in relation to the maturation of somatic embryos using a new liquid chromatography-ELISA analysis method

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SUMMARY

The levels of abscisic acid (ABA), its conjugate ABA-GE, and IAA were determined in embryogenic calli of *Vitis vinifera* L. (cv. Mencía) cultured in DM1 differentiation medium, to relate them to the maturation process of somatic embryos. To achieve this goal, we developed an analytical method that included two steps of solid-phase extraction, chromatographic separation by HPLC, ABA-GE hydrolysis, and sensitive ELISA quantification. Because the ABA immunoassay was based on new polyclonal antibodies raised against a C4′-ABA conjugate, the assay was characterized (detection limit, midrange, measure range, and cross-reaction) and validated by a comparison of the ABA data obtained with this ELISA procedure and with a physicochemical method (LC–ESI–MS/MS). Radioactive-labeled internal standards were initially added to callus extracts to correct the losses of plant hormones, and thus assure the accuracy of the measurements. The endogenous concentration of ABA in the embryogenic callus cultured in DM1 medium was doubled at the fifth week of culture, concurring with the maturation process of somatic embryos, as indicated by the accumulation of carbohydrates observed through histological analysis. The ABA-GE content was higher than ABA, decreasing at 21 days of culture in DM1 medium but increasing thereafter. The data suggest the involvement of the synthesis and conjugation of ABA in the final stages of development in grapevine somatic embryos from embryogenic callus. IAA levels were low, suggesting that auxin plays no significant role during the maturation of somatic embryos.

In addition, the lower ABA levels in calli cultured in DM differentiation medium with PGRs, a medium presenting high precocious germination and deficiencies in somatic embryo development indicate that an increase in ABA content during the development of somatic embryos in grapevine is necessary for their correct maturation.

Introduction

Although somatic embryogenesis is currently available for a number of cultivars in *Vitis vinifera* L., it is not yet a routine technology (Martinelli and Gribaudo, 2009). One of its main limitations is the abnormal and/or incomplete development of somatic embryos that occurs during the culture of embryogenic callus in differentiation medium. Processes of precocious germination that hamper the maturation of somatic embryos and reduce the rates of normal plant conversion have been reported (Coutos-Thévenot et al., 1992; Perrin et al., 2001; Prado et al., 2010). However, the use of differentiation media lacking plant growth regulators (PGRs) can help overcome this problem and improve the efficiency of plant regeneration (Prado et al., 2010).

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; ABA, abscisic acid; AP, alkaline phosphatase; ABH, *p*-aminobenzoyl hydrazone; BA, benzyladenine; HPLC, high-performance liquid chromatography; IAA, indole-3-acetic acid; MRM, multiple reaction monitoring mode; NOA, naphthoxyacetic acid; PBS, phosphate-buffered saline; PGR, plant growth regulator; PcAbs, polyclonal antibodies; SPE, solid-phase extraction; TBS, Tris-buffered saline; UPLC, ultra-performance liquid chromatography.

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Abscisic acid (ABA), a key plant hormone that regulates the final phases of somatic embryo development, is among the many factors that may be involved. The inclusion of ABA in culture media promotes the transition from the proliferation to mature phase, but also enhances embryo quality by increasing desiccation tolerance and preventing precocious germination (see Rai et al., 2011 for a review). Specifically, it has been documented that high levels of endogenous and applied ABA in grapevine stimulate the maturation and inhibit the germination of premature somatic embryos (Goebel-Tourand et al., 1993). Moreover, Faure et al. (1998) related low levels of endogenous ABA to a high percentage of precocious germination of grape somatic embryos compared to zygotic embryos. Nevertheless, no report exists regarding ABA metabolic profiles in grapevine somatic embryos or embryogenic cultures, which could provide information on the importance of ABA synthesis and catabolism and its relationship to the development of somatic embryos. In this sense, ABA catabolism can occur by several routes, including oxidation, reduction, and conjugation. The major glucose conjugate is ABA-glucosyl ester (ABA-GE), which exhibits little or no biological activity but appears to be a transported form of ABA in plants. Glucosyl esters of ABA also function as storage products that can be hydrolyzed to release free ABA (Piotrowska and Bajguz, 2011).

Another class of PGR that affects somatic embryogenesis is auxin. It is generally accepted that exogenous and endogenous auxins play key roles in the acquisition of embryogenic competence by cultured tissues and in the proliferation of embryogenic cultures (Jiménez, 2005). Further development of somatic embryos occurs if the endogenous auxin levels are low, which usually requires the reduction or removal of auxin from the culture medium (Jiménez, 2005). Consequently, it has been shown that the endogenous levels of indole-3-acetic acid (IAA), the major endogenous auxin, remained steady or declined during the development of somatic embryos in various systems (reviewed by Jiménez, 2005). This is also the case in grapevine, in which the levels of IAA decreased in somatic embryos during their development in a medium without PGRs (Faure et al., 1998).

Plant hormones and their metabolites are difficult to analyze because they occur in very low amounts in plant extracts, which are very rich in interfering substances. To overcome this problem, the plant extract must undergo several purification steps using different separation mechanisms, such as solid-phase extraction (SPE) and high-performance liquid chromatography (HPLC), to increase the purification efficiency and measurement accuracy. Fortunately, ABA and IAA have similar chemical properties that can be exploited for extraction and SPE purification because these compounds very often accumulate in the same fraction (Dobrev et al., 2005). Once the compounds of interest have been purified, they must be detected and quantified by a sensitive and selective procedure. In this sense, the most popular methods for analyzing ABA and IAA are (i) HPLC-tandem mass spectrometry (LC–MS/MS) in multiple reaction monitoring (MRM) mode and (ii) HPLC combined with immunoassays based on the use of antibodies reacting specifically with the hormones. Both have advantages and limitations (Bai et al., 2010; JiHong et al., 2011), thus the choice depends heavily on the availability of spectrometric equipment or antibodies and the quality of the latter. In our case, we developed the anti-ABA (C4′) polyclonal antibodies (PcAbs) named C4′-I, which were made to react specifically against to the ABA free form (Centeno and Granda, 2009). These PcAbs were developed in the framework of a project dealing with water stress that required performing ABA analysis in a huge number of samples in a timely, simple and cost-effective manner. The C4′-I PcAbs were then applied to quantify ABA in the samples by radioimmunoassay (Granda et al., 2011). At this moment, it was desirable to develop and optimize an enzyme-immunoassay (ELISA) based in C4′-I PcAbs, because this kind of assay avoids using radioactive isotopes increasing safety at work.

