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Salt tolerance and alterations in cytosine methylation in the interspecific hybrids of *Fraxinus velutina* and *Fraxinus mandshurica*

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Abstract For cross-pollination trees, the optimal breeding method is hybridization. Tree heterosis is commonly present and is the main research focus in tree crossbreeding. Salt stress and interspecific hybridization may lead to DNA methylation changes. The study crossed *Fraxinus mandshurica* (female parent) with *Fraxinus velutina* (male parent) to obtain interspecific F1 hybrid progenies that could obtain the good characters of parents. The results showed that growth and survival rate of the interspecific hybrid progenies (F1 hybrids of *F. mandshurica* × *F. velutina*) were significantly higher than those of intraspecific open pollinated plants from parental *F. mandshurica* and *F. velutina*. Salt tolerance and cytosine methylation in interspecific F1 hybrids and the intraspecific open pollinated plants from parents were examined. Membrane permeability, ROS and antioxidant activity, malondialdehyde, and photosynthesis were measured after salt treatment and genomic methylation was analyzed using a methylation-sensitive amplified polymorphism protocol. F1 hybrids exhibited heterosis for growth in normal as well as high salt conditions. DNA methylation in the F1 hybrids was lower than the intraspecific open pollinated plants from parents. Salt treatments changed DNA methylation patterns in F1 hybrids. Genomic DNA of the intraspecific open pollinated plants from parents had internal cytosine methylation (average of 13.22 %), whereas F1 hybrid seedlings had external cytosine methylation (average of 7.34 %). Such changes in DNA methylation patterns in F1 hybrids suggest a connection between salt tolerance and epigenetic mechanisms in plants. We observed that alteration of DNA methylation was closely correlated with the adaptation to the salt stress and provided epigenetic mechanisms of salt tolerance in the interspecific hybridization of trees.

Keywords *Fraxinus mandshurica* · *Fraxinus velutina* · Interspecific hybrids · Saline stress · Heterosis · DNA methylation

Abbreviations

APX	Ascorbate peroxidase
CAT	Catalase
DHAR	Dehydroascorbate reductase
GR	Glutathione reductase
GSH	Reduced glutathione
mC	Methylcytosine

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MDAR	Monodehydroascorbate reductase
POD	Peroxidises
ROS	Reactive oxygen species
SE	Standard error
SOD	Superoxide dismutase
Pn	Net photosynthetic rate
Gs	Stomatal conductance
Ci	Intercellular CO_2 concentration
Tr	Transpiration rate
MSAP	Methylation-sensitive amplified polymorphism

Introduction

Plants respond to severe environmental changes or stresses, such as high salt, low temperature, or drought, via physiological and developmental changes. Salt stress affects plant metabolism by reducing water potential, perturbing ion balance, reducing CO_2 assimilation, and production of reactive oxygen species (ROS) (Bohnert and Jensen 1996; Hasegawa et al. 2000; Zhu 2001, 2002, Munns and Tester 2008). Plants have developed various protective strategies to minimize saline stress, such antioxidant systems to quench ROS (Apel and Hirt 2004; Kočová et al. 2009). For example, superoxide dismutase (SOD) is a major scavenger of superoxide (O_2^-), producing H_2O_2 and O_2. Hydrogen peroxide is then scavenged by catalase (CAT) and various peroxidises (PODs) (Misra and Gupta 2006). Enzymes involved in the ascorbate–glutathione (GSH) cycle include ascorbate peroxidase, dehydroascorbate reductase, monodehydroascorbate reductase, and GSH reductase also contribute to stress response (Noctor and Foyer 1998; Hernández et al. 2000; Farmer and Mueller 2013).

Fraxinus mandshurica (Manchurian ash) is a dioecious, wind-pollinated, cold-adapted, and broad-leaved tree species that is susceptible to drought and saline stress (Hu et al. 2008). Even though it comprises one of the most important components of cool-temperate forest ecosystems in Northeast China, *F. mandshurica* has been declared an endangered tree species in China due to its extensive overexploitation and widespread deforestation (Hu et al. 2008). *Fraxinus velutina* is a species native to southwestern North America, and it is widely distributed in coastal areas because of its rapid growth rate and tolerance to alkaline soils (Griffin and Critchfield 1972; Liu et al. 2003). *Fraxinus velutina* does not adapt well to cold weather in Northeast China, such as in the Heilongjiang Province. Thus, we crossed *F. mandshurica* (female parent) with *F. velutina* (male parent) to obtain interspecific F1 hybrid progenies that could obtain the good characters of parents. Heterosis refers to the phenomenon in which the hybrid F1 offspring exhibit phenotypic characteristics that are superior to the mean of the two parents (mid-parent heterosis), or the better of the two parents (better parent heterosis) (Springer and Robert 2007). Heterosis for various defense-related enzymes and physiological changes depends on the susceptibility or tolerance of a plant to saline stress (Noctor and Foyer 1998; Hernández et al. 2000). Hybridization between cultivated and wild lettuce (*Lactuca serriola*) resulted in a moderate to high heritability for the vigour traits and many of the hybrids showed improved vigour over the wild parent under non-stress and stress conditions (Hooftman et al. 2007, 2009). King et al. (1997) reported the crosses between letraploid wheat, *Triticum durum*, and *Th. bessurabicum* and demonstrated that they are more tolerant to high salt concentrations than their wheat parents. Heterosis was apparent under saline (NaCl) conditions in the elongation of stems in hybrids of *Lycopersicon esculentum* produced with three wild species (*L. cheesmanii*, *L. peruvianum*, *and L. pennellii* = *Solanum pennellii*) by Tal and Shannon (1983). Total dry matter production of another F_1 hybrid, between *L. esculentum* and *L. pennellii*, showed hybrid vigour (SarangaY et al. 1991) under saline conditions. 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. In-depth analysis of heterosis in response to saline stress is essential for the production of new salt-tolerant interspecific progenies of *F. mandshurica and F. velutina*.

Previous studies suggest that cytosine DNA methylation 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 2002; 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). The objective of this study was to obtain further insights into the relationship of salt tolerance and DNA methylation of interspecific hybrids between *F. mandshurica* and *F. velutina*. The alterations of different parameters (growth and survival rate, photosynthetic parameters, defense-related enzymes, and physiological functions) of *Fraxinus* hybrids grown under optimal and salt stress conditions were analyzed. Moreover, we investigated cytosine methylation patterns using a methylation-sensitive amplified polymorphism (MSAP) analysis in F1 hybrids and parents after salt treatment. We conclud that a connection exists between salt tolerance and altered epigenetic mechanisms in plants. Studies of salt tolerance and DNA methylation variation of interspecific hybrids at salt stress may provide information on epigenetic mechanisms of salt tolerance in the interspecific hybridization of trees. Such information also could be useful to breeders in developing appropriate selection and breeding strategies.

