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The Effects of DNA Methylation Inhibition on Flower Development in the Dioecious Plant *Salix Viminalis*

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Abstract: DNA methylation, an important epigenetic modification, regulates the expression of genes and is therefore involved in the transitions between floral developmental stages in flowering plants. To explore whether DNA methylation plays different roles in the floral development of individual male and female dioecious plants, we injected 5-azacytidine (5-azaC), a DNA methylation inhibitor, into the trunks of female and male basket willow (*Salix viminalis* L.) trees before flower bud initiation. As expected, 5-azaC decreased the level of DNA methylation in the leaves of both male and female trees during floral development; however, it increased DNA methylation in the leaves of male trees at the flower transition stage. Furthermore, 5-azaC increased the number, length and diameter of flower buds in the female trees but decreased these parameters in the male trees. The 5-azaC treatment also decreased the contents of soluble sugars, starch and reducing sugars in the leaves of the female plants, while increasing them in the male plants at the flower transition stage; however, this situation was largely reversed at the flower development stage. In addition, 5-azaC treatment decreased the contents of auxin indoleacetic acid (IAA) in both male and female trees at the flower transition stage. These results indicate that hypomethylation in leaves at the flower transition stage promotes the initiation of flowering and subsequent floral growth in *Salix viminalis*, suggesting that DNA methylation plays a similar role in vegetative–reproductive transition and early floral development. Furthermore, methylation changes during the vegetative–reproductive transition and floral development were closely associated with the biosynthesis, metabolism and transportation of carbohydrates and IAA. These results provide insight into the epigenetic regulation of carbohydrate accumulation.

Keywords: dioecious plant; DNA methylation; flower development; carbohydrates; hormones

1. Introduction

Flower initiation and development are regulated by complex networks of endogenous signals, including carbohydrates, phytohormones, polyamines and small RNAs. Endogenous phytohormones are a key factor in flower initiation, although their effects vary between plant species. For example, gibberellic acid (GA) induces flower initiation in *Arabidopsis thaliana* (L.) Heynh. [1] but suppresses the formation of flowers in woody plants such as apple (*Malus domestica* Borkh.) [2], mango (*Mangifera indica* L.) [3] and peach (*Prunus persica* (L.) Batsch.) [4]. The expression levels of abscisic acid (ABA)-related genes gradually increase in the buds and leaves of apple during floral induction, indicating that ABA may play a key role in regulating flowering in this species [5]. The localization and transportation patterns of auxin may also contribute to the formation of floral primordia, including boundary formation, organ polarity and initiation of the floral meristem [6–8].

The endogenous nutritional status of plants is another essential factor controlling flowering [9]. Sugars produced in the leaves are transferred to the shoot apical meristem and act as signaling molecules that regulate flower induction [3,10]. The accumulation of carbohydrates in the leaves represses photosynthesis, an inhibition that is enhanced by the presence of high nitrogen levels in these tissues [11]. Studies in Arabidopsis, white mustard (*Sinapis alba* L.), tomato (*Solanum lycopersicum* L.) and grape (*Vitis vinifera* L.) have indicated that a high ratio of endogenous carbohydrates to nitrogen promotes flowering, whereas the opposite conditions maintain vegetative growth [9,12]. Branch bending in apple was found to increase the carbon:nitrogen ratio in the terminal shoot and therefore to increase the proportion of flowering buds relative to total buds [13].

A growing body of evidence has indicated that DNA methylation, an important epigenetic modification, also plays a role in the flowering process. Studies in Arabidopsis have revealed that extensive *de novo* methylation and demethylation occur during flower development [14]. The loss of methylated cytosine in the *FLOWERING WAGENINGEN* (*FWA*) promoter activates the expression of the associated gene, which results in the late-flowering phenotype observed in the *fwa* epialleles [15]. In apples, 90.6% of differentially expressed 24-nt siRNAs are required for the RNA-directed DNA methylation (RdDM) pathway. In plants, de novo DNA methylation is mainly mediated through the RdDM pathway in which the siRNAs are loaded onto ARGONAUTE4 (AGO4) protein. AGO4 interacts with DOMAINS EARRANGED METHYLASE2 (DRM2), which catalyzes *de novo* DNA methylation in a sequence-independent manner [16]. The 24-nt -siRNAs are predominantly expressed in the flower buds, whereas only 0.4% of them are predominantly expressed in vegetable buds [17].

DNA methylation also alters the effects of other factors regulating floral development [18]. The epigenetic changes caused by exogenous auxins were shown to affect flower development [19]. The cytokinin (CK) kinetin induces DNA hypomethylation, while the synthetic auxin naphthylacetic acid causes DNA hypermethylation, resulting in the contrasting phenotypes observed in plants treated with these two hormones [19–21]. In Arabidopsis, mutants with a reduced level of S-ADENOSYL-L-HOMOCYSTEIN HYDROLASE (*SAHH*) expression, which maintains DNA methylation, have altered expression levels in their CK-signaling genes and delayed flowering [22]. In azalea (*Rhododendron sp.*), the application of the GA inhibitors chlormequat and paclobutrazol changes the levels of GAs, CKs and DNA methylation, which were all correlated with flower development [23]. In trifoliate orange (*Poncirus trifoliate* L./(L.) Raf.), the application of 5-azaC promoted the expression of *CiLFY*, the homolog of *AtLEAFY,* during the GA-induced transition from vegetative to reproductive growth [24,25].

