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Molecular analysis of the hull-less seed trait in pumpkin: expression profiles of genes related to seed coat development[11]

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Abstract

We undertook a comparative study of molecular changes during development of seed coats in the wild-type and a recessive hull-less mutant of pumpkin (*Cucurbita pepo* L.), with the goal of identifying key genes involved in secondary cell wall development in the testa. The mature mutant testa has reduced amounts of cellulose and lignin as compared to the wild type. The expression patterns of several genes involved in secondary cell wall biosynthesis during the development of the testa are described. These genes are: *CELLULOSE SYNTHASE*, *PHENYLALANINE AMMONIA-LYASE*, *4-COUMARATE-CoA LIGASE*, and *CINNAMOYL-CoA REDUCTASE*. Additionally, the expression patterns of a few genes that were differentially expressed in the two genotypes during testa development (*GLUTATHIONE REDUCTASE*, *ABSCISIC ACID RESPONSE PROTEIN E*, a *SERINE-THREONINE KINASE*, and a *β-UREIDOPROPIONASE*) are presented. The results show a coordinated expression of several genes involved in cellulose and lignin biosynthesis, as well as marked differences in the level of their expression between the two genotypes during testa development. There is generally a higher expression of genes involved in cellulose and lignin biosynthesis in the wild-type testa as compared to the mutant. The molecular data presented here are consistent with anatomical and biochemical differences between the wild-type and the mutant testae. An understanding of the genes involved in cell wall development in the testa will facilitate the manipulation of seed coat development in *Cucurbita* and other species for diverse commercial applications.

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Introduction

The cucurbit (*Cucurbita pepo* L.) seed coat (testa), which is derived from the ovular integuments, is relatively hard and leathery and possesses five tissue layers: the epidermis, hypodermis, sclerenchyma, parenchyma and chlorenchyma (see Fig. 1) (Singh and Dathan, 1972; Loy, 2000). In the late 19th century, a hull-less seed ('naked-seed') mutation in normal field pumpkins was discovered (reviewed in Teppner, 2000) that displays reduced cellulose and lignin content in the hypodermis, sclerenchyma and parenchyma tissues of the seed coat (Stuart and Loy, 1983; Loy, 2000, 2004). As the hull-less seed matures, the epidermis collapses and, along with reduced cellulose and lignin contents of the other tissues, results in a papery thin, translucent seed coat. The growth habit and other phenotypic characteristics of the plant are apparently unaffected.

Based on extensive inheritance studies, Schöeniger (1950, 1952, 1955) and others (Weilung and von Becherer, 1950; Mudra and Neumann, 1952) concluded that two factors influenced seed coat thickening, a main 'H' gene controlling development of the median seed surface and an associated 'N' gene affecting the seed coat margin. Later reviews of the work on the hull-less trait (Whitaker and Davis, 1962; Robinson *et al.*, 1976) and additional inheritance studies (Stuart, 1983) led to the conclusion that the hull-less mutant testa is the result of a single recessive gene (designated by the symbol 'n'), with other modifying genes having lesser influence on testa development. The hull-less seed trait has been beneficial for the development of oil-seed pumpkins and pumpkin snack seed (Winkler, 2000; Loy, 2000, 2004).

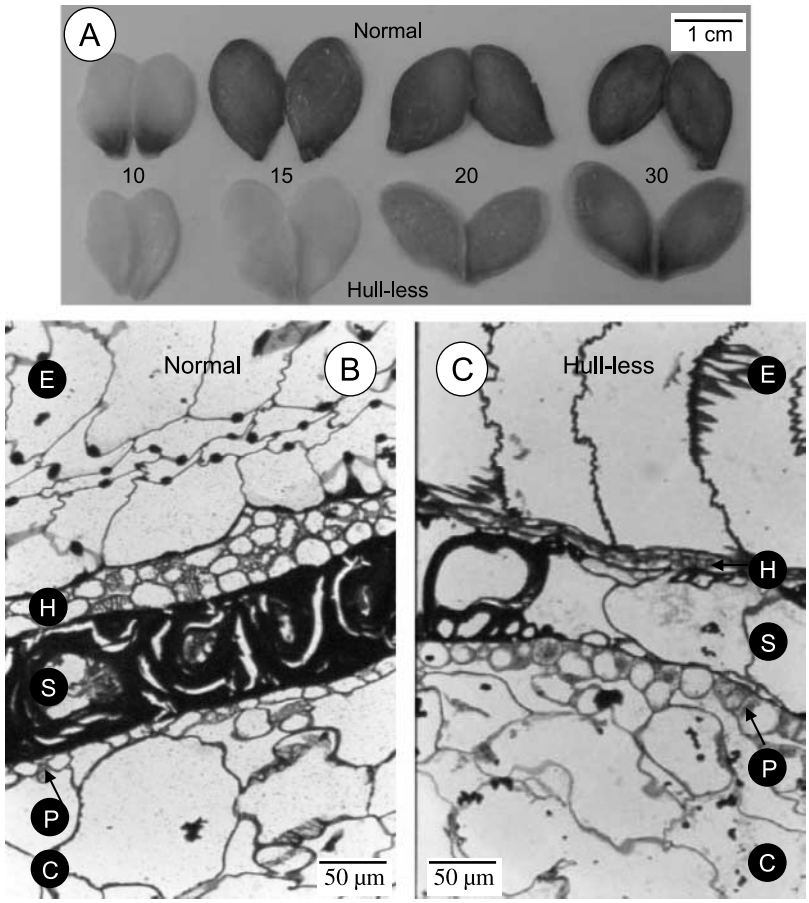

Figure 1. (A) Phloroglucinol-stained normal (wild type) and hull-less fresh, dissected testae from 10 to 30 days post-anthesis (dpa). Testae were stained for 30 min in 1% phloroglucinol in 20% HCl. (B, C) Toluidine blue-stained, methacrylate-embedded sections of wild-type and hull-less testae at maturity. Staining of hypodermis (H), sclerenchyma (S) and parenchyma (P) tissues in wild-type testa indicates lignification. E, epidermis; C, chlorenchyma.

