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Corresponding Author	Family Name	**Cebulj**
	Particle	
	Given Name	**Anka**
	Suffix	
	Division	
	Organization	Agricultural Institute of Slovenia
	Address	Hacquetova ulica 17, 1000, Ljubljana, Slovenia
	Phone	
	Fax	
	Email	anka.cebulj@kis.si
	URL	
	ORCID	http://orcid.org/0000-0002-9915-0345
Author	Family Name	**Halbwirth**
	Particle	
	Given Name	**Heidi**
	Suffix	
	Division	Institute for Chemical Engineering
	Organization	Technical University of Vienna
	Address	Getreidemarkt 9/1665, 1060, Vienna, Austria
	Phone	
	Fax	
	Email	
	URL	
	ORCID	
Author	Family Name	**Mikulic-Petkovsek**
	Particle	
	Given Name	**Maja**
	Suffix	
	Division	Chair for Fruit, Vine and Vegetable Growing, Department of Agronomy, Biotechnical Faculty
	Organization	University of Ljubljana
	Address	Jamnikarjeva 101, 1000, Ljubljana, Slovenia
	Phone	
	Fax	
	Email	

	URL	
	ORCID	
Author	Family Name	**Veberic**
	Particle	
	Given Name	**Robert**
	Suffix	
	Division	Chair for Fruit, Vine and Vegetable Growing, Department of Agronomy, Biotechnical Faculty
	Organization	University of Ljubljana
	Address	Jamnikarjeva 101, 1000, Ljubljana, Slovenia
	Phone	
	Fax	
	Email	
	URL	
	ORCID	
Author	Family Name	**Slatnar**
	Particle	
	Given Name	**Ana**
	Suffix	
	Division	Chair for Fruit, Vine and Vegetable Growing, Department of Agronomy, Biotechnical Faculty
	Organization	University of Ljubljana
	Address	Jamnikarjeva 101, 1000, Ljubljana, Slovenia
	Phone	
	Fax	
	Email	
	URL	
	ORCID	

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Abstract

Although many aspects of scald have been investigated, we are still far away from a general understanding of the disorder. Here, we tried to elucidate the role of the phenolic compounds and their fate during scald development. The study was performed on ‘Granny Smith’ apples. After 5 months in cold storage, scald developed rapidly at room temperature, giving us the opportunity to study unaffected, partly scalded, and fully scalded apple peels. We analyzed the phenolic content, enzyme activity, and gene expression of the flavonoid pathway as well as peroxidase and polyphenol oxidase activity. We demonstrate that scald incidence is mostly dependent on hydroxycinnamic acids, namely, chlorogenic acid and its increased accumulation during scald development, while other phenolic compounds do not seem to be relevant. Expression of genes of the flavonoid pathway was strongly increased during disorder development, whereas a decreased activity of most analyzed flavonoid pathway enzymes was observed. The results presented here help elucidate the role of phenolic content during scald development, which will be useful for future studies of scald development.

Keywords (separated by '-')	*Malus domestica* Borkh. - Scald - Gene expression - Hydroxycinnamic acids - Color - Enzyme activity
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1 RESEARCH REPORT

2 The impact of scald development on phenylpropanoid metabolism 3 based on phenol content, enzyme activity, and gene expression 4 analysis

5 Anka Cebulj[1] · Heidi Halbwirth[2] · Maja Mikulic-Petkovsek[3] · Robert Veberic[3] · Ana Slatnar[3]

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11 performed on 'Granny Smith' apples. After 5 months in cold storage, scald developed rapidly at room temperature, giving
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19 **Keywords** *Malus domestica* Borkh. · Scald · Gene expression · Hydroxycinnamic acids · Color · Enzyme activity

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20 1 Introduction

21 Scald, a physiological disorder mainly affecting apples and
22 pears, is an important storage disorder (Lurie and Watkins
23 2012) that greatly reduces the income of fruit growers and
24 handlers (Wang and Dilley 1999). Scald incidence is local-
25 ized to the few layers of hypodermis cells; its first signs are
26 light-brown lesions, which subsequently transform into dark-
27 brown lesions (Bain and Mercer 1963). The cause behind
28 scald development is still not completely understood. How-
29 ever, studies largely support the hypothesis of an underlying
30 α-farnesene oxidation (Meigh and Filmer 1969; Anet 1972;
31 Bramlage et al. 1993; Lurie and Watkins 2012). On the other
32 hand, α-farnesene is present in resistant and non-resistant
33 apple cultivars, thus pointing to the presence of further
34 mechanisms affecting scald development.

35 A significant role of antioxidant systems in scald develop-
36 ment has also been suggested (Barden and Bramlage 1994).
37 Phenolic compounds are an important part of these antioxi-
38 dant systems, which are frequently upregulated in reaction
39 to different types of stress (Dixon and Paiva 1995). A higher
40 phenolic content mediates enhanced protection, which was
41 also demonstrated by Abdallah et al. (1997). On the other
42 hand, membrane disintegration releases phenolic compounds
43 from the vacuoles, thereby providing additional substrate
44 for polyphenol oxidase (PPO) activity, which enhances skin
45 browning (Ju et al. 1996). Some earlier papers indicated an
46 indirect relationship between phenolic compounds and scald
47 incidence as the phenolic content decreases during scald
48 development (Golding et al. 1998). Polymeric phenols can
49 be irreversibly bound to polymeric matrices in brown apple
50 skin (Piretti et al. 1994, 1996). Ju et al. (1996) and Rudell
51 and Mattheis (2009) both confirmed that sun-exposed sides
52 of apples developed less scald symptoms than shaded sides.

A1 Communicated by Eun Jin Lee, Ph.D.

