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Full Length Research Paper

Different patterns of transcriptomic response to high temperature between diploid and tetraploid *Dioscorea zingiberensis* C. H.

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Polyploidy is an important evolutionary force in plants and may have significant impact on plant breeding. In this study, expression changes between diploid and tetraploid *Dioscorea zingiberensis* C. H. under control and high temperature conditions were investigated by sequence-related amplified polymorphism (SRAP)-cDNA display approach. Up to 2.7% of the expression changes induced by genome doubling were detected in the tetraploid *D. zingiberensis* relative to its diploid progenitor. Under high temperature stress, a "random transcriptome response" pattern employed with 6.3% of the expression changes were detected in diploid plants, while, an "activation transcriptome response" pattern developed with 6.9% expression changes were detected in tetraploid plants. This result indicated that there might be ploidy dependent pattern of transcriptomic response to high temperature environment, which might contribute to the evolutionary success of polyploids.

Key words: *Dioscorea zingiberensis* C. H., high temperature, polyploidy, sequence-related amplified polymorphism -cDNA.

INTRODUCTION

Dioscorea zingiberensis C. H. Wright belonging to the genus of *Dioscorea* is mainly distributed in the mountain areas of southern China. Previous investigations have identified that the rhizome of *D. zingiberensis* contains a high concentration of diosgenin, which is an extremely important basic compound for the synthesis of sex hormones and corticosteroids in the pharmaceutical industry (Ding et al., 1981; Sautour et al., 2007). The *D. zingiberensis* is not only a dominant natural resource for the production of diosgenin but also a traditional Chinese herb with curative effects of alleviating the symptoms of cough and pain, in detoxification, and in reducing swelling (Huang et al., 2010). For its economic importance, *D. zingiberensis* has been over-intensively harvested in the past, which resulted in rapid extinguish of its wild populations. Now, *D. zingiberensis* has been cultivated on scale for diosgenin production. However, it has been found that field grown *D. zingiberensis* plants are easily affected by various biotic and abiotic stresses (Liao et al., 2004; Zhang et al., 2005; Zhu et al., 2009). High temperature which results in damage of photosystem and sexual reproduction is an important limiting factor for crop production (Allakhverdiev et al., 2008; Zinn et al., 2010). Previous studies have shown that both temporary and long period exposure of *D. zingiberensis* to high temperature will result in physiological injures and production loss (Zhang et al., 2005, 2010).

The vulnerability of *D. zingiberensis* will be increased with a projected global average surface temperature increase of 2.0 to 4.5°C and the possibility of increased variability about this mean by the end of this century (IPCC, 2007). Hence, in the future, *D. zingiberensis* will be grown in a warmer environment. To improve the high temperature tolerance of *D. zingiberensis* and to investigate its mechanism are of profound significance. Polyploidy is well known to have a better chance to survive under various stress and is also considered as a

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useful approach for developing superior varieties in plant breeding (Dubcovsky and Dvorak, 2007; Fawcett et al., 2009; Udall and Wendel, 2006). For it is a high content in secondary metabolites, tetraploid *Isatis indigotica* Fort. (Lu et al., 2006), *Datura stramonium* (Berkov, 2001) and *Atropa belladonna* (De Jesus-Gonzalez and Weathers, 2003) have been successfully used in medical plant production. It is interesting that tetraploid *Zingiber officinale* (Sanwal et al., 2010; Shang et al., 2003) and tetraploid Chinese cabbages (*Brassica rapa* L.) (Liu et al., 2002) possess higher high temperature tolerance than their corresponding diploids. Recent studies also demonstrated that tetraploid *D. zingiberensis* possess a higher antioxidant defense system and increased heat tolerance than those of the diploid progenitors (Zhang et al., 2005, 2010). Polyploidy often leads genome wide changes in genetic structure, epigenetic patterns and gene expression, which have been observed in *Arabidopsis* (Beaulieu et al., 2009; Madlung et al., 2002), *Brassica* (Gaeta et al., 2007; Xu et al., 2009), *I. indigotica* (Lu et al., 2006), *Paspalum notatum* (Martelotto et al., 2005), *Eragrostis curvula* (Cervigni et al., 2008), potato (*Solanum phureja*) (Stupar et al., 2007) and other polyploids (Doyle et al., 2008; Parisod et al., 2010).

