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Research

Divergent gene expression networks underlie morphological diversity of abscission zones in grasses

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Summary

- Abscission is a process in which plants shed their parts, and is mediated by a particular set of cells, the abscission zone (AZ). In grasses (Poaceae), the position of the AZ differs among species, raising the question of whether its anatomical structure and genetic control are conserved.
- The ancestral position of the AZ was reconstructed. A combination of light microscopy, transmission electron microscopy, RNA-Seq analyses and RNA *in situ* hybridisation were used to compare three species, two (weedy rice and *Brachypodium distachyon*) with the AZ in the ancestral position and one (*Setaria viridis*) with the AZ in a derived position below a cluster of flowers (spikelet).
- Rice and *Brachypodium* are more similar anatomically than *Setaria*. However, the cell wall properties and the transcriptome of rice and *Brachypodium* are no more similar to each other than either is to *Setaria*. The set of genes expressed in the studied tissues is generally conserved across species, but the precise developmental and positional patterns of expression and gene networks are almost entirely different.
- Transcriptional regulation of AZ development appears to be extensively rewired among the three species, leading to distinct anatomical and morphological outcomes.

Introduction

Plants have developed specific and sophisticated mechanisms for shedding parts, a process known as abscission. In general, the plant forms a specialised set of cells known as an abscission zone (AZ), marking the point at which the cells of the falling organ separate from those of the parent (Patterson, 2001). Abscission has received considerable attention in cereal grains because loss of abscission (retention of seeds on the parent plant, also called loss of shattering) was one of the earliest steps in plant domestication (Fuller & Allaby, 2009). Cereals are all members of the grass family (Poaceae), a monophyletic group of *c.* 12 000 species with global distribution (Kellogg, 2015; Soreng *et al.*, 2015). In grasses, the AZ rarely forms below the fruit (caryopsis or grain), but rather in a position that allows the fruit to be dispersed with various floral and inflorescence parts. Grasses bear their tiny flowers in clusters known as spikelets, consisting of one to many flowers and associated surrounding bracts (glumes, lemmas and paleas). Spikelets are borne on stalks known as pedicels and, within the spikelet, flowers are borne on a central stalk known as the rachilla. The AZ may form below the flower in the rachilla, below sets of flowers in the pedicel or rachis, or even below the entire inflorescence (Fig. 1a) (Doust *et al.*, 2014b; Yu & Kellogg, 2018).

AZs may differ anatomically. Often, the AZ is composed of one to a few layers of small, cytoplasmically dense and meristem-like cells that are distinct from the surrounding cells (Sexton & Roberts, 1982; Patterson, 2001). For example, the AZ of rice (tribe Oryzeae) is located in the rachilla and is composed of small cells with thin nonlignified walls surrounded by larger lignified cells. By contrast, the AZs of *Setaria viridis* (tribe Paniceae) and wild barley (*Hordeum vulgare* subsp. *spontaneum*; tribe Triticeae) are located in the pedicel and inflorescence stalk (rachis), respectively, and are histologically indistinguishable from adjacent cells (Pourkheirandish *et al.*, 2015; Hodge & Kellogg, 2016). Differences in the position and anatomy of the AZ raise the question of whether different grass species share the same underlying genetics and how molecular regulation generates morphological diversification.

Because evolution generally operates by modifying existing structures, we hypothesised that AZs in grasses would be developmentally and genetically similar despite their positional and anatomical differences. Genetic studies on the AZ have been most extensive in rice, and a number of genes have been identified that, when mutated, lead to loss of abscission. These include a myb transcription factor (TF) *Shattering4* (*SH4*) (Li *et al.*, 2006; Wu *et al.*, 2017), BEL1-like homeodomain TFs *qSH1* (Konishi *et al.*, 2006) and *SH5* (Yoon *et al.*, 2014), the KNOX TF *OSH15* (Yoon *et al.*, 2017), the APETALA2 (AP2) TF *SHATTERING ABORTION1* (*SHAT1*) (Zhou *et al.*, 2012), the YABBY TF *YAB2/ObSH3* (Lv *et al.*, 2018) and the carboxy-terminal domain phosphatase-like gene *OsCPL1* (Ji *et al.*, 2010). Data on species other than rice remain scarce. Only two genes are

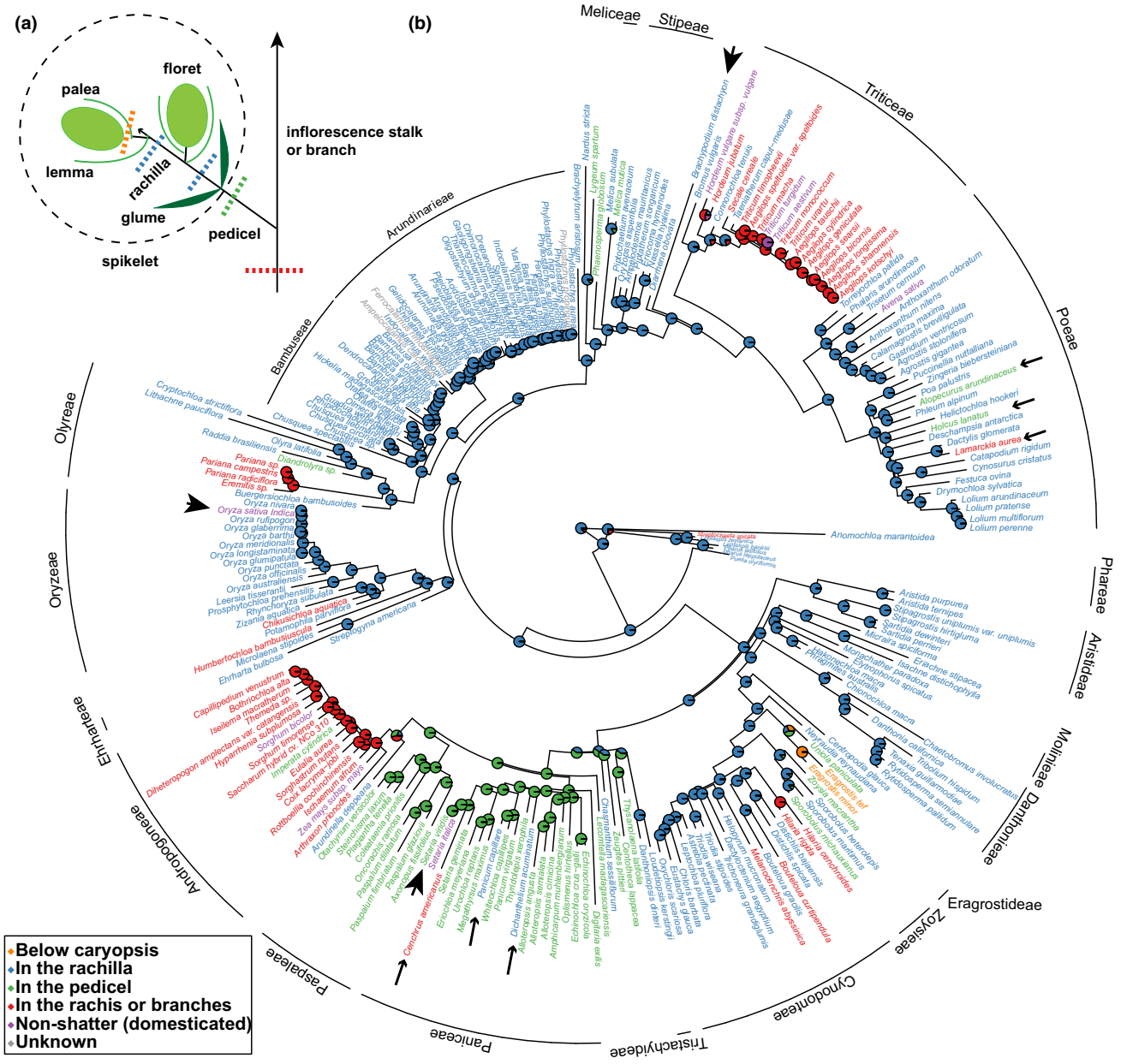

Fig. 1 The abscission zone (AZ) position may shift from rachilla down to pedicel and rachis during evolution. (a) A diagram illustrating different AZ positions: below caryopsis and above lemma and palea (orange), in the rachilla below floret and above the glumes (blue), in the pedicel below the glumes (green), or in the rachis or inflorescence branches (red). A floret includes caryopsis and floral bracts (palea and lemma). A spikelet (marked by dotted circle) includes florets and the subtending pair of glumes. (b) Ancestral state reconstruction of the AZ positions in the grass family. Big arrowheads point to rice, *Brachypodium* and *Setaria*. Small arrowheads point to examples of species with AZ positions different from other species within the tribe.

known to control AZ development in species with different AZ positions. *YAB2*/*OsSH3* (also called *Shattering1*, *SH1*) leads to loss of shattering not only in rice (Lv *et al.*, 2018), but also in *Setaria* (Doust *et al.*, 2014a; Odonkor *et al.*, 2018) and *Sorghum* (Lin *et al.*, 2012), where AZs are located in the pedicel and rachis, respectively. Similarly, the wheat AZ forms in the rachis, which is a different position from rice, and yet orthologous *AP2* (called *SHAT1* in rice and *Q* in wheat) loci in both species play pleiotropic roles in inflorescence development, including AZ development (Simons *et al.*, 2006; Zhou *et al.*, 2012). *OsAP2* is highly expressed in the AZ in rice and a null mutation completely abolishes the small and nonlignified AZ cells (Zhou *et al.*, 2012), while the specific role of *TaAP2* in wheat AZ development is unknown.

