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# Investigating the effect of plant growth regulators in the endodormancy release in apricot by a metabolomic approach

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## Abstract

Endodormancy is one of the most studied physiological processes in perennial plants like apricot. This period is vital both for the tree survival against the adverse climatic conditions of winter and for obtaining a proper flowering and fruit set. Many studies have remarked the importance of chill accumulation as the limiting factor for endodormancy release. The increase of mean temperatures caused by climate change has been seriously endangering this process during the last decades. Because of this, plant growth regulators for promoting endodormancy release have spread worldwide. However, due to the toxicity and the irregular efficiency, there is a great necessity of developing new environment-friendly regulators for promoting endodormancy release. In this 3-year study, we applied four different commercial plant growth regulators to the Flopría apricot cultivar. Two of them, Broston® and Erger® were the most effective ones to advance endodormancy release. The physiology and biochemistry behind these treatments were studied by a non-target metabolomic and expression analysis in flower buds. Metabolic groups, like phospholipids, only varied in treated samples, whereas others like by-products of L-Phe metabolism, or ABA significantly varied in both types of samples throughout endodormancy release. Finally, to validate these results, solutions of phospholipids, phenylpropanoids, or ABA, among others, were applied for the first time to apricot trees, showing, i.e., that phospholipids treated-trees released from endodormancy two weeks earlier than control. This study aims to be an initial stage for the elaboration of environmentally safe regulators in apricot, with a potential in other *Prunus* and temperate fruit tree species.

**Keywords** Apricot · Endodormancy release · Phospholipids · Gene expression · Metabolomics · Plant growth regulators · UPLC-QToF

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## Introduction

Worldwide 15 million tons of apricot (*Prunus armeniaca* L.) are produced per year, with a high commercial significance for agriculture in the Mediterranean regions, such as Turkey, Italy, Spain, etc. This production is being endangered by the decrease of chill during winter necessary for a correct endodormancy release, and a direct consequence of global warming (Luedeling et al. 2009; Campoy et al. 2019). Endodormancy in *Prunus* species like apricot is considered a survival mechanism and required phase, because of two main reasons: (1) harsh low temperatures can only be handled when metabolism is slowed down and no growth occurs and (2) flowering and correct fruit set depends directly on endodormancy release, which relies on cold weather conditions (Sánchez-Pérez et al. 2012). Therefore, the accumulation of a certain amount of chill, named chill requirements (CR), is mandatory for a correct endodormancy release and depends on the species and cultivar, which means that is also genetically inherited (Campoy et al. 2011; Ruiz et al. 2007; Sánchez-Pérez et al. 2012).

In order to mitigate the effects of global warming, farmers have, for more than 30 years, been applying different agrochemicals or plant growth regulators (PGRs), such as Dormex® (cyanamide) or Dropp® (thidiazuron) to activate endodormancy release, synchronize flowering time, and recover to some extent the normal yield expected (Ferreira et al. 2019b; Guillamón et al. 2022; Ionescu et al. 2017a, b). The majority of these PGRs are based on high organic nitrogen-content solutions (Guillamón et al. 2022) and, for some of them, their mechanism of action is mostly still unknown, despite being used for more than three decades. On top of that, over the past few years, the toxicity demonstrated by some of agents, like Dormex® towards farmers, and to the environment has led to their ban in many countries (Sheshadri et al. 2011). Owing to this fact, alternative PGRs for promoting endodormancy release with similar mechanisms of action, like Erger® + Activ Erger® or Syncron® + NitroActive®, are being tested with the aim of palliating the effects of low-chill accumulation during winter in temperate regions. Nevertheless, these new PGRs are not as effective as required for guaranteeing proper endodormancy release and flowering time, synchronizing harvest and keeping the yield (Ardiles and Ayala 2017). Moreover, given their mechanism of action, the application of these PGRs presents a high difficulty, since any mistake in their application time or concentration can lead to an extreme increase of ROS in the plant, causing phytotoxicity (Guillamón et al. 2022).

Regarding molecular and biochemical studies, endodormancy release has always been described as a complex physiological process from both the genetic and metabolic point of view (Guillamón et al. 2020; Prudencio et al. 2020). The phytohormone abscisic acid (ABA) is well known for its key role in the endodormancy control in species like Japanese pear (*Pyrus pyrifolia* [Burm.f.] Nakai) and *Populus* species, among others (Howe et al. 2015; Ito et al. 2019). A drop of this metabolite is necessary for releasing endodormancy.

As a key phytohormone, many other metabolites depend on the ABA concentration. For example, in almond and sweet cherry, an increase of the cyanogenic glucoside prunasin, coupled with a drop of ABA levels was detected during endodormancy release (Guillamón et al. 2020; Ionescu et al. 2017a, b). Moreover, previous works in sweet cherry have unveiled that ROS levels increased during the early and mid-stages of endodormancy, decreasing prior to endodormancy release (Takemura et al. 2015). Antioxidant metabolites like ascorbic acid have also been described as the responsible of the ROS decrease in species like almond and sweet cherry (Baldermann et al. 2018a; Guillamón et al. 2020).

Furthermore, in grapevine (*Vitis vinifera* L.), Dormex® treatment causes an ABA drop during the late endodormancy (Zheng et al. 2015). Indeed, previous studies in Japanese pear unveiled the importance of redox metabolism in this physiological process. Accordingly, in sweet cherry and almond, the role of ascorbic acid was reported as being a key antioxidant compound, decreasing the levels of reactive oxygen species (ROS) before endodormancy release (Baldermann et al. 2018a; Guillamón et al. 2020). In addition, an increase of prunasin has been detected in almond and sweet cherry some days after treating branches with Dormex® (Del Cueto et al. 2017), as well as in almond samples during endodormancy release (Guillamón et al. 2020). In kiwi flower buds [*Actinidia deliciosa* (A.Chev.) C.F.Liang & A.R.Ferguson], the application of Erger® + Activ Erger® displayed a significant gene expression drop related to many physiological processes such as seed germination and abiotic stress during endodormancy release (Hoeberichts et al. 2017).

Despite the works mentioned above, the biochemistry insights of endodormancy release in apricot have not been focused on the metabolic rearrangements caused by the promotion of endodormancy release. In addition, apricot is a recalcitrant species, whose genetic transformation has not been replicable through different experiments (Ricci et al. 2020). This fact limits the number of genetic validations that could be carried out in this species. The discovery and validation of the main pathways involved in the promotion of endodormancy release would produce a great advance guiding the discovery of new targets for modulating this process. These new targets may be the key for the formulation of new environment-friendly PGRs that may deal with the effects of low-chill accumulation and global warming with

no associated toxicity, saving not only the global stone fruit production but also other economically important species like grapevine, kiwi, apple, among others. In addition, the knowledge gained from this work could also give light to the dormancy release in deciduous woody plants like poplar (*Populus* sp.)

For these reasons, in this research, we have studied the effects on phenology, metabolism, and gene expression caused by the application of Erger® + Activ Erger®, Broston® + NitroActive®, Sitofex, or a mix of nitrates in apricot flower buds from endo- to ecodormancy period. The non-target metabolomic analysis showed a great number of individual metabolites and metabolic pathways that significantly varied during endodormancy release. Last but not least, we validated these biochemistry pathways as candidate targets for modulating endodormancy release through the application of pure standards of metabolites from these pathways. To the best of our knowledge, this is the first time that natural compounds have been successfully used to modulate endodormancy release in a temperate fruit species like apricot.

