New Phytologist

Research

# Sex-biased gene expression in dioecious garden asparagus (*Asparagus officinalis*)

**Alex Harkess[1], Francesco Mercati[2,3], Hong-Yan Shan[4], Francesco Sunseri[2], Agostino Falavigna[5] and Jim Leebens-Mack[1]**

[1]Department of Plant Biology, University of Georgia, Athens, GA 30602, USA; [2]Dipartimento di AGRARIA, Università Mediterranea di Reggio Calabria, Salita Melissari, 89124 Reggio Calabria (RC), Italy; [3]CNR – National Research Council of Italy, Institute of Biosciences and Bioresources, Corso Calatafimi 414, 90129 Palermo, Italy; [4]State Key Laboratory of Systematic and Evolutionary Botany, Institute of Botany, Chinese Academy of Sciences, 20 Nanxincu, Xiangshan, Beijing 100093, China; [5]Consiglio per la Ricerca e la Sperimentazione in Agricoltura – Research Unit for Vegetable Crops, Montanaso Lombardo, 26836 Lodi, Italy

Author for correspondence:
*Jim Leebens-Mack*
*Tel: +1 706 583 5573*
*Email: jleebensmack@uga.edu*

Received: *15 November 2014*
Accepted: *7 February 2015*

*New Phytologist* (2015) **207:** 883–892
**doi**: 10.1111/nph.13389

**Key words:** dioecy, garden asparagus (*Asparagus officinalis*), RNA-sequencing (RNA-Seq), sex chromosomes, transcriptome.

## Summary

- Sex chromosomes have evolved independently in phylogenetically diverse flowering plant lineages. The genes governing sex determination in dioecious species remain unknown, but theory predicts that the linkage of genes influencing male and female function will spur the origin and early evolution of sex chromosomes. For example, in an XY system, the origin of an active Y may be spurred by the linkage of female suppressing and male promoting genes.
- Garden asparagus (*Asparagus officinalis*) serves as a model for plant sex chromosome evolution, given that it has recently evolved an XX/XY sex chromosome system. In order to elucidate the molecular basis of gender differences and sex determination, we used RNA-sequencing (RNA-Seq) to identify differentially expressed genes between female (XX), male (XY) and supermale (YY) individuals.
- We identified 570 differentially expressed genes, and showed that significantly more genes exhibited male-biased than female-biased expression in garden asparagus. In the context of anther development, we identified genes involved in pollen microspore and tapetum development that were specifically expressed in males and supermales.
- Comparative analysis of genes in the *Arabidopsis thaliana*, *Zea mays* and *Oryza sativa* anther development pathways shows that anther sterility in females probably occurs through interruption of tapetum development before microspore meiosis.

## Introduction

In contrast with vertebrates, species with separate sexes are relatively rare in flowering plants. Nearly 90% of angiosperms are hermaphroditic, with bisexual flowers that harbor both male (staminate) and female (pistillate) organs (Ainsworth, 2000). At the same time, a diverse set of alternative mating strategies has evolved across the angiosperms, including the temporal separation of male and female organ function in a bisexual flower (dichogamy), the separation of unisexual male and female flowers on a single plant (monoecy), the production of both hermaphrodite and male flowers on the same plant (andromonoecy) and entirely distinct male and female plants (dioecy). Dioecy is a rare, yet phylogenetically widespread, phenomenon in flowering plants; it has been described in < 6% of angiosperm species and in nearly half (43%) of all angiosperm families, but it has evolved at least 100 independent times (Charlesworth, 2002).

From a developmental standpoint, the differentiation of males and females in dioecious systems is highly variable across flowering plant species, with many species forming both stamen and pistil primordia that are selectively aborted before maturity; pistil development is arrested in male flowers, and stamens abort in female flowers. Given the repeated evolution of dioecy and the genetic complexities of androecium and gynoecium development, the timing and genetic mechanisms governing the cessation of sex organ development in ancestrally bisexual flowers may well be lineage specific, with alterations in the function of different genes spurring the transition from hermaphroditism to dioecy (Diggle *et al.*, 2011). A closer genetic dissection of the male and female gametophyte development pathway in various model systems reveals a complex assortment of genes (Ma *et al.*, 2008; Ma & Sundaresan, 2010; Chang *et al.*, 2011; Chettoor *et al.*, 2014; Zhang & Yang, 2014), hormonal controls (Wilson *et al.*, 2011; Orozco-Arroyo *et al.*, 2012), small RNAs (Guo *et al.*, 2009) and epigenetic marks (Song *et al.*, 2012) that interact to contribute to proper flower development, ultimately complicating the identification of the mutations responsible for the evolutionary transition from hermaphroditism to dioecy. Recently, a small RNA producing gene has been identified as a sex-linked candidate for the regulation of anther development in dioecious persimmon species (*Diospyros* sp.; Akagi *et al.*, 2014), but this gene has not been implicated as influencing female function.

In dioecious plant and animal systems, a sex chromosome pair can genetically control the differentiation of male and female sexes. Although the genetic basis of this mechanism has not yet been fully elucidated for any dioecious plant species, theory predicts that at least two dominant genes, one suppressing female organogenesis (i.e. pistil development) and one promoting male organogenesis (i.e. anther and pollen production), must be tightly linked in a nonrecombining region on the Y/W chromosome (Charlesworth & Charlesworth, 1979). The identification of these sex-linked gender determination genes is difficult given the complexity of floral development paired with the highly repetitive, transposon-rich nature of nonrecombining sex determination regions. As a consequence, even in dioecious species with sequenced genomes, including poplar (*Populus trichocarpa*; Tuskan *et al.*, 2006), papaya (*Carica papaya*; Ming *et al.*, 2008), cannabis (*Cannabis sativa*; Van Bakel *et al.*, 2011) and date palm (*Phoenix dactylifera*; Al-Dous *et al.*, 2011; Al-Mssallem *et al.*, 2013), the causal sex determination genes remain elusive. The recent identification of a putative regulator of anther development in persimmon (Akagi *et al.*, 2014) is consistent with the two-gene model of the chromosome Charlesworth & Charlesworth (1978) in that there is no evidence for the gene having any influence on female function. However, the existence and identity of a female suppressor has yet to be described in any dioecious plant species.

Garden asparagus (*Asparagus officinalis*, $2n = 2x = 20$) is a particularly useful system for the investigation of the early stages of sex chromosome evolution. It is a dioecious species with an XX/XY sex determination system, where the presence of a Y chromosome in males dominantly suppresses female organogenesis and promotes complete development of fertile anthers. X and Y are cytologically homomorphic (Flory, 1932; Löptien, 1979; Telgmann-Rauber *et al.*, 2007; Deng *et al.*, 2012), suggesting that the conversion of an autosome pair to a sex chromosome is a recent event. Co-dominant markers verify a nonrecombining, male-specific region on the Y chromosome that distinguishes males from females (Jamsari *et al.*, 2004; Nakayama *et al.*, 2006; Kanno *et al.*, 2014). Moreover, the viability of the YY 'supermale' genotypes derived through anther culture or selfing of andromonoecious plants (Franken, 1970; Peng & Wolyn, 1999; Falavigna & Casali, 2002) suggests an early stage of sex chromosome evolution in *Asparagus*.

