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Induction, identification and genetics analysis of tetraploid *Actinidia chinensis*

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Actinidia chinensis is a commercially important fruit, and tetraploid breeding of *A. chinensis* is of great significance for economic benefit. In order to obtain elite tetraploid cultivars, tetraploid plants were induced by colchicine treatment with leaves of diploid *A. chinensis* 'SWFU03'. The results showed that the best treatment was dipping leaves 30 h in 60 mg l⁻¹ colchicine solutions, with induction rate reaching 26%. Four methods, including external morphology comparison, stomatal guard cell observation, chromosome number observation and flow cytometry analysis were used to identify the tetraploid of *A. chinensis*. Using the induction system and flow cytometry analysis methods, 187 tetraploid plants were identified. Three randomly selected tetraploid plants and their starting diploid plants were further subjected to transcriptome analysis, real-time quantitative polymerase chain reaction (RT-qPCR) and methylation-sensitive amplification polymorphism (MSAP) analysis. The transcriptome analysis results showed that there were a total of 2230 differentially expressed genes (DEG) between the diploid and tetraploid plants, of which 660 were downregulated and 1570 upregulated. The DEGs were mainly the genes involved in growth and development, stress resistance and antibacterial ability in plants. RT-qPCR results showed that the gene expression levels of the growth and stress resistance of tetraploid plants were higher than those of diploid ones at the transcriptome level. MSAP analysis of DNA methylation results showed that tetraploid plants had lower methylation ratio than diploid ones. The present results were valuable to further explore the epigenetics of diploid and tetraploid kiwifruit plants.

1. Introduction

Actinidia chinensis is a commercially important fruit, and its chromosomes are small, but a lot [1]. It seems to have a lot of ploidy level variation [2,3], including from diploid to octoploid and there may be chromosome races within individual taxa [4]. The basic chromosome number of *A. chinensis* is 29, and most of the *A. chinensis* plants are diploid ($2n = 2x = 58$). It is hard to find tetraploid, hexaploid and octoploid plants, yet, they do exist in nature [1,4,5]. Differences in ploidy level have made hybridization difficult due to infertility of polyploidy; however, it can offer significant opportunities for creating novel types [6]. Tetraploid fruit trees usually have better stress resistance, bigger fruit and faster growth speed compared to diploid ones [6–8], hence induction and identification of tetraploids are very important for fruit tree breeding. Researchers had attempted ploidy level manipulation on *A. chinensis* [6–8].

Treatment of plant tissues with colchicine is the most commonly used method for inducing tetraploidy [9,10]. In practice, colchicine induction of tetraploidy was commonly used with immersion or dropping method [11,12]. *In vitro* induction method has a lot of advantages, not only experimental conditions can be artificially controlled, easy operation, low cost, but also the results of the experiment can be replicated [13].

Current common methods for ploidy identification were chromosome counting [14,15] and flow cytometry testing [16,17]; external morphology observation [18,19] and stomatal guard cells microscopic observation were auxiliary appraisals. Although these methods were used widely, each method has its disadvantages. For example, external morphology observation and stomatal guard cell microscope observation were simple but not accurate enough; chromosome counting was accurate but time-consuming and difficult to conduct; flow cytometry testing was fast and high flux, but just relatively accurate [20].

Transcriptome sequencing (RNA-seq) is based on the whole genome of high-throughput sequencing technologies [21,22]. RNA-seq is a modern tool of transcriptome analysis. A new generation of sequencing technology can be used for fast and accurate transcriptome profiling [23]. This technology can detect any species of the overall transcription activity; based on the analysis of the transcriptome, gene structure and expression level can be described. This method can also detect novel transcript and rare transcript, providing an experimental study more comprehensive set of transcription information. The results of RNA-seq are highly reproducible, having made RNA-seq an increasingly attractive and popular method for studying transcriptomes [24–27].

Real-time fluorescent quantitative polymerase chain reaction (RT-qPCR) refers to adding fluorescent groups in the PCR reaction system, using the fluorescent signal cumulative to monitor the entire process of PCR. It achieves the leap from qualitiveness to quantitiveness, with strong specificity, high sensitivity, good repeat-ability, accuracy and quickness, having become an important tool in molecular biology research [28,29]. The expression levels of specific genes in transcriptome of some species were successfully confirmed by RT-qPCR [30–32].

DNA methylation has been reported as one of the most common covalent modifications of DNA, and has both epigenetic and mutagenic effects causing specific gene expression, cell differentiation, chromatin inactivation and embryo growth [33,34]. Methylation-sensitive amplification polymorphism (MSAP) combined with the appealing features of amplified fragment length polymorphism (AFLP) is based on the restriction enzyme and PCR amplification, and is highly efficient for large-scale detection of cytosine methylation in the genome [35,36].

Colchicine-induced chromosome-doubling tends to increase stress resistance of plants [37,38]. However, there is no evidence to show if the resistance of *A. chinensis* would increase the gene expression level after chromosome-doubling induction using colchicine or not. To lay the foundation of *A. chinensis* tetraploid breeding and understand what has changed on the gene expression levels in the *A. chinensis* tetraploid, it is necessary to carry out research on the induction, identification and genetics analysis of *A. chinensis* tetraploid plants. Here, we present the results of using young plants of *A. chinensis* 'SWFU03' as materials for inducing tetraploid plants and employing four methods including external morphology comparison, stomatal guard cell observation, chromosome number observation and flow cytometry analysis to identify the tetraploid. Then transcriptome analysis, fluorescence quantitative PCR and MSAP analysis were further employed to access the diploid and tetraploid plants.

2. Material and methods

2.1. Plant materials

Young plants of 'SWFU03' were used as the materials, and leaf blades were used as explants. Leaves were taken from Key Laboratory of Biodiversity Conservation in Southwest China, Southwest Forestry

University. 'SWFU03' is an excellent *A. chinensis* breeding clone selected by Southwest Forestry University. Donor diploid plants was used as references for phenotypic and genetic analysis of resulted tetraploids, and the culture room conditions were as following: the temperature was $25 \pm 1^\circ\text{C}$, humidity was 40–80%, the light intensity was 2000 LX, and lighting time 16 h light/8 h darkness, with the incandescent light source.

2.2. Colchicine treatment

For initiation of *in vitro* culture, leaves were sliced into 1×1 cm pieces on the clean bench, and then were put into sterilized colchicine solution. The six colchicine concentrations were 0, 20, 40, 60, 80 and 100 mg l^{-1} , and treatment times were 6, 12, 18, 24 and 30 h, each treatment time had a control with sterile water treatment. The orthogonal experimental design was employed in the study. Every group had 20 explants, 30 groups in total and three repeats. In total, 500 ml glass bottles with plastic cap were used as culture vessels; every glass bottle had four explants. Using MS medium [39] + 0.5 mg l^{-1} NAA (1-naphthylacetic acid) + 5 mg l^{-1} 6-BA (6-benzyladenine) + sucrose 30 g l^{-1} + agar 5 g l^{-1} , pH 5.8, autoclaved at 121°C for 20 min before use. Randomized block was used to arrange the culture vessels on the culture shelf, after cultured for 30 d, and total contamination rate [(total contamination/total explant) $\times 100\%$], dieback rate [(total dieback/total explant) $\times 100\%$] and polyploidization efficiency [(number of polyploidizations/explant of survival number) $\times 100\%$] were determined.

For subculturing, regeneration and shoot propagation, the medium for regeneration was the same as initiation culture. The medium for shoot propagation was $\frac{1}{2}$ MS + 0.7 mg l^{-1} IBA (3-Indolebutyric acid) + 0.1 g l^{-1} AC (activated carbon) + sucrose 30 g l^{-1} + agar 5.5 g l^{-1} .

2.3. Phenotypic evaluation

Diploid and tetraploid *A. chinensis* plants with the same successive generation were randomly selected. In 10 tetraploid and 10 diploid plants, two leaves were collected from each plant, and a total of 40 leaves were measured. The third and fourth leaves from each plant after subculturing for rooting for one month were sampled. Twenty centimetres of straight edge was used to measure the leaf length and width, respectively, and leaf thickness was measured with a vernier caliper. Finally, the average value was generated and compared.

Fifteen diploid and 15 tetraploid *A. chinensis* leaves were randomly selected from the same successive generation plants. The differences of stomatal density, length, and width of guard cell between diploid and tetraploid leaves were observed using a Leica DMR-X microscope (Leica, Stuttgart, Germany) at $40\times$. Data were analysed using SPSS 17. Descriptive statistics, one-way ANOVA test, and independent *t*-test were used [40].

The root tips of 10 diploid and 10 tetraploid *A. chinensis* plants *in vitro* culture were collected, and put into 0.002 mol l^{-1} 8-hydroxyquinoline solution for 1–3 h, then stored at 4°C for 16 h. Subsequently, they were fixed using a solution of alcohol:glacial acetic acid = 3:1 for 12–24 h, hydrolysed in 15% HCl solution for 3–5 min. Meanwhile, carbonic acid magenta was used for staining 15–25 min and the chromosome number observed using an optical microscope after the roots were placed on the slides. Finally, photos were taken using a camera for counting chromosome number [41,42]. Five cells were used for chromosome counting in each sample.