The demethylation agent 5-azacytidine (5-azaC) is incorporated into DNA and prevents the function of DNA methyltransferase. Its inhibitory effect on methylation has allowed it to be widely used to investigate the roles of DNA methylation in flower development and sex determination [26], among other traits. The application of 5-azaC promotes the flowering of photoperiod- and vernalization-dependent plants in non-inducing environments [27,28]; for example, the treatment of nonvernalized late-flowering Arabidopsis ecotypes with 5-azaC induced them to flower significantly earlier than the untreated controls [28]. Treatment with 5-azaC also promoted flowering in the long-day flowering plant Sweet William catchfly (*Silene armeria* L.) under short-day conditions and promoted flowering in short-day plants such as the beefsteak plant (*Perilla frutescens* var. *crispa*) and Japanese morning glory (*Pharbitis nil* (L.) Choisy) under long-day conditions [29,30]. In addition, 5-azaC treatments have been used to reveal the epigenetic mechanisms of DNA methylation in sex determination in dioecious plants. In white campion (*Silene latifolia* Poir.)—a dioecious plant—5-azaC induced a sex change to andromonoecy in about 21% of male plants; however, a 5-azaC-mediated sex reversal was not observed in female plants [31]. Treatments with 5-azaC also increased the percentage of monoecious plants in spinach (*Spinacia oleracea* L.), which was largely a result of sex reversals in the female plants [32]. The 5-azaC-mediated changes in the sexual phenotypes of *Spinacia oleracea* and *Silene latifolia* suggested that DNA methylation has various functions in sex determination among dioecious plants.

Most studies of the effects of 5-azaC on flowering and sex determination have been performed using annual herb plants; however, woody plants and annual herbs have different developmental processes. Perennial woody plants typically have yearly growth cycles; for example, apple and pear (*Pyrus sp.*) trees usually bloom in early spring, using flower buds produced during the previous growing season [33]. The terminal meristems in bourse shoots, short vegetative shoots produced beneath a flower bud, are converted into inflorescence meristems in the early summer and continue to produce floral meristems until the autumn [34]. The new flower buds lie dormant over the winter and bloom in the early spring [34,35].

Developmental programming also differs between female and male individuals in dioecious woody plants; the fertilized egg cell in female individuals requires a large amount of nutrients to support its further development and growth, while the male individuals can immediately transition back to vegetative growth after flowering, to prepare for the next floral initiation [36–38]. The differing mechanisms by which DNA methylation functions in male and female individuals of woody plants are yet to be elucidated.

A methylation-sensitive amplified polymorphism (MSAP) analysis is performed by digesting DNA with methylation-sensitive restriction enzymes, followed by a modified amplified fragment length polymorphism (AFLP) analysis [39,40]. The isoschizomers *Hpa*II and *Msp*I recognize the sequence 5′-CCGG-3′ and have different sensitivities to cytosine methylation. The respective combination of these restriction enzymes with *Eco*RI can produce four types of bands depending on the differing methylation patterns of the 5′-CCGG-3′ sequences: (I) HpaII(+)/MspI(+), unmethylated, indicating that none of the cytosines were methylated; (II) HpaII(+)/MspI(-), hemimethylation, indicating hemimethylated external cytosine sites; (III) HpaII(-)/MspI(+), internal cytosine methylation, indicating full and hemimethylation of internal cytosine sites; and (IV) HpaII(-)/MspI(-), full methylation, indicating full methylation of external cytosine/both cytosine, hemimethylation of both cytosines or the absence of the target sequence [41,42]. MSAP is widely used to detect differences in DNA methylation patterns in non-model plants, such as perennial ryegrass (*Lolium perenne* L.) [43], maize (*Zea mays* L.) [44], rapeseed (*Brassica napus* L.) [45], black wattle (*Acacia mangium* Willd.) [46] and Japanese larch (*Larix kaempferi* (Lamb.) Carr.) [47].

Osier willow (*Salix viminalis* L.), a dioecious shrub, rapidly produces large amounts of biomass and is an important bioenergy species in addition to being used for phytoremediation in Europe because of its outstanding capacity to extract heavy metals from the soil [48,49]. *Salix viminalis* seedlings can complete the transition from the juvenile to adult stage in two years, much less than the majority of woody plants, which facilitates its use in the study of reproductive development and breeding in woody plants. Adult individuals of *Salix viminalis* undergo an annual reproductive growth cycle, with flower bud differentiation taking place before the cessation of apical growth [50]. To investigate the role of DNA methylation in the flower development of this dioecious woody plant, we injected the DNA methylation inhibitor 5-azaC into the male and female trunks of *Salix viminalis* prior to the initiation of flower bud development and analyzed the subsequent changes in the levels of sugars, hormones and DNA methylation in the leaves, as well as the morphological indicators of the flower buds.

2. Materials and Methods

2.1. Materials and Treatments

The experiments were conducted in the Saihanwula National Nature Reserve, Inner Mongolia, China (44°14′48″ N, 118°20′17″ E). Both male (six plants) and female (six plants) individuals of *Salix viminalis* originating from the same population were selected for use. All 12 selected individuals were uniformly distributed across an ~200-m^2 area in the same environmental conditions.

An electric hand drill was used to drill a hole (ϕ = 4 mm) into the trunk at a height of 1.5 m and at a 45° angle. A syringe containing a 2 mM solution of the methylation inhibitor 5-azaC (in water) was inserted into the hole and fixed into position and a dosage of 3 mL per centimeter of stem diameter

was allowed to soak into the trunk. The syringe was not removed until the solution was absorbed completely, after which the hole was sealed with plastic wrap. The control individuals were injected with water. These treatments were implemented on 26 May 2016, before the initiation of flower bud differentiation. The three individual male or female plants were considered to be three independent replicates of each treatment. A total of 10 randomly selected annual shoots of the same length were labeled on each tree and their morphologies were observed. The leaves of the observed shoots were harvested and frozen in liquid nitrogen at both the flower transition stage (26 June 2016) and the flower development stage (1 August 2016). The number and size of the flower buds in the labeled shoots were measured after the leaves had fallen (Figure 1b).

