Identification and analysis of an outer-seed-coat-specific promoter from Arabidopsis thaliana

Article *in* Plant Molecular Biology · November 2012

DOI: 10.1007/s11103-012-9984-0 · Source: PubMed

Plant Mol Biol (2013) 81:93–104
DOI 10.1007/s11103-012-9984-0

Identification and analysis of an outer-seed-coat-specific promoter from *Arabidopsis thaliana*

Elahe Esfandiari · Zhaoqing Jin · Ashraf Abdeen · Jonathan S. Griffiths · Tamara L. Western · George W. Haughn

Received: 9 March 2012 / Accepted: 25 October 2012 / Published online: 1 November 2012

Abstract Differentiation of the *Arabidopsis thaliana* (*Arabidopsis*) seed coat epidermal cells involves pronounced changes highlighted by the synthesis and secretion of copious amounts of dispensable, pectinaceous mucilage followed by a thick cellulosic secondary cell wall. This cell type, therefore, represents an excellent molecular-genetic model to study the biosynthesis and modification of cell wall components, particularly pectin. To support such research, we sought to identify a promoter that drives expression specifically in the *Arabidopsis* seed coat epidermis. *Arabidopsis* seed coat microarray data was analysed for genes expressed in the wild type seed coat but not the seed coat of the *apetala2* mutant where the epidermal cells fail to differentiate. Of 14 candidate genes, 9 showed a seed-specific expression pattern by reverse transcriptase-PCR. Transcriptional regulatory region-β-*glucuronidase* (*GUS*) reporter gene fusions introduced into *Arabidopsis* identified one promoter, that of the *DIRIGENT PROTEIN1* (*DP1*) gene, as seed coat specific. The specificity of the expression was confirmed using a second reporter gene, *Citrine YFP*. Expression of both reporter genes was limited to the epidermal and palisade cell layers of the seed coat. Quantitative PCR data using wild type seed coat RNA suggested that the promoter is particularly active at 7 days post anthesis. The *DP1* promoter was able to direct transcription of *GUS* in a similar pattern in the *Brassica napus* seed coat. Thus, in addition to its application in studying the plant cell wall, this promoter will provide an experimental tool for expressing high-valued recombinant proteins as well as modifying seed coat traits in economically important crops.

Keywords *DIRIGENT PROTEIN1* · Seed coat-specific promoter · *Arabidopsis thaliana* · *Brassica napus*

Abbreviations

GUS	β-Glucuronidase
bp	Base pair
DPA	Days post anthesis
AP2	APETALA2
DP1	DIRIGENT PROTEIN1

Introduction

Seed coats play important roles in seed dormancy, germination, dispersal and protection from pathogens and mechanical and chemical damage (Boesewinkel and

Elahe Esfandiari and Zhaoqing Jin have contributed equally to this work and should be considered co-first authors.

Electronic supplementary material The online version of this article (doi:10.1007/s11103-012-9984-0) contains supplementary material, which is available to authorized users.

E. Esfandiari · Z. Jin · J. S. Griffiths · G. W. Haughn (✉)
Botany Department, University of British Columbia, Vancouver, BC V6T1Z4, Canada
e-mail: george.haughn@ubc.ca

Present Address:
Z. Jin
Department of Entomology, University of Maryland, College Park, MD 20472, USA

A. Abdeen · T. L. Western
Biology Department, McGill University, Montreal, QC H3A1B1, Canada

Present Address:
A. Abdeen
Hebron University, P.O Box 40, Hebron, West Bank, Palestine

Bouman 1995). They are comprised of several layers of specialized cell types that differentiate from the ovule integuments in response to fertilization. In some species, including *Arabidopsis thaliana* (*Arabidopsis*), differentiation of the seed coat epidermal cells includes the synthesis and secretion of large quantities of mucilage comprised primarily of pectin (myxospermy; Frey-Wyssling 1976). Upon exposure of myxospermous seed to water, the mucilage expands, ruptures the primary cell wall and extrudes to encapsulate the seed, providing protection and moisture prior to germination (Western et al. 2001).

The *Arabidopsis* seed coat has three distinct cell types arranged in layers, the mucilage-containing epidermis, a sub-epidermal palisade layer with a secondary cell wall, and a pigmented layer that synthesizes proanthocyanidins (Beeckman et al. 2000; Western et al. 2000; Windsor et al. 2000). Two other cell layers derived from the inner ovule integument undergo cell death early in seed coat development (Nakaune et al. 2005). Differentiation of the *Arabidopsis* seed coat epidermal cells has been well studied (Beeckman et al. 2000; Western et al. 2000; Windsor et al. 2000; reviewed in Haughn and Chaudhury 2005; Young et al. 2008). Following fertilization, the cells undergo a growth phase until about 3–4 days post anthesis (DPA). At approximately 5 DPA the epidermal cells synthesize and secrete large quantities of mucilage at the junction of the outer tangential and radial cell walls, thus forming a donut-shaped apoplastic pocket. Deposition of the mucilage shapes the cytoplasm into a volcano-shaped column. Subsequently (8–11 DPA), a secondary cell wall is synthesized along the membrane surfaces of the outer and radial sides of the cell until the cytoplasm is completely replaced by a thick cellulosic secondary cell wall termed the columella (Mendu et al. 2011). During a similar time frame, the sub-epidermal layer (palisade layer) also synthesizes a secondary cell wall on the inner tangential and radial cell walls (Beeckman et al. 2000; Western et al. 2000). Differentiation of both the epidermal and palisade layers is dependent on the function of the transcription factor APETALA2 (Jofuku et al. 1994; Western et al. 2001).

Since the *Arabidopsis* seed coat epidermal cell synthesizes both pectinaceous mucilage and a thick cellulosic secondary cell wall (columella), and is dispensable under normal laboratory conditions (Western et al. 2001), it has been begun to be exploited as a model system for molecular genetic analysis of the biosynthesis, secretion, modification, and metabolism of plant cell wall carbohydrates (reviewed in Arsovski et al. 2010; Haughn and Western 2012). Much of the research on this cell type, to date, has been focussed on the identification of genes through the isolation of mutants defective in mucilage. However, there is potential for probing the consequences of manipulating cell wall structure through targeting the expression of specific carbohydrate-active enzymes to the epidermal cells. To accomplish this goal, promoters driving expression at different times during seed coat epidermal differentiation are required. Seed coat specific promoters are preferred for this purpose to avoid potential deleterious effects of changing cell walls in other parts of the plant. In addition to their use as research tools, seed coat specific promoters could also be employed for crop improvement either through manipulation of seed coat properties or the deposition of commercially important recombinant proteins.

Relatively few seed coat specific genes have been identified in plants. The *SEED COAT SUBTILISIN1* (*SCS1*) gene, expressed specifically in the thick-walled parenchyma cells of the outer integument in soybean seed coat (Batchelor et al. 2000), and *CYSTEINE PROTEASE1* (*CYSP1*; Wan et al. 2002) gene, expressed in the inner integument cells of the developing seed coat of *Brassica napus* (*B. napus*), are examples. Several *Arabidopsis* genes expressed specifically in the seed coat have also been described. The *δVACUOLAR PROCESSING ENZYME* (*δVPE*) transcript is specific to the outer two cell layers of the inner integument (Nakaune et al. 2005). It has been shown that *TRANSPARENT TESTA2* (*TT2*) is expressed in the outer two cell layers as well as the innermost pigmented layer of the seed coat (Nesi et al. 2001; Gonzalez et al. 2009). Furthermore, promoter regulatory regions of the *Arabidopsis* genes *BAN* and *LACCASE15* (*LAC15*), have been shown to drive expression of a reporter gene in the pigmented layer in *Arabidopsis* (Devic et al. 1999; Debeaujon et al. 2003; Liang et al. 2006). No *Arabidopsis* promoters have been reported to be specific to the outer layers of the *Arabidopsis* seed coat. However, the *GAMMA INTERFERON-RESPONSIVE LYSOSOMAL THIOL REDUCTASE* (*AtGILT*) promoter described as specific to the *Arabidopsis* seed coat (Tiwari et al. 2006) was shown to drive expression of a reporter gene in the outer seed coat layers of *B. napus* (Wu et al. 2011). In addition, unlike its expression in *Arabidopsis*, the promoter of the *Arabidopsis* gene *LAC15* also directed expression specifically in the outer integument layers of *B. napus* seed coats (El-Mezawy et al. 2009).

