

Effects of long-term successive subculture on phenotype, organogenesis capacity, and genetic stability in pear

Qi Liu

Qingdao Agricultural University

Jianlong LIU

Qingdao Agricultural University

Dingli Li

Qingdao Agricultural University

zhenhua Cui

Qingdao Agricultural University

chunhui Ma

Qingdao Agricultural University

Jiankun Song

Qingdao Agricultural University

chenglin Liang

Haidu, Qingdao Agricultural University

Ran Wang

Qingdao Agricultural University

Yingjie Yang

wuhuaguoyj@163.com

Qingdao Agricultural University

Research Article

Keywords: pear, long-term subculture, somaclonal variation, genetic stability

Posted Date: September 5th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-10672817/v1>

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Additional Declarations: No competing interests reported.

Abstract

To study the effect of long-term successive subculture on pear somaclonal variation, so as to provide a theoretical basis for the control and utilization of variation *in vitro* culture and reproduction. Here, we compared newly established (3-month) tissue-cultured plantlets of the dwarfing rootstock 'Qingzhen D1' (*Pyrus communis* × *P. bretschneideri*) with those subcultured for five years, using an integrated approach that combined phenotypic and anatomical evaluation, endogenous hormone quantification, flow cytometry (FCM), whole-genome resequencing (WGS), and functional validation of candidate mutations. Long-term subcultured plantlets exhibited notable phenotypic changes, with approximately 20% of individuals developing lobed leaves; the shoot proliferation coefficient increased from 2.7 to 5.4, and the rooting rate rose from 75% to 100%. Plant growth regulator and endogenous hormone quantification revealed elevated 6-benzylaminopurine (6-BA), indole-3-butyric acid (IBA), and zeatin (ZT), and decreased indole-3-acetic acid (IAA) in long-term subcultured plantlets. FCM confirmed that no ploidy variation occurred during subculture. WGS identified 452,432 SNPs and 44,892 Indels, yielding a mutation frequency of approximately 5.0×10^{-4} per base pair. Based on the KEGG enrichment results and the observation that long-term subcultured plantlets exhibited elevated endogenous ZT levels, we selected the cytokinin oxidase/dehydrogenase (*CKX*) gene (*pycom07g02010*) located on chromosome 7 for functional validation. A non-synonymous SNP was identified in this gene, corresponding to a T-to-C transition on the coding strand, which results in a leucine-to-proline substitution at position 34 (L34P). Transient expression in tobacco demonstrated that the L34P variant partially impaired the ability of *CKX* to degrade ZT. Overall, long-term successive subculture altered morphological and physiological traits linked to shifted growth regulator and endogenous hormone levels, while maintaining relatively stable genetic integrity. These findings suggest a mechanistic link between somaclonal variation and cytokinin homeostasis, providing a scientific basis for optimizing pear micropropagation strategies.

Introduction

In vitro propagation using tissue culture technology has the advantage of rapid multiplication, which has been widely used for the large-scale production of clonal plants. In the *in vitro* propagation process, subculture is an important procedure and plays an essential role in optimization of micropropagation systems, multiplication and large-scale plantlet production^[1]. However, in the long-term subculture process, the phenotype and organogenesis capacity of explants may change. For instance, the leaves of pear (*Pyrus betulaefolia*) and apple (*Malus xiaojinensis*) showed lobed starting from the third and fifteenth generation of subculture, with subsequent generations showing increasing severity of lobing^[2, 3]. In *Vanilla planifolia*, subculture generation also affected the competence to produce shoots, with the fewest shoots per explant being noted during the initial one to five subcultures, whereas the highest multiplication rate was observed during subcultures six to ten^[4]. Similar results were reported in *Aloe vera*, where shoot production in the first subculture was lower compared to that obtained in the sixth subculture^[5]. Nevertheless, this unidirectional rising trend does not persist indefinitely during prolonged subculturing for woody plants such as pears. The shoot proliferation rates of 'Jinhua' and 'Jinshui' pears increased progressively with increasing subculture generation, but declined gradually after 40–46 passages^[6]. At the same time, long-term subculture generation can also affect the rooting capacity of shoots. With increasing subculture generation, the adventitious rooting abilities of cherry and *Malus xiaojinensis* was significantly improved^[7, 3]. However, research on *Cedrela fissilis* Vellozo demonstrated that long-term subculture significantly suppressed rooting performance, causing significant declines in root induction rate, root numbers, root elongation, and the survival rates of regenerated plantlets^[8].

Somaclonal variation, which refers to genetic or epigenetic alterations during cell and tissue culture, was first proposed in 1981^[9]. Somaclonal variation can induce gene polymorphism. So one hand, it can produce new genotypes or traits, which have great value for the breeding of new varieties; on the other hand, it has adverse effects on the genetic stability of clonal offspring. Genetic instability poses a significant challenge in commercial micropropagation, undermining the uniformity and genetic fidelity of propagated plantlets^[10]. One key factor driving somaclonal variation is long-term subculture. Studies have demonstrated that somaclonal variation frequency increases with successive subculture generations^[11], including chromosomal structural variation^[12], changes in chromosome number^[13], base mutations^[14, 15], and so on. For example, genetic variation was observed to initiate at the 6th passage during tissue culture of roselle^[16]. In coffee suspension cell culture, aneuploidy occurs after 4 months of subculture^[17]. SSR molecular marker analysis demonstrated that extended subculture generations led to increased polymorphic band variation in 'Gala' apple callus^[18]. The variation rate of banana (*Musa spp.*) was 1.3% after three cycles of *in vitro* culture, and it increased to 3.8% after 11 cycles of *in vitro* culture^[19]. The DNA-level variation frequency of fourth-subculture pitaya plantlets was

as high as 24% ^[20]. Beyond DNA-level alterations, long-term subculture can also perturb endogenous hormone homeostasis—for instance, IAA accumulation has been correlated with increased somaclonal variation frequency ^[21], which in turn modulates organogenic capacity and phenotypic traits *in vitro*.

Pear is an important horticultural crop with high economic value. As a highly heterozygous woody species, sexual reproduction leads to obvious trait segregation. Meanwhile, asexual propagation techniques, including layering and cutting, are limited by poor rooting ability. Therefore, tissue culture is widely used for rapid propagation in production. However, it remains unclear whether long-term subculture alters the phenotype, organogenesis capacity, and genetic stability of pear.

In this research, a dwarfing rootstock cultivar, 'Qingzhen D1' (*Pyrus communis* L. × *Pyrus bretschneideri* Rehd.) was taken as material. Taken newly established tissue-cultured plantlets as controls, analyzed the changes of the morphology, organogenesis capacity, and endogenous hormone contents in the plantlets that were successive subcultured for 5 years. At the same time, genetic stability was assessed through flow cytometry (FCM) and whole-genome sequencing (WGS). And a missense mutation (L34P, Leu→Pro) at a conserved residue of the cytokinin-degrading gene *CKX* (*pycom07g02010*) was analyzed through transient expression in *Nicotiana benthamiana*, confirming that L34P partially impairs CKX-mediated ZT degradation. This study helps to further understand the rules of somaclonal variation of pear during long-term successive subculture, and provides a theoretical basis for the application of tissue culture in pear propagation.