2.2. Genomic DNA Extraction and Methylation-Sensitive Amplified Polymorphism Analysis

Genomic DNA was extracted from the leaves using the cetyltrimethylammonium bromide (CTAB) method [51]. A NanoDrop 2000 was used to analyze the concentration and quality of the genomic DNA. The MSAP analysis was then performed using the protocol described by Diao *et al*. [47]. Briefly, 450 ng of genomic DNA from each sample was digested using pairs of restriction enzymes, either *Eco*RI/*Hpa*II or *Eco*RI/*Msp*I. An *Eco*RI adapter and a *Hpa*II or *Msp*I adapter (Table 1) were ligated to the digested products. The forward primer, E-pre and the reverse primer, HM-pre (Table 1), were used for the preamplification reaction. The preamplification products were diluted 20-fold and used as the template for a selective amplification (primers listed in Table 1). The products of the selective amplification were separated using capillary electrophoresis on an ABI 3730 (Thermo Fisher Scientific, Waltham, MA, USA). The raw data were analyzed using a fragment analysis module of Gene Marker V2.2.0. Bands under 50 bp were disregarded for their fuzzy appearance and fragments ranging from 50 to 500 bp were marked and exported with a 1 (presence) or 0 (absence) score for cytosine methylation, which was used in a matrix for the following data analysis. The matrix was categorized into four types of loci: Type I (1, 1) for unmethylated loci, Type II (1, 0) for hemimethylated loci, Type III (0,1) for internal cytosine methylation and Type IV (0, 0) for fully methylated loci or the absence of a target.

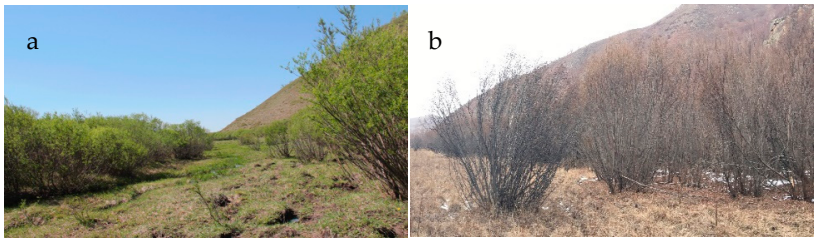

Figure 1. *Salix viminalis* in its growing and dormant seasons. (**a**) *Salix viminalis* in the growing season. (**b**) *Salix viminalis* in the dormant season.

Table 1. Sequences of adapters and primers used in the methylation-sensitive amplified polymorphism (MSAP) analysis.

EcoRI Primer Sequences (5′–3′)		**HpaII/MspI Primer Sequence (5′–3′)**	
Adapter	CTCGTAGACTGCGTACC AATTGGTACGCAGTCTAC	Adapter	GACGATGAGTCTCGAT CGATCGAGACTCAT
E-pre	GACTGCGTACCAATTCA	HM-pre	ATGAGTCTCGATCGG
E1	GACTGCGTACCAATTCAGG	HM1	ATGAGTCTCGATCGGAAT
E2	GACTGCGTACCAATTCACG	HM2	ATGAGTCTCGATCGGACG
E3	GACTGCGTACCAATTCAAC	HM3	ATGAGTCTCGATCGGATC
E4	GACTGCGTACCAATTCACA	HM4	ATGAGTCTCGATCGGATT
E6	GACTGCGTACCAATTCACT	HM6	ATGAGTCTCGATCGGTCA
E7	GACTGCGTACCAATTCAAG	HM7	ATGAGTCTCGATCGGTGC
		HM8	ATGAGTCTCGATCGGTCCA

Note: Six *Eco*RI adapter primers (E1, E2, E3, E4, E5 and E6) and seven *Hpa*II/*Msp*I adapter primers (HM1, HM2, HM3, HM4, HM6, HM7 and HM8) were designed for selective amplification. The primer combinations used in MSAP were E1HM6, E1HM7, E2HM1, E2HM3, E3HM1, E3HM8, E4HM1, E4HM3, E4HM8, E6HM2, E6HM4, E6HM7, E6HM8, E7HM1 and E7HM2.

2.3. Phytohormone Extraction and Measurement

High-performance liquid chromatography (HPLC) was used to measure the phytohormone levels in the leaves, following the method of Pan [52]. Frozen leaves were ground into powder using a mortar and pestle in liquid nitrogen. A 300-mg sample of the powder was transferred into a 10-mL tube containing 5 mL of extraction solvent (80:20 methanol:deionized water (v/v) and 30 mg/L sodium diethyldithiocarbamate) and then shaken for 12 h at 4 °C in darkness. Following a 15-min centrifugation at 10,000 rpm and 4 °C, the supernatant was collected and the sediment was reextracted twice with 1 mL of the extraction solvent. The supernatants from the two extracts were combined and centrifuged and then HPLC hybrid triple quadrupole-linear ion trap mass spectrometry (API 3200 Q-TRAP liquid mass combination; Thermo Fisher Scientific) was performed to analyze the phytohormone contents [53].

2.4. Carbohydrate Measurement

A 300-mg sample of powdered leaves sampled at the floral transition and flower development stages was analyzed to determine the levels of soluble sugars, starch and reducing sugars. The soluble sugar and starch contents were determined using anthrone colorimetry, as described by Clegg [54], while the reducing sugar content was measured using 3,5-dinitrosalicylic acid colorimetry [55].

