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RESEARCH PAPER

Epigenetic aspects of floral homeotic genes in relation to sexual dimorphism in the dioecious plant *Mercurialis annua*

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Abstract

In plants, dioecy characterizes species that carry male and female flowers on separate plants and it occurs in about 6% of angiosperms; however, the molecular mechanisms that underlie dioecy are essentially unknown. The ability for sex-reversal by hormone application raises the hypothesis that the genes required for the expression of both sexes are potentially functional but are regulated by epigenetic means. In this study, proteomic analysis of nuclear proteins isolated from flower buds of females, males, and feminized males of the dioecious plant *Mercurialis annua* revealed differential expression of nuclear proteins that are implicated in chromatin structure and function, including floral homeotic proteins. Focusing on floral genes, we found that class B genes were mainly expressed in male flowers, while class D genes, as well as *SUPERMAN*-like genes, were mainly expressed in female flowers. Cytokinin-induced feminization of male plants was associated with down-regulation of male-specific genes concomitantly with up-regulation of female-specific genes. No correlation was found between the expression of class B and D genes and the changes in DNA methylation or chromatin conformation of these genes. Thus, we could not confirm DNA methylation or chromatin conformation of floral genes to be the major determinant regulating sexual dimorphisms. Instead, determination of sex in *M. annua* might be controlled upstream of floral genes by one or more sex-specific factors that affect hormonal homeostasis. A comprehensive model is proposed for sex-determination in *M. annua*.

Keywords: Chromatin, cytokinin, dioecy, DNA methylation, epigenetics, feminization, floral homeotic gene, *Mercurialis annua*, nuclear proteome, sex-determination.

Introduction

The majority of angiosperms are hermaphrodites and monoecious (sexually monomorphic), whereby both male and female organs are found on the same individual plant. In contrast, only about 6% of the angiosperms are dioecious (sexually dimorphic), where male and female flowers are carried on separate individual plants (Renner and Ricklefs, 1995; Charlesworth, 2002). Obviously, the question of cost of sexual reproduction in dioecious species has been considered by evolutionary biologists, since there is a greater cost when two individuals are required for production of offspring in contrast to hermaphrodites and monoecious plants, where one individual is sufficient. This question has puzzled botanists for generations, including Darwin (1877). Various theoretical considerations, definitions, and models have been proposed over the years to explain dioecy, but mechanistic studies to understand the regulation of sex-determination in dioecious species

at the molecular level have failed to provide a comprehensive model of the process (Obeso, 2002). In this study we do not ask the question of why dioecy exists, but rather of how? Genetic aspects related to sex-determination in dioecious species have been studied quite intensively, including our own search for molecular markers in dimorphic species (Golan-Goldhirsh *et al.*, 2001; Khadka *et al.*, 2002, 2005; Yakubov *et al.*, 2005). Dioecious plants show diversity in sex-determination systems that range from a single locus to heteromorphic chromosomes, indicating the independent origin of dioecy in various plant families (Charlesworth and Charlesworth, 1978; Akagi *et al.*, 2014; Harkess *et al.*, 2017; Puterova *et al.*, 2018).

The annual dioecious *Mercurialis annua* has been used as a model plant for dioecy because it has a short life cycle that makes it amenable to molecular-genetic studies, in contrast to most dioecious plants that are woody perennials. In addition, *M. annua* is also amenable to sex conversion by hormonal treatment, which allows a myriad of experimental designs for particular biological questions of sex determination. Phytohormones play a role in sex determination in plants, often acting in a species-specific manner to specify gender (reviewed in Golenberg and West, 2013). Ethylene and gibberellins contribute to sex differentiation in cucumber and maize, respectively (Hansen *et al.*, 1976; Trebitsh *et al.*, 1997). Hormone-induced sex change has also been shown in *Spinacia oleracea* (West and Golenberg, 2018). In *M. annua*, exogenous application of auxins has been shown to induce masculinization while cytokinins induce feminization of male plants (Delaigue *et al.*, 1984; Durand and Durand, 1991). Identification of male-specific molecular markers and recent genetic analyses have revealed that male *M. annua* possess homomorphic XY chromosomes, but which genetic components are responsible for sex determination and floral dimorphism is not yet fully known (Khadka *et al.*, 2002, 2005; Russell and Pannell, 2015; Veltsos *et al.*, 2018). The genome of *M. annua* has recently been assembled and contains over 34 000 genes, of which about third have been assigned to linkage groups, with the sex chromosome appearing as the largest group. Based on genetic mapping and exome resequencing, it has been estimated that about one-third of the Y chromosome has lost recombination capacity, which might facilitate divergence between the sexes in *M. annua* (Veltsos *et al.*, 2019). Furthermore, transcriptome analysis of males and females has revealed differential gene expression between them at the first leaf stage, while expression of sex-biased genes peaks just prior to, and after, flowering (Cossard *et al.*, 2019).

Most studies related to the regulation of flower development have been performed in hermaphroditic model species such as *Arabidopsis thaliana* that have four whorled flowers. *Mercurialis annua* belongs to type II dioecious species and has three apparent whorls, without the rudiment whorl of the opposite sex (Mitchell and Diggle, 2005). Dioecious species are considered to be amenable for identification of the genetic and epigenetic components involved in dimorphic flowers and sex determination. Therefore, in this study we have used this species to examine several functional classes of floral homeotic, MADS box-containing transcription factors (TFs) that regulate organ identity in various whorls, which are described by the ABCDE model (Coen and Meyerowitz, 1991; Krizek and Fletcher, 2005; Theißen *et al.*, 2016). Thus, the class A proteins APETALA1 (AP1) and AP2 together with the class E proteins SEPALLATA1 (SEP1) to SEP4 specify sepals, class B proteins such as AP3 and PISTILLATA (PI) together with class A and class E proteins specify petals, class C AGAMOUS (AG) together with class B and class E proteins specify stamens, class C and class E proteins specify carpels, and class D proteins such as SHATTERPROOF1 (SHP1)/AGAMOUS-LIKE1 (AGL1) together with class E proteins specify ovules (Theißen and Saedler, 2001; Soltis *et al.*, 2007). The class B–E proteins play the key role in the development of reproductive whorls, i.e. the stamens and carpels, whilst the SUPERMAN (SUP) transcription factor has been proposed to act as a negative regulator of class B genes to maintain boundaries between the two whorls (Bowman *et al.*, 1992; Yun *et al.*, 2002; Wuest *et al.*, 2012; Ó'Maoiléidigh *et al.*, 2013; Stewart *et al.*, 2016; Prunet *et al.*, 2017).

Multiple studies that have examined the expression patterns of MADS-box floral genes in type I dioecious plants such as *Silene latifolia* and *Rumex acetosa* (Hardenack *et al.*, 1994; Ainsworth *et al.*, 1995) as well as in type II dioecious plants such as *Thalictrum dioicum* and *S. oleracea* (Di Stilio *et al.*, 2005; Pfent *et al.*, 2005) have shown that their expression in dioecious plants essentially follows the classical ABC model of flowering (Coen and Meyerowitz, 1991). Consistent with these findings, Sather *et al.* (2010) showed that silencing of class B genes in *S. oleracea* is sufficient to alter the floral gender of males into hermaphrodites or females due to transformation of stamens into carpels. However, the genetic and/or epigenetic regulation of the sexually dimorphic expression of floral genes is poorly understood. The ability of hormone application to cause sex-reversal suggests sexual bi-potency in *M. annua*, and that the genes required for the development of both sexes are present in both genders but they may be restrained by various factors, including epigenetics in the floral primordia, to bring about dioecy. Epigenetics refers to changes in heritable phenotypes that do not involve changes in the DNA sequence but instead involve changes in the regulation of gene expression. This is brought about by multiple mechanisms that control chromatin structure and function, including DNA methylation and histone modification, which are often controlled by sRNA-based mechanisms (Gibney and Nolan, 2010).

The capacity for hormonal sex-reversal in *M. annua* prompted us to examine the expression pattern its floral homeotic genes and to determine whether their epigenetic regulation represents the major constituent in sex determination. We found that differential expression of floral homeotic genes was associated with sexual dimorphism in *M. annua* and that cytokinin was involved in their transcriptional control. Furthermore, cytokinin-induced feminization of males was accompanied by extensive changes in nuclear proteins that are involved in chromatin structure and function. However, the relationship between sexual dimorphism and epigenetic regulation of floral homeotic genes could not be confirmed in the present work. Based on our results, a model is proposed for sex determination in *M. annua*.

Materials and methods

Plant growth conditions

Dioecious *Mercurialis annua* (Euphorbiaceae) of Belgian origin was used in this study. Seeds were sown in trays containing standard gardening soil and seedlings were transplanted into 2.5-l pots and grown in a controlled climate growth chamber at 27 °C with photoperiod of 14/10 h light/dark and light intensity of ~400 µmol m^{-2} s^{-1}.

