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Research article

A TRANSCRIPTIONALLY ACTIVE *copia*-LIKE RETROELEMENT IN *Citrus limon*

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Abstract: The plant nuclear genome is largely composed of mobile DNA, which can rearrange genomes and other individual gene structure and also affect gene regulation through various promoted activities: transposition, insertion, excision, chromosome breakage, and ectopic recombination. Ty1-*copia*-like retrotransposon is a widespread class of transposable elements in the plant kingdom, representing a large part of the total DNA content. Here, a novel retrotransposon-like sequence was isolated and identified as the Ty1-*copia*-like reverse transcriptase domain (named here CLCoy1), based on the homology of known elements. Fluorescence *in situ* hybridization, revealed that CLCoy1 was mainly located in telomeric and sub-telomeric regions along the *Citrus* chromosomes. CLCoy1 composes 3.6% of the genome and, interestingly, while transposons are mostly specific to a species, this element was identified in other Citrus species such as *Citrus aurantium*, *Fortunella margarita* and *Citrus paradisi*, but undetected in *Poncirus trifoliata*. We also determined that wounding, salt and cell culture stress produced transcriptional activation of this novel retroelement in *Citrus limon*. The novel Ty1-*copia*-like element CLCoy1 may have played a major role in shaping genome structure and size during *Citrus* species evolution.

Key words: Ty1-*copia*-like, *Citrus*, Transcription, Biotic and abiotic stress

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Abbreviations used: *C. limon* – *Citrus limon*, PCR – polymerase chain reaction; RT – reverse transcriptase

INTRODUCTION

Transposable elements are found throughout the plant kingdom, and can represent about 50% of the total DNA in some species [1]. Transposons could be considered an elegant survival strategy of plant biology or stress-induced generators of genomic diversity.

Plant genomes expand by several mechanisms, including polyploidization, transposition, and duplication. The high proportion of retroelements within plant genomes is a consequence of their replicative mode (copy-and-paste) of transposition, which produces new copies of the element each time it is retrotransposed. Retrotransposition is followed by insertion of reverse transcripts into the genome at new sites. New sites of homology for unequal crossing over arise from the new copies of transposons and retrotransposons [2, 3]. As a result, transposition might be a major cause of plant genome expansion. The detailed characterization of different plant taxa with respect to the content, variability, and physical distribution of retrotransposons would therefore make a major contribution to the understanding of host genome organization and evolution [4]. Retrotransposons are subdivided into three types: long terminal repeat (LTR) retrotransposons, short interspersed-like elements (SINE-like elements), and long interspersed-like elements (LINE-like elements). The Ty1-*copia*-like element is an LTR retrotransposon that shows striking similarities in structure to retroviruses. The main difference is that LTR retrotransposons lack the *env* genes, which code for the viral coat proteins in retroviruses. LTR retrotransposons are further divided into two groups: Ty1 or *copia,* and Ty3 or *gypsy*. The major structural difference between the *copia* and *gypsy* groups is in the order of the reverse transcriptase (RT) and integrase domains in their pol genes. Both *gypsy*-like and *copia*-like retrotransposons were first identified in the maize genome, and subsequently in many other plant genomes [5, 6].

The presence of *copia*-like retrotransposons in plants, insects, fungi and protisti suggests that this class of elements might be a universal component of the eukaryotic genome. The Ty1-*copia*-like retrotransposon, which has been the subject of extensive research and has been identified in over 100 plant species, is thought to be present in all plants [3]. Moreover, there is speculation that certain forms of "genomic stress" may stimulate the activation of transposons [7]. It has been reported that biotic and abiotic stresses are correlated to an increase in transposon activity [8]. Studies performed on rice and tobacco have shown that growth by tissue culture, for instance, can lead to the activation and transcription of these elements [8, 9].

The *Citrus* genus, which includes the most widely producing fruit species in the world, is highly polymorphic. The lemon (*Citrus limon* (L.) Burm.) belongs to the *Rutaceae* (*Citrinae* subtribal group C) , and originates from China, having been cultivated for thousands years. Lemon is one of the most economically important plants in the Mediterranean (Italy) and in other mid-latitude regional economies (Florida, Texas, California and Argentina), because it is utilized both

as a food crop and an industrial product. Some highly repetitive and dispersed DNA sequences have been isolated in *Citrus* genera, and their genomic organization has been studied [10-14]. However, it is likely that novel repetitive sequences involving the transposable elements could increase the amount of molecular data for understanding the genomic evolution and function of *Citrus limon* and related genera. In fact, transposons can be considered natural vectors for evolutionary force in shaping the genome.

In our research on *Citrus limon*, we scanned its genome using PCR targeting of the reverse transcriptase and ribonuclease H domains of retrotransposons [15-19]. The multiple alignments showed that we had isolated a novel type of Ty1-*copia*-like reverse transcriptase (named here CLCoy1) from *Citrus limon*, leading to investigation of the genomic and chromosomal organization, the CLCoy1 abundance, and the transcription activity during stress and under cultured cell conditions. We also examined the genomic organization of these sequences in other related *Citrus* species, such as *C. sinensis*, *C. paradisi*, *Fortunella margarita* and *Poncirus trifoliata*. The analysis of transposable element activity and genomic distribution might contribute to our understanding of their potential impact on *Citrus* evolution and diversity.