A2 ✉ Anka Cebulj
A3 anka.cebulj@kis.si

A4 [1] Agricultural Institute of Slovenia, Hacquetova ulica 17,
A5 1000 Ljubljana, Slovenia

A6 [2] Institute for Chemical Engineering, Technical University
A7 of Vienna, Getreidemarkt 9/1665, 1060 Vienna, Austria

A8 [3] Chair for Fruit, Vine and Vegetable Growing, Department
A9 of Agronomy, Biotechnical Faculty, University of Ljubljana,
A10 Jamnikarjeva 101, 1000 Ljubljana, Slovenia

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Whereas Ju et al. (1996) ascribed this mostly to anthocyanins, Rudell and Mattheis (2009) suggested that quercetin glycosides are mainly responsible for a higher scald resistance. Both anthocyanins and quercetin glycosides have antioxidative effects, and their protective role is especially well researched with respect to sunburn (Felicetti and Schrader 2008; Yuri et al. 2010; Zupan et al. 2014). Ju and Bramlage (1999) reported a higher total antioxidant activity (activity of free phenolics in the cuticle plus activity of lipid-soluble antioxidants in fruit peel) in apple cultivars that are more resistant to scald incidence.

Recent studies on scald and phenolic compounds were carried out by Busatto et al. (2014) and Busatto et al. (2018). In the first study, they examined the variation of some phenolic compounds and related gene expression during superficial scald development. They observed a higher expression of *MdPAL* and *MdC3H* genes and a consequently higher accumulation of chlorogenic acid, which is subsequently further oxidized by PPO. The second study, by Busatto et al. (2018), focused on an integrated screen, including an analysis of volatiles, phenols, and lipids, together with a large-scale transcriptome study to elucidate the mechanism of superficial scald development. The results showed the induced gene flavonoid 3′ monoxygenase, a phenylalanine ammonia lyase, and a flavonol synthase along with increased levels of chlorogenic acid and its isomers. However, there are no reports of enzyme activity downstream in the phenylpropanoid pathway.

Besides phenolic compounds, antioxidant enzymes also contribute to cell protection. Shaham et al. (2003) studied the role of peroxidase (POX), superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), and glutathione reductase (GL) activities in scald development. No direct relationship between antioxidant content and the above-mentioned enzyme activities during scald development could be found. Other studies, such as Ahn et al. (2007) did not observe a direct involvement of APX, SOD, and POX in scald susceptibility or resistance. While Abbasi et al. (2008) did not find any connection of POX activity to scald development either, they did observe a possible correlation between PPO activity and antioxidant level. Rao et al. (1998) found that lower H_2O_2 concentrations are associated with higher POX and catalase activities, and consequently lower scald susceptibility. A higher H_2O_2 content was confirmed in ‘Granny Smith’ apples in comparison to a scald-resistant cultivar, which suggests direct regulation of transcription of the antioxidant enzymes catalase and GL and monodehydroascorbate reductase (Zubini et al. 2007). Apart from their antioxidant role, phenolic compounds are also involved in brown coloration of the peel after cell membranes disintegrate due to increased PPO activity (Nicolas et al. 1994; Abbasi et al. 2008). Yihui et al. (2018) reported that laccase catalyzes the reaction between epicatechin to cause the browning of apple peels. Laccases are dioxygen oxidoreductases that are, together with catechol oxidase, part of the PPO category (Pourecl et al. 2006). Busatto et al. (2014) described a higher PPO gene expression level in apple skin on the fourth day outside cold storage.

The role of phenolic compounds in scald development and the improved phenol content determination techniques prompted us to perform a thorough study on the phenolic content and on quantitative expression of genes of the flavonoid pathway. We also included for the first time measurements of selected enzyme activities (phenylalanine ammonia lyase—PAL, chalcone synthase—CHS, chalcone isomerase—CHI, flavanone 3-hydroxylase—FHT, dihydroflavonol 4-reductase—DFR and phloretin 2′-*O*-glycosyltransferse—P2′GT) and the activities of PPO and POX to elucidate the fate and role of phenolic compounds in scald development. We hypothesize that phenol content is not entirely connected to phenylpropanoid and flavonoid enzyme activity.

2 Materials and methods

2.1 Plant material

A cultivar prone to scald, ‘Granny Smith’, was used for this experiment. Apples were harvested in the autumn of 2015 at optimal technological ripening stage and stored in cold conditions for 5 months. On February 22nd, only the apples without visible signs of scald were selected and placed at room temperature (21–23 °C), where they were further evaluated and analyzed. Fruits were sampled on the following dates: 22nd (T0), 23rd (T+1), 24th (T+2), and 26th (T+4) of February. On T0, only the peel of apples without scald was sampled (K); on T+1, two types of peel were sampled from the same fruit: unaffected peel (K) and peel with first visible signs of scald (B); on T+2 and T+4, three types of peel were sampled: K, B, and peel with fully developed scald (S) (Fig. 1A). A thin layer of peel was peeled and immediately immersed in liquid nitrogen and stored at -80 °C until further analyses.

