Using 23S rDNA to identify contaminations of Escherichia coli in Agrobacterium tumefaciens cultures

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Abstract

Cross contamination of *Agrobacterium tumefaciens* stocks with *Escherichia coli* are difficult to identify by microbiological techniques, leading to false negative results in transformation experiments. We have developed a genotyping assay for *A. tumefaciens* and *E. coli* lab strains based on amplification of 23S rDNA by PCR. *Agrobacterium* strains LBA 4404, C58 and EHA105 and E. coli DB3.1, DH5α and XL1-Blue can be identified separating the corresponding PCR amplicons in 2.5% agarose gels. However in crossed contaminations, interpretation of results is improved using melting point analysis on a quantitative PCR machine.

Keywords: Bacteria; Genetic transformation; PCR;

1. Introduction

Handling of different microbial strains in laboratory practice can turn into a problem when different bacteria become contaminated with each other. In most cases, contamination of bacterial strains is the result of sharing plasticware, pipettes and solutions. Furthermore, although different bacterial genera can be grown on specific media, simplicity has led to a generalized use of common growth media as long as they do not interfere with bacterial properties. Two types of bacterial genera are routinely used in plant biology laboratories, *Escherichia coli* used for general cloning and *Agrobacterium tumefaciens* to obtain transgenic plants.

Recent advances in cloning strategies based on recombination cloning coupled with virus-induced gene silencing (VIGS) [1] allow research projects where hundreds to thousands of genes can be tested for loss of function. The standard procedure is the construction of cDNA libraries and development of recombinant libraries in vectors suitable for VIGS. These recombinant libraries are then transferred to *Agrobacterium* that is used to obtain transiently transformed plants expressing the cDNA cloned as double stranded RNA that causes post transcriptional gene silencing. This technology has been shown to work in different plants like *Papaver* [2], potato [3] or petunia [4].

But one obvious problem is that if *Agrobacterium* strains are contaminated with *E. coli*, then some of the colonies processed will not transform plants giving as a result a false negative. Although *E. coli* and *Agrobacterium* can be differentiated by microbiological techniques, it is much faster to establish a genotyping protocol based on PCR that will allow the identification of *E. coli* contaminations in those cases where there is doubt of the purity of the *Agrobacterium* strain. In this study we report the development of a genotyping assay to distinguish between *E. coli* and *Agrobacterium*.

2. Materials and Methods

2.1 Strains

The strains used were in the case of *E. coli* DH5α, and DB3.1 from Invitrogen and XL1-Blue from Stratagene. Whereas that for *Agrobacterium* the strains used were LBA 4404, C58 and EHA105.

2.2 Bacterial culture

We grew on LB medium to complete saturation the *E. coli* strains DH5α, and DB3.1 and XL1-Blue at 37ºC while the *Agrobacterium* strains LBA 4404, C58 and EHA105 were grown at 28ºC.

2.3 DNA extraction

Extraction of bacterial genomic DNA was performed starting from 1.5mL of saturated culture. Samples were centrifuged and the

bacterial pellets were resuspended in 1 mL of TE buffer (10 mM Tris HCL, 1mM EDTA, pH 8.0) and lysed with 200 µg of lysozyme and 1% SDS. Protein was digested with 500 µg of proteinase K incubating at 65ºC for one hour. Genomic DNA was purified from the samples by standard phenol:chloroform extraction and precipitation [5]. The resulting pellets were resuspended in 100 µL of TE buffer.

2.4 PCR

Amongst the possible genome regions valuable for genotyping, the intergenic transcribed spacer (ITS) has been found useful in plants [6], the 23S rDNA and the 16S-23S rDNA spacer in bacteria [7], or 5.8S-ITS region to identify yeast contamination in food samples [8]. We used specific primers that amplify the bacterial 23S rDNA (23S-forward ACCAGGATTTTGGCTTAGAAG and 23S-reverse CACTTACCCCGACAAGGAAT) [7] in a standard PCR experiment performed with Taq polymerase from Promega. The cycling conditions consisted in thirty cycles of 30 seconds at 94ºC, 30 seconds at 62ºC and 30 seconds at 72ºC. Reaction products were separated using 2.5% agarose gels containing ethydium bromide, and visualized with a UV transilluminator.