Developmentally, the temporal separation of male and female organ abortion in *A. officinalis* suggests that at least two genes are involved in sex determination. Early in floral development, female (XX), male (XY) and supermale (YY) genotypes have indistinguishable hermaphroditic floral meristems. The first observable gender difference occurs early in XY male and YY supermale development, when the tricarpellate gynoecium fails to fully elongate and fuse to form a mature stylar tube. Later in XX female development, anther degeneration occurs when the tapetum breaks down before the completion of microsporogenesis, ultimately leading to anther sterility (Caporali *et al.*, 1994).

The genes responsible for the observed differences in male and female asparagus flower development remain unknown. One approach to their identification is to sequence and assemble whole-genome shotgun data, identify gender-specific nonrecombining and hemizygous regions, annotate genes in these regions and experimentally test the functions of these genes. This work is underway for garden asparagus, but the transposon-rich complexity of the *A. officinalis* genome, particularly in the nonrecombining sex determination region, poses challenges (Telgmann-Rauber *et al.*, 2007). An alternative approach is to use RNA-sequencing (RNA-Seq) to identify gender-specific single nucleotide polymorphisms (SNPs) within coding regions and genes exhibiting gender-biased expression (Muyle *et al.*, 2012) that are known to play some role in the anther and ovule developmental pathways. Here, we assembled a comprehensive transcriptome for garden asparagus including transcripts from supermales, males and females, and used it to identify expression differences among these three gender types. Genes exhibiting gender-biased expression were compared with known anther and ovule development genes in order to narrow the window during which sex-linked gender determination genes may act to promote anther development and gynoecium suppression in males or to regulate suppression of anther development and promotion of gynoecium maturation in females.

## Materials and Methods

### Spear tip RNA isolation and sequencing

Recently emerged (> 15 cm spears) *Asparagus officinalis* L. spear tips were harvested from male, female and supermale plants of four distinct breeding lines. Total RNA was isolated from the spear tips using a Qiagen RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and treated with Qiagen RNase-free DNase. RNA quality (RNA Integrity Number (RIN) > 8.0) was assessed using an Agilent Bioanalyzer RNA nanochip (Agilent, Wilmington, DE, USA). Sequencing libraries were prepared using a TruSeq RNA Sample Preparation Kit v2 (Illumina, San Diego, CA, USA), according to the manufacturer's specifications, and quality and insert size distribution were assessed using an Agilent Bioanalyzer DNA 1000 chip. Sequencing libraries were qPCR quantified, pooled in equimolar concentration and analyzed on an Illumina HiSeq 2000, producing 2 × 100-nucleotide paired-end reads. All RNA-Seq generated for this project has been deposited in the National Center for Biotechnology Information (NCBI) BioProject 259909.

### *De novo* transcriptome assembly and annotation

Raw reads were adapter clipped and assessed using FASTQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc) to identify adapter contamination. We trimmed the 3′ ends of reads with < 20 Phred scores using FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/), requiring that the resulting read should be > 40 nucleotides in length. Following end trimming, reads were filtered by applying a threshold of Phred score > 20 over at least 80% of the read length. To reduce memory and runtime, redundant paired reads were normalized to 30× coverage with *normalize_by_kmer_coverage.pl*, Trinity's *in silico* read

normalization script (Grabherr *et al.*, 2011). This reduced the dataset to nearly 40 million normalized paired reads. Unpaired reads were added to the normalized paired reads and the entire set was assembled in Trinity (version r2012-10-05) with default parameters. Functional annotation of transcript assemblies was performed using the included Trinotate pipeline (http://trinotate.sourceforge.net/) to identify open reading frames and assign best hits to UniprotKB (1e-03), PFAM-A (1e-03), gene ontology (GO) and eggNOG categories. GO over-representation analysis was performed using the DAVID web server, utilizing a custom background gene set derived from the nonredundant *A. officinalis* whole-transcriptome UniprotKB annotations.

### Transcript abundance estimation and differential expression

Cleaned reads from each library were aligned to the assembly using Bowtie (v0.12.8; Langmead *et al.*, 2009) wrapped by Trinity's *alignReads.pl* script, and transcript abundance was estimated in the RSEM program v1.2.0 (Li & Dewey, 2011). Gene-level abundance estimations from RSEM were rounded up to the nearest integer and used as input for differential expression analysis in edgeR (Robinson *et al.*, 2010). Initial filtering required transcripts to be represented in at least three libraries, each with > 1 read count per million mapped reads (CPM). Given the nested experimental design, an additive model (line + sex genotype) was fitted in edgeR to remove the underlying differences between lines and to identify the genes that consistently displayed differential expression in multiple lines for a given pairwise sex comparison. Generalized linear model (GLM) common dispersions, trended dispersions and tagwise dispersions were estimated before a GLM was fitted. In order to simplify and visualize multidimensional patterns in our expression data, multidimensional scaling (MDS) analysis was performed using the edgeR function plotMDS with pair-wise log fold-changes estimated on read counts (normalized to library size) for the 250 most heterogeneously expressed genes. Differentially expressed genes (DEGs) with a false discovery rate (FDR) < 0.05 are reported in Supporting Information Table S1.

### Isolation and RNA-Seq of developmentally staged flower buds

*Asparagus officinalis* flower buds were harvested from female (XX) and double haploid androgenetic supermale (YY) clones. The flower buds were sampled on several plants of each clone at three approximate developmental stages: pre-meiotic, microspore and pollen. Total RNA was isolated from flower buds using a NucleoSpin RNA Plant kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) and treated with RNase-free DNase. RNA quantity and purity were assessed using a Nanodrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA); the RNA integrity (> 8.0) was determined using a Bioanalyzer 2100 (Agilent) with an RNA 6000 nanochip (Agilent). Synthesis of cDNA and library preparation were carried out starting with 2.5 μg of total RNA using the TruSeq RNA Sample Prep Kit v2 (Illumina) according to the manufacturer's instructions. Size selection of cDNA libraries was performed by excising and eluting 350–550-nucleotide fragments using a MinElute Gel Extraction kit (Qiagen). Following size selection, cDNA was quantified using qPCR with a KAPA Library Quantification kit (KAPA Biosystems, Woburn, MA, USA), and RNA-Seq libraries were pooled and sequenced on an Illumina HiSeq 2000, generating 2 × 100-nucleotide paired-end sequences.

## Results and Discussion

### Transcriptome sequencing and assembly

We generated RNA-Seq to assemble transcripts and quantify spear tip gene expression patterns in all females, males and supermales. Whole-transcriptome Illumina shotgun sequencing of each gender type in four inbred lines yielded nearly 648 million paired-end reads. These data were combined with two existing YY supermale spear tip libraries for a total of nearly 763 million paired-end reads (Table 1). Given the large number of sequenced reads, we used Trinity to digitally normalize and *de novo* assemble the reads into 276 556 transcripts representing 120 061 subcomponents (referred to here as genes or loci) with an N50 of 2386 nucleotides (Fig. S1).

