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ORIGINAL PAPER

Drought physiology and gene expression characteristics of *Fraxinus* interspecific hybrids

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Abstract In this study, we investigated the physiological and gene expression responses of *Fraxinus* interspecific hybrids seedlings to short-term artificially-applied drought stress. Experiment results showed that there were differences in physiological and gene expression responses between hybrids and their parents. Hybrid D110 kept higher levels of photosynthetic parameters and antioxidant enzyme activities than its female parents, as well as ABA contents and expressions of ABA-related genes. These results showed that hybrid D110 improved the suitability which could be due to the change of ABA signal transduction under drought conditions. The expression characteristics of circadian clock gene *TOC1* and *LHY* in *Fraxinus* hybrid plants and their parents under drought conditions showed that altered amplitude of circadian gene expression might be a possible molecular mechanism for drought-advantage in hybrids. Combined with the physiological response of each hybrid under drought stress, these more sensitive responses of *NCED*, *PYR1* and *SnRK2.6* expressions in hybrid D110 compared to its female parent and other hybrids, along with the higher levels of *PYR1* and *SnRK2.6* expressions might coursed the drought-advantage in hybrid D110.

Keywords *Fraxinus* · Hybrid · ABA · Circadian clock · Drought stress

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Abbreviations

FP	Female parent
MP	Male parent
F1	Hybrid plant
HGR	Height growth rate
BGR	Base diameter growth rate
MPV	Mid-parent value
ZT	Zeitgeber time
P_n	Net photosynthetic rate
g_s	Stomatal conductance
C_i	Intercellular CO_2 concentration
E	Net transpiration rate
GA	Gibberellin
ABA	Abscisic acid
IAA	Indoleacetic acid
ZR	Zeatinriboside

Introduction

As sessile organisms, plants must cope with multiple environmental stress factors commonly referred as "abiotic stress". This term includes drought, salinity, extreme temperatures or hypoxia (Valdes et al. 2013). Drought stress is one of the most life-threatening conditions, affecting processes such as photosynthesis, respiration, carbohydrate metabolism or ion uptake (Chaves et al. 2009; An et al. 2015), which eventually lead to stress-mediated responses in plant growth and development that are crucial for the individual survival. Such responses include a wide spectrum of physiological and molecular programs evolved to face unfavorable conditions to efficiently perceive changes and adapt accordingly (Ahuja et al. 2010).

Abscisic acid (ABA) is known as a systemic mediator and its accumulation after drought sensing acts as an initial signal for long-term acclimation reactions, which eventually involve the differential expression of genes leading to changes in transcript and protein patterns (Shinozaki and Yamaguchi-Shinozaki 2007; Hirayama and Shinozaki 2010; Fulda et al. 2011). Like other abiotic stresses, drought is accompanied by an increased production of reactive oxygen species (ROS) such as superoxide radical (O_2^-), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^·$) (Choudhury et al. 2013). The excess ROS caused by environmental stress can damage plant cells irreversibly by oxidation of cellular components such as lipids, proteins and DNA (Apel and Hirt 2004). Besides their destructive effects in cells, ROS can also act as signalling molecules in many biological processes such as stomatal closure, growth, development, and stress signalling (Demiral et al. 2011). Due to this dual role of ROS, plants are able to fine-tune their concentrations between certain thresholds by means of production and scavenging mechanisms (Askim et al. 2014). Since this ROS homeostasis is disrupted under stress in favour of production, constitutive and induced enzymatic antioxidant defences are considered a crucial component of plant stress tolerance (Suzuki et al. 2012).

Estimates indicate that 10 % of animal and 25 % of plant species hybridized with at least one other species (Mallet 2004). The common occurrence of hybrids shows an evolutionary advantage in having additional genetic materials for growth and adaptations (Ni et al. 2009). Hybrid cultivars have been used commercially in many crop plants, and have made great contributions to the world food supply (Duvick 1999). The genetic basis for hybrid vigor or heterosis has been debated for over a century, but little consensus has been reached. Several hypotheses including dominance, overdominance, and pseudo-overdominance are available to explain the phenomenon of hybrid vigor (Jeffrey 2010). Understanding of molecular basis underlying heterosis will facilitate more predictive development of heterotic hybrids (Somerville and Somerville 1999; Somerville 2000). Based on studies of evolution of plant form, it is suggested that phenotype evolution often proceeds through changes in the spatial and temporal patterns of gene expression (Doebley and Lukens 1998). The genetic architecture of hybrid F1 is contributed from its two parents, with the cytoplasm derived from female parent (Sun et al. 2004). Earlier researches demonstrated that some proteins and mRNAs are differentially expressed in root tissues between a maize hybrid and its parents (Romagnoli et al. 1990), and the mean mRNA expression for 35 tested genes were higher in a highly heterotic hybrid cultivar than in a non-heterotic hybrid cultivar and their parents (Doebley and Lukens 1998; Tsaftaris and Kafka 1998). By using differential display of mRNA (Liang and Pardee 1992), differences in mRNA expressions and patterns between one heterotic hybrid and its parents were also detected in maize and rice (Cheng et al. 1996, 1997).

Circadian clocks are complex timekeeping systems that generate endogenous rhythms with periods of about a day (Green and Tobin 2002). Circadian clocks affect many physiological and developmental processes, including various metabolic pathways and adaptation traits in animals and plants, as well as photosynthesis and starch metabolism in plants (Dodd et al. 2005; Wijnen and Young 2006; Panda et al. 2002; Michael et al. 2003). At least 15 % of genes, including those involved in photosynthesis and starch metabolism (Harmer et al. 2000; Smith et al. 2004), and up to 90 % of transcriptome (Covington et al. 2008) are affected by the circadian clock regulators. Studies have revealed that the core of the circadian clock system is an oscillator based on a transcriptional–translational negative feedback loop. There are evidences that CIRCADIAN CLOCK ASSOCIATED 1 (*CCA1*)/LATE ELONGATED HYPOCOTYL (*LHY*) and TIMING OF CAB 1 (*TOC1*) are oscillator components and comprise the core negative feedback loop (Wang and Tobin 1998; Schaffer et al. 1998; Strayer et al. 2000). According to this model, the partly redundant transcription factors *CCA1* and *LHY* (Mizoguchi et al. 2002; Alabadí et al. 2002) function as negative components that participate in the repression of *TOC1* (Alabadí et al. 2001) by directly binding to the Evening Element (EE) motif present in the *TOC1* promoter (Harmer et al. 2000). Increased *TOC1* expression was predicted to close the feedback loop by activating the transcription of *CCA1* and *LHY* (Alabadí et al. 2001). Earlier researches demonstrated that altering the clock amplitude but maintaining the rhythmic phase increases growth vigor in the hybrids (Jeffrey 2010). Hybrids simply exploit epigenetic modulation of parental alleles and homologous loci of the internal circadian regulators and use this convenient mechanism to change the amplitude of gene expression and metabolic flux and gain advantages from clock-mediated photosynthesis and carbohydrate metabolism (Ni et al. 2009).