Feminization of male plants by treatment with 6-benzylaminopurine

At the onset of flowering (plants ~25 d old), male and female plants were separated. Feminization of the isolated male plants was done by spraying 1 mg l^{-1} 6-benzylaminopurine (BAP) three times daily as described previously (Durand and Durand, 1991; Khadka *et al.*, 2005). Samples from three biological replicates were collected and either used immediately for isolation of nuclei or stored at –80 °C until analysis. Each biological replicate consisted of a pool of flower buds from 3–5 plants with >20 flower buds from each plant.

Isolation of nuclei

Flower buds were cut into small pieces in ice-cold nuclei isolation buffer (NIB; Saxena *et al.*, 1985) supplemented with protease inhibitor cocktail (Sigma). The homogenates were gently rotated at 4 °C for 1 h and then filtered through a 100-µm nylon mesh followed by passing through a 30-µm nylon mesh. The filtered homogenate was then centrifuged 300 *g* at 4 °C for 8 min, the supernatant was discarded, and the nuclei pellet was gently washed to remove the upper chloroplast layer. The pellets were washed twice with NIB and inspected under a confocal microscope to ensure that they were of high quality.

Proteomic analysis

The nuclei isolated from the flower buds were subjected to analysis by the proteomic services of The Smoler Protein Research Center at the Technion, Israel. Briefly, the samples were digested with trypsin, analysed by LC-MS/MS on a Q-Exactive (ThermoFisher Scientific), and identified using the Discoverer1.4 software against the *Ricinus communis*, *Jatropha curcas*, and Arabidopsis protein databases (http://uniprot.org). All the identified peptides were filtered with high confidence, top rank, and mass accuracy. High-confidence peptides were passed the 1% false discovery rate (FDR) threshold. The peak area on the chromatogram of a protein was calculated from the average of the peptides from each protein. The PANTHER classification tool was used for categorization of differentially expressed proteins (Mi *et al.*, 2013). The proteomics analysis was repeated, and the two datasets were compared and showed ~60% repeatability; low repeatability and reproducibility is often seen in proteomics (Tabb *et al.*, 2010).

Nucleic acid extraction and cDNA synthesis

Genomic DNA was extracted using a PureLink Genomic DNA Mini Kit (ThermoFisher Scientific) according to the manufacturer's protocol. Total RNA was extracted using a RNeasy Mini Kit (Qiagen). The first strand of cDNA was synthesized from 1 µg total RNA treated with DNase (Epicentre) using a Verso cDNA Synthesis Kit (ThermoFisher Scientific).

Isolation of genes and partial promoter sequences

Floral homeotic cDNA clones were prepared by PCR using *M. annua* flower cDNA as the template and appropriate degenerate primers (based on conserved regions of *A. thaliana*, *R. communis*, and *J. curcas*; Supplementary Table S1) for the recovery of class B (*AP3*, *PI*, *TM6*), class C/D (*AG*, *AGL5*, *AGL11*), and two *SUPERMAN*-like (*SUP*-like) gene products. Touchdown PCR conditions were as follows: 95 °C for 2 min; 40 cycles of 95 °C for 30 s; 65–45 °C for 30 s; 72 °C for 60 s; 72 °C for 5 min. The PCR products were cloned into the pJET1.2 plasmid vector (ThermoFisher Scientific) and sequenced at the Biotechnology Center, Ben-Gurion University of the Negev, Beer-Sheva, Israel. To obtain the full cDNA sequence, 3′- RACE was performed as described by Yadav *et al.* (2012) and 5′-RACE was performed using a 5′-Full RACE Core Set kit (Takara). The class B orthologs were designated as *MaPI* (for *PISTILLATA*), *MaAP3* (for *APETALA3*), *MaTM6* (for *TOMATO MADS-box 6*). The AGAMOUS-like orthologs were designated as *MaAG1* (for *AGAMOUS*, class C), *MaAGL1* (for *AGL11/STK*, class D), and *MaAGL3* (for *SHP2/AGL5*, class D). The *SUP*-like genes were designated as *MaSL1* and *MaSL2*.

The upstream promoters of *MaAP3*, *MaAGL1*, *MaPI*, *MaSL1*, and *MaSL2* were isolated by a semi-random sequence walking strategy modified from Aquino and Figueiredo (2004). Briefly, a gene-specific primer was used for linear amplification of the specific DNA segment for 20 high-stringency cycles (95 °C for 30 s, 60 °C for 30 s, 72 °C for 2 min). The random walking primer was then added and a low-stringency cycle (95 °C for 30 s, 35 °C for 30 s, 72 °C for 2 min) was used for unspecific binding and amplification. Following this, 30 high-stringency cycles were used for exponential amplification. The desired fragments were screened by semi-nested PCR using an asymmetrical ratio (1:5) of walking primer and nested gene-specific primer. The products of interest were purified, cloned, and sequenced as described above. The sequences are available under NCBI GenBank accession numbers KR781112-KR781116 and MN068012-MN068021.

For reference, 135 bp of the *Actin* gene was amplified using primers designed from conserved region of mRNA of *J. curcas*, *R. communis*, and *Populus trichocarpa*. The amplified product of the *M. annua ACTIN* (*Act*) gene was confirmed by direct sequencing from both ends.

Gene expression analysis

Quantification of gene expression was done by quantitative or semi-quantitative RT-PCR analysis using gene-specific primers. qPCR reactions were carried out using Perfecta SYBR green supermix (Quanta Biosciences) on an Applied Biosystems 7500 Real-Time PCR System. All reactions were performed for three biological samples, each with three technical replicates. The PCR conditions were as follows: 94 °C for 15 s, 40 cycles of 94 °C for 5 s, 60 °C for 30 s. Each reaction was normalized against the expression of the *Actin* gene. The relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

MNase assays

Nuclei prepared from male and feminized *M. annua* flower buds were used for micrococcal nuclease (MNase) assays essentially as described by Zhao *et al.* (2001). MNase assays were performed for three biological replicates, each consisting of nuclei derived from flower buds from at least three individual plants. The recovery of DNA after MNase treatment was checked by PCR and separated on agarose gel containing ethidium bromide.

DNA methylation analysis

For cytosine methylation analysis, chop-PCR (methylation-sensitive enzyme digestion followed by PCR) and bisulfite sequencing were performed as described previously (Yadav *et al.*, 2018). For the chop-PCR, genomic DNA was treated with the methylation-sensitive restriction enzymes *Hpa*II or *Msp*I and subjected to PCR to amplify various gene fragments containing the restriction site CCGG. Bisulfite conversion was done by adding a mixture of sodium bisulfite, hydroquinone, and urea and incubating at 55 °C for 16 h. The samples were desalted using a PCR purification kit and desulfonated by adding NaOH to a final concentration of 0.3 M. The DNA was then purified using a QIAquick PCR purification kit (Qiagen). The bisulfite-converted DNA was used for PCR amplification of promoter and gene-body fragments. The PCR products were cloned into the pJET1.2 vector. At least 10 individual clones from each region were sequenced by Macrogen, Netherlands. The sequences were analysed and scored using the Kismeth online service (Gruntman *et al.*, 2008).

Results

Feminization of male Mercurialis annua*: setting-up the experimental system*

Female and male *M. annua* plants have distinct inflorescence morphologies (Fig. 1A, B). In female plants, flowers develop directly at the leaf axils with short pedicels, while in male plants clusters of flowers develop on long pedunculated inflorescences. Feminization of male flowers by the cytokinin BAP caused development of female flowers that yielded fertile seeds on male inflorescences (Fig. 1C; see also Khadka *et al.*, 2005).