2.4. Flow cytometry analysis

Fresh leaves of three diploid and 187 *A. chinensis* plants which were suspected to be tetraploid according to the morphological indexes were used as the material for flow cytometry analysis of the relative nuclear DNA content according to a previous report [43]. Samples were calibrated against standards of diploid and tetraploid *A. chinensis* for which the ploidy had been checked previously by morphological indexes like thicker leaves, more leaf pubescence, bigger stems and larger veins. Using *Oryza sativa* as internal standard, and Germany Partec Flow cytometry (CyFlow-Space-3000) for detection, at least 5000 nuclei were collected in each measurement and repeated at least three times for each of the species.

2.5. Transcriptome analysis

To compare the differential expression of genes (DEGs) in three diploid and three tetraploid plants, the fresh leaves were used as the material and next-generation sequencing was employed to access the RNA samples. RNA-seq was performed by Nextomics Biosciences Ltd using Illumina HiSeq™ 2500.

Table 1. Primer sequence of target genes (TG) and reference genes (RG) used in RT-qPCR.

gene name	primer	sequence (5'–3')	length (bp)
elongation factor EC 3.6.5.3 (RG)	forward primer	ACAAGCTGGTGACAATGTGG	127
	reverse primer	CGACCACCTTCATCCTTGT	
β -1,3-glucosidase (TG)	forward primer	ATGCTCGTGACAGGAAACGC	109
	reverse primer	GCAATGCCAATGTAACACCTGC	
triacylglycerol lipase (TG)	forward primer	ATGCTCGTGACAGGAAACGC	124
	reverse primer	GCAATGCCAATGTAACACCTGC	
Achn295071 (TG)	forward primer	CGTCGAAGCAGGGTCATTTA	96
	reverse primer	TTGAGCCTCTGGATTGGTAAAG	
Achn247401 (TG)	forward primer	CTGCCGGAATAACCTAACC	113
	reverse primer	GTGACCAGGAACATGACTATCC	
Achn140601 (TG)	forward primer	CGGTGTTCTCGTGGATGTATAG	117
	reverse primer	CCGCCTTGTCCTTCATCTC	
Achn047951(TG)	forward primer	CTTGAAGGCTCGCTCTATG	103
	reverse primer	TCCCTCTGGGAACACAATG	
Achn048611(TG)	forward primer	GTATTGGAAGGCACGCTCTA	105
	reverse primer	TCCCTCTGGGAACACAATG	
Achn217451(TG)	forward primer	TGAATGTTGGAGACGGAAAGG	109
	reverse primer	TCAGGAACTGATGGTGTGAAG	
Achn125011(TG)	forward primer	TGGGAGGTCACCTACATACTT	94
	reverse primer	CATCGCAAACCTCCAACATTC	
Achn009011(TG)	forward primer	GACACAAAGCTAGGGACGATAC	111
	reverse primer	CTCTCCTTGCTTTAGGCTCTTC	
Achn007531(TG)	forward primer	CTACGGTGTCGTGATCCTAGA	114
	reverse primer	GTCTCACTGCCCTCACAAA	

FastQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) software was used for clean data quality control. The Kiwifruit reference sequence was downloaded from <http://bioinfo.bti.cornell.edu/cgi-bin/kiwi/download.cgi>, and transcriptome analysis was performed using DEGseq and DESeq softwares [44]. Blast2GO [45] was applied to obtain GO annotations of unigenes; after the GO annotation of each unigene was obtained, WEGO software [46] was used to classify and count the GO functions of all unigenes. Pathway annotations of the unigenes based on the KEGG database were also obtained.

2.6. RT-qPCR analysis

Tissue culture shoots leaves of three diploid and three tetraploid *A. chinensis* plants were used as the materials. The total RNA was extracted using the Qiagen RNeasy Mini Kit (Qiagen Inc., Valencia, CA), and then the RNA was reverse transcribed into cDNA by random primers. The RT-qPCR was conducted according to a previous report [47].

The transcriptional expression level of 11 candidate genes including two known resistance genes which were β -1,3-glucosidase [48] and triacylglycerol lipase [49] were checked by RT-qPCR in diploid and tetraploid kiwifruit plants. Gene specific primers were designed using Primer Premier 5.0 software, and the primers used for RT-qPCR analyses are listed in table 1. The $2^{(-\Delta\Delta Ct)}$ method [47] was employed to analyse the data. Statistical significance was evaluated by the two-sample *t*-test (independent variable) at the 95% confidence level.

Table 2. Adapter and primer sequences.

adapter and primer	sequences
EcoRIAdapter 1	CTCGTAGACTGCGTACC
EcoRIAdapter 2	AATTGGTACGCGATC
HM Adapter 1	GACGATGAGTCTAGAA
HM Adapter 2	CGTTCTAGACTCATC
PreAmp primer E-A	GACTGCGTACCAATTCA
PreAmp primer HM-T	GATGAGTCTAGAACGGT
selective primers E32	GACTGCGTACCAATTCAAC
selective primers E45	GACTGCGTACCAATTCATG
selective primers Msp39	GATGAGTCTGAGCGGAGA
selective primers Msp40	GATGAGTCTGAGCGGAGC
selective primers Msp41	GATGAGTCTGAGCGGAGG
selective primers Msp44	GATGAGTCTGAGCGGATC
selective primers Msp48	GATGAGTCTGAGCGGCAC
selective primers Msp50	GATGAGTCTGAGCGGCAT
selective primers Msp61	GATGAGTCTGAGCGGCTG

2.7. MSAP analysis

Three deplod and three tetraploid plants which confirmed by chromosome number observation and flow cytometry analysis were subjected to analysed DNA methylation using MSAP. The MSAP system had three major parts, digestion and ligation reactions, preamplification and selective amplification reactions, and detection reactions. The designed adapters and primers for *EcoR* I and *Hpa* II-*Msp* I listed in table 2 were fixed as described by Lu *et al.* [50], with some modifications. We used 16 pairs of selective primers obtained from four *EcoR* I primers (E32 and E45) in combination with four *Hpa* II/*Msp* I primers (Msp39, Msp40, Msp41 Msp44, Msp48, Msp50 and Msp61), to analyse DNA methylation in CCGG sites of diploid and tetraploid tissues.

3. Results

3.1. Polyploidization efficiency

Multiple comparisons of the fatality rate and mutagenesis rate showed that the fatality rate and mutagenesis rate were significantly different among the treatment combinations. The treatment combination 7 (12 h + 20 mg l⁻¹) and 9 (24 h + 20 mg l⁻¹) had the lowest dieback rate, and the treatment combination 15 (30 h + 60 mg l⁻¹) had the highest polyploidization efficiency (table 3).

As shown in table 3 and figure 1, when colchicine concentration was 20 mg l⁻¹, the mutagenesis rate and dieback rate were the lowest. When colchicine concentration was greater than 20 mg l⁻¹, the change between dieback rate and colchicine concentration was not obvious. The polyploidization efficiency first increased and then declined with the increase of colchicine concentration, and reached the highest at 60 mg l⁻¹.

To sum up, polyploidization efficiency rose with increasing concentration of colchicine and gradually appeared to be stable to a certain degree. For increasing the polyploidization efficiency, a high concentration of colchicine and long processing time were required. Meanwhile, a low concentration of colchicine led to a low polyploidization efficiency, and was not suitable for induction of tetraploid *A. chinensis* plants.

When treatment time was brief, the polyploidization efficiency increased gradually with an increase of colchicine concentration. When treatment time was long, the polyploidization efficiency tended to decrease with an increase of colchicine concentration. However, with the same concentration, the treatment time did not significantly affect the polyploidization efficiency. These results showed that the main influence factor in the induction of tetraploid plants was the concentration of colchicine. Exploiting this system, a total of 187 independent tetraploid plants were generated.

