

## Activation of Ty1-copia Group Retrotransposons of *Dendrobium officinale* under Abiotic Stress Conditions

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### Abstract

By using universal primer Ty1-copia retrotransposon reverse transcriptase (RT), the conserved reverse transcriptase domain of about 260 bp, which was induced by cold stress and osmotic stress, was amplified by RT-PCR from the *Dendrobium officinale* in this study, indicating that the retrotransposon could be activated by stress conditions. The amplicons were recovered and cloned for sequencing and analyzing by related bioinformatics software. 78 Ty1-copia like retrotransposon RTs transcriptionally activated were obtained with high heterogeneity. The length of these sequences varied from 245 to 265 bp, all the sequences were rich in AT and homology ranged from 42.9% to 99.2%. The same to Ty1-copia like retrotransposon RT of genome, different cis-acting regulatory elements induced by stress conditions and the starting transcription signals, corresponding to CAAT box, TATA box conserved sequences and some other regulatory elements. The cis-acting regulatory elements induced by stress conditions of reverse transcriptase transcriptionally activated of Ty1-copia retrotransposons were significantly increased than Ty1-copia like retrotransposon RT of genome. When translated into amino acids, some sequences presented stop codon mutation or/and frameshift mutation, and all sequences presented mutation in conserved sequence "SLYGKQ". Four categories were identified through phylogenetic analysis after alignment analyses of their amino acid sequences, and with Ty1-copia like retrotransposon RT of genome having low homology, which indicated that reverse transcriptase transcriptionally activated of Ty1-copia retrotransposons, which induced under stress conditions, had significantly differences with Ty1-copia like retrotransposon RT of genome.

**Keywords:** *Dendrobium officinale*; Cold; Osmotic; Ty1-copia-like retrotransposon; Reverse Transcriptase (RT); Transcriptionally activated

### Introduction

*Dendrobium officinale* Kimura et Migo (Orchidaceae) is a traditional and rare Chinese medicinal plant species and was ranked "the first of the nine Chinese fairy herbs". *Dendrobium officinale* [1,2] has been acknowledged for its unparalleled curative effects, such as nourishing yin and clearing away toxic materials accumulated in human tissues, benefiting the stomach, promoting the production of body fluid, reducing blood sugar levels, enhancing the body's immunity and prolonging life [3,4]. Unfortunately, in recent years, its habitats are susceptible to the deterioration by logging and overexploitation [5] which threaten the survival of these species [6]. Protected cultivation must be used to cultivate this plant to ensure its continued availability. However, environmental stress is a serious problem for the cultivation of *D. officinale* on an industrial scale. Therefore, elucidation of the molecular mechanisms how *D. officinale* tolerates environmental stresses is necessary for breeding and genetic engineering approaches to improving *D. officinale* performance under stress conditions.

Retrotransposons are the most common class of mobile genetic elements in eukaryotes. Recent evidences have shown that they are a common feature of plant genome [7-9] and can constitute a very large part of some of them [10-12]. Retrotransposons, termed as Class I elements, transpose via reverse transcription of an RNA intermediate. The retrotransposons contains long terminal repeats (LTR) and non-LTR retrotransposons. LTR retrotransposons can be further classified

as either Ty1-copia or Ty3-gypsy group and the most studied group is the Ty1-copia group. In particular, Ty1-copia group has been characterized from a wide variety of plant taxa, both in monocotyledonous and dicotyledonous plants [13,14], namely, Tnt1 [15] and Tto1-3 [16] in tobacco, Tal3 [17] in Arabidopsis, RIRE1 [18] and Tos17 [19] in rice, and so on. In spite of being both ubiquitous and abundant, most retrotransposons are thought to be transcriptionally inactive or in a quiescent state during normal growth and development [20]. In all eukaryotic systems, the retrotransposition of retrotransposons is controlled by the element itself, signals of the host organism and some external factors. Many studies have indicated that some retrotransposons can be activated by abiotic and biotic stresses [20].

