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Effects of Plant Growth Regulators, Soil Moisture Contents, and Carbon/Nitrogen Ratios on Sex Differentiation in Persimmon (*Diospyros kaki* Thunb.) Flowers

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Abstract

The sex types of persimmon (*Diospyros kaki* Thunb.) flowers are occasionally alterable. The feminizing agents such as ethrel, 5-azacytidine, a mixture of exogenous zeatin (ZT), abscisic acid (ABA), and indole-3-acetic acid (IAA) were injected into androecious wild persimmon trees. Different concentrations of masculinizing exogenous gibberellin (GA_3) were injected into gynoecious 'Jiro' cultivar trees. 'Jiro' trees were also grown in soils differing in moisture content and carbon/nitrogen (C/N) ratio, to determine their effects on floral development and sex differentiation in persimmons. Injection of ethrel significantly decreased the diameter of pollen grains and the length of pollen tubes, thereby demonstrating a feminizing effect. Injection of GA_3 significantly increased the length of arrested stamens in gynoecious trees, demonstrating a masculinizing effect. High soil moisture levels (55% and 45% volumetric moisture contents) significantly increased the length of pistils compared to the control (20% moisture content) in gynoecious trees; a high C/N ratio markedly increased the length of both pistils and arrested stamens. Many of the treatments also significantly changed the expression patterns of crucial genes, the contents of assimilates and stress markers, and the activities of antioxidative enzymes in floral buds. These treatment responses may relate to changes in phenotypes. Although the sex types of persimmon flowers were not completely transformed in our study, the treatments significantly influenced pistil and stamen length. Improved artificial treatments may be used to control sex type in persimmons in the future.

Keywords *Diospyros kaki* Thumb. · Plant growth regulators · Soil moisture · Carbon/Nitrogen ratio · Floral development · Sex differentiation

Introduction

The persimmon (*Diospyros kaki*) is an important fruit tree that occurs most frequently in temperate East Asia. On the basis of the level of astringency loss in fruits, Kajiura (1946) classified persimmons into four types: pollination-constant non-astringent (PCNA), pollination-constant astringent (PCA), pollination-variant non-astringent (PVNA), and pollination-variant astringent (PVA). Yonemori et al. (1993) divided persimmon into three types at the individual level sex expression: (i) bear only female flowers (gynoecious-type), (ii) both pistillate and staminate flowers on the same plant (monoecious-type), and (iii) hermaphroditic, pistillate, and staminate flowers on the same plant (polygamo-monoecious-type). Pistillate (female) flowers, locating in the axils of leaves, are usually solitary with a central flower per inflorescence and two lateral aborted flowers. Male

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(staminate) flowers are presented in cymose clusters of three to five flowers with fertile stamens but rudimentary arrested carpels, and smaller than female flowers (pistillate) (Akagi et al. 2014; Wang et al. 2018). At present, we also found a small number of wild persimmon trees (*D. kaki*) are androecious and andromonoecious, i.e., bear staminate flowers only (Wang et al. 2018) and bear both staminate and hermaphroditic flowers (Li et al. 2019a, b), respectively. 'Youhou' (a cross-breeding product of occasionally blooming male flowers of 'Jiro' and 'Fuyu' cultivars) is a Japanese pollination-constant non-astringent (J-PCNA) persimmon of good commercial value (Yamane et al. 1991). Zhang et al. (2016) showed that the Chinese androecious persimmon 'Male 8′ is a potential male hybrid for PCNA-type cultivation because it has a dominant gene controlling natural deastringency. These observations support the further use of appropriate androecious persimmon germplasm resources for cross- and polyploid-breeding by controlled pollination to produce improved cultivars (Wang et al. 2018). However, most domesticated persimmon cultivars are gynoecious and only a few are monoecious. As far as we know, androecious types are found only occasionally in the rare wild persimmon populations (Zhang 2006; Fu et al. 2017). Due to a strong parthenocarpic ability of most persimmon cultivars, pollen is dispensable for production of persimmon fruits, leading to an indifferent attitude on androecious types. This situation restricts the breeding of new persimmon cultivars by cross-pollination (Wang et al. 2018). In addition, the breeding value of androecious persimmon types is always ambiguous because no fruit is born on the trees. Therefore, the development of procedures for inducing (i) the male (or hermaphrodite) flowers from gynoecious persimmons to produce active pollen, and (ii) the female (or hermaphrodite) flowers from androecious persimmons to bear fruit would be very valuable from a theoretical and practical point of view.

Gynoecious persimmon trees may occasionally bear male flowers (Yakushiji et al. 1995), indicating that the sexuality of persimmon flowers is labile. According to Akagi et al. (2016), sexuality in persimmons is controlled by the methylation level of a promoter of the sex determination gene *MeGI*, and high DNA methylation level of the *MeGI* promoter determines the development of male flowers. Gene methylation is one of the mechanisms underlying epigenetic regulation; it may be affected by methylation inhibitors, such as 5-azacytidine (Kondo et al. 2010) and zebularine (Akagi et al. 2016), and environmental factors, such as moisture (Wang et al. 2011), nutrition (Kucharski et al. 2008), temperature (Choi and Sano 2007), radiation (Aypar et al. 2011), heavy metals (Ou et al. 2012), and salinity (Marconi et al. 2013). Thus, the sexuality of persimmons is controlled by the methylation level of a promoter of the *MeGI* gene, but may also be influenced by environmental factors. This premise appears to be supported by the following observation: the gynoecious PCNA persimmon cultivar 'Jiro' occasionally bear male flowers in some years. These events may be related to particular environmental factors, such as soil moisture and nutrition, in the years in which male flowers are formed (Nishida and Ikeda 1961; Yamane et al. 1991; Yakushiji et al. 1995). Environmental stress can increase the plasticity of sexual expression, a phenotypic response that is likely advantageous for individuals located in habitats that are sometimes physiologically stressful (Golenberg and West 2013). Thus, for example, the DNA methylation level of the pea genome is significantly elevated under drought stress (Labra et al. 2002). This response to stress is a common masculinizing mechanism. Drought stress also increases the frequency of male flowers in *Solanum carolinense* (Solomon 1985) and *Elaeis guineensisi* (Adam et al. 2011). In contrast, Nanami et al. (2004) showed that the frequency of female flowers produced by *Acer rufinerve* was increased by drought stress. DNA methylation in honeybees can also be altered by their nutrition, and epigenesis is therefore an important mechanism in the expression of reproductive status (Kucharski et al. 2008). Kraus and Kraybill (1918) put forward a classic carbon/nitrogen (C/N) ratio theory based on their studies on tomatoes, i.e., the vegetative growth and reproductive development of plants are controlled by the C/N ratio. Flowering is stimulated when carbohydrate accumulation in plants exceeds their tissue nitrogen content.

Sexuality in persimmons is also closely related to endogenous phytohormone levels (Sun et al. 2017), which are important regulators of sex differentiation in many plants (Golenberg and West 2013). Ethylene has a major role in female expression in different species (Boualem et al. 2008; Martin et al. 2009). Sufficient levels of ethylene in *Cucumis sativus* activated the signaling cascade by inhibiting activities of CTR proteins, leading to the development of female flowers (Pawełkowicz et al. 2019a). Gibberellins (GAs) are considered to be masculinizing hormones (Irish and Nelson 1989). They have obvious masculinizing effects on *Mercurialis annua* (Boissay et al. 1996), *Populus tomentosa* (Song et al. 2013) and *Cucumis sativus* (Pawełkowicz et al. 2019a). Indole-3-acetic acid (IAA), a natural auxin, is a regulator of flower sex differentiation that influences the crosstalk with cytokinins (CTKs) in the regulation of sex determination in diverse plants (Irish and Nelson 1989). Thus, for example, genotypically male mercury flowers develop female characteristics following exogenous application of CTK, but the application of auxin has the reverse effect (Louis and Durand 1978). IAA is also reportedly correlated with early floral initiation; increases in free IAA concentrations and decreases in IAA conjugates promote the differentiation of female floral buds in *Polianthes tuberosa* (Ding et al. 1999). Jasmonate (JA) and brassinosteroid (BR) act together to suppress tassel development in maize (Acosta et al. 2009; Hartwig et al. 2011). GA is required for suppression of stamens in maize

ears (Bensen et al. 1995). Abscisic acid (ABA), which has a feminizing effect on *Solanum carolinense* (Solomon 1985) but promotes maleness in *Cucumis sativus* (Pawełkowicz et al. 2019a), and jasmonic acid (JA) which has a masculinizing effect on *Zea mays* (Browse 2009), also have roles in sex differentiation.