To better assess the number of full-length transcripts assembled, we compared the transcripts with 768 340 peptide annotations from 22 sequenced and annotated plant genomes (*Amborella* Genome Project, 2013) using blastx (1e-10, best hit only), and found that 13 336 unique peptides aligned over at least 80% of their length to an *A. officinalis* transcript (Fig. S2). In addition, we performed a gene family analysis to determine the diversity of sampled transcripts. Using OrthoMCL-derived estimated gene families from these 22 genomes,

**Table 1** Sequencing read counts and alignment statistics for all sequenced garden asparagus (*Asparagus officinalis*) individuals

| Line | Sex | Tissue type | Clean pairs | % Mapped |
|---|---|---|---|---|
| 8A | Female | Spear tip | 11506758 | 88.72 |
| 8A | Male | Spear tip | 33433776 | 87.53 |
| 8A | Supermale | Spear tip | 37066238 | 88.37 |
| 8B | Female | Spear tip | 33711613 | 87.84 |
| 8B | Male | Spear tip | 6677641 | 87.43 |
| 8B | Supermale | Spear tip | 24710491 | 87.81 |
| 10 | Female | Spear tip | 40348458 | 87.50 |
| 10 | Male | Spear tip | 63871037 | 88.39 |
| 10 | Supermale | Spear tip | 1463330 | 87.18 |
| 9 | Female | Spear tip | 36909297 | 87.41 |
| 9 | Male | Spear tip | 28551654 | 86.68 |
| 1 | Supermale | Pre-meiotic* | 25929261 | 82.44 |
| 1 | Supermale | Meiotic* | 21421286 | 80.36 |
| 1 | Supermale | Post-meiotic* | 28222100 | 79.84 |
| 2 | Female | Pre-meiotic* | 28077734 | 82.50 |
| 2 | Female | Meiotic* | 27316690 | 82.59 |
| 2 | Female | Post-meiotic* | 18451370 | 81.34 |

Mapped read percentages only include those reads with concordant, paired alignments.
*Flower buds.

we translated and sorted 121 704 *Asparagus* transcripts into 13 498 distinct gene families using a pipeline described in McKain *et al.* (2012).

We annotated transcripts through comparisons with the Swiss UniprotKB complete proteome databases and other databases using the Trinotate pipeline (see the Materials and Methods section for details). Nearly 49% of the assembled transcripts had a blastx (e-value ≤ 1e-03) hit against the UniprotKB database. Similarly, 51% of the transcripts had open reading frames > 100 amino acids in length. We extracted 7774 nonredundant GO terms from 41% of the transcripts and summarized them into 103 GOslim plant categories using CateGOrizer (Hu *et al.*, 2008) (Table S2). The complete list of transcript annotations is presented in Table S3. Taken together, these analyses suggest that we sequenced and accurately assembled a diverse set of transcripts expressed in *A. officinalis* spear tips which include developing vegetative and reproductive organs.

## Diverse lines reveal male-biased gene expression

Following transcript annotation, transcript abundances for each library were calculated using RSEM. We filtered the transcriptome assembly by requiring that each tested gene be expressed at > 1 CPM in at least three libraries, which reduced the number of tested genes to 19 986. The first two dimensions of the MDS analysis on the 250 most heterogeneously expressed genes revealed clustering of individuals by breeding line rather than gender (Fig. 1). At the same time, there was substantial among-gender transcriptional variation within lines (coefficient of variation = 0.33).

After accounting for the underlying differences between lines (see the Materials and Methods section), tests for differential gene expression in pairwise male : female, supermale : female and supermale : male comparisons identified a total of 570 DEGs (Fig. 2). Almost 91% of DEGs were upregulated in males and supermales compared with females, indicating that gene expression is generally male biased in spear tip tissue. In addition, nearly 34% of DEGs were found in both male and supermale transcript pools (Fig. 3). All DEGs and their annotations are presented in Table S1.

Sex-biased gene expression is a common phenomenon in both dioecious plant and animal species, as documented in humans (Dimas & Nica, 2012), *Drosophila* (Zhang *et al.*, 2007), emus (Vicoso *et al.*, 2013) and *Silene latifolia* (Zemp *et al.*, 2014), among others. Genes that exhibit sex-biased expression might be under unique evolutionary constraints, evolving more rapidly in terms of both sequence and expression (Meiklejohn *et al.*, 2003). The developmental stage in which sex-biased genes are expressed may also impact their rate of evolution, given that they often have reproductively and developmentally specialized expression domains (Perry *et al.*, 2014).

Sex-biased gene expression in dioecious species is strongly influenced by the developmental stage at which gynoecium develop is arrested in male flowers and male function is interrupted in anthers of female flowers. In garden asparagus, anthers are initiated in female flowers and develop to a stage just before microspore meiosis (Caporali *et al.*, 1994). Therefore, pollen development genes functioning downstream of Y-linked sex determination genes are expected to be expressed in male but not female flowers. Males form a tricarpellate gynoecium with development of the stylar tube typically arrested before the stigma forms. Ovule development and abortion in males are not well characterized, but variation in male style lengths (Galli *et al.*,

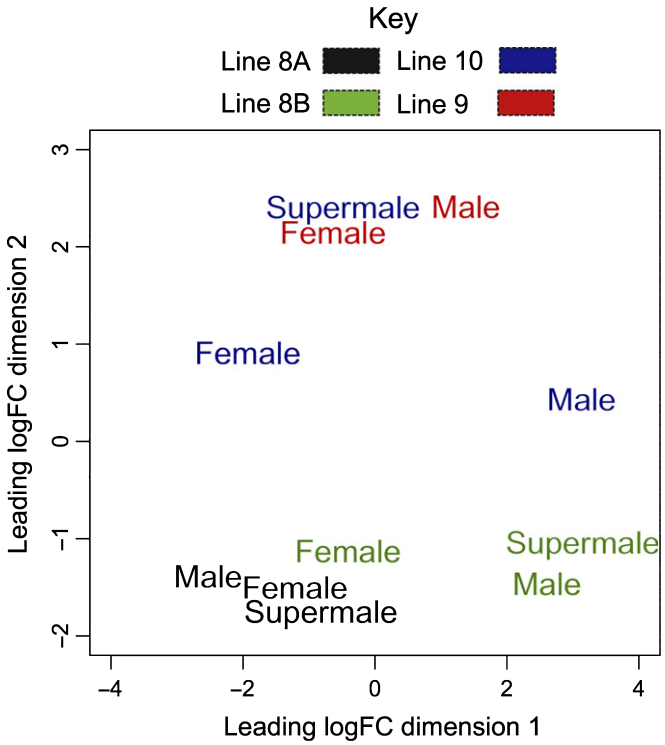


**Fig. 1** Multidimensional scaling (MDS) plot showing the distance between samples as the root-mean-square deviation (Euclidean distance) for the top 250 most heterogeneously expressed genes in independent, unsupervised pairwise comparisons between all garden asparagus (*Asparagus officinalis*) samples. FC, fold change.

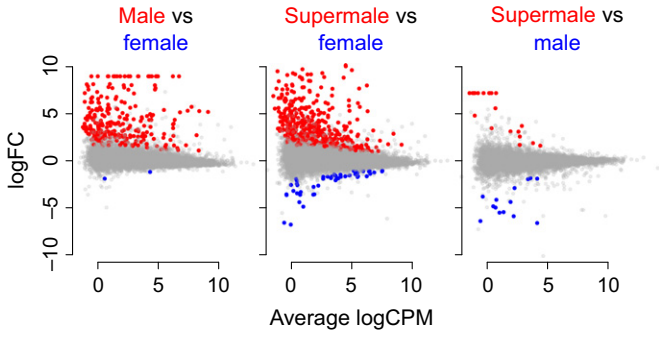


**Fig. 2** Pairwise comparisons of gene expression (average log fold change (FC) vs average log of counts per million mapped reads, CPM) among female, male and supermale garden asparagus (*Asparagus officinalis*) genes across line replicates. Red and blue dots indicate genes exhibiting significantly biased expression levels at a false discovery rate (FDR) of < 0.05. Colored dots spread horizontally at the *y*-axis upper limit represent genes not expressed in one comparison, and are plotted at the minimum CPM across all samples. Gray dots represent genes that are not significantly differentially expressed.