The expression of the Tto1 elements and Tnt1 of tobacco [16,21-23] and that of the rice Tos17 elements [19] are greatly increased by stresses, including pathogen attacks or stimulations during protoplast isolation or cell culture [24]. This phenomenon agrees with McClintock's original model, which postulates transposable elements are involved in genome restructuring in response to environmental challenges ('genomic shocks') [25]. One of the best characterized plant retrotransposons is the copia-like Tnt1 element of tobacco (*Nicotianatabacum*) [15]. Tnt1 is induced by a range of abiotic elicitors such as wounding, freezing or heavy metals [26,27], as well as by compounds involved in the transduction of the stress signal, such as reactive oxygen species [28] or salicylic acid [29-31], which all have in common the ability to elicit the plant defence response [26].

At present, little is known on the behavior of retrotransposons of *D. officinale* when the plant suffers stresses. In our previous study, we isolated and characterized the reverse transcriptase (RT) gene

sequences of Ty1-copia retrotransposons from the *D. officinale* genome [32]. Further, we investigated effects of cold stress and osmotic stress on the activity of RT gene sequences of Ty1-copia retrotransposons and characterized the heterogeneity of transcriptionally activated RT gene sequences in *D. officinale*.

## Materials and Methods

### Plant materials

*D. officinale* used in this study was originally planted in Lin'an (119°12'E 30°31'N) of Zhejiang province in China. The germplasm was identified by one of our co-authors, J. Si, The Natural Medicine Research and Development Center at Zhejiang Agricultural and Forestry University.

### Plant growth conditions and stress treatments

*D. officinale* seedlings were grown and maintained in the greenhouse of Nurturing Station for the State Key Laboratory of Subtropical Silviculture, Zhejiang Agriculture and Forestry University, with a 16-h light/ 8-h dark photoperiod at  $25 \pm 2$ . Stress treatments for gene expression analyses were performed as follows. For cold stress, 6-month-old seedlings of *D. officinale*, which were well grown under an optimal condition, were incubated at 4 in low temperature incubator for 4, 8, 12, and 24 h, respectively. For osmotic stress, the 5-month-old seedlings of *D. officinale* which were well growth under an optimal condition were transplanted to new culture medium at different sucrose levels: 20, 30, 40, and 50 g/L, respectively for one month. Seedlings grown in normal conditions were used as control. After each treatment, leaves were collected and frozen in liquid nitrogen for the isolation of total RNA.

### RNA extractions

Total RNAs were extracted using the modified CTAB [33]. Finally, the extracted RNA was assayed using DU800 spectrophotometer (Beckman, USA), and was compared with standard markers by 1.0% agarose gels.

### Semi-quantitative RT-PCR

Reverse transcription was carried out using PrimeScript™ II 1st Strand cDNA Synthesis Kit (TaKaRa, Japan), following the manufacturer's instructions. Degenerate primer pairs were used to amplify RT domains of Ty1-copia retrotransposons: Ty1-F (5'-ACNGCNTTYTTCNCAAYGG-3') and Ty1-R (5'-ARCATRTCTCNCACRTA-3') [34]. The ACTIN gene of *D. officinale* was used as the internal control: actin F(5' TTGTGTTGGATTCTGGT GATGGTGT 3') and actin R(5' TTTCCCGTTCTGCTGTTGTTGTGA A -3'). The Ty1-copia reaction system (20  $\mu$ L) contained 4 uL of cDNA solution derived from the mRNA samples, 2  $\mu$ L 10  $\times$  PCR buffer, 1.5 mmol $\cdot$ L<sup>-1</sup> MgCl<sub>2</sub>, 0.1 mmol $\cdot$ L<sup>-1</sup> dNTPs, 0.4  $\mu$ mol $\cdot$ L<sup>-1</sup> of each primer and 0.5 U Taq DNA polymerase (TaKaRa, Japan). The PCR reaction programme included an initial denaturation step at 94 for 1 min followed by 35 cycles at 94 for 1 min, 46.5 for 1 min 45 s, and 72 for 1 min, and then a final elongation step at 72 for 5 min. As for ACTIN, 20 PCR cycles were used to visualize whether stress-specific variation occurred (a higher number of cycles led to signal saturation).

## Purification, cloning, and sequencing

RT-PCR products were purified and recovered by using the Agarose Gel DNA Recovery Kit (TaKaRa, Japan), cloned into the pMD<sup>+</sup>18-T vector (TaKaRa, Japan) and then transformed into DH5 $\alpha$  strain of competent cells. DNA sequencing reactions were performed on an automated DNA sequencer (ABI PRISM3730) with M13 primers by using the Big Dyeterminatorver software for analysis. This work was done by Sangon Biological Engineering and Technology and Service, Shanghai, China.

