The Role of Threonine Deaminase/Dehydratase in Winter Dormancy in Sweet Cherry Buds

Klaus-Peter Götz [1],*, Chibueze Ene [2] , Joerg Fettke [2] and Frank-M. Chmielewski [1]

[1] Agricultural Climatology, Faculty of Life Sciences, Humboldt-University of Berlin, Albrecht-Thaer-Weg 5, 14195 Berlin, Germany; chmielew@agrar.hu-berlin.de
[2] Biopolymer Analytics Group, University of Potsdam, Karl-Liebknecht-Str. 24/25, 14476 Potsdam, Germany; ene@uni-potsdam.de (C.E.); fettke@uni-potsdam.de (J.F.)
* Correspondence: klaus-peter.goetz@agrar.hu-berlin.de

Abstract: The determination of the endodormancy release and the beginning of ontogenetic development is a challenge, because these are non-observable stages. Changes in protein activity are important aspects of signal transduction. The conversion of threonine to 2-oxobutanoate is the first step towards isoleucine (Ile) biosynthesis, which promote growth and development. The reaction is catalyzed by threonine deaminase/dehydratase (TD). This study on TD activity was conducted at the experimental sweet cherry orchard at Berlin-Dahlem. Fresh (FW), dry weight (DW), water content (WC), and the specific TD activity for the cherry cultivars Summit, Karina and Regina were conducted from flower bud samples between October and April. The content of asparagine (Asn), aspartic acid (Asp), Ile, and valine (Val) were exemplarily shown for Summit. In buds of Summit and Karina, the TD activity was one week after the beginning of the ontogenetic development (t_1*), significantly higher compared to samplings during endo- and ecodormancy. Such "peak" activity did not occur in the buds of Regina; TD tended for a longer time (day of year, DOY 6–48) to a higher activity, compared to the time DOY 287–350. For the date "one week after t_1*", the upregulation of TD, the markedly increase of the Ile and Val content, and the increase of the water content in the buds, all this enzymatically confirms the estimated start of the ontogenetic development (t_1*) in sweet cherry buds.

Keywords: endodormancy; ecodormancy; sweet cherry; buds; threonine deaminase/dehydratase; ontogenetic development; isoleucine; valine

1. Introduction

Deciduous trees enter into a period of winter rest in fall, called dormancy, until growth and development restarts in spring. The phases of dormancy, endo- and ecodormancy, and the subsequent phase of ontogenetic development [1] are thereby accompanied by anabolic and catabolic processes of, e.g., carbohydrates, organic acids, amino acids, amines, and phytohormones [2,3]. The result of an untargeted global metabolite profiling in sweet cherry buds [2], to detect relevant metabolites which are related to the induction, maintenance, and release of dormancy phases, revealed that during the endo- and ecodormancy, the energy metabolism in the form of glycolysis and the tricarboxylic acid (TCA) cycle are shut down to a minimum. However, the beginning of ontogenetic development was closely related to the up-regulation of carbohydrate metabolism and thus to the regeneration of energy for growth and development processes of sweet cherry buds. As a result of a targeted global metabolite profiling over nine years in sweet cherry buds [4], only abscisic acid (ABA) showed a clear change in the temporal course after the date of endodormancy release. It appears to be the key metabolite explaining the end of endodormancy (t_1) and the beginning of ontogenetic development (t_1*). Such metabolites would be of particular interest for phenological modeling, because they would allow modeling of the non-observable stages

of t_1, indicating the fulfillment of the cultivar-specific chilling requirement, and t_1*, the latter which must start a few weeks before any visible changes in the buds are detectable [1].

Amino acids (AAs) have many important functions in plants. Besides their usage for protein biosynthesis, they also act as precursors for other biosynthesis pathways (carbohydrate/secondary metabolism) and play pivotal roles in plant stress response and in signaling processes as well [5,6].

The occurrence of metabolites, small molecule substrates, intermediates, and "end products", is a result of cell metabolism. Cells are able to react to a complex and ever-changing mix of signals. Changes in protein level, protein localization, protein activity, and protein–protein interactions are important aspects of signal transduction. Cells respond highly specifically to a nearly limitless set of cues. Signal-dependent changes of levels of gene expression and protein synthesis play an important role in the regulation of protein levels, whereas post-translational modifications of proteins regulate their degradation, localization, and functional interactions [7].

