

Allopolyploidization induced the activation of Ty1-*copia* retrotransposons in *Cucumis hytivus*, a newly formed *Cucumis* allotetraploid

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ABSTRACT. Allopolyploidy is an important process of species evolution that delivers a huge ‘genomic shock’ to the host genome. To investigate the effect of allopolyploidy on the expression of retrotransposons in a new synthetic allopolyploid, *Cucumis hytivus*, RT-PCR strategy was carried out to amplify reverse transcriptase (RT) genes from allotetraploid *C. hytivus* and its diploid parents, *C. hystrix* and *C. sativus*, using degenerate oligonucleotide primers corresponding to conserved RT domains of Ty1-*copia* retrotransposons. Only the allotetraploid yielded a specific product with expected size. After recovering and sequencing, 18 unique clones with significantly high heterogeneity were obtained. All of these clones were different from each other and could be divided into at least eight groups. The synonymous (dS) and nonsynonymous (dN) substitution analysis suggested that the RT sequences had been under constraint or purifying selection. A comparative maximum likelihood (ML) tree was constructed based on the deduced amino acids of the 18 RT sequences and those of other species acquired from the GenBank database. These results showed that the cloned sequences had high homology with both related and unrelated species, implying that they shared a common ancestor. The expression analysis of the cloned reverse transcriptase in the first four generations of allotetraploid further proved that the activation of retrotransposons was induced by allopolyploidization. These findings provide important information for polyploid evolution and will be of great importance for further epigenetic studies.

Keywords: Allopolyploidization; *Cucumis*; Retrotransposon; Reverse transcriptase; RT-PCR.

Abbreviations: gPCR, Genomic PCR; IRAP, Inter-retrotransposon amplified polymorphism; LTR, Long terminal repeat; REMAP, Retrotransposons-microsatellite amplified polymorphism; RT, Reverse transcriptase; PCR, Polymerase chain reaction; UV, Ultraviolet light.

INTRODUCTION

Allopolyploidy is a well-known process in plant speciation where two or more genomes are joined into the same nucleus through interspecific or intergeneric hybridization followed by chromosome doubling. Many important crop plants, such as wheat, oat, cotton, canola, and tobacco are allopolyploids (Masterson, 1994). The process of allopolyploidy has thus played a key role in the origin of many species and has driven and shaped plant evolution. Newly-formed allopolyploids undergo a huge ‘genomic shock’ (McClintock, 1984), which triggers wide genomic and epigenetic changes in the early stages of allopolyploid formation (Wendel, 2000; Adams et al., 2003). Ge-

nomes of newly-formed allopolyploids exhibit obvious instabilities, including major structural, cytogenetic, and functional changes to the genome, which potentially lead to new phenotypes and to reproductive isolation (Song et al., 1995; Ozkan et al., 2001; Shaked et al., 2001; Kashkush et al., 2002, 2003; Adams and Wendel, 2005; Chen and Ni, 2006).

Retrotransposon is ubiquitous in the plant kingdom (Flavell et al., 1992; Voytas et al., 1992). It contributes to increasing genome size and genome evolution in the plant kingdom (Kumar and Bennetzen, 1999; Feschotte et al., 2002). Retrotransposons are mostly inactive during normal plant development, but can be activated by biotic and abiotic stresses (Grandbastien, 1998). Wide hybridization and polyploidy can also induce activation of retrotransposons. Transcriptional activation of retrotransposons has been reported in response to wide crosses in wheat, *Arabidop-*

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sis and rice (Kashkush et al., 2002, 2003; Liu et al., 2004; Madlung et al., 2005). Newly-formed allopolyploid species *Spartina anglica* and *Gossypium*, however, inherited all their parents' genomes, which indicated that retrotransposon activation did not occur in the allopolyploid genomes (Liu and Wendel, 2000; Liu et al., 2001; Baumel et al., 2002). Consequently, more elaborate research is needed to pierce what effects polyploidy has on retrotransposons in different species.