MATERIALS AND METHODS

Plant material and genomic DNA isolation

Plant material was collected from *Poncirus trifoliata* (Flying Dragon) and other species from the *Rutaceae* family, namely *Citrus limon* (L) Burm. (lemon), *Citrus aurantium L.* (sour orange), *Citrus paradisi* (grapefruit) and *Fortunella margarita* (Lour.) Swingle (kumquat). The plants were fron the collection of the Botanical Garden of Naples, Italy.

Seeds of *C. limon* (L) Burm. (lemon) were germinated and grown in Murashige and Skoog nutrient medium [20], 3% saccarose (without and with 0.7% NaCl, i.e. the lowest toxic concentration of the salt) in a regime of 12 h light/12 h dark at 25ºC until the plants were 3 months old. The leaves were wounded by cutting them into small pieces, then incubated at room temperature for 24 h. The total genomic DNA was extracted from the young leaves using the CTAB method [21].

Isolation of retrotransposon sequences by PCR

Partial reverse transcriptase and RNase H sequences were amplified from LTR and non-LTR retrotransposons using 100 ng of *C. limon* DNA as a template. The PCR conditions and primers are given in Tab. 1, and the primer concentration was 0.5 μM. In our analysis of retrotransposon elements in lemon, we obtained a conspicuous DNA band of about 270 bp. It was obtained using the primers Ty1-R (5'ARCATRTCRTCNACRTA) and Ty1-F (5'ACNGCNTTYYTNCAYGG), as described by Flavell *et al.* [16]. It was electrophoresed on 1.5% agarose gels containing ethidium bromide, cut from the gel, purified with the Qiaquick Gel Extraction Kit (Qiagen), cloned into the pMOSBlue vector (pMOSBlue blunt-

ended cloning kit, Amersham Pharmacia Biotech), and used to transform the DH5α competent cells (Stratagene).

Tab. 1. Degenerate primers used for the amplification of retrotransposon domains by PCR.

Primer pair sequences	Reference	Retrotransposon types
Retro3 5' car atg gar gtn aar ac 3'	[17]	*copia* LTR retrotransposons
Retro4 5' cat rtc rtc nac rta 3'		
GyRT1 5'MRNATGTGYGTNGAYTAYMG	[19]	*gypsy* LTR retrotransposons
GyRT4 5' RCAYTTNSWNARYTTNGCR 3'		
GyRT3 5'YKNWSNGGNTAYCAYCARAT 3'	[19]	
GyRT4 5' RCAYTTNSWNARYTTNGCR 3'		
BEL1MF 5' RVNRANTTYCGNCCNATHAG 3'	[18]	non-LTR retrotransposons
BEL2MR 5' GACARRGGRTCCCCCTGNCK 3'		
NLTR1 5' ggg atc cng gnc cng ayg gnw t 3'	[20]	
NLTR2 5' att cgg nsw narn ggr ymn ccy tg 3'		
NLTR1 5' ggg atc cng gnc cng ayg gnw t3'	[20]	
Ty1-R 5' ARCATRTCRTCNACRTA 3'	[16]	*copia* LTR retrotransposons
Ty1-F 5' ACNGCNTTYYTNCAYGG 3'		

Inverse PCR

To obtain the full Ty1-*copia*-like Reverse Transcriptase domain, 2 μg of genomic DNA was digested with 20 U *EcoRI* (Roche) in a 50-μl volume at 37ºC for 3 h. The restricted DNA was extracted with phenol:chloroform and precipitated with a 0.1 volume of 3 M sodium acetate and 2.5 volumes of ethanol. The DNA was recovered and dissolved in TE at a final concentration of 0.1 μg/μl. The ligation was performed with cleaved template DNA at 2.5 ng/μl and 1 U of T4 DNA ligase (Fermentas) in its commercial buffer, then incubated at room temperature for 3 h ON. After circularization, the DNA was diluted again up to 10 ng/μl. A 5-μl aliquot was amplified in a 50-μl PCR reaction containing 400 nM of each of the outward primers, 0.4 mM of each dNTP, 2.5 mM $MgCl_2$ and 2.5 U of Taq High Fidelity DNA polymerase (Roche) in its reaction buffer. PCR was carried out using the following conditions: 3 min at 94ºC; followed by 30 cycles of 1 min at 94ºC, 1 min at 61ºC, 4 min at 72ºC (+5 s/c); and a final extension step at 72ºC for 7 min. The primers, designed based on the cloned *C. limon* RT domain sequence, were: 5'-CAACCTTTCTTCGGTAATCCA-3' and 5'-CAAAAGGCCCAGAAACCG-3'.

Sequences and phylogenetic analysis

Recombinant clones were sequenced from both strands using the Big Dye Terminator Cycle Sequencing Kit via the automatic sequencing system ABI PRISM 310 DNA Sequencer (Applied Biosystems). The cloned sequences were analyzed for homology to known sequences to DNA (BLASTN) and proteins

(BLASTX) using the NCBI and PlantSat database (http://w3lamc.umbr.cas.cz/PlantSat). The nucleotide sequences were translated for all six possible reading frames, using the ExPASy translation tool. The closest matches to known sequences from the NCBI blast searches were retrieved and aligned to those sequenced using the Clustal W program [22]. A phylogenetic tree was constructed from the evolutionary distance matrix based on the Kimura two-parameter algorithm using the neighbour-joining method [23]. The analysis was performed with the Mega4 program [24], and gap sites in the alignment were excluded in the pairwise comparison.