Materials and methods

Plant materials

Male flowering branches of *F. velutina* were collected in mid-April in Tianjin City (China). The branches were cultivated in water, and their pollen was collected in test tubes and stored at 4 °C. Hybridization was performed in May using *F. mandshurica* (Dailing, Heilongjiang Province) and *F. velutina* as the female and male parents, respectively. Four individuals of *F. mandshurica* were used and labelled as follows: *F. m* 2, *F. m* 5, *F. m* 7 and *F. m* 8; four individuals of *F. velutina* were used and labelled as follows: *F. v* 3, *F. v* 6, *F. v* 9 and *F. v* 10. We made six combinations for crossing: *F. m* 2 × *F. v* 3; *F. m* 5 × *F. v* 3; *F. m* 5 × *F. v* 9; *F. m* 7 × *F. v* 3; *F. m* 7 × *F. v* 10; *F. m* 8 × *F. v* 6. Prior to pollination, *F. velutina* pollen was treated with a high-voltage electrostatic field (HVEF) for 30 min. The interspecific hybrid progenies and the intraspecific open pollinated plants from parental *F. mandshurica* and *F. velutina* were planted in the Maoershan experimental forest farm of the Northeast Forestry University under natural conditions (45.14°N, 127.55°E) in a randomized complete block design. Each region had 30 plants with 5 replicates. After 2 years, the tree height, ground diameter and survival rate were analyzed.

Salt treatment

Healthy 2 year-old plants were grown in the plastic buckets containing humus-sand mixture (3:1) in greenhouse with nature light. The seedlings were randomly divided into control and treatment groups. Treatment groups received 200 mmol/L salt and three replicates were taken from each group. Seedlings were administered salt solution twice a day, then irrigated daily with water. Physiological parameters of each plant were measured on the tenth day after treatment. Seedling height was measured before and after treatment; survival percentages and symptoms of stress were recorded everyday after treatment. Stress symptoms included the time and degree of leaf wilting, withering, and shedding. The same plants were given water without salt for 3 d after 10 d of salt treatment.

Photosynthesis

Net photosynthetic rate (Pn), stomatal conductance (Gs), intercellular CO_2 concentration (Ci), and transpiration rate (Tr) were measured with a LI-6400XT portable photosynthesis system (Li-COR, USA) using the following measuring cell (6 cm^2) parameters: 26 °C, 800 μmol photons m^{-2} s^{-1}, and an air flow of 300 μl s^{-1}, as previously described (Mittler et al. 2001; Pnueli et al. 2003).

MDA, Electrolyte leakage and ROS steady-state measurement

Thiobarbituric acid (TBA)-reactive substance-MDA adducts were was 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 temp and centrifuged (11, 000×*g*) 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 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 measurement 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 (L1) was subsequently determined. Samples were then autoclaved at 120 °C for 20 min and the final electrical conductivity (L2) was determined after equilibration at 25 °C. The EL was defined as follows: EL (%) = (L1/L2) × 100. Total indirect ROS was measured twice using four replicates as described by Causevic et al. (2006) using a Bioxytech H_2O_2-560 assay kit (TEBU-BIO, Le Perray en Yvelines, France) according to the manufacturer's instructions.

Enzyme assays

Enzyme extracts were prepared by homogenizing leaves in pre-chilled 50 mM sodium phosphate buffer (pH 7.0) containing 1.0 mM (EDTA), 0.5 % (v/v) Triton X-100 and 1 % (w/v) polyvinyl-pyrrolidone (PVPP). Ascorbate (5 mM) was added to the extraction buffer for determination of ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR). Homogenates were centrifuged at 18,000×*g* for 20 min at 4 °C, and their total soluble protein content was measured by Bradford's assay (Bradford 1976). Supernatants were immediately used for standard enzyme assays and three replicates were measured for each enzyme assay. DHAR activity was measured according to the protocol by Doulis et al. (1997). The assay mixture contained 90 mM phosphate buffer (pH 7.0), 0.1 mM EDTA, 5.0 mM reduced GSH, and approximately 30 µg extracted protein. The reaction was initiated with the addition of freshly made 0.2 mM dehydroascorbate (DAsA). Activity was measured by the reduction of DAsA at 265 nm after accounting for the non-enzymatic reduction of DAsA by GSH. MDHAR activity was assayed in a reaction mixture consisting of 90 mM potassium phosphate (pH 7.5), 0.01 mM EDTA, 0.0125 % Triton X-100, 2.5 mM ascorbate, 0.25 units ascorbate oxidase (units as defined by Sigma Chemical Co.), 0.2 mM NADH, and up to 50 µg protein extract (Hossain et al. 1984). The reaction was monitored by measuring the decrease in absorbance at 340 nm due to NADH oxidation. GR activity was determined according to the procedure by Foyer and Halliwell (Foyer and Halliwell 1976). The reaction mixture contained 80 mM Tris-HCl (pH 8.5), 1.5 mM EDTA, 2.5 mM oxidized GSH (GSSG), and up to 100 µg extracted protein. The reaction was initiated with the addition of 0.5 mM NADPH in 1 % $NaHCO_3$ and then oxidation of NADPH was read at 340 nm. APX was measured using methods described by Nakano and Asada (1987). Briefly, the assay mixture containing 90 mM potassium phosphate (KP) buffer (pH 7.0), 0.1 mM EDTA, 0.65 mM ascorbate, and 1.0 mM H_2O_2 was initiated with the addition of approximately 40 µg extracted protein. Activity was measured via H_2O_2-dependent decomposition of ascorbate at 290 nm. CAT activity was assayed in a reaction mixture containing 100 mM KP buffer (pH 6.5), 1.0 mM EDTA, 60.0 mM H_2O_2, and approximately 40 µg extracted protein according to the method described by Aebi (1983). Activity was measured by following the decomposition of H_2O_2 at 240 nm. Gamma glutamyl transferase (indicator of GSH) was measured according to Smith (1985). Briefly, 1 g (fresh weight) of leaves were homogenized in 5 ml 5 % sulfosalicylic acid; homogenates were centrifuged at 1000×*g* for 10 min and the supernatant was neutralized with 1.5 ml 0.5 M potassium phosphate buffer (pH 7.5) to assay total GSH. The standard incubation mixture contained: 0.5 ml 0.1 M sodium phosphate buffer (pH 7.5) containing 5 mM EDTA, 0.2 ml 6 mm 5, 5′-dithiobis-(2-nitrobenzoic acid), 0.1 ml of 2 mM NADPH, and 0.1 ml (1 unit) of yeast GSH reductase type III (Sigma Chemical Co.). The reaction was initiated by the addition of 0.1 ml GSH standard (50–400 ng) or extract. The change in absorbance at 412 nm (A_{412}) was monitored for 650 s at 25 °C or until A_{412} reached 0.5.

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).

Nucleic acid isolation and MSAP analysis

Genomic DNA was isolated from fresh leaves using the cetyl trimethyl ammonium bromide (CTAB) method described by Porebski et al. laboratory (1997). 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 (location). Overall, one pair of pre-selective primers and 20 pairs of selective primers were used for amplifications (Supplementary Table 1). Silver-stained sequencing PAGE was used to resolve and visualize amplification products. *Hpa*II and *Msp*I recognize the same sequence (CCGG) but have different sensitivities to DNA methylation. *Hpa*II cleaves sequences with hemi-methylated external cytosines (mCCGG), whereas, *Msp*I can only cleave sequences that are fully methylated at internal cytosines (CmCGG) (Ashikawa 2001). Only clear and reproducible bands that appeared in two independent PCR amplifications were scored. The scored MSAP bands represented three major cytosine methylation states: (1) hemi-methylation of the external cytosine, which were bands present in *Hpa*II-digest but absent in the corresponding *Msp*I-digest, such as pattern H/M = ±; (2) full methylation of the internal cytosine, which were bands absent in *Hpa*II-digest but present in the corresponding *Msp*I-digest, such as pattern H/M = ∓; and (3) non-methylation of both cytosines, which were bands absent in both *Hpa*II- and *Msp*I-digest, such as pattern H/M = −/−.

