CARE1, a TY3-gypsy long terminal repeat retrotransposon in the food legume chickpea (Cicer arietinum L)

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Abstract

We report a novel Ty3-gypsy long terminal repeat retrotransposon CARE1 (*Cicer arietinum* retro-element 1) in chickpea. This 5920-bp AT-rich (63%) element carries 723-bp 5' and 897-bp 3' LTRs respectively flanking an internal region of 4300-bp. The LTRs of CARE1 show 93.9% nucleotide identity to each other and have 4-bp (ACTA) terminal inverted repeats. A 17-bp potential tRNA<sup>met</sup> primer binding site downstream to 5' LTR and a 13-bp polypurine tract upstream to 3' LTR have been identified. The order of domains (*Gag*-proteinase-reverse transcriptase-RNaseH-integrase) in the deduced amino acid sequence and phylogenetic tree constructed using reverse transcriptase sequences places CARE1 in the *gypsy* group of retrotransposons. Homologues of a number of *cis*-elements including CCAAT, TATA and GT-1 have been detected in the regulatory region or the 5' LTR of CARE1. Transgenic tobacco plants containing 5' LTR:GUS construct show that its 5'-LTR is inactive in a heterologous system under normal as well as tissue culture conditions. Genomic Southern blot experiments using 5'LTR of the element as a probe show that CARE1 or its related elements are present in the genomes of various chickpea accessions from various geographic regions.

Key words  Retrotransposon  *gypsy*  LTR  reverse transcriptase  *Cicer arietinum*

Abbreviations

*CARE1*  *Cicer arietinum* retro-element 1

| Abbreviation | Description                        |
|--------------|------------------------------------|
| LTR          | Long terminal repeat               |
| RT           | reverse transcriptase               |
| ORF          | open reading frame.                |
Introduction

Retrotransposons are a class of transposable elements that encode reverse transcriptase and like retroviruses propagate via a RNA intermediate but are non-infectious. These elements are widely distributed and comprise a significant fraction of eukaryotic genomes. Based on the presence or absence of LTRs retrotransposons are divided into LTR and non-LTR retrotransposons. The LTRs do not encode any known proteins, but they contain the promoters and terminators associated with the LTR retrotransposons (Kumar and Bennetzen 1999). The internal region of retrotransposons consists of gag (group-specific antigen) and pol (polyprotein) regions involved in the maintenance of the retroviral life cycle (Bishop and Varmus 1985). The pol gene of retrotransposon consists of four internal domains encoding the enzymes protease, reverse transcriptase, RNase H and integrase (or endonuclease). On the basis of the arrangement of the internal domains in pol gene, LTR retrotransposons have been classified into Ty1-copia and Ty3-gypsy groups (Doolittle et al. 1989; Xiong and Eickbush 1990). In copia group integrase domain is at 5’ to the reverse transcriptase domain, while in gypsy it is located 3’ to the RT domain. The terminology has been derived after the elements gypsy and copia in Drosophila and yeast respectively.

The great difference in genome size among the members of grass family is attributed to the presence of large amount of retroelements in plants having large
genomes (Bennetzen 2000). For example maize genome contains over 80% and barley genome more than 70% of its sequences as retroelements (Messing et al. 2004; SanMiguel and Bennetzen 1998; Vicent et al. 1999). Analysis of the recently sequenced Arabidopsis genome reveals that, despite its small size, it has as many as 2109 retroelements including representatives of all the categories of retroelements (Arabidopsis genome initiative 2000). Thus “C-value-paradox” i.e. non-correspondence between structural complexity to the functional complexity could largely be explained by the proportion of retroelements in the genomes. Some retrotransposons are shown to have contributed to genome evolution by changing structure, gene expression patterns and are also known to cause gene activation by inserting near or within genes or by causing alterations in transcript processing and/or stability. In some cases, insertion of retrotransposon has been shown to alter the spatial and temporal pattern of gene expression or the structure of the resulting gene product (Flavell et al. 1994; Wessler et al. 1995).

The retroelements are known to be transcriptionally and transpositionally activated in response to a variety of biotic and abiotic stresses. In that sense, they are also considered to have some role in stress alleviation phenomena in plants (Mhiri et al. 1997; Takeda et al. 1998).