Southern hybridizations

Genomic DNA was digested with *MboII* restriction enzyme. After electrophoresis, the genomic DNA restriction fragments on agarose gels were denatured, transferred onto nylon membranes (Osmotics), and finally cross-linked to the membrane by exposure to ultraviolet light according to the standard Southern protocol. The cloned full length *C. limon* Ty1-like reverse transcriptase (CLCoy1) was used as a probe. Digoxigenin labeling of the probe, filter hybridizations, and detection of the hybridization signals were performed according to the manufacturer's instructions (Roche). The post-hybridization washes were 2 × 5 min in 2 × SSC and 0.1% SDS at room temperature, and 2 × 15 min in 0.1 × SSC and 0.1% SDS at 65ºC. The probe was specific for CLCoy1, as assessed by ruling out cross-hybridization with another retroelement already reported for *Citrus* (data not shown).

Estimation of the CLCoy1 DNA percentages in the *C. limon* genome

The percentage of CLCoy1 in *Citrus* species was estimated by dot-blot hybridization. DNA and RT Ty1-*copia*-like DNA dilutions were blotted onto membranes, denatured with 0.5 N NaOH and cross-linked to the membrane by exposure to ultraviolet light. The CLCoy1 clone was used as a probe in the dot-blot. The hybridization signals from four different amounts of RT Ty1-*copia*-like DNA (from 2 to 12 ng) were compared with the signals from four different amounts of genomic DNA (from 20 to 60 ng) to determine the percentage of the RT Ty1-*copia*-like DNA in the genome. Post-hybridization washes were performed as outlined above for Southern hybridization. The dot-blots were scanned and then analyzed with Fotoplot software. The software estimates the percentage of RT Ty1-*copia* DNA in the genomic DNA by measuring the hybridization signal of the RT Ty1-*copia* DNA probe from the different genomic DNA spots using the RT Ty1-*copia* DNA standard curve (hybridization signals of the different amounts of RT Ty1-*copia* DNA).

RNA extraction and RT-PCR

The total RNA from roots, young and wounded leaves, and normal and salt-stressed cultured cells was extracted using a High Pure RNA isolation kit (Roche), according to the manufacturer's instructions. Traces of contaminated DNA were removed with a DNAse I treatment. 100 ng of RNA template was

reverse-transcribed in a 50-µl reaction mix containing 200 µM dNTPs, 100 mM DTT, 0.25 µl RNAse inhibitor, 1.5 mM $MgCl_2$, and 1 µl enzyme mix (Titan One-tube RT-PCR kit, Roche). The solution was incubated for 30 min at 50ºC in an automated DNA thermal cycler (GeneAmp 2400 Perkin Elmer). To perform RT-PCR under optimal conditions and to stay within the logarithmically linear product formation, 30 cycles were chosen (30 s at 94ºC, 30 s at 59ºC, 60 s at 68ºC), followed by a final extension for 7 min at 68ºC. The primer pairs were designed on the novel *C. limon* Ty1-*copia* Reverse Transcriptase sequence CLCoy1, to yield a PCR product of 270 bp.

Parallel amplification of a 560-bp fragment of the *C. limon* 26S ribosomal RNA was performed, as a control of cDNA synthesis and PCR amplification, using the primers 5'-CGAGAGACCGATAGCAAACAAG-3' and 5'-GGGAGCG CGCAGGTGCGCGA-3'. To rule out genomic DNA contamination, we also had a negative control which contained RNA instead of cDNA for each sample.

Quantitative reverse transcription-PCR (real time-PCR)

100 ng of the total RNA was amplified in the presence of a mixture containing 5 pmol of specific primers designed on the novel *C. limon* Ty1-*copia* reverse transcriptase sequence CLCoy1, sense 5'-CTATATGGTC CACTGGATAA-3' and antisense 5'-ACAAAGTTCT GTTTGGGATT-3', and the LightCycler-FastStart DNA Master SYBR Green I kit (Roche). The mixture was incubated for 30 min at 50ºC and the cDNA product was amplified by 40 cycles of PCR (30 s at 94ºC, 30 s at 57ºC, and 1 min at 72ºC). The incorporation of the dye into the amplified products was monitored using a LightCycler (Roche), and the concentration of a specific transcript in the sample was analyzed by the associated software based on the standard curves predetermined with known amounts of target transcripts. The results were normalized to those for *C. limon* 26S ribosomal RNA. Quantifications of each cDNA sample were averaged from three independent RT-PCR analyses, and the consistent results from at least two separately prepared RNA samples were used.