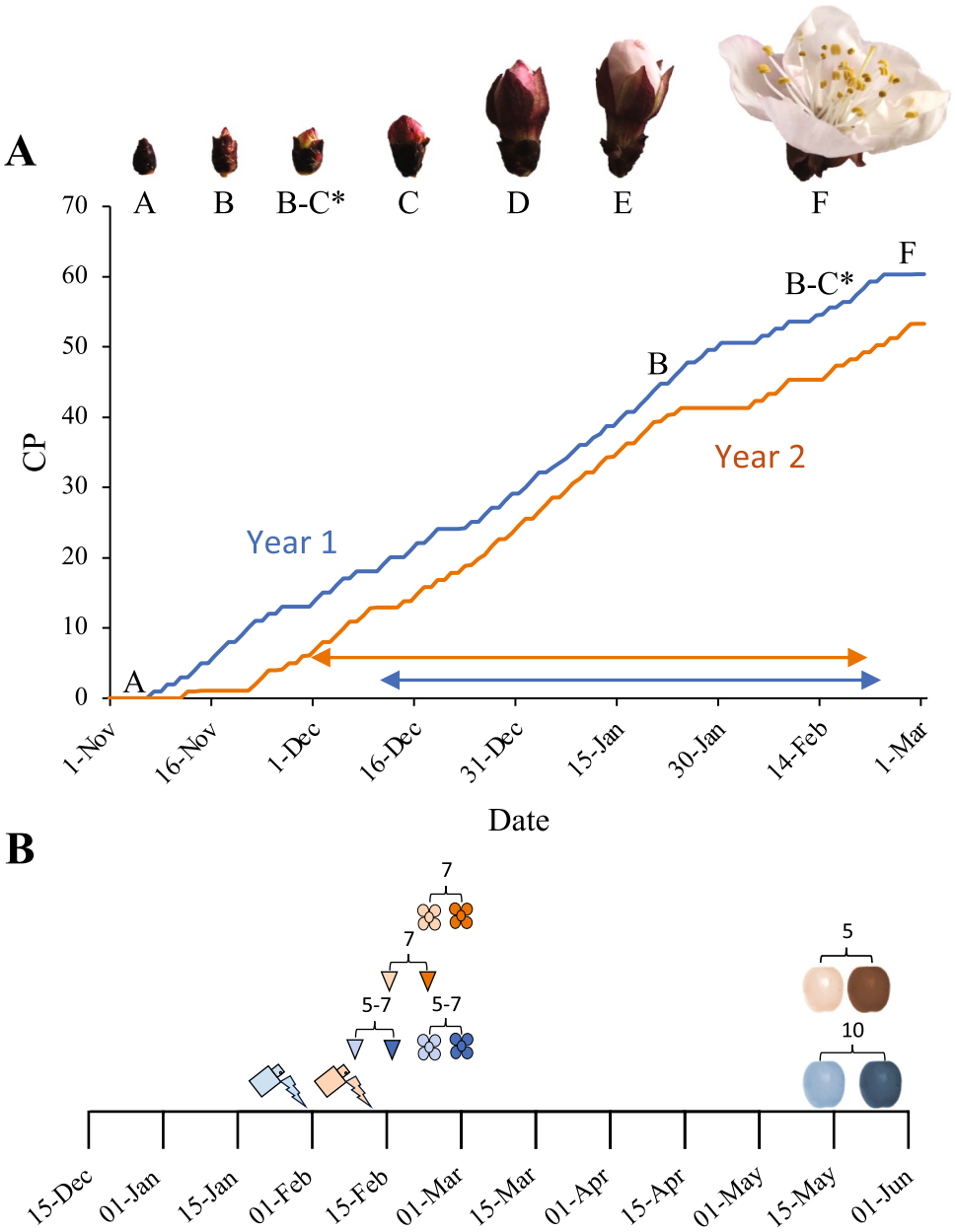



**Fig. 1** Set-up of the research. **A** Chill Portions (CP) accumulated during the three years of study: year 1 (blue line, 2019/2020) and year 2 (orange line, 2020/2021). The sampling period is marked with an horizontal double-arrow line. Flower bud phenological scale (A, B, B-C, C, D, E and F) is disposed at the top of the figure. Phenological states according to date are labeled in the chill accumulation chart. Asterisk means the phenological stage where endodormancy is released. **B** Timeline of the events, after the plant growth regulator treatments, are indicated by a light blue or orange spray symbol (year 1 and year 2, respectively). Treatment B (TB) was performed with Broston® + NitroActive®, treatment E (TE) with Erger® + Activ Erger®, treatment S (TS), with Sitofex® + mineral oil and treatment N (TN), by a mix of nitrates + mineral oil. Light blue/orange colors indicate the dates of TB and TE experiments, while dark blue/orange colors indicate the control, TN and TS dates, as these last two treatments did not work. The traits measured were: endodormancy release (endoR), flowering time and harvest date, shown by a triangle, flower or by an apricot, respectively. For each trait measured, numbers above the square bracket indicates the days between the successfully treated (with TB or TE) and control trees

## Results

### Chill accumulation, endodormancy release, and flowering time determination in response to the plant growth regulators treatments

During two years, from the deep endodormancy to flowering time, chill accumulation was calculated in chill portions (CP) (Fig. 1A). In parallel, every week, the efficiency of each treatment was determined by calculating the percentage of bud break after 10 days in the growth chamber. Remarkable differences in the CPs necessary to reach 50% of bud break (B–C stage) were only observed in two out of the four treatments applied (Fig. 1A). Broston® + NitroActive® − treated (TB) flower buds reached endodormancy release seven days earlier (53 and 46 CP) than the control (56 and 50 CP) in both years of study, whereas Erger® + Activ Erger® − treated (TE) flower buds advanced five and seven days (54 and 46 CP) the endodormancy release in the first and second year, respectively, when compared to the control. On the contrary, no differences were observed between TS (Sitofex®) and TN (mix of nitrates)-treated flower buds and the control.

The effects of the treatments also changed the flowering time. In the first year, TE and TB advanced 5 and 7 days flowering time, respectively, while they both produced a 7-day advance in the second year (Fig. 1B). Finally, the maturation time was also modified by the TB and TE, especially in the first year of study (Fig. 1B).

### Metabolomic profiling

To monitor the TB and TE effects on the whole metabolome, a non-target metabolomic analysis was conducted from the samples where the treatments advanced the endodormancy release at least a week. This means that TB samples were analyzed from both years (TB1 and TB2), whereas TE samples analyzed were only analyzed the second year (TE2). Overall, a total of 6958 features with a signal-to-noise ratio of > 3 for both ESI (+) and ESI (−) was obtained from the three groups of study (TB, TE and control). In the first year, when compared deep endodormant (stage A) with endodormancy release (stage B–C50, when 50% of flower buds are in stage B–C) samples, the statistical analysis remarked the significant variation of 16 metabolites in the control and 131 in the TB group. Similarly, 49 metabolites significantly varied in the control, 53 in the TB, and 56 in TE groups throughout the second year of study (Supplementary tables S1 and S3).

The Partial Least-Square Discriminant Analysis model (PLS-DA) created with the raw data from the two years showed how similar or dissimilar the different groups of study were at the different stages. For example, control deep endodormant groups (Cendo1 and Cendo2), showed high similarity, but displayed a considerable separation during endodormancy release (CendoR1 and CendoR2), as well as with both endodormant groups (Cendo1 and Cendo2) (Fig. 2A). These differences between years and the endodormancy stage were even more pronounced during the endodormancy release of all treated groups (TBendoR1, TBendoR2, and TEendoR2), which showed a huge gap with the rest of the groups (Fig. 2A). The explained variance of the model $R^2$ was 0.83, the prediction of the model $Q^2$ was 0.81, and accuracy was 0.88.

### Pathway and enrichment analysis

To summarize all the significant metabolic differences involved in the endodormancy release process observed in the non-target metabolomic study, we carried out a pathway and enrichment analysis. In the first one, the glycerophospholipid metabolism, phenylpropanoid, amino acid, and carotenoid biosynthesis showed the lowest *p* value and the highest pathway impact, being the most relevant pathways in the two years of study (Fig. 2B). In this sense, the enrichment analysis showed that phospholipids and hydroxyflavonoids were the two most over-represented groups in the non-target metabolomic study (Supplementary figure S1). Based on these analyses, we are going to show the main results of the following pathways.

### By-products of L-Phe and L-Tyr

Phenylpropanoids are produced using L-Phe and L-Tyr as precursors. The increase of phenylpropanoid species has been tightly associated with the endodormancy release process in many species of the Rosaceae family and the *Populus* genus (Conrad et al. 2019; Howe et al. 2015). In our work, most studied phenylpropanoids significantly increased throughout endodormancy release in all studied groups (FC > 2) (Supplementary figures S2–S6). Among all of them, coumaric acid and its by-product, tricoumaroyl spermidine, showed the most significant rise in all the studied groups during both years of study (Fig. 3A). On the contrary, phenylpropanoids like N1,N10-dicoumaroylspermidine, just increased in certain sample groups, whereas a few complex species, such as 3-caffeoyl-4-feruloylquinic acid, decreased in treated groups from the first year of study (Supplementary table S1).

In order to understand the molecular basis behind these pathway changes, we analyzed the behavior of *4-coumarate-CoA ligase 1* (*4CL1*), a gene involved in the biosynthesis of complex phenylpropanoids (Fig. 3B). An up-regulation

