Journal of Horticulture
ISSN: 2376-0354

Chmielewski et al., J Hortic 2018, 5:1
DOI: 10.4172/2376-0354.1000221

Research Article Open Access

Abscisic Acid Related Metabolites in Sweet Cherry Buds (*Prunus avium* L.)

Chmielewski FM[1]*, Baldermann S[2,3], Götz KP[1], Homann T[2], Gödeke K[4], Schumacher F[2,5], Huschek G[4] and Rawel HM[2]

[1]Agricultural Climatology, Faculty of Life Sciences, Humboldt-University of Berlin, Albrecht-Thaer-Weg 5, 14195 Berlin, Germany
[2]Institute of Nutritional Science, University of Potsdam, Arthur-Scheunert-Allee 114-116, 14558 Nuthetal, Potsdam, Germany
[3]Leibniz Institute of Vegetable and Ornamental Crops (IGZ), Theodor-Echtermeyer-Weg 1, 14979 Großbeeren, Germany
[4]IGV Institute of Grain Processing GmbH, Arthur-Scheunert-Allee 40/41, D-14558, Nuthetal OT Bergholz-Rehbrücke, Germany
[5]Department of Molecular Biology, University of Duisburg-Essen, Hufelandstr, 55, 45122 Essen, Germany

Abstract

As our climate changes, plant mechanisms involved for dormancy release become increasingly important for commercial orchards. It is generally believed that abscisic acid (ABA) is a key hormone that responds to various environmental stresses which affects bud dormancy. For this reason, a multi-year study was initiated to obtain data on plant metabolites during winter rest and ontogenetic development in sweet cherry buds (*Prunus avium* L.). In this paper, we report on metabolites involved in ABA synthesis and catabolism and its effect on bud dormancy in the years 2014/15-2016/17. In previous work, the timings of the different phases of para-, endo-, ecodormancy and ontogenetic development for cherry flower buds of the cultivar 'Summit' were determined, based on classical climate chamber experiments and changes in the bud's water content. Based on these time phases, we focused now on the different aspects of the ABA-metabolism. The results show that there is a continual synthesis of ABA about 5 weeks before leaf fall, and a degradation of ABA during ecodormancy and bud development until the phenological stage 'open cluster'. This is confirmed by relating the ABA content to that of the total precursor carotenoids, neoxanthin and violaxanthin. The tentative monitoring of individual intermediate metabolites revealed that dihydroxyphaseic acid is the most abundant catabolite of ABA and ABA glucosyl ester is in terms of mass intensity, the most abundant ABA metabolite observed in this study. The results suggest that the direct route for ABA biosynthesis from farnesyl pyrophosphate may also be relevant in cherry flower buds.

Keywords: Dormancy; Abscisic acid; Synthesis; Catabolism, *Prunus avium* L.; Flower buds

Introduction

In many European countries within the temperate latitudes, the varying timing of dormancy release poses a major obstacle to commercial agriculture of fruits and berries [1]. The transition between dormancy and flowering also represents a critical stage in the life cycle of higher plants and is therefore also an important ecological trait. Abscisic acid (ABA) is involved in a variety of plant development and environmental stress responses including dormancy and growth regulation, leaf senescence and desiccation tolerance [2-5]. ABA belongs to the class of secondary plant metabolites and can be structurally classified to the group of isoprenoids, derived from the common five-carbon (C5) precursor, isopentenyl phosphate [6]. Many studies document that ABA content and reduced seed dormancy is very closely related [2,6-8]. In this context, ABA has also been shown to be involved as one of the central regulator of bud dormancy [1]. There are some similarities, but also differences between seed and bud dormancy, suggesting that there might be different molecular mechanisms contributing to ABA synthesis during winter rest [9]. ABA deficiency and lack of its synthesis, or interference in ABA signaling pathway, may result in a dormancy loss, whereas a down regulation of ABA inactivation may cause an increased depth of dormancy [1,6,10].

ABA synthesis is effectively influenced by the preceding epoxy-carotenoid synthesis. Initially a two-step epoxidation of zeaxanthin occurs via antheraxanthin to form all-trans-violaxanthin catalyzed by zeaxanthin epoxidase (ZEP) [2]. Mutants impaired in ZEP have been shown to accumulate zeaxanthin and show a significant reduction in ABA content [6]. The next intermediately processes include the formation of cis-isomers of violaxanthin and neoxanthin. The following key regulatory step has been attributed to 9-cis-epoxycarotenoid dioxygenase (NCED) which induces a carotenoid cleavage resulting in the modulation of ABA synthesis, which in turn may affect the bud dormancy. NCED specifically catalyzes thereby the oxidative cleavage of xanthophylls, 9-cis-violaxanthin and/or 9'-cis-neoxanthin to produce xanthoxin (Figure 1A) [2]. After the cleavage of 9-cis-epoxycarotenoids, xanthoxin is converted to ABA in the cytosol and three possible pathways have been proposed via abscisic aldehyde, xanthoxic acid or abscisic alcohol (Figure 1A) [2]. For example, an overexpression of the enzyme NCED in tomato seeds was shown to induce an increased ABA level and dormancy [11]. An induction of NCED seems also sufficient to suppress germination of imbibed seeds even when conditions for a dormancy release are realized [12]. Recent studies show that besides NCED, a RING H2-type zinc-finger protein XERICO up regulation may also cause a dramatic increase in cellular ABA level and has been reported to be closely related to drought tolerance [5].

With regard to bud dormancy, ABA levels have also been discussed to increase in autumn as a signal of shorter day-length [1]. The onset of autumn causes an inactivation of metabolism which is consequently re-configured toward the acquisition of cold tolerance and the accumulation of storage compounds [13]. Light (day length/light intensity), ethylene, and ABA have been discussed as major signal elements that act consecutively to control bud development by setting,

***Corresponding author:** Chmielewski FM, Professorship of Agricultural Climatology, Agricultural Climatology, Faculty of Life Sciences, Humboldt-University of Berlin, Albrecht-Thaer-Weg 5, 14195 Berlin, Germany, Tel: +49 30 2093 46395; Fax: +49 30 2093 46488; E-mail: chmielew@agrar.hu-berlin.de

Received January 22, 2018; **Accepted** February 05, 2018; **Published** February 12, 2018

Citation: Chmielewski FM, Baldermann S, Götz KP, Homann T, Gödeke K, et al. (2018) Abscisic Acid Related Metabolites in Sweet Cherry Buds (*Prunus avium* L.). J Hortic 5: 221. doi: 10.4172/2376-0354.1000221