Cucumis hytivus ($2n=4x=38$) is a newly synthesized allotetraploid species obtained after successful interspecific hybridization and chromosome doubling of *C. hystris* ($2n=2x=24$) and *C. sativus* cv. *Beijingjietou* ($2n=2x=14$) (Chen et al., 1997; Chen and Kirkbride, 2000). Our previous studies showed that allopolyploid formation in *Cucumis* can induce various changes, including flowering time, fruit shape, morphological traits, cytogenetic and rapid genetic variations (Chen et al., 1997, 2002, 2003, 2007). In *Cucumis*, numerous sequences of LTR retrotransposons were isolated and characterized from melon, and it was reported that the transcription of a *cop* retrotransposon Remel was induced by UV light, but not by wounding or by water stress (Ramallo et al., 2008). In our current study, we focused mainly on the effect of allopolyploidy on the transcription of Ty1-*cop* retrotransposons and analyzed the characterization of RT domains of active retrotransposons in the newly-synthesized allotetraploid species. In addition, we investigated the expression of activated retrotransposons in the first four generations of the allotetraploid.

MATERIALS AND METHODS

Plant materials

Plant materials consisted of the two diploid parents, cucumber cultivar [*C. sativus* cv. *Beijingjietou* (genome CC, $2n=2x=14$)], a wild species [*C. hystris* (genome HH, $2n=2x=24$)], and the S_1 - S_4 generations of the synthetic allotetraploid [*C. hytivus* (genome HHCC, $2n=4x=38$)]. The primary allotetraploid (S_0) was previously obtained from interspecific hybridization, through embryo rescue and chromosome doubling (Chen and Kirkbride, 2000). The S_1 - S_4 generations constitute the first four self-pollinated generations. All the plants were grown in a plastic greenhouse under standard conditions.

DNA and RNA extraction

Total genomic DNA was extracted from young leaves of seedlings by the CTAB method described by Murray and Thompson (Murray and Thompson, 1980). Total RNA was isolated from 1 g of young leaves using the Trizol kit (Promega) and RNA quality was checked by running the samples on a formaldehyde agarose gel. The residual DNA was removed by DNase I (RNase-free) (TaKaRa), and RNA concentration was estimated using a spectrophotometer. The first cDNA strand was synthesized according to instructions of the Kit (TaKaRa).

Cloning of RT domain of retrotransposons

RT-PCR was used to amplify a conserved region in the RT domain of Ty1-*cop* retrotransposons from the allotetraploid *C. hytivus* and its diploid parents using the degenerative primers (5'ACNGCNTTPyPyTNCAPyGG3' and 5'APuCATPuTCPuTCNACPuTA3'), corresponding to the conserved RT peptide motifs of the Ty1-*cop* group retrotransposons TAFLH and YVDDM, respectively (Kumar et al., 1997). 1 μ l cDNA solution was added into a 20 μ l reaction mixture containing 50 pmol of each primer, 0.2 mmol/L of dNTP, 2.5 mmol/L of $MgCl_2$ and 1 U of Taq polymerase (TaKaRa). PCR amplification consisted of the following conditions: 3 min initial denaturation at 94°C; 35 cycles of 1 min at 94°C, 1 min at 45°C, 1 min at 72°C; followed by 10 min at 72°C.

Purifying, cloning and sequencing

RT-PCR products were purified with Gel Extraction Mini kit (Bio Spin) from agarose gel and directly ligated to pGEM-T-Easy vector (Promega). The DH5 α strain of competent cells was used as a bacterial host. Plasmids from selected colonies were isolated by standard alkaline lysis and digested with ECOR I. Sequencing of the clones containing anticipated product was performed by Shanghai Bioasia Biological Engineering Technology & Service CO., Ltd.

Sequence and phylogenetic analyses

We tested the authenticity and homology of the clones by blastX at the NCBI website (<http://www.ncbi.nlm.nih.gov/>). These amino acid sequences were aligned using ClustalW (Thompson et al., 1994) with other RT sequences obtained from GenBank: G53226 in *Arabidopsis thaliana*; CAH56518 in *Brassica juncea*; CAD11841 in *Brassica napus*; CAD11830 in *Brassica rapa*; CAJ09747 in *Camellia sinensis*; CAJ41394 in *Citrus sinensis*; GU569971 in *Cucumis hystris*; CAJ76068 in *Cucumis melon*; EU162122 in *Cucumis sativus*; BAB47218 in *Diospyros kaki*; ABS11056 in *Malus $\times$ domestica*; AAK55317 in *Oryza sativa*; AAA33849 in *Platanus occidentalis*; ABD19061 in *Phelipanche ramosa*; ABD19073 in *Phelipanche tunetana*; ABF57076 in *Prunus mume*; AAL36463 in *Setaria adhaerans*; AAC34606 in *Solanum lycopersicum* and AAK84849 in *Zea mays*. A protein maximum likelihood (ML) phylogenetic tree was constructed based on the deduced amino acid sequences of the RT region using TREE-PUZZLE 5.2 (Schmidt et al., 2002), as well as MEGA 4.0 software (Tamura et al., 2007). Values that support the internal branches within the ML tree were obtained from a total of 1000 puzzling steps.