The aim of the present work was to relate the endogenous levels of ABA, ABA-GE, and IAA in *V. vinifera* (cv. Mencía) embryogenic callus tissue with the maturation process of somatic embryos using calli cultured in DM1 differentiation medium (DM) (Prado et al., 2010). To achieve this goal, a sensitive LC-ELISA method coupled to the PcAbs C4′-I reacting selectively with (+)ABA was developed, validated, and applied. The ELISA assay had to be optimized because it used a tracer of new development and the previously produced PcAbs named C4′-I which had been only applied to quantify (+)ABA by RIA (Granda et al., 2011). In addition, the endogenous levels of ABA were determined in grapevine embryogenic callus tissue cultured in DM differentiation medium. As this medium contains a complex phytohormone composition producing higher levels of precocious germination in developing grapevine somatic embryos (Prado et al., 2010), these data will provide additional information on the hormonal regulation of the maturation process of grapevine somatic embryos.

Materials and methods

Plant material and culture conditions

Adult field-grown plants of *Vitis vinifera* L. cv. Mencía were selected for this study from the grapevine collection at the "Centro de Formación y Experimentación de Viticultura y Enología de Ribadumia" (Galicia, north-western Spain). Inflorescences at stage H on the Baggiolini phenological scale, corresponding to separated clusters, were collected over a one-week period during April–May 2008. Flowers at developmental stage R3 (late binucleate microspore stage, Prado et al., 2010), as determined by the microsporogenesis stage observed microscopically, were selected for initiation of the cultures. The flower clusters were collected, washed, and sterilized, and the immature anthers were isolated for use as explants for the induction of somatic embryogenesis in CIM1-A medium containing 4.5 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 9 μM benzyladenine (BA), as described by Prado et al. (2010).

Embryogenic calli after 6 or 12 months in induction medium were subcultured to DM1 and DM embryo differentiation media, respectively (Prado et al., 2010), the first without PGR and the second supplemented with 20 μM IAA, 10 μM naphthoxyacetic acid (NOA), and 1 μM BA. The calli were sampled at the beginning (0 days) and after 14, 21, 28, and 35 days of culture to examine the variations in the ABA, ABA-GE, and IAA contents in the material cultured in the DM1 medium and the variation in the ABA content in the material cultured in the DM medium. Pieces of callus were collected, washed with distilled water to remove medium residue, and dried with filter paper, and the fresh mass was determined to attain samples of at least 0.8–1 g of callus per incubation period and culture medium. The samples were then frozen in liquid N_2, powdered, lyophilized, and reweighed to determine their dry mass and water content. These data were further used to calculate the μM concentrations of plant hormones. Samples were collected to repeat each analysis three times.

Preparation of tracer and enzyme-linked immunosorbent assay (ELISA) to measure (+)ABA

The tracer was prepared by (+)ABA conjugation to alkaline phosphatase (AP) (E.C.3.1.3.1) via an aminobenzoyl hydrazone (ABH) bridge according to the method for the synthesis of ABA-4′-protein conjugates described by Quarrie and Galfre (1985) and modified by Centeno and Granda (2009). Briefly, this method consists of the

following: (1) coupling of (+)ABA to ABH through the C4′ carbonyl group of the (+)ABA molecule under acidic conditions and in a dark, oxygen-free atmosphere to obtain (+)ABA-4′-aminobenzoyl hydrazone (ABA-4′-ABH); (2) ABA-4′-ABH purification and concentration, followed by diazotization with sodium nitrite; and (3) linkage of the activated hydrazone to previously dialyzed AP through tyrosine residues of the enzyme. The resulting (+)ABA-AP tracer was dialyzed against phosphate-buffered saline (PBS) (8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.14 M NaCl, and 2.7 mM KCl, pH 7.2), and the protein concentration was determined by the method of Bradford (1976). Sodium azide at 0.1% (w/v) was added to the dialyzed conjugates, which were then stored in 50% (v/v) glycerol at −80 °C.

Maxisorp (NUNC™; eBioscience, San Diego, CA, USA) 96-well microtitre plates were coated with 200 μL per well of a 1:1000 dilution of the C4′ ABA purified PcAbs (C4′-I, Centeno and Granda, 2009) in 50 mM $NaHCO_3$ buffer (pH 9.6), excluding the outer wells. After incubation at 4 °C overnight, the liquid in the wells was decanted, and the wells were washed three times with 250 μL Tris-buffered saline (TBS) (25 mM Tris, 100 mM NaCl, and 1 mM $MgCl_2$, pH 7.5). Then, 250 μL of 0.1% (w/v) bovine seroalbumin in TBS were added per well and the plate was maintained for 30 min at room temperature. The plate was again emptied and washed. A 100-μL aliquot of each sample dissolved in TBS (or 100 μL of the (+)ABA solutions prepared in TBS for the calibration curve) was added to each well, and the plate was incubated another 30 min at 4 °C in darkness. Afterwards, 100 μL of a 1:40,000 dilution of the (+)ABA-AP tracer dissolved in 0.1% gelatine (w/v) in TBS were added to each well, and the plate was incubated again in darkness at 4 °C for 3 h. After the immunological reaction was complete, the liquid in the plate was decanted, and the wells were washed. The enzymatic reaction was initiated by adding 200 μL of p-nitrophenylphosphate (1.4 mg mL^{-1}) in DEA buffer (0.9 M diethanolamine and 0.3 M $MgCl_2 \cdot 6H_2O$, pH 9.8) per well. After incubation for 1 h at 37 °C, the absorbance was measured at 405 nm using a plate spectrophotometer Synergy HT (BioTek, Winooski, VT, USA).

