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Dynamic DNA-Methylation of Retrotransposons in Rue under Drought Stress

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Research Article

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ABSTRACT

Ruta graveolens (rue) is plant native to the Mediterranean region and presents in traditional medicine of this region since ancient times. There is poor information about the genome of *Ruta* and repetitive sequences and active mobile genetic elements have not been identified yet. Since rue genome is still mostly unexplored, and proliferative capability and large size of transposons make them key contributors to genome size and evolution, we aimed to isolate and characterize transposon sequences with a view to better understanding rue genome. We have isolated novel types of Ty1-copia like LTR reverse transcriptase from *Ruta graveolens*, leading to investigation of the genomic organization and phylogenetic relationships. Since the activation of transposable elements in response to environmental changes represents a form of adaptive response to biotic stress, we investigated if drought stress could influence transposon methylation and if the extent of methylation has been related to expression level. The results can have implications for rue genome understanding and their potential impact on *Ruta* evolution.

INTRODUCTION

Ruta graveolens is a plant belonging to genus of *Rutaceae* family. It features mainly shrubby plants, native to the Mediterranean region and presents in traditional medicine of this region since ancient times. It is native to Southern Europe, with a rich localization in the Mediterranean region, but widely distributed into all the temperate and tropical regions, it can be also found on dry rocky and stony sites. The three most important species are *Ruta chalepensis L., Ruta graveolens L. and Ruta montana,* even if they are morphologically poorly differentiated and until today, phylogenetic relationships are supported by few molecular data. *Ruta graveolens L. (Rutaceae*), the Common Rue, also known as Herb-of-grace, is a species of “rue”, or 'ruda', grown as an herb. Rue has several therapeutic purposes worldwide. Recently, much interest is focusing on potential anti-inflammatory and anticancer action of Ruta extract for human therapeutic purposes. A significant inhibitory effect on several inflammatory mediators unravels a novel anti-inflammatory action of this plant is documented the ruta extract capacity to modulate on inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) gene expressions and their antioxidant capacity on murine RAW 264.7 macrophage challenged with lipopolysaccharide (LPS) through the inhibition of the nuclear factor-κB (NF-κB) activation [1-3]. Besides, *Ruta graveolens* is one of the few plants producing furanocoumarins, which are molecules with established therapeutic properties. Furanocoumarins, *in vivo*, play a protective role against insect attack. Despite *Ruta graveolens* application as potential anti-inflammatory and anticancer for human therapeutic purposes, research on molecular aspects of rue are still insignificant, considering the restricted documentation on rue genome, and also that so far, only 146 sequences have been deposited in GenBank. At the moment, transposable elements have not been identified yet in rue.

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Transposable elements (TEs) are sequences of DNA able to move from one location to another in the genome. TEs have been identified in all organisms, prokaryotic and eukaryotic, and can occupy a high proportion of a species' genome. Transposable elements make up a large fraction of the genome and are responsible for much of the mass of DNA in a eukaryotic cell. has been shown that TEs are important in genome function and evolution [4]. Retroelements, class I transposable elements, are ubiquitous and an abundant part of plant genomes, and are mainly responsible for plant genome size variation [5]. Autonomous LTR retrotransposons play important roles in centromere functions and in chromatin structures, beside they regulate gene expressions in their host genomes [6]. However, a large number of LTR-RTs can be inactivated by DNA methylation and small RNA-mediated silencing mechanisms. DNA methylation can also influence plant regulation in response to environmental stresses [7]. Abiotic stresses such as chilling, planting density, rubbing, cutting, and successive rounds of subculture generally modify the levels of DNA methylation [8].