Maximum likelihood (ML) estimates of nonsynonymous substitutions (dN) and synonymous substitutions (dS) between pairwise alignment were obtained with PAML (Yang, 2000) using a codon-based model of sequence evolution with dN, dS, and transition-transversion bias as the data at each codon position (Goldman and Yang, 1994; Yang, 2000).

Genomic PCR and RT-PCR analysis

To verify the expression of the cloned reverse transcriptase sequences in the first four generations of allotetraploid, genomic PCR (gPCR) and RT-PCR using specific primers were carried out respectively. We tried to design specific primers based on all the 13 intact clones. RT3 and RT10 belonging to different groups of retrotransposons in *C. hytrix* were chosen as target fragments for gPCR and RT-PCR. The PCR condition was followed as: 3 min at 94°C; 30 sec at 94°C, 30 sec at 55°C, 30 sec at 72°C, followed by 35 cycles. *Actin* gene was used as positive control in the RT-PCR reactions.

RESULTS

Identification of active retrotransposons in *Cucumis*

Transcriptional active retrotransposons were tested by RT-PCR from allotetraploid and its two diploid parents, *C. hystrix* and *C. sativus*. Only the allotetraploid yielded a specific product with expected size (260 bp) (Figure 1). The amplified fragment was recovered and cloned into a pGEM-T vector, and 20 clones were obtained. Sequence analysis revealed that 18 clones contained the RT domains of the retrotransposons (named as RT1 to RT18), while the remaining 2 didn't. These sequences have already been submitted to GenBank with the accession numbers of HMO036494-HMO036511.

RT sequence alignments

Comparing the 18 newly-isolated sequences with each

other, we confirmed that these sequences were highly heterogeneous. The 18 sequences were conceptually translated and where necessary edited for frameshift, based on published residue landmarks of plant retrotransposons' RT domains. The amino acid alignments had properly translated primer sequences at both ends. Among these 18 sequences, 5 (27.8%) contained premature stop codons and/or frameshift in their coding regions (RT1, RT5, RT7, RT12, and RT13). The remaining 13 sequences (72.2%) were not affected by either stop codons or frameshift. The homology ranged from 49.4% (RT4 and RT9) to 98.1% (RT3 and RT14) when amino acid sequences of these RTs

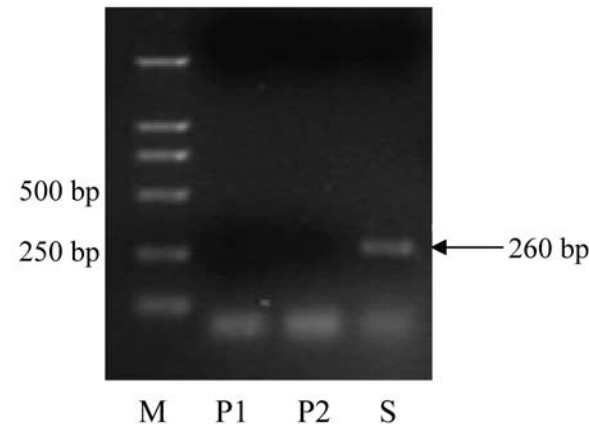


Figure 1. RT-PCR amplification of reverse transcriptase (RT) domains of Ty1-copia retrotransposons from genomes of allotetraploid and its two diploid parents. M: 2000 bp Marker; P₁: *Cucumis hystrix*; P₂: *C. sativus*; S: *C. hytivus*.

Table 1. Homology matrix of the 18 reverse transcriptase (RT) sequences of active retrotransposons amplified from allotetraploid *C. hytivus*.

