The promoter of the *Arabidopsis thaliana* *BAN* gene is active in proanthocyanidin-accumulating cells of the *Brassica napus* seed coat

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Abstract As part of an ongoing research program dedicated to the understanding of proanthocyanidin (PA) accumulation in *Brassica napus* seed coat, transgenic rapeseed plants carrying a 2.3-kb fragment of the *Arabidopsis thaliana* *BAN* promoter (*ProAtBAN*) fused to the *uidA* reporter gene (GUS) were generated. Analysis of these plants revealed that *ProAtBAN* was activated in *B. napus* seed coat, following a spatio-temporal pattern that was very similar to the PA deposition profile in rapeseed and also to the one previously described in *Arabidopsis*. *ProAtBAN* activity occurred as soon as the early stages of embryogenesis and was restricted to the cells where PAs were shown to accumulate. Therefore, the *Arabidopsis*

BAN promoter can be used to trigger gene expression in *B. napus* seed coat for both genetic engineering and functional validation of candidate genes. In addition, these data strongly suggest that the transcriptional regulatory network of the *BAN* gene is conserved between *Arabidopsis* and rapeseed. This is consistent with the fact that similarity searches of the public rapeseed sequence databases allowed recovering the rapeseed homologs for several *BAN* regulators, namely TT1, TT2, TT8, TT16 and TTG1, which have been previously described in *Arabidopsis*.

Keywords *Brassica napus* · Seed coat · Proanthocyanidins · *BAN* promoter · Regulation of flavonoid metabolism

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Abbreviations

<i>BAN</i>	<i>Banyuls</i>
BuOH–HCl	Butanol–HCl
PAs	Proanthocyanidins
Dap	Day after pollination
DMACA	4-Dimethylamino cinnamaldehyde
GUS	β-Glucuronidase
Hyg	Hygromycin B
TBO	Toluidine blue O
<i>TT</i>	<i>Transparent Testa</i>
<i>TTG</i>	<i>Transparent Testa Glabrous</i>
35S	Cauliflower mosaic virus 35S

Introduction

Oilseed rape (*Brassica napus* L.) is an important oil crop that also provides a source of high quality proteins for animal feed. The molecular bases of seed development and

maturation have been extensively covered in rapeseed, with a focus on the accumulation of seed storage compounds (reviewed in Hills 2004). On the contrary, data about the molecular and cellular events that underlie seed coat development are rather scarce although the testa significantly contributes to seed mass (Li et al. 2006) and contains undesirable compounds that reduce the energy value of the oil-free seed cake (reviewed in Marles et al. 2003b). Indeed, the presence of oxidized dark pigments has anti-nutritive implications both in monogastric and ruminant livestock feeds (Martin-Tanguy et al. 1977; Kumar and Singh 1984). Since un-pigmented natural variants of *B. napus* have never been observed, yellow-seeded (YS) rapeseed lines with reduced pigment contents were developed through introgressions of the trait from related *Brassica* species for which YS genotypes naturally exist (*B. rapa*, *B. juncea*, *B. carinata*) (Rahman 2001). The resulting YS rapeseed genotypes display a reduction of their testa thickness that is correlated with higher contents in both oil and proteins, a lower content in fibres and therefore an improved meal nutritional value compared to their black-seeded counterparts (Simbaya et al. 1995; Slominski et al. 1999).

In *Brassicaceae*, the seed coat originates from the outer and the inner integuments of the ovule that subsequently go through different fates from fertilization onwards (reviewed by Haughn and Chaudhury 2005; Moïse et al. 2005). In *B. napus* seeds, the outer integument is composed of three parenchymatous cell layers surrounded by a one-layer epidermis (Wan et al. 2002). In very young seeds (around 6 days after pollination), the inner integument consists of a maximum of eight to ten cell layers that quickly undergo a reduction to one or two layers in the later developmental stages in association with programmed cell death (Wan et al. 2002). The innermost layer of the inner integument, known as the endothelium, accumulates phenolic compounds starting from the early stages of embryo development (Iwanowska et al. 1994). Seed pigments in mature dry seed of *Brassica* species are primarily oxidised proanthocyanidins (PAs; Fig. 1) (Durkee 1971) of the insoluble type (Naczek et al. 2000) constituted by cyanidin derivatives as basic units (Leung et al. 1979), very similar to those detected in seeds of the closely related model crucifer *Arabidopsis thaliana* (Devic et al. 1999; Routaboul et al. 2006). PAs originate from the flavonoid metabolic pathway

Fig. 1 Simplified overview of the flavonoid metabolic pathway with an emphasis on the proanthocyanidin (PA) biosynthetic branch. **a** PAs are derived from the flavonoid pathway. When applicable, the colours of the flavonoid compounds are indicated with or without chemical treatment. *Dashed lines* represent steps that were not detected in the testa of *Brassicaceae* seeds (Routaboul et al. 2006 and this study). *ANR* anthocyanidin reductase, *ANS* anthocyanidin synthase, *CE* putative

condensing enzyme, *CHI* chalcone isomerase, *CHS* chalcone synthase, *DFR* dihydroflavonol reductase, *F3H* flavanone-3-hydroxylase, *F3'H* flavonoid-3'-hydroxylase, *FLS* flavonol synthase, *GT* glucosyl transferase, *LAR* leucoanthocyanidin reductase, *PPO* polyphenol oxidase. **b** PAs encountered in the *Brassica napus* seed coat are *n*-polymers of epicatechin

that also leads to the accumulation of pigments in vegetative parts (e.g., anthocyanins, flavonols) (Fig. 1a). During seed maturation, PAs form oxidized complexes with cell wall polysaccharides, other phenolics and/or proteins, thus imparting a dark colour to the seed tissues (reviewed in Moïse et al. 2005). A similar structure and development of the seed coat have been described for other *Brassica* species (Ren and Bewley 1998; Marles 2001; Zeng et al. 2004) and *Arabidopsis thaliana* (Devic et al. 1999; Beeckman et al. 2000; Debeaujon et al. 2003; Haughn and Chaudhury 2005), except that the number of cell layers per integument and the contribution of the seed coat to the total seed mass differ from one species to the other (Li et al. 2006).

Unravelling the molecular basis of seed coat development in *B. napus* is of particular interest due to its economic importance for the final crop products. It requires genes with expression patterns exclusively restricted to the seed coat, and even ideally to a unique cell layer of the testa. For instance, deciphering and engineering PA metabolism will require endothelium-specific promoters that are active from the early stages of seed development onwards (Devic et al. 1999; Debeaujon et al. 2003). Although the use of *Arabidopsis* seed mutants has greatly improved our knowledge on testa development and biology (reviewed in Haughn and Chaudhury 2005), there are still only few reports about seed coat-specific genes in literature. To date, specific genes that have been identified in this field are the following ones: members of the barley germin gene family (Wu et al. 2000), subtilisin-like protease genes from soybean (*SCSI*; Batchelor et al. 2000) or rapeseed (Dong et al. 2004), a BURP-domain containing gene of soybean (*SCBI*; Batchelor et al. 2002), an aquaglyceroporin from pea (*PsNIP-1*; Schuurmans et al. 2003), several cystein endopeptidases associated with programmed cell death of either the outer integument in orchid (Nadeau et al. 1996) or the inner integument in rapeseed (*BnCysPI*; Wan et al. 2002) and in *Arabidopsis* (*δVPE*; Nakaune et al. 2005), an epicatechin-specific glucosyltransferase in *Medicago* (*UGT72L1*; Pang et al. 2008), and some of the PA-related genes in *Arabidopsis* (*TRANSPARENT TESTA* [*TT*]-like genes; reviewed in Lepiniec et al. 2006). Among the 23 known *TT*-like loci, the *BANYULS* (*BAN*) gene is of particular interest. It encodes an anthocyanidin reductase (ANR) that constitutes the core enzyme of the PA biosynthetic pathway (Devic et al. 1999; Xie et al. 2003; Fig. 1a). In addition, activation of the *BAN* promoter is restricted to PA-producing cells and is triggered by fertilization in the endothelium (Debeaujon et al. 2003). Therefore, the *BAN* promoter is a powerful tool to investigate not only the regulation of PA-accumulating cell differentiation but also seed coat development in *Arabidopsis*. Since *Arabidopsis thaliana* is closely related to *B. napus*, one could assume that the basic genetic regulation

that governs *Arabidopsis* seed coat development, and especially PA metabolism, will also apply to rapeseed. For instance, recent studies have shown that PA-related genes are highly conserved between *Arabidopsis* and rapeseed (Xu et al. 2006; Wei et al. 2007; Chai et al. 2008).

