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Research article

Differential hormonal and gene expression dynamics in two inbred sunflower lines with contrasting dormancy level

Paula L. Roselló [a], Ana E. Vigliocco [b], Andrea M. Andrade [b], Natalí V. Riera [b], Mario Calafat [a], María L. Molas [a], Sergio G. Alemano [b, *]

[a] Laboratorio de Fisiología Vegetal, Fac. de Agronomía, Universidad Nacional de La Pampa (UNLPam), 6300, La Pampa, Argentina
[b] Laboratorio de Fisiología Vegetal, Fac. de Ciencias Exactas, Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto (UNRC), 5800, Río Cuarto, Córdoba, Argentina

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ABSTRACT

Seed germination and dormancy are tightly regulated by hormone metabolism and signaling pathway. We investigated the endogenous content of abscisic acid (ABA), its catabolites, and gibberellins (GAs), as well as the expression level of certain ABA and GAs metabolic and signaling genes in embryo of dry and imbibed cypselas of inbred sunflower (*Helianthus annuus* L., Asteraceae) lines: B123 (dormant) and B91 (non-dormant). Under our experimental conditions, the expression of *RGL2* gene might be related to the ABA peak in B123 line at 3 h of imbibition. Indeed, *RGL2* transcripts are absent in dry and early embedded cypselas of the non-dormant line B91. ABA increase was accompanied by a significant ABA-Glucosyl ester (ABA-GE) and phaseic acid (PA) (two ABA catabolites) decrease in B123 line (3 h) which indicates that ABA metabolism seems to be more active in this line, and that it would be involved in the imposition and maintenance of sunflower seed dormancy, as it has been reported for many species. Finally, an increase of bioactive GAs (GA_1 and GA_3) occurs at 12 h of imbibition in both lines after a decrease in ABA content. This study shows the first report about the *RGL2* tissue-specific gene expression in sunflower inbred lines with contrasting dormancy level. Furthermore, our results provide evidence that ABA and GAs content and differential expression of metabolism and signaling genes would be interacting in seed dormancy regulation through a mechanism of action related to embryo itself.

1. Introduction

Sunflower is an oil-seed species widely spread in the world, being Argentina one of the first oil global exporters. Seed dormancy can be a major problem for seed companies. This event represents an important delay for immediate sowing and makes it difficult the commercialization, mainly for counter seasonal production. In sunflower breeding programs, for instance, dormancy limits the number of crop cycles per year; it also leads to asynchronous blossom times, restricting the opportunity to make crosses between plants (Marchetti, 2012). Interestingly, sunflower is an excellent model for understanding seed dormancy since seeds are deeply dormant at harvest time, reducing progressively this state during dry storage (Corbineau and Côme, 2003). In sunflower, dormancy is controlled either by embryo, surrounding seed layers (seed coat + pericarp) or a combination of both (Brunick, 2007). Coat-enhanced dormancy acts on oxygen availability for the embryo through the phenolic compounds effects, usually at high temperatures. At temperatures below 15 °C, the embryo is the main responsible for impairing seed germination restriction (Corbineau et al., 1990). Often, embryo dormancy includes a short period (4–8 weeks), while dormancy imposed by seed coat and pericarp generally persists for more than 32 weeks (Brunick, 2007). On the other hand, it is known that during sunflower seed development, dormancy is significantly impacted by mother plant growing environment (Fenner, 1991).

Seed germination and dormancy are physiological events regulated by simultaneous action of different plant hormones such as Abscisic Acid (ABA), Gibberellins (GAs), Jasmonic Acid (JA), Ethylene (ET), Indol-3-acetic Acid (IAA) and Brassinosteroids (BRs) that play an important role in promoting or inhibiting those processes. It has been reported that plant hormones metabolisms depend one from each other, and changes in endogenous levels of some of these compounds eventually modify the content of other

* Corresponding author.
E-mail address: salemano@exa.unrc.edu.ar (S.G. Alemano).

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hormones (Preston et al., 2009; Miransari and Smith, 2014). Particularly, ABA-GAs balance plays an important role in dormancy-germination regulation in most species (Yamaguchi, 2008). ABA content is high in dry seeds and it is reduced through the imbibition process whereas GAs content increases during phase II of imbibition in germinating seeds (Preston et al., 2009). Thereafter, a diminution in ABA content is needed in order to germination arise. This decrease occurs by suppression of ABA *de novo* biosynthesis and/or by activation of ABA catabolism (Liu et al., 2009), accompanied by an increase in GAs biosynthesis (Ogawa et al., 2003; Nonogaki et al., 2010). Therefore, a dynamic equilibrium exists between ABA and GAs biosynthesis and catabolism pathways determining a specific ABA:GAs balance. In addition, transition from dormant to non-dormant state is characterized by both a decrease in ABA sensitivity and an increase in GAs sensitivity (Cadman et al., 2006). Seed dormancy-germination processes are not only regulated by plant hormones. In fact, Šírová et al. (2011) reported a crosstalk between plant hormones and reactive oxygen species (ROS) in regulating those processes. Numerous studies have provided evidence that H_2O_2 treatment can modify the plant hormone contents (Bailly et al., 2008; Barba-Espin et al., 2010, 2011; Díaz-Vivancos et al., 2013). For example, exogenous H_2O_2 not appear to alter ABA biosynthesis and signaling in barley seeds, but has a more pronounced effect on GAs signaling, inducing a change in hormonal balance that results in germination (Bahin et al., 2011). Additionally, novel evidences about role of hormones in dormancy regulation have been brought by El-Maarouf-Bouteau et al. (2015) who demonstrated an interaction between ABA and ET in the regulation of sunflower seed dormancy, which are tightly interacting with ROS in this process.

