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Short Communication

Quantitative Transcript Analysis in Plants: Improved First-strand cDNA Synthesis

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Abstract The quantity and quality of first-strand cDNA directly influence the accuracy of transcriptional analysis and quantification. Using a plant-derived α-tubulin as a model system, the effect of oligo sequence and DTT on the quality and quantity of first-strand cDNA synthesis was assessed via a combination of semi-quantitative PCR and real-time PCR. The results indicated that anchored oligo dT significantly improved the quantity and quality of α-tubulin cDNA compared to the conventional oligo dT. Similarly, omitting DTT from the first-strand cDNA synthesis also enhanced the levels of transcript. This is the first time that a comparative analysis has been undertaken for a plant system and it shows conclusively that small changes to current protocols can have very significant impact on transcript analysis.

Key words cDNA; real-time PCR; RT-PCR; transcript analysis

In the past 10 years, there was a very rapid development in fluorescence-based techniques for the detection and monitoring of transcript abundance. Two methods that have gained rapid popularity are microarray and real-time PCR. The microarray allows parallel analysis of thousands of genes from two differentially labelled RNA populations [1], while real-time PCR provides simultaneous expression analysis of a limited number of genes from many different samples [2–5]. Both techniques lend themselves to high-throughput format, offering speed and the potential for automation. Despite these advantages, the techniques also increase the number of manipulations and therefore the potential sources of technical error. The implication of the technical error associated with microarray has been discussed by Yang and Speed [6].

The first step in any transcriptional study is the reliable quantitative extraction of total RNA from the relevant tissue. This is followed by first-strand cDNA synthesis. There are two basic formats: a one-step procedure or a two-step procedure. Both have their own merits. The one-step procedure, although often being preferred as it is assumed that the reduced number of manipulations will decrease the variation, does have inherent limitations and technical problems. Vandesompele *et al.* have reported extensive primer-dimer formation in non-template control reactions [7]. Furthermore, because the cDNA is derived from a specific primer, it is not possible to carry out amplification of multiple targets from the same cDNA/RNA population, and clearly this will make the normalization impossible by using house-keeping genes. The two-step procedure, however, eliminates primer-dimer formation using an oligo dT primer and also allows multiple targets to be amplified from the same RNA population including a house-keeping gene.

Thus, it would appear that two-step RT-PCR has a significant number of advantages over one-step RT-PCR. However, a number of issues relating to cDNA synthesis from oligo dT have been raised recently. Wang *et al.* have observed the selective loss of low-abundance transcripts during normalization and subtraction reactions because of the formation of double-stranded poly dA and poly dT hybrids [8]. The model proposed by Wang *et al.* for the dT and dA interaction had significant implications for the

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efficiency of reverse transcription and therefore led to under-representation of the individual transcripts within an RNA population. The problem was apparently overcome by using a combination of five anchored oligo dT primers. The same primer combination has also been used to address the frequency of truncated mRNA species generated by a conventional oligo dT primer that were found to use internal poly(A) sites instead of the 3′ poly(A) at a high frequency [9]. While the authors were primarily concerned with the effect of oligo type on the generation of ESTs and gene identification, truncated cDNAs can have a serious impact on RT-PCR and real-time PCR.

Lekanne Deprez *et al*. have investigated the factors that could potentially influence the sensitivity and reproducibility of real-time PCR [10]. To improve the sensitivity of two-step reverse transcription reactions, they analyzed the effect of the type of oligo dT primer sequence. The anchored oligo dT_{VN} and gene-specific primer gave the best results, although the conventional oligo dT and anchored primer were not compared. In addition to studying the effect of primers on cDNA synthesis, their work also illustrated that DTT, while it is included in first-strand cDNA reactions, actually reduced the sensitivity of real-time PCR.

Thus, from the studies of mammalian systems described in published work, it has been shown that DTT and the type of dT oligo could impact the quantity and quality of cDNA produced from first-strand synthesis. Our research objective is to develop a model system that enables the quantitative analysis of heterologous gene expression in a plant. Therefore, we have undertaken the comparison of two techniques, semi-quantitative PCR and real-time PCR, which enable levels of transcript to be analyzed. This was done in conjunction with studying the effects of using anchored oligo dT primers and removing DTT from the first-strand cDNA synthesis.

This article reports the implications of the modified protocol on the quantity and quality of the first strands of cDNA produced from plant RNA using both techniques.

Experimental Procedures

Trifolium repens cv Rivendel, genotype 10, a line characterized in our laboratory, was grown under glasshouse conditions. Plant materials were sampled and freeze-dried before RNA extraction. Total RNA was prepared from 5 mg of freeze-dried material using Tri reagent (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions.

