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RESEARCH PAPER

Differential activity of F-box genes and E3 ligases distinguishes sexual versus apomictic germline specification in *Boechera*

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Abstract

Germline specification is the first step during sexual and apomictic plant reproduction, and takes place in the nucellus of the ovule, a specialized domain of the reproductive flower tissues. In each case, a sporophytic cell is determined to form the sexual megaspore mother cell (MMC) or an apomictic initial cell (AIC). These differ in their developmental fates: while the MMC undergoes meiosis, the AIC modifies or omits meiosis to form the female gametophyte. Despite great interest in these distinct developmental processes, little is known about their gene regulatory basis.
To elucidate the gene regulatory networks underlying germline specification, we conducted tissue-specific transcriptional profiling using laser-assisted microdissection and RNA sequencing to compare the transcriptomes of nucellar tissues between different sexual and apomictic *Boechera* accessions representing four species and two ploidy levels. This allowed us to distinguish between expression differences caused by genetic background or reproductive mode. Statistical data analysis revealed 45 genes that were significantly differentially expressed, and which potentially play a role for determination of the reproductive mode. Based on annotations, these included F-box genes and E3 ligases that most likely relate to genes previously described as regulators important for germline development. Our findings provide novel insights into the transcriptional basis of sexual and apomictic reproduction.

Keywords: Apomixis, *Boechera*, E3-ligase, F-box, laser-assisted microdissection, megasporogenesis, nucellus tissue, plant reproduction, RNA-seq, tissue-specific transcriptome.

Abbreviations: *AGO9*, *ARGONAUTE 9*; AIC, apomictic/aposporous initial cell; *RMA3*, *RING MEMBRANE-ANCHOR 3*; *AtPXG2*, *Arabidopsis thaliana* PEROXYGENASE 2; CUL3, CULLIN3; *CYP*, cytochrome P450; DEG, differentially expressed gene; FMS, functional megaspore; GH3, GRETCHEN HAGEN 3; GO, Gene Ontology; KRP, KIP-RELATED PROTEIN; LAM, laser-assisted microdissection; MMC, megaspore mother cell; *OSD1*, *OMISSION OF SECOND DEVISION 1*; *RDR6*, *RNA-DEPENDENT RNA POLYMERASE 6*; SCF, Skp1-Cullin1-F-box protein ligase complex; *SGS3*, *SUPPRESSOR OF GENE SILENCING 3*; *SPO11-1*, *SPORULATION 11-1*; TRAF-like, Tumor Necrosis Factor Receptor (TNF-R) Associated Factor-like; TRAMGaP, TRAF Mediated Gametogenesis Progression; *UBC28*, *UBIQUITIN-CONJUGATING ENZYME 28*.

Introduction

The acquisition of reproductive or germline fate is a key step in the life cycle of higher plants, as it mediates the transition from the sporophytic to the gametophytic generation. The formation of the female germline in sexual plants initiates with the selection of a single sporophytic cell of the nucellus for reproductive fate, the megaspore mother cell (MMC). During megasporogenesis, the MMC undergoes meiosis to generate the functional megaspore (FMS) as the first cell of the gametophytic generation. Subsequently, three rounds of mitosis starting from the FMS followed by cellularization (gametogenesis) lead to the formation of the mature gametophyte. This consists of two female gametes (egg and central cell), and accessory synergid cells and antipodals (Sprunck and Gross-Hardt, 2011). Double-fertilization of the egg and central cell, which gives rise to the embryo and endosperm, respectively, completes the life cycle by starting a new sporophytic generation.

Apart from sexual reproduction through seeds, more than 400 angiosperm species produce seeds asexually in a process called gametophytic apomixis (hereafter referred to simply as apomixis; Asker and Jerling, 1992; Carman, 1995; Spillane *et al.*, 2001). Apomixis comprises three consecutive developmental processes that differ from sexual reproduction. It begins with the formation of an unreduced female gametophyte (apomeiosis), which subsequently enables fertilization-independent embryogenesis (parthenogenesis), and fertilization-dependent or autonomous endosperm development (Nogler, 1984; Asker and Jerling, 1992). Generally, two major types of apomixis can be distinguished based on how the female germline is established, namely diplospory and apospory, (Nogler, 1984; Asker and Jerling, 1992; Koltunow, 1993; Bicknell and Koltunow, 2004). In diplospory, a single apomictic initial cell (AIC) is specified instead of the MMC. It typically omits meiosis, or enters a form of meiosis that is modified to proceed as a mitotic-like division, thus leading to the formation of an unreduced FMS. In contrast, apospory is characterized by an aposporous initial cell (also termed AIC), which specifies adjacent to a sexual MMC. This cell directly differentiates into an unreduced FMS without undergoing meiosis. In each case, the FMS develops further into a mature gametophyte as during sexual reproduction; however, gametes typically remain genetically identical to the mother plant. Subsequently, clonal offspring is formed by parthenogenesis.

As it is foreseen that complex genotypes from hybrid crops can be fixed over subsequent generations by harnessing apomixis, it holds great potential for use in agriculture. Hence, the understanding of germline specification in both sexual and apomictic reproduction is not only of particular scientific interest, but also can have practical implications in the future. Despite increasing interest in the developmental processes that lead to apomixis, knowledge about the underlying gene regulatory mechanisms is still scarce.

A widely accepted hypothesis states that apomixis results from asynchronous expression of genes of the sexual pathway (Carman, 1997). In accordance with this, there is increasing evidence to suggest that apomixis evolved from deregulation of the sexual reproductive pathway several times independently (Koltunow, 1993; Grimanelli *et al.*, 2001; Grossniklaus *et al.*, 2001; Koltunow and Grossniklaus, 2003; Tucker *et al.*, 2003; Sharbel *et al.*, 2009, 2010).

Studies in sexual model species such as Arabidopsis, *Zea mays*, and *Oryza sativa* have provided strong evidence that complex regulatory networks are required for megasporogenesis (Schmidt *et al.*, 2015; Nakajima, 2018). Interestingly, mutations in a number of genes important for sexual germline development lead to aposporous- or diplosporous-like phenotypes in sexual species (Barcaccia and Albertini, 2013; Hand and Koltunow, 2014; Schmidt *et al.*, 2015). These include regulatory components involved in restricting reproductive fate (Hand and Koltunow, 2014; Schmidt *et al.*, 2015). In contrast to aposporous apomicts where the AIC typically forms adjacent to the sexual MMC, the formation of only one germline in each ovule is tightly controlled in sexual species (Albertini *et al.*, 2005; Albrecht *et al.*, 2005; Colcombet *et al.*, 2005; Olmedo-Monfil *et al.*, 2010; Schmidt *et al.*, 2011; Singh *et al.*, 2011, 2017; Wang *et al.*, 2012; Zhao *et al.*, 2017b; Cao *et al.*, 2018). Developmental alterations resembling apospory have been described for mutations that disrupt members of epigenetic regulatory and small RNA pathways, for example Arabidopsis *ARGONAUTE 9* (*AGO9*), *RNA-DEPENDENT RNA POLYMERASE 6* (*RDR6*), and *SUPPRESSOR OF GENE SILENCING 3* (*SGS3*) (Ravi *et al.*, 2008; Garcia-Aguilar *et al.*, 2010; Olmedo-Monfil *et al.*, 2010; Singh *et al.*, 2017). In contrast, phenotypes resembling diplospory are induced in maize mutant lines of *AGO104*, a homologue of Arabidopsis *AGO9*, in addition to Arabidopsis *DYAD*, and in certain combinations of mutants in meiotic genes, for example in *SPORYLATION 11-1* (*SPO11-1*), which is involved in pairing and recombination of homologous chromosomes, in addition to *REC8*, which takes part in chromatid segregation, and *OMISSION OF SECOND DEVISION 1* (*OSD1*) (Ravi *et al.*, 2008; d'Erfurth *et al.*, 2009; Singh *et al.*, 2011). However, it remains to be determined whether alterations in these genes and pathways underlie reproductive development in natural apomicts.

The genus *Boechera* is increasingly being appreciated as a model for studying apomictic reproduction (Sharbel *et al.*, 2009, 2010; Aliyu *et al.*, 2010, 2013; Corral *et al.*, 2013; Lovell *et al.*, 2013; Schmidt *et al.*, 2014; Mau *et al.*, 2015; Rojek *et al.*, 2018). While apomixis is almost exclusively restricted to polyploids in most species, it occurs at low ploidy levels, including diploid and triploid, throughout *Boechera* (Bicknell and Koltunow, 2004; Windham and Al-Shehbaz, 2007; Lovell *et al.*, 2013). Furthermore, both apospory and diplospory are represented (Mateo de Arias, 2015; Rojek *et al.*, 2018). Most frequently diplospory is of the *Taraxacum*-type, which is characterized by first division restitution and normal meiosis II, but the *Antennaria*-type also occurs where the AIC directly adopts FMS identity without meiosis (Naumova *et al.*, 2001; Mateo de Arias, 2015; Rojek *et al.*, 2018).

