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Short communication

Selection and validation of reference genes for transcript normalization in gene expression studies in *Catharanthus roseus*

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ABSTRACT

Quantitative Real-Time PCR (qPCR), a sensitive and commonly used technique for gene expression analysis, requires stably expressed reference genes for normalization of gene expression. Up to now, only one reference gene for qPCR analysis, corresponding to *40S Ribosomal protein S9* (*RPS9*), was available for the medicinal plant *Catharanthus roseus*, the only source of the commercial anticancer drugs vinblastine and vincristine. Here, we screened for additional reference genes for this plant species by mining *C. roseus* RNA-Seq data for orthologs of 22 genes known to be stably expressed in *Arabidopsis thaliana* and qualified as superior reference genes for this model plant species. Based on this, eight candidate *C. roseus* reference genes were identified and, together with *RPS9*, evaluated by performing qPCR on a series of different *C. roseus* explants and tissue cultures. NormFinder, geNorm and BestKeeper analyses of the resulting qPCR data revealed that the orthologs of *At2g28390* (SAND family protein, *SAND*), *At2g32170* (N2227-like family protein, *N2227*) and *At4g26410* (Expressed protein, EXP) had the highest expression stability across the different *C. roseus* samples and are superior as reference genes as compared to the traditionally used *RPS9*. Analysis of publicly available *C. roseus* RNA-Seq data confirmed the expression stability of *SAND* and *N2227*, underscoring their value as reference genes for *C. roseus* qPCR analysis.

1. Introduction

The medicinal plant *Catharanthus roseus* (Madagascar periwinkle) is a species of flowering plants native to Madagascar. Because of its long flowering period and its tolerance to drought and nutrient deficiency, *C. roseus* is widely cultivated as an ornamental plant in parks and gardens in regions with a (sub)tropical climate (Kumar et al., 2012). As a medicinal plant, *C. roseus* synthesizes over 130 different monoterpenoid indole alkaloids (MIAs), including the pharmaceutically important molecules ajmalicine and serpentine. Furthermore, it is the only source of the commercial drugs vinblastine and vincristine, MIAs that are commonly used in the treatment of several types of cancer, including leukemia and lymphoma (El-Sayed and Verpoorte, 2007; Van Der Heijden et al., 2004; Verma et al., 2012). The combination of complex MIA mixtures naturally occurring in *C. roseus* leaves, low production amounts of the individual MIAs and environmental instability in *C. roseus* cultivating countries leads to high market prices of the purified compounds and makes them excellent targets for breeding, metabolic engineering or synthetic biology programs that aim to enhance or alter their production (Miettinen et al., 2014; Van Der Heijden et al., 2004).

For any metabolic engineering effort or study of *C. roseus*, it may be necessary to investigate the expression of a target gene by quantitative Real-Time PCR (qPCR), a sensitive technique for gene expression analysis that is used in many research fields. The most appropriate normalization strategy for qPCR is the use of experimentally validated reference genes (Bustin et al., 2009), i.e., genes that are stably expressed among the different analyzed samples and that are unaffected by an experimental treatment. Furthermore, it is often mandatory to use more than one reference gene to achieve accurate normalization of gene expression (Vandesompele et al., 2002). For several studies with *C. roseus*, qPCR was used to assess the expression of a particular gene. For most of them, normalization was done using a single reference gene, *40S Ribosomal protein S9* (RPS9), originally described as a control gene for Northern blot analysis (Menke et al., 1999). Hence, this gene may not be the most suitable reference gene for normalization of

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C. roseus qPCR data. In this study, we used the geNorm, NormFinder and BestKeeper algorithms to evaluate the expression stability of *RPS9* and eight other candidate *C. roseus* reference genes that were identified by mining of *C. roseus* RNA-Seq data for orthologs of a set of 22 known reference genes for transcript normalization from the model plant *Arabidopsis thaliana* (Czechowski et al., 2005).

2. Materials and methods

2.1. Plant materials, cultivation and treatments

Ten different *C. roseus* plant materials were generated to use for the qPCR analysis. Cultivation of *C. roseus* plants and generation of calli and hairy roots were carried out as described (Häkkinen et al., 2012; Van Moerkercke et al., 2013). Elicitation of *C. roseus* shoots with 1 mM methyl jasmonate (MeJA) was performed as described (Van Moerkercke et al., 2013). An overview of the different *C. roseus* tissues and treatments is given in Table 1.

