

Heterogeneity in *Ty1-copia* group of retroelements in chickpea (*Cicer arietinum*) genome

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Retrotransposons constitute a major fraction of plant genomes and these elements may have played a significant role in evolution and sequence organization of genomes. In order to access the diversity of *Ty1-copia* group of retroelements, reverse transcriptase (RT) sequences were amplified from chickpea genome, using the primers derived from two conserved domains of RT region. Thirty-six RT regions from independent amplicons were cloned and sequenced. On the basis of homology of deduced amino acids, the RT sequences could be grouped into three major families. The intra-family divergence at amino acid level ranges from 2 to 19%. Though intra-family RT sequences were conserved but no two sequences were identical. The results indicate a high degree of heterogeneity among the *Ty1-copia* group of retroelements from chickpea. It was possible to isolate RT specific sequences from RNA isolated from stressed seedlings, indicating that some of the retroelements may be functional under certain stress conditions.

Key words: Retrotransposon, *Ty1-copia*, reverse transcriptase, chickpea, *Cicer arietinum*

Abbreviations: RT – reverse transcriptase, CART – *Cicer arietinum* retrotransposon, CARE – *Cicer arietinum* retroelement, cF – *copia* family, csF – *copia* sub-family.

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Retrotransposons constitute a large proportion of plant genome ranging from 14% in *Arabidopsis* to over 70% in maize. On the basis of presence or absence of long terminal repeats (LTRs), retrotransposons are classified into LTR or non-LTR retroelements. LTR-retrotransposons contain “gag” (group antigen), endonuclease and reverse transcriptase domains. Depending on the internal placement of RT and endonuclease domains, the LTR-retroelements have been named *Ty1-copia* or *Ty3-gypsy* group of retrotransposons (Dolittle et al., 1989; Xiong and Eickbush, 1990). *Ty1/Ty3* or *copia/gypsy* denotes the similarities to the retroelements present in yeast or *Drosophila* genomes. In *copia*-like retrotransposons, the endonuclease domain is positioned 5′ to the reverse transcriptase domain, while in the *gypsy* type; it is at the 3′ end of the reverse transcriptase domain, but functionally both are similar. All the known plant retrotransposons including active ones are largely quiescent during normal development but some of them are to be activated transcriptionally as well as transpositionally in response to various biotic and abiotic stresses (Grandbastien, 1998; Beguiristain et al., 2001). Transcriptional activation of an element in response to stress indicates that it may have a role in stress alleviation phenomena in plants (Hirochika, 1993, 1996; Moreau-Mhiri et al., 1996). Due to their high copy number in highly heterogeneous populations and dispersal throughout the genome, retrotransposons are now being utilized as

molecular markers in DNA finger printing, genetic linkage mapping and phylogenetic analyses (Ellis et al., 1998; Kumar and Hirochika 2001). The “C-value-paradox” i.e. non-correspondence between structural and functional complexity could in part be explained by presence of retroelements (Kumar and Bennetzen, 1999). Retroelements have also been implicated in genomic expansion during evolution (Kalendar et al., 2000; Feschotte et al., 2002).

Chickpea (*Cicer arietinum* L) is one of the most important legume crops in the Indian subcontinent and ranks third in the world for pulse production. This self-pollinating annual diploid crop with a somatic chromosome number of $2n = 16$ has a genome size of ~738 Mb. Such a large plant genome is expected to have a significant fraction of retroelements. In order to analyze the heterogeneity among the *copia* family of retroelements in the chickpea genome, we have isolated reverse transcriptase (RT) sequences by PCR amplification using primers designed from the conserved regions of RT domain and analyzed the sequences for heterogeneity.