Sample preparation

Extraction and purification of ABA, ABA-GE, and IAA was performed following the method described by Dobrev et al. (2005), with minor modifications. For the three replicates of each sample, 50 mg (DW) of callus were extracted twice with 5 and 3 mL of extraction solvent, which consisted of a solution of methanol/water/formic acid (75:20:5, v/v/v) containing 0.01% (w/v) butylated hydroxytoluene, for 12 and 3 h, respectively, by repeated inversion at 4 °C in darkness. At the beginning of the extraction, radioactive standards of [^{3}H]ABA (1.7 TBq/mmol; GE Healthcare, Barcelona, Spain) and [^{3}H]IAA (1 TBq/mmol; GE Healthcare) were added to monitor the losses during purification. The homogenates were cleared by centrifugation (10,000 × *g*, 4 °C, 20 min), and the supernatants were first combined and then divided in two identical 4-mL fractions to avoid exceeding the capacity of the SPE column. Each fraction was then transferred onto a 5-mL C_{18} cartridge (reverse phase, Sep-Pack PlusC_{18}, 400 mg; Waters), previously washed with 5 mL of methanol and equilibrated with 5 mL of extraction solvent. Afterwards, 2 additional mL of methanol/water/formic acid (75:20:5, v/v/v) were passed through each of the two columns. The eluates from the two cartridges were combined (total volume of 12 mL), and the methanol was removed under an N_2 stream. Formic acid at 1 M was then added to adjust the volume to 5 mL. The extract was applied to a column filled with a mixed-mode reverse-phase anion-exchange polymeric sorbent (Oasis MAX, 150 mg/6 cc; Waters, Milford, MA, USA), which was pre-conditioned with 5 mL of methanol and 5 mL of 1 M formic acid. After loading the samples, the column was sequentially washed with 5 mL of 10 mM KH_2PO_4 (pH 7) and 5 mL of water. The ABA, ABA-GE, and IAA retained were eluted from the column by applying 5 mL of 1% (v/v) formic acid in methanol. The ABA-GE behavior in the Oasis MAX matrix was studied through experiments described in the Results and Discussion section. The solvent was evaporated using a Savant Speed-Vac centrifugal evaporator by vacuum (Thermo Fisher Scientific, Madrid, Spain), and the dry fractions were stored at −20 °C until further processing.

LC-ELISA analysis of (+)ABA, ABA-GE, and IAA

An LC-ELISA method was performed for (+)ABA, ABA-GE, and IAA detection and quantification of the grapevine embryogenic callus purified extracts. The HPLC system consisted of a Waters 2690 Alliance chromatograph equipped with a 996 photodiode array detector (UV/vis-PDA) coupled to a fraction collector. For the analysis of the extracts, a Kromasil 100–5C_{18} reverse-phase column (25 × 0.46 mm, 0.5 μm; Teknokroma, Barcelona, Spain) was used with a C18 XTerra guard column (3.9 × 20 mm; Waters). The samples were dissolved in 200 μL of the mobile phase, filtered through a 0.45-μm PVDF filter (Teknokroma), and injected via the autosampler in volumes of 50 μL. The mobile phase was methanol/water acidified to pH 3.5 with formic acid (50:50, v/v). The analytes were eluted from the column over 22 min using a linear gradient from 50% to 90% methanol; the flow rate was 0.6 mL min^{-1}. Standards (1 μg) were injected into the system after each three samples to verify the (+)ABA, ABA-GE, and IAA retention times. The data were registered in chromatograms at 270 nm. Those fractions with times matching the retention times of the (+)ABA, ABA-GE, and IAA standards were collected, dried under vacuum, and stored at −20 °C.

The fractions containing (+)ABA were dissolved in 600 μL of TBS buffer. Two 50-μL aliquots were taken, mixed with 150 μL of distilled water, and 2 mL of scintillation liquid (Ecoscint A; National Diagnostics, Atlanta, GA, USA), and the radioactivity was measured using a liquid scintillation counter (Tri-Carb 2900 TR; Packard, Meriden, CT, USA) to determine the [^{3}H]ABA recovery. To quantify (+)ABA, 4 replicates of 100 μL were assayed by ELISA, as described above.

The fractions containing IAA were methylated via diazomethane, dried, and dissolved for quantification according to the protocol of the Phytodetek® IAA test kit (Agdia, Elkhart, IN, USA). As described for ABA, the radioactivity corresponding to [^{3}H]IAA was measured in aliquots taken from the fractions before the IAA quantification by ELISA.

The alkaline hydrolysis of ABA-GE was performed according to the protocol of Zhou et al. (2003). Fractions containing the glucoside were dissolved in 800 μL of TBS buffer, alkalinized to pH 11 with 0.1 N NaOH, and incubated at 23 °C for 1 h; the pH was then neutralized by adding 0.1 N HCl, and the volume fractions was brought to 1.5 mL with TBS buffer. Free ABA resulting from hydrolysis was quantified by ELISA, as described above.

The content of ABA and IAA measured in each sample was corrected according to the recoveries of the radioactive standards. For ABA-GE, we assumed that the recovery was the same as that of ABA. μM concentrations were calculated taking into account the water content of each sample.

LC–ESI–MS/MS analysis of (+)ABA

To validate the use of the C4′-I polyclonal antibodies and the ELISA developed to analyze (+)ABA, three control samples of grapevine embryogenic callus tissue were extracted and purified as described above and then quantified by LC–ESI–MS/MS and by ELISA.

