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Identification of reference genes for normalizing RNA expression in potato roots infected with cyst nematodes

Authors

Patricio Castro-Quezada, Jawad Aarrouf, Michel Claverie, Bruno Favery, Didier Mugniéry, Véronique Lefebvre and Bernard Caromel

Abstract

Potato cyst nematodes induce changes in plant host gene expression following root invasion. For accurate comparison of gene expression by reverse transcription quantitative real-time PCR (RT-qPCR), internal reference genes are necessary for transcript normalization. Very few experimental data on suitable reference genes are available for interactions between plant and root pathogens. In this study, we tested eight potential candidate reference genes, for normalizing levels of potato gene transcripts by RT-qPCR, after inoculation by nematodes. Ranking of candidate reference genes was performed using RefFinder WEB-based software. Four reference genes, RPN7 (26S proteasome regulatory subunit), UBP22 (Ubiquitin-specific protease 22), OXA1 (OXA1 protein) and MST2 (mercaptopyruvate sulfurtransferase), were stably expressed in roots of susceptible or resistant potato plants, infected or uninfected by *Globodera pallida*. A normalization factor based on data from these four genes, highly homologous between potato and tomato, was used to normalize the expression of a chitinase gene, which was induced by nematodes in roots of potatoes carrying the resistance allele at a low effect QTL, $GpaXI_{spl}$.

Keywords: RT-qPCR; transcript normalization; compatible-incompatible interactions; *Globodera pallida; Solanum;* chitinase

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P. Castro-Quezada, M. Claverie, V. Lefebvre and B. Caromel

INRA, UR1052 GAFL Génétique et Amélioration des Fruits et Légumes, Domaine Saint Maurice, Allée des Chênes, CS 60094, F-84143 Montfavet Cedex, France

J. Aarrouf

UAPV, Laboratoire de Physiologie des Fruits et des Légumes, Pôle Agrosciences

BP 21239, F-84916 Avignon cedex 9, France.

B. Favery

INRA, UMR INRA 1355 – UNS - CNRS 7254 Institut Sophia-Agrobiotech, 400 route des Chappes,

F-06903 Sophia Antipolis, France

D. Mugniéry

INRA, UMR 1349 IGEPP INRA/Agrocampus Ouest/Université Rennes1,

F-35653 Le Rheu, France

Corresponding author: B. Caromel

Fax number: 33 (0)4 32 72 27 02

E-mail address: Bernard.Caromel@avignon.inra.fr

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Introduction

Gene expression analysis has become an essential method for understanding many processes in plant biology. Reverse transcription quantitative real time PCR (RT-qPCR) is the main technique used to investigate gene expression changes due to its high sensitivity, specificity and reproducibility (Bustin and Nolan 2004; Vandesompele et al. 2002). To accurately measure the variation of expression levels between samples for a gene of interest, the most accepted approach is to normalize its expression level to reference genes used as internal controls. A good reference gene should be stably expressed between the different conditions investigated. The reference gene is used to correct for the different amounts of starting RNA and for the variable efficiency of the reverse transcription between samples. Genes involved in basic cellular processes, such as those encoding ubiquitins, actins, tubulins, GAPDH and 18S rRNA have been used as reference genes (Guo et al. 2012, Gupta et al. 2011, Inigo et al. 2012, Stolf-Moreira et al. 2011, Yang et al. 2011). However, using putative "housekeeping genes" as references is no longer considered as good practice (Gutierrez et al. 2008) as it has been recently demonstrated that genes from multigenic families i.e. actins have specific spatio-temporal expression and that no genes are universally stable (Hruz et al. 2011). Statistical algorithms and associated programs like NormFinder, BestKeeper, geNorm or comparative delta-Cq method have been developed to select the most suitable reference gene(s) for transcript normalization from a panel of candidate genes putatively stably expressed (Andersen et al. 2004; Pfaffl et al. 2004; Vandesompele et al. 2002, Silver et al. 2006). Depending on the software used, the ranking of candidate reference genes can be slightly different (Goulao et al. 2012, Qi et al. 2010, Rivera-Vega et al. 2012). Methods and tools have recently been developed to integrate the rankings provided by different software (Goulao et al 2012, Xie et al. 2012).

We are interested in plant-nematode compatible and incompatible interactions. In potato, oligogenic resistances to the cyst nematode *Globodera pallida* have been introgressed from wild species into the

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cultivated species *Solanum tuberosum*. The resistance originating from the wild species *S. sparsipilum* is conferred by two quantitative trait loci (QTL): one major effect QTL named $GpaV_{spl}$ and one low effect QTL named $GpaXI_{spl}$. Depending on the combination of alleles at both QTLs, the resistance reaction of the plant varies between a strong necrotic reaction to a shift of the sex-ratio of nematode populations (Caromel et al. 2005). The resistance takes place approximately two days after the penetration of the nematode in the root, once the feeding cell, named syncytium, is initiated (Caromel et al. 2005; Jolivet et al. 2007; Mugniéry et al. 2001). Several genes coding for pathogenesis-related (PR) proteins are induced in response to pathogen infections (Grover 2012, Jolivet et al. 2007). Among PR proteins, several chitinase genes have been demonstrated to be induced upon nematode infection (Rahimi et al. 1998).

