The OCL3 promoter from Sorghum bicolor directs gene expression to abscission and nutrient-transfer zones at the bases of floral organs

Krishna K. Dwivedi1,2,3, Dominique J. Roche1,4, Tom E. Clemente5, Zhengxiang Ge5 and John G. Carman1,3,†

1 Caisson Laboratories, Inc., 1740 Research Park Way, North Logan, UT 84322-4820, USA, 2 Crop Improvement Division, Indian Grassland and Fodder Research Institute, Jhansi (UP) 284003, India, 3 Plants, Soils and Climate Department, Utah State University, Logan, UT 84322–4820, USA, 4 PhytoGen Seed Co. LLC, Western Research Station, 850 Plymouth Avenue, Corcoran, CA 93212, USA and 5 Department of Agronomy and Horticulture, Center for Plant Science Innovation, University of Nebraska, Lincoln, NE 68588, USA

† For correspondence. E-mail john.carman@usu.edu

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The results suggest that the HD-Zip IV transcription factor SbOCL3 regulates kernel nutrition and abscission. The latter is consistent with evidence that members of this transcription factor group regulate silique abscission and dehiscence in Arabidopsis thaliana. Collectively, the findings suggest that processes of floral organ abscission are conserved among angiosperms and may in some respects differ from processes of leaf abscission.

Key words: Abscission zone, apoplast, closing layer, epidermis-specific, fruit abscission, kernel development, nutrient transport, OCL3, promoter, seed fill, Sorghum bicolor, symplast.

INTRODUCTION

The megagametophyte of angiosperms (embryo sac) is unique among Plantae in that it produces a central cell, in addition to the egg, which upon fertilization produces endosperm. It is also unique because of its reduced cell number, usually eight-nucleate, and its location within two sporophytic structures, the ovule and the ovary. In this respect, gametophytes of Plantae have evolved from a many-celled structure of anatomical complexity and nutritional autonomy, as in ferns and lower plants, to a structure that depends entirely on the sporophyte for nutrition (Linkies et al., 2010; Qiu et al., 2012). Physiological processes unique to angiosperms transport nutrients from the sporophyte to the absorptive surfaces of the megagametophyte, and later to those of the developing endosperm and embryo. Eventually, this nutrient flux is terminated by processes that include fruit and seed abscission. Physiologically, these processes are reasonably well understood (Patrick and Offler, 2001; Stadler et al., 2005), but they remain poorly understood at the gene network level (Ingram, 2010; Waters and Sankaran, 2011; Lucas et al., 2013).

Ovule meristems form on surfaces of vasculate placentae within immature ovaries. They may initially produce a vasculate and elongated stock-like funiculus, as in brassicas and legumes, which extends the ovule body away from the placenta and into the ovary cavity. Alternatively, the vasculate funiculus may remain short, as in grasses, such that the ovule body remains close to the ovary placenta (Johri et al., 1992). In either case, the apical meristem of the elongating funiculus becomes partitioned into two zones. The distal zone produces the nucellus, which is a largely transient and non-vasculate tissue. Almost simultaneously, the lateral meristematic zone produces one or two integuments, which are leaf-like structures that grow around the nucellus, eventually defining the micropyle. The integuments are non-vasculate in most plants but vasculate in some large-seeded plants (Corner, 1976). Eventually, the integuments, often in participation with the nucellar epidermis, produce the non-living seed coat (Johri et al., 1992).
The terminology used in the literature to define ovule structures often lacks specificity. Herein, we use the terminology of Esau (1977). Accordingly, the region of the funiculus that gives rise to the nucellus and the integuments is referred to as the chalaza (Esau, 1977). We do not use pedicel, placenta or placenchochala to define this region.

The cellular boundary between the chalaza and the nucellus is indistinguishable early in ovule differentiation (Esau, 1977). During this early phase, a single megasporocyte (megaspore mother cell) differentiates from a centrally located nucellar cell and undergoes meiosis. One of four megaspores survives to form a genetically reduced multicellular megagametophyte (Ma and Sundaresan, 2010; Yang et al., 2010). In Zea mays (maize), as the megagametophyte matures, the boundary between the nucellus and the chalaza becomes identifiable, and cellular and physiological activities in the nucellus, particularly during seed fill and maturation, diverge from those that occur in the chalaza (Kladnik et al., 2004; Tang and Boyer, 2013).