Table 3. Polyploidization efficiency using *in vitro* colchicine treatment of leaf explants of *A. chinensis*. Mean separation within column by one-way ANOVA. Duncan's multiple comparison analysis was performed. The means ($n = 20$) followed by the same letter do not differ at $p = 0.05$.

colchicine concentration (mg l ⁻¹)	time (h)	contamination rate (%)	dieback rate (%)	polyploidization efficiency (%)
0	6	20.0	0.0	0.00
	12	20.0	0.0	0.00
	18	20.0	0.0	0.00
	24	20.0	0.0	0.00
	30	20.0	0.0	0.00
20	6	0.0	10.0 ^D	0.00
	12	0.0	5.0 ^D	0.0012
	18	20.0	6.3 ^D	0.00
	24	0.0	5.0 ^D	0.00
	30	20.0	12.5 ^{CD}	0.00
40	6	20.0	50.2 ^A	0.00
	12	20.0	12.5 ^{CD}	9.52 ^C
	18	20.0	37.5 ^{BC}	10.00 ^{BC}
	24	0.0	30.0 ^{BC}	7.14 ^C
	30	40.0	16.7 ^{CD}	10.00 ^{BC}
60	6	0.0	38.3 ^{BC}	16.22 ^{AB}
	12	0.0	43.3 ^{AB}	14.71 ^B
	18	20.0	37.5 ^{BC}	20.00 ^A
	24	40.0	33.3 ^{BC}	25.00 ^A
	30	0.0	16.7 ^C	26.00 ^A
80	6	40.0	36.1 ^{BC}	13.04 ^{BC}
	12	20.0	31.3 ^B	12.12 ^{BC}
	18	0.0	20.0 ^C	10.42 ^{BC}
	24	20.0	56.3 ^A	19.05 ^{AB}
	30	0.0	20.0 ^C	14.58 ^B
100	6	20.0	43.8 ^{AB}	22.22 ^A
	12	20.0	25.0 ^C	16.67 ^{AB}
	18	20.0	37.5 ^B	20.00 ^A
	24	20.0	35.4 ^{BC}	9.68 ^C
	30	0.0	40.0 ^B	13.89 ^{BC}

3.2. Differences in the morphologies and stomatal guard cells between diploid and tetraploid plants

Table 4 shows differences between leaves of diploidy and tetraploidy. The average leaf length of diploid was 3.57 cm, and that of tetraploid was 3.72 cm and the difference was significant. The average leaf width of diploidy (2.26 cm) was not significantly from that of tetraploid (2.35 cm). The average leaf thickness of diploidy (0.52 mm) was significantly ($p = 0.004$) lower than that of tetraploid (1.04 mm). Tetraploid plants exhibited thicker leaves, more leaf pubescence, bigger stems and larger veins (figure 2a,b). According to morphological indexes like thicker leaves, more leaf pubescence and so on, 187 suspected tetraploid plants were selected.

There was a major difference between the stomatal characteristics of the leaves of the diploid and tetraploid plants. Stomatal size of tetraploid was obviously larger than that of diploid, and the

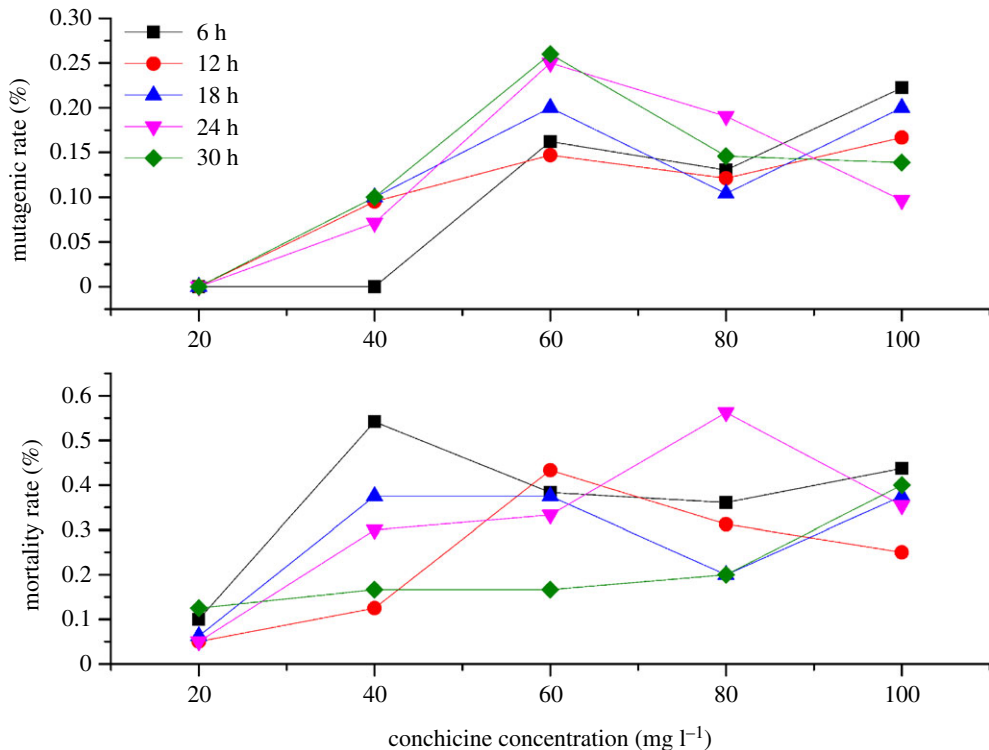


Figure 1. The change rule of mutagenic rate and mortality rate.

Table 4. Leaf characteristics of diploid and tetraploid plants. Mean separation within column by independent sample *t*-test. The means ($n = 20$) followed by the same letter do not differ at $p = 0.05$.

	diploid	tetraploid
leaf length (cm)	3.57	3.72
	0.003	0.005
	b	a
leaf width (cm)	2.26	2.35
	0.01	0.002
leaf thickness (m)	0.52	1.04
	0.004	0.026
	B	A
stomatal guard cells (μm)	18	12
	1.778	3.556
	A	B

number of stomata on the unit leaf area decreased as the density decreased (figure 2*c,d*). The results showed that the average number of the diploid stomatal guard cells within a field of vision was significantly higher than the average number of tetraploid stomatal guard cells ($p = 0.00001795$, table 4).

3.3. Chromosome number and nuclear DNA content

Chromosome numbers of diploid *A. chinensis* and tetraploid *A. chinensis* plants were $2n = 2x = 58$ and $2n = 4x = 116$, respectively. Parts of the chromosome number observation results are shown in figure 2*e,f*.

As shown in figure 3, the average DNA content of the tetraploid *A. chinensis* plants was about twice that of diploid plants. All 187 suspected tetraploid plants according to morphological indexes were confirmed to be tetraploid.

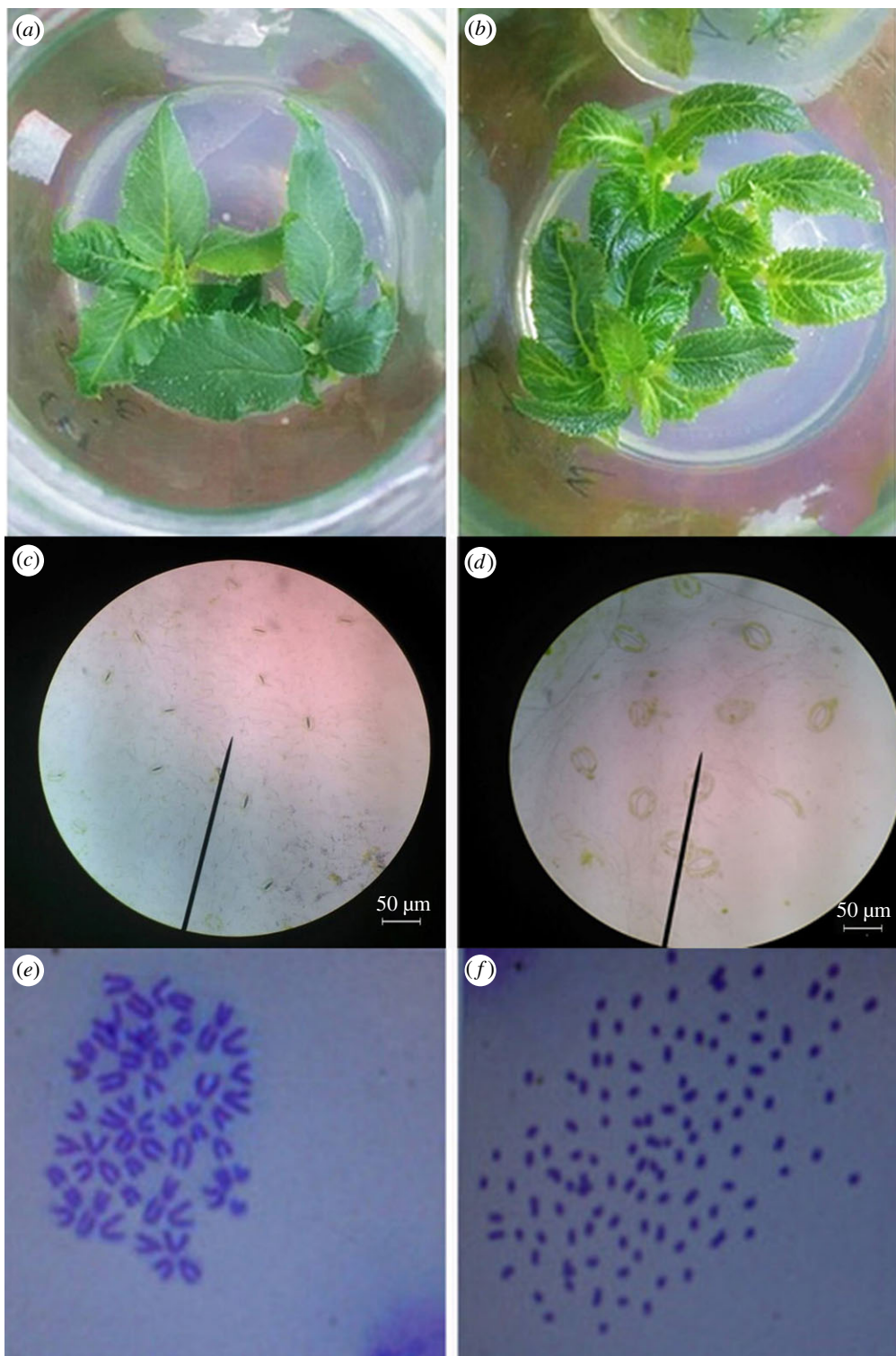


Figure 2. The morphological differences, stomatal guard cells and chromosome number of diploid and tetraploid plants. (a) diploid plants; (b) tetraploid plants with thicker leaves and larger veins; (c) stomatal guard cells of diploidy with average length of 15.7 ± 1.44 (μm); (d) stomatal guard cells of tetraploidy with average length of 42.1 ± 6.07 (μm); (e) chromosome number of diploidy (58); (f) chromosome number of tetraploidy (116).

