Plant Physiology and Biochemistry 126 (2018) 163–172

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

β-Glucosidase activity in almond seeds

Jorge Del Cueto[a,b,c], Birger Lindberg Møller[b,c], Federico Dicenta[a], Raquel Sánchez-Pérez[a,b,c,*]

[a] Department of Plant Breeding, CEBAS-CSIC, P.O. Box 164, 30100 Campus Universitario de Espinardo, Murcia, Spain
[b] University of Copenhagen, Faculty of Science, Plant Biochemistry Laboratory, DK-1871 Copenhagen C, Denmark
[c] VILLUM Research Center for Plant Plasticity, DK-1871 Frederiksberg C, Denmark

ARTICLE INFO

Keywords:
β-glucosidases
Agroinfiltration
Bitterness
Cyanogenic glucosides
Prunasin hydrolase
Prunus dulcis

ABSTRACT

Almond bitterness is the most important trait for breeding programs since bitter-kernelled seedlings are usually discarded. Amygdalin and its precursor prunasin are hydrolyzed by specific enzymes called β-glucosidases. In order to better understand the genetic control of almond bitterness, some studies have shown differences in the location of prunasin hydrolases (PH, the β-glucosidase that degrades prunasin) in sweet and bitter genotypes. The aim of this work was to isolate and characterize different PHs in sweet- and bitter-kernelled almonds to determine whether differences in their genomic or protein sequences are responsible for the sweet or bitter taste of their seeds. RNA was extracted from the tegument, nucellus and cotyledon of one sweet (Lauranne) and two bitter (D05–187 and S3067) almond genotypes throughout fruit ripening. Sequences of nine positive *Phs* were then obtained from all of the genotypes by RT-PCR and cloning. These clones, from mid ripening stage, were expressed in a heterologous system in tobacco plants by agroinfiltration. The PH activity was detected using the Feigl-Anger method and quantifying the hydrogen cyanide released with prunasin as substrate. Furthermore, β-glucosidase activity was detected by Fast Blue BB salt and Umbelliferyl method. Differences at the sequence level (SNPs) and in the activity assays were detected, although no correlation with bitterness was found.

1. Introduction

Bitterness in almonds (*Prunus dulcis* Miller D.A. Webb syn. *Prunus amygdalus* Batsch) is one of the most important and widely studied traits in this species. Almond bitterness is a monogenic trait, and the sweet taste allele is dominant over the bitter taste allele (Heppner, 1923, 1926; Dicenta and García, 1993; Dicenta et al., 2007). The gene responsible for bitterness in almonds is denoted *Sweet kernel* (*Sk*), and it is located in linkage group five, out of the eight that almond has (Sánchez-Pérez et al., 2007; 2010; Joobeur et al., 1998; Bliss et al., 2002). Commercial sweet genotypes can be homozygous (*SkSk*) or heterozygous (*Sksk*) for this trait, while slightly bitter genotypes are always heterozygous (*Sksk*), and bitter genotypes are homozygous recessive (*sksk*) (Grasselly et al., 1980; Vargas et al., 2001). The bitter trait is maternally controlled (Heppner, 1923; Dicenta and García, 1993; Kester and Gradziel, 1996; Socias, 1998; Sánchez-Pérez et al., 2010). As a result, all the fruits of an almond will be sweet, slightly bitter or bitter, and the influence of both progenitors will be seen in the next generation (Kester et al., 1975; Dicenta et al., 2000; Arrázola et al., 2012).

Cyanogenic glucosides (CNGLcs) are β-glucosides of α-hydroxinitriles (Conn, 2007; Zheng and Poulton, 1995; Morant et al., 2008). These compounds are present in more than 3000 plant species (Bak et al., 2006a; Sánchez-Pérez et al., 2008; Gleadow and Møller, 2014) like sorghum, cassava and Rosaceous stone fruits. Their main function is to defend plants against pathogens and predators (Bak et al., 2006b; Zagrobelny et al., 2008).

There are two cyanogenic glucosides present in almonds: prunasin (mono-glucoside of R-mandelonitrile) and amygdalin [di-glucoside of R-mandelonitrile with a β-(1,6) bond] (Conn, 2007; Sánchez-Pérez et al., 2008; McCarty et al., 1952; Frehner et al., 1990; Møller and Seigler, 1991; Swain et al., 1992a; Poulton and Ping Li, 1994; Dicenta et al., 2002; Franks et al., 2008). These two compounds are *de novo* synthesized in the almond seed (Sánchez-Pérez et al., 2008), and the amygdalin content is significantly higher in mature bitter almond seeds than in slightly bitter or sweet seeds (between 200 and 1000-fold higher, respectively). On the other hand, there is no clear relationship between the prunasin levels in the vegetative parts of the plant and the bitterness of the fruit (Dicenta et al., 2002).

The almond fruit consists of maternal tissues (endocarp, mesocarp, tegument – also called the seed coat – and nucellus) and parental tissues endosperm (2n (mother) + n (father)) and cotyledon (n (mother) + n (father)). The first difference between sweet and bitter almonds can be found in the tegument, as prunasin only accumulates in the bitter

* Corresponding author. Department of Plant Breeding, CEBAS-CSIC, P.O. Box 164, 30100 Campus Universitario de Espinardo, Murcia, Spain.
E-mail address: rasa@plen.ku.dk (R. Sánchez-Pérez).

https://doi.org/10.1016/j.plaphy.2017.12.028
Received 29 September 2017; Received in revised form 12 December 2017; Accepted 15 December 2017
Available online 16 December 2017

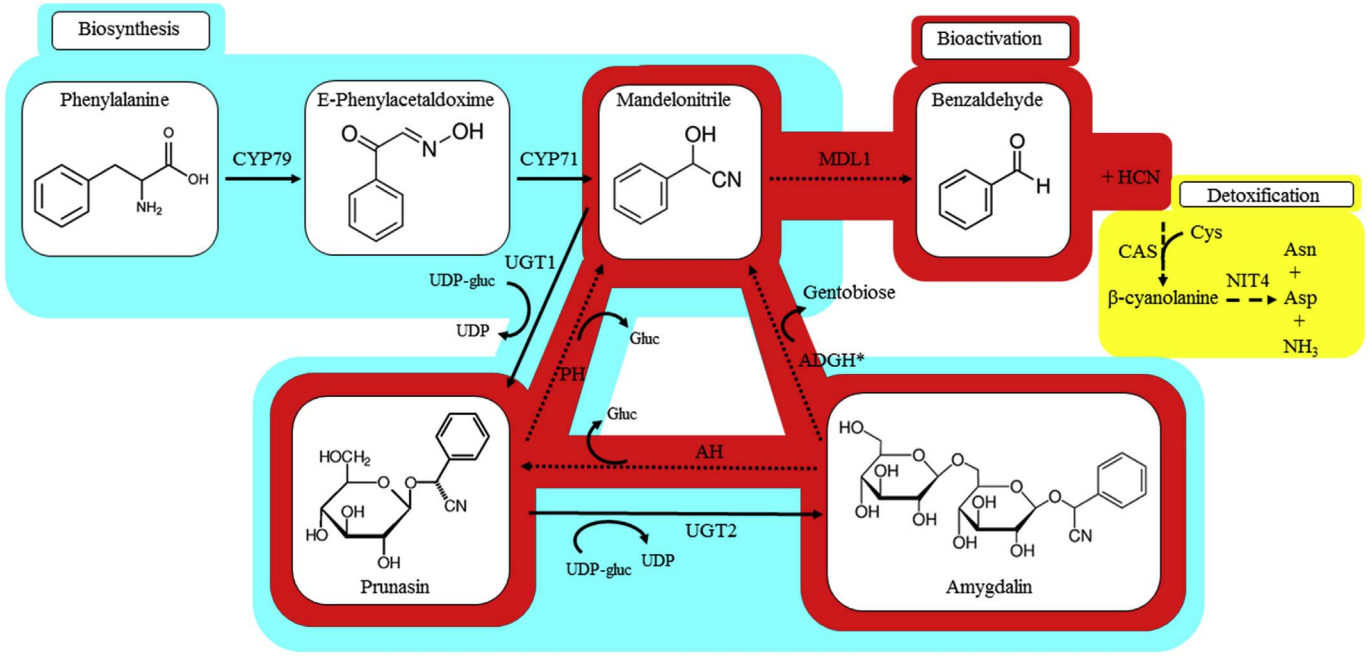

Fig. 1. The metabolic pathways for the synthesis and catabolism of the cyanogenic glucosides prunasin and amygdalin in almonds. Biosynthetic enzymes (solid lines): CYP79 and CYP71, Cytochrome P450 monooxygenases, UGT1, UDPG-mandelonitrile glucosyltransferase; UGT2, UDPG-prunasin glucosyltransferase. Catabolic enzymes (dotted lines): AH, amygdalin hydrolase; PH, prunasin hydrolase; MDL1, mandelonitrile lyase; ADGH*, amygdalin diglucosidase (putative). Detoxification enzymes (dashed lines) CAS: β-cyanoalanine synthase; NIT4: nitrilases 4. Source: adapted from Sánchez-Pérez et al. (2008) and Del Cueto et al. (2017). Blue indicates biosynthesis, red indicates bioactivation and yellow indicates a detoxification pathway. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

teguments throughout seed development (Sánchez-Pérez et al., 2008).

Fig. 1 describes the metabolism of prunasin and amygdalin, which can be divided into the following three parts: biosynthesis, bioactivation and detoxification.