Retrotransposons are now being utilized as molecular tools in DNA fingerprinting, genetic linkage mapping, phylogenetic studies and molecular breeding. The presence of retroelements in high copy number and in heterogeneous populations; their dispersion throughout the genome; and their insertion into new genomic sites without loss of parental copies are some of the properties which make them suitable candidates for generating molecular markers in various crop plants (Kumar and Bennetzen 1999).

Although legumes belong to the third largest family of angiosperms, they are least explored in terms of their genome organization (Pearce et al. 1996; Sant et al. 2000; Lall
et al. 2002; Neuman et al. 2003). Chickpea (*Cicer arietinum* L), the world's third most important food legume, is currently grown on about 10 m ha worldwide, with 95% cultivation in the developing countries like India. This self-pollinating annual diploid crop with a somatic chromosome number of 2n = 16 has a genome size of ~931 Mb. Such a large genome is expected to have a significant fraction of retrotransposons. Here we report isolation and characterization of a typical *gypsy*-like retrotransposon *CARE1* from chickpea. The 5′-LTR of *CARE1* appears to be inactive in normal and tissue culture condition in a heterologous plant system.

**Materials and Methods**

*Isolation of reverse transcriptase probe*

DNA from chickpea (*Cicer arietinum*) variety Pusa 362 was isolated using standard procedures (Ausubel et al., 1994). Polymerase chain reaction (PCR) was performed in 50 µl volume with 0.5 µg of genomic DNA, each dNTP @ 200 µM, 100 pmoles of each *gypsy* RT-specific primer 5′-TAC CCN TTN CCN CGN ATH GAY GAT-3′ and 5′-GTC GTN TTY ATH GAY GAY ATH CTA-3′, 2.5 units of *Taq* DNA polymerase (New England Biolabs) and 1.7 mM MgSO₄. Temperature cycling was performed on MJ Research thermal cycler with the following profile: 94°C for 5 min, followed by 35 cycles of: 94°C for 1 min, 48°C for 1 min, 72°C for 1.5 min followed by 72°C for 10 min. The expected amplified product (270-bp) was purified from 1.2% agarose gel, cloned into pGEM-Teasy vector (Promega) and the nucleotide sequence was determiner by DNA sequencer (Applied Biosystem).

*Screening of chickpea genomic library*

A genomic library constructed using partially digested and size-fractionated genomic DNA of chickpea, into the EcoRI site of λZAP vector, was screened for retrotransposon complementary sequences using the cloned RT-specific sequence as a
probe. Approximately 2 lakh plaques were plated on host *Escherichia coli* XL-1 blue, blotted onto nylon membrane and hybridized (Sambrook et al. 1989). DNA from one of the positive λ clones was isolated and the sub-fragments of retrotransposon, CARE1 were cloned into pBluescript vector.

**Sequencing of CARE1**

The nucleotide sequence was determined using DNA sequencer (Applied Biosystem). The nucleotide sequence was compared with the sequences in databases using either BLASTN or BLASTX programs of NCBI (Altschul et al. 1990).

**In silico analysis (Generation of dendrogram and Phylogenetic analysis)**

The phylogenetic tree construction and sequence alignments using RT regions were carried out using clustal W (1.83) (http://www2.ebi.ac.uk/clustalw; Thompson et al., 1994). The method used is the neighbour joining method based on algorithm of Saitou and Nei (1987). The ORFs were determined by using NCBI ORF finder software (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The cis-acting regulatory DNA elements were scanned using PLACE database as a reference (Higo et al., 1999).

**Southern analysis**

Chickpea seeds (Accessions) were procured form ICRISAT (International Crop Research for Semi Arid Tropics), Patancheru, Andhra Pradesh, India. The genomic DNA digested with appropriate enzyme (Hind III) 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 5'-LTR sequence of the retrotransposon CARE1 as a probe (Sambrook et al., 1989).