3.4. Analysis of differential gene expression and functional annotation

Approximately 47.26 and 41.36 million clean reads were generated for the tetraploid and diploid samples, respectively. Mapping rates of the tetraploid and diploid were 88.01% and 86.53%, respectively, as determined by genome comparison.

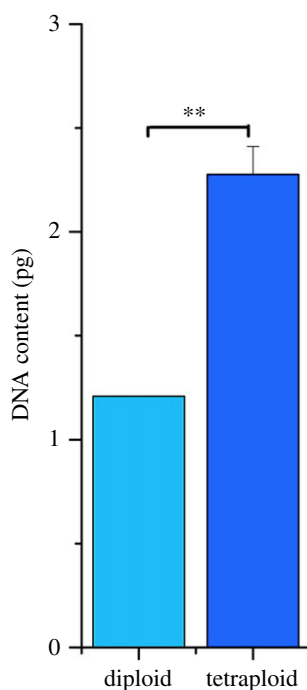


Figure 3. Nuclear DNA contents in *A. chinensis* diploid and tetraploid plant leaves determined by flow cytometric analysis. Average DNA content of the diploid *A. chinensis* plants was significantly lower than that of tetraploid plants ($p = 0.000254$). Data were compared using one-way ANOVA and Fisher's post hoc comparisons. $**p < 0.01$.

Table 5. DEGs annotation number.

sample	trend	number of DEGs significantly annotated	the total number of DEGs annotated
diploid versus tetraploid	up	660	16 106
	down	1570	17 943
	total	2230	34 049

There involved a total of 34 049 differentially expressed genes between diploid and tetraploid, 16 106 of them were downregulated, and 17 943 of them were upregulated. Among these differentially expressed genes, 2230 were significantly annotated, of which 660 genes downregulated, 1570 genes upregulated in the tetraploid plants opposed to the diploid ones (table 5).

The differentially expressed genes (table 6) were selected from three replicates for which differentially expressed levels were very significant, and the unannotated genes were removed. Most of the 16 ascending genes were related to growth development and resistance. The gene annotation of mannan synthase [51], vacuolar protein sorting-associated protein [52], auxin efflux carrier protein [53] and cytochrome P450 [54] were related to plant growth and development, and the gene annotation of glycosyltransferase family 2 protein [55] and 3-ketoacyl-CoA synthase [56] were related to plant resistance. In the five descending genes, the three genes, ferritin [57], anthranilate *N*-benzoyltransferase protein [58] and chitinase protein [59] were related to antibacterial activity. The results indicated that the growth and stress resistance of tetraploid plants was better than those of diploid ones, while their antibacterial ability was worse than diploid ones.

3.5. Gene ontology classification analysis of DEGs

The differentially expressed genes were subjected to GO (gene ontology) analysis, and the results were reflected in three main categories: cellular components, molecular function and biological process. These DEGs were mainly enriched in GO categories of catalytic activity, binding, transporter activity, membrane and metabolic process.

Table 6. DEGs after filtered of tetraploid and diploid *A. chinensis*.

gene_id	gene annotation	p-value	regulation	logFC
Achn022281	Patatin T5	0.00069357	up	2.45684171
Achn072311	Centrin-1	4.88×10^{-9}	up	5.54193126
Achn075911	putative citrate efflux MATE transporter	0.000880555	up	2.88589185
Achn086711	mannan synthase	6.91×10^{-5}	up	4.99262803
Achn107571	glycosyl transferase family 2 protein	3.58×10^{-7}	up	5.99001591
Achn133241	2-oxoglutarate-dependent dioxygenase	2.11×10^{-8}	up	4.45289714
Achn150071	vacuolar protein sorting-associated protein	0.000797167	up	4.41027292
Achn162311	reductase 1	0.000745493	up	4.41027292
Achn176611	cellulose synthase	1.67×10^{-12}	up	6.62819319
Achn181151	putative ROX1	1.88×10^{-8}	up	5.32241374
Achn207191	lactoylglutathione lyase	0.000139736	up	2.97970391
Achn211741	auxin efflux carrier protein	0.00013422	up	4.24269787
Achn263191	2-oxoisovalerate dehydrogenase alpha subunit	2.79×10^{-6}	up	4.46772330
Achn285441	1-aminocyclopropane-1-carboxylate oxidase	4.24×10^{-11}	up	6.02373138
Achn311381	3-ketoacyl-CoA synthase	1.69×10^{-6}	up	3.83116076
Achn329921	cytochrome P450	6.68×10^{-9}	up	5.70327952
Achn036721	ER glycerol-phosphate acyltransferase	0.000128046	up	2.94934454
Achn103301	beta-1,3-glucanase	0.000359838	down	-2.49679123
Achn175101	ferritin	2.48×10^{-8}	down	-4.20769625
Achn196611	anthranilate <i>N</i> -benzoyltransferase protein	0.000963949	down	-2.25811355
Achn273491	chitinase protein	2.85×10^{-11}	down	-5.58243403
Achn334521	inorganic phosphate transporter	1.50×10^{-8}	down	-4.80873117

Using the diploid as the control, the upregulated and downregulated genes in the GO classification were subjected to comparative analysis, and only the genes with altered expression levels were chosen. The results showed that the number of upregulated genes in each GO-term was greater than the downregulated genes (figure 4).

3.6. KEGG pathway analysis of DEGs

These DEGs were mainly enriched to Kyoto Encyclopedia of Genes and Genome (KEGG) pathways of fatty acid elongation (ko00062), retinol metabolism (ko00830), linoleic acid metabolism (ko00591), terpenoid backbone biosynthesis (ko00900), sesquiterpenoid and triterpenoid biosynthesis (ko00900), phenylpropanoid biosynthesis (ko00940) and bile secretion (ko04976).

Two complete upward and downward pathways were chosen for analysis (figures 5 and 6). In the pathway of retinol metabolism, there just was upregulated gene, Achn329921. The gene was related to cytochrome P450, which might be highly associated with biosynthesis of terpenoids. The upregulated pathway might be highly related to better growth and development in tetraploid plants than in diploid ones. In the pathway of fatty acid elongation, there were one upregulated gene and two downregulated genes. The upregulated gene Achn311381 was related to 3-ketoacyl-CoA synthase, which was involved in biofilm lipid synthesis, and was a precursor to the formation of waxy organisms in keratoplasm. The two downregulated genes Achn141891 and Achn001331 were related to palmitoyl (protein) and hydrolase.

3.7. RT-qPCR validation

To confirm that the differentially expressed unigenes and genes obtained from Illumina sequencing were truly transcribed at different levels between diploid and tetraploid plants, two known genes (figure 7a) associated with stress resistance, three other upregulated genes (figure 7b) and six downregulated genes

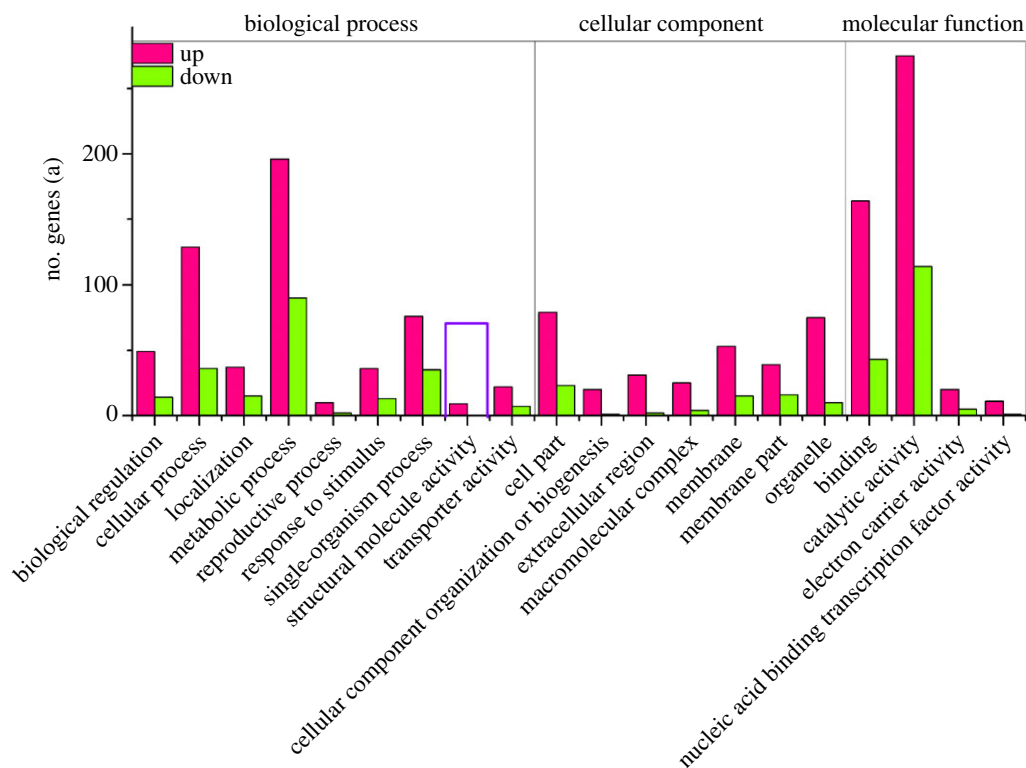


Figure 4. Ontology classification of DEGs.

