Selection of housekeeping genes for normalization by real-time RT–PCR: Analysis of *Or-MYB1* gene expression in *Orobanche ramosa* development

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Abstract

Real-time PCR has become the method of choice for accurate and in-depth expression studies of candi- date genes. To avoid bias, real-time PCR is referred to one or several internal control genes that should not fluctuate among treatments. A need for reference genes in the parasitic plant *Orobanche ramosa* has emerged, and the studies in this area have not yet been evaluated. In this study, the genes *18S rRNA, Or-act1, Or-tub1,* and *Or-ubq1* were compared in terms of expression stability using the BestKeeper soft- ware program. Among the four common endogenous control genes, *Or-act1* and *Or-ubq1* were the most stable in *O. ramosa* samples. In parallel, a study was carried out studying the expression of the transcrip- tion factor *Or-MYB1* that seemed to be implicated during preinfection stages. The normalization strategy presented here is a prerequisite to accurate real-time PCR expression profiling that, among other things, opens up the possibility of studying messenger RNA levels of low-copy-number-like transcription factors.

Introduction

Orobanche (Broomrape) is a plant that has lost its photosyn- thetic ability and subsequently presents a vestigial root system. This parasitic plant, therefore, is totally reliant on its host for the supply of both reduced carbon and nitrogen and also inorganic nutrients. Among *Orobanche* species, *O. ramosa* attacks a wide range of host plants, including tobacco, tomato, and potato, and causes important yield losses [1]. The main difficulty that currently limits the development of successful control measures is the ability of the parasite to produce thousands of tiny seeds (0.3 x 0.2 mm) per plant that can remain viable in the soil for many years and the development of a subterranean phase in which *Orobanche* parasitizes the host before it emerges and becomes evident.

The best long-term strategy for limiting damage by *Orobanche* is the development of *Orobanche*-resistant crops, but traditional plant breeding has generally failed to produce resistance that is stable across time or variations of location and parasite pressure [2]. In addition to conventional breeding, optimal parasitic weed control could be achieved by crops genetically engineered for resistance [3]. In this sense, transgenic host plants expressing the sarcotoxin IA or a silencing construct against the mannose 6-phosphate reductase (M6PR)[1] gene have shown a reduced *Orobanche* infection [4,5]. However, more research is needed to understand the mecha- nism of sarcotoxin IA selectivity toward *Orobanche* and to optimize this mechanism for engineering parasite-resistant crop species.

The identification of new key *Orobanche* genes is needed to ob- tain totally resistant genotypes. Understanding patterns of ex- pressed genes during the parasitic plant development may provide insight into complex regulatory networks and help to identify genes implicated in pathogenesis to be used in *Orobanche* control. Transcription factors (TFs) could result in more interesting parasitic plant control because these proteins normally affect whole sets of target genes. In an attempt to select candidate genes, the MYB family TF *Or-MYB1* was cloned in *O. ramosa* [6]. However, the kinetic expression pattern of this gene could not be determined because of its undetectable transcript levels by Northern analysis. A more sensitive technique, in situ hybridization, showed expres- sion of this gene in parenchymatic cells proximal to the vascular vessels. However, in situ hybridization could not be applied to pre- infection stages because of sample limitation. Thus, a more precise technology must be applied to carry out the *Or-MYB1* expression analysis during different developmental stages. In this sense, real-time PCR technology has opened the way for more precise, reproducible, and sensitive gene expression studies becoming the predominant technique for measuring messenger RNA (mRNA) levels of low-copy-number-like TFs. An additional benefit over conventional methods is the reduction of the employed RNA quan- tity and of the time consumed [7].

To develop reliable experiments with real-time PCR, several variables need to be controlled, including RNA integrity or input concentration of complementary DNA (cDNA), enzymatic efficiencies, and differences between tissues or cells in overall transcriptional activity [8,9]. To cover all of these variables, a reference gene is commonly used in the relative quantification and is selected on its supposedly equal expression in each cell of a specific tissue and under different treatments and designs. Nevertheless, the expression level of many commonly used internal controls fre- quently vary across tissue type or experimental conditions [8,10]. For these reasons, a robust strategy for normalization takes into consideration a prior study of the expression levels of different housekeeping genes simultaneously in the studied organism. How- ever, there are no reports regarding housekeeping gene expression in parasitic plants, and very little research has been done for pho- tosynthetic plants [11–15]. Most of the studies deal with human and animal tissues [16–22] as well as with bacteria and viruses [23–26]. Therefore, research on *O. ramosa* is needed for the identification of accurate housekeeping genes to be used in gene expres- sion studies.

