



*Genetic Resources*

## **Detection of Transgenes in Crop Plants Using Molecular Beacon Assays**

R. KOTA\*, T.A. HOLTON and R.J. HENRY

*Centre for Plant Conservation Genetics, Southern Cross University, P.O. Box 157, Lismore NSW, Australia 2480*

**Abstract.** Molecular beacons are oligonucleotide probes that form a stem-and-loop structure and possess an internally quenched fluorophore. When they bind to complementary targets, they undergo a conformational transition that turns on their fluorescence. These probes recognise their targets with higher specificity than linear probes and can easily discriminate targets that differ from one another by a single nucleotide. As a model system to test the applicability of molecular beacons in crop plants, we have designed a molecular beacon to detect the *bar* transgene in barley. Results from this experiment indicate that molecular beacons can be successfully employed in detecting transgenes, simultaneously combining the benefits of being highly reproducible and sensitive. The molecular beacon assay is suitable for diagnostics, simultaneously being employed in the development of rapid DNA-based assays for analysing single nucleotide polymorphisms (SNPs).

**Key words:** barley, molecular beacons, PCR, SNP, transgenic

**Abbreviations:** PCR, polymerase chain reaction; SNP, single nucleotide polymorphism.

### **Introduction**

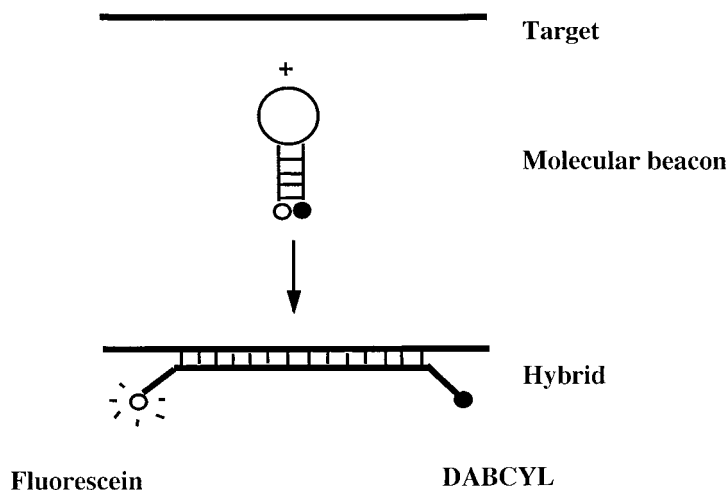
Many methods used for genomic DNA analysis rely on amplification of target sequences by PCR, followed by separate product analysis. Post-PCR analysis of amplification products is commonly performed by hybridisation analysis using blots, gel electrophoresis or enzyme assays in microtitre plates. Despite their advantages, PCR-based methods are not widely employed for routine analysis of transgenic plants. This is primarily because existing assays are labour-intensive, relatively time-consuming and expensive. In addition, gel-based assays are also not ideally suited for automation procedures.

\*Author for correspondence. e-mail: rkota@scu.edu.au; fax: 61-2-66222080; ph: 61-2-66203457

An ideal system for high-throughput testing would involve a 'homogeneous PCR assay', i.e., one in which the processes of amplification and detection are performed simultaneously. A homogeneous assay would allow specific PCR products to be monitored within a closed reaction tube which would allow post-PCR automation. Higuchi et al. (1992, 1993) developed the first homogeneous real-time detection of PCR products. They exploited the fact that fluorescence of the intercalator dye ethidium bromide increases in the presence of double-stranded DNA (dsDNA). By monitoring fluorescence during amplification, they could continuously follow the progress and kinetics of a PCR reaction. Despite the obvious advantages of this intercalator dye system, its major disadvantage is its non-specificity i.e., it cannot discriminate between the PCR fragment of interest and the common PCR background from mis-priming and primer-dimer artefacts leading to false positive results.

In recent years, assays such as molecular beacons have been making an impact on the development of rapid non-gel based assays. Molecular beacons are single-stranded nucleic acid molecules that possess a stem-and-loop structure (Tyagi and Kramer, 1996). The loop portion of the molecule serves as a probe sequence that is complementary to a target nucleic acid. The stem is formed by the annealing of the two complementary arm sequences that are on either side of the probe sequence (Figure 1). A fluorescent moiety is attached to the end of one arm and a nonfluorescent quenching moiety is attached to the end of the other arm. The stem hybrid keeps the fluorophore and the quencher so close to each other that the fluorescence does not occur (Tyagi and Kramer, 1996). The fluorophore-quencher pairing represents a unique system in which the energy received by the fluorophore is directly transferred to the quencher and dissipated as heat, rather than being emitted as light resulting in the fluorophore being unable to fluoresce (Tyagi et al. 1998). When the molecular beacon encounters a target sequence, it forms a probe-target hybrid that is stronger and more stable than the stem hybrid. The probe undergoes spontaneous conformational reorganisation that forces the arm sequences apart, separating the fluorophore from the quencher, and permitting the fluorophore to fluoresce (Bonnet et al., 1999). The power of molecular beacons lies in their ability to hybridise only to target sequences that are perfectly complementary to the probe sequence, hence permitting detection of single base differences (Tyagi et al., 1998).