**Generation of CARE1 LTR:GUS tobacco plants**

The 5'-LTR region of the element was amplified using λ clone containing the full CARE1 element as a template, 20 pmoles each of the LTR-specific primers (with added restriction sites HindIII and BamHI) 5'-AAG CTT ACT ATT GGG AAG TTG GGA
GAC T-3' and 5'-GGA TCC ACT AAA ATT GAA AAA AAG TTC C-3' in a 50 µl PCR reaction containing 100 µM dNTPs, 2 mM MgSO₄ and 2.5 units Taq DNA polymerase (New England Biolabs). Temperature cycling was performed on MJ Research thermal cycler with the following profile: 94°C for 5 min, followed by 35 cycles of: 94°C for 1 min, 53°C for 1 min, 72°C for 1.5 min followed by 72°C for 10 min. The amplified fragment (723 bp) was cloned into pGEM Teasy vector. This 723-bp fragment containing the entire 5'-LTR was excised from the plasmid and further cloned into the plant transformation vector, pBI101.

The recombinant plasmid or the construct harbouring 5'-LTR was transferred to Agrobacterium strain LBA4401 by freeze-thaw method. The transformed Agrobacterium colonies were selected on YEM-agar (yeast extract mannitol) medium supplemented with 50 µg/ml kanamycin and 25 µg/ml rifampicin. The transformed Agrobacterium cells were used for tobacco (Nicotiana tabacum cv. Xanthi) leaf-disc transformation (Horsch et al. 1985). The regeneration was carried out on MS medium supplemented with 1.0 mg/l BAP, 0.1 mg/l NAA, 250 µ g/ml cefotaxime and 300 µg/ml kanamycin. After 18-25 days, well-developed independent shoots were excised and placed upright in the MS basal medium (MS medium with 0.6% agar) for rooting. The kanamycin resistant plants were tested for histochemical GUS assay. For histochemical localization of GUS, intact plants were treated with 1 mg/ml X-gluc in phosphate buffer (pH 7.2) and incubated overnight at 37°C (Jefferson 1987). The stained material was subsequently treated with 70% ethanol with several changes of solution. Southern blotting using the 5'-LTR sequence as a probe was performed to confirm the structural integrity of the construct among the individual transformants.
Results and Discussion

Isolation of retrotransposon CARE1

This is the first report of any complete retrotransposon from the food legume chickpea. A 270-bp reverse transcriptase sequence was used as a probe to screen chickpea genomic library for retrotransposon. The 5920-bp isolated retrotransposon was named CARE1 (*Cicer arietinum* Retro-element1) and submitted to database under accession no. DQ239702 (Fig. 1).

![Diagram of CARE1 retrotransposon](image)

**Fig 1.** Structural features of retrotransposon CARE1 (Acc.no. DQ239702).

- LTRs are shown by green rectangles at 5’ and 3’ ends.
- Various internal domains are shown in the yellow rectangle.
- The primer binding site for minus strand DNA synthesis is shown in black.
- The sequence of proposed methionyl tRNA primer showing complementarity to Primer binding site is shown in light brown.
- Putative polypurine tract is shown in pink.
- Each LTR contains inverted terminal repeats of 4 bp (shown in enlarged boxes).

\[
\text{ACCGUAACACAAGTAAACUA} - \text{proposed Methionyl-tRNA} \\
\text{PBS} - \text{TGGTATTGTGTTCATTGAT}
\]
Structure of CARE1