Outside Poaceae, evolution of the AZ has been studied extensively in fruits of Brassicaceae. Despite their diverse shapes, most studies support a conserved genetic regulatory pathway composed of several TFs identified in *Arabidopsis thaliana*, including the

MADS-box TFs *FRUITFULL* (*FUL*) (Ferrándiz *et al.*, 2000), *SHATTERPROOF1* (*SHP1*) and *SHP2* (Liljegren *et al.*, 2000), the basic helix-loop-helix (bHLH) TFs *ALCATRAZ* (Rajani & Sundaresan, 2001) and *INDEHISCENT* (*IND*) (Liljegren *et al.*, 2004), and BEL1-like homeodomain TF *REPLUMLESS* (*RPL*) (Roeder *et al.*, 2003). Altered expression of these genes in other Brassicaceae species, including the naturally nonshattering *Cakile lanceolata* (Avino *et al.*, 2012) and transgenic plants of shattering *Lepidium campestre* (Lenser & Theißen, 2013; Mühlhausen *et al.*, 2013), changes abscission capability. The functions of *FUL* and *SHP* are also conserved in Solanaceae, a eudicot family distantly related to Brassicaceae. Overexpression of *FUL* and suppression of *SHP* in *Nicotiana* species reduces shattering as in Arabidopsis (Smykal *et al.*, 2007; Fourquin & Ferrándiz, 2012). Furthermore, *FUL2* is expressed in the AZ of *Streptochaeta angustifolia* (Preston *et al.*, 2009), a lineage sister to the remainder of the grass family, suggesting deep conservation of *FUL* in AZ development, even though the organs where abscission occurs are not homologous between Brassicaceae and Poaceae: the former is between the carpels and the latter is along the inflorescence axis.

If the genetic pathway controlling AZ development is conserved and only shifts from one position to another, the differences in AZ position within Poaceae and even between Brassicaceae and Poaceae can be interpreted as heterotopy, an evolutionary change of position of a developmental process or program (Bateman, 1994; Zelditch & Fink, 1996). Activating a developmental process in a new position often leads to novel structures, and therefore heterotopy could be a means of evolutionary modification (Hall, 1999; Baum & Donoghue, 2002). Only a few examples of heterotopy have been demonstrated, however, including development of the jaw in gnathostomes (Shigetani *et al.*, 2002), shell formation of turtles (Nagashima *et al.*, 2009) and leaf-borne inflorescences in some angiosperms (Baum & Donoghue, 2002).

To test whether variation in AZ position and anatomy fits the definition of heterotopy, we first need to understand the phylogenetic distribution of the different AZ positions and then to examine AZs in different positions to determine if they share similar anatomy, histology and underlying gene networks. In this study, we compared AZ development of three distantly related grass species, a shattering weedy rice (*Oryza sativa, Os*), purple false brome (*Brachypodium distachyon, Bd*) and green foxtail (*Setaria viridis, Sv*). Rice and *Brachypodium* are both members of one major clade of the grass family and have an AZ located in the rachilla, while *Setaria* belongs to the other major clade and its AZ is located in the pedicel. We show that the AZs of all three species differ in anatomy, cell wall properties and gene expression as shown by co-expression network analyses and *in situ* hybridisation, suggesting divergence of regulatory mechanisms.

Materials and Methods

Ancestral state reconstruction

AZ position was assessed for 250 grass taxa by new observations of specimens at the herbarium of the Missouri Botanical Garden, supplemented with information from Barkworth *et al.* (2003, 2007) and Wu *et al.* (2006). AZ position was classified as either below the fruit, in the rachilla below the floret, in the pedicel below the spikelet, or in the rachis or branches (Fig. 1a). The ancestral AZ position was reconstructed on the grass phylogeny of Saarela *et al.* (2018) using *phytools* (Revell, 2012) in R (v.3.5.0) with an equal rates model.

Plant materials and growth conditions

De-domesticated US weedy rice (*Oryza sativa*), straw-hulled (Os-SH) (Thurber *et al.*, 2010), was provided by Dr Kenneth M. Olsen at Washington University at St Louis. *Brachypodium distachyon* (Bd21-0) was provided by Dr Malia Gehan at Donald Danforth Plant Science Center. *Setaria viridis* (ME034V) was from seeds available in the Kellogg lab (Layton & Kellogg, 2014). Each species was grown under its own optimal growth conditions at the Danforth Center Plant Growth Facility. Rice was grown in Turface MVP soil (Turface Athletics, Buffalo Grove, IL, USA) in a glasshouse with 30–70% humidity and 15 h : 9 h, light : dark with temperatures of day : night, 24–28°C : 23–25°C. *Brachypodium* was grown in a growth chamber with 50% humidity, light : dark, 20 h : 4 h, with light intensity of 200 μmol m^{-2} s^{-1} and temperatures of day : night, 24°C : 18°C. *Setaria viridis* was grown in Metro-Mix 360 (Sun Gro) in a growth chamber with 50% humidity, light : dark 12 h : 12 h, with light intensity of 250 μmol m^{-2} s^{-1} and temperatures of day : night, 31°C : 22°C.

Histology

At least three inflorescences per species were collected and dissected (Supporting Information Fig. S1). Post-anthesis spikelets (seeds are half filled, corresponding to the 'old stage' described below) (Fig. S1d–i) were fixed in ice cold FAA (37% formaldehyde : ethanol : H_2O : acetic acid = 10 : 50 : 35 : 5), and dehydrated in 50%, 70%, 85%, 95%, 100%, 100% and 100% ethanol and then 25%, 50%, 75%, 100%, 100% and 100% Histo-Clear II (National Diagnostics, Atlanta, GA, USA) with ethanol as solvent, following Ruzin (1999). Paraplast® (Leica Biosystems, Wetzlar, Germany) was then added to each vial of samples and kept overnight, heated at 42°C, and placed in a 60°C oven. Solution was replaced with molten Paraplast® twice a day for 3 d. Samples were embedded in paraffin using a Leica EG1150 tissue embedder, sectioned into 10-μm serial slices with a Leica RM2255 automated microtome, and mounted on microscope slides at 37°C on a Premiere XH-2001 Slide Warmer overnight.

Sections were deparaffinised, rehydrated, stained with 1% (w/v) Safranin O for 3 h and 0.05% (w/v) Fast Green for 5–15 s following Ruzin (1999). Specimens were mounted with Permount, and images taken using a Leica DM750 LED Biological microscope with ICC50 camera module and Leica Acquire v.2.0 software (Leica Microsystems).

Transmission electron microscopy (TEM)

Old stage tissues from two biological samples per species were dissected and fixed in 2% glutaraldehyde in 0.1 M PIPES buffer

for 90 min under vacuum. After three rinses in 0.1 M PIPES buffer for 7 min each, tissues were post-fixed in 2% osmium tetroxide in 0.1 M PIPES for 90 min, followed by three rinses in water and dehydrated in 5%, 10%, 20%, 30%, 50%, 75%, 95% EtOH for 15 min, 100% EtOH for 30 min, 100% acetone for 15 min and a second 100% acetone for 45 min. Tissues were infiltrated in a series of Spurr's resin of 5% and 10% for 12 h, 25%, 50%, 75% and 100% for 24 h with acetone as solvent, and embedded in Spurr's resin at 60°C for 48 h. Specimens were sliced into 90-nm sections using a Leica Ultracut UCT, mounted on a copper grid, stained in uranyl acetate for 12 min and lead salts for 3 min, and imaged using a LEO 912AB TEM.

RNA isolation, RNA-Seq library construction, sequencing and mapping

Spikelets were sampled when anthers were at late sporogenesis and gametogenesis but the AZ lacked obvious secondary cell wall formation (the 'young stage'), and also after anthesis, when seeds were approximately half filled (the 'old stage'). The AZ (A) plus tissues immediately above (upper, U) and below (lower, L) were collected for transcriptomic analysis (Fig. S1). Each of the three biological replicates used spikelets from at least two plants per species per replicate at each developmental stage. Tissues were dissected in RNAlater™ Stabilisation Solution (ThermoFisher Scientific, Waltham, MA, USA) using a scalpel under a dissecting microscope. The upper tissues included the lowermost parts of the rachilla and bracts (lemma, palea, and/or glumes depending on species) but excluded reproductive organs, and lower tissues were rachilla or pedicel, again depending on species (Fig. S1). Dissected tissues were immediately transferred to the extraction buffer of PicoPure RNA Isolation Kit (Thermo Fisher Scientific), ground with pellet pestles, total RNA isolated using the kit with in-column DNase I treatment following manufacturer's instructions, and quantified using a Qubit 3.0 Fluorometer (Life Technologies). Next, *c.* 300 ng total RNA was used for generating 3′ mRNA-Seq libraries using QuantSeq 3′ mRNA-Seq Library Prep Kit FWD for Illumina (Lexogen) (Moll *et al.*, 2014). In total, 54 libraries were single-end sequenced at 150 nt in two lanes on an Illumina HiSeq 4000 instrument at the Roy J. Carver Biotechnology Center at the University of Illinois at Urbana-Champaign. Sequences were deposited in the Sequence Read Archive (SRR8635261-SRR8635314).

Sequencing adaptors, polyA and low quality sequences were removed using Trimmomatic (v.0.35) (Bolger *et al.*, 2014). Reads from rice, *Brachypodium* and *Setaria* were mapped to an *indica* rice genome Shuhui498 (R498) (Du *et al.*, 2017), *Brachypodium distachyon* v.3.1 (International *Brachypodium* Initiative, 2010) and *Setaria viridis* ME034V v.1.0 (P. Huang *et al.* unpublished), respectively, using HISAT2 software (v.2.0.6) (Pertea *et al.*, 2016). Mapped read counts were calculated using HTSEQ-COUNT (HTSEQ v.0.9.1) (Anders *et al.*, 2015). Raw counts were input into DESEQ2 (v.1.22.2) in R (v.3.5.0) (Love *et al.*, 2014). Genes with < 10 normalised counts in all of the libraries were filtered out before subsequent analyses.

Self-organising map (SOM) clustering, weighted gene co-expression network analysis (WGCNA) and gene co-expression visualisation

Differentially expressed genes (DEG) were identified using a threshold of absolute fold change of 1.5 between A vs L or A vs U at young or old stage, and adjusted *P*-value < 0.05, using DESEQ2 (Love *et al.*, 2014). Genes with at least one significant difference in the comparisons were used for SOM analysis using Multiple Experiment Viewer (v.4.8.1) with the bubble neighbourhood method (radius = 0.7).