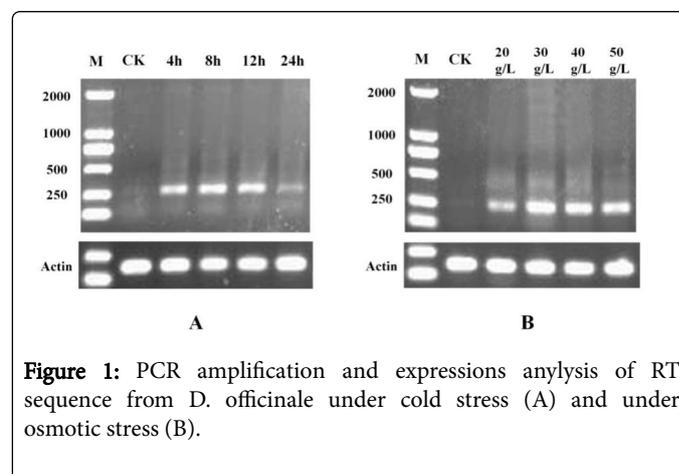
## Sequence analysis of reverse transcriptase

The nucleotide sequence of each clone was compared online with the database sequences using BLASTN and BLASTX searching tools on the National Center of Biotechnology Information (NCBI) website (<http://www.ncbi.nlm.nih.gov>). Sequence display was made with DNAMAN.full.version 5.2.2. The neighbor-joining trees were generated by using MEGA 5.0 software [35] and ClustalX 1.83. A bootstrapping test was performed with 1,000 times.

## Results

### Isolation of transcriptional active sequences under stress conditions

PCR amplification with degenerate primer pairs flanking the RT sequences of Ty1-copia retrotransposon yielded the expected sizes of about 260 bp in *D. officinale* induced by either cold stress (Figure 1A) or osmotic stress (Figure 1B). The targeted PCR products of Ty1-copia retrotransposons were eluted from the gel, purified, and then cloned to DH5 $\alpha$ . Fifty randomly selected clones, which showed positive insertion of 260 bp PCR products, were used for sequencing. Our sequencing results revealed that 39 independent clones were obtained from two different treatments respectively. For clarification, the clones obtained from cold-stress treated leaves were named as COOFC- (COOFC stands for copia retrotransposon of *D. officinale* under cold stress) and that from osmotic-stress treated leaves as COOFO- (COOFO stands for copia retrotransposon of *D. officinale* under osmotic stress). These seventy eight DNA sequences of RT gene of Ty1-copia retrotransposons were categorized according to the difference of stress treatments and deposited respectively in the GenBank database under accession nos. KJ849241-KJ849279 and KJ831125-KJ831163.



**Figure 1:** PCR amplification and expressions analysis of RT sequence from *D. officinale* under cold stress (A) and under osmotic stress (B).

### Characterization of RT sequences in *D. officinale*

Similar to RT sequence of Ty1-copia retrotransposons isolated from genome of *D. officinale* (GenBank accession KF999787-KF999807), most clones contain PCR fragments ranging from 259 to 265 bp, while two of them COOFO-22 and COOFO-25 were 245 and 248 bp, respectively (Table 1), indicating that deletion mutations existed in the RT sequence of *D. officinale*. These clones were AT-rich in nucleotide composition (Table 1) The cloned sequences were highly heterogeneous with homology varying from 42.9 to 99.2%. Among them, the sequences COOFO-22 and COOFO-15 with the highest homology shared 99.2% in identity, while the sequences COOFO-30 and COOFC-4 have the lowest homology, only 42.9% (data not shown), implying that the transcription activity of many RT sequences of Ty1-copia retrotransposons in *D. officinale* genome were induced under stress treatments. Analysis by using the software plantCARE revealed that, similar to RT sequences of Ty1-copia retrotransposons isolated from genome of *D. officinale* [26], multiple regulatory elements were founded in these sequences. These regulatory elements were related to stress conditions, such as many conserved promoter elements, e.g. TATA box and CAAT box, and some other regulatory elements.