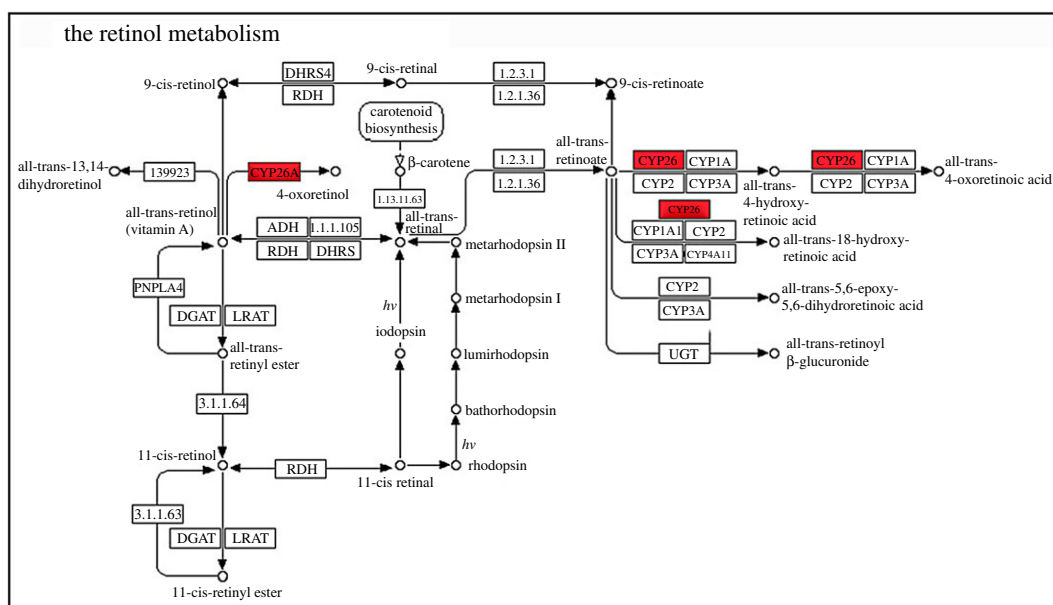


Figure 5. The retinol metabolism pathway of *A. chinensis* diploid and tetraploid plants. Red, upregulated genes; green, downregulated genes; blue, both.

(figure 7c) were chosen for RT-qPCR analysis. The results showed that the three upregulated genes still exhibited enhanced expression in RT-qPCR analysis. Five downregulated genes were confirmed to be consistent with the transcriptome results; however, one gene (Achn007531) was not consistent with it. The reason for this might be the individual differences between initial sequencing and late detection. Overall, the results of RT-qPCR were coherent with the Illumina sequencing. The findings suggested that there were statistically significant differences between the expression levels of these genes in diploid and tetraploid plants.

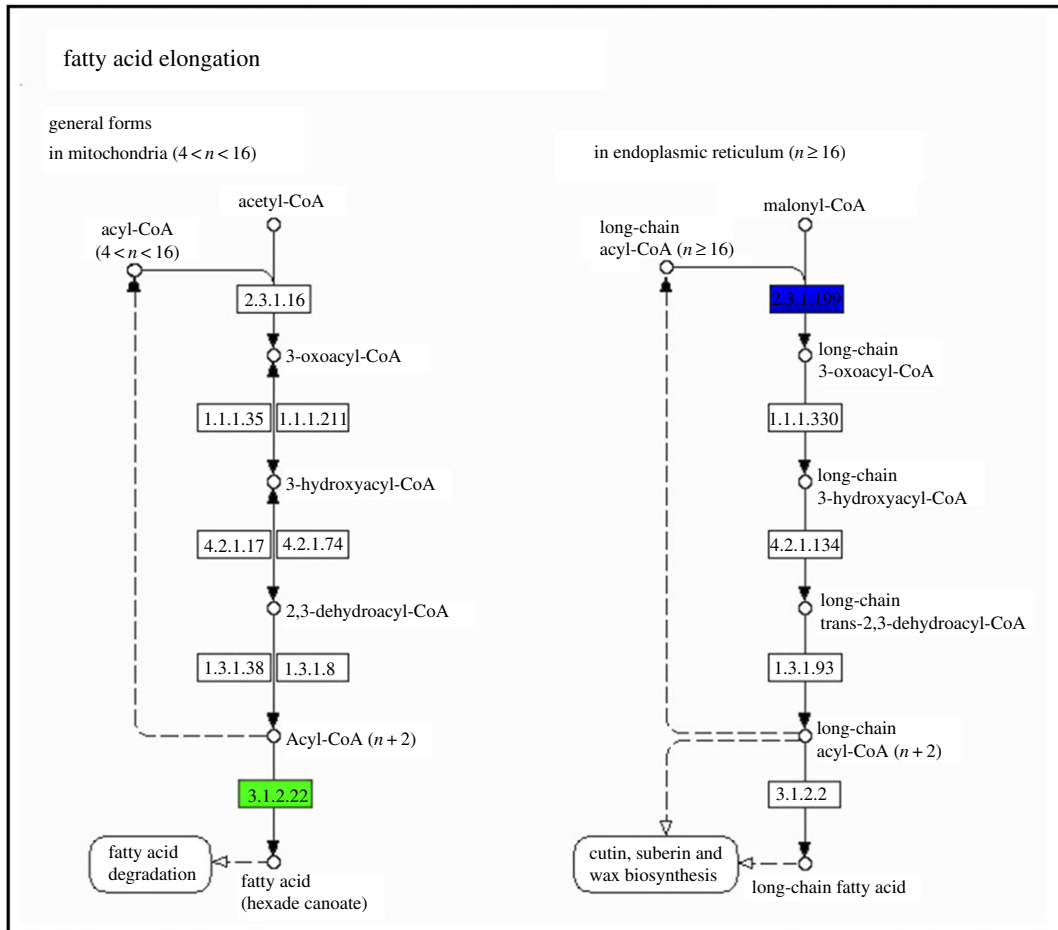


Figure 6. The fatty acid elongation pathway of *A. chinensis* diploid and tetraploid plants. Red, upregulated genes; green, downregulated genes; blue, both.

3.8. DNA methylation levels of diploid and tetraploid plants

Methylation levels of total amplified sites in diploid and tetraploid tissues were 49.5% and 42.35%, respectively (table 7). Therefore, there was a certain degree of difference among the diploid and tetraploid tissues.

4. Discussion

Shoot-immersion method which immersed the shoots with colchicine solution has been used in kiwifruit [60], wheat [61], *Vitis davidii* [62], maize [63], garlic [64] and other plants to induce polyploidy. The dropping method which dropped colchicine solution on cotton ball binding to the top of shoots was used in *Secale*, *Haynaldia*, *Aegilops*, *Brassica* spp. and mulberry [65,66]. These two methods have their advantages and disadvantages in the induction of tetraploidy. In the study, shoot-immersion method was considered to be better because the tissues of immersion could contact more fully, so the mutation rate would be higher. In practice, the specific conditions of species should be considered in the selection of a suitable method for the induction of tetraploidy.

A previous study showed that using micro shoots treated with 0.05 or 0.1% colchicines effectively induced tetraploidy in *A. chinensis*, and the induction rates were 30% and 20%, respectively [6]. Our results showed that using leaf segments treated with 60 mg l^{-1} (0.006%) also could effectively induce tetraploidy, and the induction rate was about 26%. And we found that using the concentration of 100 mg l^{-1} colchicines (0.01%) the tetraploid induction efficiency was lower than that of 60 mg l^{-1} (0.06%). The concentration of colchicine was 10 times lower than previously reported. It indicated that the different varieties of *A. chinensis* required using different concentrations of colchicine to reach the higher induction rate.