Materials and Methods

DNA from chickpea variety Pusa 362 was isolated using standard procedures (Ausubel et al., 1995). Two conserved regions (DVKTAF and YVDDMDP) of the reverse transcriptase domain were used to design forward and reverse primers for PCR amplification (Voytas and Ausubel, 1988; Flavell et al., 1992). In our lab using same set of RT primers a *Ty1-copia*-like retrotransposon, *panzee* has been isolated from pigeonpea (Lall et al., 2002). PCR was performed in a 50- μ l reaction mixture, with 500 ng of the genomic DNA, 100 μ m each of dNTPs, 50 pmol of each primer (5' - GGGATCCAYRTCRTCACRTANARNA-3' and 5' - ATTCGAYGTNAARCANGCNTTYT-3'), and 2U of *Taq* DNA polymerase

(Promega) using 1.5 mM MgCl₂. PCR was performed in a thermal cycler (MJ Research) with the following parameters: 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 47°C for 1 min and 72°C for 1.5 min, followed by a final extension step at 72°C for 10 min. The expected amplified product was eluted from the agarose gel, purified and cloned into pGEM-Teasy vector (Promega). Nucleotide sequencing was done using an automated DNA sequencer (Applied Biosystem).

Chickpea genomic DNA digested with appropriate enzymes was fractionated on 0.7% agarose gel in TAE buffer and blotted onto Hybond N+ nylon membrane (Amersham). Hybridization was performed under conditions of high stringency using the clone CART77 sequences as a probe (Sambrook et al., 1989).

Abiotic stress was given to 10-day old seedlings by keeping them at 25°C for 5 days without water to get a state between temporary and permanent wilting. Total RNA from leaves was extracted using RNeasy Plant Minikit of QIAGEN. RT-PCR was carried out with 1 µg of total RNA with the *copia* family of RT-specific degenerate primers using Promega's AccessQuick RT-PCR system. The primer annealing was done at 52°C for 90 sec.

Pair-wise and multiple sequence alignments were carried out using clustalW (1.83) (<http://www2.ebi.ac.uk/clustalw>; Thompson et al., 1994). The sequences were classified according to the method of Feng and Doolittle (1990). The ORFs were determined by using NCBI ORF finder software (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>).

Accession numbers and Source of Sequences used for comparative analysis

Following RT sequences were used for comparison of the chickpea sequences: AAF37865 (*Ipomoea batatas*), BAA11674 (*Nicotiana tabacum*), CAJ09744 (*Camellia sinensis*), CAA13065 (*Solanum tuberosum*), ABD19095 (*Phelipanche tunetana*), X13777 (*Nicotiana tabacum*), AAG44306 (*Aegiceras corniculatum*), ACC34608 (*Lycopersicon*

esculentum), CAD11851 (*Brassica oleracea*), ABD19043 (*Phelipanche bungeana*), AAT73707 (*Populus ciliata*), AAG44354 (*Spiranthes sinensis*).

Results and Discussion

Structurally *Ty1-copia* retrotransposons contain 5' - and 3' - LTRs, *gag* and *pol* regions. The *pol* region encoding reverse transcriptase (RT) contains certain domains that show high degree of conservation. These conserved domains have been used to design oligonucleotide primers for amplification of RT sequences from a large spectrum of *copia* family of elements (Voytas and Ausubel, 1988). The amplification using chickpea DNA as a template resulted in an amplicon of ~280 bp (Fig. 1). Considering the expected high copy number of retroelements in the chickpea genome, it was conjectured that the amplicon would contain different RT sequences from a wide spectrum of *copia* elements. Therefore, the amplified sequences were eluted from the gel, purified and cloned into pGEM-Teasy vector. Sequences of 36 independent clones were determined.

All the RT regions contain sequences complementary to the primers used. A comparison of nucleotide sequences reveals variation among the RT regions. The RT regions of all the clones were translated into amino acid sequence and analyzed for ORF. Of the 36 RT regions, 26 showed uninterrupted reading frames. The other 10 clones (approximately 17%) contained one or more chain terminating codons in all the three reading frames. A continuous reading frame in 6 of the clones could be generated by a single nucleotide frame-shift. It has been proposed that the elements could derive a functional translational product by mechanism of frame shifting (Dinman et al., 1991). However, the presence of multiple chain terminating codons in a single ORF may reflect the non-functionality of the element. Since the retroelements are normally present in high

copy numbers within plant genomes it is likely that only a few copies of a particular element may be functional.

Southern hybridization of chickpea genomic DNA digested with EcoRI or HindIII, with the CART77 sequences as a probe shows the existence of a large population of *Ty1-copia*-like retrotransposons in the chickpea genome (Fig. 2). The presence of hybridizing bands across the entire lane possibly indicates dispersal of *Ty1-copia*-like sequences throughout the chickpea genome.