Transcription factors (TF) play central roles in regulating ovule development (Agarwal et al., 2011; Ruan et al., 2012), and some played important roles in land plant evolution. In particular, the plant-specific homeodomain-leucine zipper (HD-Zip) family of TFs regulates many developmental and physiological traits that originated during land plant evolution, traits that differentiated land plants from their aquatic ancestors and enabled them to tolerate terrestrial stresses (Floyd et al., 2006; Mukherjee et al., 2009; Zalewski et al., 2013).

The HD-Zip TFs contain a homeodomain (HD) for DNA binding and an adjacent leucine zipper domain (Zip), which enables enzyme function through dimerization. These domains occur in all HD-Zip TFs, and alone they define the HD-Zip I subfamily. Additional subfamilies are defined based on additional motifs: HD-Zip II TF contain a CPSCE motif, HD-Zip III and IV TFs contain a stereoidogenic acute regulatory protein-related lipid transfer domain (START) and a START-adjacent domain (HD-SAD), and HD-Zip III TFs also contain a MEKHLA motif. Collectively, HD-Zip TFs have been implicated in maintaining meristem cell layer identity, leaf development, vascular patterning and differentiation, stomata, trichome and cuticle formation, anthocyanin accumulation and possibly abscission zone (AZ) and dehiscence zone formation (Ingram et al., 2000). Beyond this, only general locations of OCL IV TF expression have been reported (Javelle et al., 2011).

In Arabidopsis, the HD-Zip IV TF HOMEODOMAIN GLABROUS1 (HDG1) regulates cuticle formation on leaf surfaces, but there is also evidence that it regulates processes of AZ and dehiscence zone formation in siliques (Wu et al., 2011). In the present study, we provide evidence that the Sorghum bicolor (sorghum) HD-Zip IV TF SbOCL3 may also regulate processes of AZ formation in kernels and other floral organs of sorghum. To accomplish this, we cloned the promoter of SbOCL3 (PSbOCL3) and transformed it into sorghum as a promoter–GUS (β-glucuronidase) construct. PSbOCL3 activity was strong in kernel closing layers (kernel AZ) and in the AZ of anthers and lodicules, but it was absent from the nucellar epidermis, immediately distal from the kernel closing layer, and from the AZ of lemmas, paleas and glumes.

**MATERIALS AND METHODS**

**Promoter cloning and in silico analyses**

Genomic DNA was isolated from Sorghum bicolor seeds (DNeasy Plant Mini kit, Qiagen Inc., Valencia, CA, USA), and DNA 5′ of SbOCL3 was amplified and cloned (Universal Genome Walker Kit; Clontech, Palo Alto, CA, USA) using primers designed from the ZmOCL3 sequence (Primer3; Rozen and Skaletsky, 2000; Supplementary Data Table S1). A 2.0 kb fragment was then cloned (pGEMT, Promega, Madison, WI, USA) and sequenced (Sequtech, Mountain View, CA, USA), and is referred to herein as the SbOCL3 promoter (PSbOCL3). This promoter was then compared by BLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi; Altschul et al., 1990) with the AtHDG1 promoter (PAHZDG1) and with maize whole-genome shotgun sequences. Putative transcription start sites and cis-regulatory elements in the largely homologous sorghum and maize promoter regions and in PATHDG1 were identified using NNPP2-2 (http://www.fruitfly.org/seq_tools/promoter.html; Reese, 2001) and PLACE (http://www.dna.afrc.go.jp/PLACE/signalscan.html; Higo et al., 1999), respectively. The numbers of copies of specific cis-regulatory elements were compared among the three species by linear regression (SYSTAT, 2004; http://www.systat.com/).

**Construct preparation and transformation**

The cloned PSbOCL3 and a pPTN289 vector were digested with HindIII and EcoRI, and the PSbOCL3 fragment and a fragment containing the Tobacco etch virus (TEV) leader, GUSPlus™ reporter gene (Broothaerts et al., 2005) and terminator were eluted. A pPZP211 binary vector was then digested with HindIII and dephosphorylated, and the linear fragment was eluted. The fragments were ligated to obtain the PSbOCL3::GUSPlus™ (PSbOCL3::GUS) construct, which was confirmed by sequencing. This construct was transformed into Agrobacterium tumefaciens strain NTL4/pKPSF2 by electroporation (Palanichelvam et al., 2000; Luo et al., 2001), and the engineered A. tumefaciens was used to transform sorghum “TX-430” (Howe et al., 2006). Selfed seed (T1) of all putative T0 plants were obtained.
Integration patterns of the transgenic allele were ascertainment for 11 of 31 putative events generated from sorghum transformations involving the P_{SbOCL3}::GUSPlusTM binary vector. Bulk DNA was extracted from 15 T1 seedlings per event. Total genomic DNA was extracted from bulk samples following the protocol outlined by Dellaporta et al. (1983). Genomic DNA (10 μg) was restriction digested with EcoRV, a cut site of which resides within the binary vector P_{SbOCL3}::GUSPlusTM. The DNA were then separated on a 0.8 % agarose gel. Gel processing and hybridization conditions were carried out as previously described ( Howe et al., 2006). The membrane was hybridized with a [32P]dCTP-labelled GUSPlus element. The positive control lane contained 50 pg of P_{SbOCL3}::GUSPlusTM digested with EcoRV. The negative control lane contained 10 μg of non-transgenic TX-430 genomic DNA.