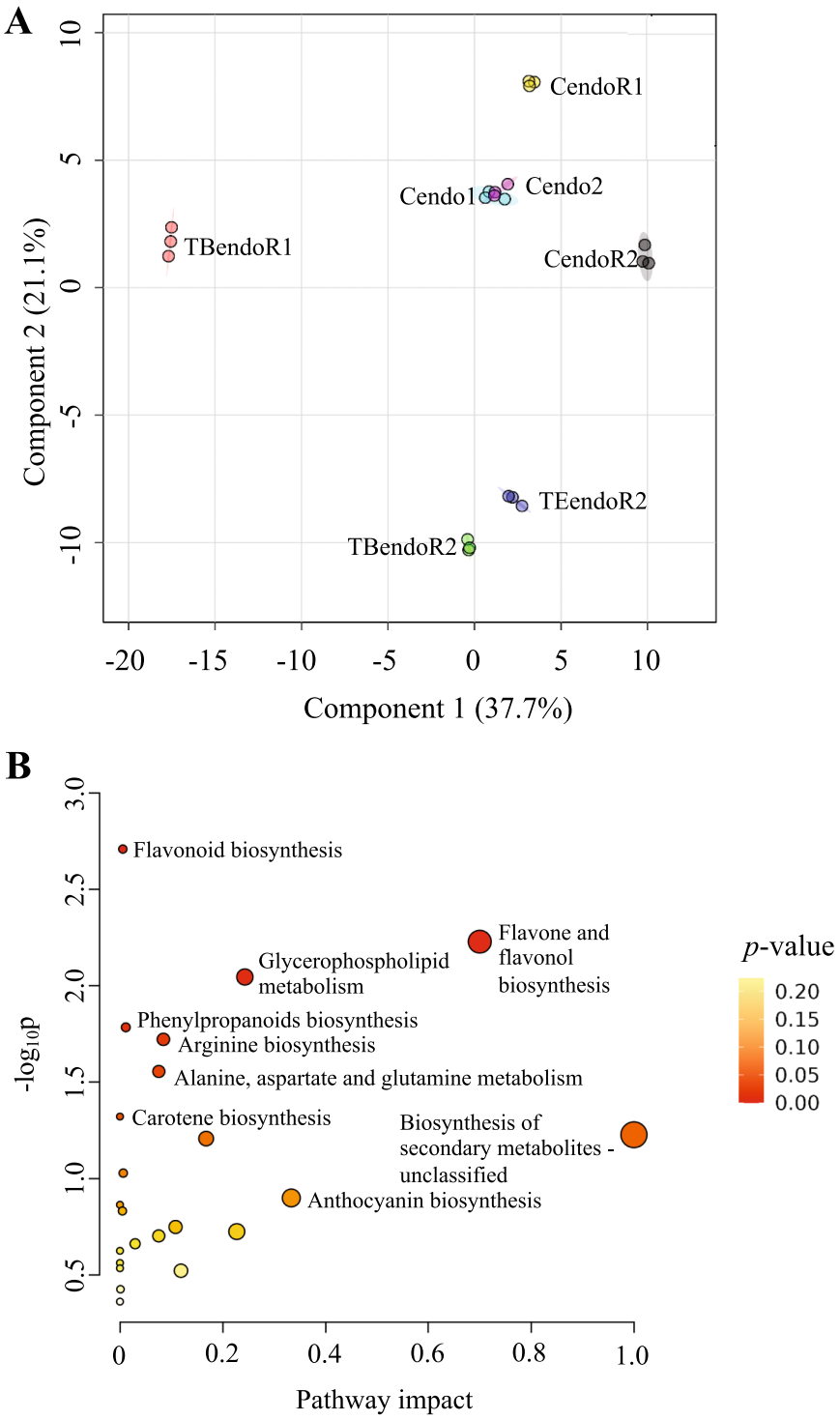
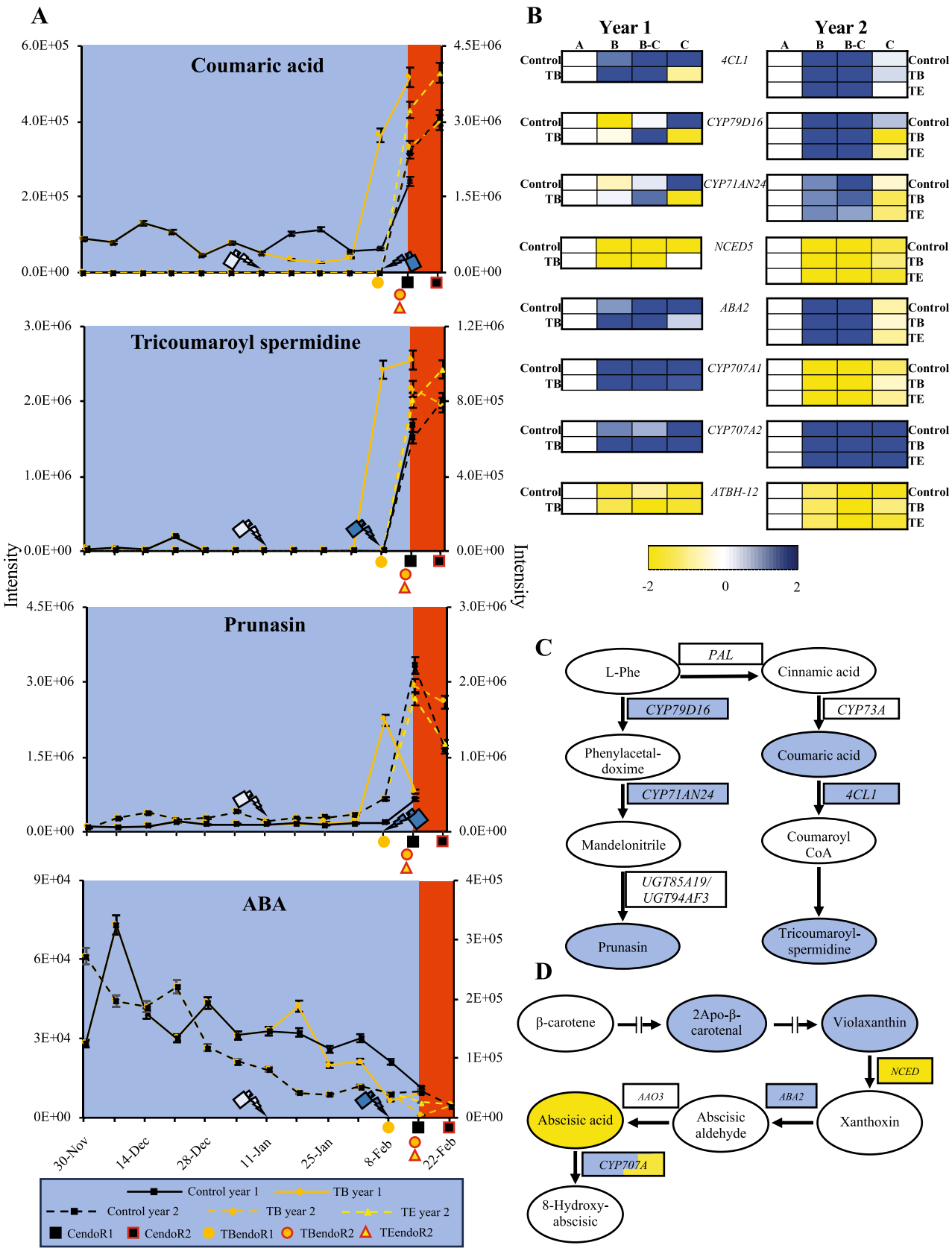



**Fig. 2** **A** PLS-DA gathering the two years of study. In this model, we can observe the differences between treated with Broston® + Nitroactive® (TB), with Erger® + Activ Erger® (TE) and control samples. The groups were classified as endodormant control samples from years 1 and 2 (Cendo1 and Cendo2), endodormancy release control samples (CendoR1 and CendoR2), endodormancy release samples treated with B (TBendoR1 and TBendoR2) and endodormancy release samples treated with E only in year 1 (TEendoR1). The explained variance of the model R2 was 0.83, the prediction of the model Q2 was 0.81 and the accuracy of the model was 0.88. **B** Pathway analysis of the two years of study. Node size indicates the impact of each pathway. Node color from white to red indicates the significance *p* value of each pathway. These charts were created using the data from three biological replicates

of this gene in all groups from both years was detected right before and during endodormancy release (stage B-C), decreasing in the late ecodormancy (stage C) (Fig. 3B).

Another class of natural products derived from amino acids is the cyanogenic glucosides. L-Phe is the precursor for prunasin and amygdalin, the most common cyanogenic glucosides present in *Prunus spp.* (Thodberg et al. 2018). A deep association with prunasin and endodormancy release has been previously observed in species from this genus, like almond and sweet cherry (Del Cueto et al. 2017; Guillamón et al. 2020). In our work, prunasin significantly increased during endodormancy release in every group from both


A
Coumaric acid
Tricoumaroyl spermidine
Prunasin
ABA
Intensity
30-Nov
14-Dec
28-Dec
11-Jan
25-Jan
8-Feb
22-Feb
Control year 1
TB year 1
Control year 2
TB year 2
TE year 2
CendoR1
CendoR2
TBendoR1
TBendoR2
TEendoR2
B
Year 1
Year 2
A
B
B-C
C
Control
TB
TE
4CL1
CYP79D16
CYP71AN24
NCED5
ABA2
CYP707A1
CYP707A2
ATBH-12
-2
0
2
C
L-Phe
PAL
Cinnamic acid
CYP79D16
CYP73A
Phenylacetal-doxime
Coumaric acid
CYP71AN24
4CL1
Mandelonitrile
Coumaroyl CoA
UGT85A19/ UGT94AF3
Prunasin
Tricoumaroyl-spermidine
D
β-carotene
2Apo-β-carotenal
Violaxanthin
NCED
AAO3
ABA2
Abscisic acid
Abscisic aldehyde
Xanthoxin
CYP707A
8-Hydroxy-abscisic