A comparative anatomical analysis of the wild-type and the hull-less pumpkin seeds showed that all tissue layers were completely formed in the testa of both genotypes by 10 d post-anthesis (dpa), and there was considerably reduced deposition of both cellulose and lignin in the mutant testa after 20 dpa (Heinisch and Ruthenberg, 1950; Stuart and Loy, 1983; Loy, 2000, 2004). Mature seeds of both genotypes have relatively low pectin and hemicellulose (as compared to cellulose and lignin) contents, with the latter being reduced further in the hull-less seeds (Stuart and Loy, 1988). Pectin and hemicellulose are typically degraded during seed maturation and assimilated by the growing embryo along with starch and lipids (Stuart and Loy, 1988; Brett and Waldron, 1996; Vining and Loy, 1998).

Several mutations involving altered polysaccharide or lignin content have provided useful information about biochemical and deposition processes of cell wall macromolecules in plants. Mutations have been identified that exert their influence on cell wall formation through diverse mechanisms: reduced generation of cellulose or lignin precursors, altered cytoskeletal organization or movement of secretory vesicles to the plasma membrane, failure to synthesize specific polysaccharides, or the loss of post-translational modification of cell wall proteins (Ruan and Chourey, 1998; Taylor *et al.*, 1999; Fagard *et al.*, 2000; Turner *et al.*, 2001). Another category of cell wall developmental mutants includes those that affect the signalling and regulatory pathways involved in the transition from primary to secondary cell wall deposition (Turner and Hall, 2000). Mutations that affect cell wall development by altering cellulose or lignin production may exhibit pleiotropic effects (Fagard *et al.*, 2000; Turner *et al.*, 2001).

We undertook a comparative study of molecular changes during development of seed coats in wild-type

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and the hull-less genotype of pumpkin, with the goal of identifying key genes involved in secondary cell wall development in the testa (Bezold, 2002). The hypothesis was that the hull-less phenotype might be associated with either a reduction or a temporal shift in expression of the genes involved in secondary cell wall biosynthesis. The pumpkin seeds provide some unique advantages for molecular and biochemical analysis of seed coat development, e.g. the multi-carpel fruits contain over 100 seeds, and the large seed size provides ample tissue for analysis. Endosperm and embryo are easily separated, and sufficient tissue mass is available for study starting at 10–20 dpa.

The present report describes the expression pattern of several genes involved in cell wall development in the testa of the wild-type and the hull-less seeds. In addition, the expression patterns of a few genes that were identified through a suppression-subtractive hybridization approach, and were shown to be differentially expressed in the two genotypes during testa development, are also described. The results show differences in the levels of expression of several genes between the two genotypes during the period of testa development from 10 to 30 dpa, including a higher expression of genes involved in cellulose and lignin biosynthesis in the wild-type testa, as compared to the mutant.

Materials and methods

Tissue collection

Seeds of two *Cucurbita pepo* genotypes, wild type (NH 32-31-20-18-2-4-1) and a recessive single-gene mutant (NH 32-31-20-18-3-1-4), used in this study were from F_8 inbred populations that are approximately 97% isogenic (isogenecity established at F_6; Brent Loy, unpublished). The plants were grown at the UNH Kingman and Woodman Research farms. Upon anthesis (near the end of July), open-pollinated female flowers were tagged early in the morning. Seeds were collected from fruits at 10, 15, 20 and 30 dpa, the endosperm and embryo were removed, and the entire testae were quickly frozen in liquid nitrogen and stored at −80°C. For anatomical analysis, the developing seeds were saturated with 25% glutaraldehyde immediately after isolation. Preparation of testa tissues for methacrylate embedding and staining of cross-sections and whole tissues has been described previously (Bezold *et al.*, 2003).

RNA isolation and cDNA cloning

Total RNA was isolated using a modified guanidine thiocyanate method (Puissant and Houdebine, 1990), except that after the addition of phenol and chloroform, the samples were incubated on ice for 15 min. Following chloroform:isoamyl alcohol (24:1) treatment, total RNA was precipitated with ethanol and checked for quality by spectrophotometric analysis and non-denaturing agarose gel electrophoresis.

Degenerate primers (Table 1) were designed for several genes involved in cell wall biosynthesis, using published sequence information for at least three plant species and the NCBI database and MegAlign program (DNASTAR Inc., Madison, Wisconsin, USA). Synthesis of cDNA followed protocols of the SMART PCR cDNA synthesis kit (PT3041-1; Clontech, Palo Alto, California, USA) using DNase-treated total RNA. *ACTIN* and *G3PDH (GLUCOSE-3-PHOSPHATE*

Table 1. Degenerate primers used for amplification of specific genes from *C. pepo.* Primers were designed using sequence information for each gene from at least three species (source NCBI database). D = A or g or T; K = g or T; M = A or C; N = A or C or g or T; R = A or g; S = C or g; W = A or T; Y = C or T; guanine residues are in lower case for clarity

Gene name	Primer name	Primer sequence	Annealing temperature (°C)
ACTIN	ACT(5′)	5′-TgYgACAATggAACWggAATg-3′	
	ACT(3′)	5′-CATCTgYTggAARgTgCTgAg-3′	63
G3PDH	G3PDH(5′)	5′-TCSAAggAYgCSCCNATgTTYgT-3′	
	G3PDH(3′)	5′-TANCCCCAYTCDTTDTCgTACCA-3′	68
CCR	CCR.F	5′-CAARgTSCgRCgDgTKgTgTTCAC-3′	
	CCR.R	5′-TCYYTYAgCTTYTggTTKgAgAAYT-3′	58
CES	CES.F	5′-TCYTgggTgYTKgAYCAgTTC-3′	
	CES.R	5′-CYTggAMRACRgCAAARAgATg-3′	60
4CL	4CL.F	5′-AgCgTKgCMCARCARgTYgATg-3′	
	4CL.R	5′-ACYTgRAAKCCYTTRTATTTgAT-3′	50
PAL	PAL.F	5′-ggCTATTCCggTATTAggTTT-3′	
	PAL.R	5′-ATTTgAAggCAATCCATTgTT-3′	62

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DEHYDROGENASE) cDNAs were cloned from reverse-transcribed leaf mRNA (PolyA–T tract mRNA isolation kit; Promega, Madison, Wisconsin, USA). Total RNA from wild-type 20 dpa testae was used to construct first-strand cDNA by reverse transcriptase-polymerase chain reaction (RT-PCR), for amplification of specific genes involved in cellulose and lignin biosynthesis. The resulting PCR products were ligated into the TOPO™ TA Cloning® vector pCR2.1 and transformed into TOP10 eletrocompetent *Escherichia coli* (Invitrogen, Carlsbad, California, USA). Plasmids were isolated using the Wizard Miniprep DNA Purification System (Promega). Cloned amplified fragments were sequenced in both directions and analysed by BLASTX (Altschul *et al.*, 1990).