Chromosome preparation and *in situ* hybridization

The seeds were germinated on moistened paper in a growth chamber at 20ºC. When the primary roots were 1-2 cm long, they were excised and incubated in an aqueous solution of 0.029% 8-hydroxyquinoline for 5 h at room temperature to arrest the cells in their metaphase, rinsed in water, and fixed in absolute ethanol-acetic acid (3:1) (v/v) for at least 2 h. The root tips were hydrolyzed in 5 N HCl for 55 min at room temperature, stained with Schiff's reagent for 2 h, and squashed in a drop of 45% acetic acid. The slides were made permanent with Entellan (Merck) after dehydration in ethanol and subsequently in xylol. To localize *C. limon* RT-Ty-*copia* sites, fluorescent *in situ* hybridization (FISH) was applied on interphase nuclei and mitotic chromosomes. FISH was applied to roots pretreated as reported previously, fixed for 10 h, and stored in ethanol at -20ºC. The cloned full-length *C. limon* Ty1-like RT (CLCoy1) was used as

a probe. The probe was labeled with digoxigenin-11-dUTP (Roche) and FITC-conjugated sheep antidigoxigenin antibody (Roche) was used for detection. The slides were mounted with 5 μl 4'6-diamino-2-phenylindole (DAPI), 10 μl citifluor and 10 μl glycerol, and were examined with a fluorescence microscope. The images were acquired with a highly sensitive CCD camera (10^{-5} lux) and processed using Adobe Photoshop software.

RESULTS AND DISCUSSION

The structural characteristics of the Ty1-*copia*-like reverse transcriptase domain in *Citrus limon*

The search for mobile elements in *Citrus limon* led to the isolation of a novel Ty1-*copia*-like reverse transcriptase (RT) domain, performed using specific oligonucleotides for a conserved region in the Ty1-*copia* reverse transcriptase protein [16]. In each case, this led to the detection of an about 270-bp fragment on the agarose gel, which is the expected size of the internal RT domain of the *copia*-like retrotransposon. An analysis of the ten clones obtained showed a high level of overall similarity within the sequences; we called CLCoy the most putative cloned sequence.

To determine the entire nucleotide sequence of the Ty1-*copia*-like reverse transcriptase domain of lemon, we used the inverse PCR approach to obtain sequences flanking the CLCoy sequence. Finally, different overlapping fragments covering the entire reverse transcriptase sequence were obtained. The full nucleotide sequence of a representative Ty1-*copia*-like RT was the result of joining two contiguous nucleotides sequences, representing the entire reverse transcriptase (RNA-dependent DNA polymerase) of approximately 616 bp (200aa), which we called CLCoy1 (Genbank Accession number: EU812120).

The *C. limon* CLCoy1 sequence was compared to homologous sequences found within the NCBI nucleotide sequence database. The potential correct frame for translation of the RT domain was inferred using the tBLASTX search tool and the alignment using Clustal W.

This CLCoy1 sequence is a typical *copia* retrotransposon RT, sharing a high similarity with the *copia* reverse transcriptase reported for other plants (Fig. 1). 100% similarity was found between the amino acid sequences of the RT encoded by CLCoy1 from *Citrus limon* and the putative reverse transcriptase from *Zingiber officinale* (ABK60177). 98% similarity was found with the *copia*-type pol polyprotein-like from *A. thaliana* (BAA97087).

The amino acid sequence of CLCoy1 and various plant RT was used to derive a dendrogram tree by the neighbour-joining method. As shown in Fig. 2, the CLCoy1 elements were clearly clustered within several dicotyledonous *copia* retrotransposon groups with a high bootstrap value, which strongly supported the grouping.