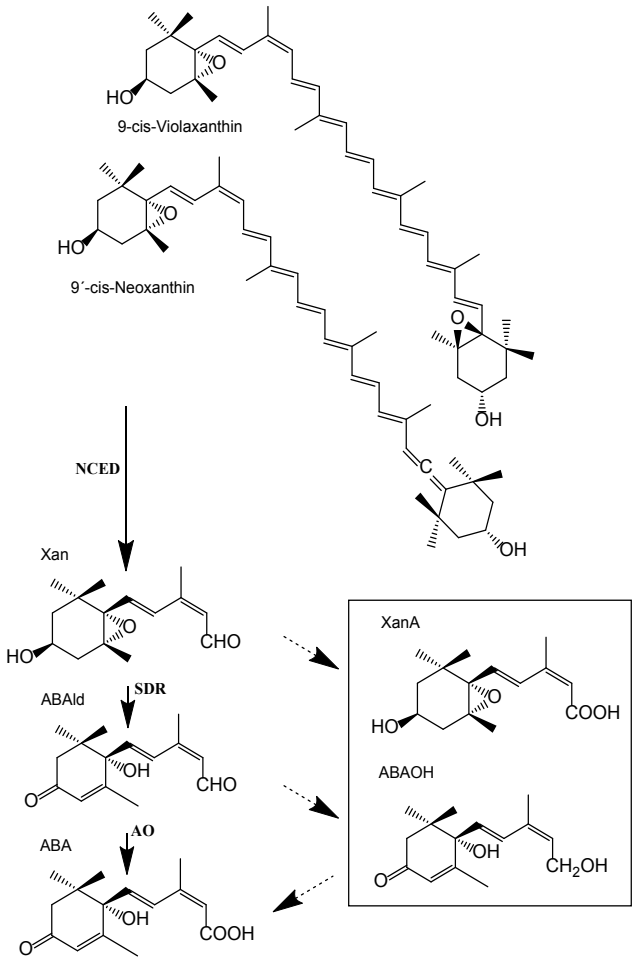

Figure 1A: Synthesis of abscisic acid adapted from Seo & Koshiba [2]. Abbreviations: ABA: abscisic acid, ABAld: abscisic aldehyde, ABAOH: abscisic alcohol, AO: aldehyde oxidase, NCED: 9-cisepoxycarotenoid dioxygenase, SDR: short-chain dehydrogenase/reductase, Xan: xanthoxin, XanA: xanthoxic acid.

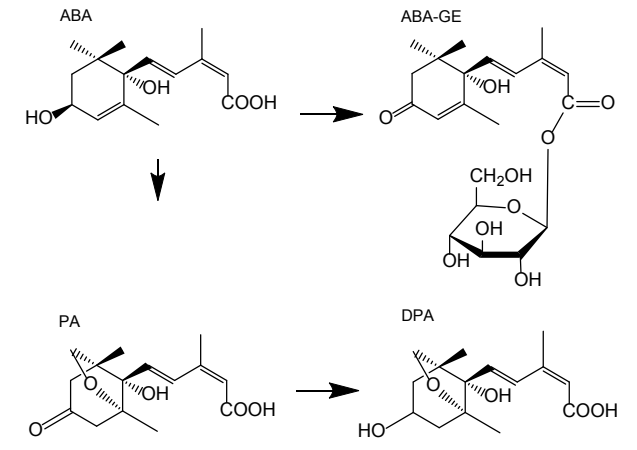

Figure 1B: Catabolism and conjugation of abscisic acid adapted from Nambara & Marion-Poll [6]. Abbreviations: ABA: abscisic acid, ABA-GE: ABA glucosyl ester, DPA: dihydroxyphaseic acid, PA: phaseic acid.

modifying, or terminating these processes [13]. The role of ABA and ABA-responsive factors in dormancy maintenance/modulation in buds has been mainly addressed in poplar trees [14]. Corresponding data on poplar buds reveal that with increasing levels of ABA, the regulators of ABA biosynthesis and ABA signal transduction components were also positively modulated [13,15,16]. Accordingly, a decrease in endogenous ABA level also preceded the consequent release of bud dormancy in birch, grapevine, and potatoes [1,17-20]. In potato, the diminishing ABA content throughout the dormancy cycle was correlated with a decreased expression of NCED [21]. Incubation of single-node cuttings of grapevine buds with exogenous ABA, resulted in a significant decrease of the bud-break percentage relative to the control population – the results testify that exogenous ABA delays bud break of dormant buds [1]. The inhibitory effect of ABA on bud burst also seems to be sensitive to seasonal changes and depends on the developmental stage of the bud as well as endogenous ABA catabolic metabolism (Figure 1B) [1]. The endogenous ABA level is modulated by the precise balance between biosynthesis and catabolism of this hormone [6]. The ABA catabolism is thought to proceed through a major pathway involving hydroxylation to 8'-hydroxy ABA by cytochrome P450, followed by spontaneous isomerization into phaseic acid (PA) and reduction to dihydrophaseic acid (DPA) [6,22-24]. On the other hand, ABA is inactivated by conjugation with sugars. ABA glucosyl ester (ABA-GE) is one of the predominant ABA conjugates [1,24,25]. ABA 8'-hydroxylase is most likely the major regulatory enzyme in many physiological processes involved in the ABA catabolism [6]. The regulation of ABA metabolism is not merely restricted to specific steps in ABA metabolism (i.e., NCED and ABA 8'-hydroxylase), but is also coordinated with the upstream metabolism (genes encoding regulatory enzymes for the 2-C-methyl-d-erythritol-4-phosphate – MEP) pathway (1-deoxy-d-xylulose 5-phosphate synthase), carotenoid biosynthesis (phytoene synthase), and xanthophyll cycle (zeaxanthin epoxidase – ZEP) [6]. The application of ABA also indicates that many biosynthetic and catabolic genes are also upregulated, suggesting that ABA might regulate its own accumulation [1,6].

In this study we focused on the different aspects of ABA-metabolism and its effect on bud dormancy. We further focused on cherry trees of the cultivar 'Summit' and here we report on data derived for the seasons 2014/15-2016/17. The cultivar 'Summit' was chosen due to the expected larger fruit size, of potentially 35 mm diameter, having attractive potential for practical cultivation resulting in high yields [26].

Material and Methods

Chemicals

Phytohormones (indol-3-acetic acid – IAA, indol-3-butryicacid – IBA, 1-naphtylacetic acid – NAA, 6-benzyladenine – BAP, gibberellic acid – GA, abscisic acid – ABA) were from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). Neoxanthin and violaxanthin were purchased from Carote Nature GmbH (Switzerland). Liquid chromatography–mass spectrometry (LC-MS) grade solvents were used for the liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. All the other chemicals used were of analytical grade (VWR, Darmstadt, Germany).

Study design and sampling

The study was carried out in the Experimental Sweet Cherry Orchard at Humboldt-University in Berlin-Dahlem (52° 28′ Northern latitude and 13° 18′ Eastern longitude). The long-term (1981–2010) average annual air temperature and precipitation are 9.9°C and 562

mm, respectively. The details of the growth conditions and sampling are recently given in Götz et al. [26].

Bud and twig sampling

Four trees in the middle of the orchard were selected to collect the buds. Sampling of 3 clusters from each tree (n=4) were taken weekly at random locations over the whole tree starting 30 September 2014 – 10 March 2015, 29 September 2015 – 22 March 2016, 04 October 2016 – 28 February 2017 and after beginning of different bud development stages at 'swollen bud' (SB), 'side green' (SG), 'green tip' (GT), 'tight cluster' (TC) until 'open cluster' (OC, BBCH 56). After cutting, clusters were immediately placed in plastic bags on ice in a polystyrene box. They were consequently frozen in liquid nitrogen and stored at -80 °C until freeze-drying. Leaf fall (LF, BBCH 97; all leaves have fallen) was determined by visual observation.