2.2 Color measurements

Ten apple fruits per sampling date were used for color measurements. The color was measured in the same way as tissue was sampled: On T0, the color of K peel; on T+1, the color of K and B; and on T+2 and T+4 the color of K, B, and S were measured. Measurements were carried out with a portable colorimeter (CR-10 Chroma; Minolta, Osaka, Japan) in the CIELAB scale system; therefore, parameters a*, b*, lightness of color (L*), and hue angle (h°) were measured. Parameter L* provides the information of relative lightness of colors with the range from 0 to 100, where 0 represents

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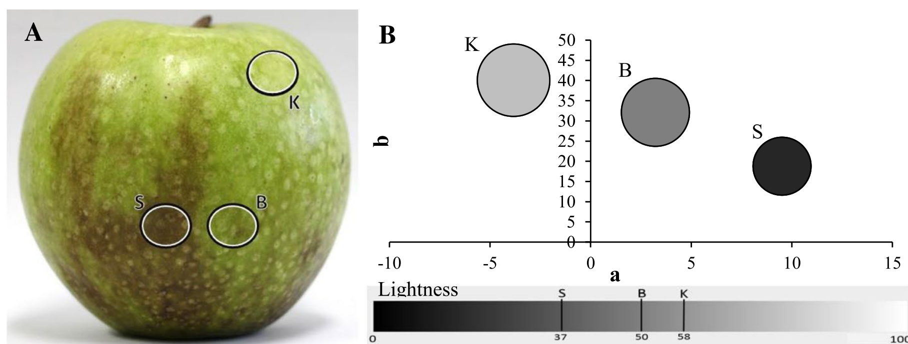

Fig. 1 **a** Marked treatments: K—no visible signs of scald; B—first signs of scald; S—fully developed scald. **b** Apple peel color parameters a, b, hue angle (°), and relative lightness of colors for all three sample types (K, B, and S) at sampling day T + 2. Parameters a and b are presented on the x and y axes, respectively; the size of the bubbles represents the hue angle, and marked values on the bar below the graph represent the relative lightness of colors

black and 100 represents white. Coordinates a* and b* locate the color on a rectangular-coordinate grid perpendicular to the L* axis (positive a* indicates a hue of red-purple and negative bluish-green; positive b* indicates yellow and negative blue).[25] Hue angle (h°) tells us the basic tone of color and is expressed in degrees from 0 to 360.

2.3 Extraction and determination of individual phenolic compounds

Frozen apple peels were ground in a mortar to a fine powder, and 0.6 g was extracted with 5 mL of methanol. Further extraction and HPLC analysis were performed as described by Zupan et al. (2014). The identification of phenolic compounds was carried out using an HPLC-Finnigan MS detector and an LCQ Deca XP MAX instrument (Thermo Finnigan, San Jose, CA) with an electrospray interface (ESI) operating in negative ion mode. The content was calculated in mg kg^{-1} (fresh weight) and in relation to the control K, which was set as 100% for each sampling date. Content of 100% (control) values for each date are shown in Fig. 2.

2.4 Enzyme assays

Shock-frozen peel was ground into powder using liquid nitrogen, and 0.5 g of the fine powder was homogenized in a mortar with 0.25 g quartz sand (Sigma-Aldrich), 0.25 g Polyclar AT (Serva Electrophoresis), and 3 mL of extraction

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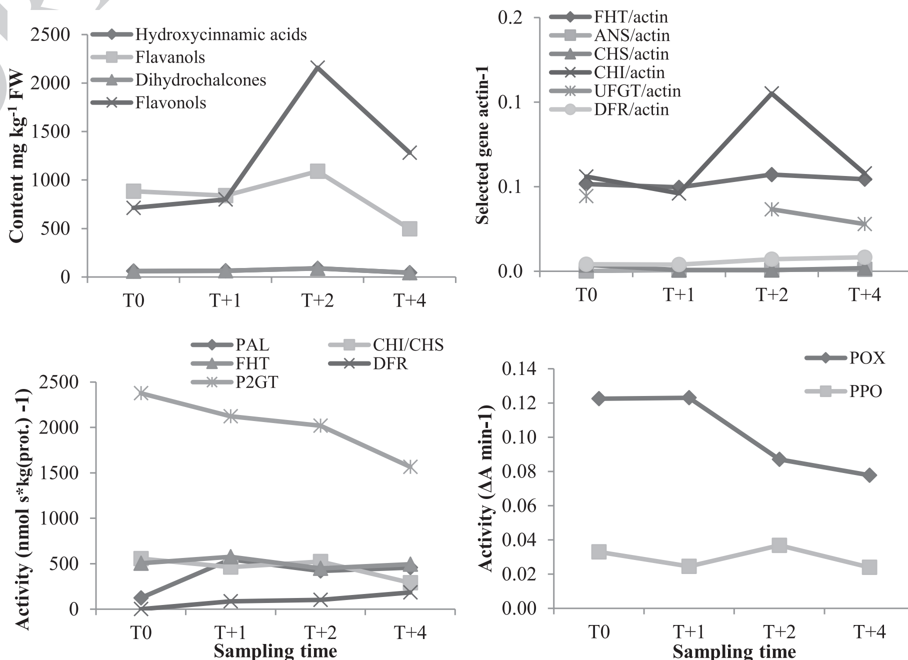

Fig. 2 Content of hydroxycinnamic acids, flavanols, dihydrochalcones, and flavonols (mg kg^{-1} FW); expression of *MdCHS*, *MdCHI*, *MdFHT*, *MdDFR*, *MdANS*, and *MdUFGT* normalized to *actin*; changes in specific enzyme activity (nkat g^{-1} protein) of PAL, CHS/CHI, FHT, DFR, and P2′GT; and peroxidase (POX) and polyphenol oxidase (PPO) activity ($_{\Delta}A$ min^{-1}) in unaffected control peel (K) on four sampling days (T0 – T + 4)

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177 buffer. The homogenate was poured into micro tubes and
178 centrifuged for 10 min at 4°C (12,857 *x g*). To remove low
179 molecular compounds, supernatant (400 µl) was added on
180 gel chromatography columns (Sephadex G25 medium;
181 Sigma-Aldrich). The protein solution eluted in the excluded
182 volume of the column (crude extract) was used for enzyme
183 assays.