1.1. Biosynthesis

Biosynthesis starts with the amino acid L-phenylalanine (Phe) (Mentzer and Favre-Bonvin, 1961), which is N-hydroxylated and converted to prunasin by two cytochromes P450 (CYP79 and CYP71) and an UDP-glucosyltransferase (UGT1) (Franks et al., 2008). Finally, prunasin is converted to amygdalin through another unknown UDP-glucosyltransferase (UGT2).

1.2. Bioactivation

Amygdalin can be hydrolyzed by amygdalin hydrolase (AH) to produce prunasin and glucose. Prunasin is subsequently hydrolyzed by prunasin hydrolase (PH), releasing mandelonitrile and glucose (Zheng and Poulton, 1995; Sánchez-Pérez et al., 2008; Del Cueto et al., 2017; Kuroki and Poulton, 1987; Li et al., 1992; Zhou et al., 2002; Sánchez-Pérez et al., 2009, 2012). Both enzymes (AH and PH) are β-glucosidases, which belong to family 1 of the glycoside hydrolases. This family of enzymes (β-glucosidases) catalyzes the hydrolysis of the β-glycosidic bonds between carbohydrates or between a carbohydrate and an aglycone (Morant et al., 2008). Both cyanogenic glucosides and β-glucosidases are separated in different compartments of the cell. Only when tissue is damaged by an attack of an herbivore for example, cyanogenic glucosides are bioactivated by β-glucosidases, releasing, among other products, hydrogen cyanide (Morant et al., 2003; 2008; Zhou et al., 2002; Conn, 1969; Zagrobelny et al., 2004), providing an immediate chemical defence against herbivores (Ketudat et al. 2015). The compartmentalization of cyanogenic glucosides and β-glucosidases prevents any premature HCN release prior to tissue disruption (Poulton and Ping Li, 1994; Poulton, 1990; Swain et al., 1992b). A similar organization has been observed in other species like *Plantago lanceolate* and *Plantago major* between iridoid glycosides and β-glucosidases (Pankoke et al., 2013). Finally, mandelonitrile lyase 1 (MDL1) hydrolyzes mandelonitrile (Swain and Poulton, 1994; Suelves and Puigdoménech, 1998) producing benzaldehyde (which gives the bitter taste) and releasing hydrogen cyanide (which is toxic) (Evreinoff, 1952). Other plants and organisms like insects are able to release this toxic compound in a process called cyanogenesis (Poulton, 1990).

1.3. Detoxification

The operation of a cyanide-detoxification pathway based on nitrilases results in simultaneous production of a nitrogen source for the plant. This route consists of the following two steps: a) β-cyanoalanine synthase catalyzes the reaction between HCN and L-cysteine to synthesize β-cyanoalanine (Floss et al., 1965) and b) nitrilases catalyze the production of ammonia, L-aspartic acid and L-asparagine from H_2O and β-cyanoalanine (Piotrowski et al., 2001; Piotrowski and Volmer, 2006; Jenrich et al., 2007; Kriechbaumer et al., 2007).

In almond the majority of the above-mentioned enzymes have not yet been characterized, except for UGT1 (UGT85A19 (Franks et al., 2008)); MDL1 (Suelves and Puigdoménech, 1998); and two *Phs* have been identified in almond (*Ph691* and *Ph692*) (Sánchez-Pérez et al., 2012). To date, however, no *Ph* gene has been expressed and had its protein activity tested.

As already mentioned, the bitter flavour in almond is produced by the presence of amygdalin in the almond kernel, so it would be possible that the difference between sweet and bitter almonds would be related to differences in biosynthesis, degradation or detoxification that would or not allow the accumulation of amygdalin in the seed. However, it was observed that all enzymes and substrates necessary to produce or degrade prunasin and amygdalin were present in both bitter and sweet almonds (Sánchez-Pérez et al., 2008). Why then do only bitter almond kernels accumulate amygdalin?

From the genetic point of view, given that bitter is the original trait in wild almonds, the alteration of just one allele prevents the accumulation of amygdalin. The question is thus whether this change would

prevent the synthesis of amygdalin or favour its hydrolysis. In the literature, there are two theories to explain why an almond is sweet or bitter, the first based on biosynthesis and the second on hydrolysis.

In the first theory, it was proposed that the anabolic enzyme glucosyltransferase was responsible for the accumulation of amygdalin (Frehner et al., 1990). However, since we know that the bitter trait is recessive, an anabolic enzyme cannot be directly responsible for bitterness. In the second theory, it was focused on the catabolic enzymes, suggesting the absence of a catabolic mechanism resulting in the presence of amygdalin in bitter almond kernels, based on the β-glucosidase activity and the different location of prunasin hydrolases in the apoplast or symplast of the tegument (Sánchez-Pérez et al., 2008, 2009, 2012). For this reason, we have focused in prunasin hydrolases in this study.

The aim of this work was to isolate and characterize different tissue-localized PHs in sweet- and bitter-kernelled almonds to determine whether differences in their genomic or protein sequences are responsible for the sweet or bitter taste of their seeds.

2. Materials and methods

2.1. Plant material

The almonds (*Prunus dulcis* (Miller) D.A. Webb) used in this study, from the genotypes Lauranne (*SkSk*, sweet), D05-187 (*sksk*, bitter) and S3067 (*sksk*, bitter), were provided by the Almond Breeding Program of the "Centro de Edafología y Biología Aplicada del Segura" (CEBAS-CSIC, Murcia, Spain).

2.2. The evolution of PHs during kernel development

In order to study PHs synthesis during fruit ontogeny, fruits from the Lauranne, D05–187 and S3067 genotypes were harvested during the beginning (JD89), middle (JD130) and final (JD167) stages of kernel development. "JD" stands for Julian Days, meaning days after the first of January. The teguments, nucellus + endosperms (difficult to separate) and cotyledons were isolated, ground in liquid nitrogen, stored at −80 °C and analyzed separately. RNA extraction was carried out with 100 mg of each tissue using an Ultra Clean Plant RNA Isolation kit (MO BIO) according to manufacturer's instructions.

As previously shown, *Ph691* and *Ph692* are present in both sweet and bitter almonds (Sánchez-Pérez et al., 2012). PH691 was selected for the present study due to its high homology (73%–94% protein similarity) compared to other PHs previously described and characterized in *P. serotina* (Zhou et al., 2002). PH692, on the other hand, showed between 71% and 79% protein similarity with other PHs.

RT-PCR was performed to obtain cDNA using SuperScript III Taq DNA Polymerase (Invitrogen, http//www.invitrogen.com/), where 1 μg of total RNA was used with the primers 691ATG and 691UGA (Table 1).

The amplification conditions were as follows: 30 min at 55 °C, 30 cycles of (2 min at 94 °C, 15 s at 94 °C, 30 s at 59 °C) and one cycle of 2.30 min at 68 °C, and one cycle of 5 min at 68 °C. PCR products were analyzed by electrophoresis in 1% agarose gel with a 1 kb Plus Ladder (Invitrogen) or Lambda Hind III (Thermofisher Scientific). The expected band size was 1638 bp, which was cut and purified following the protocol of the Ultra Clean DNA Purification Kit (MO BIO).

2.3. Comparison of PH sequences

2.3.1. Cloning in Escherichia coli

The amplified cDNA was cloned into pGEM-T easy Vector (Promega). After overnight ligation at 4 °C, chemical transformation took place by adding 2 μL of ligation to *E. coli* competent cells in a total volume of 50 μL. The mixture was incubated in ice for 20 min and then subjected to heat shock for 45 s at 42 °C, followed by an additional 2 min in ice. Immediately afterwards, 950 μL SOC medium was added, and then the mixture was incubated for 1.5 h at 37 °C with shaking. Finally, 100 μL of each transformation culture was spread on LB agar plates with kanamycin (50 μg/mL) and incubated overnight at 37 °C. Nested PCR was performed with the internal primer pairs 27F and 27R (Table 1) to check the positive clones using the following PCR conditions: 2 min at 95 °C, 30 cycles of 30 s at 95 °C, 30 s at 57 °C and 1 min at 72 °C, and one cycle of 5 min at 72 °C. The expected band size was 1050 bp.

2.3.2. Minipreps and sequence analyses

Once positive colonies were identified by nested PCR, plasmid was purified using a Pure Link Quick Plasmid Miniprep Kit (Invitrogen). DNA was quantified based on A280 (Nanodrop; Thermo Scientific, http://www.thermofisher.com) and sent to sequence Secugen (http://www.secugen.es/) for sequencing. Sequences were visualized with the software BioEdit and BlastX versus National Center for Biotechnology Information (NCBI).

2.3.3. Phylogenetic tree, signal peptide and motif analyses

Neighbor-joining phylogenetic trees were constructed using MEGA 5.5 software (Tamura et al., 2011); (http://www.megasoftware.net/index.html) with 1000 bootstrap trials performed. For signal peptide prediction, three aspects were analyzed: the peptide size, cleavage site and the place the protein would be led to. To do this, we used the following two web servers: SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/) and WoLF PSORT (http://wolfpsort.org/). For the N-glycosylation sites, the NetNGlyc 1.0 Server was used (http://www.cbs.dtu.dk/services/NetNGlyc/).

2.4. Detection of β-glucosidase activity

The β-glucosidase activity was determined in *Nicotiana benthamiana* plants transformed with *Agrobacterium tumefaciens*. Clones used for this experiment came from the mid ripening stage (JD 130) as in this stage the *Ph691* was expressed in all the tissues studied.