Materials and Methods

Plant material and establishment of tissue culture system

Plant material was obtained from the pear experimental orchard of Qingdao Agricultural University. The plant material was the pear dwarfing rootstock, 'Qingzhen D1', the offspring of *P. communis* L. dwarf variety 'Le Nain Vert' and '*P. bretschneideri* Rehd.'. One-year-old branches with full vegetative buds were harvested in March and placed in tap water. The water was changed daily until the vegetative buds germinated and grew up to about 0.5 cm long. The buds were surface-disinfected with 75% ethanol (30 s) and 2.0% NaClO (10 min), then rinsed with sterile distilled water for 3-5 times. The explants were cultured on MS ^[22] medium supplemented with 0.1% activated carbon and 1.0 g·L⁻¹ polyvinylpyrrolidone (PVP). The culture conditions were set as follows: temperature at 25±1 °C, the light intensity at 100 μmol·m⁻²·s⁻¹ and 16 h light / 8 h dark photoperiod. After about 20 d, survival explants were cultured on MS medium supplemented with 1.0 mg·L⁻¹ 6-BA and 0.1 mg·L⁻¹ IBA for subculture. The plantlets were subcultured once every 4 weeks. The plantlets continuously subcultured for 3 months and 5 years were considered as newly established tissue cultured plantlets and long-term subcultured ones, separately.

Morphology and anatomical structure analysis

The newly established and long-term subcultured plantlets, as well as their rooting and transplanted plants were used for morphological observation and analysis. The leaf length, width, leaf area and area index were analyzed using professional WinFOLIA (Legentsys Co. Ltd., Canada) analysis software. The plant height and stem diameter were measured with ruler and vernier caliper, respectively. Each type of subcultured plantlets were taken 30 plants randomly.

For anatomical structure analysis, newly established and long-term subcultured plantlets were taken 30 plants randomly, separately. The paraffin sections of mature leaves and stems were first prepared according to the method reported by Wang ^[23]. Then the paraffin section samples were observed and took photos using microscope (Leica RM 2235). Use ImageJ (version 1.53e) software measurement analysis system to determine the thickness of palisade tissue and sponge tissue, as well as the palisade sponge ratio (P/S, palisade tissue thickness / sponge tissue thickness). The areas of phloem, xylem and pith were measured by digimizer software, and the timber ratio (ratio of xylem and pith to phloem) was calculated.

For leaf epidermal stomata observation, colorless nail polish were applied evenly on the abaxial surface of the mature leaves for 2-3 minutes to form a film, then the film was peeled off gently with tweezer and immediately observed under a microscope. Each type of subcultured plantlets were taken 30 plants randomly, and 2 mature leaves were used for epidermal stomata observation. Three fields of view on each leaf were picked randomly for observation and photograph. Then the stomata shapes and densities were calculated using the ImageJ software.

Comparison of proliferation ability and rooting ability

To analyze the effect of long-term subculture on proliferation ability, the newly established tissue cultured plantlets and long-term subcultured ones with strong and uniform growth were inoculated on the subculture medium for 5 weeks. Then the multiplication index was calculated. The multiplication index = the total number of multiplied shoots / the number of inoculated plantlets. In this experiment, three repeats were adopted per treatment, and 10 explants were used in every repeat.

To analyze the rooting ability of long-term subcultured plantlets, the 1.5 cm height newly established and long-term subcultured plantlets with strong and uniform growth were inoculated on rooting medium (1/2MS medium supplemented with $0.3 \text{ mg} \cdot \text{L}^{-1}$ IBA), respectively. The rooting rate, average root length and number were observed and counted 30 days later. The rooted plantlets were then acclimatized and transplanted to plastic pots under greenhouse conditions, and the survival rate was counted 20 days after transplantation. The rooting rate = the number of rooting plantlets / the number of inoculated plants $\times 100\%$. Number of roots = the total number of adventitious roots / the number of rooting plantlets. In this experiment, three repeats were adopted per treatment, and 10 explants were used in every repeat.

Determination of hormone content

The hormone contents of newly established and long-term subcultured plantlets were detected using 6 biological replicate samples, separately. 0.5 g fresh plant samples were ground and dissolved in 1 mL solution of methanol/water/formic acid (15:4:1, v/v/v). 10 μL internal standard mixed solution ($100 \text{ ng} \cdot \text{mL}^{-1}$) was added into the extract. And the mixture was vortexed for 10 minutes, then centrifuged for 5 min ($12000 \text{ r} \cdot \text{min}^{-1}$). The supernatant was transferred to clean plastic microtubes and evaporated to dryness, which then dissolved in 100 μL 80% methanol (V/V) and filtered through a $0.22 \mu\text{m}$ membrane filter. Phytohormones contents were detected by MetWare (<http://www.metware.cn/>) based on the AB Sciex QTRAP 6500 LC-MS/MS platform.

Analysis of ploidy variation by flow cytometry

The ploidy levels of 100 randomly chosen newly established and long-term subcultured plantlets were determined using a flow cytometer (Partec GmbH, Germany) as described by Liu ^[24]. And the newly established plantlets were taken as control.

DNA isolation and genome resequencing

Total genomic DNA was isolated from newly established and long-term subcultured plantlets (six biological replicates per group, each replicate consisting of a pooled sample from five individual plants) using a Plant Genomic DNA Extraction Kit (Sangon Biotech, Shanghai, China). DNA quality and concentration were assessed by NanoDrop 2000 spectrophotometry (Thermo Fisher) and 1% agarose gel electrophoresis. Qualified DNA samples (amount $> 1.5 \mu\text{g}$, concentration $> 50 \text{ ng} \cdot \mu\text{L}^{-1}$, and free of RNA and protein contamination) were randomly fragmented to $\sim 350 \text{ bp}$ using a Covaris ultrasonicator, purified with Agencourt AMPure XP magnetic beads, and used for library construction. Library quality was evaluated by Qubit 3.0 fluorometry for preliminary quantification, NGS3K/Caliper for insert size determination, and qPCR for accurate quantification of the effective library concentration ($> 3 \text{ nM}$). Finally, the qualified libraries were sequenced on the Illumina HiSeq™ platform using the PE150 strategy at Novogene Co., Ltd. (Beijing, China).

Data quality control and filtering

The original sequence data which was obtained by Illumina platform was transformed into raw data by Base recognition (Base Calling). Then the raw data was filtered through removing single-end sequencing read pairs with adapters or error rates exceeding 10%, as well as single-end sequencing reads with more than 50% low-quality bases relative to the read length, and the clean data was finally obtained.

Mutation site detection and annotation

The raw sequence data were converted into raw reads through base calling. Quality control was performed by calculating the proportion of high-quality reads with a quality score greater than Q30 and the GC content in the raw reads. The clean reads were then aligned to the reference genome (*Pyrus communis* Bartlett DH Genome v2.0) using BWA software. Duplicate reads were removed using SAMtools. SNPs and InDels of less than 50 bp in length were detected in individual samples following the

method described by Lilly et al. [25]. Variants consistently present across all six replicates (30 plantlets per replicate) of long-term subcultured plantlets were compared with those shared across all six replicates of newly established cultures. Variants unique to the long-term subcultured group were defined as long-term subculture-specific and selected for downstream analysis. Using BCFtools, the SNPs and InDels were categorized into intergenic regions, gene regions (exons, introns, and UTRs), upstream regions (within 1500 bp upstream of the start codon), and downstream regions (within 1500 bp downstream of the stop codon).

Mutation sites effect prediction

The effects of mutation sites were predicted utilizing the SnpEff ver 4.3T program [26]. And ANNOVAR program was used to annotate the polymorphic loci with High-impact (HI) on amino acid and protein. Then the annotated genes with High-impact (HI) were functionally classified and predicted based on Kyoto Encyclopedia of Genes and Genomes (KEGG; <https://www.kegg.jp/>) databases.

Among the KEGG-classified genes, those assigned to the zeatin biosynthesis were of particular interest. After reviewing the functional annotations, two genes were selected as candidates for validation. To validate the identified SNPs of these genes, primers were designed (Table S1). PCR amplification was performed using total DNA extracted from long-term subcultured plantlets and newly established ones, and the products were sent to Sangon Biotech (Shanghai) Co., Ltd. for Sanger sequencing. Sequence alignment results were compared with the VCF file from whole-genome sequencing data, and the sequences were simultaneously translated into amino acids for comparison.