In turn, transposon activity is linked to a decrease of genome stability, which is further associated to hypomethylation levels [9]. The correlation between hypomethylation and stress exposure has been identified for cold stress, considering that it was shown a tissue-specific hypomethylation in plant genome under cold condition, including alterations in the retrotransposon sequences [10]. Several specific examples of TE influence on the expression of nearby genes have now been documented, TE insertions near genes may influence gene expression through several potential mechanisms, including inserting within cis-regulatory regions, contributing an outward reading promoter from the TE into the gene, or providing novel cis-regulatory sequences that can act as enhancers/repressors by positive modulation of transcription binding factor, or influence the chromatin state of gene promoter regions [11,12]. Some TEs exhibit stress-responsive transcription or movement [4]. For example, expression of the tobacco Tnt1 element can be induced by biotic and abiotic stress [13]. The genome of *Ruta graveolens* has not been studied yet in relation to retrotransposon activity and in response to stress or adaptation to environmental conditions. Since retrotransposons play a significant contribution to the size, organization and genetic diversity of genomes, characterization of rue retrotransposons is important. In this background, we have analyzed the heterogeneity, and transcriptional activity of Ty1-copia in the genome of rue. Herein, we isolated and sequenced part of the RT gene of copies-like retrotransposons from *Ruta graveolens*, and compared them with published sequences. The multiple alignments allowed us to isolate 11 novel types of Ty1-copia like LTR reverse transcriptase from *Ruta graveolens*, leading to investigate the genomic organization, phylogenetic relationships, transcriptional activity and copy numbers. Besides, whit the aim of investigate DNA methylation changes in response to drought stress in rue copia-like sequences, we performed COBRA analysis. Interestingly, we found substantial alterations in the expression of several RT sequences, which was paralleled by alterations in sequence specific methylation in *Ruta graveolens* leaves and roots under drought stress. The results allowed us to obtain a preliminary understanding of rue DNA methylation, exposed to drought stress, and also the analysis of genomic distribution and sequence of transposable elements might contribute to our understanding of their potential impact on *Ruta* evolution.

MATERIALS AND METHODS

Plant Material and Genomic DNA Isolation

Plant leaves were collected from species from the *Rutaceae* family, namely *Ruta graveolens, Ruta chalipensis*, *Citruslimon (L) Burm*. (lemon), *C. reticulata*; *C. sinensis*; *C. aurantium L.*, *Citrus paradisi*, *Fortunella margarita* (Lour.) Swingle (kumquat) and *Poncirus trifoliata*. Plants have been obtained from the collection of the Botanical Garden of Naples, Italy. Plant leaves were ground into a fine powder in liquid nitrogen, and total genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen) following the manufacturer's protocol. DNA concentrations were determined by measuring the absorbance of the samples at 260 nm.

Drought Stress Treatment and Evaluation

For the drought experiments, seeds of rue plant were planted in 12 pots on October and the pots (30 cm diameter) were filled with loam clay soil using a standard method described by Klute in 1996. Briefly, clay 37%, silt 35% sand 25% with pH 7.7, E.C. 0.59, organic matter 1.30, soluble anions (maq/100 g soil) HCO^{3-} 0.69, Cl^{-} 2.1, SO^{-} 1.12 and soluble cations (meq/100 g soil) Ca++ 1.11, Mg++ 0.88, Na+ 2.23, K+ 0.19. Every pot contained three plants, of which two plant leaf and two root samples were taken, the first at the beginning of flowering stage (at end of March) and the second during flowering stage (on June). Rue plants from 6 pots were put under drought stress by withholding water until soil humidity decreased down to 12%. After recovery with re-watering, the growth parameters and Ty-copia expression values of each plant were measured. The values obtained under drought stress conditions were means ±SE (n=48) statistically different from the normal conditions values (n=48) (p<0.05).