The so-called branched-chain amino acids (BCAAs) leucine, isoleucine, and valine contain small, branched, hydrocarbon residues. In plants, the conversion of threonine to 2-oxobutanoate is the first and also the committed step towards isoleucine biosynthesis. This reaction is catalyzed by threonine ammonia-lyase, also commonly referred to as threonine deaminase/dehydratase (EC 4.3.1.19, TD) [6]. BCAA homeostasis is strictly regulated, and understanding of such regulation comes mostly from the studies of allosteric control of three enzymes, TD, acetohydroxyacid synthase (AHAS) and isopropylmalate synthase (IPMS) [6]. The activity of TD is feedback inhibited by its end product, isoleucine (Ile). Results of a study on an Arabidopsis mutant, low in isoleucine biosynthesis, that has defects in both cell proliferation and cell expansion, reveal an important role for Ile in plant development [8].

During bud development of the sweet cherry cultivar Summit, the content of Ile increased markedly in four seasons at "green tip", 14 days after the stage "swollen bud (SB)", at which the water content rose significantly compared to the endo- and ecodormancy, but from none of eight free amino acids (asparagine (Asn), aspartic acid (Asp), Ile, glutamine (Gln), glutamic acid (Glu), arginine (Arg), alanine (Ala), or histidine (His)) could a clear determination of the date of endodormancy release (t_1) or the beginning of the ontogenetic development (t_1*) be derived [9]. For this reason, as first to our knowledge, the role of the enzyme TD during phenological development was studied in sweet cherry buds of three cultivars and evaluated on the basis of specific enzyme activity.

2. Materials and Methods

The description of the experimental site, phenological observations in the orchard, determination of ecodormancy phase (t_1–t_1*), sampling of sweet cherry buds and preparing for analysis has already been extensively published [1–4,9,10].

2.1. Experimental Site

Briefly, this study was conducted for the season 2014/15 at the experimental sweet cherry orchard at Berlin-Dahlem (52.47° N, 13.30° E, h = 51 m), which was established in 1999. The orchard (980 m^2) comprises 80 cherry trees of the cultivars Summit, Karina, Regina (developed in British Columbia, introduced 1973; bred in Jork, Germany, 1957, went on sale in 1993; bred in Jork, Germany, 1957, commercially cultivated since 1987, respectively) growing in 8 rows with 10 trees each, aligned in a N–S direction. All trees are grafted on Gisela-5 rootstocks and pruning, fertilization, and watering was done on demand. The long-term annual mean air temperature and precipitation (1991–2020) are 10.4 °C and 562 mm, respectively.

2.2. Sampling of Sweet Cherry Buds

Fresh (FW) and dry weight (DW), and the water content (WC) for Summit, Regina, and Karina were conducted from flower bud samples of three trees of each cultivar (biological

replication n = 3) between October 2014 and April 2015. For each cultivar, three bud clusters were taken weekly in the orchard at random locations over the whole tree. After the beginning of bud development, analysis was done for the stage 'side green' (SG). For determining the TD activity, twenty clusters were collected from each tree at day of year (DOY) 287, 329 (one week after leaf fall (LF)), 336, 343 (t_1), 350 (one week after t_1), 6, 27, 48 (1 week after t_1*), 86 (SG). After cutting, clusters were immediately placed in plastic bags on ice and were frozen in liquid nitrogen and stored at $-80\,^{\circ}$C until TD measurement.

2.3. Extraction and Enzyme Activity Assay for Threonine Deaminase/Dehydratase (TD) from Sweet Cherry Samples

A total of 1.5 g of cherry samples were crushed into fine powder in liquid nitrogen. Extraction buffer (10 mL) was added and the sample vortexed. The extraction buffer contained 20 mM K_2HPO_4 (pH 8.0), 2 mM Na_2EDTA, 2 mM DTE, 0.4 mM pyridoxal phosphate, 1 mM isoleucine, 1 mM PMSF. The homogenates were centrifuged at 20,000 g for 15 min at $4\,^{\circ}$C and the resulting supernatant passed through 0.04 mm filter. An equal volume of saturated ammonium sulphate was added to the supernatant and kept at $4\,^{\circ}$C for 30 min. The samples were then centrifuged as before, the supernatant decanted and the sediment resuspended in 2 mL of equilibration buffer. Equilibration buffer contained 1 M K_2HPO4 (pH 9.0), 1 mM Na_2EDTA, 1 mM dithiothreitol (DTT). The resuspended samples were desalted by passing through a 10 kDa filter. The retained solution is the TD extract and was stored at $-80\,^{\circ}$C.