Our previous study showed that the developmental processes in male and female flowers of the monoecious persimmon cultivar 'Zenjimaru' can be separated into 11 stages during the period from June (floral ontogeny) through May of the following year (blooming) (Fig. 1) (Li et al. 2016; Sun et al. 2017). We identified two crucial morphological time points in floral sex differentiation during this time period. The first occurred in mid-June (stage 2 of the 11 stages of floral development) when the male flower formed a three-flower cyme primordium, and the female floral bud developed a terminal floral primordium. The second crucial time-point occurred in the following mid-April (stage 8 of the 11 stages of floral development), when development of the stamen primordium in the female floral bud and the pistil primordium in the male floral bud were arrested, leading to the formation of unisexual flowers (Li et al. 2016). Our previous study also showed that high levels of endogenous GA_3 at stage 2 (mid-June) and stage 8 (mid-April), and high ABA/zeatin (ZT) in stages 6–11 (from early April to early May) might promote the development of stamen primordia. However, high levels of endogenous IAA, ZT, and ABA in April might accelerate the development of pistil primordia, in turn leading to the development of female unisexual flowers (Sun et al. 2017). Additionally, we identified (through transcriptome sequencing and comparative analyses of persimmon male and female floral buds) 25 differentially expressed genes functionally involved in phytohormone biosynthesis and signaling transduction, development of floral organs, transcription regulation and other processes, as crucial elements in the regulation of sex differentiation (Li et al. 2019a, b).

In the present study, we injected (i) the previously reported feminizing regulators ethrel, 5-azacytidine, and a mixture of exogenous ZT, ABA, and IAA into androecious wild persimmon trees, and (ii) masculinizing exogenous GA_3 at different concentrations into gynoecious 'Jiro' trees. In addition, 'Jiro' trees were exposed to gradients of soil moisture and C/N ratios. After terminating the treatments, we determined the phenotypic traits, the expression levels of eight crucial genes, the contents of assimilates (including soluble carbohydrate and sucrose) and stress markers (including proline and malondialdehyde (MDA)), and the activities of antioxidative enzymes (including superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT)) in floral buds at different developmental stages. Our findings allowed us to assess the effects of plant growth regulators, soil moisture contents, and C/N ratios on floral development and sex differentiation in persimmon.

Materials and Methods

Plant Materials

Eight-year-old androecious 'Mulan wild persimmon' (*D. kaki*) trees, eight-year-old gynoecious 'Jiro' trees grown in the fields, and five-year-old 'Jiro' trees grown in pots were used as experimental subjects. Specimens from all of the trees were deposited in the Persimmon Germplasm Repository (Yuanyang County, Henan Province, China; 34°55.30′–34°56.45′ N, 113°46.24′–113°47.59 E), which is owned by the Non-timber Forestry Research & Development Center, Chinese Academy of Forestry.

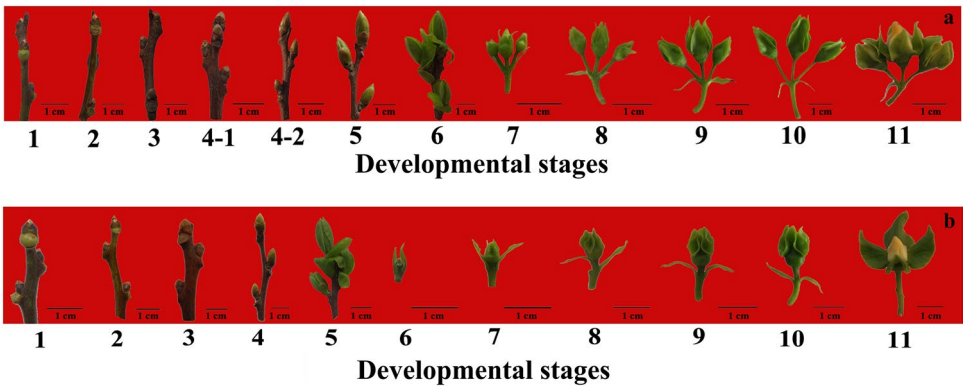

Fig. 1 Male (**a**) and female (**b**) floral buds at different developmental stages (Li et al. 2016; Sun et al. 2017). The crucial stages for development and sex differentiation of flowers were highlighted as below. Stage 2: inflorescence primordia initiation; stage 3: petal primordia initiation; stage 4: stamen primordia initiation; stage 5: carpel primordia initiation; stages 6 and 7: rapid development of stamen and pistil primordia; stage 8: arrest of inappropriate carpel and stamen primordia; stage 11: anthesis

Injections of Plant Growth Regulators

Ethrel, 5-azacytidine and a mixture of exogenous ZT, ABA, and IAA were injected into the 8-year-old androecious wild persimmon trees. Different concentrations of masculinizing exogenous GA_3 were injected into the 8-year-old gynoecious cultivar 'Jiro' (Table 1). Water was injected into control trees. The injection holes were drilled into the tree stems at an angle of 45° above horizontal. Each hole was 5 mm in diameter, 40 mm deep, and located 40 cm above ground level. Each treatment was assigned to five replicate trees. Trees were injected once a month from stage 2 (on June 15, 2017, when male floral buds formed three-flower cyme primordia, and the female buds formed terminal floral primordia) through stage 3.5 (on March 14, 2018, the buds were still in dormancy at this stage and it is six days prior to stage 4, when the stamen primordia initiated in both female and male floral buds), and once a week from stage 4 (on March 22, 2018, when it is immediately after bud burst and stamen primordia initiated) through stage 11 (on May 3, 2018, at anthesis) (Fig. 2). Male buds, female buds, and flowers from all samples were collected at stage 3.5 (on March 14, 2018, as gene expression levels and physiological conditions at this stage—6 days before stage 4—may be important for the occurrence of stage 4, when stamen primordia initiated), stages 6 and 7 (on April 1 and April 14, 2018, when the stamen and pistil primordia were developing quickly during this period, and gene expression levels and physiological conditions at these stages—prior to stage 8 when stamen and pistil primordia were arrested in female and male floral buds respectively—may be important for the occurrence of stage 8), and stage 11 (on May 3, 2018, which is the anthesis and the mature pollen/embryo sac formation stage) for real-time quantitative polymerase chain reaction (RT-qPCR) analyses and physiological index measurements (detail information was shown in the part of Measurement of the Physiological Index). Male and female flowers from all treatments were collected at stage 11 for phenotypic measurements (including lengths of pistils and stamens in gynoecious trees, and lengths of stamens and pollen tubes, diameters of pollen grains, and pollen viability in androecious trees). Approximately 20 g of male and female floral buds/flowers were collected from each tree at each developmental stage.

Soil Moisture Treatments

Five-year-old gynoecious 'Jiro' trees grown in pots were subjected to soil moisture treatments. The volumetric

Table 1 Plant growth regulator concentrations and types of trees treated

Growth regulator	Concentration	Sex type of treated trees
Ethrel	100 mg/L	Androecious 'Mulan wild persimmon'
Mixture	ABA (2.25 mg/L) + IAA (0.03 mg/L) + ZT (0.03 mg/L)	Androecious 'Mulan wild persimmon'
5-Azacytidine	24.42 mg/L	Androecious 'Mulan wild persimmon'
GA_3	6.0 mg/L	Gynoecious cultivar 'Jiro'
GA_3	12.0 mg/L	Gynoecious cultivar 'Jiro'

ABA abscisic acid, *IAA* indole-3-acetic acid, *GA* gibberellin, *ZT* zeatin

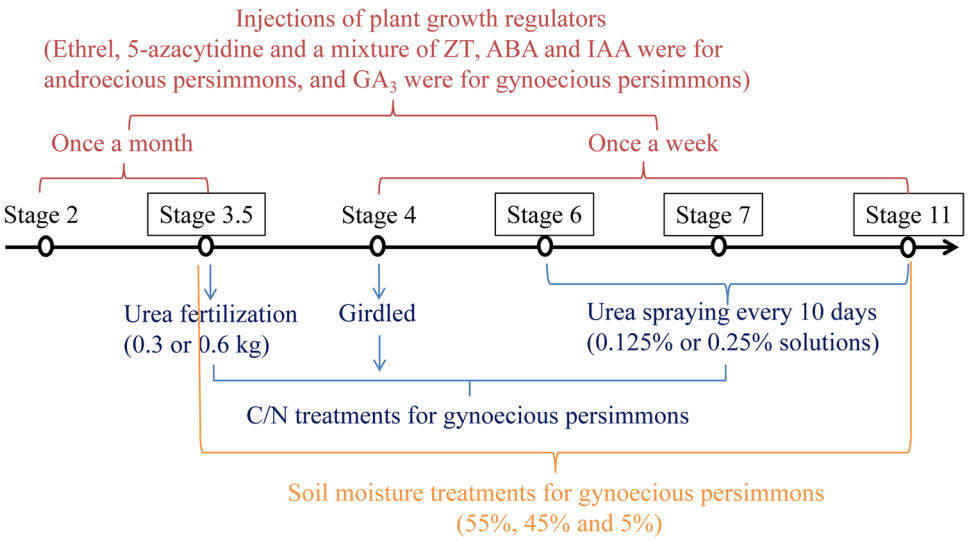

Fig. 2 Overview of the time scheme for the overall research. Black arrow in the middle represents timeline of the overall research scheme. Circles on the timeline represent developmental stages of treatments, and the developmental stages with frames represent time points for floral buds collection, which were used for phenotypic measurements, real-time quantitative polymerase chain reaction (RT-qPCR) analyses and physiological index measurements. Words with different colors in the figure represent different treatments

moisture contents of soil in pots (determined with a soil moisture meter; Field Scout TDR 300; Spectrum Technologies, Aurora, IL, USA) were maintained in 55%, 45% and 5% amounts in the respective treatments. A control treatment with an intermediate 20% moisture content was also established. The treatments were applied from stage 3.5 (on March 14, 2018) through stage 11 (on May 3, 2018) (Fig. 2). Each treatment was applied to five replicate trees. Approximately 20 g of female floral buds/flowers were collected from each tree at stage 3.5, stage 6, stage 7, and stage 11 in 2018. After collection, half of the flowers obtained at stage 11 were stored in formalin–acetic acid–alcohol (FAA, a kind of biochemicals used for the storage of samples while maintaining their morphological characteristics) for later phenotypic measurements (including the lengths of pistils and stamens). The remaining samples were immediately frozen in liquid nitrogen and stored at –80℃ prior to RT-qPCR analyses and physiological index measurements.