Tetraploid plants show some typical morphological characteristics, like stunted growth and presence of larger, thicker, dark green and larger stomata [67,68]. Morphological difference was also found between

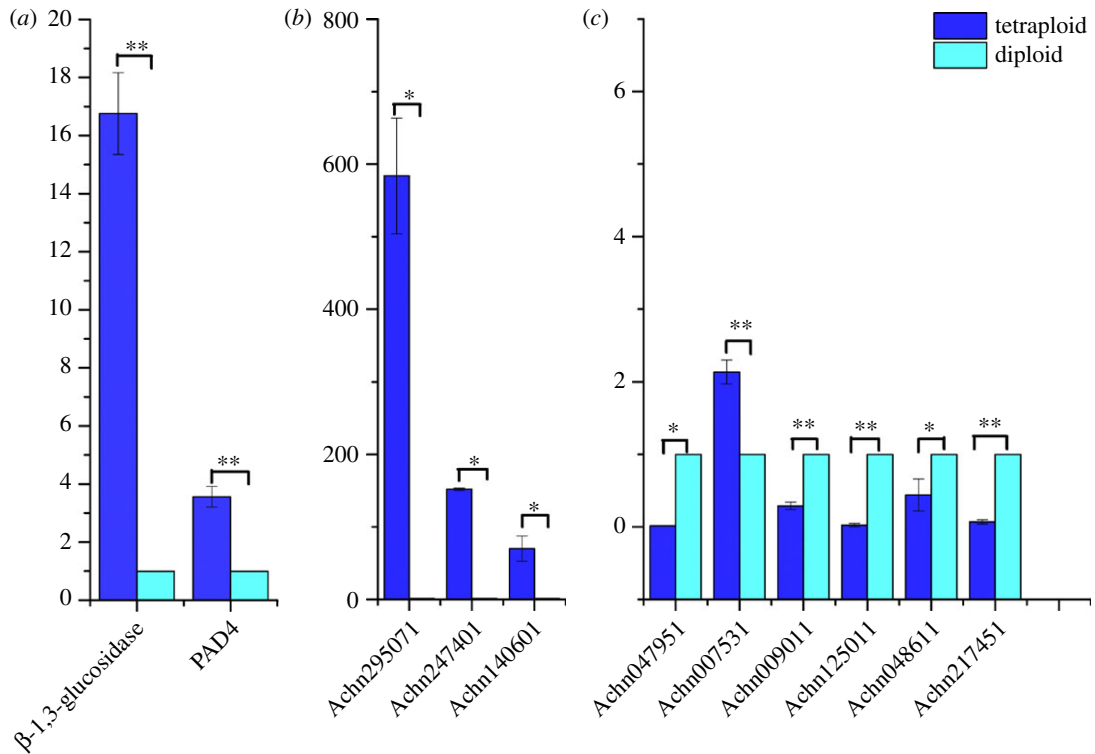


Figure 7. The expression levels of the selected genes in diploid and tetraploid plants. (a) Two resistance genes; (b) three up genes from transcriptome; (c) six down genes from transcriptome. Data were compared using one-way ANOVA and Fisher's *post hoc* comparisons. Values were mean \pm s.e. of $2^{(-\Delta\Delta Ct)}$ ($n = 3$). * $p < 0.05$; ** $p < 0.01$.

Table 7. The ratio of patterns of genomic DNA methylation of the diploid and tetraploid tissues. Total methylated bands = II + III + IV; fully methylated bands = III + IV.

types	diploidy	tetraploid
I	366	410
II	22	22
III	216	171
IV	61	63
total amplified bands	604	602
total methylated bands	299	255
MSAP (%)	49.5	42.35
fully methylated bands	277	234
fully methylated ratio	45.86	38.87

diploid plants and their tetraploid plants in the other research of kiwifruit [8,60]. These external morphologies could be used as evidence for the identification of tetraploid plants. Size and density of stomatal guard cells, chromosome number and flow cytometric analysis were used to identify tetraploid plants in previous study [69,70]. Here, external morphology observation, stomatal guard cell observation, chromosome number observation and flow cytometry analysis were employed to identify the tetraploid of *A. chinensis*. A combination of these methods was found to be effective in the identification of tetraploidy of *A. chinensis*.

There are usually some special characteristics in a tetraploid fruit tree, such as stronger growth with bigger fruit and fewer or no seed, stronger adaption ability and better stress resistance [71]. To our knowledge, there continue to be no reports on investigating the reason for the phenomena at the molecular level. In this study, upregulated genes in DEGs were related to growth and stress resistance. The downregulated genes were related to antibacterial activity. In the GO annotation, all functional genes concerning structural molecule activity were upregulated, indicating that the growth of tetraploid *A. chinensis* plants was stronger than

diploid ones. Meanwhile, almost all the functional genes of membrane and membrane part were upregulated genes, and upregulated expression of the membrane and membrane part-related genes indicated that stress-resistant ability might increase, because the functional genes of biological membrane play very important roles in plant resistance [72]. In the KEGG pathway, the genes associated with stress resistance were upregulated, while those related to leaf senescence were downregulated. Leaf senescence was associated with proteolytic enzymes, and the activity of the proteolytic enzyme increased gradually with leaf senescence [73]. Therefore, opposed to diploid plants, the decreased expression of proteolytic enzyme-related genes could contribute to more robust leaves in tetraploid plants. The transcriptomes would be determined by the full message RNA expression in which the transcription varies depending on many factors; the tetraploids with high transcriptions could be a response to the genomics interaction inside of the new high levels of genomics of the new organize, which could be a natural response [13].

An important development in understanding the influence of chromatin on gene regulation was the finding that DNA methylation and histone post-translational modifications would lead to the recruitment of protein complexes that regulated transcription [74]. DNA methylation might lead to the expression levels of functional genes being increased or decreased. DNA methylation does not alter nucleotide sequences but regulates gene transcription, and it plays an important part in gene expression regulation during development and differentiation in plants [50]. This study adopted methylation-sensitive amplification polymorphism to compare the levels of DNA cytosine methylation in diploid and tetraploid tissues, and the result showed that tetraploid tissues had lower methylation rate than diploid tissues. Here, the increased expression of growth and stress resistance-related genes in the *A. chinensis* tetraploid plants might be caused by DNA methylation too.

The study has, for the first time, found that growth and stress resistance-related genes were upregulated, and antibacterial activity-related genes were downregulated at the transcriptome level in tetraploid plants compared to diploid ones, and tetraploid plants had lower methylation ratio than diploid ones [75,76], which might explain why tetraploid plants usually have better resistance than diploid ones.

5. Conclusion

- (1) The best treatment for tetraploid induction of *A. chinensis* SWFU 03 was soaking 30 h in 60 mg l⁻¹ colchicine solutions. Using the induction system combined with the identification methods including external morphologies comparison and flow cytometry analysis, 187 tetraploid *A. chinensis* plants were obtained in the study.
- (2) Compared with diploid plants, the stress tolerance-related genes of tetraploid plants were upregulated. We have, for the first time, found that the growth and stress resistance of tetraploid plants was stronger than those of diploid ones at the transcriptome level.
- (3) The tetraploid plants had lower methylation ratio than diploid ones. DNA methylation was important in gene expression regulation during biological development in *A. chinensis* plants.

Data accessibility. RNA-seq data were presented at the short read archive (SRA) database of NCBI (accession number PRJNA535344).

Authors' contributions. S.L. analysed the experimental data and drafted the manuscript. S.L., X.Z. and Q.Y. conducted the study. X.L. helped analyse the experimental data. H.L. participated in the coordination of the study. H.Z. conceived of the study and contributed to writing the manuscript. All authors approved the final version of the manuscript.

Competing interests. There is no any ethical/legal conflicts involved in the article.

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References

1. Yan G, Ferguson AR, McNeillage MA. 1994 Ploidy races in *Actinidia chinensis*. *Euphytica* **78**, 175–183. (doi:10.1007/BF00027515)
2. Zeng H, Li DW, Huang HW. 2009 Distribution pattern of ploidy variation of *Actinidia chinensis* and *A. deliciosa*. *J. Wuhan Bot. Res.* **27**, 312–317.
3. Rao JY, Liu YF, Hong-Wen AH. 2012 Analysis of ploidy segregation and genetic variation of progenies of different interploidy crosses in *Actinidia chinensis*. *Acta Hortic. Sin.* **39**, 1447–1456.
4. Wu JH, Ferguson AR, Murray BG. 2011 Manipulation of ploidy for kiwifruit breeding: *in vitro* chromosome doubling in diploid *Actinidia*

