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ARTICLE

A Detailed Survey of Seed Coat Flavonoids in Developing Seeds of *Brassica napus* L.

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Proanthocyanidins (PAs) are seed coat flavonoids that impair the digestibility of *Brassica napus* meal. Development of low-PA lines is associated with a high-quality meal and with increased contents in oil and proteins, but requires better knowledge of seed flavonoids. Flavonoids in *Brassica* mature seed are mostly insoluble so that very few qualitative and quantitative data are available yet. In the present study, the profiling of seed coat flavonoids was established in eight black-seeded *B. napus* genotypes, during seed development when soluble flavonoids were present and predominated over the insoluble forms. Thirteen different flavonoids including (−)-epicatechin, five procyanidins (PCs which are PAs composed of epicatechin oligomers only) and seven flavonols (quercetin-3-*O*-glucoside, quercetin-dihexoside, isorhamnetin-3-*O*-glucoside, isorhamnetin-hexoside-sulfate, isorhamnetin-dihexoside, isorhamnetin-sinapoyl-trihexoside and kaempferol-sinapoyl-trihexoside) were identified and quantified using liquid chromatography coupled to electrospray ionization-mass spectrometry (LC−ESI-MS^n). These flavonol derivatives were characterized for the first time in the seed coat of *B. napus*, and isorhamnetin-hexoside-sulfate and isorhamnetin-sinapoyl-trihexoside were newly identified in *Brassica* spp. High amounts of PCs accumulated in the seed coat, with solvent-soluble polymers of (−)-epicatechin reaching up to 10% of the seed coat weight during seed maturation. In addition, variability for both PC and flavonol contents was observed within the panel of eight black-seeded genotypes. Our results provide new insights into breeding for low-PC *B. napus* genotypes.

KEYWORDS: *Brassica napus*; Brassicaceae; flavonoids; procyanidins; flavonols; seed coat; seed development; LC−ESI-MS^n

INTRODUCTION

Flavonoids are plant secondary metabolites that belong to different subclasses according to the position of the linkage of the aromatic ring, the saturation level and the oxidation degree of the C-ring (**Figure 1B**). The diversity of the substitutions and/or of the degree of polymerization lead to a multitude of compounds since more than 9,000 flavonoids have been already identified (*1*). Among flavonoids, flavonols, anthocyanins, phlobaphenes, isoflavones and proanthocyanidins (PAs that are flavan-3-ol polymers) accumulate at high levels in distinct plant seeds and grains (reviewed in refs *2* and *3*; **Figure 1A**), where they participate to the seed coat pigmentation. Seed flavonoids are involved in defense against biotic and abiotic stresses and contribute to physiological processes such as reinforcement of seed longevity and dormancy (reviewed in ref *2*). The nature and amounts of seed flavonoids depend on plant species, genotype, and environmental growth conditions (*4*, *5*).

The flavonoid biosynthetic pathway has now been well established especially in the model Brassicaceae *Arabidopsis thaliana* L., where a wide range of mutants is available. These mutants are generally called *transparent testa* (*tt*) since their seeds display a pale-brown to yellow pigmentation due to a modification in the flavonoid composition (*6*). Most of the *tt* mutants have been characterized at the molecular and/or at the biochemical levels (reviewed in ref *3*). *Arabidopsis* seeds accumulate epicatechin and procyanidin oligomers (PCs, solely constituted of epicatechin units) that are specific of the seed coat, and flavonols that are found in both seed coat and embryo tissues (*5*). PCs accumulate first as colorless compounds and turn brown upon seed desiccation. In addition, there is a natural variability for PC content among natural accessions of *Arabidopsis* (*5*).

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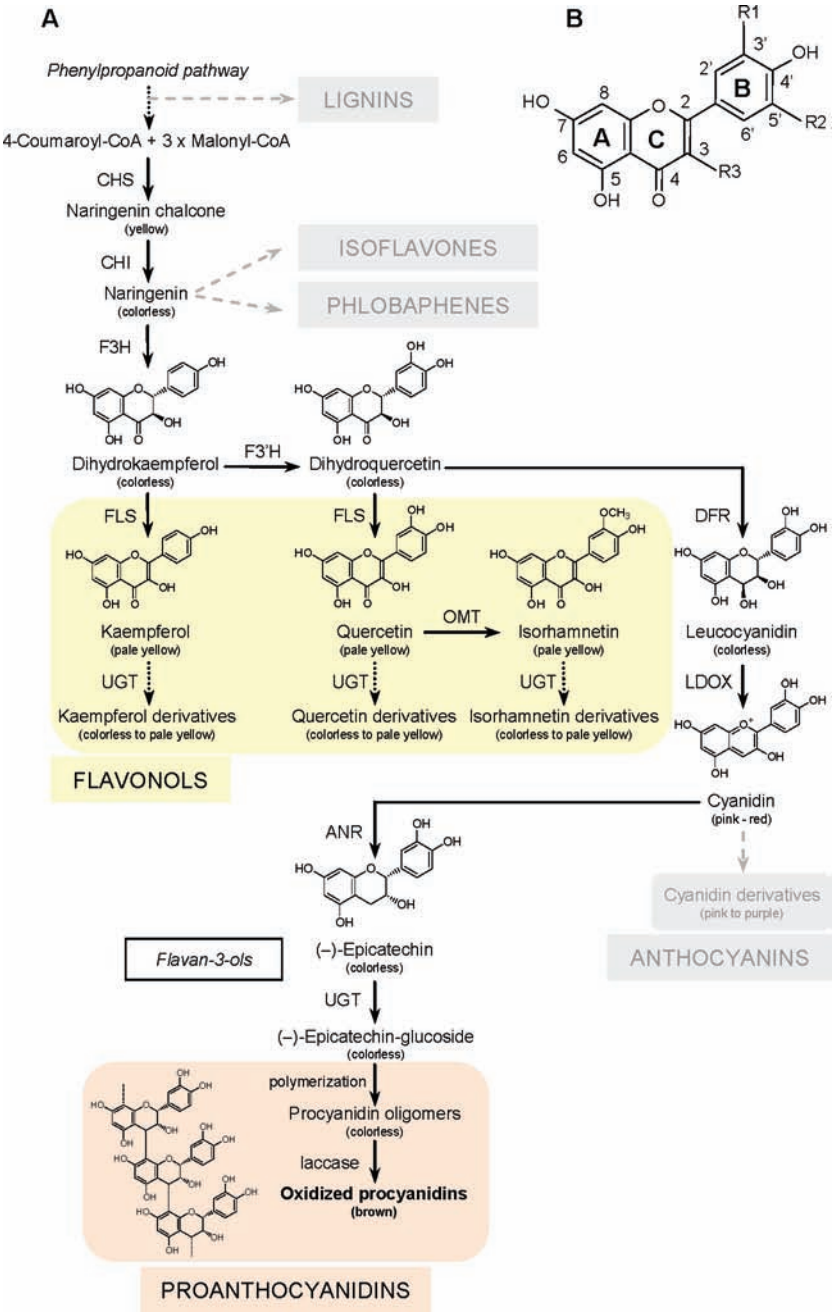

Figure 1. Schematic overview of the flavonoid biosynthetic pathway in seeds. (**A**) Flavonoid biosynthetic pathway in the seed coat of the Brassicaceae. Gray dashed arrows indicate sub-branches that were not identified in seed coats of the Brassicaceae. ANR, anthocyanidin reductase; CHS, chalcone synthase; CHI, chalcone isomerase; DFR, dihydroflavonol 4-reductase; F3H, flavanone 3-hydroxylase; F3′H, flavonoid 3′-hydroxylase; FLS, flavonol synthase; OMT, *O*-methyl transferase; LDOX, leucoanthocyanidin dioxygenase; UGT, uridine diphosphate glycosyltransferase. (**B**) Basic structure of flavonoids. Carbons on the A, C and B rings are numbered according to their acetate (unprimed numbering) or shikimate (primed numbering) origin. Substitutions at positions R1, R2 and R3 generate a very large diversity of flavonoids.