2.4.1. Cloning in Agrobacterium tumefaciens

Nine (three genotypes times three tissues) cDNA sequences of the *Ph691* gene were amplified with the primers *691B1F* and *692B2R* containing the attB1 and attB2 Gateway cloning sites (Table 1) using PCR Hot Master under the following conditions: 2 min at 94 °C, 35 cycles of 20 s at 94 °C, 20 s at 57 °C and 2 min at 65 °C, and one cycle of 5 min at 65 °C. The PCR products were cloned by Gateway recombination in two cloning steps: 1) using Gateway BP Clonase II Enzyme Mix (Invitrogen), where the PCR products were introduced into the pDONOR207 entry clone, and 2) using LR Clonase II Enzyme Mix

Table 1
Designed primers for almond cDNA, annealing temperatures, and expected product size.

Primer	Sequence (5′-3′)	Tm (°C)	Expected size (bp)
691ATG	ATGGCATTGCAATTCCGCTCTTTGCTCTTGTG	57	1638
691UGA	TCAAATTTGATACACAAATTTGGTAGCCCTA	57	1638
27F	AATTATGAAGGATATGGGGTTGG	57	1050
27R	CACCTCTGTTACCTCCAAGCA	57	1050
691B1F	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGCATTGCAATTCCGCTCTTTGCTCTTGTG	57	1667
691B2R	TCAAATTTGATACACAAATTTGGTAGCCCTATCAAATTTGATACACAAATTTGGTAGCCCTA	57	1668

(Invitrogen) into pJAM1502 as the destination vector (Luo et al., 2007). The bacteria strains used in the transformation were *E. coli* DH5α chemically competent cells (Invitrogen) and AGL1 (*A. tumefaciens*) electro-competent cells (2.2 V, 25 μF, 400Ω in a BIORAD machine). Finally, AGL1 cells from the different tissues containing *Ph691* were selected on LB agar + rifampicin (25 μg/mL) + kanamycin (50 μg/mL).

2.4.2. Agroinfiltration

Overnight cultures of *A. tumefaciens* containing *Ph691* from the different tissues (tegument, nucellus and cotyledon) from the three genotypes in pJAM1502 and the gene-silencing inhibitor protein p19 (Voinnet et al., 2003) were grown in LB-containing kanamycin (50 μg/mL) and rifampicin (25 μg/mL), while p19 was grown alone with kanamycin, rifampicin and tetracycline (10 μg/mL). Cultures were harvested by centrifugation at 3000 rpm for 10 min and resuspended to an OD600 of 2.0 in 1 mL of acetosyringone solution: 50 mL water, 0.5 mL MES 10 mM, 0.5 mL $MgCl_2$ 10 mM and 5 μL acetosyringone 100 μM. After 2 h of incubation at room temperature, a combined solution was made in a total volume of 2 mL: 0.5 mL of p19, 0.5 mL of Agro solution (OD600 of 2.0) containing *Ph691* from each genotype and 1 mL of acetosyringone solution. *N. benthamiana* leaves were agroinfiltrated using a 1 mL syringe. After 4–5 days, leaf discs (1 cm diameter) were cut from infiltrated leaves. To check if the nine transformed clones expressing *Ph691* had β-glucosidase activity, the following two methods were used.

2.4.3. Fast Blue BB salt assay

This assay for measuring β-glucosidase activity was based on of Sánchez-Pérez et al. (2009), consisting in the incubation of 6-bromo-2-naphthyl-β-D-glucopyranoside (BNG) (Sigma Aldrich) as a general substrate for β-glucosidases in the presence of Fast Blue BB salt (Sigma-Aldrich). BNG gives a reddish color when hydrolyzed. Firstly, agroinfiltrated *N. benthamiana* leaf samples were ground in liquid nitrogen. Then, 20 mM MES buffer pH 6 was added in a 1:3 ratio. Protein extracts were centrifuged at 20,000 g for 30 min at 4 °C. The supernatant was collected and quantified based on A280 (Nanodrop; Thermo Scientific, http://www.thermofisher.com). The entire process was carried out at 4 °C. The protein composition was analyzed by SDS–PAGE (12% gel) following the application of protein (18 μL, concentration around 10 mg/mL) combined with 10% bromo-phenol blue (2 μL), 100% glycerol (10 μL) and 0.5% SDS (10 μL) in a final volume of 40 μl. At the end of electrophoresis (2 h, 175 V, 4 °C), the gels were washed (2 × 10 min) in Fast Blue BB buffer [50 mM sodium citrate, 100 mM phosphate (pH 5.8)]. Solution A (15 mg Fast Blue BB salt + 20 mL Fast BB Buffer) and solution B [20 mg BNG + 200 μL DMF (dimethylformamide)] were mixed and the gel incubated with the solution at 37 °C for 2 h with gentle shaking in foil paper.

2.4.4. Umbellyferyl substrate

This second method uses umbelliferyl 4-methyl-β-D-glucoside (Sigma Aldrich), which is an excellent fluorogenic substrate for detecting β-glucosidases. Leaf discs were positioned in a tube with 150 μL MES buffer (Sigma Aldrich) 20 mM pH 6 and 6 μL of the general substrate 4-methyl-umbelliferyl-β-D-glucoside (Sigma Aldrich) 25 mM. Control was made with a p19 infiltrated leaf disc. The tubes were incubated for 15 min at 37 °C and observed under UV light. The β-glucosidase activity was detected by fluorescence.

2.5. Verification of PH activity

Clones used for this experiment came from the mid ripening stage (JD 130), as in this stage *Ph691* was expressed in all the tissues studied.

2.5.1. Feigl-Anger

The Feigl-Anger method is a qualitative assay used to detect hydrogen cyanide released. If a putative PH cleaved prunasin, then hydrogen cyanide could be detected by blue staining of the Feigl-Anger paper (Feigl and Anger, 1966). To prepare the paper, we separately dissolved 5 g of copper ethylacetoacetate (Alfa Aesar, http://www.alfa.com/) and 4.4-methylenebis (N, N-dimethylaniline) (Sigma-Aldrich) in 0.5 L of water each and then combined both solutions. We then wetted Whatman 3 MM paper (Sigma-Aldrich) (8 × 12 cm), with the solution and then dried it. The paper was stored at 4 °C until use. A total of 200 μL of 20 mM MES buffer pH 6.5 plus 15 μL substrate 4 mM were added to a 96-well microtiter plate. The following substrates were used: prunasin, dhurrin, linamarin, lotaustralin (cyanogenic monoglucosides), amygdalin and linustatin (cyanogenic diglucosides). An agroinfiltrated leaf disc from a *N. benthamiana* plant was added and triturated in each well; three ELISA well-discs were made per *Ph691*construct. Feigl-Anger paper was positioned between the plate and the lid. Cyanogenesis was detected after 2 h of exposure by blue staining of the Feigl-Anger paper.

2.5.2. Hydrogen cyanide release

The hydrogen cyanide released from the reaction of crude protein extracts and substrate was determined using the colorimetric method described by Lambert (Lambert et al., 1975), as modified by Halkier and Møller (1989). The reaction mix (total volume: 100 μL) contained 10 μL of substrate 1 mM, 10 μL of protein (1 mg/mL) and 80 μL of MES 20 mM buffer pH 6. Samples without substrate were used as control. Following incubation (10 min, 30 °C, 300 rpm shaking), the samples were frozen in closed Eppendorf tubes with the presence of liquid nitrogen to avoid loss of volatile HCN formed. While samples thawed at room temperature, 40 mL of 6 M NaOH was added. A 60-mL aliquot from each sample was transferred to a 96-well microtiter plate. Immediately, the following reagents were added to each well: 12.5 μL of 100% glacial acetic acid (Sigma-Aldrich), 50 μL of reagent A (made by dissolving 50 mg of *N*-chlorosuccinimide (Sigma-Aldrich) and 125 mg of succinimide (Sigma-Aldrich) in 50 mL of water) and 50 μL of reagent B (made by mixing 15 mL of pyridine (Sigma-Aldrich) and 3 g of barbituric acid (Fluka) in 50 mL of water). After 5 min of incubation at room temperature, the wells were scanned between 450 and 700 nm with a peak reading made at 584 nm. The hydrogen cyanide released was calculated against KCN standards made in 1 M NaOH. For the standard deviation, at least, two replicates per genotype were analyzed.

3. Results

3.1. PH evolution during kernel development

Our findings regarding the presence or absence of PHs in each tissue are described below.

3.1.1. Stage 1: beginning of development (JD 89) (Fig. 2 a)

At this time point, the immature kernel was composed of a nucellus and tegument. Cotyledons had not yet developed. In this stage, the DNA band characteristic of the *Phs* (1638 bp) was not detected in the sweet genotype. In the bitter genotype (S3067), it was more intense in the tegument than in the nucellus. At the beginning we could only amplify S3067 since it was the earliest genotype.

3.1.2. Stage 2: halfway through development (JD 130) (Fig. 2 b)

At this time point, endosperm and cotyledons began to develop inside of the tegument, and the nucellus was still visible. The amplified DNA bands of *Ph691* from the bitter genotype S3067 were weaker in the tegument than in Stage 1, but were similar in the nucellus. For the first time, *Ph691* was detected in the cotyledon, being so strong that the band migrated a bit further when compared to the bands amplified in the other tissues. In the sweet Lauranne, *Ph691* was also amplified in the three tissues.