184 Enzyme assays for PAL (EC 4.3.1.24), CHS (EC
185 2.3.1.74), CHI (EC 5.5.1.6), DFR (EC 1.1.1.219), FHT
186 (EC 1.14.11.9), and FLS (EC 1.14.11.23) were performed
187 according to Halbwirth et al. (2002) using the assay condi-
188 tions optimized for apple peel (Table 1). The assays were
189 incubated for 30 min at 30 °C. PAL and CHS/CHI assays
190 were stopped with 200 µl ethyl acetate. To PAL assays 10 µl
191 of acetic acid was also added. Products were quantified
192 on a scintillation counter. The FHT and DFR assays were
193 terminated by adding 70 µl of ethyl acetate and 10 µl of
194 acetic acid. Organic phases were transferred to precoated
195 cellulose plates (Merck, Germany) for thin-layer chroma-
196 tography (TLC) in chloroform/acetic acid/H_2O (10:9:1,
197 v/v/v) for FHT and DFR assays. The conversion rates were
198 then determined with a TLC linear analyzer (Berthold, Ger-
199 many). P2′GT was terminated by adding 10 µl of acetic acid
200 and 25 µl of methanol. The mixture was chromatographed on
201 Schleicher and Schüll 2043b paper, using water as a solvent
202 system. Zones containing the labelled products were cut out,
203 and radioactivity was quantified on a scintillation counter.
204 All products were identified as described using authentic
205 substances (Fischer et al. 2003).

206 POX and PPO activity determination was carried
207 out according to Slatnar et al. (2010). For POX enzyme
208 measurements, the preparation was incubated with *o*-dian-
209 isidine (Sigma-Aldrich, Germany) in buffer F (Table 1),
210 and time-dependent changes in the absorption at 460 nm
211 were determined in comparison with a blank containing
212 only *o*-dianisidine and buffer F measured with a spectro-
213 photometer (Perkin Elmer, UV/VIS Lambda Bio 20). For
214 PPO measurements, the enzyme preparation was incubated
215 with 0.2 m pyrocatechol solution (Merck, Germany) in
216 buffer G, and the time-dependent changes in absorption at
217 410 nm were determined in comparison with a blank con-
218 taining only buffer G and pyrocatechol solution. Enzyme
219 activity was calculated in $_{\Delta}A\ min^{-1}$. At each sampling
220 date, the results were calculated in relation to the control
221 K, which was set as 100%. Content of 100% (control) val-
222 ues for each date are shown in Fig. 2.

223 Buffers used: Extraction buffer for activity assays of
224 PAL, CHS/CHI, DFR, FHT, and FLS was a 0.1 m HEPES
225 0.1 m, pH 7.3; PEG 1500, 1.5%; sucrose, 10%: dithio-
226 erythritol, 1 mM; ascorbic acid, 100 mM: calcium chlo-
227 ride, 25 mM. Extraction buffer for activity assays of POX
228 and PPO was 0.1 m Tris/HCl (Merck). Buffer A (PAL
229 assays): 0.1 m KPi + 0.4% Na-ascorbate, pH 8.5. Buffer
230 B (CHS/CHI assays): 0.1 m KPi + 0.4% Na-ascorbate, pH
231 6.0. Buffer C (FHT assays): 0.1 m Tris/HCl + 0.4% Na-
232 ascorbate, pH 7.25. Buffer D (P2′GT, assays): 0.1 m Tris/
233 HCl + 0.4% Na-ascorbate, pH 7.5. Buffer E (DFR assays):
234 0.1 m KPi + 0.4% Na-ascorbate, pH 5.75. Buffer F (POX
235 assays): 0.1 m KPI, pH 6.0, 0.1 m H_2O_2 pH 6.5. Buffer G
236 (PPO assays): 0.1 m Na_2HPO_4, pH 6.5.

Table 1 Optimised conditions for assays of main enzymes of the flavonoid pathway in apple peel

enzyme	crude extract after gel chromatography (µl)	Substrate(s)	(µl)	Cofactor and cosubstrate solutions	(µl)	Buffer	Buffer (µl)	Final volume (µl)
PAL	40	(^{14}C)-phenylalanine (0.063 nmol, 548 Bq)	5	–		A	55	100
CHS/CHI	40	*p*-CuCoA (1 nmol) (^{14}C)-malonyl-CoA (1.5 nmol, 1300 Bq)	5 5	–		B	50	100
FHT	40	(^{14}C)- naringenin (0.036 nmol, 100 Bq)	–	2-oxoglutarate (1.46 mg ml^{-1}) $FeSO_4 \times 7\ H_2O$ (1.46 mg ml^{-1})	5 5	C	50	100
DFR	20	(^{14}C)-dihydroquercetin (0.036 nmol, 100 Bq)	–	NADPH (41.86 mg ml^{-1})	5	E	25	50
P2′GT	30	Phloretin (0.3 nmol)	2.5	(^{14}C)-UDPG (0.206 nmol, 2300 Bq)	2.5	D	15	50
POX	50	*o*-dianisidin (0.410 nmol)	10			F	1.050	1.110
PPO	100	Pyrocatechol solution (1.1 g pyrocatechol and 50 mL buffer G)	170			G	330	600

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2.5 Gene expression studies

Total RNA prepared according to Chang et al. (1993) was used for the isolation of mRNA via the μMACS mRNA isolation kit (Miltenyi Biotech, Auburn, CA). cDNA was prepared using RevertAid H Minus MuLV reverse transcriptase (Fermentas Life Science, St. Leon-Rot, Germany) with the oligo(-dT) anchor PrimerGACCACGCGTATCGATGT-CGAC$(T)_{16}$V.