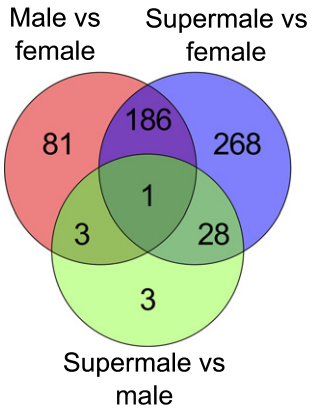


**Fig. 3** Venn diagram showing the overlap of differentially expressed garden asparagus genes (*Asparagus officinalis*) between the three pairwise comparisons.

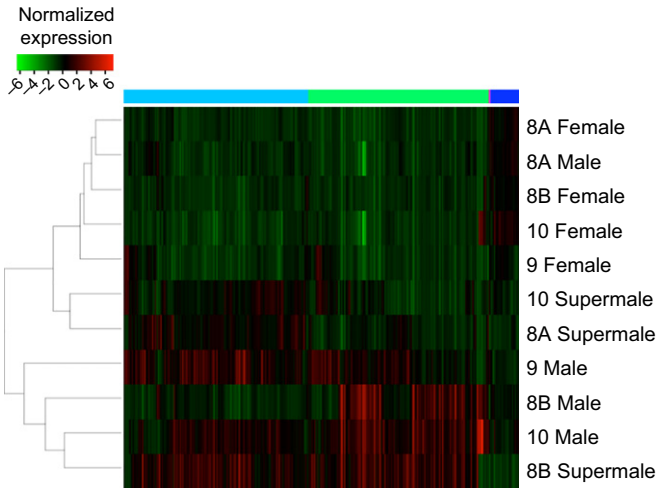


**Fig. 4** Heatmap clustering of the 570 differentially expressed garden asparagus (*Asparagus officinalis*) genes in all spear tip tissues. Trimmed mean of M-values (TMM)-normalized fragments per kilobase of transcript per million fragments sequenced (FPKM) values were log transformed before ward clustering, cutting the tree into $k = 4$ clusters.

1993) and the occurrence of andromonecy in some genotypes (Sneep, 1953) suggest a continuum of points over which female development is interrupted in male flowers. The expression profiles of many genes vary over the course of development of both male and female flowers, underscoring the need for developmentally staged sampling of transcripts.

## Heterogeneous gene expression among lines for each gender

As indicated in the MDS plot of the 570 most DEGs (Fig. 1), there is significant among-line gene expression variation for each gender. This is especially true for males and supermales. Whereas both axes of the MDS plot exhibit variation among males and supermales, all females for all four lines have similar values on the first axis of the MDS plot. Heterogeneous gene expression among males and supermales is also evident in a heatmap (Fig. 4). Strikingly, the line 8A male and supermale expression profiles cluster with the four female individuals (Fig. 4). We hypothesized that the observed male and supermale expression heterogeneity is probably related to developmental differences between the lines and the sexes; despite all spear tips being collected at the same vegetative height (*c.* 15 cm above ground), males and supermales will usually flower earlier than females (Kahara *et al.*, 1940; Caporali *et al.*, 1994). To better understand this heterogeneity, we sectioned and imaged spear tips from female, male and supermale spears of the same spear height. Unfortunately, we were unable to section the lines included in the gene expression study (they are no longer available), but our observations matched those of previous studies (Kahara *et al.*, 1940; Caporali *et al.*, 1994). All gender types exhibited a developmental gradient along the length of the spear tip, with young floral buds at the top and more mature buds towards the bottom. Moreover, supermale spear tips at each height showed the most developmentally advanced flower buds, female flower buds were least developmentally advanced at each height and males were intermediate (Fig. S3). We surmise that similar developmental variation exists among lines and that flower bud development is slow in line 8A relative to the other lines.

In order to better resolve whether the heterogeneous expression patterns among males and supermales might be a result of differences in flower bud development, we generated additional targeted RNA-Seq derived from flower buds at three developmental stages (pre-meiotic, meiotic and post-meiotic) in one XX female and one YY supermale line (Table 1). Among-sample correlations in the 570 most DEGs identified in the spear tip gene expression profiles suggest that the among-line variation for each gender may indeed be a result of variation in the frequency of flower buds at each stage (Figs 5, S4). The female pre-meiotic and meiotic flower bud expression profiles are similar to those of the line 8A supermale spear tips, whereas the female spear tip transcript profiles are more correlated with the line 8A male spear tip profile. Interestingly, the supermale pre-meiotic flower bud expression profile is quite distinct from the other flower bud profiles and most similar to lines 10 and 8B male profiles (Fig. 5).

Comparisons of the flower bud and spear tip expression profiles show that future investigations of gender-based expression should focus on flower buds and flower bud primordia rather than spear tips. Nonetheless, informative gender-biased expression patterns are detected in the spear tip RNA-Seq data. Further, the among-line variation is also of interest for breeders wanting to develop male lines with more slowly developing flower buds and lateral branch primordia.

## Differential expression in the anther development pathway

Gene expression patterns in all samples were compared with expression profiles for known *Arabidopsis*, *Zea* and *Oryza* anther development genes (Ma *et al.*, 2008; Ma & Sundaresan, 2010; Wilson *et al.*, 2011; Zhang & Yang, 2014). Previous developmental studies in garden asparagus have revealed that anther development in female anthers arrests after the initiation of tapetum development, but before the formation of tetrads in microspore meiosis (Caporali *et al.*, 1994). By placing the differences

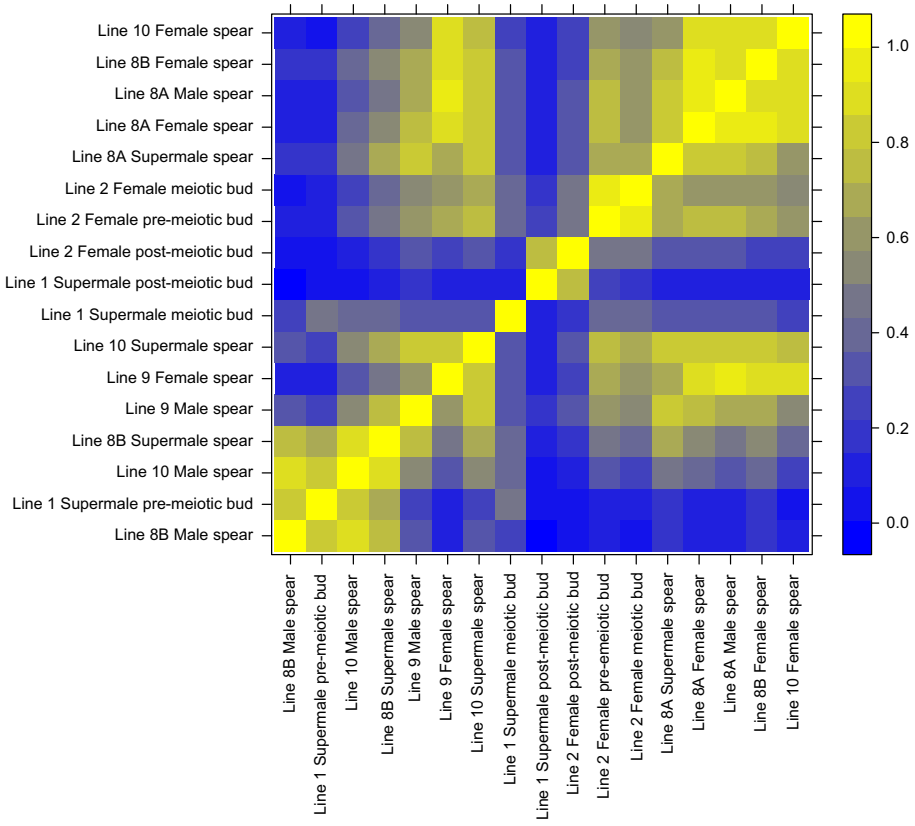


**Fig. 5** Spearman correlation matrix of the 570 differentially expressed genes in all comparisons for spear tip tissues and the developmentally staged garden asparagus (*Asparagus officinalis*) flower buds. Trimmed mean of M-values (TMM)-normalized fragments per kilobase of transcript per million fragments sequenced (FPKM) values were used to generate a Spearman correlation matrix, and individuals were ordered according to a complete clustering dendrogram using that matrix (Supporting Information Fig. S4).

between male and female gene expression within the context of the known anther development pathway (Fig. 6), we aimed to distinguish differentially expressed sex determination genes from downstream male function genes.