Transcriptional profiling has previously been used to identify genes involved in apomictic development. Studies in *Boechera* have compared entire ovules of sexual and apomictic

plants, while cell type-specific investigations have compared the sexual MMC of Arabidopsis and the apomictic AIC of triploid *B. gunnisoniana* (Sharbel *et al.*, 2009, 2010; Schmidt *et al.*, 2011, 2014). This has revealed several hundreds of genes that are differentially expressed in apomictic versus sexual germlines, which are involved in cell-cycle control, and epigenetic, transcriptional, and hormonal regulation (Schmidt *et al.*, 2014).

To narrow down candidate genes of putative importance for apomixis and to minimize confounding effects of ploidy or species differences, we conducted a comprehensive investigation of two sexual and four apomictic *Boechera* accessions. We used laser-assisted microdissection combined with RNA sequencing to perform a comparative transcriptome analysis of nucellus tissues harbouring the MMC or AIC. This study therefore allowed us to examine germline specification in a tissue-specific manner. Particularly with regard to the female germline that develops inside of sporophytic flower tissues, cell or tissue type-specific investigations have great potential to identify important genes that might otherwise be overlooked due to an over-abundance of sporophytic tissues in the sample (Wuest *et al.*, 2010; Schmid *et al.*, 2012; Schmidt *et al.*, 2012; Florez Rueda *et al.*, 2016). Moreover, any relevant signalling processes involving cells of the surrounding nucellus tissue can simultaneously be detected. In this way, we identified 45 genes that were consistently differentially regulated between sexual and apomictic accessions, pointing to the involvement of genes and pathways previously described as regulators for germline development, namely cell-cycle regulation and stress responses.

Material and methods

Plant material

Seeds of *Boechera* Á.Löve & D.Löve were kindly provided by Timothy F. Sharbel (Global Institute for Food Security, University of Saskatchewan, Canada) and Thomas Mitchell-Olds (Duke University, USA), or were obtained from the laboratory of Ueli Grossniklaus (University of Zürich, Switzerland). Seeds were stratified for at least 1 week at 4 °C before surface-sterilization and germination as described previously (Wuest *et al.*, 2010). About 2 weeks after germination, seedlings were transferred to a mixture of soil and sand as described previously (Schmidt *et al.*, 2014). Plants were germinated and grown in a growth chamber with 55% relative humidity and under 16/8 h light/darkness at 20–22 °C. For each apomictic plant, ploidy was confirmed by flow cytometry using a CyStainUV Precise P Kit (Sysmex Partec, Görlitz', Germany) on a Partec CyFlow Space instrument following the manufacturer's instructions.

Preparation of plant material for laser-assisted microdissection

For laser-assisted microdissection (LAM), flower buds with ovules were harvested that harboured either a MMC or AIC, predominantly before meiosis or apomeiosis, respectively, but covering a range of developmental stages until approximately prophase of meiosis I. Tissue fixation and manual embedding was done as described by Wuest *et al.* (2010), except that we applied a xylene gradient with 25%, 50%, 75%, and twice 100% xylene in EtOH. After blocking in Paraplast X-tra (Leica Biosystems, Nussloch, Germany), samples were stored at 4 °C until further use. Thin sections of 7 µm were prepared from embedded tissues using a RM2255 microtome (Leica Biosystems), mounted on 1.4 µm PET-membrane frame slides (Leica Microsystems, Wetzlar, Germany), and de-waxed according to Schmidt *et al.* (2011).

Laser-assisted microdissection

LAM was performed using an Eclipse Ti microscope (Nikon Instruments) equipped with a mmiCellCut instrument (Molecular Machines & Industries, Eching, Germany). Nucellus tissue was isolated from 7 µm thin sections using a 40× SPF Ph2 ELWD objective. As controls for RNA quality corresponding to the samples, ~10 ovary sections from the same slides as the nucelli sections were isolated afterwards. All samples were stored at −80 °C until RNA extraction.

RNA extraction and amplification

RNA extraction from LAM samples including DNA digest was done using PicoPure RNA isolation kits (ThermoFisher Scientific) following the manufacturer's instructions, except that 15 µl extraction buffer was used. Each sample contained pooled individuals from the same accession to obtain 38–226 nucelli sections per sample (Supplementary Table S1 at *JXB* online). The RNA integrity of controls was determined using Agilent 2100 Bioanalyzer RNA Pico Chips. Good and reproducible RNA integrity was consistently achieved (Supplementary Fig. S1A).

A SMARTseq v4 Ultra Low Input RNA Kit for Sequencing (Takara Bio USA) was used for linear amplification of mRNA derived from nucelli samples following the manufacturer's instructions (Supplementary Fig. S1B, C). Amplified cDNA was purified using AMPure Sample Purification Beads (Beckman Coulter, Brea, USA) and eluted in nuclease-free H_2O and stored at −20 °C until subsequent library preparation.

RNA sequencing

A Nextera XT DNA Library Prep Kit (Illumina) was used to prepare libraries for RNA-seq of the nine samples (Supplementary Table S1) according to the manufacturer's instructions. The quality of individual libraries and equimolar library pool was determined using Qubit (ThermoFisher Scientific) and Bioanalyzer High Sensitivity DNA assays (Agilent) (Supplementary Fig. S1D).

RNA-seq was conducted on one lane of a flow cell on a NextSeq 500 platform (Illumina) by the Deep Sequencing Core Facility of Heidelberg University at the EMBL Genomics Core Facility (EMBL, Heidelberg). The 75-bp single-end protocol was applied with 94 cycles (including index read). Original data files are deposited in the NCBI SRA database with the accession number SRP159014.

Data analysis

Pre-analysis and processing of raw reads

The quality of raw reads was assessed with FastQC version 0.11.2. For trimming with cutadapt version 1.14 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc; Martin, 2011) parameters were set to hard trimming of final base, and quality trimming at the 3′-end with phred score threshold 20. Adapter sequences were provided as fasta files. Trimmed reads shorter than 30 bases were filtered out. The *B. stricta* genome assembly and annotations from Lee *et al.* (2017) were used for read-mapping using STAR version 2.5.3a_modified (Dobin *et al.*, 2013). The number of reads mapped per gene was determined with featureCounts as described previously (Liao *et al.*, 2014; Schmid, 2017).

As quality controls, mapping statistics and gene body coverage were assessed with the modules bam_stat.py and geneBody_coverage.py from the RseQC package version 2.6.4 (Supplementary Fig. S2) (Wang *et al.*, 2012).

Differential gene expression analysis

Read counts derived from featureCounts were used to analyse differential gene expression with the Bioconductor package edgeR version 3.12.1 (Robinson *et al.*, 2010). Samples were grouped by accession and reproductive mode, low-expressed genes were filtered out (>1 counts per million bases in ≥2 libraries). Normalization factors for libraries were calculated with the TMM-method (Robinson and Oshlack, 2010). Normalized read counts were used for statistical analysis. For pairwise

comparisons between apomictic and sexual accessions, the biological coefficient of variation was set to 0.8 as previously described (Schmidt *et al.*, 2014). For comparisons treating groups of sexual or apomictic accessions as biological replicates, common and tagwise dispersions were estimated by edgeR. To identify genes differentially expressed between any of the accessions, dispersions were estimated using edgeR and a generalized linear model was applied to model all contrasts by grouping samples per accessions. All comparisons were performed with exact test and Benjamini–Hochberg adjustment of the false discovery rate (FDR). Differential expressed genes (DEGs) with FDR≤0.05 were considered significant. To identify genes that were differentially expressed in all comparisons of sexual against apomictic accessions, an intersection of gene lists was calculated in R. DEGs between any of the accessions were afterwards searched for core cell-cycle genes and meiotic genes (Vandepoele *et al.*, 2002; Gutierrez, 2009; Mercier *et al.*, 2015).

Validation of gene expression by comparison to published datasets

Our datasets were compared with published cell- and tissue type-specific transcriptome analyses of megasporogenesis in Arabidopsis and the triploid apomict *B. gunnisoniana* (Schmidt *et al.*, 2011, 2014). Genes were defined to be expressed if they had either ≥10 mapped reads or present calls in ≥3 of 4 microarray replicates (Schmidt *et al.*, 2011, 2014; this study).