Bisulfite Conversion and COBRA (Combined Bisulfite Restriction Analysis)

To study the methylation extent of p1c3 sequence, bisulfite conversion was performed using 217 the EZ DNA Methylation-Gold™ kit according to the manufacturer's protocol (ZYMO RESEARCH, Integrated Sciences, Pvt. Ltd, Chatswood, Australia). An amount of 500 ng genomic DNA was used for bisulfite conversion. Bisulfite converted genomic DNA was subjected to polymerase chain reaction (PCR) using Platinum® Taq DNA Polymerase (Invitrogen Pty. Ltd., Blackburn, Australia). Primers were designed using Primer 3 Primer3 program (http://www.bioinformatic.nl/cgibin/primer3plus/primer3plus.cgi). PCR was set up as follows: 1x Platinum Taq reaction buffer, 1 mM MgCl2, 100 nM dNTP mix, 400 nM of primers for each RT TY-copia and 0.05 units of Taq

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polymerase made up with Ultra-Pure™ distilled water (Invitrogen Pty. Ltd., Blackburn, Australia) to a final volume of 20 µl. PCR was performed by initial denaturation at 94.0°C for 5 min, followed by 40 cycles of 94°C for 30 secs, 58°C for 30 secs, 72°C for 45 sec, and finally by amplicon elongation at 72°C for 10 min. The PCR product (size 217 bp) was visualized in a 1.7% agarose gel. Restriction fragment length analysis was performed on PCR products incubated with the restriction endonucleases MspI (methylated 199 bp+107 bp, 229 unmethylated 306 bp), (New England BioLabs Inc., Genesearch Pvt Ltd., Arundal, Queensland, Australia). Genomic DNA isolated from Ruta served as an unmethylated COBRA control. Genomic DNA from the same sample treated with 231 CpG methyltransferase M.SssI (New England BioLabs Inc., Genesearch Pvt Ltd.) served as the positive methylation control.

RESULTS AND DISCUSSION

Mobile sequences are described in most of plants contributing to genome plasticity, hence, we aimed to prove the presence of RT sequences of Ty1-copia group retrotransposons in the *Ruta graveolens* genome too. Using degenerate oligonucleotide primers specific for Ty1-copia retrotransposons, the amplified PCR product of expected size, 260 bp, was obtained from rue genomic DNA. The targeted PCR product of *copia* RT domain was cut and eluted from the gel, purified, cloned and then sequenced. One hundred and twenty-four independent clones were isolated after cloning the rue genomic DNA band amplified with copia-specific primers and sequenced. Of these, 96 clones (70% of the total) showed their sequence homology with theTy1-copia RT sequences from plants belonging to different taxonomic groups. When compared to the RT-Ty1 region of known retrotransposons from plant genomes, the amino-acid similarities ranged from 58.0% to 73% (data not shown). Thirteen clones (15%) had stop codons while 17 clones (19%) possessed frame-shifts with or without stop codons. Thus, a total of 30 clones (34% of the putative RT clones) were found to be defective. After manual corrections of frame 252 shifts in some clones, 70 clones were finally selected for further phylogenetic analysis. The phylogenetic analysis showed a high level of sequence heterogeneity among rue clones with an overall average of 38% that they could be grouped into at least eleven distinct families **(Figures 1 and 2)**. Alignments of rue copia RT sequences and construction of NJ-tree with sequences from other plants revealed very interesting results **(Figure 1)**. Eight Ty1-copia RT family in rue were more strictly related to the representative elements present in other plant species far from *Rutaceae* family. For example, several RT Ty- copia *Ruta* sequences (HM210869, HM210871, HM210872, HM210873, HM217180, HM217182 and HM217183) were more closely related to RT Ty1-copia like present in parasitic flowering plants, such as *Orobanche* (ABD18986) and *Phelipanche* (ABD19090) genus **(Figure 1)** with 77% to 88% aminoacidic similarity. *Orobanche* and *Phelipanche* are non-photosynthetic (holoparasitic) plants, which lack any photosynthetic activity and are completely dependent on their hosts. Furthermore, one copia-RT sequence showed 69% 265 similarities with reverse transcriptase isolated in *Medicago truncatula* (ABN06064) **(Appendix S1)**.