At molecular level, most of the biochemical and physiological modifications that occur during germination-dormancy processes are governed by the expression of different genes (Weitbrecht et al., 2011). Moreover, changes in the expression of genes encoding ABA and GAs metabolic enzymes are considered main regulators of hormone metabolism. Studies with *Arabidopsis thaliana* and *Triticum aestivum* seeds have demonstrated that ABA and GAs biosynthetic and catabolic genes are mutually regulated in dormancy maintenance and release (Liu et al., 2013). For instance, the catabolic gene ABA-8'-hidroxylase is highly expressed during imbibition and causes ABA inactivation and dormancy release (Liu et al., 2009). In the same line, GAs content is regulated through GA_3-oxidase (GA_3-ox) and GA_{20}-oxidase (GA_{20}-ox) enzymes, both involved in GAs biosynthesis pathway (Yamaguchi, 2008). Nevertheless, when GAs endogenous concentration is excessive, they can be inactivated through GA_2-ox enzymatic action, consequently GAs content decreases and germination is negatively affected (Bewley et al., 2013). On the other hand, GAs signal-transduction pathway is very important for seed germination control. One of the key signaling elements is RGL2 (RGA-like 2), a DELLA protein that represses germination in *A. thaliana* (Piskurewicz et al., 2008). These proteins are accumulated at low GAs concentrations and act as repressors of GA-activated processes (Gallego-Bartolomé et al., 2010). Thereby, if GAs biosynthesis does not occur, RGL2 is persistently accumulated in imbibed seeds and promotes the ABA endogenous accumulation. On the contrary, when GAs content increases, its molecules are perceived by a nuclear receptor named GID1 (GA-INSENSITIVE DWARF 1). This interaction facilitates a ternary complex formation between GA, GID1 and DELLA protein amino-terminal domain (RGL2). This complex triggers DELLAs recruitment by a specific F-box protein, known as SLY1 (SLEEPY 1) in *Arabidopsis* and GID2 (GA-INSENSITIVE DWARF 2) in rice (Ariizumi et al., 2008), that transports DELLA proteins to an E3-ubiquitin-ligase complex where they are polyubiquitinated and degraded. DELLA proteins disappearance enhances GA-responsive process, such as germination (Hirsch and Oldroyd, 2009).

In sunflower crop, seed germination is seriously impaired by dormancy compromising agronomic yield. To better understand the physiological mechanism underlying these processes, we studied ABA-GA metabolism and signaling during dormancy and germination in sunflower. More precisely, we investigated ABA and GAs endogenous contents in correlation with the expression level of certain ABA and GAs metabolic and signaling genes in dry and imbibed cypselas of dormant sunflower line (B123) and non-dormant line (B91).

2. Material and methods

2.1. Plant material

Cypselas of inbred B123 and B91 *Helianthus annuus* lines, supplied by the breeder MSc. Daniel Álvarez, were sown in the experimental field of EEA-INTA Manfredi (latitude 31°51′9.00″ S, longitude 63°44′55.91″ W), Córdoba, Argentina. At harvest, the different phenotype of the cypselas of both lines is showed in supplementary data (Supplemental Fig. 1). In previous studies in our laboratory, the germination of mature and dry sunflower cypselas of above inbred lines was monitored during dry storage (25 ± 1 °C, relative humidity 50%) at different days following harvest (0, 11, 22, 33 and 44) to determine its germination capacity and dormancy level. These studies have allowed us to conclude that B123 is a dormant line (near 0% germination at harvest) and B91 is a non-dormant line (approx. 100% germination at harvest) (Andrade et al., 2015).

2.2. Germination assay

The germination test was performed on mature dry cypselas of B123 and B91 inbred lines obtained at harvest. Four biological replicates (each 25 cypselas) were sown in 16 × 12-cm pots between filter paper moistened with 25 ml deionized water. The pots were kept under controlled growth conditions in a walk-in rooms GR48 (Conviron Winnipeg, Canada) programmed with a cycle of 16 h light (200 μmol/m2/seg), at 28 °C and 70% relative humidity, and 8 h dark, 20 °C and 80% relative humidity. To avoid any effect due to the position of pots in the walk-in rooms, each pot was rearranged at random every day. Then, at different imbibition times (3, 6, 12 and 24 h) the cypselas were collected and embryos (embryonic axis and cotyledons) were carefully removed by hand to avoid mechanical damage. The dry cypselas were considered as 0 h of imbibition. The embryos isolated were immediately frozen in liquid N_2 and lyophilized for hormonal determination. For expression gene analysis, the embryos were frozen in liquid N_2 and immediately stored at −80 °C. The germination assay was performed for quadruplicate.

2.3. Extraction and purification of endogenous phytohormones

ABA, PA, ABA-GE, GA_1, and GA_3 were extracted from 0.2 g dry weight (DW; g $plant^{-1}$) embryos by the method of Durgbanshi et al. (2005) with some modifications. Plant material was homogenized in Ultraturrax T25 basic (IKA; Staufen, Germany) with 5 ml deionized water. D_6-ABA, D_3-PA, D_5-ABA-GE (NRC-Plant Biotechnology Institute, Saskatoon, Canada), D_2-GA_1 and D_2-GA_3 (OlChemIm Ltd, Olomouc, Czech Republic), were used as internal standards. Fifty ng of each was added to samples. Samples were centrifuged at 1540 x *g* for 15 min. The supernatant was adjusted to pH 2.8 with 15% (v/v) acetic acid and extracted twice with diethyl ether. The organic fraction was evaporated under vacuum. The dried extracts were dissolved in 1 ml methanol, and filtered

on a vacuum manifold at a flow rate <1 ml min^{-1}. The eluate was evaporated at 35 °C under vacuum in a Speed Vac SC110 (Savant Instruments; New York, NY, USA). Experiments were performed in quadruplicate.