2.5. Data Analysis

The data were analyzed using SPSS 21.0 software (IBM, Armonk, NY, USA). The results are presented as means ± SE. Least significant difference (LSD) tests were used to determine any significant differences between the treatment and control groups ($p < 0.05$, significant; $p < 0.01$, highly significant). The figures were drawn using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA).

3. Results

3.1. DNA Methylation during the Flower Transition and Flower Development Stages

To analyze the changes in DNA methylation caused by 5-azaC, the differences in methylation between the treatment and the control were detected using MSAP. A total of 825 and 835 5′-CCGG-3′ loci were detected in the female and male plants, respectively, at the flower transition stage, which increased to 1142 and 1160, respectively, during flower development (Table 2). All of these loci were divided into four types by their methylation states: type I, unmethylated sites; type II, hemimethylated sites; and types III and IV, fully methylated sites. The numbers of methylated loci (types II, III and IV) increased from the flower transition stage to the flower development stage in both the male and female plants; however, the relative ratios of the various methylation categories did have sex-specific differences (Table 3). The ratios of unmethylated (type I) loci decreased by 11.68% and 13.79% in the female and male controls, respectively, between the flower transition stage and the flower development stage and by 6.31% and 5.95%, respectively, in the female and male 5-azaC-treated plants (Table 3). On the contrary, the ratios of fully methylated (type III and type IV) loci in the male and female 5-azaC-treated plants both increased between the flower transition and floral development stages. In comparison with the slight changes in the relative abundance of hemimethylated (type II) loci in the female plants during the same period, the relative abundance of type II loci increased dramatically in males between the flower transition stage and the flower development stage. The 5-azaC treatment significantly increased the ratios of unmethylated loci and significantly decreased the ratios of total methylated loci, particularly for the fully methylated loci, at the flower development stage (Table 3); however, no significant differences were observed between the ratios of the total methylated (type II, type III and type IV) loci of the treatment and control plants at the flower transition stage.

Table 2. Number of DNA methylated loci identified using MSAP.

Stage	Sex	Material	Type I	Type II	Type III	Type IV	Total Loci
			HPA+/MSP+	HPA+/MSP-	HPA-/MSP+	HPA-/MSP-	
FTS	Female	Control	412 ± 9b	78 ± 4b	157 ± 7b	177 ± 7c	825
		5-azaC	423 ± 9b	75 ± 4bc	151 ± 4b	185 ± 8c	825
	Male	Control	440 ± 8b	58 ± 7c	153 ± 3b	184 ± 1c	835
		5-azaC	432 ± 10b	58 ± 2c	158 ± 5b	187 ± 4c	835
FDS	Female	Control	437 ± 19b	118 ± 4a	195 ± 16a	391 ± 4a	1142
		5-azaC	513 ± 11a	104 ± 7a	156 ± 11b	369 ± 2b	1142
	Male	Control	452 ± 14b	115 ± 11a	206 ± 12a	387 ± 13a	1160
		5-azaC	532 ± 18a	108 ± 5a	193 ± 6a	327 ± 11b	1160

Type I, unmethylated loci; Type II, hemimethylated loci; Type III and Type IV, fully methylated loci. The total number of methylated loci = type II + type III + type IV. A least significant difference (LSD) test was used to identify statistically significant differences between samples. Data are means ± SE, $n = 3$. Different lowercase letters in each column indicate a significant ($p < 0.05$) difference between samples.

Table 3. Effects of 5-azaC on methylation level in female and male *Salix viminalis*.

Stage	Sex	Material	Unmethylated (%)	Hemimethylated (%)	Fully Methylated (%)	Total Methylated (%)
FTS	Female	Control	49.98 ± 1.05a	9.49 ± 0.44ab	40.53 ± 0.89c	50.02 ± 1.05c
		5-azaC	51.23 ± 1.93a	8.08 ± 0.53bc	40.69 ± 1.39c	48.77 ± 1.11c
	Male	Control	52.73 ± 0.93a	6.99 ± 0.84c	40.28 ± 0.33c	47.27 ± 0.93c
		5-azaC	51.78 ± 1.18a	6.99 ± 0.21c	41.24 ± 0.98c	48.22 ± 1.18c
FDS	Female	Control	38.30 ± 1.67c	10.36 ± 0.36a	51.34 ± 1.75a	61.70 ± 1.67a
		5-azaC	44.92 ± 0.96b	9.11 ± 0.61ab	45.97 ± 0.89b	55.08 ± 0.96b
	Male	Control	38.94 ± 1.21c	9.91 ± 0.98ab	51.15 ± 0.49a	61.06 ± 1.21a
		5-azaC	45.83 ± 1.54b	9.34 ± 0.42ab	44.83 ± 1.21b	54.17 ± 1.54b

Type I, unmethylated loci; Type II, hemimethylated loci; Type III and Type IV, fully methylated loci. A least significant difference (LSD) test was used to identify statistically significant differences between samples. Data are means ± SE, $n = 3$. Different lowercase letters in each column indicate a significant ($p < 0.05$) difference between samples. Total methylated ratio = [(II + III + IV)/(I + II + III + IV)] × 100%; Fully methylated ratio = [(III + IV)/(I + II + III + IV)] × 100%; Hemi-methylated ratio = [(II)/(I + II + III + IV)] × 100%; Unmethylated ratio = [(I)/(I + II + III + IV)] × 100%