The *MdCHS*, *MdCHI*, *MdFHT*, *MdDFR*, *MdANS*, and *MdUFGT* gene expression was analyzed by qRT-PCR using a StepOnePlus system and SYBRW Green PCR Master Mix (Applied Biosystems, Darmstadt, Germany) according to the supplier's instructions. Primer sequences are provided in Table 2.

The analysis was carried out in triplicates for all mentioned primers. The efficiency of the primers was determined based on standard curves, which were obtained by applying different DNA concentrations. Results were calculated in relation to the housekeeping gene *actin*. At each sampling date, the results were calculated in relation to the control K, which was set as 100%. Content of 100% (control) values for each date are shown in Fig. 2.

2.6 Statistical analysis

For phenolic compounds, peels of two fruit were combined into one sample, with eight samples for each treatment (T0: 8K; T + 1: 8K and 8B; T + 2: 8K, 8B and 8 s; T + 4: 8K, 8B and 8 s; in total 72 samples).

The peels were also collected from two fruits for enzyme activity determination, but there were three replicate samples made for each treatment (T0: 3K; T + 1: 3K and 3B; T + 2: 3K, 3B and 3 s; T + 4: 3K, 3B and 3 s; in total 27 samples).

A one-way analysis of variance (ANOVA) and a Duncan test were used to determine the difference among treatments (K, B, and S). Data were analyzed with the Statgraphics Centurion program (Manugistics Inc.; Maryland, USA). Error bars are shown where pertinent.

3 Results

Apples started to show visible signs of scald after only 1 day of storage at a temperature of about 22 °C. After 7 days, apples were so badly scalded that control samples from the same fruit were impossible to obtain. Therefore, we collected samples on four sampling dates in 1 week (T0, T + 1, T + 2, and T + 4). The color of fruit was also measured on those days. When the statistical analysis was performed, three distinct groups of scald incidence could be distinguished (Fig. 1, B), of which the control peel (K) was the lightest and peel with fully developed scald (S) was the darkest. The hue angle was the highest for control peel and the lowest for peels with fully developed scald, which shows the color shift from green-yellow to yellow-red, respectively.

Twenty-eight individual phenolic compounds were quantified in the peel of cv. 'Granny Smith'. For a better illustration of the results, phenolic compounds are presented in four groups (Fig. 3): total hydroxycinnamic acids (3-, 4-, and 5-caffeoylquinic acid; *p*-coumaric acid hexoside 1 and 2; sinapic acid glycoside, and 4-*O*-*p*-coumaroylquinic acid), total flavanols (catechin, epicatechin, and procyanidins), total dihydrochalcones (3-hydroxy-phloretin-2′-*O*-xyloglucoside, phloretin-2′-*O*-xylosylglucoside, and phloretin-2-*O*-glucoside (phloridzin)) and total flavonols (quercetin glycosides).

In scald-affected peels (B and S), an increase in hydroxycinnamic acids was noticed, with the highest increase in peels with fully developed scald from T + 2 to T + 4, where hydroxycinnamic acids content increased by more than twofold. About 90% of hydroxycinnamic acids on T + 4 in peels with fully developed scald are represented by chlorogenic acid and 4-caffeoylquinic acid, whereas their content in the peel with first visible signs of scald and in controls was considerably smaller, 76% and 44%, respectively. In control peel, the content of chlorogenic acid and 4-caffeoylqunic acid varied from 41 to 47% from T0 to T + 4 and in peel with first visible signs of scald from 53–76% from T + 1 to T + 4.

Table 2 Forward and reverse primers used in qRT-PCR analysis

Gene identifier (Genbank)	Name	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)
CN938023	Actin	TGACCGAATGAGCAAGGAAATTACT	TACTCAGCTTTGGCAATCCACATC
CN944824	CHS	GGAGACAACTGGAGAAGGACTGGAA	CGACATTGATACTGGTGTCTTCA
CN94654	CHI	TCCTCCAACACTTTGTTCCTC	CGGTCTTACCCTTCCACTTAAC
CN491664	FHT	TGGAAGCTTGTGAGGACTGGGGT	CTCCTCCGATGGCAAATCAAAGA
AF117268	DFR	GATAGGGTTTGAGTTCAAGTA	TCTCCTCAGCAGCCTCAGTTTTCT
AF117267	UFGT	CCACCGCCCTTCCAAACACTCT	CACCCTTATGTTACGCGGCATGT
AF117269	ANS	TGAGTGGGAGGACTACTTCTT	GCGGTTGCCTCAATGTAATC

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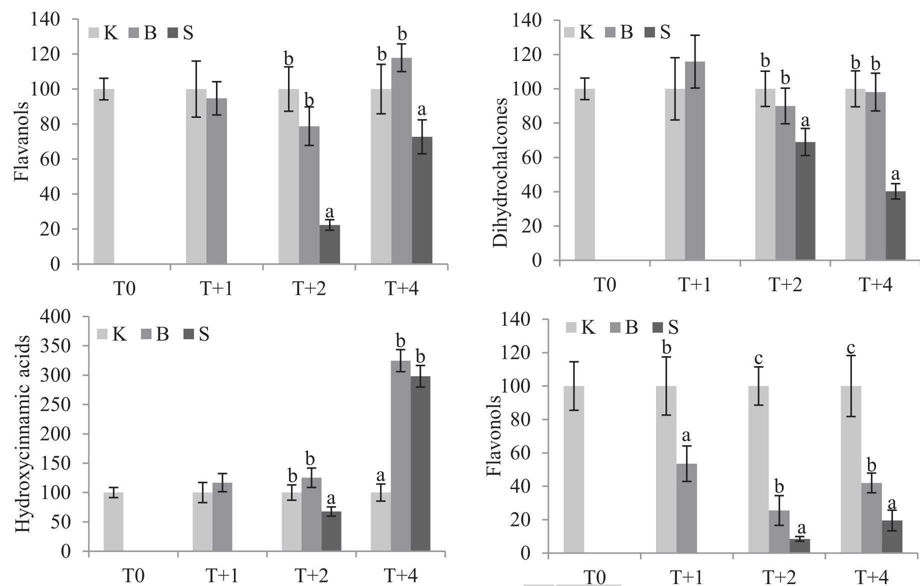