Overall evaluation of salt resistance and statistical analysis

Fuzzy subordination method was adopted to evaluate salt resistance and measure greater hybrid vigor. The subordinate function adopts the method of membership functions in fuzzy mathematics for different combinations to determine their average index subordinate function values and identify their resistance. If a positive correlation exists between the index and resistance, then the formula is $X(u) = (X - X_{min})/(X_{max} - X_{min})$. If a negative correlation exists between the index and resistance, then the formula is $X(u) = 1 - (X - X_{min})/(X_{max} - X_{min})$. In the two formulas, X is the index determination value, X_{max} is the maximum index determination value, and X_{min} is the minimum index determination value.

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

Adaptability and growth of F1 hybrids in field under natural conditions

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 the interspecific hybrid progenies (F1 hybrids of *F. mandshurica* × *F. velutina*) were significantly higher than those of intraspecific open pollinated plants from parents. *F. velutina* died in the winter and was regenerated from the root in the spring, suggesting that *F. velutina* cannot live through the winter (Fig. 1). These findings suggest that interspecific hybrid progenies (F1 hybrids of *F. mandshurica* × *F. velutina*) exhibit heterosis for growth and survival rate.

Effect of salt stress on seedling growth of F1 hybrids and intraspecific open pollinated plants from parents

To study the effect of salt 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 salt stress. Seedling growth was greatly affected

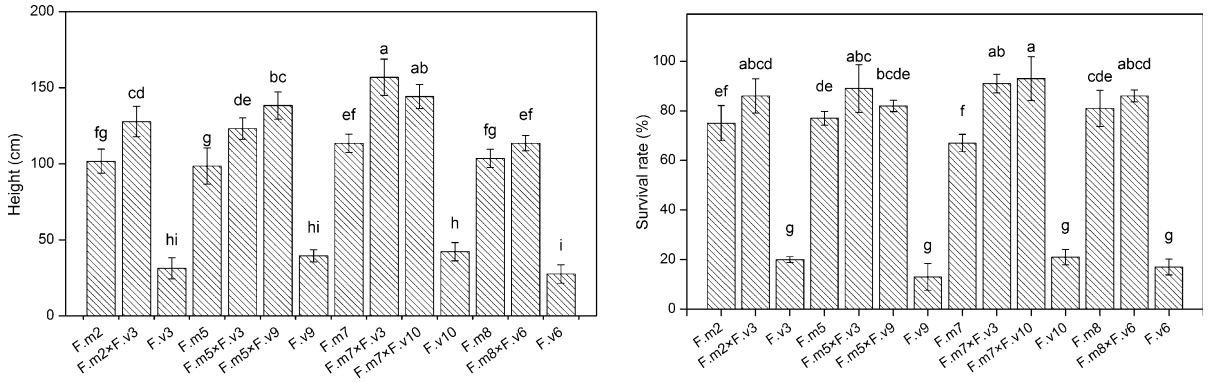

Fig. 1 Growth and survival rate of different F1 generation in the field *F. m* 2, *F. m* 5, *F. m* 8, *F. v* 3, *F. v* 10, *F. v* 6 represented the intraspecific open pollinated plants from parental *F. mandshurica* 2, *F. mandshurica* 5, *F. mandshurica* 8, *F. velutina* 3, *F. velutina* 10, *F. velutina* 6, respectively. Data are mean ± SE from three independent replicates. The different normal letters indicate significant difference among variance ration after salt stress of F1 at 0.05 levels as determined by one-way ANOVA

by stress, and this may reflect plant adaptation to their environments. Two days after treatment with 200 mmol/L salt solution, F1 hybrid seedling leaves gradually curled, wilted, and discolored (yellow), and salt crystals appeared on the leaf surfaces. Five days after treatment, the top leaves of all F1 hybrids gradually recovered their normal color; however, the bottom leaves of *F. m* 2, *F. m* 5, *F. m* 7 and *F. m* 8 had brownish patches and were shedding. Ten days after treatment, the top leaves of *F. m* 8 × *F. v* 6 and the intraspecific open pollinated plants from parents had brownish patches, none of the other F1 hybrids had these characteristics. The relative growth of F1 hybrids and the intraspecific open pollinated plants from parents under no salt stress (controls) and 200 mmol/L salinity is shown in Table 1. Except for *F. m* 8 × *F. v* 6, the relative growth of the other F1 hybrids was greater than growth of the the intraspecific open pollinated plants from parents when seedlings were not salt treated. *F. m* 7 × *F. v* 10 had the highest relative growth (164.83 %), followed by *F. m* 2 × *F. v* 3 (150.49 %), *F. m* 8 × *F. v* 6 had the lowest relative growth (95.17 %). Ten days after salt treatment, F1 hybrid growth decreased compared with untreated controls. Specifically, *F. m* 8 × *F. v* 6 growth was only 29.95 % of that of the untreated control. With the exception of *F. m* 8 × *F. v* 6 and *F. m* 5 × *F. v* 3, the relative growth of all F1 hybrids was higher than those of their respective parents, whether they were treated with salt or not, indicating increased F1 hybrid vigor compared to their parents.

Effect of salt stress on cell membrane permeability, MDA content, and steady-state ROS in seedlings

Relative conductivity, MDA content, and steady-state ROS are important physiological indices for examining injured/stressed cells as they reflect the variety and degree of cell membrane injury (Puckette et al. 2007). Conductivity measurements of cell membrane revealed that different F1 hybrids had significant differences in permeability in response to salt stress (Fig. 2). Compared with their untreated controls, cell membrane permeability increased in all hybrid F1 progeny. Cell membrane permeability of *F. m* 7 × *F. v* 10 increased only 4.17 % compared with its untreated control. Similarly, salt stress did not significantly affect membrane permeability of *F. m* 5 × *F. v* 9, indicating that *F. m* 7 × *F. v* 10 and *F. m* 5 × *F. v* 9 had greater hybrid vigor with respect to resisting cell injury after salt stress. The cell membrane permeability of *F. m* 5 × *F. v* 3 increased 304.15 % compared with its untreated control. MDA content and ROS followed similar trends, with differences between treatment and control groups in the same combination being significantly different (Fig. 2).

Salt treatment increases antioxidant activity

APX, GR, DHAR, and MDHAR are essential antioxidants involved in the ascorbate-GSH pathway.