The LC–ESI–MS/MS analyses were performed according to López-Carbonell et al. (2009). The LC system consisted of an Acquity ultra-performance liquid chromatography (UPLC) (Waters) with quaternary pump, thermostated (4 °C) autosampler, and UV detector and was linked to an API 3000 (PE Sciex, Concord, Ontario, Canada) triple quadrupole mass spectrometer equipped with a Turbo Ionspray interface. For the analysis of the extracts, a reverse-phase Xbridge C18 column (50 × 2.1 mm, 3.5 μm; Waters) was used with a C18 Phenomenex precolumn (4 × 3 mm). A 98:2 (v/v) mixture of water with 0.05% acetic acid (solvent A) and acetonitrile 100% (solvent B) was applied at a constant flow of 0.4 mL min^{-1}. The separation was performed as follows: isocratic conditions (98% A: 2% B) were applied during the first 5 min, followed by a linear gradient from 2% to 100%-B for 5 min; the final solvent was then continued for 1 min. The return to the initial conditions occurred in 2 min. The column was equilibrated for 7 min after each injection. To reduce the residual matrix effect reaching the mass spectrometer, a divert valve (Valco, Houston, TX, USA) drained off the LC eluent during the time that the interfaces were detected to avoid the contamination of the mass spectrometer.

The MS and MS/MS experiments were performed in negative ion mode following the settings optimized by López-Carbonell et al. (2009). A capillary voltage of −3.5 kV and a declustering potential of −30 V were applied. The MS/MS product ions were produced by collision-activated dissociation (CAD) of the ABA deprotonated molecular ion $[M-H]^-$ (m/z = 263) at −30 V of collision energy using N_2 (4 arbitrary units) as the collision gas. The m/z 153 ion was the most abundant product ion; thus the 263/153 transition was selected and monitored to acquire the data and to quantify (+)ABA by the MRM method.

The extracts of control samples were reconstituted in 200 μL MilliQ water:acetonitrile:acetic acid (90:10:0.05; v/v/v), stirred, vortexed, filtered by centrifugation using Amicon Ultrafree-MC filters (0.22 μm; Millipore, Madrid, Spain), and 10 μL were injected into the LC–MS/MS system. Quantification was done by the standard addition method by spiking control callus samples with (+)ABA solutions (ranging from 0.5 to 5 ng μL^{-1}). Furthermore, 20 or 40 μL of these ABA solutions in the matrix samples were evaporated and redissolved in 500 μL of 25 mM TBS (pH 7.5), and the (+)ABA present was assayed by ELISA (4 replicates of 100 μL). Data from MRM and ELISA quantification were corrected by the corresponding dilution factor and converted to the same units (nmol) to compare the results obtained by the two methods.

Histological analysis

Pieces of embryogenic callus were fixed in 4% paraformaldehyde in PBS at 4 °C. After fixation, the samples were dehydrated in acetone series and embedded in Technovit 8100 resin (Kulzer, Germany). The blocks were polymerized at 4 °C, and Technovit semithin sections (2 μm) were obtained.

To observe the general structure of the tissues, the sections were stained either with toluidine blue or calcofluor. The sections were stained with 0.075% (w/v) toluidine blue (Panreac, Barcelona, Spain) in 0.1 M acetate buffer (pH 4.4) for 5 min and then rinsed with distilled water. For the calcofluor staining, the sections were treated with 1% calcofluor in 0.1 M Tris–HCl buffer (pH 8.5) for 30 min in darkness and then rinsed twice for 1 min with distilled water. For determining the presence of carbohydrates, the sections were stained for 1.5 h in darkness with 0.5% (w/v) periodic acid-Schiff reagent (PAS) and then rinsed for 10 min with running tap water.

All stained sections were mounted in Eukitt mounting medium (Kindler GmbH, Freiburg, Germany) and observed under bright-field illumination using an E800 microscope (Nikon, Tokyo, Japan) equipped with a Bio-Rad MRC 1024 confocal system (Bio-Rad Lab., Hercules, CA USA).

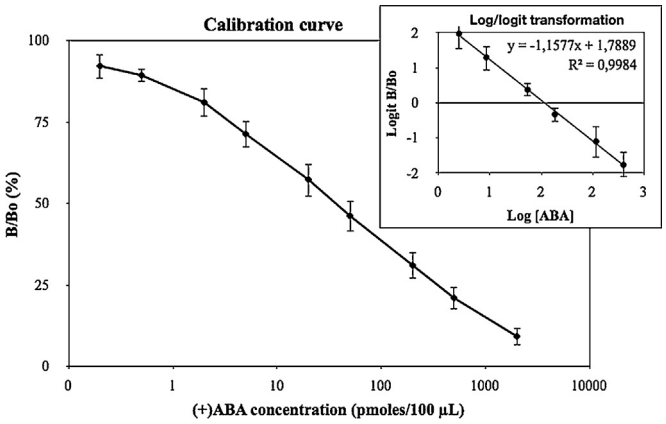

Fig. 1. Typical standard curve obtained using the enzyme-linked immunosorbent assay with ABA polyclonal antibodies. B/Bo is the relative binding (%) of tracer ((+)ABA-AP conjugate) in the presence (B) relative to the absence (Bo) of respective (+)ABA standards. The bars indicate the standard deviations (n = 20). The inset shows the linearized log/logit plot of the same data (logit B/Bo = ln[B/Bo: (100 − B/Bo)]).

