Morphology and Characterization of Abscission Zone Development and its Role in Domestication in Setaria viridis and Setaria italica

John Gerard Hodge

University of Missouri-St. Louis, jgeradhodge@gmail.com

Follow this and additional works at: https://irl.umsl.edu/thesis

Recommended Citation
https://irl.umsl.edu/thesis/37

This Thesis is brought to you for free and open access by the UMSL Graduate Works at IRL @ UMSL. It has been accepted for inclusion in Theses by an authorized administrator of IRL @ UMSL. For more information, please contact marvinh@umsl.edu.
Morphology and Characterization of Abscission Zone Development and its Role in Domestication in *Setaria viridis* and *Setaria italica*

John G. Hodge  
M.S., Biology, University of Missouri - St. Louis, 2015  
B.S., Biology, University of Missouri - St. Louis, 2012

A Thesis Submitted to the Graduate School at the University of Missouri - St. Louis in partial fulfillment of the requirements for the degree  
Master of Science in Biology

August 2015

Advisory Committee

Elizabeth A. Kellogg, Ph.D.  
Chairperson

Peter F. Stevens, Ph.D.

Bethany K. Zolman, Ph.D.
ABSTRACT

Many critical agronomic traits have been selected over the course of domestication of various crops and of these, one of the most crucial for cereal crops (Poaceae) is increased seed retention or “reduced shattering”, although the underlying mechanisms remain poorly understood. *Setaria viridis* was selected to identify the underlying patterns related to abscission zone development within the previously uncharacterized tribe Paniceae (Panicoideae). For this study one line of the wild species *S. viridis* and two lines of *S. italica*, a derived domesticate of *S. viridis*, were used. Consistent differences were found in the phenotypic patterns and morphology of *S. viridis* abscission layers compared to either *S. italica* accession. In addition, the abscission zone itself forms completely only in *S. viridis* whereas the analogous region in *S. italica* is poorly formed or absent. Among the currently known shattering genes, *qSH1* and *SH1* were expressed in both *S. viridis* and *S. italica* during flowering. It seems likely that the mechanism for abscission in *Setaria* is morphologically distinct from the shattering mechanisms seen in other grasses although they may all utilize similar underlying pathways.
INTRODUCTION

Crop domestication was critical in early human history, reducing the likelihood of food scarcity and allowing for the development of society. Of the domesticated crops, cereals remain the most important to humans because they can rapidly convert solar energy into calories. For their successful domestication various desirable traits have been selected to increase harvest efficiency and yield per acre: the more notable being to reduce tillering, to enhance dwarfing, and to increase grain size or number (Glémin & Bataillon 2009). Loss of seed shattering is also among these traits. Shattering is the release of diaspires (seeds including immediately subtending organs) through the activation of specialized layer(s) of cells known as an abscission zone that separate from their neighbors, creating a weak point. Selection of non-shattering lines is often one of the earliest stages of cereal domestication and is critical for harvesting grains effectively (Glémin & Bataillon 2009).

Studies based within eudicots have indicated abscission zones develop in a stereotypical manner over the course of organ development and senescence (Addicott 1982). This same view of a stereotypical mechanism has also been held for grasses and their shattering mechanism. However, a variety of forms of abscission zone insertion or shattering has been described in Poaceae ranging from along inflorescence branches (Zea and Triticum), beneath spikelets (Sorghum and Setaria), within the spikelet axis (Oryza and Brachypodium), or immediately subtending the fertile ovary (Sporobolus) (Doust et al. 2014). In part this is due to
the complexity both of grass inflorescence architecture as well as the terminal unit of the grass inflorescence, the spikelet. The spikelet is subtended by two bracts known as glumes, above which additional bracts known as lemmas are borne on the central axis, the rachilla. In the axils of the lemmas floret meristems are borne (Fig. 1A). The architecture of the spikelet itself can vary considerably across the family (Doust et al. 2014). Early work characterizing abscission zone development heavily emphasized eudicot lineages noting various cytological changes (Addicott 1982). However, the degree to which this pioneering work applies to grasses, or monocots in general for that matter, remains uncertain. Certain general patterns seem to be shared between eudicots, the patterns of lignification around the abscission zone boundaries being among them although the exact pattern of lignification varies by lineage (Addicott 1982). This pattern is consistent within the grasses, with several studies in rice utilizing either phloroglucinol-HCl or acridine orange revealing the abscission zone consists of an un lignified boundary of cells bordered on either side by multiple lignified cell layers (Li et al. 2006, Zhou et al. 2012, Yoon et al. 2014). The relationship between the senescence of organs and their abscission also seems to be shared between Poaceae and eudicots although the mechanism driving this process within Poaceae is still poorly understood (Addicott 1982).

The genes and physiological processes involved in the grass abscission zone are perhaps best understood in rice (*Oryza sativa*). Within *Oryza*, a specialized layer of cells forms immediately below the point of attachment of the lowermost florets to the rachilla just prior to anthesis (Fig. 1; Li et al. 2006, Konishi et al.
This distinctive layer is generally one cell thick and extends from the epidermis to the vascular bundle in the rachilla (Li et al. 2006, Konishi et al. 2007). The abscission zone does not fully develop in domesticated *Oryza* lines, with parenchymatous cells often intercalated through the abscission zone, causing difficulty in diaspose release (Li et al. 2006).

This loss of abscission zone integrity also manifests itself as diminishing tensile strength through development (Li et al. 2006, Zhou et al. 2012). The reduction in tensile strength over time agrees well with anatomical data. In wild, free-shattering rice lines, the tensile strength of the rachilla is around 150 grams of force as the inflorescence emerges from the sheath (heading), but after abscission zone activation at 10-12 days from heading tensile strength precipitously drops to a free-shattering state of zero grams of force (Zhou et al. 2012). However, in domesticated lines with poorly developed abscission zones, tensile strength diminishes over time but rarely reaches a free-shattering state (Konishi et al. 2007, Zhou et al. 2012).