Taken together, these data prompted us to analyse the activity of the *Arabidopsis thaliana* *BAN* promoter in *B. napus* seeds. In the present study, seeds of *B. napus* transgenic plants carrying a 2.3-kb fragment of the *Arabidopsis* *BAN* promoter fused to the *uidA* reporter gene (GUS) were analysed. The activation pattern of the *Arabidopsis* *BAN* promoter was consistent with the accumulation of PAs in *B. napus* and showed conservation between the two *Brassicaceae*. This demonstrated that the *Arabidopsis* *BAN* promoter could be used to trigger endothelium-restricted gene expression in rapeseed for genetic engineering as well as for functional genomics approaches. Finally, we present preliminary molecular data about the regulatory system that is likely to govern the activation of the *Arabidopsis* *BAN* promoter in rapeseed.

Materials and methods

Plant material

Brassica napus L. cv. Westar (spring genotype) was used for transformation assays in this study. Plants were grown in the greenhouse (16 h light at 22°C/8 h dark at 18°C; 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity at the plant level), using a mixture of 20% black peat, 70% white peat and 10% perlite as substrate (Haasnoot Substraten, Zaltbommel, The Netherlands). Plants were vernalised for 4 weeks to ensure correct and homogenous flowering. Individual flowers on the primary raceme were manually pollinated and tagged on the day of pollination. Inflorescences were subsequently covered with a selfing bag to avoid cross pollination. Siliques were collected from individual plants at different stages of seed development from 5 to 35 days after pollination (dap). Seeds were carefully removed from siliques for histochemical and microscopic analyses.

Mature seeds were harvested at 50–60 dap (depending on the moisture level in the greenhouse), taking care to avoid precocious germination. For in vitro culture, seeds were surface-sterilised 20 min in 25% sodium hypochlorite plus 0.1% Tween-20 added as a surfactant, and rinsed three times for 5 min in sterile deionised water. Sterile seeds were sown on full strength Murashige and Skoog (MS) medium (pH = 5.7) with 30 g L⁻¹ sucrose solidified with 2 g L⁻¹ Gelrite (Duchefa Biochimie, Haarlem, The Netherlands), and placed into a growth room (22 ± 2°C; 16 h/8 h light/dark; 70 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity) to allow germination.

Plasmids and *Agrobacterium* strains

Rapeseed explants were transformed with *Agrobacterium tumefaciens* C58C1Rif(pmp90) disarmed strain (Koncz et al. 1984) carrying the *ProAtBAN:uidA/pBIB-Hyg* binary vector or with *Agrobacterium tumefaciens* C58C1 pGV2260 bearing the *Pro35S:uidA-intron/pBIN+* binary vector (Vancanneyt et al. 1990) as a control. For *ProAtBAN:uidA/pBIB-Hyg*, the T-DNA contains the hygromycin B phosphotransferase gene (*hpt*) for in planta selection and a 2.3-kb fragment of the *Arabidopsis BAN* promoter (hereafter named *ProAtBAN*) fused to the *uidA* gene that corresponded to the *ProBAN1:uidA* fusion described by Debeaujon et al. (2003). The bacterial strain was grown on LB supplemented with 50 mg L⁻¹ rifampicin, 25 mg L⁻¹ gentamycin and 50 mg L⁻¹ kanamycin. For *Pro35S:uidA-intron/pBIN+*, the T-DNA contains the neomycin phosphotransferase II gene (*nptII*) for plant selection. The bacterial strain was grown on LB supplemented with 50 mg L⁻¹ rifampicin, 50 mg L⁻¹ carbenicillin and 50 mg L⁻¹ kanamycin.

Agrobacterium-mediated rapeseed transformation and plant regeneration

Rapeseed genetic transformation was carried out according to Cardoza and Stewart (2003), using hypocotyls as explants. Hypocotyls were excised from 10-day-old seedlings grown in vitro, cut into 1-cm pieces and pre-conditioned for 72 h on MS medium with 1 mg L⁻¹ 2,4-dichlorophenoxyacetic acid (2,4-D), 30 g L⁻¹ sucrose and 2 g L⁻¹ Gelrite. The pre-conditioned hypocotyl segments were dipped into an overnight suspension of *Agrobacterium* (OD_{600 nm} = 0.8) that have been previously pelleted and re-suspended in liquid MS with 1 mg L⁻¹ 2,4-D and 30 g L⁻¹ sucrose. The co-cultivation was conducted for 48 h on MS medium with 1 mg L⁻¹ 2,4-D, 30 g L⁻¹ sucrose and 2 g L⁻¹ Gelrite. For selection of transformed cells, the explants were transferred for 2 weeks onto the co-cultivation medium supplemented with 400 mg L⁻¹ timentin and 20 mg L⁻¹ hygromycin (*ProAtBAN:uidA/pBIB-Hyg*) or 200 mg L⁻¹ kanamycin (*Pro35S:uidA-intron/pBIN+*). To promote organogenesis, all explants were then transferred to the MS medium with 4 mg L⁻¹ 6-benzylaminopurine (BAP), 2 mg L⁻¹ zeatin, 5 mg L⁻¹ silver nitrate, 30 g L⁻¹ sucrose, 2 g L⁻¹ Gelrite and antibiotics as mentioned previously. After a further 2 weeks, shoot development was induced by transferring the explants to MS medium with 3 mg L⁻¹ BAP, 2 mg L⁻¹ zeatin, 30 g L⁻¹ sucrose, 2 g L⁻¹ Gelrite and antibiotics. The developing shoots were transferred to MS medium with 0.05 mg L⁻¹ BAP, 30 g L⁻¹ sucrose, 2 g L⁻¹ Gelrite and antibiotics, for shoot elongation promotion. The elongated shoots were excised from calli and transferred on rooting medium

(half-strength MS salts, MS vitamins, 10 mg L⁻¹ sucrose, 5 mg L⁻¹ indole-3-butyric acid [IBA], 1 g L⁻¹ Gelrite and 4.1 g L⁻¹ plant agar, supplemented with 400 mg L⁻¹ timentin and 20 mg L⁻¹ hygromycin [*ProAtBAN:uidA/pBIB-Hyg*] or 200 mg L⁻¹ kanamycin [*Pro35S:uidA-intron/pBIN+*]). Shoots were maintained on this medium until rooting. Finally, resistant rooted shoots were transferred to the greenhouse where they were maintained under propagator during the first 2 weeks to ensure correct acclimatization. All plant growth regulators and antibiotics were from Duchefa Biochimie.

Molecular techniques

Genomic DNA templates were prepared from resistant rooted plantlets grown in vitro by a simplified extraction protocol based on alkali treatment (Klimyuk et al. 1993) and adapted for rapeseed. Leaf pieces of 0.5 cm diameter were punched into an Eppendorf containing 200 µL NaOH 0.25 M and quickly crushed before being boiled for 30 s. The samples were incubated in 200 µL HCl 0.25 N and subsequently neutralised with 100 µL of 0.5 M Tris–HCl pH 8.0; 0.25% (v/v) Triton X-100 before being boiled for a further 2 min. Templates were then centrifuged and the supernatant was recovered and used for PCR analysis.