Functional analysis of genetic variants

To validate the effect of the point mutation on cytokinin degradation, overexpression constructs for the wild-type *CKX* (*CKX-WT*) and the point-mutated variant (*CKX-MUT*), together with an empty vector (EV) control, were generated. *Agrobacterium tumefaciens* strain GV3101 harboring each construct was used to transiently transform *N. benthamiana* leaves by agroinfiltration essentially as described by Sparkes et al. [27]. The presence of the transgene at the DNA level was confirmed by PCR, and its expression at the RNA level was verified by RT-qPCR. Total RNA was extracted from *N. benthamiana* leaves using the RNAPrep Pure Plant Kit (Tiangen, Beijing, China). First-strand cDNA was synthesized using the PrimeScript RT Reagent Kit (TaKaRa, Dalian, China). qRT-PCR was performed using SYBR Green Master Mix (Takara) on a CFX96 system (Thermo Fisher Scientific, USA). The *N. benthamiana* actin gene was used as the internal reference. Primer sequences are listed in Table S1. Relative expression levels were calculated using the 2^{-ΔΔCt} method. The ZT contents were quantified as described above.

Statistical analysis

SPSS version 25 software (IBM) was used to conduct the statistical analyses. One-way analysis of variance followed by Duncan's Multiple Range Test (DMRT, *p* < 0.05) was done to test the significance of difference. Data represented the mean of different replications, and the means with the same letters are not significant.

Results

Effect of long-term successive subculture on the phenotypic characteristics of ‘Qingzhen D1’

We first analyzed the growth phenotype differences of newly established (subcultured for 3 month) and long-term subcultured (subcultured for 5 year) ‘Qingzhen D1’ plantlets. As shown in Table S2, whatever under *in vitro* culture condition or transplanted and cultured for 30 d after rooting, no significant differences in plant height, stem diameter were detected. But approximately 20% of long-term subcultured plantlets developed lobed leaves (Fig.1).

Anatomical observation showed that no significant differences were detected in leaf palisade tissue thickness, spongy tissue thickness, palisade-to-spongy tissue ratio, as well as stem phloem area, xylem area and pith area between newly established and long-term subcultured pear plantlets (Table S3). We also observed and measured the leaf epidermal stomata. As shown in Fig.2 and Table 1, the stomatal density of long-term subcultured plantlets showed significantly higher than newly established plantlets. But the differences of stomatal length and width were not significant.

Table 1 Stomatal analysis of *in vitro* plantlets

Tissue-cultured plantlets	Stomatal length /um	Stomatal width /um	Stomatal density No./mm ²	Stomatal aperture ratio
Newly established <i>in vitro</i> plantlets	30.7±3.29 a	27.2±2.95 a	156.4±33.2 b	0.84±0.14 a
Long-term subcultured <i>in vitro</i> plantlets	32.8±2.33 a	29.9±6.58 a	329.8±34.8 a	0.89±0.15 a

Note: Different lower-case letters showed significant difference (P < 0.05).

Effects of long-term successive subculture on the shoots proliferation and rooting ability of ‘Qingzhen D1’

Though shoot proliferation and rooting ability analysis of newly established and long-term subcultured plantlets, we found that the multiplication index of long-term subcultured plantlets was 5.4, about twice as much as newly established plantlets (Table 2). Concerning the rooting ability, cultured on the same rooting medium, 75% of the newly established plantlets could be induced adventitious roots, while 100% rooting rate was observed in long-term subcultured ones (Table 2). At the same time, the average root number of newly established plantlets was 8, which was significantly lower than that (18) of long-term subcultured ones. But there was no significant difference in root length (Table 2). Although a slight difference in survival rate was observed between the two types ‘Qingzhen D1’ transplanted plants, both were above 80%. In summary, long-term subculture increased the organogenesis capacity of ‘Qingzhen D1’ explants to some extent.

Table 2 Effect of long-term subculture on shoots proliferation and rooting ability of *in vitro* plantlets

Variety	Multiplication index	Rate of rooting (%)	Number of roots	Root length (cm)	Transplant survival rate (%)
Newly established <i>in vitro</i> plantlets	2.6±1.3 b	75	8±3b	3.05±0.95 a	80
Long-term subcultured <i>in vitro</i> plantlets	5.4±1.7 a	100	18±5a	4.54±1.25 a	87

Note: Different lower-case letters showed significant difference (P < 0.05).

Effects of long-term successive subculture on endogenous hormone content of ‘Qingzhen D1’

Considering the hormone and plant growth regulators play roles in plant proliferation and rooting abilities. We detected the endogenous hormone contents of newly established and long-term subcultured plantlets. As shown in Fig.3, comparing with newly established plantlets, the contents of 6-BA, ZT and IBA in long-term subcultured plantlets were significantly higher, while the IAA content decreased. We speculated that the continuous long-term subculture on MS medium supplemented with 1.0 mg·L⁻¹ 6-BA and 0.1 mg·L⁻¹ IBA might lead to a certain accumulation of exogenous 6-BA and IBA in the plantlets. And the changes of auxin-cytokinin balance may disturb normal organogenesis and growth homeostasis, thereby inducing variations in phenotypic traits and organogenesis capacity in long-term subcultured pear plantlets.

Ploidy analysis of long-term subcultured ‘Qingzhen D1’ plantlets

In order to investigate whether long-term subculture affect genetic stability, we first detected the chromosome ploidy of the two types of plantlets. As shown in Fig.S1, through analysis of DNA contents by FCM, the peaks in the fluorescence histograms displayed resemblance between the two types of plantlets, and no statistically significant difference (p<0.05) was obtained in the geometric means of fluorescence histograms (Table S4). The result proved that the long-term subculture process did not change the ploidy of ‘Qingzhen D1’.

Genomic variation characteristics induced by long-term subculture

Through genome resequencing technique, 463~714 million reads per sample were obtained. And for the samples, the sequencing depth ranged from 8.50 × to 10.30 ×, the GC contents were 37.85 % - 38.31%, and the Q30 all above 90.63%. Through mapping with reference genome, the mapping rate of all samples ranged from 97.04% to 97.36%, and the cover ratio ranged from 94.27 % to 95.04 %. The result suggested that the resequencing data were dependable and can be used for further analysis.

Through resequencing dates comparing of long-term subcultured plantlets to newly established ones, a total of 452,432 SNPs and 44,892 Indels were detected. Through statistical analysis, the single site variation frequency of long-term subcultured plantlets was 5.0×10^{-4} .

Among these SNPs, 282,175 transitions (Ti), including both interchanges of purines (A-G) and pyrimidines (C-T), 170,221 transversions (Tv), the interchanges between purine and pyrimidine bases, were detected. Transitions were more frequent than transversions, Ti/Tv ratio was 1.66. The most common mutation type was C:G > T:A (34.2%), followed by T:A > C:G (31.3%). The C:G > G:C mutation type was the rarest, representing less than 10% (Fig.S2).

Through correlation analysis between the mutation sites and the neighboring bases, we found that SNPs adjacent to adenine (A) and thymine (T) were more frequent than those adjacent to guanine (G) and cytosine (C). And the frequency of 5'-A [N] N-3' (30.08%), 5'-N [N] A-3' (29.48%), 5'-T [N] N-3', and 5'-N [N] T-3' (30.13%) were all similar. But the frequency of 5'-C [N] N-3' (23.95%) was similar with 5'-N [N] G-3' (23.93%), which were obviously higher than 5'-N [N] C-3' (16.46%) and 5'-G [N] N-3' (16.48%) (Table 3).