2.4. Phytohormone identification and quantification by liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI/MS-MS)

ABA and their catabolites (PA and ABA-GE), GA_1, and GA_3 were separated from tissues by reversed-phase high-performance liquid chromatography (HPLC). An Alliance 2695 separation module (Waters; Milford, MA, USA) equipped with a Restek C_{18} column (100 mm × 2.1 mm, 3-μm) was used to maintain performance of the analytical column. Fractions were separated using a gradient of increasing methanol concentration, constant glacial acetic acid concentration (0.2% in water), and initial flow rate 0.2 ml min^{-1}. The gradient was increased linearly from methanol/water-acetic acid (40/60, v/v) at 25 min to methanol/water-acetic acid (80/20, v/v). After 1 min, the initial conditions were restored, and the system was allowed to equilibrate for 7 min. The identification and quantification of all hormones were performed by quadruple tandem mass spectrometer (Quattro Ultima, Micromass, Manchester, UK) fitted with an electrospray ion (ESI^-) source, in multiple reactions monitoring mode (MRM) using precursor ions and their transitions (*m/z)* to ABA (*m/z* 263/153), D_6-ABA (*m/z* 269/159), PA (*m/z* 279/139), D_3-PA (*m/z* 282/142), ABA-GE (*m/z* 425/263), D_5-ABA-GE (*m/z* 430/268), GA_1 (*m/z* 348/242), D_2-GA_1(*m/z* 350/244), GA_3 (*m/z* 345/221), and D_2-GA_3(*m/z* 347/223) with retention times of 8.25, 5.30, 3.15, 3.20, 5.46 min respectively. The spectrometry software used was MassLynx V. 4.1 (Micromass).

2.5. Extraction of total RNA, cDNA synthesis and PCR analysis

Total RNA was extracted from embryo using the Trizol reagent (Invitrogen). Embryo were ground with liquid nitrogen and homogenized in Trizol. The homogenate was incubated for 5 min at room temperature, then 120 μl of chloroform were added and each tube was vigorously skaken for 15 s. The samples were incubated at room temperature for 3 min and then centrifuged at 8870.4 x *g* for 15 min at 5 °C. The aqueous phase was transferred to a new tube and 250 μl of isopropanol were added. Samples were mixed by inversion, incubated for 10 min at −20 °C and centrifuged for 10 min at 8870.4 × *g* at 5 °C to obtain the RNA pellet. The pellet was washed with 70% ethanol, dried, re-suspended in 60 μl of water and incubated at 60 °C for 10 min. RNA was quantified with a spectrophotometer (Ocean Optics CHEM 2000) at 260 nm and 280 nm cDNA was synthesized from 1 μg of RNA using the Reverse Transcriptase (Promega M1701) in a 20 μl reaction volume. PCR amplification was conducted using the following gene-specific primers: *AAO* (ABA aldehyde oxidase) (pF AGAACCATCTAACCGCTTCC, pR GAGCCACCGTTACTTCTTGC); *ABA-8′-OH* (pF CTGCTGCTGCTAAGCTTGTG, pR GTTTAATCGATTCGGGCGTA); *β-glucosidase* (pF CCGCACGAGAGAAGAAAAC, pR TGCCTCCAAATCGCCAATCC); *GA20-ox* (pF CGCTATGGAACGCCACTC, pR GAGATGCCAAAAACCAGACC); *GA3-ox* (pF CGCGTAAACTCTGGCCTAAC, pR AAAAGGGTTGAATCGGTGTG); *GA2-ox* (pF CCAATAACACTTCTGGGCTTG, pR CACCACCCTATGCTTCACAC); *SLY1* (pF GACTGCTGTTCTGCTGGATG, pR GATATTCGCCCAGTGTTTGG) and *RGL2* (pF CGGAACCAGAATCAAAACCA, pR ACATACCGGCTCGGCTTACT). Samples were subjected to 35 cycles of denaturation for 1 min at 95 °C, annealing for 1 min at 55 °C and extension for 1 min at 72 °C, followed by a final extension for 5 min at 72 °C. House-keeping gene *Actin* was amplified as a control. PCR products were subjected to electrophoresis on a 1% agarose gel containing GelRed dye for an hour at 90 V. Transcript levels were analyzed using ImageJ software and were normalized with house-keeping gene transcript.

2.6. Statistical analysis

Data were analyzed by one-way ANNOVA. For phytohormone determination, the data were subjected to Multiple Range Test (Tukey's HSD test), and statistical software used was Statgraphics Plus, V. 3. For gene expression analysis, the Multiple Range Test (LSD test) was performed with the statistical InfoStat software (2008 Version).