Whether or not genome doubling would cause some transcriptional changes in tetrapoid, *D. zingiberensis* is still an open question. Furthermore, expression partitioning between duplicated genes were observed in cotton polyploid under abiotic stress (Liu and Adams, 2007). Previous study displayed different antioxidant defense system between tetraploids and diploid *D. zingiberensis* (Zhang et al., 2010) which indicated possible changes in gene expression between diplods and tetraploids under high temperature stress. It is very interesting to compare the patterns of transcriptomic response to high temperature between diploid and tetraploid *D. zingiberensis*. The objectives of this study were: (i) to shed light on how genome doubling affects gene expression in *D. zingiberensis* by comparing expression changes between diploid and tetraploid plants; (ii) to see whether there is ploidy specific transcriptomic response pattern under high temperature in *D. zingiberensis*.

MATERIALS AND METHODS

Plant materials

The seeds of *D. zingiberensis* were obtained from Shiyan mountain area, Hubei province, China. The seeds were surface sterilized with 0.1% (w/v) $HgCl_2$ for 5 min and then washed thoroughly with distilled water three times. The sterilized seeds were germinated for 7 days in the dark at 25 ± 1°C on MS medium (Murashige and Skoog, 1962), then incubated under a 16 h photoperiod with 1200 Lux light intensity for 7 days. The seedings were then transferred to shoot multiplication medium, which consisted of MS medium supplemented with 1.0 mg/L benzyladenine and 0.2 mg/L naphthalene acetic acid (NAA). After about 45 days, the bud clusters were excised from *in vitro* grown cultures and transferred into liquid MS medium supplemented with 2.0% (w/v) dimethyl sulfoxide (DMSO) and 0.2% colchicine (w/v) for 24 h. The diploid control was handling in the same fashion without colchicine treatment. Buds were washed three times with sterile distilled water and transferred to the shoot multiplication medium for 2 weeks. Then, all of the living buds were placed on the rooting medium (1/2 MS medium supplemented with 2.0 mg/L indole butyric acid and 0.4 mg/L NAA) for 30 days.

Flow-cytometric analysis of ploidy level

Flow-cytometric analysis was performed according to published method (Li et al., 2005). Leaves were collected from colchicines treated samples, finely chopped with a sharp sterile scalpel blade in a Petri dish containing 0.5 ml ice-cold freshly prepared nucleus isolation buffer containing 10 mM $MgSO_4$, 50 mM KCl, 5 mM Hepes, 1 mg/ml dithiothreitol (DTT), 30 mg/ml propidium iodide (PI) and 0.2% Triton X-100. The suspension of nuclei was filtered through a 30 mm nylon mesh. RNase A was added to the suspension to the final concentration of 10 µg/ml for 30 min at 37°C. The colchicines untreated samples were used as control. The ploidy level was determined with a FACSC alibur flow cytometry (Becton Dickinson, Franklin Lakes, NJ) equipped with an argon-ion laser, using the 488 nm laser line for excitation. CellQuest and Modfit softwares were used for data acquisition and data analysis. For each analysis, three replicates were carried out.

High temperature stress treatment

Rooted tetraploid and diploid plantlets were planted on sterile soil in a growth chamber at 25 ± 1°C/ 20 ± 1°C (day/night) under a 16 h photoperiod with 1200 Lux light intensity. After approximately 60 days, healthy and consentaneous developmental plantlets were used for high temperature treatment. The aforementioned growth condition was used as control. The high temperature treatment was performed in a growth chamber at 39 ± 2°C/ 30 ± 2°C (day/night) for 24 h with the same photoperiod and light intensity as control. After the high temperature stress, leaves were immediately collected from the treated plants. The treatment was performed for three replicates and three individuals were used for each replicate.