Remarkably, three isolated RT sequences, p3c11, p3c1, p3c4 clones (Genebank accession numbers HM210870, HM210869, HM210872 respectively), were related to retrotransposons found in several genera belonging to the Citrus subfamily, *Aurantioideae*, a sister group to *Ruta graveolens* **(Figure 2)**. Such sequences shared 87% to 99% sequence similarity with copia-RT sequence of Citrus plant species, *Citrus sinensis* (CAJ41389), *Citrus limon* (AAC02552), *Citrus grandis* (AM1177429), *Citrus paradise* (DQ414756). Relation between R. *graveolens* and *C. sinensis* sequences has been previously reported for genes as chalcone synthases and acridone synthases showing that chalcone synthases translated nucleotide sequence from *Ruta* had about 90% identity with *Citrus sinensis* homologue, while acridone synthases sequences shares 75% to 85% polypeptide sequence identity with CHSs from other plant families [14]. Supporting the relation between transposable elements identified in our study and other *Rutaceae* plants, translated *Ruta* sequences have been found to be closely related to C. *sinensis* and *C. clementine* transposon-similar proteins. Genetic element mobility has been described as a fundamental mechanism leading to phenotypic variation in Citrus plants [15]. Report that Sicilian blood orange originated from a retrotransposition of a copia-like mobile element adjacent transcriptional activator of anthocyanin synthesis, controlling activator expression through a cold-mediated mechanism [16-20].

This raises the question as to why a Ty1-copia RT sequence is still conserved in *Ruta* and *Citrus* plants, which belong to two different subfamilies in *Rutaceae.* To explain this phenomenon, we could theorize some additional function for this element that provides a selective advantage to *Rutaceae*. 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 [21,22]. *Ty1-copia* sequences in the *Ruta* and *Citrus* genus might contribute to the diversity of gene function and regulation and be a source of biodiversity. Despite their proliferative capacity, transcriptional and/or transpositional activity of most retroelements is repressed by the host genome through a combination of epigenetic mechanisms involving both transcriptional and posttranscriptional controls [23]. However, under certain genomic and environmental conditions, such as hostile environment, pathogens, and wounding, host repression may fail, resulting in large-scale and episodic activation and proliferation [24-30]. DNA methylation varies in different tissues and during developmental stages, under environmental stress and in experimental conditions as shoot regeneration in tissue cultures and callus induction. The level of 5-methylcytosine (5 mC) is strongly different among plants, accounting for up to 25% of cytosine in maize [31]. Every plant studied shows high levels of genomic methylation restricted to repeats and transposable elements [32-41]. Cytosine methylation in the sugar beet genome was studied in repetitive sequences, including retrotransposons and DNA transposons, revealing a differential methylation among leaves and other tissues in rice, the transposition of Tos17 in callus is

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associated with DNA hypomethylation [42]. DNA methylation has a central role in the inactivation of transposons. Genome-wide approach uncovered unique aspects of stress-induced dynamic DNA methylation changes, including a remarkable relationship among hypomethylation, biogenesis of specific siRNAs and transcriptional derepression at transposon sequence. We asked ourselves if, as other plants, rue could utilize a particular series of defense mechanisms under stress and transposon sequences could be modulated by DNA methylation, and mutants globally lacking CG or non-CG methylation displaying inducible expression.