A total of 516 genes was identified as exhibiting male-biased (XY and YY) expression relative to females. Of these, 46 displayed no expression in any XX female spear (Table S1). Genes with male-specific expression in *A. officinalis* included homologs of Quartet1 (QRT1), polygalacturonase (PG) and other pollen-related genes. QRT1 is a pectinesterase expressed in *A. thaliana* anthers shortly after microspore meiosis is completed. QRT1 is required for proper microspore separation (Francis *et al.*, 2006). The *qrt1* mutant will properly deposit and degrade callose on the secondary pollen mother cell (PMC) wall, but the primary PMC is left mostly intact (Preuss *et al.*, 1994; Rhee & Somerville, 1998). PG, a member of a family of cell wall-loosening enzymes that are specifically involved in the degradation of pectin and cell walls, is involved in pollen exine development. Several PG homologs in *Brassica napus*, *Zea mays* and *Lillium longiflorium* are specifically expressed in late-stage pollen development (Niogret *et al.*, 1991; Robert *et al.*, 1993; Chiang *et al.*, 2006). Several other pollen cell wall-related genes are significantly more abundant in males and supermales relative to females, including genes encoding Laccase 7, pollen-specific leucine-rich repeat extension-like protein 1, oligopeptide transporter 5 (OPT5) and glucan endo-1,3-β-glucosidase. This transcriptional pattern suggests that genes required for proper pollen wall exine formation are expressed weakly in female anthers relative to males, which agrees with previous microscopic analysis of anther degeneration in XX female plants (Caporali *et al.*, 1994; Mariziani *et al.*, 1999). Although each of these genes could be considered as promoters of male function, their reduced transcription in female flowers does not necessarily imply that they are ultimately responsible for gender determination.

As the expression data suggested that the gene(s) ultimately responsible for female anther sterility occurs upstream of pollen exine formation, we next identified genes that tended to show high expression in *A. thaliana* tapetal tissues. *Male Sterility 2* (*MS2*) exhibits male-specific expression in *A. officinalis*, encoding a fatty acid reductase that is primarily expressed in the *A. thaliana* tapetum during pollen tetrad release (Aarts *et al.*, 1997). Another tapetum-specific gene is *Aborted Microspores* (*AMS*), a gene that encodes a MYC class basic helix–loop–helix (bHLH) transcription factor that is expressed specifically in the tapetum of *A. thaliana* anthers. Tapetal tissue in *ams* mutants degenerates prematurely (Xu *et al.*, 2010), similar to developmental defects found in *A. officinalis* females (Caporali *et al.*, 1994). AMS can also modulate the expression of a suite of anther development genes, binding to promoter regions *in vivo* (Xu *et al.*, 2010). Although exhibiting significantly higher expression in males, we also found female expression for *AMS*. Similar patterns of male- and supermale-biased expression emerge for other tapetum-related transcription factors, such as bHLH89, which has been shown to interact with AMS during anther development (Ma *et al.*, 2012).

Finally, we identified genes expressed further upstream of tapetum and pollen development, with the expectation that they would be expressed in both males and females. Indeed, we found

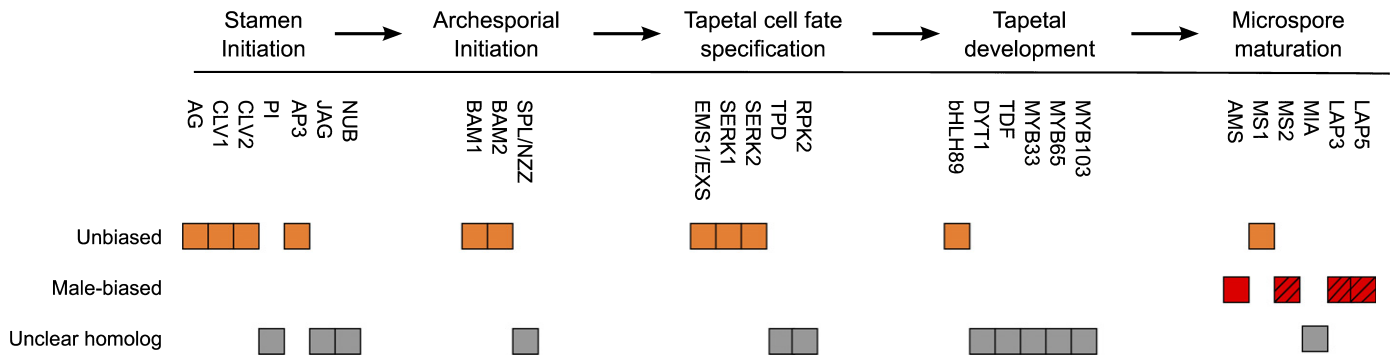


**Fig. 6** A sampling of genes involved in the *Arabidopsis thaliana* anther development pathway and their expression patterns in female and male/supermale *Asparagus officinalis*. Using 0.1 fragments per kilobase of transcript per million fragments sequenced (FPKM) as a cut-off for the identification of a gene as expressed, we identified genes that showed no gender-biased expression between female, male and supermale sexes (orange squares). Genes involved in stamen initiation, archesporial initiation and tapetal cell fate specification show expression in all genders. Later in tapetal development and microspore maturation, some genes, such as *Aborted Microspores* (*AMS*), show male-biased expression (red blocks without hatching), whereas others, such as *Male Sterility 2* (*MS2*), *LAP3* and *LAP5*, show male-specific expression (red blocks with hatching). Genes that were unable to be confidently annotated in our transcriptome assembly are represented by gray blocks.

very similar female, male and supermale expression levels for genes involved in development at multiple stages far upstream of microsporogenesis (stamen initiation, archesporial initiation, tapetal cell fate specification and tapetal development; Fig. 6). Given the developmental patterns described by Caporali *et al.* (1994) and earlier, genes involved in initiation and early development of male and female organs were expected to show similar expression patterns. For example, in agreement with earlier work (Park *et al.*, 2003) no difference was observed between male and female flower buds in expression of homeotic MADS box genes.

## Differential expression in the phenylpropanoid biosynthesis pathway

We also found numerous genes in the phenylpropanoid biosynthesis pathway that were differentially expressed between sexes. The general phenylpropanoid and flavonoid pathway is well described, and has been shown to affect pollen development and male sterility (Van der Meer *et al.*, 1992). Significant male- or supermale-biased expression occurs in genes encoding cinnamic 4-hydroxylase acid (C4H), chalcone synthase (CHS), flavanone-3-hydroxylase (F3H), stilbene synthase 2 (STS2), stilbene synthase 4 (STS4) and shikimate O-hydroxycinnamoyltransferase (HCT). Several genes in the phenylpropanoid pathway exhibit significantly higher transcript abundance in females as well, including cinnamoyl-CoA reductase 1 and 2 (CCR1/CCR2) and leucoanthocyanidin deoxygenase (LDOX).