Fig. 3 Content of hydroxycinnamic acids, flavanols, dihydrochalcones, and flavonols (%) in apple peel in three sample types unaffected: control peel (K), peel with first visible signs of scald (B), and peel with fully developed scald (S) on four sampling days (T0 – T + 4). Different letters denote significant differences (Duncan test, $p < 0.05$) between treatments (K, B, and S). At each sampling date, the results were calculated in relation to the control K, which was set as 100%. Control values for each date are shown in Fig. 2

All other groups of phenolic compounds showed a different behavior than the hydroxycinnamic acids and remained unchanged with a decrease mostly at the last sampling date (Fig. 3). Fully scalded peel had significantly lower dihydrochalcone and flavanol content in comparison to the healthy control and the peel with beginning symptoms. In the group of flavanols, nine procyanidins were determined besides the monomers catechin and epicatechin. Epicatechin, procyanidin trimer, and procyanidin tetramer were the prevalent compounds in the total flavanol content; thus, their pattern is about the same as total flavanols. Even more evident changes were in flavonol content, where not only S peel, but also B peel differed from K.

Expression of genes *MdCHS*, *MdCHI*, *MdDFR*, *MdFHT*, and *MdUFGT* increased in B peel and that of *MdCHS*, *MdCHI*, *MdDFR*, *MdANS*, and *MdUFGT* increased in S peels (Fig. 4). The gene expression in S peel on the last sampling date (T + 4) was the most definite.

The activity of five enzymes from the phenylpropanoid/flavonoid pathway was determined (Fig. 5). Apart from phloretin 2′-*O*-glucosyl transferase (P2′GT), we observed a decrease in S peels compared to the control, which is consistent with the decrease in the majority of phenolic compounds but in contrast to the enhanced gene expression observed. Activity of DFR in B peel at T + 1 and T + 4 was evidently higher in comparison with K and S peel.

POX and PPO activity is presented in Fig. 6. While POX activity was less affected and generally decreased slightly, PPO activity increased significantly with scald symptoms, particularly on the last sampling date. Furthermore, correlation analysis revealed a negative correlation − 0.755 ($R^2 = 56.9\%$) between the two enzyme activities.

4 Discussion

The results of the shift in color is well presented and fits in with results obtained by Pesis et al. (2007), which measured higher $h°$, $L*$, and $C*$ at the blossom end in comparison to the stem end of fruit; the blossom end showed signs of scald. This shift from lighter to darker color could be used to aid the monitoring of stored fruits.

Hydroxycinnamic acids are well known for their accumulation in stress situations such as wounding (Mayr et al. 1994; Slatnar et al. 2013), fungal infection (Slatnar et al. 2010; Mikulic-Petkovsek et al. 2011), and physiological disorders (Felicetti and Schrader 2009; Zupan et al. 2014). The increase in hydroxycinnamic acids was also noticed in our present study on scald development. The increase in hydroxycinnamic acids in the tissue developing scald is mainly due to increased chlorogenic acid and 4-caffeoylquinic acid contents. This is in accordance with the results from Busatto et al. (2018), who noticed the extremely high increase in chlorogenic acid content in apples with developed scald. Furthermore, they observed a negative correlation between induced genes by scald incidence (such as flavonoid 3′ monoxygenase, PAL, and flavonol synthase), which resulted in an increase in chlorogenic acid (and its isomers) and other compounds such as phloridzin, catechin, quercetin, and rutin. The latter were correlated with the set of genes triggered by 1-MCP. Our results also show a decrease in content of these compounds. Busatto et al. (2014) also reported decreased accumulation of phloridzin in apples that were out of storage for 4 or 8 days showing visible signs of scald. They found a decrease in the content of some flavanols after 4 days;

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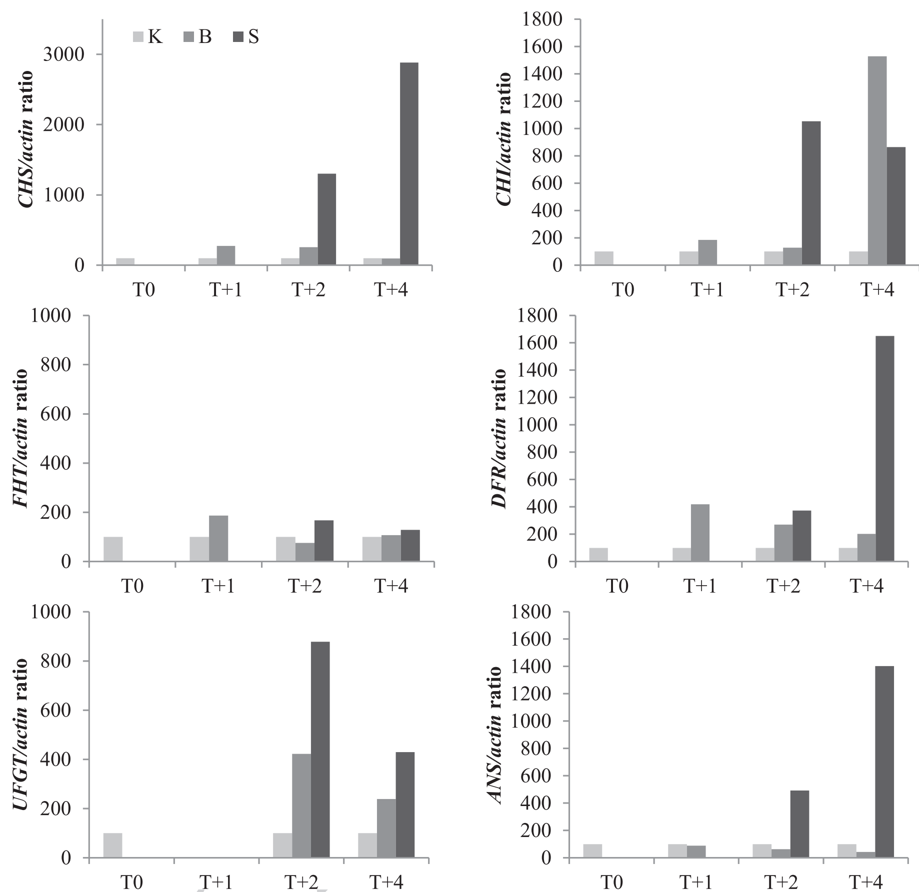