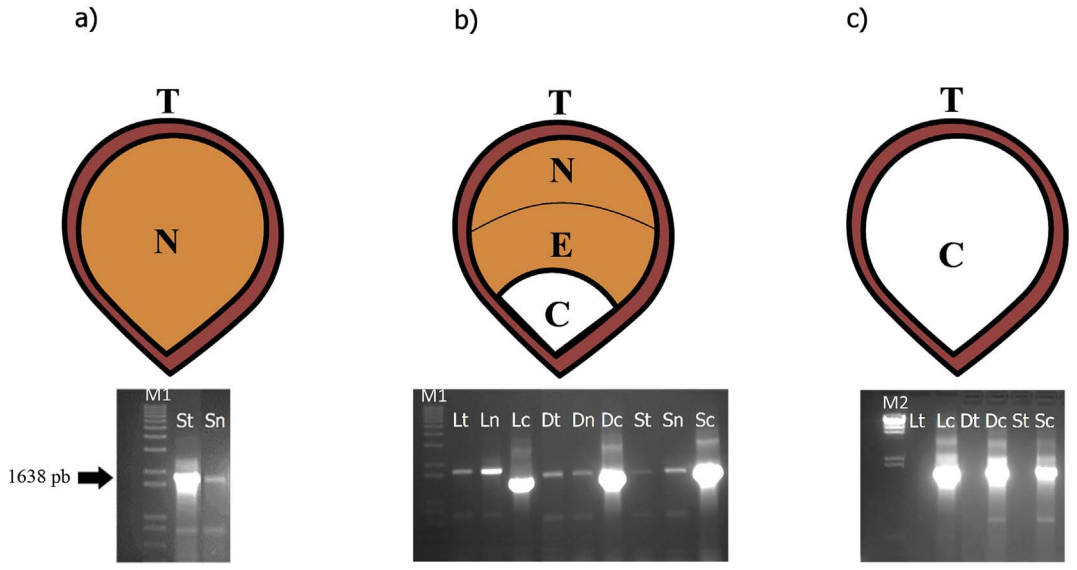

Fig. 2. Kernel development and detection of prunasin hydrolases encoding genes by RT-PCR in the three development stages: a) beginning (JD 89); b) halfway (JD 130); c) final (JD 167). T: tegument; N: nucellus; E: endosperm; C: cotyledon. The arrow indicates the band size of 1638 pb expected for amplification of *prunasin hydrolase 691* (*Ph691*). M1, Ladder 1 kb+; M2: Ladder Lambda HindIII. L: Lauranne (sweet); D: D05-187 (bitter); S: S3067 (bitter).

3.1.3. Stage 3: final development (JD 167) (Fig. 2 c)

At this time point, the kernel was only made up of the cotyledons and the dried tegument. The *Ph691* gene was amplified from the cotyledons and but not from the tegument, in both the sweet and bitter genotypes. This makes sense, as at this stage the tegument is a very dry tissue surrounding the cotyledon.

3.2. Comparison of PH sequences

The amplified sequences of the *Ph691* gene contained 13 exons and 12 introns (Fig. 3). The full-length cDNA in the 9 clones contained 1635 bp encoding 544 amino acid proteins, which contained 5 putative N-glycosylation sites (Table 2). The PH691s studied here had ITENG and NEP motifs characteristic of the active site of the glycoside hydrolase family 1 and the INKKGIEYY motif specific for PH (Fig. 3). No differences were detected between the active sites in sweet and bitter

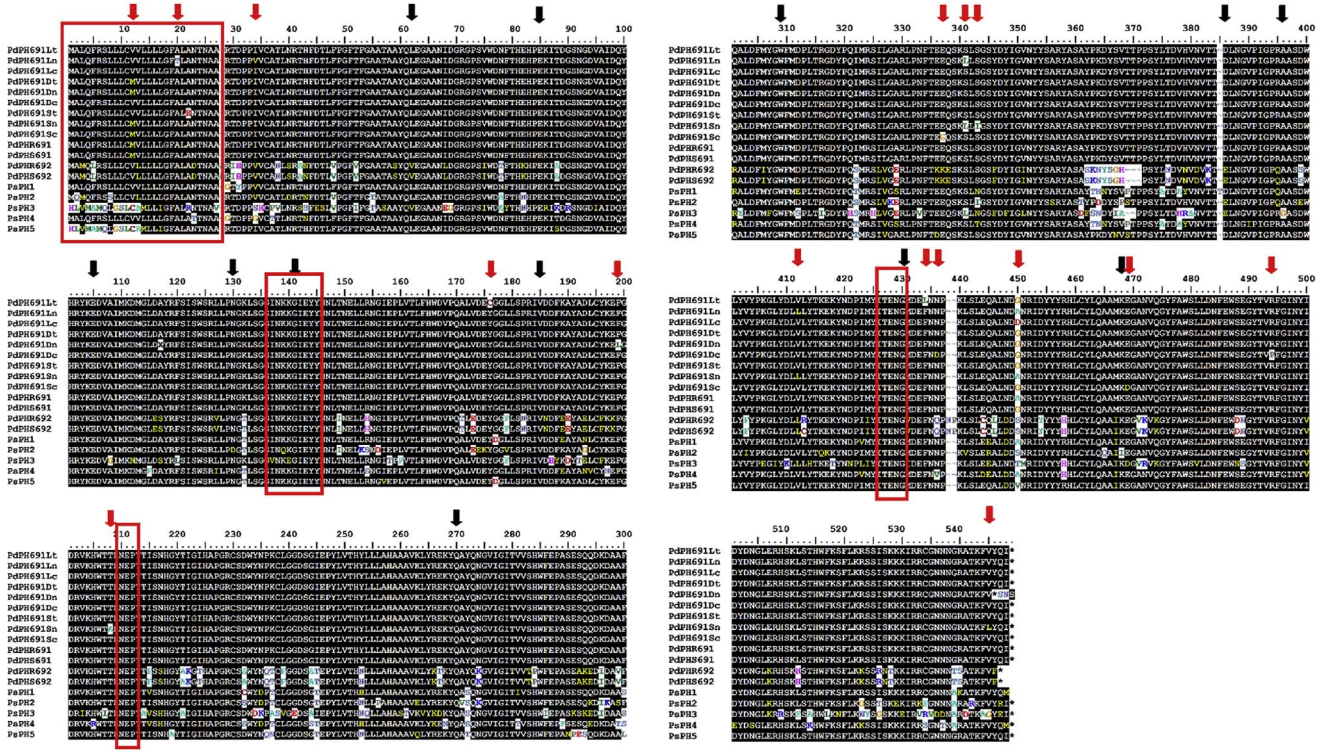

Fig. 3. Amino acid sequences of prunasin hydrolases (PHs) from *P. serotina* (Ps) and *P. dulcis* (Pd). Differences in amino acids are marked in color. Red frames: signal peptide and NEP and ITENG motif of the active site and INKKGIEYY motif of PH. Black arrows: introns. Red arrows: SNPs. L: Lauranne (sweet); D: D05-187 (bitter); S: S3067 (bitter); t: tegument; n: nucellus; and c: cotyledon. PsPH1, PsPH2, PsPH3, PsPH4 and PsPH5 (Zhou et al., 2002). PdPHR691, PdPHS691, PdPHR692 and PdPHS692 (Sánchez-Pérez et al., 2012). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Comparison of major features of prunasin hydrolases from *P. dulcis* and *P. serotina* based on nucleotide and amino acid sequences. Sequence length, polypeptide length, N-glycosylation sites, signal peptide and location of the enzyme are indicated. L: Lauranne (sweet); D: D05-187 (bitter); S: S3067 (bitter); t: tegument; n: nucellus; and c: cotyledon. *PsPh1, PsPh2, PsPh3, PsPh4* and *PsPh5* (Zhou et al., 2002). *PdPhR691*, *PdPhS691, PdPhR692 and PdPhS692* (Sánchez-Pérez et al., 2012).

	Sequence length	Polypeptide length	N-glycosylation sites	Signal peptide	Location
PdPh691Lt	1635	544	5	23	Vacuolar
PdPh691Ln	1635	544	5	27	Vacuolar
PdPh691Lc	1635	544	5	23	Vacuolar
PdPh691Dt	1635	544	5	27	Vacuolar
PdPh691Dn	1635	544	5	27	Vacuolar
PdPh691Dc	1635	544	5	23	Vacuolar
PdPh691St	1635	544	5	27	Extracellular
PdPh691Sn	1635	544	5	27	Vacuolar
PdPh691Sc	1635	544	5	27	Vacuolar
PsPh1	2056	549	8	23	Vacuolar
PsPh2	2059	544	2	23	Vacuolar
PsPh3	1960	537	6	27	Vacuolar
PsPh4	1911	545	7	23	Vacuolar
PsPh5	1819	542	5	23	Vacuolar
PdPhR691	1635	544	5	27	Vacuolar
PdPhS691	1635	544	5	27	Vacuolar
PdPhR692	1629	542	5	23	Vacuolar
PdPhS692	1629	542	5	23	Vacuolar

genotypes.

Regarding the signal peptide (Table 2), the length was 27 amino acids for most of the genotypes and tissues analyzed but only 23 amino acids in the Lauranne tegument, the Lauranne cotyledon and the D05-187 cotyledon PH sequences. According to signal peptide sequences found with the SignalP 4.1 Server program, the PH proteins were always predicted targeted to the vacuole except in the tegument of S3067, in which case the protein was predicted to be extracellular.

Table 3 shows amino acid differences between the nine PH sequences studied. Although some nucleotide differences were detected in the exons, they were only translated into an amino acid change in 17 cases (in exons 1, 6, 7, 9, 11, 12 and 13). Three of these changes were positioned in the signal peptide at amino acids 12, 20 and 22. The differences observed were not consistent between the sweet and bitter genotypes or between tissues. However, that small differences of each genotype could have a consequence in the activity of the protein expressed.