Proteome analysis of flower-bud nuclei

To identify the regulatory genes involved in BAP-induced sex alteration of *M. annua*, we performed proteome analysis of nuclear proteins derived from the flower buds of females, males, and males treated with BAP for 4, 8, 12, and 16 d. The proteome data indicated that the core histone proteins H2A, H2B, H3, and H4 showed the highest intensities among the proteins identified (Supplementary Tables S2, S3). The occurrence of cytoplasmic proteins such as rbcS resulted from contamination of nuclei during preparation. The major difference between the genders was that 52 proteins present in female flowers were absent in males, while 244 proteins present in male flowers were absent in females. This was consistent with a recent transcriptome analysis in which a higher number of male-biased genes (1385) were found compared to female-biased genes (325) (Veltsos *et al.*, 2019). Among the 52 proteins expressed only in female flowers, 49 were up-regulated in feminized males (Supplementary Table S4). Out of the 244 male-specific proteins, 84 were disappeared from the proteome during the course of feminization (Supplementary Table S5), as follows: 39 proteins were absent after 4 d of BAP treatment, a further 15 after 8 d, a further 12 after 12 d, and a further 18 after 16 d. Multiple classes of proteins were identified by categorization analysis of the differentially expressed proteins in the feminized males. The major up-regulated protein classes were nucleic acid-binding proteins, transcription factors, and cytoskeleton proteins (Fig. 2A), and the major down-regulated classes were hydrolases, nucleic acid-binding proteins, ligases, and transferases (Fig. 2B). Among the differentially expressed proteins, four floral organ identity MADS-box transcription factors were identified. The class E proteins SEP1 and SEP3 and the class D protein SHP2/AGL5 were up-regulated during feminization, reaching their highest levels at day 16 (Fig. 2C–E). In contrast, the class B protein PI was down- regulated within 4 d of BAP treatment and could not be detected thereafter (Fig. 2F). The proteome data for floral homeotic proteins were confirmed by RNA analysis (see below). In addition to floral homeotic proteins, other chromatin and transcription regulatory proteins were up-regulated in BAP-treated males. These included DNA TOPOISOMERASE 2 (TOP2), HISTONE H1, ATP-dependent DNA helicase 2 subunit KU80, ZINC-FINGER HOMEODOMAIN 9 (ZHD9), WRKY39.1, RINGLET2 (RLT2), and the bromodomain-containing protein, GTE4. The bZIP transcription factor related to Arabidopsis BZIP21 as well as the protein related to Arabidopsis DEFECTIVE IN EXINE FORMATION 1 (DEX1) were down-regulated in BAP-treated males. The proteome data also revealed 132 proteins not found in male and female flowers that were present during the course of feminization (Supplementary Table S6). Among them were the histone acetyltransferase GCN5, which is related to the Arabidopsis histone acetyltransferase of the GNAT family that is involved in transcriptional activation, and MINICHROMOSOME MAINTENANCE (MCM) proteins, which function as components of the MCM2-7 complex that is implicated in seed development in Arabidopsis (Herridge *et al.*, 2014). There were also 70 proteins present in male and female flowers that were gradually down-regulated during the course of feminization (Supplementary Table S7), including a histone H2B variant related to Arabidopsis HTB5 and HTB6, which are presumed to be involved in chromatin compaction, and COMPASS component SWD2, a homolog

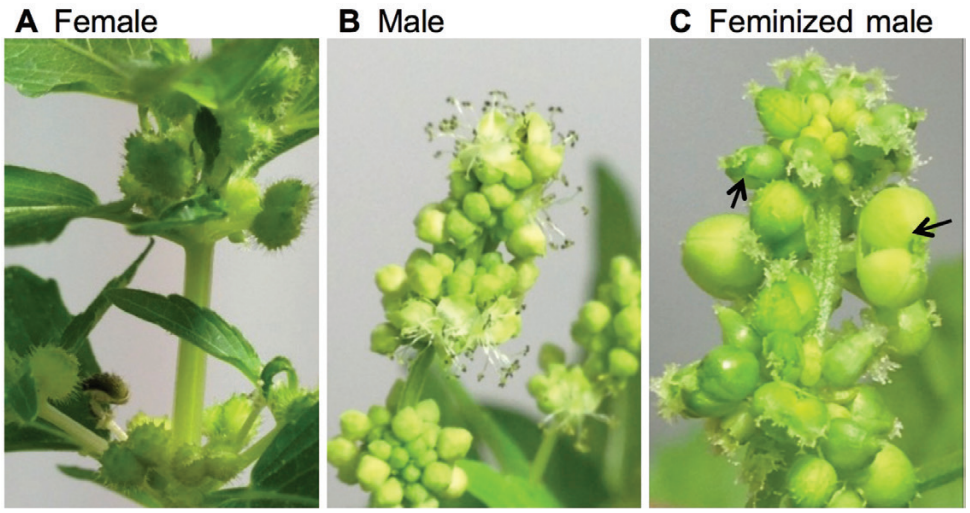

Fig. 1. Morphology of dioecious *Mercurialis annua*. (A) Female plant, (B) male inflorescence, and (C) feminized male inflorescence, induced by spraying plants with 6-benzylaminopurine three times daily for 4 weeks. Feminized males produced bi-carpellet flowers, some of which are indicated by arrows.

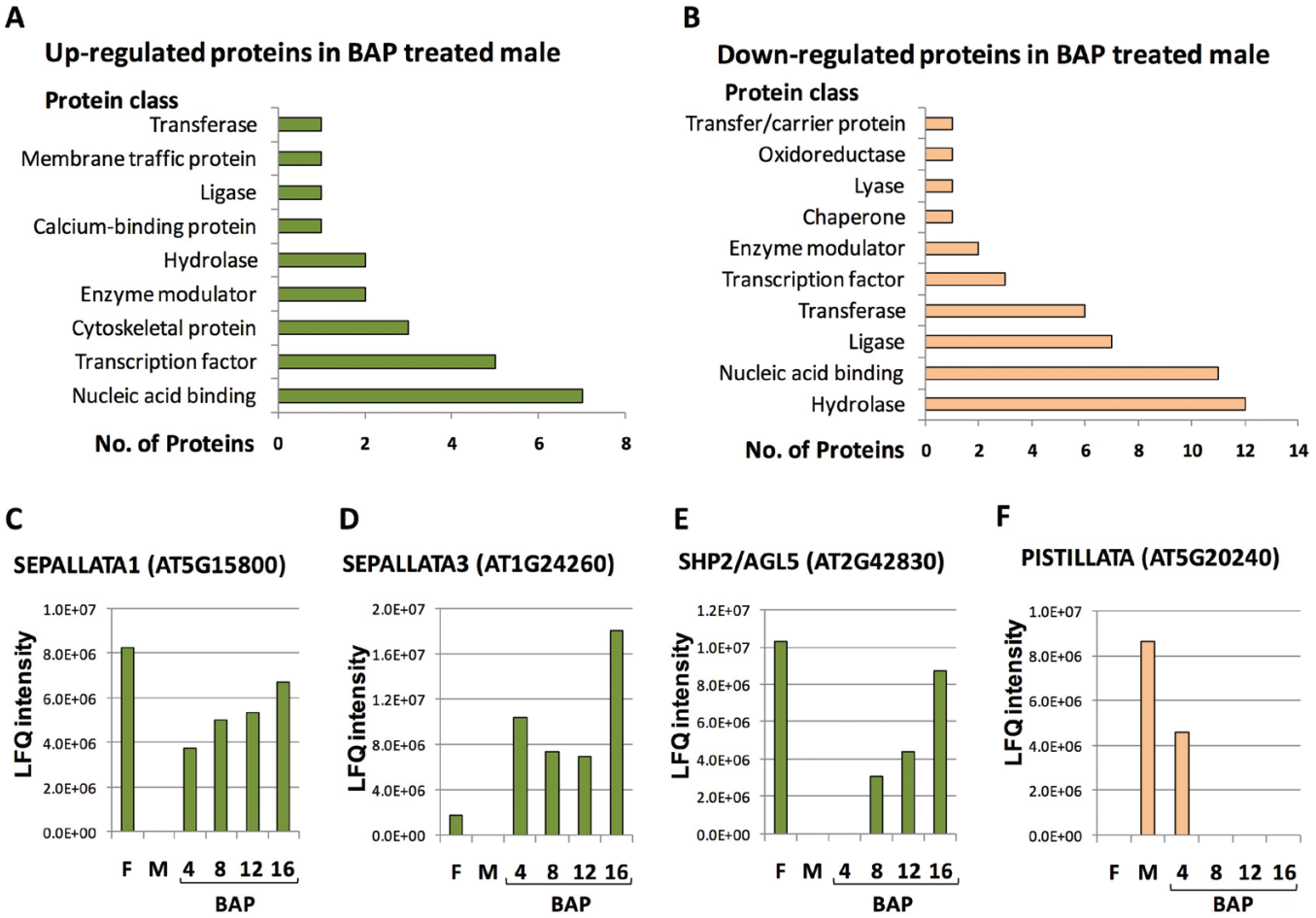

Fig. 2. Proteome analysis of nuclei of *Mercurialis annua* isolated from flower buds of female, male, and feminized male plants (treated with 6-benzylaminopurine, BAP). (A) Down-regulated and (B) up-regulated proteins following male feminization. (C-F) The label-free quantification (LFQ) intensity reflecting the relative amounts of the indicated proteins, which were calculated using peptide intensities normalized between the samples (the corresponding Arabidopsis homolog gene ID is given in brackets). F, female; M, male. (This figure is available in colour at *JXB* online.)

of the Arabidopsis ANTHESIS POMOTING FACTOR 1 (APRF1) that is implicated in promoting flowering under long-day conditions (Kapolas *et al.*, 2016).