Compared to the genomic RT sequences of Ty1-copia retrotransposons, the abundance of these regulatory elements

increased significantly probably due to activation of RT sequences under stress conditions. The proportion of the transcriptionally active RT sequences under cold and osmotic stress containing cis-acting element involving in defense and stress responsiveness increased from 4.76% (in the genome) to 28.21% and 33.33% respectively. The cis-acting element involving in low-temperature responsiveness increased from 9.52% to 12.82% and 15.38% respectively. what's more, the cis-acting element involving in heat stress responsiveness decreased to 2.56% and increased to 10.26% respectively from 4.76%. The putative 78 RT sequences were then translated into their amino acids (Figure 2) to check the presence of stop codons, frameshifts, and deletion mutations in their coding regions. Of these sequences, thirty six (46.15%) sequences contained premature stop codons and/or indels, which disrupt the open reading frame, while the remaining forty two (53.85%) sequences had potentially functional RT fragments. One or more amino acid mutations occurred within the SLYGLKQ sequences, which are the characteristic of plant retrotransposons. All these results indicated that the heterogeneity of the transcriptionally active RT sequences under stress conditions was higher than that of the RT sequences isolated from the genome of *D. officinale* [33].

Sequence No.	Total	AT/GC	Sequence No.	Total	AT/GC	Sequence No.	Total	AT/GC
COOFC-1	265	1.57	COOFC-27	262	1.26	COOFO-14	261	1.29
COOFC-2	262	1.54	COOFC-28	265	1.48	COOFO-15	261	1.21
COOFC-3	261	1.39	COOFC-29	261	1.33	COOFO-16	261	1.64
COOFC-4	259	1.82	COOFC-30	261	1.49	COOFO-17	262	1.88
COOFC-5	265	1.6	COOFC-31	263	1.8	COOFO-18	261	1.33
COOFC-6	264	1.61	COOFC-32	261	1.25	COOFO-19	261	1.84
COOFC-7	260	1.71	COOFC-33	261	1.39	COOFO-20	261	1.58
COOFC-8	262	1.43	COOFC-34	264	1.51	COOFO-21	265	1.57
COOFC-9	261	1.56	COOFC-35	265	1.55	COOFO-22	245	1.72
COOFC-10	264	1.67	COOFC-36	264	1.61	COOFO-23	262	1.62
COOFC-11	261	1.18	COOFC-37	264	1.59	COOFO-24	262	1.4
COOFC-12	263	1.83	COOFC-38	263	1.41	COOFO-25	248	1.67
COOFC-13	255	1.43	COOFC-39	264	1.61	COOFO-26	261	1.42
COOFC-14	263	1.8	COOFO-1	263	1.74	COOFO-27	262	1.54
COOFC-15	263	1.77	COOFO-2	262	1.08	COOFO-28	262	1.59
COOFC-16	265	1.55	COOFO-3	261	1.39	COOFO-29	261	1.37
COOFC-17	262	1.62	COOFO-4	262	1.45	COOFO-30	259	1.82
COOFC-18	263	1.96	COOFO-5	262	1.3	COOFO-31	266	1.61
COOFC-19	265	1.6	COOFO-6	265	1.6	COOFO-32	262	1.7
COOFC-20	261	1.42	COOFO-7	265	1.52	COOFO-33	264	1.78
COOFC-21	261	1.81	COOFO-8	262	1.38	COOFO-34	264	1.4