3. Results

3.1. ABA and catabolites in embryo of dry and imbibed cypselas

Endogenous free ABA, ABA-GE and PA were determined in embryo of dry and imbibed cypselas of B123 dormant and B91 non-dormant lines. In embryo of dry cypselas, B123 presented higher ABA content than that B91. In 3 h-imbibed cypselas of B123 line, an important increase in ABA content (1.5-fold, $P < 0.05$) was detected. From 6 h, ABA significantly decreased and then it remained stable during the course of imbibition. In B91 line, no significant differences in ABA content were found among 0 (dry cypselas), 3 and 6 h of imbibition. However, at 12 and 24 h a noticeable reduction (12-fold) was observed; for both times of imbibition, the ABA content was the same in two lines ($P < 0.05$, Fig. 1A). In terms of catabolic pathway, ABA-GE catabolite was more abundant than PA (Fig. 1B, C). In embryo of dry cypselas (0 h of imbibition), the endogenous ABA-GE content was higher in B123 compared to B91. In 12 h-imbibed cypselas of dormant line ABA-GE peaked. However, in non-dormant line ABA-GE did not change until 12 h. At 24 h, ABA-GE content was significantly reduced in both lines, reaching the lowest value among all imbibition times analyzed (B123: 438.6 pmol g^{-1} DW; B91: 3211.15 pmol g^{-1} DW) ($P < 0.05$, Fig. 1B). In embryo of dry cypselas, the PA content was similar between both lines. At 6 h of imbibition, a remarkable PA increase was detected in B123 embryo, and then its content decreased to reach the value recorded for embryo of dry cypselas at 24 h. In 3 h-imbibed cypselas of B91 line, a significant increase in PA content was observed in relation to embryo of dry cypselas (Fig. 1C).

3.2. GAs in embryo of dry and imbibed cypselas

Bioactive GA_1 and GA_3 were detected in embryo of dry and imbibed cypselas. In embryo of dry cypselas, endogenous content of both GAs were lower than ABA content (Fig. 1D, E). In embryo of imbibed cypselas for 3 h, GA_1 increased 2-fold in B123 and one-fold in B91 compared to embryo of dry cypselas (0 h of imbibition) ($P < 0.05$); comparing between lines, B123 showed a higher GA_1 content than that B91. At 12 h, a 3-fold increase was observed in both lines, and after this point, a subsequent decrease occurred (Fig. 1D). Regarding GA_3 content, it was similar to GA_1 in embryo of dry cypselas. No differences between lines were found at 0 (dry cypselas), 3, 6 and 24 h of imbibition. At 12 h, GA_3 increased 5-fold in B91 and 2-fold in B123 in relation to embryo of dry cypselas (0 h of imbibition) ($P < 0.05$, Fig. 1E).

3.3. Expression of ABA metabolic genes

Relative abundance of ABA biosynthetic and catabolic gene transcripts was evaluated in embryo of dry and imbibed cypselas of B123 and B91 lines. Regarding ABA biosynthesis, *AAO* gene expression was studied (Fig. 2A). In the dormant line B123, the *AAO*