Various genes are active as the abscission layer in rice develops. Among these is the bHLH domain BEL1-like homeobox transcription factor *qSH1*, which was first identified by QTL mapping near-isogenic lines of *O. indica-sativa* hybrids as a locus which explained the highest percentage of variance associated with the shattering phenotype (Konishi et al. 2006). Expression of *qSH1* occurs in two distinct phases within *Oryza* inflorescences. Initial *qSH1* expression is ubiquitous across the tunica of young inflorescence meristems before spikelet meristem
differentiation while in the second phase \textit{qSH1} is expressed in the anthers and abscission zones during floret development (Konishi et al. 2006, Zhou et al. 2012). In addition, it has also been suggested from functional studies that \textit{qSH1} acts in a pathway with \textit{SHATTERING ABORTION1} and another prominent shattering locus, \textit{SH4} (Zhou et al. 2012). Recently \textit{SH5}, a paralogue of \textit{qSH1} that was likely produced during the Poaceae whole genome duplication event, was described as also being expressed in the abscission zone where it appears to suppress lignin biosynthesis (Yoon et al. 2014). \textit{SH5} was shown to have similar patterns of expression within the developing spikelet to \textit{qSH1} and was also associated with shattering based on transgenic studies utilizing both RNAi and overexpression. This creates a single layer of un lignified cells that are bordered on either side by lignified layers (Yoon et al. 2014).

Most research on seed shattering in grasses has been focused on rice within the subfamily Ehrhartoideae. The subfamily Panicoideae contains various grain crops, notably maize (\textit{Zea mays ssp. mays}) and sorghum (\textit{Sorghum bicolor}), as well as foxtail millet (\textit{Setaria italica}) and pearl millet (\textit{Pennisetum glaucum}). The abscission zone of \textit{Oryza} forms beneath the lemma of the lowermost floret so that the diaspose contains only the spikelet floret axes and their immediately subtending lemma and palea (Fig. 1). Within \textit{Setaria}, however, the abscission zone forms immediately beneath the insertion of the lower glume so that the entire spikelet is released as a diaspose. The differences of abscission zone insertion between \textit{Oryza} and \textit{Setaria} leaves some room to question how much overlap there is between their
respective abscission pathways (Fig. 1A, B). QTL studies in various domesticated grass lineages have identified several genes that have undergone human mediated selection against shattering. Presently, the only locus that was shown to have undergone convergent selection between separate cereal lineages is the YABBY2 gene SH1 (Poncet et al. 2002, Lin et al. 2012). SpWRKY was also identified in Sorghum, but whether it is functional elsewhere remains uncertain (Lin et al. 2012, Tang et al. 2013).

Several features are notable within the domesticate S. italica that have been enhanced in comparison to those of S. viridis. Central to its use as a grain crop is its increased inflorescence branch size and the heightened branching of its axillary meristems. Ultimately, S. italica itself is not a conducive system for study given, like the inflorescence, the plant as a whole is correspondingly larger (being comparable to juvenile Z. mays) and the generation time has been greatly increased. The C₄ annual, green millet (Setaria viridis) is in the lineage from which the domesticate S. italica (both of which will hereafter be referred to as the Setaria species complex) is derived and has quickly developed into a model system for various aspects of growth and development in Panicoideae. The small size of S. viridis, short generation time and diploid genome have made it an ideal system for the study of genetics and development (Brutnell et al. 2010). An initial S. viridis x S. italica cross identified linkage groups to help elucidate the underlying genetics of the Setaria species complex (Wang et al. 1998). Recombinant inbred lines generated from these crosses have since been used in genetic studies to identify loci
involved in agronomically important traits such as inflorescence architecture and
tillering (Doust et al. 2004, Doust et al. 2005). Comparisons with previous mapping
studies have indicated that the genomic positions of SH1 orthologues are syntenic
and they localize within known shattering QTLs across multiple systems including
Sorghum, rice, and Setaria (Lin et al. 2012, Doust et al. 2014). However, a basic
description of abscission zone formation is lacking for S. viridis, and it is unclear
how the development of this region differs in the non-shattering domesticated
relative S. italica. Accordingly, this study uses developmental and molecular
approaches to characterize abscission zone formation and activity in wild type and
domesticated lines.

MATERIALS AND METHODS

Plant Material for Study - Three genetic backgrounds were used in this study,
one free shattering S. viridis (A10) and two non-shattering S. italica (B100 and
Yugu). The line A10 is a Canadian collection of S. viridis and has been widely
adopted as a model line within the Setaria community (Brutnell et al. 2010). The S.
italica lines B100 and Yugu are domesticated lines. Yugu has been a focal point
for research and development in S. italica and currently has an annotated genome
available from the Department of Energy’s Joint Genome Institute (Bennetzen et al.
2012, Brutnell & Mockler, unpublished). The S. italica line B100 is one parent of
the cross between S. viridis and S. italica whose offspring have been used for
various mapping and QTL studies (Wang 1998, Doust et al. 2004, Doust et al.
2005).
**Tensile Strength Phenotyping** - The tensile strength of the pedicel-spikelet junction was measured using a Mark-10 Model M3-2 force gauge. An inflorescence was cut off the plant and hung from the force gauge; spikelets were then pulled off one at a time and the tensile strength in grams of force (gF) recorded (Supp. Fig. 1). Caryopsis maturation and senescence occur within roughly 20 days after inflorescence emergence in *Setaria* suggesting this period of time would be sufficient to characterize shattering. Measurements were taken beginning when the apex of the panicle emerged from the sheathing leaves (day 0), and continuing at 2-day intervals, for a total of 10 time points in 20 days. In addition, samples were also measured at senescence to provide a phenotypic end point. For each time point, 5 different panicles were harvested and 20 tensile strength measurements were recorded from each for a total of 100 measurements.

**Sectioning and Microscopy** - Spikelet branches and subtending pedicels were harvested from one inflorescence at each time point surveyed in the force gauge study. For anatomical study samples were fixed in FAA (formalin 37%/acetic acid 100%/ ethanol 55% = 5:10:85) solution for 12-24 hours. After fixation samples were run through an ethanol series (50%/70%/85%/95%/100%/100%/100%) followed by a Histoclear series (25%/50%/75%/100%/100%/100%). After samples were in pure Histoclear half of the volume was replaced with paraplast chips and samples were stored in a 55-60°C oven. Samples were then taken through a paraplast series by replacing half the volume of Histoclear/Paraplast with molten paraplast 2 or 3 times a day for 3 days. After being fully infiltrated samples were
then embedded in embedding rings and stored at 4°C. Serial sections were made utilizing a Microm HM 355S microtome at a 10 micron depth. Older stages with particularly hard caryopses and well-developed endosperm were first frozen at -70°C for a minimum of 10-15 minutes to harden the paraffin prior to sectioning. Ribbons were then floated in a 50°C water bath and mounted on Fisher Probe-On Plus slides. Slides were dried on slide warmers for a minimum of 48 hours. Sections were then stained with acridine orange (AO), 4′6-diamidino-2-phenylindole (DAPI), phloroglucinol-HCl, or toluidine blue.