For gene expression analysis, tissue samples were ground in liquid nitrogen and total RNA was extracted with the SV Total RNA Isolation System (Promega, Madison, WI, USA) according to the instructions of the manufacturer. The extracts were treated with RNase-free DNase I (Promega) and eluted with 100 µL of nuclease-free water. RNA integrity was checked onto a 1.5% (w/v) agarose electrophoresis gel and RNA concentration was measured with an Eppendorf BioPhometer. Absence of genomic DNA contamination was confirmed by PCR on 40 ng of RNA using primers BnEF1-UP1 and BnEF1-RP1 (see below). For reverse transcription (RT)–PCR studies, 2.5 µg of DNA-free RNA extract was converted into first-strand cDNA by using the SuperScriptII preamplification system for first-strand synthesis kit (Invitrogen, Glasgow, UK) and oligo(dT)_{12–18}, following the manufacturer's recommendations. The cDNA samples were diluted tenfold and 1 µL of dilution was tested by PCR with primers BnEF1-UP1 and BnEF1-RP1.

PCR reactions were conducted in a 20 µL mixture containing 10–50 ng of DNA, 0.25 mM of dNTPs (Promega), 0.5 µM of each primers (Eurogentec, Angers, France) and 0.5 U of Taq DNA polymerase (Promega) in the appropriate buffer (1 X) supplemented with 2.5 mM MgCl₂. The amplification program was as following: 35 cycles of denaturation at 94°C for 30 s (3 min for the first cycle), annealing at 55–60°C for 30 s, and elongation at 72°C for 1 min 30 s (10 min for the last cycle).

Primers used in this study were as following : uidA-UP1 (5'-AATGGTGATTACCGACGAAAAC-3') and uidA-RP1 (5'-ATCAGCACGTTATCGAATCCTT-3') were designed from the *E. coli UidA* sequence and used to check for transgene insertion; T7 (5'-GTAATACGACTCACTA TAGGGC-3') and SP6 (5'-ATTTAGGTGACACTATAG AATA-3') were used to amplify the cDNA inserts from clones BN15023K07, BN20042P19 and BN25061A20; BnTTG1-ATG (5'-ATGGACAACCTCAGCTCCGGACTC -3') and BnTTG1-Stop (5'-TCAAACCTCTAAGGAGCTG CATTG-3') were used to amplify BnTTG1 coding sequences and to analyse the expression profile of BnTTG1; BnTT8-UP (5'-ATACTACAACGGCGCAATAAAG-3'), BnTT8-RP (5'-GATGTGGAACTGAATCTGAAAG-3'), BnTT16-ATG (5'-ATGGGAAGAGGGAAGATCGAAA T-3') and BnTT16-Stop (5'-TTAATCATCTCTGGGCC GTTGGATC-3') were used to monitor the expression of BnTT8 and BnTT16 respectively; BnEF1-UP1 (5'-TCGA GACCACCAAGTACTACTGCAC-3') and BnEF1-RP1 (5'-GATCCTTGACAGCAACATTCTTAAC-3') were designed on the sequence of the BnEF1 EST (GenBank accession nb. CD827228), which encodes an ortholog from the *Arabidopsis* translation elongation factor EF-1 alpha chain (At5g60390) and were used as an internal control in gene expression analyses.

Colorimetric assays for qualitative detection of flavonoids in *B. napus* seeds

The DMACA histochemical assay (Li et al. 1996) is specific for detection of (epi)-flavan-3-ols and PA polymers. Immature seeds or deparaffined seed section were incubated in a freshly prepared solution of 0.3% (w/v) DMACA (4-dimethylamino cinnamaldehyde; ref. D-4506, Sigma-Aldrich Chemical) in a cold mixture of ethanol/12 N HCl (1/1 : v/v) at room temperature for 1 h. The samples were rinsed with 70% ethanol. A resulting blue coloration of the stained tissue is indicative of the presence of PAs. Observations and photographs were made under a low-power stereo microscope (SMZ-U, Nikon, Japan) equipped with a digital camera (Coolpix 950, Nikon, Japan).

For a rapid detection of both anthocyanins and PAs, a simplified butanol-HCl (BuOH-HCl) protocol was adapted from Porter et al. (1986). Around 20 rapeseed integuments were crushed into an Eppendorf and flavonoids were extracted by 500 µL of a mixture of methanol/acetone/water/trifluoroacetic acid (40/32/28/0.05 : v/v/v/v) during 15 min at RT. Three mL of BuOH/12 N HCl (95/5 : v/v) and 100 µL of the ferric reagent (2% ferric ammonium sulphate in 2 N HCl) were added to the samples that were subsequently boiled for 15 min and cooled on ice for a further 15 min. A pink or orange coloration before boiling is consistent with the presence of cyanidin- or pelargonidin

derivatives, respectively. A red to purple coloration after boiling indicates the presence of (epi)-catechin derived PAs (acid-hydrolysis cyanidin products).

GUS assays

GUS staining was performed according to Jefferson et al. (1987). Tissues were collected in 0.1 M phosphate buffer pH 7.2 (PB) and then prefixed by incubation in glacial 90% (v/v) aqueous acetone for 30 min at 4°C. After three rinses in 0.1 M PB, samples were transferred into a fresh PB solution containing 2 mM X-gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide; Duchefa Biochimie), 0.1% (v/v) Triton X-100, 2.5 mM each of potassium ferricyanide and potassium ferrocyanide, and 10 mM Na₂-EDTA. Samples were vacuum infiltrated for 1 h, incubated overnight at 37°C in the dark, and depleted of chlorophyll by incubation at room temperature in 70% ethanol. Stained samples were observed under a low-power stereo microscope.

Microscopy

Samples for resin embedding were directly fixed in 2% (v/v) glutaraldehyde and 1% (v/v) paraformaldehyde in 0.1 M PB under vacuum for 1 h and transferred to fresh fixative for 16 h at 4°C. After fixation, the material was rinsed twice in PB, dehydrated through a series of graded ethanol solutions (10, 30, 50, 70, 90, 100%), and embedded in Technovit 7100 resin (Heraeus Kulzer Histo-Technik, Wehrheim, Germany) according to the manufacturer's instructions. The Technovit-embedded samples were sectioned to a thickness of 4 µm using a rotary microtome (Jung RM 2055, Leica, Germany) equipped with metallic blades (Heraeus Kulzer). Sections were observed with a microscope (Axioskop2 Plus, Zeiss, Germany) under bright-field optics.

Selected sections were stained for 30 s in 0.5% (w/v) toluidine blue O (TBO) in 0.1 M PB, according to O'Brien et al. (1964), rinsed abundantly with water and observed under a microscope. The metachromatic stain TBO develops a blue-green colour in association with polyphenols (e.g., PAs, lignins) and stains the pectic substances in pink and the nucleic acids and proteins in purple.

Sequence analyses

Similarity searches of the databases were conducted according to Altschul et al. (1990) via the NCBI BLAST server (<http://www.ncbi.nlm.nih.gov/blast/>), where 458,264 *B. napus* expressed hits have been released (last update : 11 July 2008) and assembled through 26,733 clusters (Unigene Bna Build #17). BLASTN option was used to recover rapeseed ESTs starting from *Arabidopsis* sequences.

Similarity between probe and hit sequences was deemed significant if the BLASTN e-value was lower than $1.e^{-68}$. Alignments were performed with the ClustalW algorithm and visualized with the BOXSHADE server (http://www.ch.embnet.org/software/BOX_form.html). The CAP3 DNA sequence assembly program (Huang and Madan 1999) available at <http://pbil.univ-lyon1.fr/cap3.php> was used to create EST contigs.

The cDNA clones BN15023K07, BN20042P19 and BN25061A20 were obtained from Genoplante rapeseed cDNA libraries BN15, BN20 and BN25 (Lib. IDs = Lib. 13977, 13978 and 13979, respectively) and used for sequencing with the following primers: T7 and BnTT16-UP5 (5'-TCCGTCTGCGTCCATACCATGG-3') for BN15023K07 and BN20042P19; T7, BnTT8-UP2 (5'-CAACGAACAAGGTCAAAGAGAGTG-3') and BnTT8-UP3 (5'-GATGTTTTGTTAGAGATGAGATG-3') for BN25061A20. All DNA sequences were performed by Genome Express (Grenoble, France).