Abscisic acid is a major phytohormone that regulates a wide range of plant processes and is especially important for adaptation to environmental conditions (Umezawa et al. 2010; Zhang et al. 2015). Our understanding of ABA signal transduction indicates that the earliest events occur via a central regulatory module made up of proteins belonging to three protein classes: Pyracbactin Resistance (PYR)/Pyracbactin Resistance-like (PYL)/Regulatory Component of ABA Receptor (RCAR) which act as the ABA receptors, PP2Cs (Protein Phosphatase 2Cs) which act as negative regulators and SnRK2s (SNF1-related protein kinase 2s) which act as positive regulators (Park et al. 2009;

Umezawa et al. 2010; Yan et al. 2012). Although the transcriptional regulation of this ABA signalling module has been studied in some species, little is reported about ABA signal transduction in trees. Recently, Wang et al. (2015) reported their study on three *PaPYLs*, six *PaPP2Cs* and six *PaSnRK2s* which were identified from sweet cherry (*Prunus avium* L.). In the aspect of ABA signal transduction, the PYR–PP2C–SnRK2 pathway may play a important role in regulating of sweet cherry fruit development and ripening. Moreover, Shao et al. (2014) indentified 14 putative sequences encoding 12 deduced SnRK2 proteins within the apple genome. The expression analysis showed that some family members were up-regulated in response to drought, salinity, or ABA treatments. This suggested their possible roles in plant response to abiotic stress.

Fraxinus mandshurica Rupr. 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. 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* L. 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* L. cannot live through winter in the high-latitude cold regions (Abrams and Mostoller 1995). *F. sogdiana* Bunge is one of the oldest broadleaf trees that mainly distributed in the northwest of China, and it showed the same difficulties with *F. americana* L. of not able to live through winter in the high-latitude cold regions when we conducted the introduction experiment in the Northeast China. So we crossed *F. mandshurica* Rupr. (female parent) with *F. americana* L. and *F. sogdiana* Bunge (male parent) to obtain interspecific F1 hybrid progenies that could obtain the good characters of parents.

Previous studies had been taken to evaluate the drought tolerance of various *Fraxinus* interspecific hybrids (Dong 2012). Various physiological indexes of *Fraxinus* hybrids under drought stress were measured, to study the potential heterosis obtained from the interspecific hybridization. For the *Fraxinus* interspecific hybrids under drought stress, which had varying degrees of heterosis on physiological indexes, such as growth, photosynthesis, photosynthetic pigment content, SOD activity and POD activity, plant performance varied depending on female parent family lines. We conducted a comprehensive evaluation, and 39 *Fraxinus* hybrids were sorting in terms of drought tolerance. The *Fraxinus mandshurica* Rupr. × *Fraxinus Americana* Linn. D110 turned to be the most drought resistant hybrid.

This study selected *Fraxinus* interspecific F1 hybrids from three different Lines which showed different drought tolerances and the Random Pollination Progeny of their parents as materials based on the preliminary research (Dong 2012). The similarities and differences of expression levels of circadian genes and ABA signal transduction related genes, photosynthetic efficiency, peroxidation and antioxidant enzyme activities between hybrids and their parents (especially female parents due to the low survival rate of male parents) were studied. Based of these results, we analyze relations between endogenous ABA content and circadian gene expression under stress. So far, functions of circadian genes in the mechanism of the tree heterosis has not been reported. The study will provide some evidences for the molecular mechanism of the drought-advantage in interspecific hybrids of *Fraxinus*.

Materials and methods

Plant material and water stress treatments

Plants of four *Fraxinus* hybrids (hybrid seedling group) and the Random Pollination Progeny of their parents (parent seedling group; Table 1) were subjected to water withholding followed by re-watering during 2012 summer in the glasshouse at Northeast Forestry University. The temperature in the glasshouse during the study ranged from 20 °C (night) to 30 °C (day). Briefly, 5-year-old plants were used, each plant grew in a 90 L pot filled with a mixture of clay soil and gravel (3:1; v/v). There were three lines of *Fraxinus* hybrids contained ten different biotypes of seedlings. Control plants were irrigated daily to field capacity, while treatment plants were subjected to progressive drought by withholding water 12 days to a limit extent of soil moisture was 25 % of the field capacity to assess possible acclimation. After 12 days of water withholding, all plants were re-watered to field capacity and recovery was followed for 3 days.

Determination of relative water content

The fresh weight, dry weight and saturated weight of treated leaves were measured. Relative water content (RWC) of leaves was calculated according to formula: [(fresh weight − dry weight)/(saturated weight − dry weight)] × 100 % (Barrs and Weatherly 1962).

Plant sampling and determination of growth rate

Three samplings were collected from each treatment: after 12 days of water restriction and 3 days of rehydration. Leaves fully expanded were harvested, frozen immediately in liquid N_2, and kept at −80 °C until used. To determine the growth rate, the height and base diameter of all seedlings were measured at the first and final days of water

Table 1 The combinations of *Fraxinus* hybrids

Hybrid combination	Line1: *F. mandshurica* Rupr. × *F. Americana* L.	Line2: *F. mandshurica* Rupr. × *F. sogdiana* Bunge	Line3: *F. mandshurica* Rupr. × *F. Americana* L.
Female parent seedling group	D113	M8	M2
Hybrid seedling group	D110	D15	D47
		D16	
Male parent seedling group	4–3	2–1	3–1

restriction. Relative height growth rate (HGR) and relative base diameter growth rate (BGR) were calculated from the increase in height (H) and base diameter (B) at the beginning and after each treatment, using the equation $HGR = [Ln(H_f) - Ln(H_i)]/(T_f - T_i)$; $BGR = [Ln(B_f) - Ln(B_i)]/(T_f - T_i)$, where T is the time and the subscripts denote the final and initial sampling (Sánchez-Rodríguez et al. 2010).

Air-exchange measurements

Values of net photosynthetic rate (P_n), stomatal conductance (g_s), intercellular CO_2 concentration (C_i) and net transpiration rate (E) were determined between 9 and 11 a.m. local time, on nine leaves from different plants of each treatment, using a gas-exchange system (LI-6400XT; Li-Cor). Photosynthesis was induced with 1000 μmol photons m^{-2} s^{-1} and 400 μmol mol^{-1} CO_2 surrounding the leaf. The methods are modified from Zhao et al. (2011).

Measurement of peroxidation

For the malondialdehyde (MDA) assay, fresh leaves were homogenized in 5 mL of 100 g L^{-1} trichloroacetic acid containing 250 g L^{-1} thiobarbituric acid, and centrifuged at 12,000 rpm for 25 min (4 °C). The mixture was heated to 100 °C for 30 min, and then cooled quickly in an ice-bath. Subsequently, samples were centrifuged at 12,000 rpm for 10 min (4 °C) and the supernatant absorbance was read at 532 nm. The value for the non-specific absorption at 600 nm was subtracted from the 532 nm reading. The concentration of MDA was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1} (Rosales et al. 2012).

Determination of antioxidant enzyme activities

For extraction of antioxidant enzyme, about 0.5 g of tissue was ground in liquid nitrogen with a pre-cooled pestle and mortar, and homogenized in 5 mL of extraction buffer containing 50 mmol L^{-1} phosphate buffer (pH 7.8) and 1 % polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 12,000 rpm for 20 min at 4 °C and the resulting supernatant was collected for enzyme activity measurement (Liu et al. 2013). SOD activity was assayed by the photochemical NBT method. 300 μL supernatant was added to 2.7 mL reaction mixture containing 50 mM potassium phosphatebuffer (pH 7.8), 13 mM L-methionine, 75 μM NBT, 2 μM riboflavin and 1 mM EDTA. Then the reaction was illuminated for 20 min in white fluorescent light (100 μmol^{-2} s^{-1}), and the absorbance was recorded at 560 nm. One unit of SOD activity was defined as the quantity sufficient to inhibit the reduction of NBT by 50 % per min per mg protein (Sun et al. 2013). POD activity was estimated from the absorbance change at 470 nm caused by the oxidation of guaiacol. One unit of POD activity was defined to be equivalent to the amount of enzyme required to degrade 0.01 μM of substrate per min per mg protein (He et al. 2011).