AO stain (acridine orange/dH₂O 100%/acetic acid 100% = 0.1:99.899:0.001) was used following the protocol: dH₂O (2min), AO (30 min), 0.5% Glacial Acetic acid/95.5% EtOH (2 min), 100% EtOH (2 min), Histoclear (2 min). Fluorescent microscopy was then employed using a Leica SP-8 confocal microscope. DAPI staining was also employed following the protocol: Histoclear (4 min), 100% EtOH (2 min), 95% EtOH (2 min), 70% EtOH (2 min), 30% EtOH (2 min), dH₂O (2 min), 1xPBS (2 min), DAPI incubation (15 min), 1x PBS (1 min). Images for both AO and DAPI staining were taken using a Leica SP-8 confocal fluorescent microscope. White light at a 500nm wavelength at 1-2% intensity was used for AO excitation with the emissions wavelengths in either the range of 515-535nm (false-colored green) or 640nm-660nm (false-colored red) being recorded. UV light at 1-2% was used for DAPI excitation intensity with emission wavelengths in the range of 431-486nm being recorded. For both AO and DAPI samples, images were recorded at 200-400Hz using line averaging of 4-8, and when
optical sectioning methods were used the optimal Z-stack slice intervals were calculated by the Leica Application Suite.

Toluidine blue staining followed the protocol: dH₂O (2min), Toluidine blue (8 min), dH₂O (2min), dH₂O (2min), dH₂O (2min), 95% EtOH (2 min), 100% EtOH (2min), 100% Histoclear (4 min). Phloroglucinol-HCl staining was also conducted to detect lignin deposition following the protocol: 100% Histoclear (4 min), 100% EtOH (2 min) 95% EtOH (2 min), 70% EtOH (2 min) 50% EtOH (2 min), dH₂O (2 min), 1% phloroglucinol/20% HCl. Slides stained with either toluidine blue or phloroglucinol-HCl were imaged under brightfield microscopy using either an Olympus BX40 microscope mounted with a Canon Powershot A640 camera or a Leica DM 750 microscope mounted with a Leica ICC50 HD camera using the Leica Application Suite.

For SEM imaging a Hitachi TM1000 unit was used on materials of each of the three lines. Senescent materials were gathered and diasporas were removed in a manner similar to the technique used for force gauge measurements. The pedicels were then mounted on carbon tape and photographed.

Image processing of both confocal fluorescent and bright field micrographs was completed in FIJI (Schindelin et al. 2012). For confocal images the recorded red and green color channels were merged into a single channel and for cases where optical sectioning was used samples were subsequently Z projected into a single plane. Scale bars were created based on pixel/micron conversions stored within the confocal metafile. For bright field micrographs scale bars were generated using a
calibration slide imaged at the same magnification as their corresponding sections. Brightness and contrast of micrographs were adjusted as needed.

**Phylogenetic Analysis and Ortholog Identification**- Assembled and annotated genomes are currently available for the *S. italica* line Yugu (Bennetzen et al. 2012) and the *S. viridis* line A10 (Brutnell & Mockler, unpublished). Candidate genes from *Oryza sativa* and *Sorghum bicolor* were identified on Phytozone version 9 ([http://www.phytozone.net/](http://www.phytozone.net/)) and the coding sequences of homologs were aligned based on their amino acid translations using MUSCLE version 3.8.31 (Edgar 2004). The orthologue of the *O. sativa* locus SH4 was not annotated in the *Setaria* genomic databases, so BLAST searches were performed on genomic scaffolds and the CDS regions were extracted from genomic sequences after being aligned to the existing CDS alignment matrices. In cases where MUSCLE alignments were disrupted by incorporation of more disparate sequences an iterative approach was used in which alignments were adjusted in a text editor or Geneious 6.1.6 (Kearse et al. 2012) and rerun through MUSCLE. Alignments were then used for phylogenetic tree estimation using RAxML version 8.0.22 (Stamatakis 2014) set to a GTR + gamma model and run for 500 bootstrap replications. From these gene trees sequences orthologs to the candidate genes were identified and used in developing primer sets for gene expression studies.

**Gene Expression Study**- Inflorescences collected from days 0 and 10 (see above) were stored at -70C before extraction. Tissue was immersed in liquid nitrogen and ground in a mortar and pestle and total RNA was extracted using a
Qiagen RNeasy Plant Mini-kit using the manufacturer’s protocol. Extracts were then diluted to a standardized concentration and treated with RNase-free Roche recombinant DNase I. Targeted cDNA synthesis of mRNA templates used an anchored poly-T primer [Table 1] and the Life Technologies® Superscript® II Reverse Transcriptase Kit.

Forward primers were designed utilizing NCBI’s Primer-Blast database (Ye et al. 2012) and targeted the 3’ ends of cDNA sequences. Of the potential primers obtained, those with the highest specificity for the intended target based on the Refseq mRNA database and closest to the 3’ end of the template were selected. For genes with unannotated 3’UTR initial screens for gene expression were performed by PCR using the Promega GoTaq® DNA Polymerase Kit with gene specific forward primers and an anchored poly-T reverse primer. PCR products were then size selected via electrophoresis run at 40-60V for 1-2 hours. Products of the expected size were excised and purified via the QIAquick Gel Extraction Kit and were then ligated into a pGEM® -5Zf(+) vector utilizing the Promega pGEM®-T Easy Vector System. Vectors were then transformed into Promega JM109 *E. coli* utilizing a heat shock method: 4°C 20 min, 42°C 45 sec, 4°C 3 min. Transformed *E.coli* were subsequently grown in SOC media for 1-2 hours at 37°C with shaking and were plated on LB plates (with X-Gal and Ampicillin) which were then placed in a 37°C incubator to grow overnight. Based on the number of expected templates that could be targeted by the gene specific forward primers 2 to 8 clones were Sanger sequenced. From these sequencing products reverse gene-specific primers
were developed to target the 3’UTR region of each gene to enable targeted amplification of specific genes. An anchored poly-T primer was used for cDNA synthesis and gene specific primers were utilized to test for expression patterns of each gene.