2.2. RNA extraction and cDNA synthesis

Total RNA was extracted from 100 mg of homogenized *C. roseus* plant material using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). To avoid genomic DNA contamination, an on-column DNase digestion was performed during RNA extraction according to the manufacturer's instructions. The concentration and purity of the obtained RNA was determined using a NanoDrop® ND-1000 UV–Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and the RNA integrity was assessed by gel electrophoresis on a 1.2% agarose gel stained with SYBR Safe™ (Life Technologies, Carlsbad, CA, USA). cDNA was synthesized from 1 μg of total RNA using the iScript cDNA synthesis kit (Biorad, Hercules, CA, USA) according to the manufacturer's instructions in a final volume of 20 μL. The iScript cDNA synthesis kit uses a blend of oligo(dT) and random hexamer primers for cDNA synthesis. After synthesis, the template for qPCR was prepared by diluting the obtained cDNA eightfold with ultrapure water.

2.3. qPCR

qPCR primers were designed using Beacon Designer version 4.0 (Premier Biosoft International, Palo Alto, CA, USA) with default parameters. qPCR reactions were carried out in triplicate in 384-well plates in a final volume of 5 μL. Each 5 μL reaction contained 0.5 μL qPCR template, 2.5 μL SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA, USA) and 2.0 μL of primer mix (1.25 μM for each primer, leading to a final concentration of 0.5 μM for each primer in the 5 μL reaction). The reactions were prepared and dispensed in the 384-well plates using a JANUS Automated Workstation (Perkin Elmer, Waltham, MA, USA) and qPCR was carried out with a LightCycler 480 (Roche Applied Science, Penzberg, Germany). The qPCR reaction profile consisted of an initial activation of 10 min at 95 °C, followed by 45 cycles of 95 °C for 10 s, 60 °C for 15 s and 72 °C for 15 s. Finally, a melting curve was generated by increasing the temperature from 65 °C to 95 °C with continuous monitoring of the SYBR Green fluorescence. For each reaction, the crossing point (Cp) value was determined using the Second Derivative Maximum method with the LightCycler 480 software with default parameters. After converting the data to the required input files, stable reference genes were selected using the geNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004) and BestKeeper (Pfaffl et al., 2004) algorithms.

Table 1
C. roseus plant materials used for analysis of candidate reference genes.

Sample	Tissue	Cultivar	Treatment
1	Callus	Würzburg	–
2	Callus	Leiden	–
3	Hairy roots	Würzburg	–
4	*In vitro* plants	Würzburg	–
5	Roots	Würzburg	–
6	Buds	Würzburg	–
7	Shoots	Würzburg	6 h DMSO
8	Shoots	Würzburg	6 h MeJA
9	Shoots	Würzburg	24 h MeJA
10	Shoots	Würzburg	24 h DMSO

3. Results and discussion

3.1. Selection of candidate reference genes

Previously, we described the assembly of CathaCyc, a *C. roseus* metabolic pathway database built from Illumina HiSeq2000 RNA-Seq data (Van Moerkercke et al., 2013). The *C. roseus* reference transcriptome used to create this pathway database contains a set of 31,450 contigs that are accessible for analysis through the web interface of the ORCAE database (http://bioinformatics.psb.ugent.be/orcae). These contigs were assembled by combining RNA-Seq data from two research consortia, SmartCell (http://www.smartcell.org/) and the Medicinal Plant Genomics Resource (MPGR) consortium (http://medicinalplantgenomics.msu.edu/). The SmartCell dataset comprises two independent experiments in which *C. roseus* suspension cells and shoots treated or not with MeJa were used (Van Moerkercke et al., 2013). The MPGR dataset comprises RNA-Seq data from 23 different *C. roseus* tissues and cultures grown in different conditions (Góngora-Castillo et al., 2012).

To screen for candidate qPCR reference genes, TBLASTX searches using the nucleotide sequences of 22 genes from *A. thaliana*, shown to be superior reference genes in this model plant (Czechowski et al., 2005), were performed in the *C. roseus* reference transcriptome, revealing orthologs for 20 of them. Subsequently, candidate qPCR reference genes were withheld based on three criteria: (1) they were stably expressed in both the suspension cells and shoots datasets from SmartCell; (2) only one unique ortholog is present in the *C. roseus* reference transcriptome; and (3) the orthologs show >99% sequence identity between the SmartCell and MPGR datasets. Based on these criteria, eight candidate reference genes were retained.