◂**Fig. 3** **A** Coumaric acid, tricoumaroyl spermidine, prunasin and ABA intensity variations during endodormancy release in control (black squares), Broston® + NitroActive® (B, orange circles) and Erger® + Active Erger® (E, yellow triangles) groups. First (left y axes) and second (right y axes) year intensities are represented by continuous and discontinuous lines and first and second year treatments by light and dark blue sprays, respectively. Endodormancy release (EndoR) date is marked for each treatment with the same marker as the displayed in the chart. When red border is present, the EndoR dates correspond to the second year of study. The background color in the chart indicates the endodormancy (in blue) and ecodormancy (in orange) period for the control group in year 1, as an example. These charts were created using the mean value from three biological replicates. Error bars represent the standard error. **B** Heatmap of the relative expressions of the studied genes in both years. A (deep endodormancy), B (medium endodormancy), B-C (endodormancy release) and C (ecodormancy). *4-Coumarate ligase 1* (*4CL1*), *Cytochrome P450 79D16* (*CYP79D16*), *Cytochrome P450 71AN24* (*CYP71AN24*), *Xanthoxin dehydrogenase* (*ABA2*), *9-cis-Epoxycarotenoid dioxygenase* (*NCED5*), *HomeoBox-Leucine Zipper Protein ATHB-12* (*ATBH-12*), *Cytochrome P450 707A1* (*CYP707A1*) and *Cytochrome P450 707A2* (*CYP707A2*). **C** Phenylalanine metabolism. Metabolites are represented by ovals and genes by rectangles. Blue indicates an increase and white indicates no significant variation observed. PAL: phenylalanine ammonia lyase. **D** Abscisic acid biosynthetic pathway. Yellow color indicates a decrease in the gene expression or metabolite concentration. *AAO3* Abscisic aldehyde oxidase 3. In case of *CYP707A*, as one of its isoforms exhibited an upregulation in the first year and a significant downregulation in the second year, it is colored in both blue and yellow

years of study (FC > 2), being more pronounced in treated groups (FC > 5) (Fig. 3A).

With the aim of studying the biosynthetic pathway of prunasin, and due to the difficulty in detecting some of the intermediates because of metabolic channeling or turn-over, we decided to study some of the genes involved in their biosynthetic pathway (Table 1). The first and second genes from this pathway, named *CYP79D16* and *CYP71AN24*, are two cytochrome P450 monooxygenases responsible for the conversion of L-Phe to mandelonitrile (Thodberg et al. 2018). Both genes presented similar trends in all treated samples, peaking during endodormancy release (stage B-C) in both years of study. This trend was also followed by control samples only in the second year, but with less intensity, whereas control samples from the first year peaked during ecodormancy (stage C) (Fig. 3B).

## ABA metabolism

The phytohormone ABA has been described as one of the most important metabolites involved in the endodormancy release process (Li et al. 2021). In our study, ABA showed a significant and pronounced decrease throughout endodormancy release in all the studied groups from both years (Fig. 3A, Suppl. Figures S2–S6). Consequently, the concentration of ABA precursors like violaxanthin and 2'-apo-beta-carotenal increased during endodormancy release in TB samples from the first year and in TE samples from the second year, respectively (Supplementary tables S1 and S3).

Given the great variations found in this group (FC > 3) and the relevance of ABA in the endodormancy release process, we decided to study some of the genes involved in the biosynthesis, catabolization, and response to ABA. The *9-CIS-EPOXYCAROTENOID DIOXYGENASE* (*NCED5*) and *XANTHOXIN DEHYDROGENASE* (*ABA2*) genes are both related to ABA biosynthesis. *NCED5* displayed a significant drop during the entire period analyzed in all groups from both years (Fig. 3B). Parallelly, *ABA2* showed a huge increase during the late endodormancy (stage B) and endodormancy release (stage B-C) in both years of study, decreasing throughout ecodormancy in all samples, but for the control group from the first year, in which its expression peaked during ecodormancy (Fig. 3B). In terms of ABA catabolism, we analyzed the expression pattern of *CYP707A1* and *CYP707A2* genes, involved in the abscisic acid to 8-hydroxi abscisic acid step. The *CYP707A2* gene showed a steep increase during late endodormancy (stage B) and endodormancy release (stage B–C) peaking in ecodormancy (stage C) in all the studied groups from both years (Fig. 3B). On the contrary, the *CYP707A1* gene exhibited a huge difference between both years, following the same trend as *CYP707A2* gene in the first year of study, while it suffered a great downregulation in the second (Fig. 3B). Regarding genes from the ABA response, *Arabidopsis thaliana* homeobox 12 (*ATBH-12*) gene exhibited the same trend as *NCED5* and ABA, decreasing during the late endodormancy and endodormancy release in all studied groups from both years (Fig. 3B).

## Antioxidant species during endodormancy release

ROS levels play a crucial role during the whole endodormancy phase, being extremely important for its release (Takemura et al. 2015). During this physiological process, the metabolomic analysis showed a great increase of several ascorbic acid precursors, such as spermidine and spermidine by-products. Among all of them, tricoumaroyl spermidine, which is a precursor of both ascorbic acid and simple phenylpropanoids, showed an extremely significant rise in all studied groups from both years of study (Supplementary tables S1 and S3).

Flavonoids are a metabolic group composed by several heterogeneous subgroups. In our study, they could be detected among the top 25 enriched metabolic groups (Supplementary figure S1), with one of the highest enrichment ratio. Indeed, most flavonols and flavones that significantly varied during endodormancy release increased during the first year in all the studied groups (Supplementary table S1). In the second year, flavonols exhibited significant increases or decreases depending on each particular metabolite. On the

**Table 1** Primer features of the genes analyzed by RT-qPCR

| Gene | Forward primer | Reverse primer | Amplicon size (pb) | Melting temperature (°C) | Efficiency (%) | $R^2$ |
|---|---|---|---|---|---|---|
| *4-Coumarate ligase 1 (4CL1)* | GTTCAGCAGGAGCCACTT | ACAAGAATGGATGGCTTCA | 123 | F: 59.10; R: 56.00 | 90.38 | 0.97 |
| *Glutathionylspermidine synthase (GSS)* | TCCTCAGCCCTCCCAACTTC | CGGTTGGTTGCTGGACATTTT | 118 | F: 61.40; R: 68.84 | 84.94 | 0.99 |
| *CYP707A1* | TCCCATCTGTTCAAGCCAACC | TGAAGGCACGGAGGACAAG | 120 | F: 60.58; R: 60.11 | 81.33 | 0.98 |
| *CYP707A2* | ATGTTTGCCGCTCAGGAC | TGGCCTTCTGCTCAATCTGG | 105 | F: 60.61; R: 60.22 | 103.88 | 0.99 |
| *CYP707A4* | AGGTTGTGTTGGAGAGTTTGAG | GGCATCACCTTCCAACCTTT | 114 | F: 60.29; R: 60.29 | 84.05 | 0.99 |
| *Xanthoxin dehydrogenase (ABA2)* | GAGTTGGGAGTGCATGGCAT | CAGCCGTCATATCCATTTTCCC | 111 | F: 62.94; R: 61.04 | 86.87 | 0.99 |
| *9-CIS-EPOXYCAROTENOID DIOXYGENASE (NCED5)* | CTTTGGGGTGTGTTTTTCT | CTCTTCCTCCTCTTCTTCT | 80 | F: 55.64; R: 56.00 | 118.53 | 0.99 |
| *HOMEOBOX-LEUCINE ZIPPER PROTEIN ATHB-12* gene (*ATBH-12*) | CTGCAACTGAATAACCAAGG | TACAGCATACTCAGAGCC | 90 | F: 56.01; R: 54.84 | 107.85 | 0.99 |
| *CYP79D16* | ATGAAGCGTTATTGGCACC | TTATGTCTGGTACACGTGAGCTGGC | 90 | F: 60.00; R: 60.00 | 84.05 | 0.99 |
| *CYP71AN24* | CACCATGGCTCTTCTAACACTT | TCAAGGGGAGTATGGTGTTGG | 90 | F: 60.00; R: 60.00 | 86.82 | 0.99 |
| *The 60S ribosomal protein (60S)* | ATACCAGTTGAAGGATCGT | GGAATATGAGTTGCTAAGAAGG | 99 | F: 55.63; R: 54.77 | 99.06 | 0.99 |
| *Outer envelope pore protein 16 chloroplastic (OEP16)* | CCACGGACCCTTTCTAAA | TGAGCACACTTTGAAGAAG | 105 | F: 55.45; R: 55.44 | 99.14 | 0.99 |
| *Elongation factor 1-alpha (TEF2)* | GGTGTGACGATGAAGAGTGATG | TGAAGGAGAGGGAAGGTGAAAG | 129 | F: 57.00; R: 57.00 | 106.34 | 0.99 |

contrary, all the chalcones that showed significant variations steeply decreased throughout this period (Supplementary table S2). Regarding catechins, these metabolites have been described as essential antioxidant species for many physiological processes (Henning et al. 2003). Contrary to the most of the other flavonoids, these metabolites showed a significant decrease during endodormancy release in both years of study (Supplementary table S1 and S2).