**RESULTS**

*Tensile Strength Phenotyping* - Force-gauge measurements indicate that spikelets of all three lines are retained at roughly the same tensile strength at emergence from the sheathing leaves (day 0) (Fig. 2). Over subsequent sample times *S. viridis* maintains an average tensile strength around 30 grams of force (gF) whereas the two *S. italic* lines B100 and Yugu steadily increase in tensile strength, reaching ca. 50-60gF by 8 days, the peak of tensile strength in *Setaria* developmental time. This increase in tensile strength corresponds to the elongation of spikelets borne on the higher order branches of *S. italic* inflorescences which are often underdeveloped at emergence. In contrast, the spikelets of *S. viridis* are almost entirely at a mature size by day 0 and never display this climb in tensile strength (Fig. 2). Tensile strength is far less variable in the *S. viridis* accession than in either *S. italic* line. Broad variance becomes especially notable in the later stages of *S. italic* B100 development and especially at senescence where spikelets within an inflorescence varied from being highly resistant to removal (>100gF) to nearly free-shattering (~10gF). By comparison, the tensile strength of *S. italic* Yugu was more consistent than B100 but more variable than *S. viridis* A10 (Fig. 2).

*Anatomy* - Anatomical sections across developmental time assessed
development of the abscission zone relative to stages of floral development (Figs. 3, 4). At days 0 and 2 post-emergence, the abscission zone is first discernable in younger spikelets as a lip-like structure immediately subtending the outer glume along the surface of the epidermis (Figs. 3D-F; black arrows). In all three lines this expansion of the pedicel apex into a cup-like structure coincides with the initiation of the androecial primordia in the upper floret of the spikelet (Figs. 3A-C). At this stage of development the epidermal cells of the pedicel that immediately surround the abscission zone develop dense walls, especially on the external surface, compared to their immediate neighbors and display a golden or bronze hue when viewed unstained under magnification. Due to their distinctive morphology, these cells will hereafter be referred to as gilded. The gilded cells appear only after the archesporial cells have differentiated from the thecae in *S. viridis* (Fig. 4D) whereas the gilded cells are already prominent when the androecial primordia are differentiating in B100 and Yugu (Figs. 3E, F).

The different lines develop at different rates. Therefore, all spikelets were staged according to both absolute time (days from emergence) and relative time (developmental landmarks in fruit and flower). Because *S. italica* develops many more orders of branching than *S. viridis*, at any given time point spikelets may be at a broader range of developmental stages, so we used development of the gynoecium and embryo for staging relative age of the spikelet as a whole. In *S. viridis*, globular stage embryos can be found at day 16 (Fig. 5A) whereas in *S. italica* such embryos can be found at day 16 (Fig. 5B), or as early as day 8 (Fig. 5C). At days 8
(S. *viridis*, Fig. 6A), 20 (*S. italic*ca B100, Fig. 6B), or 12 (*S. italic*ca Yugu, Fig. 6C),
the embryo has a clearly developed scutellum, with an obvious cleft between the
base of the scutellum and coleorhiza. Fully developed embryos were also found in
*S. viridis* by day 16 (Fig. 7A), and in *S. italic*ca by day 20 (Figs. 7B, C). Preliminary
measurements indicate that the embryos of both *S. italic*ca lines were roughly double
the size of those seen *S. viridis* at maturity although the numbers of embryonic
tissue layers were comparable between them (Fig. 7). On closer inspection, this
increase in size corresponded well with the increased volume of the *S. italic*ca
embryonic cells which are notably larger than those of *S. viridis*, particularly in
B100. In addition, more rounds of cell division appear to occur in both the
hypocotyl and root meristem with the effect being most prominent in B100. This
proportionate increase in size can also been seen in the gynoecium which is also
notably larger in the domesticates.

Despite the differences demonstrated in tensile strength of the pedicels and
abscission zones, pedicel anatomy differs very little within each line even late into
seed maturation (Figs. 5-7). At senescence, disarticulation in *S. viridis* occurred
along a plane between the abscission zone and the gilded cells with often only the
lower two transverse layers of the abscission zone remaining attached to pedicel
apex (Fig. 8).

When stained with DAPI (Fig. 9), *S. viridis* spikelets late in maturity had
abscission zones that were composed entirely of cells with stainable nuclei that,
combined with other data, suggest these cells are maintained throughout the
abscission process (Figs. 5-9). Conversely, S. _italica_ lines had fewer DAPI 
stainable cell nuclei visible (Fig. 9). Within S. _italica_ B100 the nuclei had a fainter 
DAPI signal when compared to S. _viridis_ (Fig. 9). Within Yugu the pattern seemed 
even further perturbed with only a single layer of cells being vaguely discernable all 
of which appeared to have malformed nuclei (Fig. 9).

Combined, the Toluidine Blue and DAPI micrographs suggest that S. _viridis_
has layers of cells at the boundary between the pedicel apex and glume insertion 
that are more obvious than in S. _italica_. In both lines of S. _italica_ these cell layers 
fail to develop fully. The boundary of these cell layers closely corresponds to the 
abaxial side of the glume vascular supply in the pedicel.

Toluidine Blue staining suggested that lignification might be occurring in both 
the gilded cells around the abscission cup and the cells immediately above the 
abscission layer in the later developmental stages of S. _viridis_ (Figs. 5-7). Lignin 
deposition was thus assayed with phloroglucinol-HCl on day 20 samples from all 
three lines (Fig. 10). Phloroglucinol did not stain either the gilded cells or the cells 
immediately above the abscission zone indicating lignin biosynthesis is unlikely at 
these later stages (Fig. 10). The upper lemma and palea of the spikelet as well as 
their corresponding points of insertion do stain strongly with phloroglucinol 
suggesting these tissues and their nodes along the rachilla are highly lignified and 
verifying that the staining system was working as expected (Fig. 10A).

To investigate if a more sensitive staining system would show differences 
between S. _viridis_ and S. _italica_, spikelets were sectioned at day 12, the time at
which tensile strength begins to diverge, and stained with an alternative lignin and nuclei acid stain, acridine orange (Figs. 11A-C). The fluorescence micrographs show a distinctive transverse band of red-orange (~650nm) cells immediately below of insertion position of the glumes in *S. viridis*, which passes from the epidermis to the vasculature (Fig. 11A). This region is not seen in either *S. italica* line instead being intercalated with the yellow-green (~525nm) characteristic of lignified cells (Figs. 11B, C). Also characteristic of AOs staining pattern, many of the cells within the red-orange region of *S. viridis* also have visible nuclei, as would be expected (Fig. 11A).