We identified two DEGs annotated as CHS. One of these transcript assemblies (comp32958_c0) was annotated on the basis of a match to a *Malus* × *domestica* gene model in UniprotKB, and showed even closer similarity to the *A. thaliana LESS ADHESIVE POLLEN 5* (*LAP5*) gene, a chalcone and stilbene synthase family member. LAP5 is essential for proper pollen exine development and shows co-expression with other asparagus male anther-specific genes, such as *MS2*, providing additional transcriptional evidence for the lack of proper pollen development in female asparagus plants (Dobritsa *et al.*, 2010). Further, the differential expression of numerous phenylpropanoid genes in dioecious asparagus is intriguing, given that disruptions in the phenylpropanoid and downstream anthocyanin pathways have classically been implicated in nuclear and cytoplasmic male sterility (CMS) phenotypes (e.g. Van der Meer *et al.*, 1992; Matsuda *et al.*, 1996).

Overall, the observed patterns of gender biases in gene expression lead us to hypothesize that a Y-linked male promoting gene, perhaps complementing a deficiency on the nonrecombining portion of the X chromosome, plays some role in the late development of the tapetum in early microsporocyte development, probably before or during pollen wall exine formation and disassociation of pollen tetrads. This inference is consistent with the developmental investigations of Caporali *et al.* (1994) and the model of sex chromosome evolution proposed by Charlesworth & Charlesworth (1978).

## Differential expression in ovule development genes

Gynoecium and female gametophyte development pathways have been investigated through the analysis of homeotic mutants (see Alvarez & Smyth, 1999; Ferrándiz *et al.*, 1999). A small number of asparagus transcripts with *Arabidopsis* homologs influencing ovule development exhibited female-biased expression relative to males or supermales. In total, just 51 genes exhibited significantly higher transcript abundance in females relative to males or supermales. The smaller number of transcripts exhibiting female-biased expression relative to those exhibiting male-biased expression is not surprising, given the massive diversity of genes expressed during anther and pollen (the male gametophyte) development (Ma *et al.*, 2008). GO term enrichment for transcripts with female-biased expression included phenylpropanoid biosynthesis and cellular amino acid derivative processes (Table S4). Among these DEGs, we found *AINTEGUMENTA* (*ANT*) homolog transcripts as significantly more abundant in females than males or supermales. *ANT* is an APETALA2 (AP2) domain containing ethylene-responsive transcription factor that regulates many aspects of female growth, including ovule and gynoecium development, inner and outer integument

development, and petal epidermal cell identity (Elliott *et al.*, 1996; Klucher *et al.*, 1996; Mizukami & Fischer, 2000). Previous studies in *Arabidopsis* have shown that *ant* mutants are female sterile, with undeveloped or malformed integuments and a failure for megasporogenesis to proceed past the tetrad stage (Elliott *et al.*, 1996).

Several other genes with female-biased expression and homologs involved in female gametophyte development and flowering time were also identified, including *PIN2* and *FD*. The *PIN* gene family is intriguing, given its involvement in auxin transport, and some members show expression early in ovule development in *A. thaliana* (Fraga *et al.*, 2002; Ceccato *et al.*, 2013). *FD* is another interesting gene involved in the transition from vegetative to floral development, encoding for a bZIP transcription factor that directly regulates *FLOWERING LOCUS T* (*FT*), the floragen gene which promotes the transition to flowering at the shoot apex (Abe *et al.*, 2005).

### Conclusions

*De novo* RNA-Seq transcriptome assembly and expression analysis can inform the investigation of gender determination in dioecious species, but, although many genes may exhibit sex-based expression, few are expected to regulate gender determination. Most genes exhibiting male and female-biased expression function downstream of gender specification in the developing flower bud. Here, we placed genes exhibiting gender-biased expression within anther and carpel developmental pathways in order to identify the earliest points in these pathways that are influenced by Y-linked *Asparagus* gender determination genes. As expected, we observed male-specific expression for many known pollen development genes, but similar expression levels in male and female spear tips for organ-specifying genes and several tapetum development genes, corroborating previous findings (Caporali *et al.*, 1994) in suggesting that female asparagus anthers initiate the abortion process during tapetum and young microspore development. The DEGs placed within the anther and gynoecium developmental pathways will guide ongoing work to identify Y-linked genes ultimately responsible for sex determination in dioecious *Asparagus* species.

Unsurprisingly, *Asparagus* homologs of the HD-Zip genes, hypothesized to regulate gender differences in anther development in dioecious persimmon species (Akagi *et al.*, 2014), were not implicated as possible gender determination genes. Class I HD-Zip transcripts were observed in the transcript assembly, but none exhibited sex-biased expression. Given that dioecy has evolved independently in many flowering plant lineages (Charlesworth, 2002; Renner, 2014), and suppression or loss of function mutations of many genes in the anther development pathway would convert hermaphroditic flowers to female, we expect that the identity of sex determination genes will vary among unrelated dioecious species.

## Acknowledgements

This work was supported by funding from the National Science Foundation grants DEB-0951071 and DEB-0841988, the University of Georgia and Limgroup (Horst, the Netherlands). We are particularly grateful for the insights provided by Ron van der Hulst of Limgroup. Sequencing was performed through the Georgia Genomics Facility (www.dna.uga.edu) and computational resources were provided by the Georgia Advanced Computing Resource Center (www.gacrc.uga.edu).

## References

**Aarts M, Hodge R, Kalantidis K. 1997.** The Arabidopsis *MALE STERILITY 2* protein shares similarity with reductases in elongation/condensation complexes. *Plant Journal* **12**: 615–623.

**Abe M, Kobayashi Y, Yamamoto S, Daimon Y, Yamaguchi A, Ikeda Y, Ichinoki H, Notaguchi M, Goto K, Araki T. 2005.** FD, a bZIP protein mediating signals from the floral pathway integrator FT at the shoot apex. *Science* **309**: 1052–1056.

**Ainsworth C. 2000.** Boys and girls come out to play: the molecular biology of dioecious plants. *Annals of Botany* **86**: 211–221.

**Akagi T, Henry I, Tao R, Comai L. 2014.** A Y-chromosome-encoded small RNA acts as a sex determinant in persimmons. *Science* **346**: 646–650.

**Al-Dous EK, George B, Al-Mahmoud ME, Al-Jaber MY, Wang H, Salameh YM, Al-Azwani EK, Chaluvadi S, Pontaroli AC, DeBarry J *et al.* 2011.** *De novo* genome sequencing and comparative genomics of date palm (*Phoenix dactylifera*). *Nature Biotechnology* **29**: 521–527.

**Al-Mssallem IS, Hu S, Zhang X, Lin Q, Liu W, Tan J, Yu X, Liu J, Pan L, Zhang T *et al.* 2013.** Genome sequence of the date palm *Phoenix dactylifera* L. *Nature Communications* **4**: 2274.

**Alvarez J, Smyth D. 1999.** *CRABS CLAW* and *SPATULA*, two genes that control carpel development in parallel with *AGAMOUS*. *Current Topics in Developmental Biology* **45**: 155–205.

