Journal of Integrative Agriculture 2017, 16(4): 809–819

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RESEARCH ARTICLE

Identification of suitable reference genes in leaves and roots of rapeseed (*Brassica napus* L.) under different nutrient deficiencies

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Abstract

Nutrient deficiency stresses often occur simultaneously in soil. Thus, it's necessary to investigate the mechanisms underlying plant responses to multiple stresses through identification of some key stress-responsive genes. Quantitative real-time PCR (qRT-PCR) is essential for detecting the expression of the interested genes, of which the selection of suitable reference genes is a crucial step before qRT-PCR. To date, reliable reference genes to normalize qRT-PCR data under different nutrient deficiencies have not been reported in plants. In this study, expression of ten candidate reference genes was detected in leaves and roots of rapeseed (*Brassica napus* L.) after implementing different nutrient deficiencies for 14 days. These candidate genes, included two traditionally used reference genes and eight genes selected from an RNA-Seq dataset. Two software packages (GeNorm, NormFinder) were employed to evaluate candidate gene stability. Results showed that *VHA-E1* was the highest-ranked gene in leaves of nutrient-deficient rapeseed, while *VHA-G1* and *UBC21* were most stable in nutrient-deficient roots. When rapeseed leaves and roots were combined, *UBC21*, *HTB1*, *VHA-G1 and ACT7* were most stable among all samples. To evaluate the stabilities of the highest-ranked genes, the relative expression of two target genes, *BnTrx1;1* and *BnPht1;3* were further determined. The results showed that the relative expression of *BnTrx1;1* depended on reference gene selection, suggesting that it's necessary to evaluate the stability of reference gene prior to qRT-PCR. This study provides suitable reference genes for gene expression analysis of rapeseed responses to different nutrient deficiencies, which is essential for elucidation of mechanisms underlying rapeseed responses to multiple nutrient deficiency stresses.

Keywords: reference genes, rapeseed (*Brassica napus* L.), nutrient deficiency, leaves, roots

1. Introduction

Quantitative real-time PCR (qRT-PCR) is regarded as a preferred technique for rapid detection and quantification of gene expression due to its outstanding accuracy, specificity and convenience (Ginzinger 2002). However, the accuracy of qRT-PCR results can be affected by several factors, such as variable RNA quality, nonspecificity or bias in cDNA synthesis, inefficient PCR amplification, and inaccurate data

Received 26 April, 2016 Accepted 17 June, 2016
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doi: 10.1016/S2095-3119(16)61436-3

analysis (Czechowski *et al.* 2005). In addition, selection of one or more suitable reference genes is crucial prior to qRT-PCR (Czechowski *et al.* 2005). In short, choosing reliable reference genes as internal controls to normalize gene expression in qRT-PCR is extremely important for reducing errors and determining accurate expressions of target genes.

Traditionally used reference genes exhibiting relatively stable expression among plant tissues are typically housekeeping genes, such as 18S ribosomal RNA (*18S rRNA*), actin (*ACT*), tubulin (*TUB*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and elongation factor1-α (*EF1*α) (Hao *et al.* 2014). Ideal reference genes would be constitutively and stably expressed regardless of growth conditions or developmental stage. However, several recent studies have found that expression of housekeeping genes are not always stable, with variation in expression detected among species and experimental treatments (Artico *et al.* 2010; Qi *et al.* 2010; Pollier *et al.* 2014; Roberta *et al.* 2015; Ferradás *et al.* 2016). In recent years, researchers have selected several reference genes under different stress conditions, such as drought, salinity, heat, cold, and boron toxicity (Wang *et al.* 2014; Ferdous *et al.* 2015). These results strongly suggest that it is necessary to perform systematic verification of reference genes under specific experimental conditions before using them for qRT-PCR normalization.

Rapeseed (*Brassica napus* L.) is one of the most important crops for vegetable oil, biofuel and animal feed worldwide. Rapeseed production is often limited by various abiotic stresses, such as drought, salinity, heavy metal toxicity and nutrient deficiency (Jensen *et al.* 1996; Ashraf and McNeilly 2004; Zhang *et al.* 2010; Mourato *et al.* 2015). Among them, nutrient deficiency is a major constraint to the production of rapeseed.

In recent years, investigating molecular mechanisms underlying rapeseed responses to various nutrient deficiencies has attracted increasing attentions in the world (Shi *et al.* 2013; Avice and Etienne 2014). Although, many nutrient deficiency stresses can occur simultaneously in plant, studies to date have only focused on individual nutrient. Plants might exhibit different responses to multiple nutrient stresses than to deficiency of any one nutrient. Recently, plant growth has been found to be influenced by a complex tripartite interaction between phosphate, zinc and iron homeostasis (Bouain *et al.* 2014a), and the underlying mechanism was also revealed in rice (Saenchai *et al.* 2016).

In order to investigate the mechanisms underlying rapeseed responses to nutrient deficiency stress, first is to accurately normalization of stress-responsive genes expression by using suitable reference genes. To date, the selection of reliable reference genes for qRT-PCR normalization in rapeseed under various nutrient deficiencies has not been reported. In the current study, ten candidate reference genes including two traditional reference genes and eight genes selected from an RNA-Seq dataset were chosen for further analysis. The expression of these reference genes were determined in leaves and roots of rapeseed plants suffering from nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), sulfur (S), boron (B), iron (Fe), copper (Cu) and zinc (Zn) deficiency stresses for two weeks. Two major software packages (GeNorm and NormFinder) were employed to evaluate the stability of candidate reference gene's expression, and to determine suitable reference genes for qRT-PCR of different nutrient-deficient samples. Finally, two target genes were used to validate the stability of selected reference genes. Thus, our data provide valuable information for gene expression analysis of rapeseed responses to different nutrient deficiencies.

2. Materials and methods

2.1. Plant materials and stress treatments

A national authorized rapeseed cultivar, Zhongshuang11 (No. 2008030) was used in this study, which was bred by the Oil Crops Research Institute, Chinese Academy of Agricultural Sciences (CAAS). It had high oil content as well as high resistance to shattering and lodging. Rapeseed seeds were geminated in 1/4 strength Hoagland's nutrient solution with slight modification (the ratio of NH_4^+-N/NO_3^--N was adjusted to 1:9 to replace the complete NO_3^--N in Hoagland's nutrient solution) for 5 days, and then the uniform seedlings were separately transferred into 1/4 strength nutrient solution modified for various nutrient deficiencies in plastic boxes (in 1/2 strength nutrient solution for second week with the same treatments in the same plastic boxes). Treatments including N, P, K, Ca, Mg, S, B, Fe, Cu, Zn deficiency stresses, the concentrations of each treatment were verified by preliminary experiment and listed as follow, N (237.5 μmol L^{-1}), P (10 μmol L^{-1}), K (10 μmol L^{-1}), Ca (166.67 μmol L^{-1}), Mg (10 μmol L^{-1}), S (12.5 μmol L^{-1}), B (0.5 μmol L^{-1}), Fe (1 μmol L^{-1}), Cu (0 μmol L^{-1}), Zn (0 μmol L^{-1}). Other nutrients in solution were adjusted according to each treatment. Rapeseed seedlings in hydroponic cultures were grown in a plant growth room with 24°C/20°C (day/night) and 16 h/8 h (day/night) photoperiod with a daytime photon flux density of 300 μmol m^{-2} s^{-1}. Leaves and roots from four biological replicates were separately collected after 2 weeks of treatment and immediately frozen in liquid nitrogen and stored at –80°C for RNA isolation.

2.2. RNA isolation and cDNA synthesis

Total RNA was isolated from 100 mg of plant materials using