	RT1	RT2	RT3	RT4	RT5	RT6	RT7	RT8	RT9	RT10	RT11	RT12	RT13	RT14	RT15	RT16	RT17
RT2	82.2																
RT3	60.4	63.5															
RT4	86.4	83.8	61.7														
RT5	67.8	68.8	60.5	66.5													
RT6	53.3	57.0	53.6	53.2	54.8												
RT7	86.0	89.5	63.2	85.7	65.0	54.4											
RT8	57.3	57.3	57.3	58.4	58.0	52.9	57.6										
RT9	51.7	53.6	53.2	49.4	51.7	90.1	53.2	51.3									
RT10	86.4	81.6	59.8	85.3	67.7	52.5	83.1	56.9	49.0								
RT11	67.4	68.4	63.9	65.4	91.0	55.5	65.0	56.1	54.4	66.2							
RT12	53.6	55.9	56.3	53.6	56.7	51.5	55.1	68.8	51.5	53.2	58.2						
RT13	59.1	59.4	56.8	57.1	57.1	52.5	59.0	56.1	48.7	54.1	57.9	56.3					
RT14	61.0	63.5	98.1	62.4	60.9	53.6	63.9	57.6	53.2	60.5	62.8	55.9	58.6				
RT15	57.6	54.9	55.6	53.4	53.0	52.9	56.0	57.6	51.7	53.4	54.1	52.1	51.9	56.4			
RT16	69.7	69.9	63.2	66.9	90.6	55.1	66.5	56.9	52.9	66.9	94.4	59.7	59.8	63.2	54.5		
RT17	58.6	60.4	57.7	57.7	60.4	57.3	59.2	59.8	55.7	57.4	59.2	55.0	61.5	57.7	58.1	58.9	
RT18	89.4	83.8	62.4	91.7	65.0	52.9	86.1	59.5	51.3	86.1	66.9	54.8	56.8	62.0	55.6	66.2	59.2

compared with each other (Table 1). Alignment of the deduced amino acid sequences showed that they had characteristic amino acid motifs at both ends of RT gene (5'-TAFLHG and 3'-YVDDM). The YGLKQ located in the central region of RT gene (Figure 2).

Phylogenetic analysis of RT sequences

To investigate the relationships among the RT domains representing the active Ty1-copia retrotransposons of *C. hystrix* and further comparison with those acquired from the GenBank database, a 50% majority consensus tree from maximum likelihood (ML) phylogenetic was constructed using the TREE-PUZZLE 5.2 (Schmidt et al., 2002) and MEGA 4.0 software (Tamura et al., 2007). Eight groups were clearly separated in the tree based on its branching patterns (Figure 3). Five groups (II, V, VI, VII, and VIII) were constituted; those containing a single sequence were considered independent groups due to their distant relations to all the other sequences. Figure 3 also showed that the sequence of group II was clustered with a *D. kaki* clone (BAB47218) as well as with a *C. melon* clone (CAJ76068). Group III seemed to be highly homologous with Ty1-copia RT sequences of *C. sativus* (EU162122) and *C. hystrix* (GU569971). The sequence of group V was in the same clade as *Malus × domestica* (ABS11056).

The numbers of synonymous and nonsynonymous substitutions per site were estimated for three groups (I, III and IV), which contained at least two intact RT sequences (Table 2). The ratios of nonsynonymous to synonymous substitutions (dN/dS) were 0.12, 0.12 and 0.18 for group I, III and IV respectively, which suggested that the three groups had been under constraint or purifying selection.

Expression of RT gene in the first four generations of allotetraploid and their diploid parents

Genomic PCR and RT-PCR were carried out to verify the expression of RT gene in the first four generations of allotetraploid and their diploid parents using specific primers based on the RT3 and RT10 sequences. These sequences belonged to different groups of retrotransposons in *C. hystrix*. gPCR results showed that RT3 existed in the S₁-S₄ generations and in one of their parents (*C. hystrix*). However, RT-PCR results revealed that although it was inactive in *C. hystrix*, it was activated in the S₁-S₄ generations (Figure 4A). In the meantime, though RT10 existed in both of the two diploid parents and in all the four generations of allotetraploid, it was inactive in the parents but activated in the allotetraploid (Figure 4B). Since all plants were grown in the same condition, it is conceivable that the expression change of RT3 was induced by allopolyploidization.

DISCUSSION

Retrotransposons are mostly inactive under normal conditions, but can be activated by biotic and abiotic

Table 2. Number of synonymous and nonsynonymous substitutions per site within the RT gene domain of two *C. hystrix* Ty1-copia retrotransposon groups.