Further different trees of 'Summit' cultivar were selected for twig sampling. Since the release of endodormancy (t_1) is not easily determinable, we estimated it by observing twigs under controlled conditions, which would thus indicate the release of endodormancy (t_1) [27,28]. For this purpose. young, multi-branched twigs (length about 25 cm, diameter 5 mm) with 2-3 clusters of 'Summit' were cut weekly each year in November–December to visually observe the beginning of blossom of the cluster under controlled conditions [26,27]. After cutting, twigs were placed in demineralized water and kept in a climate chamber (RUMED, Rubarth Apparate GmbH, Germany) (air temperature ~20°C/15°C day/night, 12 h light intensity, relative humidity 70%). The chilling requirement was assumed to be sufficient if 3–4 flowers per twig opened completely on the first and the following samplings [26,27]. The first indication for the transition from the dormant stage to the beginning of ontogenetic development (t_1*) was obtained by observing the bud's water content [28]. The increase of the water content through the three was from ~50% to ~80% in the buds during the ontogenetic development and was related to a remarkable rise in air temperature from t_1* to open cluster.

Targeted analysis of phytohormones by LC-MS/MS

Freeze dried samples (25 mg, n=4 for each sampling) were extracted with 0.75 mL 60% methanol using an ultrasonification treatment (Sonorex RR 100, Bandelin electronic GmbH & Co. KG, Berlin, Germany) for 3 min followed by incubation at 4°C overnight (AEG Santo 60240 DT 28, Electrolux Hausgeräte GmbH, Nürnberg, Germany). The extracts were centrifuged at 9300 × g for 10 min at 4°C, the extraction repeated, and the supernatants pooled together and stored at -20°C until needed [26,29]. Determination of phytohormones was adapted using the high-performance liquid chromatography and tandem mass spectrometry [30-32] and has been described previously in detail [29]. Shortly, a Dionex Ultimate 3000 high performance liquid chromatography (HPLC) system (Thermo Fisher GmbH, Idstein, Germany) equipped with a Phenomenex Luna C18 column (Phenomenex Inc., Aschaffenburg, Germany; 150 × 3 mm; 3 µm) incubated at 40°C and a C18 pre-column containing the same material was used for chromatographic separation at a flow rate of 0.32 mL min^{-1}. The eluent A was composed of ultrapure water with 0.1% formic acid and eluent B consisted of methanol with 0.1% formic acid. The gradient used was as follows: 50% eluent B, 0–1 153 min; 50–75% eluent B, 1–12 min; 75% eluent B, 12–13 min; 75–50% eluent B, 13–13.1 min; 50% eluent B, 13.1–17 min. Total run time amounted to 17 min with an injection volume of 10 µL.

Concentrations of 0.01–10 mg mL^{-1} of selected phytohormones in a mixture (IAA, IBA, NAA, BAP, GA, ABA) were used for external calibration. Negative ion mass spectra of the samples were recorded in the multiple reaction monitoring (MRM) mode of LC-MS/MS using an API 4000 Q Trap mass spectrometer (Sciex Germany GmbH, Darmstadt, Germany). The ion source temperature was set at 600°C with a dwell time of 120 ms for negative ionization.

Targeted analysis of the carotenoids neoxanthin and violaxanthin by UHPLC-DAD-ToF-MS

A HPLC method using a Time-of-Flight LC/MS (ToF-LC/MS) equipped with an APCI ion source in positive ionization mode was applied as previously described [33]. Shortly, the carotenoids from each freeze dried sample (n=4 for each week) were extracted three times from 5 mg of the sample powder using 0.5 mL of Methanol/tetrahyhdrofuran solution (1:1, v/v, 3 x). The extracts were shaken at 1000 rpm for 5 min at room temperature and centrifuged at 4000 × g for 5 min at 20°C. The extracts were evaporated in a stream of nitrogen (evaporator VLM GmbH, Heideblüchenweg, Bielefeld) and then dissolved in 0.02 mL of dichloromethane and 0.08 mL of isopropanol. Prior to further analysis, the solutions were filtered through a 0.2 µm polytetrafluorethylen (PTFE) membrane and kept at 4°C in the auto sampler. The carotenoids were separated on a YMC C30 column (100 × 2.1 mm, 3 µm, YMC Co. Ltd., Japan). Methanol, methyl tert-butyl ether, and water were mixed in different volume ratios (solvent A, 81:15:4; and solvent B, 6:90:4), and utilized as mobile phases at a flow rate of 0.2 mL min^{-1}. To increase the ionization 20 mM ammonium acetate was added to the mobile phases. The separation was performed in a gradient mode from 100% A (10 min isocratic), 100% A to 80% A in 7 min (28 min isocratic), 80% A to 0% A in 10 min (5 min isocratic). Simultaneous, qualitative and quantitative characterization was performed on an Agilent 1290 Infinity UHPLC coupled to an Agilent 6230 ToF-LC/MS equipped with an atmospheric pressure chemical ionization (APCI) source in positive ionization mode. The gas temperature (325 °C) had a flow rate of 8 L min^{-1}, the vaporizer temperature was 350°C, and the nebulizer pressure was 35 psi. The voltage was set to 3500 V. The fragmentor voltage (175 V) was applied at a corona current of 6.5 µA. Identification was achieved by co-chromatography with reference substances and on the basis of the mass-to-charge ratio of the pseudo molecular ions [M – H_2O + H] + 583.415 and violaxanthin [M + H] + 601.425 [33]. External standard calibration curves with authentic standards were used for quantification by dose–response curves.

Detection of ABA metabolites by LC-MS/MS analysis

ABA-metabolites (Xanthoxin – Xan, Abscisic aldehyde – ABAld, Abscisic-alcohol – ABAOH, Xanthoxic acid – XanA, Phaseic acid – PA, Dihydroxyphaseic acid – DPA, Neo-Phaseic acid – Neo-PA, ABA glucosyl ester – ABA-GE) were monitored for the following selected development stages for the season 2014/15: Leaf fall (LF), release of endodormancy (t_1), beginning of ontogenetic development (t_1*), 'swollen bud' (SB) and 'open cluster' (OC). The extraction procedure was identical as described for the phytohormones (25 mg, n=4 for each development stage).

A 1260 Infinity LC system (Agilent Technologies, Waldbronn, Germany) coupled to a 6490 triple quadrupole MS (Agilent Technologies, Waldbronn, Germany) was used. The column (Poroshell 120 EC-C18, 3.0 × 150 mm, 2.7 µm) and pre-column (4.6 × 5.0 mm, 2.7 µm) utilized for the separations were both from Agilent Technologies. The column temperature was set at 30°C and the injection volume at 10 µL. The mobile phases were ultrapure water (eluent A) and acetonitrile

(eluent B) both containing 0.01% formic acid. A flow rate of 0.4 mL min^{-1} with following gradient was applied: 98% eluent A, 0–3 min; 98-50% eluent A, 3–15 min; 50-15% eluent A, 15–20 min; 15% eluent A, 20–25 min; 15-0% eluent A, 25–27 min; 0% eluent A; 27-30 min; 0-98% eluent A, 30-31 min; and finally re-equilibration of the column was from 31-40 min with 98% eluent A. The ion source parameters for MS were set as following: Polarity – Negative (electrospray ionization, ESI-); gas temperature –290°C; gas flow –11 L min^{-1} of nitrogen; nebulizer – 15 psi; sheath gas heater – 300°C; sheath gas flow –10 L min^{-1} of nitrogen; capillary voltage – 4000 V; nozzle voltage – 500 V. The settings for ion funnel were for high pressure RF at 150 V and for low pressure RF at 60 V. The MRM settings were partly adapted from Zheng et al. [1] and details are given in Supplementary Table S1. Since the specific standards were not commercially available to confirm the retention times, the evaluation was done on basis of the known fragmentation pattern and fold change as compared to the development stage LF.