Table 3 Context of SNPs

Context (5'→3')	Percentage	Context (5'→3')	Percentage	Context (5'→3')	Percentage	Context (5'→3')	Percentage	Context (5'→3')	Percentage
A[N]A	9.30	C[N]A	7.14	G[N]A	4.89	T[N]A	8.15	N[N]A	29.48
A[N]C	4.91	C[N]C	3.82	G[N]C	2.86	T[N]C	4.87	N[N]C	16.46
A[N]G	7.57	C[N]G	5.43	G[N]G	3.80	T[N]G	7.13	N[N]G	23.93
A[N]T	8.30	C[N]T	7.56	G[N]T	4.92	T[N]T	9.34	N[N]T	30.13
A[N]N	30.08	C[N]N	23.95	G[N]N	16.48	T[N]N	29.50	N[N]N	100.00

These detected indels (18,769 insertions and 26,123 deletions, with In/Del ratio was 0.7) varied in length, including mononucleotides, dinucleotides, trinucleotides, and tetranucleotides, etc., even to 20 nucleotides in length. Mononucleotide indels were the most common mutation types, accounting for 30% of total indels, followed by dinucleotide and trinucleotide indels, each accounting for 15%-20%. Indels longer than tetranucleotides represented only a small portion (Fig.S3).

Through the distribution analysis of SNPs and Indels on chromosomes, we found that on average the SNP and Indel on most chromosomes occurred once every 0.84 - 1.23 kb and 8.4 - 11.98 kb, separately, except for chromosome 4, with occurrence once every 14.6 kb and 141.65 kb (Table 4). The result suggests that different chromosomes display different sensibility for mutations, and the chromosome 4 had higher stability.

Table 4 SNP and Indel distribution on each chromosome

chromosome	Chromosome length (Mb)	SNP density ^a (kb)	Indel density ^a (kb)
chr1	21.57	0.98	9.85
chr2	24.78	0.84	8.4
chr3	23.58	1.00	9.97
chr4	23.00	14.6	141.65
chr5	32.25	0.91	8.82
chr6	24.26	1.07	10.79
chr7	27.73	1.102	11.18
chr8	20.86	0.96	9.35
chr9	21.22	0.94	9.52
chr10	29.59	0.85	8.47
chr11	29.99	1.23	11.98
chr12	24.69	1.18	11.72
chr13	26.82	0.90	9.49
chr14	21.74	0.92	9.55
chr15	39.17	0.85	8.79
chr16	28.94	0.94	9.46
chr17	26.287	1.00	10.37

^aSNP density is presented as the average physical distance between two adjacent SNP loci.

Meanwhile, we analyzed the distribution of the SNPs and Indels in genic and intergenic regions. As shown in Fig.4A, 156,945 (34.69%) and 295,487 (65.31%) SNPs distributed in genic and intergenic regions, separately. In the genic region, 66073, 15.35% of total SNPs, were detected in exons region. Among the SNPs in exons region, 33,749 SNPs were synonymous, which would not affect protein function in general. And 32,954 SNPs (7.56% of total SNPs) were non-synonymous mutations, including 32,641 missense and 313 nonsense mutations.

Concerning the Indels, 12,439 (27.71%) and 32,453 (72.29%) ones distributed in genic and intergenic regions, separately. Meanwhile, 2537 Indels, which included 950 nonframeshifts mutations, 1543 frameshifts mutations and some unknown types of mutations, located in exons, only accounting for 5.65% of those in total Indels, separately. These results suggest that most of the variations occurred in intergenic regions, and in the exons of genic regions, the number of non-synonymous SNP mutations and frameshifts Indel mutations are relatively fewer.

According to the SnpEff software, we further analyzed the SNPs and Indels in exonic regions. As shown in Table 5, 65.096% of SNPs and 65.796% of Indels were classified as having modifier effects. A total of 496 SNPs and 1,528 Indels were predicted to have high effects, accounting for 0.262% of the exonic SNPs and 20.75% of the exonic Indels, respectively.

Table 5 SNPEFF annotation classification of mutation sites

Type	SNP Count	SNP Percent %	Indel Count	Indel Percent %
High	496	0.262	1,528	20.75
Moderate	31,779	16.788	883	11.99
Low	33,798	17.854	0	\
Modifier	123,226	65.096	4953	67.26

The high-impact (HI) polymorphisms were annotated by ANNOVAR software, identifying 2,066 mutant genes. KEGG pathway enrichment analysis revealed that the mutant genes were significantly enriched in pathways related to "folding, sorting and degradation", followed by "carbohydrate metabolism" and "translation" (Fig. 5).

Functional validation of the *CKX* gene mutation

Considering our physiological observation that cytokinin contents were markedly elevated in long-term subcultured plantlets. Based on the KEGG enrichment results, we further manually screened genes involved in the zeatin biosynthesis pathway from all high-impact variant genes. Among these candidates, *pycom07g02010* harboured a non-synonymous SNP: a T-to-C transition at nucleotide position 1,615,312 on chromosome 7, resulting in a Leu-to-Pro substitution, which may affect protein function (Fig.6A). So *pycom07g02010* was selected for subsequent functional characterization.

To functionally validate the effect of this *CKX* point mutation, the wild-type (*CKX-WT*) and point-mutated (*CKX-MUT*) constructs were cloned and verified by Sanger sequencing. Following *Agrobacterium*-mediated transient infiltration into *N. benthamiana* leaves, PCR detection confirmed the presence of the transgene in all infiltrated tissues. qRT-PCR analysis confirmed that both *CKX-WT* and *CKX-MUT* were successfully overexpressed in tobacco leaves at comparable levels (Fig.6B). To assess the functional consequence of the *CKX* point mutation on cytokinin metabolism, ZT content was quantified in leaves expressing *CKX-WT*, *CKX-MUT*, and the empty vector (EV) control. Compared with the EV control (197.8 ± 54.6 ng/g FW), overexpression of *CKX-WT* significantly reduced ZT content to 134.9 ± 21.6 ng/g FW ($\downarrow 31.8\%$), confirming the cytokinin-degrading function of the wild-type *CKX* enzyme. In contrast, *CKX-MUT* expressing leaves exhibited ZT content of 165.4 ± 12.8 ng/g FW, which was intermediate between the EV control and *CKX-WT*, and was higher than that in *CKX-WT* expressing leaves, suggesting a partial impairment of *CKX* activity caused by the point mutation (Fig.6C). These results provide a potential mechanistic basis for the altered cytokinin homeostasis observed in long-term subcultured pear plantlets.

Discussion

In vitro culture serves as a key technology for clonal propagation, germplasm preservation, and genetic improvement of perennial woody fruit trees such as pear [28]. However, long-term successive subculture inevitably increases the risk of somaclonal variation [29]. While the phenotypic and organogenesis capacity alterations, genome-wide variation characteristics, and corresponding molecular mechanisms underlying long-term successive subculture remain poorly understood in pear rootstock.

In our study, compared with 3-month newly-established pear plantlets, 5-year long-term subcultured lines displayed partial leaf lobing and increased stomatal density (Fig. 1; Table 1). During *in vitro* subculture, phenotypic changes in plantlets frequently occur, with leaf morphology alteration being the most commonly observed phenotype, which has been documented in a wide range of species, including caladium, cannabis, strawberry, cherry, and date palm [30, 31]. There are also several reports concerning the effects of *in vitro* culture on stomata. For example, stomatal opening and density were reported increasing in *Philodendron* 'Con-go' plantlets during *in vitro* culture [32]. And Santos et al. [33] reported that *Billbergia zebrina* plantlets cultured under *in vitro* conditions exhibited higher stomatal density. Notably, vegetative growth traits such as plant height and stem diameter remained stable (Table S2), while only leaf-related phenotypes were altered, consistent with the widely documented observation that leaf morphology is among the most frequently affected traits in somaclonal variation, whereas variation in plant architecture-related traits is typically lower and species-dependent [34, 35]. It should be noted that this study used only plantlets subcultured for 3 months and 5 years as experimental materials; including intermediate time points would help to more comprehensively reveal the dynamic effects of culture duration on phenotypic variation.