The surface of the abscission layers of each line after diaspore release clearly differs (Figs. 12A-C). The surface of the abscission cup in *S. viridis* (Fig. 12A) was smooth, most likely being the lowermost transverse layer of cells that make contact with the gilded cells (Fig. 8). In contrast, the abscission zone cells appeared torn in both *S. italica* lines. In B100, the cells that immediately surround the central vasculature were ripped from the pedicel and the abscission cup itself completely lost integrity being partially removed with the diaspore in Yugu (Figs. 12B-C). Parenchymatous cells protruded from the torn surface in Yugu suggesting the point of release within the pedicel was well below the plane of the abscission layer (Fig. 12C).

*Gene Expression-* Genes for expression study were first identified by homology utilizing maximum likelihood based tree estimation of cDNA alignments (Fig. 13). From these trees the rice *qSH1* ortholog and BEL1-like homeobox gene
family member, Si000753, and the *Sorghum SH1* ortholog and YABBY2 gene family member, Si037789 were identified for study. Comparing sequence annotations for both of these independent gene families suggests that domains for both of these genes are functionally intact when compared to their orthologs in other species with direct comparisons between A10 and Yugu sequences showing only minor allelic variation within their introns. From total RNA evidence both genes are expressed in *S. viridis* and *S. italic* inflorescences although the manner of expression appears to differ (Fig. 14). Expression of the *Setaria* BEL1-like gene coincided well with the expression patterns characterized in rice being strongly expressed during the period of AZ differentiation and becoming much weaker by day 10 (Fig. 14A). This pattern was clearly visible in the expression studies of *S. viridis* which show expression at day 0 (where the younger spikelets along the tertiary, quaternary, and subsequent axes are still developing) which has reduced sharply by day 10 (when all functional spikelets are fully formed). Interestingly, the expression patterns between *S. viridis* and *S. italic* appeared to be fairly consistent in terms of relative intensity between days 0 and 10 within each background. Presently there is only coarse expression data currently existing in the literature to describe the patterns of the YABBY2 gene in *Sorghum* although it is known to be expressed in young inflorescence tissues (Fig 14B). Based on the expression patterns from *Setaria*, the YABBY2 gene appears to show similar patterns of high expression at day 0 and reduced expression at day 10. Unlike the BEL1-like gene where there was consistent expression between replicates the
YABBY2 expression is less consistent between replicates in B100 and Yugu (Fig. 14B).

**DISCUSSION**

*Shattering in the Cereals*- Selection against shattering has been critical for the domestication of all cereal crops. Despite its necessity for modern agriculture the manner in which shattering occurs and its relationship to abscission is still poorly understood in all but rice. QTL and fine mapping studies have implicated various loci in different cereals, however, there is often very little overlap between the “shattering domestication” genes that have been mapped in different lineages (Li et al. 2006, Konishi et al. 2006, Lin et al. 2012, Tang et al. 2013, Doust et al. 2014). This result is unsurprising given the diversity of shattering mechanisms within Poaceae (Doust et al. 2014). The position of the abscission zone often differs between the independent cereal species the domesticates were derived from suggesting that many of them have independently arisen within their respective clades (Doust et al. 2014). In particular, the position of the abscission zone in *Setaria* is below the glumes, whereas in *Oryza* it is immediately above the glumes (Doust et al. 2014).

*Differences in Abscission Zone Patterning in Oryza and Setaria*- The data presented here show that the development of the abscission zone and its loss of function in domestication are not directly comparable to the patterns found in rice. The climb in the abscission zone’s tensile strength in both *S. italica* lines to twice that of the free shattering *S. viridis* is unlike anything previously described in *Oryza*
which instead operates through diminishing the rate at which the tensile strength
decreases in its abscission zone (Konishi et al. 2006, Zhou et al. 2012).

Anatomical patterns are somewhat similar to *Oryza*, the abscission zones of
*Setaria* appear as a distinctive transverse layer of cells which can be identified by
AO staining in free-shattering backgrounds suggested that there is differential lignin
deposition occurring between the abscission zone and the bordering cells on either
side of it (Briggs & Morris 2008, Zhou et al. 2012). This pattern of lignification is
not as consistent in *Setaria* given these same cells which are stainable with AO
often cannot be marked with phloroglucinol-HCl as they could in rice (Briggs &
Morris 2008, Ruzin 1999). This may suggest that either the lignin groups in the
secondary cell wall are less abundant and hinders their ability to be detected with
phloroglucinol or that there is a different process occurring within these cell walls
over developmental time compared to *Oryza*. The position and timing of abscission
zone formation also differs in *Oryza* and *Setaria*. *Setaria* abscission zone
development is first recognizable as expansion of the pedicel apex immediately
beneath the insertion of the outer glume. This expansion in *Setaria* roughly
coincides with the initiation of the androecial primordia. Abscission zone
differentiation often occurs at a later stage in *Oryza*, corresponding instead to the
differentiation of the androecial tapetum and archesporial cells (Konishi et al. 2006,

This lack of congruity between the two species may also hint at other factors
as well. In rice, the regulatory genes involved in shattering such as *qSH1*, *SHAT1*,

and SH4 are expressed in both the anthers and the abscission zone often simultaneously and have been suggested to interact with each other in a shared pathway (Konishi et al. 2006, Zhou et al. 2012). It remains unclear if and how the genes of this rice pathway interact with the other shattering regulators that have been identified in other species such as SH1 from Sorghum. Orthologs of qSH1 and SH1 found within Setaria are both expressed and appear to have overlapping expression during the development of Setaria based on coarse surveys. More broadly, the patterns found within Setaria appear to correspond well with patterns observed for both genes in Oryza and Sorghum in which either the BEL1-like or YABBY2 gene respectively are expressed early in development during the differentiation of the abscission zone and cease to be expressed after the regions identity has been established (Konishi et al. 2006, Lin et al. 2012, Zhou et al. 2012).

**The Non-shattering Phenotype in Domesticated Setaria** - Various traits in S. italica have most likely been selected for to lead to a loss of shattering. Among these is the increase in tensile strength over the first week after heading in S. italica which was entirely absent in S. viridis. While S. viridis inflorescences at emergence predominantly bear mature spikelets which are nearing anthesis their counterparts in S. italica are often notably younger. This is due largely to the additional orders of branching in S. italica which occurs while the inflorescence is still in the sheath so that the inflorescences consist primarily of underdeveloped spikelets at emergence. When this disparity is taken into account and spikelets are compared between S. viridis and S. italica at similar anatomical stages S. italica spikelets and
their pedicels are often considerably larger and consequently have a higher tensile strength. Because of this, the elongation of these immature spikelets can be observed over the first week post-heading as a sharp climb in tensile strength. The sum of these allometrically proportionate differences in *S. italica* and *S. viridis* suggest that heterochrony may be the driving mechanism which was selected for during the domestication of *S. italica*. Other factors contribute to shattering success as well. For example, the loss of axillary branch pulvini in *Oryza* results in condensed inflorescences at maturity that serve to increase seed retention (Ishii et al. 2013). In the dense inflorescences of *S. italica* the spikelets are closely packed together due to the additional orders of branching and this, too, may increase seed retention at maturity.

