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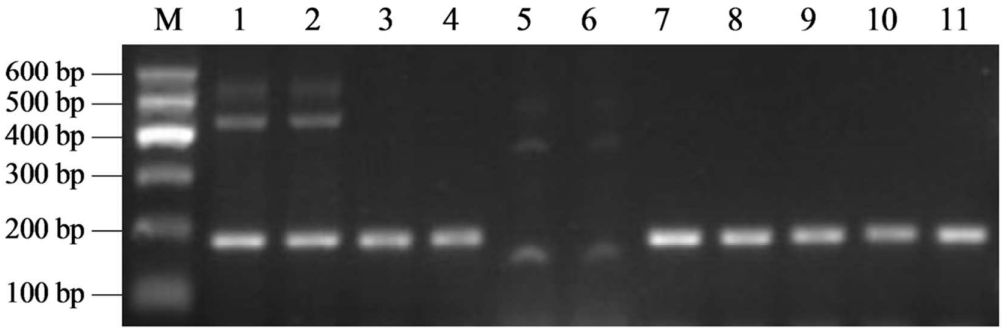

5′-end 6-mercapto-1-hexane-labeled single-stranded DNA (HS-ssDNA) can dramatically enhanced the specificity of DNA duplication during the polymerase chain reaction (PCR).

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ARTICLE TYPE

The regulation of PCR by thiolated single-stranded DNA for enhanced specificity

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In this study, we observed that 5′-end 6-mercapto-1-hexane-labeled single-stranded DNA (HS-ssDNA) dramatically enhanced the specificity of DNA duplication during the polymerase chain reaction (PCR). The regulation of PCR by HS-ssDNA is both intriguing and useful, and such findings further our understanding of the biochemical characteristics and potential applications of HS-ssDNA.

Because of its high efficiency, the polymerase chain reaction (PCR) has been used in several branches of biological science since its invention three decades ago. These include medical science, zoology, botany, microbiology and virology[1]. However, the remarkable ability of PCR to amplify specific DNA sequences, along with the obvious benefits this confers, is often compromised by various issues such as amplification of undesired sequences, low yield, primer self-ligation and self-amplification, or even complete amplification failure (no product)[2]. There is therefore still scope to improve PCR specificity and yield. Herein, we found that 5′-end 6-mercapto-1-hexane-labeled single-stranded DNA (HS-ssDNA) can improve the specificity of DNA duplication during PCR.

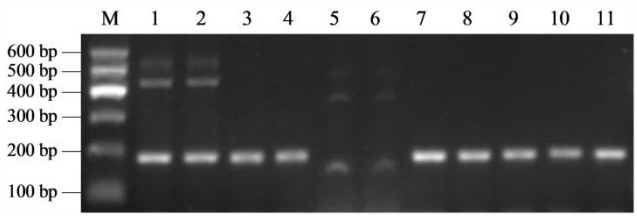

Fig. 1 The effect of HS-ssDNA on the specificity of PCR. Lane M shows the markers; lanes 1–2 show the results of duplicate PCRs performed in the absence of HS-ssDNA; lanes 3–4 show the results of duplicate PCRs performed in the presence of HS-ssDNA; lanes 5–6 show the results of duplicate PCRs performed in the presence of ssDNA alone; lanes 7–11 show the results of PCR performed in the presence of HS-ssDNA1, HS-ssDNA2, HS-ssDNA3, HS-ssDNA4 and HS-ssDNA5, respectively.
HS-ssDNA: HS-C_6H_{12}-GTATGTGCCCATGTG; ssDNA: GTATGTGCCCATGTG; HS-ssDNA1: HS-C_6H_{12}-CATACGCTCCAGACC; HS-ssDNA2: HS-C_6H_{12}-GCCCTCTACTCCACC; HS-ssDNA3: HS-C_6H_{12}-ACAGCCTCACTGGAA; HS-ssDNA4: HS-C_6H_{12}-TGACTCCATCATCTGTT; HS-ssDNA5: HS-C_6H_{12}-TAGGACAATCCGTATCT. The sequences of all HS-ssDNAs were random and the concentrations of the HS-ssDNAs or ssDNA in PCRs were 20 μM each.