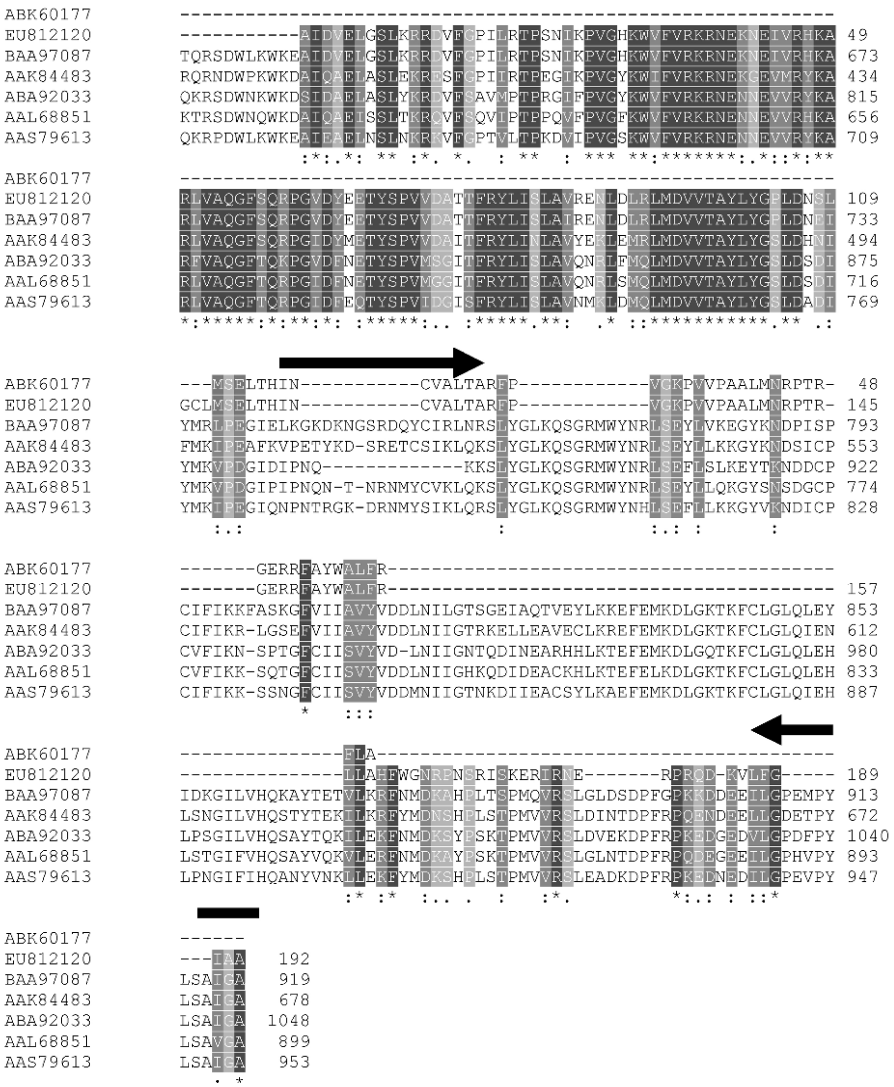

Fig. 1. A comparison of the amino acid sequences of the *Citrus limon* Ty1-*copia*-like reverse transcriptase full domain (CLCoy1) with the most similar *copia* RT amino acid sequences from other plants. The regions of high homology between CLCoy1 and the other Ty-*copia* RT elements are highlighted. Identical residues are indicated with "*"; conserved substitutions are indicated with ":"; and semi-conserved substitutions are indicated with ".". The arrows indicate amino acids encompassed by the set of PCR primers used (see Materials and Methods and [16]).

EU812120. CLCoy1 *Citrus limon* Ty1-*copia*-like RT (this study), BAA97087. *Copia*-type pol polyprotein-like from *Arabidopsis thaliana,* AAK84483. Putative *copia*-like polyprotein from *Lycopersicon esculentum,* AAS79613**.** Putative *copia*-like polyprotein from *Ipomoea trifida,* ABA92033. Retrotransposon protein, putative, Ty1-*copia* subclass from *Oryza sativa,* AAL68851. Putative *copia* polyprotein from *Sorghum bicolour,* ABK60177. Putative reverse transcriptase from *Zingiber officinale*.

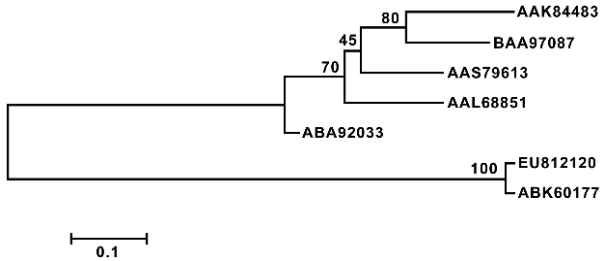

Fig. 2. Phylogenetic analysis based on the *Citrus limon* CLCoy1 Ty1-*copia*-like reverse transcriptase full domain and the nearest neighbors derived from maximum parsimony analysis. The identities of plant *copia* RT correspond to the identities given in the databases; accession numbers are given in parenthesis after the taxonomic assignment. Numbers given below the branches are the frequencies (expressed as percentages) with which a branch appeared in 1000 bootstrap replicates. The branch lengths are proportional to nucleotide differences, as indicated by numbers on branches.
EU812120. CLCoy1 *Citrus limon* Ty1-*copia*-like RT (this study), BAA97087. *Copia*-type pol polyprotein-like from *Arabidopsis thaliana*, AAK84483. Putative *copia*-like polyprotein from *Lycopersicon esculentum*, AAS79613. Putative *copia*-like polyprotein from *Ipomoea trifida*, ABA92033. Retrotransposon protein, putative, Ty1-*copia* subclass from *Oryza sativa*, AAL68851. Putative *copia* polyprotein from *Sorghum bicolour*, ABK60177. Putative reverse transcriptase from *Zingiber officinale.*

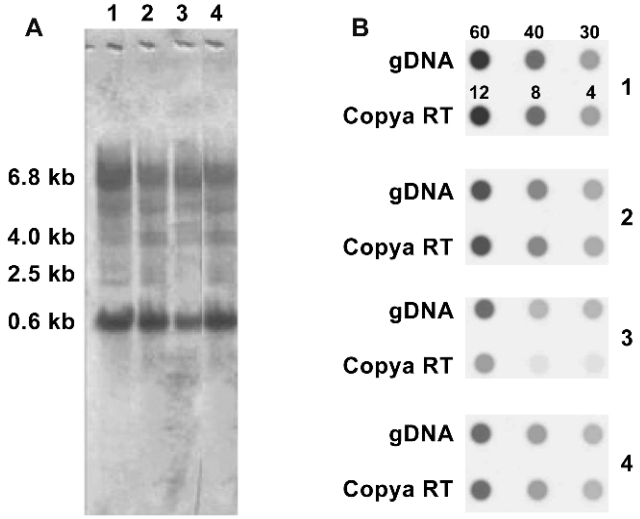

Fig. 3. The Southern blot and dot-blot analysis of CLCoy1 in different *Citrus* species. A – Southern blot hybridization. Genomic DNA from *C. limon* (1), *C. sinensis* (2), *C. paradisi* (3) and *Fortunella margarita* (4) was digested with *MboII* and hybridized with the digoxigenin-labelled CLCoy1 clone as a probe. The DNA fragment sizes (1-kb Ladder) are indicated on the left. B – Dot-blot hybridization. The CLCoy1 clone was used as a probe for the quantification of the Ty1-*copia*-like reverse transcriptase full domain to genomic DNA in *C. limon* (1), *C. sinensis* (2), *C. paradisi* (3) and *Fortunella margarita* (4). The numbers indicate the amount of DNA found, in nanograms.

