Gene 538 (2014) 113–122

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Selection and evaluation of novel reference genes for quantitative reverse transcription PCR (qRT-PCR) based on genome and transcriptome data in *Brassica napus* L.

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ARTICLE INFO

Article history:
Accepted 27 December 2013
Available online 7 January 2014

Keywords:
B. napus
Transcriptome
Quantitative reverse transcription PCR
Reference gene
Normalization
Stability

ABSTRACT

Selection of reference genes in *Brassica napus*, a tetraploid (4×) species, is a very difficult task without information on genome and transcriptome. By now, only several traditional reference genes which show significant expression differentiation under different conditions are used in *B. napus*. In the present study, based on genome and transcriptome data of the rapeseed Zhongshuang-11 cultivar, 14 candidate reference genes were screened for investigation in different tissues, cultivars, and treated conditions of *B. napus*. These genes were as follows: *ELF5*, *ENTH*, *F-BOX7*, *F-BOX2*, *FYPP1*, *GDI1*, *GYF*, *MCP2d*, *OTP80*, *PPR*, *SPOC*, *Unknown1*, *Unknown2* and *UBA*. Among them, excluding *GYF* and *FYPP1*, another 12 genes, were identified to perform better than traditional reference genes *ACTIN7* and *GAPDH*. To further validate the accuracy of the newly developed reference genes in normalization, expression levels of *BnCAT1* (*B. napus catalase 1*) in different rapeseed tissues and seedlings under stress conditions were normalized by the three most stable reference genes *PPR*, *GDI1*, and *ENTH* and little difference existed in normalization results. To the best of our knowledge, this is the first time *B. napus* reference genes have been provided with the help of complete genome and transcriptome information. The new reference genes provided in this study are more accurate than previously reported reference genes in quantifying expression levels of *B. napus* genes.

1. Introduction

Due to its outstanding accuracy, sensitivity and convenience, real time quantitative reverse transcription PCR (qRT-PCR) is a preferred method for quantifying gene transcript abundance in many plant research fields including growth development, response to pathogen infection and abiotic stress (Lee et al. 2012; Tanaka et al. 2012; Yan et al. 2012). Based on the studies aimed at improvement of reliability and reproducibility using qRT-PCR, appropriate application of qRT-PCR in such studies requires the use of reference gene(s) as an internal control to normalize mRNA levels between different samples for the purpose of an exact comparison of gene expression level (Guenin et al. 2009; Gutierrez et al. 2008; Huggett et al. 2005). Therefore, choosing an appropriate internal control is most important for quantifying gene expression.

Ideally, a perfect reference gene should be constantly transcribed in all types of cells and tissues and its RNA transcription level should not be regulated or influenced by internal and external factors (Schmittgen and Zakrajsek 2000). However, it becomes especially difficult to choose reference genes because the transcriptomes differ strongly in different samples, developmental processes, or materials (Czechowski et al. 2005; Linkies et al. 2009; Wei et al. 2010). In the pre-genomic era, the best known and most frequently used reference genes in plants and animals included *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), 18S rRNA, *actin* (*ACT*), *ubiquitin* (*UBQ*), *elongation factor-1a* (*EF-1a*), and *tubulin* (*TUA* and *TUB*, respectively) genes (Andersen et al. 2004; Deloffre et al. 2012; Dheda et al. 2004; Kim et al. 2003; Nicot et al. 2005; Radonic et al. 2004). However, many reports agreed that those traditional reference genes varied considerably under different experimental conditions and thus were unsuitable for gene expression analysis (Czechowski et al. 2005; Gutierrez et al. 2008; Radonic et al. 2004; Thellin et al. 1999). Some studies suggested that several reference genes should be used as internal standards because the use of a single

Abbreviations: ABA, abscisic acid; *ACT*, *actin*; *B. napus*, *Brassica napus* L.; *BnCAT1*, *B. napus catalase 1*; *CAT1*, *catalase 1*; CT, cycle threshold; CV, coefficient of variation; DAF, day after flowering; *EF-1a*, *elongation factor-1a*; GA3, gibberellin A3; *GAPDH*, *glyceraldehyde-3-phosphate dehydrogenase*; IAA, indole-3-acetic acid; MeJa, methyl jasmonate; NaCl, sodium chloride; OD, optical density; PEG, poly (ethylene glycol); qRT-PCR, quantitative reverse transcription PCR; SD, standard deviations; *UBQ*, *ubiquitin*.