RT-PCR was performed with 10, 15, 20 and 30 dpa total RNA (5 μg) samples using the SuperScript II (GIBCO BRL, Grand Island, New York, USA) protocol. The single-strand cDNA (sscDNA) was used as a template for PCR co-amplification, using *ACTIN* primers along with the gene-specific primers in the same reaction tube for 20 cycles.

Preparation of labelled probes and Southern hybridization of cDNA

Digoxigenin (DIG)-labelled probes were constructed from PCR-amplified fragments using gene-specific primers (Table 2) with the DIG-High Prime Labeling system (Roche, Indianapolis, Indiana, USA). Analysis of probe concentration followed the manufacturer's protocol. The same total RNA samples were used for construction of cDNA for both amplified and non-amplified procedures for Southern hybridizations. Following electrophoresis on 1.0% agarose gel, DNA was transferred to a 0.45 μm SuperCharged nylon membrane using the TurboBlotter system (Schleicher and Schuell, Keene, New Hampshire, USA). Amplified cDNAs and non-amplified sscDNAs were run at 5 V cm^{-1} and 2.5 V cm^{-1}, respectively (Jaakola *et al.*, 2001).

Hybridization and post-hybridization procedures followed the protocol recommended by the DIG-Easy Hyb system (Roche) using 25 ng ml^{-1} probe solutions. Following post-hybridization washes, the membranes were processed for chemiluminescent detection (Roche). Fuji Super RX medical X-ray film (Fuji Film Co. Ltd, Tokyo, Japan) was exposed to membranes for 3–4 h for non-amplified and 5–15 min for amplified cDNAs. Membranes were stripped [sterile water for 1 min; 37 °C in 0.2 M NaOH, 0.1% sodium dodecyl sulphate (SDS) twice; 5 min in 2 × SSC buffer] for re-probing, and stored for up to 3 d in 2 × SSC at 4°C. Densitometry was used to determine the intensity of bands, using a Dot/Slot Blot ELISA analysis procedure (GelExpert 3.5; NucleoTech Inc., San Mateo, California, USA). The values obtained from intensity/area of specific genes were normalized to those of the control (*ACTIN* or *UBIQUITIN*).

Subtractive hybridization and identification of clones

Synthesis of cDNA for subtractive hybridization followed protocols outlined in the SMART PCR-select cDNA synthesis kit (Clontech), using 1.0 μg total RNA and the Superscript II Reverse Transcriptase RNase H$^-$ (GIBCO BRL). Optimization of long-distance PCR to prepare dscDNA for subtraction procedures followed the Clontech protocol. *Rsa*I digestion of cDNAs from both wild-type and mutant lines was performed to generate short, blunt end fragments. The 'tester' was constructed by dividing the cDNA into two pools and ligating the ends of each with a different adapter containing internal and external

Table 2. Primers used for preparation of labelled probes for sequences isolated through differential screening, These primers were based upon the sequence of the cloned PCR fragment resulting from the use of degenerate primers. Guanine residues are in lower case for clarity

Gene name	Primer name	Primer sequence	Annealing temperature (°C)
ABARp	ABARp (5′)	5′-ACCACAATgCCCTCATCTTTAAC-3′	
	ABARp (3′)	5′-CAATTTgggTCCTTTCATCAAg-3′	62
GR	GR (5′)	5′-CgggCAggTACATATAAATACAg-3′	
	GR (3′)	5′-TAACCATTCCCCATCTTTATTATTT-3′	58
β-URI	β-URI (5′)	5′-ggAAAgCCTggAggACAAAATCAT-3′	
	β-URI (5′)	5′-AgAATTgCTCCgCTggTTTTACAAT-3′	58
UBQ	UBQ (5′)	5′-ACgCggggAAggCAgAgTTgCTAAC-3′	
	UBQ (3′)	5′-ACTTCCTAgCCAAAgCCATCAAAgAAg-3′	72
STK	STK (5′)	5′-CTCCAAAggCATACACATC-3′	
	STK (3′)	5′-gCACTCCAAgCATTCACAT-3′	64

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priming sites (Diatchenko *et al.*, 1996). 'Driver' cDNA lacked these adapters. Subtractive hybridization followed the protocol of Clontech PCR-Select Subtraction kit. Briefly, the driver cDNA was mixed separately with the two tester cDNA pools at a 30:1 ratio, denatured, and allowed to form tester/tester or driver/tester hybrids. Following the first hybridization (8–12 h), a second round of hybridization (15–16 h) was performed by adding more denatured driver to one tester pool and then mixing both pools. Amplification of the subtracted and the unsubtracted cDNAs for primary PCR was performed for 27 cycles using external priming sites, and a secondary PCR (nested PCR) for 10 cycles using internal priming sites, as per manufacturer's recommendations (Clontech). Subtracted wild-type testa cDNAs were ligated into 10 μg of TOPO™ TA Cloning® vector pCR2.1 (Invitrogen) for 30 min, using 3 μl of cDNA. Ligation products were purified by ethanol precipitation. TOP 10 eletrocompetent *E. coli* (Invitrogen) were used for electroporation, and the transformants were screened through blue–white selection.

The selected white and light-blue colonies were used to inoculate 96-well plates containing 200 μl of LBK (Luria–Bertani broth with 50 μg ml^{-1} kanamycin) per well, and were incubated overnight on a shaker at 37 °C. Four identical plates were prepared by spotting 0.5 μl of overnight cultures and incubating them at 37°C for 4 h. Two positive controls (*ACTIN* and *G3PDH*) and the two Clontech negative controls were also applied. Colony transfer to MagnaGraph (0.45 μm) nylon membranes (MSI, Westboro, Massachusetts, USA) followed the colony and plaque hybridization protocol from the DIG Application Manual for Filter Hybridization (Roche).