The GenBank accession numbers for the sequences mentioned in this study were as follows: M14641 (*Escherichia coli* UidA, β -glucuronidase); At1g61720 (*Arabidopsis thaliana* BAN, ANR); At1g34790 (*Arabidopsis thaliana* TT1, WIP-type Zn-Finger); At5g35550 (*Arabidopsis thaliana* TT2, AtMYB123); At4g09820 (*Arabidopsis thaliana* TT8, AtbHLH042); At5g23260 (*Arabidopsis thaliana* TT16/ABS, AtGL32); At5g24520 (*Arabidopsis thaliana* TTG1, WD-repeat protein); BN000703 (*Medicago truncatula* LAR); EU192027 (*B. napus* BnTT8.1-mRNA), EU192028 (*B. napus* BnTT16.1-mRNA), EU192029 (*B. napus* BnTT16.2-mRNA), EU192030 (*B. napus* BnTTG1.1-CDS) and EU192031 (*B. napus* BnTTG1.2-CDS).

Results

Nature, localization and time-course accumulation of pigments in developing *B. napus* seeds

Development of *B. napus* seed took around 45–50 days under our plant growth conditions, with the embryo reaching its final size between 20 and 25 days after pollination (dap; Fig. 2). Sequential embryo developmental stages can be characterized throughout seed development (Fig. 2, right column). At 5 dap, the embryo was at the globular stage (data not shown) and reached the heart to torpedo stage at 10–15 dap. A dramatic increase of embryo size between 15 and 18 dap led to the U shape stage (20 dap). At 25 dap, full size embryos were observed that was consistent with the end of *sensu stricto* embryogenesis. Maturation period included stick cotyledon embryo stage (30 dap). At 35 dap, embryos were fully mature. The first spots of brown pigmentation appeared on the seed body

starting from 35 dap. At the basis of the seed close to the funiculus, a region appeared darker from early developmental stages onwards and corresponds to the micropyle-chalaza area (Fig. 2).

Differentiation of integuments occurred at the same time as embryo development. Seed sections stained with toluidine blue O (TBO) displayed the different cell layers of the seed integuments, namely the outer integument and the inner integument (Fig. 3a). The outer integument includes one epidermis (oi3), two-to-three parenchymatous cell layers (oi2) and one thick-walled palisade layer (oi1) (Fig. 3c). The outer integument persists as such until the appearance of the first pigmentation spots around 35 dap, when oi2 begins to crush down (Fig. 3m). Mature outer integument primarily consists of oi3 stuck to oi1 (Fig. 3m). On the opposite, the inner integument is composed by five to seven parenchymatous cell layers (ii2; Fig. 3f) that rapidly crush down upon seed development (Fig. 3i, j), and by one cell layer called the endothelium (ii1; Fig. 3f), which is the innermost cell layer of the inner integument. Mature inner integument consists of only ii1 (Fig. 3j–m). Variable numbers of parenchymatous cell layers in immature oi2 and ii2 depends (1) on the seed stage, since it is difficult to collect seeds exactly at the same stage and the number of cell layers rapidly decrease all along seed development especially for inner integument, and (2) on the location in the same seed (micropyle-chalaza pole versus seed body; Fig. 3a, b). Seed polyphenols accumulated within the endothelium as revealed by the blue-green colour observed from 15 dap (Fig. 3e, f) onwards (Fig. 3h–m) as well as within the chalaza region (Fig. 3b, d, e, g), referred to as the pigment strand according to Debeaujon et al. (2003). The accumulation of polyphenols in the pigment strand occurred earlier than in the endothelium cell layer, starting from 10 dap (Fig. 3b, d).

Developing seeds from *B. napus* were stained with DMACA reagent and the blue coloration obtained with this chemical was indicative of the presence of PAs (Fig. 4). These assays showed that PA deposition was restricted to the seed coat as the embryo remained unstained throughout seed development (Fig. 4e). In addition, when the seed was opened in two parts, the DMACA blue coloration seemed to be localised within the inner integument of the seed coat (Fig. 4d). Finally, although the seed body was uniformly stained with DMACA, the micropyle-chalaza region always appeared darker (shown by arrows in Fig. 4), even during the early stages of seed development when the embryo is at the globular to torpedo stage (10 dap; Fig. 4a). These findings were confirmed by staining a seed section with DMACA. A blue coloration appeared in the endothelium as well as within the pigment strand (Fig. 4f). During the time course

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Fig. 4 Spatio-temporal pattern of proanthocyanidin deposition in *B. napus* immature seed. Proanthocyanidins were detected as blue coloration after staining with the DMACA reagent. Seeds were harvested at 10 dap (a), 18 dap (b, f), 20 dap (c, d) and 35 dap (e) and observed under a low-power stereo microscope after staining. Embryos were removed from the seed for samples collected at 20 dap (d) and 35 dap (e). Arrows point to the micropyle-chalaza region

that always stained darker than the seed body. Double arrow in (d) shows the seed coat transversal section. Seed section in panel (f) (10 μ m) was deparaffined, stained with DMACA and observed under the low-power stereo microscope. Arrowheads in (f) indicate PA accumulation. EM embryo, II inner integument, INT integuments, OI outer integument, PS pigment strand, S whole seed. Bar 0.4 mm (a); 0.6 mm (b, d, e); 1 mm (c)

of seed development, CT accumulation started from around 15–18 dap (Fig. 4b), which almost corresponded to mid-to-late embryogenesis under our plant growth conditions (refer to Fig. 2), and persisted during later stages of seed development (Fig. 4c–e).

A butanol–HCl (BuOH–HCl) colorimetric assay was conducted on isolated seed coats to qualitatively evaluate the nature and amounts of PAs throughout seed development (Fig. 5). Samples turned to red-to-purple coloration after boiling (Fig. 5b), which indicated the presence of PAs derived from (epi)-catechin units. A very faint staining was observed starting from 15 dap, increased during mid-to-late embryogenesis (18–20 dap) and reached its maximum during seed maturation (25–45 dap). On the contrary, the BuOH–HCl treated samples did not reveal any colour before boiling (Fig. 5a), suggesting that anthocyanins did not accumulate in *B. napus* seed coat.

To get further insights into the nature of PA units, we screened the *B. napus* genomic resources available through sequence databases using coding sequences from *Arabidopsis* *BANYULS* (BAN) and *Medicago* *LAR* genes, which encode ANR and leucoanthocyanidin reductase (LAR) respectively (Fig. 1a). Our preliminary results revealed the presence of at least four *BAN* sequences in the rapeseed genome whereas no *LAR* homolog was identified (data not shown).

Taken together these data showed that pigments found in *B. napus* seed coat were primarily PAs constituted by

Fig. 5 Flavonoid detection in *B. napus* seed coat using BuOH–HCl assays. Flavonoid extracts from *B. napus* integuments were submitted to a BuOH–HCl colorimetric test to detect anthocyanins and proanthocyanidins. Observations were made before (a) and after (b) boiling the samples. The purple coloration after boiling indicates the presence of (epi)-catechin derived PAs

epicatechin units (Fig. 1b) whereas no anthocyanins were detected. In addition, PA deposition was restricted to the inner integument of the seed coat and to the micropyle-chalaza region. Finally, PA accumulation started from early embryogenesis (10 dap) in the micropyle-chalaza region and from mid-to-late embryogenesis (around 15–18 dap) in the seed body. It displayed a maximum level during seed maturation (25–35 dap).

Activation pattern of *Arabidopsis thaliana* *BAN* promoter in *B. napus* seeds

Previous studies in *Arabidopsis* showed that ANR encoded by the *BAN* gene played a central role in PA accumulation in seed (Devic et al. 1999; Debeaujon et al. 2003). In addition, the detailed analysis of the *BAN* promoter using deletions revealed that its activation was restricted to PA-producing cells (Debeaujon et al. 2003). Taking advantages of this well-known system, we used a 2.3-kb fragment of the *Arabidopsis* *BAN* promoter fused to the reporter gene *uidA* (named *ProAtBAN:uidA* fusion, see “Materials and methods”) to introduce into rapeseed. This promoter fragment involves the 5'-untranslated region of the *BAN* gene.