## Phospholipids

Only a few works have pointed out the importance of the rearrangements in the plasma membrane during the endodormancy release process (Stillwell et al. 1990; Wang et al. 1988). However, as previously shown in Fig. 2B, phospholipids (found within the glycerophospholipid metabolism group) were one of the biological groups where more metabolites significantly varied during this physiological process. Indeed, dialcylglycerophosphoserines were the most enriched metabolic group in the enrichment analysis (Figure S1). In total, when we compared endodormancy (stage A) with endodormancy release (stage B–C), we found significant increases in six different species of phospholipids: phosphatidyl glycerols (PG), phosphatidic acids (PA), phosphatidyl cholines (PC), phosphatidyl ethanolamines (PE), phosphatidyl inositols (PI), and phosphatidyl serines (PS) (Fig. 4). Among all of them, PSs and PEs were the two groups where more metabolites varied. All these phospholipids presented significant increases in the treated groups, while they remained constant in the control groups from both years of study (Fig. 4). Glycerophosphocholine, a phospholipid precursor of, e.g., phosphatidyl choline, displayed the opposite trend to all phospholipids detected, decreasing during endodormancy release in the treated samples from the first year of the study (Supplementary table S1).

## Validation of significant metabolites identified

In order to determine the importance and the capacity of modulating endodormancy release of those metabolic pathways which metabolites significantly varied during endodormancy release, we performed a third-year experiment

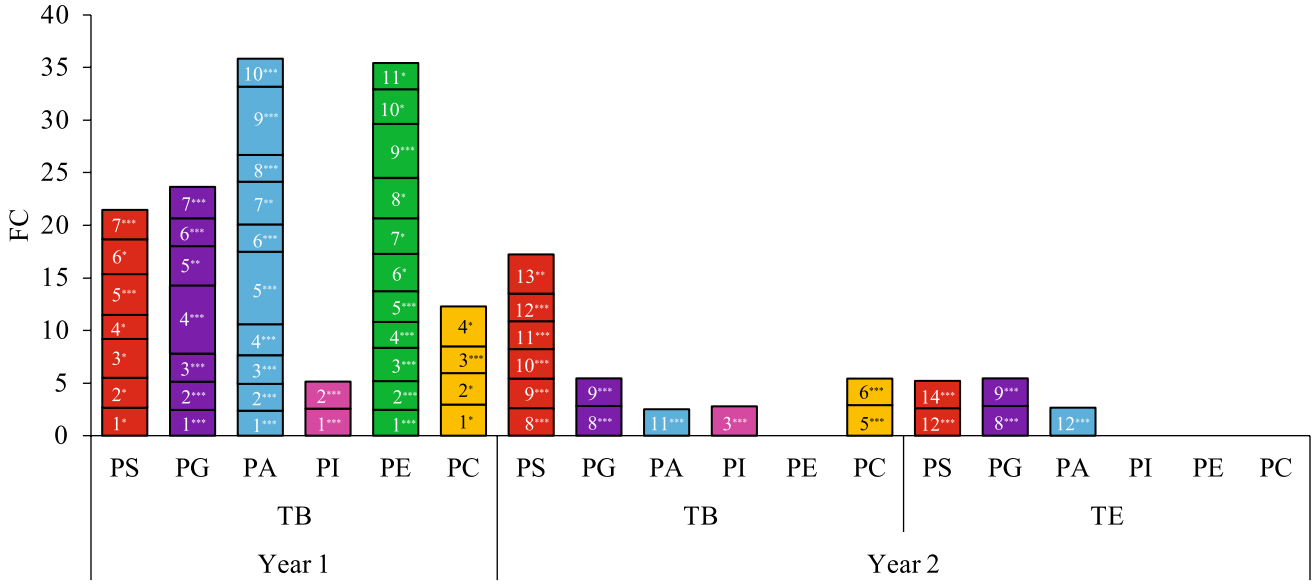



**Fig. 4** Phospholipid variation during endodormancy release. Chart of the fold changes (FC) of each phospholipid species when deep endodormancy (A stage) and endodormancy release (B–C stage) were compared in treated samples. Colored bars indicate the phospholipid specie: red for phosphatidyl serines (PS), purple for phosphatidylglycerols (PG), blue for phosphatidic acids (PA), pink for phosphatidyl inositols (PI), green for phosphatidyl ethanolamines (PE), and yellow for phosphatidyl cholines (PC). Treatment B (TB) was performed with 1% Broston®+5% NitroActive® and treatment E (TE) with 3% Erger®+5% Activ Erger®. Significant differences in the phospholipid variation were stablished through a Volcano plot based on the FC and the *p*-value. The statistical threshold was set to a FC > 2 and a *p* value < 0.05.Three different significance levels were stablished: *p* value < 0.05 indicated with an asterisk, *p* value < 0.01 indicated with two asterisks and *p* value < 0.005 indicated with three asterisks. Each phospholipid detected were labelled from 1 to 14 (e.g. PS) or from 1 to 3 (eg. PI) and so on. This labelling corresponds to the data presented in Supplementary tables S1 and S2, eg. PS1, here displayed in the bottom of the PS red bar from the first year matches with PS1 in Supplementary table S1. No significant differences in the phospholipid content were detected in control samples in the any of the two years. This chart was created using the data from three biological replicates

based on seven new treatments. The validation, done for three consecutive weeks, were completed using pure standards of phospholipids, ABA, coumaric acid, cinnamic acid, caffeic acid, L-Phe, and spermidine. The reason why we selected those standards, among all the metabolites that were found significant during endodormancy release, was a balance between the level of importance of that metabolic pathway (enrichment analyses, Fig. S1), the availability in the market and its prize. Among them, the greatest advance of endodormancy release was obtained with the phospholipid treatments, as in the field/lab they produced a three and two-week advance of endodormancy release, respectively (Fig. 5). To the best of our knowledge, this is the first time a phospholipid has been used to advance endodormancy release in a perennial plant like this fruit species.

## Discussion

Global warming is affecting stone fruit production every year. For instance, in this last winter, the extremely high temperatures, coupled with the low rainfall have ruined the production of most of the late-flowering cultivars in warm areas (Benetó et al. 2023). With the aim of palliating this, fruit breeding programs are focusing on the obtention of low-chill necessity cultivars. This is a long-time objective due to the long juvenile period (about 3–4 years) that species like apricot has, demanding more than 10 years to develop a new variety. Consequently, growers, breeders, plant physiologists, and biochemists are working together to counteract the effects by either developing new PGRs more effective and environmentally friendly than previous ones or by developing biomarkers that help the breeders and growers to know when is the best time window for the most suitable application of available PGRs. Indeed, the spray of these products would cost much less than the re-design of the orchard with a new cultivar. Furthermore, in the last years, the expansion of climate mapping in the cultivated areas, coupled with the chill accumulation monitoring and the weather prediction models facilitates the timely application of these PGRs (Do et al. 2020). In this work, we have, for two consecutive years, applied commercial PGRs to advance endodormancy release and used this as an experimental system to study the molecular mechanisms behind it in a fruit species like apricot, where knowledge is scarce.

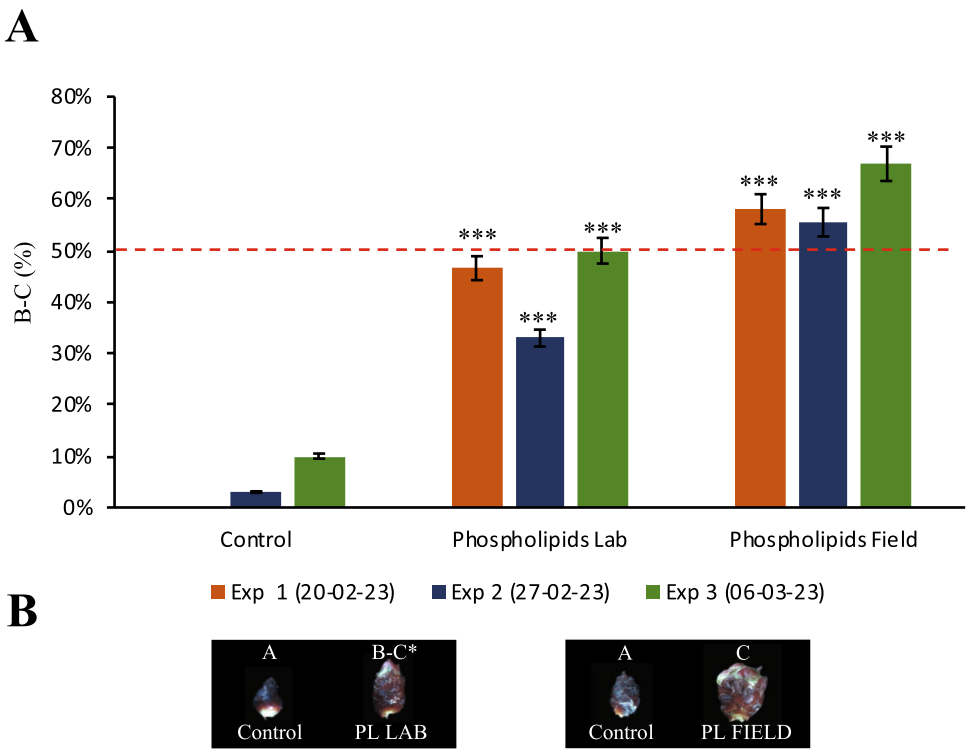



**Fig. 5** Validation of phospholipids applied in apricot flower buds. **A** Mean percentage of B-C flower buds in control and treated branches with pure phospholipids after 10 days in the growth chamber. Phospholipids applied in the field and the laboratory (lab) varied endodormancy release date with respect to control samples. Red dashed line marks the endodormancy release threshold (50% of B-C). Orange, blue and green bars correspond to experiments (Exp) 1, 2 and 3, respectively, performed for three consecutive weeks. The level of significance, calculated by a t-test with respect to the control, is marked with asterisks: one for a *p*-value of 0.05, two for 0.01 and three for 0.005. This chart was created using the data from six biological replicates, from two different trees. **B** Comparison of flower buds control (C) and treated with phospholipids in the laboratory (PL LAB), and in the field (PL FIELD). The pictures of the comparison between C, PL LAB and PL FIELD were performed on March 9th (after 10 days in the growth chamber). All pictures were taken with the same magnification. Phenological stages are labeled at the top of each flower bud. Endodormancy release is marked with an asterisk