Extraction and determination of phytohormone

From each treatment, the youngest fully expanded and exposed leaves were randomly selected for the gibberellin (GA), abscisic acid (ABA), indoleacetic acid (IAA) and zeatinriboside (ZR) analyses. The samples were weighed, frozen in liquid nitrogen. For extraction, leaf samples [0.5 ± 0.01 g, fresh weight (DW)] were homogenized in 4.5 mL cold 50 mmol L^{-1} phosphate buffer (pH 7.2–7.4). The extracts were centrifuged at 3000 rpm at 4 °C for 30 min, and the supernatant was subsequently frozen at −20 °C. Approximately 100 μL was used when analyzing for GA, ABA, IAA and ZR by ELISA using assay kits (made by Shanghai Enzyme-inked Biological Technology Co.), according to the manufacturer's instructions. Detection of different hormones was carried out at 450 nm within 15 min after the reaction was terminated. A standard curve was established for each micro-titer plate. The GA, ABA, IAA and ZR contents were determined three times for each sample. The GA, IAA and ZR levels were calculated as ng g^{-1} fresh sample, and the ABA levels were calculated as μg g^{-1} fresh sample.

Gene-expression analysis

Total RNA samples were extracted using CTAB Reagent and treated with RNase-free DNase I (Promega) to remove contaminated DNA. cDNA was synthesized by adding

100 ng total RNA into 10 μL reaction with random hexamers and oligo dT primers provided by using PrimeScript™ RT reagent Kit (Takara). Quantitative-real time PCR was performed in ABI7500 system with SYBR Premix Ex Taq™ II (Takara). An aliquot (1/100) of RT-reaction product (2 μL) was used as template in a 25 μL PCR mixture. The following program was used for PCR amplification: Initial denaturation at 95 °C 30 s. followed by 40 cycles of 95 °C 5 s. and 60 °C 34 s. The α-Tubulin gene was used as endogenous reference gene to estimate the relative expression levels in three biological replications. The primers used in real time PCR were as follows: *LHY* (LATE ELONGATED HYPOCOTYL): 5′-AGAGGAGGAGCACAATAGGTTT-3′ (forward) and 5′-TATGTCCTACTGGAACTCCTTTAAT-3′ (reverse); *TOC1* (TIMING OF CAB EXPRESSION 1): 5′-AAGTTGACCTTCCTATGTCTAAA-3′ (forward) and 5′-TTACAATGTCCTTCTCTGCTAGT-3′ (reverse); *NCED* (NINE-CIS-EPOXYCAROTENOID DIOXYGENASE): 5′-ACAACCCGTCAGGCAGAGT-3′ (forward) and 5′-TTCACCAATGGCTTTAGGA-3′ (reverse); *PYR1* (PYRABACTIN RESISTANCE 1): 5′-TGGTGGGGAGCATAGATTG-3′ (forward) and 5′-CTTCACAACTGTATCGGCAA-3′ (reverse); *SnRK2.6* (SNF1-RELATED PROTEIN KINASE 2.6): 5′-CCGTAAGACAATACAGCGAATC-3′ (forward) and 5′-TCGGGAATACTTATCCTCTCTG-3′ (reverse); *TU* (α-tubulin): 5′-AGGACGCTGCCAACAACTTT-3′ (forward) and 5′-TTGAGGGGAAGGGTAAATAGTG-3′ (reverse). The primer sequences were determined on the 5′ regions of the genes with putative amplicons of 150–250 bp. All RT-PCR expression assays were performed and analyzed according to the method by Ni et al. (2009) at least three times in independent experiments.

Statistical analysis

All data on physiological (RWC, HGR, BGR and leaf air-exchange) and biochemical (MDA, antioxidant enzyme activities, qPCR) measurements are represented as mean ± standard deviation (n = 3). Effects of drought treatment, biotypes, and interactions between the two factors were determined by analysis of variance (ANOVA) followed by Duncun's multiple range test (with a probability level of 0.05 treated as statistically significant) using the software in Excel and SPSS Inc. (Chicago, IL, USA).

Results

Relative water content

Leaf relative water content (RWC) of all seedling treated by drought stress declined by 12 days of water restriction, reaching 39.1 % for the lowest and 46.1 % for the highest, respectively (Fig. 1). The male parent groups maintained higher RWC than the hybrid groups and female parent groups. Moreover, we found that the hybrid plants from Line1 (D110) and Line2 (D16) maintained significantly higher RWC than their female parents respectively ($P < 0.05$). However, leaf relative water content of most seedling treated by drought stress recovered to the well-watered control level by 3 days of rehydration.

Growth rate

Although the HGR and BGR of most treated seedlings, to a certain extent, were restricted by drought stress, the differences of HGR and BGR between most treatment and control groups had no significances (Fig. 2). After drought stress, the HGR of hybrid (D16) and its male parent (2–1) from Line2 and the BGR of parents (M8 and 2–1) from Line2 and hybrid (D47) and its male parent (3–1) from Line3 significantly reduced. Moreover, there were differences of HGR and BGR between some hybrids and their parents in face with drought stress. After drought stress, the HGR of hybrid (D15) from Line2 was 87.9 % higher its male parent (2–1), while the BGR of hybrid (D15) from Line2 was 88.2 and 20.2 % higher than its female (M8) and male (2–1) parents, and hybrid (D16) from Line2 was 52.9 % higher than its female parent (M8), and hybrid (D110) from Line1 was 37.8 % lower than its male parent (4–3). The differences of HGR and BGR between other hybrid and their parents had no significances.

CO_2-exchange measurements

Drought treatment induced a significant reduction of net photosynthetic rate (P_n), stomatal conductance (g_s) and net transpiration rate (E) in most hybrid combinations, as well as a notable increase of Intercellular CO_2 concentration (C_i) (Fig. 3). In this study, most hybrids maintained a higher photosynthetic level than their female parents. After drought stress, the hybrid (D110) seedling group from Line1 showed a significantly reduction in g_s, E and P_n by 27, 16 and 25 %, respectively ($P < 0.05$). Correspondingly, the female parents (D113) seedling group from Line1 showed a considerable reduction in g_s and E, 47.9 and 39.1 %, respectively, leading to a 45.1 % decrease in P_n. The hybrid (D110) from Line1 maintained significantly higher levels of P_n, g_s and E than that in its female parent (D113) after drought stress. Moreover, the hybrid (D16) from Line2 maintained significantly higher levels of g_s and E than that in its female parent (M8) while the hybrid (D47) from Line2 maintained significantly higher levels of P_n and g_s than that in its female parent (M2) after drought stress.