Orthologues among rice, *Brachypodium* and *Setaria* were identified using OrthoFinder (Emms & Kelly, 2015). Normalised counts of one-to-one orthologues expressed in all three species were imported to WGCNA (v.1.66) in R for co-expression network construction (Langfelder & Horvath, 2008). A minimal module size of 30 genes, cutHeight of 0.14, soft-thresholding power of 16 and deepSplit of 2 were used for all three species. Degree distributions in each network followed the power law and satisfied the scale-free topology criterion. Module preservation analysis used the R function module PRESERVATION in the WGCNA package, and the composite statistic Zsummary value (average of module density and module connectivity) was calculated for evaluating module preservation between species (Langfelder *et al.*, 2011). Co-expressed genes were defined as a weight of interaction > 0.05, and those with weight > 0.1 were visualised using CYTOSCAPE v.3.7.1 with edge-weighted spring embedded layout (Smoot *et al.*, 2010).

In situ hybridisation

RNA probes were designed from the 3′ end of the transcript of each gene (primers and probe lengths in Table S1). Sequences were amplified from each species and cloned into the Zero Blunt™ TOPO™ PCR Cloning Kit (ThermoFisher). Digoxigenin-UTP-labeled sense and antisense probes were synthesised by *in vitro* transcription using T7 or SP6 RNA polymerase (Promega) and hydrolysed in carbonate buffer (pH 10.2) at 60°C into 150 nt.

In situ hybridisation followed published methods (Yang *et al.*, 2018). Sections were deparaffinised with Histo-Clear II (National Diagnostics) for 10 min twice, rehydrated in 100%, 100%, 90%, 70%, 50% and 30% ethanol series and DEPC treated water for 2 min each, followed by 5 μg ml^{-1} proteinase K (Roche) at 37°C for 20 min, refixation in 4% (w/v) formaldehyde for 10 min, and acetic anhydride for 10 min. Sections were then dehydrated with an ethanol series and kept under vacuum for 1–2 h. Hybridisation was performed at 50°C for 16–18 h in buffer composed of 0.5 ng ml^{-1} probe, 50% formamide, 1 mg ml^{-1} tRNA, 0.5 mg ml^{-1} polyA, 30 mM DTT, 0.3 M NaCl, 10 mM Tris-HCl, 1 mM EDTA, 10% dextran sulfate, and 1 × Denhardt's solution. On the second day, slides were washed with 4 × SSC (0.6 M NaCl and 60 mM trisodium citrate) at 50°C for 15 min, treated with 30 μg ml^{-1} RNase A in RNase buffer (0.5 M NaCl and

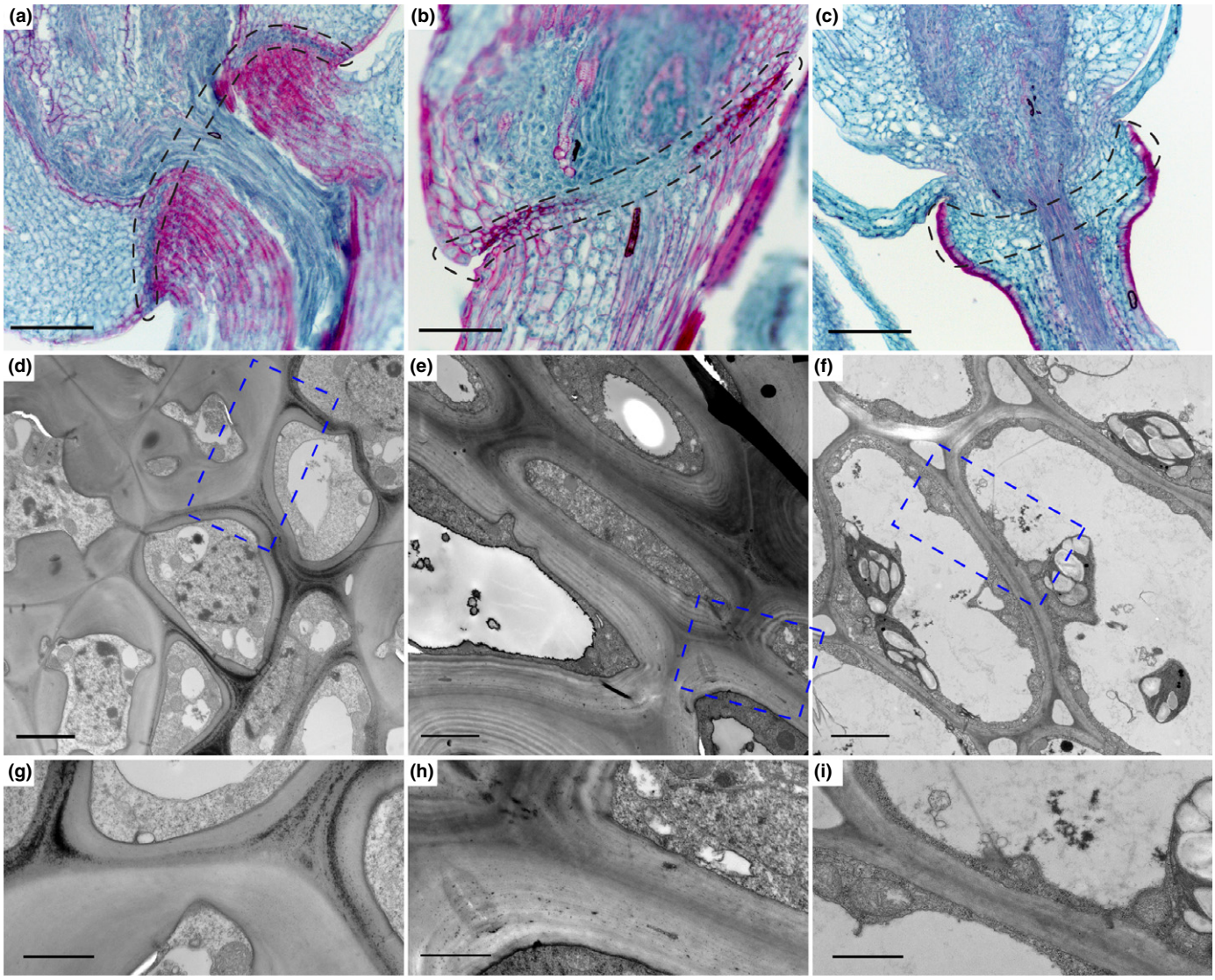

Fig. 2 The abscission zones (AZ) of rice, *Brachypodium* and *Setaria* differ in histology and cell wall structures. (a–c) Fast green/Safranin O staining of mature spikelets in (a) rice, (b) *Brachypodium* and (c) *Setaria*. Safranin O stains secondary cell wall including lignin, suberin and cutin with a magenta colour, and fast green stains primary cell wall and cytoplasm with a green colour. The AZ is marked with black dotted lines. (d–f) Transmission electron microscopy images of the AZ and surrounding cells in (d) rice, (e) *Brachypodium* and (f) *Setaria*. (g–i) Zoomed in images of the blue dotted rectangles shown in (d-f). Bars: (a–c) 100 μm; (d–f) 2 μm; (g–i) 1 μm.

0.01 M Tris-HCl, pH 7.5) at 37°C for 30 min, washed with RNase buffer for 5 min three times, 0.5 × SSC for 20 min twice, and buffer1 (0.15 M NaCl and 0.1 M Tris-HCl, pH 7.5) for 5 min twice, and incubated in blocking reagent (Roche) for 30 min. Sections were incubated with 1 : 1000 anti-digoxigenin-AP antibody (Roche) for 1 h at room temperature, washed with buffer 1 for 10 min three times and coloration buffer (0.1 M NaCl, 0.05 M $MgCl_2$ and 0.1 M Tris-HCl, pH 9.5) for 5 min. Alkaline phosphatase signal was visualised by incubating the slides in 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (Roche) in coloration buffer overnight at 37°C. Imaging used a Leica DM750 LED Biological microscope with ICC50 camera module and Leica Acquire v.2.0 software.

Results

AZ position shifts from the rachilla down to pedicel and rachis in evolutionary time

The ancestral position of the AZ is likely in the rachilla (below the floret), while the position shifts to the pedicel in tribes Paniceae and Paspaleae and to the rachis in tribes Andropogoneae and Triticeae (Fig. 1b). Additional shifts also occurred between closely related species in a number of tribes. For example, in the tribe Poeae, where most species have an AZ in the rachilla, the position shifted to the pedicel in *Alopecurus arundinaceus* and *Holcus lanatus*, and to the base of the inflorescence branches in *Lamarckia aurea*. Similarly, in the tribe Paniceae, with the AZ

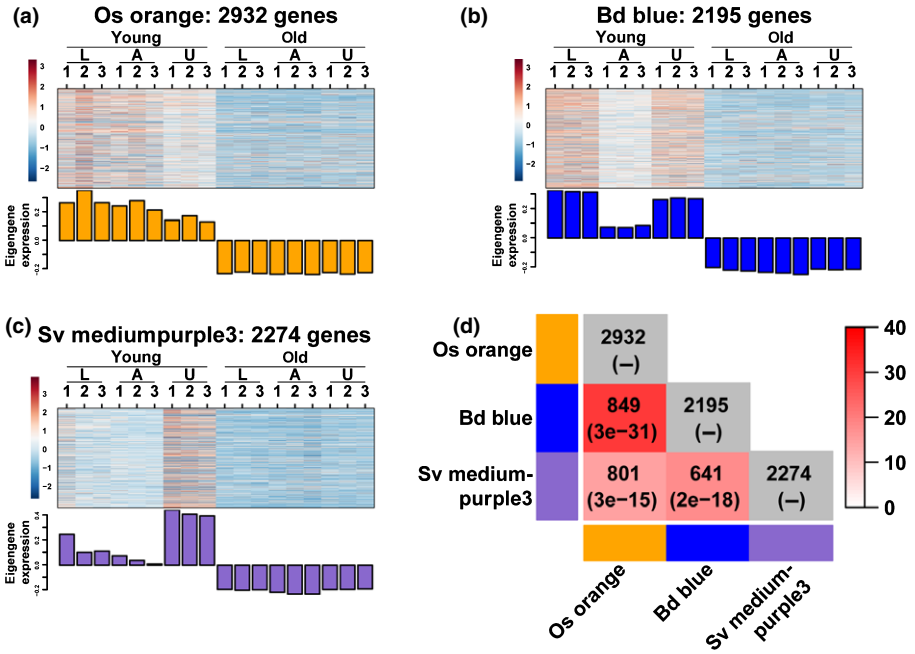

Fig. 3 WGCNA modules with differential expression at the two developmental stages have substantial gene overlap among the three species. (a–c) Heatmap and eigengene bar graph of (a) rice orange module, (b) *Brachypodium* blue module and (c) *Setaria* mediumpurple3 module showing higher expression at the young stage than that at the old stage. (d) Pairwise comparisons of the number of overlapping genes between modules. The numbers in parentheses are *P*-values from Fisher's exact test to test statistical significance between two modules. White to red colour key indicates $-\log_{10}$(*P*-value). Module comparisons within species are not meaningful and indicated with grey colour.

mostly located in the pedicel, the AZs of *Panicum capillare* and *Dichanthelium acuminatum* form in the rachilla, and in *Cenchrus americanus* at the base of the inflorescence branches (Fig. 1b; Table S2). The positional shift of the AZ in closely related species suggests that heterotopy of AZ development might be governed by simple genetics and few mutations.