Routine PCR followed by gel electrophoresis and detection has previously been used to detect transgenes in plants (Abedinia et al., 1997). However, the application of molecular beacon assays for detecting transgenes in plants has not been tested. To test the applicability of assays such as molecular beacons for discriminating between transgenic and non-transgenic plants, we developed a molecular beacon probe to detect the presence or absence of



*Figure 1.* Principle of molecular beacon assay. The hairpin stem formed by the complementary arm sequences cannot coexist with the double helix that is formed when the probe hybridises to its target. Consequently, the molecular beacon undergoes a conformational change that forces the arm sequences apart and causes the fluorophore to move away from the quencher, thereby allowing fluorescence.

the transgene *bar* in barley. The *bar* transgene contains the coding region of *Streptomyces hygrosopicus* phosphinothricin acetyltransferase gene which has been extensively used as a selectable marker in genetic transformation studies. In this study, we demonstrate that molecular beacons have the potential to be applied for large-scale screening of transgenic plants and also envisage its applicability to the development of assays for rapidly analysing single nucleotide polymorphisms (SNPs) in plants.

### Materials and Methods

The transgenic barley plants were generated by transformation of Golden Promise variety with plasmid pTO134 via *Agrobacterium tumefaciens*-mediated transformation using the method of Tingay et al. (1997).

To determine the presence of the *bar* gene, we combined the sensitivity of PCR with the specificity of molecular beacons. Molecular beacon specific to the *bar* gene (5'-FAM-ccgacgTCAACTTCCGTACCGAGCCGcgtcgg-DABCYL-3') was synthesised at Research Genetics (USA). PCR primers BarF (5'-ACCATCGTCAACCACTACATCG-3') and BarR (5'-GAGGTCGTCCGTC CACTCCTG-3') were selected from the region flanking the molecular beacon sequence. Real-time monitoring of PCR was done by measuring the fluorescence generated by the hybridisation of the molecular beacon to its

perfectly matching target during PCR. Before performing the PCR, thermal denaturation profiles of the hybrids formed by the molecular beacon and their perfect and mis-match oligonucleotide targets was determined. To achieve this, we monitored the fluorescence of a 50  $\mu$ l solution containing 0.6  $\mu$ M molecular beacon probe, 2.4  $\mu$ M oligonucleotide target, 1 mM  $MgCl_2$  and 20 mM Tris-HCl (pH 8.0), as a function of temperature. The temperature was increased from 30 °C to 80 °C in 1 °C increments, with each temperature being held for 1 min. Fluorescence was monitored during each period by using a fluorescence reader with a programmed temperature control (PE/ABI 7700; Perkin-Elmer, USA).

To perform the PCR, primers (BarF and BarR) were designed to amplify a 86 bp region within the *bar* gene. PCR conditions used consisted of 1  $\times$  PCR buffer (Perkin-Elmer, USA), 4 mM  $MgCl_2$ , each of the oligonucleotides (0.25 mM), 2.5 U of AmpliTaq Gold DNA polymerase (Perkin-Elmer, USA), 0.5  $\mu$ M of each primer, 0.3  $\mu$ M of the molecular beacon and 50 ng genomic DNA. Cycling was preceded by 10 min at 95 °C for activation of the AmpliTaq Gold DNA polymerase, followed by 45 cycles of 30 s at 95 °C, 60 s at 55 °C and 45 s at 72 °C. Fluorescence was measured during the annealing phase of the PCR and plotted automatically for each sample. For visual detection of the amplicons, the molecular beacon was used at 0.6  $\mu$ M. After the completion of the PCR reaction, the tubes were placed on a UV light source and photographed. All experiments were repeated to confirm reproducibility of results.

## Results and Discussion

To detect the temperature range in which the molecular beacon is able to discriminate between transgenic and non-transgenic barley, we determined the DNA melting profiles of hybrids between the molecular beacon and either perfect or single base mis-match oligonucleotide targets (Figure 2). Results indicated that at the temperature of 55 °C, the target oligonucleotide elicited strong fluorescence, whereas the mis-match oligo did not, thus allowing a clear discrimination between the samples. On the basis of the thermal denaturation profile, we chose 55 °C as the annealing temperature for the PCR.