Alignment of the deduced amino acid sequences of the clones reveals the presence of two highly conserved domains (DVKTAF and YVDDMDP), which have been used for designing oligonucleotide primers (Voytas and Ausubel, 1988). The alignment of all the 36 RT sequences is presented in Fig. 3. Even though all the RT sequences isolated show strong homology to the conserved RT domains of *Ty1-copia* family of retrotransposons, no two sequences are identical. The sequences do not show any significant homology to the RT domains of their closest group *gypsy* or to any of the retroviruses. Based on amino acids homology (Fig. 3) and clustering in the phylogenetic tree (Fig. 4), the 36 chickpea sequences could be grouped into three major families named cF1, cF2 and cF3. The family cF3 could further be divided into eleven sub-families i.e. csF3.1 to csF3.11 (Table 1). Within the family, the sequences that show branching from a common nearest point in the phylogenetic tree have higher amino acid homology than the others. The intra-family and intra-subfamily degree of divergence at the amino acid level ranges from 2 to 19%. Degree of divergence between most similar sequences i.e. CART14 and CART98 is 2% both at nucleotide as well as amino acid levels. Since individual copies of an element generally show 2% divergence from the original sequence at both amino acid and nucleotide levels (Mount and Rubin 1985; Emori et al., 1985), the two clones (CART14 and CART98) of the sub-family csF3.3 may be copies originated from a same element. In other cases, where the degree of divergence

at the amino acid level is 2% and at the nucleotide level is 3, they are likely to be derived from different elements. Amino acid identities of the isolated chickpea RT sequences with the corresponding regions of *Ty1-copia*-like retrotransposons from various plants calculated using the method of Higgins and Sharp (1998) are given in Table 2. One of the RT sequences, CART329 of the family cF1 shows 86% homology to BAA11674 (*Nicotiana tabacum*), CAJ09744 (*Camellia sinensis*) and ABD19095 (*Phelipanche tunetana*). The CART55 RT sequences of the sub-family csF3.6 has 87% homology to BAA11674 (*Nicotiana tabacum*) and 86% to CAJ09744 (*Camellia sinensis*).

The RT sequences from the RNA isolated from desiccated plants were amplified using the *copia* RT-specific primers in an RT-PCR assay. An amplified product of around ~280 bp (Fig. 5) was eluted from the gel, purified, cloned into pGEM Teasy vector (Promega) and then sequenced. In addition to a ~280-bp amplicon, two bands of higher molecular weight were also amplified. They were also cloned and sequenced. However, the sequences of these amplicons revealed that they were not specific to the RT regions of retroelements. Some of these sequences showed homology to protein kinase-like sequences. The reason of such amplified sequences using specific primers is not clear.

The RT-specific amplicons from RNA isolated from stressed seedlings contain seven different sequences. No amplified products could be detected if the RNA isolated from the plants grown under normal conditions is used for RT-PCR. The RT-transcripts CARE3, CARE4 and CARE7 contain stop codons while clone CARE8 possessed a frame shift. All transcripts show significant homology (41-95%) to the cF1, cF2 and cF3 families and to the conserved RT domains of *Ty1-copia* family of retrotransposons from a variety of plants. The clone CARE3 shows an amino acid identity of 98% with CART157 possibly indicating that CARE3 and CART157 might be transcripts of two

similar elements. The clone CARE5 shows 90% homology with AAT90482. The amino acid identities of all transcripts except CARE7 with RTases of active retrotransposons *Tnt1* and *Tto1* range from 51 to 87% (Grandbastien et al., 1989). The range in homology among transcripts and between transcripts and genomic RT sequences is almost same. In the phylogenetic tree CARE3 and CARE7 clustered with the sub-family csF3.10; CARE2 and CARE5 with the sub-family csF3.6; and CARE4, CARE8 and CARE9 with the subfamily csF3.1. The detection of RT specific transcripts in the RNA isolated from desiccation stressed treated plants show that the *Ty1-copia*-like retrotransposons in chickpea may be activated in response to abiotic stress (Kimura et al., 2001). The absence of any detectable level of RT-specific amplification using the RNA isolated from the plants grown in normal conditions indicates that under normal conditions *Ty1-copia*-like retrotransposons may be inactive or show very low level of activation (Hirochika, 1993, 1996; Moreau-Mhiri et al., 1996; Takeda et al., 1998).