Group	Synonymous substitutions (dS)	Nonsynonymous substitutions (dN)	Ratio (dN/dS)
I	0.078	0.0092	0.12
III	0.85±0.36	0.1±0.05	0.12
IV	3.84	0.36	0.18

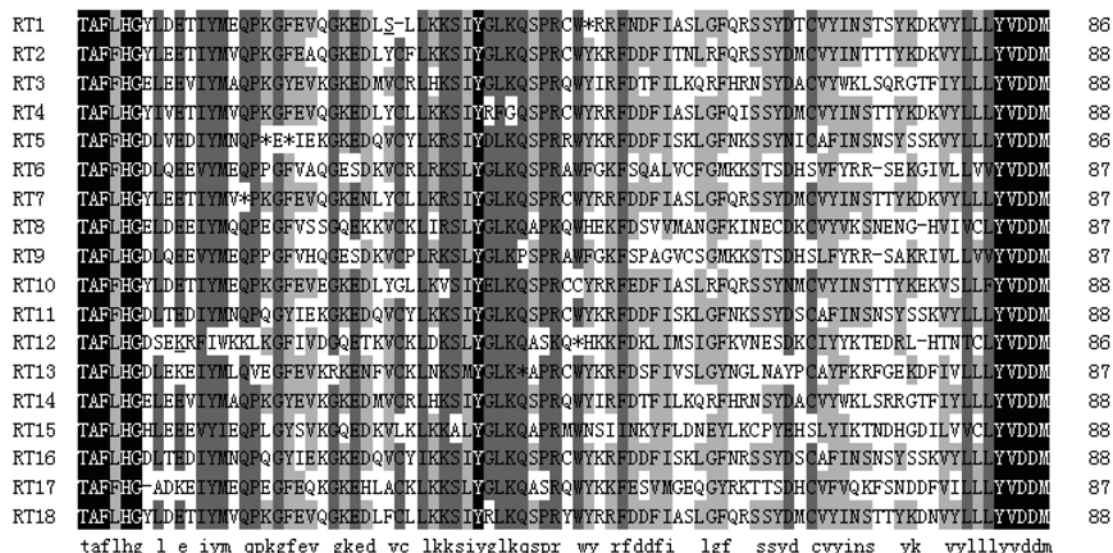


Figure 2. Sequence alignment of deduced amino acid corresponding to the RT domains of transcriptional active retrotransposons from allotetraploid *C. hystrix*. The four shading levels indicate degree of residue conservation: black (100% conserved), dark gray (75% or greater conserved), light gray (50% or greater conserved), and no shading (<50% conserved). Gaps are indicated as (-) and stop codons are presented as (*). The amino acid of frameshift is underlined. The numbers of amino acid residues are displayed on the right hand of each sequence.