C/N Treatments

Five-year-old gynoecious 'Jiro' trees grown in pots were subjected to C/N treatments. The C/N levels were controlled by a combination of girdling and urea application (Table 2). Each treatment was assigned to five replicate trees. The girdles were 1 cm wide and cut deep into the xylem. The cuts, which were located 40 cm above ground level, were made at stage 4 (on March 22, 2018). Pot soil fertilization (0.3 or 0.6 kg urea) was performed at stage 3.5 (on March 14, 2018). Sprays containing 0.125% or 0.25% urea solutions were applied every 10 days from stage 6 (on April 1, 2018) through stage 11 (on May 3, 2018) (Fig. 2). Girdling reportedly increases the C/N ratio level (Asao and Ryan 2015), and the application of urea lowers the C/N ratio. Hence, the C/N ratios in Treatment 1 (girlding only) floral buds were predicted to be highest among all treatments. Conversely, the C/N ratios in Treatment 4 (fertilized and sprayed with the highest concentration of urea) floral buds were predicted to be lowest among all treatments. Approximately 20 g of female floral buds/flowers were collected from each tree at stage 3.5, stage 6, stage 7, and stage 11 in 2018. After collection, half of the floral flowers obtained at stage 11 were stored in FAA prior to phenotypic measurements (including the lengths of pistils and stamens). The remaining samples were immediately frozen in liquid nitrogen and stored at –80℃ prior to RT-qPCR analyses and physiological index measurements.

Measurements of Phenotypes

The lengths of pistils and stamens from gynoecious and androecious (pistils barely visible) persimmons were measured with a Vernier caliper in mature flowers at stage 11. Pollen viability was assessed at 4.5 h after transferring grains onto 20% sucrose/0.001% boric acid/0.5% agarose medium held at 25 °C. Pollen grains with pollen tubes longer than the width of the grains were scored as "activated". The lengths of the pollen tubes and the diameters of pollen grains were measured and averaged for batches of > 100 pollen grains observed from five different viewing angles (i.e., biological replicates). Observations were made with a compound light microscope (BX51; Olympus, Tokyo, Japan).

Real-Time Quantitative Polymerase Chain Reaction Analyses

Our previous study used transcriptome sequencing and comparative analysis of male and female floral buds of the persimmon to identify 25 differentially expressed genes functionally involved in phytohormone biosynthesis, signaling transduction, development of floral organs, transcription regulation, and other processes that are crucial in the regulation of sex differentiation (Li et al. 2019a, b). The raw short reads were available in the NCBI SRA database under the accession number: SRP151715. Based on this previous work, eight genes homologous to *MeGI*, *AMC9*, *BAG5*, *AifA*, *IAA32*, *GA20OX2*, *ACO,* and *WRKY28* were selected for determination of expression patterns from stage 3.5 to 11 across the different treatments. The eight genes selected are described in Table 3. Primer Premier 5.0 (Premier, Inc., Canada) was used to design PCR primers.

Table 2 Carbon/nitrogen (C/N) ratio treatments applied to potted 5-year-old gynoecious 'Jiro' trees

Treatment code	Urea fertilization at stage 3.5 in 2018	Urea spraying every 10 days from stage 6 to stage 11 in 2018	Girdled at stage 4 in 2018
Treatment 1	None	None	Yes
Treatment 2	Urea (0.3 kg)	Urea (0.125%)	No
Treatment 3	Urea (0.3 kg)	Urea (0.125%)	Yes
Treatment 4	Urea (0.6 kg)	Urea (0.25%)	No
Treatment 5	Urea (0.6 kg)	Urea (0.25%)	Yes
Control	None	None	No

Table 3 Sequences of primers used for the genes analyzed in this study

Gene ID (Homologous gene)	Gene function	Primer sequence (5′-3′)	Size of PCR fragment (bp)
GAPDH	Reference gene	F:AGCTCTTCCACCTCTCCAGT R:TGCTAGCTGCACAACCAACT	157
MeGI (c100711_g1)	Female growth promoter and male growth inhibitor gene in *Diospyros* (Akagi et al. 2014, 2016)	F:GGAGTTGAACTTTGGGAACG R:AAGGCGACACTTGTGGACGA	199
AMC9 (c109216_g1)	High expression of this gene promotes programmed cell death (PCD) (Uren et al. 2000)	F:CTGCTCCTTCTCCTTGTCTATG R:GTGGACTTCCGTCAACTGG	105
BAG5 (c115962_g1)	High expression of this gene inhibits PCD (Guo et al. 2015; Gupta et al.2016)	F:GATACCTGTTCGCTTCGT R:TCAACCGCCTCAACCTCT	138
AifA (c86489_g1)	A gene that leads to large-scale DNA fragmentation and cell apoptosis when translocated to the nucleus (Savoldi et al. 2008; Dinamarco et al. 2010)	F:GTAACCTTTCACCCTCAA R:CTATCAGAGCCGATTGTAT	137
IAA32 (c92877_g1)	A negative regulator of auxin signaling (Reed 2001; Tian et al. 2002)	F:ATCGCCCACTGTCCTCCA R:ACTCTTCCCTCGCCCTTC	134
GA20OX2 (c105233_g1)	A gene promoting GA biosynthesis (Oikawa et al. 2004; Rieu et al. 2008)	F:GGATGAGCCTGGGAGTAAG R:TGTGCCTAAGGTCTGGTCT	116
ACO (c104037_g1)	A gene promoting ethylene biosynthesis (Adams and Yang 1979; Trivellini et al. 2011)	F:CGATGTCCCACCATTGAA R:CGGGTTGTAGAAAGAGGC	148
WRKY28 (c102065_g1)	A transcription factor negatively regulating ABA signal transduction (Bakshi and Oelmüller 2014; Wu et al. 2018)	F:TCTCCACTCGCTTCTTCA R:GTTCGCTTTCATGACCAA	152

GA gibberellic acid, *ABA* abscisic acid, *F* forward, *R* reverse, *bp*, base pairs

Total RNA was extracted from male/female buds/flowers in samples collected across different developmental stages using EZ-10 DNAaway RNAMini-Preps (Sangon Biotech, Shanghai, China), following the manufacturer's instructions. The first strand of cDNA was synthesized with a TRUEscript 1st Strand cDNA Synthesis Kit (Kemix). All RT-qPCR experiments described here were performed with the 2 × Sybr qPCR Mix (Kemix) using a CFX96™ Real-Time System (Bio-Rad, Hercules, CA, USA) following the manufacturers' instruction. The RT-qPCR expression analyses of genes related to sex differentiation (primers in Table 3) were performed in the following sequence: 2 min at 94 °C for denaturation, followed by 40 cycles at 94 °C for 20 s, 55–60 °C for 20 s, and 72 °C for 30 s. Three technical replicate analyses were performed on each sample, and *GAPDH* was used as the internal control to indicate that the amount of template in the PCR had been normalized. The relative changes in target gene transcripts were calculated as fold changes relative to a selected sample.

Measurement of the Physiological Index

All assays were performed with test kits purchased from the Nanjing Jiancheng Bioengineering Institute (Jiangsu, China); we followed the manufacturer's instructions in all cases. The soluble carbohydrate content was assayed using a plant soluble sugar content test kit. The sucrose content was assayed using a sucrose measurement kit. The proline content was assayed using a proline assay kit. The MDA content was assayed using a plant MDA assay kit. The SOD activity was assayed by the hydroxylamine method using a total superoxide dismutase (T-SOD) assay kit. The POD activity was assayed using a POD assay kit. The CAT activity was assayed colorimetrically (visible light) with a CAT assay kit.

C/N Measurement

After termination of the C/N treatments, we used female flowers harvested at stage 3.5 and stage 11 to determine C/N ratios. The crushed samples were dried to constant weight at 55 °C. We wrapped each 50 mg dried sample in foil and determined C/N ratios with an elemental analyzer (vario MACRO cube; Elementar, Langenselbold, Germany) in the CN mode. We averaged three separate replicates to calculate means ± SE.