Results and discussion

Development and validation of an LC-ELISA method to analyze ABA, ABA-GE, and IAA

We optimized a new competitive ELISA to quantify (+)ABA by utilizing purified C4′-I PcAbs for ABA and authentic standards of (+)ABA and ABA isomers and conjugates. These C4′-I PcAbs were previously produced in the framework of a project dealing with water stress (Centeno and Granda, 2009), and they were applied to quantify (+)ABA by RIA in samples from tissues of forest tree species (Granda et al., 2011). In the ELISA developed in the present work, the C4′-I PcAbs were used in conjunction with the (+)ABA-AP conjugate as a tracer, the appropriate dilution (1:40,000) for which was estimated by performing the assay in the absence of antigen. An advantage of this ELISA was that only amounts as low as 3.08 ng antibodies and 1.28 ng tracer, both measured by the Bradford assay (Bradford, 1976), were needed per assay. Naturally occurring (+)ABA was chosen as a reference standard because the immunoreactivity of C4′-I PcAbs with the (−)ABA enantiomer was close to zero. A mean standard curve was obtained from 20 batches of assays, and the corresponding log/logit plot is shown in Fig. 1. The assay had a reliable working range from 2 to 500 pmol of (+)ABA, which was linear over three orders of magnitude, with a midrange (50% B/Bmax) of 35.1 pmol. These values were similar to those obtained by Banowetz et al. (1994) using a competitive fluorescence ELISA based in the use of monoclonal Abs also prepared against the C4′ conjugate, which had a linear range of 0.11–379 pmol and a midrange of 10.98 pmol for (+)ABA. Thereafter, the detection limit of the ELISA using the C4′-I PcAbs was 2 pmol, which was higher but close to those obtained by other researchers analyzing (+)ABA using LC–ESI–MS/MS methods (0.13 pmol, López-Carbonell et al., 2009; 0.1 pmol, Tureckova et al., 2009). The chromatography combined with mass spectrometry has become the most commonly employed method for hormone analysis because of the excellent accuracy, favorable precision and high-throughput capability. Nevertheless, immunological methods remain a good alternative to LC–MS/MS techniques to quantify plant hormones, provided that the samples are subjected to previous purification to avoid cross-reactivity of the antibodies. Their advantages are a low detection limit, high selectivity, the speed

Table 1
Molar cross-reactivities of the C4′-I polyclonal antibodies to (+)ABA and some structurally related compounds in comparison with other anti-ABA antibodies previously published. Varying concentrations of the compounds were tested in ELISA for their ability to displace AP-labeled ABA from the antibody. The data presented are expressed as the percentage ratio of molar concentration of (+)ABA and competitor giving 50% binding.

	Antibody			
	C4′-I	D5[a]	MAC-62[b]	C4′-145[c]
(±)-cis,trans Abscisic acid methyl ester	0	<1	0.4	0
(±)-cis,trans Abscisic acid glucosyl ester	0.47	<1	0.1	0.2
(+)-cis,trans Abscisic acid	100	100	100	100
(−)-cis,trans Abscisic acid	0.91	0	1.39[d]	5.7
(±)-cis,trans Abscisic acid	50.5	50	51 (49[d])	51

[a] D5 antibody data from Banowetz et al. (1994).
[b] MAC-62 antibody data from Quarrie et al. (1988).
[c] C4′-145 antibody data from Weiler (1980).
[d] MAC-62 anti (±)-cis,trans abscisic acid antibody data from Walker-Simmons et al. (1991).

of the assay, and the lack of a requirement for sophisticated and expensive equipment (Bai et al., 2010; JiHong et al., 2011).

The specificity of the C4′-I PcAbs was determined by examining its cross-reactivity (Table 1). For comparison, the cross-reactions of two high-affinity monoclonal Abs against (+)ABA, named D5 (Banowetz et al., 1994) and MAC-62 (Quarrie et al., 1988; Walker-Simmons et al., 1991), and a PcAb against ABA, named C4′-145 (Weiler, 1980), are also shown. The cross-reactivities with ester derivatives of the ABA carboxyl group were very low or null, which was in agreement with the previously described results for antibodies in response to immunization with ABA conjugated to a carrier protein through the ring ketone group. Despite the C4′-I PcAbs being produced with a conjugate of (±)ABA, they sharply discriminated between the (+) and (−)ABA isomers, to an even better degree than the C4′-145 PcAb and in a similar way as both monoclonal Abs produced with a conjugate of (+)ABA. Taking into account the similarities between C4′-I and D5 and MAC-62 and C4′-145, very low cross-reactions of C4′-I PcAbs are expected with other ABA derivatives and metabolites, such as phaseic acid and dihydrophaseic acid, which we did not test, though they usually occur in plant extracts. Furthermore, the accuracy of the ELISA method developed in this study was validated by comparison of the levels of (+)ABA in three purified samples of grapevine embryogenic callus quantified by LC–ESI–MS/MS and by the ELISA using C4′-I PcAbs. The measurements were essentially identical, as shown by the standard deviation of the means obtained using the two procedures (Table 2). In addition to the proven high specificity of the C4′-I PcAbs, we chose to include an HPLC purification step in the method to ensure the accuracy of the measurements.

The extraction and purification of grapevine embryogenic callus tissue was performed following the procedure described by Dobrev et al. (2005). The use of an acidic solvent for hormone extraction from the plant material prevents the breakdown of ABA-GE, which leads to an overestimation of free ABA (Zhou et al., 2003). Moreover, we made two extractions from each sample to obtain efficient recoveries of hormones (Zhou et al., 2003; López-Carbonell and Jáuregui, 2005). The purification of IAA and ABA utilizing C18 and Oasis MAX SPE supports was previously optimized by Dobrev et al. (2005), but these authors did not test the behavior of ABA-GE in the second SPE step. For that, we applied a standard solution of ABA-GE prepared in 1 M formic acid to an Oasis MAX column to produce 0.8 UA. The column was then sequentially eluted as described by Dobrev et al. (2005), and the absorbance of each eluate and that of the standard solution was measured. The data of the absorbance readings at 270 nm were directly used for the recovery calculations. As predicted by Dobrev et al. (2005) for neutral hydrophobic analytes, the glucoside was completely retained on the Oasis MAX matrix because no losses were observed in washes with KH_2PO_4 and water and 100% of the ABA-GE input was recovered with methanol. Thus, the elution with 1% formic acid (w/v) in methanol allowed the recovery of IAA, ABA, and ABA-GE in the same purified and enriched fraction. Further separation of the three compounds was accomplished by HPLC, eluting ABA-GE, IAA, and ABA from the column at 7.9 ± 0.08 min, 9.87 ± 0.09 min, and 11.68 ± 0.11 min, respectively (values are the mean ± standard deviations). These data were obtained by injecting a mixed standard solution on five different days (4 injections per day), with very little shift in retention times being recorded. Similar results were found using other LC systems (López-Carbonell and Jáuregui, 2005; Hou et al., 2008). Nevertheless, we routinely injected the standard solution after each three samples to guarantee the collection of the correct HPLC fractions.