The objective of this study was to identify novel *Arabidopsis* seed-coat-specific promoters expressed in the seed coat epidermis. To do this, we made use of *Arabidopsis* seed coat microarray data (Dean et al. 2011) to identify potential candidate genes and tested the specificity of their promoters using reporter constructs. We report here the identification of a novel *Arabidopsis* seed coat specific promoter that can be used to target proteins to the seed coat epidermis of both *Arabidopsis* and *B. napus*.

Materials and methods

Plant material and growth conditions

Arabidopsis Columbia-2 (Col-2) ecotype was used as wild type in this study. The *Pro*$_{35S}$*:GUS* transgenic plants used were a gift from the Kunst laboratory (Dept. of Botany, University of British Columbia) transformed with a pGREEN0000 binary vector containing the *Pro*$_{35S}$*:GUS* cassette from the John Innes Centre plasmid pJIT166 (http://www.pgreen.ac.uk/a_cst_fr.htm). Seed germination and plant growth conditions were the same as described previously by Dean et al. (2007). Flowers were staged as described by Western et al. (2000). *Brassica napus* seeds were planted directly on soil enriched by AT medium (Haughn and Somerville 1986) and were grown in growth chambers under 24 h of light (100–120 μE m^{-2} s^{-1}) at 20 °C. To improve seed set hand-pollination (using a painting brush) was performed. The day of pollination was considered as 0 days post anthesis (DPA).

RNA extraction, reverse transcriptase-PCR (RT-PCR) and quantitative RT-PCR (qPCR)

Total RNA for RT-PCR was extracted from different plant tissues of *A. thaliana* ecotype *Col-2* including seedlings, roots, rosette leaf, stems, cauline leaf, inflorescence tips, intact siliques at 4, 7, 10 DPA and seeds at 4, 7, 10 DPA. DNaseI-treated RNA was used for first strand cDNA synthesis using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and oligo $(dT)_{18}$ primers according to the manufacturer's protocol. Between 25 and 30 cycles of PCR amplification was performed using the primers listed in supplementary Table 2 (Table S2). *CYTOSOLIC GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE* (*GAPC*) was used as an internal control.

For extracting seed coat RNA for qPCR, seed coats were separated from the developing embryos at 7 and 10 DPA using a Leica M80 stereomicroscope. First, the seeds of 1–5 siliques were removed using tweezers and the embryo removed on a wet filter paper by applying pressure on each seed until the embryo was separated from the seed coat. Because the embryo is very small at 4 DPA, whole seeds were used for RNA extraction at this stage. Following dissection tissues were placed on dry ice until enough material was gathered. Tissues were submerged in liquid nitrogen and ground using a Kontes pellet pestle, followed by total RNA extraction using the RNAqueous-Micro kit (Ambion) as explained in the manufacturer's instructions. For first strand cDNA synthesis SuperScript III (Invitrogen) was used according to the manufacturer's protocol. qPCR reactions were done as described by Dean et al. (2007). Data were analyzed by Bio-Rad iQ5—Standard Edition (version 2.0; Bio-Rad). *GAPC* was used as an internal control.

Plasmid construction and transformation

Transcription regulatory regions were amplified by PCR from genomic DNA (for the complete list of primers, see Table S2, and for a map of transcriptional regulatory sequences selected for each gene, see Fig. 2). Transcription regulatory regions of At4g11180b were introduced into the pBI101.1 binary vector (Jefferson et al. 1987) to generate promoter-β-*glucuronidase* (*GUS*) chimeric genes. The promoters of At1g09550 and At5g45770 were cloned into pGreen0029 and fused to GusPlus (amplified from pCambia 1305.1). *Pro*$_{At1g02720}$*:GUS* seeds were kindly provided by Dr. Michael Hahn (Complex Carbohydrate research Center, Univ. of Georgia, Athens GA, USA). Transcription regulatory regions of At2g23550, At2g43050, At2g47750, At3g14630, At3g52550 and At4g11180a were cloned into pDONR207 vector (Invitrogen) using the Gateway BP recombination reaction (Invitrogen) and were then subcloned into pMDC 163 vector (Curtis and Grossniklaus 2003) by the Gateway LR recombination reaction (Invitrogen) according to the manufacturer's directions.

To generate the *Pro*$_{DP1}$*:Citrine YFP* (At4g11180c; Griesbeck et al. 2001) chimeric gene, genomic DNA was amplified using the primers Pro-F3 and Pro-R3, located 1,107 base pairs (bp) upstream and one codon after the ATG start codon of the *DP1* gene (At4g11180), respectively. The amplicon was introduced into pAD (modified pGreen0029) binary vector (DeBono 2011) between the XhoI and PstI cloning sites to create a translational fusion with the *Citrine YFP* coding region. After sequencing, the plasmid was transformed into *Agrobacterium tumefaciens* GV3101 containing the pSoup plasmid.

Arabidopsis thaliana (Col-2) was transformed by the floral dip (Clough and Bent 1998) or the floral spray (Chung et al. 2000) methods.

Brassica napus line DH12075 was transformed using *Agrobacterium* as described in Moloney et al. (1989) with the exception that explants were inoculated in bulk and co-cultivated without medium. Batches of 50 to 60 cotyledons from 5-day-old seedlings were pooled in a 7 cm petri dish. The cotyledons were immersed in 4.5 mL inoculation medium (MS, 3 % sucrose, 0.5 mg/L benzyl adenine, pH 5.8). *Agrobacterium* was grown approx. 20 h in 5 mL LB medium supplemented with appropriate antibiotics (28 °C, 250 rpm). The cells were pelleted (2,000 g, 10 min), re-suspended in 5 mL inoculation medium, and then added to the cotyledons. After mixing to insure all cotyledons were inoculated, as much fluid as possible was removed by aspiration. Plates were sealed and wrapped in foil. Co-cultivation took place first at 25 °C for 2 days, then at 4 °C

for 2 days. Shoot induction, selection, elongation and rooting were essentially as in Moloney et al. (1989). All solid media were supplemented with 300 mg/L Timentin (GlaxoSmithKline) to kill the *Agrobacterium* and 25 mg/L kanamycin to select for transformed shoots.

Histochemical GUS assay

For detection of GUS activity, fresh samples from various tissues at different developmental stages were incubated in a solution containing 100 mM Phosphate buffer (sodium dihydrogen phosphate and disodium hydrogen phosphate) pH 7.0, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 20 mM Na_2EDTA, 0.1 % (v/v) Triton X-100 supplemented with 1 mg/mL 5-bromo-4-chloro-3-indolyl-b-D-glucuronide (Gold BioTechnology, St. Louis, Missouri, USA). The samples were incubated at 37 °C for 2–18 h (Jefferson et al. 1987). The staining buffer was gently removed and the samples were washed several times with 75 % (v/v) ethanol and stored in 75 % ethanol. Tissues were viewed and photographed using Olympus stereomicroscope SZX10.

Sectioning of developing *Arabidopsis* seeds

Upon dehydration, GUS stained seeds of Pro_{DP1}:*GUS* transgenic line were embedded in standard Spurr's resin as described by Kaneda et al. 2011. Using a Leica Vibratome (series 1000), 5–8 µm cross sections of the samples were cut and placed on glass slides. Tissues were viewed and photographed using Olympus stereomicroscope SZX10.

Sectioning of developing *B. napus* seeds

Brassica napus developing seeds were assayed for GUS activity as described above and stored in 75 % ethanol. Fixed seeds were embedded in 5 % low melt agarose and 20–40 µm sections generated using a LeicaVibratome (series 1000). Tissues were viewed and photographed using Olympus stereomicroscope SZX10.

Fluorescent microscopy

For fluorescent microscopy, a Leica stereomicroscope (M216FA) equipped with a Leica DC500 digital camera was used. The images were obtained using a YFP filter (510–520 nm) with exposure time of 5.1 s, using 50 % lamp intensity.

Confocal microscopy

Cut seeds were incubated in 2 % propidium iodide (Sigma-Aldrich) and shaken for 10–15 min at room temperature followed by rinsing twice with distilled water. Confocal images were acquired using a Perkin Elmer Ultraview VoX Spinning Disk Confocal microscope (PerkinElmer; Waltham Massachusetts). YFP was excited with the 514 laser, and emissions were detected with a 540/30 nm filter. Propidium iodide was excited using a 488 nm laser, with an emission filter of 527/55 nm. Images were processed with Volocity 4.3.2 (Improvision).