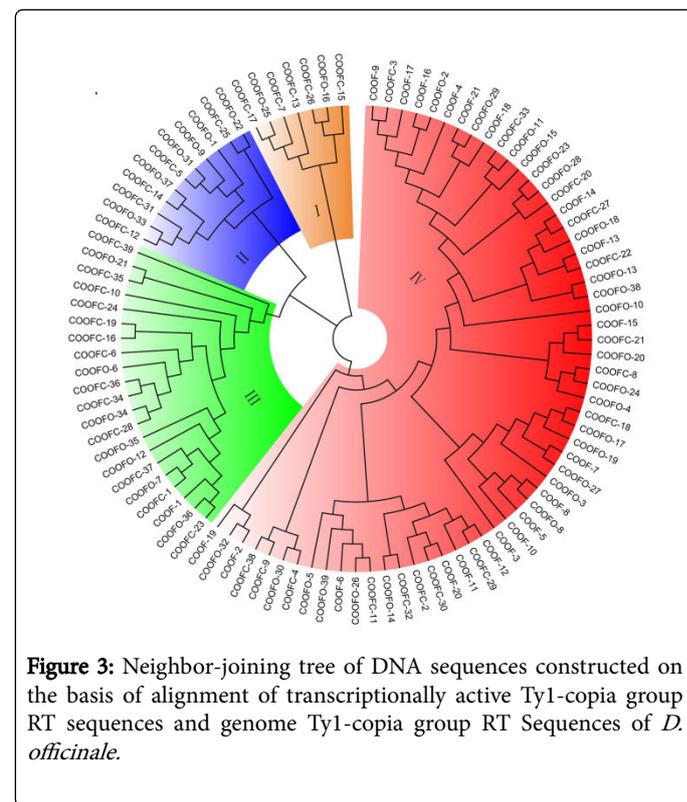
COOFC-22	261	1.31	COOFO-9	265	1.57	COOFO-35	264	1.56
COOFC-23	265	1.57	COOFO-10	262	1.45	COOFO-36	264	1.54
COOFC-24	264	1.54	COOFO-11	261	1.39	COOFO-37	263	1.68
COOFC-25	262	1.59	COOFO-12	264	1.61	COOFO-38	261	1.35
COOFC-26	261	1.33	COOFO-13	262	1.5	COOFO-39	261	1.69

**Table 1:** The nucleotide composition of reverse transcriptase transcriptionally activated of Ty1-copia retrotransposons in *D. officinale*.



**Figure 2:** The amino sequence alignment of reverse transcriptase transcriptionally activated Ty1-copia retrotransposons in *D. officinale*.

from genome, and the IV group included the other transcriptionally active RT sequences and almost all of genomic RT sequences without COOF-1. It, therefore, appeared that there existed great differences between broadly transcriptionally active RT sequences and the genomic RT sequences. Although the populations of RT gene sequences induced under cold stress and osmotic stress were much more variable than those of genome, they couldn't be differentiated from one another by phylogenetic analysis. Therefore, different treatments did not generate particular subsets of transcriptionally active RT sequences but could increase the level of closely related RT sequences transcripts.



**Figure 3:** Neighbor-joining tree of DNA sequences constructed on the basis of alignment of transcriptionally active Ty1-copia group RT sequences and genome Ty1-copia group RT Sequences of *D. officinale*.

**Phylogenetic analysis of *D. officinale* RT sequences**

The phylogenetic relationship between these transcriptionally active RT sequences and 21 RT sequences from the genome [32] was represented as a neighbour-joining-tree (Figure 3). Our work confirmed that there contained the high heterogeneity among these 99 RT sequences, and the sequences could be further grouped into four distinct families (I to IV). The tree showed that the I and II groups contained only transcriptionally active RT sequences, and had a distant phylogenetic relation with genomic RT gene sequences. The III group contained only transcriptionally active RT sequences except COOF-1

**Expression of RT genes under stress conditions**

The expression level of RT genes was confirmed to be higher in cold stress or osmotic stress treated leaves than that without any treatment. At the same time, it showed that the expression of RT genes was first up-regulated and then down-regulated subsequently under the two treatments, with the highest expression level shown on 8 h under cold stress treatment (Figure 1A) and 30 g/L sucrose level under osmotic stress treatment (Figure 1B), implying that many RT sequences of Ty1-

copia retrotransposons in *D. officinale* genome could be activated under stress treatments and plant cells were able to monitor different levels of stress intensity by modulating corresponding RT gene expression.

## Discussion

Ty1-copia retrotransposons are transposable element through a "DNA-RNA-DNA" mechanism. Transcription is the first step of the retrotransposition process, and also a major controlling step for plant retrotransposons [36]. Transcription initiation and termination signals and some regulatory elements in LTRs are related to the induction of their expression [37]. Plant Ty1-copia like retrotransposon transcriptional activity does not express under normal conditions, but can be activated by a variety of stress conditions [38], which are the results of retrotransposons mimicking the host gene expression under stress conditions in the long-term evolution [39,40]. There may exist a certain relationship between activation of retrotransposons and plant defense mechanisms [36]. It has been proposed that retrotransposons could have captured plant defence promoters from normal genes or inversely provided their own inducible promoters to some plant defence genes [38,41].