Three primer sets for the first-strand cDNA synthesis were used: (1) oligo(dT_{16}) (B26), 5′-GACTCGAGTCGACATCGATTT16-3′ (500 ng per reaction, as described by Frohman *et al.* [11]); (2) a mixture of five anchored dT_{16} oligos (ComboB26-250), 5′-GACTCGAGTCGACATCGATTT$_{16}$-A, -G, -CA, -CG and -CC-3′ (250 ng of each oligo was used per reaction here); and (3) the same anchored oligo set as (2), but with the amount of each oligo increased to 500 ng per reaction (ComboB26-500). First-strand cDNA was synthesized, with 500 ng total RNA as the template, using Superscript II (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions, except when DDT was omitted (DTT minus).

Semi-quantitative PCR was performed on first-strand cDNA prepared from *T. repens*. Both 3′-RACE and nested PCR were performed. For 3′-RACE, a primer complementary to the adaptor sequence that forms part of the oligo dT as described by Frohman *et al.* [11], referred to as the B25 primer (5′-GACTCGAGTCGACATCGA-3′), was used in conjunction with a sense primer (5′-128 bp to 147 bp-3′) designed for the α-tubulin (accession No. AY192359). The resulting product was 1154 bp. For nested PCR, sense and antisense primers designed for the same accession were used to amplify a 493 bp cDNA fragment of α-tubulin. The sense primer used was (5′-ATCTTGAACCTACCGTCATC-3′, 5′-128 bp to 147 bp-3′) and the antisense primer used was (5′-TGAGAGACAAGGCGATAA-3′, 5′-603 bp to 620 bp-3′). The PCR reaction was carried out in a total volume of 50 μl with 2.5 U of Advantage™ 2 (BD) polymerase, 10×Advantage 2 PCR buffer, 200 μM dNTP, 250 nM primers, 1 μl of diluted first-strand cDNA as the template. The thermo cycling conditions were: (1) 95 °C for 2 min; (2) 30 cycles at 95 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min; and (3) 72 °C for 10 min.

The α-tubulin PCR products derived from the cDNA samples were first resolved on a 1% agarose gel before being subjected to Southern blot hybridization using Hybond N^+ according to the manufacturer's instructions (Amersham Pharmacia Biotech, Little Chalfont, England). Pre-hybridization, hybridization and washing were performed according to the procedure described by Sambrook *et al.* [12]. The probe was an α-^{32}P-labeled α-tubulin fragment derived from the clone and primers described above. Membranes were exposed to an Imaging screen K (Bio-Rad Laboratories, CA, USA) and subsequently scanned using a molecular Imager® FX phosphoimager (Bio-Rad Laboratories) for comparative analysis.

Real-time PCR amplification and analysis were carried

out using ABI Prism® 7000 sequence detection systems and ABI Prism 7000 SDS software (version 1.0), respectively. The reaction mixture consisted of 3 μl of cDNA, 300 nM concentration of each primer, 10 μl of 2×SYBR® green I mixture (Roche). The thermo cycling conditions were: (1) 50 °C for 2 min; (2) 95 °C for 10 min; and (3) 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The sense primer (5′-TCTACTCATTCCCTCCTTG-3′) and antisense primer (5′-GCTGTTACTTCGGTAGA-3′) were designed using Primer Express software (version 1.1). The selected primers were expected to produce a fragment of 63 bp. The data was quantified against a standard curve derived from the same cloned α-tubulin. A combination of ANOVA and *t*-test was used to analyze the results using the software package SAS 6.12 (SAS Institute Inc.).

Results

The effect of the oligo dT on the total amount of cDNA synthesized was assessed using semi-quantitative PCR in conjunction with Southern blot hybridization (**Fig. 1**). An image analysis technique was employed to compare the pixel intensity of the corresponding hybridization of the probe with the PCR products; this is referred to as band analysis. The experiment was done twice for a total of 10 replicates. The average of all the data points (i.e. plus and minus DTT) revealed that both the ComboB26-250 and ComboB26-500 primers consistently yielded significantly