Results

Identification of genes with putative seed-coat specific expression using *A. thaliana* seed coat microarray data

To identify genes specifically expressed in the differentiating seed coat epidermis of *Arabidopsis* during the period of mucilage biosynthesis, we utilized *Arabidopsis* wild type and *apetala2* (*ap2*) mutant seed coat microarray data (Dean et al. 2011). In wild type seed coat epidermal cells, mucilage is synthesized between 4 and 9 DPA. However, in an *ap2* mutant, mucilage is not synthesized because the epidermal cells of the seed coat fail to differentiate. Consequently, we screened for genes with higher expression at 5–7 DPA compared to 3 DPA, and with low expression in *ap2* mutant seed coats at 7 DPA compared to wild type seed coat of the same age. Eighty-seven genes were found to match these criteria. To determine which of these genes might be transcribed specifically in the seed coat, we examined the expression of each in public databases using the *Arabidopsis* eFP Browser at the University of Toronto BioArray Resource (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi; Winter et al. 2007, data source: developmental map and seed). Fourteen of the 87 genes showed expression specifically in seeds approximately 5–7 DPA (bent cotyledon stage of embryo development). The list of these genes, fold changes in their expression level in Col-2 at 7 DPA compared to 3 DPA and in Col-2 compared to *ap2* at 7 DPA from the seed coat microarray data (Dean et al. 2011) are presented in Table 1.

Expression pattern of potential seed coat epidermal-specific genes

We investigated the expression pattern of the 14 potential seed coat epidermal-specific genes in *Arabidopsis* using RT-PCR. Total RNA was isolated from 7 to 12 day old seedlings, roots, rosettes, cauline leaves, stems, inflorescences, whole siliques and seeds at different developmental stages (4, 7 and 10 DPA). As shown in Fig. 1, transcripts of five genes (At1g02720, At2g43050, At3g14760, At4g37520 and At5g39130) were detected in tissues other than seeds and siliques. The transcripts of the remaining nine genes (At1g09550, At1g62070,

Table 1 Fold changes in gene expression in Col-2 compared to *ap2* at 7 DPA and in Col-2 at 7 DPA compared to 3 DPA

Gene name	Col(07)/Col-2(03)	Col-2(07)/ap2(07)
At1g02720	8.87	4.96
At1g09550	2.61	3.29
At1g62070	9.57	7.58
At2g23550	3.82	3.14
At2g43050	9.61	9.44
At2g47750	3.73	2.35
At3g14630	3.77	2.04
At3g14760	5.61	2.49
At3g52550	7.56	4.94
At4g11180	7.06	5.74
At4g37520	4.28	3.69
At5g07200	4.26	2.03
At5g39130	2.59	2.09
At5g45770	4.33	3.14

At2g23550, At2g47750, At3g14630, At3g52550, At4g11180, At5g07200 and At5g45770) were found exclusively in seeds and whole siliques. However, two of the latter group (At1g62070 and At5g07200) appear to have higher transcript levels in whole siliques at 10 DPA compared to seeds at the same developmental stage, suggesting that these genes are expressed in the silique wall. Therefore, genes At1g09550, At2g23550, At2g47750, At3g14630, At3g52550, At4g11180 and At5g45770 were selected as potential genes with seed coat epidermal specific expression. In addition, At1g02720 and At2g43050 were examined further despite of their expression in inflorescence and/or stem because of their high expression level in seeds at 7 DPA and low expression in seeds and whole siliques at 10 DPA (Fig. 1).

The *DIRIGENT PROTEIN1* (*DP1*) promoter drives seed coat-specific expression

In order to identify promoter and regulatory sequences that drive expression specifically in the seed coat, we cloned the putative transcriptional regulatory region of each of the nine candidate genes (Fig. 2) upstream of the promoterless β-*glucuronidase* (*GUS*) gene and transformed each construct into *Arabidopsis*. The sequences of the transcriptional regulatory regions of selected genes were obtained

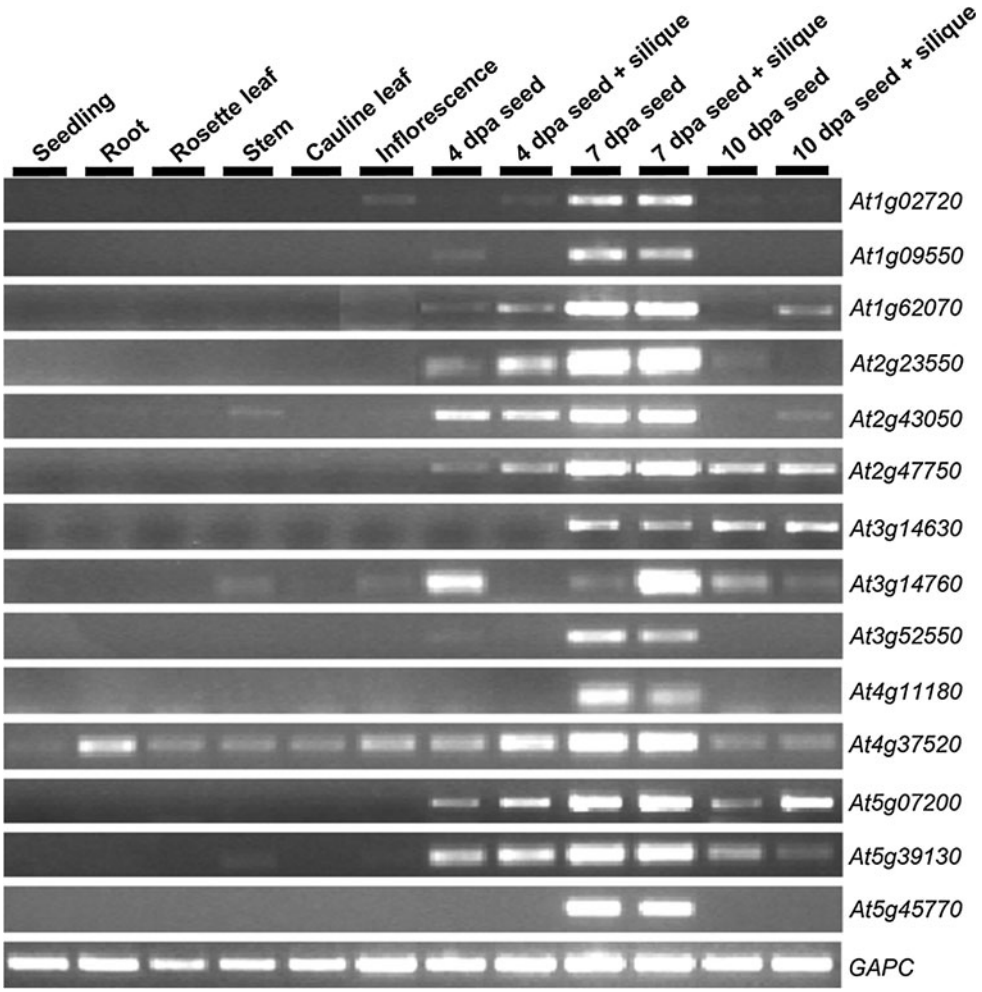

Fig. 1 Expression analysis of seed coat-specific candidate genes in *Arabidopsis* by RT-PCR. Total RNA was extracted from seedling, root, rosette and cauline leaves, stem, inflorescence, whole siliques and seeds at three different developmental stages. RT-PCR was used to determine expression of candidate genes listed in the *column* on the *right*. The expression of the *GAPC* gene was used as a control