The aim of this work was to select and evaluate, for the first time, the stability of four *O. ramosa* housekeeping genes for relative expression analyses and to assess their value as internal controls. The selected control genes were used to study the expression pat- tern of the *Or-MYB1* TF along different developmental stages (ger- mination, attachment organ, tubercle, and flower) and under sucrose treatment. In this study, we show that actin *Or-act1* and ubiquitin *Or-ubq1* genes are suitable for *O. ramosa* gene expression studies, and we provide the first evidence of the *Or-MYB1* implica- tion during the preinfection stages of *O. ramosa.*

Materials and methods

Plant material

Germinated seeds and seeds with attachment organs were obtained as proposed by González-Verdejo and coworkers [27]. *O. ramosa* seeds collected from plants parasitizing tobacco in fields lo- cated in Granada (Spain) were surface sterilized by treating them for 2 h with a solution of 0.5% formaldehyde and 0.1% Tween 20 fol- lowed by a 20-min incubation at 50 °C. Subsequently, seeds were rinsed three times with sterile distilled water. Approximately 4000 *O. ramosa* surface-sterilized seeds were sown in 9-cm Petri dishes containing an autoclaved moist glass fiber filter paper (What- man) and were maintained at 24 °C in the dark for an 8-day condi- tioning period before adding 0.5 ml of a 0.034-mM solution of the synthetic germination stimulant GR24 [28]. GR24 was routinely purchased from the University of Nijmegen, The Netherlands.

To obtain *O. ramosa* tubercles, the parasitic plant was grown on tomato (*Lycopersicon esculentum* cv. Moneymaker) by using the Petri dish system described by Pérez-de-Luque and coworkers [29]. To- mato seeds were germinated in Petri dishes on wet glass fiber filter papers and kept in the dark at 20 °C for 5 days. When the radicle reached 4 to 5 cm in length, seedlings were transferred to new dishes (15 cm diameter) with perlite and glass fiber papers (Whatman GF/A, Sigma–Aldrich, Munich, Germany). *O. ramosa* seeds (8 mg) were pre- viously spread on the paper after being disinfected with a formalde- hyde solution as described above and placed in darkness at 20 °C for 8 days. The dishes were sealed with parafilm, covered with alumi- num foil to exclude the light, and placed vertically with the germi- nating host plant upward in trays with Hoagland nutrient solution [30]. Plants were grown in a controlled environment chamber with a day/night temperature of 21 °C and 16-h photoperiod. Tubercles of *O. ramosa* were removed from the host roots 20 days after inocu- lation. *O. ramosa* flowers were obtained from mature plants parasit- izing tomato plants. Tomato seeds were surface sterilized by immersing them first in 70% ethanol for 2 min and then in 3.5% sodium hypochlorite containing 0.1% Tween 20 for 10 min before final- ly rinsing three times with sterile distilled water. Tomato plants and *O. ramosa* disinfected seeds were maintained in pots under growth chamber conditions (21 °C, 16 h light).

According to the sucrose treatment, a stronger seed disinfection protocol was used to avoid contamination by microorganisms. Seeds were treated for 10 min with 5% sodium hypochlorite con- taining 0.1% Tween 20 and were rinsed three times with sterile dis- tilled water [27]. Disinfected *Orobanche* seeds were transferred onto 6-cm Petri plates with solid Murashige–Skoog medium [31] supplemented with 3% (w/v) sucrose (Panreac Qu´ımica, Valencia, Spain). Seeds were maintained in the dark in controlled growth chambers at 24 °C, and after 10 days of conditioning they were treated for 1 day with GR24 [32].

DNA and RNA isolation

Genomic DNA from *O. ramosa* young floral buds, as well as total RNA from germinated seeds, seeds with attachment organs, tuber- cles, flowers, and seeds treated with sucrose, were isolated from frozen tissues. The extraction methods used DNAzol or Trizol as re- agent (Invitrogen, Carlsbad, CA, USA) according to the manufac- turer's protocol. Nucleic acid concentrations were measured at 260 nm. The integrity of the total RNA was determined by electro- phoresis on 1% (w/v) agarose gels. The purity of the total RNA ex- tracted was determined as the 260/280-nm ratio with expected values between 1.7 and 2.1.