Table 1 Relative growth and growth velocity of seedlings under salt stress

Hybrid combination	Non-treatment (control) for 10 d		Salt treatment for 10 d	
	A (mm)	B (%)	C (mm)	−D (%)
F. m 2	9.17 ± 0.21	–	3.57 ± 0.19	38.93
F. m 2 × *F. v* 3	13.8 ± 0.36	150.49	7.54 ± 0.43	54.64
F. v 3	11.71 ± 0.47	–	5.71 ± 0.32	48.76
F. m 5	9.00 ± 0.14	–	3.00 ± 0.22	33.33
F. m 5 × *F. v* 3	12.71 ± 0.89	141.22	4.71 ± 0.13	37.06
F. m 5 × *F. v* 9	10.86 ± 1.02	120.67	3.86 ± 0.26	35.54
F. v 9	7.14 ± 0.08	–	3.14 ± 0.12	43.98
F. m 7	12.31 ± 0.92	–	3.71 ± 0.16	30.14
F. m 7 × *F. v* 3	16.69 ± 1.32	135.58	8.29 ± 0.22	49.67
F. m 7 × *F. v* 10	20.29 ± 0.87	164.83	10.35 ± 0.31	51.01
F. v 10	14.86 ± 1.41	–	5.86 ± 0.14	39.43
F. m 8	6.00 ± 0.65	–	3.00 ± 0.16	50.00
F. m 8 × *F. v* 6	5.71 ± 0.72	95.17	1.71 ± 0.19	29.95
F. v 6	7.14 ± 0.23	–	4.14 ± 0.12	57.98

A height of seedlings of non-treatment measured on day 10, *C* height of seedlings of salt treatment measured on day 10, *B* height of F1 Hybrid seedlings/height of the intraspecific open pollinated plants from corresponding female *Fraxinus mandshurica* parent × 100 %, *D* height of seedlings under salt stress/height of seedlings without salt treatment (control) × 100 %

APX, GR, MDHAR, and DHAR increased in plants treated with 200 mM salt compared to controls (Fig. 3). POD and CAT activity and GSH content also increased after salt treatment (Fig. 4). POD activity in *F. m* 7 × *F. v* 10 was the highest of the groups, increasing 58.92 % from 16.82 U/g before treatment to 26.73 U/g after treatment. POD activity was lowest in *F. m* 5. GSH and CAT activity followed similar trends. We compared antioxidant activity between interspecific hybrid progenies and the intraspecific open pollinated plants from parental *F. mandshurica* and *F. velutina*. With the exception of *F. m* 8 × *F. v* 6, antioxidant activity in interspecific hybrid progenies of all other families was greater than that measured in the intraspecific open pollinated plants from female parent *F. mandshurica*. Antioxidant activity of *F. m* 7 × *F. v* 10 hybrid exceeded their respective parents after saline stress and normal growth conditions. These findings suggest that *F. m* 7 × *F. v* 10 hybrid exhibits heterosis for antioxidant enzymes.

Effect of salt treatment on photosynthesis

Significant differences in photosynthetic parameters were observed between untreated controls and treated groups. Compared with other seedlings, *F. m* 5 × *F. v* 3 had a lower net photosynthetic rate (Pn), but the intercellular CO_2 concentration (Ci) was high under stress (Fig. 5). Salt stress significantly decreased Pn and stomatal conductance (Gs), indicating an inhibitory effect on these parameters. The most significant reduction in Pn and Gs was observed in *F. m* 7 × *F. v* 10, which decreased by 84.39 and 89.88 % compared with its untreated control. F. m2, *F. m* 2 × *F. v* 3, *F. m* 7 × *F. v*10, F. v10 had a lower Ci rate compared to their untreated controls, illustrating their more efficient CO_2 utilization. The seedlings consumed more water to restrain their growth and increase their Tr. Data indicate that Pn and Gs were reduced in all of the interspecific hybrid progenies in response to salt stress. Furthermore, with the exception of *F. m* 2 × *F. v* 3 and *F. m* 5 × *F. v* 9, other F1 hybrids had a lower Tr than those of their intraspecific open pollinated plants from parental *F. mandshurica* and *F. velutina* seedlings under salt stress. These results reveal that the interspecific F1 hybrid progenies underwent heterosis for their photosynthetic functions under salt stress.

Overall evaluation of salt resistance

We adopted the most widely used method "fuzzy subordination method" to evaluate salt resistance and measure greater hybrid vigor. The subordinate function adopts the method of membership functions in fuzzy mathematics for different combinations to determine their average index subordinate function

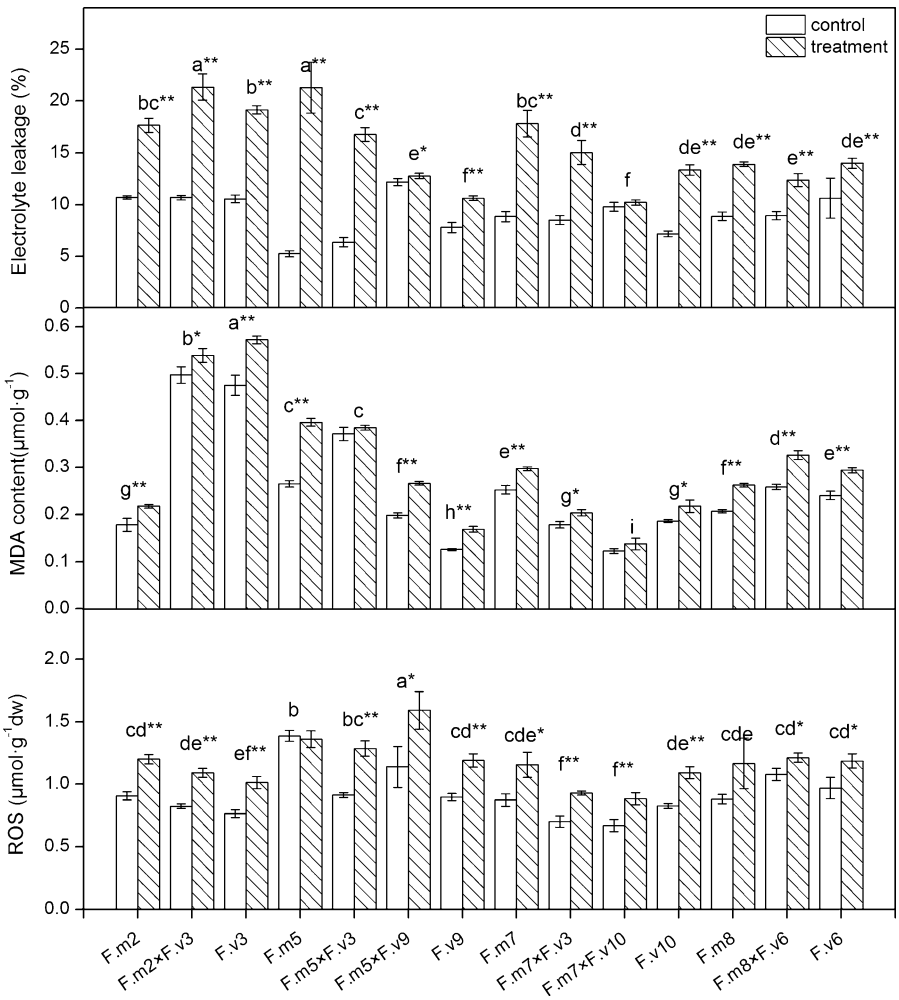

Fig. 2 Cell membrane permeability, MDA content, and steady-state ROS in seedlings treated with/without salt. Data are mean ± SE of three independent replicates (n = 3). * (0.01) and ** (0.05) indicate significant differences between treatment and control in the same combination. Ten days after treatment with/without salt, MDA, electrolyte leakage and steady-state ROS in the seedlings were measured as described in Materials and methods. *Dw* dry weight, *fw* fresh weight

values and identify their resistance. Based on fuzzy subordination method, the average index subordinate function values (Δ) with salt treatment for each line were calculated. The higher the total index subordinate function values, the stronger the salt resistance of a particular line. Figure 6 showed overall evaluation of salt resistance in seedlings by fuzzy subordination method. F1 hybrids *F. m* 7 × *F. v* 10, *F. m* 2 × *F. v* 3, and *F. m* 7 × *F. v* 3 had the highest salt resistance, and their subordinate function values were 10 % greater than average, whereas *F. m* 8 × *F. v* 6 had the least salt tolerance in F1 hybrids.