Our results showed a seven-day advance in the endodormancy release of Broston® + NitroActive® (TB)-treated trees throughout both years of study (Fig. 1B). In the same way, Erger® + Activ Erger® (TE)-treated trees advanced endodormancy release five and seven days in the first and second year of study, respectively. This advance was even greater than that previously described in apricot, in which trees treated with the same or different PGRs (like Erger® + Activ Erger®, Syncron® + NitroActive® and Tidiazuron®) released from endodormancy four days earlier than non-treated trees (López Alcolea 2018). Moreover, we could observe considerable CR differences between both years of study, differing with the theoretical CR of this cultivar during the second year. This coincides with a previous study in almond, in which the CR from all the studied cultivars was much lower than usual throughout a warm year with a low-chill accumulation (Prudencio et al. 2018).

## Metabolomic profiling

Just a couple of works have focused in the molecular mechanisms behind the effects of the PGRs in fruit species (Hoeberichts et al. 2017; Pérez et al. 2009). For example, in grapevine, the application of PGRs with a high nitrogen concentration triggers the production of ATP—needed for endodormancy release—as a consequence of the increase of the glycolysis and fermentation rates (Pérez et al. 2009). Moreover, a study in sweet cherry explained that the application of Dormex® caused a rise of both jasmonic acid and prunasin during endodormancy release (Ionescu et al. 2017a, b). Both Erger® + Activ Erger® and Broston® + NitroActive® are mainly based on high concentrations of organic nitrogen (Table 2). However, their mechanism of action remains unknown. To address this question, we carried out a non-target metabolomic analysis coupled with gene expression analysis, with a final validation of the best metabolites significantly found with the potential of being candidate targets or by-products to

**Table 2** Composition of the most used biostimulants worldwide

| Agrochemical | Composition |
|---|---|
| Dormex® | 52% Hydrogen cyanamide |
| Erger® | 3.1% Ammoniacal nitrogen + 5.8% Nitrate nitrogen + 6.1% Urea nitrogen + 3.3% Calcium |
| Activ Erger® | 9% Nitric nitrogen + 6% Ammoniacal nitrogen + 6.5% CaO |
| Syncron® | 2% Free aminoacids + 0.3% Total nitrogen + 80% Total organic matter |
| NitroActive® | 11.5% Nitric nitrogen + 5.5% Ammoniacal nitrogen + 12.3% CaO |
| Broston® | Under trade protection (request to the authors) |
| Sitofex® | 0.12% Forchlorfenuron |
| DROPP® | 42.4% Thidiazuron |

modulate endodormancy release. The statistical analyses showed that the number of metabolites that significantly varied throughout endodormancy release was much higher in treated than in control samples. This difference was even more remarkable in the first year, probably due to the differences in chill accumulation between years (Fig. 1A). The results from the PLS-DA model displayed a huge gap between endodormancy release treated groups in the two years of study (Fig. 2A). This fact coincides with a study in sweet cherry, in which gene expression and metabolism were deeply influenced by chill accumulation over the years (Rothkegel et al. 2020). This difference was also observed in control endodormancy release group from both years. However, the gap between these groups was much lower, probably due to the PGR effect. This fact is directly associated with the number of metabolites that significantly varied in both years, which was always lower in control groups.

## L-Phe and L-Tyr metabolism

Phenylpropanoids are a complex group of natural products, formed from L-Phe and L-Tyr. A lot of research has pointed out the importance of phenylpropanoids in the endodormancy release process (Garighan et al. 2021; Guillamón et al. 2020; Leida et al. 2012a; Rothkegel et al. 2020). For example, in peach, the accumulation of phenylpropanoids is a required step to obtain endodormancy release (Leida et al. 2012a). In pear (*Pyrus communis* L.) and orchid (*Paphiopedilum callosum* [Rchb.f.] Stein), an enhance of the phenylpropanoid metabolism was observed at the genetic and epigenetic level during endodormancy release (Gao et al. 2021; Yin et al. 2022). In our work, the results from the pathway analysis showed that phenylpropanoids was one of the most impactful pathways (Fig. 2B). Indeed, coumaric acid and its derivative tri(*p*-coumaroyl) spermidine as well as the *4CL1* gene increased their content or expression level in all the studied groups from both years (Fig. 3A, B and C). This rise renders both metabolites candidate biomarkers of endodormancy release in apricot.

Regarding prunasin, this is a cyanogenic glucoside derived from L-Phe (Sanchez-Perez et al. 2008; Sánchez-Pérez et al. 2019). The importance of prunasin and its biosynthetic pathway throughout endodormancy release after the application of Dormex® was studied in sweet cherry (Ionescu et al. 2017a, b). In this previous research, both prunasin and several genes responsible of its metabolization were enhanced during endodormancy release as shown here (Fig. 3C). These results align with the detected increase of prunasin in our study (Fig. 3A). The variation of this metabolite, detected for the first time in apricot, in all our studied groups, coupled with the previous study in sweet cherry and almond suggest that this metabolite represents a valid biomarker for endodormancy release in *Prunus* species (Del Cueto et al. 2017; Guillamón et al. 2020; Ionescu et al. 2017a, b).

## Rearrangements in the ABA biosynthetic pathway

ABA is a phytohormone involved in a great number of physiological processes such as growth and stress responses (Miret et al. 2018). In many *Rosaceae* species like apple, Japanese pear, and almond, ABA is a key player, promoting endodormancy maintenance during its early stages (Guillamón et al. 2020; Yamane et al. 2019). In our study, the pathway analysis showed carotenoid biosynthesis, as part of which ABA is biosynthesized, as one of the most significant pathways during this physiological process (Fig. 2B). In the same way, Dormex® application in grapevine flower buds produced a strong up and down regulation of many ABA biosynthetic genes (Ophir et al. 2009). Recent studies in apple have pointed out that the biosynthesis of ABA should be inhibited upstream due to the *NCED* silencing by the action of small RNAs (Garighan et al. 2021). This inhibitory mechanism is in concordance with the decrease

of ABA detected in all groups from both years of study (Fig. 3A) and with the accumulation of some ABA precursors during endodormancy and endodormancy release in treated groups from the second year of study (Fig. 3D). Moreover, the down- and up-regulation of *NCED5* and *ABA2* genes respectively—both involved in ABA biosynthesis—observed in both years of study indicates that *NCED* might be the principal target of the small RNA inhibition also in apricot, causing the downregulation of genes from the ABA response like *ATBH-12* (Fig. 3B). This hypothesis still needs to be validated in further studies including small RNA analyses. Regarding ABA catabolism, the up-regulation displayed by *CYP707A1* and *CYP707A2* also aligns with the ABA drop observed in all groups of study (Fig. 3B). However, in the second year of study, the *CYP707A1* gene suffered a significant downregulation during endodormancy release in all samples. The different behavior displayed by this gene may be because of the extremely different chill accumulation between the two years of study or even from the different dynamics of chill accumulation (Fig. 1A). Indeed, in other *Prunus* like sweet cherry, a huge chill accumulation variation between years can produce significant differences in the expression of some particular genes during endodormancy release (Rothkegel et al. 2020). This fact might mean that *CYP707A1* gene may be one of these genes, whose expression is highly influenced by chill accumulation, making it suitable for monitoring whether chill accumulation has been adequate or not, whereas *CYP707A2* might be used as a biomarker of endodormancy release in apricot.