Nicot et al. (2005) described reference genes for normalization in RT-qPCR experiments in potato. They investigated seven putative reference genes, particularly in the compatible interaction between the oomycete *Phytophthora infestans* and potatoes, and highlighted that EF1α (elongation factor 1α) and 18S rRNA, followed by β-tubulin, were the most stable genes in the compatible interaction between *P. infestans* and potato leaves. Nevertheless, the stability of those genes was not tested, either in roots, or in an incompatible plant-pathogen interaction. Furthermore, EF1α was reported as being differentially expressed in a few plant-nematode interactions (Hofmann and Grundler, 2007, Vaghchhipawala et al. 2001). In addition, the relevancy of using 18S rRNA as reference gene in RT-qPCR experiment has been questioned, because of their high abundance relative to the genes of interest (Vandesompele et al. 2002).

Analysis of microarray data usually allows a set of candidate reference genes to be chosen. Several transcriptomic analyses of plant-nematode interactions have been published (for review see Escobar et al. 2011), but genes whose expression is stably expressed are rarely reported. Jammes et al. (2005) published nine genes stably expressed in the interaction between root-knot nematodes (*Meloidogyne incognita*.) and *Arabidopsis thaliana*. In the present work, we used this latter study as starting point to evaluate the stability of candidate reference genes in compatible and incompatible potato-nematode interactions. We added the β-tubulin gene to compare our results with those obtained by Nicot et al. (2005). The best ranked reference

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genes were used for normalizing the expression level of a chitinase gene, which was regulated by a low effect resistance QTL upon nematode infection.

Materials and Methods

Biological material and experimental design

Potato clones, issued from the QTL mapping progeny described in Caromel et al. (2005), were chosen according to their allelic combinations at both QTLs determining resistance to *G. pallida*. For each of the four allelic combinations at QTLs, three clones were selected as being identical at QTL positions and as different as possible on the rest of the genome. Thus, a total number of 12 potato clones were analyzed. For each clone, 30 sprouting tubers were grown in Petri dishes. Two to three emerging roots per tuber, from 15 tubers, were inoculated with juveniles of *G. pallida*, population Chavornay, as described by Mugniéry and Person (1976). Roots from the 15 remaining tubers per clone were used as mock-inoculated controls. Samples consisting of pools of five root sections of 5 mm each around the inoculation point were excised from two tubers per sample (two and three root sections per tuber), at 6 hours post-inoculation (hpi) and 2 or 4 days post-inoculation (dpi). Pools of five root sections of 5 mm each around the mock-inoculated point were sampled similarly from control tubers at each time-point. The experimental design consisted of 72 samples: four allelic combinations at resistance QTLs represented each by three potato clones considered as biological replicates, inoculated or mock-inoculated and sampled during a three-point time-course.

Sample processing

After sampling, pools of root sections were immediately frozen in liquid nitrogen, and stored at -80°C. RNAs from each sample were extracted with the RNeasy plant mini kit (Qiagen), following the manufacturer's

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instructions. RNA concentration was measured with the Quant-iT RiboGreen kit (Invitrogen). RNA integrity was checked following agarose gel electrophoresis of 1 µl of RNA extracts, by evaluating the integrity of the 28S and 18S ribosomal RNA bands and the absence of smears (Supplementary figure S1). The purity of RNA extracts was assessed by absorption ratios, measured with a NadroDrop ND-1000 spectrophotometer. A260/280 ratios were comprised between 1.98 and 2.24 and A260/230 ratios between 2.10 and 2.32, indicating that RNA samples were free of proteins and organic compounds.

Potential contaminating DNA was removed from 1 µg of RNA per sample, by one hour treatment at 40 °C with 1 unit of recombinant Shrimp DNAse (USB) in 12.5 mM Tris-HCl pH 8.0, 2.5 mM $MgCl_2$. RNAse reaction was stopped by an incubation step of 20 min at 65 °C. To ensure that all contaminating DNA was digested, a PCR reaction with primer MST2 (see below) was performed with 40 ng of total RNA as template. No RNA sample produced PCR amplification, contrary to the DNA positive control, revealing an absence of DNA contamination in RNA samples.

An aliquot of 250 ng of DNAse-treated RNA was primed with 2.5 µM of oligo-d$(T)_{18}$VN by a heating step at 65°C for 5 minutes followed by an incubation step on ice for one minute. Primed RNA was reverse-transcribed in 20 µl, using one unit of SuperScript III reverse transcriptase (Invitrogen) in the manufacturer's recommended buffer, in the presence of one unit of RNasin (Promega), for two hours at 50 °C. The reaction was inactivated by heating at 70°C for 15 minutes. From each reverse-transcribed RNA sample, 5 µl were removed and pooled to prepare a seven point four-fold serial dilution. The remaining 15 µl were diluted 10 fold before being subjected to qPCR.

Candidate reference gene selection and primer design

Seven candidate reference genes were chosen from Jammes et al (2005). Arabidopsis protein sequences of the seven genes were retrieved from the MIPS database (http://mips.helmholtz-muenchen.de/plant/athal/) and a homology search was made against the potato Tentative Consensus (TC) located in the Gene Index Project

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database (http://compbio.dfci.harvard.edu/tgi/), using the TBLASTN algorithm (Altschul et al. 1990). For each gene, the corresponding potato TC with the best hit was selected. A reciprocal homology search (BLASTX) to the Arabidopsis protein database allowed the correspondence between the original candidate gene from Arabidopsis and the selected potato TC to be checked. The reciprocal blast was successful for the seven candidate reference genes (Table 1).