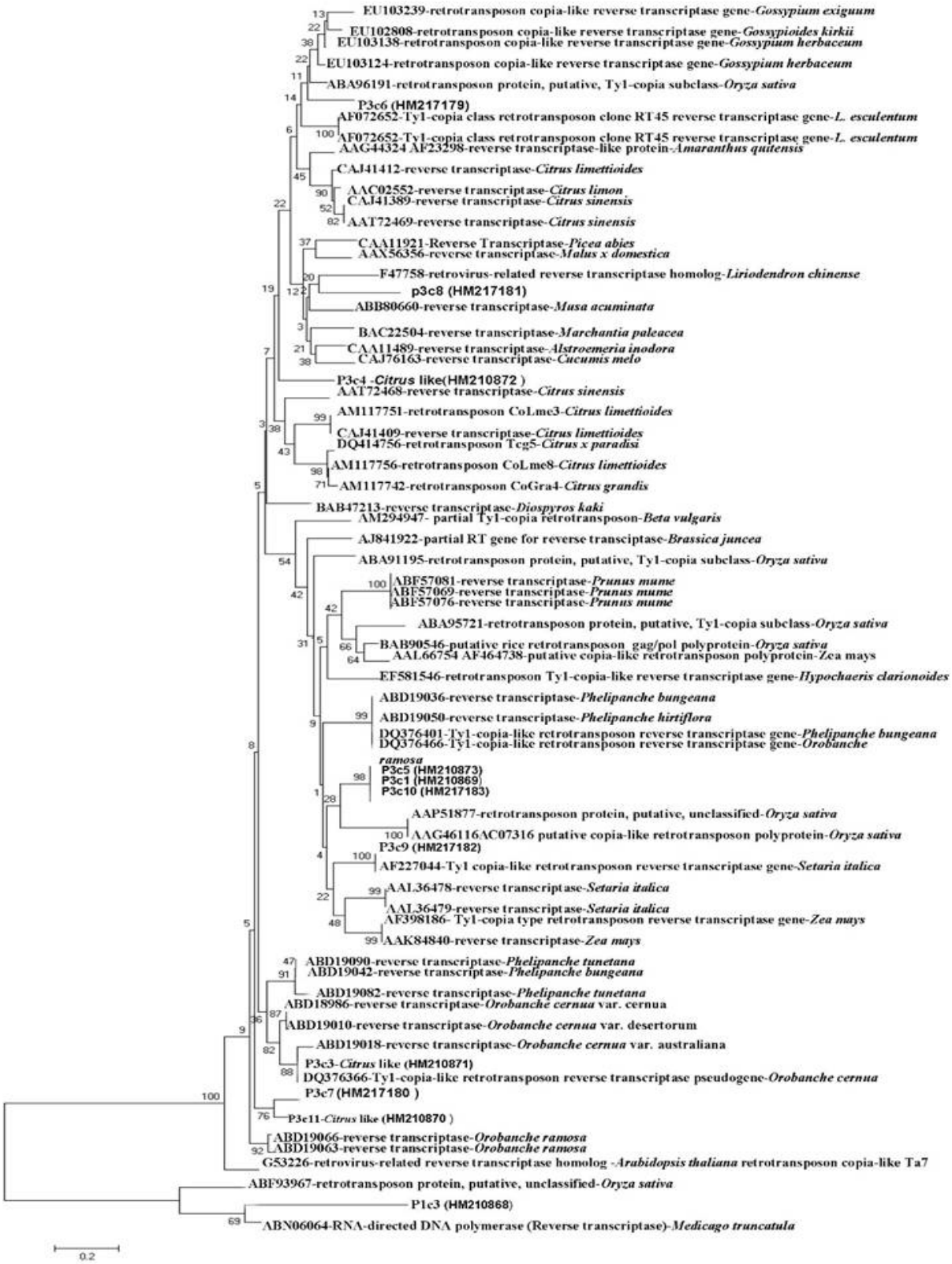

Figure 1. Phylogenetic analysis of translated amino acid sequences representing the *Ty*1-copia-like reverse transcriptase (RT) PCR fragments isolated from *Ruta graveolens*. Numerals at the branch nodes indicate the bootstrap support out of 1000 replications method by Saitou and Nei in 1987.