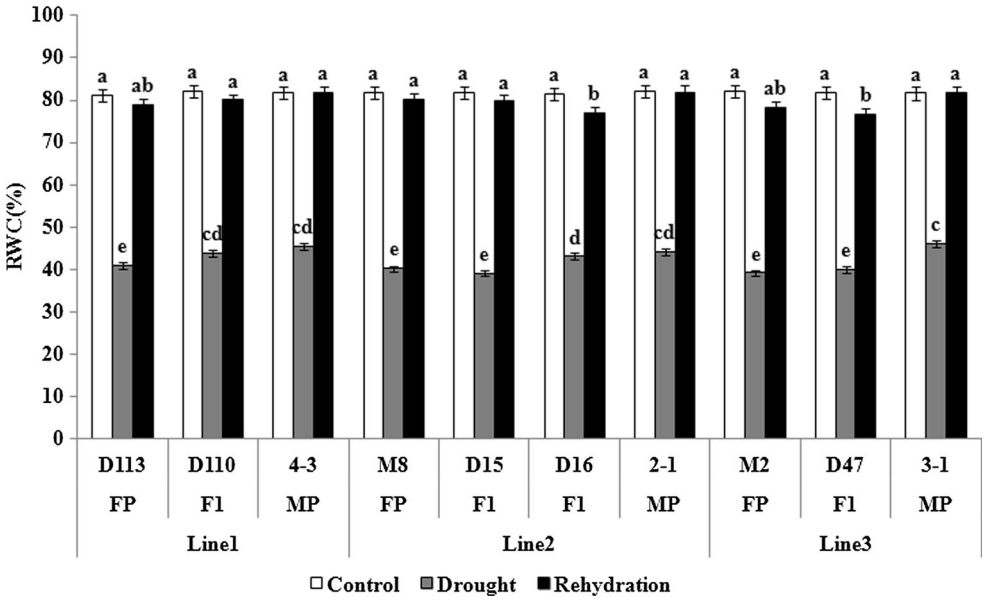

Fig. 1 The effect of drought stress and rehydration on relative water content measured before drought stress, after drought stress and rehydration. *Each data point* represents the average of three replicates, and three seedlings were used for each experiment. *Error bars* represent mean ± SE. *Different lowercase letters* indicate significant differences at $P < 0.05$

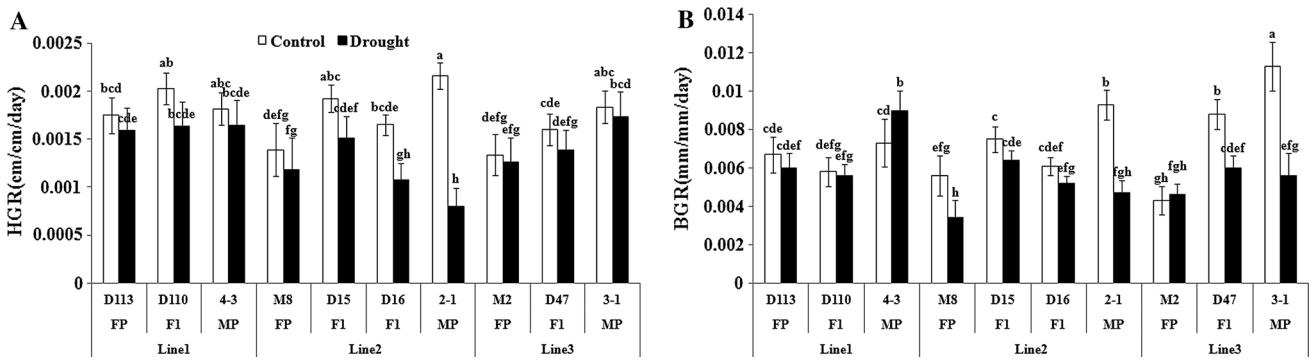

Fig. 2 The effect of drought stress on relative growth rate. Height and base diameter of both treatment and control groups were measured at the beginning and after drought stress treatment. *Each data point* represents the average of three replicates, and three seedlings were used for each experiment. *Error bars* represent mean ± SE. *Different lowercase letters* indicate significant differences at $P < 0.05$

After 3 days of re-watering, net photosynthetic rate (P_n), stomatal conductance (g_s), intercellular CO_2 concentration (C_i) and net transpiration rate (E) of stressed plants in each hybrid combinations were recovered to different levels.

Analysis of antioxidant enzyme activities and peroxidation levels under drought stress

Malondialdehyde (MDA) is one of the most important products of membrane lipid peroxidation. As an indicator of oxidative stress, MDA concentrations were determined in leaves. A significant increase in the concentration of MDA was detected in each hybrid combinations tested under drought conditions ($P < 0.05$). However, As shown in Fig. 4, most hybrid seedling groups contained lower levels of MDA as compared to the female parent seedling groups after drought treatment. After drought stress, MDA concentration in the hybrid (D16) from Line2 was 18.9 % lower than its female parent (M8), and MDA concentration in the hybrid (D47) from Line3 was 24.9 % lower than its female parent (M2). The MDA contents of most treated seedlings showed a significant decrease after rehydration but maintained significantly higher than control groups. Similarly, most hybrid seedling groups contained lower levels of MDA as compared to the female parent seedling groups after rehydration.

Antioxidant enzymes play significant roles in ROS scavenging and influence the cellular ROS level. To assess the antioxidant response of these plants, SOD and POD activities were determined. In all drought conditions tested, an increase in these enzymatic activities was detected in all seedlings. Under drought stress, the differences in enzyme activities between F1 and their parents became more pronounced. However, the elevated multiples of SOD and