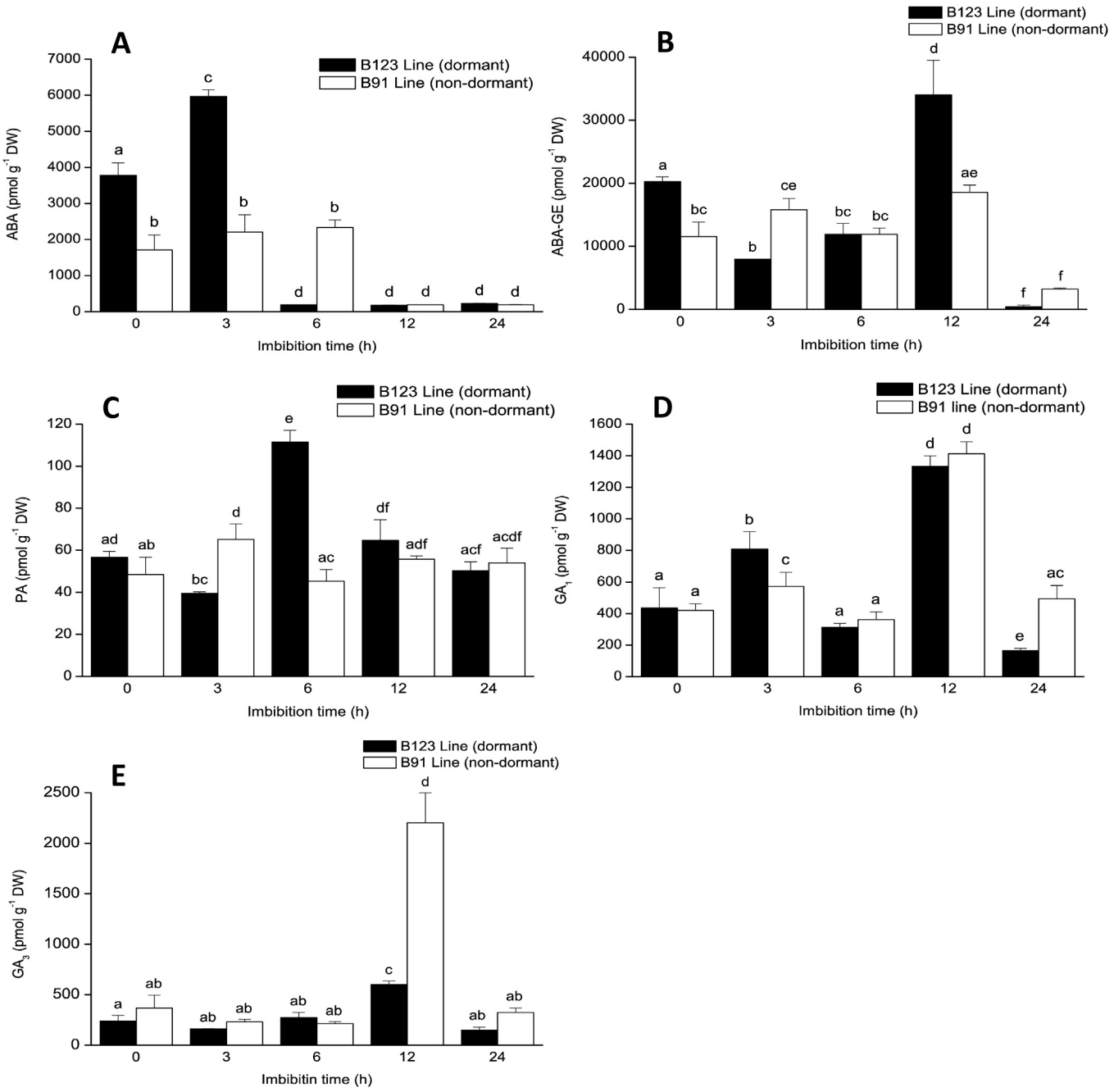

Fig. 1. Hormonal content in embryo of sunflower cypselas: A) Abscisic acid (ABA), B) ABA-Glucosyl ester (ABA-GE), C) Phaseic acid (PA), D) Giberellin A_1 (GA_1), E) Giberellin A_3 (GA_3); B123 line (black bars) and B91 line (white bars) in dry cypselas (0 h) and during imbibition time course (3, 6, 12 and 24 h). Data are means of four replicates with SEs. Values with the same letter are not significantly different at $p \leq 0.05$.

transcripts level remained stable during imbibition whereas in the non-dormant line B91 these transcripts decreased at 3 h ($P < 0.05$). To address ABA catabolism, we measured the level expression of ABA 8-hydroxylation gene, *ABA-8′-OH* (Fig. 2B). In B123, an increase in transcript amounts was observed at 3 h of imbibition, which was correlated with an increase of PA detected at 6 h. In addition, ABA de-conjugation pathway -intervening in bioactive ABA recycling from conjugated ABA-was also analyzed through *β-glucosidase* gene expression (Fig. 2C). No significant differences between lines in *β-glucosidase* transcript level were observed during the course of imbibition. Nevertheless, in B123 line a significant decrease in transcript level was observed at 6 and 12 h until to reach the value recorded for embryo of dry cypselas at 24 h. On the other hand, differences in *β-glucosidase* expression were not registered in B91 line along the imbibition period analyzed, suggesting that the de-conjugation pathway does not significantly contribute to recycling free ABA pool during B91cypselas imbibition process (Fig. 2C).

3.4. Expression of GAs metabolic genes

In order to research bioactive GAs biosynthesis, the *GA20-ox* (Fig. 3A) and *GA3-ox* (Fig. 3B) gene level expression was evaluated. A significant increase in the *GA20-ox* transcripts level was detected from 6 h of imbibition in both lines. Interestingly, at 12 h a