Gene ontology analysis.

For gene ontology (GO) analysis we used the Bioconductor package topGO v.2.36.0 (http://bioconductor.org/packages/release/bioc/html/topGO.html). Over-representation of biological processes was done using Fisher's exact test combined with the function 'weight'. Genes with annotations for biological processes in the *B. stricta* genome were used as the gene universe (Lee *et al.*, 2017).

Data visualization

Venn diagrams of overlapping gene expression were constructed using Venny 2.1 or Biovenn for comparison of four or fewer datasets, respectively (http://bioinfogp.cnb.csic.es/tools/venny/index.html; Hulsen *et al.*, 2008).

Heatmaps showing expression levels of DEGs were generated using the R package gplots version 3.0.1. (https://CRAN.R-project.org/package=gplots). Heatmaps were based on $\log_2$-transformed, TMM-normalized read counts generated by NOISeq (Tarazona *et al.*, 2011).

In situ hybridizations

Flower tissues for *in situ* hybridizations were prepared as for LAM, except that 8 µm thin sections were mounted on glass slides and dewaxed with Histoclear (Carl Roth, Karlsruhe, Germany). For cloning of *in situ* probes, RNA was extracted from *B. stricta* acc. LTM buds using an RNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions and reverse-transcribed to cDNA using oligo dT12-dT18 primers and Superscript IV (ThermoFisher Scientific) following the manufacturer's instructions. Sequences for probe synthesis were amplified using the primers listed in Supplementary Table S2. Probe synthesis, labelling with T7 RNA polymerase, and hybridizations were performed as described by Medzihradszky *et al.* (2014). An Axio Imager M1 (Zeiss) was used to capture images, which were cropped and processed in Adobe Photoshop CS2 Version 9.0.

Results

Transcriptome profiling identifies genes expressed in sexual and apomictic nucelli

To gain new insights into the gene regulatory networks that underlie germline specification during sexual and apomictic reproduction, tissue-specific RNA-seq was performed. The study design comprised four obligatory apomictic and two sexually reproducing accessions of the genus *Boechera* (Table 1) (Schranz *et al.*, 2005; Aliyu *et al.*, 2010; Mau *et al.*, 2015; Lee *et al.*, 2017). We selected accessions based on reproductive mode but also to include four different species at two ploidy levels to account for part of the genetic diversity of *Boechera* (Mau *et al.*, 2015). Specifically, we included the sequenced accession *B. stricta* LTM, *B. divaricarpa* as a hybrid of *B. stricta*, together with *B. williamsii* and *B. pallidifolia* (Table 1) (Dobeš *et al.*, 2004a; Lee, 2017). The origin of apomixis in *Boechera* is typically associated with hybridization or intraspecific crosses (Lovell *et al.*, 2013), which might result in broader changes in gene regulation. Therefore, our study design enabled us to distinguish differential expression based on reproductive mode from effects of ploidy or genetic background.

LAM was used to isolate nucelli from developing ovules at the onset of megasporogenesis. Tissues were precisely dissected from dry sections of young flower buds (Fig. 1B); however, minor cross-contamination with surrounding tissue could not be avoided completely. In order to identify genes consistently differentially regulated among all sexual and all apomictic accessions included in the analysis, a total of nine samples was prepared (Supplementary Table S1).

For transcriptional profiling, RNA was extracted, amplified, and subjected to library preparation (Supplementary Fig. S1). RNA-seq was then performed and resulted in individual libraries that comprised 28 396 884–99 921 406 reads of overall good quality (Supplementary Table S1).

After quality controls and trimming, reads were mapped to the *B. stricta* reference genome using STAR (Dobin *et al.*, 2013; Lee *et al.*, 2017). On average, ~98% of the reads

Table 1. *List of Boechera accessions included in this study*

***Boechera* Species**	**Accession no.**	**Reproductive mode**	**Ploidy**	**Reference**
B. divaricarpa	ES517	Apomictic	2*x*	Aliyu *et al.* (2010)
B. pallidifolia	B12-1578	Apomictic	2*x*	Mau *et al.* (2015)
B. pallidifolia	B12-1599	Apomictic	3*x*	Mau *et al.* (2015)
B. williamsii	B12-1524	Apomictic	2*x*	Mau *et al.* (2015)
B. williamsii	B12-558	Sexual	2*x*	Mau *et al.* (2015)
B. stricta	LTM	Sexual	2*x*	Schranz *et al.* (2005), Lee *et al.* (2017)

All accessions have been previously described as indicated, and represent different reproductive modes (sexual, apomictic), ploidy levels (2x, 3x), and species.

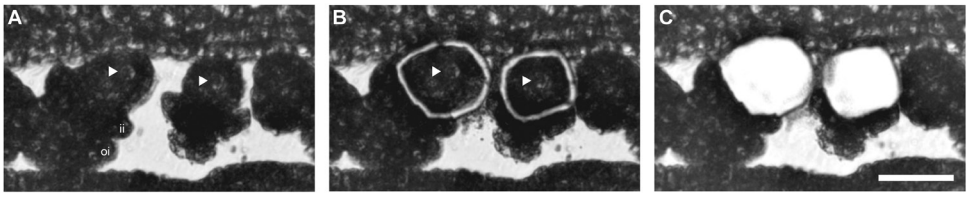

Fig. 1. Precise sampling of nucelli using laser-assisted microdissection (LAM). Nucelli harbouring apomictic/aposporous initial cells (AICs) in ovary of apomictic *Boechera pallidifolia* (B12-1578) (A) before and (B) after LAM, which allows precise isolation with only ~2 µm cutting width of the laser beam. (C) After sample collection. Nuclei of AICs are marked with arrowheads. ii, inner integument; oi, outer integument. The scale bar is 30 µm.

could be mapped per library (Supplementary Table S1). Unique reads constituted 95% of total reads on average, and gene bodies were covered homogenously by mapped reads (Supplementary Fig. S2). Further, 85% of total reads mapped to exonic regions, when counted with featureCounts (Liao *et al.*, 2014). Taken together, the quality controls performed during processing of the RNA-seq reads, the general composition of read sequences, and the library statistics demonstrated an overall good quality of the data obtained (Table S1, Supplementary Figs S1–S3).

Global gene expression in Boechera *nucelli widely overlaps*

Nucellus tissues at similar developmental stages from related sexual and apomictic *Boechera* accessions were profiled. This provided a good basis to identify genes consistently transcribed and of general importance independent of the reproductive mode.

In total, we identified 24 197 genes that are expressed (≥10 normalized read counts) in at least one sample, thus representing 88% of the 27 416 genes in *B. stricta* (Supplementary Fig. S4) (Lee *et al.*, 2017). Expression of 15 511 genes was shared among all the samples from apomicts, while 11 236 genes were expressed in all samples from sexual accessions (Fig. 2, Supplementary Fig. S4). Overlapping expression in all samples was detected for 10 526 genes, representing 38% of the genes in the genome (Fig. 2A).

To classify biological processes that were up-regulated during germline specification independently of the reproductive mode, we applied a GO enrichment analysis on the consistently active set of 10 526 genes using topGO. Based on the available annotations for 33% of the genes of the genome, 37 GO terms were significantly enriched ($P<0.01$, Supplementary Table S3) (Lee *et al.*, 2017). In agreement with previous findings for Arabidopsis MMCs (Schmidt *et al.*, 2011), terms related to 'translation', 'ubiquitin dependent protein catabolism', and 'ribosome biogenesis' were amongst the significantly enriched processes, in addition to terms pointing towards the importance of redox homeostasis, signal transduction, cell-cycle, and different metabolic processes.

Despite similar regulation for a large number of genes, variation of expression between accessions of the same reproductive mode was also observed. However, only seven genes were identified to be expressed in all sexual accessions and not in any apomict, while 82 genes were exclusively active in all samples from apomicts (Supplementary Fig. S4A, B).

Overall, our analyses revealed widely overlapping gene expression patterns in nucelli of apomictic and sexually reproducing *Boechera*. Nevertheless, the comparisons indicated distinct regulation of a subset of genes according to the reproductive mode.

Comparisons to the transcriptomes of Arabidopsis MMCs and B. gunnisoniana *AICs indicate the overall accuracy of the dataset*

Cell and tissue type-specific transcriptome analyses have previously been described for Arabidopsis MMCs and surrounding nucellus tissues isolated separately, and the AICs of the triploid apomict *B. gunnisoniana* (Schmidt *et al.*, 2011, 2014). To determine how much these datasets conform with ours, the transcriptomes were compared. Generally, comparisons were restricted to annotated Arabidopsis homologues (Fig. 2).