The standard prehybridization solution [5 × SSC, 0.1% *N*-laurylsarcosine, 0.2% SDS, 1.0% blocking reagent in maleic acid buffer (100 mM maleic acid, 150 mM NaCl, pH 7.5)] was prepared from the prehybridization solution (Clontech PCR-Select Differential Screening Kit). The Clontech solution (50 μl) was denatured in 50 μl 20 × SSC and added to the preheated (65°C) prehybridization solution. Following prehybridization (1 h, 72°C), membranes were probed overnight at 70 °C with unsubtracted and subtracted hull-less and wild-type DIG-labelled cDNA probes (25 ng ml^{-1}). Preparation of membranes for detection of the signal by Anti-DIG AP-conjugate antibody and colorimetric reaction followed the manufacturer's protocol (Roche).

The size of the cDNA insert in the TOPO 2.1 vector was determined by *Eco*RI digestion. The cDNA clones were sequenced in both directions using M13 and T7 promoter primers of the cloning vector, as well as gene-specific primers, with the Dyenamic ET Terminator Sequencing kit (Pharmacia Biotech Inc, Piscataway, New Jersey, USA) at the UNH Hubbard Center for Genome Analysis. Sequences were analysed using SeqEd v1.0.3 program (Applied Biosystems Inc., Foster City, California, USA). Sequenced clones were identified using BLASTX with the GenBank database.

Results

Seed coat development

The development of the seed coat begins with the ovule, a maternal tissue. The testae of both the wild-type and the hull-less seeds were soft and easily dissected until 20 dpa; beyond this period both became rigid. Lignification hardened the wild-type testa, impeding dissection at later developmental stages. The seed coat of the hull-less seed became more flexible relative to that of the wild-type seed. At 10 dpa, a noticeable beige pigmentation developed at the micropylar end of the wild-type testa; this region was slightly yellow in the hull-less seed. When stained for lignin with phloroglucinol (Clifford, 1974), only the micropylar region of the wild-type testa stained red at this time. This staining covered the entire wild-type testa at later developmental stages but was weakly dispersed in the mutant testa (Fig. 1A).

At 20 dpa, toluidine blue staining of methacrylate-embedded sections of wild-type testa produced a deep blue tint in the hypodermis, sclerenchyma and parenchyma layers, indicating lignification (as per O'Brien *et al.*, 1964). Also, the hypodermal cells of wild-type testa were reticulated. The mutant 20-dpa testa did not stain blue (instead it stained purple), and hypodermal cells were not reticulated (data not shown). The mutant testa was noticeably thinner and transparent at 30 dpa compared to wild-type testa. At maturity, the hull-less testa mostly lacked the thick, lignified walls seen in the wild type (Fig. 1B, C). The reduced lignification and collapsed hypodermal tissue contributed to the overall transparency of the outer seed coat of the mature mutant testa.

Analysis of gene expression

The approach of modified Southern hybridization with cDNA was used to study the expression of two groups of genes that could potentially be involved in (or responsible for) the hull-less phenotype in the mutant seeds: (1) genes known to be involved in cell wall biosynthesis; and (2) genes identified by modified subtractive hybridization to be differentially expressed in the two genotypes. Included in the first approach were *CELLULOSE SYNTHASE* (*CES*), *PHENYLALANINE AMMONIA-LYASE* (*PAL*), *4-COUMARATE-CoA LIGASE* (*4CL*), and *CINNAMOYL-CoA REDUCTASE* (*CCR*) genes, with

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UBIQUITIN or *ACTIN* as an internal control. The selection of genes was based upon their known roles in cellulose and lignin biosynthesis (Fagard *et al.*, 2000; Turner *et al.*, 2001). The genes identified by subtractive hybridization, expression of which was followed in detail, included: *GLUTATHIONE REDUCTASE* (*GR*), *ABSCISIC ACID RESPONSE PROTEIN* E (*ABARp*), *SERINE THREONINE KINASE* (*STK*), *β-UREIDOPROPIONASE* (*β-URI*) and *UBIQUITIN* (internal control).

Expression of cell wall biosynthesis-related genes

Total RNA isolated from testa tissues collected in the summers of 1999 and 2000 at 10, 15, 20 and 30 dpa was converted into cDNA (using degenerate primers), which was then used for semi-quantitative Southern hybridization. The conversion was done to achieve better stability during quantification. Transcript abundance was estimated directly by Southern hybridization of the sscDNA or, in some cases, after PCR amplification of the resulting cDNA. For normalization of densitometry data, *UBIQUITIN* was used as a standard for quantification of non-amplified cDNA, while *ACTIN* was used as internal standard for co-amplification, where needed. The selection of whether amplified or non-amplified cDNA was used was based upon the results of preliminary hybridization experiments involving co-amplification of the *ACTIN* versus the *UBIQUITIN* genes, along with the specific gene being studied, and estimation of the signal strength (data not shown). The two approaches gave similar results.

Relative expression of *CES* transcripts was compared using amplified as well as non-amplified cDNA methods. For amplified cDNA, the control was *ACTIN* and for non-amplified cDNA, the control was *UBIQUITIN*; this was because only *ACTIN* could be co-amplified with this gene. The patterns of changes in CES transcript were similar using the two approaches, with higher sensitivity of detection by the amplified cDNA approach (Fig. 2). The *CES* signal was not detectable in the mutant testa on any day of analysis using non-amplified cDNA (Fig. 2A, B). Using this method, the maximum expression of *CES* in the wild-type testa was seen at 20 dpa. However, on amplification, a signal became visible at 10, 15, 20 and 30 dpa; the signal strength was lower in the mutant testa on all days (Fig. 2C, D). For amplified DNA, the ratio of *CES* to *ACTIN* showed earlier as well as greater signal intensity in the wild-type testa compared to the mutant testa; the maximum expression in the wild-type testa was at 15 dpa in contrast to 20 dpa for the mutant. In both genotypes, the expression of this gene declined by 30 dpa. Fig. 2D also shows the appearance of a smaller transcript (15–20 dpa) along with the major transcript in the wild-type testa following amplification of the cDNA; this transcript was not seen in the mutant testa. The size of the dominant *CES* band detected through hybridization of the non-amplified cDNA was determined to be around 3.0 kb; this size