**Histology**

Pre-fertilization-staged pistils of non-transformed sorghum were fixed and cleared for differential interference contrast (DIC) microscopy (Crane and Carman, 1987). Additional sets of pre-fertilization-staged pistils and post-fertilization-staged kernels at 15 and 30 days post-anthesis (DPA) as well as leaves, stems and roots were excised from transformed and control plants. Tissues were stained for GUS as in Jefferson et al. (1987) with modifications. Specifically, tissues were incubated for 60 min in 90 % acetone at 4 °C followed by 20 min at 20 °C. They were then incubated for 20 min at 4 °C in GUS staining buffer consisting of 50 mM sodium phosphate buffer (pH 7), 0.2 % Triton X-100, 2 mM potassium ferricyanide and 2 mM potassium ferrocyanide (Sigma, St. Louis, MO, USA). Specimens were then vacuum infiltrated at 4 °C for 60 min with staining buffer that contained 2 mM X-Gluc (Sigma) and incubated overnight in the dark at 37 °C. The specimens were examined for GUS staining using a dissecting microscope. A set of GUS-stained pistils and kernels from transformed and control plants were dehydrated in an ethanol series, embedded in paraffin (Paraplast, Sigma), sectioned, mounted on slides and deparaffinized (Weigel and Glazebrook, 2002). Mounted specimens were observed using a BX51 microscope (Olympus, Center Valley, PA, USA) equipped with DIC optics and a MicroFire 599809 camera (Olympus).

**RESULTS**

### Functional analyses of P_{SbOCL3}

To characterize P_{SbOCL3} activity, a P_{SbOCL3}::GUS construct (Fig. 1) was engineered into sorghum. Seventy-five putatively transgenic plants were regenerated from a separate immature embryo-derived callus, were selected for transgene verification (Supplementary Data Table S2). Fully mature, harvested seeds from five of the 11 T0 plants tested positive for GUS. In each case, GUS expression was restricted to the integuments of the seed coat (maternal tissue), and its expression was most prominent at the ovule base adjacent to where abscission had occurred (Fig. 2A–D). The transgenic status of these five plants was further verified by Southern hybridization (Supplementary Data Fig. S1). An additional three plants (from the 11 selected) were verified as being transgenic by Southern analyses, but seeds of these plants did not express a GUS phenotype (Supplementary Data Table S2, Fig. S1). The T-DNA in these cases may have integrated into regions of silenced chromatin. The remaining three plants were not transformed, as determined by absence of GUS-stained seeds and negative Southern analysis results (Supplementary Data Table S2, Fig. S1).

Detailed functional analyses were performed using tissues from non-transformed control plants and from the transformed plant ZG 60-1-2a (Supplementary Data Table S2). P_{SbOCL3}::GUS activity was not detected in vegetative or floral tissues of control plants or in vegetative tissues (leaves, stems and roots) of the transformed plant. However, floral organs, including pistils, filaments and lodicules, were GUS positive in the transformant. Staining in this plant was somewhat diffuse before anthesis (Fig. 2E, F) but became more restricted to the base of these floral organs shortly thereafter (Fig. 2G, H). GUS was not detected at the base of lemmas, paleas or glumes. By 10 DPA, GUS staining in kernels was most prominent at the basal (chalazal) end of the ovule. This halo of staining was retained during kernel maturation (Fig. 2I–L).

