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ORIGINAL PAPER

Drought resistance and DNA methylation of interspecific hybrids between *Fraxinus mandshurica* and *Fraxinus americana*

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Abstract

Key Message **The advantages of F1 hybrid progenies of *Fraxinus mandshurica* and *Fraxinus americana* were analyzed under drought conditions in the field and greenhouse.**

Abstract For cross-pollination trees, the optimal breeding method is hybridization. Tree heterosis is commonly present and is the main research focus in tree cross-breeding. In this study, *Fraxinus mandshurica* was used as female and *Fraxinus americana* was used as male parents to generate F1 hybrid generation. The adaptability and overwintering survival of F1 hybrids were evaluated under natural conditions. The advantages of F1 generation were analyzed under drought conditions in the field and greenhouse. The results showed that hybrid F1 generation (IH-10 and IH-15) had significantly higher growth and survival rate than the intraspecific progenies from either *F. mandshurica* or *F. americana* (MA-13 and FA-43, respectively) in the field. After 12 days of drought stress, growth, chlorophyll *a* and *b*, superoxide dismutase and peroxidase of IH-10 and IH-15 were higher than those of MA-13 and FA-43, suggesting an advantage of drought resistance for the hybrid progenies. Relationship of drought resistance and DNA methylation of interspecific hybrids was analyzed. Without drought treatment, methylation degree in the hybrid progenies (IH-10 and IH-15) was lower than that in the paternal or maternal progenies (MA-13 and FA-43). Drought stress enhanced the methylation rate in both hybrid and parental progenies, with higher increases in MA-13 and FA-43 than those in IH-10 and IH-15. Methylation in MA-13 and FA-43 mainly occurred in the full methylation of the internal cytosine, while in IH-10, methylation occurred in both full methylation of the internal cytosine and hemi-methylation of the external cytosine. These results showed that DNA methylation pattern was reconstructed in the hybrid progenies. Further analysis indicated that methylated DNA fragments were associated with growth and cell metabolism. Southern blot and transcriptional analysis showed that DNA methylation in *FmaHSP70*, *FmaCO*, *FmaWRKY2* and *FmaEF1A* altered their gene expression. We observed that alteration of DNA methylation is closely correlated with the adaptation to the drought stress and provided epigenetic mechanisms of drought resistance in the interspecific hybridization of trees.

Keywords *F. mandshurica* × *F. americana* · Interspecific hybrid · DNA methylation · Drought stress

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Introduction

Heterosis is a common and important biological phenomenon in which F1 hybrid progenies from two different populations confer growth, reproduction and adaptation

advantages compared to their parents (Birchler et al. 2010; Riedelsheimer et al. 2012). Tree heterosis is commonly present and is the main research focus in tree crossbreeding. For cross-pollination trees, the optimal breeding method is hybridization (Shepherd et al. 2008; Li et al. 1998). However, the mechanisms of heterosis have not been fully elucidated. Interspecific hybridization can alter the epigenetic status (Chen 2010; Ishikawa and Kinoshita 2009; Wang et al. 2005; Zhang et al. 2007a). Previous studies suggest that cytosine DNA methylation, an important epigenetic modification of eukaryotic chromatin, is critical for orchestrating gene expression during plant development (Rangwala and Richards 2004; Chan et al. 2005; Yan et al. 2010) and in maintaining genome integrity (Cao and Jacobsen 2002a; Rapp and Wendel 2005). Recent studies implicate cytosine DNA methylation in plant salt-stress responses (Boyko and Kovalchuk 2011; Mirouze and Paszkowski 2011; Karan et al. 2012; Bilichak et al. 2012; Song et al. 2012; Bräutigam et al. 2013).

Interspecific hybridization may lead to chromosomal rearrangements (Shivaprasad et al. 2011), transposable element mobilization (Liu and Wendel 2000; Shan et al. 2005) and DNA methylation changes (Salmon et al. 2005). DNA methylation is involved in gene regulation; thereby hypermethylation status is related to gene silencing while hypomethylation and demethylation are associated with gene expression. DNA methylation is mainly affected by the combined effects of three types of DNA methylases (maintaining methyltransferase enzyme MET1; chromomethylase CMT; de novo methyltransferase DRM) (Cokus et al. 2008). Alteration of DNA methylase expression affects DNA methylation degree and pattern in plants (Finnegan et al. 2000). Ni et al. (2009) showed that heterosis in *Arabidopsis* was closely correlated with the epigenetic status in the promoter region of circadian rhythm genes (*CCA* and *LHY*). Studies on sorghum (*Sorghum bicolor*), rice (*Oryza sativa*) and maize (*Zea mays*) also indicate that heterosis may be associated with alterations of DNA methylation in hybrid F1 generation (Michalak 2009; Shan et al. 2005; Wang et al. 2010). However, few studies are available on the epigenetic alterations during tree crossbreeding (Valledor et al. 2007).

Fraxinus mandshurica is distributed in the northwest and northeast of China, the Russian Far East, the northern part of the Korean Peninsula and northern Japan. It is one of the valuable hardwood species in the forest areas of northeast China and has been listed as endangered species. *Fraxinus mandshurica* has short taproot and well developed lateral and capillary roots with preferences for cold and wet climate, light and fertile soil. It can endure low temperature (−40 °C), but the high-growth period is short with 40–50 days in the Northeastern China (Hu et al. 2008). *F. americana* has a long life, strong drought resistance and is easy to breed. It is an excellent tree species for soil and water conservation. However, *F. americana* cannot live through winter in the high-latitude cold regions (Abrams and Mostoller 1995). We crossed *F. mandshurica* (female parent) with *F. americana* (male parent) to obtain interspecific F1 hybrid progenies that could obtain the good characters of parents.

Drought is a major environmental stress factor that affects the growth and development of plants. Drought tolerance has been associated with alteration of antioxidant metabolisms in various plant species (DaCosta and Huang 2007; Jiang and Huang 2001). Antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) are the major defense systems of any organism against ROS injury. Malondialdehyde (MDA) content is a commonly used measurement for assessing lipid peroxidation and oxidative damage in plants (Queiroz et al. 1998; Zhou and Zhao 2004), and its maintenance of low levels has been associated with increased drought stress resistance in many plant species (Lima et al. 2002; DaCosta and Huang 2007). Leaf chlorophyll fluorescence reflects the integrity of photosynthetic apparatus or photochemical efficiency of photosystem II system in the light reaction of photosynthesis (Xu et al. 2011). Other reports have shown that SOD activity and chlorophyll content can be used as an indirect selection criterion for screening drought-resistant durum wheat (*Triticum durum*) (Zaefyzadeh et al. 2009). Cell membrane stability plays a critical role in maintaining cell turgor and physiological functions, particularly during plant dehydration, and electrolyte leakage (EL) has been widely used to estimate cell membrane stability (Blum and Ebercon 1981; Xu et al. 2011). In-depth analysis of heterosis in response to drought stress is essential for the production of new drought-tolerant interspecific progenies of *F. mandshurica and F. americana*. The aim of the present paper was therefore to obtain further insights into the relationship of drought resistance and DNA methylation of interspecific hybrids between *Fraxinus mandshurica* and *Fraxinus Americana*. To this end, drought-stress responses of F1 hybrids of *F. mandshurica* × *F. Americana* were studied. We observed growth and survival rate, defense-related enzymes and physiological functions between F1 hybrids of *F. mandshurica* × *F. americana* and intraspecific progenies of parents under drought stress. Moreover, we investigated DNA methylation patterns using a methylation-sensitive amplified polymorphism (MSAP) analysis after drought stress. Gene expression of *FmaCMT*, *FmaMET* and *FmaDRM* and genes that showed DNA methylation patterns altered under drought stress also were evaluated.