The eight genes that were retained are the orthologs of *At5g46630* (clathrin adaptor complex subunit, *CACS*), *At4g26410* (expressed protein with unknown function, *EXP*), *At5g12240* (expressed protein with unknown function, *EXPR*), *At4g33380* (unknown protein F17M5, *F17M5*), *At*5g15710 (F-box domain containing protein, *Fbox*), *At2g32170* (N2227-like family protein, *N2227*), *At2g28390* (SAND family protein, *SAND*), and *At4g34270* (TIP41-like family protein, *TIP41*). An overview of the names and the *C. roseus* reference transcriptome (Caros) accession numbers of the retained genes is provided in Table 2.

3.2. Analysis of expression stability of the candidate reference genes

3.2.1. Primer design and qPCR analysis

By performing qPCR on ten different *C. roseus* samples (Table 1), including callus, hairy roots, in vitro plants, roots, buds and shoots treated or not with MeJa, Cp values were obtained for the eight candidate reference genes and for *RPS9* (Menke et al., 1999), that was previously used as a reference gene for *C. roseus* expression analysis. Analysis of the melting curves and agarose gel electrophoresis indicated that for each specifically designed primer pair

Table 2
The selected candidate reference genes, qPCR primers and product length.

At-code	Annotation	Gene ID	Caros accession number	qPCR primers	Product length
At2g28390	SAND family protein	*SAND*	Caros010066.1	Fw: TGCTGTGGAGGAGGAAGAAG Rv: ACTGGCGGAACTACTACTACC	88
At2g32170	N2227-like family protein	*N2227*	Caros011588.1	Fw: TCCTTACGCCGCATTATCAG Rv: AGATGAGACAGTAACGCCTTG	122
At4g26410	Expressed protein (unknown function)	*EXP*	Caros010480.1	Fw: ACAATACCATCGCCATCAC Rv: AAGAGGACTGCTGGAAGG	172
At4g33380	unknown protein F17M5	*F17M5*	Caros008946.1	Fw: CGGCTTCCTCCTGAATGTC Rv: GCTCATACGGGCAATAAACC	181
At4g34270	TIP41-like family protein	*TIP41*	Caros003969.1	Fw: CGGTGAGAAAGAACTGAAGG Rv: GATGAAGGGACTTGAGAATGG	119
At5g12240	Expressed protein (unknown function)	*EXPR*	Caros004934.1	Fw: CGCATTCTCAACCTCTTCC Rv: ATCACCACGGTCACTTCC	168
At5g15710	F-box domain containing protein	*Fbox*	Caros012244.1	Fw: TTGGGTTGAGATAAGTCGGATG Rv: CTGGCTGTTGTATGATTGAAGAG	199
At5g46630	Clathrin adaptor complex subunit	*CACS*	Caros003381.1	Fw: GCGGCGATGTCCTCATCAATC Rv: GCATCCTCCAATCTGACGAACTG	128
–	40S ribosomal protein S9	*RPS9*	Caros004092.1	Fw: GGTTGTCAATGTTCCTTCCTTC Rv: TCTTCATCCTCTTCATCTCCATC	168

(see Table 2 for the sequences), only a single product was amplified (Fig. 1). The average Cp values for the candidate reference genes ranged between 23 and 30 cycles (Fig. 2 and Table 3), with *N227* having the lowest (average Cp = 29.024) and *EXPR* the highest (average Cp = 23.791) transcript levels. The obtained Cp values were used to calculate the expression stability of the candidate reference genes with the geNorm (Vandesompele et al., 2002), NormFinder (Andersen et al., 2004) and BestKeeper (Pfaffl et al., 2004) algorithms.

3.2.2. geNorm analysis

A first algorithm that is commonly used to predict the most suitable qPCR reference genes is geNorm (Vandesompele et al., 2002), which works on the assumption that the expression ratio of two ideal reference genes is constant in different tested templates. For each candidate reference gene, geNorm calculates a gene expression stability value (*M*) that is the average of the pairwise variation for that candidate reference gene with all other candidate reference genes. As a result, genes with a stable expression