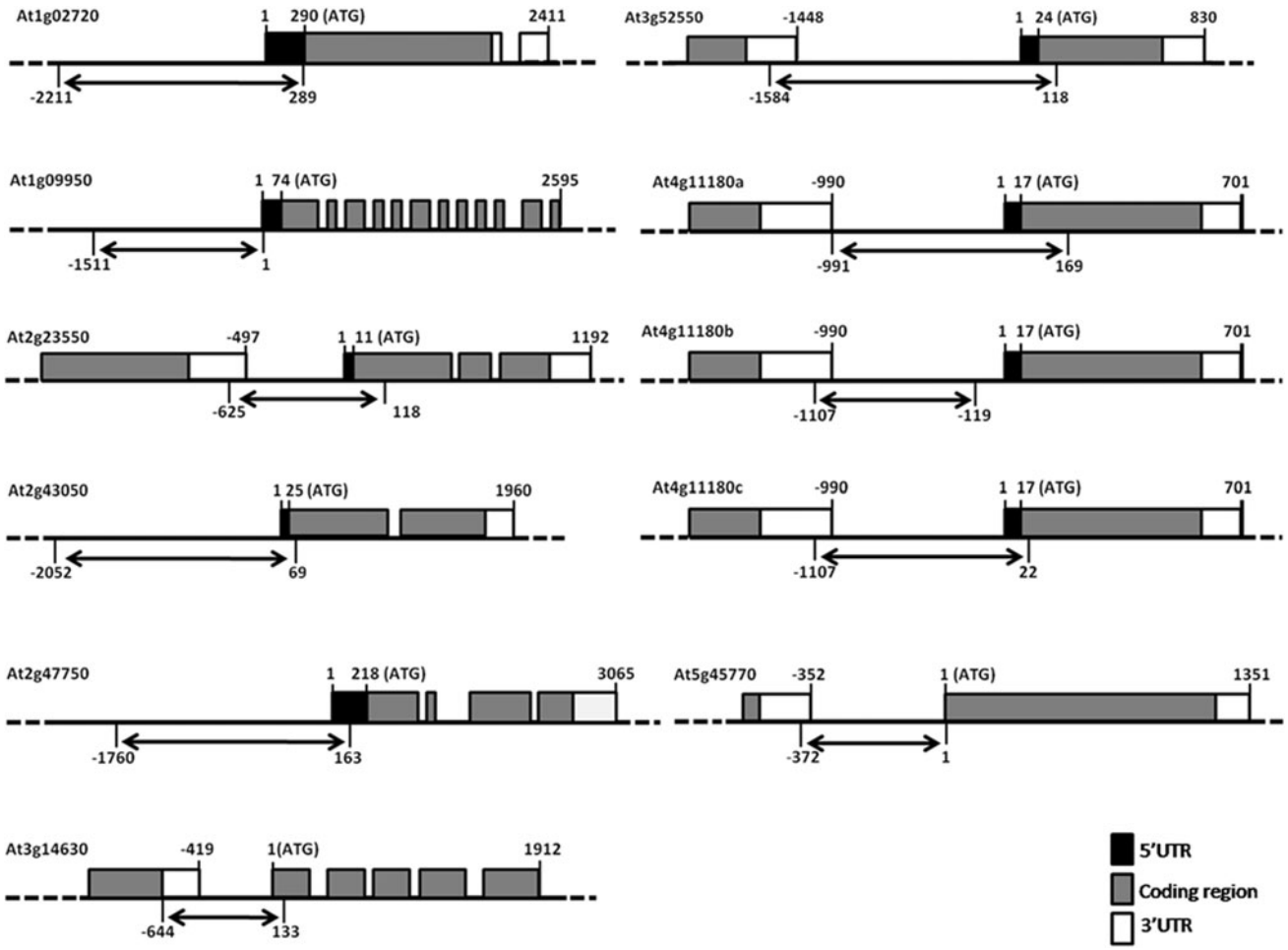

Fig. 2 Schematic representation of the transcriptional regulatory sequences of nine genes cloned upstream of a promoterless *GUS* reporter gene. The fragment used is indicated by a double-headed arrow. The *numbers* indicate the nucleotide position relative to the predicted transcription start (+1 for At1g09950, At2g23550, At2g43050, At2g47750, At3g52550, At4g11180) or translation start codon (+1 for At3g14630, At5g45770). For five of the genes, all or part of the neighbouring gene immediately upstream is shown to the left

from The *Arabidopsis* Information Resource (TAIR) website at http://www.arabidopsis.org/ using SeqViewer tool.

Several transformants for each construct were selected (Supplementary Table S1) and a histochemical GUS assay performed using seedlings, leaves, inflorescences, developing seeds and embryos. Transformants of five constructs showed a distinct pattern of *GUS* expression (Fig. 3) while those of the other four constructs lacked expression in all transformants examined. As shown in Fig. 3A(a–c) and B(a–d), *GUS* expression under the control of the promoter of At1g02720 was detected in root tissue, seeds at different developmental stages and in the embryo. The promoter of At1g09550, At3g52550 and At5g45770 caused *GUS* to be expressed in seedlings, roots, rosette and cauline leaves, and whole seeds at several developmental stages. The promoter of At4g11180, the *DIRIGENT PROTEIN1* gene (*DP1*; Matsuda et al. 2010; sequences 1,160 bp upstream of the putative *DP1* start codon [−991 to +169, see Fig. 2 At4g11180a]), was the only promoter tested that resulted in seed coat specific *GUS* expression (data not shown), consistent with *Arabidopsis* eFP Browser (Winter et al. 2007, data source: developmental map and seed; data not shown) and RT-PCR results (Fig. 1). Transgenic lines carrying a smaller fragment containing 988 bp of sequence from the same region but lacking the −119 bp immediately upstream of the putative transcription start site (−1,107 to −119; see Fig. 2 At4g11180b; Pro_{DP1b}) showed a similar seed coat specific pattern of expression [Fig. 3A(j–l), B(m–p)] and were analyzed in more detail (see below). The transgenic lines transformed with $Pro_{At2g23550}$:*GUS*, $Pro_{At2g43050}$:*GUS*, $Pro_{At2g47750}$:*GUS* and $Pro_{At3g14630}$:*GUS* constructs did not show GUS activity in any examined tissues (data not shown).

The expression of *GUS* under the control of the *DP1* promoter (Pro_{DP1b}) was examined in more detail (Fig. 3C). There