To measure IAA and ABA recoveries, known amounts of radioactive standards of both were added to the grapevine callus samples, and the recoveries were calculated from the radioactivity measured in the corresponding HPLC fractions. The results showed recoveries (%) of 56.7 ± 2.5 and 42.8 ± 4.8 for ABA and IAA, respectively. Compared to the data reported by Hradecka et al. (2007), testing different purification procedures for ABA with the same samples, our method exhibited ABA recovery similar to their immunopurification/LC–ESI–MS approach (between 41% and 54%) and a better recovery than their combined DEAE Sephadex-C18/LC-ELISA procedure (from 20% to 29%). In a similar study, Tureckova et al. (2009) found recoveries of 57% for ABA and 59% for ABA-GE using Oasis HLB columns and immunopurification, followed by UPLC–ESI–MS/MS quantification. Therefore, good recoveries were obtained with the coupled C18-Oasis MAX/LC-ELISA method based on the use of C4′-I PcAbs.

Table 2
Evaluation of the LC-ELISA method used to extract and quantify ABA from samples of *Vitis vinifera* L. embryogenic callus by comparison of the ABA values obtained after applying this analytical method and the LC–ESI–MS/MS method, as described in the Materials and Methods section, using three different samples.

	ABA (nmol)			
Sample	LC-ELISA	LC–ESI–MS/MS	S.D.	RSD (%)[a]
1	0.88	0.96	0.07	6.2
2	0.83	0.64	0.13	18.3
3	1.59	1.62	0.02	1.3

[a] RSD is the relative standard deviation and is given as a percentage.

Measurements of ABA, ABA-GE, and IAA and comparison of ABA levels in the embryogenic callus of grapevine

The endogenous ABA, ABA-GE, and IAA contents were determined in the embryogenic calli of grapevine cv. Mencía during culture in a differentiation medium without PGRs (DM1) to obtain some insight into the role of these plant hormones during the maturation of somatic embryos, a critical phase for plant regeneration in grapevine (Prado et al., 2010). To achieve this goal, we applied the LC-ELISA method described above to callus samples collected at 0, 14, 21, 28, and 35 days of culture on DM1 medium. We also performed a histological analysis of the callus to evaluate the progression of the callus and somatic embryo differentiation stages.

Histological studies revealed that the embryogenic callus of grapevine maintained its appearance during the two first weeks of culture in DM1 medium (Fig. 2A). Microscopically, the callus appeared heterogenous at this stage, being formed of large parenchymatous cells typical of an undifferentiated callus and of cells with a more densely stained cytoplasm (Fig. 2B). Pro-embryo

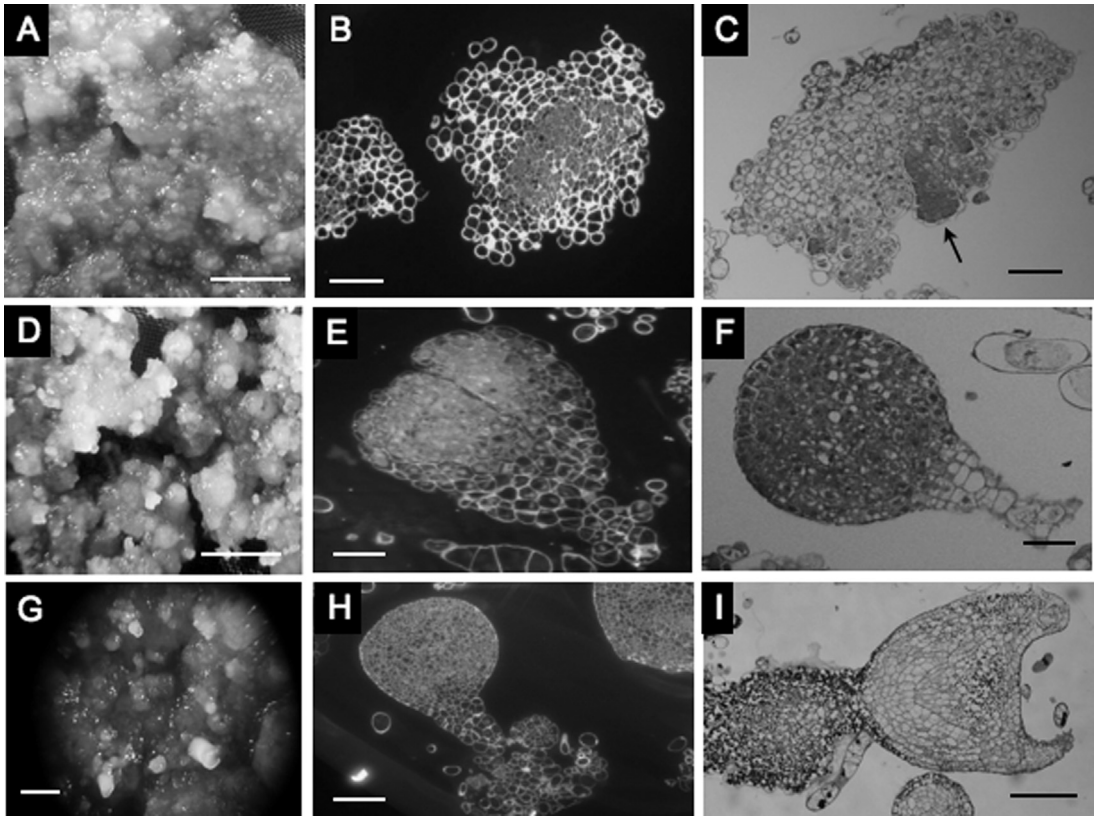

Fig. 2. Morphological (A, D and G) and microscopic (B, C, E, F, H and I) analysis of the development of somatic embryos of grapevine cv. Mencía after 14 days (A–C), 28 days (D–F), and 35 days (G–I) of culture in DM1 differentiation medium. The embryogenic callus increased its granular aspect from 14 to 28 days of culture in A and D, respectively, with somatic embryos visible on the surface of the callus after 35 days of culture in G. Calcofluor (B, E and H) and toluidine blue (C and F) staining showed the development of somatic embryos from pro-embryogenic cells in B and pro-embryo structures (arrow) in C to well-developed, heart-shaped somatic embryos in E, H, and F. (I) Mature, cotyledonary somatic embryo showing the accumulation of carbohydrate storage materials (dark blue inclusions) after PAS staining. Bars = 1 mm (A, D and G), 50 μm (B, C, E and F), 100 μm (H), and 500 μm (I).