In dry mature seeds of *Brassica napus* L. (oilseed rape, another Brassicaceae), seed coat is dark brown to black and represents up to 17% of total seed weight (*7*). It accumulates high amounts of insoluble PAs that are difficult to characterize (*8*−*10*). Henceforth, the nature of PA units still needs to be established in oilseed rape. We recently demonstrated that soluble PA accumulation started from early embryogenesis in the micropyle-chalaza area and went on from mid to late embryogenesis in the seed body. Thus, soluble PA content was maximal during seed maturation (*11*). On the other hand, the presence of flavonols in *Brassica* seeds was reported only recently with the identification of four kaempferol conjugates (*12*, *13*), but there was no indication on the *in situ* localization of these compounds. Flavonols were also detected in vegetative parts and inflorescences of *Brassica* spp (*14*−*16*). Finally no anthocyanins were detected in *Brassica* seeds (*11*, *13*, *17*). Lignins, as flavonoids, derive from the phenylpropanoid pathway (**Figure 1A**). Previous work reported the presence of high amounts of thioglycolic lignin in the seed coat within a large panel of black-seeded *Brassica* spp (*17*). However, these results need to be confirmed since the method used for lignin extraction (*18*) was not specific to these compounds and, thus, PAs may have interfered with the measurement.

The presence of PAs in oilseed rape meal has antinutritive effects in both monogastric and ruminant livestock feeds (*19*, *20*).

Indeed, PAs are able to form soluble and insoluble complexes with proteins, polysaccharides and other macromolecules from the diet, thus reducing their bioavailability (reviewed in ref *21*). Therefore, reduction of PA content constitutes one breeding objective to improve oilseed rape quality. Yellow seediness that is associated with a low PA content naturally exists in several *Brassica* spp. including *B. rapa*, *B. juncea* or *B. carinata* and has been introduced into *B. napus* through interspecific crosses (*22*). Yellow-seeded germplasms display lower dietary fiber content resulting in an improved meal quality (*17*, *23*). Concomitantly, yellow-seeded lines show an increase in seed oil and protein contents (*23*). Despite these advantages, a very small number of yellow-seeded oilseed rape varieties have been commercialized to date (*24*, *25*). Indeed, the yellow-seeded phenotype is highly variable depending on the environmental growth conditions and yellow-seeded *Brassica* often show precocious germination as well as a low agronomic value. One could hypothesize that reduction in PAs within these yellow-seeded germplasms is accompanied by a modification in the content or composition of other flavonoids or even of phenylpropanoids.

Up to now, the evaluation of PA amount in *B. napus* seed is subjected to many discrepancies in the literature (*10*, *17*, *26*) that could be explained by two reasons. On the one hand, all the published data were obtained with dry mature seeds where insoluble PAs predominate over soluble PAs (unextractable PAs constitute 70−95% of total PAs in mature seeds (*10*)), thus, lowering the total PA amount. Insolubility of PAs increases with polymerization, oxidations, and formation of complexes with fiber and/or protein fractions of the seed (*10*). On the other hand, solvent systems used for PA extraction as well as quantification methods were very diverse (*10*, *17*, *26*).

Acidolysis in the presence of an excess of a nucleophilic molecule (usually phloroglucinol or toluene-α-thiol) allows the depolymerization of PA oligomers and polymers. When combined with liquid chromatography−mass spectrometry (LC−MS) analysis of the reaction media, this method can be used to characterize PA oligomers (*27*) and is relevant to overcome the problems linked to their polymeric nature. For instance, PAs from grape (*28*), apple (*29*), beer (*30*) and many other plant materials have been characterized and quantified on the basis of their flavan-3-ol constitutive units and their mean degree of polymerization (mDP). However as far as we know, it was never used to characterize PAs from oilseed rape.

In order to conduct accurate breeding programs to reduce PAs in *B. napus* seed, it is necessary to get a better knowledge of the exact nature and quantities of seed coat flavonoids. This prompted us to conduct a detailed survey of seed coat flavonoids in *B. napus* during seed development. Flavonoid extracts from seed coat samples were analyzed through high performance liquid chromatography (HPLC) coupled to diode array detection and to mass spectrometry with electrospray ionization source (LC/DAD/ESI-MS) to determine PA and flavonol contents. Thirteen different flavonoids were identified and quantified during seed development. Most of these compounds were described for the first time in the seed of *B. napus*. Additional information on PA accumulation was obtained after hydrolysis of polymers to assess total PA content and mDP. All the results were first acquired for *B. napus* Jet Neuf genotype and confirmed for seven additional black-seeded accessions.

MATERIALS AND METHODS

Chemicals and Reagents. Methanol, acetic acid and trifluoroacetic acid (TFA) (VWR, West Chester, PA) as acetone and hexane (Sigma Aldrich, St. Louis, MO) were HPLC grade quality. Butan-1-ol, ethanol and hydrochloric acid (VWR) were analytical grade quality. Water was purified using a Milli-Q system (Millipore, Molsheim, France). 4-Dimethylamino cinnamaldehyde (DMACA), diphenylboric acid 2-aminoethyl ester (DPBA), phloroglucinol and ammonium iron(III) sulfate·12H_2O were purchased from Sigma Aldrich, sodium acetate·3H_2O and KOH from Merck (Darmstadt, Germany), and L(+)-ascorbic acid from VWR. The commercial standards were PC B2, isorhamnetin-3-*O*-glucoside, kaempferol-3-*O*-glucoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-rhamnoside, cyanidin chloride (Extrasynthèse, Genay, France), (−)-epicatechin, (+)-catechin (Sigma Aldrich) and sinapic acid (Fluka, Buchs, Switzerland). PC B5 was prepared by Dr. Pascal Poupard (INRA Rennes).

Plant Material and Sample Collection. Aviso (00), CMB1039 (00), Doublol (00), Jet Neuf (0+) and PR3984 (00) were winter type oilseed rape genotypes whereas Tanto (00), Westar (00) and Yudal (++) were spring type genotypes. All oilseed rape genotypes were lines with yellow petals from the Brassica Genetic Resources Center in INRA Rennes (France). Plants were grown as described previously (*11*) with a vernalization period of 4 or 8 weeks for spring or winter genotypes, respectively. Plant feeding with the nutritive solution was stopped at 45 days after pollination (dap) to avoid precocious germination. Individual flowers of the primary raceme were manually pollinated and tagged on the day of pollination. Developing seeds were harvested every five days from 5 to 60 dap for the different analyses. For seed flavonoid analyses, four pods were collected from one plant and five seeds were carefully removed from each pod. The seed coats (20 per sample in total) were manually separated from the embryos and immediately frozen into liquid nitrogen. The sampling was done in triplicate for each developmental stage, considering three independent plants grown together. Three independent biological replicates were conducted for the whole seed development kinetic. Seeds for colorimetric assays were collected on the same pods as those used for metabolic analyses.

Colorimetric Assays for Qualitative Detection of Seed Flavonoids. The DMACA histochemical assay (*31*) is specific for detection of flavan-3-ols and PAs (**Figure 1A**). It was conducted on immature seeds or deparaffined seed sections as described previously (*11*). Samples were observed under a low-power stereomicroscope (SMZ-U, Nikon, Tokyo, Japan).

Flavonol detection was performed in the presence of DPBA (*32*). Samples that consisted of batches of 20 seeds, seed coats or embryos, were incubated for 2 h under vacuum in 1 mL of DPBA, and then manually crushed. The supernatant was transferred into a new tube and observed under a UV lamp (230 V, 50 Hz; Fisher Bioblock Scientific, Illkirch, France). For flavonol localization, resin-embedded seed sections were mounted in a drop of DPBA and fluorescence images were captured using a CoolSnap HQ camera (Photometrics, Tucson, AZ) on an Axioplan 2 microscope (Zeiss, Oberkochen, Germany) equipped with filter sets for fluorescence (Ex BP 365/12; Em LP 397 for DAPI and Ex BP 450−490; Em LP 515 for FITC). Images were analyzed using MetaVue (Universal Imaging, Downington, PA).