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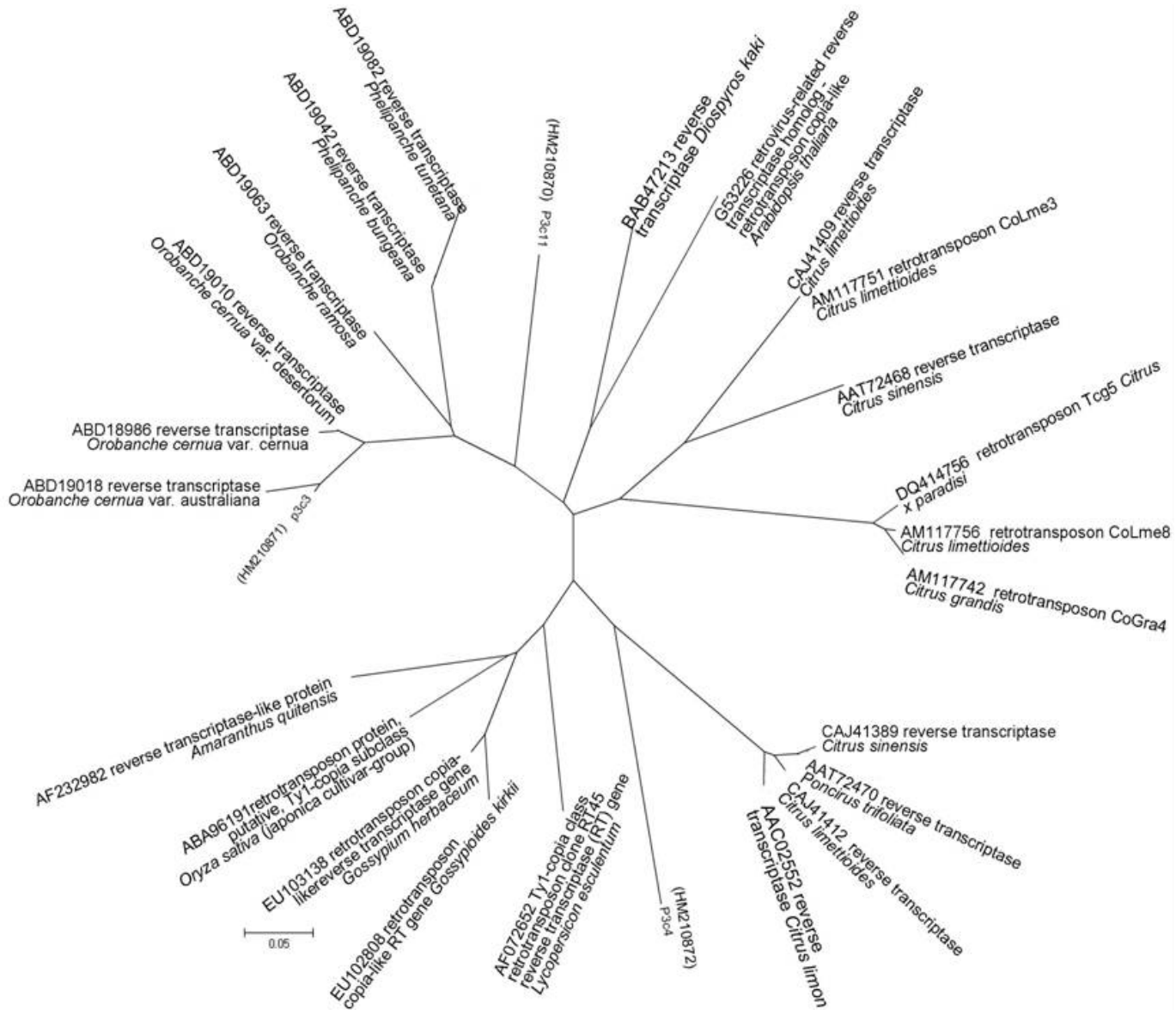

Figure 2. Unrooted phylogenetic tree of Ty1-copia -like RT sequences (300 bp) isolated from *Ruta graveolens*. Bootstrap values (>50%) are shown for branches defining major lineages as well as for deeper internal branches and are based on 1,000 replications. Phylogenetic analysis was conducted using neighbor joining.