As one of the primers of the primer pair published by Nicot et al. (2005) for β-tubulin (GenBank accession Z33382, formerly 609267) did not match any potato Express Sequence Tag (EST) in GenBank, we retrieved from GenBank three sequences (Z33382, Z33402 and EU935742) corresponding to β-tubulin mRNAs. These sequences were aligned with Muscle algorithm implemented in Mega5 software (http://www.megasoftware.net/) and two additional primer pairs were designed in the conserved region (see below).

Correspondences between the eight candidate reference genes and *S. phureja* genome were searched for, using the BLASTN algorithm against the PGSC *S. phureja* DM loci database version 3.4 (http://potatogenomics.plantbiology.msu.edu/blast.html). BLASTN default parameters were used except that the EXPECT e-value threshold was fixed at e^{-100}. TCs for which no hit was returned were blasted similarly against the PGSC *S. phureja* DM pseudomolecules version 2.1.11. Sequences of the candidate reference genes were blasted against tomato unigenes using the same parameters (http://solgenomics.net/).

Two to three primer pairs were designed per potato TC and β-tubulin sequences, using Primer 3 software (http://frodo.wi.mit.edu) with default parameters, except a melting temperature comprised between 59.5°C and 63.5°C, a maximum difference of 2°C between the Tm of both primers, a length of 18 to 25 bases; a CG content of 35 to 60 %, and a product length between 70 and 180 base pairs (bp).

Specificity of primer pairs was checked using the BLASTN algorithm with parameters adjusted for short input sequences, against the PGSC *S. phureja* DM loci version 3.4 and against the PGSC *S. phureja* DM pseudomolecules version 2.1.11 at the PGSC WEB-site, against the tomato WGS chromosomes version SL2.40 and against the tomato and the potato unigenes at the Solanaceae Genomic Network (SGN) WEB-

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site, and against *Solanum* ESTs at the NCBI WEB-site. One primer pair was considered as suitable for qPCR experiments in one species (potato or tomato) if i) it matched one EST or unigene of the species without mismatch on the whole sequence and ii) the two primers of a primer pair matched together one single location in the genome of the considered species (in this latter case, several unigenes for one single location on the genome were considered as the result of alternatively spliced transcripts).

Real-time quantitative PCR

qPCR experiments were carried out in 96 well plates (Thermo Scientific) heat-sealed with ThermalSeal RT film (Excel Scientific), in a final volume of 10 µl, containing 5 µl of SYBR *Premix Ex Taq* (Tli RNaseH Plus) kit (Takara Bio), 0.2 µM of each primer and 1 µl of cDNA. cDNA added for each sample corresponded approximately to 1.25 ng of reverse-transcribed RNA. Three serial dilutions, covering a range of 12.5 to 0.003 ng of reverse-transcribed RNA, were used to calculate PCR amplification efficiency for each primer pair (see below). A typical qPCR experiment comprised in one single PCR plate: the 72 samples, one serial dilution, and one no-template control (NTC). Reactions were run in a Mx3005P Thermocycler (Agilent Technologies), using the following parameters: 95°C for 20 s, followed by 40 cycles at 95°C for 5 s; 60°C for 20 s; 72°C for 10 s, and finally one cycle at 95°C for 1 min; 60°C for 30 s and 95°C for 30 s to generate dissociation curves. Fluorescence values were acquired at the end of the elongation phases. Dissociation curves and electrophoresis of PCR products on a 2.0 % agarose gel were used to check that one single PCR product was amplified. One single peak on the dissociation curves (Supplementary figure S2) and one single band on agarose gels indicated that one single PCR product was amplified with each chosen primer pair.

A seven point four-fold dilution series was used to generate standard curves. For each primer pair, the PCR amplification efficiency was calculated from the slope obtained by plotting the raw Cq values of three serial dilutions against the log of the initial template quantity in the standard wells. PCR efficiencies of primer pairs were calculated using the following equation: PCR Efficiency % = $(10^{(-1/\text{slope})} - 1) \times 100$. For each

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candidate reference gene, the primer pair with the best efficiency was retained. The sequences of primers are given in Table 2. Primers of the chitinase gene STMEO91 were described in Vuorinen et al (2010).

Data analysis

Two PCR plates were run for each primer pair, corresponding to two technical replicates. One serial dilution was added to each plate to ensure that PCR efficiency was similar for each qPCR run. For each sample, the mean Cq value was calculated based on Cq values of both replicates. Individual Cq values were considered as valid only if the difference between both replicates was less than one.

The stability of each candidate gene expression was analyzed using RefFinder (Xie et al. 2012, http://www.leonxie.com/referencegene.php). RefFinder is a user-friendly web-based tool, developed for evaluating the stability of reference genes and ranking them using the most commonly used programs dedicated to this task: geNorm (Vandesompele et al. 2002), NormFinder (Andersen et al. 2004), BestKeeper (Pfaffl et al. 2004), and the comparative delta-Cq method (Silver et al. 2006). Mean Cq values were entered to the RefFinder WEB page and based on the ranking obtained with each program, RefFinder calculates an overall final ranking.