- chinensis* Planch. *Plant Cell Tiss. Org.* **106**, 503–511. (doi:10.1007/s11240-011-9949-z)
5. Liu C, Sun X, Dai H, Zhang Z. 2011 *In vitro* induction of octoploid plants from tetraploid *Actinidia arguta*. *Acta Hort.* **913**, 185–190. (doi:10.17660/ActaHortic.2011.913.23)
 6. Wu JH, Ferguson AR, Murray BG, Jia Y, Datson PM, Zhang J. 2012 Induced polyploidy dramatically increases the size & alters the shape of fruit in *Actinidia chinensis*. *Ann. Bot.-London.* **109**, 169. (doi:10.1093/aob/mcr256)
 7. Wang XH, Xiong L, Qu YH, Yang J, Gu ZJ. 2006 The polyploid induction and identification of *Platycodon grandiflorus* (Campanulaceae) in China. *Acta Bot. Yunnan.* **28**, 593–598.
 8. Wu JH, Ferguson AR, Murray BG, Gejkskes RJ, Lakshmanan P, Taji A. 2009 *In vitro* induction of autotetraploid *Actinidia* plants and their field evaluation for crop improvement. *Acta Hort.* **829**, 245–250. (doi:10.17660/actahortic.2009.829.35)
 9. Wang LJ, Sheng MY, Wen PC, Du JY. 2017 Morphological, physiological, cytological and phytochemical studies in diploid and colchicine-induced tetraploid plants of *Fagopyrum tataricum* (L.). *Gaertn. Bot. Stud.* **58**, 2. (doi:10.1186/s40529-016-0157-3)
 10. Babil PK, Funayama C, Iijima K, Irie K, Shiwachi H, Toyohara H, Hiroshi F. 2011 Effective induction of polyploidy by *in vitro* treatment with colchicine and characterization of induced polyploid variants in water yam (*Dioscorea alata* L.). *Jpn. J. Trop. Agric.* **55**, 142–147.
 11. Kobayashi T, Shimamura T. Morphological and cytological studies on induced polyploidy in *Sesamum indicum* L. *Jpn. J. Genet.* **27**, 157–171 1952. (doi:10.1266/jjg.27.157)
 12. Schiffno MT. 1987 Induction of polyploidy & cytological characterization of autotetraploids of *Trifolium riograndense* Burkart (Leguminosae). *Euphytica* **36**, 863–872. (doi:10.1007/BF00051871)
 13. Wu JH. 2018 Establishment of *in vitro* culture systems for breeding new types of kiwifruit and for *Actinidia* genomics studies. *Acta Hort.* **1224**, 195–202. (doi:10.17660/actahortic.2018.1224.26)
 14. Du X, Sun Y, Yuan S, Li Q, Gong Z. 2011 Identification of colchicine-induced polyploid plants in two species of *Impatiens balsamina*. *Acta Agric. Bor-Occid. Sin.* **7**, 014.
 15. Fatima B, Usman M, Khan MS, Khan IA, Khan MM. 2015 Identification of citrus polyploids using chromosome counts, morphological and SSR markers. *Pak. J. Agr. Sci.* **52**, 107–114.
 16. Kadota M, Niimi Y. 2002 *In vitro* induction of tetraploid plants from a diploid Japanese pear cultivar (*Pyrus pyrifolia* N. cv. Hosui). *Plant Cell Rep.* **21**, 282–286. (doi:10.1007/s00299-002-0509-1)
 17. Xing SH, Guo XB, Wang Q, Pan QF, Tian YS, Liu P. 2011 Induction and flow cytometry identification of tetraploids from seed-derived explants through colchicine treatments in *Catharanthus roseus* (L.) G. Don. *J. Biomed. Biotechnol.* **2011**, 793198. (doi:10.1155/2011/793198)
 18. Schween G, Schulte J, Reski R, Hohe A. 2005 Effect of ploidy level on growth, differentiation, and morphology in *Physcomitrella patens*. *Bryologist* **108**, 27–35. (doi:10.1639/0007-2745(2005)108[27:EOPL0G]2.0.CO;2)
 19. Tang ZQ, Chen DL, Song ZJ, Yu CH, De TC. 2010 *In vitro* induction and identification of tetraploid plants of *Paulownia tomentosa*. *Plant Cell Tiss. Org.* **102**, 213–220. (doi:10.1007/s11240-010-9724-6)
 20. Beck SL, Visser G, Dunlop RW, Hare PD. 2005 A comparison of direct (flow cytometry) and indirect (stomatal guard cell lengths and chloroplast numbers) techniques as a measure of ploidy in black wattle, *Acacia mearnsii* (de Wild). *S. Afr. J. Bot.* **71**, 354–358. (doi:10.1016/S0254-6299(15)30109-5)
 21. Fahlgren N *et al.* 2009 Computational and analytical framework for small RNA profiling by high-throughput sequencing. *RNA* **15**, 992. (doi:10.1261/ma.1473809)
 22. Benjamini Y, Speed TP. 2012 Summarizing and correcting the GC content bias in high-throughput sequencing. *Nucleic Acids Res.* **40**, e72. (doi:10.1093/nar/gks001)
 23. He J, Jiao Y. 2014 Next-generation sequencing applied to flower development: RNA-seq. *Methods Mol. Biol.* **1110**, 401. (doi:10.1007/978-1-4614-9408-9_23)
 24. Wenbin L. 2015 Gene expression profiling of development and anthocyanin accumulation in kiwifruit (*Actinidia chinensis*) based on transcriptome sequencing. *PLoS ONE* **8**, e0136439. (doi:10.1371/journal.pone.0136439)
 25. Zhang JY, Huang SN, Mo ZH, Xuan JP, Jia XD, Wang G, Guo ZR. 2015 De novo transcriptome sequencing and comparative analysis of differentially expressed genes in kiwifruit under waterlogging stress. *Mol. Breed.* **35**, 208. (doi:10.1007/s11032-015-0408-0)
 26. Tang P, Zhang Q, Yao X. 2017 Comparative transcript profiling explores differentially expressed genes associated with sexual phenotype in kiwifruit. *PLoS ONE* **12**, e0180542. (doi:10.1371/journal.pone.0180542)
 27. Tao W, Gang W, Zhan-Hui J, De-Lin P, Ji-Yu Z, Zhong-Ren G. 2018 Transcriptome analysis of kiwifruit in response to *Pseudomonas syringae* pv. actinidiae infection. *Int. J. Mol. Sci.* **19**, 373. (doi:10.3390/ijms19020373)
 28. Wang GH, Zhao WC, Cheng JA. 2008 Real-time fluorescent quantitative PCR technique and its applications in entomological studies. *Acta Entomol. Sin.* **51**, 1293–1303.
 29. Huang Y, Yin X, Zhu C, Wang W, Grierson D, Xu C, Chen KS. 2013 Standard addition quantitative real-time PCR (SA-qPCR): a novel approach for determination of transgene copy number avoiding PCR efficiency estimation. *PLoS ONE* **8**, e53489. (doi:10.1371/journal.pone.0053489)
 30. Mygind T, Birkelund S, Falk E, Christiansen G. 2001 Evaluation of real-time quantitative PCR for identification and quantification of *Chlamydia pneumoniae* by comparison with immunohistochemistry. *J. Microbiol. Meth.* **46**, 241–251. (doi:10.1016/S0167-7012(01)00282-2)
 31. Taylor JMG, Paterson LJ, Havis ND. 2010 A quantitative real-time PCR assay for the detection of *Ramularia collo-cygni*, from barley (*Hordeum vulgare*). *Let. Appl. Microbiol.* **50**, 493–499. (doi:10.1111/j.1472-765X.2010.02826.x)
 32. Faize M, Faize L, Burgos L. 2010 Using quantitative real-time PCR to detect chimeras in transgenic tobacco and apricot and to monitor their dissociation. *BMC Biotechnol.* **10**, 53. (doi:10.1186/1472-6750-10-53)
 33. Meaburn E, Schulz R. 2012 Next generation sequencing in epigenetics: insights and challenges. *Semin. Cell Dev. Biol.* **23**, 192–199. (doi:10.1016/j.semcdb.2011.10.010)
 34. Lavelle D, Vaitkus K, Hankewych M, Singh M, Desimone J. 2006 Developmental changes in DNA methylation and covalent histone modifications of chromatin associated with the ϵ -, γ -, and β -globin gene promoters in *Papio anubis*. *Blood Cell Mol. Dis.* **36**, 269–278. (doi:10.1016/j.bcmd.2006.01.004)
 35. Cervara MT, Ruin GL, Martinez-Zapater JM. 2002 Analysis of DNA methylation in *Arabidopsis thaliana* based on methylation-sensitive AFLP markers. *Mol. Gen. Genet.* **268**, 543–552. (doi:10.1007/s00438-002-0772-4)
 36. Suzuki MM, Bird A. 2008 DNA methylation landscapes: provocative insights from epigenomics. *Nat. Rev. Genet.* **9**, 465. (doi:10.1038/nrg2341)
 37. Pereira RC, Davide LC, Techio VH, Timbó AL. 2012 Chromosome doubling of grasses: an alternative to plant breeding. *Ciência Rural.* **42**, 1278–1285. (doi:10.1590/S0103-84782012000700023)
 38. Plamenov D, Spetsov P. 2011 Synthetic hexaploid lines are valuable resources for biotic stress resistance in wheat improvement. *J. Plant Pathol.* **93**, 251–262.
 39. Murashige T, Skoog F. 1962 A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol. Plantarum.* **15**, 473–497. (doi:10.1111/j.1399-3054.1962.tb08052.x)
 40. Salameh E, Alshaarani F, Hamed HA, Nassar JA. 2015 Investigation of the relationship between psychosocial stress and temporomandibular disorder in adults by measuring salivary cortisol concentration: a case-control study. *J. Indian. Prosthodont. Soc.* **15**, 148–152. (doi:10.4103/0972-4052.158075)
 41. Watanabe KN, Orillo M. 1993 An alternative pretreatment method of mitotic chromosome observation in potatoes. *Am. J. Potato Res.* **70**, 543–548. (doi:10.1007/BF02846755)
 42. Preeda N, Yanagi T, Sone K, Taketa S, Okuda N. 2007 Chromosome observation method at metaphase and pro-metaphase stages in diploid and octoploid strawberries. *Front Microbiol.* **114**, 133–137. (doi:10.1016/j.scienta.2007.06.001)
 43. Cousin A, Heel K, Cowling WA, Nelson MN. 2009 An efficient high-throughput flow cytometric method for estimating DNA ploidy level in plants. *Cytometry A* **75**, 1015–1019. (doi:10.1002/cyto.a.20816)
 44. Anders S, Huber W. 2010 Differential expression analysis for sequence count data. *Gen. Biol.* **11**, R106. (doi:10.1186/gb-2010-11-10-r106)
 45. Conesa A, Götz S, García-Gómez JM, Terol J, Talón M, Robles M. 2005 Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinform* **21**, 3674–3676. (doi:10.1093/bioinformatics/bti610)
 46. Ye J, Fang L, Zheng H, Zhang Y, Chen J, Zhang Z, Wang J. 2006 WEGO: a web tool for plotting