Differential expression of floral homeotic genes

Based on the results of the proteome analysis, we cloned and examined the expression of *M. annua* orthologs of floral homeotic MADS-box genes. The homology of the isolated genes was confirmed by phylogenetic analyses (Supplementary Figs. S1–S3). We examined the patterns of RNA expression of the isolated genes in female and male flowers at the bud and opened-flower stages. The class B genes *MaPI* and *MaAP3* were highly expressed in male flowers and poorly expressed in female flowers (Fig. 3A, B) whilst the class C gene *MaAG1* was highly expressed at similar levels in both female and male flowers (Fig. 3C). In contrast to the class B genes, the class D genes *MaAGL1* and *MaAGL3* were highly expressed in female flowers and poorly expressed in male flowers (Fig. 3D, E). The expression levels of most of floral homeotic genes were significantly different between the floral bud and open-flower stages in a sex-specific manner. There was higher expression in open flowers of *MaPI* in males and of *MaAGL3* in females, while there was higher expression in flower buds of *MaAP3* in males and of *MaAG1* in both females and males (Fig. 3A–C, E).

Examination of the organ specificity of gene expression showed that *MaPI* and *MaAP3* were almost exclusively expressed in male flowers (Fig. 4), although *MaPI* was also strongly expressed in male peduncles. Expression of *MaTM6* was evident in flowers of both females and males, but its expression in vegetative organs was very low in female plants. In the males, *MaTM6* expression was high in flowers, moderate in leaves and peduncles, and very low in stems and roots. *MaAGL1* and *MaSL1* were exclusively expressed in flowers of female plants. *MaAG1* was expressed at moderate level in flowers of both sexes, and at a lower level in the peduncle of male plants. *MaAGL3* was highly expressed in flowers of females and had slightly lower expression in flowers and peduncles of male plants.

BAP-induced feminization of male plants resulted in changes in the expression patterns of floral genes (Fig. 5). The expression of the class B identity gene *MaTM6* was not significantly affected by feminization, while *MaPI* and

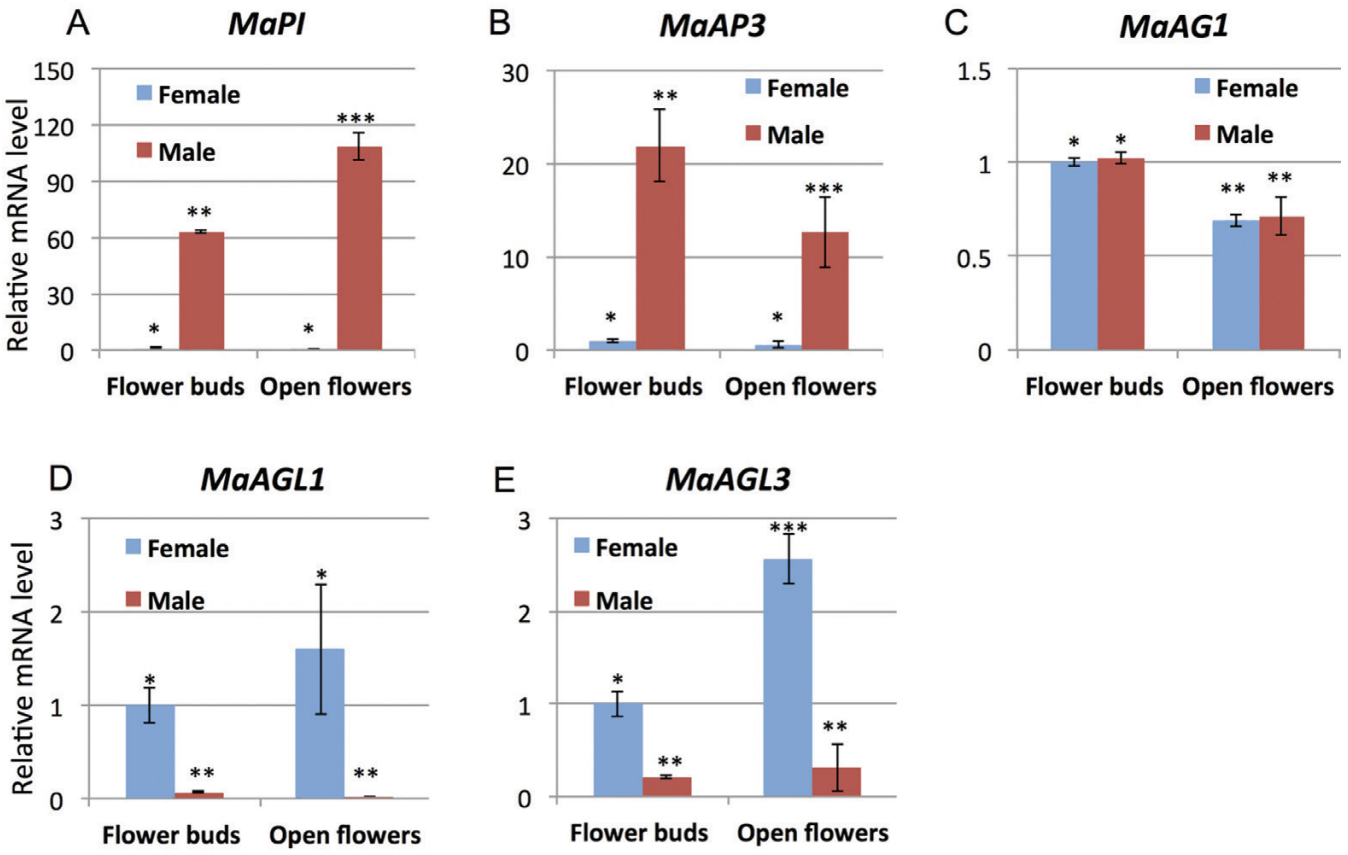

Fig. 3. Expression of MADS-box genes in flower buds and open flowers of female and male plants of *Mercurialis annua*. Relative expression of (A) *MaPI*, (B) *MaAP3*, (C) *MaAG1*, (D) *MaAGL1*, and (E) *MaAGL3* determined using RT-qPCR. The relative transcript levels are normalization to the *Actin* gene. Data are means (±SE) of three biological replicates. Significant differences between means are indicated by different numbers of asterisks as determined by Tukey's HSD test ($P<0.05$). (This figure is available in colour at *JXB* online.)

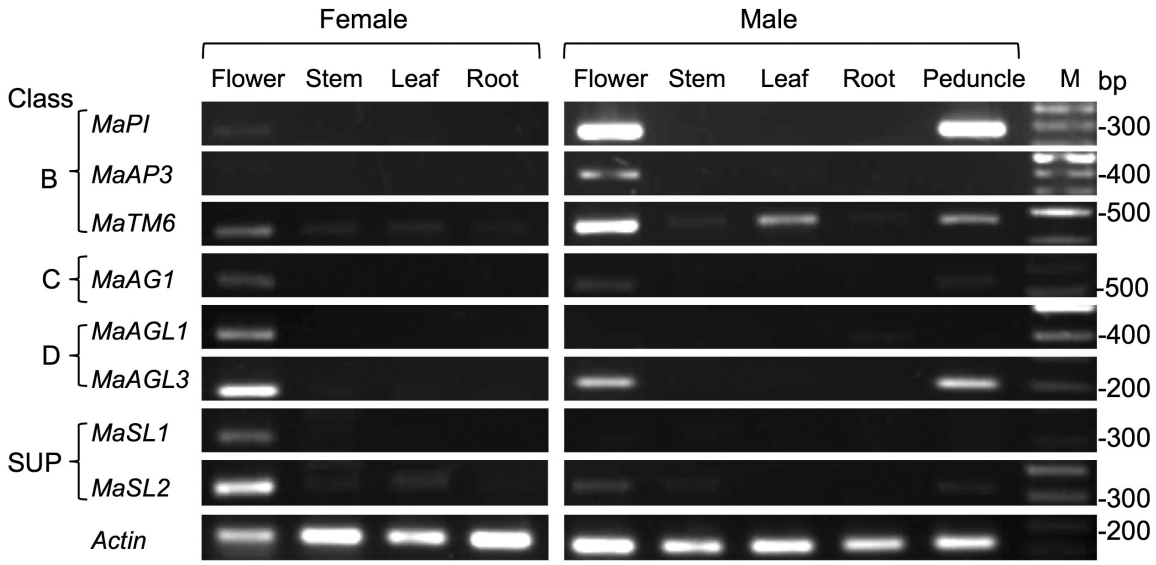

Fig. 4. Expression patterns of floral homeotic genes in different organs of female and male plants of *Mercurialis annua*. Expression of class B, C, D, and SUPERMAN-like (SUP) genes was determined using semi-quantitative PCR using cDNAs derived from RNA extracted from the various plant organs. *Actin* was used as a ubiquitously expressed reference gene. M, molecular size markers.

MaAP3 were down-regulated. In contrast, the expression of the class C/D floral genes *MaAG1*, *MaAGL1*, *MaAGL3*, and *MaSL1* was up-regulated by feminization. A significant up-regulation of class C/D genes was observed at 8–11 d of BAP treatment. Thus, the RNA results confirmed the proteome data with respect to the down- and up-regulation of male and female identity proteins, respectively, during the course of feminization.