**Caporali E, Carboni A, Galli M. 1994.** Development of male and female flowers in *Asparagus officinalis*. Search for point of transition from hermaphroditic to unisexual developmental pathway. *Sexual Plant Reproduction* **7**: 239–249.

**Ceccato L, Masiero S, Sinha Roy D, Bencivenga S, Roig-Villanova I, Ditengou FA, Palme K, Simon R, Colombo L. 2013.** Maternal control of PIN1 is required for female gametophyte development in Arabidopsis. *PLoS ONE* **8**: e66148.

**Chang F, Wang Y, Wang S, Ma H. 2011.** Molecular control of microsporogenesis in Arabidopsis. *Current Opinion in Plant Biology* **14**: 66–73.

**Charlesworth B, Charlesworth D. 1978.** A model for the evolution of dioecy and gynodioecy. *American Naturalist* **112**: 975–997.

**Charlesworth D. 2002.** Plant sex determination and sex chromosomes. *Heredity* **88**: 94–101.

**Charlesworth D, Charlesworth B. 1979.** The evolutionary genetics of sexual systems in flowering plants. *Proceedings of the Royal Society of London. Series B. Biological Sciences* **205**: 513–530.

**Chettoor AM, Givan SA, Cole RA, Coker CT, Unger-Wallace E, Vejlupkova Z, Vollbrecht E, Fowler JE, Evans MM. 2014.** Discovery of novel transcripts and gametophytic functions via RNA-seq analysis of maize gametophytic transcriptomes. *Genome Biology* **15**: 414.

**Chiang JY, Shu SW, Ko CW, Wang CS. 2006.** Biochemical characterization of a pollen-specific cDNA encoding polygalacturonase in *Lilium longiflorum*. *Plant Science* **170**: 433–440.

**Deng C-L, Qin R-Y, Wang N-N, Cao Y, Gao J, Gao W-J, Lu L-D. 2012.** Karyotype of asparagus by physical mapping of 45S and 5S rDNA by FISH. *Journal of Genetics* **91**: 209–212.

**Diggle PK, Di Stilio VS, Gschwend AR, Golenberg EM, Moore RC, Russell JRW, Sinclair JP. 2011.** Multiple developmental processes underlie sex differentiation in angiosperms. *Trends in Genetics* **27**: 368–376.

**Dimas A, Nica A. 2012.** Sex-biased genetic effects on gene regulation in humans. *Genome Research* **22**: 2368–2375.

**Dobritsa A, Lei Z, Nishikawa S-I, Urbanczyk-Wochniak E, Huhman DV, Preuss D, Sumner LW. 2010.** *LAP5* and *LAP6* encode anther-specific proteins with similarity to chalcone synthase essential for pollen exine development in *Arabidopsis thaliana*. *Plant Physiology* **153**: 937–955.

**Elliott R, Betzner A, Huttner E. 1996.** AINTEGUMENTA, an APETALA2-like gene of Arabidopsis with pleiotropic roles in ovule development and floral organ growth. *Plant Cell* **8**: 155–168.

**Falavigna A, Casali PE. 2002.** Practical aspects of breeding program of asparagus based on *in vitro* anther culture. *Acta Horticulturae* **589**: 201–210.

**Ferrándiz C, Pelaz S, Yankofsky MF. 1999.** Control of carpel and fruit development in Arabidopsis. *Annual Reviews of Biochemistry* **68**: 328–354.

**Flory WS. 1932.** Genetic and cytological investigations on *Asparagus officinalis* L. *Genetics* **17**: 432–467.

**Fraga MF, Rodríguez R, Cañal MJ. 2002.** Genomic DNA methylation-demethylation during aging and reinvigoration of *Pinus radiata*. *Tree Physiology* **22**: 813–816.

**Francis KE, Lam SY, Copenhaver GP. 2006.** Separation of Arabidopsis pollen tetrads is regulated by *QUARTET1*, a pectin methylesterase gene. *Plant Physiology* **142**: 1004–1013.

**Franken AA. 1970.** Sex characteristics and inheritance of sex in asparagus (*Asparagus officinalis* L.). *Euphytica* **19**: 277–287.

**Galli MG, Bracale M, Falavigna A, Raffaldi F, Savini C, Vigo A. 1993.** Different kinds of male flowers in the dioecious plant *Asparagus of officinalis* L. *Sexual Plant Reproduction* **6**: 16–21.

**Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, Amit I, Adiconis X, Fan L, Raychowdhury R, Zeng Q *et al.* 2011.** Full-length transcriptome assembly from RNA-seq data without a reference genome. *Nature Biotechnology* **29**: 644–652.

**Guo X, Su B, Zhou Z, Sha J. 2009.** Rapid evolution of mammalian X-linked testis microRNAs. *BMC Genomics* **10**: 97.

**Hu Z-L, Bao J, Reecy J. 2008.** CateGOrizer: a web-based program to batch analyze gene ontology classification categories. *Online Journal of Bioinformatics* **9**: 108–112.

**Jamsari A, Nitz I, Reamon-Büttner SM, Jung C. 2004.** BAC-derived diagnostic markers for sex determination in asparagus.. *Theoretische und angewandte Genetik* **108**: 1140–1146.

**Kahara K, Hagiwara T, Hamada K, Tamura S. 1940.** A study of sex in young asparagus plantings. *Journal of the Horticular Association of Japan* **11**: 150–161.

**Kanno A, Kubota S, Ishino K. 2014.** Conversion of a male-specific RAPD marker into an STS marker in *Asparagus officinalis* L. *Euphytica* **197**: 39–46.

**Klucher KM, Chow H, Reiser L, Fischer RL. 1996.** The AINTEGUMENTA gene of Arabidopsis required for ovule and female gametophyte development is related to the floral homeotic gene APETALA2. *Plant Cell* **8**: 137–153.

**Langmead B, Trapnell C, Pop M, Salzberg S. 2009.** Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biology* **10**: R25.

**Li B, Dewey CN. 2011.** RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics* **12**: 323.

**Löptien H. 1979.** Identification of the sex chromosome pair in asparagus (*Asparagus officinalis* L.). *Zeitschrift fur Pflanzenzüechtung* **82**: 162–173.

**Ma H, Sundaresan V. 2010.** Development of flowering plant gametophytes. *Current Topics in Developmental Biology* **91**: 379–412.

**Ma J, Morrow DJ, Fernandes J, Walbot V. 2008.** Comparative profiling of the sense and antisense transcriptome of maize lines. *Genome Biology* **7**: R22.

**Ma X, Feng B, Ma H. 2012.** AMS-dependent and independent regulation of anther transcriptome and comparison with those affected by other *Arabidopsis* anther genes. *BMC Plant Biology* **12**: 23.

**Mariziani G, Caporali E, Spada A. 1999.** Search for genes involved in asparagus sex determination. In: Ainsworth CC, ed. *Sex determination in plants*. Oxford, UK: BIOS Scientific Publishers, 149–162.

**Matsuda N, Tsuchiya T, Kishitani S, Tanaka Y, Toriyama K. 1996.** Partial male sterility in transgenic tobacco carrying antisense and sense PAL cDNA under the control of a tapetum-specific promoter. *Plant and Cell Physiology* **37**: 215–222.

**McKain MR, Wickett N, Zhang Y, Ayyampalayam S, McCombie WR, Chase MW, Pires JC, dePamphilis CW, Leebens-Mack J. 2012.** Phylogenomic analysis of transcriptome data elucidates co-occurrence of a paleopolyploid event and the origin of bimodal karyotypes in Agavoideae (Asparagaceae). *American Journal of Botany* **99**: 397–406.