DNA and RNA extraction and cDNA synthesis

Total DNA was isolated from leaves of control and treated plants using the CTAB procedure. Total RNA was extracted from leaves of treated and control plant using TRIzol reagent (Invitrogen, Carlsbad, CA). Before reverse transcription, total RNA was treated with RNase-free DNase I (Promega, Beijing, China) at 37°C for 30 min to avoid genomic DNA contamination. First-strand cDNA was synthesized from total RNA using oligo-$(dT)_{18}$ primer and reverse transcriptase (RT) SuperScript (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. Second-strand cDNA was synthesized using 10 U DNA polymerase I (Takara, Dalian, China) and 3 U RNase H (Takara, Dalian, China) according to standard protocols (Sambrook et al., 2001). The resulting double-stranded cDNA was purified by phenol–chloroform extraction and ethanol precipitation, and resuspended in a final volume of 40 µl ddH_2O. Half of this volume was checked on an agarose gel.

If the expected smear between 100 and 2000 bp was observed, the rest of the cDNA was stored at -20°C for future use.

SRAP-cDNA and SRAP analysis

The sequence-related amplified polymorphism (SRAP)-cDNA and

Table 1. Sequences of primers used for SRAP-cDNA and SRAP analyses.

Primer name	Sequence (5' to 3')
SRAP-F1	TGAGTCCAAACCGGATA
SRAP-F2	TGAGTCCAAACCGGAGC
SRAP-F3	TGAGTCCAAACCGGAAT
SRAP-F4	TGAGTCCAAACCGGTCC
SRAP-F5	TGAGTCCAAACCGGTGC
SRAP-F6	TGAGTCCAAACCGGCTT
SRAP-F7	TGAGTCCAAACCGGCAG
SRAP-R1	GACTGCGTACGAATTAAT
SRAP-R2	GACTGCGTACGAATTTGC
SRAP-R3	GACTGCGTACGAATTGAC
SRAP-R4	GACTGCGTACGAATTTGT
SRAP-R5	GACTGCGTACGAATTAGC
SRAP-R6	GACTGCGTACGAATTCGA
SRAP-R7	GACTGCGTACGAATTGGT

SRAP display technique was carried out following published method (Li and Quiros, 2001). Equal amounts cDNA or DNA from three different individuals per each treatment (or control) was mixed, respectively. The amplification step was carried out with 30 ng cDNA or DNA as template, 0.2 µM primers, in a final volume of 10 µl containing 1 x PCR buffer, 2.0 mM $MgCL_2$, 0.2 mM dNTP, and 1 U *Taq* polymerase (Fermentas, Vilnius, Lithuania). The PCR reactions were performed with the following program: the first five cycles were run at 94°C, 1 min, 35°C, 1 min and 72°C, 1 min, for denaturing, annealing and extension, respectively, then the annealing temperature is raised to 50°C for another 35 cycles. The sequences of primers are listed in Table 1. To ensure that there is no DNA contamination in our RNA samples, a negative control was prepared without reverse transcriptase (RNA samples treated with RNase-free DNase I). A clear SRAP-cDNA gel with no bands was obtained. For all primer combinations, a non-template control was included to rule out the presence of any unwanted bands caused by primer dimers or contamination.

PCR products were mixed with 10 µl of formamide dye (98% formamide, 10 mM EDTA, 0.05% w/v bromophenol blue and xylene cyanol), denatured at 95°C for 5 min and separated by electrophoresis on 6% denaturing polyacrylamide (37.5:1 acrylamide: bisacrylamide, 7.5 M urea, and 1 × Tris-borate-EDTA buffer, pH 7.8). The gels were pre-run at 100 W for about 30 min before 8 µl of the mix was loaded, and run at 65 W for about 2 h, then silver-stained according to the DNA silver staining system procedure. Each pair of primer combination was run for three biological replicates (cDNA from three independent treatment and control). Only clear and reproducible bands between the three replicates were used for scoring. Moreover, the upper part and the lower part of the SRAP-cDNA gel, where resolution is not satisfactory, was not used for band scoring. The scored SRAP-cDNA bands were transformed into a binary character matrix, using "1" and "0" to indicate the presence and absence of a band at a particular position, respectively. Chi square test of independence was carried out by SPSS 10.0 software.