Meanwhile, long-term subcultured pear plantlets exhibited stronger shoot multiplication and adventitious rooting capacities compared with newly established ones (Table 2). Similar results were reported in *Castanea sativa*, where repeated subcultures progressively enhanced shoot multiplication, rooting competence, and plantlet survival [36]. However, the response of organogenic competence to subculture generation is highly species- and genotype-dependent. For instance, in cherry, plum, as well as certain pear genotypes, shoot multiplication capacity generally decreased with increasing subculture generation [37], whereas in apple cultivars, shoot proliferation and rooting remained relatively stable after long-term subculture [38].

Plant growth regulators play central roles in organogenesis [29]. In our study, the subculture medium was supplemented with IBA and 6-BA. After years of successive subculture, exogenous IBA and 6-BA accumulated in plantlets, accompanied by increased endogenous ZT and decreased IAA (Fig. 3), which may explain the enhanced organogenesis capacities. The resultant increase in the CTK/IAA ratio promotes shoot induction and cell proliferation [39]. Concurrently, accumulated IBA sustains adventitious root formation. The peroxisomal conversion of IBA to IAA is coupled with NO release, and the combined action of IBA-derived IAA and NO effectively promotes root initiation [40, 41]. Collectively, a high CTK/IAA ratio favouring shoot proliferation, and a sustained IBA supply supporting rooting—account for the enhanced organogenic capacity of pear plantlets following prolonged subculture. In addition, cytokinin positively regulates stomatal development progression and final stomatal number in *Arabidopsis* [42]. And transgenic manipulation of *CKX* activity directly alters leaf margin morphogenesis: enhanced cytokinin degradation reduces leaf serration [43, 44], whereas elevated cytokinin promotes lobed leaf morphogenesis [45, 46]. So the characteristic alterations in leaf morphology, elevated stomatal density, and improved organogenic competence observed in long-term subcultured pear plantlets may largely be associated with the pronounced increase in cytokinin levels. Although these hormonal changes can explain the enhanced organogenic capacity and phenotypic alterations, the genetic basis remains to be further explored.

To explore the genetic basis of the hormone contents and phenotypic alterations observed in long-term subcultured pear plantlets, we analyzed the genomic stability and somaclonal mutation frequency through whole-genome resequencing. In our study, the mutation frequency of long-term subcultured pear plantlets was approximately 5.0×10^{-4} . In *Arabidopsis*, *in vitro* cultured lines exhibited mutation rates of 4.2×10^{-7} to 24.2×10^{-7} [14]. Rice clones maintained *in vitro* for 20 years accumulated mutations at 2.9×10^{-3} to 3.6×10^{-3} [47]. In sweet orange callus subcultured for over 30 years, the mutation frequency reached 1.7×10^{-7} , approximately 100-fold above the natural background [48]. This comparison suggests that pear displays a moderate somaclonal mutation level among these tested species. Although the overall mutation frequency was relatively high, only 0.262% of exonic SNPs and 20.75% of exonic InDels were predicted to have high-impact effects on gene function. These findings suggest that the genetic stability of long-term subcultured pears generally acceptable, with a low risk of widespread functionally consequential mutations. In addition, the mutation characteristics observed in this study, such as the mutation frequency ($\sim 5.0 \times 10^{-4}$), the predominance of C:G > T:A transition, the highest SNP frequency in 5'-T[N]T-3' and lowest in 5'-G[N]C-3', and uneven variant distribution across chromosomes with chr4 exhibiting markedly lower mutation density, were consistent with genomic variation signatures observed in our previous work on leaf regeneration process [24]. The consistent mutational patterns observed across two distinct *in vitro* culture systems indicated that these sequence mutational features might represent conserved features of somaclonal variation in pear.

Due to the upregulation of endogenous ZT content in plantlets subjected to five years of long-term subculture, the T-to-C transition identified in a cytokinin oxidase/dehydrogenase (*CKX*) gene of these long-term subcultured plantlets is particularly noteworthy. *CKX* catalyzes the irreversible degradation of cytokinins, and mutations impairing its enzymatic activity can directly alter endogenous cytokinin homeostasis [49]. Our transient expression assay in *Nicotiana benthamiana* further indicated that the L34P substitution partially blocks CKX-dependent ZT breakdown, triggering excessive accumulation of endogenous ZT, which in turn may further destabilize the genome and promote additional variation. But the functional evidence relies on a heterologous transient system, which cannot perfectly reflect the endogenous regulatory context of pear. Subsequent stable transformation or CRISPR editing in pear are essential to validate the effect of the L34P mutation on endogenous ZT accumulation. In addition, it has been reported that exogenous 6-BA application could increase total endogenous cytokinins in *Medicago arborea* callus [50], and similar response was observed in *Amelanchier alnifolia* [51]. So we speculated that the L34P mutation of *CKX* gene together with sustained accumulation of exogenous 6-BA account for the elevated ZT content in long-term subcultured plantlets. Nevertheless, stable transformation is still required to confirm the role of the *CKX* L34P mutation, which will be the focus of our further research.

Somaclonal variation arises from diverse abiotic stresses imposed during *in vitro* culture. Long-term exposure to exogenously supplied cytokinins is regarded as a critical hormonal stress and induces somaclonal variation. For example, supplementation with 6-BA and NAA markedly increased somaclonal variation in *African violet* [52]; excessive 6-BA concentrations promoted somaclonal variation in banana [53]; and 8–10 mg·L⁻¹ 6-BA induced red-leaf variants in *anthurium* at rates of 3%–7% [54].

Accordingly, we speculated that long-term subculture on 6-BA-containing medium results in the persistent absorption and retention of exogenous 6-BA, which may induce endogenous ZT biosynthesis. Then the higher level of cytokinin stress drives the emergence

of the CKX L34P mutation. This mutation may weaken CKX-mediated ZT catabolism and elevate cytokinin concentration, thereby contributing to the enhanced organogenic competence and the characteristic alterations in stomatal density and leaf architecture in long-term subcultured pear plantlets. Meanwhile, a self-reinforcing feedback loop may establish: high cytokinin levels induce CKX variants, which in turn exacerbate cytokinin overaccumulation; the higher hormonal stress continuously impairs genome stability and accelerates the generation of additional somaclonal variation throughout long term *in vitro* subculture. It is worth noting that whether the high cytokinin levels and the phenotypic changes will affect growth after transplanting to the field remains to be tested through long-term field trials.

Conclusions

Long-term subculture of pear dwarf rootstock 'Qingzhen D1' altered leaf morphology, increased stomatal density and greatly improved shoot multiplication and rooting abilities, accompanied by accumulation of exogenous 6-BA and endogenous ZT as well as reduced IAA. Genome resequencing analysis showed that the overall mutation frequency was approximately 5.0×10^{-4} per site. Most variants distributed in intergenic regions, and only a tiny fraction of exonic mutations exerted high functional impacts. A functional missense mutation in CKX (L34P) was identified, which partially impairs ZT degradation and may contribute to cytokinin accumulation. This research provides a basis for monitoring somaclonal variation in pear micropropagation.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The whole-genome sequencing data supporting the conclusions of this article are available in the NCBI Sequence Read Archive (SRA) under the BioProject accession PRJNA1511452 (<https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1511452>). The raw sequence data are available under the SRA accessions SRR40127118–SRR40127129.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by the National Key R&D Program of China (2018YFD1000102) and the China Agriculture Research System (CARS-28-07).

Authors' contributions

LQ, YYJ and WR conceived and designed the experiments. LQ performed the experiments. LQ and YYJ wrote the manuscript. LJL, LDL, CZH, MCH, SJK and LCLassisted with the experiments. All authors read and approved the final manuscript.

Acknowledgements

The authors thank Novogene Co., Ltd. for assistance with sequencing and bioinformatics analysis. Additionally, I thank my husband for his invaluable assistance with data uploading and his wholehearted support with computer software throughout this study.