The genomic and chromosomal organization of *copia*-like retrotransposons and transcriptional activity in *Citrus limon*

The genomic distribution of CLCoy1 in *Citrus* was obtained by Southern blot analysis using genomic DNA digestions from *Citrus limon* hybridized with the Ty1-like RT (CLCoy1) probe (Fig. 3A). The strong multimeric pattern at high stringency conditions suggested that a high number of copy sequences homologous to the probe were integrated throughout the *Citrus* genome, and that the presence of superimposed fragments could be related to the clustering of these sequences. The pattern of distribution of *copia*-like RT in this genome shows four hybridization bands of 0.6, 2.5, 4 and 6.8 kb respectively. The observation of different band intensities within a single track could be attributed

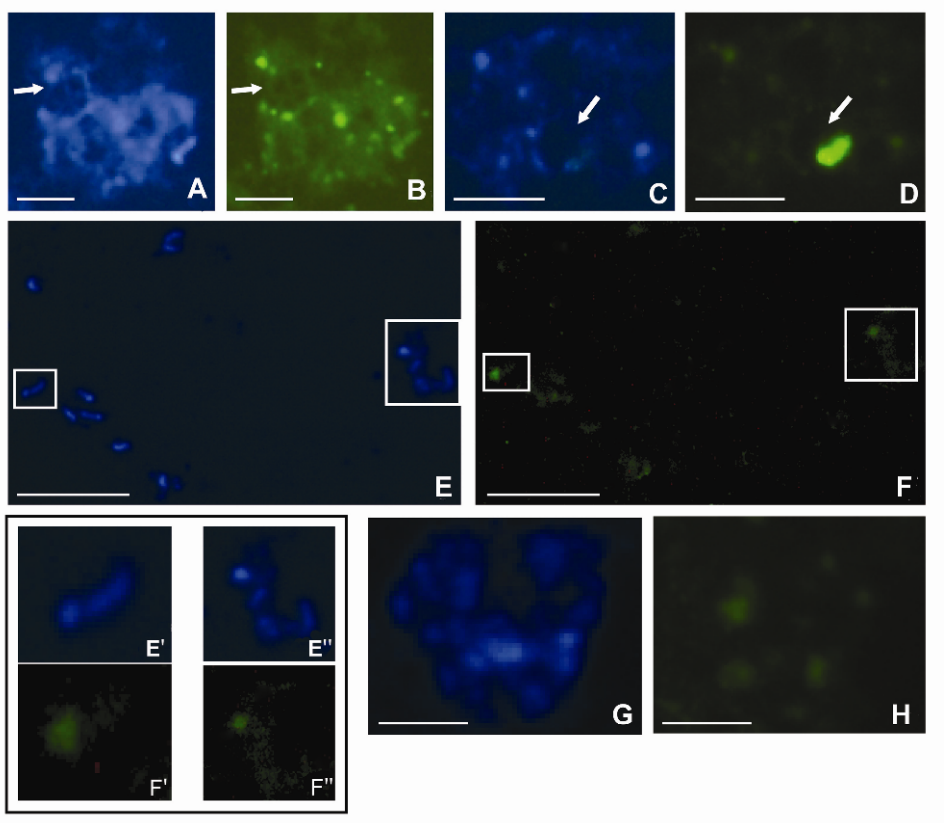

Fig. 4. FISH using the digoxigenin-labeled CLCoy1 clone of *Citrus limon* as a probe on cytological preparations of *C. limon*. A-D – Interphase nuclei stained with DAPI (A, C) and FITC (B, D) showing FISH signals. The arrows indicate the nucleolus. E, F – A group of mitotic metaphase chromosomes stained with DAPI (E) and FITC (F) showing the Ty localization at a telomeric/subtelomeric region in two chromosomes, enlarged 3x in E', F' and 2x in E'', F'', respectively. G, H – Prometaphase nucleus stained with DAPI (G) and FITC (H) showing three major FISH spots together with minor signals. The bar represents 3 μm.

to the copy number of *copia*-like RT with a canonical structure within the genome. Major and minor FISH signals of CLCoy1 were observed in the *C. limon* interphase (Fig. 4A, B) and prometaphase (Fig. 4G, H) nuclei. Some of these were clearly close to the nucleolus, and visible during the interphase, presenting a strong signal depending upon the chromatin condensation (Fig. 5C, D). Two major sites of CLCoy1 were located at a telomeric/subtelomeric region (Fig. 4E, F) in metaphase chromosomes. To detect if the Ty1-*copia* RT was transcriptionally active under stress conditions in *Citrus limon*, we performed RT-PCR with the specific primers of an internal domain of RT using total RNA from *Citrus limon* roots, young and wounded leaves, and normal and salt-stressed (0.7% NaCl) regenerated plants. We detected an amplification product of 270 bp corresponding to the expected size of the *C. limon* Ty1 RT domain in