RESULTS

Ploidy level determined by flow cytometry

Clearly defined histograms were obtained following flow cytometric analysis of intact leaf nuclei. The ploidy level was estimated by comparing the mean fluorescence intensity of nuclei of sample material with that of the diploid controls. Diploid possessed a small percentage of nuclei with a tetraploid complement of DNA (Figure 1a), which represents nuclei at the G2 or M phase of the cell cycle. The fluorescence intensity of the flow peak of the tetraploids was corresponding to the G2 or M phase cells of the diploids, which confirmed the ploidy level of the tetraploid plants. Similarly, tetraploids (Figure 1b) possessed octaploid nuclei, which represent nuclei at the G2 or M phase of the cell cycle.

Expression band patterns of SRAP-cDNA

To investigate how genome doubling and high temperature affects gene expressions in *D. zingiberensis*, SRAP-cDNA analysis was performed on the diploid and tetreaploid plants grown in both the control and treated environments. The band patterns in the tetraploids were expected to be similar to the diploids, and all cases of deviation from such additivity were scored as expression changes induced by genome doubling. And the band patterns in the diploids and the tetraploids under high temperature were expected to be similar, any deviation from this additivity were regarded as ploidy specific response to high temperature stress. From 49 primer combinations, 19 primer combinations (Table 2), which display consistent amplifications and clear banding patterns were selected for SRAP-cDNA analysis. Representative examples of SRAP-cDNA profiles are shown in Figure 2. The frequency of each band pattern calculated for the diploid in the control environment (DC), diploid in the high temperature (DH), tetraploid in the control environment (TC) and tetraploid in the high temperature (TH) are listed in Table 2. In all, 14 types of