3.2. Effects of 5-Azac on Flower Bud Development

Axillary buds in the annual shoots of *Salix viminalis* include both leaf and flower buds. The formation of flower buds requires an appropriate exogenous environment and specific endogenous signaling. To estimate the influence of DNA methylation on the development of flower buds and annual shoots, we injected the trunks of male and female *Salix viminalis* trees with the DNA methylation inhibitor 5-azaC prior to floral bud initiation. We counted the number of buds produced per annual shoot and measured the length and diameter of both the first bud and the annual shoots after the cessation of growth (Figure 2). The total number of leaf and flower buds produced by the female plants was not significantly affected by 5-azaC (Figure 2a); however, the number of flower buds and the ratio of flower buds to leaf buds in the females treated with 5-azaC were significantly higher ($p < 0.05$) than the control (Figure 2b,c). Furthermore, the 5-azaC treatment increased the diameter and lengths of the flower buds in the female trees (Figure 2d,e).

In male individuals, the total number of buds per annual shoot did not differ between the treatment and control groups (Figure 2a). In contrast, the number of flower buds and the ratio of flower buds to vegetative buds were significantly reduced by the 5-azaC treatment ($p < 0.05$), reaching just 65.9% and 61.0%, respectively, of the control (Figure 2b,c). The 5-azaC treatment significantly suppressed the diameter of the flower bud (Figure 2e) but did not significantly influence their lengths (Figure 2d). The length and diameter of the annual shoots were similar in the treated and control trees in both the male and female groups (Figure 2f,g).

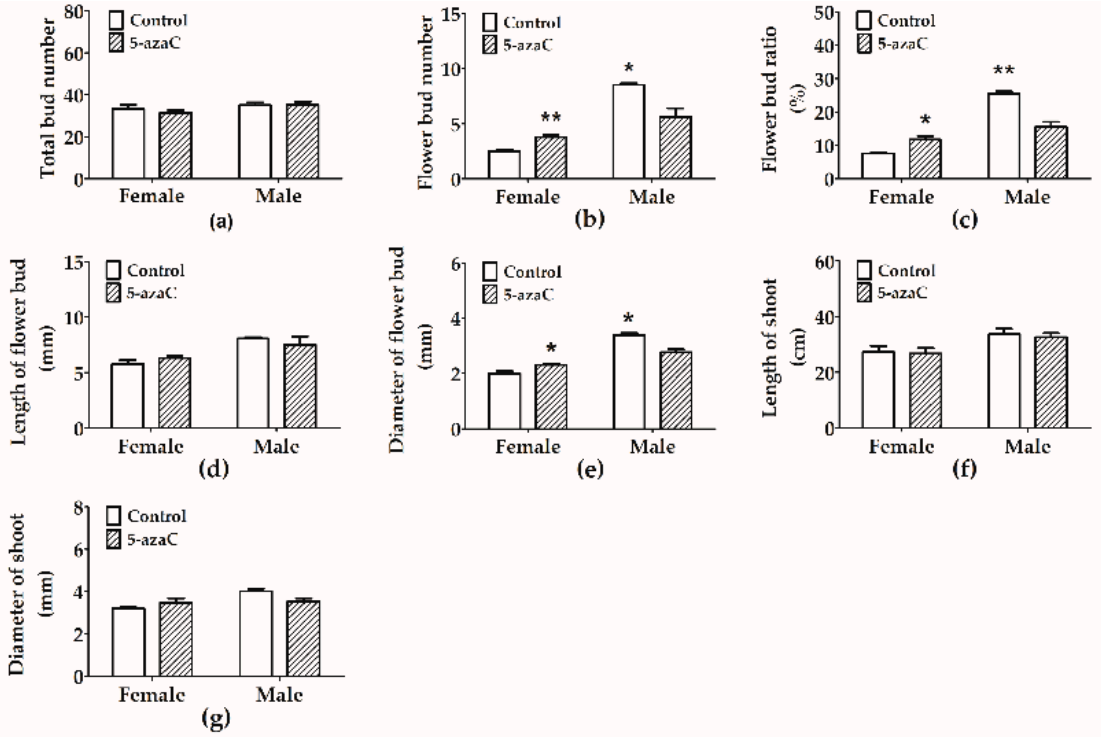

Figure 2. Development of flower buds and annual shoots in male and female plants treated with 5-azaC. (**a**) Total (vegetative and floral) bud numbers in the annual shoots. (**b**) Flower bud numbers in annual shoots. (**c**) The ratio of flower buds to total buds in annual shoots. (**d**) Length of flower buds. (**e**) Diameter of flower buds. (**f**) Length of annual shoots. (**g**) Diameter of annual shoots. Least significant difference (LSD) tests were used to determine any significant differences between the treatment and control groups. Data are mean ± SE, $n = 3$. *, Significant difference between treatment and control ($p < 0.05$); **, significant difference between treatment and control ($p < 0.01$).

3.3. Effects of 5-Azac on Carbohydrate Contents

Carbohydrates are important molecular signals regulating floral initiation, as well as vital energy sources during floral development. To determine whether DNA methylation affects floral initiation and development through the regulation of carbohydrate contents, we determined the soluble sugar, reducing sugar and starch contents of the leaves of 5-azaC-treated trees at the flower transition and flower development stages. In both male and female trees, the amount of soluble sugars, starch and reducing sugars in the leaves decreased from the flower transition stage to the flower development stage, except that the starch levels in the female individuals treated with 5-azaC did not decrease and the soluble sugars of the male control plants were similar at both stages (Figure 3). The effects of 5-azaC on the carbohydrate contents differed between the male and female plants (Figure 3); for example, in the females, 5-azaC significantly ($p < 0.05$) decreased the contents of the three kinds of carbohydrate at the flower transition stage (Figure 3a–c), whereas the soluble and reducing sugars at the flower transition stage were significantly ($p < 0.05$) higher in male plants treated with 5-azaC than the male controls (Figure 3d,f). At the flower development stage, the starch contents of the female 5-azaC-treated plants were significantly ($p < 0.05$) higher than those of the controls, while the starch contents of the males in the treatment group were slightly but not significantly higher than their respective controls (Figure 3b,c). These results show that the effect of 5-azaC on the contents of soluble sugars, starch and reducing sugars differed between the male and female plants.