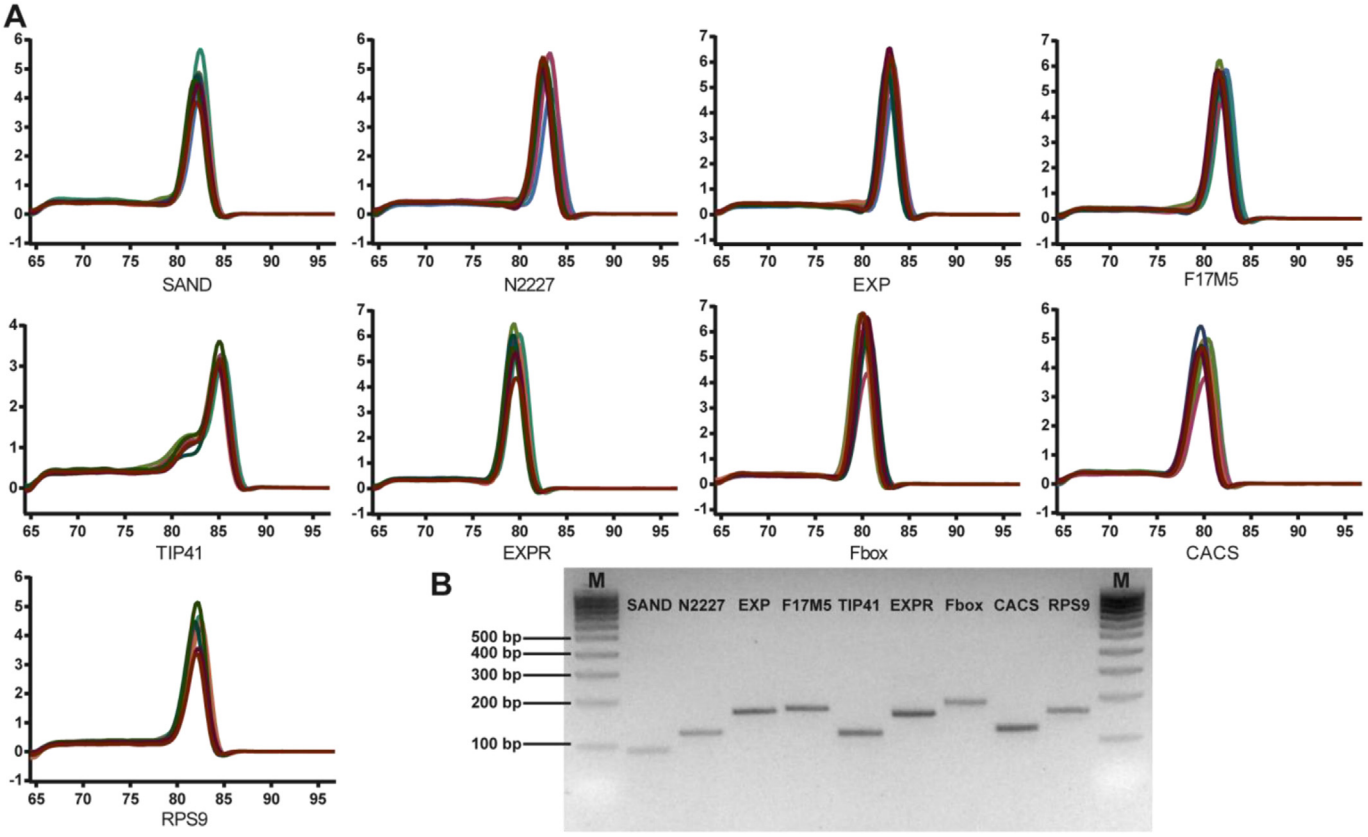

Fig. 1. qPCR primer specificity and amplicon length. A. Melting curves generated for all genes. The curves were generated using the first repeat of each of the ten *C. roseus* samples. B. Agarose gel electrophoresis of the amplified products revealed a single band of the expected size for each primer pair. M, DNA size marker (Eurogentec SmartLadder SF).

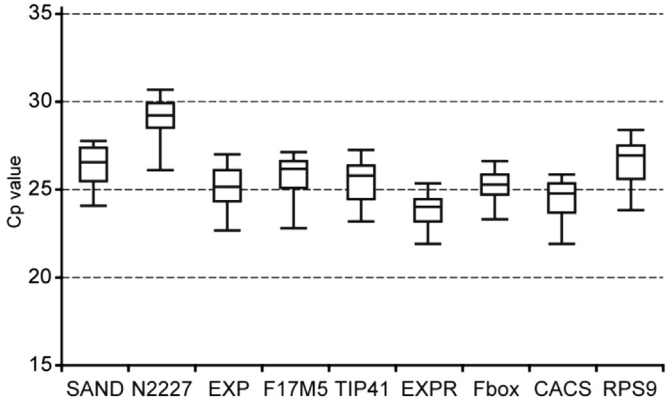

Fig. 2. Box and whisker plot displaying the Cp values for the candidate reference genes in all *C. roseus* samples. The lower and upper edges of the box represent the 25th and the 75th percentiles, respectively; the line inside the box indicates the median value; the whiskers represent the maximum and minimum values.