To characterize the cellular locations of P_{SbOCL3}::GUS activity, GUS-stained kernels of ZG 60-1-2a were embedded in paraffin and sectioned, and the sections were observed by bright-field microscopy. What had appeared as a halo of GUS staining in the chalazal side of ovules (Figs 2J–L and 3B) was restricted to several layers of GUS-expressing cells in the chalaza adjacent to the nucellar epidermis. The latter did not express GUS (Fig. 3A, C–F). Kladnik et al. (2004) observed that the layers of chalazal cells in maize immediately adjacent to the nucellus produce a unique set of phenolics and flavonoids prior to their early death and lysis. They also determined that these layers of chalazal cells, which were GUS stained in the present study, are compressed during the later phases of seed fill, and this results in the formation of the kernel closing or black layer (Fig. 2M) where kernel abscission occurs.

At 15 DPA, chalazal cells adjacent to the nucellar epidermis were broken and compressed, and their components, including GUS protein, had spilled into the apoplasm. A layer of GUS protein, typically a cytoplasmic component (Jefferson et al., 1987), accumulated in the apoplasm against the nucellar epidermis (Fig. 3E). This protein apparently was unable to pass through the nucellar epidermis but instead accumulated against it, possibly as a consequence of selective nutrient flow from the cell lysis-enriched chalazal apoplasm into the nucellar symplasm.

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**Fig. 1.** P_{SbOCL3}::GUS construct used for sorghum transformation. Cloned P_{SbOCL3} was fused to GUSPlusTM. The neomycin phosphotransferase selectable marker NPTII was driven by the CaMV35S promoter. Restriction sites and T-DNA left (LB) and right (RB) borders are shown.
Nucellar epidermis differentiation

The stage at which the nucellar epidermis differentiates from adjoining layers of chalazal cells was studied using DIC microscopy of cleared pistils. Evidence for nucellar epidermis or closing layer formation from layers of chalazal cells was not detected in immature ovules during megasporogenesis or early megagametophyte formation. At these early stages, layers of chalazal cells appeared to be morphologically homogeneous with layers of nucellar cells, i.e. there were no visible signs of divergent developmental differentiation between them (Fig. 4A, B). However, by the mature pre-fertilization megagametophyte stage, the nucellar epidermis had begun to differentiate and was detected as a layer of cells that formed a continuum with the more distinct nucellar epidermis that had previously differentiated laterally, adjacent to the integuments, and distally, at the micropylar end (Fig. 4C). The nucellus at this stage contained thousands of cells, nearly all of which had disintegrated by 15 DPA, except for the nucellar epidermis. By 15 DPA, the nucellar epidermis adjacent to the chalaza was highly differentiated. It consisted of large cells (Fig. 3D, E) that were consistent in gross morphology with the transfer cells that constitute the nucellar epidermis in wheat (Zheng and Wang, 2011), rice (Krishnan and Dayanandan, 2003) and Brachypodium (Opanowicz et al., 2011). In these species, and presumably in sorghum, nucellar transfer cells transport nutrients from the chalaza through the multicellular symplasm of the nucellus and into the endosperm apoplasm.

In silico analyses of promoter regions

Using BLASTn, three regions of homology were found between the 2.0 kb P_{SbOCL3} (GenBank accession no. HM035541) and a larger region from maize chromosome 7 that flanks the P_{ZmOCL3} sequence (GenBank accession no. AEC001002594-1). Percentage identities for these three regions (Supplementary Data Fig. S2), from 5′ to 3′, were 92, 73 and 74 %, respectively. A predicted transcription start site was found in the P_{ZmOCL3} region that was highly homologous with one of five start sites found in the P_{SbOCL3} region. Also noteworthy were (1) a strong homology between sequenced exons of SbOCL3 and their corresponding exons in ZmOCL3 and (2) a poor homology between sorghum and maize for the sequenced intron (Supplementary Data Fig. S2). BLASTn failed to identify regions of homology between P_{SbOCL3} and P_{AhHDG1}. The latter possessed two transcription start sites, but these were not homologous with the start site in P_{ZmOCL3} or with any of the five start sites in P_{SbOCL3} (data not shown).

Cis-regulatory elements found in P_{SbOCL3}, P_{ZmOCL3} and P_{AhHDG1} were arranged into four loosely partitioned categories: stress, seed, phenolics and miscellaneous (Supplementary Data...