Overlapping expression in nucelli from all analysed sexual *Boechera* accessions (B12-558, LTM) compared with the MMCs and nucellar tissue without the MMCs of Arabidopsis was determined to comprise 5139 of 6650 (77%) and 7267 of 10 081 (72%) genes, respectively (Fig. 2B). In addition, ~91% (10 940) of 11 967 genes expressed (≥10 reads) in the AICs of *B. gunnisoniana* were also identified to be active in the triploid *B. pallidifolia* included in this study (B12-1599; Fig. 2C) (Schmidt *et al.*, 2014). This high overlap of gene expression indicated an overall high accuracy of the dataset presented in this study.

We also checked the activity of the homologue of *KNUCKLES*, a marker gene for MMC identity in Arabidopsis (Tucker *et al.*, 2012), and *APOLLO*, which shows allelic variants linked to apomixis in *Boechera* (Corral *et al.*, 2013). In contrast to *APOLLO*, *KNUCKLES* was either expressed at low levels or was absent in all samples (Supplementary Fig. S5, Supplementary Table S4).

Few core cell-cycle and meiotic genes are differentially regulated between any accessions

As the sampled tissues harboured MMCs or AICs, we analysed transcription of meiotic genes and core cell-cycle regulators (Vandepoele *et al.*, 2002; Gutierrez, 2009; Mercier *et al.*, 2015). Consistent with previous findings (Schmidt *et al.*, 2014), homologues of 16 out of 24 selected meiotic genes were active in samples from all accessions (Supplementary Fig. S5, Supplementary Table S4). To identify which of the genes were differentially regulated between accessions, we searched DEGs obtained using edgeR to apply a generalized linear model to

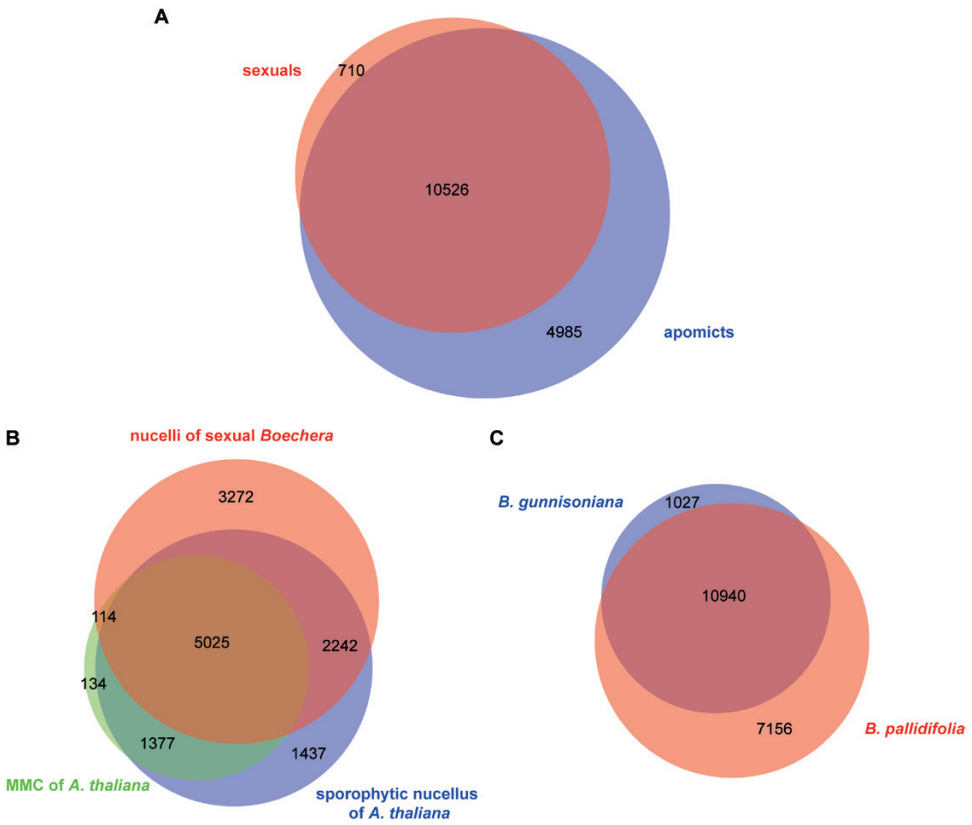

Fig. 2. Comparison of gene expression between different reproductive modes. (A) Venn diagram representing the overlap of genes expressed commonly in either all samples from apomictic or all samples from sexual accessions of *Boechera* (≥10 normalized read counts). (B) Venn diagram representing the intersections of genes expressed in isolated megaspore mother cells (MMCs) or sporophytic nucellar tissue of Arabidopsis (present calls in at least three of four of microarray replicates; Schmidt *et al.*, 2011) and nucelli of all analysed samples of sexual *Boechera* accessions in the current study (LTM, B12.558). (C) Venn diagram representing the overlap of expressed genes (≥10 reads) in apomictic initial cells (AICs) of triploid *B. gunnisoniana* (Schmidt *et al.*, 2014) and nucelli of *B. pallidifolia* (B12.1599, this study). Each comparison was based on annotated Arabidopsis homologues of corresponding *Boechera* genes, with available data having been generated on Affymetrix ATH1 microarrays for Arabidopsis or mapped using the reference transcriptome of *B. gunnisoniana* (B, C).

compare all accessions (Supplementary Table S5) (Robinson *et al.*, 2010). Interestingly, this identified *SPO11-1* and *REC8* in addition to the cell-cycle regulators *PROLIFERATING CELL NUCLEAR ANTIGEN*, *CYCLIN D3;3*, *CYCLIN A2;3*, *CYCLIN B2;1*, and *E2F TRANSCRIPTION FACTOR 3* (Supplementary Fig. S5). However, the differences observed were not consistent between all sexual and all apomictic accessions.

Statistical analysis reveals genes consistently differentially expressed between apomictic and sexual nucelli

Despite the large number of genes active in all samples, differential expression would be expected for genes important for the determination of the reproductive mode or for processes specific to apomictic or sexual development. To identify such genes, statistical analysis of differential gene expression between apomictic and sexual nucelli samples was applied using edgeR (Robinson *et al.*, 2010).

First, we compared the transcriptomes of all apomictic against all sexual samples, treating samples of same reproductive mode as biological replicates. Using this approach, 43 genes were identified to be significantly differentially expressed (FDR≤0.05 after Benjamini–Hochberg adjustment; Fig. 3, Supplementary Tables S6, S7). Of these DEGs, 28 were significantly up-regulated in apomictic and 18 in sexual samples. Hierarchical clustering and heatmap representation of the expression levels demonstrated a clear distinction between reproductive modes and a close relation between biological replicates of the same accession (Fig. 3). Moreover, the expression patterns of samples from the *B. pallidifolia* accessions (B12-1578 and B12-1599) clustered together, as did those of the apomictic *B. divaricarpa* (ES517) and apomictic *B. williamsii* (B12-1524) accessions.

In a second approach, the ploidy and genetic backgrounds of the analysed accessions were taken into account. To this end, DEGs were identified by individual pairwise comparisons of sexual and apomictic accessions (FDR≤0.05 after Benjamini–Hochberg adjustment; Supplementary Table S7). Relevant candidate genes for germline specification were narrowed down by taking the intersection of all DEGs identified in individual pairwise comparisons. This resulted in the identification of seven common DEGs, which were all more highly expressed in apomictic than in sexual accessions (Supplementary Fig. S6). Five of these were consistently identified in both analyses (Table 2, Supplementary Table S8).