The size of CARE1 (5920 bp) falls in average length range of LTR-retrotransposons. The gypsy-like retrotransposon *Grande-1* (13.5 kb) is the largest LTR-retrotransposon, which has ever been found (Martinez-Izquierdo et al., 1997). Other gypsy-like retrotransposons having size 10 kb are *Athila* (10.5 kb; Pelissier et al., 1995), *Athila1.1* (12 kb; Wright and Voytas, 1998), *Cereba* (10 kb; Presting et al., 1998), *Cyclops-1* (12.3 kb; Chavanne et al., 1998) and *RIRE3* (10.5 kb; Kumesawa et al., 1999a). The AT rich (63%) element CARE1 carries 723-bp and 897-bp 5' and 3' LTRs respectively flanking an internal region of 4300 bp with two successive domains, both showing homology to retroviral genes *gag* and *pol*. The pol region of CARE1 contains sequence motifs related to the enzymes protease (PR), reverse transcriptase (RT), RNase H (RH) and integrase (IN) in the order 5'-PR-RT-RH-IN-3' characteristic for a gypsy-like retrotransposons and retroviruses. Like certain retroelements such as marY1 and Tnt1, CARE1 also lack typical canonical sequence 5'-TG…..CA-3' (Grandbastien et al., 1989; Murata and Yamada, 2000). Since a typical canonical sequence is not present in CARE1 LTRs, it is probable that (CA) might have mutated to (GA). Both LTRs of CARE1 have perfect 4-bp (ACTA) inverted repeats (IRs), which are thought to be important for integration or insertion. In CARE1 we identified a potential 17-bp (TGTTATTGTGTTCCATTG) non-overlapping tRNA\textsuperscript{met} primer binding (site for minus strand DNA synthesis which is located just down stream to the 5'LTR and a 13-bp (AAAAGAGGAGAAA) non-overlapping polypurine tract just upstream to the 3'LTR, which could be primer binding site for plus-strand DNA synthesis. In most of retroelements including CARE1 a 16-21 nucleotide stretch of PBS sequence begins with TGG and shows best matching to cytosolic tRNA\textsuperscript{met} of plants suggesting, that cytosolic tRNA\textsuperscript{met} from chickpea is used to prime the minus-strand DNA synthesis. The other gypsy-like retroelements with non-overlapping PBS and PPT are
412, *Micropia*, *Ulysses*, *SURL*, *Mag*, *Del* and *Ty3*. The PBS of CARE1 also shows significant homologies to the PBS of gypsy-like retrotransposons *LORE1*, *Del-1*, *Magellan* (Madsen et al., 2005; Smyth et al., 1989; Purugganan et al., 1994) and most other LTR-retrotransposons. Reverse transcription of retroelement transcripts has always been found to proceed from a tRNA primer bound at the 3' end of the 5'LTR. For the LTR-retrotransposons, the process of reverse transcription can be conveniently separated into minus-strand and plus-strand reverse transcription. In the former, the mRNA of the retrotransposon serves as a template for reverse transcription but, in the latter the minus-strand reverse transcript (cDNA) serves as template (Boeke and Cores, 1989).

**Structure of long terminal repeats (LTRs) of CARE1**

The 3′-LTR is longer by 174 bp. This 174-bp stretch contains 165 duplicated segment and two additional sequences of 5 and 4 bp. The 5-bp (GTAAT) sequence in 3′-LTR is located as an intervening sequence between the 165-bp duplicated segment, and the 4-bp (TCAG) is located 9-bp upstream to the end of the 3′-LTR (*Fig. 2a & 2b*). Excluding the duplicated segment, the two LTRs show a homology of ~94% at the nucleotide level. One deletion of a single nucleotide (indel) is present in both the LTR’s. The 165-bp segment that is duplicated in the 3′-LTR shows a homology of 87.8% with the corresponding region in the 5′-LTR. The duplicated region shows ~90% homology and contains 12 nucleotide substitutions, which includes 7 transversions (4 T to G, one G to T, one G to C and one T to A) and 5 transitions (2 T to C, 2 C to T and one G to A). The 12 nucleotide changes possibly indicate that the duplication event may not be a recent one.

| 5′ LTR                              | ACTATTGGGAAAGTTGGGAGACTTGCCCTAACTGAG 37 |
| 3′ LTR                              | ACTATTGGGAAATTTGGGAGACTTGCCCTAGTAGAG 37 |
|-------------------------------------|----------------------------------------|
| ************* ** ********** *****   ***|                                        |
**Figure 2a.** Alignment of nucleotide sequences of 5’LTR and 3’LTR.
The stars denote identical bases. In red duplication of internal sequence of 3’LTR is shown (bases in red are those which are different in duplicated sequence).

| 5’LTR | TAATCTCTATCTTTTCTATCTATGTCATGAGTAACTAAA 157 |
| 3’LTR | TAATCGCTATCTTTTCTATCTCTATCATGAGTAATTTAAA 157 |
| 3’LTR duplication > | 289 TAATCTCTATCTTTTCTATCTCTATCATGAGTAATTTAAA 328 |

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5’LTR                  3’LTR                  3’LTR duplication >
TAATCTCTATCTTTTCTATCTATGTCATGAGTAACTAAA  TAATCGCTATCTTTTCTATCTCTATCATGAGTAATTTAAA  TAATCTCTATCTTTTCTATCTCTATCATGAGTAATTTAAA
***** *************** * ********** ****
*   *       *               *       *             *