For rapeseed transformation, we used the spring genotype Westar that displayed a high shoot regeneration potential ($80 \pm 2\%$ explants giving a shoot) and a high susceptibility to *Agrobacterium tumefaciens* ($45 \pm 3\%$)

when compared to a large panel of rapeseed genotypes under our experimental conditions (M.-O. Lucas et al. unpub.). During the course of this study, a total of 400 explants were prepared for co-infection with *Agrobacterium tumefaciens* carrying the *ProAtBAN:uidA*/pBIB-Hyg binary vector. Thirteen hygromycin-resistant primary transformants (T1) were recovered of which eight set seeds in progeny. Transformation efficiency therefore reached 2% in this study. Presence of the transgene was double checked by PCR analysis using a piece of T1 plant leaf and by GUS staining of immature T2 seeds. In addition, T2 progeny from the eight transformants exhibited different segregation patterns on hygromycin selection medium (data not shown), indicating that they were produced as independent transformation events.

Six T1 plants were selected for a detailed analysis of *ProAtBAN:uidA* activation pattern in different organs and the GUS profiles were compared to those obtained with *Pro35S:uidA-intron* transgenic rapeseeds (Fig. 6 a–d). Our

Fig. 6 Activation pattern of *ProAtBAN* in *B. napus* transgenic plants. GUS staining of various organs from Westar plants transformed with either *ProAtBAN:uidA*/pBIB-Hyg (a left, b left, c, e–j) or *Pro35S:uidA-intron*/pBIN+ (a right, b right, d). **a** 10-day-old seedlings; **b** open flowers; **c, d** young siliques (around 1–2 dap); **e–j** immature seeds collected at 5 dap (**e**), 10 dap (**f**), 15 dap (**g**), 20 dap (**h**), and 25 dap (**i, j**). Seeds in (**h**) and (**j**) were opened to separate the integuments from each other and from the embryo. *Double arrow* in **h** shows the seed coat transversal section. Note that flowers (**b**) and seeds (**c–j**) originate from T1 plants while seedlings (**a**) were selected from the T2 generation after seed germinating using accurate selective agent. *EM* embryo, *F* funiculus, *II* inner integument, *INT* integuments, *M* micropyle, *OI* outer integument, *SIL* silique. Bars 1 cm in **a**; 0.5 cm in **b**; 1 mm in **c–g**; 1.25 mm in **h–j**

results demonstrated that GUS activity was undetectable in vegetative parts as well as in floral organs from *ProAtBAN:uidA* plants (Fig. 6a, b) but was restricted to the seed (Fig. 6c). On the contrary, *Pro35S:uidA-intron* transgenic plants displayed GUS staining in whole seedlings, flowers and pod valves whereas the young seeds remained unstained except the funiculus (Fig. 6a, b, d).

Activation of *ProAtBAN:uidA* in *B. napus* seed was observed starting from very early developmental stages, as soon as 1–2 dap (Fig. 6c), and persisted during the course of seed formation (Fig. 6e, f, g, i). At 25 dap, GUS staining appeared to be less homogenous from one seed to the other and even at the surface of a single seed (Fig. 6i), which

could be explained by a reduced seed coat permeability towards chemicals.

Seeds at 20 and 25 dap were dissected to separate the integuments from the embryo. Samples showed that *ProAtBAN:uidA* expression was restricted to the seed coat as no staining was observed in the embryo (Fig. 6j). In addition, our results showed that all T2 seeds from *ProAtBAN:uidA* T1 plants were GUS positive within the silique (Fig. 6c–g), which was consistent with the fact that GUS expression was linked to the integuments. Finally, more precise observations suggested that GUS activity was specifically located within the inner integument (Fig. 6h, j), where PAs were shown to accumulate (Fig. 4f).

Fig. 7 Spatio-temporal pattern of *ProAtBAN* activity in *B. napus* seeds. Sections (4 μ m) of developing seeds from Westar plants transformed with *ProAtBAN:uidA/pBIB-Hyg* and stained for GUS activity were visualised with a microscope under bright-field optics. Seeds were harvested at 5 dap (a), 15 dap (b–d), and 25 dap (e, f). c, d and f represent enlargements of b and e, respectively. The expression of *ProAtBAN* is restricted to the endothelium layer as shown by the arrowheads as well as to the chalaza region. For abbreviations refer to Fig. 3. Bars 125 μ m in a; 250 μ m in b; 80 μ m in e

To monitor the tissue-specific location of *ProAtBAN:uidA* activation pattern, GUS-expressing seeds taken at different developmental stages were resin-embedded and sections were produced for microscopic observations. GUS expression was localised in the innermost cell layer of the integuments (Fig. 7) as well as in several cell layers at the basis of the seed body (Fig. 7c). Comparison of GUS- and TBO-stained sections (Figs. 3 and 7, respectively) thereby demonstrated that GUS activity was restricted to the endothelium layer within the seed body and to the pigment strand within the chalaza area, where the PAs accumulated.

Altogether our results revealed that *ProAtBAN* was activated in *B. napus* seed in a fully consistent manner to the spatial PA accumulation pattern observed in this species. Indeed, *ProAtBAN* expression was restricted to PA-accumulating cells. Nevertheless, it is worthwhile to note that *ProAtBAN* expression occurred as soon as the globular embryo stage (around 5 dap; Fig. 7a) whereas PA

accumulation began during mid-to-late embryogenesis (15–18 dap; Fig. 4).

Identification of rapeseed homologs for *BAN* regulatory genes

In *Arabidopsis*, correct expression of *BAN* requires at least five molecular components of which are the transcription factors TT1 (WIP-type Zn-Finger; Sagasser et al. 2002), TT2 (AtMYB123; Nesi et al. 2001), TT8 (AtbHLH042; Nesi et al. 2000) and TT16 (MADS AtAGL32; Nesi et al. 2002) as well as the regulatory factor TTG1 (WDR; Walker et al. 1999). Although *ProAtBAN* activation is altered in both *tt1* and *tt16* mutant seeds (Nesi et al. 2002; Debeaujon et al. 2003), the corresponding regulatory genes are likely to control cellular or tissue differentiation rather than PA metabolism directly (Nesi et al. 2002; Sagasser et al. 2002). On the contrary, Baudry et al. (2004)

Table 1 Identification of rapeseed homologs for three *Arabidopsis* PA regulatory genes

<i>A. thaliana</i> gene	<i>B. napus</i> EST cluster (Unigene acc. nb.) ^a	<i>B. napus</i> ESTs (GenBank acc. nb.) ^b	<i>B. napus</i> transcripts ^c
TT8	Bna.6320 [3; <i>seed</i>]	<u>CD825535</u> , EV027089, EV080565	BnTT8.1-mRNA (EU192027)
TT16	Bna.13305 [25; <i>seed</i>]	EE451062, EE453231, EE453989, EE454628, EE455950, EE456757, EE458134, EE458355, EE462069, EE462705, EE474660, EE476345, EE478700, EE479023, EE564845, EE566135, EV012102, EV021584, EV021863, EV022963, EV025773, EV028865, EV035172, EV057167, ES968264	BnTT16.1-mRNA (EU192028)
	Bna.13731 [8; <i>seed</i>]	EE455946, EE474346, EE474551, EE485285, ES992355, EV023710, EV039605, EV070646	BnTT16.2-mRNA (EU192029)
	unclustered seq. [2; <i>seed</i>]	<u>CD814606</u> , <u>CD817735</u>	
TTG1	Bna.15494 [11; <i>seed, leaf, microspore-derived embryo</i>]	EE475976, EE541697, EE560829, ES268686, ES921044, ES922727, EV047092, EV172875, EV206821, EV207090, EV226364	BnTTG1.1-CDS (EU192030)
			BnTTG1.2-CDS (EU192031)

^a Clusters are those defined through the Unigene Bna Build #17 using 458,264 rapeseed ESTs. Number of ESTs per cluster is given into brackets as well as original tissues used to generate the corresponding cDNAs (written in italics)

^b ESTs that correspond to cDNA clones obtained from Genoplante and used for further sequencing are underlined. CD825535 correspond to cDNA clone BN25061A20; CD814606 to cDNA clone BN15023K07 and CD817735 to cDNA clone BN20042P19

^c Expressed sequences that were deposited into GenBank correspond to the longest sequences obtained during the course of this study. GenBank accession numbers are given into parentheses

demonstrated that TT2, TT8, and TTG1 form a ternary complex that is likely to directly activate *BAN* expression *in planta* and that TT2 and TT8 are able to bind to the *BAN* promoter when simultaneously expressed in yeast. These published data combined with the fact that *ProAtBAN* was active in *B. napus* seeds suggested that the regulatory network that controls *BAN* expression is conserved between *Arabidopsis* and rapeseed. Recent studies demonstrated that three *TT2* transcripts accumulated in *B. napus* seed (Wei et al. 2007). On the same line, several *TT1* transcripts were found in rapeseed (B. Weisshaar pers. comm.). In this study, we searched for additional rapeseed homologs of the *BAN* regulatory genes by investigating public EST databases with *Arabidopsis* *TT8*, *TT16* and *TTG1* coding sequences as *in silico* probes.