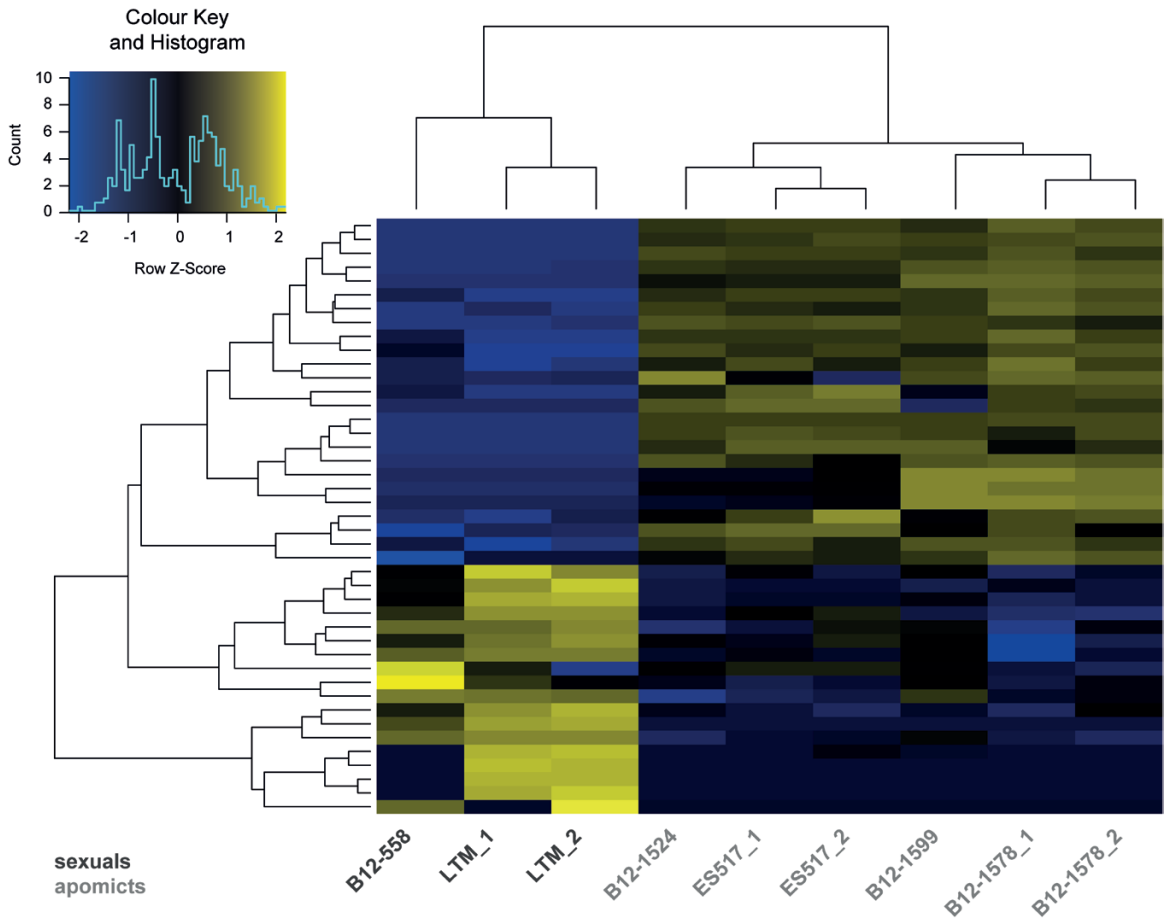

Fig. 3. Heatmap of $\log_2$-transformed read counts of differentially expressed genes (DEGs) in *Boechera* accessions. Expression levels are shown for 43 DEGs in all sexual as compared to all apomictic samples. The heatmap is based on TMM-normalized and $\log_2$-transformed read counts. The hierarchical clustering of samples and genes was based on Euclidean distance and hierarchical agglomerative clustering. The colours within the heatmap are scaled per row with blue indicating low expression and yellow high expression.

Despite the large overlap of global gene expression, our statistical data analysis identified 45 genes that were significantly differentially expressed in all sexual versus all apomictic samples (Supplementary Tables S6, S7). By comparing closely related *Boechera* accessions we reduced the influence of genetic backgrounds on our ability to identify promising new candidate genes with enriched expression in sexual or apomictic reproduction.

Annotations of DEGs suggest roles in protein degradation, transcriptional regulation and stress responses

Because it has been used as a model only relatively recently, knowledge about specific gene functions is scarce in the genus *Boechera*. However, its close relation to Arabidopsis and genome annotations of *B. stricta* provide a useful basis for investigations (Lee *et al.*, 2017).

Arabidopsis homologues have been annotated for 21 890 genes in the *B. stricta* genome, equal to ~80% of the total of 27 416 genes (Lee *et al.*, 2017). Based on this, DEGs could be attributed to the following different functional categories: protein degradation, transcriptional and post-transcriptional regulation of gene expression, redox processes and stress responses, and phytohormones and cell signalling (Table 2, Supplementary Tables S7, S8).

The first group, functionally related to protein degradation, in particular comprised genes coding for proteins with F-box and associated interaction domains (e.g. cyclin-like and Skp2-like domains), an F-box/RNI-like superfamily protein, or a protein related to F-box proteins. These genes were mostly up-regulated in apomictic nucelli (Table 2A, Supplementary Table S7). In addition, two E3 ligases were identified as DEGs: Bostr.7867s0569, the homologue of the E3 ligase *RING MEMBRANE-ANCHOR 3* (*RMA3*), was consistently identified in both comparisons, and a gene encoding a Tumor Necrosis Factor Receptor Associated Factors-like (TRAF-like) family protein, Bostr.25993s0534, was found to have significantly higher expression levels in apomictic nucelli. In addition, two genes related to ubiquitination were represented in the set of DEGs: Bostr.29223s0097 and Bostr.2983s0066, the homologues of *UBIQUITIN-CONJUGATING ENZYME 28* (*UBC28*), and of a ubiquitin-like superfamily protein, respectively.

In the second group, two DEGs related to transcriptional and post-transcriptional regulation processes were identified. A putative endonuclease, Bostr.3279s0010, was significantly up-regulated in apomictic nucelli in both analyses, whilst a B3

Table 2. *List of 43 genes differentially expressed between all samples originating from all apomictic versus sexual nucellar tissues isolated by laser-assisted microdissection*

Gene locus*	GO terms	Arabidopsis gene locus	Arabidopsis gene name	Arabidopsis gene/ protein description
(A) DEGs up-regulated in apomictic accessions				
Bostr.26527s0134	GO:0030246, GO:0005975, GO:0004553	At5G20710	*BGAL7*	Beta-galactosidase 7
Bostr.7867s0569	**GO:0046872**	**At4G27470**	*AtRMA3*, *RMA3*	**RING membrane-anchor 3**
Bostr.25375s0042	NA	At1G64290	NA	F-box protein-related
Bostr.15697s0319	NA	At1G30790	NA	F-box and associated interaction domains-containing protein
Bostr.15697s0321	NA	At1G32660	NA	F-box and associated interaction domains-containing protein
Bostr.13129s0275	**NA**	**NA**	**NA**	**NA**
Bostr.29044s0001	NA	At5G36710	NA	NA
Bostr.3288s0214	**GO:0033177, GO:0015991, GO:0015078**	**At1G19910**	*AtVHA-C2*, *AVA-2PE*, *AVA-P2*	**ATPase, F0/V0 complex, subunit C protein**
Bostr.2983s0066	GO:0005515	At4G05310	NA	Ubiquitin-like superfamily protein
Bostr.15697s0461	GO:0046872	At1G31480	*SGR2*	Shoot gravitropism 2 (SGR2)
Bostr.0568s0078	GO:0008270	At5G17890	*CHS3*, *DAR4*	DA1-related protein 4
Bostr.0697s0107	NA	At4G10400	NA	F-box/RNI-like/FBD-like domains-containing protein
Bostr.26833s0971	NA	NA	NA	NA
Bostr.19424s0775	**NA**	**NA**	**NA**	**NA**
Bostr.10058s0023	GO:0008168	At4G00740	NA	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein
Bostr.13158s0243	GO:0055114, GO:0009396, GO:0004488, GO:0003824	At4G00620	NA	Amino acid dehydrogenase family protein
Bostr.26959s0357	GO:0055114, GO:0009396, GO:0004488, GO:0003824	At4G00620	NA	Amino acid dehydrogenase family protein
Bostr.7305s0030	NA	At5G52390	NA	PAR1 protein
Bostr.18351s0192	GO:0005515	At2G17310	*SON1*	F-box and associated interaction domains-containing protein
Bostr.3279s0010	**GO:0010468, GO:0005777**	**At4G30760**	**NA**	**Putative endonuclease or glycosyl hydrolase**
Bostr.26833s0821	NA	At5G54510	*DFL1*, *GH3.6*	Auxin-responsive GH3 family protein
Bostr.5022s0132	NA	At2G21140	*AtPRP2*, *PRP2*	Proline-rich protein 2
Bostr.25993s0534	NA	At2G42480	NA	TRAF-like family protein
Bostr.13158s0246	GO:0055114, GO:0009396, GO:0004488, GO:0003824	At4G00620	NA	Amino acid dehydrogenase family protein
Bostr.13671s0276	GO:0000062	At1G31812	*ACBP*, *ACBP6*	Acyl-CoA-binding protein 6
(B) DEGs up-regulated in sexual accessions:				
Bostr.13129s0114	GO:0005515, GO:0003677	At5G08430	NA	SWIB/MDM2 domain; Plus-3; GYF
Bostr.26959s0127	NA	At1G67800	NA	Copine (Calcium-dependent phospholipid-binding protein) family
Bostr.7867s0507	GO:0005975, GO:0004553	At4G26830	NA	O-Glycosyl hydrolases family 17 protein
Bostr.12396s0001	GO:0055114, GO:0051287, GO:0016616	At1G26570	*ATUGD1*, *UGD1*	UDP-glucose dehydrogenase 1
Bostr.7867s1023	GO:0003677	At4G31680	NA	Transcriptional factor B3 family protein
Bostr.18351s0250	GO:0007165, GO:0000155, GO:0000160	At2G17820	*AHK1*, *ATHK1*, *HK1*	Histidine kinase 1
Bostr.7867s1594	GO:0055114, GO:0020037, GO:0016705, GO:0005506	At4G37330	*CYP81D4*	Cytochrome P450, family 81, subfamily D, polypeptide 4
Bostr.0697s0084	GO:0055085, GO:0016021	At3G54700	*PHT1;7*	Phosphate transporter 1;7
Bostr.26833s0862	GO:0016758, GO:0008152	At3G21790	NA	UDP-Glycosyltransferase superfamily protein
Bostr.15774s0342	GO:0055114, GO:0016614, GO:0050660	At5G51950	NA	Glucose-methanol-choline (GMC) oxidoreductase family protein
Bostr.2021s0098	NA	NA	NA	NA
Bostr.29223s0097	NA	At1G64230	*UBC28*	Ubiquitin-conjugating enzyme 28