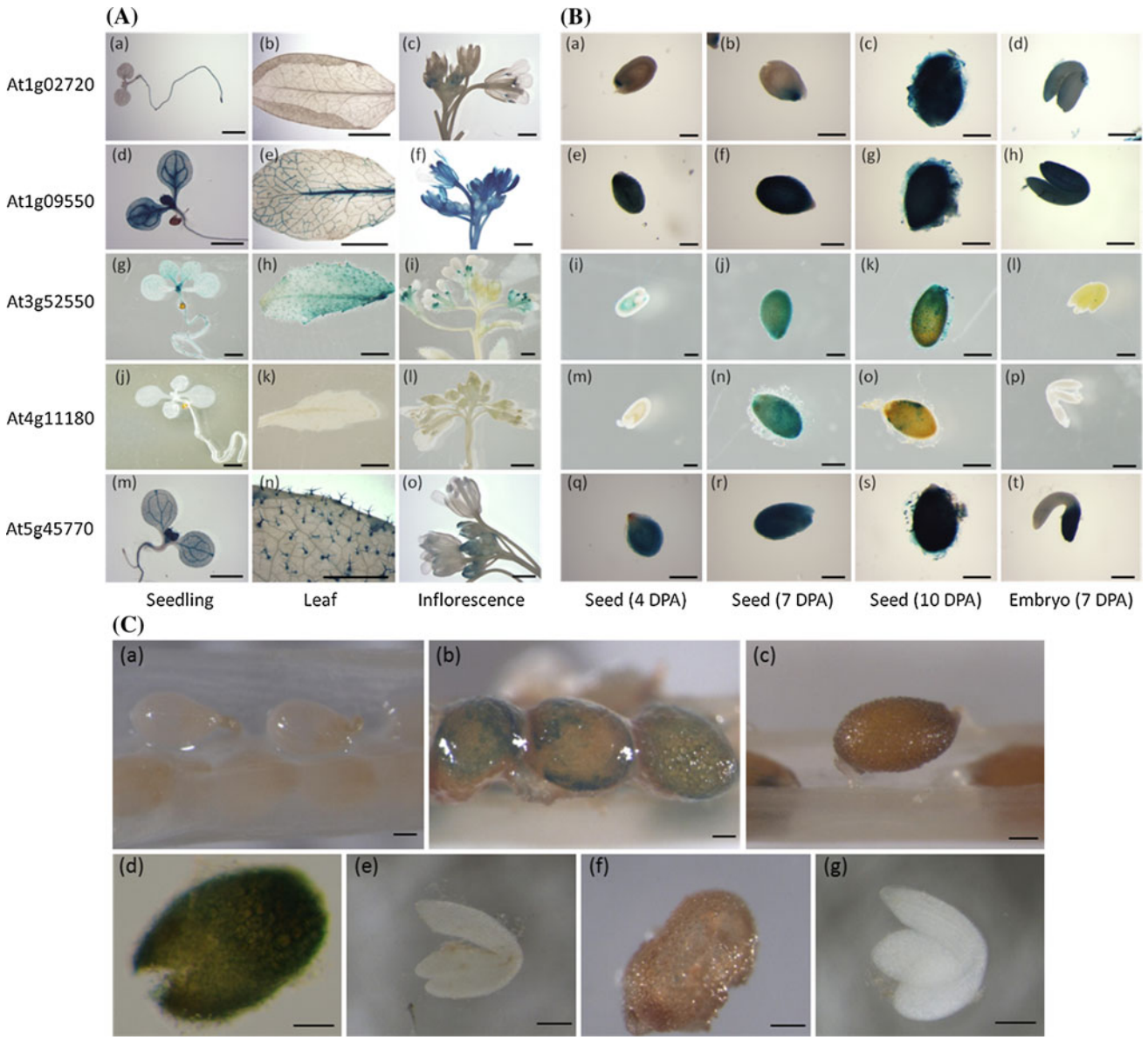

Fig. 3 Expression of the *GUS* reporter gene driven by transcriptional regulatory regions of putative seed coat-specific genes. **A**, **B** Transgenic *Arabidopsis* plants were assayed histochemically for GUS activity. **A** Seedlings, leaves (*Scale bars* 5 mm) and inflorescences (*Scale bars* 0.5 mm). **B** Developing seeds at three developmental stages and embryos at 7 DPA. *Scale bars* 0.5 mm. **C** More detailed histochemical GUS assay of tissues of *Arabidopsis* transformed with Pro_{DP1b}:*GUS*. *a–c* Seeds at 4, 7 and 10 DPA, respectively. *Scale bars* 100 µm. *d* Seed coat at 7 DPA (embryo removed). *e* Embryo at 7 DPA. *f* Seed coat at 10 DPA (embryo removed). *g* Embryo at 10 DPA. *Scale bars* 100 µm

was no *GUS* expression detected in developing silique walls. *GUS* was expressed strongly on the seed coat surface at 7 DPA but not at 4 or 10 DPA [Fig. 3C(a–c)]. To examine whether the expression of *GUS* is limited to the seed coat, seed coats were separated from embryos at 7 and 10 DPA prior to performing the GUS assay. *GUS* expression was specifically found on the outer side of the seed coats but not in the embryos at 7 DPA [Fig. 3C(d, e)], and no GUS activity was observed in the seed coats and embryos at 10 DPA [Fig. 3C(f, g)]. In Pro_{DP1b}:*GUS* transgenic flowers, the anthers stained weakly but this was also true for wild type flowers (Suppl Fig. 1A, B). The staining in flowers of both lines was much weaker than in the positive control Pro_{35S}:*GUS* transgenic plants as shown in supplementary figure 1C. To confirm this observation, RT-PCR analysis using RNA extracted from inflorescences did not detect *GUS* transcript in Pro_{DP1b}:*GUS* transgenic flowers (Suppl. Fig. 1D).

In order to confirm the seed coat specificity of the Pro_{DP1} expression, the Pro_{DP1} regulatory region (from −1107 to +22; including the putative *DP1* start codon;

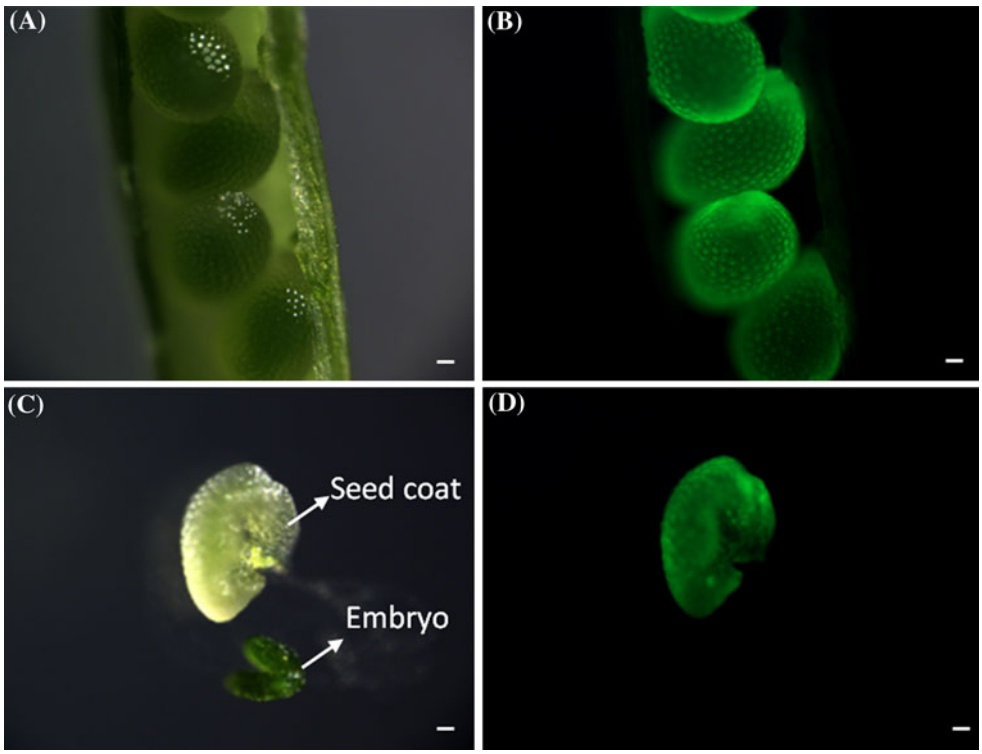

Fig. 4 Expression of the *Citrine YFP* reporter gene driven by the *DP1* promoter. Seeds at 7 DPA from *Arabidopsis* transgenic lines expressing Pro_{DP1c}:*Citrine YFP* are shown. Plants were imaged using **A** light microscopy and **B** fluorescent microscopy where *Citrine YFP* expression was found in the seed coat. The embryo was dissected from the seed coat and imaged with **C** light microscopy and **D** fluorescent microscopy to showing that expression of *Citrine YFP* was limited to the seed coat. *Scale bars* 100 μm

Fig. 2 A4g11180c) was cloned in front of a promoterless *Citrine YFP* gene (Griesbeck et al. 2001) in the pAD binary vector. Consistent with the results using *GUS* as a reporter, *Citrine* driven by Pro_{DP1c} was found only in the seed (Fig. 4A, B). The expression of *Citrine* under the control of Pro_{DP1c} was first observed at 6 DPA and was absent by 9–10 DPA (data not shown). Fluorescence intensity suggested that the maximum expression of the reporter gene occurs at approximately 7 DPA. This hypothesis was confirmed by qPCR measurements (see below). Separation of the embryo and seed coat at 7 DPA showed that *Citrine* expression was found only in the seed coat (Fig. 4C, D).

DIRIGENT PROTEIN1 transcript distribution correlates well with *DP1* promoter activity

RT-PCR and qPCR were used to determine if the amount of *DP1* mRNA in the seed coat during development is similar to that observed for *GUS* under the control of Pro_{DP1b}. Seed coats were separated from developing embryos at 4, 7 and 10 DPA, total RNA was extracted and first strand cDNA was synthesized using *DP1* specific primers (Supplementary Table S2). Since the endosperm layer tightly adheres to the seed coat, we presume that the samples also contained some endosperm RNA. Consistent with the results using reporter genes (Figs. 1, 3, 4), *DP1* transcript was detected in seed coats at 7 DPA but not at 4 and only slightly at 10 DPA (Fig. 5A, B). This expression correlates with the period of mucilage biosynthesis. qPCR was used to determine the quantity of *DP1* mRNA in seed coats relative to expression in embryos and silique walls (including the suspensor and funiculus) at 7 DPA. As shown in Fig. 5C, *DP1* expresses mainly in the seed coat. Very low expression was found in silique walls and almost no expression was detected in embryos (Fig. 5C).