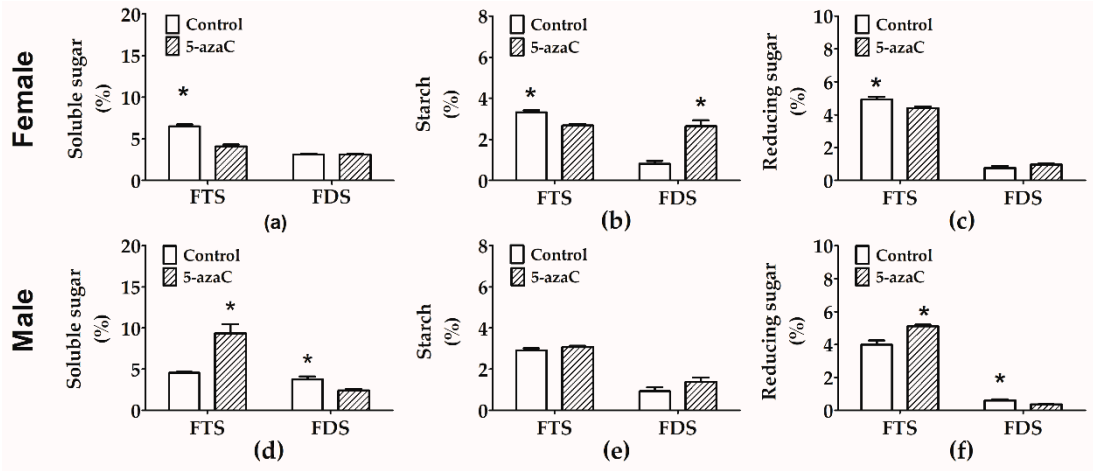

Figure 3. Effects of 5-azaC on the carbohydrate contents of male and female *Salix viminalis* trees. (**a**–**f**) Contents of soluble sugars (**a**,**d**), starch (**b**,**e**) and reducing sugars (**c**,**f**) in the female (**a**–**c**) and male (**d**–**f**) trees. FTS, flower transition stage; FDS, flower development stage. Least significant difference (LSD) tests were used to determine any significant differences between the treatment and control groups. Data are mean ± SE, $n = 3$. *, Significant difference between treatment and control ($p < 0.05$); **, significant difference between treatment and control ($p < 0.01$).

3.4. Effect of 5-Azac on Phytohormone Levels

Phytohormones are plant-growth-regulating substances with essential roles in the formation and development of floral organs. To analyze the relationships between DNA methylation, phytohormones and floral development, we determined the levels of phytohormones in the leaves of *Salix viminalis* trees treated with 5-azaC, both at the flower transition and flower development stages (Figure 4). The IAA levels decreased sharply between the flower transition stage and the flower development stage (Figure 4a,b) but the GA3 and GA4 levels increased rapidly (Figure 4g–j). The main GA, GA4 [56], was undetected in the flower transition stage but a high level of GA4 was present in leaves during the flower development stage. The ZT and ABA contents changed slightly between the flower transition stage and the flower development stage (Figure 4c–f). No significant differences in hormone levels were detected between the male and female plants (Figure 4). The 5-azaC treatment significantly decreased the IAA contents of male and female individuals by 42.6% and 36.0% relative to their controls, respectively, at the flower transition stage (Figure 4a,b); however, it did not influence the ABA, ZT, GA3 or GA4 levels of the male or female plants at either stage (Figure 4c–j).