Fig. 4 Expression of *MdCHS*, *MdCHI*, *MdFHT*, *MdDFR*, *MdANS*, and *MdUFGT* in three sample types: unaffected-control peel (K), peel with first visible signs of scald (B), and peel with fully developed scald (S) on four sampling days (T0 – T+4) normalized to *actin.* At each sampling date, the results were calculated in relation to the control K, which was set as 100%. Control values for each date are shown in Fig. 2

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however, the content was again increased on the eighth day outside of storage. In older studies, which cover only some flavanols, a decrease in flavanol content was reported after the apples were taken out of storage and scald developed (Piretti et al. 1994; Ju et al. 1996). In a more recent study, Rudell and Mattheis (2009) performed a PCA study of the peel metabolome of bagged fruit after artificial UV exposure at harvest in connection to scald incidence and reported that catechin and epicatechin levels increased in unexposed peel and decreased with increased scald severity.

On the gene expression level we observed a pronounced activation of all selected genes, except for *MdFHT*, in the fully scalded peels. Busatto et al. (2014) determined higher expression of genes *MdCHS*, *MdF3H*, *MdFLS*, and *MdANR* after 1 month of storage and a higher expression of genes *MdPAL* and *MdC3H* after 2 months of storage in apples with scald. Similar results were obtained also in the study of Busatto et al. (2018). It is evident that scald development activates genes in the phenylpropanoid and flavonoid pathways. For some genes, expression is higher already in the B peel, especially *MdCHI* and *MdUFGT*, but mostly it is more pronounced in S peels. The up-regulation could be a defense reaction to stress, which is the result of scald formation.

The activity of selected enzymes of the flavonoid pathway did not show a clear correlation to phenolic content and gene expression. We assume that this can be partially explained by cell destruction and increased oxidation of phenolic compounds, which—as quinoid structures—can interact with enzymes and result in a denaturation of the enzymes. However, this is not the case for P2′GT, which remains almost unchanged if fully scalded peels are compared to controls or increased in B peels. It could be speculated that a portion of the observed in vitro activity is derived from unspecific ß-glucosidases released during cell deterioration, as the higher P2′GT activity is not reflected by higher phloridzin content. The lack of correlation between gene expression and enzyme activity could also be explained in the following way: General activation of the flavonoid pathway in response to disorder development did not occur early enough to be reflected in the flavonoid concentrations in the peels.

While the role of PPO is generally related to phenol oxidation (Nicolas et al. 1994), and thus its role in scald development is clear, the role of POX is a little more complicated. POX can generate or regulate ROS, POX also regulates the H_2O_2 level and oxidize various substrates (Passardi et al. 2005). The correlation to scald is not completely clear; however, it seems that apples with scald show lower POX activity in comparison to sound