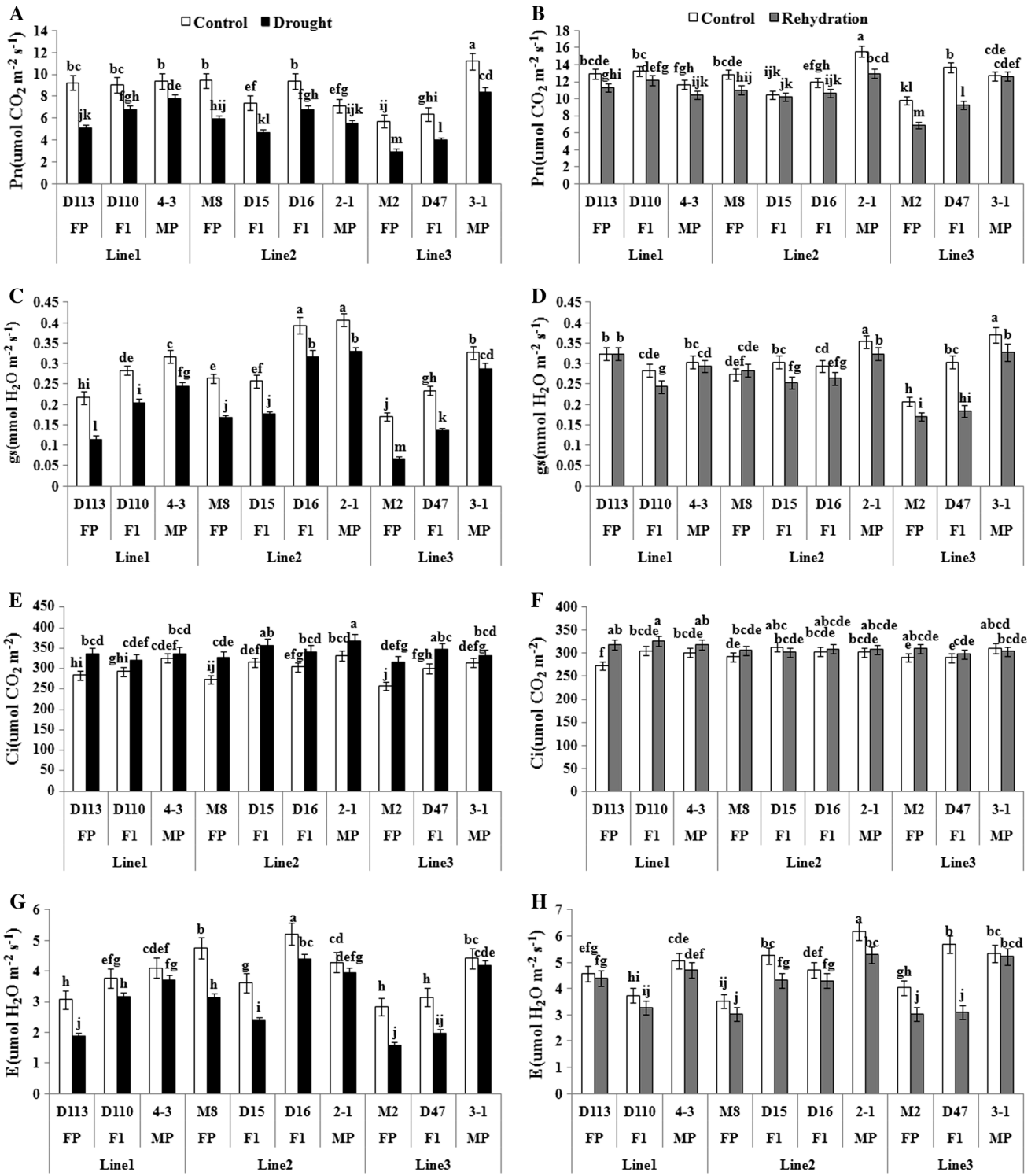

Fig. 3 The effect of drought stress and rehydration on photosynthetic parameters. Net photosynthetic rate (P_n), Stomatal conductance (g_s), Intercellular CO_2 concentration (C_i) and Net transpiration rate (E) of both treatment and control groups were measured by LI-6400XT after drought stress and rehydration. **a**, **b** Mean of the P_n, **c**, **d** mean of the g_s, **e**, **f** mean of the C_i, **g**, **h** mean of the E. *Each data point* represents the average of three replicates, and three seedlings were used for each experiment. *Error bars* represent mean ± SE. *Different lowercase letters* indicate significant differences at $P < 0.05$

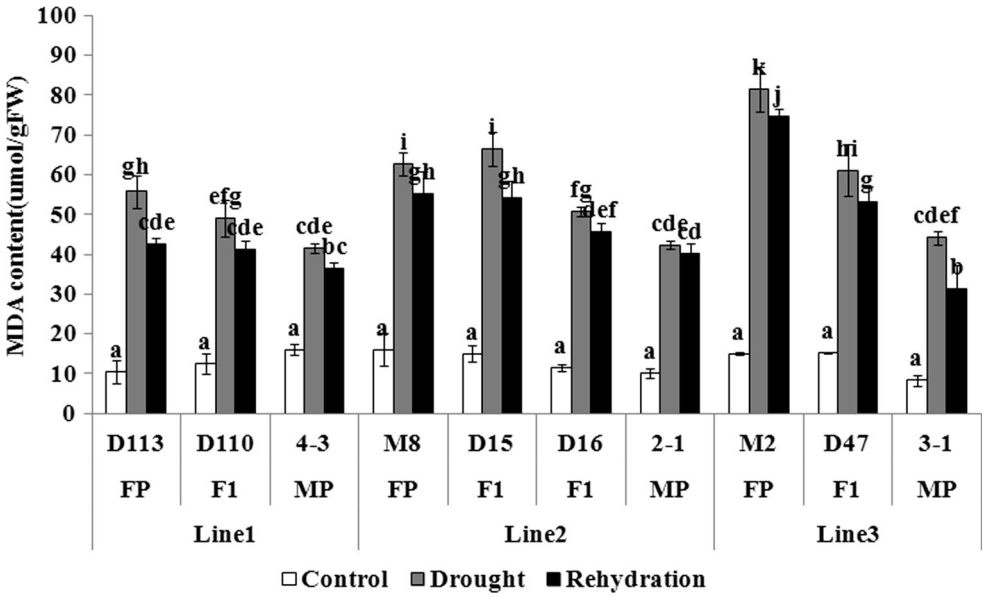

Fig. 4 The effect of drought stress and rehydration on MDA contents measured after drought stress and rehydration. *Each data point* represents the average of three replicates, and three seedlings were used for each experiment. *Error bars* represent mean ± SE. *Different lowercase letters* indicate significant differences at $P < 0.05$

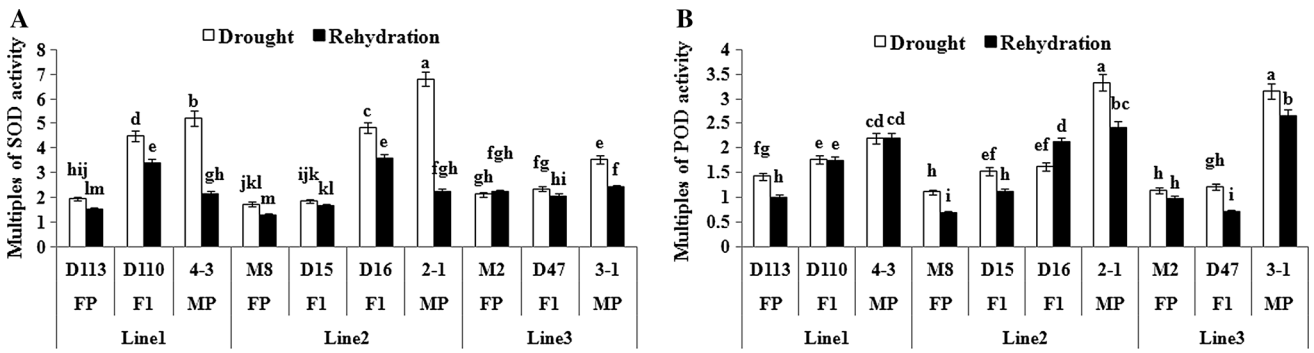

Fig. 5 The effect of drought stress and rehydration on antioxidant enzyme activities. The SOD and POD activities of both treatment and control groups were measured after drought stress and rehydration. **a** Multiples of SOD activities in treatment groups compared to the control groups, **b** multiples of POD activities in treatment groups compared to the control groups. *Each data point* represents the average of three replicates, and three seedlings were used for each experiment. *Error bars* represent mean ± SE. *Different lowercase letters* indicate significant differences at $P < 0.05$

POD activities were higher in the hybrid (D110) from Line1 and the hybrid (D16) from Line2 than that in their female parents, respectively (Fig. 5). The most obvious difference was observed for SOD, which was increased more than fourfold in hybrid (D16) seedling group from Line2 compared with a 1.7-fold increase in female parent (M8) seedling groups and a 6.8-fold increase in male parent (2–1) seedling groups under drought treatment. After rehydration, SOD and POD activities of each hybrids combination were decreased in varying degrees, but still maintained a level higher than that in control of water group. These data show that hybrid plants from different line had different capabilities to enhance the activity of antioxidant enzymes to against severe water condition than their female parents.

Effect of drought stress on endogenous phytohormones

The endogenous ABA content increased under drought stress (Fig. 6). After drought stress, the concentrations of ABA were increased by 5.6 % in the leaf tissue of the hybrid (D110) seedling group from Line1 compared to control group, which was higher than that of their parent seedling groups and increased 4.1 and 7.8 % compared to their female and male parent (D113 and 4–3) seedling groups, respectively. After rehydration, the concentrations of ABA were increased by 13.4 and 20.8 % in the leaf tissue of the hybrid seedling groups from Line1 and Line3 (D110 and D47) compared to the control, respectively.