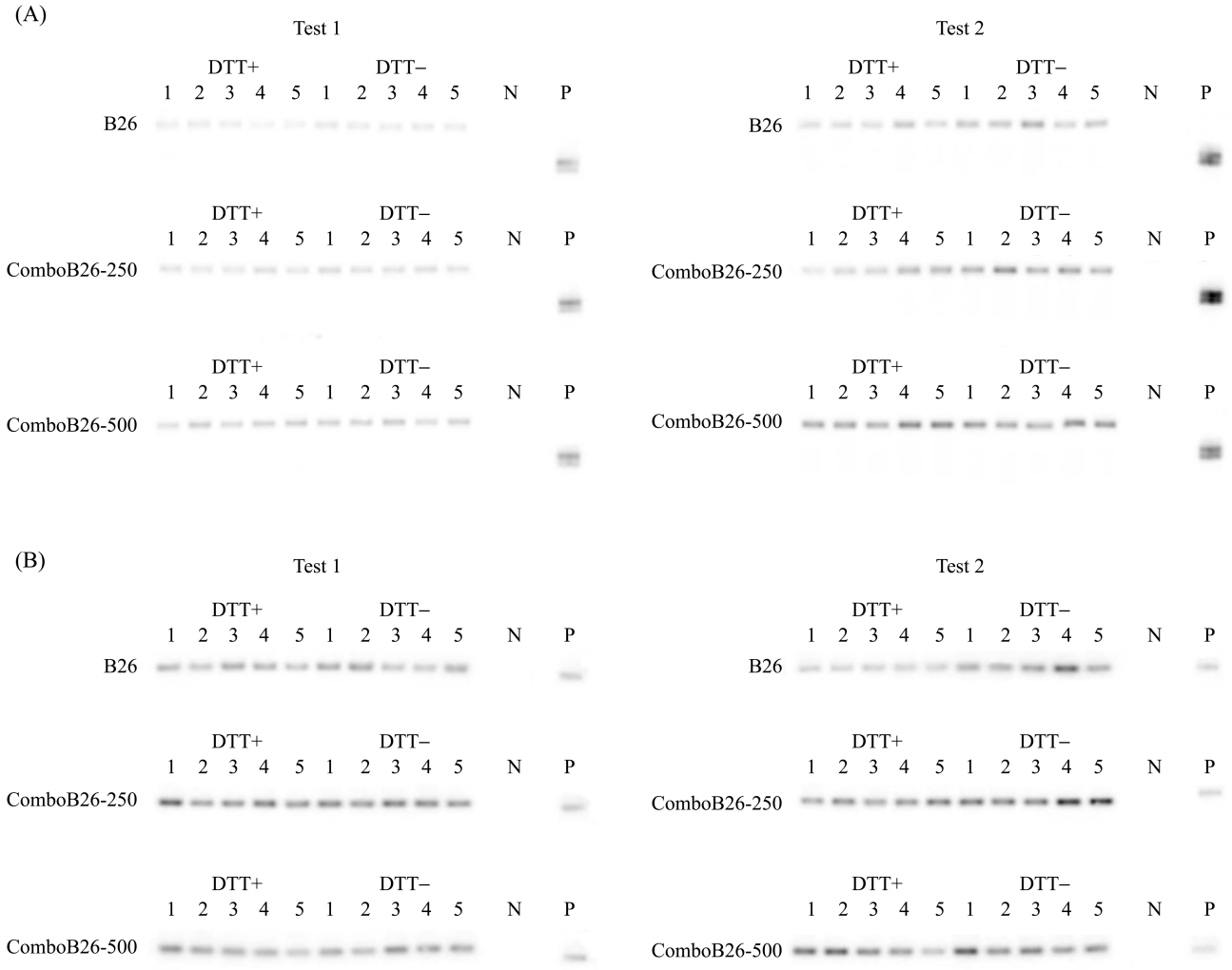

Fig. 1 Southern blot hybridization of α-tubulin products from 3′-RACE and nested PCR

(A) 3′-RACE. (B) Nested PCR. Test 1 and 2 refer to the results of two duplicate experiments. 1–5, the five replicates in a single experiment; DDT+, plus DTT; DTT–, minus DTT; N, negative control; P, positive control.