Statistical analysis

The data were analyzed (mean, standard deviation, Tukey's multiple comparisons test, $p \leq 0.05$, n=4) using statistical software Prism 6 for Windows (Vers. 6.01; GraphPad Software, Inc.).

Results and Discussion

The timing of the different phases of para-, endo-, ecodormancy and ontogenetic development for cherry flower buds of the cultivar 'Summit' was determined as recently reported [28]. The period of paradormancy was identified as the preliminary stage of bud development, where the days become shorter and temperatures finally decreases in autumn, ultimately inducing complete LF. The period (PR-LF, PR: picking ripeness) is characterized by inability of the flower buds to bloom. In average this phenophase lasts for about 4 months and is followed by the endodormancy (LF-t_1) [28]. The date of endodormancy release (t_1) was obtained by observing the harvested twigs under controlled conditions in a climate chamber experiment (Table 1). This period generally lasts for 21-28 days as observed for the three years 2014/15 to 2016/17 (Table 2). In the following, a relatively long period of ecodormancy (t_1-t_1*; 63-98 days, Table 2) was usually witnessed, where no visible growth occurred (suppressed by the accompanying low temperatures) and is terminated by the beginning of the ontogenetic development (t_1*). This date was defined by a steady and continuous increase of the water content of the buds, which stayed stable during endo- and ecodormancy [28]. After beginning of ontogenetic development, development-related 'bud swelling' (SB), 'side green' (SG), 'green tip' (GT), 'tight cluster' (TC) and 'open cluster' (OC) dates were documented (Table 1). The duration (d) and the average temperatures for the different stages are given in Table 2. The mean air temperature decreased in the dormancy phases, reaching its lowest value (2.4°C) in the longest phenophase of ecodormancy (t_1-t_1*, mean=77 d). Thereafter, it again increased, but not homogeneously and was then mainly influenced by the annual weather variability (Table 2). In the following, the focus has been laid on the metabolism of ABA during these phases and for ABA catabolism for selected development stages.

Stage	2014/15	2015/16	2016/17
LF	322	307	313
t_1	343	328	341
t_1*	41	61	45
SB	76	89	66
SG	86	92	76
GT	92	97	82
TC	105	102	90
OC	107	105	94

Table 1: Timing of different phenological development stages from leaf fall (LF) in DOY (day of the year) for the seasons 2014/15 to 2016/17 [27]; t_1: endodormancy release, t_1*: beginning of ontogenetic development, SB: swollen bud, SG: side green, GT: green tip, TC: tight cluster, OC: open cluster.

Stage	2014/15		2015/16		2016/17		x ± s	
	D (d)	T (°C)	D (d)	T (°C)	D (d)	T (°C)	D (d)	T (°C)
LF-t_1	21	2.6	21	9.4	28	3.0	23.3 ± 4.0	5.0 ± 3.8
t_1-t_1*	63	2.8	98	3.7	70	0.6	77.0 ± 18.5	2.4 ± 1.6
t_1*-SB	35	4.1	28	4.7	21	5.8	28.0 ± 7.0	4.9 ± 0.9
SB-SG	10	6.7	3	7.4	10	5.8	7.7 ± 4.0	6.6 ± 0.8
SG-GT	6	6.6	5	12.8	6	7.6	5.7 ± 0.6	9.0 ± 3.3
GT-TC	13	8.3	5	9.2	8	9.4	8.7 ± 4.0	9.0 ± 0.6
TC-OC	2	13.5	3	11.4	4	13.7	3.0 ± 1.0	12.9 ± 1.3

Table 2: Duration (D) in days and average air temperature (T) in °C observed during the different development stages for the seasons 2014/15 to 2016/17; t_1: endodormancy release, t_1*: beginning of ontogenetic development, SB: swollen bud, SG: side green, GT: green tip, TC: tight cluster, OC: open cluster; x: mean, s: standard deviation.

Targeted phytohormone analysis

Initially, we screened for the presence of different phytohormones (IAA, IBA, NAA, BAP, GA, ABA) and the results indicated that only ABA was detectable in the investigated cherry flower bud samples under the applied conditions. The ABA content was determined on basis of the negative ion mass spectra generated and the signals recorded in the MRM mode during the HPLC analysis of the samples. The mass transition m/z 263 → 153 was utilized together with an external calibration applying an ABA standard to quantify the content. The data thus collected is given in Figure 2A-C for the seasons 2014/15 to 2016/17. The results indicate that the concentration of ABA lies between 1.25 – 8.24 µg/g DM for the season 2014/15, between 1.64 – 5.39 µg/g DM for 2015/16 and in the range of 0.43 – 3.53 µg/g DM for 2016/17. The period S1-LF (S1: first sampling date), shows a continual increase in the content of ABA for all three seasons, which is followed by plateau of maximum ABA contents characterizing the endodormancy phase (LF-t_1). The maximum contents were found at DOY 329 (2014/15), DOY 321 (2015/16) and at DOY 334 (2016/17), always being in vicinity of the t_1. The lowest values were found for the phase TC-OC of the ontogenetic development. These results confirm our findings which were based on 5 seasons 2011/12-2015/16 [34] and also the course of ABA concentration in peach buds (*Prunus persica* L.) during the dormancy phases [9]. The role of ABA in context of bud dormancy has been often implied but is not yet completely clear [9,35].

Mediation of free ABA levels through conjugation has been discussed [35], but here also very few studies have directly focused on ABA metabolism related to bud dormancy in the Prunus family. Endogenous ABA levels also increased during onset of grape bud dormancy consistent with observations made here [1]. ABA therefore seems to play a central role in modulating the endodormancy stage and its content has been reported to increase in autumn as a possible reaction to the shorter day lengths [1]. An overexpression of the enzyme NCED has been frequently associated with an increased ABA level [11] and since its increase also stimulates a carotenoid cleavage (Figure 1A) resulting in the modulation of ABA synthesis, a connection of the observed response in ABA concentrations as illustrated in Figure 2A-C was sought in the corresponding changes in the concentrations of the two relevant carotenoid precursors (neoxanthin, violaxanthin) over the development stages.