AZs of rice, *Brachypodium* and *Setaria* differ in anatomical structure

We hypothesised that AZs in the same position should be anatomically similar and differ from those in different positions. To test this hypothesis, we compared the AZ anatomy of a shattering weedy rice and two wild shattering species, *Brachypodium* and *Setaria*. The AZ of rice and *Brachypodium* is located in the rachilla, although *Brachypodium* has multiple fertile florets and therefore multiple AZs within a spikelet while rice has only one. The AZ of *Setaria* is in the pedicel (Fig. 1b). Consistent with our hypothesis, the AZs of rice and *Brachypodium* are composed of one or two layers of cells that are much smaller than the adjacent ones (Fig. 2a,b), while the cells in the AZ of *Setaria* are not distinguishable from adjacent cells (Fig. 2c) (Hodge & Kellogg, 2016). The cells of the rice AZ lack the characteristic magenta colour of lignin stained with safranin O (Johansen, 1940; Ruzin, 1999) (Fig. 2a), whereas the AZ of *Brachypodium* is more strongly stained by safranin O than the surrounding cells (Fig. 2b), indicating different cell wall composition between the AZ of rice and *Brachypodium* despite their similar position and cell size. In *Setaria*, cells in the AZ lack lignin entirely except in the epidermis, confirming previous data (Fig. 2c) (Hodge & Kellogg, 2016).

TEM shows that cells in the rice AZ are thin-walled and cytoplasmically dense, while the adjacent cells are thick-walled, indicating secondary cell wall deposition (Fig. 2d,g). In *Brachypodium*, AZ cells also have dense cytoplasm but the wall is much thicker than that in rice, although thinner than that of the adjacent cells (Fig. 2e,h). *Setaria* exhibits only thin-walled cells with large vacuoles and extensive intercellular spaces in the region of the AZ (Fig. 2f,i). In summary, the AZs of rice, *Brachypodium* and *Setaria* all differ in anatomy and cell wall structure, although rice and *Brachypodium* are more similar to each other than either is to *Setaria*.

Gene co-expression patterns are conserved at similar developmental stages across three species

After filtering out genes with low expression, we identified 19 990, 20 408 and 20 381 genes expressed in at least one of AZ, upper or lower tissues in rice, *Brachypodium* and *Setaria*, respectively (Tables S3–S5).

Overall gene expression was similar among the three species. Among 12 951 one-to-one orthologues identified, 11 333, 11 235 and 11 456 genes are expressed in rice, *Brachypodium* and *Setaria*, respectively, and 10 273 genes are expressed in all three species (90.6% of Os, 91.4% of Bd and 89.7% of Sv expressed orthologues), suggesting substantial overlap and extensive conservation of gene expression in the examined tissues as a whole. The expressed one-to-one orthologues were then used for WGCNA

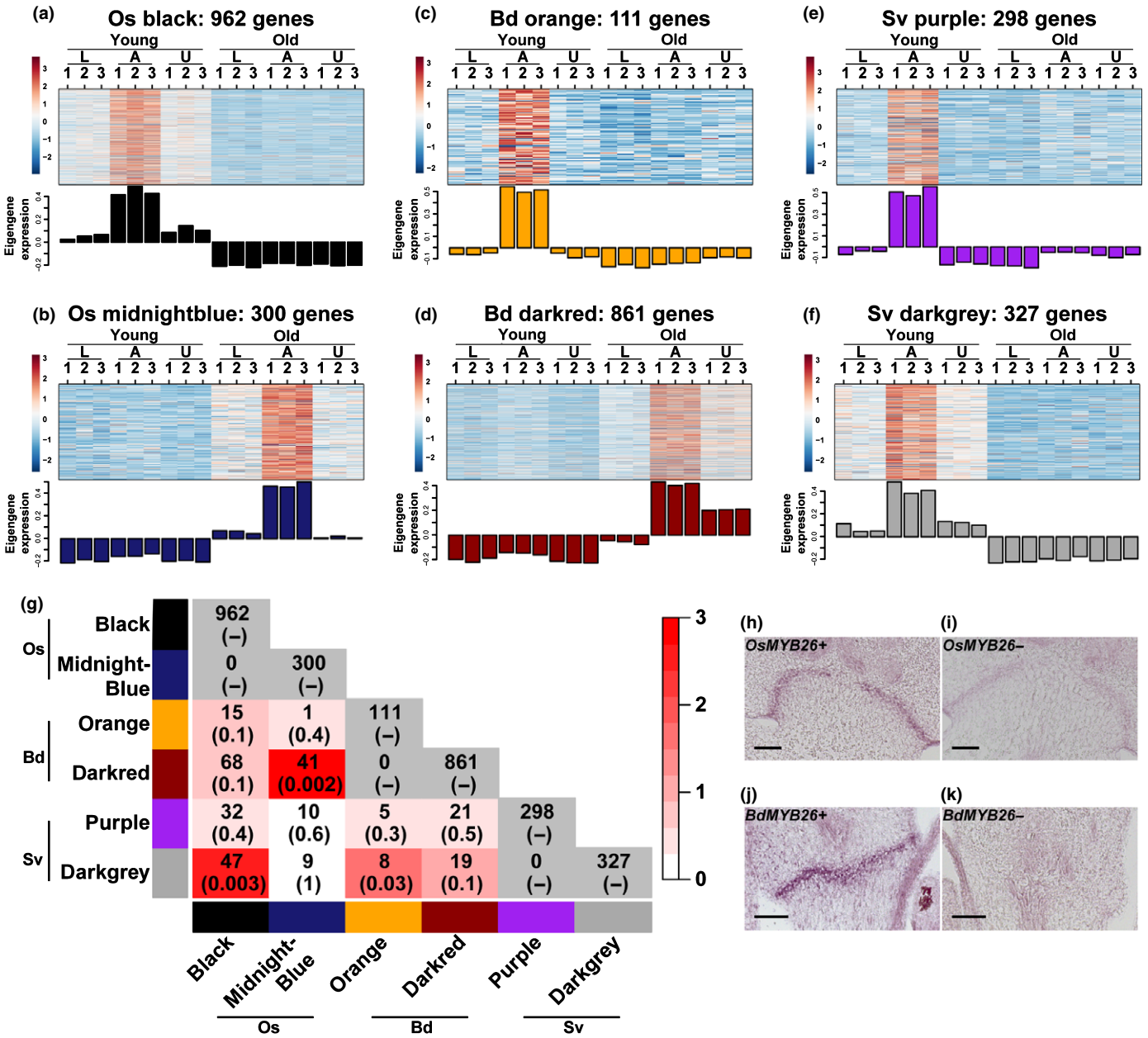

Fig. 4 The number of overlapping genes between abscission zone (AZ) modules is small among the three species. (a–f) Heatmap and eigengene bar graph of (a) rice young AZ module (black), (b) rice old AZ module (midnightblue), (c) *Brachypodium* young AZ module (orange), (d) *Brachypodium* old AZ module (darkred) and (e, f) *Setaria* young AZ modules (purple and darkgrey). (g) Pairwise comparisons of the number of overlapping genes between modules. The numbers in parentheses are *P*-values from Fisher's exact test to test statistical significance of overlap between two modules. White to red colour key indicates $-\log_{10}$(*P*-value). Module comparisons within species are not meaningful and indicated with grey colour. (h–k) *In situ* hybridisation of *MYB26* in (h, i) rice and (j, k) *Brachypodium*. (h, j) antisense probes; (i, k) sense probes. Bars, 50 μm.

in individual species. Overall, 15, 17 and 18 co-expression modules were found in rice, *Brachypodium* and *Setaria*, respectively, including a module of unassigned genes (grey module) in each species (Figs S2–S4; Table S6). WGCNA designates modules by colours, which here apply only within individual species; they are not comparable among species.

To test whether any modules in one species were preserved in the other two, we calculated the preservation statistic $Z_{summary}$ using the modules in one species as a reference network and the other two as test networks (Langfelder *et al.*, 2011). Most modules were only weakly to moderately preserved ($2 < Z_{summary} < 10$), and only three to five modules were highly preserved ($Z_{summary} > 10$) (Fig. S5). For instance, when using rice as reference, the orange module in *Brachypodium* and the blue module in *Setaria*, preferentially expressed at young and old stages, respectively, were highly preserved (Fig. S5a,b). We observed the same pattern when using *Brachypodium* and *Setaria* as reference (Fig. S5c–f). Therefore, a partially conserved set of genes differentiates young and old stages of development in the study tissues.