DNA samples from the transgenic and non-transgenic barley were used to determine the efficiency of the molecular beacon to distinguish between the two samples. During PCR cycles, at 95 °C the molecular beacons are denatured and have a random coil structure, allowing full fluorescence. Decreasing of the temperature to 55 °C during the annealing step enables the formation of hairpins, which causes a drop in fluorescence. However, in the presence of

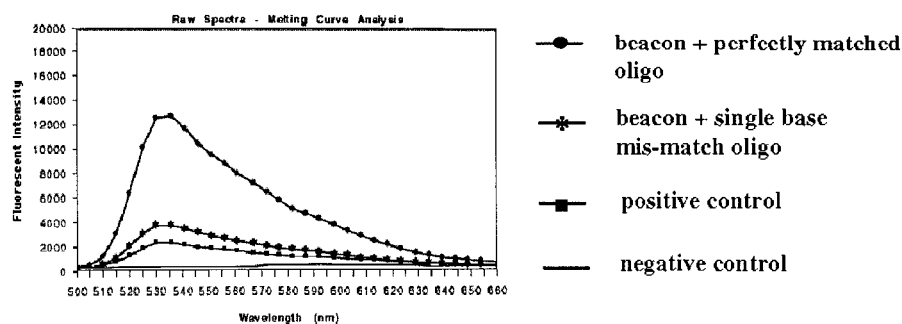


Figure 2. Thermal denaturation profiles of the molecular beacon used in this study. The above profile displays that at approximately 55 °C, the molecular beacon allows a clear discrimination between perfect or mismatch oligonucleotide targets respectively.

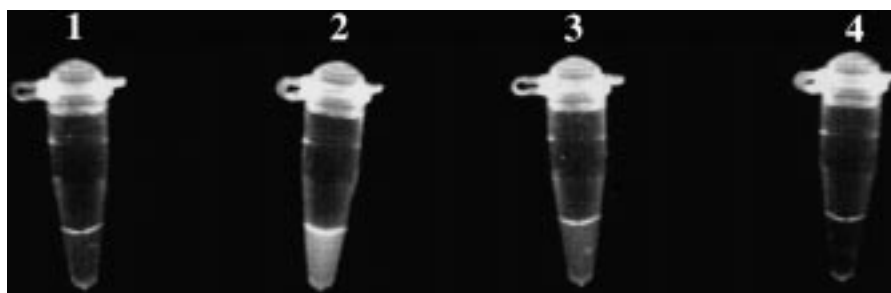


Figure 3. Illumination of the PCR reactions by an ultraviolet source. Tube 1: wild-type genomic DNA, tube 2: transgenic genomic DNA containing *bar*, tube 3: positive control, tube 4: negative control.

a genomic DNA target, the probe sequence of the molecular beacons bind to their complementary sequences and their fluorescence increases and it is at this step that the fluorescence is measured. During primer elongation at 72 °C, molecular beacons are dissociated from their target.

Initial experiments employing the molecular beacon were performed on a standard thermal cycler (PE9700; Perkin-Elmer, USA) with the results displayed in Figure 3. Although the *bar*-specific molecular beacon was present in tubes 1, 2 and 3; fluorescence was visibly detected only when the molecular beacon formed a hybrid with the target sequence. These results demonstrate the potential for molecular beacon assays to be performed on samples without the need for real-time fluorescent measurements. This would allow laboratories with limited resources to employ molecular beacon assays for rapid discrimination among samples.

Further experiments carried out on a PE7700 (Perkin-Elmer, USA) indicated that the molecular beacons could successfully be employed in detecting transgenes in plants (Figure 4). The fluorescent signal was generated by the

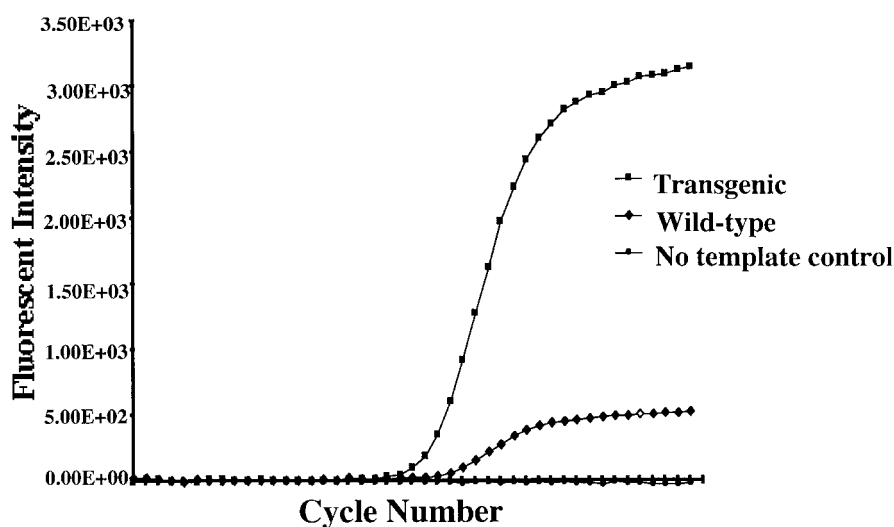


Figure 4. Results obtained after PCR with a *bar* specific molecular beacon for a transgenic individual containing the *bar* gene (top), a wild-type individual (middle) and a no-template control (bottom).