Fig. 2. Presence and absence of P_{SbOCL3}::GUS expression in florets and kernels of sorghum. (A–C) GUS-stained mature seeds representative of five independently transformed plants (Supplementary Data Table S1). Staining was restricted to the narrow integument layer of the seed coat (arrows), ZM-60-1-2a (A), ZM-60-2-22b (B), ZM-60-2-25d (C). (D) Absence of GUS staining in the seed coat of a non-transformed control. The tops of (A-D) represent the ventral side of the seeds just below the closing layer. (E, F) Excised floret (E) and pistil (F) of ZM-60-1-2a at the late megagametophyte development stage. (G, H) Excised floret (G) and pistil (H) of ZM-60-1-2a at the early post-fertilization stage. (I) Excised hand-sectioned kernel from a non-transformed plant at 10 day post-anthesis (DPA). (J–L) Excised hand-sectioned kernels from ZM-60-1-2a at 10, 15 and 25 DPA, respectively. (M) Naturally abscised (shattered) mature sorghum kernels showing the ventrally located closing (or black) layer (arrow) and the dorsally located embryo (bottom kernel).
Table S3). Stress- and seed-associated elements were common in promoter regions of all three species. Stress-associated elements were most common in \( P_{SbOCL3} \) and \( P_{ZmOCL3} \), while seed-associated elements were most common in \( P_{AtHDG1} \) (Table 1). Regions of homology shared by \( P_{SbOCL3} \) and \( P_{ZmOCL3} \) (Supplementary Data Fig. S2) contained similar distributions of the four categories of cis-regulatory elements (Table 1), but differences were observed with regard to numbers of specific elements. In this respect, 240 elements were found in the largely homologous regions shared by \( P_{SbOCL3} \) and \( P_{ZmOCL3} \), but only 136 of these (57\%) were shared by both. The linear regression, based on numbers of specific cis-elements in \( P_{SbOCL3} \) vs. \( P_{ZmOCL3} \), was highly significant \( (P < 0.001) \), but the \( r^2 \) was only 0.50. Only regions of \( P_{SbOCL3} \) that were recognized as being highly homologous with \( P_{ZmOCL3} \) were used in this analysis. The percentages of cis-regulatory elements falling within the four general categories (Table 1) were also similar between \( P_{SbOCL3} \) and \( P_{AtHDG1} \). Here, 545 elements were found in the promoters of either \( SbOCL3 \) or \( AtHDG1 \), but only 204 of these (37\%) were shared by both. Again, the linear regression was highly significant, \( P < 0.001 \), but the \( r^2 \) was also low (0.57). There were no regions recognized as highly homologous between \( P_{SbOCL3} \) and \( P_{AtHDG1} \). Hence, this \( r^2 \) was determined based on numbers of specific cis-elements found in the entire \( P_{SbOCL3} \) and \( P_{AtHDG1} \) sequences.

### DISCUSSION

The chalaza--nucellus interface is only vaguely recognizable cytologically during megagametophyte formation (Esau, 1977; Rudall, 1997; Fig. 4A). However, in cereals, by early seed fill, an epidermis forms from the proximal cell layer of the nucellus, which lies adjacent to the distal cell layer of the chalaza. This newly formed epidermis is symplasmically contiguous with the previously differentiated nucellar epidermis that resides inward from the integuments. Additionally, cell layers of the chalaza (proximal to the nucellar epidermis) tend to be contiguous with cell layers of the integuments (Kladnik et al., 2004; Figs 4C and 5A). Interface activities between the chalaza and the nucellus facilitate (1) nutrient transfer during active seed fill and (2) kernel AZ (closing layer) formation at the end of seed fill. The closing layer performs at least three functions: it terminates nutrient flow to mature kernels, it produces a cuticle that protects filial tissues from pathogens and desiccation, and it produces a cleavage plane, parallel to the nucellar epidermis, where kernel abscission occurs (Daynard and Duncan, 1969).

#### The nucellus during kernel development

Megagametophyte growth before anthesis and embryo and endosperm growth after anthesis occur rapidly and require nutrients released from the digestion of adjacent nucellar cells (Figs 4B, C and 5A, B). During early to mid seed fill in cereals, multiple layers of nucellar cells near the chalaza persist, but the patterns of this persistence and the functions that the nucellar cells perform vary extensively among species (Thorne, 1985). In wheat, the vascular furrow and its associated layers of chalazal and nucellar cells run the length of the kernel. Collectively, these tissues are referred to as the nucellar projection, and the nucellar cells in this projection differentiate morphologically and functionally into transfer cells. These absorb nutrients...
proximally and deposit them distally (Offler et al., 2002; Zheng and Wang, 2011). During seed fill in wheat, nutrients and hormones pass through these nucellar transfer cells and into the nutrient- and hormone-rich endosperm cavity (Wang et al., 1995; Carman et al., 1996; Patrick and Offler, 2001; Hess et al., 2002).