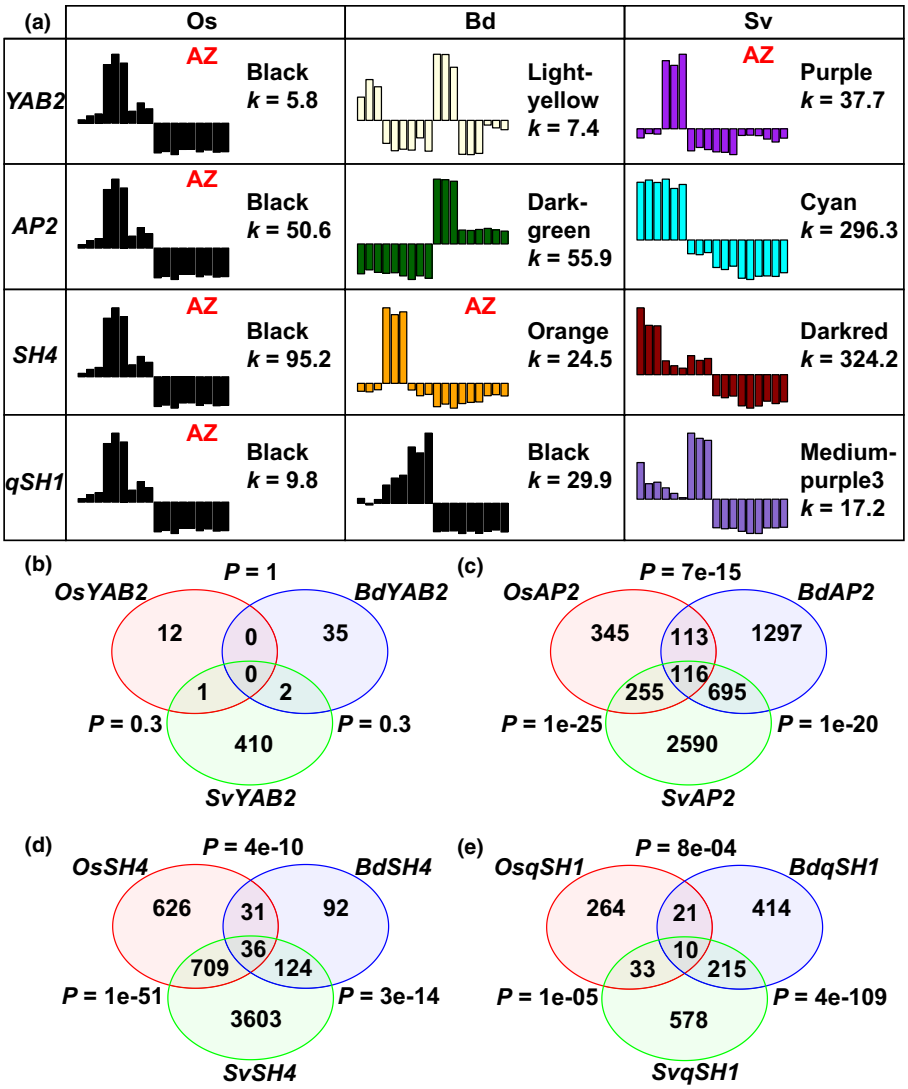

Fig. 5 Previously identified abscission zone (AZ) genes are in different co-expression modules in different species. (a) Modules in which *YAB2*, *AP2*, *SH4* and *qSH1* are classified. k stands for the total connectivity of a gene with all other expressed genes. (b–e) The number of overlapping co-expressed genes of (b) *YAB2*, (c) *AP2*, (d) *SH4* and (e) *qSH1* in rice, *Brachypodium* and *Setaria*. *P*-values are calculated from Fisher's exact test to test statistical significance of overlap between species.

We next calculated gene overlap between all pairs of modules (Fig. S6) and found significant overlap among all three species for modules upregulated at the young stage, again supporting conserved expression patterns for developmental stages (Fig. 3). In addition, modules highly expressed in the region below the AZ (L) at the old stage also overlap significantly among three species (Fig. S7a–f), and two pairs of modules with highly significant overlap are observed between *Brachypodium* and *Setaria* (Fig. S7g–l). Modules with highly significant overlap across species also have similar expression patterns (Figs 3, S7), suggesting that these genes may play fundamental roles in development of these tissue types.

AZ-enriched co-expression modules are largely nonoverlapping between species

Each species had AZ-specific co-expression modules. Rice had one AZ module (black, 962 genes) at the young stage and one (midnightblue, 300 genes) at the old stage (Fig. 4a,b). *Brachypodium* also had one at each stage (young: orange, 111 genes; old: darkred, 861 genes) (Fig. 4c,d). *Setaria* had two AZ modules at the young stage (purple and darkgrey, 298 and 327 genes, respectively) (Fig. 4e,f), but none at the old stage (given our module identification criteria). No module is upregulated in the AZ at both young and old stages in any species, suggesting different gene co-expression networks controlling AZ development at the two stages (Fig. 4a–f).

We then compared shared genes between the AZ modules. By contrast with the conserved developmental stage modules (Fig. 3), AZ modules share many fewer genes (Fig. 4g; Table S6). Only three pairs of comparisons show significant overlap, including the young stage AZ modules Os black vs Sv darkgrey (Fisher's exact test, $P = 0.003$) and Bd orange vs Sv darkgrey (Fisher's exact test, $P = 0.03$), and the old stage modules Os midnightblue vs Bd darkred (Fisher's exact test, $P = 0.002$), with 47, 8 and 41 shared genes, respectively (Fig. 4g). Surprisingly, the set of genes preferentially expressed in the AZ was no more similar between rice and *Brachypodium* than between those species and *Setaria*, despite the similar position and anatomy of the AZ in the former two species (Figs 1, 2).

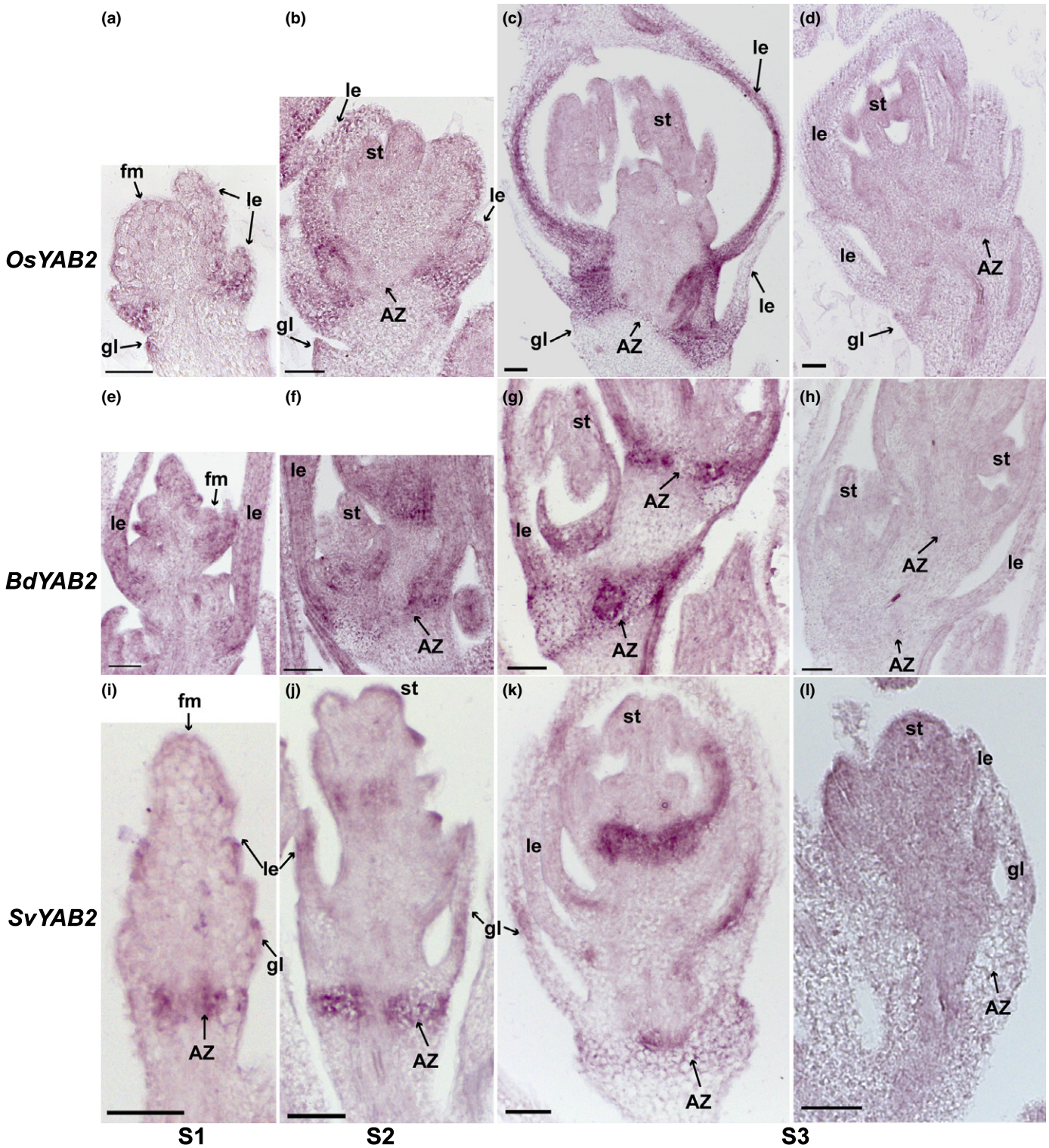

Fig. 6 *YAB2* is expressed in the abscission zone (AZ) and floral bracts by *in situ* hybridisation. *In situ* hybridisation of *YAB2* in (a–d) rice, (e–h) *Brachypodium* and (i–l) *Setaria* was performed at (a, e, i) floret meristem stage (S1), (b, f, j) anther differentiation stage (S2) and (c, g, k) a stage when anthers were fully developed (S3). (d, h, l) are sense probe controls of *YAB2* in (d) rice, (h) *Brachypodium* and (l) *Setaria*. fm, floral meristem; gl, glume; le, lemma; st, stamens. Bars, 50 μm.

Only three genes are shared among the young stage AZ modules of all three species: *MYB26*, *4-COUMARATE:CoA LIGASE 3* and a gene with a possible lysine decarboxylase domain (Table S7). The first two of these may regulate lignification or other aspects of secondary cell wall development (Yang *et al.*, 2007; Li *et al.*, 2015), while the role of the putative lysine decarboxylase is unknown.