Here, combined bisulfite and restriction analysis (COBRA) was performed to determine the methylation status within Ty1-copia sequences in *Ruta* under drought stress. The Combined Bisulfite Restriction Analysis (COBRA) is a method that quantifies DNA methylation in a specific gene region by PCR amplification from bisulfite-treated DNA. The results showed substantial levels of DNA hypomethylation in the investigated CpG site **(Figure 3)**. This experiment supports our hypothesis that, under drought, stress triggers loci-specific methylation changes in rue genome. The epigenetic landscape can be dynamically modified; indeed, widespread alterations in DNA methylation can be elicited by stress, plants subjected to heat stress display transient changes in nucleosome density, as well as transcriptional depression, at some repetitive elements and mobile elements [43].

Subsequently, we investigated whether the extent of DNA Ty1-copia methylation was negatively related to the differential expression levels of Ty-copia among rue under drought stress expression [44-51]. The characteristic of retrotransposon transcriptional activity in rue were studied by the quantitative real-time polymerase chain reaction (qRT-PCR) approach for a total of 12 individuals under drought stress and 12 individuals under normal humidity conditions. Transcriptional analysis of rue *Ty1-copia* like sequences (named here p3c4, p1c3, p3c5 and p3c10) has revealed differential gene expression in *Ruta* tissues under drought stress compared to controls **(Figure 4)**. As shown in **Figure 4**, some Ty1-copia sequences showed higher transcription levels in leaves and in roots under drought stress compared to controls. Indeed, the results showed substantial levels of DNA hypomethylation in the investigated CpG sites **(Figure 4)**, were consistent with the qPCR data, showing a significantly increased expression in leaves and in roots under drought stress compared to controls.

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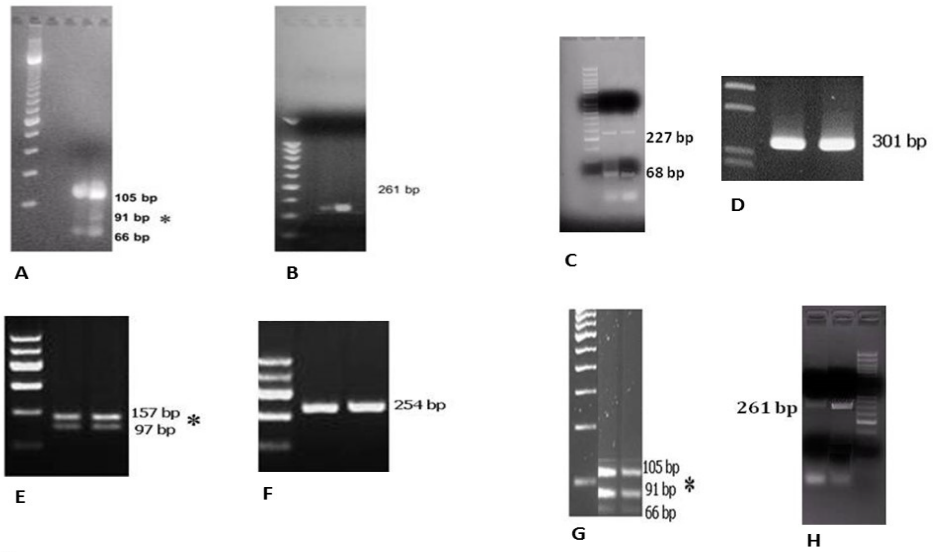