Sequence comparisons of the tissues (tegument, nucellus and cotyledon) from the different genotypes are presented in Supplemental Fig. 1. Four SNPs were identified in the codons for amino acids 12, 340, 410 and 446. In the four cases, the difference was detected in the nucellus in comparison with tegument and cotyledon.

When we compared the *Phs* studied here, the percentage of nucleotide similarity ranged between 98 and 99 in our 9 sequences (Table 4). No differences were found between sweet and bitter genotypes. As expected, there was high homology (99%) with the two sequences of *Ph691* described in almond (Sánchez-Pérez et al., 2012). Compared to black cherry sequences, *PsPh1* and *PsPh5* were the most similar (94% and 96%, respectively), and *PsPh3* showed a greater difference than the others (Table 4).

Phylogenetic analysis clearly separated one group of sequences from the rest based on the positions of the clones in the tree and the percentage of bootstrap support (Fig. 4). This group contained the nine *Phs* characterized in this study and, as expected, the two *PdPh691*s from a sweet (Ramillete, R) and a bitter (S3067, S) genotype (Sánchez-Pérez et al., 2012). According to the PHs from black cherry, *Ps*PH5 had the closest amino acid sequence to the PHs from almond, which agrees with the nucleotide similarity described before. In this analysis, AH from *P. serotina* appears as a distinct branch, so we clearly observed the differences between all the PHs and the AH.

3.3. Detection of β-glucosidase activity

3.3.1. Fast blue BB method

With this method, the nine PHs showed β-glucosidase activity, except in D05-187 tegument (Fig. 5a). Different bands of different sizes (between 50 and 75 kD) were observed. Moreover, some clones showed more than one band (Lauranne nucellus and cotyledon), which could indicate the presence of different PH isoforms. These differences were not related to the sweet or bitter phenotype.

3.3.2. Umbelliferyl method

With this method, β-glucosidase activity was detected in all the clones except in the D05-187 tegument, which showed low fluorescence, similar to the p19 control (background) (Fig. 5b). The results were thus similar to the Fast BB results described above.

Table 3
Amino acid differences between sequences of nine PHs. Exon, gDNA, cDNA and aa positions of the differences are indicated. These nine PHs are compared with five PHs described in *P. serotina* by Zhou et al. (2002) and with two PHs described in sweet (PdPHR691 and PdPHR692) and bitter (PdPHS91 and PdPHS92) almonds (Sánchez-Pérez et al., 2012) (L: Lauranne, D: D05–187, S: S3067).

Difference	Position	Tegument				Nucellus			Cotyledon			*Prunus serotina*					*Prunus dulcis*			
	EXON	aa	L	D	S	L	D	S	L	D	S	Ps PH1	Ps PH2	Ps PH3	Ps PH4	Ps PH5	PdPH R691	PdPH S691	PdPH R692	PdPH 692
V/M/C	EXON 1	12	V	M	V	V	M	M	V	V	M	V	V	C	V	C	M	M	V	V
A/T	EXON 1	20	A	A	A	T	A	A	A	A	A	A	A	A	A	A	A	A	A	A
A/E/R	EXON 1	22	A	A	E	A	A	A	A	A	A	A	A	R	A	A	A	A	A	A
I/V/S/G	EXON 1	34	I	I	I	V	I	I	I	I	I	V	V	S	G	I	V	I	V	V
Y/C	EXON 6	176	C	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
F/L	EXON 7	199	F	F	F	F	L	F	F	F	F	F	F	F	F	F	F	F	F	F
T/A	EXON 7	208	T	T	T	T	T	A	T	T	T	T	T	T	T	T	T	T	T	T
E/G/K	EXON 9	337	E	E	E	E	E	E	E	E	G	E	E	E	E	E	K	E	K	K
S/L	EXON 9	341	S	S	S	L	S	L	S	S	S	S	S	L	S	S	S	S	S	S
S/I/N/T	EXON 9	343	S	S	S	S	S	I	S	S	S	N	I	N	T	S	S	S	S	S
V/L	EXON 11	412	V	V	V	L	V	L	V	V	V	V	V	L	V	V	L	V	L	L
F/L	EXON 12	434	L	F	F	F	R	F	F	F	F	F	F	F	F	F	F	F	F	F
N/D/Q	EXON 12	436	N	N	N	N	N	N	N	D	N	N	N	N	V	N	Q	N	Q	Q
G/A/D/S/T/V	EXON 12	450	G	G	G	A	G	A	D	G	G	A	S	T	A	V	S	G	S	S
E/D	EXON 13	469	E	E	E	E	E	E	E	E	D	E	E	D	E	E	E	E	E	E
R/P	EXON 13	494	R	R	R	R	R	R	R	P	R	R	R	R	R	R	R	R	R	R
V/L/G	EXON 13	545	V	V	V	V	V	L	V	V	V	V	V	G	V	V	V	V	V	V

Table 4
Percentage of nucleotide similarity between our nine *Phs* and other sequences of *P. serotina* and *P. dulcis*. L: Lauranne (sweet); D: D05-187 (bitter); S: S3067 (bitter); t: tegument; n: nucellus; and c: cotyledon. *PsPh1, PsPh2, PsPh3, PsPh4* and *PsPh5* (Zhou et al., 2002). *PdPhR691, PdPhS691*, *PdPhR692* and *PdPhS692* (Sánchez-Pérez et al., 2012). *Lt* stands for *PdPh691Lt*, *Ln* means *PdPh691Ln*, and so on.

	Sweet			Bitter					
	Lt	*Ln*	*Lc*	*Dt*	*Dn*	*Dc*	*St*	*Sn*	*Sc*
PdPh691Lt	100.00	98.90	99.69	99.45	99.33	99.79	99.27	98.96	99.57
PdPh691Ln	–	100.00	98.90	98.96	98.71	98.90	98.78	99.45	98.84
PdPh691Lc	–	–	100.00	99.39	99.27	99.72	99.20	98.96	99.51
PdPh691Dt	–	–	–	100.00	99.39	99.59	99.27	99.14	99.51
PdPh691Dn	–	–	–	–	100.00	99.45	99.14	98.99	99.39
PdPh691Dc	–	–	–	–	–	100.00	99.24	99.04	99.60
PdPh691St	–	–	–	–	–	–	100.00	98.83	99.20
PdPh691Sn	–	–	–	–	–	–	–	100.00	99.02
PdPh691Sc	–	–	–	–	–	–	–	–	100.00
PdPhR691	99.00	99.00	99.00	99.00	99.00	99.00	99.00	99.00	99.00
PdPhS691	99.00	99.00	99.00	99.00	99.00	99.00	99.00	99.00	99.00
PdPhR692	79.00	79.00	79.00	79.00	79.00	79.00	79.00	79.00	79.00
PdPhS692	79.00	79.00	79.00	79.00	79.00	79.00	79.00	79.00	79.00
PsPh1	94.00	94.00	94.00	94.00	93.00	94.00	94.00	94.00	94.00
PsPh2	91.00	91.00	91.00	91.00	91.00	91.00	91.00	91.00	91.00
PsPh3	82.00	83.00	82.00	82.00	82.00	83.00	82.00	83.00	82.00
PsPh4	92.00	92.00	92.00	92.00	92.00	92.00	92.00	92.00	92.00
PsPh5	96.00	96.00	96.00	96.00	96.00	96.00	96.00	96.00	96.00

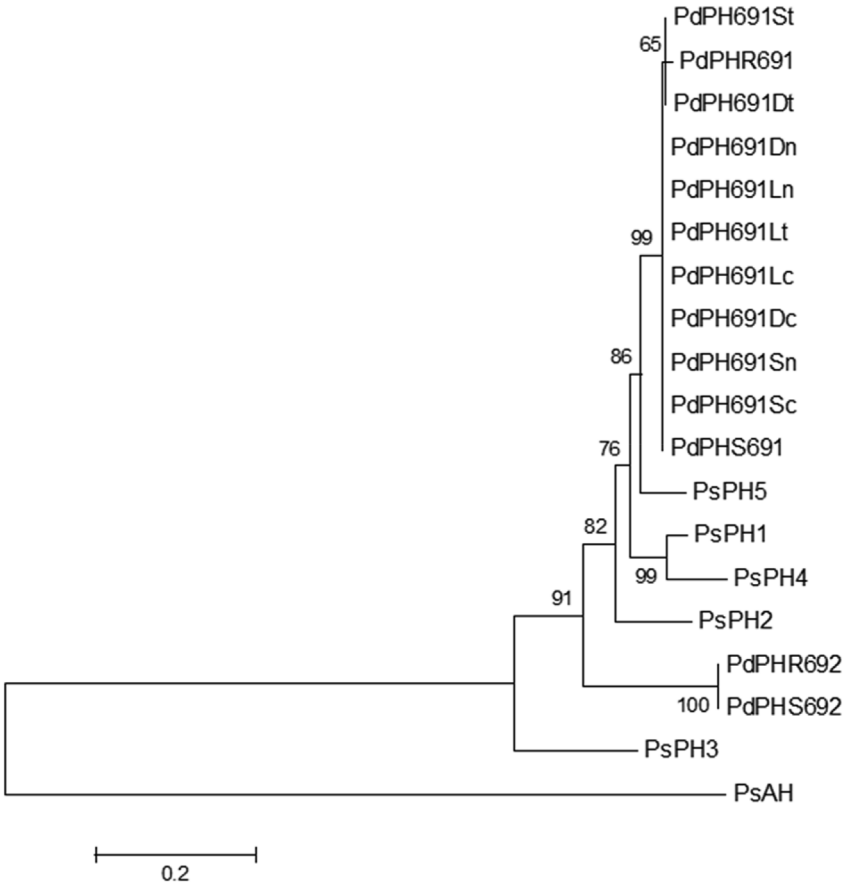

Fig. 4. Phylogenetic tree of the prunasin hydrolases (PHs) characterized in this study and other PHs from *P. serotina* (Ps) and *P. dulcis* (Pd). The numbers represent percentage bootstrap support (1000 replicates). L: Lauranne (sweet); D: D05-187 (bitter); S: S3067 (bitter); t: tegument; n: nucellus; and c: cotyledon. PsPH1, PsPH2, PsPH3, PsPH4 and PsPH5 (Zhou et al., 2002). PdPHR691, PdPHS691, PdPHR692 and PdPHS692 (Sánchez-Pérez et al., 2012), and PsAH (Li et al., 1992).