In addition to the ranking of a set of reference genes, the geNorm software calculates the optimal number of reference genes required for building an accurate normalization factor. To exploit this ability of the program which was not implemented in RefFinder, raw Cq values were transformed in relative quantities, taking into account PCR efficiencies, with the highest expression level set to 1 as described in the geNorm manual (2008). Relative quantities were loaded in geNorm software in order to determine the optimal number of reference genes necessary to measure gene expression in our experiment and to calculate a normalization factor.

The values of the normalization factor were used to normalize of the chitinase gene STMEO91 relative quantities. Standard errors were calculated as described in the geNorm manual (2008) to draw figure

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4. Differences in STMEO91 expression were compared by the way of a 10000 pairwise fixed reallocation randomization test, computed by the REST 2009 program. As we were working with a small number of samples in each class, randomization tests have the advantage of being more powerful than non-parametric tests and of making no assumption on distribution (Pfaffl et al. 2002). For the gene of interest STMEO91, and the reference genes RPN7, UBP22, OXA1 and MST2, the PCR efficiencies and the mean quantification cycle (Cq) value obtained for each sample were used as inputs. Differences were considered statistically significant at $p < 0.05$. Analyses were first done on the three biological replicates per allelic combination at both QTLs. As no significant effect was associated with the allelic phase at the major effect $GpaV_{spl}$ QTL, samples carrying resistance or susceptibility alleles at the minor effect QTL $GpaXI_{spl}$ were gathered whatever their allelic phase at $GpaV_{spl}$, giving a total number of six biological replicates for each condition.

Results and discussion

Strategy to select candidate reference genes and associated primer pairs

In studying gene regulation in potato-nematode interaction, we were looking for candidate reference genes suitable for normalization in RT-qPCR experiments. Thus, we selected a subset of putatively stably expressed genes from published studies, dedicated to biotic interactions in potato or to plant-nematode interactions. Following a microarray analysis, Jammes et al. (2005) validated nine genes by RT-qPCR, as not being differentially expressed between galls collected on roots infected by root-knot nematodes and uninfected roots of *A. thaliana*. We chose seven of these genes as starting point to select candidate reference genes suitable for expression studies in cyst nematode-potato interactions. We retrieved potato ortholog sequences, following a TBLASTN analysis, from the Gene Index Project database (Table 1). We added β-tubulin to compare our results with those published by Nicot et al. (2005) in the interaction between *P. infestans* and potato leaves.

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The genome of a potato-related species, *S. phureja*, has recently been sequenced and loci have been annotated (Xu et al. 2011). To identify the loci corresponding to candidate reference genes, the sequences of the seven potato TCs and the sequence of the β-tubulin (TUB) accession EU935742 were blasted to the *S. phureja* loci database (Table 1). OXA1, ATPase, OVA6 and RPN7 matched one single locus with more than 97 % identity. MST2 matched one single locus with 92 % identity. KAS1 matched two loci, one with 99.61 % identity and the second one with 88.81 % identity. TUB matched 10 loci in *S. phureja* database, the first one with 99 % identity on the whole sequence and the following one with 80 % identity. Thus, as KAS1 and TUB matched several loci in the potato genome, we were particularly cautious in that the primers designed on these genes matched only the locus with the best hit. UBP22 did not match any locus with the chosen expect threshold of e^{-100}, but matched with the same threshold, one single location on *S. phureja* chromosome 7 with 98 % identity on the whole sequence, revealing a lack of annotation of this locus.

Two to three primer pairs were designed for each candidate reference gene. For each candidate reference gene, the primer pair with the best efficiency was retained. The efficiencies of the chosen primer pairs ranged between 93 % and 100 % (Table 2). Specificity of primer pairs was checked using a BLASTN analysis as described in M&M. All primer pairs matched one single potato gene without mismatch and were suitable for candidate reference gene selection in potato. Interestingly, primer pairs designed on RPN7, MST2, OXA1, KAS1, OVA6 and TUB also matched one single tomato gene without mismatch, and were then also suitable for candidate reference gene selection in tomato.

Selection of reference genes for potato-nematode interaction analysis

Testing all the candidate reference genes on the whole set of samples, in which the expression of a gene of interest will be measured, is the best way to chose a set of reference genes and build an accurate normalization factor dedicated to this experiment (Guenin et al. 2009). To investigate gene regulation determined by resistance QTLs to *G. pallida* originating from *S. sparsipilum* (Caromel et al. 2005), we

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selected 12 potato clones from the QTL mapping progeny, three clones per allelic combination at both QTLs. Because the resistance phenotype appears shortly after the induction of the syncytium by the nematode (Mugniéry et al. 2001), inoculated and mock-inoculated root fragments were excised at three time points: 6 hpi corresponding to the migratory phase of the nematode toward the central cylinder, 2 dpi corresponding to the induction of the syncytium in roots of both resistant and susceptible clones, and 4 dpi corresponding to the degeneration of the syncytium in roots of clones carrying resistance alleles at both minor and major QTLs. qPCR analyses with the eight candidate reference genes were then carried out on 72 cDNA samples and were run twice, in two separated PCR plates.