In addition to gross differences in phenotype and panicle architecture, clear anatomical differences also distinguish the abscission zones of *S. viridis* and *S. italica*. In *S. viridis* there is a distinct transverse region running across the pedicel/spikelet junction made up of small cells in 1 to 4 layers which are distinctive both due to their lack of lignification and their maintenance of nuclei late into flowering. By comparison the abscission layers of B100 and Yugu are less distinguishable with lignified cells intercalating through their abscission layers. In *S. italica* B100, nuclei are visible by DAPI staining although there is not a strong boundary between the transverse cell layers and the parenchymatous cells on either side. Interestingly, the nuclei in *S. italica* B100 often appear fainter than those seen in *S. viridis* perhaps suggesting that there is a difference in DNA quantity and that
endoreduplication has occurred in *S. viridis*. By contrast, *S. italica* Yugu parenchymatous cells are readily apparent throughout the abscission zone and few if any discernible nuclei were observed later in development. Other structural differences in the abscission zone were observed. SEM imaging of the abscission cup post-shattering revealed clear differences in ease of diaspore release between *S. viridis* and either *S. italica*. Both *S. italica* lines release with difficulty along the abscission zone, but the phenotype of Yugu was by far the most severe. Differences are also visible early in development one of the most prominent being the expansion of the pedicel apex when the abscission zone layers are laid down through successive rounds of cell division. While this process of abscission zone formation is prominent in *S. viridis* it is often less distinguishable in both *S. italica*’s. Whereas some moderate degree of abscission zone pattern is recognizable in B100 at least, this period being hardly recognizable in Yugu at all which, by maturity, not only lacks a clearly discernible abscission zone but also fails to maintain viable cells in this region. Based on these observations it appears that the cell division which occurs early in the formation of the abscission zone in *Setaria* corresponds to its ability to maintain an abscission zone identity within these cells.

The genetic basis of abscission zone patterning in *Setaria* could shed more light on the underlying mechanisms of this pathway. Two of the known shattering loci in rice, *qSH1* and *SH1*, are actively transcribed within the spikelets of *Setaria*. *SH1* also belongs to the YABBY transcription factor family, many of which are involved in specifying abaxial identity of leaves and floral structures (Sarojam et al.
2010). Despite this pattern many of the other YABBY genes that have been characterized in the grasses have been shown to be expressed either adaxially or more generally around the vascular insertion of axillary organs (Juarez et al. 2004, Ishikawa et al. 2009). In this context, it is interesting that the abscission zone of Setaria is so close to the abaxial side of the glume vascular insertion. Although future work will be required to disentangle the expression patterns of both loci within Setaria, SHI in particular seems like a strong candidate for future study of shattering based on the combination of gene expression and anatomical data.

**Seed Shattering Evolution across the Grasses**- Few previously described features of grass shattering correspond to what has been found in Setaria. Indeed, in grasses as a whole it is remarkable how variable abscission zone positioning within the inflorescence is given that it serves such a critical function. This may in fact hint that abscission zone development like other seemingly complex traits such as C$_4$ photosynthesis, may somehow still manage to be convergent (Grass Phylogeny Working Group II 2012). A recent association mapping study performed across Chinese S. *italica* cultivars suggested that the orthologs of at least two shattering genes known from rice, $qSH1$ and $SH1$, display little allelic diversity possibly indicating a selective sweep (Jia et al. 2013). The expression of both loci during flowering of S. *viridis* and S. *italica* suggests these genes are actively expressed during the period of abscission zone activity. In addition, the intensity of the sweep at these two loci is less severe than what was noted for other domestication genes suggesting that selection on $qSH1$ and $SH1$ may have occurred
at a later period over the course of *Setaria* domestication in a manner analogous to *SH4* in rice.

Interestingly, the rachilla node at which the upper lemma and palea attach is ligninified. In rice the node at which the lowermost lemma attaches is lignified, but neither species does lignification occur below the glumes. Furthermore, the cells immediately below the lignified node in *Setaria italica* function as a secondary break during threshing (data not shown). A similar plane of disarticulation subtends the insertion point of the lemmas in *Brachypodium* (Barkworth et al. 2007). It is thus possible that the shattering position beneath the insertion of the lemma may be the pleisomorphic condition for Poaceae. Given the scarcity of anatomical data on abscission zone development across the majority of grass lineages the ancestral state of diaspore release and its diversification can only be guessed at presently.

The shattering position of *Setaria* beneath the insertion of the outer glume is a synapomorphy for the Panicoideae so that many members of the subfamily, such as switchgrass (*Panicum virgatum*) also share this shattering pattern (Hitchcock 1971). Despite this widely shared pattern, many of the core Andropogoneae have a shattering inflorescence branch forming an abscission zone at the base of their axillary branches (Barkworth et al. 2007, Doust et al. 2014). This area of the Poaceae family may prove to be a fruitful area of study to better understand the degree to which shattering patterns are both conserved and independently evolved within the grasses.
Literature Cited


Jia, G., Huang, X., Zhi, H., Zhao, Y., Zhao, Q., Li, W., Chai, Y., et al. (2013). A


two domesticated x wild pearl millet (*Pennisetum glaucum* L., Poaceae)
crosses. *Theoretical and Applied Genetics* 104: 965-975

Ruzin S.E. (1999). Plant microtechnique and microscopy, Oxford University Press,
New York, New York, USA.

Sarojam R., Sappl, P.G., Goldshmidt, A., Efroni, I., Floyd, S.K., Eshed, Y.,
Bowman, J.L. (2010). Differentiating *Arabidopsis* shoots from leaves by

Schindelin J., Arganda-Carreras I., Frise E., Kaynig V., Longair M., Pietzsch, T.,

Stamatakis, A. (2014). RAxML Version 8: A tool for phylogenetic analysis and
post-analysis of large phylogenies. *Bioinformatics*, doi:
10.1093/bioinformatics/btu033

(2013). Seed shattering in a wild *Sorghum* is conferred by a locus unrelated to
domestication. *Proceedings of the National Academy of Sciences* 110:
15824-15829.