5’LTR                  3’LTR                  3’LTR dup.
CCCTATTTGTTAGGAATGAGTGTAACAAACTAAAACCCTTATTTTTATGATTTGATTTCT  CCCTATTTGTAAGGGAGGAGTGTAATAAGATGAAACTCTTATTTTTCTGATTTGATTTCT  CCCTATTTTTTATGGATGAGCGTAATAAGATGAAACTCTTATTTTTCTGATTTGATTTAT
 ******** * * * * *** **** **  * **** ******* * *********** *
*   *       *               *       *             *

5’LTR                  3’LTR                  3’LTR dup.
AGCTATATGAATGAGTTTATTGAATTATTTTTCTCATCTCTGTGCTTAATGCTTATTATT  AGTTATATGAATGAGTTTATTAAATTATTTTTCTCATCTCTGTGCTTAATACTTTTTATT  AGTTATATGAATGAGTTTATTGAATTATTTTTCTCATCTTTGGTGCTTAATACTTTTTATT
** ****************** *****************  ********* *** ****
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**Figure 12b.** Alignments of duplicated sequence with its original in 3’LTR and homologous sequence in 5’LTR.

Black * shows bases matching in all three sequences.
Red * shows bases matching in 5’LTR and duplicated 3’LTR.
Blue* shows bases matching in 5’LTR and original 3’LTR.

shows bases matching in original 3’LTR and duplicated 3’LTR.

The potential functionality of a retrotransposon depends on its regulatory sequences (promoter) present in its 5’-LTR; however, the transposability of a retrotransposon depends on the functionality of the translated polyprotein as well as on the activity of regulatory sequences or cis-elements. While scanning the 5’-LTR of CARE1 for cis-elements using PLACE (Plant cis-acting elements) database as a reference (Higo et al., 1999) three putative TATA boxes, CCAAT box, GT-1 consensus, copper
responsive element GATC, ethylene responsive element ERELEE4 and various MYB related cis-elements were detected. The 5′ -LTR contains homologues of MYB related elements which are shown to be induced by wounding and elicitors in tobacco retrotransposon Tto1 and defense-related genes (Sugimoto et al., 2000), ethylene responsive element ERELEE4 of retrotransposon TLC1.1 of Lycopersicon chilense (Tapia et al., 2005), a copper responsive element GATC (Quinn and Merchant, 1995).

When 5′-LTR of CARE1 is compared with 5′-LTRs of active retrotransposons LORE1, Tnt1 and Tto1, it is found that LTRs of CARE1 contain almost all cis-elements which are present in their LTRs (Grandbastien et al., 1994; Sugimoto et al., 2000). It may be possible that some of these cis-elements could be binding sites for repressors, which after binding may stop the recruitment of transcription factors to the promoter.
Although most gypsy-like retrotransposons show no divergence between their two LTRs, such sequence differences have been observed in many gypsy-like elements (Smyth et al., 1989; Chavanne et al., 1998). The 723-bp 5′ -LTR and 897-bp 3′ -LTR of CARE1 with 93.9% inter-LTR sequence identity fall within the average size range of LTRs of most of the gypsy-like retrotransposons (Smyth et al., 1989; Pelissier et al., 1996; Chavanne et al., 1998; Wright and Voytas, 1998; Kumekawa et al., 1999). This suggests that LTRs may diverge very frequently, presumably because LTRs are non-coding regions. The analyses of the LTRs of CARE1, Del-1, Micropia, Zeon-1, Cyclops-2, Tnt1, Tto1, Tos17 and LORE1 indicate that there appears be relation between functionality and inter-LTR homology for a given retroelement (Grandbastien et al., 1989; Smyth et al., 1989; Purugganan et al., 1994; Grandbastien et al., 1994; Hirochika et al., 1996; Kumar et al. 1999; Madsen et al., 2005). Till date only a few active retrotransposons are known, three of them Tnt1 (tobacco), Tto1 (tobacco), Tos17 (rice) are copia-like (Grandbastien et al., 1994; Lucas et al., 1995; Hirochika et al., 1996; Mhiri et al., 1997; Takeda et al., 1998; Kumar et al. 1999) but, recently discovered active element LORE1 (Lotus japonicus) belongs to the gypsy group (Madsen et al., 2005). The homology between 5′ -LTR and 3′ -LTR of these active retroelements is very high.