Indeed, as a phytohormone, ABA presents a close relationship with many other metabolic groups throughout endodormancy release. For example, in rice (*Oryza sativa* L.) and grapevine, the ABA–gibberellin ratio is involved in the maintenance or promotion of endodormancy through the up and downregulation of genes involved in the biosynthetic pathway of phenylpropanoids, flavonoids, as well as redox metabolism (Leida et al. 2012b; Li et al. 2021; Rubio et al. 2014; Vimont et al. 2021). Regarding *Prunus spp.*, a drop of ABA coupled with an increase of phenylpropanoid levels is a vital step in peach for the transition from endodormancy to ecodormancy. In addition, it has been observed in this species that high ABA levels cause a drop of several antioxidants, such as ascorbic acid and catechins among others, as well as the decrease of cyanogenic species like prunasin under a salt-stress scenario (Diaz-Vivancos et al. 2017). The demonstrated implication of ABA in different species suggests that these metabolites found in this study (coumaric acid and spermidine) in combination or not with the genes studied (e.g., *4CL1*) could be excellent biomarkers for monitoring endodormancy release in *Prunus*.

## Antioxidant species involved in endodormancy release

Previous studies in *Prunus* and in other species, e.g., kiwi, have unveiled the great effect that PGRs have on flowering time, fruit yield, and harvest synchronization. However, the effectiveness of these PGRs depends very closely on the concentration and the application date (Ferreira et al. 2019a; Guillamón et al. 2022; Tavares et al. 2018). Due to its molecular mechanism of action, and based on the increase of ROS levels, small variations in these parameters might cause great damages to flower buds ruining the annual yield. The importance of oxidative stress in the endodormancy release process has been widely described in species like grapevine and sweet cherry. ROS levels increase during endodormancy and decrease right before endodormancy release takes place (Baldermann et al. 2018b; Vergara et al. 2012). Indeed, as previously stated, the common mechanism of action of the PGRs is by the increase of ROS levels. A study in almond pointed towards ascorbic acid as the main antioxidant species that decrease ROS during endodormancy release (Guillamón et al. 2020). These results concord with the increases of several ascorbic acid precursors in all sample groups, such as, spermidine and tricoumaroyl spermidine (Fig. 3A).

Flavonoids are an additional class of natural products that has demonstrated its implication in the endodormancy release process in species like peony tree (*Paeonia* × *suffruticosa* Andrews). This group is divided in several subgroups, such as flavonols, flavonones, cathechins, chalcones, and anthocyanins. It has been demonstrated in peony tree that an increase in flavonoid levels is necessary to release endodormancy (Zhang et al. 2020). In our study, flavonoids presented the most significant values during the enrichment analysis (Supplementary figure S1). This fact is a consequence of the flavonol and flavone increase detected in treated samples during the first year of study, as well as the drop of catechins detected in all studied groups during endodormancy release (Supplementary Figures S2–S6). This different trend between flavonols, flavones, and catechins is in concordance with a previous study in sweet cherry, in which a drop in catechins was observed during the late endodormancy (Baldermann et al. 2018a). Like ascorbic acid, catechins have been identified as for their role as key antioxidants in many physiological processes (Henning et al. 2003). The decrease of these species during the end of endodormancy might be caused by their involvement for in diminishing the ROS levels during this period. This fact might be also correlated with the increase of the ascorbic acid precursors detected in all sample groups that would be also helping with the decrease of ROS, enabling endodormancy release.

### Phospholipids rise in treated samples

Phospholipids are a class of lipids that are essential components of cell membranes in living organisms. They are composed of two fatty acid chains, a glycerol molecule and a phosphate group (Baldermann et al. 2018a). An increase of fluidity of the plasma membrane is mandatory to gain release from endodormancy in peach (Portrat et al. 1995). The increase of phospholipids in membranes is associated with an increase of fluidity (Beauvieux et al. 2018). This is in concordance with our study, since these metabolites increased in all treated groups from both years of study (Fig. 4). The fact that these species only increased in groups where a PGR was applied indicates that the observed rearrangements in phospholipid metabolism results from the promotion of endodormancy release. Furthermore, it was observed that several phospholipid species, such as PAs, PGs, and PEs exhibited a lower increase during the second year of study. This fact is probably related with the different chill accumulation trends observed in the two studied years. Indeed, in other *Prunus* species, differences in chill accumulation affected the metabolic and genetic rearrangements throughout endodormancy release (Rothkegel et al. 2020). This hypothesis concords with a previous study in apple, in which the application of thidiazuron —a cytokinin that promotes plant growth—(Table 2) causes an increase of fatty acid levels in the membrane, as well as a significant rise of several polar lipids (Wang et al. 1988). Several studies in *Arabidopsis* pointed out that genes responsible of flowering control, such as *FLOWERING LOCUS* (*FT*), *TERMINAL FLOWER* (*TFL*), and *MOTHER OF FT and TFL* (*MFT*) present a phosphatidylethanolamine-binding domain (Colin and Jaillais 2020; Nakamura et al. 2014; Wang et al. 2015). *FT* is known for being a strong promoter of flowering, whereas *TFL* is a repressor. The expression of both genes is dependent of the circadian cycle, increasing the expression of *FT* during daytime and *TFL* during nighttime. In *Arabidopsis*, the saturation of phospholipids varies between day and night. This fact might be crucial for the specific binding of *FT* and *TFL* to PEs. Moreover, a research in this species pointed out that *FT* presents a selective binding domain to PCs, showing a much higher affinity to high saturated PCs during the day (Nakamura et al. 2014). In the same line, a study in gray poplar leaf buds (*Populus x canescens*) during endodormancy release points to PEs and PSs, as two of the main phospholipid species that significantly increase during endodormancy release (Watanabe et al. 2018). In our study, although phospholipids were detected in all sample groups, they significantly increased only in treated samples during endodormancy release, suggesting that the binding of a homolog of *FT* to PEs and PCs is enhanced in these samples. In addition, as shown in the enrichment analyses, PEs and PSs, two of the most significant species, as well as PCs, which were highly enriched, support the hypothesis of phospholipid metabolism as key player in the endodormancy release promotion (Supplementary figure S1). Given our results and the low number of previous studies pointing towards phospholipids as metabolites for modulating endodormancy release, we decided to extend our studies on the suitability of these species as candidate targets for endodormancy release modulation. The application of pure phospholipids, both in laboratory settings and in the field, resulted in a significant advancement of endodormancy release of over two weeks compared to the control trees (Fig. 5). A two-week advance in the endodormancy release date translates into a 20% decrease of the CP needed for releasing from endodormancy release. This difference is vital for ensuring endodormancy release in warm winter scenarios, where chill is poorly accumulated during the last weeks of winter. The results from these experiments identifies phospholipids as suitable targets for modulating endodormancy release. These metabolites are not associated to any kind of phytotoxicity and are, therefore, perfect candidates for the development of new environment-friendly PGRs for combating the adverse effects of climate change and global warming.

## Materials and methods

### Plant material

For this study, we analyzed the apricot cultivar Flopría located in the field "Agrícola Don Fernando" from "Frutas Esther® company" (Campotejar, Murcia, Spain; latitude: 38º 7′ 39.52'' N, longitude 1º 3′ 16.38'' W). Flopría is a late-flowering cultivar obtained by *PSB Producción Vegetal* (Spain).

### Chill accumulation

Hourly temperatures in the field were recorded by the Campotejar meteorological station during the two years of study (CR1000, *Campbell Scientific Corporation*, Canada) with the aim of measuring the chill accumulation. This was calculated using the Dynamic model (Fishman et al. 1987), which is the most accurate for mild-winter areas. On average, the chill requirements (CR) of the Flopría cultivar in Campotejar are 57 chill portions (CP).

### Plant growth regulator application

To study the metabolism behind the modulation of endodormancy release, four plant growth regulators were applied to

groups of 10 trees each (approximately 7 L per tree) using a sprayer. For two years (2019/20 and 2020/21), four PGRs were analyzed, which were made of: a) 1% Broston® + 5% NitroActive® solution (TB), b) 3% Erger® + 5% Activ Erger® (TE), c) Sitofex® 1000 ppm + 3% mineral oil solution (TS), and d) 2.5% $KNO_3$ + 2.5% $Ca(NO_3)_2$ + 3% mineral oil (TN). Based on our previous experience in the field, in which we tested different application times, all PGRs were applied when 85% of the theoretical CR of the cultivar were fulfilled (most efficient application time). This was January 28 in the first year and February 12 in the second year.

## Endodormancy release monitory

In order to evaluate the effectivity of the PGRs applied, the endodormancy release of all treated groups, as well as, control trees was monitored. For every group, three twigs from three trees (nine in total) containing flower and vegetative buds (30 cm long and 5 mm in diameter) were picked weekly and placed in a growth chamber under controlled conditions (23 ± 1 °C, RH 70% during the 16 h of light and 18 ± 1 °C, RH 70% during the dark period) (Ruiz et al. 2007). Endodormancy release was established when the 50% of the flower buds reached the phenological stage B–C according to Baggiolini (Baggiolini 1952).

## UHPLC-QToF-MS/MS analysis

From early December to the late February, flower buds from all the studied groups (1 g × 3 biological replicates per group) were weekly collected and only processed where PGRs demonstrated the highest efficiency (at least a 7-day advance of endodormancy release). In total, 23 samples (3 biological replicates each) were collected in the first year and 17 samples (3 biological replicates each) in the second year.