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Legends

Figure 1. Amplification of *Ty1-copia* RT specific sequences from the chickpea genome. Lane M contains 100-bp DNA ladder and lane A contains *Ty1-copia* RT specific amplification products.

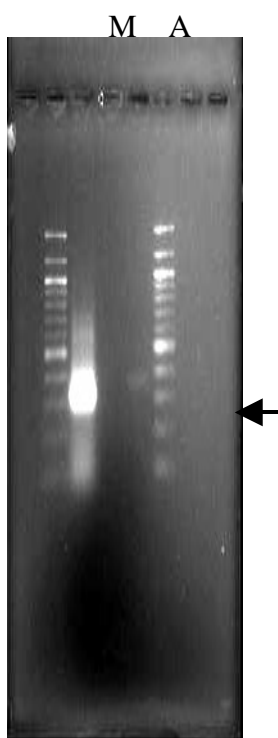


Figure 2. Southern blotting of chickpea genomic DNA digested with HindIII (Lane H) and EcoRI (Lane E) using the clone CART77 sequences as a probe. Lane M contains Hind III digested λ DNA as a marker.

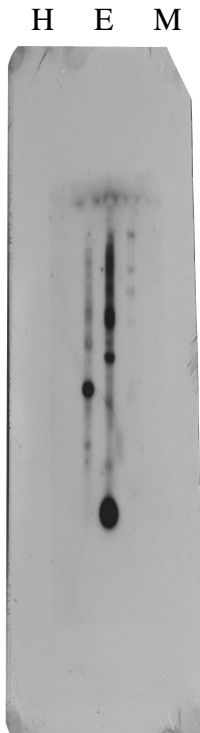


Figure 3. Amino acid sequence alignment of RT regions of chickpea.

