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RESEARCH ARTICLE

Screening internal controls for expression analyses involving numerous treatments by combining statistical methods with reference gene selection tools

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Abstract Real-time PCR is always the method of choice for expression analyses involving comparison of a large number of treatments. It is also the favored method for final confirmation of transcript levels followed by high throughput methods such as RNA sequencing and microarray. Our analysis comprised 16 different permutation and combinations of treatments involving four different *Agrobacterium* strains and three time intervals in the model plant *Arabidopsis thaliana*. The routinely used reference genes for biotic stress analyses in plants showed variations in expression across some of our treatments. In this report, we describe how we narrowed down to the best reference gene out of 17 candidate genes. Though we initiated our reference gene selection process using common tools such as geNorm, Normfinder and BestKeeper, we faced situations where these software-selected candidate genes did not completely satisfy all the criteria of a stable reference gene. With our novel approach of combining simple statistical methods such as *t* test, ANOVA and post hoc analyses, along with the routine software-based analyses, we could perform precise evaluation and we identified two genes, *UBQ10* and *PPR* as the best reference genes for normalizing mRNA levels in the context of 16 different conditions of *Agrobacterium* infection. Our study emphasizes the usefulness of applying statistical analyses along with the reference gene selection software for reference gene identification in experiments involving the comparison of a large number of treatments.

Keywords Reference genes · *Arabidopsis* · *Agrobacterium* · Stable expression · Normalization · Real-time PCR

Introduction

Studies on the response of plants towards pathogen stress are often initiated with expression profiling of host genes using techniques involving real-time PCR analysis, microarray, or transcriptome sequencing. Microarrays and transcriptome sequencing are high throughput techniques that reveal the expression response of almost whole of the genome. Real-time PCR is for specific gene expression analysis and is also used for the reconfirmation of microarray-derived and transcriptome sequencing-derived data. Real-time PCR quantifies the exponential amplification of a specific transcript by monitoring newly synthesized DNA in each PCR cycle (Higuchi et al. 1993).

One of the hindrances in doing real-time PCR is the selection of an internal control/reference gene. Reliability of real-time PCR is highly dependent on the stability of internal control, although other factors such as quality of template, and data evaluation are equally important. The use of inappropriate reference genes can lead to improper results that may draw false conclusions (Gutierrez et al. 2008; Guénin et al. 2009). Selection of an internal control often becomes a tedious task when a large number of conditions/treatments are to be considered because its expression should be stable in all the conditions/treatments under study and several genes may need to be systemically evaluated.

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