Cloning the partial sequences of Or-act1, Or-tub1, and Or-ubq1

Genomic DNA was used for PCR amplification. Degenerate primers corresponding to highly conserved regions of actin, tubu- lin, and ubiquitin proteins were designed (Table 1). The cloning of the 18S ribosomal RNA (rRNA) gene, *18S rRNA,* was not neces- sary because there are available specific primers to be used in real-time PCR assays. The following PCR conditions were used: 40 cycles with denaturation at 94 °C for 35 s, annealing at 53, 56, or 56.5 °C for *Or-act1, Or-tub1,* and *Or-ubq1,* respectively, during 35 s, and extension at 72 °C for 1 min. An initial denaturation step of 5 min at 94 °C and a final elongation step at 72 °C for 7 min were performed. The amplified DNA fragment was cloned into pGEM-T (Promega, Madison, WI, USA) and sequenced. Sequencing of both DNA strands of the clones was performed at the Servicio Centraliz- ado de Apoyo a la Investigación, University of Córdoba, using the Dyedeoxy terminator cycle sequencing kit (PE Biosystems, Foster City, CA, USA) on an ABI Prism 377 genetic analyzer apparatus (Ap- plied Biosystems, Weiterstadt, Germany). DNA and protein se- quence databases were searched using the BLAST algorithm [33] at the National Center for Biotechnology Information (Bethesda, MD,

USA). Once it was verified that the cloned sequences corre- sponded to the target genes, isolated plasmids containing the housekeeping gene PCR fragments were used for designing real- time PCR specific primers.

Two-step real-time PCR

Two-step real-time PCR was performed for evaluating gene expression profiles. cDNA was synthesized from 1 lg of RNA using the QuantiTect Reverse Transcription Kit (Qiagen, Leusden, The Netherlands) with oligo(dT) according to the manufacturer's instructions. The cDNA was diluted 1:4 with nuclease-free water. Samples were screened for genomic contamination using the pri- mer pair amplifying an intron oli-myb18 (5'-GTT CTA GGG CTG CCT TTC AAT -3')/19 (5'-TTT GGA AAT TGG AAC GGA TAA-3'), designed from the *Or-MYB1* sequence (GenBank database number DQ380234). The sequences cloned previously (*Or-act1, Or-tub1,* and *Or-ubq- 1*) and the *Or-MYB1* sequence were used to design gene-specific primers for real-time PCR. Four primers pairs were designed (150 bp maximum length, optimal melting temperature [T_m] at 60 °C, GC percentage between 20 and 80%) with Primer Express 2.0 software (Applied Biosystems) (Table 2). For 18S gene amplification, the QuantumRNA 18S Internal Standards Kit (Ambion, Austin, Texas, USA) was used. Real-time PCR using SYBR Green technology on the Stratagene LightCycler (Stratagene, La Jolla, CA, USA) was then carried out. Each cDNA sample was amplified in the same run of real-time PCR using all of the described primer sets. The RT–PCR reactions,containing 200 nM of each primer, 2.5 ll of cDNA sample (~25 ng of input RNA), and 2x SensiMix DNA master mix (Quantace, London, UK), were run in a total volume of 25 ll. The following run protocol was used: polymerase activation (95 °C for 10 min) and amplification and quantification cycles repeated 40 times (95 °C for 30 s, 60 °C for 1 min). All RT–PCR runs contained nega- tive controls with no cDNA template so as to exclude or detect pos- sible contamination. Specificity of the primer amplicons was checked by a melting curve analysis performed by the PCR ma- chine after 40 amplification cycles (55–95 °C with one fluorescence read every 0.5 °C). Data were analyzed using Mx3000P analysis software (version 3.00, Stratagene). PCR efficiencies (*E*) between 1.96 and 2.02 with R^2 values higher than 0.998 were selected using the LinRegPCR program [34]. The entire experiment was repeated, giving a total of two bio- logical replicates, with each one represented by three technical repetitions.