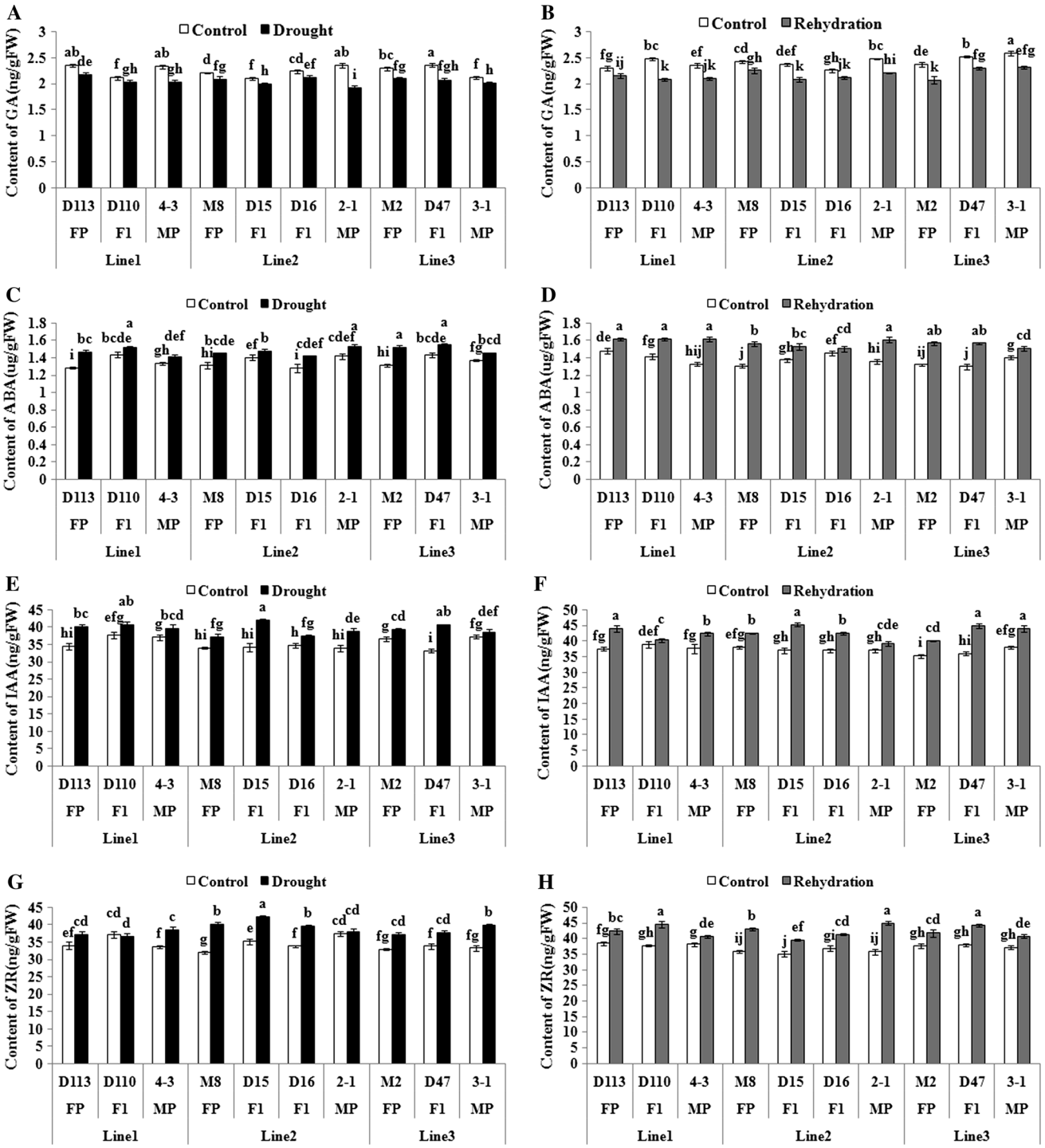

Fig. 6 The effect of drought stress and rehydration on endogenous phytohormone. The GA, ABA, IAA and ZR contents of both treatment and control groups were measured after drought stress and rehydration. **a**, **b** Mean of GA contents, **c**, **d** mean of ABA contents, **e**, **f** mean of IAA contents, **g**, **h** mean of ZR contents. *Each data point* represents the average of three replicates, and three seedlings were used for each experiment. *Error bars* represent mean ± SE. *Different lowercase letters* indicate significant differences at $P < 0.05$

In contrast, the endogenous GA content significantly decreased under drought stress. After drought stress, the concentration of GA was decreased by 14.92 % in the leaf tissue of the hybrid (D110) seedling group from Line1 compared to the control, which was significantly lower than that of its female parent (D113) seedling group and reduced 10.6 % compared to its female parent (D113) seedling group. Similarly, the hybrid (D15) from Line2

accumulated lower GA level than its female parent (M8). The GA concentrations of all seedlings were texted also showed significant reductions after rehydration. Correspondingly, the hybrid (D110) from Line1 and hybrid (D15) from Line2 accumulated lower GA levels than their female parents, respectively.

IAA and ZR concentrations were also measured in the leaf tissues of each hybrid combinations. The patterns of IAA and ZR concentrations under water-stressed condition were similar to the ABA concentration, which was increased under drought stress and rehydration treatment. The hybrid (D15) from Line2 and hybrid (D47) from Line3 accumulated higher IAA levels than their female parents, respectively, both after drought stress and rehydration. Moreover, the hybrid (D15) from Line2 accumulated higher ZR levels than both its parents after drought stress, while the hybrid (D110) from Line1 and the hybrid (D47) from Line3 accumulated higher ZR levels than both their parents, respectively, after rehydration.

Expressions of circadian clock genes and ABA-related genes

As core components of circadian system, LATE ELONGATED HYPOCOTYL (*LHY*) and TIMING OF CAB EXPRESSION 1 (*TOC1*), which were oscillating from day to night, were analyzed by quantitative-PCR to determine the expression patterns of circadian clock genes in a 24 h-period (light/dark cycles) under normal circumstances. Expression levels of *LHY* and *TOC1* within a day undergone significant changes, and these changes had obvious differences between hybrid and parents (Fig. 7). *LHY* expression peaked at ZT3 (Zeitgeber time 3), decreased 6 h after dawn (ZT6), and continued declining until dusk (ZT15). Notably, *LHY* was expressed lower in the hybrid than the mid-parent value (MPV) at ZT6–12 and higher than the MPV at dawn (ZT0). *TOC1* expression was inversely correlated with *LHY* expression, suggesting feedback regulation in the hybrid as in their parents. The expression changes of these genes from noon to dusk in the hybrid may alter the amplitude but not the phase of circadian clock. These differences might cause their different performance in responses to environment.

Plants of *Fraxinus* hybrids and the Random Pollination Progeny of their parents were used as the experimental subjects to study the effect of drought stress on the expressions of circadian clock genes *LHY* and *TOC1*, as well as ABA-related genes 9-cis-epoxycarotenoid dioxygenase (*NCED*), PYRABACTIN RESISTANCE 1 (*PYR1*) and SNF1-RELATED PROTEIN KINASE 2.6 (*SnRK2.6*).