Statistical Analyses

We used the $2^{-\Delta\Delta CT}$ method to calculate relative gene expression from the RT-qPCR data. All data from the analyses of phenotypes, RT-qPCR outputs, and physiological indices are summarized here as means ± SE. One-way ANOVAs, executed with the SPSS for Windows statistical software package (ver. 20.0; SPSS, Inc., Chicago, IL, USA) were used to identify significant differences among

the treatment and control groups. Significant differences among means ($P < 0.05$) were identified with Tukey's tests. Significantly different means are identified here by different lower case letters. Proportional differences between index values at stage 11 (on May 3, 2018) and stage 3.5 (on March 14, 2018) were calculated with the following expression: $(\text{value}_{\text{Stage 3.5}} - \text{value}_{\text{Stage 11}}) \times 100/\text{value}_{\text{Stage 3.5}}$. Proportional changes over time were either positive or negative, among which, positive values represent the decrease (%) of each physiological index at stage 11 compared with stage 3.5, while negative values represent the increase (%). Three replicate values were averaged to calculate means ± SE.

Results

Exogenous Plant Growth Regulator Injections Inhibited the Growth of Stamens in Androecious Persimmon Flowers

Stamen length, pollen tube length, and pollen tube viability were significantly different (ANOVA, $P < 0.05$) among injection treatments of ethrel, 5-azacytidine, the mixture of ABA, IAA, and ZT, and the control. Differences in pollen grain diameters among these treatments were highly significant (ANOVA, $P < 0.01$). Injections of ethrel significantly decreased the length of pollen tubes and diameter of pollen grains (Table 4).

All injections of (i) ethrel, (ii) the mixture of ZT, ABA, and IAA, and (iii) 5-azacytidine markedly increased the expression levels of the genes homologous to *MeGI*, *BAG5,* and *AifA* in male floral buds of androecious wild persimmon at stages 3.5, 6, and 7, compared to control (Fig. 3a, c, d). These three treatments also increased the expression levels of the gene homologous to *AMC9* at stages 3.5 and 7, compared to control (Fig. 3b). The treatments decreased the expression of the gene homologous to *WRKY 28* (Fig. 3e), but increased the expression of the gene homologous to *ACO* (Fig. 3g) at stage 7. The three treatments also increased the expression levels of the genes homologous to *IAA32* (Fig. 3f) and *GA20OX2* (Fig. 3h) at stages 3.5 and 6.

Figure 4 depicts the positive and negative effects of exogenous plant growth regulator injections on physiological indices of androecious persimmon buds and flowers collected at stages 3.5 and 11. The soluble carbohydrate content of the 5-azacytidine- and mixture-treated plants was lower at stage 11 than at stage 3.5, but this was not the case in the ethrel-treated and control plants (Fig. 4a). All three treatments decreased the content of proline and the activity of POD, but increased the content of MDA; these effects were reversed in untreated plants (Fig. 4b,c).

GA3 Injections Increased the Length of Stamens in Gynoecious Persimmon Flowers

Pistil lengths were significantly different (ANOVA, $P < 0.05$) among the following treatments: injections of 6 mg/L GA_3, 12 mg/L GA_3 and control. Treatment effects on stamen length were highly significant (ANOVA, $P < 0.01$). Injections of both concentrations of GA_3 significantly increased stamen length in comparison with the control (Table 5).

Injections of 6 and 12 mg/L GA_3 significantly increased the expression levels of the genes homologous to *MeGI*, *AMC9*, *BAG5*, *WRKY28*, *IAA32*, and *GA20OX2* in the female floral buds of gynoecious 'Jiro' trees from stage 3.5 to 11 (Fig. 5a–c, e, f, h). These injections also markedly increased the expression of the gene homologous to *AifA* (Fig. 5d) from stage 3.5 and 7, but strongly reduced the expression of the gene homologous to *ACO* (Fig. 5g) at stages 3.5, 7, and 11.

Injection of 6 mg/L GA_3 reduced the soluble carbohydrates content and increased the sucrose content between stages 3.5 and 11, but this effect was reversed by injection with 12 mg/L GA_3 (Fig. 6a). All GA_3 treatments decreased

Table 4 Effects of exogenous plant growth regulator injections on androecious persimmon flower traits

Treatment	Stamen length (mm)	Length of pollen tube (μm)	Diameter of pollen grain (μm)	Pollen viability (%)
5-Azacytidine	7.10 ± 1.03^{a}	171.15 ± 12.49^{a}	45.57 ± 4.35^{a}	49.87 ± 7.01^{a}
Mixture of ABA, IAA and ZT	7.37 ± 1.07^{a}	173.65 ± 10.39^{a}	45.10 ± 4.10^{ab}	45.59 ± 3.89^{ab}
Ethrel	7.15 ± 1.22^{a}	163.14 ± 8.44^{b}	43.93 ± 3.10^{b}	32.25 ± 2.18^{b}
Control	7.53 ± 0.83^{a}	175.56 ± 10.66^{a}	45.84 ± 4.62^{a}	34.70 ± 3.82^{ab}
P	*	*	**	*

Different lower case letters denote significant differences among means in columns (Tukey's test; $P < 0.05$). Values are means ± SE ($n = 3$)

ABA abscisic acid, *IAA* indole-3-acetic acid, *ZT* zeatin

*$P < 0.05$; **$P < 0.01$

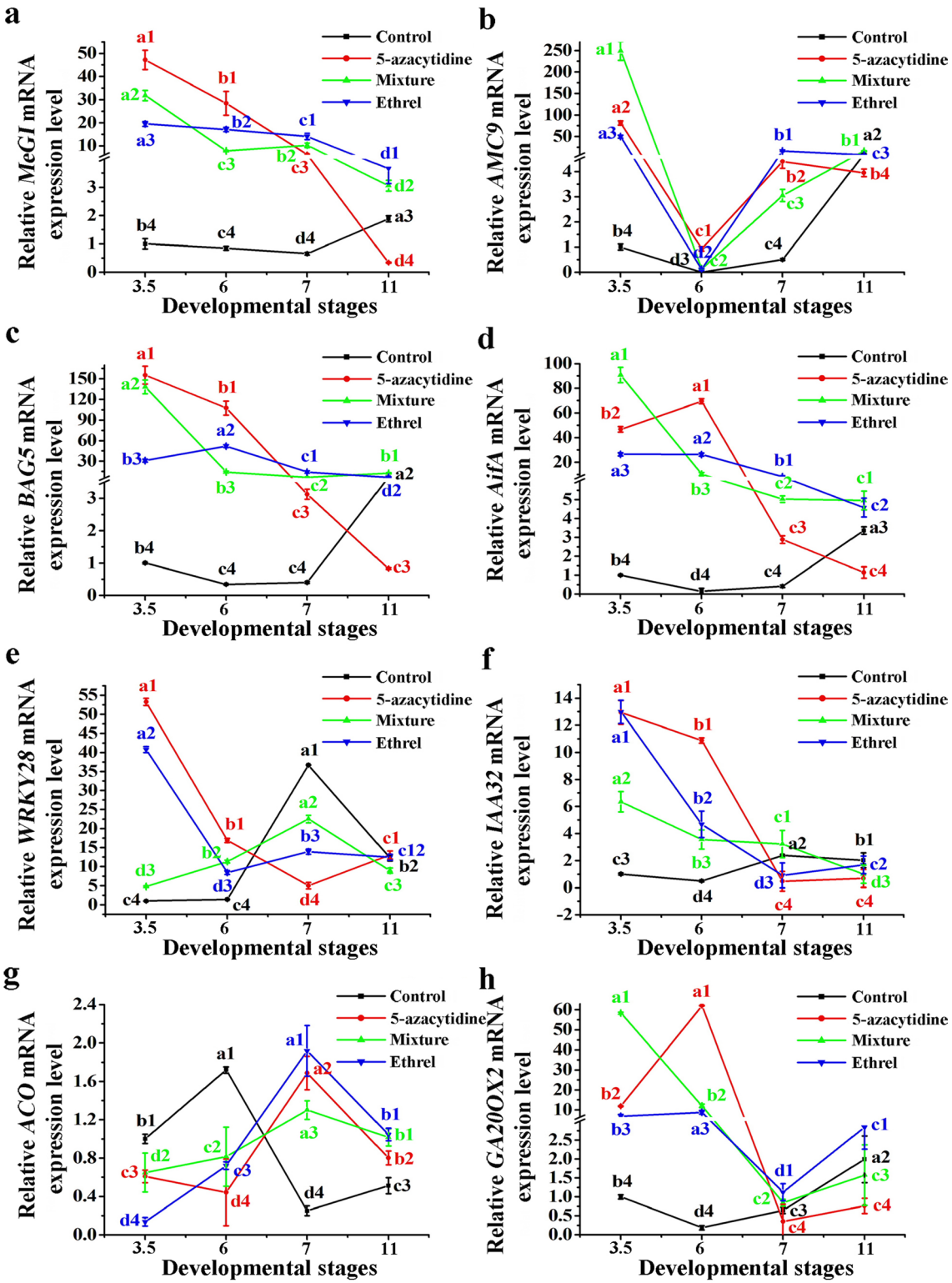

Fig. 3 Gene expression analysis of androecious flowers treated by feminizing regulators. **a**–**h**, relative expression levels of *MeGI*, *AMC9*, *BAG5*, *AifA*, *WRKY28*, *IAA32*, *ACO*, and *GA20OX2*, respectively. The value for control buds at stage 3.5 was set to 1.0. Different letters denote significant differences for specific treatments or the control among male floral bud developmental stages (Tukey's test, $P < 0.05$). Different numbers denote significant differences among treatments and controls within developmental stages (Tukey's test, $P < 0.05$). Values are means ± SE ($n = 3$). These criterions are also effective in Figs. 5, 7, and 9