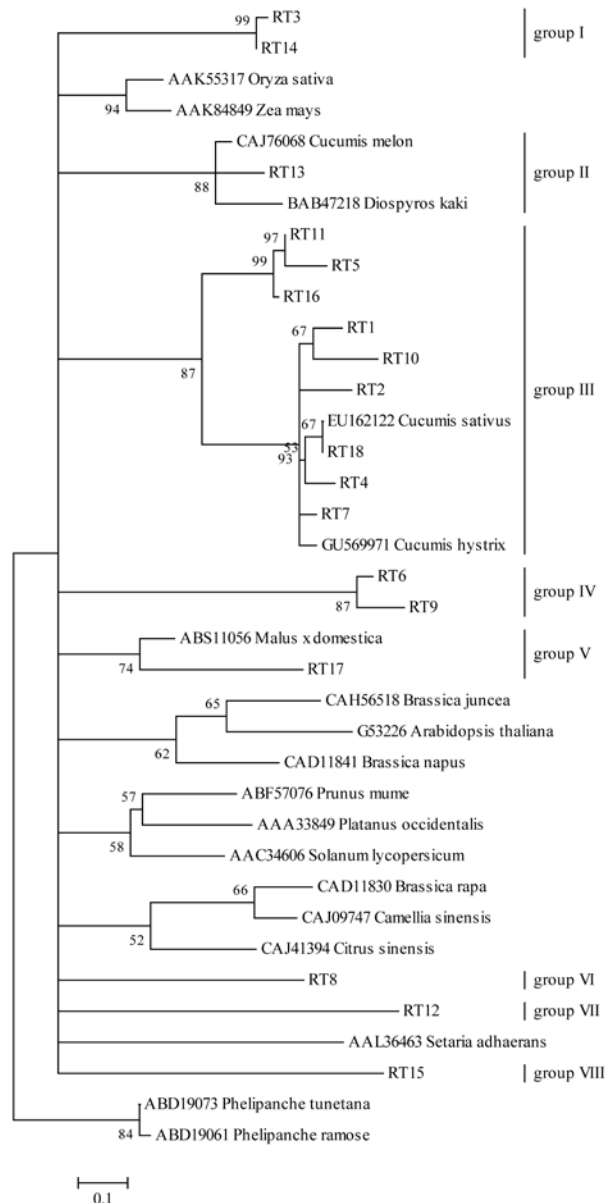


Figure 3. The 50% majority consensus tree from maximum likelihood (MP) analyses based on the deduced amino acid sequences corresponding to RT domain of active retrotransposons isolated from *C. hytivus* with other RT sequences by quartet-puzzling analysis (Schmidt et al., 2002). The tree was arbitrarily rooted using *Phelipanche ramosa* (ABD19061) as an outgroup. Numbers above branches indicate bootstrap values.

stresses (Grandbastien, 1998). Allopolyploidization causes a huge ‘genomic shock’ to the host genome and can result in activation of retrotransposon (McClintock, 1984; Kashkush et al., 2003). In wheat, it was reported that the Ty1-copia retrotransposon *Wis 2-1A* was activated by allopolyploidization (Kashkush et al., 2002). In contrast, in the young allopolyploid species *Spartina anglica*, the result of IRAP and REMAP indicated that retrotransposons were not reactivated during the formation of allopolyploid (Baumel et al., 2002). To examine whether retrotransposons present in the parental genomes have been activated in newly-synthesized allotetraploid *C. hytivus*, we used the RT-PCR strategy to amplify the reverse transcriptase (RT) region of Ty1-copia group retrotransposons from *C. hytivus* and its diploid parents, *C. sativus* and *C. hystris*. The RT primers correspond to the two conserved amino acid domains of the Ty1-copia-like retrotransposons of plant species, TAFLHG (amino-terminal) and YVDDM (carboxy-terminal) of reverse transcriptase (Kumar et al., 1997). The allotetraploid amplified the expected fragment, while the diploid parents did not. This result showed that retrotransposons were activated in *C. hytivus* by allopolyploidization, since all the seedlings were grown under the same condition, thus excluding the variances induced by surrounding conditions. We also found in our previous studies that there was very little variation in the polymorphic level between diploid and autotetraploid cucumbers as revealed by AFLP technique (Zhang et al., 2006). Extensive genomic and epigenetic changes were detected, however, in the newly formed allotetraploid *C. hytivus* (Chen et al., 2007; Chen and Chen, 2008). Consequently, we can presume to some extent that the expression variant detected here was induced by allopolyploidization. The synonymous (dS) and nonsynonymous substitution (dN) analysis showed that constraint or purifying selection acted on these elements, therefore supporting the suggestion that most of the retrotransposons obtained there were active.

Like other genes, retrotransposons can be transmitted from parent to the next generation. This is designated as vertical transmission (Doolittle et al., 1989), the main method for the genetic transmission of retrotransposons. Horizontal transmission also occurs via an asexual mode. The result of horizontal transmission leads to the wide distribution of retrotransposons in a variety of species (Doolittle et al., 1989). Phylogenetic analysis based on