The expression of Tnt1 retrotransposon in tobacco is activated by abiotic stresses and by components involved in early stress signal transduction and the expression of the Tnt1 promoter is a sensitive indicator of the plant defence response [26]. In the present study, we detected transcriptionally active RT sequences in *D. officinale* under cold stress and osmotic stress. Notably, RT gene expression was confirmed to be higher in leaves treated by cold stress and osmotic stress than that without any treatments (Figure 1), indicating that many RT sequence of Ty1-copia retrotransposons in *D. officinale* genome can be activated under stress treatments and different treatments had varying success in terms of inducing degrees. Our work, therefore, confirmed there are some links between the expression of RT sequences of Ty1-copia 241 retrotransposons and the plant defense response in *D. officinale*.

During the long-term evolution, an interacting mechanism between retrotransposons and hosts may have formed, which may be beneficial to the survival and reproduction of plants, and retrotransposons may take a variety of ways to promote the events of transposition [40]. In this study, seventy eight transcriptionally active Ty1-copia group RT sequences in *D. officinale* were successfully amplified with degenerate primers and the data showed that those sequences were highly heterogeneous, similar to those observed in other plant species [13,14,42,43]. All these sequences were different from each other with homology varying from 42.9% to 99.2%

Molecular studies in plants have shown that several genes with various functions were induced by cold stress and osmotic stress. For several stress-inducible genes, cis-acting elements in promoters regions and the corresponding transcription factors have been analyzed in Arabidopsis. For examples, the dehydration-responsive element (DRE)/C-repeat (CRT), a cis-acting element, involves in osmotic stress- and cold stress- inducible gene expression [44]. In this study, multiple regulatory elements were found in seventy eight transcriptionally active Ty1-copia group RT sequences which are related to stress conditions, such as many promoters characteristic structure of the TATA box and CAAT box conserved sequences and some other regulatory elements. However, regulatory elements, which related to stress conditions in the transcriptionally active RT sequence

increased significantly than that was isolated from genome. The results indicated that the RT sequence of Ty1-copia retrotransposons in *D. officinale* 263 containing stress regulatory elements could be activated under stress conditions.

A common character of most retrotransposons is the presence of stop codons and frame shifts in the coding regions. For example, 51% of the Ty1-copia RT clones contained stop codons and/or frame shifts in persimmon [45]. In potato, approximately 40% of the RT gene fragments of Ty1-copia group retrotransposon contained translational stop codons [46], however, a comparatively low fraction (34%) of Ty1-copia RT sequences possessed mutations in the coding regions of mungbean [47]. In this study, some the isolated RT sequences from *D. officinale* were found to contain mutations including deletion stop codons, and frame shifts. Even mutations were found in the shared character of SLGLKQ sequence of plant retrotransposons in all clones sequenced. These mutations, however, did not affect their transcriptional activity and even may not affect their transposition activity. Such phenomenon was also found in other organisms, like maize [48-51]. The most extreme example is BSI, a LTR-retrotransposon in the maize genome, which encoded a reverse transcriptase containing an unusually divergent sequence in the absence of a reverse transcriptase consensus sequence [48].

In our study, phylogenetic analysis showed that all the sequences could be divided into four different families (named I to IV) by their RT similarity in *D. officinale*. In addition to COOF-1, others RT sequences, which were isolated from genome of *D. officinale*, belong to IV, indicating that transcriptionally active RT sequences had lots of differences with genomic RT sequences. All these showed that the RT sequences populations induced under cold stress and osmotic stress were indistinguishable, suggesting that both stresses induced RT sequences through the same cis-acting elements [40]. Consequently, different stress did not generate particular subsets of transcriptionally active RT sequences but could increase the transcript level of closely related RT sequences.

It becomes clearer that retrotransposons were activated in response to various forms of stress [38]. The results in this study have provided the necessary information for future studies of retrotransposons in *D. officinale*; and provided a new starting point for the study of the relationship between the abiotic stress and the activity of Ty1-copia retransposons in *D. officinale*; and created new opportunities for further study on the role of retrotransposons in the stress response mechanisms in *D. officinale*.

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