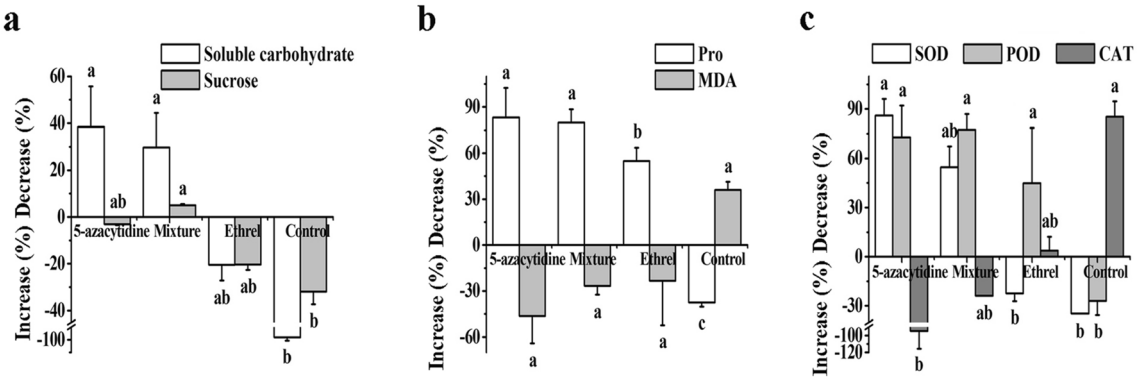

Fig. 4 Influence of feminizing regulators on assimilates accumulations (**a**), stress marker contents (**b**), and antioxidative enzyme activities (**c**). Different letters denote significant differences among treatments and control for the respective physiological indices (Tukey's test, $P<0.05$). Proportional differences between index values at stage 11 and stage 3.5 were calculated with the following expression: $(value_{Stage\ 3.5} - value_{Stage\ 11}) \times 100/value_{Stage\ 3.5}$. Proportional changes over time were either positive or negative, among which, positive values represent the decrease (%) of each physiological index at stage 11 compared with stage 3.5, while negative values represent the increase (%). Values are means ± SE ($n=3$). These criterions are also effective in Figs. 6, 8, and 10

Table 5 Effects of exogenous GA_3 injections on female flowers of gynoecious 'Jiro' trees

Treatment	Pistil length (mm)	Stamen length (mm)
GA_3, 6 mg/L	10.61 ± 1.46^a	4.80 ± 0.71^a
GA_3, 12 mg/L	10.95 ± 1.09^a	4.75 ± 0.80^a
Control	9.83 ± 0.51^a	4.37 ± 0.56^b
P	*	**

Different lower case letters denote significant differences among means in columns (Tukey's test; $P<0.05$). Values are means ± SE ($n=3$)

GA gibberellic acid

*$P<0.05$; **$P<0.01$

proline and MDA levels, which were clearly lower than that in controls (Fig. 6b), and considerably increased the activities of SOD and CAT compared to control (Fig. 6c).

High Soil Moisture Levels Increased the Length of Pistils in Gynoecious Persimmon Flowers

Differences in pistil length among treatments with volumetric soil water contents of 55%, 45%, 5% (plus control.) were highly significant ($P<0.01$), and differences in stamen length were also significant ($P<0.05$). The pistil lengths of female floral buds of 'Jiro' trees in treatments with soil moisture contents of 45% and 55% were significantly greater than in the 5% water content treatment and the control (Table 6).

We did not found remarkable change rule in the expression pattern of the eight genes in the female floral buds of 'Jiro' trees among different soil moisture treatments (Fig. 7).

Soil moisture treatments significantly decreased sucrose content compared to control (Fig. 8a). Reductions in the proline content between stages 3.5 and 11 in the 55%, 45%, and 5% soil moisture treatments were markedly larger than in the control (Fig. 8b). SOD activities were greatly downregulated in the treatment plants compared to control (Fig. 8c).

High C/N Ratios Increased the Length of Pistils and Stamens in Gynoecious Persimmon Flowers

The C/N ratios of female floral buds were highly significantly different ($P<0.01$) among treatments. The C/N ratio of floral buds in Treatment 1 was highest among treatments. The C/N ratio of floral buds in Treatment 4 was lowest among treatments. Thus, the C/N ratio of floral buds was strongly influenced by the combination of girdling and urea application. Differences ($P<0.01$) in pistil length and stamen length among treatments were highly significant. Treatments with high C/N ratios tended to have longer pistils and stamens than treatments with low C/N ratios (Table 7).

No remarkable change rule in the expression pattern of the eight genes in female floral buds of 'Jiro' trees among the different C/N treatments was detected (Fig. 9).

Treatment 4 markedly increased soluble saccharide content, and Treatment 5 greatly increased sucrose content between stages 3.5 and 11. Other treatments had no significant effects (Fig. 10a). The proline and MDA contents were not significantly different between C/N treatments and the control, but the proline content in plants in Treatments 4 and 5 was reduced by 76.29% and 68.43%, respectively (Fig. 10b). The CAT activity of plants in Treatments 3 and 4 markedly increased between stages 3.5 and 11. Differences among other treatments and the control were unremarkable (Fig. 10c).

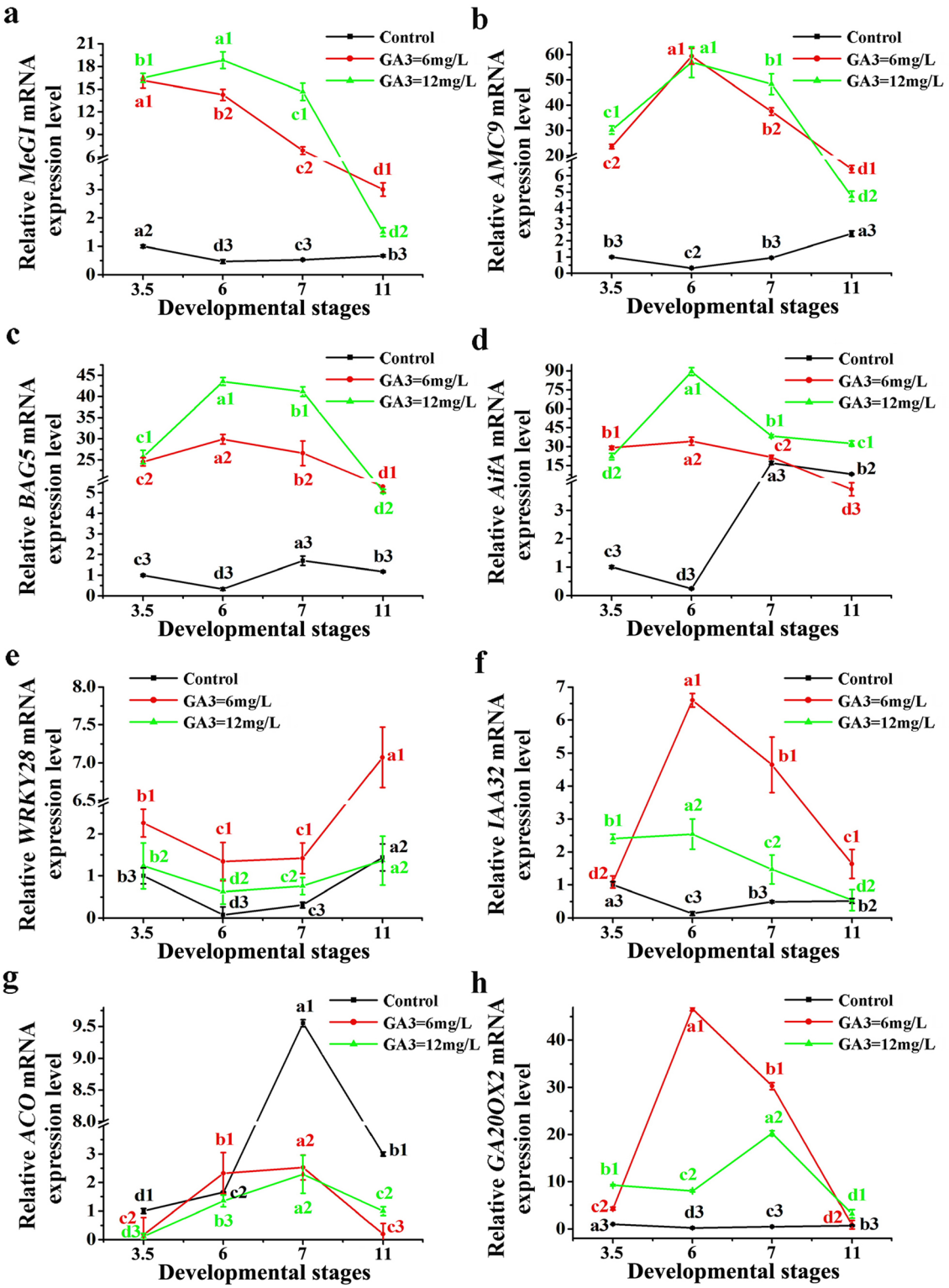

Fig. 5 Gene expression analysis of gynoecious flowers treated by exogenous GA_3. **a**–**h**, relative expression levels of *MeGI*, *AMC9*, *BAG5*, *AifA*, *WRKY28*, *IAA32*, *ACO*, and *GA20OX2*, respectively