Table 2. *Continued*

Gene locus*	GO terms	Arabidopsis gene locus	Arabidopsis gene name	Arabidopsis gene/ protein description
Bostr.2618s0047	GO:0005975, GO:0004553	At5G17500	NA	Lycosyl hydrolase superfamily protein
Bostr.15697s0040	NA	NA	NA	NA
Bostr.7867s0172	NA	At1G25290	*AtRBL10*, *RBL10*	RHOMBOID-like protein 10
Bostr.26833s0734	NA	At5G55240	*AtPXG2*	ARABIDOPSIS THALIANA PEROXYGENASE 2
Bostr.25993s0569	NA	At3G53750	*ACT3*	Actin 3
Bostr.3751s0034	NA	At5G40680	NA	Galactose oxidase/kelch repeat superfamily protein

**Boechera stricta* gene locus., Arabidopsis homologues are based on genome annotations by Lee *et al.* (2017). DEGs are ordered according to Supplementary Table S7 (sheet 1) and those identified consistently in both analyses are highlighted in bold. NA, no available information.

family transcription factor, Bostr.7867s1023, was identified as significantly lower expressed in apomictic than sexual nucelli (Table 2A, Supplementary Table S7).

The third group included DEGs related to redox processes and stress responses. Most prominently, a gene encoding a vacuolar ATPase subunit protein, Bostr.3288s0214, was found to have significantly higher expression levels in apomicts in both comparisons. Its homologue has previously been described to be related to abiotic stress responses (Kreps *et al.*, 2002) (Table 2A, Supplementary Tables S7, S8). In contrast, several genes of this group had significantly lower levels of expression in apomictic nucelli. This subset was comprised of the homologue of Arabidopsis *PEROXYGENASE 2* (*AtPXG2*), Bostr.26833s0734, a gene coding for a glucose-methanol-choline (GMC) oxidoreductase family protein, Bostr.15774s0342, and another oxidoreductase, a member of the cytochrome P450 superfamily *CYP81D4*, Bostr.7867s1594 (Table 2B, Supplementary Table S7).

In the fourth group, Arabidopsis homologues are involved in phytohormone-mediated cell signalling, or cell signalling *per se*. This group was comprised of a gene coding for an auxin-responsive GRETCHEN HAGEN 3 (GH3) family protein (GH3.6), Bostr.26833s0821, and the non-ethylene receptor HISTIDINE KINASE 1 (HK1), Bostr.18351s0250, which is a positive regulator in ABA signal transduction and drought responses (Tran *et al.*, 2007) (Table 2, Supplementary Table S7).

Interestingly, apart from *RMA3*, the putative endonuclease and the gene encoding the vacuolar ATPase subunit protein, two genes with unknown function lacking any annotation, Bostr.13129s0275 and Bostr.19424s0775, were consistently detected with both approaches (Table 2A, Supplementary Tables S7, S8).

In summary, our dataset indicated consistent differential expression related to sexual and apomictic megasporogenesis for a rather small number of genes. The differences identified mainly pointed towards involvement of protein degradation mediated by poly-ubiquitination, transcriptional and post-transcriptional regulatory mechanisms, regulation by plant hormones, signal transduction, and stress responses. In addition, a few genes sharing no significant homology to Arabidopsis genes were identified as DEGs.

Independent validation of expression supports evidence for the candidate genes

To independently confirm the expression in reproductive nucellus tissues we selected four DEGs for *in situ* hybridizations on *B. stricta* LTM or *B. divaricarpa* ES517 buds (Fig. 4). Probes were based on *B. stricta* LTM cDNA for the putative endonuclease Bostr.3279s0010 (Fig. 4A, B), the B3 transcription factor B3 TF (Bostr.7867s1023; Fig. 4C–F), *CYP81D4* (Bostr.7867s1594; Fig. 4G–K), and for Bostr.13129s0275 (Fig. 4L–O). All probes were designed to have significant homology only to the gene of interest based on the *B. stricta* reference genome (Lee *et al.*, 2017). Activity in the young reproductive nucellus tissues could be confirmed for the genes.

Discussion

Genes involved in cell-cycle regulation, transcriptional control, and stress responses are differentially regulated during sexual and apomictic germline specification

The identification of genes of putative relevance in determining the reproductive mode or in sustaining either sexual or apomictic development remains challenging. This is mainly due to the small size of the reproductive tissues. A previous comparison of gene activity between apomictic *B. gunnisoniana* and Arabidopsis identified almost 900 genes with evidence of expression that was different in the AIC compared with the MMC (Schmidt *et al.*, 2014). This study provided important insights into the gene regulatory processes that govern germline specification in apomicts as compared with sexual plants; however, species and ploidy effects probably contributed to the differential regulation of such a large number of genes, thus complicating the identification of genes important for apomixis.

In our present study, different apomictic and sexual *Boechera* accessions were compared and our design allowed us to set apart differences due to ploidy or genetic background from those relevant for reproduction (Table 1, Supplementary Table S7). This allowed the number of DEGs to be narrowed down to 45 (Table 2, Supplementary Tables S6, S7, S8). Their consistent