*Theoretical and Applied Genetics* 96: 31-36.

Primer-BLAST: A tool to design target-specific primers for polymerase chain


### Tables

**Table 1: cDNA synthesis and gene expression primers**

<table>
<thead>
<tr>
<th>anchored poly-T</th>
<th>5’-CCGGATCCTCTAGAGCGGCGGCTTTTTTTTTT3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>SiSH1_337F</td>
<td>5’-CCAGAGAAGAGGCAACGTGT-3’</td>
</tr>
<tr>
<td>SiSH1_603R</td>
<td>5’-CACGGAATCAGGATGGGTGT-3’</td>
</tr>
<tr>
<td>SiqSH1_1640F</td>
<td>5’-CTCCACCAGAAACAGGGGT-3’</td>
</tr>
<tr>
<td>SiqSH1_1816R</td>
<td>5’-AGCAGCCATATCAGCCAAC-3’</td>
</tr>
<tr>
<td>UBI_441F</td>
<td>5’-AACAGCTGAGGGCCAAGA-3’</td>
</tr>
<tr>
<td>UBI_632R</td>
<td>5’-CTGTCACCATCGACACCAAGG-3’</td>
</tr>
</tbody>
</table>

### Supplementary Table 1: BEL1 phylogenetic tree gene annotations

<table>
<thead>
<tr>
<th>Tip Name</th>
<th>Gene Annotation Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bdistachyon_23530</td>
<td>Bradi2g23530</td>
</tr>
<tr>
<td>Bdistachyon_54940</td>
<td>Bradi2g54940</td>
</tr>
<tr>
<td>Zmays_074645</td>
<td>GRMZM2G074645</td>
</tr>
<tr>
<td>Zmays_125976</td>
<td>GRMZM2G125976</td>
</tr>
<tr>
<td>Zmays_154641</td>
<td>GRMZM2G154641</td>
</tr>
<tr>
<td>Maccuminata_07020</td>
<td>GSMUA_Achr5T07020_001</td>
</tr>
<tr>
<td>Maccuminata_14960</td>
<td>GSMUA_Achr7T14960_001</td>
</tr>
<tr>
<td>Maccuminata_22520</td>
<td>GSMUA_Achr10T22520_001</td>
</tr>
<tr>
<td>Osativa_62920_qSH1</td>
<td>LOC_Os01g62920</td>
</tr>
<tr>
<td>Osativa_38120</td>
<td>LOC_Os05g38120</td>
</tr>
<tr>
<td>Pdactylifera_741631</td>
<td>PDK_30s741631g002</td>
</tr>
<tr>
<td>Pdactylifera_1069811</td>
<td>PDK_30s1069811g006</td>
</tr>
<tr>
<td>Tip Name</td>
<td>Gene Annotation Name</td>
</tr>
<tr>
<td>--------------------</td>
<td>---------------------------------------------</td>
</tr>
<tr>
<td>Pdactylifera_890901</td>
<td>PDK_30s890901g014</td>
</tr>
<tr>
<td>Sbicolor_039840</td>
<td>Sb03g039840</td>
</tr>
<tr>
<td>Sbicolor_022270</td>
<td>Sb09g022270</td>
</tr>
<tr>
<td>Sitalica_000753</td>
<td>Si000753m</td>
</tr>
<tr>
<td>Sitalica_021626</td>
<td>Si021626m</td>
</tr>
</tbody>
</table>

**Supplementary Table 2: YABBY phylogenetic tree gene annotations**
Figure Legends

**Figure 1.** Spikelet morphology of *Setaria* and *Oryza*. The spikelet axis is subtended by two bracts known as glumes (pale green) above which other bracts are born known as lemmas (moderate green) from the axils of which floret meristems develop. Florets (green ovals) include an adaxial structure known as a palea (pale green) (A) *Setaria* spikelet architecture. Often only the upper floret meristem will develop whereas the lower floret meristem aborts (black X). Abscission (red bar) occurs below insertion of glumes. (B) *Oryza* spikelet architecture. Wild *Oryza* often has the mid-vein of the lemma elongate into an awn (dark green). Often only the upper floret meristem develops whereas the lower two floret meristems never initiate (black X). Abscission (red bar) occurs below insertion of lowermost lemma.

**Figure 2.** Grams of force required to induce shattering from days 0 (heading) to 20, and at senescence for *S. viridis* (A10) and *S. italica* (B100 & Yugu). By senescence all seeds of *S. viridis* are free shattering at ~0-10gF. *S. italica* lines remain at ~50-60gF at senescence. Points = mean values, solid bars = standard error, dashed bars = 1 standard deviation, n = 5.

**Figure 3.** Sections of *S. viridis* at day 0 (A & D), *S. italica* B100 at day 0 (B & E), and *S. italica* Yugu at day 2 (C & F). Upper floret (A, B, & C) and abscission zone (D, E, & F) of a single spikelet, stained with Toluidine Blue. Expansion of the pedicel coincides with initiation of the androecial primordia (red arrow) in the upper floret. Abscission zone is first visible as transverse layers of cells.
immediately beneath the insertion of the glume creating a lip-like deformation along the epidermis (black arrow). This pattern is most easily recognizable within A10 (D), and B100 (E), whereas Yugu (F) appears far less distinctive at a comparable stage. Scale bar = 100um.

**Figure 4.** Sections of *S. viridis* at day 0 (A & D), *S. italica* B100 at day 0 (B & E), and *S. italica* Yugu at day 0 (C & F). Upper floret (A, B, & C) and abscission zones (D, E, & F) from a single spikelet, stained with Toluidine Blue. Slightly more mature spikelets from the same stage inflorescences as Figure 2. Gynoecium has enlarged and the ovule has developed integuments (red arrow). The abscission zone of A10 (D) is the most recognizable displaying 3-4 transverse cell layers, whereas B100 (E) has 2-3 poorly developed transverse layers, and Yugu (F) 1-3 highly underdeveloped layers with the abscission zone nearly unrecognizable on the left side of the pedicel (black arrow). Scale bar = 100um.