3.4. Verification of PH activity

3.4.1. Feigl-Anger assay

The addition of prunasin to the extract from agroinfiltrated *N. benthamiana* leaves showed PH activity of the nine PHs, both in the sweet and bitter genotypes. The negative control p19 did not show any activity (Fig. 6). Hydrolase activity was also detected in Ln, Lc, Dn and Dc with the monoglucoside dhurrin, but not with the diglucosides amygdalin and linustatin or with the other monoglucosides assayed (linamarin and lotaustralin) (Fig. 6), showing that the PH has a high substrate specificity for prunasin.

3.4.2. Hydrogen cyanide assay

The assay to quantify the PH activity based on hydrogen cyanide

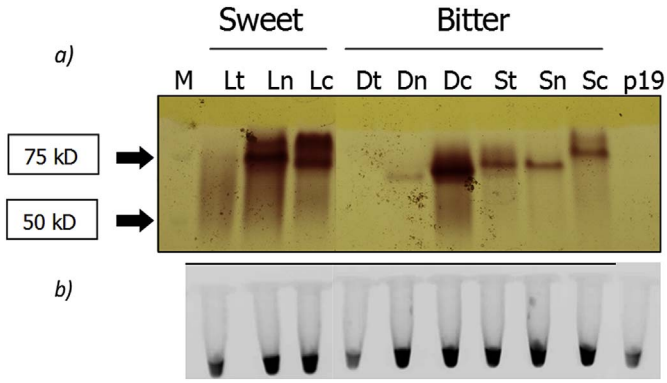

Fig. 5. β-Glucosidase activity, from agroinfiltrated *N. benthamiana* leaves expressing *prunasin hydrolase 691* belonging to sweet and bitter cultivars from mid ripening stage (JD 130), detected by the following two methods: a) *Fast Blue BB salt.* L: The band size ranged between 50 and 75 kD; and b) *Umbellifery method.* Fluorescence under UV light using the substrate 4-methyl-umbelliferyl-β-D-glucoside. Lauranne (sweet); D: D05-187 (bitter); S: S3067 (bitter); p19: negative control; t: tegument; n: nucellus; and c: cotyledon. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

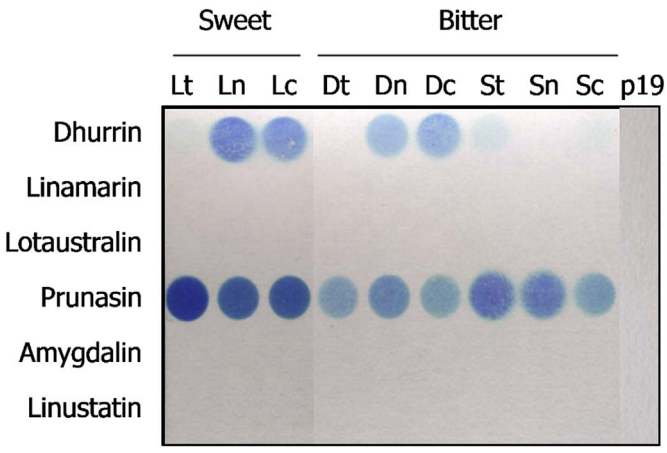

Fig. 6. Substrate specificity detected by Feigl-Anger method from agroinfiltrated *N. benthamiana* leaves expressing *prunasin hydrolase 691* from mid ripening stage (JD 130), incubated with dhurrin, linamarin, lotaustralin, prunasin, amygdalin and linustatin. p19: negative control. Blue indicates the positive reaction liberating CN from the PH691 activity. L: Lauranne (sweet); D: D05-187 (bitter); S: S3067 (bitter); t: tegument; n: nucellus; c: cotyledon. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

release was performed with the substrates representing cyanogenic mono- and diglucosides. Prunasin was recorded as the preferred substrate, low hydrolase activity was observed towards dhurrin, and no or very low activity towards amygdalin (Fig. 7).

With the substrate prunasin, the PH from the Lauranne nucellus liberated the highest amount of hydrogen cyanide, with more than 500 nmol HCN/g per agroinfiltrated leaf. The Lauranne cotyledon PH liberated the second highest amount of hydrogen cyanide, followed by the PHs of D05-187 cotyledon and the D05-187 nucellus, which liberated between 300 and 400 nmol HCN/g. The rest of the PHs liberated between 100 and 200 nmol HCN/g. Only the D05-187 tegument PH showed a very low level of hydrogen cyanide release, which agrees with the results of the Fast BB and umbelliferyl assays described above. With dhurrin, only the Lauranne tegument PH had significant hydrogen cyanide release (almost 200 nmol HCN/g), whereas the rest of the PHs showed very low activity.

4. Discussion

4.1. The evolution of PHs during kernel development

The kernel development stages described in this work were similar to those previously analyzed in almond (Martínez-Gómez et al., 2008; Abarrategui, 2010) and in black cherry (Swain et al., 1992a). *Ph691* appeared in the tegument and nucellus of the bitter S3067 at the beginning of the development period; in the tegument, nucellus and cotyledon in both the bitter and sweet genotypes halfway through the development period; and only in the cotyledon of the bitter and sweet genotypes at the final development stage.

4.2. Comparison of PH sequences

The amplified sequences of the *Ph691* contained 13 exons and 12 introns (Fig. 3), the same number of exons previously described in almond (Sánchez-Pérez et al., 2012) and in black cherry (Zhou et al., 2002). On the other hand, the five PHs identified in black cherry had between 537 and 549 amino acids, compared with the 544 amino acids in the PHs here studied. In black cherry, between 2 and 8 N-glycosylation sites were detected (Zhou et al., 2002) compared to 5 in the PHs here studied.

Regarding the signal peptide (Table 2), according to Sánchez-Pérez et al. (2012), the predicted cleavage site for PH691 was between amino acids 26 and 27 (TNA-AR), whereas for PH692 it was between amino acids 22 and 23 (ALA-DT). Therefore, the signal peptide length of 27

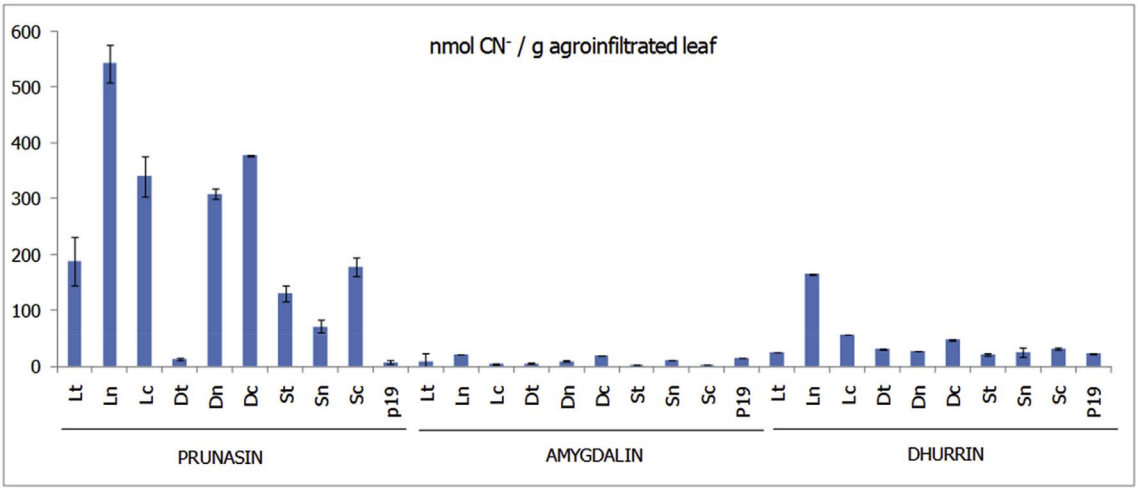

Fig. 7. Hydrogen cyanide (nmol HCN/g fresh tissue) released from agroinfiltrated *N. benthamiana* leaves from mid ripening stage (JD 130) by the PHs encoded by the nine *Ph691*s against the substrates prunasin, amygdalin and dhurrin. L: Lauranne (sweet); D: D05-187 (bitter); S: S3067 (bitter); t: tegument; n: nucellus; and c: cotyledon.

amino acids for most of the genotypes and tissues in this study was more similar to PH691 (Sánchez-Pérez et al., 2012). On the other hand, the Lauranne tegument, Lauranne cotyledon and D05-187 cotyledon had 23 amino acids, making them more similar to PH692.