Targeted analysis of neoxanthin and violaxanthin

The results of the analysis for the seasons 2014/15 to 2016/17 are presented in Figure 3A-C. Thereafter, the content of 9'-cis neoxanthin remains relatively constant from S1 until SB for the three seasons with a mean (ng/mg DW) of 9.88 ± 0.95 for 2014/15, of 8.74 ± 0.78 for 2015/16 and of 10.58 ± 0.86 for 2016/17. With the progressed ontogenetic development of the cherry flower buds after the stage of SB, the content increases continuously to a level of 21-26 ng/mg DW at the last documented stage of OC. The determined levels of violaxanthin, the second discussed carotenoid precursor for ABA-biosynthesis (Figure 1A) are also given in Figure 3A-C. The content remains similar to neoxanthin relatively constant until SB for the three seasons with a mean (ng/mg DW) of 272 7.74 ± 2.01 for 2014/15, of 4.08 ± 0.51 for 2015/16 and of 4.31 ± 0.40 for 2016/17. The content also generally

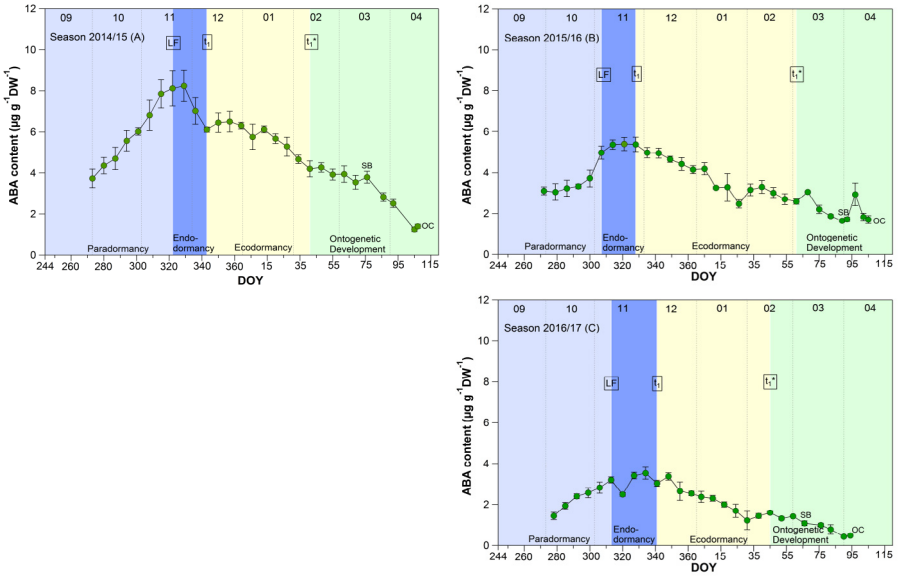

Figure 2: Weekly and development orientated changes of abscisic acid content (mean of 4 replications, error bars show the standard deviation). A: Season 2014/15, B: Season 2015/16 and C: Season 2016/17.
Abbreviations: ABA: abscisic acid, LF: leaf fall, SB: swollen bud, OC: open cluster, t_1: end of endodormancy and t_1*: beginning of ontogenetic development, according to the classic approach [28], DOY: day of year, DW: dry weight.

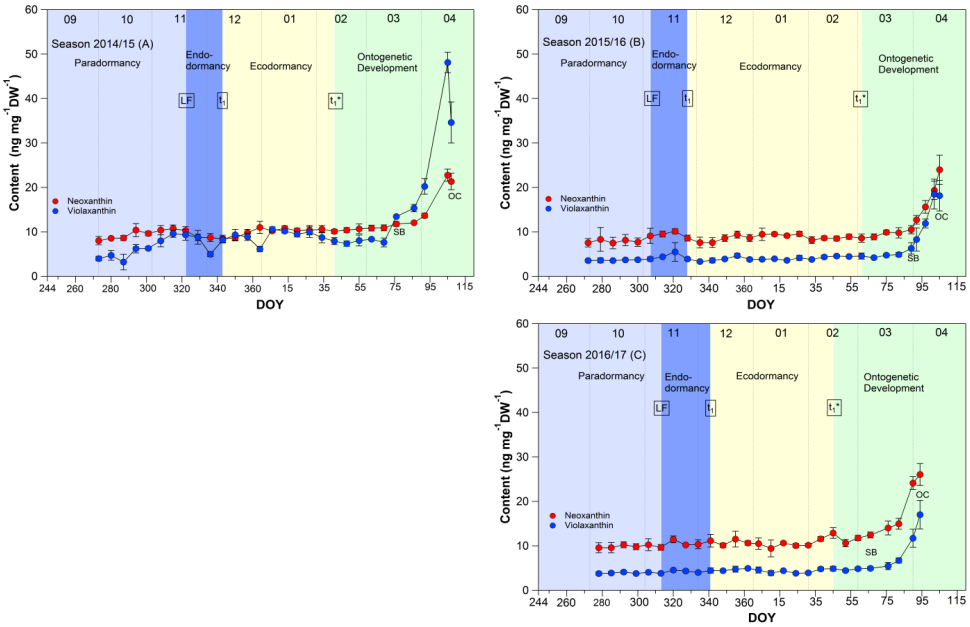

Figure 3: Weekly and development orientated changes of neoxanthin and violaxanthin content (mean of 4 replications, error bars show the standard deviation). A: Season 2014/15, B: Season 2015/16 and C: Season 2016/17.
Abbreviations: ABA: abscisic acid, LF: leaf fall, SB: swollen bud, OC: open cluster, t_1: end of endodormancy and t_1*: beginning of ontogenetic development, according to the classic approach [27], DOY: day of year, DW: dry weight.

increases after SB to a level of 17-35 ng/mg DW at the stage of OC. Considering, that these are observations over 3 years with n=4 for each data point, it seems that the content of neoxanthin and violaxanthin are unaffected over the different phases of the end of para-, endo- and ecodormancy. Their individual contribution to ABA synthesis seems to be indiscernible. Two processes may explain this observed constancy in the contents of neoxanthin and violaxanthin: Either their biosynthesis (including eventually that of ABA also) is coordinated with the upstream metabolism as discussed by Nambara and Marion-Poll [6] and/or there is a continuous supplementation to keep a constant level of the two individual carotenoids in the buds. Both these regulation processes need further investigations. The synthesis of the precursors neoxanthin and violaxanthin results from other C40 carotenoids (phytoene, ζ-carotene, lycopene and β-carotene) [2]. Of these, only β-carotene was determined (data not shown) whose mean content (in ng/mg DW) also remained constant until SB for the three seasons (15.91 ± 1.18 in 2014/15, 11.58 ± 1.31 in 2015/2016, 12.13 ± 1.17 in 2016/17) and to increase thereafter to maximum values (81-103 ng/mg DW) at OC. This indirect pathway of ABA biosynthesis from the precursor carotenoids neoxanthin and violaxanthin is reported to be the main pathway [2,6]. An alternative, route has also been suggested for ABA biosynthesis, that it is derived from the C15 compound farnesyl pyrophosphate [2]. Further investigations are necessary to confirm, if this alternative regulation is more relevant for cherry flower buds of the cultivar 'Summit'.

In order to gain more insight in the processes involved during ABA-biosynthesis and -catabolism, in the next step we investigated the role of the corresponding individual intermediate metabolites as depicted in Figure 1A-B.

Analysis of ABA metabolites

ABA-metabolites (Xan, ABAld, ABAOH, XanA, PA, DPA, Neo-PA, ABA-GE) were monitored for selected specific development stages of the season 2014/15: 'leaf fall' (LF), release of endodormancy (t_1), beginning of ontogenetic development (t_1*), 'swollen bud' (SB) and 'open cluster' (OC) and the results are presented in Figure 4A-E.