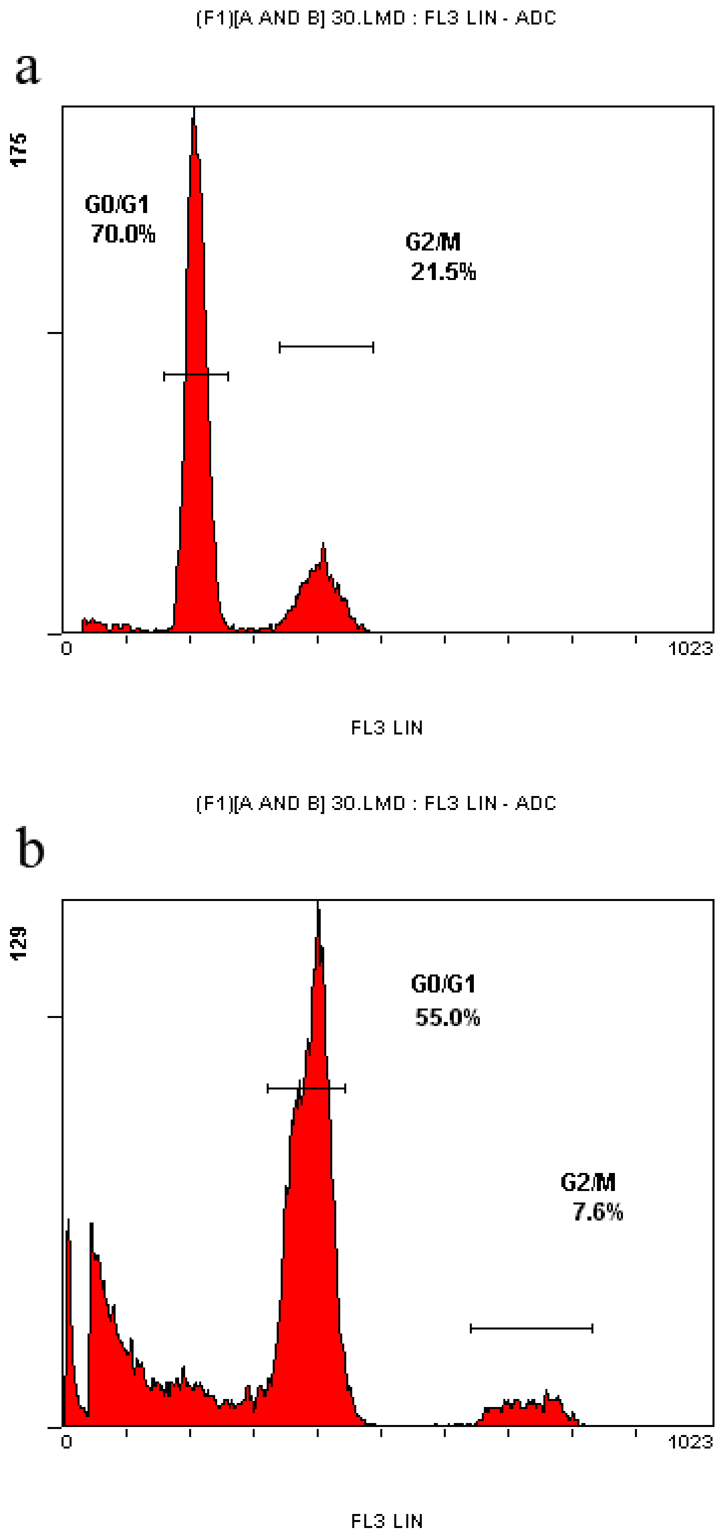

Figure 1. Flow cytometric detection of the ploidy of nuclei in *D. zingiberensis* leave cells. a) DNA-histograms of nuclei in diploid *D. zingiberensis*; b) DNA-histograms of nuclei in tetraploid *D. zingiberensis*.

SRAP-cDNA band patterns were detected (Table 2).

874 bands were monomorphic (A type), which indicated that their expression was not affected by genome doubling and high temperature stress; 13 bands (B type)

Table 2. Frequencies of SRAP-cDNA band patterns in diploid and tetraploid *D. zingiberensis* under control and high temperature conditions.

Parameter	Band pattern*	Type	Number of band for each primer combination																			Total
			F1R 2	F1R 3	F2R 3	F2R 5	F2R 6	F3R 1	F3R 5	F3R 6	F4R 3	F4R 6	F5R 3	F5R 6	F5R 7	F6R 2	F6R 6	F7R 1	F7R 3	F7R 5	F7R 6	
Additive bands	1111	A	46	45	48	47	39	42	48	45	43	46	46	44	52	50	43	47	42	48	54	875
Consensus changes to HT	1010	B	1	0	2	0	0	1	0	0	1	1	1	1	0	1	1	0	1	1	1	13
	0101	C	1	1	1	1	2	0	2	3	2	0	0	1	1	0	1	1	0	2	2	21
Diploid specific changes to HT	1011	D	1	0	0	1	1	0	1	2	0	0	2	2	0	1	0	1	0	0	1	13
	0100	E	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	1	4
Tetraploid specific changes to HT	1110	F	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	3
	0001	G	2	1	1	1	0	1	0	1	1	1	0	0	2	0	1	0	2	1	0	15
Genome doubling induced silence	1100	H	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	2
	1101	I	1	0	2	0	1	0	1	1	0	0	0	0	0	1	0	0	0	0	0	7
	1000	J	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	2
	1001	K	0	0	0	1	0	0	1	0	0	0	0	1	0	0	0	0	1	0	0	4
Genome doubling induced activation	0011	L	0	1	0	0	1	0	0	0	0	0	1	0	0	1	0	0	0	0	0	4
	0111	M	0	0	1	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	3
	0010	N	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	3
			52	50	56	52	44	46	53	52	49	49	50	49	55	54	47	50	48	53	60	969

*Band patterns in SRAP-cDNA gels as follows: diploid in the control environment (DC); diploid in the high temperature (DH); tetraploid in the control environment (TC); tetraploid in the high temperature (TH).

were found silenced and 21 bands (C type) were found activated after high temperature treatment in both the diploids and the tetraploids; 13 bands (D type) were found silenced and 4 bands (E type) were found activated after high temperature treatment only in the diploids, which indicated diploid specific response to heat stress; 3 bands (F type) were found silenced and 15 bands (G type) were found activated after high temperature treatment only in the tetraploids, which indicated tetraploid specific response to heat stress. The band patterns of H, I, J and K type referring bands were silenced in the tetraploids caused by genome doubling, and then keep silence or activation under high temperature stress. The band patterns of L, M and N type referring genome doubling induced novel expression in the tetraploids which have a variety of fates under high temperature stress.