TD enzymatic activity was measured following the method described by [8]. A 200 µL of TD extract was mixed with 300 µL of assay mixture, vortexed and incubated at $30\,^{\circ}$C for 30 min. Assay mixture contained 0.1 M Tris-HCl, (pH 9.0), 60 mM threonine, 0.3 M K_2HPO_4, 0.3 mM Na_2EDTA, 0.3 mM DTT, and 0.04 mM pyridoxal phosphate. The reaction was stopped by the addition of 200 µL of 50% (*w/v*) trichloroacetic acid (TCA). A total of 200 µL of 0.1% (*w/v*) 2,4-dinitrophenylhydrazine in 2 N HCl was added and kept at room temperature. After 20 min, 0.9 mL of 2.5 N KOH was added. Absorbance was read at 515 nm after 15 min.

2.4. Analysis of Amino Acids

The content of Asn, Asp, Ile, and Valine (Val) was conducted exemplary for Summit, applying the EZ:Faast™ Kit according to manufacturer's instructions (EZ:Faast™ User's Guide for Physiological Amino Acids, Phenomenex, Torrance, CA, USA, using liquid chromatography with mass spectrometry (LC-MS) [9].

2.5. Statistical Analysis

Standard statistical analyses (mean, standard deviation (SD), multiple comparisons of means) was performed with IBM SPSS V29, using one-way ANOVA with Tukey HSD-test, and the two-sided test, testing whether the means are different at $p < 0.05$.

3. Results and Discussion

3.1. Timing of Phenological Stages and Air Temperature

The timing of phenological stages (in DOY) was similar (± 1 day) for Summit, Karina, and Regina. Leaf fall was observed on DOY 322, t_1: endodormancy release, on DOY 343, t_1*: beginning of ontogenetic development on DOY 41, swollen bud, on DOY 76, and side green on DOY 86. The mean air temperature ($^{\circ}$C) during the phenophases endodormancy [leaf fall (LF)–end of endodormancy (t_1)], ecodormancy [t_1–beginning of ontogenetic development (t_1*)], and the ontogenetic development, between swollen bud (SB) and side green (SG) was 2.6, 2.8, 4.1, and $6.7\,^{\circ}$C, compared to the mean temperature of nine years between 2011/12 and 2019/20 with 5.3, 2.4, 4.5, and $6.7\,^{\circ}$C. The FW (Figure 1A–C), DW (Figure 1D–F) and WC (Figure 1G–I) did not significantly change between one week before LF and one week after t_1*, but was markedly increased at SG (DOY 86) for Summit, Karina and Regina (Figure 1A–I). The beginning of biological activity of the buds was visible in nine seasons