Materials and methods

Ethics statement

No specific permits were required for the described field studies.

Plant materials

Male flowering branches of *F. americana* were collected in mid-April from Beijing botanical garden (Beijing City, 40.00°N, 116.22°E). The branches were cultivated in water, and their pollen was collected in test tubes stored at 4 °C. Hybridization was performed in May using *F. mandshurica* and *F. americana* as the female and male parents, respectively, in Dailing, Heilongjiang Province (47.03°N, 129.03°E). The male and female trees were 31 and 35 years old in a natural stand. The F1 hybrids of crossing combinations *F. mandshurica* 2 × *F. americana* 10 and *F. mandshurica* 2 × *F. americana* 15 were labeled as IH-10 and IH-15, respectively. The intraspecific offspring of *F. mandshurica* and *F. americana* were labeled as MA-13 and FA43, respectively, used as control. The interspecific hybrid progenies (F1 hybrids of *F. mandshurica* × *F. americana*) and the intraspecific open-pollinated offspring of *F. mandshurica* and *F. americana* were planted in the Maoershan experimental forest farm of the Northeast Forestry University (45.14°N, 127.55°E) in a randomized complete block design with three replicates. Every replicates had 30 plants. Each hybrid progeny include 90 plants. After 2 years, the tree height, ground diameter and survival rate were analyzed.

Drought stress treatment

Healthy 2-year-old seedlings were transplanted in a 10-L pot containing humus-sand mixture (3:1) in greenhouse with nature light on 10 May. The experiment was arranged in a randomized complete block design with three replicates. Every replicates had ten plants. Each hybrid progeny include 30 plants. Plants were watered to saturation once daily prior to the initiation of the water treatments. Plants were then randomly assigned to control group or drought stress treatment group. Drought stress treatment was started by withholding water. On 10th June, drought stress treatment group plants were watered to 30 % of the soil water-holding capacity (SWC) for the stressed plants and to 100 % SWC for the control plants (pots were weighed and watered daily) throughout the experiment (Lovisolo and Schubert 1998; Talame et al. 2007). Leaf samples (third leaf) were collected from both drought stress treated and control plants, at 1000 hours of day 12, which used for MDA, electrolyte leakage, chlorophyll content, SOD, POD in each group to assess the stress level. 12 days after starting the drought-stress treatment, plants were irrigated again. All drought stress treatment samples collected at 1000 hours of day 0, 3, 6, 9, 12 and 3 days later of rewatering were snap-frozen in liquid nitrogen and stored at -80 °C, which used to detect the alteration of DNA methylation.

Malondialdehyde (MDA), electrolyte leakage and chlorophyll measurement

Thiobarbituric acid (TBA)-reactive substance-MDA adducts were extracted as described previously (Meir et al. 1992) by homogenization of 0.5 g of tissue in 5 mL of solution containing 20 % trichloroacetic acid (TCA) and 1.5 mM EDTA. TBA reactive substance-MDA adducts was assayed via the TBA testing (Kosugi and Kikugawa 1985), according to modifications of Meir et al. (1992). One milliliter of 0.67 % TBA was added to 3 mL aliquot of the supernatant and the solution was incubated at 100 °C for 1 h. The solution was then cooled to room temperature and centrifuged (8,000 rpm) for 10 min. The volume of the resultant supernatant was increased to 10 mL with distilled water, and the absorbance was read at 532 nm (A532) to measure MDA. Three biological replicates were used for each assay. Electrolyte leakage (EL) was determined according the method of Lutts et al. (1996) and Maribel and Dionisio-Sese (1998). Leaf segments (5-mm) were measured for each treatment. Briefly, samples were washed with de-ionized water to remove surface adherent electrolytes, and then samples were placed in closed vials containing 10 mL de-ionized water and incubated at 25 °C on a rotary shaker for 24 h. The electrical conductivity of the solution (L_1) was subsequently determined. Samples were then autoclaved at 120 °C for 20 min and the final electrical conductivity (L_2) were determined after equilibration at 25 °C. The EL was defined as follows: EL (%) = (L_1/L_2) × 100. Chlorophyll (Chl) was extracted from samples of cut leaf segments (0.5 g) by boiling them for 30 min in 10 mL of 80 % (v/v) ethanol, as previously described (Philosoph-Hadas et al. 1991; Meir 1992). Chl was subsequently quantified by measuring A_{652} and A_{665} (mg/Chl per gram fresh weight) as described by Arnon (1949).

Enzyme assays

After being homogenized with liquid nitrogen, 0.3 g of leaves were suspended in 3 mL of ice-cold HEPES buffer (25 mM, pH 7.8) which contained 0.2 mM EDTA and 2 % PVP. The homogenate was centrifuged at 4 °C and 12,000*g* for 20 min, and the resulting supernatant was used

for determination of SOD (Ramiro et al. 2006). Activity of SOD was assayed by measuring its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) according to the method of Hwang et al. (1999). One unit of the enzyme activity was defined as the amount of enzyme required to result in a 50 % inhibition of the reduction rate of NBT under assay conditions. The enzyme extract was used for the AsA-POD (POD activity toward ascorbate) and G-POD (POD activity toward guaiacol) assays. AsA-POD activity was assayed according to the method of Amako et al. (1994). The reaction was started by adding H_2O_2. The G-POD assay mixture contained 0.1 M phosphate buffer (pH 6.1), 4 mM guaiacol as donor, 3 mM H_2O_2 as substrate and 1.0 mL crude enzyme extract. The total reaction volume was 3.0 mL. The rate of change in absorbance at 420 nm was measured, and the level of enzyme activity was expressed as the difference in absorbance (OD).