PCR amplification of DNA within a complex plant genome was selected as the model system. A 195 bp-long fragment of the *CaMV35S* promoter gene[3], which is often used in genetically modified plants, was amplified by PCR from a mixture of DNA using 35 PCR cycles (F: GCTCCTACAAATGCCATCA; R: GATAGTGGGATTGTGCGTCA). The template DNA was prepared by mixing DNA from the genetically modified corn NK603 genome (containing the *CaMV35S* promoter gene) with DNA from conventional rice, cotton, and soybean genomes. Plant genomic DNA has a high molecular weight and interference from other genomic (rice, cotton, and soybean) DNA is often present[4]. Thus, the appearance of non-specific products in a Premix Ex Taq amplification system is expected. As shown in lanes 1–2 of Figure 1, two clear non-specific bands were observed at approximately 450 and 550 bp. Strikingly, in the presence of 20 μM 5′-thiolated single-stranded DNA (HS-ssDNA), the non-specific bands disappeared, thereby improving PCR specificity (lanes 3–4 in Fig. 1). In contrast, the specificity was not improved when using ssDNA alone; instead, the PCR amplification was negatively affected (lanes 5–6 in Fig. 1). Subsequently, we designed five other HS-ssDNAs of varying sequence with no homology to the template. These ssDNAs were modified with a thiol on the 5′ end and added to the PCR (HS-ssDNA1, HS-ssDNA2, HS-ssDNA3, HS-ssDNA4 and HS-ssDNA5). In all cases, specific amplification was obtained (lanes 7–11 in Fig. 1).

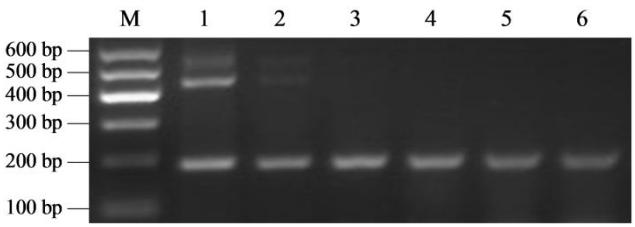

Fig. 2 The effect of HS-ssDNA T_m on PCR specificity. Lane M: markers; Lanes 1–6: Tm was 12.9°C, 30.3°C, 37.7°C, 47.4°C, 53°C, and 59.1°C, respectively. HS-ssDNA (12.9°C): HS-C_6H_{12}-GTATGTGC; HS-ssDNA (30.3°C): HS-C_6H_{12}-GTATGTGCCC; HS-ssDNA (37.7°C): HS-C_6H_{12}-GTATGTGCCCAT; HS-ssDNA (47.4°C): HS-C_6H_{12}-GTATGTGCCCATGTG; HS-ssDNA (53°C): HS-C_6H_{12}-GTATGTGCCCATGTGTTG; HS-ssDNA (59.1°C): HS-C_6H_{12}-GTATGTGCCCATGTGTTGCG. The concentrations of HS-ssDNAs in PCRs were 20 μM each.

The T_m of HS-ssDNA is critical for obtaining optimal PCR results. HS-ssDNAs of different T_m values were added separately to the PCR mixtures. We observed that non-specific bands gradually diminished by increasing the T_m of the HS-ssDNA. As shown in Fig. 2, in the presence of HS-ssDNA we obtained a single product band when the T_m of the HS-ssDNA was ≥37.7°C.