Alterations in cytosine methylation in the interspecific hybrids revealed by MSAP

Using 20 pairs of *EcoR*I + *Hpa*II/*Msp*I selective primer combinations (Supplementary Table 1), 394–726, 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. Among the intraspecific open pollinated plants from parental *F. m* 7, *F. v* 10 and *F. m* 5, total methylation (calculated by adding up the various patterns) was 25.04, 23.02 and 28.05 %, respectively. Total DNA methylation levels in interspecific hybrid progenies *F. m* 7 × *F. v* 3, *F. m* 7 × *F. v* 10, and *F. m* 5 × *F. v* 9 ranged from 18.51 to 20.79 %; the hemi-methylation of the external cytosine ranged from 10.16 to 13.36 %, and the full methylation of the internal cytosine ranged from 6.23 to 8.35 % (Fig. 7; Table 2). These results suggested that the methylation patterns between interspecific hybrid progenies and the intraspecific open pollinated plants from parental *F. mandshurica* and *F. velutina*

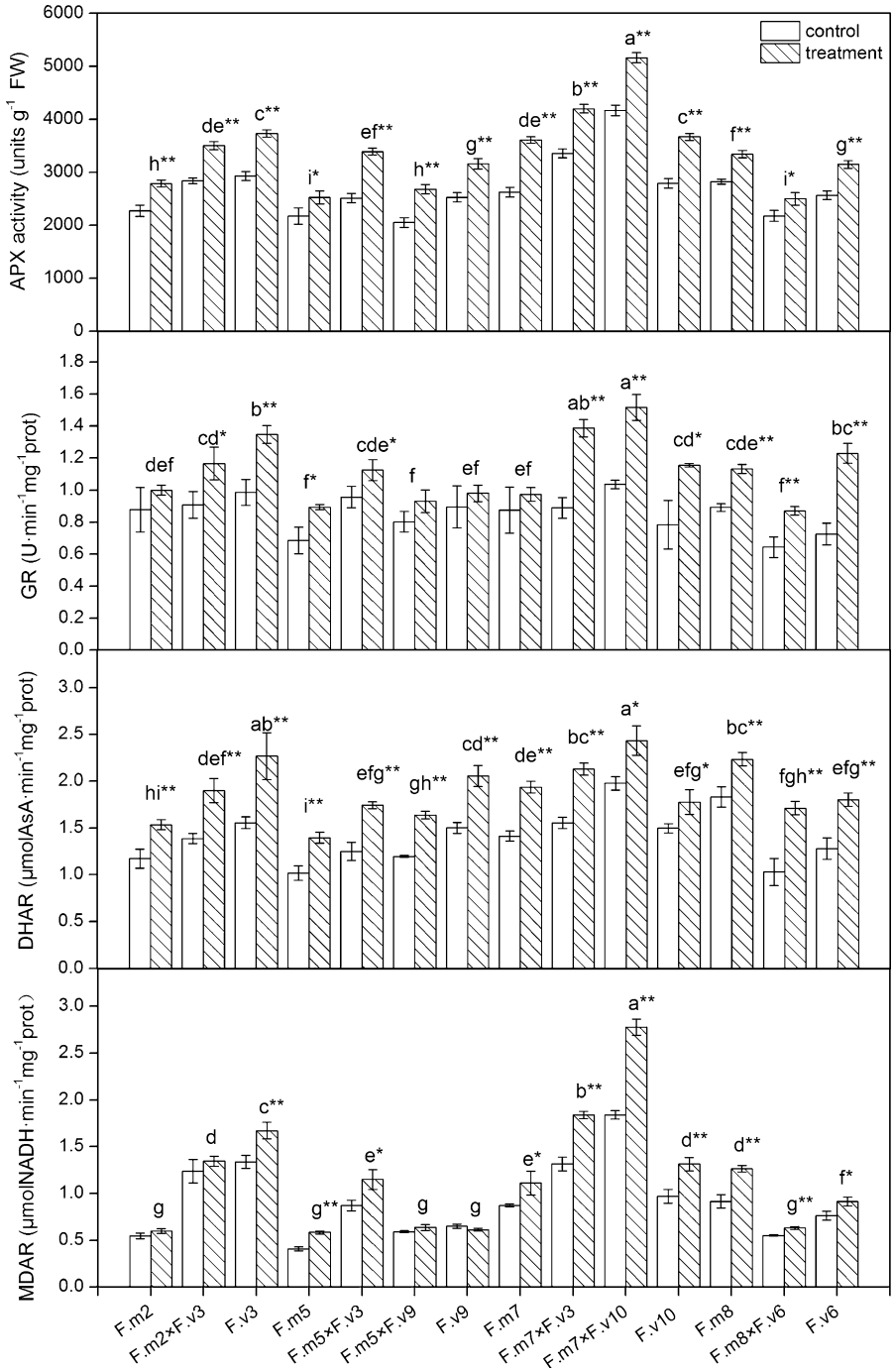

Fig. 3 Effects of salt stress on MDAR, DHAR, GH, APX activity in seedlings treated with/without salt. Data are mean ± SE (n = 3). * (0.01) and ** (0.05) indicate significant differences between treatment and control in the same combination. Ten days after treatment with/without salt MDAR, DHAR, GH, APX activity in seedlings were measured as described in Materials and Methods. *Dw* dry weight; *fw* fresh weight, *prot* protein

were different. The level of DNA methylation in interspecific hybrid progenies *F. m* 7 × *F. v* 3, *F. m* 7 × *F. v*10, and *F. m* 5 × *F. v* 9 was significantly lower than those of the intraspecific open pollinated plants from parents. Differences in DNA methylation level and patterns were observed between untreated controls and salt-treated plants. On average, DNA methylation of the intraspecific open pollinated plants *F. m* 7, *F. v10* and *F. m5* increased from 31.97 to 35.19 % 5 d after salt treatment, whereas those of interspecific hybrid progenies *F. m* 7 × *F. v* 3, *F. m* 7 × *F. v*10, and *F. m* 5 × *F. v* 9 increased from 27.11 to 28.85 %.