DNA isolation and MSAP analysis

Leaf samples were collected from the third or fourth compound leaf of the main branch per plant (5 individual pools) and stored in liquid nitrogen. Genomic DNA was isolated from fresh leaf material using the cetyl trimethyl ammonium bromide (CTAB) method with the modifications of Zeng et al. (2010). Methylation-sensitive AFLP (MSAP) analysis was used to detect the alteration of DNA methylation. MSAP analysis was conducted as reported previously (Reyna-Lopez et al. 1997; Xiong et al. 1999). The restriction enzymes *EcoR*I, *Hpa*II and *Msp*I were purchased from New England Biolabs Inc. Generally, the two isoschizomers *Hpa*II and *Msp*I are used as "frequent-cutter" enzymes for MSAP. Although *Hpa*II and *Msp*I both recognize the same tetranucleotide sequence (5′-CCGG-3′), they display different sensitivity to DNA methylation. *Hpa*II is inactive when either of the two cytosines is fully methylated, while it cleaves hemimethylated 5′-CCGG-3′ at a lower rate than the unmethylated sequence. *Msp*I cleaves 5′-C^{5m}CGG-3′, rather than 5′-^{5m}CCGG-3′ (Duan et al., 2009). Overall, one pair of pre-selective primers and 20 pairs of selective primers were used for amplifications (Supplementary Table 1). A silver-stained sequencing gel was used to resolve and visualize the amplification products. Only clear and reproducible bands that appeared in two independent polymerase chain reaction amplifications (starting from the digestion-ligation step, i.e., the first step of MSAP) were scored. The scored MSAP bands represent three major cytosine methylation states: (1) hemi-methylation of the external cytosine, which are bands present in *Hpa*II but absent in the corresponding *Msp*I-digest, such as pattern H/M = +/− ; (2) full methylation of the internal cytosine, which are bands absent in *Hpa*II but present in the corresponding *Msp*I-digest, such as pattern H/M = −/+; and (3) full methylation of the external cytosine or both cytosines, which are bands absent in both *Hpa*II- and *Msp*I-digest.

Recovery and sequencing of MSAP bands

Bands showing various patterns of methylation alteration were eluted from the silver-stained MSAP gels with the ploy gel DNA extraction kit (Omega, the USA State) following the manufacturer's instructions and re-amplified with the appropriate selective primer combinations according to Zhang et al. (2007b). Sizes of the PCR products were verified by agarose gel electrophoresis and then cloned into the AT cloning vector (Takara Biotech. Inc., Dalian, China). The cloned DNA segments were sequenced with vector primers by automated sequencing. The Advanced BlastN and BlastX programs at the NCBI website (http://www.ncbi.nlm.nih.gov/) were used for homology analysis of the cloned DNA sequences that gave quality-reads. Genes with altered DNA methylation were cloned for further southern blot analysis. 10 μg aliquots of genomic DNA were digested by either of the pair of methylation-sensitive isoschizomers, *Hpa*II or *Msp*I. Digested DNA was fractionated by running through 0.8 % agarose gels. Gels were denatured, neutralized and blotted onto Hybond-N^+ nylon membrane (Roche, the USA State) in 20× SSC transfer buffer, fixed by UV cross-linking. Cloned DNA segments (*FmaCO*, *FmaEF1A*, *FmaHSP70*, *FmaWRKY2*) representing different methylation patterns in the MSAP profile were selected as hybridization probes. Southern hybridizations were conducted with the DIG Labeling and Detection System (Roche) following the manufacturer's instructions (Zeng et al. 2010).

Gene expression analysis

Plant genomes encode three well-characterized classes of DNA methyltransferase (maintaining methyltransferase enzyme (MET) family, the chromomethylase (CMT) family, the domain-rearranged (DRM) family) (Pavlopoulou and Kossida 2007). To determine the effect of drought stress on DNA methylation of interspecific hybrids, expression pattern of methyltransferases *FmaMET*, *FmaDRM* and *FmaCMT* was analyzed. We also selected four genes (*FmaCO*, *FmaEF1A*, *FmaHSP70*, *FmaWRKY2*) obtained from DNA sequences with altered methylation to analyze the expression pattern. Gene expression was constructed using real-time RT-PCR (reverse transcription polymerase chain reaction) with specific primers (Table 1). Total RNA was extracted from leaves of plants using the

Table 1 Seleeted MSAP bands for transcriptional analysis by RT-PCR

MSAP bands	Primer sequence
FmaCO	F: 5′ AGACAACAGACGCTCTCAAC 3′
	R: 5′ ACTCGTAACATACGGCTAAA 3′
FmaHSP70	F: 5′ ACTAGCTCCTATTCCTCCCA 3′
	R: 5′ ATTTTTCCGCATCATTTATC 3′
FmaWRKY2	F: 5′ CGTATGAGGGAAAGCACACC 3′
	R: 5′ GCCTATCTATTAGAGCACCG 3′
FmaEF1A	F: 5′ ACCCACCTCTTGGTCGTTTT 3′
	R: 5′ TCACTTGGCACCCTTCTTTG 3′
FmaCMT	F: 5′ GGAATATGTCTCCATTGGCTACA 3′
	R: 5′ TCAACAACATTTTCCATCAACAC 3′
FmaDRM	F: 5′ TTTCTCTCTCTCATAAACCCACC 3′
	R: 5′ ACAAAATTGATACATCGCCTCGT 3′
FmaMET	F: 5′ GAAGCAATAAAGAGTCGGAGTCA 3′
	R: 5′ TCCATTTCAAAAAGTTCATAAAA 3′
FmaTU	F: 5′ AGGACGCTGCCAACAACTTT 3′
	R: 5′ TTGAGGGGAAGGGTAAATAGTG 3′
FmaACTIN	F: 5′ TTCATTGGAATGGAATCGTC 3′
	R: 5′ ATTTTCTCTCTGGTGGGGCA 3′

CTAB method with the modifications of Zeng et al. (2010). The extracted RNA was then treated with RNase-free DNaseI (Promega, the USA State) to remove residual DNA. Total RNA (0.5 μg) was reverse-transcribed to generate cDNA in a 20-μl volume using oligodeoxythymidine primers with a Prime-Script™ RT reagent Kit (TaKaRa) according to the instructions provided by the manufacturer. Real-time PCR analysis was performed using real-time SYBR® Green PCR MIX Kit (TaKaRa) according to the instructions provided by the manufacturer. Amplification was completed using the following cycling parameters: 94 °C for 30 s followed by 45 cycles of 94 °C for 12 s, 55 °C for 30 s, 72 °C for 45 s and 1 s at 78 °C for plate reading. The actin and TU genes were used as internal references to normalize the amount of total RNA present in each reaction. A melting curve was generated for each sample at the end of each run to serve as an assessment of purity for the amplified products. Three independent RT-PCR experiments (biological replicates) were carried out to assess the reproducibility of results.

Statistical analyses

Statistical analyses of data were performed using the SPSS statistical software package (SPSS version 19.0). Data were expressed as mean ± SE and were compared by ANOVA (significance $p \leq 0.05$). Enzyme assays and physiological parameters were all measured using three replicates.