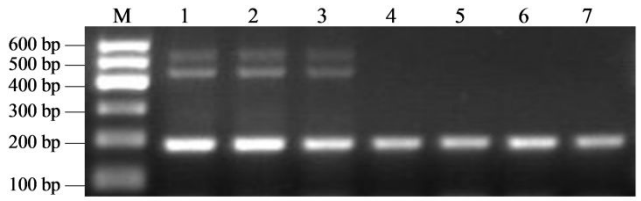

Fig. 3 The effect of HS-ssDNA concentration on the specificity of PCR: lane M: markers; lane 1: 0 μM; lane 2: 5 μM; lane 3: 10μM; lane 4: 15 μM; lane 5: 20 μM; lane 6: 25 μM; lane 7: 30 μM. (HS-ssDNA: HS-C_6H_{12}-GTATGTGCCCATGTG; Tm 47.4°C)

To test the effect of HS-ssDNA concentration, different amounts of HS-ssDNA were added to the PCR. As shown in Fig. 3, the non-specific bands gradually disappeared with increasing HS-ssDNA concentration and no non-specific bands were observed at final concentrations of 15 μM or higher.

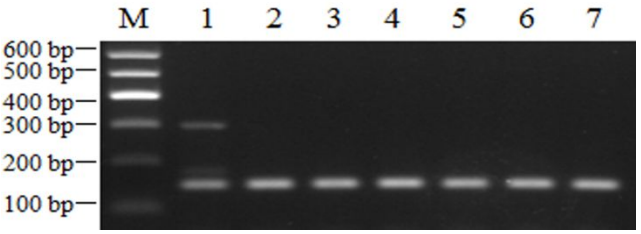

Fig. 4 The effect of HS-ssDNA on the specificity of PCR. Lane M shows the markers; lane 1 show the results of PCR performed in the absence of HS-ssDNA; lanes 2–7 show the results of PCR performed in the presence of HS-ssDNA, HS-ssDNA1, HS-ssDNA2, HS-ssDNA3, HS-ssDNA4 and HS-ssDNA5, respectively.

To confirm the reliability of the effect of HS-ssDNA on PCR specificity, we validated this approach using another complex template for PCR amplification. Primers F/R[5] (F: CTCCCAATCCTTTGACATCTGC; R: TCGATTTCTCTCTTGGTGACAGG) were used to amplify the endogenous reference gene *zSSIIb* of the glyphosate-resistant transgenic maize MON88017. In addition to the target product (151 bp), a non-specific band was observed at approximately 170 and 300 bp (Fig. 4, lane 1). As expected, upon addition of 20 μM HS-ssDNA to the PCR system, the non-specific bands disappeared, confirming the versatility of the HS-ssDNA effect.

Before adding HS-ssDNA to the PCR systems, we dissolved the HS-ssDNA in 1 mM 1,6-dithiothreitol (DTT) solution to maintain the thiol in the reduced state (In fact, in order to retain Taq polymerase activity, PCR buffers often contain 10 μM DTT). We then added DTT solution alone to control (no HS-ssDNA oligos) PCR systems to give final DTT concentrations of 0.05, 0.1, 0.15, 0.2, 0.25 and 0.3 mM. We also set up systems with un-modified 5 μM ssDNA+0.2 mM DTT, 10 μM ssDNA+0.2 mM DTT, 15 μM ssDNA+0.2 mM DTT, 20 μM ssDNA+0.2 mM DTT, 25 μM ssDNA+0.2 mM DTT and 30 μM ssDNA+0.2 mM DTT. Because no improvement was observed from using DTT alone or ssDNA+ DTT (see Fig.S1 in the Supporting Information), the possibility of this effect being because of the presence of DTT or ssDNA+ DTT could be eliminated. As shown in Fig. 2, no enhancement of specificity was observed when Tm of HS-ssDNA was 12.9°C or 30.3°C (lanes 1–2); when the Tm of HS-ssDNA was 37.7, 47.4, 53 or 59.1°C, specificity was enhanced (lanes 3–6). However, DTT concentrations in lanes 1–6 were all 0.2 mM. This result also proves that DTT had no effect on enhancing PCR specificity.