throughout the dataset (as compared to the other candidate reference genes) will have low M-values, whereas the more unstable genes will have high M-values. Subsequently, the gene with the highest M-value is eliminated from the dataset, and new M-values are calculated. This iterative process is repeated until there are only two genes left, which are considered the optimal reference genes. The M-values were calculated for all nine investigated candidate reference genes and showed that *N2227* and *CACS* had the highest expression stability throughout the dataset obtained by qPCR on the ten *C. roseus* samples and are thus the optimal reference genes according to this analysis (Fig. 3).

3.2.3. NormFinder analysis

NormFinder (Andersen et al., 2004), the second algorithm that was used to assess the stability of the set of candidate reference genes, uses a mathematical model of gene expression to identify the most stable reference genes. As output, the NormFinder algorithm provides a stability value for each gene that is reflective of its expression variation, with lower stability values corresponding to lower variation, and hence, higher stability of the gene. The NormFinder algorithm ranked the candidate reference genes in a different order as the geNorm analysis, with *SAND* and *EXP* being the most stable (Fig. 4).

3.2.4. BestKeeper analysis

The third algorithm that was used, BestKeeper (Pfaffl et al., 2004), ranks the candidate reference genes according to their coefficient of correlation (R) to the BestKeeper Index (BI). The BI is the geometric mean of the Cp values of the candidate reference genes.

Table 3
Overall ranking of the candidate reference genes after geNorm, NormFinder and BestKeeper analysis, and the observed average crossing point (Cp) and PCR efficiencies (E).

Overall rank	Gene	geNorm rank	NormFinder rank	BestKeeper rank	Total score	Average Cp	Average E
1	*SAND*	3	1	1	5	26.415	1.905
2	*N2227*	1	3	3	7	29.024	1.846
3	*EXP*	5	2	2	9	25.181	1.875
4	*CACS*	1	4	4	9	24.470	1.888
5	*TIP41*	4	5	6	15	25.626	1.869
6	*Fbox*	6	7	5	18	25.244	1.871
7	*EXPR*	7	6	7	20	23.791	1.882
8	*RPS9*	8	8	8	24	26.644	1.704
9	*F17M5*	9	9	9	27	25.796	1.855

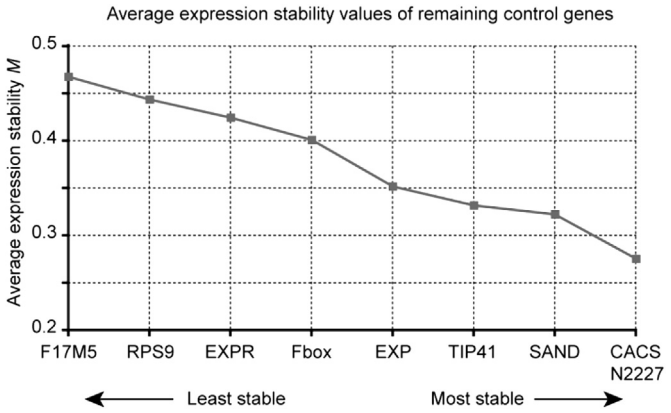

Fig. 3. Expression stability and ranking of the candidate reference genes according to the geNorm algorithm.

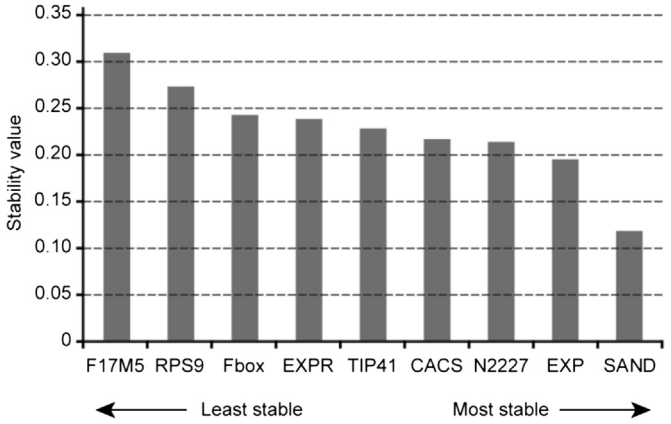

Fig. 4. Expression stability and ranking of the candidate reference genes according to the NormFinder algorithm.