by a steady rise of the water content in the buds [10] and was, for Summit, in four out of six seasons, significantly higher seven days after t_1^* [1].
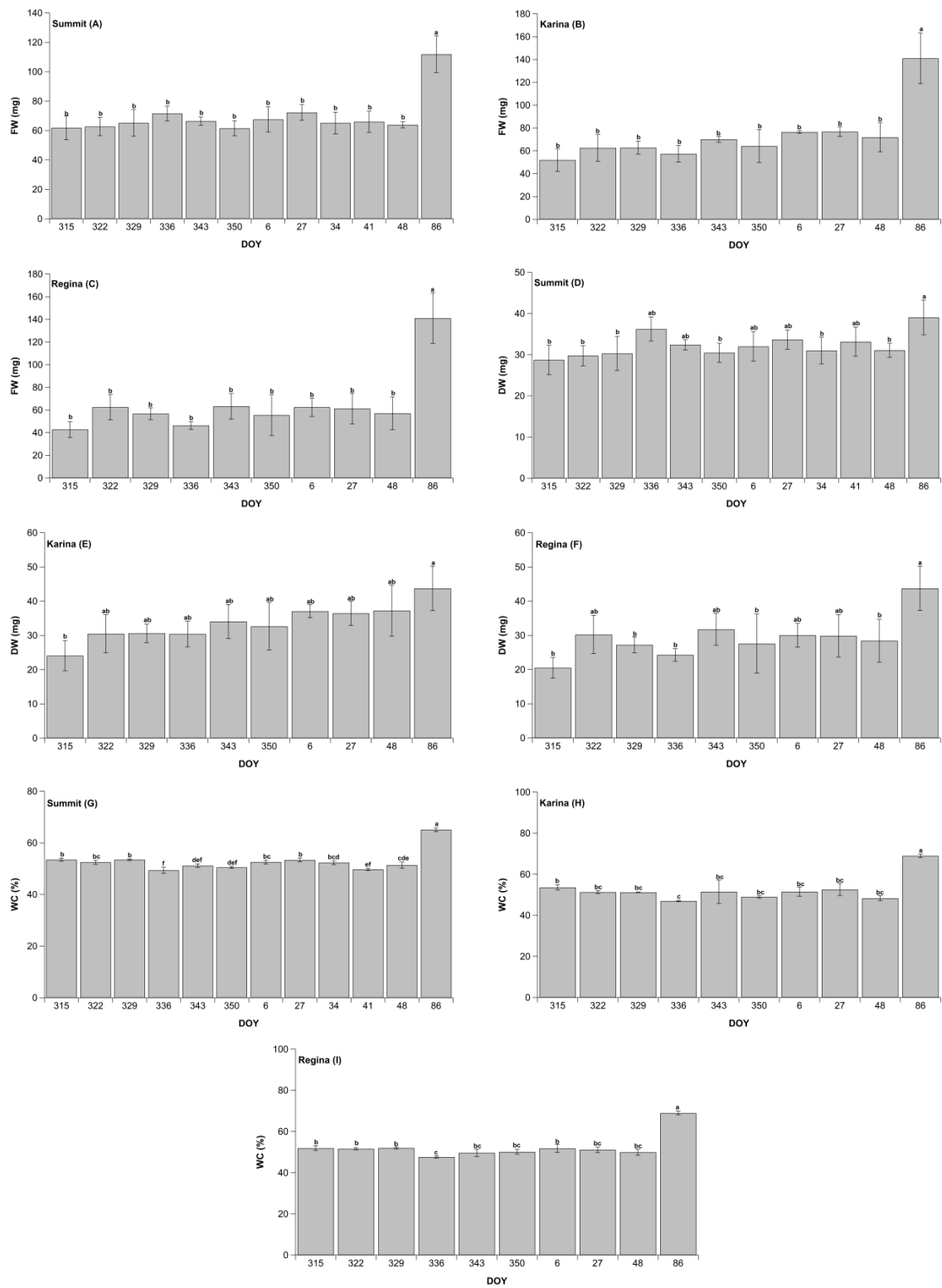

Figure 1. Mean (±SD) of fresh weight (FW, mg, (**A**–**C**)), dry weight (DW, mg, (**D**–**F**)) and the water content (WC, %, (**G**–**I**)) of the sweet cherry cultivars Summit, Karina and Regina between day of year (DOY) 315 (2014) and DOY 86 (2015). Different letters indicate significant differences at $p < 0.05$.