Extraction of Seed Flavonoids. Protocol for seed flavonoid extraction was adapted from the literature (*5*, *33*). Samples (20 seed coats) previously harvested were lyophilized, weighted and ground for 5 min in an Eppendorf tube containing four stainless steel balls (Ø 4 mm; Lemoine, Rennes, France) using a tissue lyser system. One milliliter of a methanol/acetone/water/TFA mixture (40:32:28:0.05, v/v/v/v) was added to the samples that were sonicated for 15 min at 4 °C. After centrifugation (5 min, 19000*g*), the pellet was extracted further with 1 mL of the same methanol/acetone/water/TFA mixture overnight at 4 °C under agitation (200 rpm), while the supernatant was stored at −80 °C. After a second centrifugation, the two supernatants were pooled and separated into two equal fractions: the first one (hereafter called the "flavonoid extract") for the direct analysis of extractable flavonoids and the second one (hereafter called the "PA extract") for the characterization of the flavan-3-ol constitutive PA-units (i.e., (epi)-catechin, (epi)-gallocatechin, (epi)-afzelechin and/or their galloyl esters), as well as for the quantification of soluble PAs and the estimation of their mDP. The two extracts were concentrated under a flow of nitrogen at 23 °C in a heater dry bath. To ensure the total elimination of water, 200 μL of methanol was added to the extracts two times during evaporation. Then, the dry flavonoid extract was dissolved in 1.2 mL of 1% acetic acid in methanol and stored at −80 °C before LC−ESI-MS^n analysis. The PA extract was submitted to depolymerization for

quantification of soluble PAs and estimation of mDP (see below). The pellet was stored at −80 °C before analysis of insoluble PCs.

Quantification of Soluble PAs and Determination of the mDP. The method for determination of soluble PA content and mDP is based on the release of terminal flavan-3-ol units and extension units as carbocations after cleavage of PAs under dry acidic conditions (ref *34*, see below). If present in excess in the reaction medium, a nucleophilic molecule like phloroglucinol can trap these carbocations and lead to stable flavan-3-ol-phloroglucinol adducts. Their structure strictly depends on the nature of the extension units in the genuine PA molecules. The PA extract was dissolved in 900 μL of 0.1 N HCl in methanol containing 50 g/L phloroglucinol and 10 g/L ascorbic acid. After incubation at 50 °C for 30 min, the reaction was stopped by adding one volume of 0.2 M aqueous sodium acetate. The samples were stored at −80 °C before LC−ESI-MS analysis. After LC−ESI-MS analysis using the ion trap mass spectrometer LCQ Deca (Thermo Finnigan), the molar amount of soluble PAs was quantified as the sum of phloroglucinol-derivatized extension units and free terminal units. The amount of soluble PAs was expressed in μg (−)-epicatechin equivalent (EC equiv) per mg of dry weight (DW). The mDP was calculated as described by Kennedy and Jones (*34*), using the formula mDP = (phloroglucinol-derivatized extension units + free terminal units − native flavan-3-ols)/(free terminal units − native flavan-3-ols) that allowed taking the amount of native flavan-3-ols into account.

Liquid Chromatography−Mass Spectrometry. Samples were filtered through a Teflon membrane (0.45 μm, Interchim, Montluçon, France) before analysis. HPLC was performed on a system including a SCM1000 vacuum membrane degasser (Thermo Quest, San Jose, CA), a Surveyor autosampler (Thermo Finnigan, San Jose, CA), a 1100 series binary pump (Agilent Technologies, Palo Alto, CA), and a Spectra system LP6000 DAD detector (Thermo Fisher Scientific, San Jose, CA). Four microliters of samples were separated on an XDB-C18 Eclipse column (2.1 × 150 mm, 3.5 μm; Agilent Technologies). The flow rate was 0.2 mL/min, and the oven temperature was 30 °C. Eluent A was 0.1% formic acid in water, and eluent B was 0.1% formic acid in acetonitrile. The following gradient was applied: initial, 3% B; 0−5 min, 9% B linear; 5−15 min, 16% B linear; 15−45 min, 50% B linear; 45−51 min, 90% B linear, followed by column wash and re-equilibration.

Mass analyses were carried out with the mass spectrometer LCQ Deca, equipped with an ESI source used in the negative ion mode. Source parameters were as following: spray voltage, 3.69 kV; capillary voltage, −70.85 V; sheath gas, 67.72 arbitrary units; auxiliary gas, 3.76 arbitrary units; capillary temperature, 240 °C. Nitrogen was used as sheath gas and helium as damping gas. Full scan spectra were acquired from m/z 200 to m/z 2000. Soluble flavonoid extracts were first analyzed in full MS mode to identify the major flavonoids. Then, MS^n analyses were carried out on selected m/z ions. Collision energy for MS^n analyses was from 20 to 40%. Data collection and processing were done with Xcalibur software version 1.2. Relative quantification was performed on extracted ion chromatograms of the molecular ions $[M - H]^-$ of the identified compounds, using the bracket external standard function of Xcalibur. Contents in flavan-3-ols and PA oligomers were expressed in (−)-epicatechin equivalents, relative to an (−)-epicatechin calibration curve. A standard was available to express phloroglucinol−epicatechin content. Contents in individual flavonols were expressed relative to the calibration curves of available standards, namely, isorhamnetin-3-*O*-glucoside, kaempferol-3-*O*-glucoside or quercetin-3-*O*-glucoside, depending on the aglycon core.

To confirm the structure of the identified molecules, MS and MS^n analyses were also conducted using an Alliance 2695 RP-HPLC system (Waters, Milford, MA), a Waters 2487 UV detector set at 280 nm, a triple quadrupole "Quattro LC" with an ESI "Z-spray" interface (MicroMass Co, Manchester, U.K.), and the MassLynx software (*35*). An Uptisphere C_{18} column (150 × 2 mm; 5 μm; Interchrom) was used with a mix containing solvent A acetonitrile/acetic acid (95.5:0.5, v/v) and solvent B water/acetic acid (0.5:95.5, v/v) with a gradient profile starting with 10:90, A/B, v/v, for 5 min; linear gradient up to 52.8:47.2, A/B over 30 min; a washing step 100:0, A/B for 10 min at a flow rate of 0.2 mL/min; final equilibration at 10:90, A/B for 5 min at a flow rate of 0.4 mL/min and 5 min at a flow rate of 0.2 mL/min. Column temperature was maintained at 24 °C. The ESI source potentials were as follows: capillary, 3.25 kV (positive mode) or 3.0 kV (negative mode); extractor, 2 V; sampling cone, 20 V (positive mode) or 25 V (negative mode). The source block and desolvation gas were heated at 120 and 400 °C, respectively. Nitrogen was used as nebulization and desolvation gas (100 and 500 L/h, respectively). Argon was used as collision gas and was set at 3.5×10^{-3} mbar for the collision-induced dissociation (CID) MS^n experiments (10−50 eV collision energy). Theoretical isotopic signatures of ions were established by the MassLynx software.

To determine the actual mass of ions, time-of-flight (TOF) mass spectrometry was conducted on a LCT Micromass device supplied with an ESI source (MicroMass Co). The coupled HPLC device and conditions of analysis were as described previously for the triple quadrupole, except for the sampling cone that was settled on 25 or on 40 V.

Quantification of Insoluble PCs. The method for quantification of insoluble PCs was described by Porter and co-workers (*36*). It is based on the release of pink-red cyanidin after cleavage of PCs under acidic conditions that could be quantified by comparison of the absorbance at 550 nm with a cyanidin standard curve. Three milliliters of butanol−HCl (95:5; v/v) and 100 μL of 2% ferric ammonium sulfate (w/v) in 2 N HCl were added to the extraction pellets. The tubes were heated 3 h at 95 °C in a water bath. After cooling on ice, absorbance of cyanidin was recorded at 550 nm using a SPECTRAmax M2 microplate reader (Molecular Devices, Sunnyvale, CA) and the SoftMax Pro v.4.6 software. Three cyanidin standard curves were systematically established for each spectrophotometer 96-well plate, with cyanidin-chloride amounts ranging from 0 to 30 μg/mL. The amount of insoluble PCs was expressed in μg cyanidin equiv per mg DW using the mean values given by the three cyanidin standard curves.