**Meiklejohn CD, Parsch J, Ranz JM, Hartl DL. 2003.** Rapid evolution of male-biased gene expression in *Drosophila*. *Proceedings of the National Academy of Sciences, USA* **100**: 9894–9899.

**Ming R, Hou S, Feng Y, Yu Q, Dionne-Laporte A, Saw JH, Senin P, Wang W, Ly BV, Lewis KLT *et al.* 2008.** The draft genome of the transgenic tropical fruit tree papaya (*Carica papaya* Linnaeus). *Nature* **452**: 991–996.

**Mizukami Y, Fischer RL. 2000.** Plant organ size control: *AINTEGUMENTA* regulates growth and cell numbers during organogenesis. *Proceedings of the National Academy of Sciences, USA* **97**: 942–947.

**Muyle A, Zemp N, Deschamps C, Mousset S, Widmer A, Marais GAB. 2012.** Rapid *de novo* evolution of X chromosome dosage compensation in *Silene latifolia*, a plant with young sex chromosomes. *PLoS Biology* **10**: e1001308.

**Nakayama H, Ito T, Hayashi Y, Sonoda T, Fukuda T, Ochiai T, Kameya T, Kanno A. 2006.** Development of sex-linked primers in garden asparagus (*Asparagus officinalis* L.). *Breeding Science* **56**: 327–330.

**Niogret MF, Dubald M, Mandaron P, Mache R. 1991.** Characterization of pollen polygalacturonase encoded by several cDNA clones in maize. *Plant Molecular Biology* **17**: 1155–1164.

**Orozco-Arroyo G, Vázquez-Santana S, Camacho A, Dubrovsky JG, Cruz-García F. 2012.** Inception of maleness: auxin contribution to flower masculinization in the dioecious cactus *Opuntia stenopetala*. *Planta* **236**: 225–238.

**Park JH, Ishikawa Y, Yoshida R, Kanno A, Kameya T. 2003.** Expression of *AODEF*, a B-functional MADS-box gene, in stamens and inner tepals of the dioecious species *Asparagus officinalis* L. *Plant Molecular Biology* **51**: 867–875.

**Peng M, Wolyn DJ. 1999.** Development of a microspore culture method to produce haploid and doubled-haploid asparagus (*Asparagus officinalis* L.) plants. *Acta Horticulturae* **479**: 357–363.

**Perry JC, Harrison PW, Mank JE. 2014.** The ontogeny and evolution of sex-biased gene expression in *Drosophila melanogaster*. *Molecular Biology and Evolution* **31**: 1206–1219.

**Preuss D, Rhee SY, Davis RW. 1994.** Tetrad analysis possible in Arabidopsis with mutation of the *QUARTET* (*QRT*) genes. *Science* **264**: 1458–1460.

**Renner SS. 2014.** The relative and absolute frequencies of angiosperm sexual systems: dioecy, monoecy, gynodioecy, and an updated online database. *American Journal of Botany* **101**: 1588–1596.

**Rhee SY, Somerville CR. 1998.** Tetrad pollen formation in quartet mutants of *Arabidopsis thaliana* is associated with persistence of pectic polysaccharides of the pollen mother cell wall. *Plant Journal* **15**: 79–88.

**Robert LS, Allard S, Gerster JL, Cass L, Simmonds J. 1993.** Isolation and characterization of a polygalacturonase gene highly expressed in *Brassica napus* pollen. *Plant Molecular Biology* **23**: 1273–1278.

**Robinson MD, McCarthy DJ, Smyth GK. 2010.** edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* **26**: 139–140.

**Sneep J. 1953.** The significance of andromonoecy for the breeding of *Asparagus officinalis* L. *Euphytica* **2**: 89–95.

**Song Y, Ma K, Bo W, Zhang Z, Zhang D. 2012.** Sex-specific DNA methylation and gene expression in andromonoecious poplar. *Plant Cell Reports* **31**: 1393–1405.

**Telgmann-Rauber A, Jamsari A, Kinney MS, Pries JC, Jung C. 2007.** Genetic and physical maps around the sex-determining M-locus of the dioecious plant asparagus. *Molecular Genetics and Genomics* **278**: 221–234.

**Tuskan GA, Difazio S, Jansson S, Bohlmann J, Grigoriev I, Hellsten U, Putnam N, Ralph S, Rombauts S, Salamov A *et al.* 2006.** The genome of black cottonwood, *Populus trichocarpa* (Torr. & Gray). *Science* **313**: 1596–1604.

**Van Bakel H, Stout JM, Cote AG, Tallon CM, Sharpe AG, Hughes TR, Page JE. 2011.** The draft genome and transcriptome of *Cannabis sativa*. *Genome Biology* **12**: R102.

**Van der Meer IM, Stam ME, van Tunen AJ, Mol JN, Stuitje AR. 1992.** Antisense inhibition of flavonoid biosynthesis in petunia anthers results in male sterility. *Plant Cell* **4**: 253–262.

**Vicoso B, Kaiser VB, Bachtrog D. 2013.** Sex-biased gene expression at homomorphic sex chromosomes in emus and its implication for sex chromosome evolution. *Proceedings of the National Academy of Sciences, USA* **110**: 6453–6458.

**Wilson ZA, Song J, Taylor B, Yang C. 2011.** The final split: the regulation of anther dehiscence. *Journal of Experimental Botany* **62**: 1633–1649.

**Xu J, Yang C, Yuan Z, Zhang D, Gondwe MY, Ding Z, Liang W, Zhang D, Wilson ZA. 2010.** The *ABORTED MICROSPORES* regulatory network is required for postmeiotic male reproductive development in *Arabidopsis thaliana*. *Plant Cell* **22**: 91–107.

**Zemp N, Minder A, Widmer A. 2014.** Identification of internal reference genes for gene expression normalization between the two sexes in dioecious white Campion. *PLoS ONE* **9**: e92893.

**Zhang D, Yang L. 2014.** Specification of tapetum and microsporocyte cells within the anther. *Current Opinion in Plant Biology* **17**: 49–55.

**Zhang Y, Sturgill D, Parisi M, Kumar S, Oliver B. 2007.** Constraint and turnover in sex-biased gene expression in the genus *Drosophila*. *Nature* **450**: 233–237.

## Supporting Information

Additional supporting information may be found in the online version of this article.

**Fig. S1** Length distribution of assembled garden asparagus (*Asparagus officinalis*) transcripts.

**Fig. S2** Length of assembled garden asparagus (*Asparagus officinalis*) transcripts compared with 22 plant genome peptides using blastx (1e-10).

**Fig. S3** Floral developmental stages in spear tip sections from female, male and supermale garden asparagus (*Asparagus officinalis*) spears.

**Fig. S4** Complete clustering dendrogram of Spearman correlations between all differentially expressed garden asparagus (*Asparagus officinalis*) genes.

**Table S1** Differential expression testing and annotations in pairwise comparisons

**Tables S2** Whole-transcriptome gene ontology (GO) term assignment for garden asparagus (*Asparagus officinalis*)

**Table S3** Whole-transcriptome gene annotation for garden asparagus (*Asparagus officinalis*)

**Table S4** Gene ontology (GO) enrichment for differentially expressed genes in each pairwise comparison

Please note: Wiley Blackwell are not responsible for the content or functionality of any supporting information supplied by the authors. Any queries (other than missing material) should be directed to the *New Phytologist* Central Office.