**Figure 5.** Sections of *S. viridis* at day 16 (A & D), *S. italica* B100 at day 16 (B & E), and *S. italica* Yugu at day 8 (C & F). Embryo in upper florets (A, B, & C) and abscission zones (D, E, & F) from a single spikelet, stained with Toluidine Blue. Embryo is the undifferentiated mass of tissue near the base of the embryo sac (red arrow); endosperm has become cellularized (A, B, & C). Within the abscission zone the transverse cell (black arrows) layers are most distinguishable in A10 as 2-4 layers of nuclei-bearing cells with darker staining of their cell walls (D). *S. italica* B100 has 2-3 loosely arranged transverse layers present but no obvious distinction in staining (E). *S. italica* Yugu appears to stain more poorly than either *S. viridis* or
S. *italica* B100 and has only a vaguely discernable abscission layer and correspondingly, poor expansion of pedicel apex (F). Scale bar = 100um.

**Figure 6.** Sections of *S. viridis* at day 8 (A & D), *S. italic* B100 at day 20 (B & E), and *S. italic* Yugu at day 12 (C & F). Embryo in upper florets (A, B, & C) and abscission zones (D, E, & F) from a single spikelet, stained with Toluidine Blue. A highly developed scutellum is visible in all three lines. *S. viridis* and *S. italic* Yugu (A & C) are at similar relative ages with the differentiation of the coleoptile and coleorhiza being discernable. Further endosperm development can also be distinguished occurring along the boundary of the ovule (A & C). *S. italic* B100 (B) is older than either *S. viridis* or *S. italic* Yugu with the coleoptile and coleorhiza being more distinctive and notable elongation of the root also being visible. *S. viridis* exhibits 3-4 transverse cell layers with denser cell walls (D), *S. italic* B100 displays 2-3 loosely arranged transverse layers (E), and *S. italic* Yugu displays 1-2 layers of cells (F). Note nuclei in transverse cell layers of *S. viridis* and *S. italic* B100. The transverse cells of *S. italic* Yugu closely follow the vascular trace of the outer glume (black arrow) (F). Scale bar = 100um.

**Figure 7.** Sections of *S. viridis* at day 16 (A & D), *S. italic* B100 at day 20 (B & E), and *S. italic* Yugu at day 20 (C & F). Embryo in upper florets (A, B, & C) and abscission zones (D, E, & F) from a single spikelet, stained with Toluidine Blue. Later stages of embryo patterning are readily visible across all lines (A, B, & C) with the coleoptile and coleorhiza being readily visible as well as the first leaf primordia. There is a clear difference in size between the embryo of *S. viridis* (A)
and either *S. italica* (B & C). Nuclei are also present in the transverse cells of *S. viridis* and *S. italica* B100 (D & E) as was the case in Figures 4 & 5. Darkly stained areas along the borders of some of the *S. italica* Yugu transverse cells (F) may or may not be nuclei. More generally, the transverse cells within *S. italica* Yugu stain poorly as in Figure 4. Scale bar = 100um.

**Figure 8.** Brightfield micrograph of *S. viridis* abscission zone after seed release stained with Toluidine Blue. Disarticulation has occurred along the lower layers of the transverse cells so that few cells of the abscission zone remain attached to the cup post release. Scale bar = 100um.

**Figure 9.** Confocal Micrographs of day 20 *S. viridis* (A & D), *S. italica* B100 (B & E), and *S. italica* Yugu (C & F) stained with DAPI. Nuclei are visible across the abscission zone and neighboring regions within the spikelet and pedicel (A, B, & C). Magnified images of analogous portions of the abscission layers of all three species were also generated so that individual nuclei can be distinguished (D, E, & F). For *S. italica* B100 and *S. italica* Yugu the nuclei of the magnified images are indicated by (black arrows) (E & F).

**Figure 10.** Brightfield micrographs of day 20 *S. viridis* (A), *S. italica* B100 (B), and *S. italica* Yugu (C) stained with phloroglucinol. Lignin biosynthesis is not detected immediately around the abscission zone. Staining appears higher in the rachilla at the node of the upper lemma as is seen in *S. viridis* (A) (red arrow). Scale bar = 100um.

**Figure 11.** Confocal micrographs of day 12 *S. viridis* (A & D), *S. italica* B100 (B
& E), and S. italica Yugu (C & F) stained with AO. The transverse layers of the S. viridis abscission zone exhibit acidification relative to the parenchyma of the pedicel or rachilla (A). This pattern is absent in both S. italica B100 (B) and S. italica Yugu (C).

**Figure 12.** SEM images of the abscission cup surface in S. viridis (A), S. italica B100 (B), and S. italica Yugu (C) after diaspor release at senescence. A smooth plate of cells visible along the surface of the cup in S. viridis (A). Tearing of cells along the abscission zone boundary and structural deformation of the abscission cup is instead noted in B100 (B) and Yugu (C).

**Figure 13.** Maximum likelihood trees for the immediate gene family members of the BEL1-like homeobox gene qSH1 (A) and the YABBY transcription factor SH1 (B). The full annotation name for each gene is listed in for qSH1 and SH1 in supplementary tables 1 and 2, respectively. Outgroups are Phoenix dactylifera and Musa acuminate (red branches). The immediate orthologous clade of the shattering genes colored blue and the specific Setaria ortholog to the known shattering loci colored turquoise. Numbers at nodes indicate bootstrap values.

**Figure 14.** Triplicate RT-PCR reactions in A10, B100, and Yugu at days 0 and 10 with a negative control lane. Expression patterns for qSH1 (top), SH1 (middle), and a ubiquitin as a positive control (bottom).
<table>
<thead>
<tr>
<th>qSH1 (BEL1)</th>
<th>B100 (D0)-1</th>
<th>B100 (D0)-2</th>
<th>B100 (D0)-3</th>
<th>B100 (D10)-1</th>
<th>B100 (D10)-2</th>
<th>B100 (D10)-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>A10 (D0)-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A10 (D0)-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A10 (D0)-3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A10 (D10)-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A10 (D10)-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A10 (D10)-3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sh1 (YAB2)</th>
<th>Yugu (D0)-1</th>
<th>Yugu (D0)-2</th>
<th>Yugu (D0)-3</th>
<th>Yugu (D10)-1</th>
<th>Yugu (D10)-2</th>
<th>Yugu (D10)-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>B100 (D0)-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B100 (D0)-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B100 (D0)-3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B100 (D10)-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B100 (D10)-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B100 (D10)-3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ubi</th>
<th>Yugu (D0)-1</th>
<th>Yugu (D0)-2</th>
<th>Yugu (D0)-3</th>
<th>Yugu (D10)-1</th>
<th>Yugu (D10)-2</th>
<th>Yugu (D10)-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>A10 (D0)-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A10 (D0)-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A10 (D0)-3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A10 (D10)-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A10 (D10)-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A10 (D10)-3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Neg. Con. |