RESULTS AND DISCUSSION

Flavonoids Accumulate in the Seed Coat of *B. napus*. The development of *B. napus* seed was described previously (*11*). Oxidized PAs that give the seed coat its brown to black color appeared as dark brown spots over the seed coat during seed maturation (35−45 dap). These spots enlarged to cover the whole seed body of the mature seed (data not shown). To get the first clues about flavonoid localization and composition, we treated developing seeds of *B. napus* with the DMACA reagent that specifically stained blue in the presence of PAs and their precursors. PA deposition was restricted to the seed coat since the embryo remained unstained (**Figure 2A**). In addition, DMACA-stained seed sections showed that PAs were localized into the innermost cell layer of the inner integument, namely, the endothelium (**Figure 2B**,**D**). PAs also accumulated in the micropyle-chalaza region (**Figure 2B**,**C**), referred to as the pigment strand (*11*, *37*). Staining seed samples with DPBA gave insights into the flavonol composition since DPBA-conjugated flavonols emitted a specific fluorescence under UV excitation (*32*). This fluorescence was orange with flavonol extracts from whole seeds (**Figure 2E**, left), suggesting the predominance of quercetin derivatives that fluoresce yellow to orange (**Figure 2F**). Seed coat extract also produced a faint orange fluorescence barely detectable (**Figure 2E**, middle). In contrast, embryo extract gave a greenish fluorescence (**Figure 2E**, right) that could be due to the presence of kaempferol derivatives (**Figure 2F**) and/or sinapate esters. DPBA-treated cross sections of immature seeds displayed a yellow fluorescence in the thick-walled palisade layer, which is the innermost cell layer of the outer integument (**Figure 2H**). This fluorescence was mostly localized at the basis of the palisadic cells as well as on the radial cell walls and could be due to quercetin derivatives. An orange fluorescence was also observed on the most internal part of the pigment strand (**Figure 2G**).

Taken together, these results suggested that flavonoids in the *B. napus* seed coat were primarily PAs and/or their precursors, as well as flavonols that were most probably quercetin derivatives. These two classes of flavonoid compounds were localized in distinct cell layers within the seed integuments. Localization pattern of flavonoids in *B. napus* seed coat was very similar to the one described in *Arabidopsis*, where PCs accumulated in the

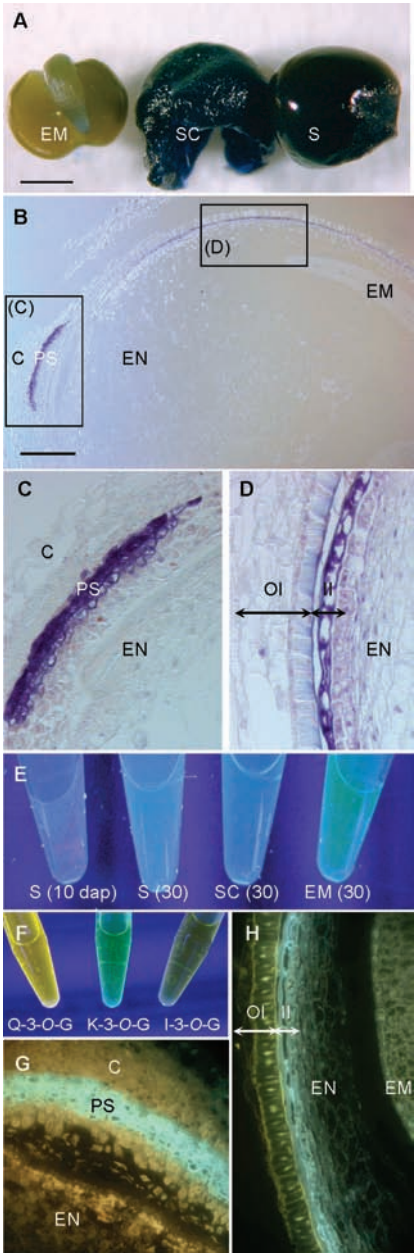

Figure 2. Histochemichal detection of flavonoids in immature seeds from *B. napus*. (**A**−**D**) DMACA stainings of immature seeds harvested at 25 dap (**A**) and of deparaffined sections from 25 dap seeds (**B**−**D**). The blue coloration observed under a low-power stereomicroscope is specific to the presence of flavan-3-ols and/or PAs. **C** and **D** represent enlargements of **B** within the pigment strand and the seed body respectively. (**E**−**H**) Detection of flavonols after DPBA assays on seeds, seed coats or embryos harvested at 10 or 30 dap (**E**), corresponding fluorescence of monoglucosylated standards of isorhamnetin-3-*O*-glucoside (I-3-*O*-G), kaempferol-3-*O*-glucoside (K-3-*O*-G) and quercetin-3-*O*-glucoside (Q-3-*O*-G) (**F**) and deparaffined sections from 25 dap seeds (G, H). Samples in **E** and **F** were observed under UV light, flavonols were detected as a dark-orange fluorescence for quercetin derivatives (seed and seed coat extracts) and a green fluorescence for kaempferol derivatives (embryo extracts). Seed sections in **G** and **H** were observed under a microscope equipped with filter sets for fluorescence and represent enlargements of the pigment strand and the seed body respectively. C, chalaza; EM, embryo; EN, endosperm; II, inner integument; OI, outer integument; PS, pigment strand; S, whole seed; SC, seed coat. Bars = 1 mm (**A**); 200 μm (**B**) and 35 μm (**G**, **H**).

endothelium and in the pigment strand layer (*37*), whereas flavonols (essentially quercetin derivatives) were found in the outer integument based on DPBA fluorescence results (*38*). This suggested that networks governing flavonoid accumulation in the seed coat were conserved between the two Brassicaceae.

PAs in *B. napus* Seed Coat Are (−)-Epicatechin Polymers. Flavonoid extracts from seed coats collected from immature seeds of *B. napus* were analyzed by LC−ESI-MS^n. Retention times (t_R), absorbance spectra and MS^n mass spectra of compounds were recorded and compared with commercial standards or published data when available, in order to characterize both flavan-3-ol monomers and PA oligomers.

The UV chromatograms and total ion currents of seed coat extracts showed an unresolved massif ranging from 14 to 28 min that corresponded to the elution of homogeneous PA oligomers (**Figure 3A**,**B**). The main ions included in this massif were (−)-epicatechin (t_R = 15.2 min and $[M - H]^-$ at m/z 289 [**Figure 3C**, **Table 1** and part 1 of the Supporting Information]) and PA oligomers (see below), which were presumably constituted by (−)-epicatechin units since no (+)-catechin nor other flavan-3-ols were detected (**Figure 3C**). This result was confirmed by LC−ESI-MS^n analyses after acidic cleavage in the presence of phloroglucinolysis, since PC oligomer extension units were unambiguously detected as phloroglucinol adduct of (−)-epicatechin only (t_R = 9.8 min and $[M - H]^-$ at m/z 413; data not shown). Thus, PAs from *B. napus* seed coat were only PCs, as shown in *Arabidopsis* (*5*). This was consistent with the presence of four genes encoding anthocyanidin reductase (ANR)-like proteins (conversion of cyanidin into epicatechin; **Figure 1A**) in the *B. napus* genome (*39*) and with the absence of transcripts for leucoanthocyanidin reductase (LAR, conversion of leucocyanidin into catechin) in *B. napus* public sequence databases (*11*). Consistent with PC localization, accumulation of ANR-like transcripts was restricted to the inner integument in *B. napus* seed coat (*39*).

Monocharged oligomers of (−)-epicatechin were identified in the flavonoid extracts by their $[M - H]^-$ signals at m/z 577, 865, 1153, 1441, and 1729 corresponding to degree of polymerization (DP) 2, DP 3, DP 4, DP 5 and DP 6 respectively (**Figure 3C**, **Table 1** and part 1 of the Supporting Information). The two isoforms B2 and B5 of DP 2 were unambiguously retrieved (**Table 1**) according to the mass spectra and t_R of the standards. Doubly charged oligomers were also detected at m/z 720, 1008, 1296, 1585, and 1873 corresponding to $[M - H]^{2-}$ of DP 5, DP 7, DP 9, DP 11 and DP 13 respectively (**Figure 3C**) as described previously (*29*). However, these signals were not intense enough to perform MS^2 analysis. For all PC oligomers from DP 3 to DP 13, the corresponding extracted chromatograms displayed one major peak and numerous broadened peaks, which likely corresponded to isomers or to fragment ions of polymers with a greater mDP and could eventually account for the unresolved massif observed between 14 and 28 min. Noticeably, although some of the flavonoid extracts originated from black seed coat samples and were thus supposed to contain oxidized PCs, no specific m/z corresponding to oxidized PCs previously characterized (P. Poupard and S. Guyot, INRA Rennes, personal communication) were clearly identified here. Finally, LC−ESI-MS^n analyses using the LCQ Deca ion trap mass spectrometer confirmed that PCs accumulated specifically in *B. napus* seed coat since no PCs were detected in flavonoid extracts from embryo, endosperm or petals (data not shown).