The *DP1* promoter is active in the epidermal and palisade layers of *Arabidopsis* and *B. napus* seed coats

For examining the spatial expression of Pro_{DP1b}:*GUS* within the seed coat of *Arabidopsis*, GUS stained 7 DPA developing seeds of the transgenic line Pro_{DP1b}:*GUS* were embedded in resin and sectioned (Fig. 6A). Pro_{35S}:*GUS* was used as a positive control (Fig. 6B). GUS activity was detected specifically in the seed coat epidermis and palisade layers of Pro_{DP1b}:*GUS* transgenic plants (Fig. 6A). This result was confirmed using confocal microscopy to examine the expression of *Citrine YFP* in 7 DPA developing seeds of Pro_{DP1c}:*Citrine* transgenic lines. Consistent with the previous results, expression is limited to the outer two cell layers of the seed coat (Fig. 6C–E). No fluorescence was detected in the transgenic line with empty vector used as a negative control (data not shown).

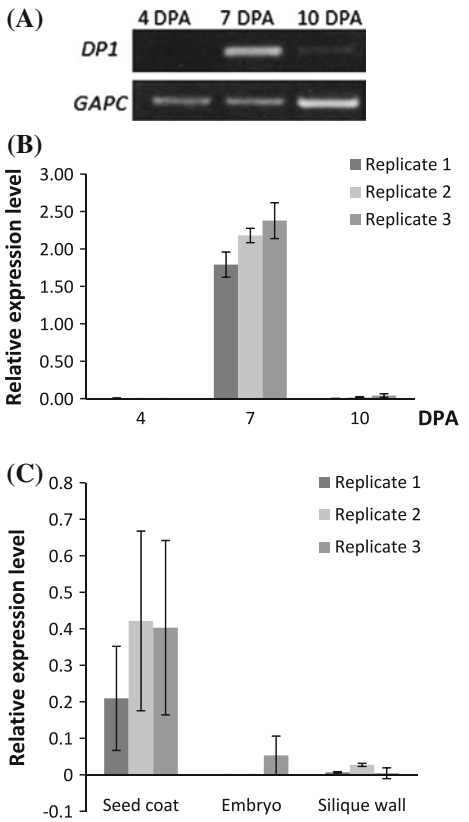

Fig. 5 *DP1* seed coat expression analysis. **A** RT-PCR and **B** qRT-PCR using RNA extracted from WT *Arabidopsis* seed coats at three developmental stages (4, 7 and 10 DPA). **C** qPCR analysis of *DP1* transcript in seed coat, embryo and silique wall at 7 DPA. RNA was extracted from seed coats, embryos and silique walls (including the suspensor and funiculus) at 7 DPA. The *DP1* transcript level determined relative to *GAPC* using three biological replicates and four technical replicates. *Bars* represent the average ± SD ($n = 4$)

To compare the activity of Pro_{DP1b} to Pro_{35S} quantitatively, *GUS* expression level was measured in 2 independent T4 Pro_{DP1b}:*GUS* lines and one Pro_{35S}:*GUS* transgenic line using qRT-PCR (Suppl. Fig. 2). Total RNA was isolated from the seed coat at 7 DPA. The expression level of *GUS* driven by Pro_{35S} was found to be fivefold to sixfold higher than those obtained with the Pro_{DP1} although it is important to consider that Pro_{DP1} is active only in the outer two cell layers whereas Pro_{35S} is active in all cell layers of the seed coat as well as in endosperm (Fig. 6a, b).

To determine whether the Pro_{DP1} behaved similarly in other *Brassicaceae*, the Pro_{DP1b}:*GUS* plasmid was used to transform *B. napus*. Based on histochemical GUS assays, no *GUS* expression was found in leaves, inflorescences, silique walls or embryos (data not shown). However, as in *Arabidopsis*, *GUS* was highly expressed in epidermis and palisade layers (Fig. 6f–h) of developing seed coats from the mid (~15 DPA) to the late developmental stages (~25 DPA).

Discussion

DP1 promoter provides a tool for studying and modifying seed coat properties

We have identified a promoter regulatory region from the *Arabidopsis DP1* gene that is sufficient to drive the expression of a reporter specifically in the two outer layers of the seed coat. Expression occurs during a narrow window of developmental time of approximately 3 days, from 6 to 8 DPA. This period corresponds closely to the period of mucilage secretion in the epidermal cells (5–8 DPA). Seed coat mucilage is being used as a model system for studying the synthesis and function of plant cell walls (Arsovski et al. 2010; Haughn and Western 2012; Western 2012). Therefore, the Pro_{DP1} could serve as a useful tool for testing the activity of carbohydrate active enzymes, engineering specific types of cell wall carbohydrates in the mucilage pocket, or for the modification of the outer cell layers of the seed coat to address questions concerning seed coat structure and function. In addition, because spatial and temporal expression driven by Pro_{DP1} is conserved in *B. napus*, the promoter could be used to modify the seed coat properties or express valuable recombinant proteins in the seed coat of *Brassica* crops.

Different fragments of *DP1* transcriptional regulatory region showed a similar seed coat specific pattern of expression

The Pro_{DP1} transcriptional regulatory region includes the 990 bp of sequence upstream of the annotated transcription start site (+1; see Fig. 2). The 119 bp immediately upstream of this putative transcription start site are not necessary for the seed coat specific promoter activity, as a fragment from −119 to −1,107 was able to promote a pattern of expression similar to ones extending from +169 to −991 and +22 to −1,107. These data suggest that either the Pro_{DP1} is positioned more than 119 bp upstream of the transcription start site and/or the transcription start site is incorrectly annotated. Promoters that initiate transcription over a broad region of 100 bp are not uncommon in eukaryotes (reviewed in Stamatoyannopoulos 2010).

The *DP1* promoter was the only promoter identified as seed coat-specific during mucilage synthesis

Our search for seed coat specific promoters identified only one. Several factors could have contributed to this low number. First, it is possible that relatively few such promoters exist. Indeed, most of the genes known to have specific roles in the seed coat, are also expressed in other tissues (e.g. Jofuku et al. 1994; Zhang et al. 2003; Dean