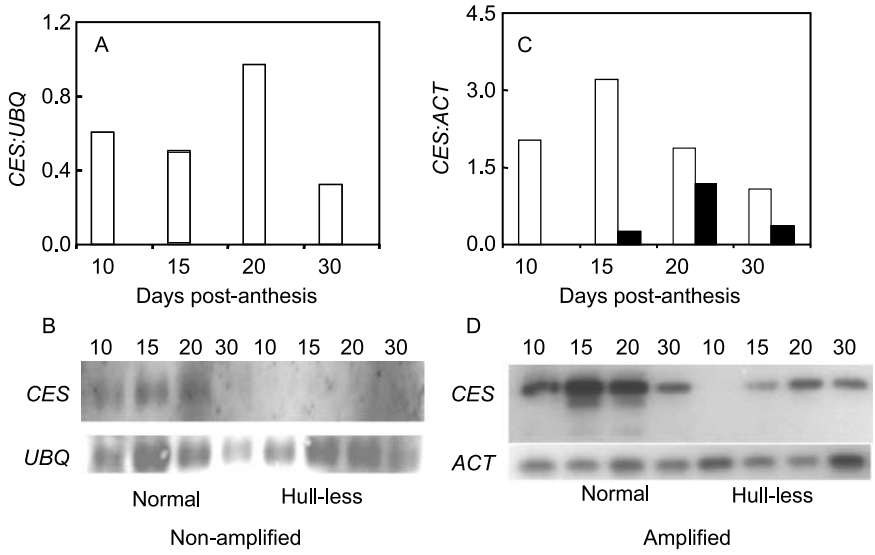

Figure 2. Densitometry results and gel images of Southern hybridization of cDNAs from normal (wild type) and hull-less testae at different days post-anthesis hybridized with *UBQ* and *CES*, and *ACTIN* and *CES* gene probes. (A) Intensity/area ratio of signal strength of *CES/UBQ*; (B) Gel images of non-amplified cDNAs; (C) Ratio of signal strength *CES/ACTIN*; (D) Gel images of amplified cDNAs. Open bars = wild type; solid bars = hull-less.

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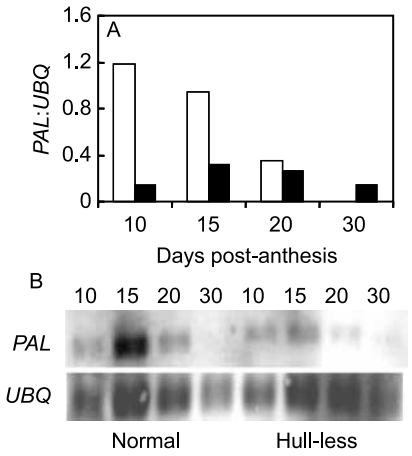

Figure 3. Densitometry results and gel images of Southern hybridization of non-amplified cDNAs from normal (wild type) and hull-less testae at different days post-anthesis hybridized with *UBQ* and *PAL* gene probes. (A) Intensity/area ratio of signal strength of *PAL/UBQ*; (B) Gel images of non-amplified cDNAs. Open bars = wild type; solid bars = hull-less.

corresponds well to the cotton *CES* mRNAs hybridized with *GhCeSA-1* and *GhCeSA-2* gene probes (Pear *et al.*, 1996).

Phenylalanine ammonia-lyase is the first enzyme in the pathway relevant to biosynthesis of lignin and other phenylpropanoids. Expression of its transcript was also studied by both the amplified and the non-amplified cDNA methods. The relative intensity of the *PAL* signal, as revealed by the amplified cDNA method, was lower in the mutant testa on all days (data not shown). Using non-amplified cDNA, the *PAL* signal was found to be several-fold greater in the wild-type testa than the mutant testa on all days of analysis except 30 dpa, when no signal was seen in the wild-type testa (Fig. 3A, B). The transcript levels were the highest at 10 dpa in the wild-type and 15 dpa in the mutant testa, and declined at 20 and 30 dpa in both cases. The size of the *PAL* cDNA, as seen on the non-amplified cDNA gels, was estimated to be 2.5 to 2.7 kb; this is similar to the transcript size of *PAL* in *Cucumis melo*, which was calculated to be 2.4 kb (Diallinas and Kanellis, 1994).

The first enzyme in the phenylpropanoid pathway committed to lignin biosynthesis is cinnamoyl-CoA reductase. The transcript level of *CCR* (non-amplified cDNA method) showed that the relative expression of this gene was the highest at 15 dpa in both types of testa (Fig. 4A, B). The signal strength for mutant testa was generally lower on all days, the differences being greater to twofold at 15 and 20 dpa, and much smaller at 10 and 30 dpa. Hybridization of the *CCR* probe to cDNA produced a 1.6 kb band, which is similar than the size of *Zea mays CCR* as seen by Northern hybridization (Pichon *et al.*, 1998).

The final enzyme in the general phenylpropanoid pathway leading to lignin biosynthesis is 4-coumarate-CoA ligase (Fig. 4C, D). The highest signal intensity for *4CL* transcript in the wild-type testa was at 15 dpa, while in the mutant it was at 20 dpa. Detectable expression of *4CL* in the mutant testa was delayed (15 dpa, versus 10 dpa in the wild-type testa), and the transcript abundance was lower as compared to the wild-type testa, except at 30 dpa, when the *4CL* transcript was very low in the wild-type testa but still visible in the mutant. The size of *4CL* cDNA was calculated to be around 2.0 kb, which corresponds well to that of poplar *4CL* (1.9 and 2.0 kb for *Pt4CL1* and *Pt4CL2*, respectively) obtained through Northern analysis (Hu *et al.*, 1998).

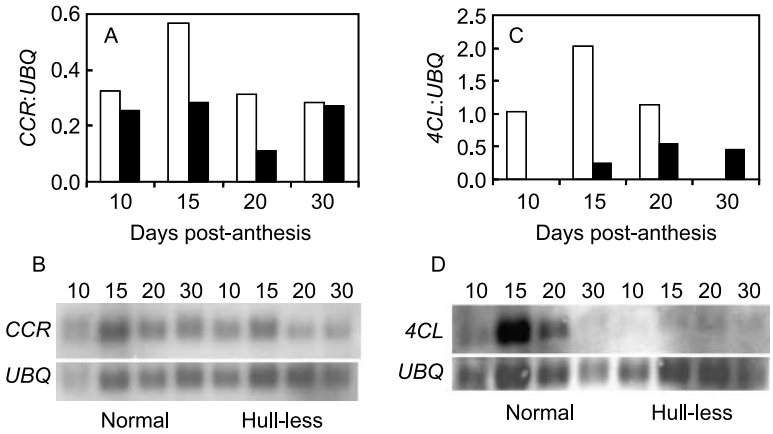

Figure 4. Densitometry results and gel images of Southern hybridization of non-amplified cDNAs from normal (wild type) and hull-less testae at different days post-anthesis hybridized with *CCR* or *4CL* and *UBQ* gene probes. (A) Ratio of signal strength of *CCR/UBQ*; (B, D) = Gel images of non-amplified cDNAs; (C) Ratio of signal strength for *4CL/UBQ*. Open bars = wild type; solid bars = hull-less.