Time-Course Accumulation of PCs in Immature *B. napus* Seed Coat Suggests That They Likely Undergo Oxidations during Late Seed Developmental Stages. Previous works reported the quantification of PAs in *B. napus* seed, but the results were acquired on mature dry seeds and therefore emphasized the predominance of

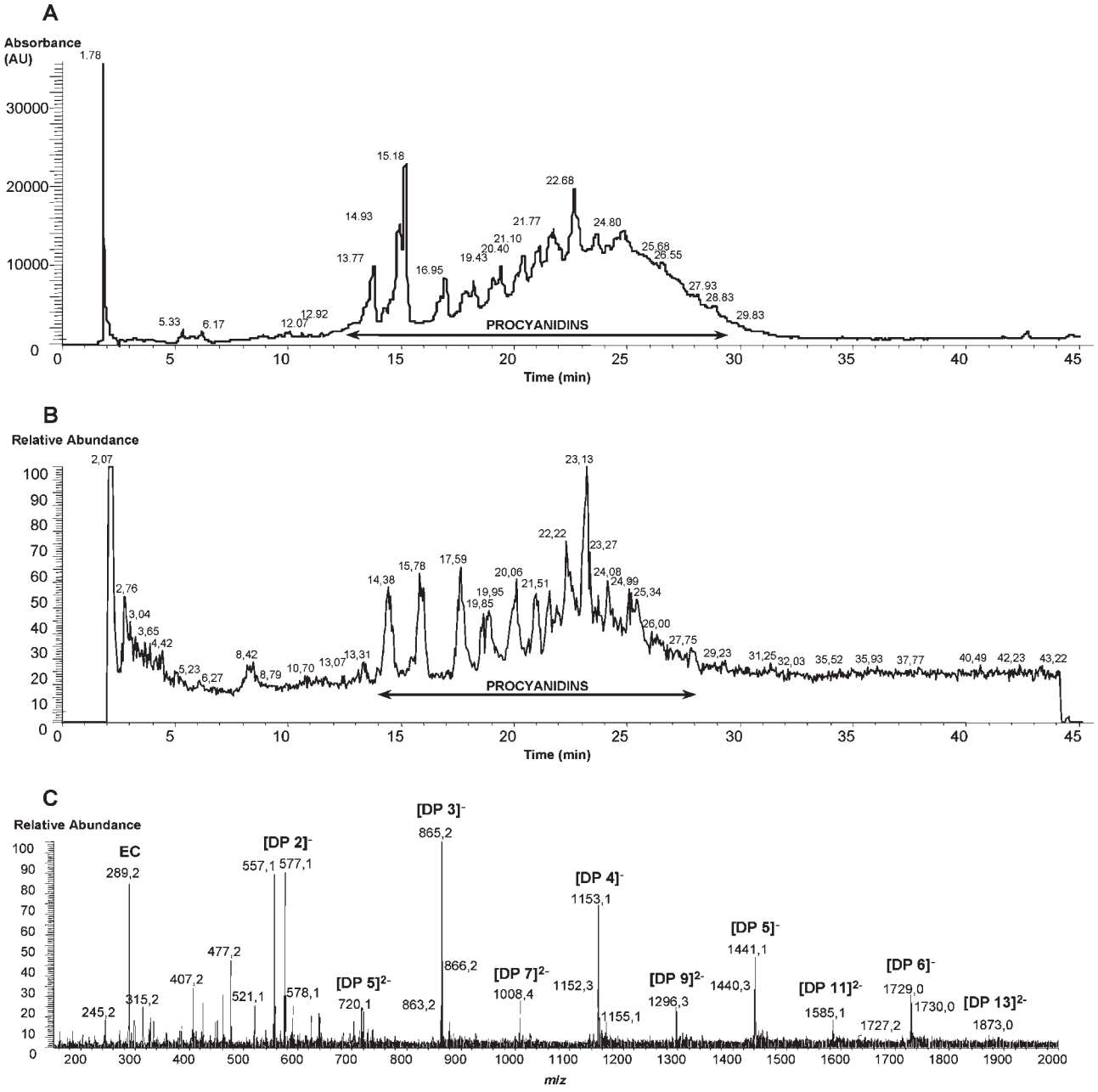

Figure 3. LC−ESI-MS profile from a 40-dap extract of seed coat flavonoids from *B. napus* Jet Neuf cv. (**A**) UV chromatogram (280 nm). Absorbance is given in arbitrary units (AU). (**B**) Total ion current showing an unresolved massif of peaks from 14 to 28 min that is characteristic of PC oligomers. (**C**) Average MS spectrum shows the relative abundance of $[M - H]^-$ ions between 14 and 28 min and displays predominance of monocharged and doubly charged PC oligomers. Data were acquired with the Xcalibur software after analysis on the ion trap mass spectrometer. EC, epicatechin; DP, degree of polymerization.

Table 1. Identification of PCs after LC−ESI-MS^2 Analysis of Seed Coat Flavonoid Extract from *B. napus* (Jet Neuf Cv.)

		mass spectrum (*m*/*z*)		
compound name[a]	t_R[b] (min)	$[M - H]^-$ [c]	$[M + H]^+$ [d]	MS^2 $[M - H]^-$ [c] (*m*/*z*)
$[DP 2]^-$ B2*	13.9	577	579	451, 425, 407, 289
(−)-epicatechin*	15.2	289	291	245, 205, 203, 179
$[DP 3]^-$	17.0	865	867	739, 695, 577
$[DP 4]^-$	18.5	1153	1155	1079, 1001, 983
$[DP 5]^-$	19.4	1441	1443	1315, 1151, 865, 737
$[DP 2]^-$ B5*	21.9	577	579	451, 425, 407, 289
$[DP 6]^-$	ND	1729	1731	nd

[a] Compounds were identified after comparison with standards (*). [b] Measured with an XDB-C18 Eclipse column and corresponding gradient profile (see Materials and Methods). [c] Obtained with an ion trap mass spectrometer LCQ Deca. [d] Obtained with a triple quadrupole mass spectrometer Quattro LC; nd, not determined.

insoluble over soluble PAs (*10*). In the present study, we chose to analyze and to quantify PCs using isolated integuments from immature seeds harvested throughout seed development.

Soluble PCs were measured as PC oligomer extension units after hydrolysis in the presence of phloroglucinol followed by LC−ESI-MS analyses. They were detected from mid-embryogenesis (15 dap) onward (**Figure 4A**) and their content increased throughout seed development to reach a maximum level during the early seed maturation stage (30 dap) (**Figure 4A**). This maximal amount was of 92.4 ± 4.7 μg EC equiv per mg of seed coat DW, which almost corresponded to 10% of the total seed coat DW. Soluble PC amount decreased dramatically and concomitantly with the onset of seed browning (from 40 dap onward) (**Figure 4A**). No extractable PCs were still detectable in the seed coat of full-brown mature seeds (50 dap) (**Figure 4A**). Quantification

of soluble (−)-epicatechin and (−)-epicatechin oligomers (DP 2 to main DP 5) in the "flavonoid extracts" revealed that these compounds accumulated with a similar pattern than the total PC content measured into the "PA extracts" during seed development (data not shown). The mDP of extractable PCs was of 6 at the beginning of their accumulation and increased up to 12 before PCs became unextractable (**Figure 4A**). Finally, when inner and outer integuments were separated from seed coats collected on 20-dap seeds, soluble PCs were primarily detected into the inner integument sample (**Figure 4A**).