Table 1. Degenerate primer pairs designed for PCR fragment cloning

Gene	Primer	Forward and reverse primers 5´-> 3'	T_m (°C)	Amplicon
Or-act1	oli-act1	GTN ARY AAC TGG GAT GAY ATG G	56.5	231bp
	oli-act2	ACA ATA CCW GTW GTR CGA CC		
Or-tub1	oli-tub1	GAA GAT GCT GCH AAY AAY TTY GC	53.0	282 bp
	oli-tub2	GAR AGH ACA CTG TTR TAA GGY TC		
Or-ubq1	oli-ubq1	GAY TAC AAC ATY CAG ARG GAG	56.0	392 bp
	oli-ubp2	GCR AAR ATC ARC CTC TGC TG		

Statistical analyses

To determine the best standards, the BestKeeper software program [35] was used. This program, as well as necessary informa- tion concerning data processing, was downloaded from http:// www.wzw.tum.de/gene-quantification/bestkeeper.html.

First, an initial estimation of gene expression stability was calculated for all individual housekeeping genes based on threshold cycle (C_t) values and was displayed as the standard deviation (SD) and coefficient of variance (CV). Any studied gene with the SD higher than 1 can be considered as inconsistent (Table 3).

Second, to estimate intergene relations of all possible house- keeping pairs, the possible pairwise correlation analyses were per- formed. Within each such correlation, the Pearson correlation coefficient (*r*) and the probability (*P*) value are calculated. All of those highly correlated housekeeping genes are combined into the BestKeeper Index. Then correlation between each candidate housekeeping gene and the index is calculated, describing the relation between the index and the contributing housekeeping gene by the Pearson correlation coefficient, the coefficient of determination (r^2), and the *P* value.

Finally, the expression level of the gene of interest, *Or-MYB1,* relative to the BestKeeper Index was calculated for each cDNA sample applying the equation as described by McGrath and coworkers [36]. We performed a one- way analysis of variance (ANOVA) followed by the Tukey HSD mul- tiple comparison test at a 0.01 probability level to calculate the sig- nificance of relative expression values between the expression levels during each developmental stage.

Results

Selection of housekeeping genes

A total of four housekeeping genes known to be involved in dif- ferent aspects of the cellular functions were evaluated for their po- tential as good internal controls: *18S rRNA, Or-act1, Or-tub1,* and *Or-ubq1.* Of these genes, *O. ramosa* actin, tubulin, and ubiquitin se- quences were not available from the public database; thus, specific PCR fragments for these genes were cloned. After their identity was confirmed by sequencing, the resultant sequence data were deposited in the GenBank database under accession numbers EU232717, EU232718, and EU232719, respectively.

PCR efficiency and amplification specificity

The efficiency values obtained for the real-time PCR amplifica- tion of the four housekeeping genes and the gene of interest, *Or- MYB1,* were in the range 1.96 to 2.02, as listed in Table 2. Amplifi- cation of the specific transcript was confirmed by the appearance of a single peak in the melting curve analysis following completion of the amplification reaction. The amplified products were also fur- ther analyzed by agarose gel electrophoresis and ethidium bro- mide staining. Only a single band with the expected size (Table 2) was detected in each experiment, indicating good specificity of all the primer sets in real-time PCR.

Primer pair oli-myb18/19 amplified a 964-bp intron when genomic DNA was used as template . The intron position was similar to that of the 3'intron conserved in GAMYBs genes [37] (data not shown).

Expression profile and expression stability of the housekeeping genes

To analyze the transcription level of each housekeeping gene during different life stages of *O. ramosa,* total RNA was isolated from a variety of samples corresponding to germinated seeds, seeds with attachment organs, tubercles, flowers, and seeds under sucrose treatment. Transcription profiles of the four genes for every individual RNA sample are shown in Fig. 1. The C_t value is de- fined as the number of cycles needed for the amplification signal to reach a specific threshold level of detection and, thus, is correlated inversely with the amount of cDNA template present in the PCR amplification reaction [38]. As shown in this figure, C_t values for *Or-ubq1* obtained from different RNA samples were usually higher than those of other genes, indicating a relatively low level of *Or- ubq1* transcription during most life stages of *O. ramosa.* The gene encoding *18S rRNA,* in contrast, was found to display low C_t values, whereas *Or-act1* and *Or-tub1* showed intermediate transcription levels.

The Excel-based tool BestKeeper was tested in biological materials. In a first estimation, *18S rRNA* was excluded because it showed high C_t variation (SD = 1.22 C_t). Nevertheless the SDs of the C_t values in various combinations of the four candidate genes (groups of $n = 4$, 3, or 2) were calculated (Table 3).