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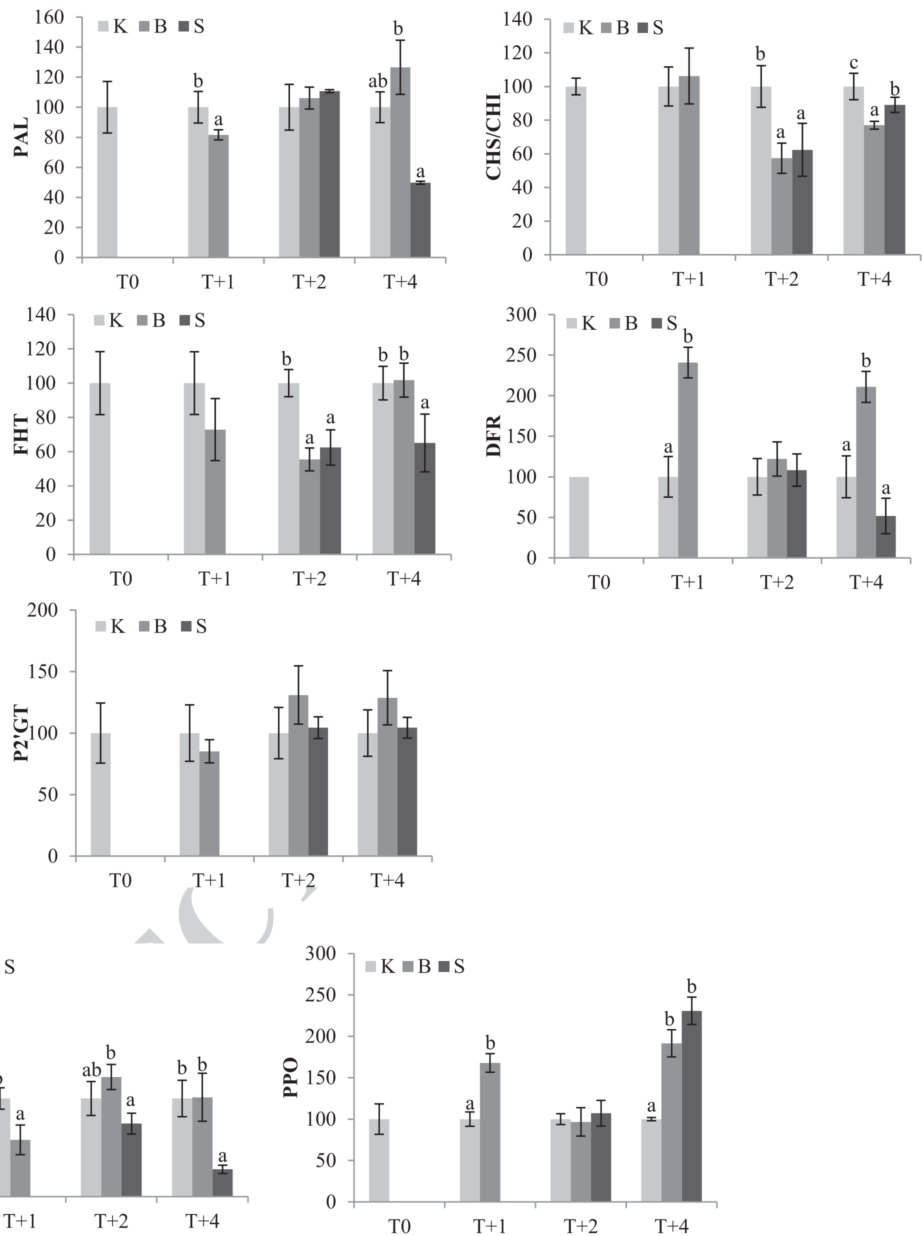

Fig. 5 Changes in specific enzyme activity (%) of PAL, CHS/CHI, FHT, DFR, and P2′GT in three sample types: unaffected - control peel (K), peel with first visible signs of scald (B), and peel with fully developed scald (S) on four sampling days (T0 – T+4). At each sampling date, the results were calculated in relation to the control K, which was set as 100%. Different letters in each sampling date denote significant differences (Duncan test, $p < 0.05$) between treatments (K, B, and S). Control values for each date are shown in Fig. 2

Fig. 6 Peroxidase (POX) and polyphenol oxidase (PPO) activity (%) in three sample types: unaffected - control peel (K), peel with first visible signs of scald (B), and peel with fully developed scald (S) on four sampling days (T0 – T+4). At each sampling date, the results were calculated in relation to the control K, which was set as 100%. Different letters in each sampling date denote significant differences (Duncan test, $p < 0.05$) between treatments (K, B, and S). Control values for each date are shown in Fig. 2

apples or even resistant cultivars (Bramlage et al. 1993; Rao et al. 1998; Ju and Bramlage 1999; Fernandez-Trujillo et al. 2003). In our present study, POX activity was also the lowest in peels with fully developed scald, especially on T+4. As mentioned above, the PPO activity was like a mirror picture of POX activity, with the highest activity in fully scalded peels and lowest in healthy control peel. The increased PPO activity in fully scalded peel and also in peel with beginning scald symptoms on T+4 can also be correlated to high hydroxycinnamic content, especially chlorogenic acid, which is a well-known substrate for PPO (Di Guardo et al. 2013). Yihui et al. (2018) claim that laccases, a subgroup of PPO category, are responsible for the brown coloration in scald formation, with their strong affinity for epicatechin as a substrate in oxidation process. While our test for PPO activity determination was not that specific, we do believe it is not just the laccase, but the whole PPO group, that is responsible for the brown coloration. However, further investigation should be conducted to make firm conclusions.

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446 ## 5 Conclusions

447 We conclude that the main reaction to scald in the phenyl-
448 propanoid pathway occurs at various levels. Whereas there
449 is a strong induction of gene expression in the flavonoid
450 pathway, probably as part of the defense reaction, most
451 polyphenol groups and enzyme activities of the flavonoid
452 pathway decrease with the development of scald symp-
453 toms, most likely due to their disintegration. Our hypoth-
454 esis was therefore confirmed. A strong up-regulation was
455 observed in hydroxycinnamic acid concentrations, namely,
456 of chlorogenic acid. The increase of chlorogenic acid con-
457 tent was followed by increased PPO activity, whereas POX
458 activity decreased. With this, we elucidated the phenolic
459 role and fate during scald development. Among all phe-
460 nolic compounds, chlorogenic acid has the most prominent
461 role in scald development. In future studies, indestructible
462 methods for phenolic content determination could be used
463 to follow the transition from healthy to scald on the same
464 fruit. This knowledge could be useful for apple sorting or
465 even for scald incidence prediction.

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472 **Author contributions** A.C.—experiment execution, extraction and
473 analyses of phenolic content, POX and PPO activity determination,
474 statistical analysis, writing of the manuscript. H.H.—results review
475 and comment (analyzes of enzyme activity and analysis of gene expres-
476 sion), manuscript supervisor. M.M-P.—MS analyses. R.V.—mentor,
477 the experiment initiator and manuscript supervisor. A.S.—determi-
478 nation and analysis of enzyme activity, analysis of gene expression,
479 statistical analysis, manuscript corrections statement.

480 ## Compliance with ethical standards

481 **Conflict of interest** The authors have no conflict of interest to declare.

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