The differences in the N-terminal sequences of the different PH691s were minor compared to the differences observed in the PH isoforms from black cherry (Li et al., 1992). The sequence of the N-terminal signal peptide may target β-glucosidases to either the symplast or apoplast (Morant et al., 2008). Previously, a different localization (symplastic versus apoplastic) for these catabolic enzymes were reported in sweet and bitter genotypes (Sánchez-Pérez et al., 2012). This was not observed in the current study. One reason could be that the PHs in the former study we localized using an antibody common to more than one PH. It would be interesting to clone *Ph692* to see how it localizes in the tegument of bitter species. The PH691s studied here possessed ITENG and NEP sequence motifs characteristic of the active site of the glycoside hydrolase family 1 and the INKKGIEYY motif specific for PH (Fig. 3). These active-site motifs have also been described in PHs (Kuroki and Poulton, 1987) and AHs (Zheng and Poulton, 1995) from black cherry. No differences were detected between the active sites in sweet and bitter genotypes.

Another difference had previously been reported in the amino acid sequences at position 414 in sweet and bitter genotypes (Zhou et al., 2002). This difference was not observed within the PH sequences here studied.

The similarity between the amino acid sequences of the almond PHs here studied and PH sequences from different species verifies that the almond PHs belong to the BGA family of β-glucosidases based on: a) sequence similarities and amino acid lengths, b) signal peptide size, c) ITENG, NEP and INKKGIEYY motifs, d) number of exons and introns, and e) N-glycosylation sites. BGAs (glycoside hydrolase family 1) includes β-glucosidases, phosphor-β-glucosidases, thio-β-glucosidases and β-galactosidases from archaebacteria, bacteria, plants and mammals.

In this study we could not assign specific sequence differences between the PHs to sweet and bitter genotypes. The next step was therefore to determine whether the putative PHs studied possessed β-glucosidase and PH activity and whether the sequence differences influenced the prunasin hydrolase activity.

4.3. Detection of β-glucosidase activity

All putative PHs here studied, except in the D05-187 tegument, possessed β-glucosidase activity. Differentiation between PHs from sweet and bitter genotypes in terms of their PH activity was not possible. In an earlier study (Abarrategui, 2010), a high level of β-glucosidase activity was reported in the cotyledons of sweet and bitter almond genotypes, very low activity in the nucellus and endosperm, and no activity in the tegument. This distribution of PH activity was not observed in the present study. One reason could be that the analyses were carried out on kernels at different stages of ripening. The localization of the PH differs from the beginning to the end of the ripening season (Sánchez-Pérez et al., 2012).

With respect to the protein bands stained for β-glucosidase activity, different masses between 50 and 75 kD were observed. A common structure for most β-glucosidases with molecular masses in the 55 to 65 kD region has previously been reported (Poulton, 1990). Four AH isoenzymes of 52 kD from black cherry have been isolated (AH I, I′, II and II') (Li et al., 1992). Furthermore, PHs of 60 kD for *P. domestica* and of 68 kD for black cherry (Dicenta et al., 2002) and five PHs in black cherry with masses between 52 and 68 kD have been described (Zhou et al., 2002).

4.4. Verification of PH activity

None of the techniques used in this study enabled identification of sequence differences related to the bitterness phenotype. We still need to determine, however, why the PH from the D05-187 tegument showed reduced β-glucosidase activity.

In accordance with our results, Li et al. (1992) also reported the specificity of PH towards prunasin in black cherry. PH and AH specificity towards prunasin and amygdalin, respectively, was demonstrated in black cherry and almond was shown (Zhou et al., 2002; Sánchez-Pérez et al., 2009). In fact, in 2009, this activity was obtained using protein extracts from almond and not from heterologous expression of a prunasin hydrolase. This current study is therefore the first to demonstrate functional expression of a prunasin hydrolase from almond.

As expected, PH is not able to catalyze hydrolysis of amygdalin, linamarin or linustatin (Del Cueto et al., 2017).

It has been observed that substitution of only a few amino acid residues may be enough to change the substrate specificity of an enzyme. It was observed that a change of two amino acids (position 220 and 394) in AH was able to confer PH activity (Sánchez-Pérez et al., 2009). Moreover, only a single amino acid polymorphism in the aglycone binding region of the active site (G211 in BGD2 and V211 in BGD4) explained the difference in substrate specificity for the cyanogenic glucosides linamarin and lotaustralin (Lai et al., 2015).

5. Conclusions

In this study, we have characterized *Ph691* from the tegument, nucellus and cotyledon during kernel development in one sweet and two bitter almond genotypes. Minor differences in the nucleotide and corresponding amino acid sequences were found between the nine PH sequences studied. None of the differences could be related to the sweet or bitter flavour. Only PH691 from the D05-187 tegument showed reduced PH activity. In order to determine whether bitterness in almond is related to PH activity, additional PHs will have to be studied. No significant differences in the expression profiles of the *Phs* between the sweet and bitter genotypes were found.

Acknowledgements

This work was financed by the projects “Mejora Genética del Almendro” (MINECO-Spain, AGL2017-85042-R), “The molecular mechanisms to break flower bud dormancy in fruit trees” to RS-P within the Villum Young Investigator Program and by the VILLUM Research Center for Plant Plasticity-Denmark and “Breeding stone fruit species assisted by molecular tools” (Fundación Séneca-Spain).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.plaphy.2017.12.028.

References

Abarrategui, L., 2010. Bitterness in Almonds. Master thesis. University of Copenhagen.
Arrázola, G., Sánchez-Pérez, R., Dicenta, F., Grané, N., 2012. Content of the cyanogenic glucoside amygdalin in almond seeds related to the bitterness genotype. Agron. Colomb. 30, 260–265.
Bak, S., Paquette, S.M., Moran, M., Morant, A.V., Saito, S., Bjarnholt, N., Zagrobelny, M., Jørgensen, K., Osmani, S., Hamann, T., Simonsen, H.T., Sánchez-Pérez, R., van Heeswijck, T.B., Jørgensen, K., Møller, B.L., 2006a. Cyanogenic glycosides: a case study for evolution and application of cytochromes P450. Phytochemistry Rev. 5, 309–329.
Bak, S., Olsen, C.E., Halkier, B.A., Møller, B.L., 2006b. Transgenic tobacco and Arabidopsis plants expressing the two multifunctional sorghum cytochrome P450 enzymes, CYP79A1 and CYP71E1, are cyanogenic and accumulate metabolites derived from intermediates in Dhurrin biosynthesis. Plant Physiol. 123, 1347–1448.
Bliss, F.A., Arulsekar, S., Foolad, M.R., Becerra, A.M., Gillen, A., Warburton, M.L., Dandekar, A.M., Kocsisne, G.M., Mydin, K.K., 2002. An expanded genetic linkage map of Prunus based on an interspecific cross between almond and peach. Genome 45, 520–529.
Conn, E., 1969. Cyanogenic glycosides. J. Agric. Food Chem. 17, 519–526.
Conn, E., 2007. Cyanogenic compounds. Annu. Rev. Plant Physiol. 31, 433–451.