CLUSTAL W (1.83) multiple sequence alignment

CART50	DVKTAFLNGDIDETIYMVQPENFVLGDPKNMVCKLRKSIYGLKQASRQWYHKFHQVILSF	60
CART366	DVKTAFFNGDIDETIYMVQPENFMLGDPKNMVCILRKSIYGLKQASRQWYHKFHQVILSF	60
CART387	DVKTAFLNGDIDETIYMVQPENFVLGDPKNMVCKLRKSIYGLKQASRQWYHKFHQVILSF	60
CART37	DVKTAFLHGDNLKNTILMQQPYGFRIQGGKEDWVCLLKRSVYGIKQSPRQWYLFKNSFMLSQ	60
CART157	DVKTAFFHGDQNETILMQQPNGFRTQGGKEDWVCLLKRSYGLKQSSR--YLRFDLSMLSQ	58
CART61	DVKTAFLYGDLEEEIYMDLPPGYSEHIAANTVCKLKKALYGLKQSPRAWFGRFARAMVGL	60
CART384	DVKTAFLHGDLEEEIYMDLPLGYSEHIAANTVCKLKKALYGLKQSPRAWFGRFARVMVGL	60
CART9	DVKTAFLHGDLEEEIYMEQLEGFEVKGKEPLVCKLKKSLYGLKQAPRQWYKKFDSFMEKH	60
CART67	DVKTAFLHGDLEEEIYMEQPEGFKVKGKEQLVCKLKKSLYGLKQAPRQWYKKFDSFMEKH	60
CART73	DVKTAFLHGDLEEEIYMEQLEGFEVKGKEQLVCKLKKSLYGLKQAPRQWYKKFDSFMQKH	60
CART120	DVKTAFLHGDLEEEIYMEQPEGFEVKGKEQLVCKLKKSLYGLKQAPRQWYKKFDSFMEKH	60
CART126	RC-DRILHGDLEEEIYMEQLEGFEVKGKEPLVCKLKKSLYGLKQAPRQWYKKFDSFMEKH	59
CART241	RR-TAFLHDDLEEEIYMEQLEGFEVKGKTKQLRRKLKKGLYGLKHAPRQWYKKFDSFMEKH	59
CART123	DVKTAFLHGDLEEEIYMKQPNGFLVKGKEDYVCRLIKSLYGLKRVPRQWYKKFESVMCEQ	60
CART55	DVKTAFLHGDLEEEIYMKQPDGFLVKGKEDYVCRLRKSLYGLKQAPRQWYKKFESVMCEQ	60
CART33	DVKTAFLHGDLEEEIYMKRPDGFVLVGKEDYVCRLRKSLYGLKQAPRQWYKKFESVMCEQ	60
CART135	DVKTAFLHGDLEEEIYMKQPDGFLVKGKEDYVCRLRKSLYGLKQAPRQWYKKFESVMCEQ	60
CART40	DVKTAFLHGDLEEEIYMKQLDGFVLVGKEDYVCRLRKSLYGLKQAPRQWYKKFESVMCEQ	60
CART364	DVKTAFLHGDLEEEIYMKQLDGFVLVGKEDYVCRLRKSLYGLKQAPRQWYKKFESVMCEQ	60
CART14	DVKTAFLHGDLEEEIYMKQPDGFLVKGKEDYVCRLRKSLYGLKQAPRQWYKKFESVMCEQ	60
CART56	DVKTAFLHGDLEEEIYMKQPDGFLVKGKEDYVCRLRKSLYGLKQTPRQWYKKFESVMCEQ	60
CART98	DVKTAFLHGDLEEEIYMKQPDGFLVKGKEDYVCRLRKSLYGLKQAPRQWYKKFESVMCEQ	60
CART155	DVKTAFLHGDLEEEIYMKQPDGFLVKGKEDYVCRLRKSLYGLKQAPRQWYKKFESVMCEQ	60
CART377	DVKTAFLHGDLEEEIYMKQPDGFLVKGKEDYVCRLRKSLYGLKQAPRQWYKKFESVMCEQ	60
CART52	DVKTAFLHGDLEEEIYMKQPDGFLVKGKEDYVCRLRKSLYGLKQAPRQWYKKFESFMEQ	60
CART54	DVKTAFLHGDLEEEIYMKQPDGFLVKGKEDYVCRLRKSLYGLKQAPRQWFKKFESIMCEQ	60
CART324	DVKTAFLHGDLEEEIYMKQPDGFLVKGKEDYVCRLRKSLYGLKQAPRQWFKKFESIMCEQ	60
CART77	RCQNCFLHGDLEEEIYMKQPDGFLVKGKEDYVCRLRKSLYGLKQAPRQWYKKFESVMCEQ	60
CART103	RCKNCFLHGDLEEEIYMKQPDGFLVKGKEDYVCRLRKSLYGLKQAPRQWYKKFESVMCEQ	60
CART105	RC-DGISHGDLEEEIYMKQPDGFLVKGKEDYVCRLRKSLYGLKQAPRQWYKKFESVMCEQ	59
CART141	RCEDGFLHGDLEEEIYMKQPDGFLVKGKEDYVCRLRKSLYGLKQAPRQWYKKFESVMCEQ	60
CART345	RC-NCIFHGDLEEEIYMKQPDGFLVKGKEDYVCRLRKSLYGLKQAPRQWYKKFESVMCEQ	59