- GO annotations. *Nucleic Acids Res.* **34**, W293–W297. (doi:10.1093/nar/gkl031)
47. Liu X, Zhang Z, Sang M, Sun X, He C, Xin P, Zhang H. 2018 Functional analysis of the *FZF1* gene in *Saccharomyces uvarum*. *Front. Microbiol.* **9**, 96. (doi:10.3389/fmicb.2018.00096)
48. Singh G, Verma AK, Kumar V. 2016 Catalytic properties, functional attributes and industrial applications of β -glucosidases. *Biotech* **6**, 3. (doi:10.1007/s13205-015-0328-z)
49. Szechyńska-Hebda M, Czarnocka W, Hebda M, Bernacki MJ, Stanisław K. 2016 *Pad4*, *Lsd1* and *Eds1* regulate drought tolerance, plant biomass production, and cell wall properties. *Plant Cell Rep.* **35**, 527–539. (doi:10.1007/s00299-015-1901-y)
50. Lu Y, Rong T, Cao M. 2008 Analysis of DNA methylation in different maize tissues. *J. Genet. Genomics* **35**, 41–48. (doi:10.1016/S1673-8527(08)60006-5)
51. Kim WC, Reza IB, Kim Y, Park S, Thomashow MF, Keegstra K, Han KH. 2014 Transcription factors that directly regulate the expression of *CSLA9* encoding mannan synthase in *Arabidopsis thaliana*. *Plant Mol. Biol.* **84**, 577–587. (doi:10.1007/s11103-013-0154-9)
52. Shimaoka T, Ohnishi M, Sazuka T, Mitsuhashi N, Hara-Nishimura I, Shimazaki KI, Mimura T. 2004 Isolation of intact vacuoles and proteomic analysis of tonoplast from suspension-cultured cells of *Arabidopsis thaliana*. *Plant Cell Physiol.* **45**, 672–683. (doi:10.1093/pcp/pch099)
53. Friml J. 2003 Auxin transport-shaping the plant. *Curr. Opin. Plant Biol.* **6**, 7–12. (doi:10.1016/S136952660200031)
54. Aranda A, Pascual A. 2001 Nuclear hormone receptors and gene expression. *Physiol. Rev.* **81**, 1269–1304. (doi:10.1152/physrev.2001.81.3.1269)
55. Vogt T, Jones P. 2000 Glycosyltransferases in plant natural product synthesis: characterization of a supergene family. *Trends Plant Sci.* **5**, 380–386. (doi:10.1016/S1360-1385(00)01720-9)
56. Hu XJ, Zhang ZB, Fu ZY, Xu P, Hu SB, Li WQ. 2010 Significance of a β -ketoacyl-CoA synthase gene expression for wheat tolerance to adverse environments. *Biol. Plantarum.* **54**, 575–578. (doi:10.1007/s10535-010-0103-2)
57. Deák M, Horváth GV, Davletova S, Török K, Sass L, Vass I, Barna B, Király Z, Dudits D. 1999 Plants ectopically expressing the iron-binding protein, ferritin, are tolerant to oxidative damage and pathogens. *Nat. Biotechnol.* **17**, 192. (doi:10.1038/6198)
58. Schomburg D, Stephan D. 2006 Anthranilate N-benzoyltransferase. In *Springer handbook of enzymes*. Berlin, Germany: Springer.
59. Medeiros SCD, Júnior JEM, Sales GWP, Grangeiro TB, Nogueira NAP. 2018 Chitinases as antibacterial proteins: a systematic review. *J. Young Pharm.* **10**, 144–148. (doi:10.5530/jyp.2018.10.33)
60. Wu JH, Mooney P. 2002 *Autotetraploid tangor* plant regeneration from *in vitro* citrus somatic embryogenic callus treated with colchicine. *Plant Cell Tissue Org.* **70**, 99–104. (doi:10.1023/A:1016029829649)
61. Taira T, Shao ZZ, Hamawaki H, Larter EN. 1991 The effect of colchicine as a chromosome doubling agent for wheat-rye hybrids as influenced by pH, method of application, and post-treatment environment. *Plant Breed.* **106**, 329–333. (doi:10.1111/j.1439-0523.1991.tb00518.x)
62. Deng J, Shi X. 2009 Mutagenic effects of colchicines on *Vitis davidii* Foex. *Subtrop. Agric. Res* **1**, 84–89.
63. Duan MX, Liu XX, Zhao JR, Zhang RY, Wang YD, Xing JF, Chen XJ. 2013 Study on the doubling effect using colchicine in maize haploid. *Seed* **32**, 19–23.
64. Kong SP, Cao QW, Sun JQ, Liu B, Kun XU. 2014 The tetraploid induction of shoot-tip plantlets with colchicine in garlic. *Sci. Agric. Sin.* **47**, 3025–3033.
65. Kondo N. 1941 Chromosome doubling in *Secale*, *Haynaldia*, and *Aegilops* by colchicine treatment. *Jpn. J. Genet.* **17**, 46–54. (doi:10.1266/jjg.17.46)
66. Vijayan K, Raju PJ, Tikader A, Saratchandra B, Alkhayri J, Subramaniam S. 2014 Biotechnology of mulberry (*Morus L.*): a review. *Emir. J. Food Agr.* **26**, 472–496. (doi:10.9755/ejfa.v26i6.18019)
67. Indira C, Abraham S. 1977 Morphological and cytological studies on a radiation induced polyploid in *Capsicum annum* Linn. *Cytologia* **42**, 371–375. (doi:10.1508/cytologia.42.371)
68. Xiao Y, Bao MH, Peng F, Li YH. 2009 Induction and identification of the polyploid seedlings of *Echinacea purpurea*. *Genomics Appl. Biol.* **1**, 149–152.
69. Lihová J, Kučera J, Perný M, Marhold K. 2007 Hybridization between two polyploid *Cardamine* (Brassicaceae) species in north-western Spain: discordance between morphological and genetic variation patterns. *Annu. Bot.* **99**, 1083–1096. (doi:10.1093/aob/mcm056)
70. Clarindo WR, Carvalho CRD, Alves BMG. 2007 Mitotic evidence for the tetraploid nature of *Glycine max*, provided by high quality karyograms. *Plant Syst. Evol.* **265**, 101–107. (doi:10.1007/s00606-007-0522-5)
71. Ye WJ, Qin YH, Ye ZX, Silva JATD, Zhang LX, Wu XY. 2009 Seedless mechanism of a new mandarin cultivar ‘wuzhishatangju’ (*Citrus reticulata* Blanco). *Plant Sci.* **177**, 19–27. (doi:10.1016/j.plantsci.2009.03.005)
72. Ashraf M, Foolad MR. 2007 Roles of glycine betaine and proline in improving plant abiotic stress resistance. *Environ. Exp. Bot.* **59**, 206–216. (doi:10.1016/j.envexpbot.2005.12.006)
73. Ray S, Choudhuri MA. 1980 Flag leaf senescence in intact rice plant: effects of hormones on the activities of ‘senescence-enzymes’ during leaf age at the reproductive development. *Biochemie und Physiologie der Pflanzen* **175**, 346–353. (doi:10.1016/S0015-3796(80)80075-8)
74. Berger SL. 2007 The complex language of chromatin regulation during transcription. *Nature* **447**, 407–412. (doi:10.1038/nature05915)
75. Li A, Hu BQ, Xue ZY. 2011 DNA methylation in genomes of several annual herbaceous and woody perennial plants of varying ploidy as detected by MSAP. *Plant Mol. Biol. Rep.* **29**, 784–793. (doi:10.1007/s11105-010-0280-3)
76. Zhu HJ, Liu WG, Zhao SJ. 2014 Comparison between tetraploid watermelon (*Citrullus lanatus*) and its diploid progenitor of DNA methylation under NaCl stress. *Sci. Agric. Sin.* **47**, 4045–4055.