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Table 3. Results of a BLASTX search using sequences from clones isolated through differential screening with high homology, based on percent identity (%id) of amino acids and e-values

Clone	Size (bp)	Accession number	Match		BLASTX e-value	Homology	Organism
			Overlap (AA)	%id			
ABARp	584	M62991	135	69	1e-75	Abscisic acid response protein E	*Craterostigma plantagineum*
β-URI	473	AB008268	46	56	3e-21	β-Ureidopropionase	*Arabidopsis thaliana*
UBQ	320	AF195224	88	100	5e-43	Ubiquitin fusion protein	*Pyrus pyrifolia*
GR	550	AB028621	69	79	7e-33	Glutathione reductase	*Arabidopsis thaliana*
STK	954	AC021893	72	50	1e-31	Serine–threonine protein kinase	*Oryza sativa*

Differentially expressed genes

Following preliminary analysis using suppression subtractive hybridization (Diatchenko *et al.*, 1996), several genes were identified that appeared to be expressed more strongly in the wild-type testa than the mutant (Table 3). The subtraction efficiency was quantified by analysing the reduction of common housekeeping genes (*G3PDH* and *ACTIN*) over a series (18, 23, 28 and 33) of PCR cycles. Amplified *G3PDH*, as well as the products from unsubtracted cDNA, appeared at 18 cycles of amplification, but from the subtracted cDNA a *G3PDH* band was seen only at 23 cycles (Bezold, 2002; data not shown here), showing a 32-fold (2^5) reduction in the normal 10 and 20 dpa cDNA. Subtracted normal testa cDNA libraries were constructed in the TOPO cloning vector. The selection of 45 clones of interest, from more than 500 that hybridized with DIG-labelled probes made from 20 dpa normal and mutant-subtracted and unsubtracted cDNAs, was based upon signal strength in five screenings; these clones were sequenced and identified by BLASTX search. Confirmed homologies of ten clones and two putative proteins were based on greater than 50% similarity of amino acid sequences and low e-value probability. The relative expression over time of four of the selected genes that showed the highest homology (*GR*, *ABARp*, an *STK* and a *β-URI*) was studied in detail, using amplified cDNA for each gene and *ACTIN* as an internal standard for co-amplification.

Based on the pair of primers (Table 1) designed using the published sequences in the NCBI database, the expected PCR product for *GR* was 550 bp, but a 350 bp band was also amplified. Both PCR products hybridized with the *GR* probe; the 350 bp putative *GR* transcript (GR_{350}) band showed a greater intensity of hybridization than the GR_{550} band (Fig. 5). Both the wild-type and the mutant testae showed increased levels of *GR* expression with the stage of development from 10 to 30 dpa, for both bands. A greater relative expression of both bands was seen in the wild-type testa as compared to the mutant testa on all days of analysis.

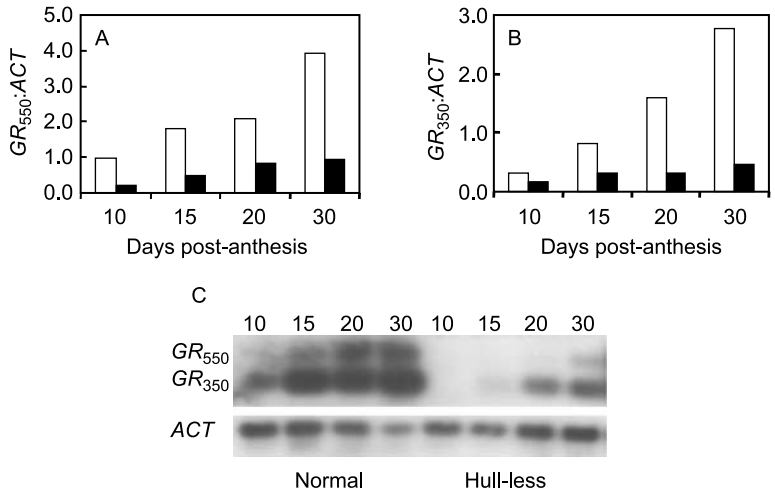

Figure 5. Densitometry results and gel images of Southern hybridization of amplified cDNAs from normal (wild type) and hull-less testae at different days post-anthesis hybridized with *GR* and *ACTIN* gene probes. (A) Ratio of signal strength of $GR_{550}/ACTIN$; (B) ratio of signal strength of $GR_{350}/ACTIN$. (C) Gel images of amplified cDNAs. Open bars = wild type; solid bars = hull-less.

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Also analysed using a similar approach were the relative expressions of transcripts whose sequences matched an *ABARp* and an *STK*. The transcripts for both these genes showed similar relative expression patterns in the two genotypes. *ABARp* transcript was detected by 15 dpa in both lines and appeared to increase with the time of testa development (Fig. 6A, B). The wild-type testa had higher transcript levels of *ABARp* than the mutant testa on all days of analysis. The hybridization signal for *STK* was quite low at 10 dpa in both lines, remained undetectable in the mutant testa at 15 and 20 dpa, and became visible again at 30 dpa. In the wild-type testa, *STK* transcript level increased steadily up to 30 dpa; on this day, the *STK* signal in the wild-type testa was three- to fourfold higher than the mutant testa (Fig. 6C, D).

A cDNA with sequence resembling a *β-URI*, an enzyme involved in the catabolism of uridine monophosphate (UMP) and uridine diphosphate (UDP), showed similar signal intensity of hybridization in both lines at 10 and 15 dpa. During the next 15 d, the signal strength appeared to increase further in the hull-less testa, while it decreased in the wild-type testa (data not shown). Thus at 20 and 30 dpa, the *URI* signal was three- to fourfold higher in the mutant testa than in the wild-type testa. In the material collected in the summer of 2000, a different pattern of transcript signal was seen in the two types of testa at 20 dpa. In those samples, the wild-type testa showed a relatively stronger signal for *URI* than the mutant testa (data not shown). The difference in the pattern of expression between the samples of 2 years of collection was unique only to the *URI* gene among those that have been studied so far and reported here.