Like the NormFinder algorithm, the best correlations with BestKeeper were obtained for *SAND* ($R = 0.986$) and *EXP* ($R = 0.982$) (Fig. 5).

3.2.5. Global ranking of the candidate C. roseus reference genes

The expression stability analysis using the geNorm, NormFinder and BestKeeper algorithms gave a different ranking of the candidate reference genes. This difference in ranking has been noted in

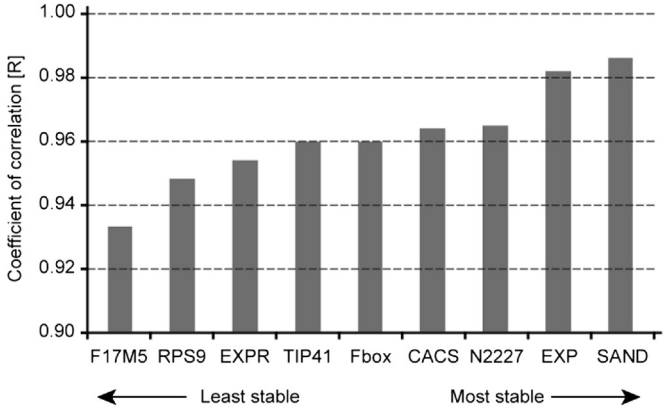

Fig. 5. Expression stability and ranking of the candidate reference genes according to the BestKeeper algorithm.

other studies (Wan et al., 2010; Xu et al., 2011) and may be attributed to the distinct statistical algorithms used by the three methods. The geNorm algorithm ranks the genes according to the gene expression stability value (M), which is a measure of the pairwise variation of the candidate genes. Hence, the two most stable genes according to the geNorm analysis, *CACS* and *N2227*, are the two genes that have the most identical expression pattern throughout the sample set. In contrast, the NormFinder and BestKeeper algorithms consider all candidate reference genes to create the ranking and both showed that *SAND* and *EXP* are the most stable reference genes, followed by *N2227* and *CACS*. When ranked according to their performance in all three tests, *SAND* is the most stable reference gene, followed by *N2227* and *EXP* (Table 3). According to all used algorithms, *RPS9* was the second most unstable of the tested reference genes.

3.3. *In silico assessment of the top reference genes in the C. roseus gene expression atlas*

As part of CathaCyc/ORCAE, the *C. roseus* RNA-Seq gene expression atlas allows to visualize the expression pattern of any gene of interest in the different experimental conditions included in the RNA-Seq atlas (Van Moerkercke et al., 2013). Currently, the *C. roseus* RNA-Seq gene expression atlas holds the expression data from the MPGR and SmartCell consortia, totaling a set of 30 different *C. roseus* plant organs and plant, suspension cell and hairy root cultures grown under various conditions and treatments. When probed for the expression pattern of *SAND* (CathaCyc/ORCAE accession number Caros010066.1), the RNA-Seq atlas revealed that this gene was stably expressed throughout the various experimental conditions (Fig. 6A). Similarly, a stable expression was