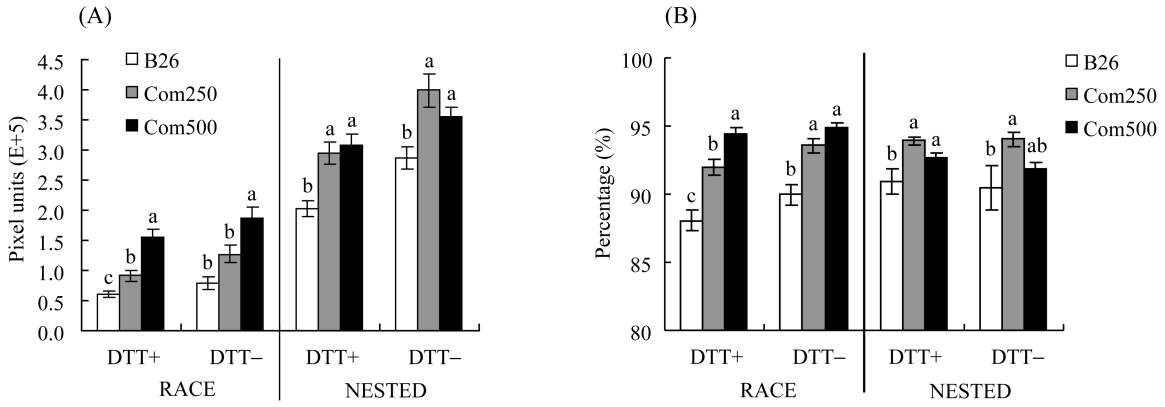

Fig. 2 Band analysis and lane analysis of the semi-quantitative PCR

(A) The mean ± SE pixel value corresponding to the hybridization intensity obtained from Southern blot analysis of the PCR products derived from first-strand cDNA synthesized according to oligo types and the presence or absence of DTT. (B) The mean pixel value of the gene of interest as a percentage of the overall background "noise" according to oligo types and the presence or absence of DTT. Different letters indicate the significance of the differences at $P<0.05$ level. Com250, ComboB26-250; Com500, ComboB26-500; DDT+, plus DTT; DTT–, minus DTT.

($P<0.05$) more α-tubulin 3′-RACE cDNA when compared to the amount of 3′-RACE cDNA produced using the conventional B26 oligo [**Fig. 2(A)**]. Similar results were obtained using nested PCR on the same cDNA template [**Fig. 2(B)**]. However, contrary to the 3′-RACE results, the nested PCR did not show significant differences between the two ComboB26 primers. This was attributed to differences in the amplification efficiencies between nested PCR and 3′-RACE.

The effect of DTT on the amount of cDNA synthesized was also evaluated using band analysis. Significant differences were observed for the 3′-RACE products derived from cDNA prepared with and without DTT ($P<0.05$). Similar trends, although not significant, were obtained using nested PCR [**Fig. 2(A)**]. Overall, when the conventional protocol (i.e. using oligo dT B26 in the presence of DTT) was compared with the use of two oligo dT variants in the absence of DTT, the ComboB26-250 and ComboB26-500 oligo dT variants without DTT yielded 2.1 and 3.1 times more α-tubulin cDNA respectively compared with the conventional method ($P<0.05$). The nested PCR results also suggested that the ComboB26-250 and ComboB26-500 oligo dT variants without DTT improved the amount of α-tubulin cDNA produced by 2.0 and 1.8 times respectively when compared to that produced by the B26 oligo dT with the addition of DTT ($P<0.05$).

The background "noise" widely associated with 3′-RACE has been attributed to non-specific amplification products. Furthermore, based on the work of Nam *et al*. [9], these products can be regarded as being truncated cDNAs which are produced because of the presence of potential internal poly(A) sites in the mRNAs. The lane analysis [**Fig. 2(B)**] showed that the percentage of α-tubulin 3′-RACE product was significantly increased as a percentage of the background "noise" when the anchored oligo dT, ComboB26, was used compared to that when the conventional oligo dT B26 was used. The effects of ComboB26-250 and ComboB26-500 on the "quality" of α-tubulin cDNA were significant in comparison with B26 ($P<0.05$). Therefore, the analysis showed that the level of "noise" in the 3′-RACE product was reduced and the quality of α-tubulin cDNA was improved by using the anchored oligo dT for the first-strand synthesis. This supports the assumption that truncated cDNA, through internal poly (A) priming, is reduced and does account for a proportion of the background. While the 3′-RACE results indicated the significant effect of oligos, the results of the nested PCR [**Fig. 2(B)**] did not. Despite the lack of statistical significance in the cDNA quality derived from the ComboB26 oligos, the trend was one of improvement. Again, the difference between the results obtained for 3′-RACE and nested PCR may be attributed to the different amplification efficiency.

Contrary to the significant effect of DTT observed when using band analysis, there was no significant difference detected using lane analysis for either 3′-RACE or nested PCR products. However, the B26 primer plus DTT showed a significant difference ($P<0.05$) compared to the ComboB26-250 and ComboB26-500 oligos minus DTT. This indicates that the quality of the cDNA can be im-

proved using the ComboB26 oligos in the absence of DTT, thus suggesting a combined effect.