Discussion

Seed coat development

The anatomical pattern of testa development in the wild-type and the mutant pumpkin seed observed in the current study was similar to that reported earlier using non-isogenic lines in which modifier genes were also present (Stuart, 1981; Loy, 2000). Differences in the wild type and the hull-less testae were seen within 10 dpa, in that phloroglucinol staining (for lignin; Clifford, 1974) of the wild-type testa showed intense red colour at the micropylar end of the seed; in contrast, the mutant testa showed no red staining at 10 and 15 dpa, and minimal dispersed positive staining at later time periods. At maturity, the entire seed coat of the wild-type seed stained red. Although chemical analysis of lignin monomers has not been performed in the frail mutant testa, it does not show the typical brown coloration, and at 10 dpa, also lacks the beige colour that develops in the micropyle region of wild-type seeds. Mutations in lignin biosynthetic genes can cause discoloration of the affected tissues due to variations in the phenolic compounds that constitute lignin (Vignols *et al.*, 1995; Halpin *et al.*, 1998; Lapierre *et al.*, 2000).

As reported by Stuart and Loy (1983), at 10 dpa, the hypodermis and parenchyma of wild-type testa showed the presence of lignin; later at 20 dpa, sclerenchyma also tested positive for lignin. Cross-sections of mature wild-type and mutant testae (Fig. 1) confirm the previous findings on lignification in the testae of the two genotypes. The cell walls in mutant testa remained thin, the hypodermis collapsed, and both the hypodermis and parenchyma lacked

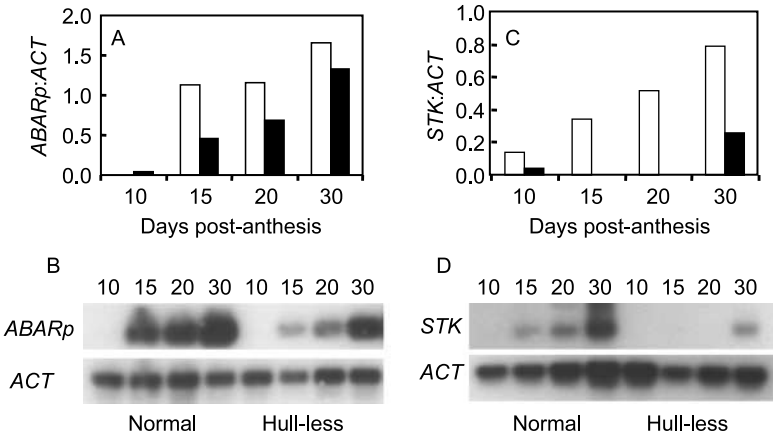

Figure 6. Densitometry results and gel images of Southern hybridization of amplified cDNAs from normal (wild type) and hull-less testae at different days post-anthesis hybridized with *ABARp* or *STK* and *ACTIN* gene probes. (A) Ratio of signal strength of *ABARp*/*ACTIN*; (B, D) Gel images of amplified cDNAs; (C) Ratio of signal strength for *STK*/*ACTIN*. Open bars = wild type; solid bars = hull-less.

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reticulation. It is known that, as the seed matures, components of the testa become assimilated by the growing embryo, resulting in a loss of mass over time (Stuart, 1983; Vining and Loy, 1998; Loy, 2000). A similar loss of testa mass with time of development was also apparent in the present study (data not shown).

Expression of cellulose and lignin biosynthesis genes

The expression of more than one *CES* family gene at the same time (e.g. *GhCESA-1* and *GhCESA-2* in cotton), and a distinct pattern of tissue-specific and developmentally regulated expression of *CESA* genes in *Arabidopsis* and *Zea mays*, have been reported (Holland *et al.*, 2000). Plant *CES* genes contain specific conserved regions that distinguish them from the bacterial counterparts and a hypervariable (HVR) region that differs among different plant genes that show tissue and temporal specificity of expression (Holland *et al.*, 2000). Based on partial sequence analysis, it appears that the pumpkin testa *CES* cDNA clone obtained in this study has highest homology with the cotton *GhCESA-1* gene (Accession #U58283; 89% identical over 556 amino acids). The relative transcript levels of *CES* were greater in the wild-type testa at all times, whether the probing was done using non-amplified cDNA or amplified DNA, showing that both approaches are effective in studying gene expression patterns in this tissue. The results were similar in the 2 years of seed collections, the highest level of the *CES* transcript being present at 15–20 dpa in the wild-type testa. The expression of *CES* in the mutant, compared to the wild-type testa, was reduced as well as temporally delayed. Moreover, the smaller transcript seen at 15 and 20 dpa in the wild-type testa (Fig. 2D) was not seen in the mutant testa. The potential importance of this *CES* transcript is not clear.

After the deposition of cellulose and hemicellulose, lignification begins in three distinct stages, with specific monolignols deposited in a time- and cell type-dependent manner (Terashima, 1990). During lignification, phenyl rings of lignin precursors align to the cellulose molecule surfaces in a controlled and ordered process. Through this interaction, polysaccharides may be capable of influencing the structure of lignin (Houtman and Atalla, 1995). Thus, a reduction in cellulose may also contribute to altering the structure as well as quantity of lignin in the hull-less pumpkin seed, and the toxic monolignols that do not polymerize could be scavenged by antioxidant compounds such as glutathione or polyamines (see also Bezold *et al.*, 2003).

Several mutations of the phenylpropanoid pathway result in altered lignin composition, showing reductions in specific monomers. The enzymes PAL, CCR and 4CL are present at key points in the lignin biosynthesis pathway, and their altered expression can markedly affect flavonoid and lignin precursor abundance (Lee *et al.*, 1997; Lapierre *et al.*, 2000; Chabannes *et al.*, 2001). The enzyme PAL regulates the first step in the general phenylpropanoid pathway, providing cinnamic acid for subsequent production of phenolic compounds, including lignin precursors. A multi-gene family comprises the *PAL* genes in plants. In *Populus kitakamiensis*, *PALG1* was expressed only in young stems, apical meristems and young leaves, suggesting that its expression is developmentally regulated (Osakabe *et al.*, 1995). The *PALG2A* was expressed in older stems where secondary xylem was developing. The *PAL* probe used in the present study was homologous to *PAL* of *Cucumis melo* (Accession #X76130; 91% identity over 248 amino acids; Diallinas and Kanellis, 1994), as well as to *P. kitakamiensis PALG2A*. Relative expression of *PAL* in wild-type testa was greatest around 10–15 dpa and was highly reduced, as well as delayed, in the hull-less seed coat. This period coincides with the onset of lignification. Since the turnover rate of PAL is not known, it is possible that the enzyme activity of PAL persists during the later stages of testa development even though the transcript levels decrease.