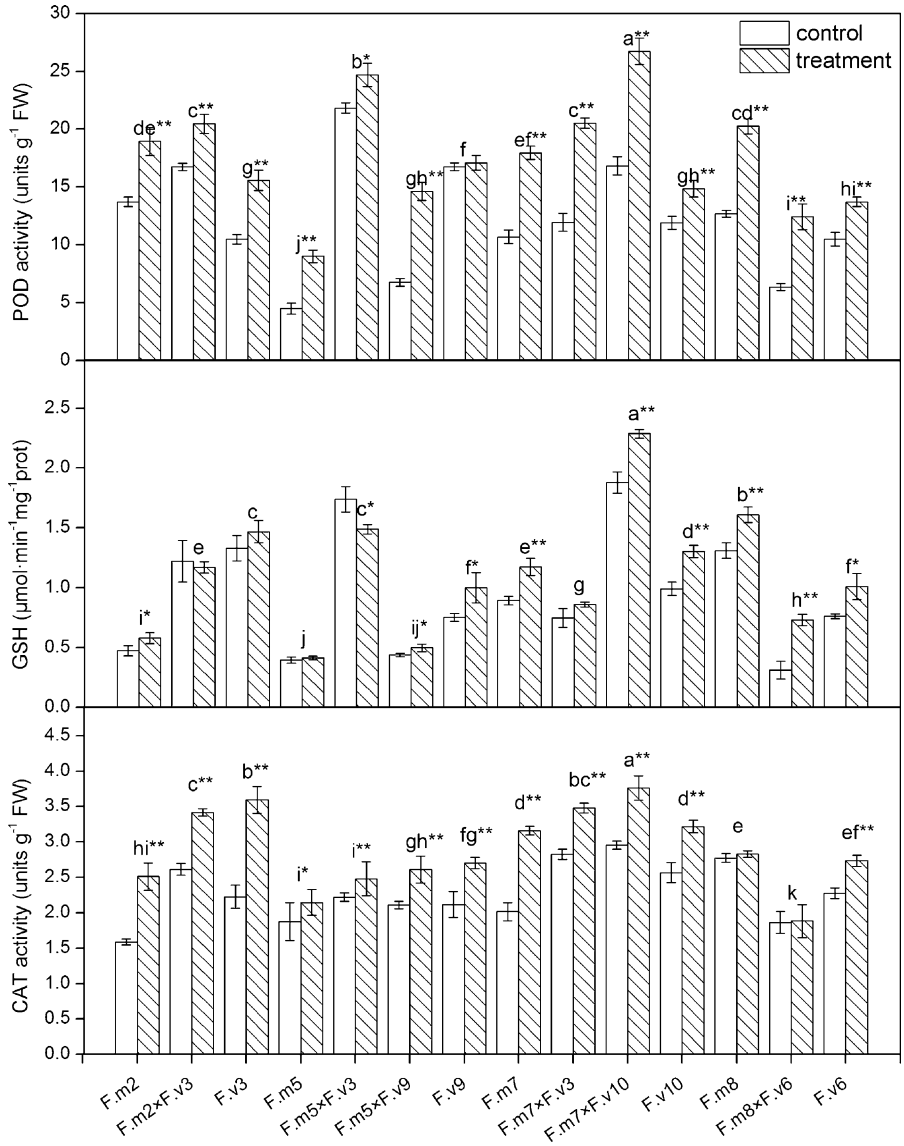

Fig. 4 Effects of salt stress on CAT, POD activity and GSH in seedlings treated with/without salt. Data are mean ± SE (n = 3). * (0.01) and ** (0.05) indicate significant differences between treatment and control in the same combination. Ten days after treatment with/without salt, CAT, POD activity and GSH in seedlings were measured as described in Materials and methods. *Dw* dry weight; *fw* fresh weight, *prot* protein

Methylation of *F. m* 7 reached 35.19 % after 5 d of salt treatment, whereas that of *F. m* 7 × *F. v*10 was only 26.09 %. DNA methylation decreased slightly 10 d after salt treatment. After rehydration for 3 d, cytosine methylation remained high (22.83–25.14 % in F1 hybrid progenies), suggesting that salt treatment can increase methylation of the plant genome.

Discussion

Compared with the intraspecific open pollinated plants from parents under normal growth conditions, *F. m* 7 × *F. v*10 had the greatest growth (164.83 %). Among hybrid combinations, family *F. m* 7 had the highest relative growth rate. Significant differences were observed among interspecific F1 hybrids of *F. velutina* and *F. mandshurica* and the intraspecific open pollinated plants from parents after salt treatment. Generally, growth suppression is one of the most significant symptoms of plants suffering saline stress (Tozlu et al. 2000). Our results showed that seedling growth was inhibited by salt stress. Leaves had brown patches and some were shed; however, with the exception of *F. m* 8 × *F. v* 6, F1 hybrids had less growth reduction than their respective parents. Our