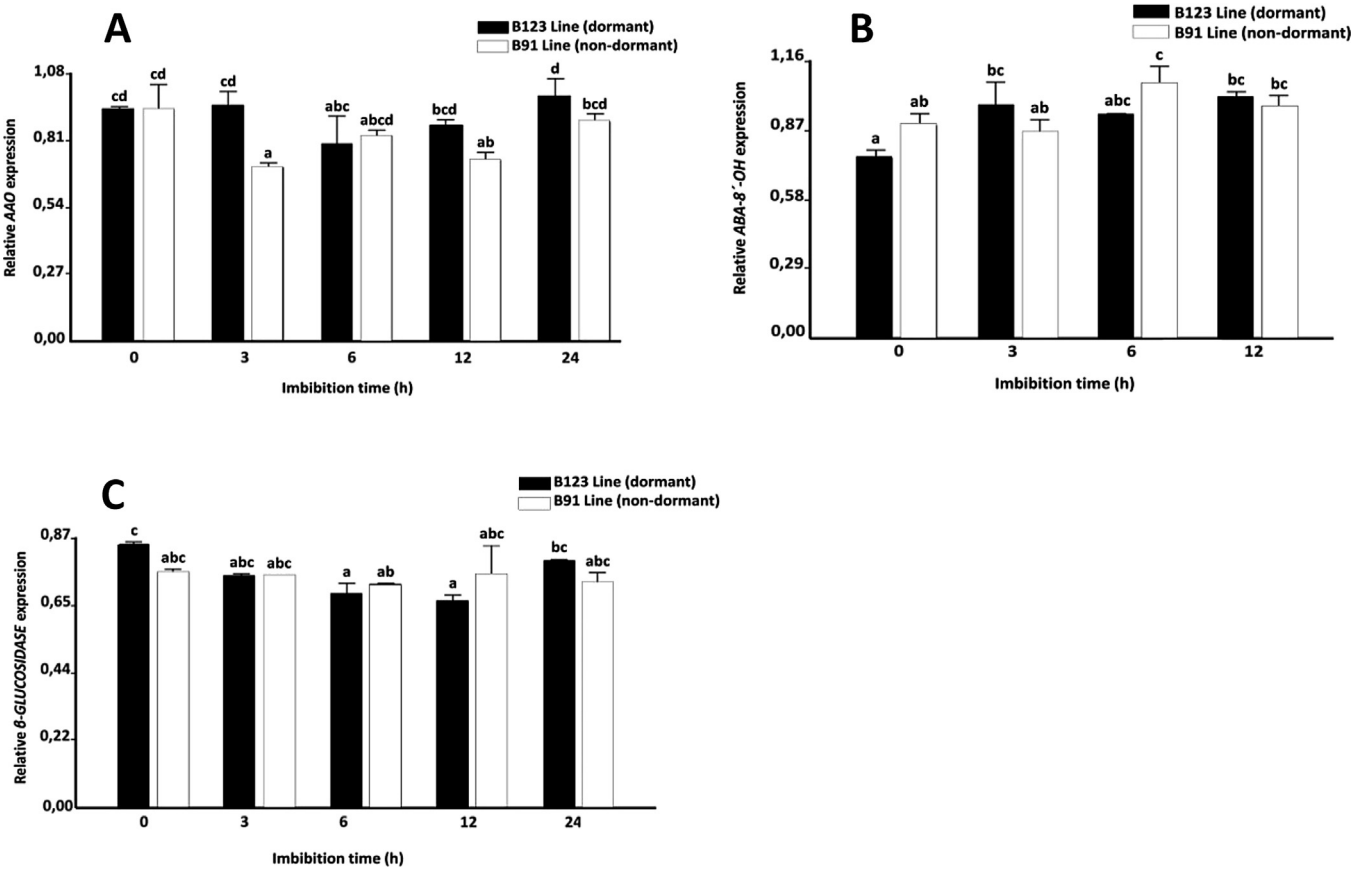

Fig. 2. Expression level of: A) AAO, B) ABA-8′-OH, C) β-glucosidase gene in embryo of B123 and B91 sunflower cypselas in dry cypselas (0 h) and during imbibition time course (3, 6, 12 and 24 h). Relative transcript level was calculated and normalized with respect to the sunflower actin transcript level. Data are means of three replicates with SEs. Values with the same letter are not significantly different at $p \leq 0.05$.

significant difference between lines was observed for *GA20-ox* gene (Fig. 3A). In 6 h-imbibed cypselas, the *GA3-ox* expression increased only in B123 line compared to embryo of dry cypselas. The expression level of this gene did not vary between two lines during the whole imbibition period (Fig. 3B). The increase in *GA20-ox* and *GA3-ox* genes transcription would be suggesting GAs *de novo* biosynthesis activation in both studied lines. This increase in the expression level of both genes was correlated with increased content of GA_1 (3 and 12 h) and GA_3 (12 h). Regarding to GAs catabolic genes, the *GA2-ox* gene expression was studied (Fig. 3C). In embryo of dry cypselas of B123 line, the transcripts amounts were 30% higher than that B91 line. At 3 h of imbibition, the *GA2-ox* transcripts level decreased in B123 while no significant changes were observed in B91 at the same imbibition time respect to embryo of dry cypselas (Fig. 3C). However, the expression level significantly increased in B91 line at 6 and 12 h. GAs augmented catabolism would be in consonance with biosynthesis activation at 6 h of imbibition, suggesting a dynamic and active metabolism.

3.5. Expression of GAs signaling genes

The GAs signaling genes assessed were *SLY1* (Fig. 3D) and *RGL2* (Fig. 3E). Relative abundance of *SLY1* transcripts did not show significant differences ($P < 0.05$) between B123 and B91 lines. In both lines, mRNA levels kept constant at 3 h, and then they increased at 6 and 12 h of imbibition. This increment was correlated with bioactive GAs levels rise at 12 h. Finally, the transcripts level returned to the value recorded for embryo of dry cypselas at 24 h (Fig. 3D). On the other hand, the transcripts level of *RGL2* gene showed a noticeable difference between lines. *RGL2* transcripts were detected in dry and 3 h-imbibed cypselas only in the dormant line, but not in the non-dormant line under the same conditions. In B123, the *RGL2* expression significantly and progressively decreased from 3 h of imbibitions, and did not recover the expression level observed for embryo dry cypselas. In B91, the first transcripts were detected at 6 h of imbibition and its expression levels significantly increased at 24 h (Fig. 3E).

4. Discussion

In another previous study, we reported the existence of different hormonal profiles in pericarp of B123 (dormant) and B91 (non-dormant) sunflower inbred lines depending on storage conditions and imbibition process (Andrade et al., 2015). Here, to get a more comprehensive view of the contributions of the different tissues that comprise a sunflower cypsela in dormancy control, we performed a physiological and molecular study on embryo of sunflower seed. Thus, the B123 and B91 embryos showed differential dynamics in hormonal endogenous content (ABA and GAs) and in gene expression related with its metabolism and signaling pathway.

In terms of ABA dynamics, it has been widely documented for numerous species such as sunflower (Le Page-Degivry and Garello, 1992) and *Arabidopsis* (Preston et al., 2009) that seed dormancy level is usually correlated with high ABA content. At harvest time, ABA content produced by the zygote in the late states of seed