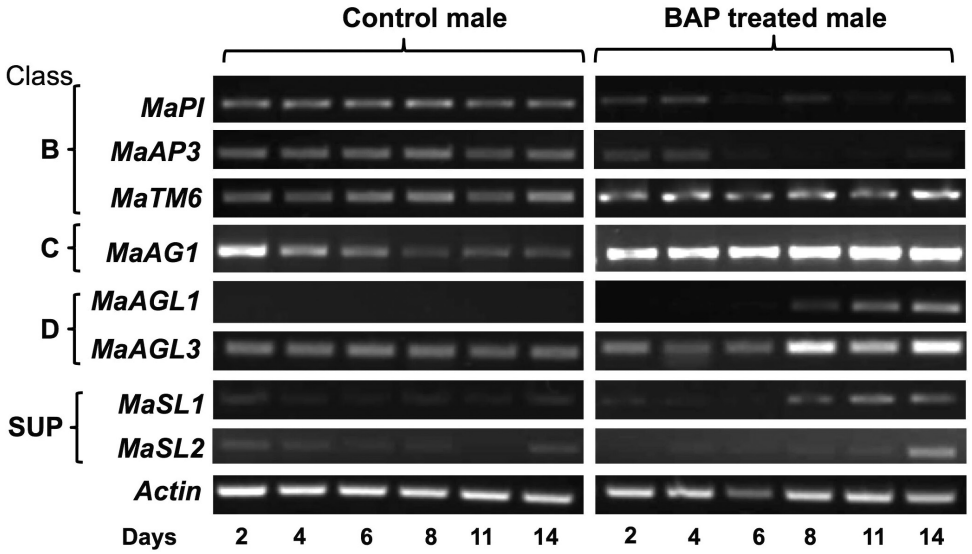

Fig. 5. Time-course of the expression of floral genes in *Mercurialis annua* during feminization induced by treatment with 6-benzylaminopurine (BAP). Plants at 25 d old were sprayed three times daily with water (Control) or with BAP and newly emerging inflorescences were collected on the days indicated. RNA was extracted and subjected to cDNA synthesis, which was then used to determine expression using semi-quantitative PCR. Class B, C, D, and SUPERMAN-like (SUP) genes are indicated. *Actin* was used as the reference gene.

Epigenetic regulation of floral genes

Epigenetics has often been implicated in sex-determination in dioecious plants (Janousek *et al.*, 1996; Bräutigam *et al.*, 2017) and therefore we investigated the involvement of epigenetic mechanisms (i.e. DNA methylation) in the regulation of floral gene expression. We examined the status of cytosine methylation at the promoter regions of the differentially expressed genes *MaSL1*, *MaSL2*, and *MaAGL1* using chop-PCR assays with the methylation-sensitive enzymes *Hpa*II and *Msp*I. Both enzymes recognize the CCGG site but differ in their sensitivity to cytosine methylation: *Hpa*II is sensitive when either of the cytosines is methylated while *Msp*I is sensitive only when the external one is methylated, thus allowing CG and CHG methylation to be distinguished. The assays indicated that there were no differences in the CpG methylation status of the genes between female and male flowers; however, CHG methylation appeared to be absent from the promoter regions of *MaAGL1* and *MaSL2* in male flowers in so far as no recovery of the PCR fragment could be detected in the *Msp*I digest (Fig. 6A). We also perform bisulfite sequencing of the *MaAP3* and *MaSL1* promoter and gene body regions that showed no differences in DNA methylation status between male and female flowers. The promoter regions of both genes were highly methylated in all cytosine contexts (CG, CHG, and CHH) while their gene bodies were essentially unmethylated (Fig. 6B).

Since no differences in DNA methylation were found between male and female flowers, we used micrococcal nuclease (MNase) assays to investigate the chromatin configuration of the promoters of several floral genes during the course of feminization. After 14 d of BAP treatment, the MNase-treated nuclei from male and feminized male flower buds showed similar progressive digestion of genomic DNA with notable nucleosomal ladders (Fig. 7A). The MNase-digested DNAs were used as templates for PCR analysis of the promoter regions of several genes, and no notable differences in digestion patterns were found between male and feminized male flowers (Fig. 7B). However, two groups of major digestion patterns reflecting open and relatively closed chromatin configuration were observed. Group I consisted of the promoter regions of the class B genes *MaPI* and *MaAP3*, and showed higher sensitivity to MNase digestion that was similar to actin, a constitutively expressed gene. Group II consisted of the class D gene *MaAGL1* together with *MaSL1* and *MaSL2*, and were more resistant to MNase digestion (Fig. 7B). Thus, it appeared that class B genes that assumed an open chromatin conformation in male flowers remained open upon feminization, while class D genes remained in a relatively closed configuration in feminized male flowers.

Discussion

The data presented here regarding the expression of floral identity genes are consistent with their known functions in determining sexual identity of floral organs in various plant species. It has been shown previously that class B genes are highly expressed in male flowers of the type-II dioecious plants *T. dioicum* and *S. oleracea* (Di Stilio *et al.*, 2005; Pfent *et al.*, 2005) and our results showed that male flowers were characterized by a strong expression of the class B genes *MaPI* and *MaAP3*, which was concomitant with suppression of female identity genes such as *MaAGL1* (class D) and *MaSL1* (Figs. 2–4). The involvement of cytokinin in sex-determination has been reported in a variety of plant species including Arabidopsis (Lindsay *et al.*, 2006) and the oilseed crops *Plukenetia volubilis* and *Jatropha curcas* (Pan and Xu, 2011; Fu *et al.*, 2014). We found that BAP-induced feminization was accompanied by increased expression of the class C gene *MaAG1* (Fig. 5) and this was concomitant with suppression and activation, respectively, of class B male-specific (e.g. *MaPI*) and class D female-specific (*MaAGL1*, *MaAGL3*) genes thus specifying female flower

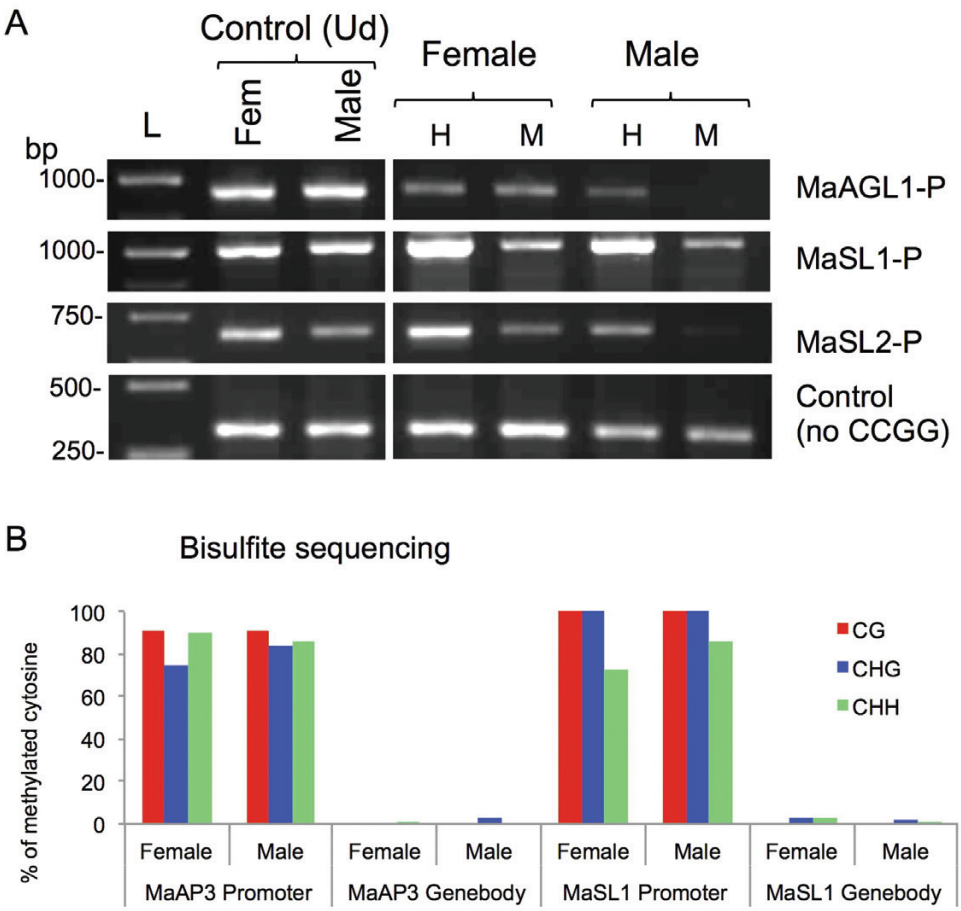

Fig. 6. Transcriptionally active floral genes are methylated in both females and males of *Mercurialis annua*. (A) Analysis of DNA methylation at the promoters (-P) of *MaAGL1*, *MaSL1*, and *MaSL2* by as determined by chop-PCR. A fragment of *MaSL1* lacking the CCGG site (no CCGG) was used as an internal control. Left panel is a control of undigested DNA (Ud). Ud, Undigested DNA; H, *HpaII*; M, *MspI*; L, molecular size markers in base pairs. (B) Analysis of methylation at the promoter and in the gene-body of *MaAP3* and *MaSL1* as determined by bisulfite sequencing. The percentage of cytosine methylation for each fragment was determined from at least 10 different clones. (This figure is available in colour at *JXB* online.)