**Internal structure of CARE1**

The deduced amino acid sequence of the internal region of CARE1 shows the presence of five open reading frames along the same strand and in the same frame. The inter-ORF regions contain many termination (stop) codons. All conserved domains (gag, proteinase, reverse transcriptase and RNase H) except integrase were detected in this +1 frame. The integrase domain was found in deduced amino acid sequence in +2 frame. The first ORF is predicted to encode a protein containing 109 amino acids with a putative central motif QGX\(_2\)EX\(_3\)FX\(_3\)LX\(_2\)H, from amino acid 109 to 126, which is common to retroviridae gag proteins, but is poorly conserved. The predicted molecular mass of this
protein is very much less than those of most gag gene products, which is generally ~60-80 kDa. In CARE1, a proteinase conserved domain LIDIGA is present from 428 to 433 amino acid positions. The aspartic proteinases of retroelements are extremely diverse and hence, little sequence conservation outside the active site is observed between CARE1 and others (Smyth et al., 1989; Purugganan et al., 1994; Chavanne et al., 1998; Kumekawa et al., 1999).

The second or the smallest ORF extends from 2545 to 2652 nucleotide positions and is followed by reverse transcriptase region with conserved domain from 2998 to 3493 nucleotide positions. The alignment of RTase conserved region of CARE1 with the corresponding regions of retroelements from various organisms (plants, animals, viruses, fungi and insects) reveals highly conserved motifs like PRID and FXXD. These regions are highly conserved and they have been used to design degenerate primers to amplify RTases of gypsy-like retrotransposons from diverse plant species (Muthukumar and Bennetzen, 2004). In CARE1, the actual conserved motif is larger which contains the core motif FIND. The third or the longest ORF starts at the 3523rd nucleotide and ends at 3906; and it is predicted to encode polypeptide of 128 amino acids with three internal methionines. This ORF is immediately followed by fourth ORF, which starts at nucleotide position, 3919 and ends at 4074. RNase conserved domain is present in this ORF from 1036 to 1039 amino acid positions. For retroviruses, an RNaseH conserved motif is represented by TDGS (thr, asp, gly, and ser) while it is TDAS (thr, asp, ala and ser) for most gypsy-like elements (Warmington et al., 1985; Smyth et al., 1989) but in case of CARE1, Cyclops-2 and Ulysses it is CDAS (cys, asp, ala and ser). The 189-bp fifth ORF exists between 4075 and 4263 nucleotides. After fifth ORF, a single nucleotide frame shift occurs which separates the integrase region from the whole element in the context of translational frame and the integrase conserved domain is present between 4259 to 4744 nucleotides and zinc finger motif in this region is from 1271 to 1292 amino
acid positions. This deduced amino acid sequence of CARE1, which corresponds to the endonuclease region of other retrotransposon, shows an amino acid configuration capable of forming a zinc-binding domain, which could interact with DNA to facilitate integration of LTR-retroelements into the host genome (Grantgenett and Mumm, 1990). The structure of conserved zinc finger motif in CARE1 is His-(7)-Lys-(10)-Cys-(1)-Cys. Although this conserved motif of CARE1 shows high homology to the corresponding regions of other retrotransposons but does not represent a typical structure. Functional polyproteins are known to form in retroelements by frame-shifting (Farabaugh et al., 1993). However, due to presence of many chain terminating codons in CARE1, it appears that this copy of the element is a non-functional one but a possibility of another functional element of the CARE family can not be ruled out.

**Phylogenetic Analysis**

The phylogenetic analysis of genomic sequences is done on the basis of their deduced amino acid homology. Among retroelements from the diverse organisms the region encoding the reverse transcriptase is highly conserved (Feng and Doolittle, 1987; Xiong and Eickbush, 1990; McClure, 1991), thus these are used to derive the phylogenetic tree along with RTase of CARE1 by neighbor joining method (Saitou and Nei, 1987). All categories of LTR retroelements viz. copia, gypsy and retroviruses were used for alignment and generation of phylogenetic trees (**Table 1**).