Changes in expression between diploid and tetraploid *D. zingiberensis*

25 of 928 (2.7%) bands were changed in expression after genome doubling with 15 bands that were silenced, 10 bands were activated in the teraploids relative to the diploids in the control conditions. While, 45 of 950 (4.7%) bans were changed in expression in the tetraploids relative to the diploids under high temperature stress, with 9 bands silenced and 36 bands were activated. It is very interesting that the divergence of the transcriptome between diploid and tetraploid *D. zingiberensis* under high temperature treatment is much more serious than that in control condition (X^2 = 4.97, P < 0.05). Transcriptional responses to high temperature stress were also compared between diploid and tetraploid *D. zingiberensis*. In

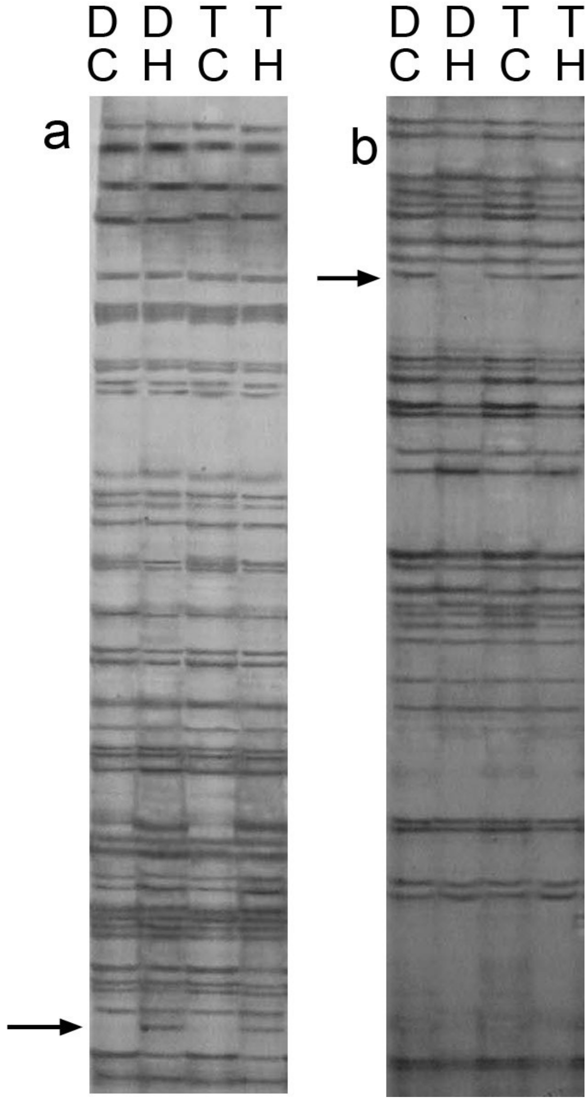

Figure 2. SRAP-cDNA profiles in diploid and tetraploid *D. zingiberensis* under control and high temperature conditions. DC lane: diploid in control condition; DH lane: diploid under high temperature stress; TC lane: tetraploid in control condition; TH lane: tetraploid under high temperature stress. (a) Expression change of C type (primer combination F7R1); (b) expression change of D type (primer combination F5R6).

the diploids, 60 of 946 (6.3%) bands were changed in their expressions, among them, 32 bands were silenced and 28 bands were activated. In the tetraploids, 66 of 960 (6.9%) bands were changed in their expressions, among them, 19 bands were silenced and 47 bands were activated. The total level of changes of transcriptome response to high temperature stress was similar (X^2 = 0.12, P = 0.73) between diploid and tetraploid *D. zingiberensis*. However, significant different transcriptomic response patterns were detected between diploid and tetraploid *D. zingiberensis*.