The vascular trace in rice is also long, like in wheat, extending the length of the ovary. Early in seed fill, a small nucellar projection persists, along the length of the chalaza, but the cells of this projection disintegrate rapidly. Thereafter, the predominant route of nutrient transfer during seed fill in rice is through the circumferential nucellar epidermis, the cells of which assume the morphology and function of transfer cells (Ellis and Chaffey, 1987; Offler et al., 2002; Krishnan and Dayanandan, 2003).

Though more closely related to wheat, *Brachypodium* also has a poorly developed nucellar projection. Hence, nutrient transport during seed fill in this genus occurs, as in rice, through its circumferential transfer cell-like nucellar epidermis (Opanowicz et al., 2011).

In the present study, we observed a rapid and extensive degeneration of internal nucellar cells during seed fill, similar to that observed in rice and *Brachypodium*, i.e. most internal nucellar cells had died by 15 DPA (Fig. 3D). However, near the chalaza, wall fragments of the dead nucellar cells did not fully disintegrate. Instead, they produced a nucellar lysate, which remained contained within the placental sac (Figs 3A, C and 5C). The formation of a placental sac among tropical cereals is
unique to sorghum (Maness and McBee, 1986). Its proximity immediately above the chalaza suggests an origin and function similar to those of the endosperm cavity in wheat. In both cases, a pocket of fluid forms between the nucellus (or nucellar epidermis) and the endosperm (Thorne, 1985; Kladnik et al., 2006; Wang et al., 2012). The large size of the placental sac in sorghum coupled with its proximal location suggests that nutrient delivery during seed fill occurs primarily through the nucellar epidermis at this location, instead of circumferentially as in rice and Brachypodium.

The terms placental sac and endosperm cavity are misleading. Sac implies a membrane-bound structure, placenta refers to a tissue not in the ovule but in the ovary where ovules originate, and endosperm cavity suggests the presence of a cavity in the endosperm. These inferences are incorrect. During seed formation in gymnosperms, a corrosion cavity forms, which is a nutrient- and hormone-rich fluid-filled cavity produced by rapid influx of nutrients and a correspondingly rapid disintegration of megagametophyte cells adjacent to the developing embryo (Carman et al., 2005). A similar rapid influx of nutrients occurs in kernels of sorghum and temperate cereals, such as wheat; however, the nutrient influx is accompanied by digestion of sporophytic nucellar cells, not megagametophytic cells. Collectively, these processes produce a cavity in sorghum and wheat that is bound on one side by endosperm and on the other side by nucellus or nucellar epidermis. Since the cavity forms where nucellar cells, not endosperm cells, have disintegrated, we suggest using the term nucellar cavity instead of the terms endosperm cavity or placental sac.

In maize, nucellar cavities and nucellar lysate do not form to an appreciable extent. Instead, several layers of dead nucellar cells immediately distal from the cup-shaped chalaza and its associated nucellar epidermis remain intact and form a dome-shaped nucellar projection that is entirely apoplasmic (Fig. 5D; Felker and Shannon, 1980; Kladnik et al., 2004). A similar apoplasmic nucellar projection forms in sorghum, but its existence is highly transitory, i.e. the cell walls disintegrate rapidly to produce the nucellar lysate-containing nucellar cavity (compare Fig. 5C with D). The rate of disintegration varies among genotypes, e.g. Wang et al. (2012) observed a greater longevity of nucellar cells in ‘KS-304’ than we observed in ‘TX-430’.

### Nutrient transport through the chalaza–nucellus interface

The conventional model of nutrient transport in cereals from phloem termini in the pericarp to pre-fertilization megagametophytes or post-fertilization filial tissues stipulates a symplasmic continuum through the chalaza–nucellus interface, a process enabled by plasmodesmata (Patrick and Offler, 2001). Carrier-mediated transport then delivers nutrients from the nucellar symplasm to the megagametophyte or endosperm apoplasms. Our observations are consistent with this model for early phases of gametophyte development, i.e. we observed during these early phases a continuum of tightly packed, morphologically similar layers of chalazal and nucellar cells that spanned the distance from the phloem termini to the apoplastic border adjacent to the developing gametophyte (Fig. 4A, B). However, there is...
evidence in maize (Kladnik et al., 2004; Tang and Boyer, 2013) and sorghum (present study, Fig. 5B) that during late gametophyte formation, a second, more proximal apoplastic barrier forms midway between the phloem termini and the gametophyte, i.e. between the chalaza and the nucellus.