Del Cueto, J., Ionescu, I.A., Pičmanová, M., Gericke, O., Motawia, M.S., Olsen, C.E., Campoy, J.A., Dicenta, F., Møller, B.L., Sánchez-Pérez, R., 2017. Cyanogenic glucosides and derivatives in almond and sweet cherry flower buds from dormancy to flowering. Front. Plant Sci. 800, 1–16.
Dicenta, F., García, J.E., 1993. Inheritance of the kernel flavor in almond. Heredity 70, 308–312.
Dicenta, F., Martínez-Gómez, P., Ortega, E., Duval, H., 2000. Cultivar pollinizer does not affect almond flavor. Hortscience 35, 1153–1154.
Dicenta, F., Martínez-Gómez, P., Grané, N., Martín, M.L., León, A., Cánovas, J.A., Berenguer, V., 2002. Relationship between cyanogenic Compounds in kernels, leaves, and roots of sweet and bitter kernelled almonds. J. Agric. Food Chem. 50, 2149–2152.
Dicenta, F., Ortega, E., Martinez-Gomez, P., 2007. Use of recessive homozygous genotypes to assess the genetic control of kernel bitterness in almond. Euphytica 153, 221–225.
Evreinoff, V.A., 1952. Quelques observations biologiques sur l'amandier. Rev. Int. Bot. Appl. Agric. Trop. 32, 442–459.
Feigl, F., Anger, V., 1966. Replacement of benzidine by copper ethylacetoacetate and tetra basis as spot-test reagent for hydrogen cyanide and cyanogen. Analyst 91, 282–284.
Floss, H.G., Hadwiger, L., Conn, E.E., 1965. Enzymatic formation of β-cyanoalanine from cyanide. Nature 208, 1207–1208.
Franks, T.K., Yadollahi, A., Wirthensohn, M.G., Guerin, J., Kaiser, B.N., Sedgley, M., Ford, C.M., 2008. A seed coat cyanohydrin glucosyltransferase is associated with bitterness in almond (*Prunus dulcis*) kernels. Funct. Plant Biol. 35, 236–246.
Frehner, M., Scalet, M., Conn, E.E., 1990. Pattern of the cyanide-potential in developing fruits. Plant Physiol. 94, 28–34.
Gleadow, R.M., Møller, B.L., 2014. Cyanogenic glycosides: synthesis, physiology, and phenotypic plasticity. Annu. Rev. Plant Biol. 65, 155–185.
Grasselly, C., Crossa-Raynaud, P., L'amandier, G.P., 1980. Maison et Larose, París.
Halkier, B.A., Møller, B.L., 1989. Biosynthesis of the cyanogenic glucoside dhurrin in seedlings of *Sorghum bicolor* (L.) Moench and partial purification of the enzyme system involved. Plant Physiol. 90, 1552–1559.
Heppner, J., 1923. The factor for bitterness in the sweet almond. Genetics 8, 390–392.
Heppner, J., 1926. Further evidence on the factor for bitterness in the sweet almond. Genetics 11, 605–606.
Jenrich, R., Trompetter, I., Bak, S., Olsen, C.E., Møller, B.L., Piotrowski, M., 2007. Evolution of heteromeric nitrilase complexes in Poaceae with new functions in nitrile metabolism. Proc. Natl. Acad. Sci. U.S.A. 104, 18848–18853.
Joobeur, T., Viruel, M.A., de Vicente, M.C., Jáuregui, B., Ballester, J., Dettori, M.T., Verde, I., Truco, M.J., Messeguer, R., Battle, I., Quarta, R., Dirlewanger, E., Arús, P., 1998. Construction of a saturated linkage map for *Prunus* using an almond × peach F_2 progeny. Theor. Appl. Genet. 97, 1034–1041.
Kester, D.E., Asay, R.N., 1975. Almonds. In: Janick, J., Moore, J.N. (Eds.), Advances in Fruit Breeding. Purdue University Press, West Lafayette, Indiana, pp. 387–419.
Kester, D.E., Gradziel, T.M., 1996. In: Moore, J.N., Janick, J. (Eds.), Almonds (Prunus) En: Fruit Breeding. Wiley, New York, pp. 1–97.
Ketudat Cairnsa, J.R., Mahongc, B., Baiyaa, S., Jeonc, J.-S., 2015. β-Glucosidases: multitasking, moonlighting or simply misunderstood? Plant Sci. 241, 246–259.
Kriechbaumer, V., Park, W.J., Piotrowski, M., Meeley, R.B., Gierl, A., Glawischnig, E., 2007. Maize nitrilases have a dual role in auxin homeostasis and β-cyanoalanine hydrolysis. J. Exp. Bot. 58, 4225–4233.
Kuroki, G.W., Poulton, J.E., 1987. Isolation and characterization of multiple forms of prunasin hydrolase from black cherry (*Prunus serotina* Ehrh.) seeds. Biochem. Biophysis. 255, 19–26.
Lai, D., Picmanová, M., Abou Hachem, M., Motawia, M.S., Olsen, C.E., Rook, Møller, B.L., Takos, A.M., 2015. *Lotus japonicus* flowers are defended by a cyanogenic â-glucosidase with highly restricted expression to essential reproductive organs. Plant Mol. Biol. 89, 21–34.
Lambert, J.L., Ramasamy, J., Paukstelis, J.V., 1975. Stable reagents for the colorimetric determination of cyanide by modified König reactions. Anal. Chem. 47, 916–918.
Li, C.P., Swain, E., Poulton, J.E., 1992. *Prunus serotina* amygdalin hydrolase and prunasin hydrolase. Plant Physiol. 100, 282–290.
Luo, J., Nishiyama, Y., Fuell, C., Taguchi, G., Elliott, K., Hill, L., Tanaka, Y., Kitayama, M., Yamazaki, M., Bailey, P., Parr, A., Michael, A.J., Saito, K., Martin, C., 2007. Convergent evolution in the BAHD family of acyltransferases: identification and characterization of anthocyanin acyltransferases from Arabidopsis thaliana. Plant J. 50, 678–695.
Martínez-Gómez, P., Sánchez Pérez, R., Dicenta, F., 2008. Fruit development in almond for fresh consumption. J. Am. Pomol. Soc. 62, 82–86.
McCarty, C.D., Leslie, J.W., Frost, H.B., 1952. Bitterness of kernels of almond 3 peach hybrids and their parents. Proc. Am. Soc. Hortic. Sci. 59, 254–258.
Mentzer, C., Favre-Bonvin, J., 1961. Sur la biogenèse du glucoside cyanogénétique des feuilles de laurier-cerise (Prunus lauro-cerasus). C. R. Acad. Sci. Ser. III Sci. Vie 253, 1072–1074.
Møller, B.L., Seigler, D.S., 1991. Biosynthesis of cyanogenic glycosides, cyanolipids and related compounds. In: Singh, B.K. (Ed.), Plant Amino Acids, Biochemistry and Biotechnology. Marcel Dekker, New York, pp. 563–609.
Morant, M., Bak, S., Møller, B.L., Werck-Reichhart, D., 2003. Plant cytochromes P450: tools for pharmacology, plant protection and phytoremediation. Curr. Opin. Biotechnol. 14, 151–162.
Morant, A.V., Jørgensen, K., Jørgensen, C., Paquette, S.M., Sánchez-Pérez, R., Møller, B.L., Bak, S., 2008. β-Glucosidases as detonators of plant chemical defense. Phytochemistry 69, 1795–1813.
Pankoke, H., Buschmann, T., Müller, C., 2013. Role of plant β-glucosidases in the dual defense system of iridoid glycosides and their hydrolyzing enzymes in *Plantago lanceolata* and Plantago major. Phytochemistry 94, 99–107.
Piotrowski, M., Volmer, J.J., 2006. Cyanide metabolism in higher plants: cyanoalanine hydratase is a NIT4 homolog. Plant Mol. Biol. 61, 111–122.
Piotrowski, M., Schönfelder, S., Weiler, E.W., 2001. The Arabidopsis thaliana isogene NIT4 and its orthologs in tobacco encode beta-cyano-L-alanine hydratase/nitrilase. J. Biol. Chem. 276, 2616–2621.
Poulton, J.E., 1990. Cyanogenesis in plants. Plant Physiol. 94, 401–405.
Poulton, J.E., Ping Li, C., 1994. Tissue level compartmentation of (R)-amygdalin and amygdalin hidrolase prevents large-scale cyanogenesis in undamaged *Prunus* seeds. Plant Physiol. 104, 29–35.
Sánchez-Pérez, R., Howad, H., Dicenta, F., Arús, P., Martínez-Gómez, P., 2007. Mapping major genes and quantitative trait loci controlling agronomic traits in almond. Plant Breed. 126, 310–318.
Sánchez-Pérez, R., Jørgensen, K., Erik Olsen, C., Dicenta, F., Møller, B.L., 2008. Bitterness in almonds. Plant Physiol. 146, 1040–1052.
Sánchez-Pérez, R., Jørgensen, K., Motawia, M.S., Dicenta, F., Møller, B.L., 2009. Tissue and cellular localization of individual β-glycosidases using a substrate-specific sugar reducing assay. Plant J. 60, 894–906.
Sánchez-Pérez, R., Howad, W., Garcia-Mas, J., Arus, P., Martinez-Gomez, P., Dicenta, F., 2010. Molecular markers for kernel bitterness in almond. Tree Genet. Genomes 6, 237–245.
Sánchez-Pérez, R., Belmonte, F.S., Borch, J., Dicenta, F., Møller, B.L., Jørgensen, K., 2012. Prunasin hydrolases during fruit development in sweet and bitter almonds. Plant Physiol. 158, 1916–1932.
Socias, i R., 1998. Company, Fruit tree genetics at a turning point: the almond example. Theor. Appl. Genet. 96, 588–601.
Suelves, M., Puigdoménech, P., 1998. Molecular cloning of the cDNA coding for the (R)-(+)-mandelonitrile lyase of Prunus amygdalus: temporal and spatial expression patterns in flowers and mature seeds. Planta 206, 388–393.
Swain, E., Poulton, J.E., 1994. Immunocytochemical localization of prunasin hydrolase and mandelonitrile lyase in stems and leaves of *Prunus serotina*. Plant Physiol. 106, 1285–1291.
Swain, E., Li, C.P., Poulton, J.E., 1992a. Development of the potential for cyanogenesis in maturing black cherry (*Prunus serotina* Ehrh.) fruits. Plant Physiol. 98, 1423–1428.
Swain, E., Li, C.P., Poulton, J.E., 1992b. Tissue and subcellular localization of enzymes catabolizing (R)-amygdalin in mature *Prunus serotina* seeds. Plant Physiol. 100, 291–300.
Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28, 2731–2739.
Vargas, F.J., Romero, M.A., Batlle, I., 2001. Kernel taste inheritance in almond. In: In: Ak, B.E. (Ed.), 11 GREMPA Seminar on Pistachios and Almonds. Cahiers Options Méditerranéennes, vol. 56. pp. 129–134.
Voinnet, O., Rivas, S., Mestre, P., Baulcombe, D., 2003. An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. Plant J. 33, 949–956.
Zagrobelny, M., Bak, S., Rasmussen, A.V., Jørgensen, B., Naumann, C.M., Møller, B.L., 2004. Cyanogenic glucosides and plant-insect interactions. Phytochemistry 65, 293–306.
Zagrobelny, M., Bak, S., Møller, B.L., 2008. Cyanogenesis in plants arthropods. Phytochemistry 69, 1457–1468.
Zheng, L., Poulton, J.E., 1995. Temporal and spatial expression of amygdalin hydrolase and (R)-(+) - mandelonitrile lyase in black cherry seeds. Plant Physiol. 109, 31–39.
Zhou, J., Hartmann, S., Shepherd, B.K., Poulton, J.E., 2002. Investigation of the microheterogeneity and aglycone specificity-conferring residues of black cherry prunasin hydrolases. Plant Physiol. 129, 1252–1264.