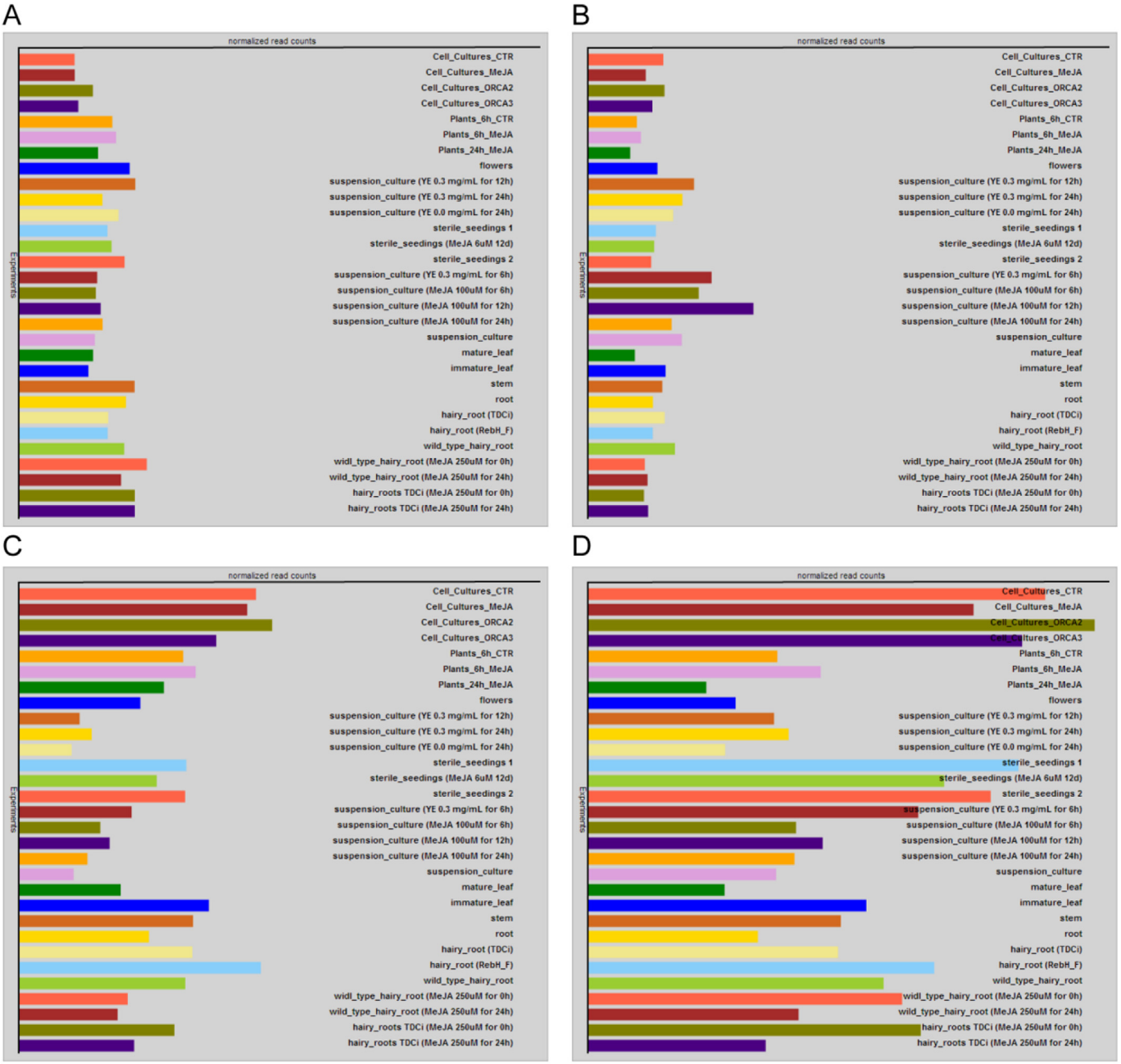

Fig. 6. Expression profile of *SAND* (A), *N2227* (B), *EXP* (C) and *RPS9* (D) according to the *C. roseus* RNA-Seq gene expression atlas.

observed for *N2227* (CathaCyc/ORCAE accession number Caros011588.1; Fig. 6B). When the RNA-Seq atlas was probed for the expression pattern of *EXP* (CathaCyc/ORCAE accession number Caros010480.1; Fig 6C), slightly more variation was observed as compared to *SAND* or *N2227*. Notably, when probed for in the *C. roseus* RNA-Seq atlas, considerable expression variation was observed for *RPS9* (Fig. 6D).

4. Conclusion

Prior to this study, *RPS9* was the only available reference gene for expression analysis in *C. roseus*. Accordingly, it has been used as such in numerous studies. Here, we evaluated the performance of this gene in parallel with eight candidate reference genes that were the *C. roseus* orthologs of known *Arabidopsis* reference genes and that were identified through mining of *C. roseus* RNA-Seq data. According to our qPCR analysis, the used algorithms, and mining of public quantitative RNA-Seq data, two reference genes, *SAND* and *N2227*, were identified as superior reference genes for transcript normalization in *C. roseus*. The use of these two genes as reference genes instead of *RPS9* may lead to more accurate normalization of *C. roseus* qPCR data.

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Author contribution

Jacob Pollier, Heiko Rischer and Alain Goossens designed the research. Jacob Pollier and Robin Vanden Bossche performed the research. Jacob Pollier and Alain Goossens wrote the paper.

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