**Table 1.** Retroelements used for alignments

| Accession no. | Organism       | Category |
|---------------|----------------|----------|
| D83003        | *Nicotiana tabacum* | copia    |
| X13777        | *Nicotiana tabacum* | copia    |
| AC087545      | *Oryza sativa*   | copia    |
| DQ105074      | *Malus sp*       | copia    |
| AY654426      | *Citrus sp*      | copia    |
| AF416818      | *Musa sp*        | copia    |
| AJ414059      | *Brassica sp*    | copia    |
| Accession  | Organism                          | Type       |
|------------|-----------------------------------|------------|
| DQ100159   | Camellia sp                       | copia      |
| DQ05417    | Pinus                            | copia      |
| DQ54416    | Ephedra                          | copia      |
| AF295692   | Anopheles                        | copia      |
| AF398212   | Zea mays                         | copia      |
| AF130856   | Phytophthora sp                   | copia      |
| AF231939   | Sweet potato                     | copia      |
| M94164     | S. cerevisiae                    | copia      |
| M94494     | H. annus                         | copia      |
| AJ966990   | Lotus japonicus                  | gypsy      |
| AJ000640   | Pism sativum                     | gypsy      |
| AJ290632   | Pinus sp                         | gypsy      |
| AJ971814   | Musa sp                          | gypsy      |
| AJ415653   | Brassica sp                      | gypsy      |
| AY959279   | Cycas sp                         | gypsy      |
| AY683037   | mungbean                         | gypsy      |
| AJ295135   | Pism sativum                     | gypsy      |
| AJ295132   | Zea mays                         | gypsy      |
| AJ295139   | Arabidopsis                      | gypsy      |
| AJ243725   | Frog                             | gypsy      |
| AB033239   | Chlamydomonas                    | gypsy      |
| X03734     | Drosophila                       | gypsy      |
| AY877248   | HIV-1virus                       | Retrovirus |
| AY454232   | lentivirus                        | Retrovirus |
| AF126467   | Simianretrovirus                 | Retrovirus |
| U34348     | Humanendovirus                   | Retrovirus |
| M25768     | Humanart                         | Retrovirus |
Figure 3. Phylogenetic tree of predicted amino acid sequences of RTase of CARE1 and other diverse retroelements. See Table 4 for source of these elements. Divergences in distance units are indicated by branch lengths.

In the phylogenetic tree CARE1 has been shown to branch from a point, which is common to other gypsy-like retrotransposons from diverse organisms (Fig. 3). Also the gypsy-like retrotransposons and retroviruses are observed to have common origin in the phylogenetic tree. This shows that retroviruses and gypsy-like retrotransposons diversified from a common ancestor. Alignments reveal that, CARE1 reverse transcriptase shows 26% to 59% amino acid sequence identity to the RT regions of other gypsy-like elements and 2-12% to the RT regions of copia-like elements and retroviruses.

The LTRs of a retroelement are very specific to the element and are quite conserved among their families but the conservation of other regions like gag and RTase is not restricted to a particular family. So the distribution, organization and presence of CARE1 family representatives in various chickpea accessions were assessed by Southern blotting using the 5′ -LTR of CARE1 as a probe. The results indicated that not only populations of copia-like retrotransposon sequences (Sant et al., 2000) are accumulated in chickpea genome but a sizable fraction of chickpea genome is also contributed by gypsy-like sequences.
Functional analysis of CARE1 promoter (5′-LTR) in heterologous plant

Considering a number of stop codons in the reading frames of the CARE1 retrotransposon, it appears that the isolated copy of the element is possibly non-functional. However, it is possible that the 5′-LTR, that contain the regulatory sequence may be functional. In order to determine the functionality of 5′-LTR, a 5′-LTR-GUS construct was made and transferred to tobacco. The structural integrity of the construct in transgenic tobacco plants were assessed by Southern blotting. The GUS expression was monitored in the transgenic plants. However, no GUS expression either histochemically or spectrofluorometrically could be detected in the normal or in response to stress. Non-expression of GUS indicates that the 5′-LTR is inactive as a promoter. This inactivity could be due to defective 5′-LTR or additional sequences are required for its functionality.

The cloning and characterization of the retrotransposon CARE1 from the legume chickpea is the first step towards the study of retrotransposons in this crop. Future studies would focus on the suitability and applicability of this retrotransposon in generating molecular markers for phylogenetic relationships among various chickpea varieties.

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