In post-pollination pre-embryo formation kernels, Tang and Boyer (2013) documented a non-selective release of sucrose and carboxyfluorescein diacetate (fed to subtending stems) from phloem parenchyma symplasm to chalazal apoplasm. Efflux of sucrose at this location could be explained by sucrose effluxers (Braun, 2012; Patrick et al., 2013), but explanations for the non-selective release of carboxyfluorescein, which the authors observed in the chalazal apoplasm only, are less apparent. Carboxyfluorescein was not found in other apoplastic regions along the phloem transport pathway. Thus, the authors suggested that non-selective membrane channels, aquaporins or H+ -ATPases may be the vehicle for this non-selective release of cytoplasmic components into the chalaza. They rejected a loss of membrane integrity as the explanation, because healthy cells, which were actively absorbing glucose, were present in this region. However, we observed in sorghum, albeit at a later stage of development, that the corresponding chalaza–nucellus region possesses attributes that implicate loss of membrane integrity, including (1) lysed and compressed chalazal cells with GUS protein in the apoplasm proximal to the nucellar epidermis and (2) symplasmic GUS in live chalazal cells proximal to the dead chalazal cells (Fig. 3E). Thus, programmed loss of membrane integrity in the chalaza may constitute a major vehicle for non-selective release of nutrients from phloem parenchyma to the chalazal apoplasm, at least from mid seed fill onward. The timing of onset of this programmed breakdown requires further investigation.

Kladnik et al. (2004) observed onset of programmed cell death in maize chalaza at 7 DPA, which increased gradually during seed fill. Hence, loss of membrane integrity might begin to occur in chalazal cells of maize as early as anthesis, possibly as one of several initial processes of cell death. Our observation, that the nucellar epidermis in sorghum starts to differentiate prior to anthesis (Fig. 4C) in a manner distinctly different from layers of chalazal cells (Fig. 3E), supports this possibility.

Tang and Boyer (2013) documented that sucrose released to the chalazal apoplasm prior to embryo was rapidly hydrolysed by cell wall invertases. Thereafter, glucose but not carboxyfluorescein was absorbed by the nucellus. This selectivity mirrors our observations. Instead of carboxyfluorescein, GUS was released to the chalazal apoplasm from dying chalazal cells where it had been produced by tissue-specific PSbOCL3::GUS activity in the chalaza. Like carboxyfluorescein (Tang and Boyer, 2013), GUS was not absorbed by the nucellar epidermis. Instead, it accumulated against it (Fig. 3E), probably as solutes moved from the chalazal apoplasm to the symplasm of the nucellar epidermis. The exclusion of GUS and carboxyfluorescein from uptake by the nucellus is evidence that an apoplastic barrier forms in the chalaza, which nutrients must cross before reaching the embryo.

SbOCL3 and floral organ abscission

Abscission of plant organs proceeds through four stages (Liljegren, 2012; Niederhuth et al., 2013). The first is AZ formation where adjacent cell layers bordering the parent plant and the abscission target diverge morphologically and functionally. For kernels of maize and sorghum, this boundary resides between the chalaza and the nucellus. In sorghum, it begins to form before seed fill (Fig. 4C). By mid seed fill, it is strikingly differentiated. The borders of the abscission plane at this stage are composed of dying chalazal cells proximally and a healthy, transfer-cell-like nucellar epidermis distally (Fig. 3E). An interesting finding of the present study is that PSbOCL3::GUS activity in the nutrient transport pathway was confined to the chalaza, where phenolics and flavonoids associated with closing layer formation are synthesized, and where programmed cell death occurs (Kladnik et al., 2004). Reporter gene expression was not observed in the adjacent nucellar epidermis, which at this location and stage had assumed a transfer-cell-like morphology and functionality (Fig. 3E).

Members of the HD-Zip IV family of TFs, including ZmOCL3, regulate epidermal development in seeds (Javelle et al., 2011; Chew et al., 2013). The occurrence of PSbOCL3 activity in the chalaza suggests that SbOCL3 also regulates specialized functions of floral organ abscission. Wu et al. (2011) provided evidence of such functions for the HD-Zip IV TF AtHDG1. This TF was shown to induce expression of two cuticle development genes, BDG and FDH, which is consistent with reported cuticle-like deposits in kernel closing layers of maize (Daynard and Duncan, 1969). In arabidopsis, BDG and FDH were downregulated when AtHDG1 synthesis was suppressed or when its function was suppressed by interactions with CURLY FLAG LEAF1 (CFL1). In both cases, cuticle development in the epidermis of leaves was impaired. Consistent with these observations, CFL1 was expressed in epidermal leaf cells, but it was also expressed in the AZ and dehiscence zone of arabidopsis siliques. The authors did not report whether silique abscission or dehiscence was impaired in AtHDG1-deficient plants or whether AtHDG1 was expressed in these tissues. In this respect, our finding that PSbOCL3 directs gene expression specifically to kernel AZ and to the AZ of stamens and lodicules (Figs 2, 3) is additional evidence of HD-Zip IV TF involvement in the regulation of floral organ abscission.