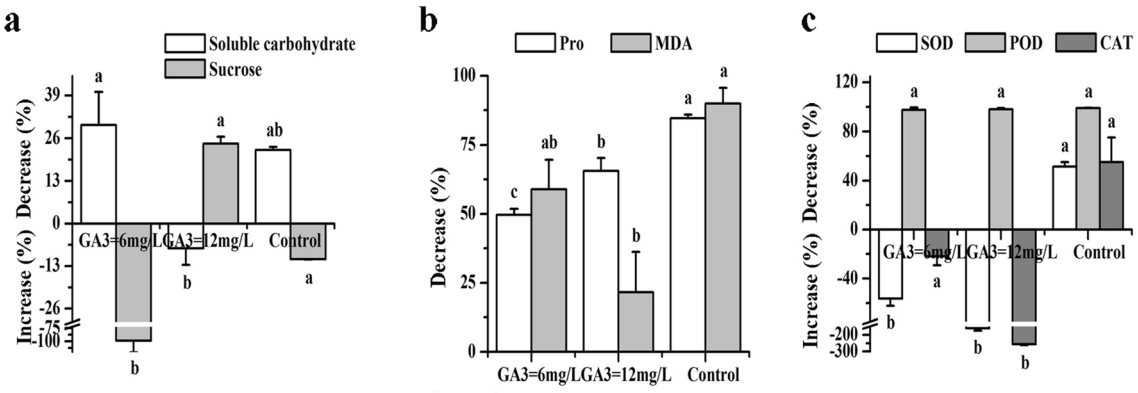

Fig. 6 Influence of exogenous GA_3 on assimilates accumulations (**a**), stress marker contents (**b**), and antioxidative enzyme activities (**c**)

Discussion

Numerous studies have investigated the effects of exogenous plant growth regulators on alternative or flexible sexual development by controlling growth regulator concentration, application time, or plant species (Golenberg and West 2013). Among key factors for success in controlling flower phenotypes is correct timing of treatments. A previous study of the persimmon cultivar 'Zenjimaru' by Li et al. (2016) showed that stage 2 (in mid-June) and stage 8 (in the following mid-April) were two crucial morphological time-points for floral sex differentiation. Thus, we injected exogenous plant growth regulators into tree stems from early June through the following early May. However, the effective control of soil moisture and C/N ratios of floral buds was time- and labor-consuming, and we consequently restricted soil moisture and C/N experimental treatments to the period from stage 3.5 to 11. This time frame spanned the period during which the stamen and pistil primordia of persimmon floral buds were initiated and developed to maturity (Li et al. 2016).

Table 6 Effect of soil moisture treatments on the flowers of gynoecious 'Jiro' trees

Volumetric soil water content	Pistil length (mm)	Stamen length (mm)
55%	10.61 ± 0.56^{a}	4.63 ± 0.63^{a}
45%	10.54 ± 0.81^{a}	4.44 ± 0.59^{a}
5%	9.49 ± 0.68^{b}	4.69 ± 0.49^{a}
Control	9.71 ± 0.80^{b}	4.41 ± 0.56^{a}
P	**	*

Different lower case letters denote significant differences among means in columns (Tukey's test; $P < 0.05$). Values are means ± SE ($n = 3$)

*$P < 0.05$; **$P < 0.01$

The abiotic stresses affect growth and development of plants by altering cell metabolism and expansion, stomatal changes, pigment contents, photosynthesis as well as respiration. Thus, the abiotic stresses have a partial or full influence on the shoot elongation, leaves growth, sexual changes of flowers, or even fruit growth and development, and crop yield (Adam et al. 2011; Yang et al. 2011; Gharache et al. 2013). In the present study, the suppression of stamen development in androecious persimmons through stem injections of exogenous plant growth regulators, especially ethrel, is likely best explained by the inhibition of cell enlargement and cell division, and reductions in the rates of metabolic processes involved in plant growth (Farooq et al. 2008). We also showed that the gene homologous to *MeGI*, a female growth promoter and male growth inhibitor gene in *Diospyros* (Akagi et al. 2016), was upregulated in male floral bud by injecting ethrel, 5-azacytidine, and a mixture of ZT, ABA, and IAA from stage 3.5 to 7, thereby promoting a feminizing effect.

We previously showed that endogenous ABA may promote the differentiation of female persimmon floral buds (Sun et al. 2017). Ethylene also has feminizing effects (Boualem et al. 2008; Martin et al. 2009). In cucumber, production of ethylene and its unimpeded signaling cascade is essential for the promotion of female floral development (Pawełkowicz et al. 2019a). In the present study, the expression level of the gene homologous to *WRKY28*, which negatively regulates ABA signal transduction (Bakshi and Oelmüller 2014; Wu et al. 2018), was decreased by injections of ethrel, 5-azacytidine, and a mixture of ABA, IAA, and ZT, at stage 7, prior to stage 8 which is the crucial morphological stage of floral sex differentiation. Meanwhile, the gene homologous to *ACO*, which promotes ethylene biosynthesis (Adams and Yang 1979; Trivellini et al. 2011), was increased after the same treatments at stage 7. Taken together, the injections likely promoted the differentiation of female floral buds.

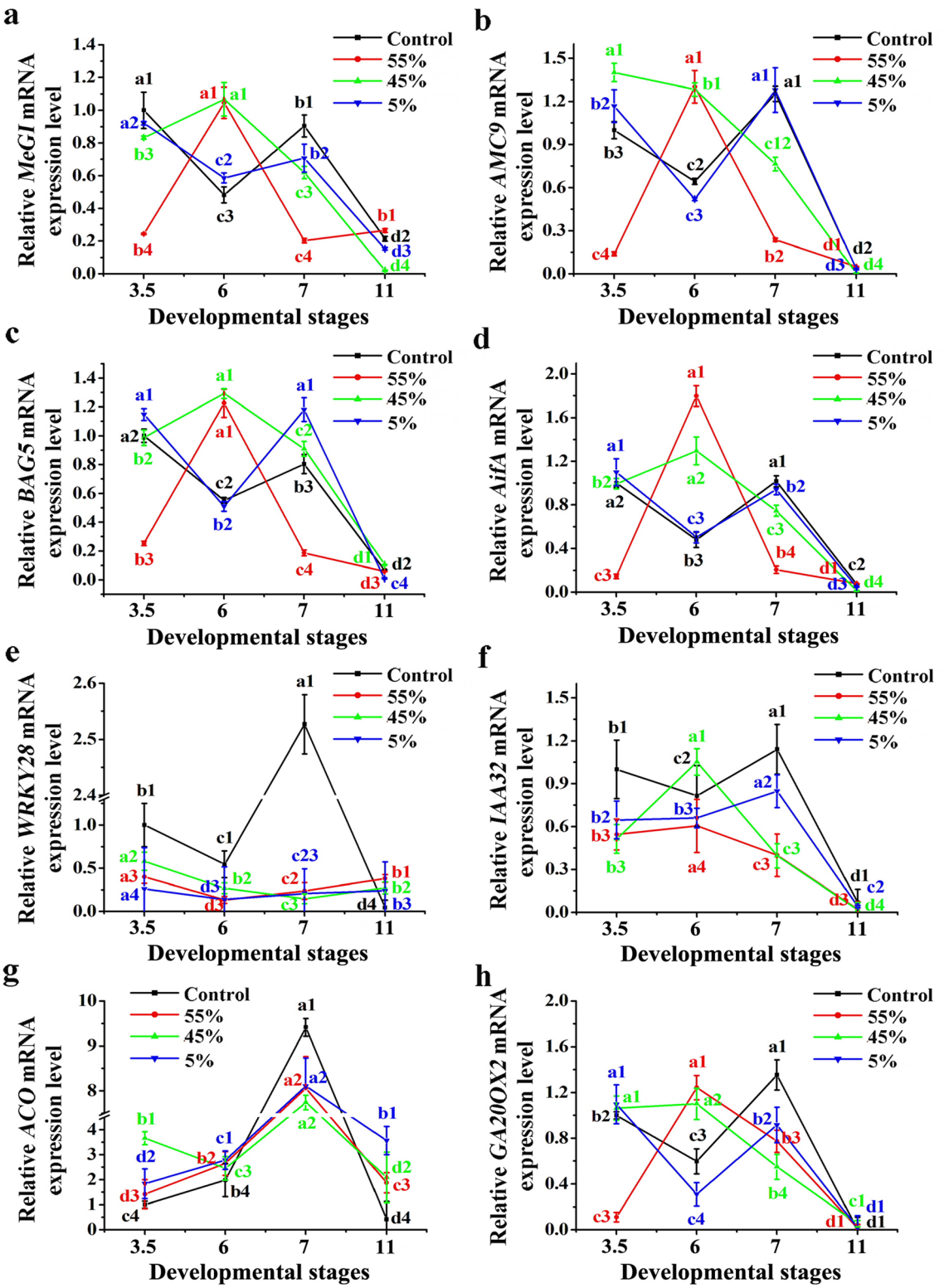

Fig. 7 Gene expression analysis of gynoecious flowers obtained from 'Jiro' trees grown in soils differing in moisture content. **a**–**h**, relative expression levels of *MeGI*, *AMC9*, *BAG5*, *AifA*, *WRKY28*, *IAA32*, *ACO*, and *GA20OX2*, respectively