During an experiment in producing nanoprobes by conjugating nano-gold and HS-ssDNA, we accidentally added HS-ssDNA to a PCR, and were surprised to achieve enhanced specificity. We then tried modifying one end of the ssDNA with other groups (N_3-, COOH-, $NH_2C_6H_{12}$-, CHCH-, and Methylene blue-), before adding the modified ssDNAs to the PCR reactions and observing their effects on PCR specificity. The results showed no specificity-enhancing effect (data not shown). In contrast, when modified with $NH_2C_6H_{12}$-, the PCR products showed more non-specific bands. Moreover, as shown in Fig. 1, lanes 5 and 6, the addition of non-thiolated ssDNA did not reduce non-specificity and instead, negatively affected the reaction. This is possibly attributable to interference by the relatively high concentration of ssDNA (20-fold or more compared to the PCR primers). Thus, tethered thiol groups play a critical role in PCR specificity optimisation by HS-ssDNA. According to the results of the HS-ssNDA T_m optimisation experiment shown in Fig. 2, an appropriate T_m value (≥37.7°C) was another important factor in the HS-ssDNA effect. In summary, a thiol end group conjugated to a random sequence of single-stranded DNA with an appropriate T_m are the two key conditions in PCR specificity optimisation by HS-ssDNA.

PCR primers obviously need to complementarily pair to the template for amplification to occur, but HS-ssDNA, having no homology to the template, cannot anneal to either of these. Despite the primer sets used in PCR amplification of the two genes (*CaMV35S* promoter gene and the endogenous reference gene *zSSIIb*) being different in sequence, the presence of HS-ssDNA increased the reaction specificity for both reactions. Therefore, we believe it to be unlikely that any interactions between the HS-ssDNA and the primers led to the observed specificity enhancement. However, it should be noted that the specific product yield decreased slightly with increasing HS-ssDNA concentration (Fig. 3). This is possibly attributable to interference by the excessd HS-ssDNA with the coupling efficiency of the primers and template.

As Taq polymerase contains an active thiol group, it could possibly bind covalently to the reduced thiol in HS-ssDNA to produce a Taq-polymerase-S-S-ssDNA dimer. The bound ssDNA may have previously unidentified biochemical characteristics which are independent of DNA sequence. This may increase the activity of Taq polymerase and greatly reduce the error rate in nucleotide pairing during DNA replication, thereby enhancing PCR specificity. This may also explain why adding thiolated oligos with various sequences produced the same increased specificity. Nevertheless, the mechanism of enhanced PCR specificity due to HS-ssDNA requires further study.

Conclusions

Because of the large size of genomic DNA and complex DNA

templates, as well as the challenge in designing perfect primers, it is difficult to avoid at least some nonspecific amplification products. In order to address this problem, we have developed a novel PCR strategy for obtaining specific DNA products *in vitro*. Thus, use of these commercially available thiolated DNAs opens up new opportunities for improving PCR, which is arguably the most important tool in molecular biology. In addition, we believe that our study on the use of HS-ssDNA in PCR suggests further avenues for exploring other biochemical effects of HS-ssDNA. It should prove very interesting to see how the scope of HS-ssDNA chemistry continues to expand in future research.

Notes and references

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Experimental Section

The transgenic maize NK603 and MON88017 were maintained and supplied by our laboratory. Genomic DNA was extracted and purified using the Plant DNA Mini Kit (Omega Bio-tek Inc., USA) according to the manufacturer's instructions. All primers and HS-ssDNAs were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The T_m values of the HS-ssDNAs were calculated using Oligo 7.4 software (Molecular Biology Insights Inc., USA). For PCR reactions, 20 μL reactions comprising 1× Premix ExTaq (containing PCR buffer, Mg^{2+}, dNTPs and ExTaq DNA polymerase; TaKaRa Biotech Co., Ltd., Dalian, China), 500 nM of each primer and 50 ng genomic DNA, were used. Aliquots of HS-ssDNA were added to the PCR system to the appropriate concentrations. To keep the HS-ssDNA thio group in the reduced state, we treated HS-ssDNA with 1 mM DTT to produce 100 μM HS-ssDNA. PCR amplifications were performed in a Bio-Rad thermal cycler (USA) using the following cycling conditions: 95°C for 5 min; 35 cycles at 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s; and a final elongation step at 72°C for 5 min. PCR products were electrophoresed for approximately 35 min at 120 V on a 1.5% (w/v) agarose gel stained with ethidium bromide.

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