3.2. Specific Activity of Threonine Deaminase/Dehydratase (TD) in Sweet Cherry Buds

In buds of Summit, as well in Karina, the specific activity of threonine deaminase/dehydratase (TD) (Figure 2) was at DOY 48, which was one week after the beginning of the ontogenetic development (t_1*), with 506.2 μmol/min/mg (±97.5), 495.2 μmol/min/mg (±114.5), respectively, significant higher by factor 1.5 and 1.8, compared to the other samplings during endo- and ecodormancy, with on average 322.8 μmol/min/mg (±21.5), 273.6 μmol/min/mg (±8.6), respectively. Such kind of increase did not occur in buds of Regina (Figure 2), but the specific activity of TD tended for a longer time between DOY 6 and DOY 48 on average with 395.7 μmol/min/mg (±41.7) to a higher activity compared to the phase between DOY 287 and DOY 350 with 312.3 μmol/min/mg (±37.3). For the cultivar Regina, therefore, there does not appear to be a direct connection between the TD activity and the transition from endodormancy to the ontogenetic development of the buds.
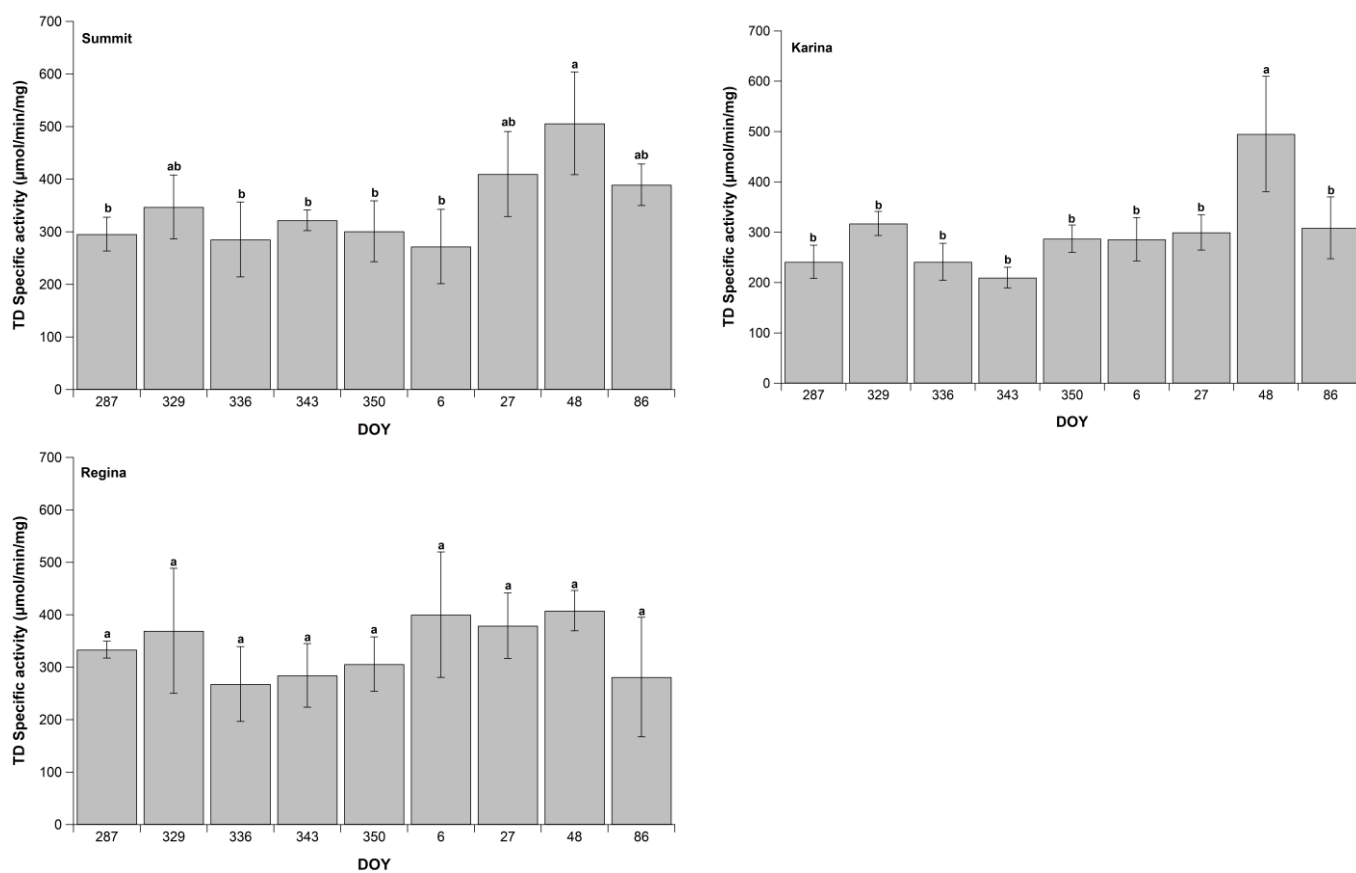

Figure 2. Specific activity (mean ± SD in μmol min^{-1} mg^{-1}) of threonine deaminase/dehydratase (TD) in sweet cherry buds of Summit, Karina, and Regina between day of year (DOY) 287 (2014) and DOY 86 (2015). DOY 329: one week after leaf fall; 343: endodormancy release (t_1); 350: one week after t_1; 48: one week after beginning of ontogenetic development (t_1*); 86: developmental stage "side green" (SG). Different letters indicate significant differences at $p < 0.05$.