For *TT8*, three rapeseed ESTs that belong to the same cluster (Bna.6320) were identified within the databases (Table 1). The *B. napus* BN25061A20 cDNA clone, which corresponded to EST CD825535, was obtained from a seed cDNA library produced by Genoplante (a major partnership French program in plant genomics). Sequencing of this cDNA clone (Jetneuf genotype) led to a 1,845-bp full length cDNA (Bn*TT8.1*-mRNA), including a 40-bp polyA tail and a 1,566-bp coding sequence (data not shown). The translated Bn*TT8.1* sequence (521 residues) shared 78% amino acid identity with At*TT8* (Fig. 8a). Residue conservation was observed all along the sequences and especially within the bHLH DNA-binding domains where identity between the two cruciferous sequences reached up to 88% (shown by the stars in Fig. 8a).

For *TT16*, a total of 35 ESTs were recovered (Table 1) and two cDNA clones (BN15023K07 and BN20042P19) were obtained from Genoplante (Jetneuf genotype). Their sequencing allowed to identify full-length transcripts of 1,036 and 1,178-bp (Bn*TT16.1*-mRNA and Bn*TT16.2*-mRNA respectively) including coding sequences of 720 and 726-bp (data not shown). The two proteins, named Bn*TT16.1* (240 residues) and Bn*TT16.2* (242 residues), shared 95.4% of residue identity among each other and 75–78% of identity with At*TT16* (Fig. 8b). Both Bn*TT16* sequences displayed a typical 56-residue MADS box located at the N-terminus, followed by conserved I- (inter) and K- (keratin) domains. In addition, the C-terminal end contained a PI motif that was shown to be conserved among the MADS domain plant transcription factors from the B-sister monophyletic group (Becker et al. 2002; Nesi et al. 2002) (Fig. 8b).

For *TTG1*, one cluster including 11 ESTs was found in rapeseed (Table 1). Assembly of these sequences using CAP3 algorithm allowed defining a 1,357-bp contig including a 19-bp polyA tail (data not shown). Two primers were designed from the contig sequence (namely Bn*TTG1*-ATG/Bn*TTG1*-Stop) and used to amplify two

Fig. 8 Identification of rapeseed homologs for three *Arabidopsis* proteins involved in the regulation of PA biosynthesis. Alignments of the deduced amino acid sequences from *Arabidopsis* *TT8* (a), *TT16* (b) and *TTG1* (c) with the corresponding rapeseed sequences retrieved after similarity searches of the public databases, sequencing of the complete cDNA clones and three-frame translation into protein sequences. Amino acids are numbered from the translation initiation codon onwards. Identical residues are highlighted in black and similar residues are shaded in grey. In a, the bHLH DNA-binding domains from both *TT8* proteins are indicated by the stars above the sequences. In b, the MADS- (asterisks), I- (solidus), K- (reverse solidus), and PI- (cross symbol) domains are noted. The MADS domain corresponds to the DNA binding domain. In c, the two residues at the end of each WD repeat are boxed in red and the dashed line above the sequences indicates a proline-rich region of 26 residues. The accession numbers of the sequences used for the alignments are given in “Materials and methods”

coding sequences of 1,014 bp each (Bn*TTG1.1*-CDS and Bn*TTG1.2*-CDS) from a cDNA preparation of Jetneuf immature seeds. The deduced Bn*TTG1.1* and Bn*TTG1.2* amino acid sequences (337 residues) displayed more than 93% identity with At*TTG1* (Fig. 8c). The four WD repeats found in At*TTG1* were retrieved in both *B. napus* homologs as well as a conserved proline-rich region of 26 residues (Fig. 8c).

These results demonstrated that homologs from *Arabidopsis* *TT8*, *TT16* and *TTG1* were present in the rapeseed genome and that the corresponding transcripts accumulated within *B. napus* seeds. In addition, rapeseed homologs displayed high sequence conservation with the corresponding *Arabidopsis* proteins.

Expression pattern of *B. napus* *TT8*, *TT16* and *TTG1* genes was monitored in different organs including roots, cotyledons, cauline leaves, floral buds and seeds from the Jetneuf genotype (Fig. 9). The present study was conducted with non-specific primers that allowed following the global expression level of *B. napus* orthologs for each one of the three target genes. Only Bn*TT16* transcripts were restricted to the seed, since Bn*TT8* and Bn*TTG1* genes were also expressed in plantlets (roots and/or cotyledons). In addition, a signal was observed in cauline leaves for Bn*TTG1*. Our results also demonstrated that seed expression of the target genes was detected as early as 5 dap (even in floral buds for Bn*TT16* and Bn*TTG1*) and was restricted to the integuments for Bn*TT8* and Bn*TT16* since no signal was observed in the embryo (Fig. 9).

Discussion

Proanthocyanidins accumulate in specific cells during *B. napus* seed development

Detection of flavonoids in *B. napus* seed coats was achieved by staining seeds collected at sequential developmental

(a) TT8 alignment (bHLH-domain transcription factor)

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BnTT8.1 1 MDELIIPLWKVIGAEKEEIQGLLKAVQSVGWTYSVFWQLCPQRKLMWSSGYNGAIKTRKTTPAEVTAAEAASERS
AtTT8 1 MDESSIIIPAEKVAGAEKKEIQGLLKTAVQSVDWITYSVFWQFCPQQRVLVWNGGYNGAIKTRKTTPAEVTAAEAALERS

BnTT8.1 81 QQIMELYETTFAGESSEARACTALSPEDLTPEWIFYLCTYSFPPSGMPGKAYARRKHVWLSGANEVDNKIFSRASIS
AtTT8 81 QQIRELYETTLAGESTSEARACTALSPEDLTPEWIFYLMCVSFSFPPSGMPGKAYARRKHVWLSGANEVDNKIFSRAIL

BnTT8.1 161 AKSAKIQTVCIPMLDGVLELGTNNKVESEFFVVEHIKSFHHPKSNKPTLSEHFINSEHEEDEFEVVEEEMTMSEE
AtTT8 161 AKSAKIQTVCIPMLDGVLELGTNNKVEDVEFVELTKSFFVDHCKINPKPALSEHSTYVHHEEADEEVEEEMTMSEE

BnTT8.1 240 IRLGSPDDDDVSNQNLSDFHIEATNSLDTHMDMMNLMEEGNYSQTVSTLLMSOPTSLSDSVSTSSYQSSFVSWRVE
AtTT8 241 MRLGSPDDEDDVSNQNLHSDFHIESTHLDTHMDMMNLMEEGNYSQTVSTLLMSHPTSLSDSVSTSSYQSSFATWRVE

*****
BnTT8.1 320 NVKEHQYQYQVVEKAAWSSSQWMLKEITLKVPLHDNTNKKRLPREELNHVVAERRRREKLNKRFITLRSVVPFVTMKDKV
AtTT8 321 NGKEHQYVKTAP----SSQWVLKQNIIPVPLHDNTDKRLPREELSHVVAERRRREKLNKRFITLRSVVPFVTMKDKV