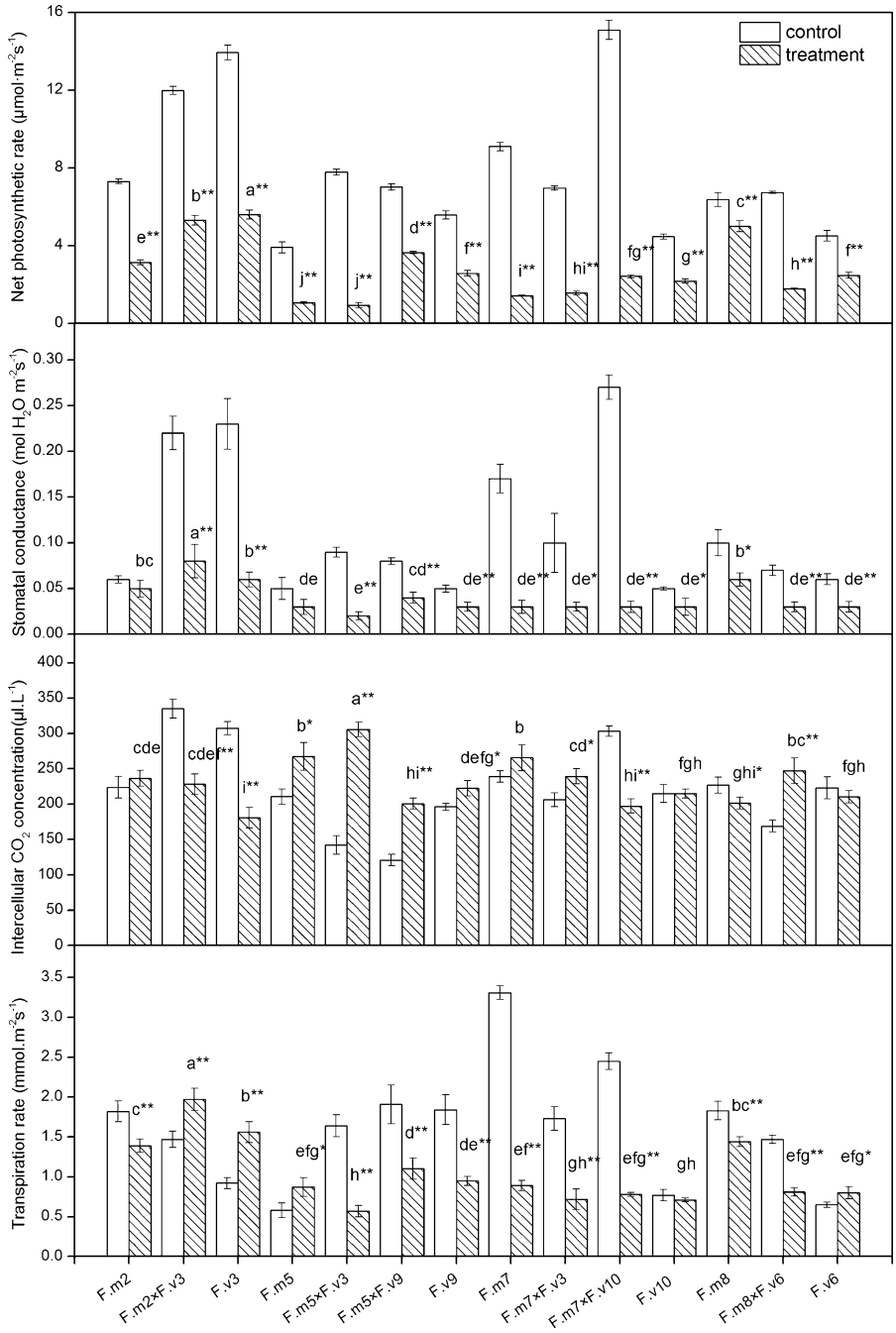

Fig. 5 Effect of salt treatment on photosynthesis in seedlings treated with/without salt. Data are mean ± SE (n = 3). * (0.01) and ** (0.05) indicate significant differences between treatment and control in the same combination. Ten days after treatment with/without salt, Net photosynthetic rate, stomatal conductance, intercellular CO_2 concentration, and Tr were measured as described in Materials and methods

results indicate that F1 hybrids had better hybrid vigor under both saline stress and normal growth conditions.

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 stress resistance in many plant species (Lima et al. 2002; DaCosta and Huang, 2007). 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

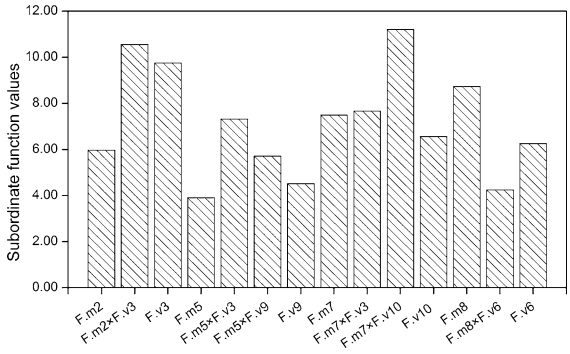

Fig. 6 Overall evaluation of salt resistance in seedlings by fuzzy subordination method

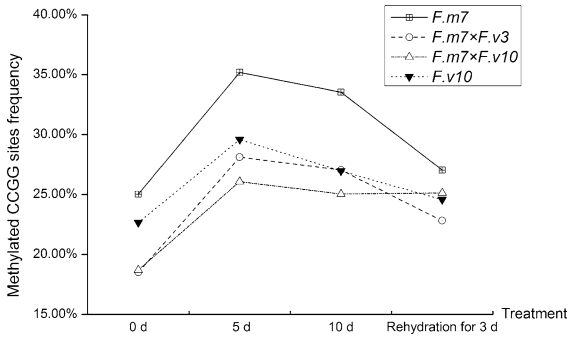

Fig. 7 Changes in cytosine methylation at CCGG sites of different lines under salt treatment. Seedlings treated with either 5 days of salt or 10 days of salt or 10 days of salt +3 days of rehydration or without salt were used for MSAP analysis

estimate cell membrane stability (Blum and Ebercon 1981; Xu et al. 2011). In this study, membrane permeability and MDA content increased to varying degrees under salt stress. whereas, cell membrane permeability and MDA content of *F. m* 7 × *F. v* 10 increased only 4.17 and 12.19 % compared with its untreated control, which indicated that *F. m* 7 × *F. v* 10 had greater hybrid vigor with respect to resisting cell injury after salt stress. The antioxidant system in plants may be a result of sequential and simultaneous actions by several antioxidant enzymes, including CAT and POD. Both CAT and POD scavenge the accumulated H_2O_2 to non-toxic levels by converting it into water and oxygen (Apel and Hirt 2004). Our experiments showed that activities of these enzymes in salt-stressed plant leaves were heightened over time when compared with the controls. Peak activities of CAT and POD were founded in *F. m* 7 × *F. v* 10 after salt treatment. Again, these increases in activities were more pronounced in our salt-tolerant hybtids, a finding in accordance with those reported with other studies (Hernandez et al. 2010; Zlatev et al. 2006; El-Mashad and Mohamed 2012). With the exception of *F. m* 8 × *F. v* 6, antioxidant activity in interspecific hybrid progenies was greater than that in the intraspecific open pollinated plants from *F. mandshurica*,which demonstrated that they are more tolerant to salt stress than their female parent. We also found here that when enzymes in the AsA-GSH cycle (APX, GR, DHAR, and MDHAR) were induced in some hybrids (*F. m* 7 × *F. v* 10 and *F. m* 7 × *F. v* 3) under salt stress, oxidative damage (EL and MDA content) could be minimized. This observation is consistent with previous research on the 'Gala' cultivar in *Malus domestica* Borkh (Ma et al. 2008). Photosynthesis is one of the physiological processes that are most sensitive to many environmental stresses, including salt stress (Zhu et al. 2012). Salinity reduced leaf photosynthesis in all species, which was consistent with other reports (Parida et al. 2003; Wang et al. 2007). Our data indicate that Pn and Gs were reduced in all of the interspecific hybrid progenies in response to salt stress. The positive relationship between stomatal conductance and net photosynthesis indicates that the primary limiting factor for net photosynthesis upon exposure to salinity is stomatal closure (Tavakkoli et al. 2010).

Based on the subordinate function method, the average index subordinate function values of all physiological parameters after salt treatment were calculated. *F. m* 8 × *F. v* 6 had weakest salt resistance among all F1 lines tested. The strongest salt tolerance was observed in family *F. m* 7, whereas *F. m* 7 × *F. v*10 had the greatest salt tolerance among all plants. The results showed that growth and survival rate of the interspecific hybrid progenies (F1 hybrids of *F. mandshurica* × *F. velutina*) were significantly higher than those of intraspecific open pollinated plants from parental *F. mandshurica* and *F. velutina*. Paternal *F. velutina* has not yet been successfully introduced in the Heilongjiang Province of China; this species has wilted shoot tips after year of growth, and these shoots re-sprout in spring. *Fraxinus velutina* cannot withstand winter in the Heilongjiang Province. Therefore, introducing these varieties into this region does not permit exploitation of their salt tolerance and growth characteristics. Interspecific hybrids could survive winter after 2 years of field trials. We compared

Table 2 Analysis of cytosine methylation at CCGG sites in the leaves of different lines