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Figures

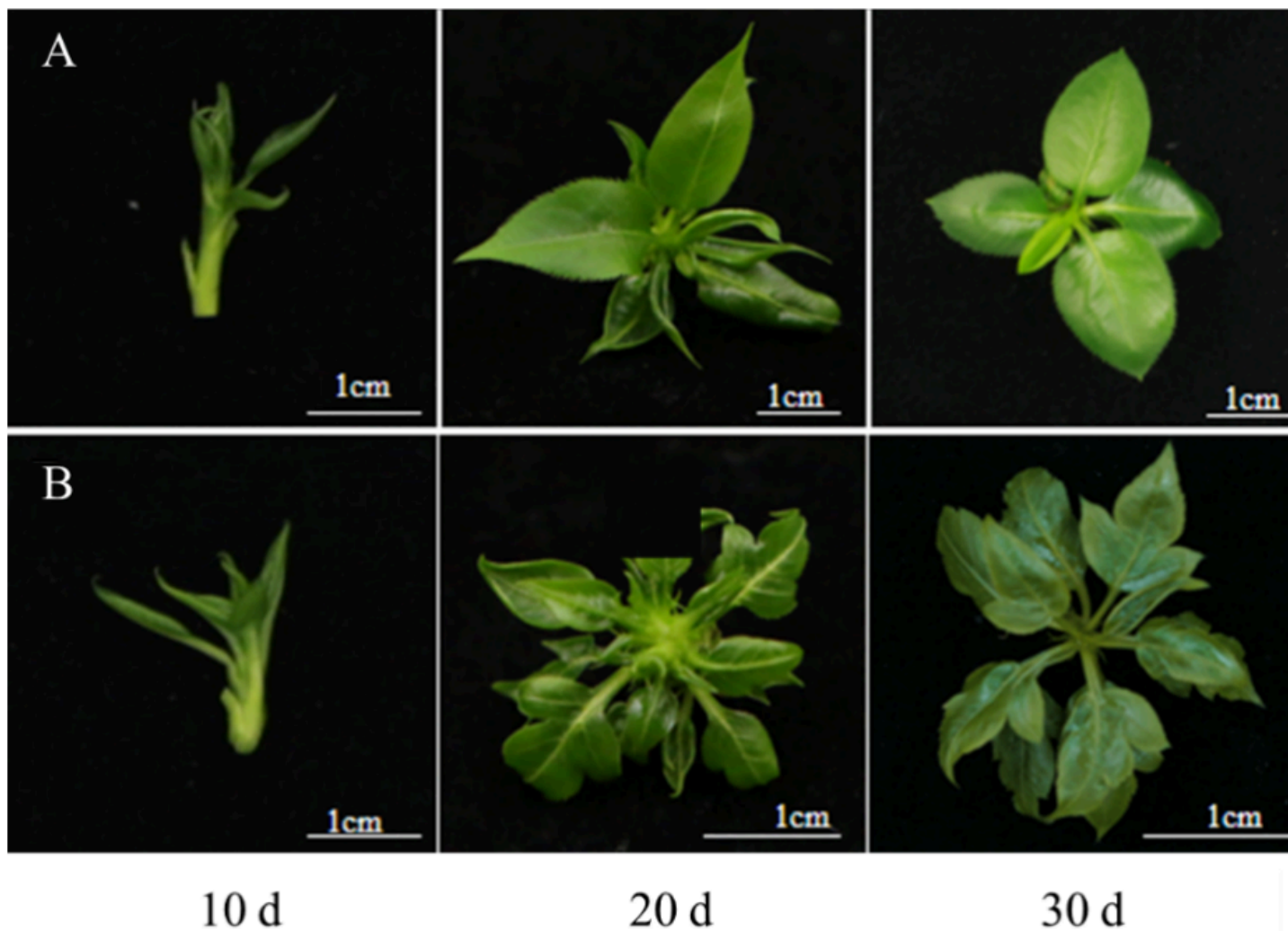


Figure 1

Growth process of in vitro plantlets with normal and lobed leaves

A: *In vitro* plantlets of normal leaves; B: *In vitro* plantlets of lobes leaves

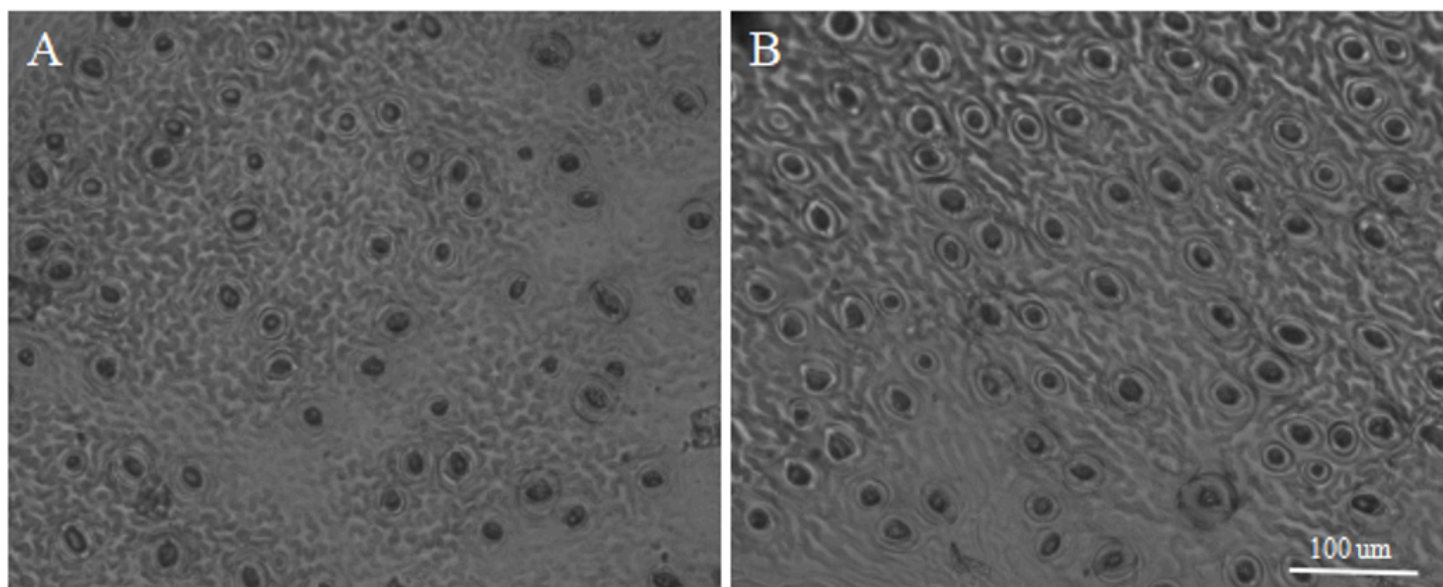


Figure 2

Stomata characteristics of newly established (A) and long-term subcultured (B) plantlets