We addressed the possibility that our result could be biased by the use of only one-to-one orthologues and WGCNA. As an alternative approach, we examined all of the expressed genes in

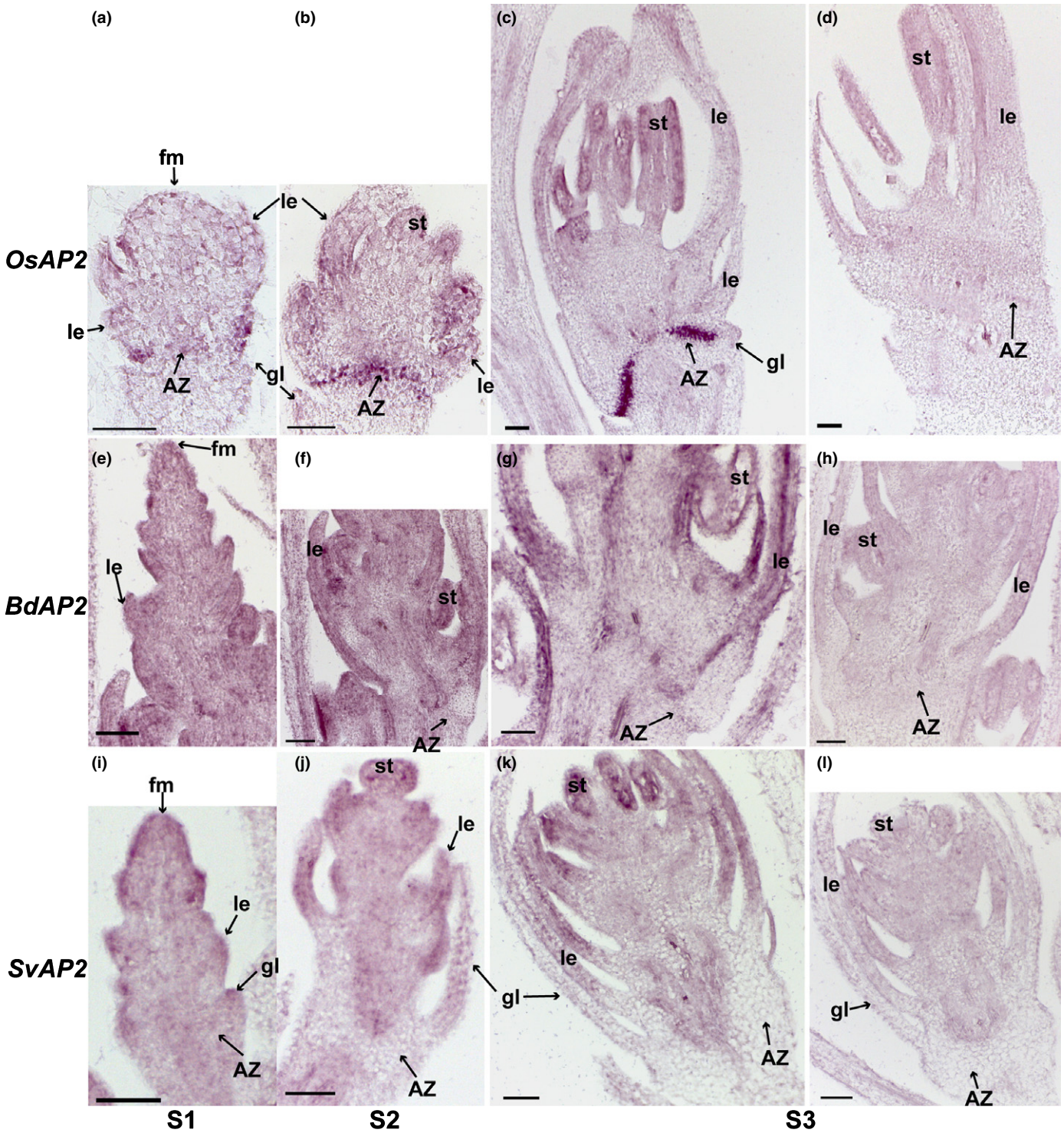

Fig. 7 *AP2* is highly enriched in the abscission zone (AZ) in rice but not in *Brachypodium* or *Setaria*. *In situ* hybridisation of *AP2* in (a–d) rice, (e–h) *Brachypodium* and (i–l) *Setaria* was performed at (a, e, i) floret meristem stage (S1), (b, f, j) anther differentiation stage (S2) and (c, g, k) a stage when anthers were fully developed (S3). (d, h, l) are sense probe controls of *AP2* in (d) rice, (h) *Brachypodium* and (l) *Setaria*. fm, floral meristem; gl, glume; le, lemma; st, stamens. Bars, 50 μm.

each individual species and identified DEG in A vs L or A vs U comparisons. Rice, *Brachypodium* and *Setaria* had 2137, 2524 and 4072 DEG, respectively (Table S8). SOM clustering identified one AZ-enriched cluster in each species at the young stage, with 318, 245 and 467 genes in rice, *Brachypodium* and *Setaria*, respectively (Fig. S8). At the old stage, rice had one AZ-specific cluster of 121 genes and *Brachypodium* had two clusters of 252 and 301 genes each (Fig. S8). Consistent with our WGCNA

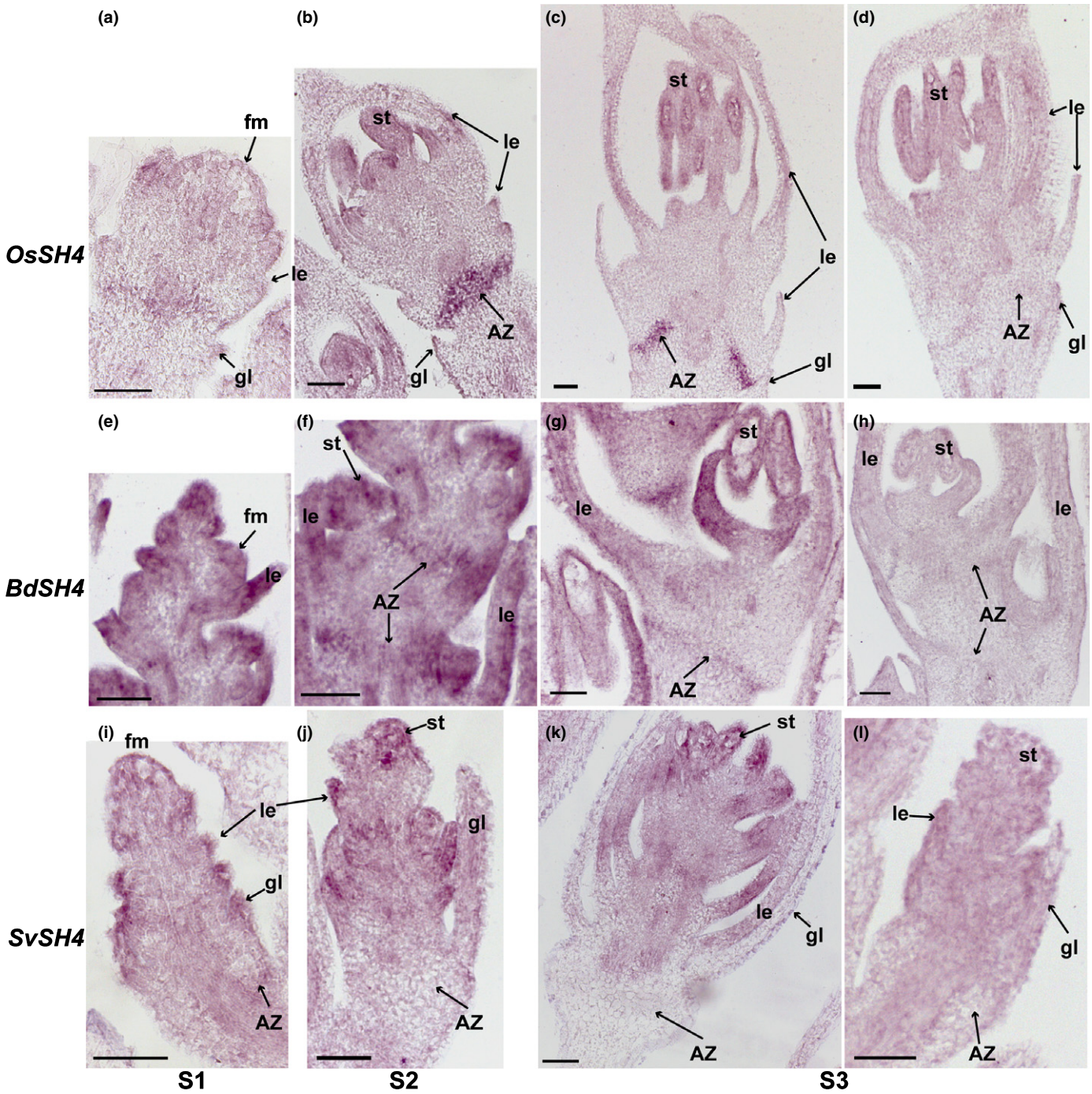

Fig. 8 *SH4* is expressed in the abscission zone (AZ) in rice and *Brachypodium* but not in *Setaria*. *In situ* hybridisation of *SH4* in (a–d) rice, (e–h) *Brachypodium* and (i–l) *Setaria* was performed at (a, e, i) floret meristem stage (S1), (b, f, j) anther differentiation stage (S2) and (c, g, k) a stage when anthers were fully developed (S3). (d, h, l) are negative controls with sense probes of *SH4* in (d) rice, (h) *Brachypodium* and (l) *Setaria*. fm, floral meristem; gl, glume; le, lemma; st, stamens. Bars, 50 μm.

results, no obvious AZ clusters are observed at the old stage in *Setaria* (Fig. S8c).

Comparing DEGs among AZ clusters again finds little overlap (Fig. S9; Table S9), suggesting that the AZ transcriptome is largely nonconserved across grass species regardless of the AZ position. However, three genes appear in the AZ clusters in all three species, two of which, *MYB26* and the gene with a possible lysine decarboxylase domain, were also identified by WGCNA (Table S7). We validated expression of *MYB26* by *in situ* hybridisation. The gene is indeed specifically expressed in the AZ of rice and *Brachypodium* (Fig. 4h–k), although expression in *Setaria* was not detectable (Fig. S10).