CART97	DVKTAFLHGDLEEEIYMKQPDGFLVKGKEDYVCRLRKSlyGLKQAPRQWYKKFESVMCEQ	60
CART311	DVKTAFLHGDLEEEIYMNQPDGFLVKGKEDYVCRLRKSlyGLKQAPRQCYKKFESVMCEQ	60
CART389	DVKTAFLHGDLEEEIYMNQPDGFLVKGKEDYVCRLRKSlyGLKQAPRQCYKKFESVMCEQ	60
CART329	DVKTAFLHGDLEEEIYMKQPDGFLVKGKEDYVCRLRKSlyGLKQAPRQWYKKFESVMCEQ	60
CARE2	DVKTAFLHGDLEEEIYMKQPDGFLVKGKEDYVCRLRKSlyGLKQAPRQWYKKFESIMCEQ	60
CARE3	DVKTAFLHGDQNETILMQQPNGFRTQGKEDWVCLLKRSlyGLKQSSR--YLRFDsLMLSQ	58
CARE4	RR-NGIPSW-FGEEIYMKQPDGFLVKGKEDYVCRLRKSlyGLKQAPRQWYKKFESVMCEQ	58
CARE5	DVKTAFLHGDLEEEIYMKQPDGFLVKGKEDYVCRLRKSlyGLKQAPRQWYKKFESVMCEQ	60
CARE7	RCQNSFFQW-SE-NNLNATTKWF-DSRK-GLGLLTKEITLWAKAITRQWYLRFDsFMLSQ	56
CARE8	RCKNAFLHGDLEEEIYMKQLDGFLVKGKEDYVCRLRKSlyGLKQAQRQWYKKFESVMCEQ	60
CARE9	NHVNHSFMV-IGGRDLHEALDGFLVKGKEDYVCRLRKSlyGLKQAQRQWYKKFESVMCEQ	59

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CART50	GFEMNTVDDYVYHK-FSGSRHIFLVLYVDDMDP	92
CART366	GFEMNTVDDCVYHK-FSGSRHIFLVLYVDDMDP	92
CART387	GFEMNTVDDCVYHK-FSGSRHIFLVFSVDDVDP	92

CART37	SYAKSNFGSCVYYKQVTSATYIYLLLYVDDVDP	93
CART157	SYVRSNFDSCVYYKQVSSATYIYMLLYVDDMDP	91

CART61	GFKQSQGDHTLFIKHSESGGVTVLLLYVDDMDP	93
CART384	GFKQSQGDHTLFIKHSESGGVTMLFLYVDDMDT	93

CART9	GYDKTTSDHCVFVKKFSGDYIILLLYVDDMDP	93
CART67	GYGKTTSDHCVFVKKFSGDYIILLLYVDDMDP	93
CART73	VYGKTTSDHCVFIKKFSEGDYIILLLYVDDMDP	93
CART120	GYGKTTSDHCVFVKKFSGDYIILLLYVDDMDP	93
CART126	GYDKTTSDHCVFVKKFSGDYIILL-YVDDMDP	91
CART241	GYGKTTSDHCVFVKKFSGDG-IILLLYVDDMDP	91

CART123	GYKKTTSdhcvfvkkfvdddfiilllyvddvdp	93
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CART55	GYKKTTSdhcvfvkkfadddfiilllyvddvdp	93
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CART33	GYRKTTSdhcvfvkkfadddfiilllyvddvdp	93
CART135	GYRKTTSdhcvfvkkfadddfiilllyvddvdp	93

CART40	GYRKTTSdhcvfvkkfadddfiilllyvddvdp	93
CART364	GYRKTTSdhcvfvkkfadddfiilllyvddvdp	93

CART14	GYRKTTSdhcvfvkkfadddfiilllyvddvdp	93
CART56	GYRKTTSdhcvfvkkfadddfiilllyvddvdp	93
CART98	GYRKTTSdhcvfvkkfadddfiilflyvddvdp	93
CART155	GYRKTTSdhcvfvkkfadddfiilllyvddvdp	93
CART377	GYRKTASdhcvfvkkfadddfiilllyvddvdp	93

CART52	GYRKTTSdhcvfvkkfadddfiilllyvddvdp	93
CART54	GYRKTTSdhcvfvkkfadddfiilllyvddvdp	93
CART324	GYRKTTSdhcvfvkkfadddfiilllyvddvdp	93

CART77	GYRKTTSdhcvfvkkfadddfiilllyvddvdp	93
CART103	GYRKTTSdhcvfvkkfadddfiilllyvddvdp	93
CART105	GYRKTTSdhcvfvkkfadddfiilllyvddvdp	92
CART141	GYRKTTSdhcvfvkkfadddfiilllyvddvdp	93
CART345	GYRKTTSdhcvfvkkfadddfiilflyvddvdp	92

CART97	GYRKTTSdhcvfvkkfadddfivlllyvddvdp	93
CART311	GYRKTTSdhcvfvkkfadddfiilllyvddvdp	93
CART389	GYRKTTSdhcvfvkkfadddfiilllyvddvdp	93