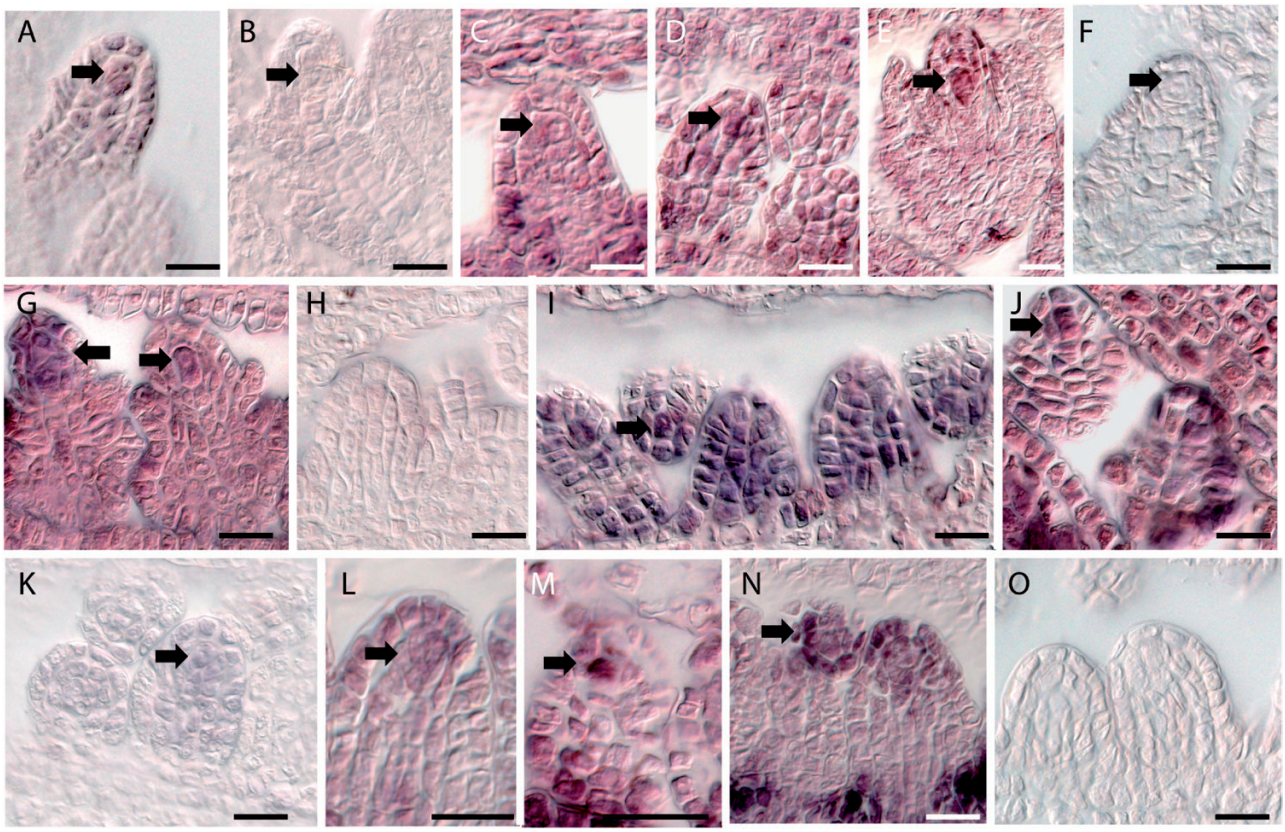

Fig. 4. Independent data validation by *in situ* hybridization. Expression in reproductive nucellus tissues of young flower buds was confirmed for Bostr.3279s0010 in *B. divaricarpa* ES517 (A, B), for Bostr.7867s1023 in *B. stricta* LTM (C–F), for Bostr.7867s1594 in *B. divaricarpa* ES517 (G, H) and in *B. stricta* LTM (I–K), and for Bostr.13129s0275 in *B. stricta* LTM (L–O). *In situ* hybridizations were performed with antisense probes (A, C–E, G, I, J, L–N) or with sense probes as controls (B, F, H, K, O). Arrows indicate the specifying megaspore mother cells (MMCs) or apomictic/aposporous initial cells (AICs), with exception of (E) where it indicates the functional megaspore. Scale bars are 50 μm.

differential regulation in all sexual versus all apomictic samples indicated putative functional importance for discrimination of sexual and apomictic development. Interestingly, the genes identified could be functionally attributed to different regulatory mechanisms, including transcriptional and post-transcriptional regulatory processes, protein degradation, cell signalling, and stress responses. This is in agreement with previous findings (Schmidt *et al.*, 2014, 2015; Mateo de Arias, 2015; Tang *et al.*, 2017).

Cell-cycle control involving protein degradation is probably of crucial importance during germline specification

Interestingly, based on homology and annotations, some of the DEGs that we identified are potentially involved in pathways that are described to have regulatory roles either during germline development or in the restriction of reproductive fate (Kim *et al.*, 2008; Gusti *et al.*, 2009; Schallau *et al.*, 2010; Schmidt *et al.*, 2014; Singh *et al.*, 2017; Tang *et al.*, 2017; Zhao *et al.*, 2017a, 2017b; Cao *et al.*, 2018). For example, six of the identified candidates encode for proteins containing F-box and/or associated interaction domains (Table 2, Supplementary Table S8). F-box proteins are involved in a multitude of biological processes in plants (Lechner *et al.*, 2006; Stefanowicz *et al.*, 2015). They are part of Skp1-Cullin1-F-box protein (SCF) ligase complexes that act in 26S proteasomal degradation mediated by polyubiquitination (Skowyra *et al.*, 1997). As an essential component of SCF complexes, they facilitate the regulation of many core cell-cycle genes and are thus responsible for the proper progression of the cycle, including the G1/S transition (Krek, 1998; Vodermaier, 2004). For example, Arabidopsis SCF^{SKP2A} complex, which contains the F-box protein SKP2A, positively regulates cell division (del Pozo *et al.*, 2002, 2006; Jurado *et al.*, 2008). One of the F-box candidate genes, Bostr.15697s0319 (Table 2), even contains a cyclin-like and Skp2-like domain. Based on this homology of the interaction domains, it is probably part of an SCF complex that guides specific protein degradation during the progression of the cell-cycle. Interestingly, the Arabidopsis SCF^{FBL17} complex, which contains the F-box-like 17 protein, has been described to act specifically in male germ cells, where it targets the cyclin-dependent kinase inhibitors KIP-RELATED PROTEIN 6 (KRP6) and KRP7 to control germ cell proliferation (Kim *et al.*, 2008; Gusti *et al.*, 2009). KRPs in turn are also required to restrict female reproductive fate to only one MMC per ovule since they repress the cell-cycle regulator RETINOBLASTOMA-RELATED 1 (RBR1) via the cyclin-dependent kinase A;1 (CDKA;1) (Zhao *et al.*, 2017b; Cao *et al.*, 2018).

These interrelated findings demonstrate that F-box proteins are essential for cell-cycle regulation during germline

development. In line with this, the F-box genes that we identified as being differentially regulated, particularly Bostr.15697s0319, might be part of the SCF complex(es) governing cell-cycle mechanisms that are distinct between apomeiosis and meiosis.

Distinct regulation of cell-cycle and meiotic genes was also observed between the *Boechera* accessions (Supplementary Fig. S5, Supplementary Table S4). Interestingly, triple-mutants in *SPO11-1*, *REC8*, and *OSD1* lead to mitotic instead of meiotic division (d'Erfurth *et al.*, 2009). However, the lack of consistent regulation observed in apomicts might relate to differences in apomeiotic development (Supplementary Fig. S5, Supplementary Table S4), and also reflect the developmental stage of sampled tissues that are predominantly before (apo) meiosis. Further studies focusing on the proteins encoded are needed to shed more light on their roles and regulation in *Boechera*.

Another DEG, Bostr.25993s0534 (Table 2), encodes a TRAF-like family protein. TRAFs comprise a class of E3 ubiquitin ligases with characteristic RING finger, TRAF, and Meprine And TRAF Homology (MATH) domains, which enable them to mediate interactions between other TRAF members, receptors, and several different intracellular signalling molecules (Ye *et al.*, 1999; Zapata, 2003; Alvarez *et al.*, 2010). In Arabidopsis, TRAF Mediated Gametogenesis Progression (TRAMGaP), which shares interaction domains with Bostr.25993s0534, is an important regulator of germline development involved in restricting reproductive fate (Singh *et al.*, 2017). The expression of several genes that act in germline specification and regulation of sporophyte-to-gametophyte transition has been shown to be dependent on TRAMGaP. Importantly, these not only include *RBR1* and the core meiotic gene *DYAD*, but also *AGO9*, *SGS3*, and *RDR6* (Singh *et al.*, 2017). While *dyad* mutants largely lead to sterility, formation of triploid offspring that fully retain parental heterozygosity has been observed at low frequencies (Ravi *et al.*, 2008). Furthermore, apospory-like phenotypes have been described for *ago9*, *sgs3*, and *rdr6* mutants (Ravi *et al.*, 2008; Olmedo-Monfil *et al.*, 2010). It is tempting to speculate that Bostr.25993s0534 might have similar functions to TRAMGaP. However, future functional studies are required to determine whether this gene mediates apospory or diplospory by targeting *DYAD* or genes active in the small RNA pathway, including *AGO9*.

Apart from this TRAF-like gene, additional DEGs with higher expression in the apomicts than in the sexual plants may be related to E3 ligases for which Arabidopsis homologs have previously been functionally described. In particular, Bostr.2983s0066 (Table 2) encodes for a ubiquitin-like superfamily protein homologous to At4G05310, for which a Cullin3A (CUL3A)-dependent expression has been reported (Dieterle *et al.*, 2005). CUL3 proteins, which form E3 ligase complexes with BTB and MATH domain proteins among others, are required for female gametogenesis and *cul3a cul3b* plants show maternal effect embryo lethality (Dieterle *et al.*, 2005; Thomann *et al.*, 2005). Bostr.7867s0569 (Table 2, Supplementary Table S8) encodes a RING E3 ligase homologous to *AtRMA3*. Notably, *AtRMA3* is expressed during pollen germination and pollen tube growth, and is co-expressed with *UBC28* (Wang *et al.*, 2008). UBC28 in turn belongs to the UBC8 group of proteins that are known to interact with RING E3 ligases (Kraft *et al.*, 2005; Stone *et al.*, 2005). Interestingly, the *Boechera* homologue of *UBC28*, Bostr.29223s0097, was also identified as a DEG in our study; however, it was more highly expressed in the sexual accessions, unlike the homologue of *AtRMA3*. HpARI7 completes the set of RING E3 ligases that have been described as potentially related to cell-cycle regulation during megasporogenesis, since it is a gene located on the apospory-linked *Hypericum APOSPORY* locus in *H. perforatum* (Schallau *et al.*, 2010).