Results

Effect of drought stress on the growth of F1 generation plants

To evaluate the adaptability and overwintering survival of F1 hybrids under natural conditions, F1 generation plants were grown in the forest field for 2 years. The results showed that growth and survival rate of IH-10 and IH-15 were significantly higher than those of MA-13 and FA-43. The stem of FA-43 dies in the winter and was regenerated from the root in the spring, suggesting that FA-43 cannot live through the winter (Fig. 1).

To study the effect of drought stress on the growth of F1 generation plants, healthy 2-year-old plants were grown in greenhouse with nature light in a randomized complete block design. The plants were treated with drought stress for 12 days followed by 3 days of rewatering. Stress response manifested as severe dehydration, wilting and burn-like withering occurred after 12 days of drought stress (Supplementary Fig. 1). After 12 days of drought stress, contents of chlorophyll *a* and *b*, SOD and POD in the hybrid F1 generation were significantly higher than those in the maternal or paternal intraspecific progenies. MDA and conductivity in hybrid progenies were significantly lower than those in the maternal or paternal intraspecific progenies (Fig. 2), suggesting a lower membrane lipid peroxidation in the hybrid progenies. Overall, under the drought stress, hybrid progenies of *F. mandshurica* × *F. americana* showed higher drought resistance than the maternal or paternal intraspecific progenies.

DNA methylation in hybrid progenies upon drought stress

Using 20 pairs of *EcoR*I + *Hpa*II/*Msp*I selective primer combinations to detect cytosine methylation at 5′-CCGG, 357–586, clear and reproducible bands were amplified by MSAP. Based on these MSAP patterns, various bands representing non-methylation, hemi-methylation of external cytosine and full methylation of internal cytosine were tabulated. Under the normal growth condition, DNA methylation degree (calculated by adding up the various patterns) was 20.12 and 24.61 % in MA-13 and FA-43, respectively. In contrast, the methylation degree in IH-10 and IH-15 was 15.48 and 16.81 %, respectively. Methylation degree was increased upon drought stress (from 15.48 to 19.45 % for IH-10, from 16.81 to 29.10 % for IH-15, from 20.12 to 31.91 % for MA-13 and from 24.61 to 32.70 % for FA-43) (Figs. 3, 4), suggesting that drought stress has dramatic effect on DNA methylation.

Methylation in MA-13 reached maximum (31.91 %) after 3 days of drought stress. Methylation in IH-15 and

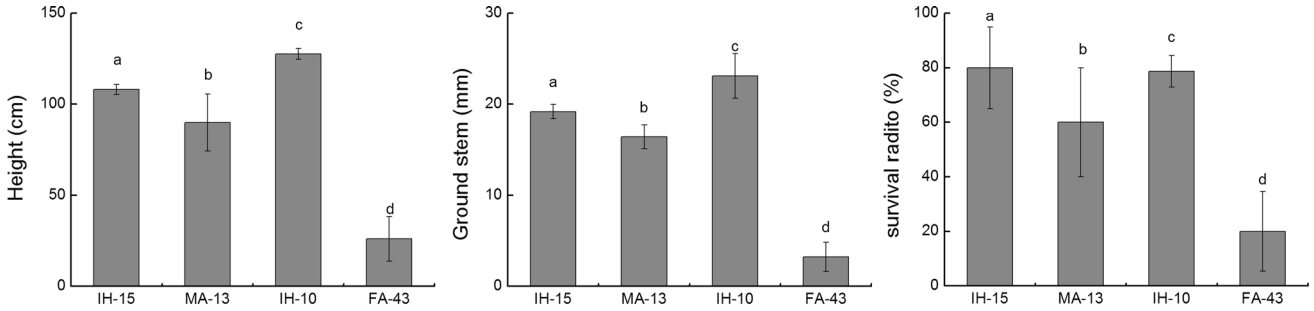

Fig. 1 Growth and survival rate of different F1 generation in the field. IH-10 and IH-15 are the interspecific hybrid progenies, MA-13 and FA-43 are the paternal or maternal progenies. Data are mean ± SE from three independent replicates. The *different normal letters* indicate significant difference among variance ration of F1 at 0.05 levels tested with least significant difference (LSD) and determined by one-way analysis of variance (ANOVA)

FA-43 reached maximum (29.10 and 32.70 %, respectively) after 9 days of drought stress. Methylation in IH-10 reached maximum (19.45 %) after 6 days of drought stress. Upon rewatering, DNA methylation was slightly decreased in all samples (11.48–22.07 %). In addition, interspecific hybridization and drought stress also altered the DNA methylation pattern. In the MA-13 and FA-43, genomic DNA methylation mainly occurred in the full methylation of the internal cytosine with an average of 13.52 ± 1.36 % and 14.10 ± 2.95 %. Semi-methylation occurred in 10.53 ± 4.64 % and 11.59 ± 4.57 % of hemi-methylation of the external cytosine in the MA-13 and FA-43. In contrast, in IH-10 and IH-15, DNA methylation occurred in 8.57 ± 1.16 % and 10.14 ± 3.61 % of full methylation of the internal cytosine. Meanwhile, 8.33 ± 0.79 % and 9.12 ± 4.15 % hemi-methylation of the external cytosine were found in IH-10 and IH-15, respectively (Table 2). These results indicated that interspecific hybridization altered DNA methylation degree and pattern.

Expression pattern of methyltransferases *FmaMET*, *FmaDRM* and *FmaCMT*

To verify the effect of drought stress on DNA methylation of interspecific hybrids, expression pattern of methyltransferases *FmaMET*, *FmaDRM* and *FmaCMT* was analyzed in maternal intraspecific progenies (MA-13) and interspecific hybrid progenies (IH-10). Under the normal growth condition, expression of *FmaMET*, *FmaDRM* and *FmaCMT* in maternal intraspecific progenies (MA-13) was significantly higher (1.3 to 1.9-folds) than that in interspecific hybrid progenies (IH-10) (Fig. 5). Drought stress increased the expression of *FmaMET*, *FmaDRM* and *FmaCMT*. The variation of methyltransferase genes in IH-10 was relatively small upon drought stress. For example, *FmaMET* expression in IH-10 was increased by 2.45 folds after 12 days of drought stress. Expression of *FmaMET* in MA-13 reached maximum after 9 days of drought stress (4.05 folds of the control). The expressions of *FmaDRM* and *FmaCMT* were similar with *FmaMET*. These results indicated that the drought had more dramatic effect on the expression of methyltransferase gene expression in MA-13 than that in IH-10.