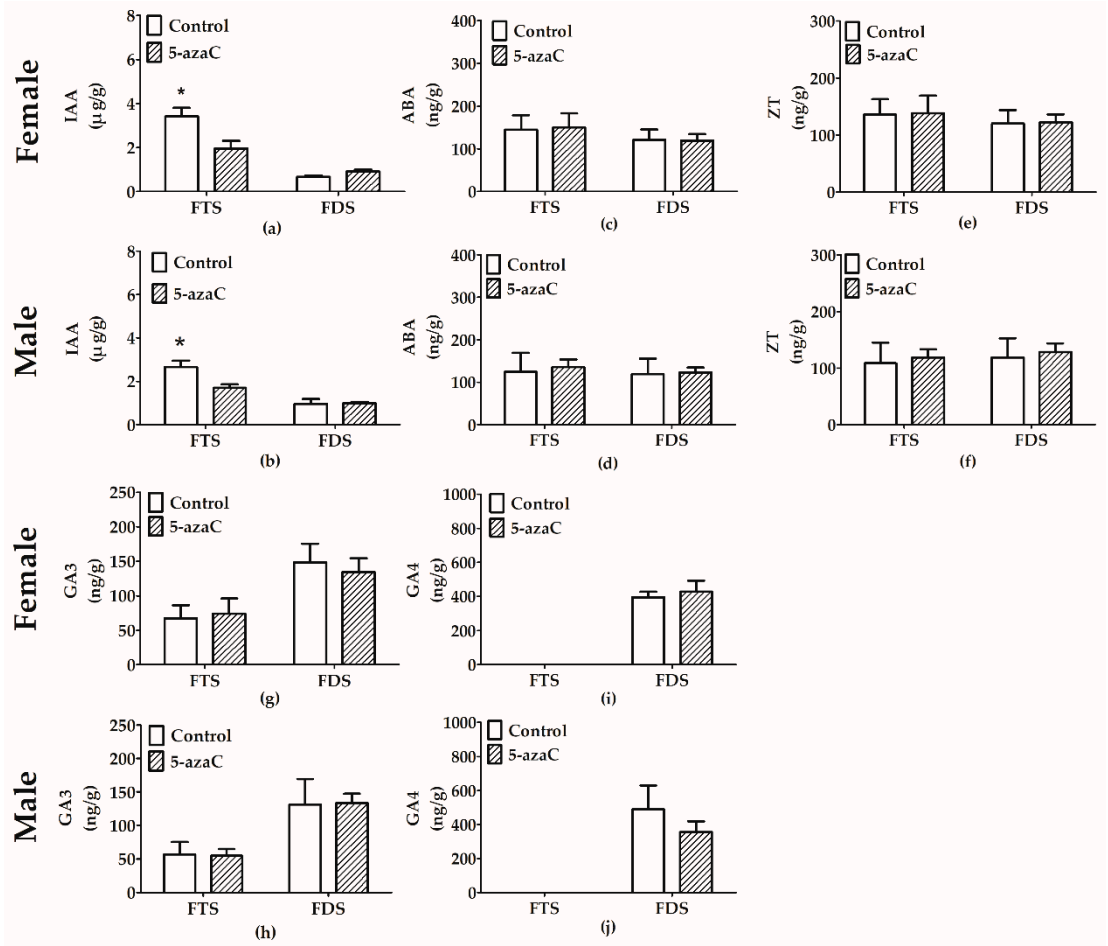

Figure 4. Effects of 5-azaC on the phytohormone contents of male and female *Salix viminalis* trees. (**a**–**j**) Contents of indoleacetic acid (IAA; **a**,**b**), abscisic acid (ABA; **c**,**d**), trans-zeatin-riboside (ZT; **e**,**f**), gibberellin 3 (GA3; **g**,**h**) and gibberellin 4 (GA4; **i**,**j**) in female (**a**,**c**,**e**,**g**,**i**) and male (**b**,**d**,**f**,**h**,**j**) trees. Least significant difference (LSD) tests were used to determine any significant differences between the treatment and control groups. Data are mean ± SE, $n = 3$. *, Significant difference between treatment and control ($p < 0.05$); **, significant difference between treatment and control ($p < 0.01$).

4. Discussion

4.1. DNA Methylation and Flower Development

Many studies have shown that DNA methylation levels change with the initiation and development of the flower bud [17,28,57,58]. In our research, the level of DNA methylation was found to increase from the flower transition stage to the flower development stage (Table 3), which is consistent with previous research in Arabidopsis [14], Monterey pine (*Pinus radiata*) [59] and sweet chestnut (*Castanea sativa*) [60,61]. The process of RdDM is recognized as the main route of *de novo* DNA methylation in plants [62,63]. In apple, the abundance of 24-nt siRNAs responsible for the RdDM pathway significantly changed as the plants transitioned from the vegetative stage to the reproductive stage [17], which suggested that large-scale *de novo* DNA methylation is involved in flower development. Here, we showed that, in the dioecious tree *Salix viminalis*, the hypomethylation of male and female leaves at the flower transition stage promotes the floral transition and flower bud growth (Figure 1, Table 3), indicating that DNA methylation plays a similar role in the floral transition and early floral development in both male and female individuals.

Previous studies have demonstrated significant morphological, physiological and transcriptional differences between the sexes of dioecious plants during flower development [36,64–66]. In our

study, the DNA methylation inhibitor 5-azaC decreased the methylation level of female leaves at the flower transition stage but these levels were increased in male leaves at the same stage (Table 3). However, both of the changes caused by 5-azaC in female and male trees at the flower transition stage were insignificant and the levels of total methylation loci in female and male were decreased by 5-azaC. Our results suggest that the inhibition of DNA methylation by 5-azaC only occurred at the flower development stage (Table 3). The reason for this may be that 5-azaC inhibits the *de novo* methylation of DNA, rather than promoting DNA demethylation because it is thought to be incorporated into DNA and covalently binds cytosine methyltransferase enzymes to prevent the function of DNA methyltransferase [26]. Another possible reason that differences in DNA methylation were not observed during floral initiation is that the MSAP technique has a relatively low resolution and may have missed the slight differences from 5-azaC. In females, the numbers and diameters of female flower buds were 50% and 15% higher, respectively, in trees treated with 5-azaC than the controls, while in the males, the flower bud number and diameters were decreased by 34% and 17%, respectively, in response to 5-azaC (Figure 1). These differing responses between males and females suggest that 5-azaC did differentially alter DNA methylation in the male and female plants. These phenotypic differences may derive from the differences in the stress responses between the male and female plants, as is consistent with previous research [67–69]. In *Hippophae rhamnoides* L., stressful environments have a more negative impact on females than on males in a variety of ways, including shoot height, leaf N concentration per unit of mass, leaf N concentration per unit area and leaf carbon isotope composition [68]. All these traits are regulated by genes. DNA methylation involves signal transduction and regulation of gene expression in response to abiotic stress [70].