Flower buds (45–55 mg) were collected and ground to a fine powder in liquid nitrogen. The powder was mixed with 200 µl of acetonitrile:$H_2O$ (80:20) with 0.1% HCOOH (*Sigma*), mechanically shaken sonicated 3 × 30 s and centrifuged for 10 min at 13,000 × *g*. Glipizide (*Sigma*) at 0.1 µg/ml was added as the internal standard. Finally, samples were passed through a filter plate (0.22 µm *Millipore*) by centrifugation (5 min, 1107 × *g*) (Guillamón et al. 2020).

UPLC–QToF–MS/MS analyses were performed using a Waters ACQUITY UPLC I-Class System (Waters Corporation, Milford, MA, USA) coupled to a Bruker Daltonics QToF- MS mass spectrometer (maXis impact Series; 55,000 FWHM) (Bruker Daltonics, Bremen, Germany). Ionization was carried out using ESI for both positive (ESI+) and negative (ESI -) ionization modes.

The separation was performed in an HSS T3 C18 100 × 2.1-mm column with 1.8 µm of size particle (Waters Corporation, Milford, MA, USA) at a flow rate of 0.3 mL/min. Water with a 0.01% of HCOOH (pH ~ 3.20) was used as the weak mobile phase (A) (PanReac AppliChem, Barcelona, Spain), while acetonitrile with a 0.01% of HCOOH was used as strong mobile phase (B) (J. T. Baker, New Jersey, USA). The separation gradient started with a 10% of B, which was gradually increased to 90% after 14 min; it was held until minute 16 and then decreased to 10% in 10 s. Finally, it was held until minute 18. Desolvation and nebulization were performed using nitrogen with a flux of 8 L/min and a pressure of 2.0 bar. Colum and drying temperatures were set to 40 and 200 °C, respectively. The voltages for ESI (−) and ESI (+) were 4,000 and 4,500 V, respectively. The MS experiment was carried out using HR-QTOF-MS, applying 24 eV for ESI (+) and 20 eV for ESI (−) and using broadband collision-induced dissociation (bbCID). The acquisition range was set on 45–1200 Da. A KNAUER Smartline Pump 100 delivered the external calibrant solution with a pressure sensor (KNAUER, Berlin, Germany). A 10 mM sodium formate solution was used for calibrating the equipment before each sequence. The mixture was prepared by adding 0.5 mL of formic acid and 1.0 mL of 1.0 M sodium hydroxide to an isopropanol/Milli Q water solution (1:1, v/v).

## Data processing and statistical analysis in the metabolomic study

Raw intensity data from the analyzed samples were obtained using Profile Analysis 2.1 software (Bruker Daltonics, USA). These data are included in four bucket tables (Supplementary tables S3–S6).

The statistical analysis was carried out using Metaboanalyst 5.0. We preprocessed our data by performing a Pareto scaling and a logarithmic transformation. To know which features presented significant variations throughout endodormancy release, we performed a Volcano Plot based on *p* value and fold change. The statistically significant threshold was established as a logarithm of fold change (FC) > 2 and a *p* value < 0.05. We also created a Partial Least-Square Discriminant Analysis model (PLS-DA) with all the samples and features detected from the two years of study to determine whether there was any difference between endodormant and ecodormant buds, and control and treated buds. To test the robustness of the model, we carried out a cross-validation to obtain its explained variance $R^2 = 0.83$, the capability of prediction $Q^2 = 0.81$ and the accuracy of the model A = 0.88.

To conclude, the top 25 metabolites that presented the highest correlation in each group were plotted in a heatmap.

The distance between them was measured using the Pearson *r* correlation coefficient.

### Metabolite identification

The MS/MS spectrum from all the features that surpassed the statistically significant threshold was acquired and searched against the following databases: METLIN, Human Metabolome Database and Lipid Maps, obtaining a tentative structure and putative identification. After that, we classified them based on their chemical nature using the KEGG PATHWAY and the ChEBI.

### Enrichment and pathway analysis

An enrichment and pathway analyses were carried out with all the identified metabolites from both years of study by Metaboanalyst 5.0 software. Regarding the enrichment analysis, the algorithm uses a generalized linear model to compute a 'Q-stat' for each metabolite set. The Q-stat is calculated as the average of the Q values calculated for each single metabolite, while the Q value is the squared covariance between the metabolite and the outcome. In terms of the pathway analysis, this was performed applying a Fisher's exact test as the enrichment method. Moreover, the topology analysis (pathway impact) was calculated based on the relative-betweeness centrality. Given this fact, the model was able to take into account the different probabilities of the different metabolic species for appearing in our samples.

### RNA extraction

RNA was extracted from the samples (three biological replicates) taken at four different dates from endodormancy to ecodormancy: deep endodormancy (A stage, early December), PGRs application day (B stage mid-January), endodormancy release day (stage B–C, 10 days after treatment, late January) and ecodormancy (C stage, 20 days after treatment, mid-February), according to Baggiolini phenological stages (Baggiolini 1952) (Fig. 1). RNA extractions were carried out following the protocol of the *NucleoSpin RNA Plant and Fungi* kit (MACHEREY–NAGEL, Düren, Germany). After that, the quality of the RNA was measured with the $A_{260\ nm}/A_{280\ nm}$ and $A_{260\ nm}/A_{230\ nm}$ ratios using a *Nanodrop One* (Thermo Fisher Scientific, Massachusetts, USA). In order to purify our RNA from gDNA and other contaminants, we cleaned it following the protocols of *DNA-free™, DNase Treatment and Removal Reagents* kit (Ambion, Life Technologies, Thermo Fisher Scientific, Massachusetts, USA) and *RNeasy® PowerClean® Pro Clean Kit* (QIAGEN, Venlo, Netherlands).

### qRT-PCR analysis

The qRT-PCR was performed in 10 µl reactions with 5 ng of RNA from each individual sample following the protocol of the *qPCRBIO SyGreen 1-Step Go Hi-ROX* kit (BIOSYSTEMS, London, UK). The qRT-PCR was performed using the *PCR StepOnePlus* system (Applied Biosystems, Thermo Fisher Scientific, Massachusetts, USA).

Primers of the candidate genes (Table 1) identified from the metabolomic analysis were designed using *CLC Genomic Workbench 5* (QIAGEN, Venlo, Netherlands). PCR product size was established between 100 and 150 bp, and primer melting temperature (Tm) was set to 60–65 °C. The other adjustments were kept in the default mode. Only primers with an efficiency > 80% were used for qRT-PCRs. Primer efficiency was tested by the standard curve method (Table 1).

Genes encoding for *60S RIBOSOMAL PROTEIN* (*60S*), the *OUTER ENVELOPE PORE PROTEIN 16 CHLOROPLASTIC* (*OEP16*), and the *ELONGATION FACTOR 1-ALPHA* (*TEF2*) were used as housekeeping genes for the normalization of the raw data obtained from the qRT-PCR analysis. Normalized relative expression was plotted in a heatmap for its analysis.

### Validation of significant metabolites in field and controlled conditions

To validate the capability of certain metabolites to modulate endodormancy release, we performed two different experiments: one in the field and the other with a set of branches from the trees taken immediately to the laboratory in a wet paper. In the field, we applied a 5% solution of phospholipids (*Merk*, Germany) to two different trees. In the laboratory, seven groups of five branches each were treated with (a) 5% of phospholipid solution; (b) 5% of cinnamic acid solution; (c) 5% of coumaric acid solution; (d) a saturated solution of caffeic acid; (e) 5% of L-phenylalanine solution; and (f) 0.1% solution of Protone®, which consisted of a 10% ABA solution. In order to increase the solubility of phospholipids, we added 0.5% of Triton™ X-100 (*Merk*, Germany). All these treatments were performed once a week from the moment where the 85% of the CR were fulfilled until endodormancy release. The endodormancy release of branches treated with pure metabolites was evaluated in the growth chamber using the method described above (Ruiz et al. 2007).

## Conclusions

Overall, the results obtained in this work show that the application of the PGRs Broston® + NitroActive® and Erger® + Activ Erger® for the promotion of endodormancy release is an efficient solution to compensate the decrease of chill accumulation observed in the last winters. With this experimental setup, we have observed a significant enhance of the secondary metabolism. Therefore, to increase the efficiency of these PGRs, endodormancy could be monitored with the set of biomarkers identified such as coumaric acid, tricoumaroyl spermidine, prunasin, or ABA. For the first time, phospholipids have been identified and validated during endodormancy release in a fruit species like apricot. The modulation of the endodormancy release opens the door of a new set of next-generation eco-friendly PGRs, which would help combat the global warming effects, the consequence of climate change in *Prunus spp.*, in other temperate fruits or in woody deciduous plants.

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**Author contributions** R.S.-P. and F.D. conceived the original screening and research plan; J.G.G. collected the samples. J.G.G., L.A-A., and R.S.-P. performed the experiments based on a UPLC–QToF metabolomics analysis; J.G.G and L.A-A. analyzed the data. J.G.G., R.S.-P., and R.L.L.-M. designed the validation experiment. J.G.G., and R.S.-P. wrote the paper, with contributions from all the authors. R.S.-P. agrees to serve as the author responsible for contact and ensures communication.

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