Insoluble PCs that remained in the extraction pellet were submitted to an oxidative cleavage under hot acidic butanol. As shown for soluble polymers, accumulation of insoluble PCs also began during mid-embryogenesis (around 15 dap). They reached

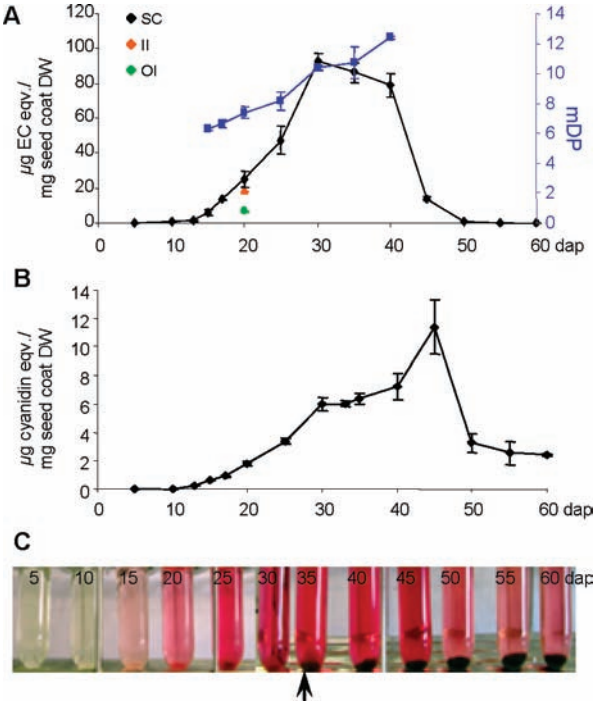

Figure 4. PC content in *B. napus* seed coat during seed development (Jet Neuf cv.). (**A**) Accumulation of soluble PCs monitored by LC−ESI-MS after acidic cleavage in the presence of excess phloroglucinol and evaluation of mDP throughout seed developmental stages. (**B**) Estimation of insoluble PC content in the extraction pellet after oxidative cleavage in hot acidic butanol. (**C**) Extraction pellets after oxidative cleavage of PCs in hot acidic butanol. The dark red color is indicative of the presence of residual PCs in insoluble material that pelleted at the bottom of the tubes from 35- to 60-dap extracts (shown by the arrow in the 35-dap extract). For quantification in **A** and **B**, mean values ± SE resulted from measurements on three plants for each of the three independent biological replicates. II, inner integument; OI, outer integument; SC, seed coat.

a maximum level of 11.4 ± 1.9 µg per mg of seed coat DW during late seed maturation (45 dap) (**Figure 4B**). Afterward, the amount of insoluble PCs decreased to 2.4 ± 0.1 µg per mg of seed coat DW in mature seeds (60 dap) (**Figure 4B**). However, results from quantification of insoluble PCs have to be modulated since extraction pellets remained dark pink stained after butanol−HCl assay, suggesting that unextractable PCs were still captured in the pellets. This was particularly obvious during the later stages of seed development (from 35 dap onward; **Figure 4C**). One could hypothesize that PCs became more and more insoluble during the end of seed development, maybe due to oxidation and subsequent formation of covalent linkages between PCs and other seed coat components. Such linkages may render their cleavage into cyanidin residues under acidic conditions more difficult. On this line, the *TT10* gene encodes a laccase-type flavonoid oxidase, which is involved in the oxidative polymerization of flavonoids in *Arabidopsis* (*38*). Parallel to the present work, we cloned the oilseed rape *TT10* orthologues (*Bna.TT10*) and showed that the *Bna.TT10* expression profile perfectly fitted with the PC accumulation pattern in the *B. napus* seed coat (Auger et al., in preparation). Taken together, these data were consistent with a putative role of *Bna.TT10* in the oxidation of PCs, thus, making the PCs less and less extractable.

***B. napus* Seed Coat Displays Original Flavonol Composition.** Seven main flavonols were detected in the flavonoid extracts from *B. napus* seed coats after LC−ESI-MS^n analysis (**Table 2** and part 1 of the Supporting Information). These compounds were identified from a comprehensive analysis of (i) MS and MS^n fragmentation products of the molecular ion in the negative and the positive modes, (ii) MS^n precursors and products of the aglycons and (iii) t_R and mass fragmentation patterns of available standards.

The most visible flavonol, from absorbance chromatogram recorded at 350 nm and total ion current, was an isorhamnetin derivative with $[M - H]^-$ at m/z 557 (t_R = 23.5 min) that gave fragments at m/z 477 $[M - H - 80]^-$, m/z 395 $[M - H - 162]^-$ and m/z 315 $[M - H - 162 - 80]^-$ under MS^2 conditions (**Table 2**). The MS^2 spectra of the m/z 315 ion in both negative and positive modes were identical to commercial isorhamnetin standard (data not shown). The loss of 162 amu likely corresponds to a hexose moiety, and the loss of 80 amu could be due to a sulfate or a phosphate moiety. The isotopic signature of the m/z 557 ion was compared to that of the predicted $C_{22}H_{21}O_{15}S$ or $C_{22}H_{22}O_{15}P$, and the $C_{22}H_{21}O_{15}S$ theoretical profile matched completely the profile of the *B. napus* flavonol (data not shown). In addition, TOF analysis indicated that this flavonol had an actual mass of 557.0601 m/z (SD = 4.6 ppm) that fitted to the $C_{22}H_{21}O_{15}S$ chemical formula. Therefore, this flavonol was tentatively identified as isorhamnetin-hexoside-sulfate. To support this hypothesis, sulfated flavonols have been already described in plant material from an 80 amu loss on LC−MS^n spectra. For instance, hexosyl luteolin sulfate was identified in

Table 2. Main Flavonols Identified by LC−ESI-MS^2 in Seed Coat Flavonoid Extract from *B. napus* (Jet Neuf Cv.)

putative identity[a]	t_R[b] (min)	$[M - H]^-$[c]	MS^2 $[M - H]^-$[c] (m/z)	MS^2 $[M + H]^+$[d] (m/z)
quercetin-dihexoside	18.3	625	463, 301	465, 303
isorhamnetin-dihexoside	19.8	639	477, 315	479, 317
kaempferol-sinapoyl-trihexoside	20.2	977	977, 815, 653, 447	817, 655, 369, 287
isorhamnetin-sinapoyl-trihexoside	20.5	1007	845, 683, 477	847, 685, 479, 369, 317
quercetin-3-*O*-glucoside*	22.5	463	301	303
isorhamnetin-hexoside-sulfate	23.5	557	477, 395, 315	479, 397, 317
isorhamnetin-3-*O*-glucoside*	25.5	477	315	317

[a] Compounds were identified after comparison with standard (*). [b] Measured with an XDB-C18 Eclipse column and corresponding gradient profile (see Materials and Methods). [c] Obtained with an ion trap mass spectrometer LCQ Deca. [d] Obtained with a triple quadrupole mass spectrometer Quattro LC.

Deglet Noor Dates from the respective losses of 80 amu (sulfate) and 162 amu (hexose) (*40*). Otherwise, isorhamnetin-hexose-sulfate was previously characterized from LC−ESI-MS^n data in the excreta of *Pieris brassicae* (*41*). MS and MS^2 mass fragmentation patterns as well as t_R of a second isorhamnetin derivative clearly corresponded to the ones of the commercial standard isorhamnetin-3-*O*-glucoside (**Table 2**). Another noteworthy isorhamnetin derivative had a $[M - H]^-$ molecular ion at m/z 1007 (t_R = 20.5 min), and its main fragment ions were detected at m/z 845 $[M - H - 162]^-$, 683 $[M - H - 162 - 162]^-$ and 477 $[M - H - 162 - 162 - 206]^-$ (**Table 2**). Furthermore, MS^2 analysis in the positive mode also revealed two fragment ions at m/z 207 and m/z 369 that were characteristic of sinapoyl-hexose moieties. This compound may thus correspond to isorhamnetin-sinapoyl-trihexoside. This was supported by the characterization of sinapoyl derivatives in several previous studies in *Brassica* spp (*12*−*16*). An additional isorhamnetin derivative corresponded to isorhamnetin-dihexoside ($[M - H]^-$ molecular ion at m/z 639, t_R = 19.8 min) that showed main fragment ions at m/z 477 $[M - H - 162]^-$ and m/z 315 $[M - H - 162 - 162]^-$. Two quercetin derivatives were also identified: a quercetin that unambiguously exhibited the same mass profile and t_R as the commercial standard quercetin-3-*O*-glucoside ($[M - H]^-$ molecular ion at m/z 463, t_R = 22.5 min) and a quercetin derivative with a $[M - H]^-$ molecular ion at m/z 625 (t_R = 18.3 min) that dissociated into fragments ions at m/z 463 $[M - H - 162]^-$ and 301 $[M - H - 162 - 162]^-$ and, thus, corresponded to quercetin-dihexoside (**Table 2**). A kaempferol derivative that matched with a kaempferol-sinapoyl-trihexoside ($[M - H]^-$ at m/z 977, t_R = 20.2 min; **Table 2**) was also recovered.