Cloning and analysis of the DNA sequences with altered methylation

A total of 181 DNA fragments (ranging from 75 to 1,200 bp) with various types of methylations in the plants under drought stress were cloned and sequenced (Supplementary Table 2). BlastN analysis indicated that 37 fragments were homologous to the genes with known functions, while majority of these fragments had unknown functions. BlastX analysis showed that DNA fragment with altered methylation upon drought stress had significant homology to known-function cellular genes. Most of these genes are metabolism-related proteins, like those involved in amino acid metabolism related proteins, cytochrome oxidase, protein kinase, DNA-binding protein, ATP-binding protein, poly pyrimidine binding protein, precursors of pectinase, helicase and glucose-6-phosphate dehydrogenase. In addition, transmembrane transporter, ABA-inducible proteins, heat shock protein 70, DNA exonuclease enzyme, mitochondrial oxidoreductase, WRKY transcription factor, elongation factor, integrase and adenosine triphosphate enzymes are also identified. Other fragments (39.23 %) had no predictable functions and these fragments could be possibly non-coding regions. These results suggest that drought stress induced changes in the DNA methylation status of many functional genes and affected a variety of biological pathways to respond or adapt to the stress of the outside environment (Supplementary Table 2).

WRKY factors have been shown to be responsive to wounding, disease, abiotic stresses including drought, salt and cold, as well as senescence (Wang et al. 2009). Heat shock proteins (HSPs) respond to environmental abiotic

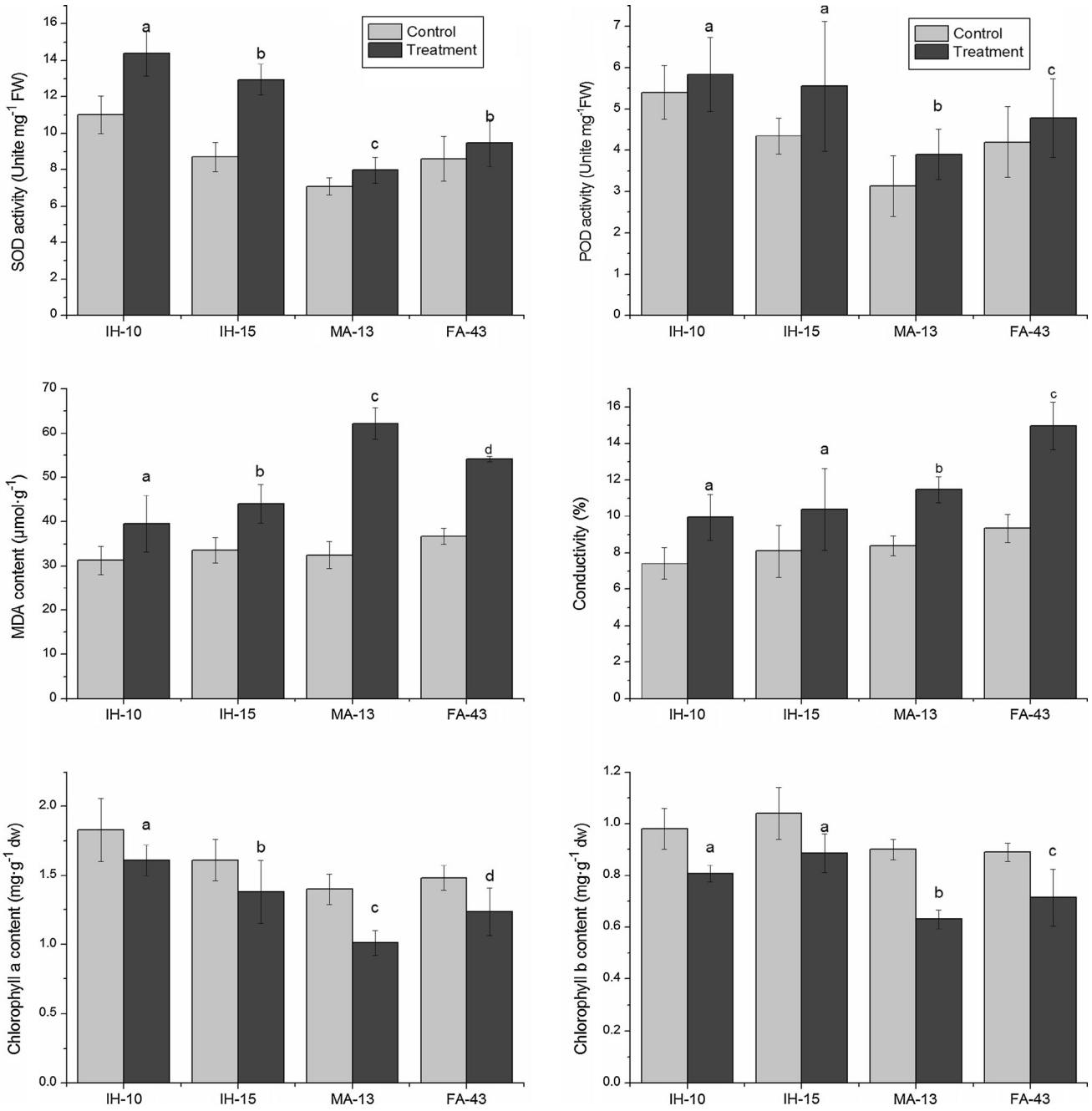

Fig. 2 Effect of drought stress on the growth and physiology of the F1. IH-10 and IH-15 are the interspecific hybrid progenies, MA-13 and FA-43 are the paternal or maternal progenies. Data are mean ± SE from three independent replicates. The *different normal letters* indicate significant difference among variance ration after drought stress of F1 at 0.05 levels tested with least significant difference (LSD) and determined by one-way analysis of variance (ANOVA). *DW* dry weight, *FW* fresh weight

stresses such as high-/low-temperature, drought and high salinity (Sung and Guy 2003; Cho and Hong 2004). Cytochrome oxidase plays a role in oxidative stress (Brand et al. 2004). Another interesting gene is eukaryotic elongation factor 1 alpha (eEF1A), which is an important component for protein biosynthesis (Andersen et al. 2003). In our study, these genes (*FmaCO*, *FmaEF1A*, *FmaHSP70*, *FmaWRKY2*) obtained from DNA sequences with altered methylation were selected to analyze the expression pattern. Variation of DNA methylation of four genes in the F1 generation and responses to drought stress analyzed by MSAP were shown in Table 3. We further used Southern blot to verify the methylation pattern. These genes were used as probes for Southern hybridization. The difference