New Phytologist

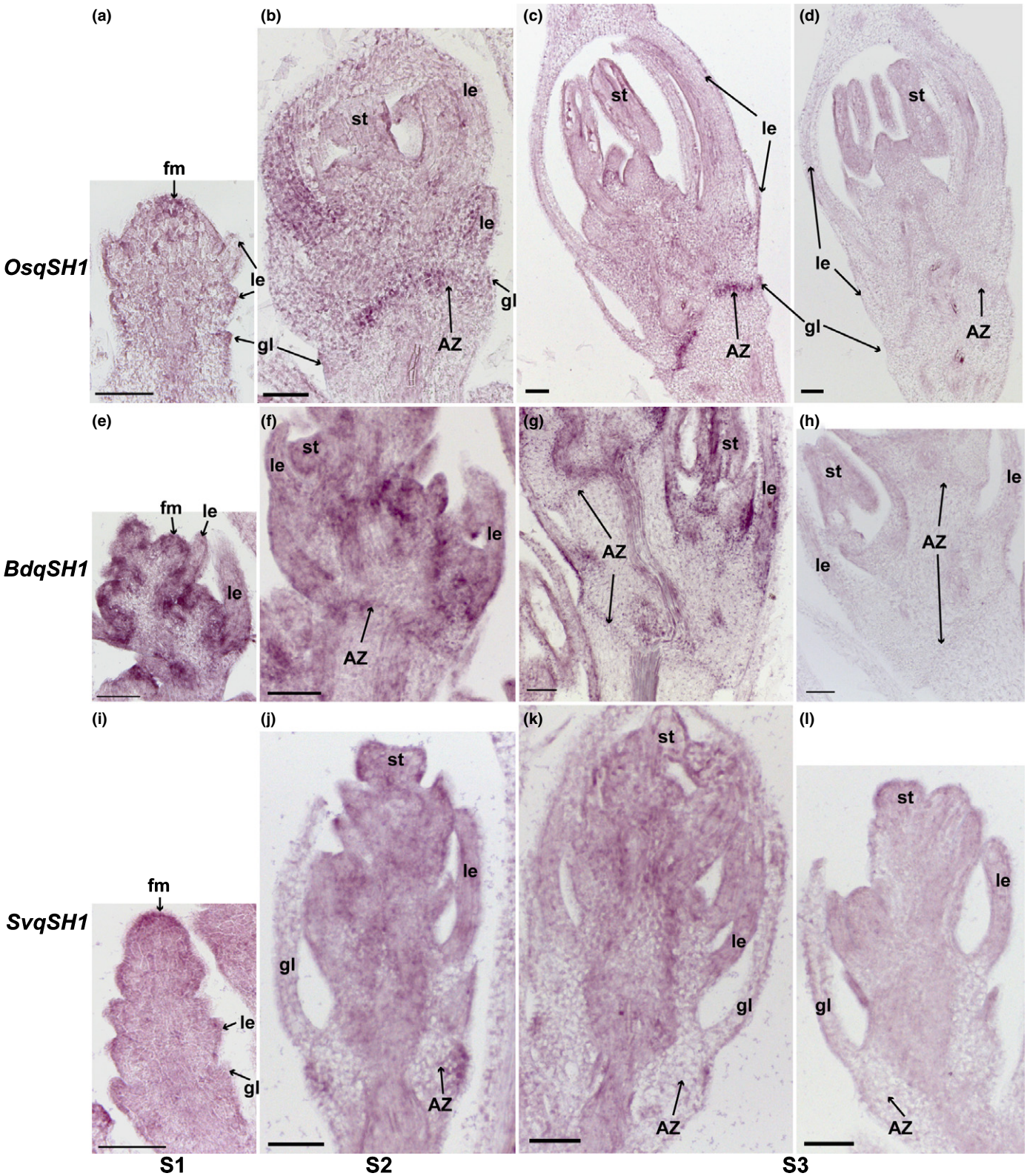

Fig. 9 *qSH1* is expressed in the abscission zone (AZ) in all three species with different expression patterns. *In situ* hybridisation of *qSH1* in (a–d) rice, (e–h) *Brachypodium* and (i–l) *Setaria* was performed at (a, e, i) floret meristem stage (S1), (b, f, j) anther differentiation stage (S2) and (c, g, k) a stage when anthers were fully developed (S3). (d, h, l) are sense probe controls of *qSH1* in (d) rice, (h) *Brachypodium* and (l) *Setaria*. fm, floral meristem; gl, glume; le, lemma; st, stamens. Bars, 50 μm.

Orthologues of previously identified AZ genes show different co-expression networks

To validate our RNA-Seq analysis, we examined the co-expression network of known AZ genes. In rice, the known AZ genes *YAB2/ObSH3/SH1* (Lin *et al.*, 2012; Lv *et al.*, 2018), *AP2/SHAT1/Q* (Simons *et al.*, 2006; Zhou *et al.*, 2012), *SH4* (Li *et al.*, 2006), *qSH1* (Konishi *et al.*, 2006) and *SH5* (Yoon *et al.*, 2014) are all assigned to the AZ-specific module at the young stage (black) of the WGCNA analysis (Fig. 5a; Table S6). *AP2*, *SH4* and *qSH1* also fall in AZ-specific clusters in the SOM analysis (Table S8). In *Setaria*, *YAB2/ObSH3/SH1* (Lin *et al.*, 2012; Odonkor *et al.*, 2018) is classified in the AZ module at the young stage in both WGCNA (purple module) and SOM analysis (Fig. 5a; Tables S6, S8). These results give credence to our RNA-Seq co-expression data. However, of the known AZ genes, only *SH4* is found in an AZ module (orange) in *Brachypodium*. Other supposedly AZ-specific genes show various co-expression patterns (Fig. 5a), suggesting that their roles in AZ development may not be conserved across species.

We next compared the co-expression networks of *YAB2*, *AP2*, *SH4* and *qSH1* in each species, using connectivity (sum of correlation strength with all other genes, k) as a measure of importance of a gene. The orthologues of these genes differ among species in connectivity and numbers of co-expressed genes (Fig. 5; Table S10). For example, although both *OsYAB2* and *SvYAB2* are in AZ modules, *OsYAB2* has a connectivity of 5.8 and 13 co-expressed genes, whereas *SvYAB2* has a connectivity of 37.7 and 413 co-expressed genes (Fig. 5a,b), implying regulatory divergence of *YAB2* in rice and *Setaria*.

We then hypothesised that the 'AZ-specific' genes might share co-expression networks, even if those networks are expressed at different positions in the plant. In other words, a subset of co-expressed genes might shift their spatial expression patterns all together. Indeed, except *YAB2*, which has many fewer co-expressed genes in rice and *Brachypodium*, networks for the other three genes all show highly significant overlap in all pairwise comparisons (Fisher's exact test, $P < 0.001$) (Fig. 5b–e), suggesting partially conserved co-expression networks. *AP2, SH4* and *MYB26* are highly connected (i.e. co-regulated) with each other in rice, and *AP2* and *SH4* are also connected in *Setaria* to a lesser degree, although not specifically enriched in the AZ (Fig. S11; Table S9).

Orthologues of known AZ genes exhibit different temporal and spatial expression patterns

Our RNA-Seq and co-expression analyses suggest that AZ transcriptomes at the same floral developmental stage differ substantially among the three species (Fig. 4). Even genes known to be critical for AZ development in one species do not have conserved expression patterns in the other two (Fig. 5). One possible explanation is that these genes might be expressed in the AZ but at times different from the two captured by our RNA-Seq experiment. We tested this hypothesis with *in situ* hybridisation on *YAB2*, *AP2, SH4* and *qSH1*, which are highly expressed in rice early in spikelet development at or before the young stage of our RNA-Seq (Zhou *et al.*, 2012; Lv *et al.*, 2018). Therefore, we used *in situ* hybridisation at three stages of development in which the oldest (Stage 3, S3 hereafter) is the young stage of our RNA-Seq experiment (Figs 6–9). Of the *YAB2* orthologues, only *SvYAB2* is specifically enriched in the AZ compared with the adjacent tissues in all three tested stages, and expression in the AZ is even higher in stage 1 (S1) and stage 2 (S2) (Fig. 6i–l), consistent with WGCNA and SOM (Tables S6, S8). *SvYAB2* is also expressed at the base of the upper floret (Fig. 6i–l), presumably the secondary AZ of *Setaria*, in which abscission occurs less frequently and later than at the pedicel AZ (Hodge & Kellogg, 2016). By contrast, *OsYAB2* and *BdYAB2* are expressed in the AZ as well as the bracts above (lemmas and paleas) so are not AZ specific, although expression in the AZ is more pronounced at stage 3 (S3) (Fig. 6a–h).

In the case of *AP2*, the rice orthologue is strongly expressed in the AZ at all three stages, and relatively weakly expressed in floral bracts at S2 and stamens at S3 (Fig. 7a–d), while expression of *BdAP2* and *SvAP2* is more obvious in bracts and stamens. *BdAP2* may be weakly expressed in the AZ at S2 and S3 (Fig. 7e–h), whereas *SvAP2* is not detectable in the AZ (Fig. 7i–l).

For *SH4*, both *OsSH4* and *BdSH4* are preferentially expressed in the AZ compared with the adjacent tissues at all three stages (Fig. 8a–h), while no expression was observed in the AZ in *Setaria* (Fig. 8i–l), consistent with our RNA-Seq results (Fig. 5a). Besides expression in the AZ, *SH4* is also expressed in floral bracts and stamens in all three species, although the signal intensity varies (Fig. 8).

qSH1 is expressed in the AZ in all three species (Fig. 9). In rice, expression of *qSH1* is specific to the AZ, especially at S3 (Fig. 9a–d), while *BdqSH1* has strong expression in the upper tissues in addition to the AZ (Fig. 9e–h). *SvqSH1* is expressed in the AZ at S2 and weakly at S3, and expression is restricted to the outer cell layers at the approximate position of the AZ (Fig. 9i–l).

In summary, all of these genes are involved in aspects of flower development and have somewhat conserved expression patterns. However, the intensity and spatial and temporal specificity of their expression in the AZ varies among species, implying relatively little conservation in genetic regulation of AZ development.