In addition, when the flavonol pattern from seed coat was compared to those from petals, embryo or endosperm, it appeared that isorhamnetin derivatives including isorhamnetin-hexoside-sulfate and isorhamnetin-sinapoyl-trihexoside were specific to the seed coat samples (parts 2 and 3 of the Supporting Information). In petals, two quercetin-trihexosides, an isorhamnetin-trihexoside, a kaempferol-trihexoside, two kaempferol-dihexosides and kaempferol-3-*O*-glucoside were identified by LC−ESI-MS^2 (part 2 of the Supporting Information). Otherwise, at least six kaempferol derivatives were specific to the embryo (part 3 of the Supporting Information). These data illustrate the huge diversity of oilseed rape flavonols and the specificity of their accumulation profiles in the different organs and tissues including the seed coat.

In this study, hexoside and acylated derivatives of isorhamnetin, quercetin and kaempferol were identified in *B. napus* seed coat. Isorhamnetin was the main flavonol aglycon core, with isorhamnetin-hexoside-sulfate the most visible flavonol that was detected. Data about flavonol composition in *Brassica* seeds are rather scarce in literature. Structure of kaempferol conjugates with a m/z at 977 was previously established as kaempferol-sinapoyl-trihexoside in the seed from *B. napus* (*12*) as well as from *B. oleracea* (*13*) and could correspond to the kaempferol derivative that was identified in our experiments. In addition, isorhamnetin-hexoside-sulfate and isorhamnetin-sinapoyl-trihexoside were described for the first time in the Brassicaceae. Excepted for these two compounds, the flavonols identified in our study were previously described in *B. napus* progenitors, *B. rapa* and/or *B. oleracea*, using vegetative parts or florets as samples. Glycoside or hydroxycinnamic acid moieties were usually esterified at positions C-3 or C-7 (*14*−*16*). In contrast to our results, flavonols in *Arabidopsis* seed coat primarily consisted of 3-*O*-rhamnoside and 7-*O*-rhamnoside derivatives, with quercetin-3-*O*-rhamnoside as the main flavonol (*5*, *35*), and acylated flavonols were not detected. Quercetin-3-*O*-glucoside was the only flavonol shared by both Brassicaceae seed coats (ref *5*; this study). Therefore,

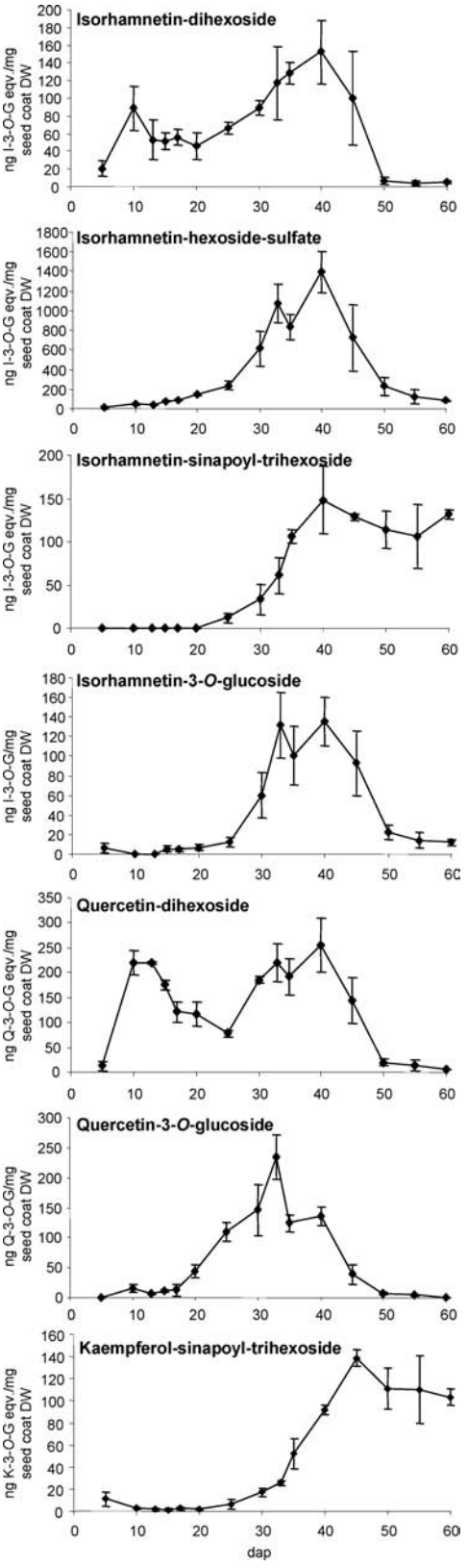

Figure 5. Time-course accumulation of the main flavonols found in the seed coat of *B. napus* (Jet Neuf cv.). Quantification was made using LC−ESI-MS. Mean values ± SE resulted from measurements on three plants for each of the three independent biological replicates. I-3-*O*-G, isorhamnetin-3-*O*-glucoside; K-3-*O*-G, kaempferol-3-*O*-glucoside; Q-3-*O*-G, quercetin-3-*O*-glucoside.

despite flavonols accumulated in similar cell layers between *Arabidopsis* and *B. napus*, their composition differs from one

plant to the other. Molecular analyses supported the absence of rhamnose derivatives in *B. napus*. Indeed, orthologues of the two *Arabidopsis* UDP-rhamnose:flavonol-rhamnosyltransferases UGT78D1 and UGT89C1, that transfer a rhamnose at positions C-3 and C-7 respectively (*42*, *43*), were not retrieved in the genome of *B. napus*.

Fifteen other compounds, for which signals were significantly different from the baseline arbitrarily fixed to an intensity of 100,000 and that displayed an accumulation kinetic in the seed coat during seed development in *B. napus* cultivar (cv.) Jet Neuf (including glucosinolates, sinapate esters and additional flavonoids incompletely characterized), are listed in part 4 of the Supporting Information.

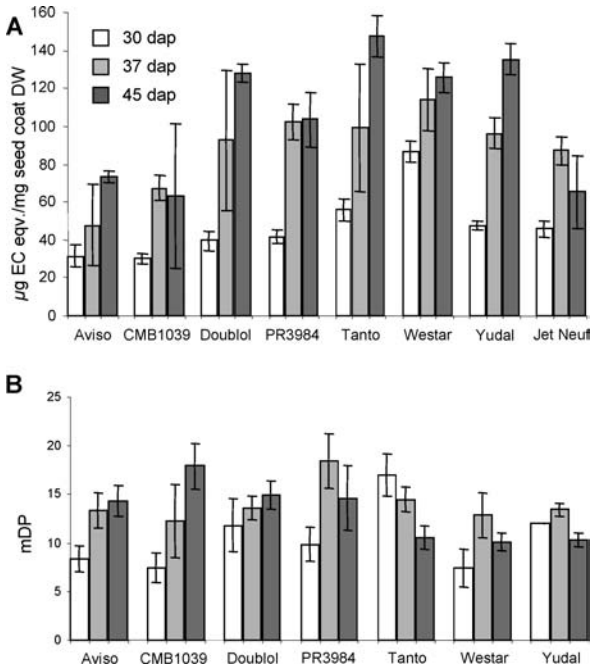

Figure 6. Accumulation of PCs in eight black-seeded genotypes of *B. napus*. (**A**) Soluble PCs were quantified through LC−ESI-MS analysis after acidic cleavage in the presence of excess phloroglucinol. (**B**) Estimation of the mDP. For results presented in **A** and **B**, seed coats were harvested on seeds at 30 dap (white histograms), 37 dap (light gray histograms) and 45 dap (dark gray histograms). Mean values ± SE of independent measurements on three plants. EC, epicatechin ; mDP, mean degree of polymerization.