ABA, which represents the biologically active form, is produced from cis-xanthoxin (Xan) by two enzymatic steps via the intermediate metabolite ABAld [6]. The enzymes (a short-chain alcohol dehydrogenase/reductase and abscisic aldehyde oxidase) involved have been prevalently identified in Arabidopsis [6]. Other possible pathways that have been proposed are via XanA (e.g. in ripening avocado fruits) or ABAOH (e.g. in selected tomato mutants) [2]. Of the four intermediates (Xan, ABAld, ABAOH and XanA) only XanA was tentatively identified on basis of the anticipated mass spectrometric fragmentation pattern (mass transition: m/z 265.1 → 155). The content tangentially decreases from LF to t_1, the decrease being not significant (Figure 4A). Relevant significant changes ($p \leq 0.05$) are noted at the development stages SB and OC. Both ABAld and ABAOH resulting from Xan are chemically very reactive moieties, such that they could have reacted further (e.g. via oxidation) thus making their identification difficult. Improved analysis methods and synthesis of relevant intermediates are needed to further confirm these preliminary observations. Although, these results also suggest that the alternative route for ABA biosynthesis, that it is directly derived from the C15 compound farnesyl pyrophosphate, is perhaps more relevant for cherry flower buds.

ABA is biologically inactivated in a stepwise manner during the course of catabolism. The mass spectrometric methods for targeting ABA catabolism are much better documented and can be utilized with more reliability [1,36,25]. Concerning ABA catabolism, three different hydroxylation pathways have been documented leading to oxidation of one of the methyl groups of the ring structure (C-7′, C-8′, and C-9′) [6]. The hydroxylation triggers further inactivation steps resulting in the corresponding formation of DPA, PA and Neo-PA. The 8′-hydroxylation is thought to be the predominant pathway for ABA catabolism [6]. In addition to hydroxylation pathways, ABA can also be conjugated to glucose resulting in the formation of ABA-GE.

The results for the relevant catabolites are presented in Figure 4B-D. Tandem analysis with mass spectrometry is widely used to identify ABA and its catabolites and can result in similar fragmentation profiles [36]. Both PA and Neo-PA have been demonstrated to have MRM transitions with identical fragmentation patterns due similar structural constellations [36]. Parameters for LC-ESI-MS/MS analysis were adapted from Zheng et al. [1] and details are given in Supplementary Table S1. For PA, a 1st transition m/z 279 → 139 and a 2nd transition m/z 279 → 205 was found, where the 1st transition is generally used to characterize the compound identity [1,36]. In case of Neo-PA, the same two transitions are also possible, where the transition m/z 279 → 205 prevails [36]. The corresponding results for these two catabolites are given in Figure 4B and C. Both follow a similar pattern, and a 2.3 to 1.9 fold increase as compared to LF is noted respectively for PA and Neo-PA at the OC stage. The increase becomes significant after reaching the end of ecodormancy t_1* ($p \leq 0.05$). PA is further catabolized to DPA by a reductase and has been shown to be relatively inactive in various bioassays. Therefore ABA inactivation is completed by reaching this stage [6]. DPA was identified using mass transition m/z 281 → 171 and Figure 4D shows that it is the most abundant catabolite of ABA, its intensity increasing to a maximum of 4.8 fold at the development stage SB, where after it decreases during further ontogenetic development to OC. The major glucose conjugate of ABA is ABA-GE, which exhibits little or no biological activity but appears to be a transported form of ABA [25]. Recently, ABA-GE was also suggested to be involved in long-distance transport of ABA [6]. The ß-D-glucosidase activity may facilitate the releases of ABA from its glycoside form. In terms of mass intensity, it was the most abundant ABA metabolite observed in this study. The identity was based on the mass transitions m/z 425 and m/z 263 → 153, where the calculated ion ratio for the transition m/z 425 → 263 was constant at 3.80 ± 0.08. An exemplary extracted ion chromatogram (XIC) depicting these transitions is provided as Supplementary Figure S1. Figure 4E shows that its content appears to follow the similar pattern as DPA, reaching a maximum of 1.3 fold increase as compared to LF at the development stage SB, where after it decreases during further ontogenetic development to OC. Fortunately, this metabolite was also recorded with the second mass transition 263 → 153 during the targeted phytohormone analysis of ABA and its course of change can be documented as ABA equivalents in Figure 5A-C for the three seasons. The content increases in trend till the end of ecodormancy (t_1*) with phases where it remains more or less constant between t_1 and t_1*, to decrease thereafter from SB to OC (except for one data point for the season 2015/16; DOY 97; the cause for this inconsistency is not clear, but could be a result of sample handling). During ecodormancy this is a graphic result of inactivation of ABA in its reversible conjugation with glucose to form ABA-GE, found in various organs of different plant species [37] and being involved in mechanism responsible for ABA increase in plants under stress.