Taken together, our study identified a number of candidate genes that code for F-box proteins, E3 ligases, or associated factors. Their Arabidopsis homologues are directly or indirectly interrelated with previously described proteasomal degradation-mediated control of cell-cycle regulators and other targets, which influence reproductive fate decisions and germline development. This provides further evidence that tightly regulated protein degradation that affects cell-cycle progression may be crucial for governing the distinct specification and differentiation of apomictic and sexual germlines.

Involvement of stress responses and cell signalling in early megasporogenesis

In addition to those considered above, other regulatory mechanisms have previously been described to be involved in megasporogenesis, including stress responses and hormonal pathways (Schmidt *et al.*, 2015), and some DEGs identified in our study were related to such pathways.

For example, oxidative stress and signalling, including reactive oxygen species (ROS), have been described to play roles in germline specification and possibly in meiosis (Schmidt *et al.*, 2015). In accordance with this, members of the cytochrome P450 (*CYP450*) superfamily of oxidoreductases are enriched in the nucellar tissue of Arabidopsis compared to other tissues (Schmidt *et al.*, 2011). Furthermore, they show cell cycle-dependent expression (Menges *et al.*, 2002). Interestingly, recent findings have demonstrated that the Arabidopsis cytochrome P450 gene *KLU* (*CYP78A5*) is involved in restricting female germline fate to only one MMC per ovule (Zhao *et al.*, 2017a). Likewise, Bostr.7867s1594 is a homologue of Arabidopsis *CYP81D4* and a member of the *CYP450* superfamily, which potentially functions in response to ROS (Table 2B). It had significantly higher expression levels in sexual than in apomictic nucelli. Thus, the detection of *CYP81D4* and other DEGs related to redox processes (Bostr.26833s0734 and Bostr.15774s0342) provides further evidence that regulation of redox homeostasis and response to oxidative stress might contribute to the determination and differentiation of female sexual and apomictic germlines.

Beside their damaging properties and the consequent triggering of oxidative stress responses, ROS are also known to function as signalling cues between and within cells, for example during male germline development (Kelliher and Walbot, 2012; De Storme and Geelen, 2014). Similarly, cell-to-cell signalling mechanisms are also required during female germline specification and differentiation (Grossniklaus and Schneitz, 1998; Koltunow and Grossniklaus, 2003; Schmidt *et al.*, 2015; Zhou

et al., 2017). In accordance with this, some of the DEGs that we found could be attributed to cell signalling mechanisms, such as the homologue of *AtHK1* (Table 2B), which potentially acts as a non-ethylene phytohormone receptor (Tran et al., 2007). Other DEGs that we found might be involved in auxin signalling in ovules, which is an important component of sporo- and gametogenesis (Li et al., 2008; Pagnussat et al., 2009; Schmidt et al., 2011; Lituiev et al., 2013; Freire Rios et al., 2014; Schaller et al., 2015). These were Bostr.7867s1023, a transcription factor of the B3 family, and Bostr.26833s0821, the homologue of *AtGH3.6*, for which auxin-responsiveness has been demonstrated previously (Nakazawa et al., 2001). Interestingly, AUXIN RESPONSE FACTORs belong to the same B3 superfamily of transcription factors and are well known as key regulators of auxin signalling (Chandler, 2016).

In summary, the putative functions of various candidate genes that we identified are consistent with the not yet fully understood complex regulatory network that governs germline specification and differentiation in plants. Notably, Tang et al. (2017) found DEGs with similar functional annotations in a comparative transcriptome analysis of entire flower buds of sexual and apomictic *Boehmeria tricuspis*. Moreover, based on a transcriptome analysis in *Hieracium praeltum*, some homologues of the DEGs that we identified were also differentially expressed between AICs as compared to the early stages of gametophyte development, or somatic cells of the ovule, or both, including homologues of *AtPXG2*, *CYP81D4*, *GH3*, and *AtHK1* (Supplementary Table S9) (Juranić et al., 2018). In contrast to our study, the work of Juranić et al. (2018) included an investigation of different cell and tissue types in one aposporous apomict, further supporting evidence for roles of the genes in reproductive development.

Taken together, the DEGs that we identified might be involved in the different apomictic and sexual fate decisions by facilitating oxidative stress responses, auxin-driven cell-signalling, and in particular ubiquitination-mediated control of the cell-cycle.

Commonly and differentially regulated genes in apomictic and sexual nucelli

In accordance with the finding that hybrid origin or polyploidy are associated with most apomicts (Bicknell and Koltunow, 2004; Lovell et al., 2013), some variation of expression was observed between the apomictic accessions that we analysed (Supplementary Fig. S4, Supplementary Table S7). This might have been caused by the genomic effects of polyploidy and hybridization and is consistent with the hypothesis that apomixis derived several times independently (Sharbel and Mitchell-Olds, 2001; Koch et al., 2003; Dobeš et al., 2004a, 2004b; Kiefer et al., 2009; Kiefer and Koch, 2012; Lovell et al., 2013). However, it is still not fully understood whether broad deregulation of the sexual regulatory programme is the cause of or only correlated with apomixis. The rather small number of DEGs consistently identified in this study tends to suggest that a small number of genes is sufficient to sustain the switch from sexual reproduction to apomixis. This is in agreement with apomixis being genetically linked to only a few loci in most species (Barcaccia and Albertini, 2013).

Despite differential regulation of a subset of genes, almost 40% of all *Boechera* genes shared expression in all the samples that we analysed, regardless of the reproductive mode (Fig. 2, Supplementary Fig. S4). These genes probably include common regulators of reproductive development during megasporogenesis. However, gene expression for either reproductive mode appeared imbalanced, with more genes being expressed commonly in apomicts than in sexual nucelli. But fewer genes were identified to be active in sample LTM_2 compared to the other samples from sexual accessions because only 38 nucelli were dissected, representing the lowest input amount used. Thus, this apparent difference is probably not of biological relevance. In addition, for certain genes marked by 'sexalleles' and 'apoalleles', such as *APOLLO* (Corral et al., 2013), read-mapping to the genome of sexual *B. stricta* (Lee et al., 2017) possibly results in underestimation of expression levels in apomicts. Overall, the comparison of transcriptomes of *Boechera* nucelli with datasets from MMCs or nucellus tissue of Arabidopsis and *B. gunnisoniana* AICs (Schmidt et al., 2011, 2014), in addition to independent validations of selected genes by *in situ* hybridizations (Fig. 4), indicated overall good accuracy of our data.

In conclusion, our in-depth comparative analysis identified 45 genes that were differentially expressed between sexual and apomictic *Boechera* accessions. Most interestingly, the newly identified genes could be associated to the control of genes and pathways previously described to relate to regulation of apomixis. The identification of these genes will provide a very good basis for future functional investigations. Thus, our study contributes to a detailed understanding of the regulatory mechanisms that govern apomixis and mediate the specification of the female germline in apomictic and sexually reproducing plants in general.

Supplementary data

Supplementary data are available at *JXB* online.

Table S1. Summary of samples and mapping statistics.

Table S2. Cloning of constructs for *in situ* probes.

Table S3. Gene ontology analysis.

Table S4. Expression of selected meiotic genes, core cell-cycle regulators, *APOLLO*, and *KNUCKLES*.

Table S5. Genes differentially expressed between any accessions.

Table S6. Transcriptome analysis of nucelli of apomictic and sexual *Boechera*.

Table S7. Differential expression analysis of nucelli of apomictic versus sexual *Boechera*.

Table S8. Table of common differentially expressed genes between apomictic and sexual nucelli.

Table S9. List of candidate genes, homologues of which are differentially expressed in *Hieracium praealtum* cells and tissue types.

Fig. S1. Quality controls of sample preparation for RNA-seq.

Fig. S2. Coverage of gene bodies by mapped reads.

Fig. S3. Assessment of read duplication rates.

Fig. S4. Visualization of overlap of gene expression.

Fig. S5. Heatmap of $\log_2$-transformed mean read counts of selected genes.

Fig. S6. Heatmap of $\log_2$-transformed read counts of common differentially expressed genes.

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Author contributions

AS conceived the project; AS and LZ planned the experiments; LZ conducted the experiments with the support of DI and AS; LZ conducted the data analysis; CV performed the *in situ* hybridizations; LZ, AS, and DI interpreted the data; LZ and AS wrote the manuscript; all authors approved the manuscript.

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