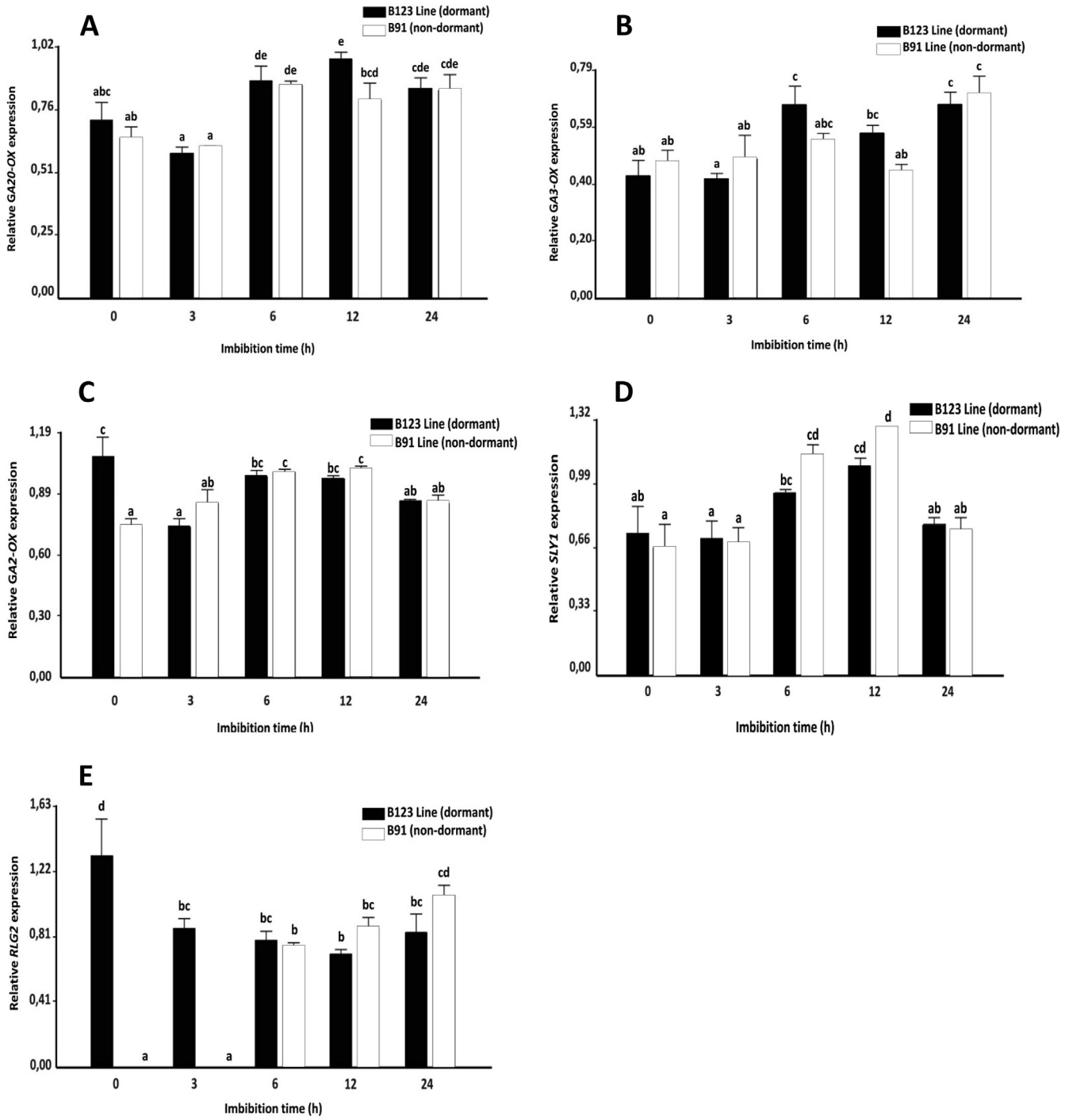

Fig. 3. Expression level of: A) GA20-ox, B) GA3-ox, C) GA2-ox, D) SLY1, E) RGL2 gene in embryo of B123 and B91 sunflower cypselas in dry cypselas (0 h) and during imbibition time course (3, 6, 12 and 24 h). Relative transcript level was calculated and normalized with respect to the sunflower actin transcript level. Data are means of three replicates with SEs. Values with the same letter are not significantly different at $p \leq 0.05$.

ripening is involved in dormancy imposition (Rodriguez-Gacio et al., 2009). However, we cannot discard the possible contribution of maternal ABA in the regulation of many aspects of seed development. Previously, we demonstrated that due to the low germination percentage obtained following pericarp removal in B123 we cannot rule out the possibility of physiological dormancy resulting from the high ABA content (~4000 pmol g^{-1} DW) in embryos of B123 line (Andrade et al., 2015). Results from this work confirm that bioactive ABA in dry embryos would be responsible for B123 dormancy imposition at harvest time.

Additionally, significant ABA-GE amounts would be indicating that this catabolite accumulates preferentially during the seed maturation and, perhaps, contributes to the dormancy level of B123 line. However, ABA-GE biosynthesis and hydrolysis implication in dormancy control has not been demonstrated yet (Arc et al., 2013). Seed dormancy level could be related to ABA endogenous content in imbibed seed rather than in dry seed in several species such as *Arabidopsis* (Ali-Rachedi et al., 2004) and tobacco (Grappin et al.,

2000). In this study, the ABA increase in B123 embryos during imbibition confirms the results obtained previously in sunflower by Le Page-Degivry and Garello (1992). In concordance, we detected high ABA accumulation in B123 pericarps during the early hours of imbibition (Andrade et al., 2015). Indeed, the ABA increase in B123 embryos at 3 h was accompanied by an important ABA-GE decrease. Therefore, either ABA *de novo* biosynthesis activation during imbibition or ABA catabolism suppression could be regulating dormancy in this line. However, in *Arabidopsis* and *Hordeum vulgare* genotypes that present dormancy, catabolism suppression would be the principal dormancy regulating process (Millar et al., 2006). In different species, ABA content reduction throughout imbibition is observed in both dormant and non-dormant seeds (Linkies et al., 2009). In embryos of B123 dormant line, a decrease in ABA at 6 h of imbibition could be indicating a biosynthesis decreasing and/or activation of catabolic pathway. In fact, this ABA decrease was correlated with a remarkable increase in ABA-GE (at 12 h) and less in PA (at 6 h) contents. Thus, the ABA catabolism could be triggered during imbibition, considering that an increase of *ABA-8′-OH* gene expression was detected at 3 h of imbibition. In this sense, it has been well demonstrated that 8′hidroxylation-pathway plays an essential role in ABA endogenous level of regulation during imbibition process (Preston et al., 2009). Such is the case of *Arabidopsis* and barley, where ABA reduction was correlated to an increase in PA level during seed imbibition (Rodriguez-Gacio et al., 2009). In this research, we observed that the decrease of ABA at 12 h in B91 non-dormant embryos was accompanied by a relevant ABA-GE increase, suggesting that bioactive ABA conjugation could be participating in later germination. In consequence, both processes -ABA synthesis deactivation and/or ABA catabolism activation-could be activated in sunflower, resulting essential for germination in B91 non-dormant line. On the other hand, in embryos of B123 dormant line ABA-GE decrease at 24 h was not correlated to ABA content increase. In this sense, it has been reported by Piotrowska and Bajguz (2011) that conjugated ABA level does not always fluctuate along with free ABA. In reference to the ABA-GE de-conjugation process, differences detected in ABA content between B123 and B91 lines would not be caused by *β-glucosidase* differential gene expression during imbibition. This could be indicating that the de-conjugation pathway would not be the principal source of bioactive ABA required for dormancy maintenance in B123 imbibed embryos. More studies need to be done to clarify how the balance between *de novo* ABA synthesized and ABA recycled via hydrolysis from ABA-GE is regulated (Piotrowska and Bajguz, 2011).