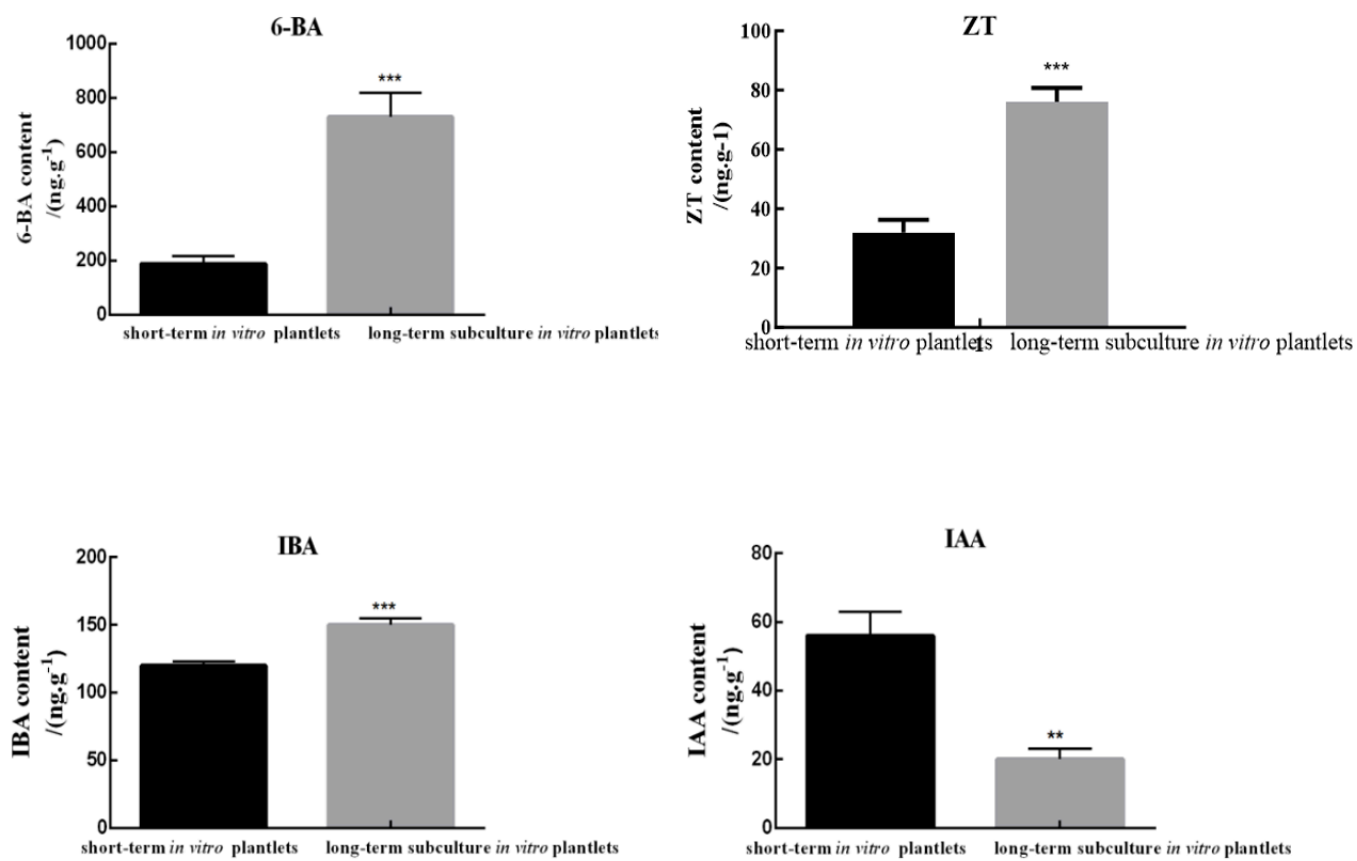


Figure 3

Hormone and plant growth regulator contents in short- and long-term subcultured *in vitro* plantlets

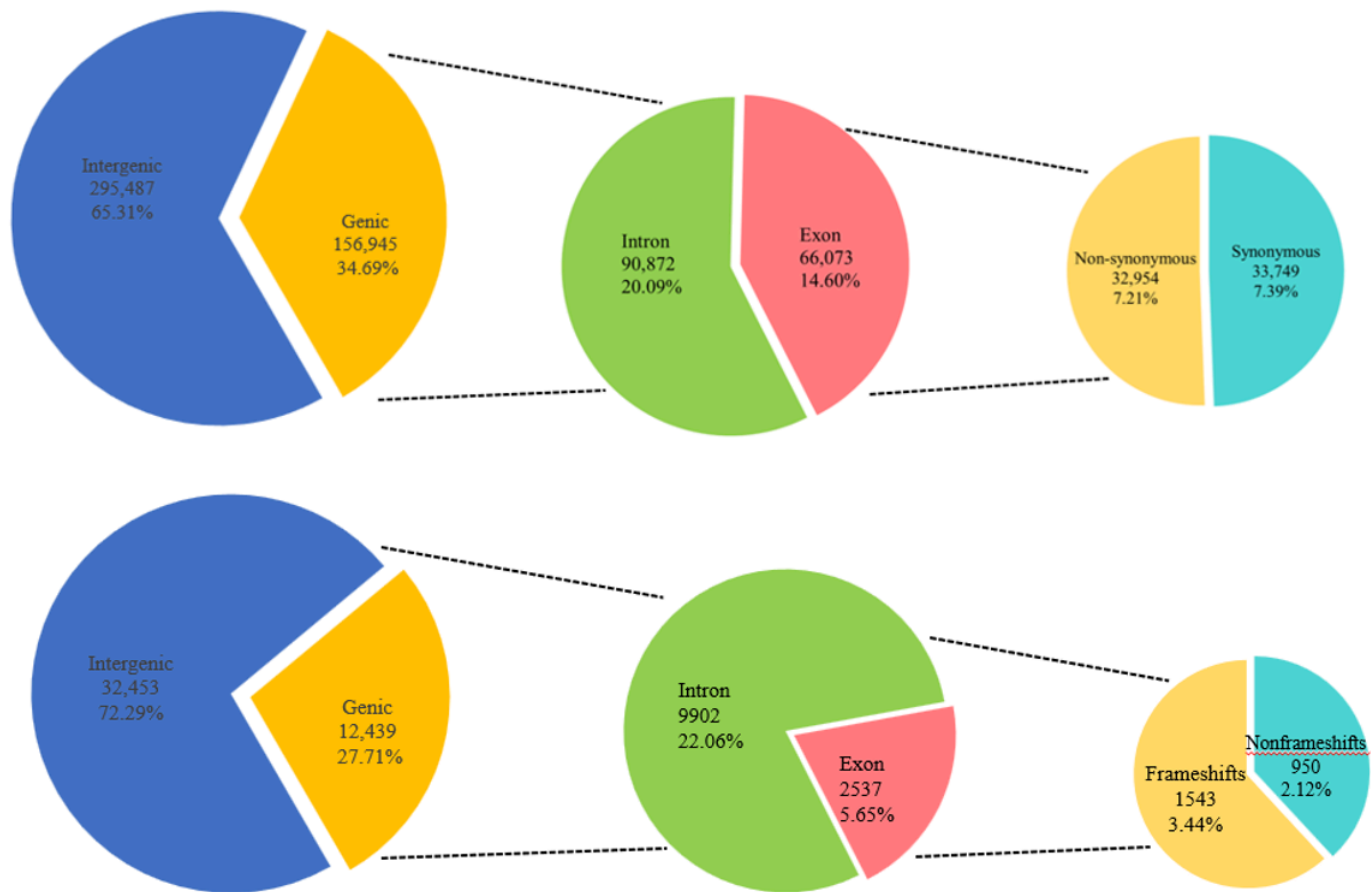


Figure 4

The number and percentage of SNPs and Indels occurring in long-term successive subcultured plantlets