In a second step to estimate intergene relations, *Or-tub1* did not correlate well with *Or-act1* or *Or-ubq1* (data not shown), and for this reason we excluded *Or-tub1* from the index calculation. Although the program clearly identified *Or-act1–Or-tub1–Or-ubq1* and *Or-tub1–Or-ubq1* as the combinations most stable regarding the SD, the low correlation of *Or-tub1* with the other genes led us to exclude it from the index. On the other hand, the analysis showed a strong correlation ($r = 0.72$) for *Or-act1* and *Or-ubq1.* For this reason, an index based on the *Or-act1* and *Or-ubq1* combi- nation was calculated, and it was selected for *Or-MYB1* normalization in this study.

Table 2. Primer sequences for real-time PCR of four housekeeping genes and one gene of interest

Gene	Primer	Forward and reverse primers 5'->3'	Amplicon size (bp)	*E*
18S		18S primer pair (Ambion)	315	1.98
Or-act1	oli-act3	CGT GAG AAG ATG ACG CAG ATT	73	1.99
	oli-act4	GAA CAG CCT GGA TAG CAA CAT AC		
Or-tub1	oli-tub3	CAT TAC ACC ATC GGC AAA GAG	108	1.98
	oli-tub4	ACC AAC AGC ATG AAA AAC CAA		
Or-ubq1	oli-ubq3	TGT CCT CTG TTT ACT TGG TGG TAT G	71	2.00
	oli-ubp4	CTT CAA GGG TAA TGG TCT TCT CAA C		
Or-myb1	oli-myb23	TGA ATC TCC CAT GTT GAG CAA	62	1.97
	oli-myb24	TTG CAG CTT CAG GTT GAC TTT C		

Note. Amplification lengths obtained, as well as PCR efficiencies (*E*) calculated with the LinRegPCR program, are shown.

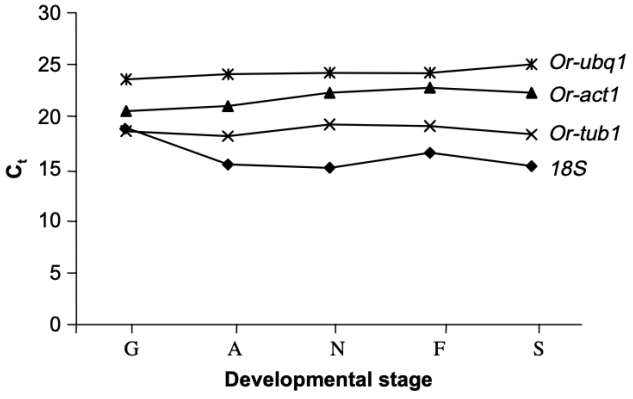

Fig. 1. RNA transcription levels of housekeeping genes tested, presented as C_t mean values in the different samples: G, germination; A, attachment organs; T, tubercles; F, flowers; S, sucrose treatment.

Normalization analysis of Or-MYB1 gene expression

The expression pattern of the gene of interest, *Or-MYB1,* was quantified during different *O. ramosa* stages using real-time PCR analysis. From the results shown in Fig. 2, it is clear that *Or- MYB1* is expressed during all of the tested stages: germinated seeds, seeds with attachment organs, tubercles, and flowers. Among these stages, very low levels of *Or-MYB1* mRNA were observed in tubercles and flowers. In contrast, the expression of *Or- MYB1* increased approximately 35- and 10-fold in germinated seeds and seeds with attachment organs, respectively. When the response of *Or-MYB1* to sucrose was studied, it appeared that this gene was increased very slightly in seedlings grown on medium containing sucrose. The C_t values for *Or-MYB1* obtained from the different RNA samples were higher (23.3–30.3) than those of the housekeeping genes. This indicates a very low level of *Or-MYB1* expression, which is not surprising for a TF whose transcripts generally are produced in low amounts.