In the annual dioecious herb *Silene latifolia*, a 5-azaC treatment was found to induce a change from male individuals to andromonoecy [31], while in *Spinacia oleracea*, 5-azaC increased the percentage of monoecious plants by changing the sex of female individuals [32]. These findings indicate that DNA methylation takes part in the sex determination of dioecious plants and its species-specific effects imply the existence of different sex determination mechanisms between species. In the present study, the 5-azaC treatment only altered the abundance of flower buds relative to vegetative buds, without altering their sex. One possible reason for this may be that 5-azaC was applied before the initiation of flowering, whereas sex differentiation is known to occur at later stages of floral development; in dioecious woody plants, inflorescence development and the differentiation of male/female primordia take place in different growing seasons [71]. In our study, the early application of 5-azaC only influenced inflorescence initiation and floral bud development, which led us to apply 5-azaC at a later development for detecting how it affects the sex of flower bud. This may help reveal the role of DNA methylation in sex determination of dioecious trees.

4.2. The Relationship between Endogenous Carbohydrates, Hormones and Floral Development

Carbohydrates are known to play essential roles in flower development; for example, sugars function as energy stores and signaling molecules that regulate flower development [9,72–74]. In the present study, the DNA methylation inhibitor 5-azaC increased the number of flower buds produced by the female plants but decreased their leaf sugar contents during the floral transition stage (Figures 2 and 3). In male plants, 5-azaC decreased the number of flower buds and increased the leaf sugar contents at the floral transition stage (Figures 2 and 3). These results suggest that the sugars in the leaves during the floral transition stage are negatively linked to the initiation of flower bud development in both female and male plants, confirming that carbohydrates play an important role in floral initiation [9]. During the transition from vegetative to reproductive growth, the carbohydrates in the leaves are transported to the floral meristem to fuel flower development [10,72]. Consistent with this, we showed that the leaf contents of soluble sugars, reducing sugars and starch decreased from the flower transition stage to the flower development stage in the control plants (Figure 3). However, the changes we observed in the leaf starch content contrasted with the dynamic changes observed in apple [5]. This difference likely occurs because the majority of apple bud growth takes place during

the early period of flower bud induction [5], while the flower buds of *Salix viminalis* continue to grow until the end of the growing season, using starch to fuel their growth. In Arabidopsis, both starch deficiencies and excess resulted in a prolonged juvenile phase because the metabolism of the plant was disturbed [9,72].

DNA methylation influences floral development by regulating the expression of the flowering genes through their epigenetic modification [14,15,75]. Consequently, the changes in the carbohydrate contents and flower bud production of the plants treated with 5-azaC suggest that DNA methylation might regulate the expression of carbohydrate-metabolism-related and transport-related genes involved in flower initiation and development. This is consistent with studies in lily (*Lilium sp.*), in which sugar contents, flowering gene expression and DNA methylation levels were all found to change during vernalization [76]. In cucumber (*Cucumis sativus* L.), the apex methylome response to lower temperatures was involved in temperature-dependent sex determination in flowers [77]; however, the flower initiation of woody plants, such as *Salix* [50], apple [5] and persimmon (*Diospyros sp.*) [71], takes place during the growing season and is largely influenced by the photoperiod. Carbohydrates in plants are the products of photosynthesis, which is driven by light and, thus, the photoperiod itself. Therefore, our results suggest that DNA methylation plays a key role in the photoperiod pathway-mediated regulation of flowering by influencing carbohydrate metabolism and transport. The genetic network by which DNA methylation, carbohydrate metabolism and photoperiod are connected is still unclear and should be elucidated in the future.

Furthermore, we analyzed the hormone levels during the flower transition stage and the flower development stage. Previous research has revealed the complex role of hormones in flower initiation and development; for example, in Arabidopsis [78] and darnel ryegrass (*Lolium temulentum* L.) [79], GA promotes flowering, while low levels of GA are required for flowering in woody plants [2,74,80]. Under short-day conditions, GA4, which is likely produced in the leaves and transported to the meristem, upregulates the expression of one or both of the genes *LFY* [24] and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*) [10], promoting flowering. In our study, the levels of GA3 were lower at the flower transition stage than during floral development and we did not detect any GA4 at the flower transition stage. These changes in GA levels imply that a low level of GA is necessary for the initiation of flowering, while higher levels of GAs promote floral development.

Auxin regulates multiple aspects of flower development, including floral meristem initiation, floral organ initiation, growth and later events that ensure reproductive success of the mature flower [81–83]. In poplars (*Populus sp.*), the males exhibited a smaller decrease in leaf IAA than females following a short-day shift [84], although the concentration of IAA was higher in the female flowers than in the male flowers [85]. In the current study, the levels of IAA in the 5-azaC-treated plants were significantly lower than the controls but the IAA levels in both the treated and control plants declined from the flower transition stage to the flower development stage (Figure 3a,b). The similar changes in the IAA level and the contrasting relationships between the IAA level and flower bud development in female and male *Salix viminalis* individuals further demonstrate the sexual differences in dioecious flower bud development in response to IAA.

5. Conclusions

Extensive *de novo* DNA methylation takes place in the transition from vegetative to reproductive growth and during flower development. Male and female individuals of *Salix viminalis* respond differently to the application of 5-azaC, a DNA methylation inhibitor. A reduced level of DNA methylation in the leaves at the floral transition stage is associated with the promotion of flower bud initiation in both male and female *Salix viminalis*, in a process that may involve the alteration of sugar metabolism and transport. We also observed potential sex-specific reactions to IAA during flower development in *Salix viminalis*. These findings highlight the dynamic interaction of multiple factors, including the physiological, molecular and environmental conditions that regulate flower bud initiation and development.

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