The *LHY* expressions of hybrid plants were up-regulated under drought condition, and the increase ratios ranged from 32.7 to 93.5 %. Furthermore, the *LHY* expressions of hybrid plants were higher than the MPV of their parents, and the increase ratio ranged from 22.8 to 90.2 %. In contrast, the *TOC1*expressions of hybrid plants down-regulated under drought condition, and the decrease ratios ranged from 20.9 to 60.1 %. Correspondingly, the *TOC1*expressions of hybrid plants were lower than the MPV of their parents, the decrease ratio ranged from 10.2 to 37.8 %. The down-regulation of *TOC1* and up-regulation of *LHY* were greater in hybrids than parents, indicated

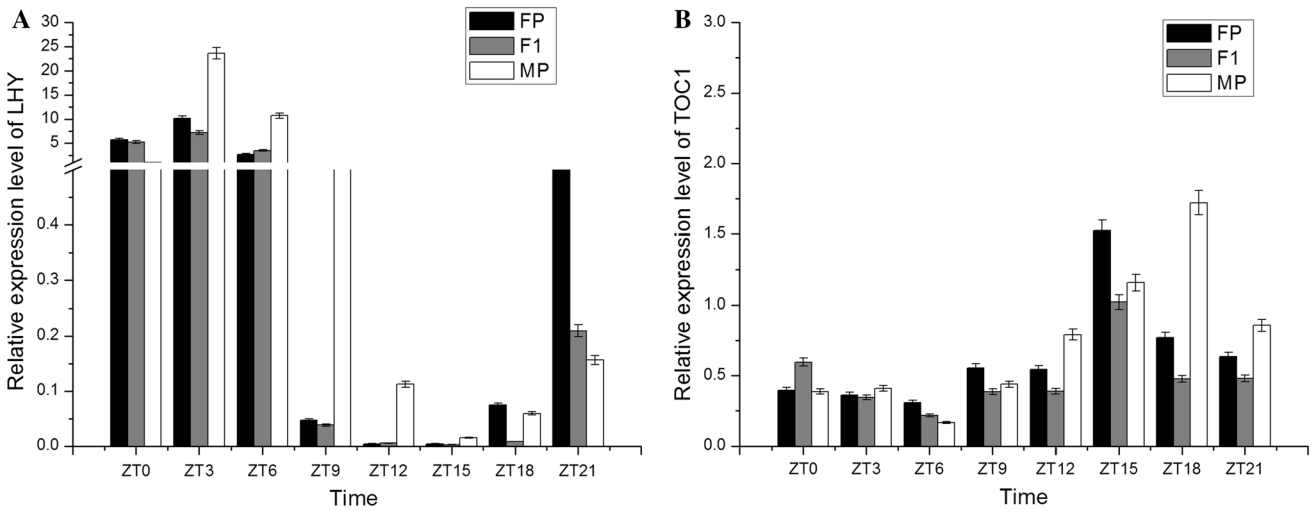

Fig. 7 Diurnal expression patterns of circadian clock genes in seedlings of Line1. Quantitative RT-PCR analysis of *TOC1* (**a**) and *LHY* (**b**) expression (α-Tubulin as a control) in a 24 h period (light/dark cycles) starting from dawn (ZT0, 3:00). FP, Line1 female parents (D113); F1, Line1 hybrid plants (D110); MP, Line1 male parents (4–3). *Each data point* represents the average of three replicates, and three seedlings were used for each experiment. *Error bars* represent mean ± SE

that altered amplitude of circadian gene expression in hybrids under drought condition might be a possible molecular mechanism for drought-advantage in hybrids (Fig. 8).

The expressions of *NCED*, *PYR1* and *SnRK2.6* in the hybrid (D110) from Line1 and the hybrid (D16) from Line2 were up-regulated under drought condition, and the increase ratios were 108.3 and 54.0, 74.2 and 167.3, 56.1 and 63.8 %, respectively (Fig. 9). Meanwhile the expressions of *NCED*, *PYR1* and *SnRK2.6* in the hybrid (D15) from Line2 and the hybrid (D47) from Line3 were down-regulated under drought condition, and the decrease ratios were 35.0 and 34.0, 43.3 and 81.3, 8.2 and −10.3 %. Furthermore, the *PYR1* and *SnRK2.6* expression level of D110 and D16 were higher than their female parents, among these the *PYR1* and *SnRK2.6* expressions of D110 were 1.9-fold and 1.1-fold compared to its female parent respectively, and the *PYR1* and *SnRK2.6* expressions of D16 were 6.5-fold and 2.0-fold compared to its female parent respectively. Combined with the physiological response of each hybrid under drought stress, the differential expressions of these ABA-related genes between different hybrid combinations might be a possible molecular mechanism for drought-advantage in hybrids, these more sensitive responses of *NCED*, *PYR1* and *SnRK2.6* expressions in hybrid D110 and D16 compared to their parents and other hybrids, along with the higher levels of *PYR1* and *SnRK2.6* expressions might coursed the fine drought physiological responses.

Discussion

We have characterized *Fraxinus* hybrids physiology and gene expression in response to drought stress by using seedlings of several hybrid combinations displaying different adaptability to drought stress. Differences between the hybrid plants and their parents (especially female parents due to the low survival rate of male parents) on the physiological responses to drought and on the recovery following re-watering were found in this study. In our experimental conditions the photosynthesis was significantly affected in most hybrid combinations under drought stress. Hybrids from different hybrid combinations had different performances in photosynthetic parameters in response to drought stress and on the recovery following re-watering. It was possible that the osmotic adjustment capacity has contributed to the maintenance of P_n, g_s and E values in the hybrid (D110) from Line1 under drought stress, as previously study showed that D110 turned to be the most drought resistant hybrid (Dong 2012).

Water deficit caused oxidative damage in plant cells. Overproductions of ROS extremely oxidize biological molecules (DNA, RNA, proteins and lipids), inactivate enzymes and decrease synthesis rate of proteins (Csiszar et al. 2012). In this study, SOD and POD activities were increased by drought stress as well as MDA content compared with that of the control group. The magnitudes of increases in the activities of SOD and POD in hybrid D110 and D16 were higher than that in their female parent seedling groups. Conversely, the magnitudes of increases in MDA contents in hybrid D110 and D16 were lower than that in female parent seedling groups. Our results demonstrated that hybrid D110 and D16 promote antioxidant enzymes activities to eliminate ROS and hybrids could decrease content of MDA to alleviate drought-induced oxidative damage.

As a generic stress hormone with multiple functions, ABA played an important role in drought adaptation. Previous studies had shown that endogenous ABA content increases rapidly under water stress, which enhances

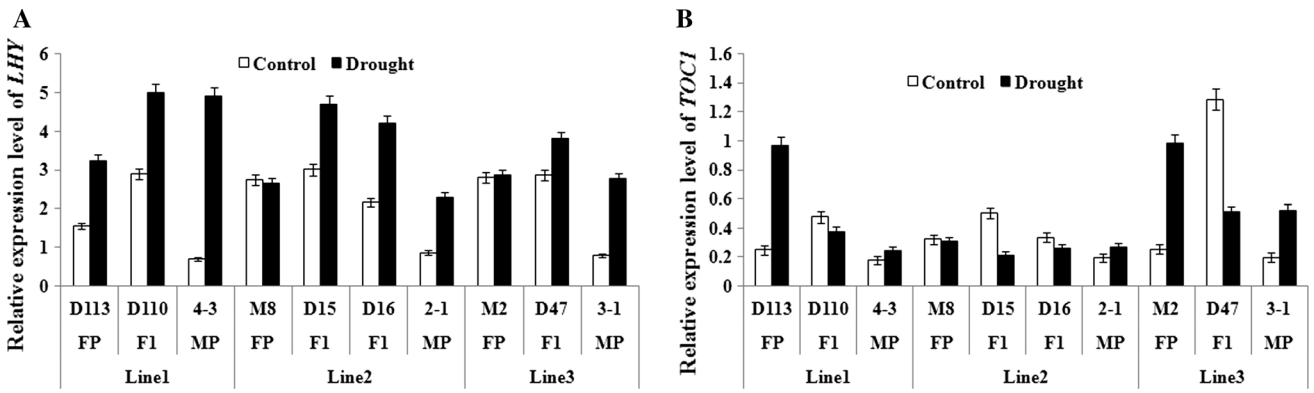

Fig. 8 The effect of drought stress on expressions of circadian clock genes. The expressions of circadian clock genes *TOC1* (**a**) and *LHY* (**b**) of both treatment and control groups were analyzed by quantitative RT-PCR (α-Tubulin as a control) during daytime (ZT6, 9:00) after drought stress. *Each data point* represents the average of three replicates, and three seedlings were used for each experiment. *Error bars* represent mean ± SE