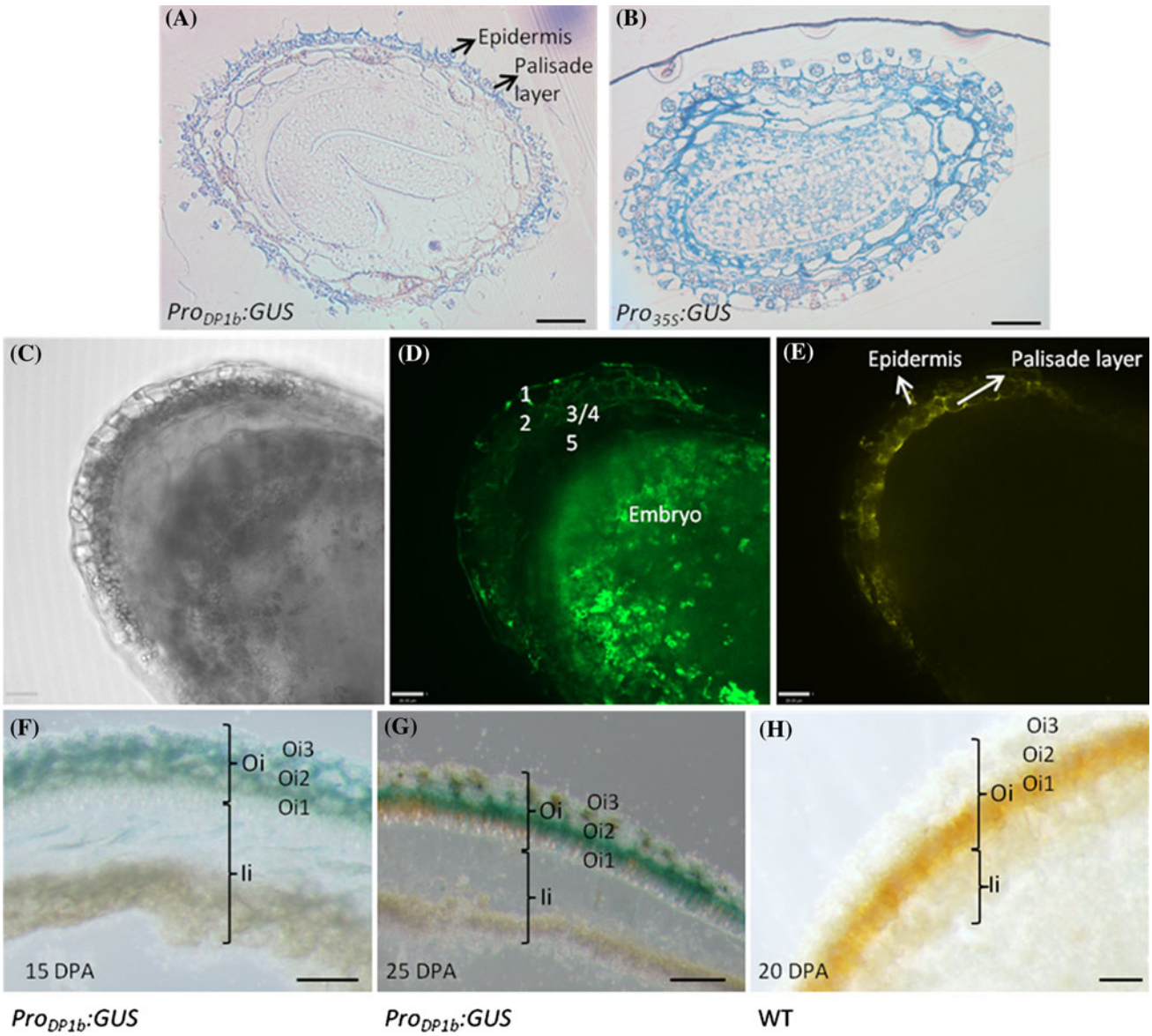

Fig. 6 *Pro$_{DP1}$* expression pattern in *A. thaliana* (**A**–**E**) and *Brassica napus* (**F**–**H**). **A**, **B** Seeds (7 DPA) of *Pro$_{DP1b}$:GUS* and *Pro$_{35S}$:GUS* transgenic plants were assayed for GUS activity embedded in resin, and sectioned. *Scale bars* 100 µm. **C**–**E** Confocal analysis of *Pro$_{DP1c}$:Citrine YFP* expression in 7 DPA seeds. **C** Transmitted light micrograph, **D** Confocal fluorescent micrograph in which cell walls are stained by propidium iodide in green. *1* epidermis, *2* palisade layer, *3/4* cells of the other two inner integument layers, *5* endothelium **E** Confocal fluorescent micrograph showing expression of *Citrine YFP* in epidermis and palisade layer of the seed coat. *Bars* 28 µm. **F**–**H** *Thick cross* sections of transgenic *B. napus*. *Pro$_{DP1b}$:-GUS* developing seeds at approximately 15 DPA and 25 DPA; wild type (20 DPA) was used as negative control. *Oi* outer integument, *Ii* inner integument, *Oi3* epidermis, *Oi2* parenchymatous cell layer, *Oi1* palisade layer. *Scale bars* 100 µm

et al. 2007). Second, because of our interest in seed coat mucilage as a model for cell wall biosynthesis, our search was designed to identify promoters with a very specific pattern of expression. Genes expressed constitutively, or at different time points, and those not regulated by AP2 would have been missed. Finally, nine genes appeared to be seed coat specific based on RT-PCR results, but four of these did not show expression when their putative transcriptional regulatory regions were tested using a reporter gene. Since these negative results could have arisen from the failure to include the entire transcriptional regulatory sequences, the sensitivity of the promoter region to the genomic insertion site following transformation or errors in chimeric gene construction, it is possible that one or more might show seed coat specific expression if re-examined.

DP1 may play a role in neolignan biosynthesis

The *DP1* gene (At4g11180) is a member of the dirigent protein gene family. Dirigent proteins have been associated with the synthesis of the phenylpropanoid compounds, neolignans (Burlat et al. 2001; Davin and Lewis 2005). Indeed, although the exact role of DP1 has not been identified, a *dp1* mutant has been shown to lack seed specific neolignans (Matsuda et al. 2010) suggesting a role in their synthesis. The presence of neolignans specifically in the outer seed coat is significant because different lignans act as antioxidant, antiviral, antibacterial, antifungal, and cytotoxic compounds (Nitao et al. 1991; Asano et al. 1996; Miyazawa et al. 1996; Day et al. 1999; Harper et al. 1999). Neolignans could, therefore, increase the efficiency of the seed coat's role as a barrier to protect embryo against pathogens. protect embryo against pathogens. In support of this hypothesis, it has been demonstrated that the expression level of a *DP1* homolog is increased in *B.napus* following infection by the necrotrophic plant pathogen *Sclerotinia sclerotiorum* (Zhao et al. 2007), suggesting a role in plant defence.

Acknowledgments We thank Dr. Michael Hahn for providing $Pro_{At1g02720}$:*GUS* transgenic plants, Kevin Hodgson and Garnet Martens of the University of British Columbia Bioimaging facility for advice and assistance with microscopy and resin embedding, Joseph Hammerlindl of the National Research Council of Canada, Plant Biotechnology Institute for technical assistance with *B. napus* transformation and Dr. Allan Debono for providing the pAD binary vector. We also thank Dr. Gillian Dean and Dr. Erin Gilchrist for comments and critical reading of the manuscript. This work was supported by a Natural Science and Engineering Research Council of Canada Strategic Grant to G.W.H. and T.L.W.

References

Arsovski AA, Haughn GW, Western TL (2010) Seed coat mucilage cells of *Arabidopsis thaliana* as a model for plant cell wall research. Plant Signal Behav 5:796–801. doi:10.4161/psb.5.7.11773

Asano J, Chiba K, Tada M, Yoshii T (1996) Antiviral activity of lignans and their glysosides from *Justicia procumbens*. Phytochemistry 42:713–717. doi:10.1016/0031-9422(96)00024-6

Batchelor AK, Boutilier K, Miller SS, Labbe H, Bowman L, Hu M, Johnson DA, Gijzen M, Miki BL (2000) The seed coat-specific expression of a subtilisin-like gene, *SCS1*, from soybean. Planta 211:484–492. doi:10.1007/s004250000320

Beeckman T, De Rycke R, Viane R, Inze D (2000) Histological study of seed coat development in *Arabidopsis thaliana*. J Plant Res 113:139–148. doi:10.1007/PL00013924

Boesewinkel FD, Bouman F (1995) The seed: structure and function. In: Kigel J, Galili G (eds) Seed development and germination. Marcel Dekker Inc, New York, pp 1–24

Burlat V, Kwon M, Davin LB, Lewis NG (2001) Dirigent proteins and dirigent sites in lignifying tissues. Phytochemistry 57:883–897. doi:10.1016/S0031-9422(01)00117-0

Chung MH, Chen MK, Pan SM (2000) Floral spray transformation can efficiently generate Arabidopsis transgenic plants. Transgenic Res 9:471–476. doi:10.1023/A:1026522104478

Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J 16:735–743. doi:10.1046/j.1365-313x.1998.00343.x

Curtis MD, Grossniklaus U (2003) A gateway cloning vector set for high-throughput functional analysis of genes in planta. Plant Physiol 133:462–469. doi:10.1104/pp.103.027979

Davin LB, Lewis NG (2005) Dirigent phenoxy radical coupling: advances and challenges. Curr Opin Biotechnol 16:398–406. doi:10.1016/j.copbio.2005.06.010

Day SH, Chiu NY, Won SJ, Lin CN (1999) Cytotoxiclignans of *Justicia ciliata*. J Nat Prod 62:1056–1058. doi:10.1021/np9900167

Dean GH, Zheng H, Tewari J, Huang J, Young DS, Hwang YT, Western TL, Carpita NC, McCann MC, Mansfield SD, Haughn GW (2007) The *Arabidopsis MUM2* gene encodes a beta-galactosidase required for the production of seed coat mucilage with correct hydration properties. Plant Cell 19:4007–4021. doi:10.1105/tpc.107.050609

Dean GH, Cao Y, Xiang D, Provart NJ, Ramsay L, Ahad A, White R, Selvaraj G, Datla R, Haughn GW (2011) Analysis of gene expression pattern during seed coat development in *Arabiopsis*. Mol Plant 4:1074–1091. doi:10.1093/mp/ssr040