3. Results and Discussion

Amplification products from *Agrobacterium* and *E. coli* could be visually differentiated on the gel, showing PCR fragments of 780 and 850bp respectively (Fig.1A). This suggests that direct analysis of PCR products is feasible. However we found that in complex mixtures of bacterial strains i.e when there is contamination of *Agrobacterium* with *E. coli*, it would be better to have a more powerful assay since the size differences between amplicons make results difficult to interpret on agarose gels (data not shown). We solved this problem by performing a qPCR on a CR-Corbett Research-quantitative PCR using TaKara Sybr Green quantitative PCR amplification kit. The PCR conditions were an initial denaturation of 5 minutes at 95ºC followed by forty cycles with a scheme of 95ºC 30 seconds, 62ºC 30 seconds, 72ºC 30 seconds, a read at 83ºC for 15 seconds and finally a melting point analysis starting at 60ºC and reaching 94ºC with reads every 0.5ºC for 15 seconds. The melting point of the 23S rDNA region of *Agrobacterium* and *E. coli* differed by 1.6ºC (87.9 and 89.5 respectively), giving clear-cut results that allow the genotyping of *Agrobacterium* and *E. coli* (Fig 1.B).

4. Conclusions

In summary, we developed a PCR assay to genotype *Agrobacterium* and *E. coli* that helps overcome situations of cross contamination between these types of bacteria. The protocol presented should be useful to test clones that do not appear to transform plants, and this lack of transformation capacity could be the result of contaminated bacterial stocks. Our approach has the additional advantage of being based on codominant PCR markers that should be more useful than *Agrobacterium* or *E. coli* specific PCR makers.

5. Acknowledgements

This work was financed by the Fundación Séneca de la Región de Murcia and BIOCARM.

6. References

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Figure

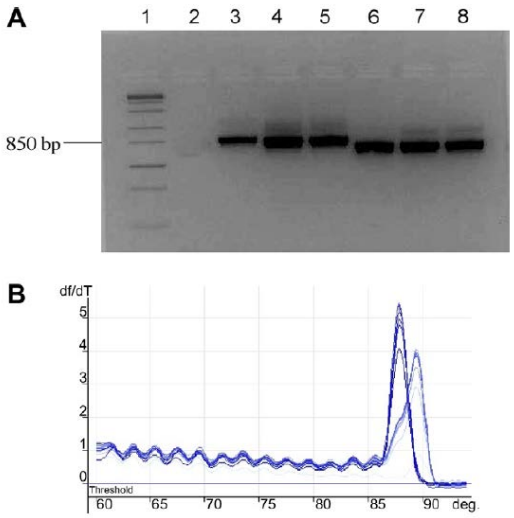

Figure 1. (A) *Amplification of 23S rDNA from bacterial samples by PCR. Lane 1 molecular weight. Lane 2 negative control; Lane 3 E. coli DB3.1; Lane 4 E.coli DH5α; Lane 5 XL1-Blue; Lane 6 A. tumefaciens LBA4404, Lane 7 C58; Lane 8 EHA105. Molecular weight markers were ZipRuler ladder-1 from Fermentas with molecular weights of 10000, 5000, 3000, 2000, 1200, 850, 500, 300 and 100 bp.* ***(B)*** *Melting curve of samples of A. tumefaciens and E. coli. The peak at 89.5 degrees corresponds to duplicated samples of DB3.1, DH5α and XL1-Blue, while the peak at 87.9 corresponds to LBA4404, C58 and EHA105 A.tumefaciens strains*