In embryos of B123 dry cypselas, the expression of catabolic gene *GA2-ox* was noticeable, in contrast to that in B91 non-dormant line. Similar results were observed in *Arabidopsis* seeds, where *GA2-ox* expression reached the maximum in dormant seed compared to non-dormant seeds (Finkelstein et al., 2008). On the other hand, in embryos of imbibed cypselas of both lines, the increase in biologically active GAs, (GA_1 and GA_3) at 12 h of imbibition corresponded to an increase in expression of biosynthetic genes *GA3-ox* and *GA20-ox* at 6 h of imbibition, suggesting that GAs *de novo* biosynthesis induction would occur simultaneously in both lines. The activation of the biosynthetic pathway was also reported in *Arabidopsis* seeds, where the increase in transcript levels of *GA20-ox* and *GA3-ox* between 8 and 24 h post-imbibition was observed (Ogawa et al., 2003). In embryos of B91 line, the increase in bioactive GAs biosynthesis (at 12 h of imbibition) was concurrent with a catabolism increase which suggests that it might exists a fine regulation in bioactive GAs concentration that effectively triggers germination. It has been demonstrated that GAs exogenous application enhances transcription of genes coding deactivating enzymes -for example GA2-ox- generating a feedback regulation (Olszewski et al., 2002).

Regarding to the GAs signaling, the DELLA proteins are key components in GAs signaling because of its function as negative regulators of the GAs signal transduction pathway. In fact, within this protein family, RGL2 is considered the mayor repressor of seed germination. Interestingly, *RGL2* was co-localized with a QTL for seed dormancy in an improved *x* wild sunflower cross (Brunick, 2007; Mandel et al., 2014). Our results showed that in embryos of B123 dry cypselas, the *RGL2* gene is expressed, but this does not occur in B91 line. A similar situation occurs at 3 h of imbibition, suggesting that *RGL2* could be blocking germination in the B123 line. In concordance, in *Arabidopsis* dormant and non-dormant seeds the *RGL2* expression stimulates ABA *de novo* biosynthesis (Piskurewicz et al., 2008). Moreover, these authors proposed a model of positive feedback between ABA and *RGL2*, whereas *RGL2* promotes dormancy by stimulation of the ABA *de novo* biosynthesis, and ABA maintains over time the expression of *RGL2* and transcription factors such as *ABI5* and *ABI3*, involved in the ABA signal transduction pathway. In our study, the *RGL2* high expression in dry and 3 h-imbibed cypselas of B123 line was correlated with high endogenous ABA level. Conversely, in B91 line, the lack of *RGL2* expression in dry cypselas and in 3 h-imbibed cypselas was related to low ABA level, which opens up the question whether ABA-RGL2 interaction can also occurs in cypselas of sunflower. It has been documented that if GAs levels increase, the RGL2 protein is degraded. This degradation is mediated by the F-box protein -SLY1-SLEEPY1 which is part of the E3 ubiquitin ligase complex in *Arabidopsis*; thus, RGL2 disappearance would trigger germination (Piskurewicz et al., 2008). In our study, *SLY1* transcript level in both lines was similar, even when *RGL2* transcripts were quite different between lines. This would indicate that the presence or absence of *RGL2* transcript is no related to *SLY1* expression. This evidence might be in line with an RGL2-independent pathway proposed for stimulating seed germination. Previously, Ariizumi and Steber (2007) have been described that *sly1 Arabidopsis* mutants continued accumulating high levels of DELLA protein RGL2 and its seeds could germinate, suggesting that RGL2 protein disappearance may not be required to de-repress GA-dependent gene expression in the *sly1* mutant. This finding supports the think that there is an RGL2-independent mechanism for GAs regulation in seed germination. This is consistent with a microarray study showing that there are GAs-regulated genes with DELLA independent expression (Cao et al., 2006).

Based on the present research we propose that the expression of *RGL2* gene and ABA *de novo* biosynthesis during imbibition could be involved in the imposition and maintenance of B123 sunflower seed dormancy. Therefore, these findings make of the *RGL2* gene a promising target for functional studies involving seed dormancy and germination in sunflower cultivated. We know the existence of new interactions between ROS and hormonal signals involved in the regulation of dormancy in sunflower such as ABA-ET (El-Maarouf-Bouteau et al., 2015) besides of classical interaction ABA-GAs. Nevertheless, further investigations on the occurrence of other phytohormones in specific tissues of sunflower cypsela (i.e. embryo and seed coat) will expand our understanding about the hormonal crosstalk on regulation of dormancy and germination processes.

Author contribution

AV, AA, MLM and SA conceived and designed research, and they wrote the manuscript. PR and MC conducted the gene expression experiments and analyzed data. NR conducted the germination assays. AV performed the hormonal determination. The manuscript has been approved by all co-authors. AV, AA, and SA re-wrote and re-submitted this manuscript.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.plaphy.2016.02.021.

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