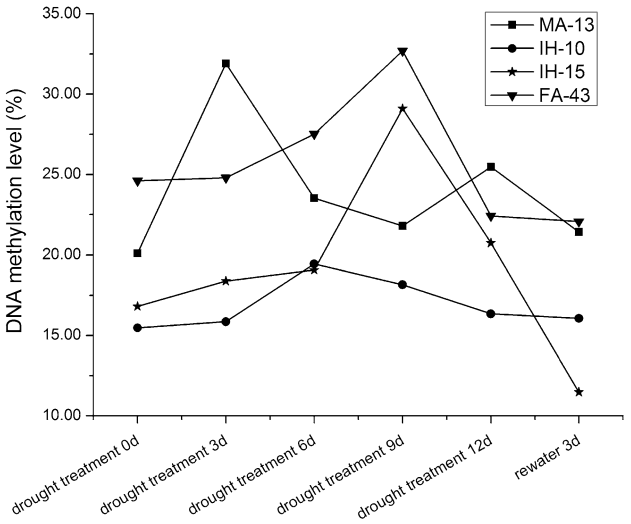

Fig. 3 Methylation of cytosine of CCGG sites in the leaves of different F1 generations. IH-10 and IH-15 are the interspecific hybrid progenies, MA-13 and FA-43 are the paternal or maternal progenies

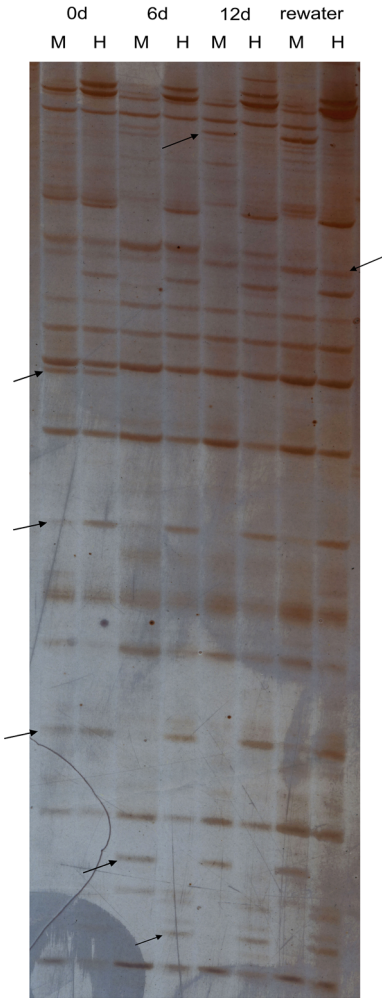

Fig. 4 MSAP amplification images of IH-10 with the primers of EA04/H + M + TAA (variation of DNA methylation of IH-10 after 0, 6, 12 days of drought stress and rewatering for 3 days. H represents the lane after digestion with *Hpa*II + *EcoR*I. M represents the lane after digestion with *Msp*I + *EcoR*I. *Arrows* indicate the bands with alterations during the drought stress)

of the detected fragments resulting from *Msp*I and *Hpa*II digestion represent different methylation patterns. The methylation patterns of four genes revealed by southern hybridization were consistent with MSAP (Fig. 6). Methylation degree of four genes in IH-10 and MA-13 altered significantly at different time points after drought stress. Among these four genes, cytochrome oxidase (*FmaCO*) and elongation factor 1-alpha (*FmaEF1A*) expression was significantly decreased upon drought stress, which is consistent with their methylation alteration. In contrast, expression of heat shock protein 70 (*FmaHSP70*) and WRKY2 (*FmaWRKY2*) was significantly increased upon drought stress, which was closely correlated with the degree and pattern of their methylation (Fig. 7). The results also suggested that the pattern of DNA methylation and expression of genes in interspecific hybrids were systematically different with their parents.

Discussion

DNA methylation variation in interspecific hybrid

Crossbreeding promotes the development of genetics and has always been the primary means to cultivate and create new varieties (Zhen et al. 2012). Because of the particularity of the forest tree breeding (e.g., dioecious, long cycle and geographic isolation), its crossbreeding also encountered a lot of difficulties (Libby et al. 1969). In this study, we collected pollen of *F. americana* and processed with high-voltage electrostatic field. After hybridization with *F. mandshurica*, we successfully obtained interspecific hybrid Fl. Excellent hybrid F1 generation IH-10 and IH15 were selected for comprehensive evaluation of drought resistance. The paternal species *F. americana* has not yet been successfully introduced in northern China (Heilongjiang Province). The stems withered after the winter and re-sprouted next spring. Thus, they cannot become timber. Drought resistance and growth advantage of the paternal species cannot be obtained directly through the plant introduction because its advantages can only be reflected in the indoor or within-year growth cycle. The plants with these advantages cannot live through the winter and thus do not have practical significance. Interspecies hybrid offsprings were able to live throughout the winter in the field for 2 years. Furthermore, hybrid F1 generation acquired the

Table 2 Methylation degree of cytosine of CCGG sites in the leaves of different plants

	MSAP total sites	Methylated sites CCGG	Methylated sites (%) CCGG	Hemi-methylation of the external sites	Hemi-methylation of the external sites (%)	Full methylation of the internal sites	Full methylation of the internal sites (%)
Drought stress for 0 day							
MA-13	512	103	20.12	35	6.84	68	13.28
IH-10	491	76	15.48	35	7.13	41	8.35
IH-15	452	76	16.81	48	10.62	28	6.19
FA-43	451	111	24.61	44	9.76	67	14.86
Drought stress for 3 days							
MA-13	470	150	31.91	85	18.09	65	13.83
IH-10	473	75	15.86	39	8.25	36	7.61
IH-15	397	73	18.39	29	7.30	44	11.08
FA-43	500	124	24.80	49	9.80	75	15.00
Drought stress for 6 days							
MA-13	544	128	23.53	59	10.85	69	12.68
IH-10	586	114	19.45	53	9.04	61	10.41
IH-15	430	82	19.07	47	10.93	35	8.14
FA-43	447	123	27.52	81	18.12	42	9.40
Drought stress for 9 days							
MA-13	532	116	21.80	47	8.83	69	12.97
IH-10	479	87	18.16	43	8.98	44	9.19
IH-15	457	133	29.10	72	15.75	61	13.35
FA-43	523	171	32.70	83	15.87	88	16.83
Drought stress for 12 days							
MA-13	424	108	25.47	56	13.21	52	12.26
IH-10	416	68	16.35	32	7.69	36	8.65
IH-15	424	88	20.75	24	5.66	64	15.09
FA-43	495	111	22.42	28	5.66	83	16.77
Rewatering for 3 days							
MA-13	448	96	21.43	24	5.36	72	16.07
IH-10	529	85	16.07	47	8.88	38	7.18
IH-15	357	41	11.48	16	4.48	25	7.00
FA-43	426	94	22.07	44	10.33	50	11.74

advantages of drought resistance. These studies achieved initial purposes of interspecific crossbreeding, while its afforestation and growth performance need to be further investigated.