Data were analyzed with a new WEB-based user-friendly software, named RefFinder (Xie et al. 2012, http://www.leonxie.com/referencegene.php). The statistical tests developed for candidate reference gene ranking, in the most popular programs geNorm (Vandesompele et al. 2002), NormFinder (Andersen et al. 2004), BestKeeper (Pfaffl et al. 2004) and the comparative delta-Ct method (Silver et al. 2006), are implemented in RefFinder, which produce an overall final ranking. RefFinder was previously used to rank candidate reference genes in papaya (Zhu et al. 2012), in leafy spurge (Chao et al. 2012) and in barley (Ovesna et al. 2012).

The ranking of our candidate reference genes was consistent between the programs, with slight differences (Table 3, Fig 1). RPN7, UBP22, OXA1 and MST2 ranked in the four first with all programs, except geNorm for which MST2 ranked sixth and KAS1 ranked fourth. By contrast, OVA6 and ATPase ranked seventh and eighth with all programs except BestKeeper, and were considered as the least stable transcripts. TUB ranked fifth with geNorm, sixth with delta-Cq method and NormFinder, and eighth with BestKeeper and was then ranked sixth in the comprehensive list calculated by RefFinder. Thus, even if TUB was one of the best ranked reference genes in the potato-*P. infestans* interaction (Nicot et al. 2005), five other genes ranked better in the potato-nematode interaction, and should be preferred for normalization. Nevertheless, the eight genes had high stabilities, with M values, calculating by geNorm, ranging from 0.67 to 1.08, far below the 1.5 maximum threshold recommended by Vandesompele et al. (2002).

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The geNorm original software (a VBA Microsoft Excel applet) offers more possibilities than the single ranking implemented in RefFinder. To use these additional possibilities, we converted raw Cq data in relative quantities, taking into account the amplification efficiencies. The ranking of the eight candidate reference genes was the same with the geNorm Excel applet as in the geNorm output returned by RefFinder. We used the geNorm software to calculate the optimal number of reference genes required for building an accurate normalization factor (NF). The program calculates the pairwise variation ($V_n/_{n+1}$) between the normalization factors NF_n and NF_{n+1} to determine the number of reference genes required for accurate normalization. A V-value below 0.15 was suggested as the limit under which it was unnecessary to add an additional gene for normalization (Vandesompele et al. 2002). Thus, with a V-value of 0.11, the optimal number of genes for normalization was four or five (Fig. 2). A NF was calculated with geNorm, using data from RPN7, UBP22, OXA1 and MST2. Despite KAS1 ranked four in the list provided by geNorm, we chose to include MST2 instead of KAS1 in the NF because the M value of MST2 (0.79) was closer to that of KAS1 (0.74) and because MST2 ranked in the four first stable genes with all the other programs.

Normalization of chitinase gene expression with the best ranked reference genes

To demonstrate the suitability of the selected reference genes in plant-nematode interaction, we used the four genes RPN7, UBP22, OXA1 and MST2 to normalize the expression level of a class II potato chitinase gene. Chitinase proteins have been classified as a subgroup of pathogenesis-related (PR) proteins that are induced when host plant cells are attacked by pathogens (Grover, 2012). Several chitinase genes are induced upon nematode infection (Rahimi et al. 1998). The potato chitinase gene STMEO91 has recently been demonstrated to be highly expressed in an incompatible interaction between potato and potato virus A, using microarray and RT-qPCR experiments (Vuorinen et al. 2010).

We wondered if STMEO91 was differentially regulated following nematode infection in roots of potato plants carrying resistance or susceptibility alleles at QTLs involved in resistance to *G. pallida*

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(Caromel et al. 2005), and if this change in expression level could be correlated with an allelic combination at resistance QTL. qPCR analyses showed that the expression level of the STMEO91 gene was low in potato roots at 6 hpi in resistant and susceptible plants, whatever the inoculated or mock-inoculated condition (Fig. 3). This observation is not surprising because at this time point, nematodes are in their migratory phase and the resistance do not appears before syncytium initiation (Mugniéry et al. 2001). For each time point, no significant difference between inoculated and mock-inoculated conditions was observed in plants carrying susceptibility alleles at both QTLs, nor in plants carrying the resistance allele at the single major effect $GpaV_{spl}$ QTL. In plants carrying the resistance allele at the low effect $GpaXI_{spl}$ QTL, whatever the allele at $GpaV_{spl}$ QTL, STMEO91 chitinase gene expression significantly increased in inoculated roots, once nematodes initiated their syncytium at 2 and 4 dpi (Fig. 3). At 2dpi the STMEO91 mean expression level was 94 times higher in roots of inoculated plants than in mock-inoculated ones. At 4dpi, the STMEO91 mean expression level slightly increased in inoculated roots of plants carrying the susceptible allele at $GpaXI_{spl}$ QTL as in mock-inoculated roots. In inoculated roots of plants carrying the resistance allele at $GpaXI_{spl}$ QTL, the STMEO91 mean expression level remained 8 times higher than in mock-inoculated roots.

Vuorinen et al (2010) observed a constitutively higher level of STMEO91 expression in potato plants carrying the resistance allele at a major dominant gene, conferring resistance to virus A (PVA), than in susceptible or partially resistant plants, but they did not observe a significant modification of STMEO91 gene expression after virus inoculation. By contrast, STMEO91 is highly up-regulated, in potato plants carrying $GpaXI_{spl}$ QTL, following nematode feeding site initiation.