Quantitative real-time PCR has become the method of choice for transcriptional analysis and it was therefore used to assess whether the observed effects of oligo type and the presence of DTT in the first-strand cDNA synthesis reaction using semi-quantitative PCR could influence quantitative analysis. There were significant differences between the ComboB26-500 and the conventional oligo dT (P<0.05), but not between ComboB26-250 and the B26 in the presence of DTT (**Fig. 3**). However, taking the average of all the data points derived from the duplicate experiments and combining the data from both plus and minus DTT (n=18), it was revealed that both ComboB26-250 and ComboB26-500 consistently yielded significantly (P<0.05) more α-tubulin cDNA compared with the conventional B26 oligo.

The real-time PCR results also confirmed that DTT affected the amount of α-tubulin cDNA produced. The amount of α-tubulin cDNA produced with DTT averaged 0.85 atto mol, which was significantly less than the 0.99 atto mol produced without DTT (P<0.05). The real-time PCR results also confirmed that both oligo and DTT affected the amount of α-tubulin cDNA produced. As illustrated in **Fig. 3**, the amounts of α-tubulin cDNA produced using ComboB26-250 and ComboB26-500 without DTT were 0.95 atto mol and 1.28 atto mol respectively, compared to 0.70 atto mol of α-tubulin cDNA produced by the conventional method of cDNA synthesis using oligo B26 plus DTT. These results indicated that 1.20 and 1.68 times more cDNA were produced using the ComboB26 primers. Therefore, it was concluded that there was a significant difference between the oligo dT variants and the effect of DTT (P<0.05). This conclusion was in agreement with the results derived from the semi-quantitative PCR analysis.

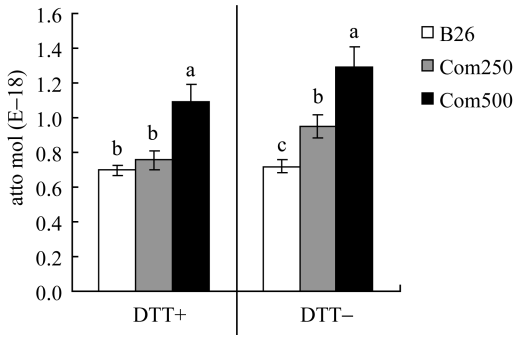

Fig. 3 Real-time PCR quantification of the amount of α-tubulin cDNA synthesized (mean±SE) according to oligo types used and the presence or absence of DTT in the cDNA synthesis protocol

Different letters indicate the significance of the differences at P<0.05 level. Com250, ComboB26-250; Com500, ComboB26-500; DDT+, plus DTT; DTT–, minus DTT.

Discussion

The objective of this study was to determine the factors that influenced the quantity and quality of first-strand cDNA prepared from plant RNA. This was done in the light of the work carried out using RNA derived from mammalian systems, which has shown that truncated cDNA products occur at a high frequency because of internal poly(A) sites [9]. In addition, the use of DTT has also been shown to affect the amount of cDNA in similar systems [10]. Therefore, these factors were assessed using plant-derived RNA populations for the first time.

In order to achieve this objective, a model cDNA system using α-tubulin as a standard template was developed, thereby allowing the potential impact of oligo dT variants and DTT to be assessed. We chose to restrict the comparison of one transcript to avoid the inherent sequence variability between different genes and therefore avoid differences in amplification efficiencies introduced by the template secondary structure. This in turn allowed the comparison of the effects of the oligo variants and DTT using two analytical techniques. It was reasoned that the effects would be genuine and not an artifact of the analytical technique if they were observed across two different platforms.

In summary, the analysis of the pooled data (thereby removing the influence of DTT) strongly suggested that using the anchored oligo dT consistently resulted in significantly higher amounts of cDNA, which in turn indicated greater efficiency with respect to reverse transcription. It was concluded that transcripts at low levels would more likely be recovered from an RNA population when using the ComboB26-500 oligo. The presence of DTT in the reaction consistently resulted in lower concentrations of cDNA, which was shown by the lower levels of product amplification either via 3′-RACE or nested PCR. The effects were more pronounced with 3′-RACE. Therefore, in addition to showing that the configuration of the oligo dT had a significant effect on the amount of cDNA synthesized, the absence of DTT from the synthesis reaction also had a very significant bearing on the efficacy of transcriptional analysis.

As stated previously, the objective was to assess the impact of oligo dT sequence and the effect of DTT on first-strand cDNA synthesis. Using 3′-RACE as an analytical tool, it was possible to clearly shown for the first time that significant improvements in the quantity and quality of the first-strand cDNA could be achieved in plant systems by using anchored primers and removing DTT from the synthesis reaction. More specifically, using ComboB26-500 consistently gave better results, clearly indicating that the amount of oligo alone was an important factor. In conclusion, incorporating the simple steps described in this article would significantly enhance sensitivity and reduce the inherent technical variability associated with microarray and other transcriptional analysis techniques.

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