development. In Arabidopsis, an increase in the number of carpels following BAP treatment is correlated with an increase in the expression of *WUSCHEL* (*WUS*) (Lindsay *et al.*, 2006; Gordon *et al.*, 2009), the protein product of which specifies stem cell identity in both the floral and the shoot apical meristems (Laux *et al.*, 1996; Mayer *et al.*, 1998). *WUS* also activates the class C homeotic gene *AGAMOUS*, which is required for specifying both stamen and carpels (Theißen *et al.*, 2016). Thus, it is possible that cytokinin may act in re-specifying the male floral meristem toward female-producing flowers by activation of *WUS-like* genes in *M. annua* concurrently with activation of *MaAG1* and suppression and activation, respectively, of male and female floral genes to bring about female flower formation.

Sex conversion: proteomic data

Proteome analysis of BAP-feminized males showed differential expression of several protein families, including nucleic acid-binding proteins and transcription factors (Fig. 2). Some of the proteins up-regulated following BAP treatment were involved in chromatin structure and function, suggesting that sex conversion is an intricate process that requires substantial genome reorganization to allow transcriptional activation and repression of genes to bring about feminization. Among these proteins were topoisomerase 2 (TOP2) that can relieve superhelical DNA (a characteristic of heterochromatin) by introducing transient double-strand DNA breaks (reviewed in Nitiss, 2009) and an ATP-dependent DNA helicase 2 subunit KU80, which is involved in DNA non-homologous end-joining (NHEJ) that is required for the repair of double-strand breaks (West *et al.*, 2002). In addition, the proteomic data indicated that BAP treatment resulted in up-regulation of a linker histone H1.1, which is involved in heterochromatin formation and regulation of gene expression (Hergeth and Schneider, 2015), as well as up-regulation of a structural maintenance of chromosomes (SMC) protein (Supplementary Table S4). SMC proteins function in a range of nuclear processes, including chromosome condensation, DNA repair, and epigenetic transcriptional silencing of genes (Harvey *et al.*, 2002). The proteomic data also indicated that multiple transcription factors were up-regulated in response to BAP treatment including WRKY transcription factor 39.1, a group II WRKY protein with a C2H2 zinc finger-like motif (Agarwal *et al.*, 2014) and the basic leucine zipper (bZIP) class transcription factors EMBP-1 the homolog of which in wheat has been implicated in the abscisic acid signaling pathway (Guiltinan *et al.*,

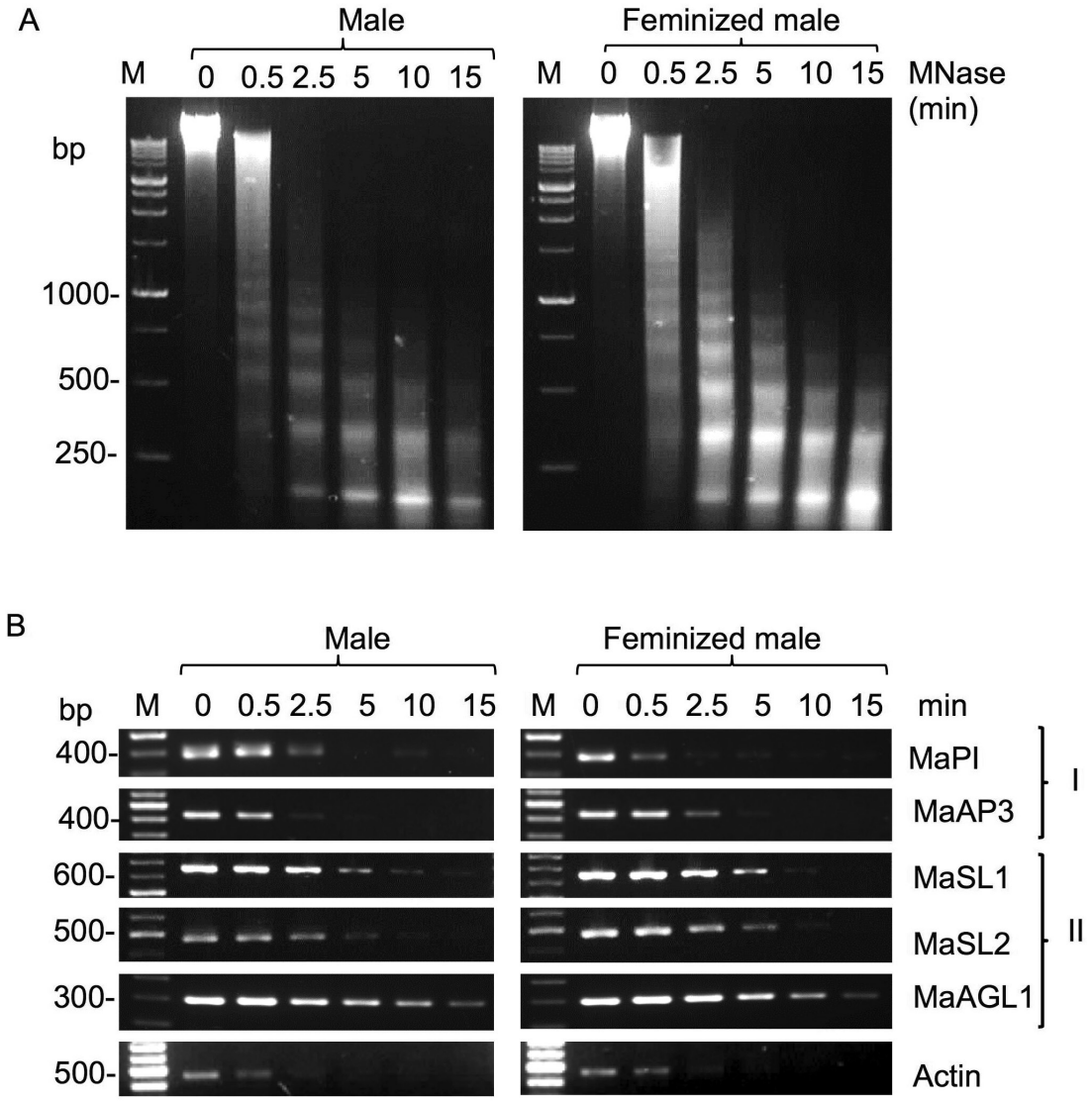

Fig. 7. Analysis of chromatin configuration of selected floral genes in *Mercurialis annua* as determined by micrococcal nuclease assays. (A) Nuclei prepared from male and feminized male flower buds (treated with 6-benzylaminopurine for 14 d, before female flowers were visible) were treated with MNase for the time periods indicated. DNA was extracted from the treated nuclei and resolved on 1.5% agarose gel. M, molecular size markers, in base pairs. (B) Assessment of chromatin configuration of promoters as determined by PCR using DNA recovered from the MNase-treated nuclei shown in (A). Group I refers to male-related identity genes and Group II refers to female-related identity genes. *Actin* was used as the reference for open chromatin configuration. M, molecular size markers.

1990) and in histone gene expression (Mikami *et al.*, 1994). Other transcription factors up-regulated in feminized males included a member of the zinc-finger homeodomain protein sub-family (ZF-HD) related to Arabidopsis ZHD9/ATHB34, the expression of which, in common with other members in this group, is elevated during floral development (Tan and Irish, 2006). Another homeodomain protein up-regulated in feminized males was a homeodomain-like transcriptional regulator RLT2, which is implicated in activation of expression of seed storage genes in Arabidopsis (Sundaram *et al.*, 2013).

Consistent with the conversion of male flowers into females, some proteins involved in male reproductive organs were down-regulated in the course of feminization (Supplementary Table S5). Among these was the bZIP transcription factor related to Arabidopsis bZIP21/TGA9, which is implicated in male gametogenesis. Plants lacking bZIP21/TGA9 and bZIP65/TGA10 are defective in anther development (Murmu *et al.*, 2010). In addition, the proteomic data showed down-regulation of the DEFECTIVE IN EXINE FORMATION protein that is related to Arabidopsis DEX1, which is required for exine pattern formation during pollen development (Paxson-Sowders *et al.*, 2001). Overall, the proteome data suggested that both the up-regulation of sex-specific proteins as well as the suppression of proteins for the opposite sex function were important for sexual dimorphism of *M. annua*.