Conclusion

In this study, we reported the identification and the validation of four reference genes as optimal for transcript normalization in both compatible and incompatible interactions between potato roots and potato cyst nematodes. These four genes RPN7, UBP22, OXA1 and MST2 were the most stably expressed genes in

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our experiment and were used to build a normalization factor. Due to a high level of homology, RPN7, UBP22, OXA1 and MST2 could be used as starting point for candidate reference gene selection in both potato and tomato interactions with additional root pathogens. Moreover, our results suggest a correlation between the up-regulation of the chitinase gene STMEO91 and the allelic phase at the low effect *GpaXI*$_{spl}$ resistance QTL, following potato-cyst nematode infection.

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Table 1 Candidate reference genes used for gene expression normalization in potato/*Globodera pallida* interactions. The potato TC number was given by the Gene Index Project (TGI) and the Arabidopsis locus name by the TAIR database. PGSC loci were identified in the PGSC potato genome database.

Gene symbol	TGI Potato TC number	Best match PGSC loci	Arabidopsis ortholog locus	Arabidopsis locus description	Biological process
OXA1	TC204983	PGSC0003DMG400005839	At5g62050	Inner membrane protein	Protein complex assembly
UBP22	TC212444	* chr07 2589188-2590066	At5g10790	Ubiquitin carboxyl-terminal hydrolase	Ubiquitin-dependent protein catabolic process
ATPase	TC201915	PGSC0003DMG400016959	At4g09650	ATP synthase delta chain	Plasma membrane ATP synthesis coupled proton transport
OVA6	TC205011	PGSC0003DMG400027526	At5g52520	Prolyl-tRNA synthetase, putative	Prolyl-tRNA aminoacylation
RPN7	TC203637	PGSC0003DMG40202421	At4g24820.2	26S proteasome regulatory subunit, putative	Protein synthesis
KAS1	TC200484	PGSC0003DMG400030158	At5g46290	3-oxoacyl-(acyl-carrier-protein) synthase I	Fatty acid biosynthetic process
MST2	TC206257	PGSC0003DMG400022232	At1g16460	Mercaptopyruvate sulfurtransferase-like protein	Unclassified proteins
TUB	EU935742	PGSC0003DMG400009938	At1g75780.1	Beta tubulin gene	Microtubule-based movement

* No PGSC locus was annotated for UBP22, but one single genome location on *S. phureja* pseudomolecules was identified with 98 % identity on the whole sequence.

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Table 2 Primer sequences and amplicon lengths for eight candidate reference genes and a chitinase gene. Amplification efficiency (*E*) and coefficient of correlation (R^2) of primer pairs were calculated with MxPro qPCR software.

Gene symbol	**Primer sequence**		**Amplicon length (bp)**	***E* (%)**	R^2
OXA1	Forward	5'-GCGATTCTGAAGCCAAAACT-3'	87	98	0.99
	Reverse	5'-TTGTTGACCTTCAGCCACAG-3'			
UBP22	Forward	5'-CAATGCAGCAACGCCAGA-3'	98	100	0.99
	Reverse	5'-AGCAGCAGTAGCACCACCAC-3'			
ATPase	Forward	5'-GGGAGCGAAAAATGTGAGAA-3'	145	93	0.99
	Reverse	5'-AATTTCAAGCTGTGCAGCAA-3'			
OVA6	Forward	5'-GCTGGTGACGAGGAAGAATC-3'	105	95	0.99
	Reverse	5'-GCCTGTCATCAGGCACTTCT-3'			
RPN7	Forward	5'-TTGGGGTGTCTGAGGATTTC-3'	123	100	0.99
	Reverse	5'-CATTCTTTGCATCAGGACGA-3'			
KAS1	Forward	5'-GCAACCTTCTGCTTCTGAGG-3'	170	100	0.99
	Reverse	5'-CTGCCTAACCAAGCCTGAAA-3'			
MST2	Forward	5'-AGTGCAGCTCGTGTATGGTG-3'	94	100	0.99
	Reverse	5'-ATCCAGAAGCACGCCATCT-3'			
TUB	Forward	5'-AGTTCACTGAAGCCGAGAGC-3'	173	96	0.99
	Reverse	5'-GGCAGAAATTGAACAAACCAAC-3'			
STMEO91	Forward	5'-ACCGCCCGTAAGAAGGAAAT-3'	70	100	0.99
	Reverse	5'-CTGCACTACCACCATTAGTTTCGT-3'			

Table 3 Ranking of eight candidate reference genes in potato-nematode interaction, according to their expression stability values as given by RefFinder, based on geNorm, NormFinder, BestKeeper and comparative Delta Cq methods.