*****
BnTT8.1 400 SILGDTIEYVNHLSKRIHELESTHHEPQKRMRIKGRTWEEVVSIIESDVLEMRCEYRDGLLLNIIQVLKELGIETT
AtTT8 396 SILGDTIAYVNHLSKRVHELENTHEEQHKRTRTCKRKTSEEVEVSIENDVLEMRCEYRDGLLLNIIQVLHELGIETT

BnTT8.1 480 AVHTAINDNHFPAEIRAKVRGKKPIAEVKKIAHQIITYNNKL-
AtTT8 476 AVHTSVNDHDFPAEIRAKVRGKKPIAEVKKIAHQVITIHDTNL

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(b) TT16 alignment (MADS-domain transcription factor)

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*****
BnTT16.1 1 MGRGKIEIKRIENRTSRQVTFSKRRSGLIKKTHELSVLCDAHIGLIVFSATGKLTGYCSDPSKMPOLIERYLQTNGLRLP
BnTT16.2 1 MGRGKIEIKRIENRTSRQVTFSKRRSGLIKKTHELSVLCDAHIGLIVFSATGKLTGYCSDPSKMPOLIERYLQTNGLRLP
AtTT16 1 MGRGKIEIKRIENRTSRQVTFSKRRSGLIKKTHELSVLCDAHIGLIVFSATGKLTGYCSDPSKMPOLIERYLQTNGLRLP

BnTT16.1 81 DPNDQOEELYQIEVLRRETCKLELRQRPYHGHDLASIPPHELDLEEQLEHSLVKVRERKNELLQQOLENLSRKRRMLE
BnTT16.2 81 DPNDQOEELYQIEVLRRETCKLELRQRPYHGHDLASIPPHELDLEEQLEHSLVKVRERKNELLQQOLENLSRKRRMLE
AtTT16 81 DPHDQOEELYQIEVLRRETCKLELRQRPYHGHDLASIPPHELDLEEQLEHSLVKVRERKNELLQQOLENLSRKRRMLE

BnTT16.1 161 VDNKNMYRRLHEHGTAMEFQQAGIETKPGEVQQFLEQVQYNN----DHQQQQP--PNSVLQLATLPSEIDPNYHLQLAQ
BnTT16.2 161 DDNKNMYRWLHEHGTAMEFQQAGIETKPGEVQQFLEQVQYNN----DHQQQQQQPNSVLQLATLPSEIDPNYHLQLAQ
AtTT16 161 DDNKNMYRWLHEHGTAMEFQQAGIETKPGEVQQFLEQVQYNN----DHQQQQQQPNSVLQLATLPSEIDPNYHLQLAQ

++++
BnTT16.1 234 PNLQNDN-----
BnTT16.2 236 PNLQNDN-----
AtTT16 241 PNLQNDPTAQND

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(c) TTG1 alignment (WD-repeat protein)

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BnTTG1.1 1 MDNSAPDSLPRSETAVTYDSPYPLYAMSF-----SSTHRIAVGSFLEDYNNRIDILSFDSDSMSLKPLPSLFEHPYPPT
BnTTG1.2 1 MDNSAPDSLPRSETAVTYDSPYPLYAMSF-----SSTHRIAVGSFLEDYNNRIDILSFDSDSMSLKPLPSLFEHPYPPT
AtTTG1 1 MDNSAPDSLPRSETAVTYDSPYPLYAMSFSSLRSSSCHRIAVGSFLEDYNNRIDILSFDSDSMVVKPLPLSFEHPYPPT

BnTTG1.1 77 KLMFSPPSLRSGGDLASSGDFLRLWEVNESSSAEPVSVLNNSKTSEFCAPLTSFDWNDVEPKRLGTCSIDTTCIIV
BnTTG1.2 77 KLMFSPPSLRSGGDLASSGDFLRLWEVNESSSAEPVSVLNNSKTSEFCAPLTSFDWNDVEPKRLGTCSIDTTCIIV
AtTTG1 81 KLMFSPPSLRSPSSGDLASSGDFLRLWEVNESSSAEPVSVLNNSKTSEFCAPLTSFDWNDVEPKRLGTCSIDTTCIIV

BnTTG1.1 157 DVERSVVETQLIAHDKVEHGIAGGEARVFASVSADGSVRIFDLRDKEHSTIIYESPQPDTPLLRLAWNKQDLRCMATILM
BnTTG1.2 157 DVERSVVETQLIAHDKVEHDIAGGEARVFASVSADGSVRIFDLRDKEHSTIIYESPQPDTPLLRLAWNKQDLRCMATILM
AtTTG1 161 DTEKSVVETQLIAHDKVEHDIAGGEARVFASVSADGSVRIFDLRDKEHSTIIYESPQPDTPLLRLAWNKQDLRCMATILM

BnTTG1.1 237 DSNKVVILDIRSPTMPVAELERHQGSVNIAIAWAPQSKKHICSGGDDAALIWEPLTMAGPNGIDPMSVYSAGSEINQLQW
BnTTG1.2 237 DSNKVVILDIRSPTMPVAELERHQGSVNIAIAWAPQSKKHICSGGDDAALIWEPLTMAGPNGIDPMSVYSAGSEINQLQW
AtTTG1 241 DSNKVVILDIRSPTMPVAELERHQGSVNIAIAWAPQSKKHICSGGDDAALIWEPLTMAGPNGIDPMSVYSAGSEINQLQW

BnTTG1.1 317 SSSLPDWIGIAFANKMQLLRV
BnTTG1.2 317 SSSLPDWIGIAFANKMQLLRV
AtTTG1 321 SSSLPDWIGIAFANKMQLLRV

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Fig. 9 Expression profiles of *BnTT8*, *BnTT16* and *BnTTG1* genes in *B. napus*. Transcript accumulation was monitored after 35 cycles of amplification using cDNA from roots, cotyledons from 10-day-old seedlings, cauline leaves, floral buds, whole seeds (*S*) at 5, 20 and 25 dap, embryos (*Emb.*) at 20 and 25 dap, and integuments (*Int.*) from 20- and 25-dap seeds. Primers used in this study were designed to amplify the two *BnTT16* or the two *BnTTG1* coding sequences at the same time. PCR products were visualised after migration onto a 1.5% (w/v) agarose electrophoresis gel. The *BnEF1* gene was used as an internal control to check for uniformity of cDNA loading

stages. Our results offered evidence that testa from *B. napus* developing seeds accumulated (epi)-catechin derived PAs, as demonstrated by the blue coloration with DMACA staining and the red-to-purple coloration of the BuOH–HCl-treated flavonoid extracts. In addition, since no LAR was found in the rapeseed genome, one could hypothesize that epicatechin rather than catechin form PAs. On the contrary, no anthocyanins were detected. This corroborates previous data obtained with mature seeds of *Brassica* species (Leung et al. 1979; Naczki et al. 1994, 2000; Marles et al. 2003a; Marles and Gruber 2004). To date, seed coat flavonoids are still poorly characterized in rapeseed due to the difficulties of pigment extraction from the mature seed. Future prospects will therefore include the precise elucidation of the PA-basic unit, the determination of the mean degree of PA polymerization as well as the identification of flavonols using developing seeds instead of mature seeds as plant material.

PA accumulation was restricted to the seed coat, as previously shown for *B. carinata* (Marles et al. 2003a), and primarily to the innermost cell layer of the inner integument, called the endothelium layer (ii1), as revealed by DMACA-staining of seed sections. PAs also accumulate in the pigment strand cell layers within the chalaza region. PA-accumulating cells in *Arabidopsis* seed were also localized in the inner integument and the chalaza area (Debeaujon et al. 2003).

B. napus seed have two integuments, namely the outer integument (OI) and the inner integument (II), that differ in their structural organisation when compared with that of *Arabidopsis thaliana* seed integuments. The outer integument was delimited by one inside palisadic cell layer (oi1)

and one outside epidermis (oi3), and also comprised two-to-three layers of parenchymatous cells (oi2), as described by Iwanowska et al. (1994). Cell walls from the palisade layer became very thick from 15–20 dap onwards. The inner integuments consisted of one endothelium (ii1) topped by numerous (>4) parenchymatous cell layers (ii2) that underwent crushing during seed development. In the future, it will be useful to determine whether seed flavonoids in rapeseed accumulate in other cell layers than in the endothelium. Indeed, Pourcel et al. (2005) reported the presence of huge amounts of flavonols within the outer integument in *Arabidopsis* seed.