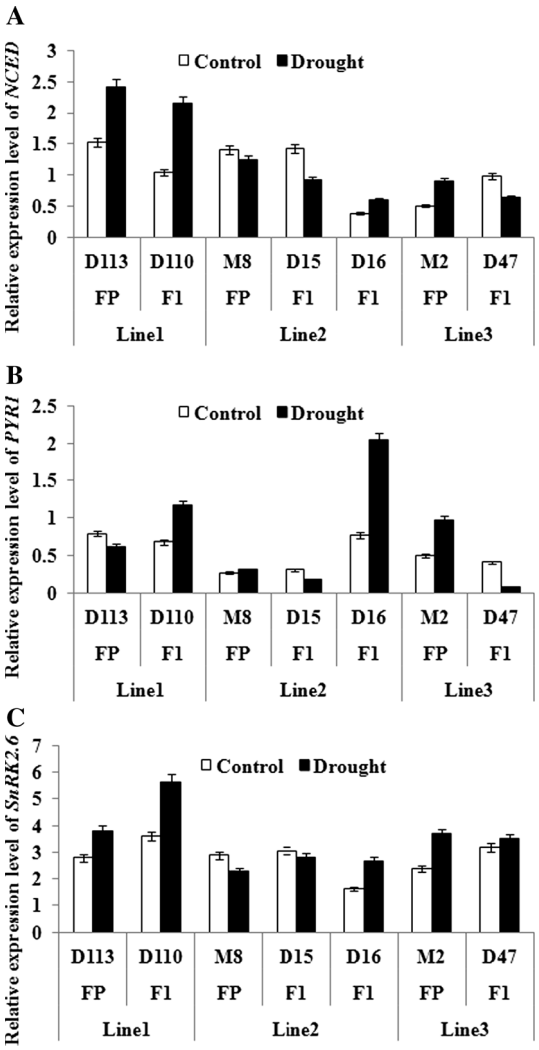

Fig. 9 The effect of drought stress on expressions of ABA-related genes. The expressions of ABA-related genes *NCED* (**a**), *PYR1* (**b**) and *SnRK2.6* (**C**) of both treatment and control groups were analyzed by quantitative RT-PCR (α-Tubulin as a control) during daytime (ZT6, 9:00) after drought stress. *Each data point* represents the average of three replicates, and three seedlings were used for each experiment. *Error bars* represent mean ± SE

drought tolerance in woody plants (Zhang et al. 2004; Hu et al. 2013; de Souza et al. 2014). IAA and ZR were major plant growth regulators affecting plant development and they were the predominant auxin and cytokinin in most plants, respectively (Garcia-Martin et al. 2005). In this study, the relationship between phytohormones and drought tolerance has been demonstrated in *Fraxinus* hybrids and their parent seedling groups. Our results showed that the changing pattern of GA was quite opposite to the one of ABA (Fig. 6), consistent with reports on the antagonist effect between ABA and GA. After exposure of seedlings of each hybrid combinations to drought stress, the ABA, IAA and ZR accumulated in the leaves. Our results regarding ABA increases in the leaves corresponded to earlier ABA synthesis in leaves in response to drought stress, which caused stomatal closure and, consequently, a reduction of leaf air exchange (Maggio et al. 2007; Toshihide et al. 2013). The relationship between the increased ABA content and the higher levels of photosynthesis maintained in hybrids was unclear. One possible explanation was that the increased ABA reduced the water loss in leaves and, consequently, a increased water content as potential substrate for photosynthesis. The evidence was that the significant higher RWC levels (Fig. 1) in the hybrids compared to their female parents under drought stress. Differences in endogenous hormones concentrations were observed between different hybrid combinations with different drought resistance. One possible explanation was that the susceptibility to water stress varied with the species of plants. Ability to tolerate drought stress resulted in different responses in different plant species when exposed to stress conditions.

In a world characterized by rhythms of light intensity and temperature cycles, there has been selection for the evolution of an internal clock that optimizes the plant's relationship with the environment (Hotta et al. 2007). Clock-mediated heterosis is probably universal because internal clocks mediate physiological and metabolic pathways in plants and animals. Moreover, this model can be extrapolated to explain superior traits of many other biological pathways (Jeffrey 2010). So we supposed that the drought-advantage in hybrids might involved in circadian clock system. In this study, the diurnal expression patterns of two major circadian gene *LHY* and *TOC1* in hybrid D110 and its parent seedling groups were analyzed, as well as the expression of *LHY* and *TOC1* in each hybrid combinations under drought stress. Quantitative RT-PCR analysis of *LHY* and *TOC1* expression in leaves of hybrids and their parent seedling groups at 9 a.m. showed that *LHY* expression was increased in hybrids while *TOC1* expression was decreased, which was consistent with previously reported inhibitions to each other of these two genes. Previously reported data in *Arabidopsis* (Tommaso et al. 2009) showed that most *TOC1*-ox plants were unable to recover from the drought stress, exhibiting a reduced survival rate (2 %) after re-watering. In contrast, approximately 50 % of the toc1–2 and 52 % of *TOC1* RNAi plants completely recovered the drought whereas WT plants showed intermediate phenotypes and about 15 % recovered after re-watering. The drought-advantage in hybrids might involved in the decreased expression of *TOC1* mediated by the increased expression of *LHY* at daytime in response to drought stress.

In this study, the expressions of ABA-related genes were also analyzed in hybrids and their female parent seedling groups under drought stress. As key genes of the ABA

pathway, these ABA-related genes which analyzed in this study played positive regulatory role in drought resistance in plant (Boneh et al. 2012). The expression of *NCED*, which encode a key enzyme in ABA biosynthetic pathway, increased in hybrid D110 with a greater rate compared to that in its female parent seedling groups under drought stress. This result described above corresponded to the data of ABA contents measured before. *PYR/PYL–PP2C–SnRK2* pathway is a major signaling network in plants under the drought condition (Fujita et al. 2011). A similar response of *PYR1* and *SnRK2.6* expressions to drought stress was found in *Fraxinus* hybrid D110. These results demonstrated that the increased expressions of *PYR1* and *SnRK2.6* in hybrid D110 compared to that in its female parent seedling groups might result in a more efficient signal transduction pathway and adaptation to drought stress.

In short, the molecular mechanism for drought-advantage in *Fraxinus* Interspecific hybrid might related to the altered circadian gene expressions in hybrids compared to their parents, inactivated the expressions of downstream genes including ABA signal transduction related genes, further regulated differential expressions of drought-related genes between hybrid and its parents, resulted in the different physiological responses between hybrids and their parent, generated the drought-advantage in *Fraxinus* interspecific hybrids.

This study might provide some insights into molecular mechanisms of drought resistance. But there were still more work to be done to explore the interactions between circadian genes and the downstream genes including ABA-related genes.

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