Expression pattern of floral genes

The *Mercurialis* orthologs of the SHP2/AGL5, SEP1, and SEP3 proteins were up-regulated in feminized males (Fig. 2C–E), suggesting that they play roles in female flower specification. This is consistent with the role of SHP in carpel development

in Arabidopsis where constitutive expression results in a partial conversion of the first whorl sepals into carpel-like structures as demonstrated by extensive proliferation of stigmatic papillae (Favaro *et al.*, 2003; Pinyopich *et al.*, 2003). Similarly, in *Gerbera hybrida* two duplicated orthologs of a SEP-like gene, *GRCD1* and *GRCD2*, are sub-functionalized for stamen and carpel identity, respectively (Zhang *et al.*, 2017). The PI protein, which was down-regulated in feminized males, is involved in controlling the development of whorls 2 and 3 in Arabidopsis, *Antirhinum*, and tomato (Tröbner *et al.*, 1992; Goto and Mayerowitz, 1994; Guo *et al.*, 2016). This suggest that cytokinin switched off male control genes (e.g. *PISTILLATA*) concomitantly with up-regulation of female identity genes, thus leading to the replacement of stamen by carpels, as in the development of normal dioecious female flowers.

Using a cell-free translation system with RNAs derived from *M. annua* male and female flowers, Delaigue *et al.* (1984) found variation in peptides between the two sexes and that cytokinin-induced feminization of male flowers led to the expression of female-specific peptides. Similarly, we found that cytokinin-induced feminization of *M. annua* male flowers was associated with up-regulation of female-specific floral genes concomitantly with down-regulation of male-specific genes (Fig. 5). In Arabidopsis, exogenous application of BAP has been reported to promote differentiation of carpeloid tissue and to suppress stamen development. This is similar to the effect obtained by overexpressing *SUP* in tobacco plants, leading to the proposition that *SUP* may regulate sex-determination pathways by promoting female organ differentiation via its effect on cytokinin signaling (Nibau *et al.*, 2011). Alternatively, cytokinin may affect male and female flower development via controlling *SUP* expression. Indeed, in *M. annua* the *SUP*-like genes exhibited female flower-specific expression (Fig. 4), as previously seen in the dioecious *Popolus tomentosa* and *Silene latifolia* (Kazama *et al.*, 2009; Song *et al.*, 2013). The *sup* mutant in Arabidopsis is associated with the ectopic expression of *AP3* in the fourth whorl (Bowman *et al.*, 1992), and therefore *SUP* has been proposed to function as a negative regulator of *AP3*. The concomitant expression of class B and SUP-like genes in male flower buds suggests that *SUP*-like gene(s) might not be a transcriptional regulator of class B genes in *M. annua*. An alternative possibility is that the expression of *SUP* in male flower buds is negatively regulated post-transcriptionally.

The expression of the class B gene *MaAP3* was restricted to male flowers, while *MaTM6* (*AP3*-related) and *MaPI* were expressed in flowers as well as in peduncles (Fig. 4). It is notable that *TM6*, which is absent in Arabidopsis, was also weakly expressed in the leaves of *M. annua* female plants and in other vegetative organs. The *TM6* ortholog *CpTM6-2* in *Carica papaya* is expressed at a low level in sepals and at a high level in leaves (Ackerman *et al.*, 2008), while the ortholog *VvTM6* in *Vitis vinifera* is expressed throughout the plant but at higher levels in flowers and berries (Poupin *et al.*, 2007). It has been proposed that a gene duplication event of the *paleoAP3* genes resulted in two types, namely the *euAP3* and *TM6* lineages that are distinguished by their C-terminal regions (Kramer *et al.*, 1998). These duplicated genes probably to some extent adopted different functions (sub-functionalization), as demonstrated by their tissue-specific patterns of expression and the differing effects of their loss-of-function on flower development (Eckardt, 2006).

The expression of the class C gene *MaAG1* was similar in male and female flowers of *M. annua* (Fig. 4), suggesting that it may not be involved in gender determination. This is consistent with a previous study that showed that the C class *AG* genes are involved in the floral quartet that specifies both stamens and carpels (reviewed in Theißen *et al.*, 2016). The class D genes *MaAGL1* and *MaAGL3* were highly expressed in female flowers, and *MaAGL3* was also expressed in male flowers and peduncles. Our results suggested that the class B genes *MaAP3* and *MaPI* together with the class C gene *MaAG1* have a role in determining the identity of male floral organs. The proteins that they encode may participate in the floral quartet that controls gene expression and the identity of the male reproductive organs (Theißen *et al.*, 2016). On the other hand, the class D genes *MaAGL1* and *MaAGL3* together with class C and class E genes may form a floral quartet that specifies female floral organs, carpels, and ovules. Notably, in seed plants the class B genes have been suggested to have a primary role in sex determination (Winter *et al.*, 1999), with expression of both class B and class C genes specifying male reproductive organs while the expression of only class C genes specifies female reproductive organs. Thus, switching from male to female and vice versa can be solely driven by changes in the spatio-temporal expression of class B genes (Winter *et al.*, 1999; Theissen and Melzer, 2007). However, our data showed that induction of feminization was associated not only with the up-regulation of female-related class C and class D genes, but also with the turning off of the expression of male-related class B genes, which might be crucial for the development of female flowers in otherwise male plants of *M. annua*.

Epigenetics and sex-determination

Our data showed that there was no clear relationship between floral homeotic genes and their epigenetic make-up (Table 1). Gene expression was primarily regulated at the chromatin level, where gene transcription requires open chromatin to allow the transcription machinery to approach the gene locus. Analysis of chromatin accessibility using MNase assays revealed that in male flowers the class B genes *MaPI* and *MaAP3* assumed an open chromatin conformation similar to the constitutively expressed gene *Actin* (Fig. 7). On the other hand, the class D gene *MaAGL1* and the SUP-like genes *MaSL1* and *MaSL2* appeared to acquire a relatively closed conformation compared with *Actin*, which was consistent with the lack of expression in male flowers. Surprisingly, however, no apparent change in accessibility of chromatin to MNase was evident upon feminization and up-regulation of *MaAGL1* and *MaSL1*. This suggests that chromatin can assume different levels of open conformation as reflected by variable sensitivity to MNase, including hyper-accessible DNA sites (Schwartz *et al.*, 2019) that which probably provide another regulatory layer for control of gene expression (Ishihara *et al.*, 2010; Kotomura *et al.*, 2015). Similarly, no change in chromatin accessibility was observed for the down-regulated class B genes *MaPI* and *MaAP3*, the transcription of which was possibly halted in an

Table 1. *Summary of the expression levels of floral genes in* Mercurialis annua *in relation to their epigenetic constraints*

Gene	Expression			DNA methylation			Sensitivity to MNase			Score		
	Female	Male	Feminized male	Female	Male	Feminized male	Female	Male	Feminized male	Female	Male	Feminized male
MaPI	–	++	–					High	High	S	O E	O S
MaAP3	–	+++	–	mALL	mALL			High	High	m S	O m E	O S
MaActin	+++	+++	+++					High	High	E	E	O E
MaAGL3	+++	+	+++							E	e	E
MaAGL1	+++	–	++	mALL	mCG			Low	Low	m E	PO m S	PO E
MaSL1	++	–	++	mALL	mALL			Low	Low	m E	PO m S	PO E
MaSL2	++	–	–	mALL	mCG			Low	Low	m E	PO S	PO S
MaAG1	+++	++	+++							E	E	E

Expression: –, no expression; +, low expression; ++/+++, high expression.
DNA methylation: mAll, methylated at all C contexts; mCG, methylated at the CG context only.
Score: S, silent; E, expressed; e, low expression; O, open chromatin; PO, partially open chromatin.

open chromatin environment by other means (e.g. suppressor proteins).

The nature of gene regulation by DNA methylation is not fully understood, but it has been generally implicated in regulating chromatin structure and function (Niederhuth and Schmitz, 2017). DNA methylation was detected at promoters but not in gene-bodies of the floral genes that we examined (Fig. 6). Interestingly, the methylation status of all the genes was similar in both sexes despite their differential expression. In Arabidopsis, gene methylation has been reported to correlate with the level of gene expression: gene-body methylation is correlated with constitutively and highly expressed genes, while promoter methylation is correlated with weakly expressed genes that are usually tissue-specific (Zhang *et al.*, 2006; Zilberman *et al.*, 2007). However, we did not find a consistent correlation between DNA methylation and expression of the floral genes in *M. annua* (Table 1, Fig. 7), with *MaAP3*, *MaAGL1*, *MaSL1*, and *MaSL2* being normally transcribed in spite of being heavily methylated at their promoters. Thus, it appears that DNA methylation at the promoter regions of *M. annua* floral genes had a positive effect on their expression, in contrast to the commonly observed effect of suppression of expression by methylation, particularly when transposable elements are concerned (Lisch, 2009). This finding may possibly be explained by a lowering of the affinity of repressors to their binding sites as a result of DNA methylation. Indeed, there are studies that have similarly shown that DNA methylation at promoters contributes to transcriptional activation of certain tissue-specific genes (Niesen *et al.*, 2005; Weber *et al.*, 2007; Rishi *et al.*, 2010; Bahar Halpern *et al.*, 2014).