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0378-1119/$ – see front matter.
http://dx.doi.org/10.1016/j.gene.2013.12.057

gene for normalization could lead to large errors (Udvardi et al. 2008). In recent years, with the development of biotechnology such as microarray and sequencing platforms (GSFLX, Solexa and SOLID), gene expression information could be easily obtained. A large number of novel and superior reference genes have been investigated in *Arabidopsis* (Czechowski et al. 2005; Graeber et al. 2011) and crops such as Chinese cabbage, zucchini, buck wheat, *Brassica juncea*, *etc.* (Chandna et al. 2012; Demidenko et al. 2011; Obrero et al. 2011; Qi et al. 2010). Additionally, development of statistical algorithms such as GeNorm (Vandesompele et al. 2002), NormFinder (Brunner et al. 2004) and BestKeeper (Pfaffl et al. 2004) has greatly simplified the selection of perfect reference genes *via* calculating expression stability.

Brassica napus L. (*B. napus*) is one of the most important vegetable oils and protein-rich meal crops in the world. Its cultivation has been increased tremendously during the last decade. By now, it represented the second largest source of vegetable oil (Tan et al. 2010). Genetic improvement to increase seed production and quality of *B. napus* is always one of the most important challenges for researchers. In *B. napus*, a great number of gene expression studies have been carried out, wherein *ACTIN* generally was the only reference gene used currently (Gao et al. 2004; Liu et al. 2010). Although in *B. juncea*, some genes were screened as candidate reference genes (Chandna et al. 2012). Unfortunately, among them, the expression pattern of most genes showed great difference in *B. napus* tissues based on transcriptome data, which meant most of new developed reference genes in *B. juncea* were not suitable in *B. napus*. Therefore, development and use of new reference genes for functional gene study is in urgent demand. As a tetraploid (4×) species, *B. napus* contains two basic genomes originating from natural diploid plants *Brassica rape* (AA) and *Brassica oleracea* (CC) and both of them underwent whole genome triplication (unpublished data). High and/or variable numbers of homologues often show differential silencing or tissue-specific expression patterns (Adams et al., 2003). Without information on genome and transcriptome, selection of reference genes in *B. napus* is a very difficult task. Although *B. napus* belongs to cruciferae, which is the same with *Arabidopsis*, a great difference exists in gene copy number and expression level between the two species. Therefore, reference genes from *Arabidopsis* could not be used in *B. napus* without identification. In the current study, based on genome sequences and transcriptome data (unpublished data) of *B. napus*, 40 genes were chosen as candidate references based on their expression levels in ten tissues. After inspecting their expression profiles with qRT-PCR, 14 reference genes showing more stable expression patterns were selected for further analysis in different tissues and cultivars, and under different conditions.

2. Material and methods

2.1. Analysis of gene copy number and expression level of transcriptome in *B. napus*

Rapeseed genes have been annotated based on genome sequence data of Zhongshuang 11, an open pollinated rapeseed cultivar in China (unpublished data), *B. oleracea* (unpublished data) and *B. rape* (Wang et al., 2011). Protein sequences were blasted with a parameter of 1e−5 and clustered into the same ortholog (Van Dongen and Abreu-Goodger, 2012). Transcriptome data of ten *B. napus* tissues were calculated by an RPKM formula (Mortazavi et al. 2008).