structures could be identified at 14 days (Fig. 2C). Thereafter, the callus showed a progressive increase in its granular aspect beginning at 21 days (Fig. 2D), with some somatic embryos microscopically identified at 28 days and showing a well-defined protodermis and a massive suspensor (Fig. 2E and F). At 35 days, well-developed, normal somatic embryos were visible on the surface of the calli (Fig. 2G), with early cotyledonary embryos being clearly observed (Fig. 2H). Thus, two stages for the expression phase of somatic embryogenesis in grapevine callus can be defined according to Jiménez (2005). The first stage comprised the initial 14 days of the culture period in DM1 medium in which considerable visible changes were not observed in the callus tissue. In the second stage, from 14 to 35 days, an extensive, asynchronous process of cell differentiation to form somatic embryos was observed. Moreover, the deposition of storage materials characteristic of embryo maturation also occurred because carbohydrate accumulation in the somatic embryos was observed using specific dyes (Fig. 2I).

The endogenous contents of ABA, ABA-GE, and IAA found in grapevine embryogenic callus are shown in Fig. 3. The concentration of IAA was low (0.2–0.44 μM) in all samples, and similar IAA levels have been found in other studies (Fujimura and Komamine, 1979; Michalczuk et al., 1992), suggesting that auxin plays no significant role during the differentiation and maturation of somatic embryos. Nevertheless, an increase in IAA levels was observed until 21 days of culture, decreasing subsequently. This is in agreement with the acquisition of embryogenic competence and the proliferation of embryogenic cultures, and the need for reduction of auxin levels to allow further development of somatic embryos (Jiménez, 2005). A decrease in IAA levels in grapevine somatic embryos was previously reported (Faure et al., 1998) and it has been related to an increase in fresh weight during embryo differentiation.

The ABA content measured at 0, 14, 21, and 28 days in grapevine callus cultured in DM1 medium remained steady at approximately 0.45 μM and only increased to 0.93 μM from 28 to 35 days (Fig. 3), coinciding with the differentiation and maturation of most of the somatic embryos, as shown by the histological analysis (Fig. 2). The endogenous ABA content in isolated somatic

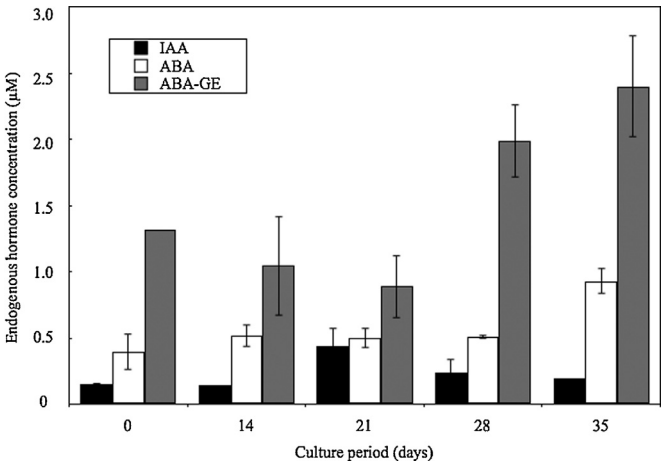

Fig. 3. Time course of ABA, ABA-GE, and IAA concentrations (μM) in grapevine embryogenic callus during a culture period of 35 days in DM1 differentiation medium. The values shown are the mean ± standard error of three sample replicates.

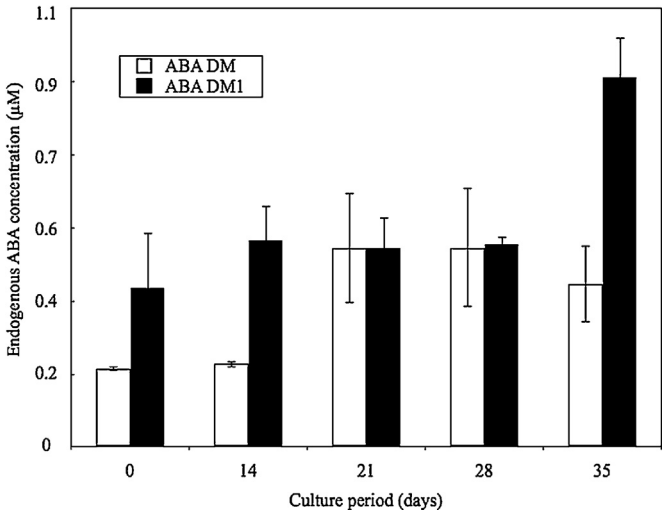

Fig. 4. Comparison of the time course of the ABA concentration in grapevine embryogenic callus during a culture period of 35 days in DM1 (without exogenous growth regulators) or DM (with exogenous growth regulators) differentiation media. The values shown are the mean ± standard error of three sample replicates.

embryos and/or embryogenic cultures of several species, including grapevine, shows a possible relationship between ABA and the accumulation of storage reserves during the late phases of embryo development (Faure et al., 1998; Jiménez and Bangerth, 2000). This was also supported by several experiments involving the exogenous application of ABA to embryogenic systems (Mauri and Manzanera, 2004; Sharma et al., 2004; Vales et al., 2007; Vahdati et al., 2008), though exogenous ABA failed to improve embryo maturation and conversion in peanut (Mhaske et al., 1998). This also occurred in experiments performed in our laboratory, where we did not find any effect of exogenous ABA in our embryogenic cultures (unpublished data).