3.3. Content of Asparagine (Asn), Aspartic Acid (Asp), Isoleucine (Ile), and Valine (Val) in Sweet Cherry Buds of the Cultivar Summit in the Season 2014/2015

The enzyme TD has two binding sites for isoleucine. One has a high affinity for isoleucine and the other has a low affinity [11]. The binding of isoleucine to the high affinity site increases the binding affinity of the low affinity site, and enzyme deactivation occurs when isoleucine binds to the low affinity site. Valine promotes enzyme activity by competitively binding to the high affinity site, preventing isoleucine from having an inhibitory effect. The combination of these two feedback methods balances the concentration of BCAAs. The content of Asn, as an optimal nitrogen transport and reserve compound due to its high nitrogen to carbon ratio of 2:4, declined clearly stepwise between DOY 315

and DOY 48 from 2.06 to 0.62 mg/g DW (Figure 3), and was again significant increased to 2.8 mg/g DW at SG. The content of Asp (Figure 3), at a lower level compared to Asn, was relatively constant, with the exception of a low content of 0.15 mg/g DW at DOY 336, one week before endodormancy release (t_1), and reached the highest content of 0.46 mg/g DW at SG. Over the observation period, both the content of Ile and that of Val were similar and markedly lower ($p < 0.05$) than at the stage SG. However, a direct comparison (two-sided test, $p < 0.05$) of the dates t_1* and one week after t_1* (DOY 41 and 48) shows a significant increase in the Ile content as well as the Val content (Figure 3), from 0.006 to 0.009, 0.006 to 0.014 mg/g DW, respectively. Thus, the existence of the combination of the two feedback responses in sweet cherry buds for the Summit variety can be proven as an example. For the date "one week after t_1*", the upregulation of the threonine deaminase/dehydratase (TD), the markedly increase of the Ile and Val content, and the changes of the water content in the buds [1], enzymatically confirms the estimated start of the ontogenetic development (t_1*) in sweet cherry buds.
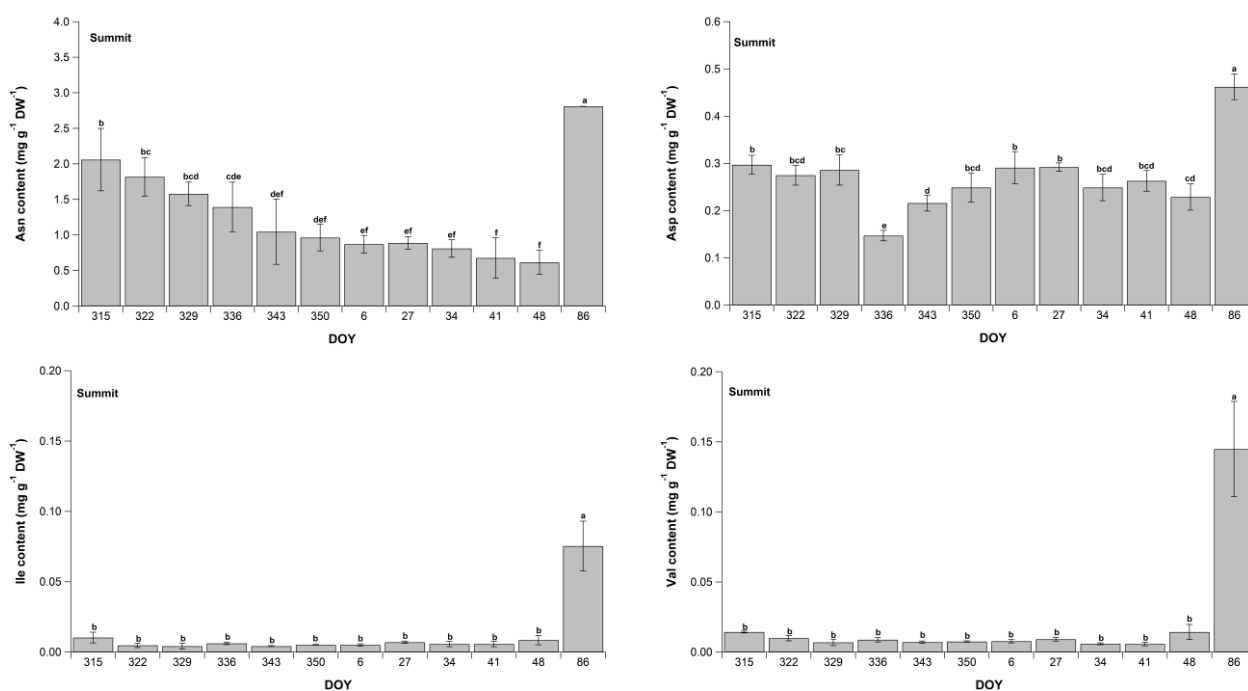