Discussion

Because the kinetic *Or-MYB1* gene expression was not possible to establish in previous works by using Northern or in situ hybrid- ization, real-time PCR was chosen to measure the mRNA levels of this gene in *O. ramosa* development. Specific PCR conditions and an appropriate internal control that shows minimal changes must be determined to establish accurate real-time PCR studies. To iden- tify housekeeping genes suitable for internal control, the expres- sion of four candidate genes at different life stages and under sucrose treatment of *O. ramosa* was analyzed by real-time PCR. Be- cause the importance of using

statistical approaches to select the best internal controls is recognized [8], we undertook an extensive evaluation with the BestKeeper software program [35] of the four housekeeping genes considered: *18S r RNA*, *Or-act1*, *Or-tub1,* and *Or-ubq1.* This program clearly identified *Or-tub1* and *Or-ubq1* as the most stable genes. Ubiquitin and tubulin genes also presented high stability in poplar and were suggested as optimal housekeep- ing genes for real-time PCR by Brunner and coworkers [14]. In addition, tubulin genes were recommended as appropriate control, but only for specific tissues in barley [13]. In spite of its stability, we could prove that *Or-tub1* did not correlate well with either *Or-ubq1* or *Or-act1.* Although there are arguments against the use of actin genes as the internal control [11,15], we found the gene *Or-act1* to be an appropriate candidate not only because of its sta- bility but also because it presented a high correlation with*Or-ubq1.* In conclusion, we established the index specific for the combina- tion of *Or-act1* and *Or-ubq1* as the most accurate for normalization of target genes in *Orobanche.*

In addition to the constant level of expression required, an internal control should be compared with the level of expression of the target genes to be analyzed. In this sense, *Or-act1* and *Or- ubq1* were also appropriate to normalize the low *Or-MYB1* gene expression because they presented most similar levels of tran- script. On the other hand, the 18S ribosomal subunit recommended as a control in potato [15] was the worst in *O. ramosa* and was re- moved from the analysis due to both its high expression and insta- bility. Taking into account the best housekeeping genes described in plants, we can conclude that they show different stability pat- terns depending on the species, and even the tissues, and that the results obtained for one plant species cannot be extrapolated to another one.

Using *Or-act1* and *Or-ubq1* as internal controls, significant quantities of *Or-MYB1* transcript were detected mainly in germinated seeds but also during attachment organ initiation. In contrast, minimal expression was found during postinfection stages (tubercles and shoots) and under sucrose treatment. This expression pattern suggests that *Or-MYB1* may play a key role during the early preinfection stages of *O. ramosa.*

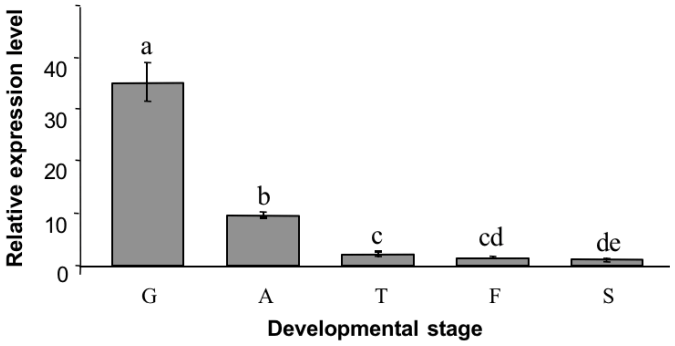

Figure 2. Expression pattern of *Or-MYB1* from *O. ramosa* at the indicated developmental stages of the parasitic life cycle: G, germination; A, attachment organs; tubercles; F, flowers; S, sucrose treatment. The gene expression level for each sa- mple was determined by the signal intensity relative to that of the most stable housekeeping genes defined by the BestKeeper analysis: *Or-act1* and *Or-ubq1.* The error bars indicate the standard errors of the means. Different letters denote exp- osure group means that are significantly different using analysis of variance ($P < 0.01$).

Usi tities of Or-MYB1 transcript were detected mainly in germinated seeds but also during attachment organ initiation. In contrast, minimal expression was found during postinfection stages (tubercles and shoots) and under sucrose treatment. This expression pattern suggests that Or-MYB1 may play a key role during the early preinfection stages of *O. ramosa.*

Table 3
Descriptive statistics of four candidate housekeeping genes based on their C_t values using BestKeeper software