Conclusions

Nutrient delivery systems supporting development of megagametophytes in angiosperms and other spermatophytes are evolutionary innovations not seen elsewhere in Plantae, and tropical cereals provide excellent models for their investigation. We show that the sorghum chalaza, sensu Esau (1977), changes radically during seed fill. Early in seed fill, these changes include programmed cell death, which produces the most proximal of three major apoplastic barriers through which nutrients must pass before reaching the embryo (Fig. 5). During seed fill, additional changes in the chalaza occur, including the synthesis of phenolics associated with closing layer formation. The adjacent nucellus also undergoes radical changes, which are entirely different from those occurring in the chalaza. These include modification of its epidermis proximally to a transfer cell morphology, which persists until late seed fill, and rapid death and disintegration of internal nucellar cells adjacent to the expanding filial tissues. The release of GUS, from chalazal symplasm to chalazal apoplasm, coupled with the exclusion of GUS from the adjacent symplasm of the transfer-cell-like nucellar epidermis is strong evidence for the presence of an apoplastic barrier at the chalaza–nucellus interface.
The TF SbOCL3 may regulate flavonoid and possibly cuticle biosynthesis in the AZ of floral organs. In this respect, \( P_{SbOCL3}^{::GUS} \) was not expressed in the AZ of subtending leaf-like structures, i.e., lemmas, paleas and glumes, or in other vegetative portions of the plant. This uniqueness of expression in the floral AZ may be a consequence of specific combinations of cis-elements (Supplementary Data Table S3) as has been proposed for the expression of the HD-Zip IV TF AtML1 (Takada and Jürgens, 2007; Chew et al., 2013). Collectively, the evidence from Arabidopsis (Wu et al., 2011) and sorghum suggests that the functioning of HD-Zip IV TFs in the AZ of floral organs may be conserved among angiosperms and may represent a divergence from AZ physiology as observed in leaves and other plant organs (Roberts et al., 2002; Liljegren, 2012). This divergence, from a normal AZ formation process, may be responsible for the evolution of the chalaza–nucellus apoplastic barrier, which is important to seed development in cereals. These are possibilities that require further investigation.

To our knowledge, \( P_{SbOCL3} \) is the first promoter identified that is specifically expressed in the AZ of floral organs. As closing layers are responsible for stopping kernel growth, this promoter may be useful for engineering grain with increased developmental longevity. Cells specifically expressing this promoter die during seed fill and contribute their contents to the chalazal apo-plast. Thus, \( P_{SbOCL3} \) might be useful in engineering cereals with transgenics that improve nutrient composition prior to uptake by the endosperm, or which might confer other kernel qualities (Patrick et al., 2013). It might also be useful for engineering biotic and abiotic stress characteristics of the closing layer, thus improving seed durability.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Table S1: primers used in promoter cloning and PCR analysis. Table S2: a list of 75 plants regenerated from 31 putative transformation (\( P_{SbOCL3}^{::GUS} \)) events. Table S3: summary of cis-regulatory elements in \( P_{SbOCL3} \), \( P_{SbOCL3} \), regions that show general homology with \( P_{ZmOCL3} \) (\( P_{ZmOCL3} \) partial), \( P_{ZmOCL3} \) regions that show general homology with \( P_{SbOCL3} \) (\( P_{ZmOCL3} \) partial), and \( P_{AtHDG1} \). Figure S1: Southern hybridization results for bulked leaf samples taken from 15 T1 seedlings per putative T0 plant and from a non-transformed TX430 sorghum control. Figure S2: \( P_{SbOCL3} \) nucleotide sequence showing regions of homology with \( P_{ZmOCL3} \).

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LITERATURE CITED


Delleda PTA, Wood J, Hick J. 1983. A plant DNA minipreparation: version partial), and \( P_{AtHDG1} \). Figure S1: Southern hybridization results for bulked leaf samples taken from 15 T1 seedlings per putative T0 plant and from a non-transformed TX430 sorghum control. Figure S2: \( P_{SbOCL3} \) nucleotide sequence showing regions of homology with \( P_{ZmOCL3} \).


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