Figure 3. Cytosine hypomethylation at CCGG sites revealed by COBRA. p1c3 RT-*Ty1copia* from *Ruta graveolens* leaves (1) and roots (2) control plants (A) and from *Ruta graveolens* under drought stress (B). The DNA was used as template for amplification and digestion with MspI. The products were fractionated by agarose gel electrophoresis. Asterisks indicate the presence of two bands (157 bp and 97 bp) in the digested DNA (A) that are missing in the DNA undigested (B) with MspI.P3c4 RT-*Ty1copia* from *Ruta graveolens* leaves (1) and roots (2) control plants (C) and from *Ruta graveolens* under drought stress (D). The DNA was used as template for amplification and digestion with MspI. The products were fractionated by agarose gel electrophoresis. Asterisks indicate the presence of two bands (68 bp and 227 bp) in the digested DNA (D) that are missing in the DNA undigested (C) with MspI P3c5 RT-*Ty1copia* from *Ruta graveolens* leaves (1) and roots (2) control plants (E) and from *Ruta graveolens* under drought stress (F). The DNA was used as template for amplification and digestion with MspI. The products were fractionated by agarose gel electrophoresis. Asterisks indicate the presence of two bands (105 bp, 91 bp and 66 bp) in the digested DNA (E) that are missing in the DNA undigested (F) with MspI. P3c10 RT-*Ty1copia* from *Ruta graveolens* leaves (1) and roots (2) control plants (G) and from *Ruta graveolens* under drought stress (H). The DNA was used as template for amplification and digestion with MspI. The products were fractionated by agarosegel electrophoresis. Asterisks indicate the presence of two bands (121 bp, 91 bp and 56 bp) in the digested DNA (G) that are missing in the DNA undigested (H) with MspI.

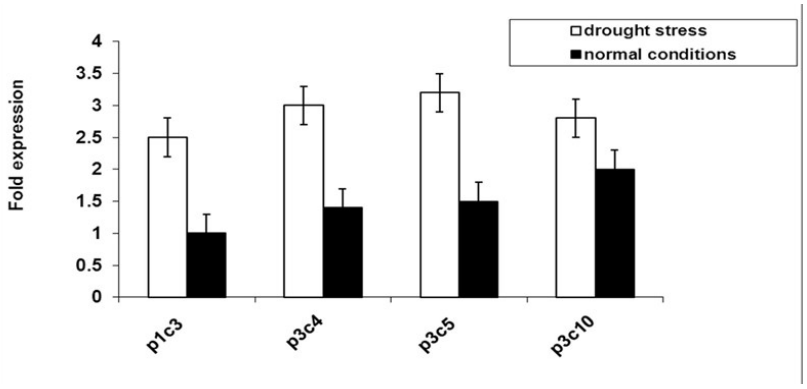

Figure 4. Real time RT-PCR showing the expression level of p3c4, p1c3, p3c5 and p3c10 RT-*Ty1copia* in *Ruta graveolens* leaves and roots under drought stress and normal conditions. 26S ribosomal RNA was used as internal control for normalization.

TEs transcription activity was only demonstrated for a few plants and only activated under stresses conditions [52]. In *Ruta,* despite mutations and cell control, TEs manage to be transcriptionally active, and transcription could be regulated at a post-transcriptional level through dynamic DNA methylation changes.

CONCLUSION

In conclusion, our study represents the first deep report of Ty1-copia retrotransposons in *Ruta*. Retrotransposons have not yet been described in rue, one of the most ancient *Rutaceae* groups. More wide analysis of transposable element sequences and genomic distribution will contribute to our understanding of their potential impact on rue evolution and diversity inside *Rutaceae* family. In our study, we demonstrate transcriptional activation of retrotransposon-like sequences in rue (*Ruta graveolens*) after exposure to drought stress which was paralleled by alterations in sequence specific methylation. Given the prominent roles played by biotic and abiotic stress in the evolutionary history, it is tempting to conclude that one or both of these factors may have been involved in these proliferations. *Ruta* species will likely emerge as an excellent group for studying the ecological and evolutionary dynamics of LTR retrotransposon activation and proliferation. Further experiments, however, will be necessary to reveal the epi-genetic regulation mechanism of the stress response in this plant.

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