2.2. Plant material and biological samples

The Zhongshuang-11 cultivar was planted at 20–22 °C in a greenhouse with a relative humidity of 50% under long day (16-hour light/8-hour dark) conditions. Zhongshuang-11 cultivar samples included ten tissues: root, leaf and stem which were taken from the plant before bolting; stamen, blossom pistil and petal which were harvested at full bloom; and bud, 10DAF (day after flowering) silique, 20DAF pericarp, and 20DAF seed. For stress treatments, 7-day old seedlings of the Zhongshuang-11 cultivar were used. Seeds were grown in half strength MS media till five days. Elicitors were added after adapting 6-day old seedlings in sucrose free liquid medium for 24 h in the dark. Thereafter, seedlings were transferred to beakers containing MS along with abscisic acid (ABA, 400 μM), GA3 (500 μM), methyl jasmonate (MeJa, 200 μM), indole-3-acetic acid (IAA, 100 μM), sodium chloride (NaCl, 300 mM), respectively, for 6 h (Biosharp, Korea). 6% PEG4000 was used for drought treatment. For cold and heat shock treatments, seedlings were kept at −5 °C and 48 °C, respectively, for 6 h. For flooding stress, seedlings were completely submerged with 200 ml of water for 12 h. Additionally, six samples including root, stem, adult leaf before bolting, buds, 20DAF pericarp, and 20DAF seed from five other *B. napus* lines (zy036, 51070, 61616, 93275, and Zhongshuang-9 cultivar) that are frequently used in our laboratory were collected. All samples were frozen

Table 1
A selection of stably expressed genes for different experimental series.

Name	Annotation	*Arabidopsis* gene ID	Primer sequences (forward, 5′–3′)	Primer sequences (reverse, 5′–3′)	Product size (bp)	Amplification efficiency
F-BOX2	F-BOX protein 2	AT5G21040	TCTCCAACGCTTGTGTGAAG	CAGCATCTGGTGACGGTAAA	228	1.17
SPOC	SPOC domain/Transcription elongation factor S-II protein	AT5G25520	AGTCGTTAATGCCACCGAAC	AAAGCTCACGGTTGGTCTACA	168	0.89
ELF5	Early flowering 5	AT5G62640	TGGACCACCACCTATGATGA	GCTGCGGATTCTCTTCTGAC	216	0.93
F-BOX7	F-BOX protein 7	AT1G21760	TCGGAAGCACAAGCAGTAAA	AGGGTTGCGAACAGTGTACC	158	0.96
PPR	Postsynaptic protein-related	AT1G61780	TGGTGTGCGATAAGTGTGAGA	GGTGTCCATCTGTTCTTCTTGG	143	0.87
MCP2d	Type II metacaspases	AT1G79340	AGACCTCTGCTGATGCGACT	CTTGCTTCTTCAACGCCTTC	150	0.92
ENTH	ENTH/ANTH/VHS superfamily protein	AT4G32285	GTTTAGACCCGTTGCTGCTC	TTGTCCATCTCAGCCATTTG	244	1.04
Unknown1	Molecular_function unknown	AT3G49601	CGCTATGGAAGGGACAGGTA	CCAGCAGCAACATCATCTCT	234	0.9
GDI1	Guanosine nucleotide diphosphate dissociation inhibitor 1	AT2G44100	GAGTCCCTTGCTCGTTTCC	TGGCAGTCTCTCCCTCAGAT	172	0.93
Unknown2	Molecular_function unknown	AT5G11680	GCGCTTAACCCTCAGTTGAT	ATTTCTCGGCGTGGATGTAA	241	0.94
OTP80	Organelle transcript processing 80	AT5G59200	CAATGTTGAGGCAGCAAGAA	GTGCAACAGCCATATCATGC	204	0.85
GYF	GYF domain-containing protein	AT1G24300	CAGCGAAGGTTGCTACACAA	TGCTGCTGCAACTGAATCTT	226	0.82
FYPP1	Phytochrome-associated protein phosphatase 1	AT1G50370	TTGGAGATACTGCACGGATG	CACAGAATGGTCCTTCATGC	164	0.94
UBA	Ubiquitin-associated (UBA) protein	AT3G56740	TGGACATCCCAGTTTCAACA	CTGAAGGACGGCAAAGAAAG	238	0.88
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase C subunit	AT3G04120	CAGTGGTCCTATGGGCTACGAT	TTGTTGTAAACTAGGCGTCCTT	182	0.89
ACTIN7	β-actin7	AT5G09810	CCCTGGAATTGCTGACCGTA	TGGAAAGTGCTGAGGGATGC	141	0.91