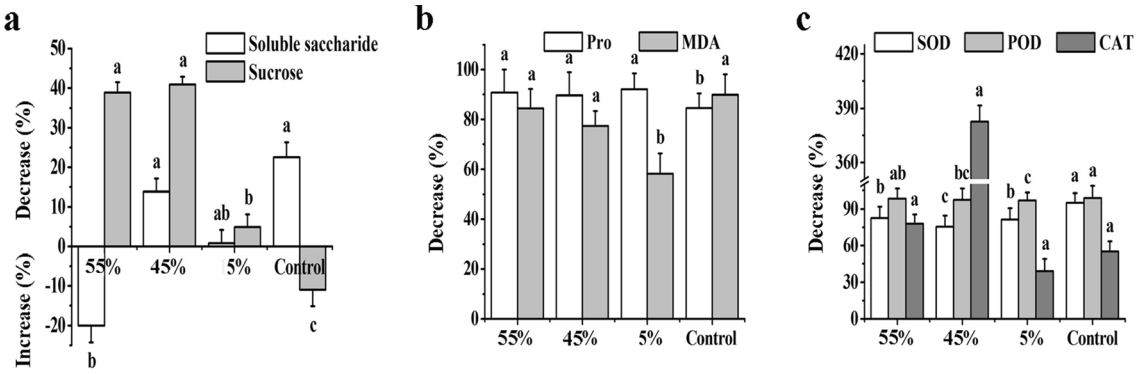

Fig. 8 Influence of different soil moistures on assimilates accumulations (**a**), stress marker contents (**b**), and antioxidative enzyme activities (**c**)

Table 7 Effect of C/N ratio treatments on the female floral buds of gynoecious 'Jiro' trees

Treatment				C/N ratio	Pistil length (mm)	Stamen length (mm)
Code	Urea		Girdling			
	Fertilization	Spray				
Treatment 1	None	None	Yes	29.69 ± 0.07^{a}	10.48 ± 0.81^{a}	4.57 ± 0.72^{ab}
Treatment 2	0.3 kg	0.125%	No	26.86 ± 0.02^{c}	10.16 ± 0.77^{ab}	4.74 ± 0.69^{a}
Treatment 3	0.3 kg	0.125%	Yes	26.97 ± 0.01^{c}	9.29 ± 0.78^{cd}	4.14 ± 0.62^{c}
Treatment 4	0.6 kg	0.25%	No	19.79 ± 0.05^{e}	9.12 ± 0.75^{d}	4.23 ± 0.58^{bc}
Treatment 5	0.6 kg	0.25%	Yes	24.02 ± 0.04^{d}	9.78 ± 0.57^{bc}	4.31 ± 0.64^{bc}
Control	None	None	No	28.39 ± 0.08^{b}	9.71 ± 0.80^{bcd}	4.41 ± 0.56^{abc}
P	–	–	–	**	**	**

Different lower case letters identify significant differences among means in columns (Tukey's test; $P < 0.05$). Values are means ± SE ($n = 3$)

*$P < 0.05$; **$P < 0.01$

Proline is an osmolyte, an antioxidant, and a universal osmoprotectant that is involved in plant recovery after responses to abiotic stresses via its function as a signaling molecule that triggers specific gene expression (Ali et al. 2011). We found that all injections into androecious persimmon were associated with decreases in proline, which may have a role in membrane protection (Santarius 1992). Hence, proline may enhance plant tolerance of exogenous plant growth regulators. The content of MDA, an indicator of membrane system injury associated with damages provoked by reactive oxygen species (ROS) due to abiotic stresses in plants (Hossain et al. 2013), increased in the male floral buds of treated androecious wild trees from stage 3.5 to stage 11, indicating that abiotic stress increased lipid peroxidation due to inadequate induction of the antioxidant system (Hossain et al. 2013). CAT and POD are both antioxidative enzymes that promote the removal of ROS and provide protection from oxidative damage caused by drought stress (Gharache et al. 2013). We detected marked decreases in POD activity in the male floral buds of androecious wild trees injected with ethrel, 5-azacytidine, or a mixture of ABA, IAA, and ZT, which may have been indicative of declined photosynthetic capacity during abiotic stress.

Our previous study showed that endogenous GA_3 may promote the differentiation of male persimmon floral buds (Sun et al. 2017). Glasziou (1969) found that GA promotes cell expansion by increasing the hydrolysis of starch and sucrose to form glucose and fructose. Interestingly, our stem injections of GA_3 increased stamen length in gynoecious persimmons, thereby providing a pivotal reference for artificially transforming female flowers into male (or hermaphrodite) flowers that produced active pollen and enhanced controlled pollination. GA_3 injections increased the expression of the gene homologous to *WRKY28*, and decreased the expression of the gene homologous to *ACO* from stage 3.5 to 11, which may explain the increases in stamen length following GA_3 injections. We also showed in our previous study that endogenous IAA may promote the differentiation of female persimmon floral buds (Sun et al. 2017). Interestingly, the gene homologous to *IAA32*, which negatively regulates auxin signaling (Reed 2001; Tian et al. 2002), was upregulated in the injected samples from stage 3.5 to 11. The gene homologous to *GA20OX2*, which is a key oxidase

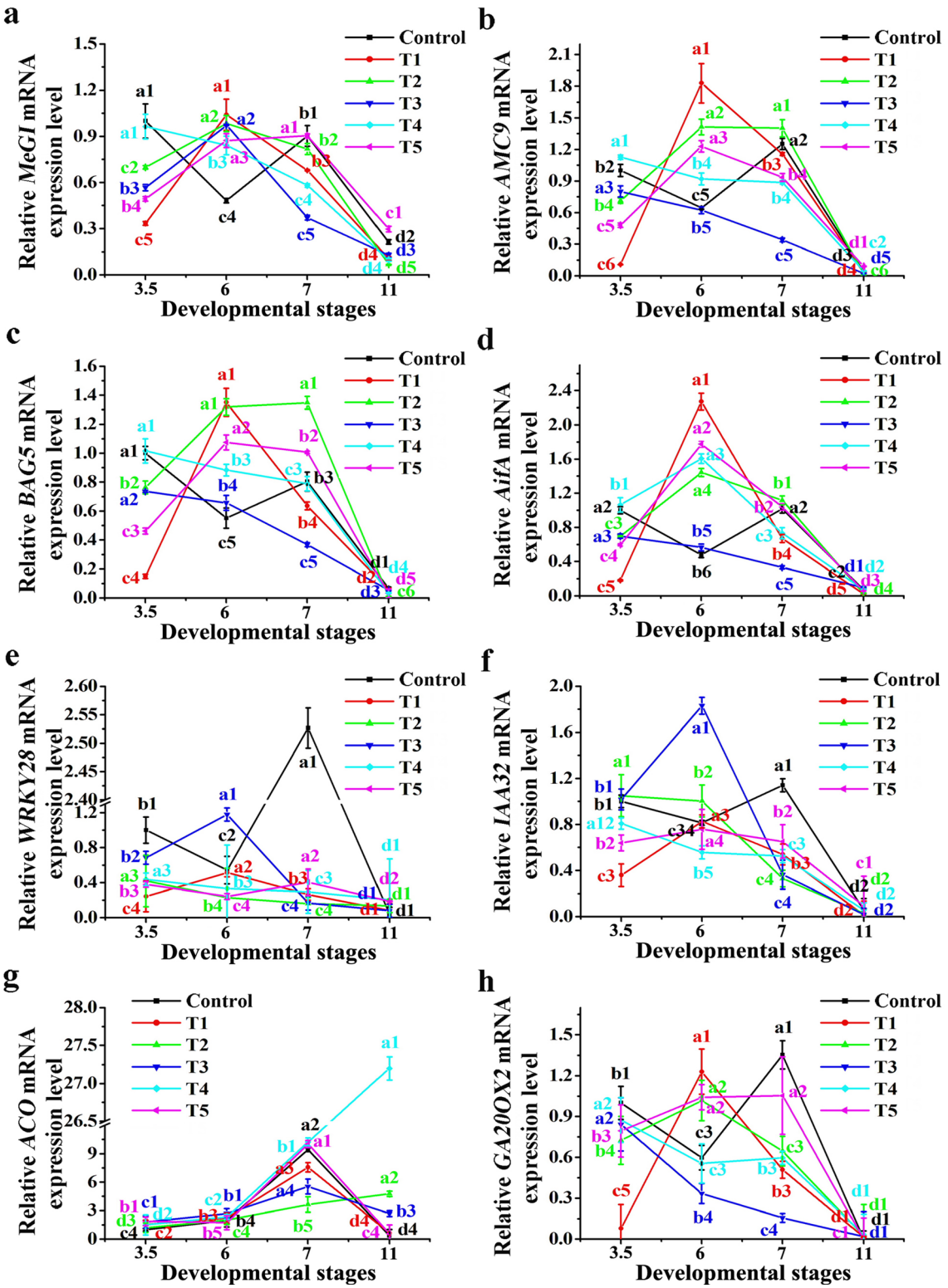

Fig. 9 Gene expression analysis of gynoecious flowers treated by different carbon/nitrogen (C/N) ratios. **a**–**h**, relative expression levels of *MeGI*, *AMC9*, *BAG5*, *AifA*, *WRKY28*, *IAA32*, *ACO*, and *GA20OX2*, respectively