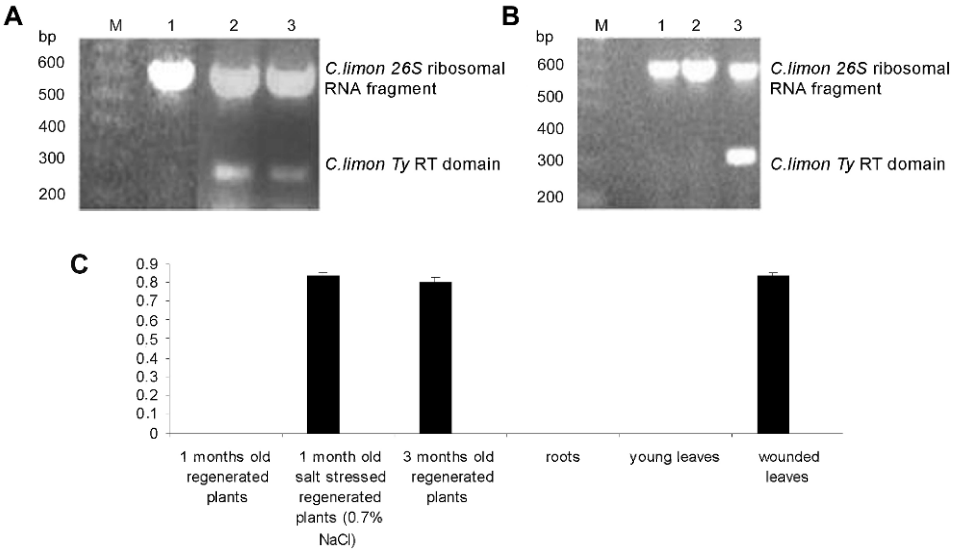

Fig. 5. The RT-PCR analysis of CLCoy1 transcriptional activity in *Citrus limon*. A – RT-PCR carried out with total RNA from 1-month old regenerated plants (lane 1), 1-month old salt-stressed regenerated plants (0.7% NaCl) (lane 2) and 3-month old (lane 3) regenerated plants. A 560-bp fragment of the *C. limon* 26S ribosomal RNA was used as a positive control of RNA amplification. B – RT-PCR carried out with total RNA from *Citrus limon* roots (lane 1), young leaves (lane 2), or wounded leaves (lane 3). A 560-bp fragment of the *C. limon* 26S ribosomal RNA was used as a positive control of RNA amplification. C – A real-time RT-PCR analysis of CLCoy1 from *Citrus limon*. Primers specific to CLCoy1 were used to screen RNA from 1-month old regenerated plants, 1-month old salt-stressed regenerated plants (0.7% NaCl), 3-month old regenerated plants, roots, young leaves and wounded leaves. Quantitation was achieved in a single reaction in all the specimens relative to the use of the *C. limon* 26S ribosomal RNA gene as an internal cDNA control. Each bar represents the mean of three independent experiments in which the ratio of the target PCR product/internal cDNA control was plotted.

RNA samples obtained from 3-month old regenerated plants and 1-month old salt-stressed regenerated plants (Fig. 5A, lanes 2 and 3, respectively). The same band was clearly detected in the wounded leaves (Fig. 5B, lane 3), but we did not observe any amplification product in the roots and young leaves and in 1-month old regenerated plants. The negative control (without RT) yielded no product and thus excluded the existence of DNA contamination in the RNA samples.

Moreover, Ty1-*copia* RT quantitative expression in *Citrus limon* roots, young and wounded leaves, and normal and salt-stressed (0.7% NaCl) regenerated plants was analyzed by real-time RT-PCR. The results confirmed that Ty1-*copia* RT expression was quite abundant in stressed tissues, but the signal of the transcript was absent in 1-month old regenerated plants, roots and young leaves. CLCoy1 expression was significantly high in wounded leaves, in salt-stressed regenerated plants and in 3-month old regenerated plants (Fig. 5C).

Our experiments provide the first evidence for the transcriptional activation of a Ty1-*copia*-like RT sequence in *Citrus limon* under stress conditions, like tissue culture, wounding and saline stress. Transposable elements are silenced during normal development, but they are activated by abiotic and biotic stress. Tissue culture, wounding, pathogen attack, allopolyploidization, and unfavourable environmental conditions, such as suboptimal temperature or water and nutrient availability, are all elicitors of genomic stress causing transposon activation.

Transcripts of plant retroelements have been found in several plants such as tobacco, barley and rice under stress [8, 25, 26]. In rice, tissue culture promotes continuing retrotransposition of *Tos*17 providing a mechanism for the higher frequency of somaclonal variation observed in plants regenerated from long-term cell cultures compared to short-term cultures [9]. Thus, culture time can play an important role in the amplification of retrotransposons within the genome. Wounding is also an important factor for the induction of biochemical changes in the process of callus formation, protoplast preparation or cell culture, in addition to the initiation of the defence reactions [7]. Indeed, it has been reported that the transcription of several retroelements is activated in response to wounding [25, 26]. Microclimatic shifts were also found to lead to transcriptional activation of retrotransposons such as BARE1 [27]. Like all known active plant mobile elements, CLCoy1 is quiescent under normal conditions and activated by stress. This is consistent with the genomic restructuring role predicted by McClintock for transposons in response to stress [28].