The concentration of ABA-GE was higher than that of ABA in all samples (Fig. 3), though it decreased from 1.42 to 0.89 μM during 21 days of culture in DM1 medium. This reduction in ABA-GE may contribute to the maintenance of free hormone levels because it is known that β-D-glucosidase releases ABA from ABA-GE (see Nambara and Marion-Poll, 2005, for a review). The concentration of ABA-GE actively increased after 21 days of culture in DM1 medium, reaching its maximum at 35 days (2.4 μM). It is conceivable that an active process of ABA synthesis occurred during the last two weeks of culture in DM1 medium, which would make necessary its further conjugation to ABA-GE for maintaining the endogenous ABA levels within the appropriate concentration range for the physiological processes involved in somatic embryo maturation. The synthesis of ABA by somatic embryos cultured in the absence of the exogenous hormone has been reported in grapevine (Faure et al., 1998) and in other species (Kong and von Aderkas, 2007).

As reviewed by Jiménez (2005), ABA plays a key role during the processes of development and maturation in somatic embryos. To gain further information about the control of grapevine somatic embryo development, we compared the ABA data described above with the ABA content in embryogenic callus cultured in DM medium supplemented with a complex mixture of PGRs (Prado et al., 2010). This comparison of endogenous ABA concentration in both types of callus is presented in Fig. 4. By day 14, the calli cultured in DM medium showed approximately half of the endogenous levels of ABA than those cultured in DM1 medium, but the level was identical in both calli by 21 and 28 days. The ABA concentration in the calli cultured in DM medium barely changed or slightly decreased from 28 to 35 days, whereas that in the calli cultured in DM1 medium strongly increased, as described above. In view of these data, we performed a histological examination of calli cultured for 35 days in DM medium (Fig. 5). Both the appearance (Fig. 5A) and microscopic structure (Fig. 5B and C) of the calli resembled that of calli cultured in DM1 medium during the first weeks of culture (Fig. 2B and C). Hence, it was evident a strong delay in the process of somatic embryo development. Taking into account that DM medium contains 20 μM IAA, it is possible that the presence of auxin in the medium impaired the initiation of the histodifferentiation process (Jiménez, 2005; Ogata et al., 2005), leading to delayed development in the somatic embryos. Supporting this idea, Senger et al. (2001) found that cultures of *Nicotiana plumbaginifolia* treated with an inhibitor of ABA synthesis, fluridone, showed disturbed morphogenesis at the preglobular stage of somatic embryo formation, an observation that was counteracted by the application of exogenous ABA. To test this hypothesis, the effect of an ABA synthesis inhibitor (norflurazon) on the development of grapevine somatic embryos is currently being tested in our laboratory.

These differences may also help to explain the previously described development of grapevine somatic embryos in DM and DM1 differentiation media (Prado et al., 2010). The low level of ABA at the end of the culture in DM medium may be related to the presence of an extensive process of precocious germination when small pieces of callus are cultured in this medium. Accordingly, the higher levels of endogenous ABA in the callus cultured in DM1 medium most likely helped to obtain well-matured and correctly developed somatic embryos, which ultimately showed less precocious germination and higher rates of conversion to normal plantlets (Prado et al., 2010). This result agrees with the lower level of ABA found in precociously germinating somatic embryos than in normally germinating zygotic embryos of grapevine cv. Grenache noir (Faure et al., 1998).

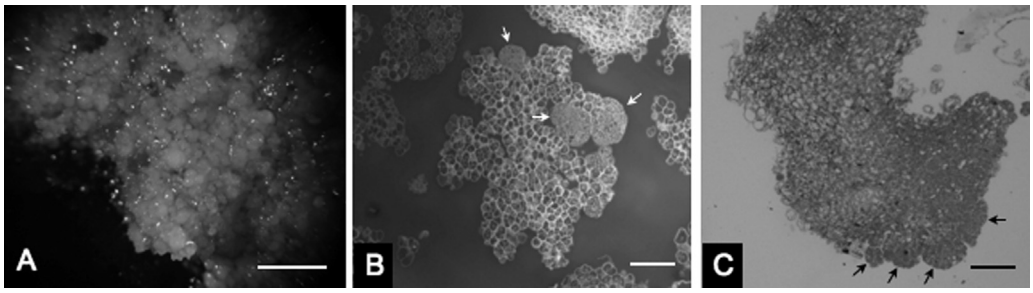

Fig. 5. Morphological (A) and microscopic (B and C) analysis of the embryogenic callus of grapevine cv. Mencía after 35 days of culture in DM differentiation medium. (A) Embryogenic callus of a granular aspect, without somatic embryos visible on its surface. Calcofluor (B) and toluidine blue (C) staining showing the formation of pro-embryo structures (arrows) on the surface of the callus. Bars = 1 mm (A) and 50 μm (B and C).

In conclusion, we present a sensitive and reliable LC-ELISA method which allowed us to measure (+)ABA and ABA-GE, the latter after being subjected to alkaline hydrolysis, in the embryogenic callus of grapevine. In this ELISA, the C4′-I PcAbs showed a high specificity for the free form of (+)ABA and barely reacted with ABA conjugates. Despite this, we considered useful to purify the crude extracts through SPE and HPLC to separate the compounds and to eliminate the concomitant interference as much as possible before ABA quantification is made. The comparison of the ABA content in calli cultured in differentiation media with and without PGRs, along with the observed histological changes in relation to somatic embryo maturation, indicates that an increase in ABA content during the development of somatic embryos in grapevine is necessary for their correct maturation. The maintenance of these ABA levels may be supported by the release of ABA from ABA-GE during the first weeks of culture, whereas an active process of ABA synthesis could be required in the last weeks, producing a concomitant increase of ABA-GE levels. In addition, the endogenous IAA content detected in grapevine callus cultured in a differentiation medium without PGRs (DM1) throughout the culture period suggests that auxin plays no significant role during the differentiation and maturation of somatic embryos.

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