In the diploids, expression silence and activation were equally affected (X^2 = 0.33, P = 0.86) by high temperature stress, which indicated a "random transcriptome response" pattern was employed in diploid against high temperature. While, in the tetraploids, more activation than silence expression changes (X^2 = 9.71, P = 0.002) were detected, which indicated an "activation transcriptome response" pattern was developed by tetraploid against high temperature.

No genetic changes were detected in tetraploid by SRAP

To assess whether expression changes in this study were correlated with genetic alterations, or not, SRAP analysis was carried out with the same primer combinations as used in SRAP-cDNA analysis in diploid and tetraploid *D. zingiberensis*. A total of 1054 bands were obtained without bands missing or novel appearance in the tetraploids. These results indicated that almost no genetic changes occurred after genome doubling in tetraploid *D. zingiberensis*.

DISCUSSION

Effect of genome doubling on gene expression

In this study, up to 2.7% of the expression changes were detected in the tetraploid *D. zingiberensis*. Similiar phenomena have been reported in autopolyploids of *Arabidopsis* (Wang et al., 2006), maize (Riddle et al., 2010), *I. indigotica* (Lu et al., 2006), *P. notatum* (Martelotto et al., 2005), *E. curvula* (Cervigni et al., 2008) and potato (*S. phureja*) (Stupar et al., 2007). Previous researches have confirmed that polyploidization effects were caused by genome doubling itself rather than by colchicines treatment (Lukens et al., 2006; Ozkan et al., 2001). Moreover, genome doubling was also reported to have effect upon gene expression changes in allopolyploids of *Senico* (Hegarty et al., 2006) and *Brassica napus* (Xu et al., 2009). Collectively, all these data supported that genome doubling per se should be responsible for some expression changes in newly formed polyploids. The plastic nature of transcriptome regulation might be programmed responses to polyploidization, and may be advantageous for adaptation and rapid establishment of successful polyploids (Doyle et al., 2008; Parisod et al., 2010). Genetic alterations have been observed in many synthetic autopolyploids (Parisod et al., 2010), which may be one of the reasons for transcription changes. If the majority of the transcription changes were caused by genetic alterations, SRAP analysis with the same primer combinations are expected to have a similar level of deviate from Mendelian expectation.

By contrast, in this study, no genetic alterations were observed in tetraploid *D. zingiberensis* by using the same primer combinations with SRAP analysis. This indicated that expression changes in tetraploid *D. zingiberensis* were not caused by genetic changes. While, epigenetic regulation mechanism, RNAi mechanism, and other post transcriptional regulation mechanism might play an important role for gene regulation in the tetraploids (Doyle et al., 2008; Parisod et al., 2010; Urano et al., 2010; Yang et al., 2010).

Ploidy dependent pattern of transcriptomic response to high temperature

High temperature induced up- and down-regulation gene expression changes have been observed in many plants (Hu et al., 2009; Urano et al., 2010). Similar phenomenon was observed in this study; about 6.3 and 6.9% expression changes were detected in diploids and tetraploids *D. zingiberensis* under high temperature stress. It is very interesting that the "random transcriptome response" pattern was employed in diploid, while, the "activation transcriptome response" pattern was developed by tetraploid against high temperature. This indicated there might be ploidy dependent pattern of transcriptomic response to high temperature stress. Despite the difference between diploid and tetraploid, plants is significance in statistics analysis, it should be noted that the number of different expressed bands detected in this study is relatively small. The point of ploidy dependent pattern of transcriptomic response to high temperature stress should be further tested on a large scale. Previous research in cotton has showed evidence for partitioning of homeologous-gene expression in response to abiotic stress (Liu and Adams, 2007). All these data indicated that rapid neofunctionalization and subfunctionalization of some duplicated genes might be evoked by stresses, which supported the hypothesis that additional set(s) of genomes may free some genes from the pressure of natural selection and allow them to develop separate functions.

The observed ploidy dependent pattern of transcriptomic response might be part of the transcription reason for high temperature tolerance in tetraploid *D. zingiberensis* reported in previous studies (Zhang et al., 2005, 2010). However, further more researches should be carried out to test this hypothesis and to develop the possibility approach by using ploidy dependent pattern of transcriptomic response to improve the high temperature tolerance of plants.

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