DNA cytosine methylation is an ancient mechanism to promote the evolution of the genome (Suzuki and Bird 2008; Cao and Jacobsen 2002a). It plays an essential role in maintaining genome stability and regulating gene expression (Tariq and Paszkowski 2004; Rangwala and Richards 2004; Chan et al. 2005). DNA cytosine methylation is widely present in plants, especially in the genes containing the transposons (Law and Jacobsen 2010; Ishikawa and Kinoshita 2009; Kakutani 2002; Rabinowicz et al. 2005). Dynamic change and pattern reconstruction of DNA methylation have been observed in Arabidopsis (*Arabidopsis thaliana*), sorghum (*Sorghum bicolor*), rice (*Oryza sativa*) and maize (*Zea mays*). Sexual reproduction can result in relatively stable epigenetic changes including the degree and pattern (Zhang et al. 2007b; Kinoshita et al. 2004; Adams 2007). This phenomenon is particularly prevalent in the interspecific hybrids and allopolyploids (Guo et al. 2006). For example, Xiong et al. (1999) crossbred two rice species (Zhenshan 97 and Minghui 63) and obtained hybrid F1 generation (Shanyou 63). MSAP analysis achieved 1076 and 721 bands from the flag leaf and seedlings, respectively. Among these bands, there were 16 and 13 fragments with altered methylations. Overall, these studies indicated that DNA methylation-based epigenetic pattern can be altered in plants. Particularly, when different genomes were gathered in the nucleus of the hybrid progenies, DNA methylation pattern could be frequently changed. Methylation

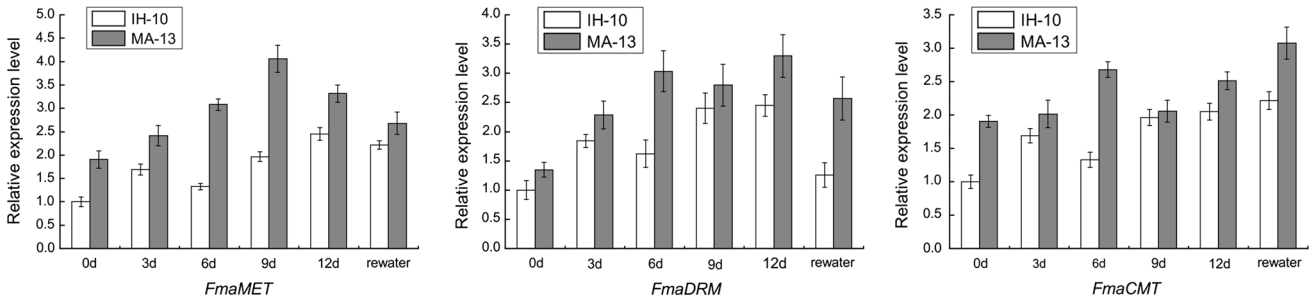

Fig. 5 Expression of methyltransferase (*FmaMET*, *FmaDRM* and *FmaCMT*) in IH-10 and MA-13 at different time points

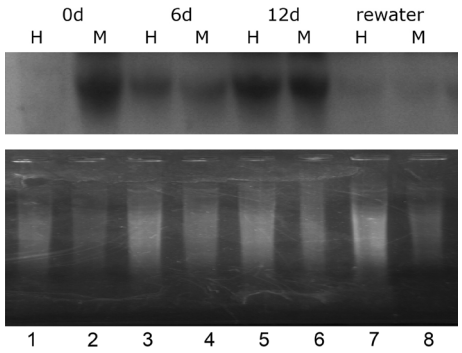

Fig. 6 Southern analysis of WRKY gene methylation in IH-10 after drought stress (*lanes 1*, *3*, *5*, *7* and *2*, *4*, *6*, *8* indicate digestion with *Hpa*II and *Msp*I and hybridization for the samples with 0, 6, 12 day of drought and 3 day of rewatering, respectively)

degree in the interspecific hybrid F1 generation of corn was 28.4 %, which was lower than that in the parental plants (Tsaftaris et al. 1999). In this study, we showed that DNA methylation degree was 20.12 and 24.61 % in MA-13 and FA-43, respectively. In contrast, the methylation degree in IH-10 and IH-15 was 15.48 and 16.81 %, respectively. DNA methylation degree in the hybrid F1 generation (IH-10 or IH-15) was lower than that in the maternal or paternal F1 generations (MA-13 or FA-43). Meanwhile, interspecific hybridization also altered the DNA methylation pattern. In the MA-13 and FA-43, genomic DNA methylation mainly occurred in the full methylation of the internal cytosine (13.28 and 14.86 %). Hemi-methylation of the external cytosine in the MA-13 and FA-43 was 7.13 and 10.26 %. In contrast, in IH-10 and IH-15, DNA methylation occurred in 8.35 and 6.19 % of full methylation of the internal cytosine. We speculated that the alteration of the methylation degree and pattern in the hybrid progenies changed the gene expression level, leading to the formation of drought resistance.

Drought stress change the methylation level and pattern

Our studies also showed that drought stress can increase the methylation degree and alter the methylation pattern in both hybrid and parental intraspecific progenies. This is probably due to the induction of DNA methylation modification and gene expression by drought stress during the process of plant growth (Ruiz et al. 2005; Portis et al. 2004). Some DNA fragments with altered methylation had predicted known functions, while other fragments did not have any homologies to genes with known functions. On the one hand, functional genomics in woody plant is still relatively limited. It is also possible that many sites with altered DNA methylation may belong to the gene regulatory regions. The cytoplasmic DNA of the progeny in sexual hybridization is mainly from the maternal chromosome, which results in the formation of a new nucleoplasm relationship and intracellular environment (Natcheva and Cronberg 2007). In the hybrids, DNA methylation patterns are largely adjusted to coordinate the expression of the cytoplasmic and nucleus genes and ensure the optimal status of both genetic systems (Grant-Downton and Dickinson 2005). Our studies also showed that alteration not only occurred in the methylation degree, but in the methylation pattern, which can be reflected by the appearance or disappearance of bands. The lowest frequency of alteration was conversion (+/−) or (−/+) of methylation sites in the full methylation of the internal and hemi-methylation of the external side of CCCGG sites.

Alterations in DNA methylation have been suggested to be involved in the process of adaptation to stress in plants (Kovalchuk et al. 2004; Urano et al. 2010). Genes methylated within the coding sequence display moderate expression levels and are less likely to have tissue-specific expression (Zilberman et al. 2007; Feng and Jacobsen 2011). Bilichak et al. (2012) also found a high degree of correlation between the levels of methylation and the level of mRNA in 12 genes (such as WRKY22, ROS1, etc.) in the progeny of salt-stressed plants. In our study, WRKY factors, cytochrome oxidase, HSPs and eukaryotic elongation factor 1 alpha (eEF1A), which have been shown to be responsive to abiotic stresses (Wang et al. 2009; Cho and Hong 2004; Brand et al. 2004), obtained from DNA sequences with altered methylation, were selected to

Table 3 Variation of DNA methylation in the F1 generation and responses to drought stress