A: Number and percentage of SNPs; B: Number and percentage of Indels

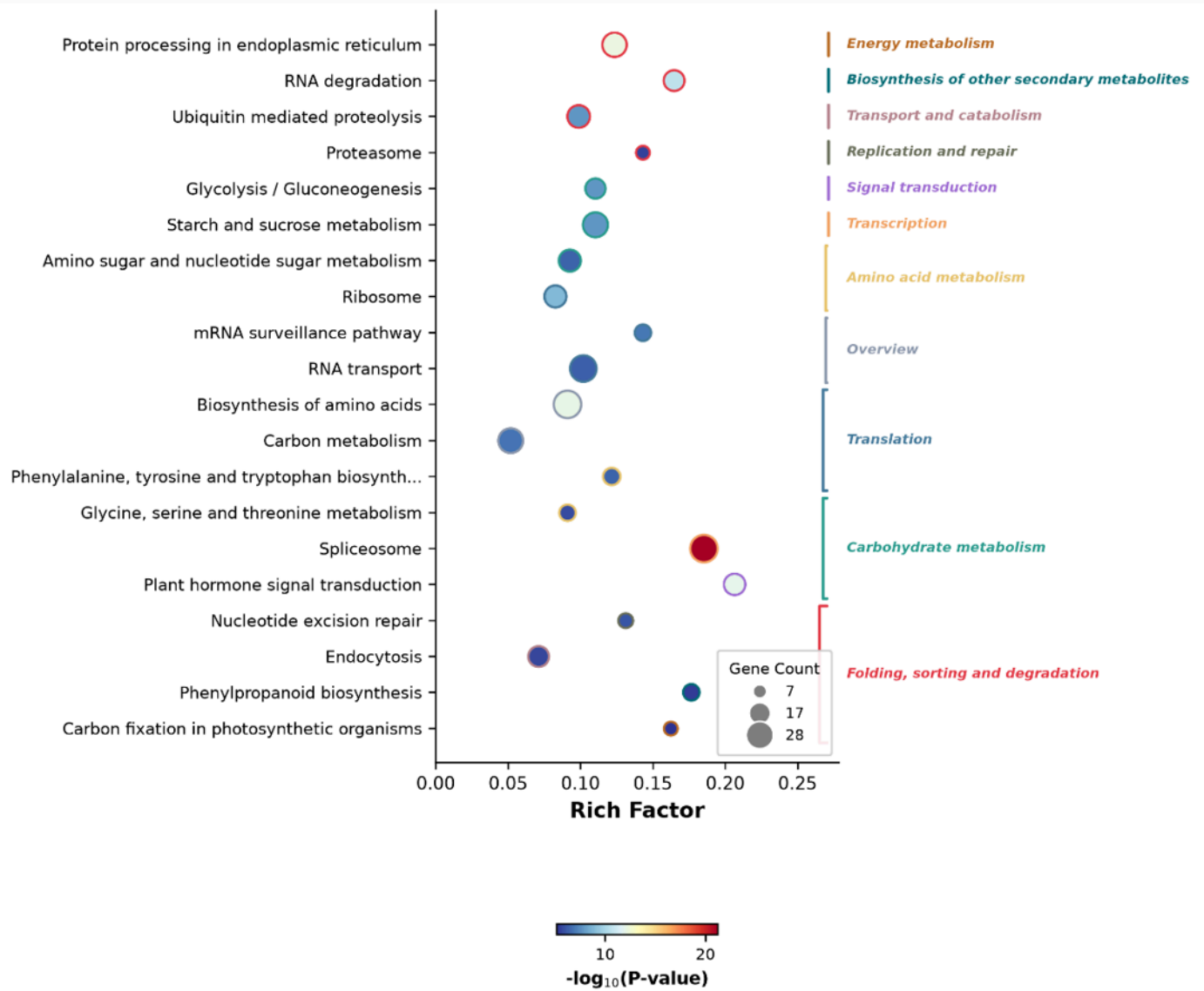


Figure 5

KEGG pathway enrichment classification of mutant genes

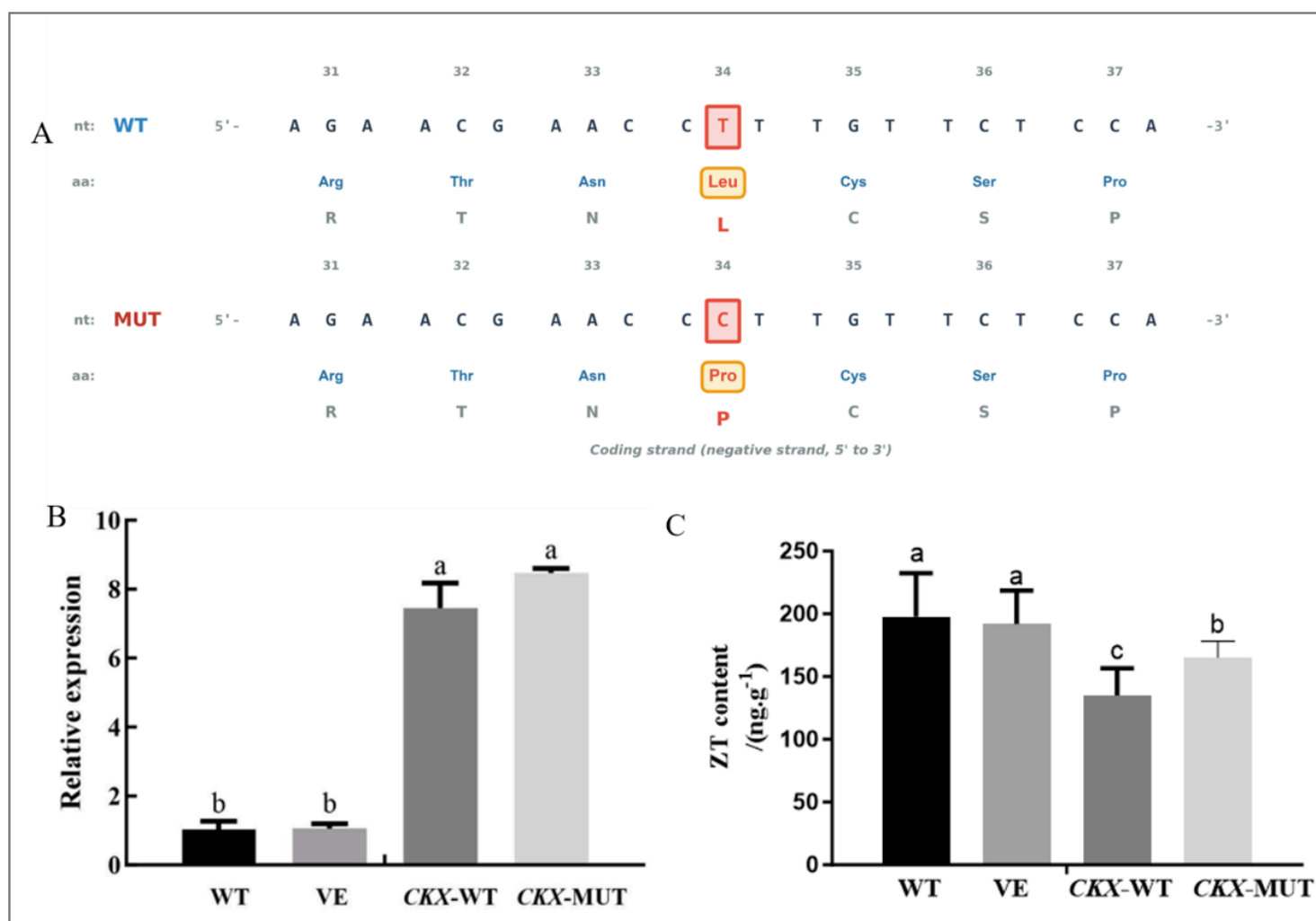


Figure 6

Identification and functional characterization of a *CKX* point mutation in transiently transformed tobacco leaves. (A) Nucleotide and amino acid sequences of the coding region around the mutation site. The wild-type *CKX* sequence contains a thymine (T) at position 34, encoding leucine (Leu, L), whereas the mutant *CKX* sequence contains a cytosine (C), encoding proline (Pro, P). The Leu-to-Pro substitution at position 34 is highlighted in red boxes. (B) Relative expression levels of *CKX* in tobacco leaves. Values are means ± SD (n = 6). (C) ZT content in tobacco leaves. Values are means ± SD (n = 6). Different lowercase letters (a, b, c) denote significant differences at $P < 0.05$, as determined by one-way ANOVA followed by Tukey's honestly significant difference (HSD) test.

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