Discussion

Divergent gene co-expression networks in a conserved background

Contrary to our initial hypothesis, we find that the AZs of rice, *Brachypodium* and *Setaria* differ in anatomy, cell wall structure and gene expression. No expression modules are completely conserved among species, and only a handful of genes are AZ specific in their expression in all cases (Figs 4, S9; Tables S6, S8). However, *c.* 90% of the expressed one-to-one orthologous genes are shared among all three species, demonstrating that the set of genes expressed in the broad region studied is largely conserved (Fig. S1). In addition, modules with differential expression

between early and late development show high preservation among species (Figs 3, S5). Therefore, the set of AZ genes is a species-specific subset of a generally conserved set of expressed genes, suggesting conservation of the overall developmental program through time but distinct spatial control, consistent with the observation of different position and anatomy of the AZ in rice, *Brachypodium* and *Setaria* (Figs 1, 2). We investigated specifically genes that had been identified in previous studies, generally in rice, as being required for shattering. We confirmed tissue specific expression with *in situ* hybridisation, and found, as with the RNA-Seq experiments, that the details of when and where the genes are deployed differs among species (Figs 6–9).

Genes controlling AZ development show both convergence and divergence at different phylogenetic scales

So far, *YAB2/ObSH3/SH1* and *AP2/SHAT1/Q* are the only two genes demonstrated by genetic studies to play conserved roles in AZ development across different tribes of Poaceae (Simons *et al.*, 2006; Lin *et al.*, 2012; Zhou *et al.*, 2012; Lv *et al.*, 2018). Interestingly, in *Arabidopsis thaliana*, *AtYAB1*, *AtYAB3* and *AtAP2* also regulate fruit AZ development (Dinneny *et al.*, 2005; Ripoll *et al.*, 2011). Similarly, the orthologous Arabidopsis *AtRPL* (Roeder *et al.*, 2003) and rice *qSH1* (Konishi *et al.*, 2006) are both involved in AZ development, although *AtRPL* is expressed in the pod replum adjacent to the AZ, while *qSH1* is expressed in the AZ (Fig. 9). The Arabidopsis homologue of *MYB26* regulates secondary cell wall thickening and anther dehiscence (Yang *et al.*, 2007), and our results suggest that *MYB26* also functions in AZ development in at least *Brachypodium* and rice (Figs 4h–k, S10). Together, these results suggest convergence or conservation of AZ genetic control with modification of expression pattern and molecular function of the genes.

Other evidence suggests mechanistic divergence. IND orthologues are confined to the Brassicaceae, indicating neofunctionalisation of IND in AZ development of that family (Pabón-Mora *et al.*, 2014). *Btr1/Btr2*, identified by their role in domestication of barley and wheat (Pourkheirandish *et al.*, 2015; Avni *et al.*, 2017), encode proteins with unknown functions. Their closest homologues in *Brachypodium* and rice only share limited sequence identity with barley and wheat, suggesting that the *Btr1/2* genes may provide novel controls of AZ development in some Triticeae species (Pourkheirandish *et al.*, 2015).

Previous studies mostly focus on one or a few genes in the genetic pathway controlling AZ development. Our transcriptomic study suggests high divergence in gene co-expression patterns in grass AZ regulation, and genes indispensable for AZ function are few.

Differential lignification may not be required for abscission

Our results provide some hypotheses regarding AZ function. Studies on AZ anatomy in eudicot leaves showed that the cells in the AZ are often small and nonlignified (Sexton & Roberts, 1982). Genetic studies of the AZ in Arabidopsis and rice also suggest that cell size and differential lignification between the AZ and the adjacent cell layers are required for abscission, possibly creating tension between cell layers and therefore promoting cell separation. For example, in AZ development of Arabidopsis fruit, *SHP1, SHP2*, and *IND* regulate AZ differentiation and lignification of adjacent cells (Liljegren *et al.*, 2000, 2004). When these genes are mutated, lignification is lost and dehiscence fails. In rice *yab2/obsh3*, *sh4*, *qsh1* and *ap2/shat1* mutants, lignin is deposited ectopically in the AZ and abscission is reduced or lost (Konishi *et al.*, 2006; Li & Gill, 2006; Zhou *et al.*, 2012; Lv *et al.*, 2018), suggesting that the lack of lignin and smaller cell size of the AZ are essential for AZ function in rice. However, we show that this pattern does not apply to all grass species. In *Brachypodium*, lignin is deposited in both the AZ and the neighbouring cells (Fig. 2b), while in *Setaria*, lignin is only observed at the epidermal layer and no cell-size difference was observed in the AZ (Fig. 2c). Similarly, lignification occurs throughout the AZ and the surrounding tissues of wild barley (Pourkheirandish *et al.*, 2015), further supporting the hypothesis that lignification pattern is not a universal prerequisite for abscission.

The differences in AZ anatomy are consistent with different expression patterns of genes controlling the anatomy. For example, *AP2* is highly enriched in the AZ of rice, and loss of *AP2* function results in loss of nonlignified and small-sized cells (Zhou *et al.*, 2012). However, as *Brachypodium* has lignin in the AZ and the *Setaria* AZ cells are not smaller than surrounding cells (Fig. 2), *AP2* may not be important for AZ development in those two species. Consistent with this interpretation, *AP2* transcripts are not enriched in the AZ of *Brachypodium* or *Setaria* (Figs 5, 7).

Change in position of the AZ in evolutionary time

Heterotopy, expressing a developmental program in a different organismal position, has been hypothesized as a mechanism for morphological change. Haeckel (1866) used the term to interpret the origin of reproductive organs from different germ layers in different organisms during embryo development, although without the advantage of knowledge of genomes and molecular biology. In its modern sense, heterotopy can be interpreted as a change in the spatial expression pattern of genes essential for a developmental process, leading to positional change of the whole developmental program (Baum & Donoghue, 2002).

Both literature and our data support a much more limited version of heterotopy, applying to only a few key genes. For example, the functional conservation of FUL/SHP/ALC/IND pathway in fruit AZ development in the Brassicaceae regardless of AZ position (Ferrándiz *et al.*, 2000; Avino *et al.*, 2012; Lenser & Theißen, 2013) may be considered as heterotopy. Similarly, the expression of *YAB2*, *qSH1* and *MYB26* in the AZ in the rachilla in rice and *Brachypodium* but in the pedicel in *Setaria* may be another example (Figs 4h–k, 6, 9; Tables S7, S9). However, heterotopy is an insufficient explanation if applied to the whole transcriptomic network. The ancestral position of the AZ is conserved in rice and *Brachypodium* and is shared by most species in Poaceae (Fig. 1). Despite this positional conservation, the underlying development and genetic control differs between the two. The AZ of rice and *Brachypodium* differ in cell wall

composition and thickness (Fig. 2). The number of shared genes enriched in the AZs of both *Brachypodium* and rice is as small as that between *Brachypodium* and *Setaria* or rice and *Setaria* (Figs 4, S9), suggesting that AZ regulatory networks are diverse regardless of position. Even in the case where genes in the same pathway shift together to a new position, such as co-expression of *SH4* and *AP2* in both rice and *Setaria* (Fig. S11), their interactions with genes expressed in the new position differ, and therefore cause distinct co-expression networks (Fig. 5).

The results presented here raise important questions about how gene regulatory networks change over time and the speed with which this change occurs. Our results show that similar AZ positions do not necessarily reflect similar regulatory networks. However, we do not know whether gene expression patterns of closely related species with different AZ positions are conserved. For example, both *Setaria* and sorghum belong to the subfamily Panicoideae, and their AZs are located in the pedicel and rachis, respectively (Fig. 1). Whether the underlying gene networks are shared is unknown. Likewise, we lack data comparing *Elymus* and *Hordeum*, two genera in the tribe Triticeae, although their histology and AZ positions differ markedly (Yu & Kellogg, 2018). Our data provide a framework for future studies on the rewiring of gene networks over evolutionary time.

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Author contributions

AND and EAK designed the research and secured funding. AND, EAK and YY designed the experimental approach and YY performed the experiments. HH and YY analysed the RNA-Seq data. EAK and YY drafted the manuscript, with input from AND and HH. All authors contributed to ideas, discussed the results and edited the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 The abscission zones (AZs) have different cell wall components at different developmental stages in rice, *Brachypodium* and *Setaria*.

Fig. S2 Weighted gene co-expression network analysis (WGCNA) identified 15 co-expression modules in rice.

Fig. S3 Weighted gene co-expression network analysis (WGCNA) identified 17 co-expression modules in *Brachypodium*.

Fig. S4 Weighted gene co-expression network analysis (WGCNA) identified 18 co-expression modules in *Setaria*.

Fig. S5 Weighted gene co-expression network analysis (WGCNA) modules in one species are moderately preserved in the other two species.

Fig. S6 Numbers of overlapping genes in pairwise comparisons of modules between species.

Fig. S7 Modules with highly significant overlapping genes have similar expression patterns.

Fig. S8 Expression patterns of self-organising map (SOM) clustering in rice, *Brachypodium* and *Setaria*.

Fig. S9 AZ clusters generated by self-organising map (SOM) have a limited number of overlapping genes between species.

Fig. S10 *MYB26* is expressed in the abscission zone in rice and *Brachypodium*.

Fig. S11 *SH4* is co-expressed with *AP2* and/or *MYB26*.

Table S1 Probe primers used in *in situ* hybridization.

Table S2 Ancestral state reconstruction of the abscission zone positions in grasses.

Table S3 DESeq2 results of rice.

Table S4 DESeq2 results of *Brachypodium*.

Table S5 DESeq2 results of *Setaria*.

Table S6 Weighted gene co-expression network analysis (WGCNA) modules of one-to-one orthologues in all three species.

Table S7 Pairwise comparisons of overlapping genes between the abscission zone modules by weighted gene co-expression network analysis (WGCNA) in rice, *Brachypodium* and *Setaria*.

Table S8 Self-organising map clusters of DEG in rice, *Brachypodium* and *Setaria*.

Table S9 Pairwise comparisons of overlapping genes between the abscission zone clusters by self-organising map analysis in rice, *Brachypodium* and *Setaria*.

Table S10 Weights of all edges of *YAB2*, *AP2*, *SH4* and *qSH1* in rice, *Brachypodium* and *Setaria*.

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