Fragments	Gene	Materials	0 day		6 days		12 days		Rewater 3 days	
			H	M	H	M	H	M	H	M
tM25-3	*FmaCO*	IH-10	+	+	−	+	−	+	−	+
		MA-13	+	+	−	+	−	+	−	+
qM15-3	*FmaHSP70*	IH-10	−	+	+	+	+	+	+	+
		MA-13	−	−	+	+	+	+	+	+
fM6-2	*FmaEF1A*	IH-10	−	+	−	+	−	−	−	−
		MA-13	−	+	−	+	−	−	−	−
BM5-2	*FmaWRKY2*	IH-10	−	+	+	+	+	+	−	−
		MA-13	−	−	+	+	−	+	−	−

Primer sequences are presented in Table 1

+ positive control, − negative control, *H Hpa*II, *H*+ band present in *Hpa*II-digest, *M Msp*I, *M*+ band present in *Msp*I-digest, *H*− band absent in *Hpa*II-digest, *M*− band absent in *Msp*I-digest, *IH-10* the F1 hybrids of crossing combinations *F. mandshurica* 2 × *F. americana* 10, *MA-13* the intraspecific offspring of *F. mandshurica*

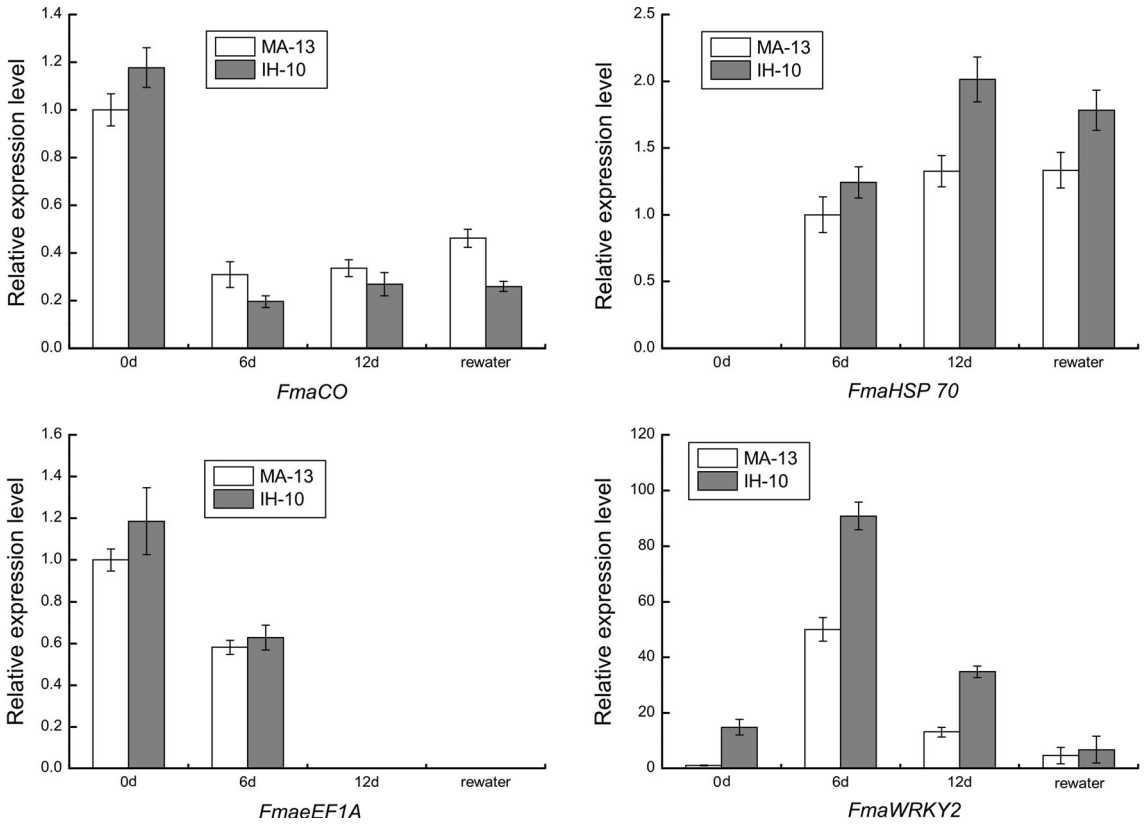

Fig. 7 Effect of drought on the expression of genes with altered methylation

analyze the expression pattern. Our results showed expression of these genes upon drought stress was closely correlated with the degree and pattern of their methylation (Fig. 7). The results also suggested that the pattern of DNA methylation and expression of genes in interspecific hybrids were systematically different with their parents.

Function of *FmaMET*, *FmaDRM* and *FmaCMT* was different under drought stress

DNA methylation is mainly affected by the combined effects of various types of DNA methylases (Cokus et al. 2008). Alteration of DNA methylase expression affects

DNA methylation degree and pattern in plants (Finnegan et al. 2000). Current studies indicate that there are three types of methyltransferase: (1) maintaining methyltransferase enzyme (MET1) with the primary function of maintaining the methylation of the CpG sites (Jackson et al. 2002; Xiao et al. 2003; Aufsatz et al. 2004), (2) chromomethylase CMT with the main function of maintaining methylation of CpNpG sites (N represents A, T, C or G) (Rival et al. 2008; Cao and Jacobsen 2002b) and (3) de novo methyltransferase (encoded by domains rearranged methyltransferase) with the main function of DNA repetitive sequence-directed and RNA-directed DNA methylation (RdDM) (Cao et al. 2000; Matzke et al. 2007). In this study, we showed that expression of *FmaMET*, *FmaDRM* and *FmaCMT* in IH-10 was significantly lower than that in MA-13. Overal, expression of *FmaMET* and *FmaDRM* in IH-10 and MA-13 was higher than that of *FmaCMT*. Drought stress had more dramatic effect on the expression of *FmaMET*, *FmaDRM* and *FmaCMT* in MA-13 than that in IH-10.

Conclusion

Our results showed interspecific hybrid F1 generation (IH-10) had significantly higher growth, survival rate and drought resistance than MA-13 and FA-43. Furthermore, interspecific hybrid F1 generation can live through the winter normally. DNA methylation degree and pattern in the interspecific hybrids was altered. Drought stress enhanced the expression of methyltransferase, but the effect on IH-10 was relatively small. Drought stress induced changes of DNA methylation of many functional genes and would affect a variety of biological pathways to respond or adapt to the stress of the outside environment. Expression of four genes was consistent with the DNA methylation variation, indicating that drought stress is closely correlated with DNA methylation pattern and degree. We observed that alteration of DNA methylation is closely correlated with the adaptation to the drought stress and provided epigenetic mechanisms of drought resistance in the interspecific hybridization of trees.

Author contributions Zhan YG, Zeng FS conceived and designed the experiments. Zeng FS, Zhou S, Dong J performed the experiments: Zeng FS analyzed the data. Zhan YG, Zeng FS contributed reagents/materials/analysis tools. Zeng FS, Zhan YG wrote the paper.

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

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