	Ranking Order							
Method	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Delta Cq	RPN7	UBP22	OXA1	MST2	KAS1	TUB	OVA6	ATPase
BestKeeper	MST2	RPN7	UBP22	OXA1	ATPase	OVA6	KAS1	TUB
NormFinder	UBP22	RPN7	OXA1	MST2	KAS1	TUB	OVA6	ATPase
geNorm	RPN7 | UBP22		OXA1	KAS1	TUB	MST2	OVA6	ATPase
RefFinder recommended comprehensive ranking	**RPN7**	**UBP22**	**MST2**	**OXA1**	**KAS1**	**TUB**	**OVA6**	**ATPase**

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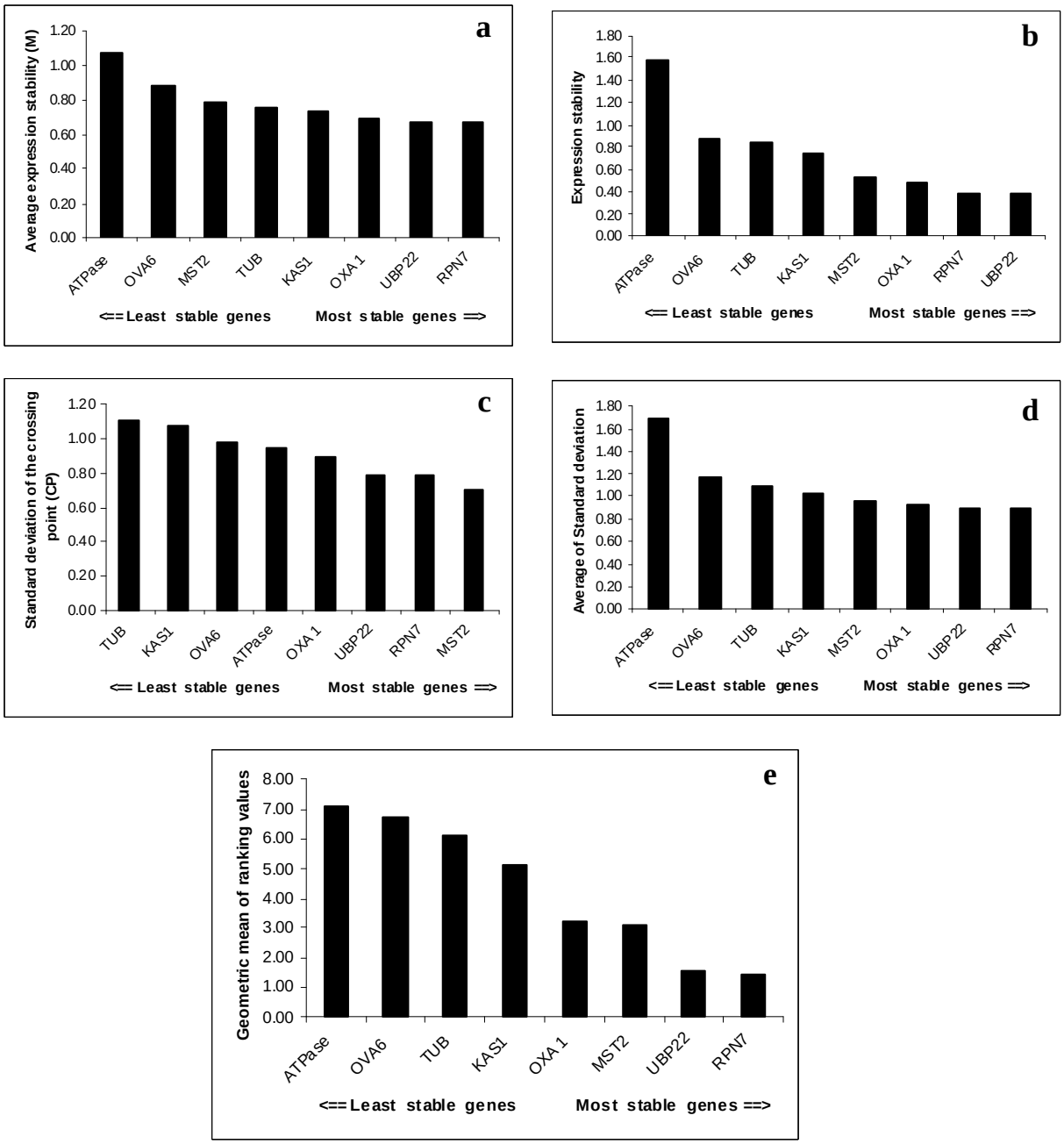


Fig. 1 Ranking of expression stability values of eight candidate reference genes in roots of potato infected or uninfected by nematodes calculated with RefFinder software, **(a)** ranking calculated with geNorm program, **(b)** ranking calculated with NormFinder program, **(c)** ranking calculated with BestKeeper program, **(d)** ranking calculated with the comparative Delta Cq method, **(e)** recommended comprehensive ranking provided by RefFinder. The lowest values indicate more stable transcript expression.