CART329	GYRKTTS DHC VLVKKFVDDDFIILLLYVDDMDP	93
CARE2	GYKKATSDQCVFVKKFADDDFIIMLLYVDDMDP	93
CARE3	SYVRSNFDSCVYYKQVSSATYIYMLLYVDDMDP	91
CARE4	GYRKTTS DHC VLVKKFADDDFIILLLYVDDMDP	91
CARE5	GYKKTTSDHC VLVKKFADDDFIILLLYVDDMDP	93
CARE7	RYARRNFNSYVYYKQVSSVTYIYLLLYVDDMDP	89
CARE8	GYRKTTS DHC VLVKKFADDDFIILLLYVDDMDP	93
CARE9	GYRKTTS DHC VLVKKFADDDFIILLLYVDDMDP	92
	: . : * : . ***:*	

Figure 4. Neighbour joining tree based on the alignment of *copia*-like elements from chickpea.

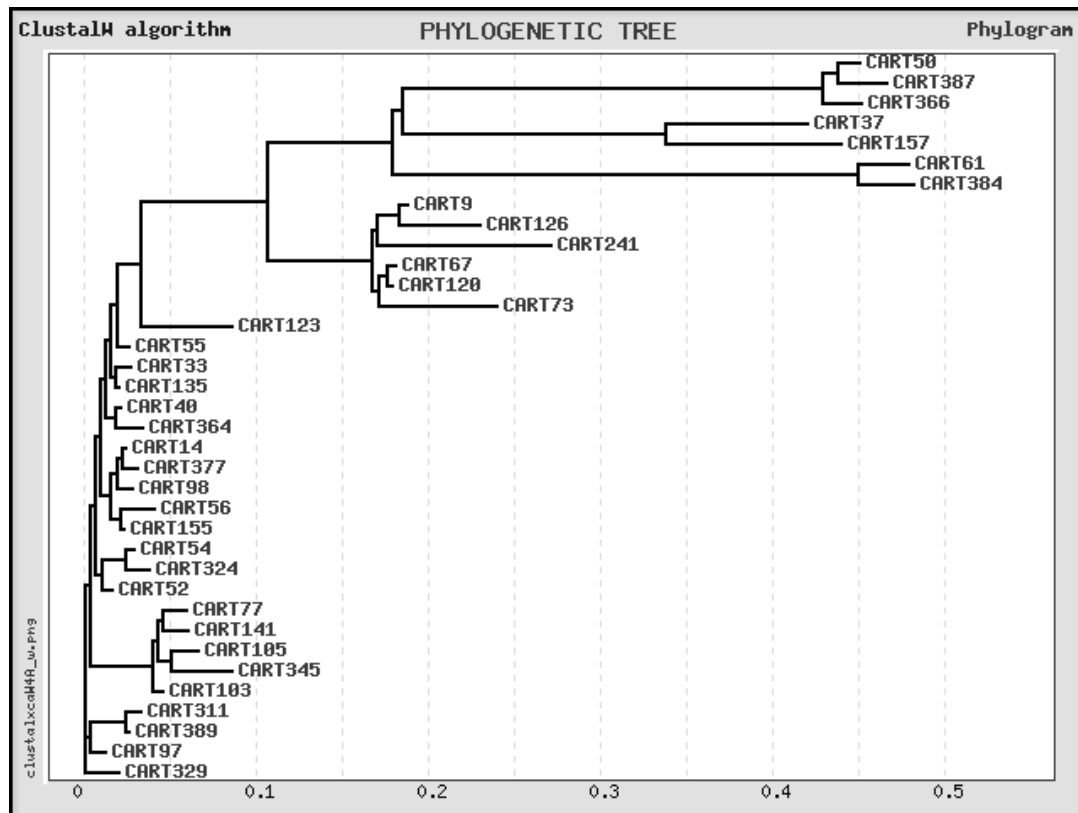


Figure 5. Shows *Ty1*-copia RT specific transcripts from stressed chickpea plants (Lane S). Lane N contains RT-PCR of RNA isolated from normal plants. Lane M contains a 100-bp DNA ladder.