Hybrid combination		Total number of sites	Non-methylated CCGG sites (number and frequency %)	Methylated CCGG sites (number and frequency %)	Average of Methylated CCGG sites (%)	Hemi-methylation of the external Cs (%)	Full methylation of the internal Cs (%)
Salt treatment for 0 d	*F. m* 7	563	422 (74.96)	141 (25.04)	25.04	59 (10.48)	82 (14.56)
	F. m 7 × *F. v* 3	551	449 (81.49)	102 (18.51)	18.61	56 (10.16)	46 (8.35)
	F. m 7 × *F. v*10	497	404 (81.28)	93 (18.71)		62 (12.47)	31 (6.23)
	F. v 10	621	478 (76.97)	143 (23.02)	23.02	94 (15.13)	49 (7.89)
	F. m 5	656	472 (71.95)	184 (28.05)	28.05	71 (10.82)	113 (17.23)
	F. m 5 × *F. v* 9	726	575 (79.21)	151 (20.79)	20.79	97 (13.36)	54 (7.43)
Salt treatment for 5 d	*F. m* 7	648	420 (64.81)	228 (35.19)	35.19	93 (14.35)	135 (20.8)
	F. m 7 × *F. v* 3	537	386 (71.88)	151(28.12)	27.11	87 (16.20)	61 (11.16)
	F. m 7 × *F. v*10	598	442 (73.91)	156 (26.09)		98 (16.39))	58 (9.69)
	F. v 10	586	399 (68.09)	187 (31.97)	31.97	121 (20.64)	66 (11.26)
	F. m 5	436	295 (67.66)	141 (32.34)	32.34	52 (11.92)	89 (20.41)
	F. m 5 × *F. v* 9	610	434 (71.15)	176 (28.85)	28.85	106 (17.38))	70 (11.48)
Salt treatment for 10 d	*F. m* 7	620	421 (66.45)	208 (33.54)	33.54	73 (11.78)	135 (21.77)
	F. m 7 × *F. v* 3	562	410 (73.21)	152 (27.05)	26.05	57 (10.14)	95 (16.90)
	F. m 7 × *F. v*10	523	392 (74.95)	131 (25.05)		49 (9.37)	82 (15.68)
	F. v 10	567	380 (67.02)	187 (32.98)	32.98	58 (10.23)	129 (22.75)
	F. m 5	489	323(66.05)	166 (33.95)	33.95	97 (19.84)	69 (14.11)
	F. m 5 × *F. v* 9	567	422 (74.43)	145 (25.57)	25.57	90 (15.87)	55(9.70)
Rehydration	*F. m* 7	536	391 (72.95)	145 (27.05)	27.05	51 (9.51)	94 (17.54)
	F. m 7 × *F. v* 3	473	365 (77.17)	108 (22.83)	23.99	53 (11.20)	55 (11.63)
	F. m 7 × *F. v*10	517	387 (74.85)	130 (25.14)		42 (8.12)	88(17.02)
	F. v 10	572	416 (72.73)	156 (27.27)	27.27	56 (9.79)	100 (17.48)
	F. m 5	394	292 (74.11)	102 (25.89)	25.89	57 (14.47)	45 (11.42)
	F. m 5 × *F. v* 9	428	330 (77.11)	98 (22.89)	22.89	61 (14.25)	37 (8.64)

The seedlings treated with salt for 5 or 10 days or without salt treatment were used to for MSAP analysis; some seedlings treated 10 days by salt were rehydrated for 3 days before subject to MSAP analysis

interspecific hybrids with their parents and observed that interspecific Fl hybrids gained the advantage of salt tolerance.

Analysis of plant genomic methylation with MSAP revealed a relationship between salt tolerance and DNA methylation. Using a well-studied model of TMV infection, Boyko et al. (2006) established a correlation between stress treatment, loci-specific epigenetic changes, and genome stability in exposed plants and their progenies. Stress can induce changes in gene expression through DNA hypomethylation or hypermethylation (Boyko and Kovalchuk 2011; Karan et al. 2012; Bilichak et al. 2012; Song et al. 2012). Osmotic stress induces transient DNA hypermethylation in two heterochromatic loci in tobacco cell-suspension cultures (Kovarik et al. 1997), and this DNA hypermethylation was also induced by drought stress in pea (Labra et al. 2002). Gene expression is influenced by chromatin structure, which in turn is governed by processes often associated with epigenetic regulation, namely, histone variants, histone post-translational modifications, and DNA methylation (Chinnusamy and Zhu 2009). Epigenetic modifications occur during the plant's exposure to stress, and initiate numerous changes in gene expression that can persist over several generations (Zhu 2008). Therefore, the epigenetic control of plant responses to stress is a complex phenomenon.

Tsaftaris and Kafka (1998) studied DNA methylation of maize hybrids and their parents and reported that cytosine methylation of the two parents was 31.4 and 28.3 %, respectively, whereas methylation of F1

hybrid was 27.4 %. Hepburn et al. (1985) studied the effect of plant DNA methylation on gene expression to analyze the relationship between hybridization and methylation. They found that the extent of genomic methylation self-hybridization led to the gradual accumulation of genomic methylation, whereas genomic methylation sites could be removed or rearranged by hybridization. In the present study, total methylation in interspecific hybrid progenies ranged from 18.51 to 20.79 %, whereas hemi-methylation of external cytosine ranged from 10.16 to 13.36 %, and full methylation of internal cytosine ranged from 6.23 to 8.35 %. DNA methylation of F1 hybrids was significantly lower than those of corresponding controls in our study, which corroborates results from previous publications (Xiong et al. 1999). Furthermore, our studies also showed that salt stress can increase the methylation degree and alter the methylation pattern in both hybrid and parental intraspecific progenies. Our results for DNA methylation patterns under salt stress in tree plants were similar to those reported in a previous model plant study (Kovalchuk et al. 2003). Dyachenko et al. (2006) studied genomic methylation changes in the *Mesembryanthemum crystallinum* plant under saline stress conditions and reported that the methylation of CCWGG (where W is A or T) sequence CpNpG (where N is any nucleotide) was twice that of control. This is probably due to the induction of DNA methylation modification and gene expression by stress during the process of plant growth (Hua et al. 2005; Ruiz et al. 2005; Portis et al. 2004). Sensing environmental changes and initiating a gene expression response is most important for plants as sessile autotrophs. Epigenetic systems must be part of the relay from sensing a change in the environment to a change in gene expression (Grant-Downton and Dickinson 2006). 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 in order to coordinate the expression of the cytoplasmic and nucleus genes and ensure the optimal status of both genetic systems (Grant-Downton and Dickinson 2005). Therefore, our results suggest an association between salt tolerance and methylation changes in plants.

Conclusion

Fraxinus velutina does not adapt well to cold weather in Northeast China, such as in the Heilongjiang Province. The study crossed *F. mandshurica* (female parent) with *F. velutina* (male parent) to obtain interspecific F1 hybrid progenies that could obtain the good characters of parents. The results showed that growth and survival rate of the interspecific hybrid progenies were significantly higher than those of intraspecific open pollinated plants from parental *F. mandshurica* and *F. velutina* in natural conditions. The F1 hybrids have also increased hybrid vigor with respect to salt stress resistance as demonstrated by measurement of photosynthesis, antioxidant enzymes, and other physiological functions. DNA methylation of F1 hybrids was lower than that measured in the intraspecific open pollinated plants from parents. Furthermore, the DNA methylation patterns of F1 hybrids can be changed by salt treatment. Thus, heterosis of F1 hybrids is associated with genomic methylation status. The study provided epigenetic mechanisms of salt stress resistance in the interspecific hybridization of trees.

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

Data archiving statement The research performed in the present study did not produce any data on nucleic acid or protein sequences, genetic maps, SNPs or gene expression that could be deposited in the public databases. Data of enzyme assays and physiological parameters is stored at Northeast Forestry University, Harbin (http://www.nefu.edu.cn/).

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