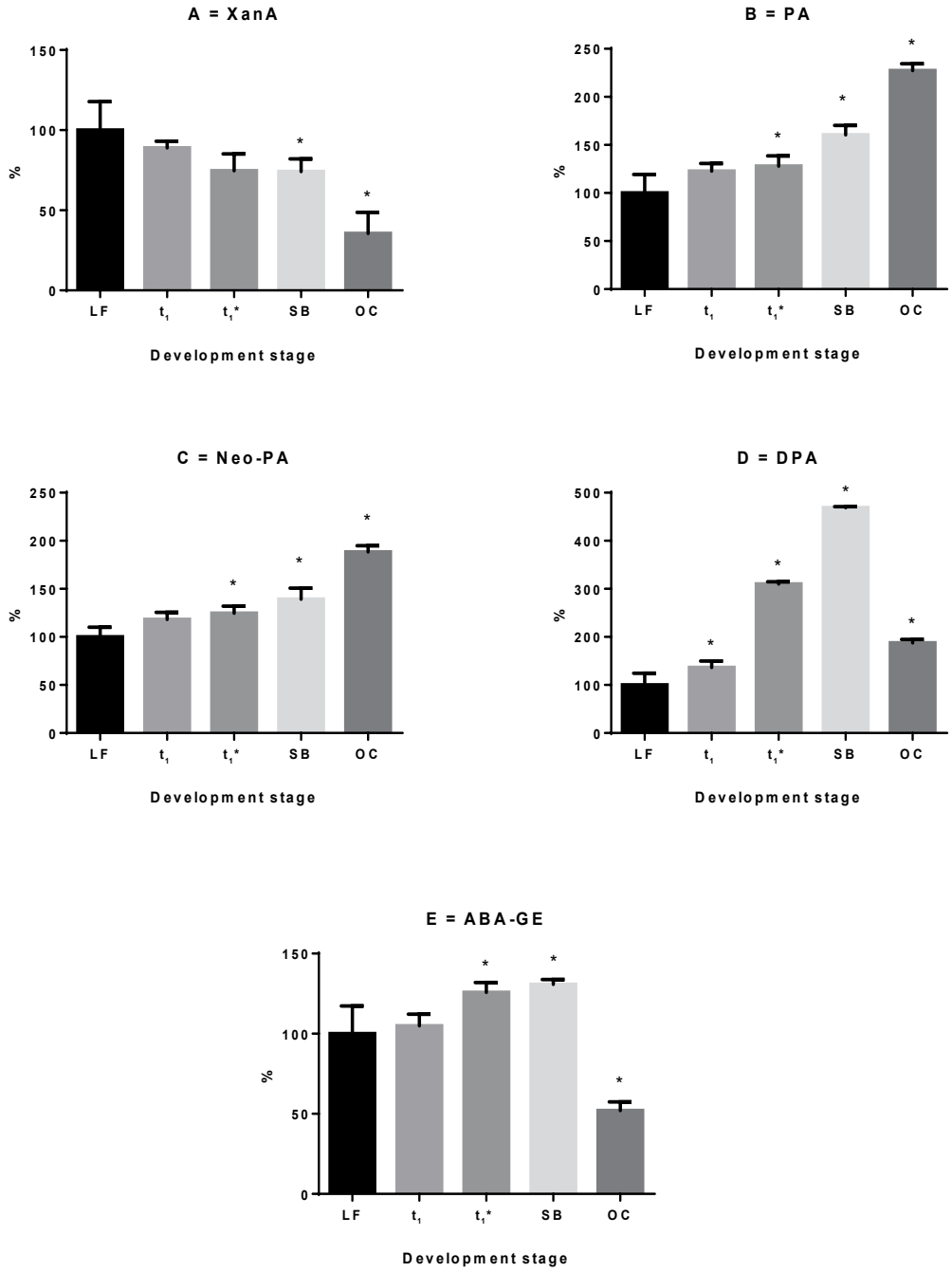

Figure 4: Monitoring of abscisic acid metabolites for selected specific development stages for the season 2014/15 (mean of 4 replications, error bars show the standard deviation). Data is presented as fold change of the intensity of mass signals in % to the timing leaf fall. A) XanA: xanthoxic acid, B) PA: phaseic acid, C) Neo-PA: neo-phaseic acid, D) DPA: dihydroxyphaseic acid and E) ABA-GE, ABA glucosyl ester.
Abbreviations: ABA: abscisic acid, LF: leaf fall, SB: swollen bud, OC: open cluster, t_1: end of endodormancy and t_1*: beginning of ontogenetic development, according to the classic approach [28].

Conclusions

As shown here and in a previous study [34], the highest ABA content was in vicinity of the end of endodormancy timing ($t_1 \pm 1$ week) and could serve as a potential metabolite marker for the timing of t_1. The content of neoxanthin and violaxanthin are unaffected over the different phases of late para-, endo- and ecodormancy. Their individual contribution to ABA synthesis is indiscernible. The results additionally suggest that the alternative route for ABA biosynthesis, that it is directly derived from the C15 compound farnesyl pyrophosphate could also be relevant for sweet cherry flower buds. Further experiments are needed to confirm this hypothesis. The results also indicate that there is a need to develop better methods capable of analyzing the different metabolites involved in the predominant synthesis phase of ABA (S1-LF), especially in encompassing Xan and XanA. The phase of dynamic degradation

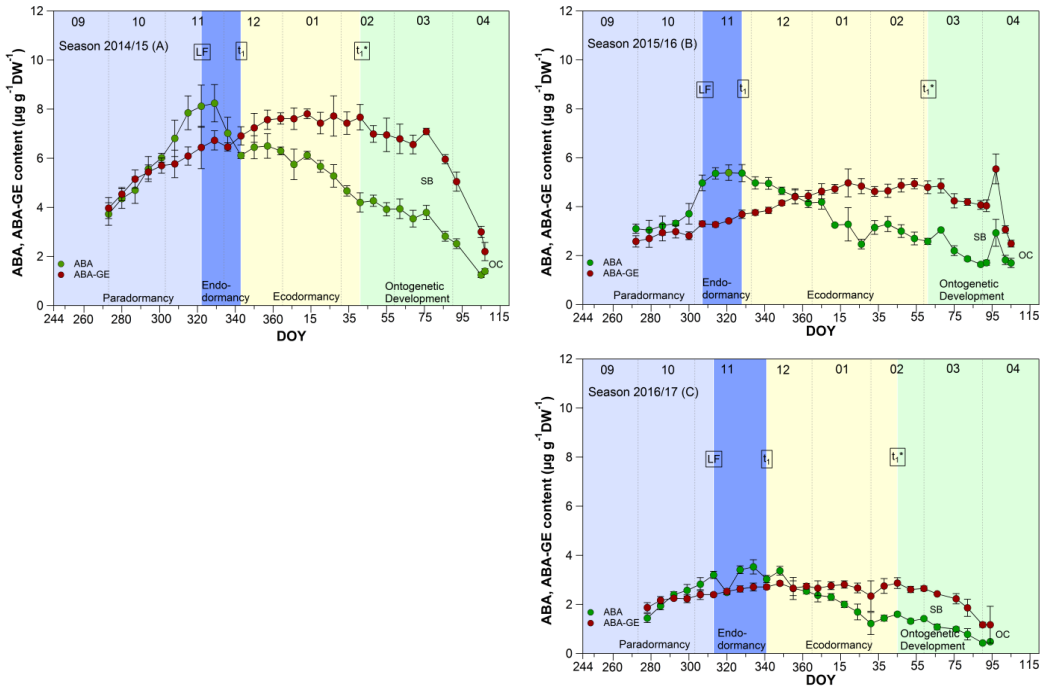

Figure 5: Weekly and development orientated changes of abscisic acid glucosyl ester and abscisic acid content (mean of 4 replications, error bars show the standard deviation). Data is presented as ABA equivalents while utilizing mass transition m/z 263 → 153 during the mass spectrometric HPLC analysis conditions given for phytohormes in the methods section. A: Season 2014/15, B: Season 2015/16 and C: Season 2016/17. Abbreviations: ABA: abscisic acid, ABA-GE: ABA glucosyl ester, LF: leaf fall, SB: swollen bud, OC: open cluster, t_1: end of endodormancy and t_1*: beginning of ontogenetic development, according to the classic approach [27], DOY: day of year, DW: dry weight.

of ABA prevailing between t_1 and SB is accompanied by a continuous level of ABA-GE (Figure 5A-C) i.e. in the synthesis of ABA-GE, which represents the reversible ABA-glucose component. The ABA-GE content starts decreasing thereafter, and since ABA concentration also decreases simultaneously, it is most likely transported to other parts of the plants – is ABA-GE also implicated here to be involved in long-distance transport of ABA? Finally, the fate of DPA (since it starts increasing after t_1) also needs to followed up – is it metabolized or transported away? Future works considering the implications of our study would contribute to a better understanding of ABA metabolism during bud dormancy and the predictions of flowering.

Acknowledgments

This work was supported by the Deutsche Forschungsgemeinschaft (DFG) in the project 'Progress in Phenological Modelling on the Basis of Metabolomic Approaches' by the grants (CH 228/5-1, RA 376 1008/9-1).