The abundance of Ty-*copia*-like RT sequences in *C. limon* and other *Citrus* species

Using the dot-blot approach, we calculated that the RT Ty-*copia*-like element represented approximately 3.6% +/- 0.27 of the *C. limon* genome (Fig. 3B). This estimate was based on quantitative dot-blot hybridization of serial dilutions of genomic DNA and control fragments (the CLCoy1 cloned sequence).

Quantitative dot-blot hybridization was also used to estimate the abundance of CLCoy1 sequences in 4 *Citrus* species. Two of these species, *C. sinensis*. and *F. margarita*, were found to contain highly amplified CLCoy1 elements, estimated to reach up to 3% +/- 0.35 and 3.2% +/- 0.26, respectively. These findings were in agreement with the results of Southern blot experiments in which only these two species showed prominent signals in addition to *C. limon* (Fig. 3B). The fact that these genomes display a similar set of *copia*-like retrotransposon-specific restriction fragments suggests that the genomic location of the *copia*-like retrotransposon could be similar within these different genomes. *C. paradisi* species produced hybridization signals corresponding to lower numbers of sequences (2.8% +/- 0.4), while *P. trifoliata* DNA did not show any hybridization signal with *C. limon* CLCoy1.

Southern hybridization analysis indicated that nested copies of the new elements are scattered along the *C. limon* genome and those of other related species such as *C. sinensis*, *C. paradisi* and *F. margarita*, but absent in *P. trifoliata*. This result could indicate that this element is an ancient component of the *Citrus* genus genome, introduced before the divergence of the species and conserved during evolution. This indicates a common ancestral transposon emerging when the genus *Citrus* arose, separating from *P. trifoliata*. The transposons' persistent localization in the host genomes could be explained if some DNA sequences are a suitable habitat for the persistence of insertion sequences. Not all transposons are equally maintained in all genomes.

Some transposable elements are selectively deleted from plant genomes, as some insertion regions show homogenization and evolution rates higher than those of transposons (so inserted copies are lost) and/or insertion in some other regions can be lethal for the plant or cause sterility. Other transposable elements are conserved in the plant genome, amplified by unequal crossing-over or via reverse transcription and re-insertion. Consequently, persistent localization of transposons in host genomes probably depends a lot on having friendly conditions for the persistence of insertion sequences. Kidwell and Lisch [29] proposed that there are "ecological niches" which different types of transposons exploit. Stress-activated transposons could give more variability to a plant, leaving it able to survive in stressful habitats, because many genes could be amplified or assembled through the action of transposable elements. The variation of new genetic variability induced by the activity of transposons may favor a more rapid adaptation. As suggested by Fedoroff [30], the preferential transmission of tandemly repeated sequences (including retroelements) through the gametes in concert with polyploidization, transposition and duplication might explain plant genome expansion.

Here, Southern blot hybridization analysis also showed that these elements are very abundant and polymorphic throughout the *Citrus* genome. This is certainly the first data available giving evidence for the transcriptional activation of transposon sequences in *C. limon* conserved in *Citrus* genome through evolution. At our acknowledgement, only a Ty-*copia* element is reported in

Citrus sinensis, activated in wounding and under hormone treatments. There is no data yet regarding transcriptional activation under cell culture and salt stress not on the evolutionary conservation of this sequence in *Citrus* species [31]. This raises the question as to why a Ty-*copia* RT sequence is still conserved in *Citrus* plants. To explain this phenomenon, one has to postulate some additional function for this element that provides a selective advantage to *Citrus*.

These observations suggest that CLCoy1 has significantly contributed to the complexity of the *Citrus* genome. Thus, the CLCyo1 element may have entered a new stage in the evolutionary life cycle of a transposon: the turning of a genomic parasite into a gene beneficial to the plant host.

Transposons are involved in the structures of chromosomes, in centromeres and telomeres, where they play an important role in chromatin modification and the stress response. The correlation between the *BARE-1* retrotransposon in barley growing in different regions of a canyon in Israel, in terms of copy number, genome size, and local environmental conditions, suggested for the first time a molecular linking of habitat with transposon induction in natural populations [27]. Therefore, CLCoy1 in the *Citrus* genus might contribute to the diversity of gene function and regulation and be a source of biodiversity. It is noteworthy that none of the Ty1-like RT showed a close correspondence to previously reported Ty1-*copia* retrotransposons in *Citrus* or to the major dispersed repeats in the *Citrus* genome, which suggests that a very complex population of such elements exists in these plants.

In conclusion, the results presented here on a novel *copia*-element are interesting. The presence of a very complex population of transposable elements and tandem repeats in *Citrus* plants [10-14], and the facts that transposons and retrotransposons can be transcriptionally activated under stress and are a target for epigenetic chromatin silencing indicate a possible correlation between domestication [32, 33] and lineage specific-conservation of *copia* retroelements. This is an intriguing matter for further studies.

We propose that the Ty-*copia*-like element played a role in the ancient *Citrus* genome, increasing genomic variability, favouring a more rapid adaptation, spreading out in stressful habitats and causing evolutionary genome expansion. Through the action of selection, only epigenetic and genetic arrangements that promote fitness are maintained in the population.

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