Most studies that have addressed epigenetic regulation of sex determination have highlighted various genes that are not related to floral homeotic genes. Bräutigam *et al.* (2017) examined the sex-determining region of *Populus balsamifera* and identified *PbRR9* as showing a clear pattern of gender-specific methylation. *PbRR9* encodes a protein that is a member of the two-component response regulator (type-A) gene family involved in cytokinin signaling. A detailed study of the occurrence of androecy in the Cucurbitaceae species *Cucumis melo* and *C. sativus* implicated the ethylene biosynthetic enzymes CmACS-7 and CmACS-11 in sex determination (Boualem *et al.*, 2009, 2015), and they are required for epigenetic repression of the male-promoting *CmWIP1* gene via induction of H3K27me3 (Latrasse *et al.*, 2017). In naturally occurring gynoecious lines of *C. melo*, the transition from male to female flowers results from a transposon insertion proximal to the *CmWIP1* promoter, which induces DNA methylation and silencing of the gene (Martin *et al.*, 2009). In maize, maintenance of the monoecious pattern of sex determination is achieved by epigenetic restriction of SILKLESS1 (SK1), a uridine diphosphate glycosyltransferase (UGT), from the apical inflorescence: *SK1* is required for female flower development, and constitutive expression of SK1 in transgenic maize results in complete feminization (Parkinson *et al.*, 2007; Hayward *et al.*, 2016). In *Diospyros lotus*, a dioecious plant with heterogametic males (XY), a Y-specific sex-determinant, *OGI*, encodes a small RNA that suppresses the *MeGI* gene that encodes a feminizing homeodomain transcription factor (Akagi *et al.*, 2014). Interestingly, our data and those obtained in other studies on a variety of dioecious species suggest that mutations leading to evolution of dioecy have not directly affected (genetically or epigenetically) floral homeotic transcription factors; rather, these transcriptional regulators seem to act as downstream effectors of sex-determining gene(s) for sex-organ specification.

Conclusions

In conclusion, we propose a comprehensive model of sex determination in dioecious *M. annua* (Fig. 8). According to this model, sex conversion in *M. annua* does not primarily involve epigenetic regulation of floral homeotic genes. Instead, sex identity in this species seems to be controlled genetically/epigenetically upstream in the regulatory pathway by a gender-specific regulator(s) that affects, at least partly, hormonal homeostasis. Thus, high cytokinin induces transcriptional activation of female identity genes and the production of female flowers, while high auxin induces transcriptional activation of male identity genes and production of male inflorescences. This is supported by recent analysis of the DNA sequences of the sex-determining region in *M. annua* that has failed to show any floral homeotic genes or other strong candidate genes for sex determination (Veltsos *et al.*, 2018). It appears that keeping

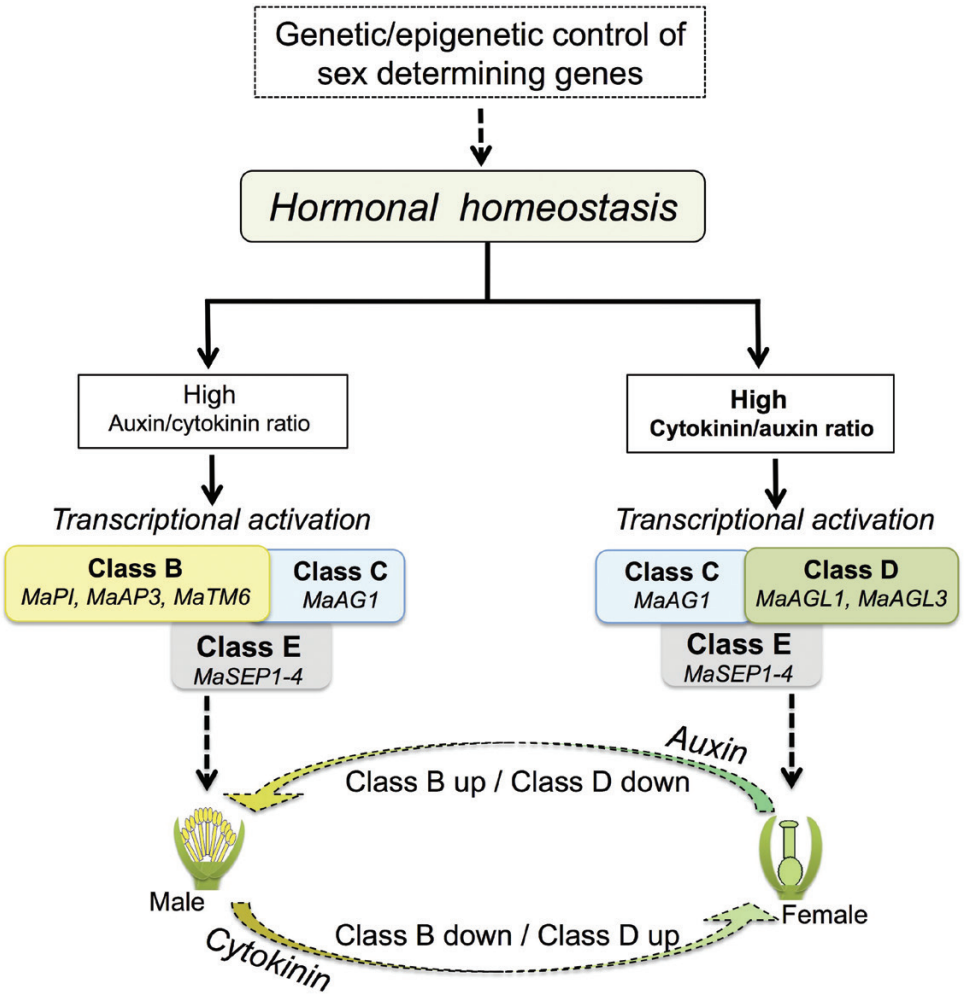

Fig. 8. A proposed model of sex determination in the dioecious plant *Mercurialis annua*. Differentiation of unisexual flowers is controlled genetically/epigenetically by as yet unknown sex-determining genes that affect hormonal homeostasis. A high cytokinin/auxin ratio activates the transcription of effector genes such as female-identity class D genes (*MaAGL1* and *MaAGL3*). The class D proteins together with class C and class E proteins promote female flower development. Alternatively, a high auxin/cytokinin ratio is presumed to lead to transcriptional activation of male-identity class B genes (*MaPI*, *MaAP3*, and *MaTM6*). The class B proteins together with class C (MaAG1) and class E (MaSEP1–4) promote male flower development. Exogenous application of cytokinin feminizes males by inducing down-regulation of class B genes and up-regulation of class D genes. Exogenous auxin masculinizes females (Delaigue *et al.*, 1984), probably via up-regulation of class B genes concomitantly with down-regulation of class D genes. (This figure is available in colour at *JXB* online.)

the functioning of the floral homeotic genes, i.e. the effector proteins that specify sex organs in dioecious plants, enables sex conversion (Fig. 8) and this might have an adaptive value, particularly in cases where the two sexes exhibit differential tolerance to stresses (Orlofsky *et al.*, 2016). The sexual bi-potency of dioecious plants may also explain their capacity for multiple cycles of transitions from dioecy to monoecy during the evolution of plants.

Supplementary data

Supplementary data can be found at JXB online.

Fig. S1. Phylogenetic analysis of class B genes from *M. annua*, Arabidopsis, and various taxonomic groups.

Fig. S2. Phylogenetic analysis of *AG*-like genes from *M. annua*, Arabidopsis, and various taxonomic groups.

Fig. S3. Phylogenetic analysis of *SUPERMAN-like* genes from *M. annua*, Arabidopsis, and various taxonomic groups.

Table S1. List of primers used in this study.

Table S2. List of proteins identified in proteomic analysis (repeat I).

Table S3. List of proteins identified in proteomic analysis (repeat II).

Table S4. Proteins exclusively present in female flower buds that appeared following BAP treatment.

Table S5. Proteins exclusively present in male flower buds that had lower expression following BAP treatment.

Table S6. Proteins normally absent in male and female flower buds were up-regulated following BAP treatment.

Table S7. Proteins present in male and female flower buds that had higher expression following BAP treatment.

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