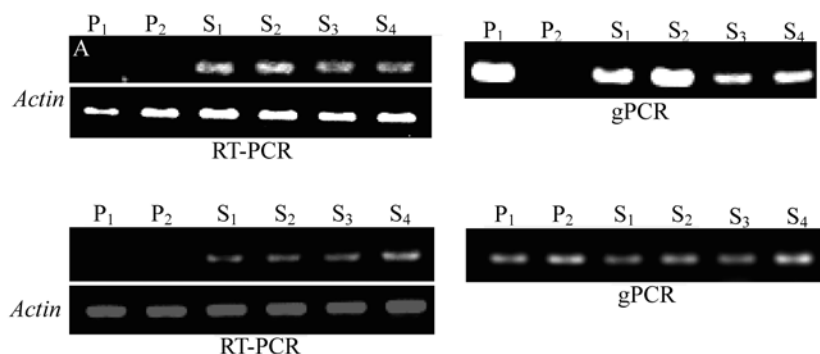


Figure 4. Expression of RT genes in the first four generations of allotetraploid and their diploid parents. A, The expression of RT3 was studied by RT-PCR using specific primers. *Actin* was used as positive control. gPCR was carried out using the same condition with RT-PCR; B, The expression of RT10 was studied using the same method with RT3. P₁: *Cucumis hystris*; P₂: *C. sativus*; S₁-S₄: the first four generations of *C. hytivus*.

nucleotide sequences of the reverse transcriptase provided evidence for the vertical transmission of retrotransposons in this study. The comparative phylogenetic analysis showed that the RT sequences obtained here had homology with related as well as unrelated species (Figure 3). Most of the RTs cluster together with sequences from *Cucumis*, such as *C. sativus* (EU162122), *C. hytivus* (GU569971) and *C. melon* (CAJ76068). In addition, some RTs showed a close phylogenetic relationship with *S. adhaerans* (AAL36463), *D. kaki* (BAB47218), *P. ramose* (ABD19061) as well as *P. tunetana* (ABD19073), which implied that they shared a common ancestor prior to speciation. Thus retrotransposons have contributed to the genetic diversity and evolution of the host genome.

To verify the expression of the cloned reverse transcriptase in the first four generations of allotetraploid and their diploid parents, gPCR and RT-PCR were carried out using specific primers corresponding to RT3 and RT10. The results showed that RT3 initially existed in *C. hystris*, that RT10 existed in both of the diploid parents, and that these two sequences could inherit into the first four generations of allotetraploid. Although these were inactive in parent material, they were activated in all four generations of allotetraploid. One might doubt that factors other than allopolyploidization induced the activation of Ty1-copia retrotransposons in *C. hytivus*. In order to minimize this doubt, all the seedlings in this study were carefully grown under the same conditions without artificial or environmental stresses. The results further prove that allopolyploidization affects the expression of Ty1-copia retrotransposons immediately after allopolyploid formation.

The transposition activity of retrotransposons conversely changed the expression of neighboring genes. For example, transcriptional activation of retrotransposon *Wis* 2-1A in wheat altered the expression of neighboring genes, leading to the activation or silence of flanking genes (Kashkush et al., 2003). Our studies suggested that there was a direct link between the expression of retrotransposons and allopolyploidy, which provided a significant foundation for further polyploid evolution in *Cucumis*. In the future, we will focus on the influence of epigenetic changes on the activation of retrotransposons in early generations of the synthesized allotetraploid - *C. hytivus*.

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異源多倍化誘導甜瓜屬新合成的異源四倍體 *Cucumis hytivus* 中 Ty1-*copia* 類逆轉座子的啟動

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異源多倍化是作物進化的一個重要過程，是宿主基因組巨大的“基因組衝擊”。為了研究異源多倍化對 *C. hytivus* 逆轉座子表達的影響，我們根據 Ty1-*copia* 類逆轉座子逆轉錄酶的保守區設計簡並引物，利用 RT-PCR 技術從異源四倍體 *C. hytivus* 及其二倍體親本 *C. hystrix* 和 *C. sativus* 中擴增逆轉錄酶基因，結果僅在異源四倍體中擴增出目的片斷。回收測序後，獲得了 18 個高度異質的克隆。這些克隆彼此不同，至少能夠分為 8 個家族。同義（dS）和非同義（dN）替換分析表明這些序列受淨化選擇的作用。根據這 18 個 RT 序列的氨基酸和從 GenBank 資料庫中獲得的其它作物序列，利用最大似然法構建進化樹，結果表明這些序列与其它作物存在高度的同源性，表明它們可能有共同起源。進一步對所獲得的逆轉錄酶序列在異源四倍體早期四個世代的表達分析證實，異源多倍化誘導了該類逆轉錄酶的表達。這些發現為多倍體進化提供了重要資訊，並對進一步表觀遺傳學研究具有重要意義。

關鍵詞：逆轉座子；逆轉錄酶；異源多倍化；RT-PCR；甜瓜屬。