Cinnamoyl-CoA reductase is involved in the first committed step of the lignin biosynthesis pathway and is considered to be a control point regulating monolignol formation, which could be used as precursors of lignin or lignans that act as antioxidants or biocides (Lacombe *et al.*, 1997). The increased expression of *CCR* in maize was directly correlated with stalk internode maturation (Pichon *et al.*, 1998). Highest expression of *CCR* in both the wild-type and the mutant testae was at 15 dpa. The mutant testa showed a significant drop in *CCR* expression at 20 dpa as compared to all other days; the transcript levels were comparable in both types of testa at 10 and 30 dpa. Similarity of *CCR* expression in the two types of testa may represent some other function of this enzyme as mentioned above, e.g. for the production of lignan precursors. It is also possible that CCR is not a limiting factor for the differences in lignification in the two types of testa. The pumpkin *CCR* showed 79% identity of bases with the *Populus tremuloides CCR* (Accession #AF217958) over 140 codons.

As the last enzyme in the general phenylpropanoid pathway, 4CL is necessary in maintaining a metabolic flux for production of flavonoids and lignin by converting hydroxylated cinnamic acids to their thioester forms (Hu *et al.*, 1998). Like *PAL*, there are several *4CL* genes in plants that show differential expression and different substrate specificities of their products (Voo *et al.*, 1995; Hu *et al.*, 1998). The *4CL* probe used in this work showed high homology (74%

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identity over 178 codons) to the *Pt4CL1* of *Populus tomentosa* (Accession #AY043494). As with *PAL*, in the wild-type testa *4CL* was expressed the greatest at 15 dpa, and its expression was reduced as well as delayed in the mutant testa.

Expression of differentially expressed genes

A number of genes were identified by subtractive hybridization of cloned cDNAs that showed preferential expression in the wild-type testa (Bezold, 2002); a few of these are listed in Table 3. One of these genes was for GR, an enzyme that is an integral part of the ascorbate–glutathione cycle that regulates H_2O_2 levels in plants (Kocsy *et al.*, 2001). H_2O_2 assists in polymerization of monolignols, but can also cause oxidative stress on the membranes. It is likely that the development of the wild-type seed coat (as opposed to the mutant) requires the production of more H_2O_2 than that needed for lignification, and GR may provide sustained levels of reduced glutathione to scavenge reactive oxygen species produced from H_2O_2 (Stevens *et al.*, 1997). Thus, in the mutant testa, which has reduced amounts of lignin, this enzyme may be down-regulated because much less H_2O_2 is being produced. At 10 dpa, when testa cells begin to develop secondary walls, *GR* transcripts were detectable in the testae of both lines (Fig. 5). Earlier reports on cotton fibre development showed the expression of *GR* concomitant with an oxidative burst of H_2O_2, which presumably acts as a signal for secondary cell wall differentiation (Wäfler and Meier, 1994; Potikha *et al.*, 1999).

As testa maturation proceeds, its tissues become desiccated (Stuart, 1983); the mutant testa, however, maintains a greater relative moisture content than the wild type. *ABARp* is probably induced as a response to cell wall desiccation (Piatkowski *et al.*, 1990). The transcript of *ABARp* showed an increase in both types of testae from 10 to 30 dpa; transcript abundance was lower in the mutant testa, possibly due to reduced desiccation. The pumpkin *ABARp* sequence showed a high degree of homology to a similar gene in *Craterostigma plantagineum* (Table 3).

Another clone isolated through differential screening resembled a *STK* (Table 3), for which an enhanced signal was visualized in the wild-type testa at all times, but in the mutant at 30 dpa only. Besides homology to rice *STK*, the pumpkin testa clone has sequence homology to the gene for a defence response protein, *PBS1*, in *Arabidopsis* (Swiderski and Innes, 2001). Other kinases possessing *STK* domains are wall-associated kinases that physically link the plasma membrane with the cell wall and may play a role in signalling cellular events. Reduction in expression levels of the *STK* cDNA in the mutant versus the wild-type testa may be an effect of underdeveloped cell walls in the former.

Conclusions

Differentiation of cells during secondary cell wall formation involves a coordinated expression of genes that control the cell cycle, as well as cell wall expansion and synthesis. For example, differentiating cells during xylogenesis show strict regulation of secondary cell wall genes, including those of cellulose and lignin biosynthesis, as well as transcription factors and other potential regulators (Hertzberg *et al.*, 2001). The results of the present study are consistent with these observations. Douglas (1996) reported that during xylogenesis, six different *MYB* genes showed different patterns of expression; these transcription factors bind to *cis*-acting elements of *PAL*, *CCR* and *CAD* and other phenylpropanoid biosynthetic genes, and are involved in coordinating the expression of genes in both these pathways. Continued or delayed or failed expression of a transcription factor such as this may alter several aspects of the cell wall structure by changing the expression pattern of many downstream genes. Based on the observation of decreased cellulose and lignin content (Stuart, 1981, 1983), and a concomitant decrease in expression of several genes that regulate both cellulose and lignin biosynthesis by a single mutation, it can be hypothesized that an element regulating gene expression in both phenylpropanoid, as well as lignin biosynthesis pathways, may be at work in the mutant pumpkin testa.

The molecular data presented here are consistent with the anatomical and biochemical differences between the wild-type and the mutant testae. It is significant that the results show only down-regulation and not complete lack of expression of several genes involved in the biosynthesis of cell wall macromolecules. Identification of the specific mutant gene or essential genes that serve as master regulators controlling the development of the wild-type seed coat will be useful in developing plant stocks that have increased cellulose or decreased lignin components. Furthermore, an understanding of the genes involved may eventually allow for the manipulation of seed coat development in *Cucurbita* and other species for diverse commercial applications.

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