To our knowledge, less is known about the time-course of PA accumulation in rapeseed since most of the studies were conducted with mature seeds only (Naczki et al. 2000; Marles et al. 2003a; Marles and Gruber 2004). Here, we provided qualitative data about the PA accumulation pattern during seed development in *B. napus*. Accumulation of PAs and/or their precursors in the seed body began during mid-to-late embryogenesis (15–18 dap under our conditions) and subsequently underwent a dramatic increase as revealed by intense colorations observed with DMACA and BuOH–HCl assays from 25 dap onwards. Pigmentation around the micropyle-chalaza region appeared to be under different genetic control than pigmentation within the seed body as it occurred earlier (as soon as 5 dap) and was darker. TBO- and DMACA-stainings of seed sections showed that polyphenolic compounds and more particularly PAs accumulated in several cell layers within the chalaza region. A very similar situation was reported for turnip rape and soybean seeds, in which one gene controlled pigment deposition in the hilum region specifically (Schwetka 1982; Todd and Vodkin 1993). In *Arabidopsis*, several of the *tt* mutant seeds with pale brown to yellow seed bodies also exhibited a dark hilum (Debeaujon et al. 2001, 2003; Nesi et al. 2002), but regulators involved in pigment accumulation specifically in this area remain to be identified in the model plant. The occurrence of genetic controls specific to the development of the micropyle-hilum region may account for its critical roles, especially in being the seed-to-pod point of attachment and also in preventing early radicle protrusion.

Activity of the *Arabidopsis thaliana* *BAN* promoter in *B. napus* seeds reflects the PA accumulation pattern

Rapeseed transgenic plants carrying an *Arabidopsis* *BAN* promoter fragment fused to the *uidA* reporter gene displayed a GUS expression restricted to PA-accumulating cells, i.e. the endothelial cells as well as the micropyle-chalaza area. This activation pattern was fully consistent with the spatial deposition profile of PAs described here for *B. napus* seeds. This demonstrated that *ProAtBAN* could be

used to trigger the expression of specific genes in *B. napus* PA-accumulating cells. Thereby, such a promoter will be very useful not only as a biotechnology tool but also to validate in planta the function of candidate genes. This will be very useful to improve our knowledge on seed coat development in *B. napus*, in particular on the formation of the pigmented cell layer. Indeed, very few seed-specific genes have been reported in literature, and especially less is known about genes that are specifically expressed in the testa.

Our results also showed that the *ProAtBAN:uidA* activation pattern was almost conserved between *Arabidopsis* and rapeseed, suggesting that functional homologs of *ProAtBAN* regulatory factors were present in rapeseed and were able to bind to and activate the *Arabidopsis thaliana* *BAN* promoter. In addition, expression of *ProAtBAN:uidA* as soon as the onset of embryogenesis suggested that the complete PA regulatory mechanism was already present in early developmental stages (1–2 dap) of *B. napus* seed, even if PAs were shown to accumulate only from mid-to-late embryogenesis (15–18 dap). This delay may be due to post-transcriptional regulation events acting either at the level of mRNA (synthesis, stability,...) or at the level of protein (synthesis, activity,...).

The *BAN* regulatory network is conserved between *Arabidopsis thaliana* and *B. napus*

To date, the development of genomic resources for *Brassica* species (e.g., ESTs, BAC libraries, *B. oleracea* and *B. rapa* genome sequencing programs) and the release of the produced data into the public databases offer the unique opportunity to quickly transfer the *Arabidopsis* knowledge to crops such as rapeseed. For instance, large sets of *B. napus* ESTs produced from numerous organs are now freely available. A total of 458,264 ESTs were released in the public databases and gathered into 26,733 clusters (Unigene Bna Build #17 in July 2008), and can be used to initiate a candidate gene approach. During the progress of this work, BLASTN searches of the databases allowed to retrieve rapeseed ESTs starting with *Arabidopsis* *TT8*, *TT16* and *TTG1* coding sequences as in silico probes. EST alignments suggested that at least two rapeseed homeologous genes were found for *TT8*, *TT16* and *TTG1*, that is consistent with the ploidy level of the *B. napus* genome. In addition, the opportunity to obtain rapeseed cDNA clones from Genoplante libraries for *TT8* and *TT16* hits allowed us to get the full-length corresponding expressed sequences that were more informative than the ESTs present in the databases. No rapeseed *TTG1* cDNA clone was available from the Genoplante libraries, however contiguing the existing ESTs led to design accurate primers for amplification of the corresponding coding sequences. In the same

time, other studies reported the molecular cloning of rapeseed *TT1* (B. Weisshaar pers. comm.) and *TT2* (Wei et al. 2007), demonstrating that the five *BAN* regulators described in *Arabidopsis*, namely *TT1*, *TT2*, *TT8*, *TT16* and *TTG1*, were identified within the rapeseed genome.

Alignments of *Arabidopsis* and rapeseed amino acid sequences displayed high residue conservation rates, ranging from 71% for *TT2* (Wei et al. 2007) to 93% for *TTG1* (this study). In addition, the protein structures and putative functional domains (e.g., DNA binding domains) were highly similar between *Arabidopsis* and rapeseed sequences (Fig. 8 and Wei et al. 2007). However, it is worthwhile to note that the sequence conservation rates between the two plants were lower for the three transcription activation factors, *TT2* (71%), *TT8* (79%) and *TT16* (75–78%), than for the regulatory factor *TTG1* (93%). This could be due to the fact that *TTG1* has a pleiotropic role, controlling not only the flavonoid biosynthetic pathway but also the development of trichomes and root hairs as well as the accumulation of seed mucilage (Koornneef 1981; Walker et al. 1999). We can therefore hypothesize that selective pressure during plant evolution was higher upon the *TTG1* sequence compared to the three other proteins.

Although the selected sequences from *Arabidopsis* and rapeseed displayed high conservation rates at the structural level, the question remains to be addressed whether the *B. napus* hits represent functional orthologs of *Arabidopsis* proteins. At least two lines of evidence argued in favour of functional orthologs between the two *Brassicaceae*. First, the results presented in the current study demonstrated that *ProAtBAN* was activated in *B. napus* seed in a similar way than this described in *Arabidopsis*. Therefore, this suggested that rapeseed proteins were able to correctly bind to and activate the *Arabidopsis* *BAN* promoter sequence. Second, in an ongoing study, we showed that at least Bn*TT2*, Bn*TT16* and Bn*TTG1* were able to restore the wild-type phenotype when introduced into the corresponding *Arabidopsis* null mutants under the control of either a constitutive or a specific promoter (Auger et al. in prep.). This definitively proved that the identified Bn*TT* proteins are functional orthologs from their *Arabidopsis* counterparts.

Finally, analysis of expression profiles for Bn*TT8*, Bn*TT16* and Bn*TTG1* in *B. napus* revealed that Bn*TT8* and Bn*TTG1* transcripts were detected in seeds as well as in vegetative tissues while Bn*TT16* expression was restricted to the seed, as shown for the *Arabidopsis* corresponding genes (Walker et al. 1999; Nesi et al. 2000, 2002; Baudry et al. 2006). Accumulation of the targeted transcripts was detected as early as 5 dap (even in floral buds for Bn*TT16* and Bn*TTG1*), consistent with the fact that *ProAtBAN* was activated as soon as early seed developmental stages (1–2 dap). In addition, expression profiles of Bn*TT8* and

BnTT16 were restricted to the seed integuments that corroborated the patterns of PA accumulation and of ProAtBAN activation in *B. napus* seed.

Future prospects of this work will report the identification of rapeseed homologs for most of the known TT-like genes, including BAN, establishing that flavonoid metabolism is active in *B. napus* seeds and highly conserved between *Arabidopsis* and *Brassica* genus.

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