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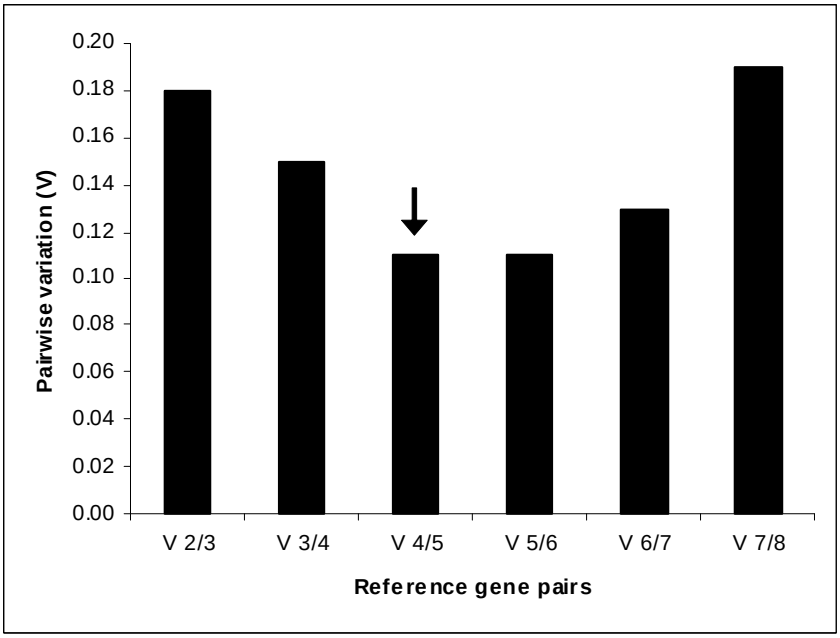


Fig. 2 Determination of the optimal number of reference genes for transcript normalization, using geNorm analysis. Calculation of pairwise variation between two sequential candidate genes (Vn/Vn+1) was used to evaluate the effect of adding an extra gene in the normalization factor. As the four best ranked reference genes (RPN7, UBP22, MST2 and OXA1) had a V value below 0.15, as recommended by Vandesompele et al. (2002), no extra gene is required. The arrow indicates the optimal number of genes for normalization.

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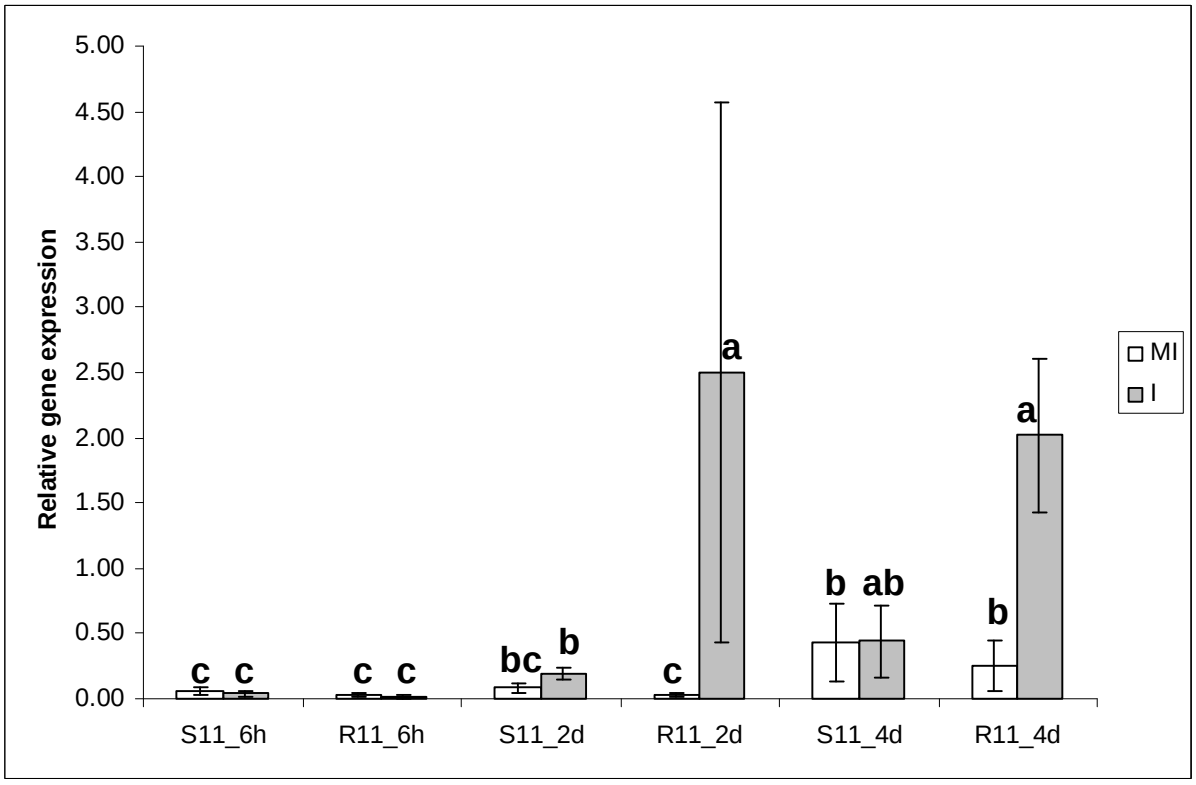


Fig. 3 Relative expression level of the STMEO91 chitinase gene, normalized with RPN7, UBP22, MST2 and OXA1 reference genes. The samples were classified according to their allele at the *GpaXI*$_{spl}$ QTL (S11 or R11 for susceptibility or resistance allele), in inoculated (I) or mock-inoculated (MI) conditions, during a three-point time-course (6h = 6hpi, 2d = 2dpi and 4d = 4dpi). Error bars are standard error of mean quantities. Different letters represent significant differences between levels of expression, as calculated with the REST software (Pfaffl et al. 2002).

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