Factor	*18S rRNA*	*Or-act1*	*Or-tub1*	*Or-ubq1*	BestKeeper ($n = 4$) all genes	BestKeeper ($n = 3$) excluding *18S rRNA*	BestKeeper ($n = 2$) *Or-act1* + *Or-tub1*	BestKeeper ($n = 2$) *Or-act1* + *Or-ubq1*	BestKeeper ($n = 2$) *Or-tub1* + *Or-ubq1*
N	10	10	10	10	10	10	10	10	10
GM (CP)	16.17	21.78	18.64	24.23	19.97	21.43	20.15	22.97	21.25
AM (CP)	16.23	21.80	18.64	24.23	19.97	21.43	20.16	22.98	21.25
Min (CP)	15.04	20.52	18.07	23.52	19.39	20.78	19.51	21.97	20.86
Max (CP)	18.98	22.73	19.20	25.06	20.42	21.91	20.81	23.65	21.56
SD (± CP)	1.22	0.80	0.38	0.35	0.32	0.46	0.51	0.59	0.29
CV (% CP)	7.50	3.67	2.06	1.46	1.58	2.15	2.51	2.56	1.38
Min (*x*-fold)	−2.18	−2.39	−1.48	−1.63	1.49	1.56	1.55	2.00	1.31
Max (*x*-fold)	7.04	1.93	1.48	1.78	1.36	1.40	1.58	1.60	1.24
SD (± *x*-fold)	2.33	1.74	1.28	1.31	1.24	1.38	1.42	1.50	1.23

Note. In the last five columns, the BestKeeper index is computed together with the same descriptive parameters either for four genes, for three genes after removal of *18S rRNA*, or for two genes. *n*, number of genes; *N*, number of samples; CP, equivalent terminology for C_t; GM (CP), geometric mean of CP; AM (CP), arithmetic mean of CP; Min (CP) and Max (CP), extreme values of CP; SD (± CP), standard deviation of CP; CV (% CP), coefficient of variance expressed as percentage of CP level; Min (*x*-fold) and Max (*x*-fold), extreme values of expression levels expressed as absolute *x*-fold over or under regulation coefficient; SD (± *x*-fold), standard deviation of absolute regulation coefficients.

Protein sequence of the *Or-MYB1* DNA-binding domain is sim- ilar to that of the *Arabidopsis thaliana* GAMYB gene *AtMYB101* (89.4%) and also to that of *Petunia hybrida* (*MYB.Ph3,* 88.5%). In addition to this, *Or-MYB1* contains four additional regions con- served in GAMYB-like gene TFs from *Arabidopsis* and cereals and, as we found in this work, the 3^0 intron conserved in GAMYB genes. The GAMYB genes have been associated with transduction of gibberellic acid (GA) signals [37]. In the case of *AtMYB101,* it may be involved in GA-regulated hypocotyl elongation during germination. The sequence similarity indicates that *AtMYB101* and *Or-MYB1* may bind similar DNA sequences in the nucleus and that both proteins may share similar biological functions [6]. For this reason, we can speculate that *Or-MYB1* could be a GAMYB-like gene with a possible role in radicle elongation during seed germination. This would fit with the finding that GA synthe- sis does occur during the conditioning period before *O. ramosa* seed germination takes place [39]. In this sense, the GA produced could activate *Or-MYB1* expression, and this might help to explain the higher levels of this transcript during the germination of seeds.

Nevertheless, the implication of this TF during the infection stages cannot be discarded because the low transcript levels found could be sufficient. In this sense, previous studies with in situ hybridization showed that low expression was also produced dur- ing the infection process in the tubercle and in the haustorium near the vascular tissues. It is likely that during this stage, *Or-MYB1* could be implicated in vessel differentiation and development, probably through the phenylpropanoid pathway [6].

We cannot conclude whether *Or-MYB1* could be implicated in the mucilage secretion that *Orobanche* species produce to attach to and penetrate into the host tissues. Although this possible role was also suggested [6], it does not appear to be likely because *Or-MYB1* expression is not increased in seeds germinated on a medium containing sucrose, as occurs in other MYB genes with this function (e.g., the *A. thaliana MYB61* gene [40]), and because of the low similarity between *Or-MYB1* and *MYB61* sequences (data not shown). In this work, we have demonstrated that real-time PCR is both highly precise, given that this technique allowed us to determine that expression during tubercle formation is very low if we compare it with that produced during germination, and sensitive, given that it is able to detect *Or-MYB1* expression levels that were unde- tectable by Northern analysis in a previous work.

Overall, our study extends our knowledge about the parasitic plant *O. ramosa.* In this sense, accurate housekeeping genes for normalization in real-time PCR experiments, as well as a possible role for the TF *Or-MYB1* in germination, have been proposed. The knowledge of *Orobanche* biology may provide advances in understanding the host–parasite interaction and, consequently, may offer opportunities for the breeding of resistant genotypes.

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