Debeaujon I, Nesi N, Perez P, Devic M, Grandjean O, Caboche M, Lepiniec L (2003) Proanthocyanidin-accumulating cells in *Arabidopsis* testa: regulation of differentiation and role in seed development. Plant Cell 15:2514–2531. doi:10.1105/tpc.014043

DeBono AG (2011) The role and behavior of *Arabidopsis thaliana* lipid transfer proteins during cuticular wax deposition. Dissertation, University of British Columbia

Devic M, Guilleminot J, Debeaujon I, Bechtold N, Bensaude E, Koornneef M, Pelletier G, Delseny M (1999) The *BANYULS* gene encodes a DFR-like protein and is a marker of early seed coat development. Plant J 19:387–398. doi:10.1046/j.1365-313X.1999.00529.x

El-Mezawy A, Wu L, Shah S (2009) A seed coat-specific promoter for canola. Biotechnol Lett 31:1961–1965. doi:10.1007/s10529-009-0098-y

Frey-Wyssling A (1976) The plant cell wall. Encyclopedia of plant anatomy, 3rd edn. Gebruder Borntraeger, Berlin

Gonzalez A, Mendenhall J, Huo Y, Lloyd A (2009) TTG1 complex MYBs, MYB5 and TT2, control outer seed coat differentiation. Dev Biol 325:412–421. doi:10.1016/j.ydbio.2008.10.005

Griesbeck O, Baird GS, Campbell RE, Zacharias DA, Tsien RY (2001) Reducing the environmental sensitivity of yellow fluorescent protein. J Biol Chem 276:29188–29194. doi:10.1074/jbc.M102815200

Harper A, Kerr DJ, Gescher A, Chipman JK (1999) Antioxidant effects of isoflavonoids and lignans, and protection against DNA oxidation. Free Radic Res 31:149–160. doi:10.1080/10715769900301661

Haughn GW, Chaudhury A (2005) Genetic analysis of seed coat development in *Arabidopsis*. Trends Plant Sci 10:472–477. doi:10.1016/j.tplants.2005.08.005

Haughn GW, Somerville C (1986) Sulfonylurea-resistant mutants of *Arabidopsis thaliana*. Mol Gen Genet 204:430–434. doi:10.1007/BF00331020

Haughn GW, Western TL (2012) *Arabidopsis* seed coat mucilage is a specialized cell wall that can be used as a model for genetic analysis of plant cell wall structure and function. Front Plant Sci 3:64. doi:10.3389/fpls.2012.00064

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: *beta-glucuronidase* as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6:3901–3907

Jofuku KD, den Boer BG, Van Montagu M, Okamuro JK (1994) Control of *Arabidopsis* flower and seed development by the homeotic gene *APETALA2*. Plant Cell 6:1211–1225. doi:10.1105/tpc.6.9.1211

Kaneda M, Schuetz M, Lin BS, Chanis C, Hamberger B, Western TL, Ehlting J, Samuels AL (2011) ABC transporters coordinately expressed during lignification of *Arabidopsis* stems include a set of ABCBs associated with auxin transport. J Exp Bot 62:2063–2077. doi:10.1093/jxb/erq416

Liang M, Davis E, Gardner D, Cai X, Wu Y (2006) Involvement of *AtLAC15* in lignin synthesis in seeds and in root elongation of *Arabidopsis*. Planta 224:1185–1196. doi:10.1007/s00425-006-0300-6

Matsuda F, Hirai MY, Sasaki E, Akiyama K, Yonekura-Sakakibara K, Provart NJ, Sakurai T, Shimada Y, Saito K (2010) AtMetExpress development: a phytochemical atlas of *Arabidopsis* development. Plant Physiol 152:566–578. doi:10.1104/pp.109.148031

Mendu V, Griffiths JS, Persson S, Stork J, Downie BA, Voiniciuc C, Haughn GW, DeBolt S (2011) Subfunctionalization of cellulose synthases in seed coat epidermal cells mediates secondary radial wall synthesis and mucilage attachment. Plant Physiol 157:441–453. doi:10.1104/pp.111.179069

Miyazawa M, Kasahara H, Kameoka H (1996) Antifungal activities of 8-O-4′-neolignans from *Myristica fragrans*. Natural Product Lett 8:271–273. doi:10.1080/10575639608044906

Moloney MM, Walker JM, Sharma KK (1989) High efficiency transformation of *Brassica napus* using *Agrobacterium* vectors. Plant Cell Rep 8:238–242. doi:10.1007/BF00778542

Nakaune S, Yamada K, Kondo M, Kato T, Tabata S, Nishimura M, Hara-Nishimura I (2005) A vacuolar processing enzyme, δVPE, is involved in seed coat formation at the early stage of seed development. Plant Cell 17:876–887. doi:10.1105/tpc.104.026872

Nesi N, Jond C, Debeaujon I, Caboche M, Lepiniec L (2001) The *Arabidopsis TT2* gene encodes an R2R3 MYB domain protein that acts as a key determinant for proanthocyanidin accumulation in developing seed. Plant Cell 13:2099–2114. doi:10.1105/TPC.010098

Nitao JK, Nair MG, Thorogood DL, Johnson KS, Scriber JM (1991) Bioactive neolignans from the leaves of *Magnolia virginiana*. Phytochemistry 30:2193–2195. doi:10.1016/0031-9422(91)83612-O

Stamatoyannopoulos JA (2010) Illuminating eukaryotic transcription start sites. Nat Methods 7:501–503. doi:10.1038/nmeth0710-501

Tiwari S, Spielman M, Day RC, Scott RJ (2006) Proliferative phase endosperm promoters from *Arabidopsis thaliana*. Plant Biotechnol J 4:393–407. doi:10.1111/j.1467-7652.2006.00189.x

Wan L, Xia Q, Qiu X, Selvaraj G (2002) Early stages of seed development in *Brassica napus*: a seed coat-specific cysteine proteinase associated with programmed cell death of the inner integument. Plant J 30:1–10. doi:10.1046/j.1365-313X.2002.01262.x

Western TL (2012) The sticky tale of seed coat mucilages: production, genetics, and role in seed germination and dispersal. Seed Sci Res 22:1–25. doi:10.1017/S0960258511000249

Western TL, Skinner DJ, Haughn GW (2000) Differentiation of mucilage secretory cells of the *Arabidopsis* seed coat. Plant Physiol 122:345–356. doi:10.1104/pp.122.2.345

Western TL, Burn J, Tan WL, Skinner DJ, Martin-McCaffrey L, Moffatt BA, Haughn GW (2001) Isolation and characterization of mutants defective in seed coat mucilage secretory cell development in *Arabidopsis*. Plant Physiol 127:998–1011. doi:10.1104/pp.010410

Windsor JB, Symonds VV, Mendenhall J, Lloyd AM (2000) *Arabidopsis* seed coat development: morphological differentiation of the outer integument. Plant J 22:483–493. doi:10.1046/j.1365-313x.2000.00756.x

Winter D, Vinegar B, Nahal H, Ammar R, Wilson GV, Provart NJ (2007) An "Electronic Fluorescent Pictograph" browser for exploring and analyzing large-scale biological data sets. PLoS ONE 2:e718. doi:10.1371/journal.pone.0000718

Wu L, El-Mezawy A, Shah S (2011) A seed coat outer integument-specific promoter for *Brassica napus*. Plant Cell Rep 30:75–80. doi:10.1007/s00299-010-0945-2

Young RE, McFarlane HE, Hahn MG, Western TL, Haughn GW, Samuels AL (2008) Analysis of the Golgi apparatus in *Arabidopsis* seed coat cells during polarized secretion of pectin-rich mucilage. Plant Cell 20:1623–1638. doi:10.1105/tpc.108.058842

Zhang F, Gonzalez A, Zhao M, Payne CT, Lloyd A (2003) A network of redundant bHLH proteins functions in all TTG1-dependent pathways of *Arabidopsis*. Development 130:4859–4869. doi:10.1242/dev.00681

Zhao J, Wang J, An L, Doerge RW, Chen ZJ, Grau CR, Meng J, Osborn TC (2007) Analysis of gene expression profiles in response to *Sclerotinia sclerotiorum* in *Brassica napus*. Planta 227:13–24. doi:10.1007/s00425-007-0586-z