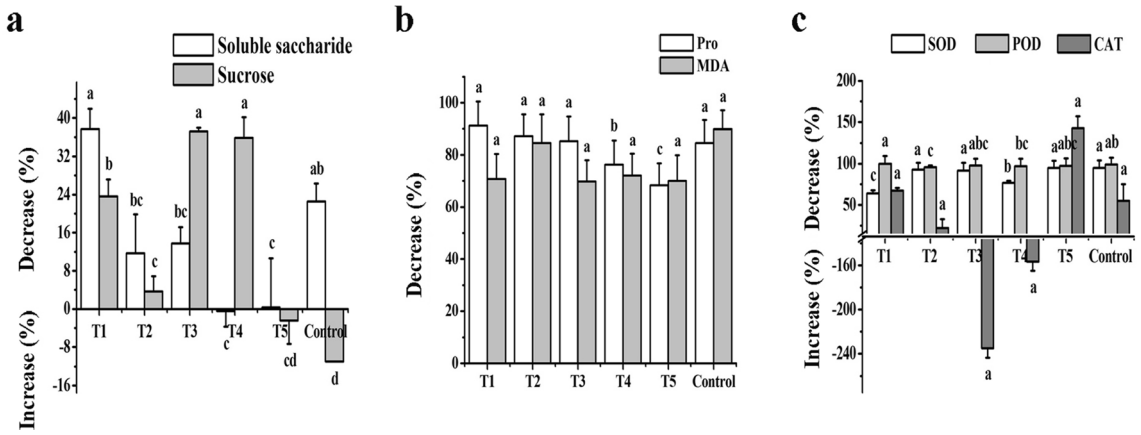

Fig. 10 Influence of different C/N ratios on assimilates accumulations (**a**), stress marker contents (**b**), and antioxidative enzyme activities (**c**)

gene in the biosynthesis of GA (Phillips et al. 1995), was also upregulated in the treated samples at the same stages. Thus, GA_3 had a masculinizing effect on persimmon floral buds. Superoxide is converted by the SOD enzyme into H_2O_2, which is transformed to O_2 and water by antioxidative enzymes, such as CAT, peroxidase (POX) and ascorbate peroxidase (APX) (Ozkur et al. 2009). The enhanced activities of SOD and CAT in female flowers following GA_3 treatments are congruent with the findings of Altuntas (2015), who showed that force feeding GA_3 to the larvae of *Galleria mellonella* resulted in a remarkable increase in the activities of both SOD and CAT.

Surprisingly, we also found that the gene homologous to the feminizing gene *MeGI* was upregulated in GA_3-treated samples of gynoecious persimmon trees from stage 3.5 to 11. The mechanism underlying this upregulation requires further investigation. Furthermore, the GA_3 injections increased the expression levels of genes homologous to *AMC9*, *BAG5*, and *AifA* at the same stages, similar to the effects of the injections of ethrel, 5-azacytidine, and the mixture of ABA, IAA, and ZT, indicating that the trees significantly responded to the injection of GA_3.

The expression of gender in some plants is occasionally sensitive to environmental factors (Aryal and Ming 2014). Nanami et al. (2004) reported that female flower production increased in *Acer rufinerve* trees subjected to drought stress. In our study, pistil lengths in the 55% and 45% soil moisture treatments exceeded that in the 5% treatment and in the control. Assimilates, including soluble carbohydrate, sucrose, starch and other carbohydrates, are important products of photosynthesis and act as energy substances for plant growth and development (Boogaard et al. 1997). Changes in carbohydrate content are especially important for reproductive growth because they are directly related to physiological processes, such as photosynthesis, translocation, and respiration (Dawood and Sadak 2014). Sucrose functions as an osmotic regulator during water uptake and seedling elongation by increasing cellular osmotic activity (Górnik and Lahuta 2017). Moreover, Miao et al. (2011) revealed that sugar metabolism was involved in the formation of cucumber female flowers in response to low temperature. We found that sucrose content decreased in the 55% and 45% soil moisture treatments from stage 3.5 to stage 11, but increased in the controls over the same time period. These results indicate that the increase of the pistil length in female flowers under higher soil moisture content may partially induced by the increase of sucrose consumption. Sánchez-Rodríguez et al. (2010) concluded that sugar metabolism in *Arabidopsis* stimulated cellulose and hemicellulose synthesis. Thus, in order to further uncover the effects of sugar metabolism on the development of female flowers, it may be valuable to determine the contents of cellulose and hemicellulose in persimmon female floral buds after soil moisture treatments in the near future.

Girdling treatment removes the bark and phloem around the stem and cuts deep into the youngest xylem. Girdling leads to the accumulation of assimilates above the level required to meet metabolic demands (Asao and Ryan 2015). Urea is an essential macroelement for plants. Urea application reportedly increases the nitrogen absorption capacity of wheat in northern China (Yang et al. 2011). We found that girdling alone, i.e., without urea application increased the plant C/N ratio to the highest among all treatments. The lowest C/N ratios were measured in plants that were sprayed or fertilized with the highest concentration of urea, but had not been girdled. Thus, the combination of girdling and urea application effectively controlled plant C/N ratios. Plants with high C/N ratios had longer pistils and stamens than plants with lower C/N ratios. Therefore, the C/N treatments influenced the development of persimmon floral buds.

Although the phenotypes of female flowers on 'Jiro' trees were changed by soil moisture and C/N treatments, we found

no associated pattern in the expression levels of eight genes, perhaps due to inappropriate timing and application of treatments. We therefore propose that, in future, the treatments can be applied to androecious and gynoecious *D. kaki* plants over the whole year from early June through the following early May to effectively control the sex expression of persimmon floral buds and enhance controlled pollination.

Abiotic stresses affect many biochemical and molecular processes, resulting in changes in gene expression, endogenous phytohormone contents, and physiological traits, thereby influencing plant growth and development (Lawlor and Cornic 2002; Hussain et al. 2010). It is important to note that endogenous substances regulating the process of reproduction may not operate alone; they directly or indirectly trigger a network of signaling pathways and crosstalk to regulate the life spans of plants (Khryanin 2002). Hence, a better understanding of the ways in which the intricate network linking the hormonal system, physiological indices, and genetic apparatus are intrinsically involved in sex differentiation in plants is crucial from both fundamental and applied points of view. However, building accurate networks is not easy, and there is almost no available such kind of network in plants. Notably, Pawełkowicz et al. (2019b) proposed a new model of regulatory mechanism network of sex development in cucumber for the first time by comparative transcriptome analysis, which is valuable for enhancing the genomic resources for cucumber as well as other economic plants and enabling the regulation of traits relevant to productivity and quality. In this study, we have provided valuable information and new directions for procedures to regulate sex differentiation in persimmon flowers. Further studies on the interaction and regulation of these internal and external mechanisms will advance understanding of the reproductive process.

Conclusions

All the persimmon trees responded significantly to injections of exogenous plant growth regulators, including ethrel, 5-azacytidine, and a mixture of ZT, ABA, and IAA, and GA_3. Compared to the untreated control, injections of ethrel significantly decreased the diameter of pollen grains and the length of pollen tubes. Injections of ethrel, 5-azacytidine, and the mixture of ZT, ABA, and IAA significantly increased the expression of the feminizing *MeGI* gene from stage 3.5 to 7, increased the expression of the gene homologous to *ACO* (a gene promoting ethylene biosynthesis), and decreased the expression of the gene homologous to *WRKY28* (a transcription factor negatively regulating ABA signal transduction) at stage 7; this highlights the feminizing effects of these plant growth regulators on persimmon flowers. In contrast, injections of GA_3 significantly increased the length of arrested stamens in gynoecious trees, increased the expression levels of genes homologous to *WRKY28*, *IAA32* (a gene that negatively regulates IAA signal transduction), and *GA20OX2* (a gene promoting GA biosynthesis), and decreased the expression of a gene homologous to *ACO* from stage 3.5 to 7, thus demonstrating the masculinizing effects of GA_3. High levels of soil moisture (55% and 45% volumetric moisture contents) significantly increased pistil length in gynoecious trees compared to the control (20% moisture content). High C/N ratios also significantly increased the length of pistils and arrested stamen growth in gynoecious trees. Although the sex types of persimmon flowers were not completely converted by these treatments, our experimental procedures significantly affected the development of pistils and stamens. Improved artificial treatments may be used in the future to control the sex types of persimmons.

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Author Contributions PS, JF, and FL conceived and designed the experiments; LW and HL carried out the experiments; LW, HL, and PS wrote the manuscript; YS, WH, SD, and YM helped to draft and review the manuscript. All authors read and approved the final manuscript.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflicts of interest.

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