molecular beacon complementary to the *bar* gene (top) whereas the sample containing the wild-type DNA (middle) did not generate any significant increase in the signal. No template control (bottom) did not display any increase in fluorescence (Figure 4). Depending upon the PCR cycling times, samples can be completely analysed in less than 3 hours with the molecular beacons, eliminating the need for post PCR experiments. This method is highly reproducible, sensitive and generates highly informative data. One of the most important advantages is that tubes remain closed during the monitoring of the fluorescence, thereby eliminating the risk of carryover contamination and the generation of false positive results. The results from these studies indicate that molecular beacons can be successfully adapted to analyse agronomically important genes in crops. More importantly, it should also be possible to employ the molecular beacon assay to determine the copy number of transgenes (Tyagi et al., 1998).

One of the main advantages of molecular beacons are their ability to discriminate between samples with single nucleotide differences. In comparison with linear probes, the high specificity of interaction of molecular beacons with their target is directly attributable to the stem structure of the molecular beacon (Tyagi et al., 1998). Hybrids formed between molecular beacons and mis-match targets dissociate at a much lower temperature than hybrids formed between linear probes and mismatch targets (Giesendorf et al., 1998). Thus a wider temperature range is observed between melting of the perfect

and mismatch hybrid. This enables a common temperature to detect several alleles or genes in a single reaction tube.

The ability of molecular beacons to analyse single nucleotide differences has been exploited to develop rapid tests for SNP analysis. For instance, Piatek et al. (1998) employed this assay for detecting drug resistance in *Mycobacterium tuberculosis*. Similarly, Giesendorf et al. (1998) used this assay to detect a point mutation in the *methylenetetrahydrofolate reductase* (*MTHFR*) gene in humans. Although limited sequence information of the many agronomically important plants is presently available, it is envisaged that the molecular beacon assays will have the potential to rapidly analyse SNPs in plants, and that these probes will find their way into nucleic acid research and diagnostics.

### Conclusions

The molecular beacon assay described here can be successfully employed in detecting transgenes in plants, and allow the processes of PCR amplification, product detection, data processing and results presentation to be automated. Using target-specific molecular probes, only amplification of the intended sequence is measured. Also, the ability to detect PCR products within closed tubes greatly reduces the risk of crossover contamination. Molecular beacons along with the recent advances in fluorogenic assays (eg. TaqMan technology) provide new and powerful tools for a wide range of PCR-based applications.

### Acknowledgements

We thank Dr Sanjay Tyagi at the Public Health Research Institute, New York for his technical assistance; Dr Don Maclean, Dr Juliane Henderson and Ms Lisa Heelan at University of Queensland for allowing the use of ABI7700. The transgene barley plants were generated by Dr Dhar Akula. This work was supported by grants from the GRDC program, Australia.

### References

- Abedinia M, Henry RJ, Blakeney AB and Lewin L (1997) An efficient transformation system for the Australian rice cultivar, Jarrah. *Aust J Plant Physiol* 24: 133–141.
- Bonnet G, Tyagi S, Libchaber A and Kramer FR (1999) Thermodynamic basis of the enhanced specificity of structured DNA probes. *Proc Natl Acad Sci USA* 96: 6171–6176.

- Giesendorf BAJ, Vet JAM, Tyagi S, Mensink EJMG, Trijbels FJM and Blom HJ (1998) Molecular beacons: a new approach for semiautomated mutation analysis. *Clinical Chemistry* 44: 482–486.
- Higuchi R, Dollinger G, Walsh PS and Griffith R (1992) Simultaneous amplification and detection of specific DNA sequences. *Bio-Technology* 10: 413–417.
- Higuchi R, Fockler C, Dollinger G and Watson R (1993) Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. *Bio-Technology* 11: 1026–1030.
- Piatek AS, Tyagi S, Pol AC, Telenti A, Miller LP, Kramer FR and Alland D (1998) Molecular beacon sequence analysis for detecting drug resistance in *Mycobacterium tuberculosis*. *Nat Biotechnol* 16: 359–363.
- Tingay S, McElroy D, Kalla R, Fieg S, Wang M, Thornton S and Brettell (1997) *Agrobacterium tumefaciens*-mediated barley transformation. *Plant J* 11: 1369–1376.
- Tyagi S and Kramer FR (1996) Molecular beacons: probes that fluoresce upon hybridisation. *Nat Biotechnol* 14: 303–308.
- Tyagi S, Bratu D and Kramer FR (1998) Multicolour molecular beacons for allele discrimination. *Nat Biotechnol* 16: 49–53.