Flavonols Accumulate in the Seed Coat throughout Seed Development. The seven major flavonols present in the seed coat were quantified throughout seed development (**Figure 5**). Accumulation of both quercetin-dihexoside and isorhamnetin-dihexoside started from very early embryogenesis (5 dap). The amount of quercetin-dihexoside increased rapidly to reach a maximum at 10 dap. Accumulation of quercetin-3-*O*-glucoside and isorhamnetin-hexoside-sulfate began at around 20 dap and was followed by accumulation of isorhamnetin-3-*O*-glucoside, isorhamnetin-sinapoyl-trihexoside and kaempferol-sinapoyl-trihexoside that occurred a little bit later during early seed maturation (25−30 dap). Contents in isorhamnetin-3-*O*-glucoside and in quercetin-3-*O*-glucoside reached their maximal levels during seed maturation (30−35 dap), whereas maximal levels in isorhamnetin-dihexoside, isorhamnetin-hexoside-sulfate, isorhamnetin-sinapoyl-trihexoside and kaempferol-sinapoyl-trihexoside were recorded during late maturation (40−45 dap). A decrease in flavonol content was observed concomitantly with seed desiccation (45 dap onward), except for the most complex forms including isorhamnetin-sinapoyl-trihexoside and kaempferol-sinapoyl-trihexoside for which the maxima persisted until the end of seed development. Among these seven flavonol compounds, isorhamnetin-hexoside-sulfate had the most intense signal from absorbance chromatogram recorded at 350 nm and total ion current. It reached a maximum content of 1394 ± 214 ng equiv isorhamnetin-3-*O*-glucoside per mg of seed coat DW (**Figure 5**). Finally, when using separated samples from inner and outer integuments from 20-dap seed coats, all the identified flavonols were shown to accumulate primarily in the outer integument (data not shown).

Content in Seed Coat Flavonoids Shows Variability among Black-Seeded Oilseed Rape Accessions. Seven additional black-seeded genotypes from *B. napus* were chosen to validate the results obtained previously with cv. Jet Neuf. These accessions were chosen to span oilseed rape variability, with some of them being from spring type (Tanto, Westar and Yudal) and the other from winter type (Aviso, CMB1039, Doublol and PR3984). All the genotypes originated from double low (00) oilseed rape varieties with the exception of Yudal (++). PR3984 was used in our laboratory as the progenitor of a doubled haploid population segregating for seed color (unpublished data).

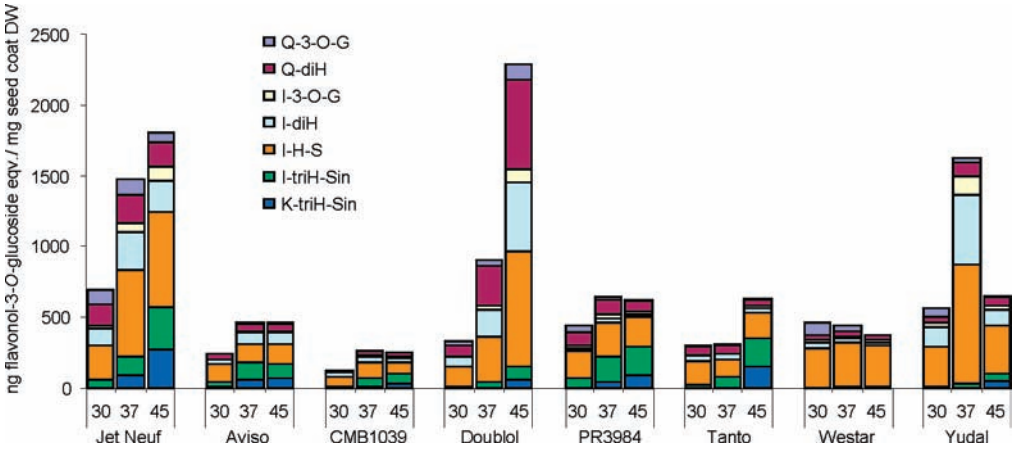

Figure 7. Flavonol content in eight black-seeded genotypes of *B. napus* measured by LC−ESI-MS. For each genotype, the three histograms correspond to seed coat samples from seeds harvested at 30, 37, and 45 dap respectively. Mean values of independent measurements on three plants. Q-3-*O*-G, quercetin-3-*O*-glucoside; Q-diH, quercetin-dihexoside; I-3-*O*-G, isorhamnetin-3-*O*-glucoside; I-diH, isorhamnetin-dihexoside; I-H-S, isorhamnetin-hexoside-sulfate; I-Sin-triH, isorhamnetin-sinapoyl-trihexoside; K-Sin-triH, kaempferol-sinapoyl-trihexoside (refer to color legend depicted on the figure).

PC content increased in all the genotypes during seed development and reached more than 100 μg EC equiv/mg seed coat DW in five of the genotypes including Doublol, PR3984, Tanto, Westar and Yudal (**Figure 6A**). The mDP of PCs found in seed coats from mature seeds ranged between 14 and 18 for winter genotypes and was around 10 for spring genotypes (**Figure 6B**). Therefore, content in PCs appeared to be higher in the seed coat of spring-type genotypes. This trait could be linked to the contrasting environments in which winter- or spring-type accessions are used to grow.

The flavonols found in Jet Neuf were retrieved in the seven other genotypes, but with variable amounts and proportions (**Figure 7**). In contrast with PCs, flavonol content did not seem to be linked with the spring- or winter-type trait. For instance, Jet Neuf, Doublol and Yudal accumulated more flavonols than the other genotypes (over 1000 ng/mg seed coat DW) and the total amount of flavonols dramatically increased between 30 and 45 dap for Jet Neuf and Doublol. On the contrary, the maximal level for flavonols in Yudal was recorded at 37 dap. Isorhamnetin-hexoside-sulfate was the only flavonol detected in all the genotypes at the three developmental stages, at an amount above 60 ng/mg seed coat DW. Quercetin-dihexoside and isorhamnetin-dihexoside also represented an important proportion of seed coat flavonols and were always detected in higher quantities than the corresponding monoglycoside forms. At 45 dap, the proportion of isorhamnetin-sinapoyl-trihexoside and kaempferol-sinapoyl-trihexoside increased in all the genotypes. These two flavonols persisted in mature seed whereas the five other flavonols accumulated transiently in the seed coat (**Figure 7**).

No obvious correlations were observed between contents in PCs and in flavonols found in the seed coat of a given genotype. Indeed, under our conditions, several genotypes accumulated low or high amounts of the two types of flavonoids whereas other accessions had a high content of only one type of flavonoids. For instance, Aviso and CMB1039 displayed low levels of both PCs and flavonols whereas Doublol and Yudal showed high levels of the two types of compounds compared to the other genotypes. Tanto, Westar and PR3984 contained more PCs than the other genotypes and in contrast Jet Neuf displayed higher levels of flavonols. It should be noted that Jet Neuf and Yudal seeds contain glucosinolates, and positive correlation between contents in glucosinolates and phenylpropanoids has already been described (*44*).

To conclude, natural variability for flavonoid content in the seed coat opens promising ways for breeding programs of low-PC oilseed rape. Follow-up of this work will include the profiling of flavonoids in *B. napus* lines producing light brown-to-yellow seeds and the identification of the *B. napus* genes involved in PC metabolism.

ABBREVIATIONS USED

amu, atomic mass unit; cv, cultivar; DAD, diode array detection; dap, day after pollination; DMACA, 4-dimethylamino cinnamaldehyde; DP, degree of polymerization; DPBA, diphenylboric acid 2-aminoethyl ester; DW, dry weight; EC, epicatechin; equiv, equivalent; ESI, electrospray ionization; HPLC, high performance liquid chromatography; LC, liquid chromatography; mDP, mean degree of polymerization; MS, mass spectrometry; PA, proanthocyanidin; PC, procyanidin; t_R, retention time; *TT*, *TRANSPARENT TESTA*; 0+, simple low oilseed rape variety (zero erucic acid); 00, double low oilseed rape variety (zero erucic acid and low glucosinolates); ++, double high oilseed rape variety (high erucic acid and high glucosinolates).

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Supporting Information Available: MS data of flavonoids that accumulate in the testa from *B. napus* seed (Jet Neuf cv.); MS data of flavonoids that accumulate in petals from *B. napus* flowers (Jet Neuf cv.); MS data of flavonoids that accumulate in embryos from *B. napus* seeds (Jet Neuf cv.); MS data of additional seed coat compounds that follow a noteworthy and reproducible kinetic during seed development in *B. napus* (Jet Neuf cv.). This material is available free of charge via the Internet at http://pubs.acs.org.

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