References

1. Zheng CL, Halaly T, Acheampong AK, Takebayashi Y, Jikumaru Y, et al. (2015) Abscisic acid (ABA) regulates grape bud dormancy, and dormancy release stimuli may act through modification of ABA metabolism. J Exp Bot 66: 1527-1542.
2. Seo M, Koshiba T (2002) Complex regulation of ABA biosynthesis in plants. Trends Plant Sci 7: 41-48.
3. Himmelbach A, Yang Y, Grill E (2003) Relay and control of abscisic acid signaling. Curr Opin Plant Biol 6: 470-479.
4. Nambara E, Kuchitsu K (2011) Opening a new era of ABA research. J Plant Res 124: 431-435.
5. Ko JH, Yang SH, Han KH (2006) Upregulation of an Arabidopsis RING-H2 gene, XERICO, confers drought tolerance through increased abscisic acid biosynthesis. Plant J 47: 343-355.
6. Nambara E, Marion-Poll A (2005) Abscisic acid biosynthesis and catabolism. Annu Rev Plant Biol 56: 165-185.
7. Lefebvre V, North H, Frey A, Sotta B, Seo M, et al. (2006) Functional analysis of Arabidopsis NCED6 and NCED9 genes indicates that ABA synthesized in the endosperm is involved in the induction of seed dormancy. Plant J 45: 309-319.
8. Pawowski TA (2010) Proteomic approach to analyze dormancy breaking of tree seeds. Plant Mol Biol 73: 15-25.
9. Wang D, Gao Z, Du P, Xiao W, Tan Q, et al. (2016) Expression of ABA metabolism-related genes suggests similarities and differences between seed dormancy and bud dormancy of peach (Prunus persica). Front Plant Sci 6: 1-17.
10. Nambara E, Okamoto M, Tatematsu K, Yano R, Seo M, et al. (2010) Abscisic acid and the control of seed dormancy and germination. Seed Science Res 20: 55-67.
11. Qin X, Zeevaart JA (2002) Overexpression of a 9-cis-epoxycarotenoid dioxygenase gene in *Nicotiana plumbaginifolia* increases abscisic acid and phaseic acid levels and enhances drought tolerance. Plant Physiol 128: 544-551.
12. Martinez-Andujar C, Ordiz MI, Huang Z, Nonogaki M, Beachy RN, et al. (2011) Induction of 9-cis-epoxycarotenoid dioxygenase in *Arabidopsis thaliana* seeds enhances seed dormancy. Proceedings of the National Academy of Sciences USA 108: 17225-17229.
13. Ruttink T, Arend M, Morreel K, Storme V, Rombauts S, et al. (2007) A molecular timetable for apical bud formation and dormancy induction in poplar. Plant Cell 19: 2370-2390.
14. Rios G, Leida C, Conejero A, Badenes ML (2014) Epigenetic regulation of bud dormancy events in perennial plants. Front Plant Sci 5: 247.
15. Arora R, Rowland LJ, Tanino K (2003) Induction and release of bud dormancy in woody perennials: A science comes of age. Hort Sci 38: 911-921.
16. Rohde A, Bhalerao RP (2007) Plant dormancy in the perennial context. Trends in Plant Science 12: 217-223.
17. Koussa T, Broquedis M, Bouard J (1994) Changes of Abscisic-Acid Level during the Development of Grapevine Latent Buds, Particularly in the Phase of Dormancy Break. Vitis 33: 63-67.

18. Or E, Belausov E, Popilevsky I, Ben Tal Y (2000) Changes in endogenous ABA level in relation to the dormancy cycle in grapevines grown in a hot climate. J Hortic Sci & Biotech 75: 190-194.

19. Li CY, Junttila O, Heino P, Palva ET (2004) Low temperature sensing in silver birch (*Betula pendula* Roth) ecotypes. Plant Sci 167: 165-171.

20. Destefano-Beltran L, Knauber D, Huckle L, Suttle JC (2006) Effects of postharvest storage and dormancy status on ABA content, metabolism, and expression of genes involved in ABA biosynthesis and metabolism in potato tuber tissues. Plant Mol Biol 61: 687-697.

21. Destefano-Beltran L, Knauber D, Huckle L, Suttle J (2006) Chemically forced dormancy termination mimics natural dormancy progression in potato tuber meristems by reducing ABA content and modifying expression of genes involved in regulating ABA synthesis and metabolism. J Exp Bot 57: 2879-2886.

22. Cutler AJ, Krochko JE (1999) Formation and breakdown of ABA. Trends in Plant Sci 4: 472-478.

23. Krochko JE, Abrams GD, Loewen MK, Abrams SR, Cutler AJ (1998) (+)-abscisic acid 8 '- hydroxylase is a cytochrome P450 monooxygenase. Plant Physiol 118: 849-860.

24. Saika H, Okamoto M, Miyoshi K, Kushiro T, Shinoda S, et al. (2007) Ethylene promotes submergence-induced expression of OsABA8ox1, a gene that encodes ABA 8 '-hydroxylase in rice. Plant Cell Physiol 48: 287-298.

25. López-Carbonell M, Gabasa M, Jáuregui O (2009) Enhanced determination of abscisic acid m(ABA) and abscisic acid glucose ester (ABA-GE) in *Cistus albidus* plants by liquid chromatography-mass spectrometry in tandem mode. Plant Physiol Biochem 47: 256-261.

26. Götz KP, Chmielewski FM, Gödeke K, Wolf K, Jander E, et al. (2017) Assessment of amino acids during winter rest and ontogenetic development in sweet cherry buds (*Prunus avium* L.). Scientia Horticulturae 222: 102-110.

27. Chmielewski FM, Götz KP (2016) Performance of models for the beginning of sweet cherry blossom under current and changed climate conditions. Agricultural and Forest Meteorology 218: 85-91.

28. Chmielewski FM, Götz KP (2017) Identification and Timing of Dormant and Ontogenetic Phase for Sweet Cherries in Northeast Germany for Modelling Purposes. J Horti 4: 205.

29. Götz KP, Chmielewski FM, Homann T, Huschek G, Matzneller P, et al. (2014) Seasonal changes of physiological parameters in sweet cherry (*Prunus avium* L.) buds. Scientia Horticulturae 172: 183-190.

30. Ma Z, Ge L, Lee ASY, Yong JWH, Tan SN, et al. (2008) Simultaneous analysis of differentclasses of phytohormones in coconut (*Cocos nucifera* L.) water using high-performance liquid chromatography and liquid chromatography-tandem mass spectrometry after solid-phase extraction. Analytica Chimica Acta 610: 274-281.

31. Hou S, Zhu J, Ding M, Lv G (2008) Simultaneous determination of gibberellic acid, indole-3- acetic acid and abscisic acid in wheat extracts by solid-phase extraction and liquid chromatography-electrospray tandem mass spectrometry. Talanta 76: 798-802.

32. Durgbanshi A, Arbona V, Pozo O, Miersch O, Sancho JV, et al. (2005) Simultaneous determination of multiple phytohormones in plant extracts by liquid chromatography-electrospray tandem mass spectrometry. J Agricul Food Chem 53: 8437-8442.

33. Errard A, Ulrichs C, Kuhne S, Mewis I, Drungowski M, et al. (2015) Single- versus Multiple-Pest Infestation Affects Differently the Biochemistry of Tomato (*Solanum lycopersicum* 'Ailsa Craig'). J Agricultural and Food Chem 63: 10103-10111

34. Chmielewski FM, Götz KP, Homann T, Huschek G, Rawel HM (2017) Identification of Endodormancy Release for Cherries (*Prunus avium* L.) by Abscisic Acid and Sugars. J Horti 4: 210.

35. Cooke JE, Eriksson ME, Junttila O (2012) The dynamic nature of bud dormancy in trees: environmental control and molecular mechanisms. Plant Cell Environ 35: 1707-1728.

36. Silva CMSd, Habermann G, Marchi MRR, Zocolo GJ (2012) The role of matrix effects on the quantification of abscisic acid and its metabolites in the leaves of *Bauhinia variegata* L. using liquid chromatography combined with tandem mass spectrometry. Brazilian J Plant Physiology 24: 223-232.

37. Korovetska H, Novak O, Tureckova V, Hajickova M, Gloser V (2016) Signalling mechanisms involved in the response of two varieties of *Humulus lupulus* L. to soil drying: II. changes in the concentration of abscisic acid catabolites and stress-induced phytohormones. Plant Growth Regulation 78: 13-20.