Figure 3. Content ($\pm$SD) (mg g^{-1} DW^{-1}) of asparagine (Asn), aspartic acid (Asp), isoleucine (Ile), and valine (Val) in Summit buds between day of year (DOY) 315 (2014) and DOY 86 (2015). DOY 322: leaf fall; DOY 329: one week after leaf fall; 343: endodormancy release (t_1); 350: one week after t_1; 48: one week after beginning of ontogenetic development (t_1*); 86: developmental stage "side green" (SG). Different letters indicate significant differences at $p < 0.05$.

4. Conclusions

These results suggest, as stated for root growth and development (cell proliferation/cell expansion), that Ile is involved in sweet cherry bud growth and development. The upregulation of the of threonine deaminase/dehydratase (TD) is obviously cultivar-specific, and can also be independent from "around" the time of the beginning of ontogenetic development (t_1*; lies in a range of 42 days, DOY 6 to DOY 48) as shown for the Regina cultivar. For the Summit cultivar, the upregulation of the TD is a further confirmation of the correctness of the determination of the beginning of the ontogenetic development (t_1*), and is an example for the interplay of water and nitrogen for growth of the plant organ "bud". The cultivar-specific TD activity of Regina should be first implemented and evaluated by determining the amino acid pattern over the observation period.

References

1. Chmielewski, F.M.; Götz, K.P. Identification and timing of dormant and ontogenetic phase for sweet cherries in northeast Germany for modelling purposes. *J. Hortic.* **2017**, *4*, 205.
2. Chmielewski, F.M.; Götz, K.P. Metabolites in cherry buds to detect winter dormancy. *Metabolites* **2022**, *12*, 247. [CrossRef] [PubMed]
3. Götz, K.P.; Chmielewski, F.M.; Tarkowská, D.; Pěnčík, A.; Novák, O. Phytohormones in sweet cherry buds during winter rest and bud development. *J. Plant Growth Regul.* **2022**, *42*, 2519. [CrossRef]
4. Chmielewski, F.M.; Götz, K.P. ABA and not chilling reduces heat requirement to force cherry blossom after endodormancy release. *Plants* **2022**, *11*, 2044. [CrossRef] [PubMed]
5. Hildebrandt, T.M.; Nesi, A.N.; Araújo, W.L.; Braun, H.P. Amino acid catabolism in plants. *Mol. Plant* **2015**, *8*, 1563. [CrossRef] [PubMed]
6. Pratelli, R.; Pilot, G. Regulation of amino acid metabolic enzymes and transporters in plants. *J. Exp. Bot.* **2014**, *65*, 5535. [CrossRef] [PubMed]
7. Lee, M.J.; Jaffe, M.B. Protein regulation in signal transduction. *Cold Spring Harb. Perspect. Biol.* **2016**, *8*, a005918. [CrossRef] [PubMed]
8. Yu, H.; Zhang, F.; Wang, G.; Liu, Y.; Liu, D. Partial deficiency of isoleucine impairs root development and alters transcript levels of the genes involved in branched-chain amino acid and glucosinolate metabolism in Arabidopsis. *J. Exp. Bot.* **2013**, *65*, 599. [CrossRef] [PubMed]
9. Götz, K.P.; Chmielewski, F.M.; Gödeke, K.; Wolf, K.; Jander, E.; Sievers, S.; Homann, T.; Huschek, G.; Rawel, H. Assessment of amino acids during winter rest and ontogenetic development in sweet cherry buds (*Prunus avium* L.). *Sci. Hortic.* **2017**, *222*, 102. [CrossRef]
10. Götz, K.P.; Chmielewski, F.M. Metabolites that confirm induction and release of dormancy phases in sweet cherry buds. *Metabolites* **2023**, *13*, 231. [CrossRef] [PubMed]
11. Wessel, P.M.; Graciet, E.; Douce, R.; Dumas, R. Evidence for two distinct effector-binding sites in threonine deaminase by site-directed mutagenesis, kinetic, and binding experiments. *Biochemistry* **2000**, *39*, 15136. [CrossRef] [PubMed]