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Reference genes for RT-qPCR analysis in *Musa acuminata* genotypes contrasting in resistance to *Fusarium oxysporum* f. sp. *cubense* subtropical race 4

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Banana (*Musa* spp.) is the most widely consumed fruit globally. Fusarium wilt, caused by *Fusarium oxysporum* f. sp. *cubense* (*Foc*), is a highly threatening disease to banana production. Resistance genes to *Foc* exist in wild *Musa* genotypes such as *Musa acuminata* subsp. *burmannicoides* var. Calcutta 4. Whilst real-time PCR (RT-qPCR) is appropriate for accurate analysis of gene expression in pathways involved in host defence responses, reference genes with stable expression under specific biotic stress conditions and host tissue types are necessary for normalization of sample variation. In this context, the stability in potential host reference genes *ACT1*, *APT*, *EF1α*, *GAPDH*, *αTUB*, *RAN*, *UBIQ1*, *UBIQ2*, *βTUB1*, *βTUB3*, *L2* and *ACTA1* was evaluated in total RNA samples from root tissues in Calcutta 4 (resistant) and *Musa* sp. cultivar Prata-anã (susceptible) extracted during interaction with *Foc* subtropical race 4 (STR4). Expression stability was calculated using the algorithms geNorm, NormFinder and BestKeeper. *βTUB3* and *L2* were identified as the most stable in Calcutta 4, with *ACTA1* and *GAPDH* the most stable in Prata-anã. These reference genes for analysis of gene expression modulation in the *Musa*-*Foc STR4* pathosystem are fundamental for advancing understanding of host defence responses to this important pathogen.

Keywords *Musa*, *Fusarium oxysporum*, Gene expression normalization

Banana (*Musa* spp.) is one of the most widely traded and consumed fruits globally and, as a basic carbohydrate component of the diet in numerous developing countries, is highly relevant for global food security[1,2]. With cultivated edible bananas and plantains derived from the fertile diploid progenitor species of *Musa acuminata* (AA) and *Musa balbisiana* (BB), many of today's commercial cultivars such as 'Cavendish' (AAA), 'Pome' (AAB), and 'Silk" (AAB) are sterile, with seedless fruits developing via parthenocarpy[3]. As genetic diversity is low across such important cultivars, resistance to evolving pathogens is limited[4].

Fusarium wilt is currently the most devastating and widely disseminated fungal disease of banana globally[2,5,6]. Vascular wilt due to pathogen advance in xylem vessels restricts water movement, resulting in leaf yellowing, pseudostem splitting and eventual plant death[7]. Fusarium wilt is caused by the soilborne fungus *Fusarium oxysporum* f. sp. *cubense* (*Foc*)[6,8], with three physiological races of *Foc* capable of infecting banana, namely race 1, race 2, and race 4, with the latter subdivided into tropical race 4 (TR4) and subtropical race 4 (STR4)[8]. Cultivars of the 'Gros Michel' group (AAA), 'Pome' (AAB), and 'Silk' (AAB) are susceptible to all races of *Foc*, while cultivars of the 'Cavendish' subgroup (AAA) are typically resistant to race 1 of *Foc* but susceptible to STR4 and TR4[6]. Breakdown of resistance to Foc race 1 (VCG0124[9], 0125 and 01220[10]) in Cavendish cultivars has, however, been reported in certain growing regions[11–14].

The fungal pathogen *Foc* can survive in soils in the absence of the host plant, with chlamydospores, which are thick-walled survival spores, able to maintain viability for periods in excess of 20 years[15]. Given such pathogen

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persistence, the employment of resistant varieties is essential for disease management. In contrast to susceptible cultivars, fertile resistant wild diploid *Musa* genotypes, that have coevolved with pathogens at the centre of origin of the genus, represent a source of defence genes and alleles that are appropriate for genetic improvement of cultivars via genetic engineering or gene modification.

For candidate gene discovery, RNAseq-based high-throughput sequencing approaches for investigation of host transcriptome responses in *Musa* materials contrasting in resistance are advancing understanding of the cellular-level defence response involved in resistance to *Foc*[16–19]. Following such investigations, in silico-derived candidate genes in the defence response require lab-based validation via the reverse transcription real-time polymerase chain reaction (RT-qPCR), which is widely employed to accurately quantify gene expression levels in cDNA derived from RNA[20–22]. The guidelines of the Minimum Information for Publication of Real-Time Quantitative PCR Experiments (MIQE)[23] recommend that for accurate measurement of target gene expression, normalization is necessary to that of a reference gene which displays stable expression for any particular tissue type or experimental treatment[24]. As reference genes are subject to correction of the effect of cDNA quality[25,26], normalization of target gene expression to reference genes also corrects for variation between sample replicates that may occur during cDNA synthesis.

Several studies have been conducted in recent years for the development of stable reference genes for different genotypes and tissue types in *Musa* sp.[27–30], with data highlighting that genes involved in basal cell activities, such as *ACT*, *UBQ*, *GAPDH*, *EF*, *L2*, *TUB*, *GAPDH*, *RAN*, and *APT*, amongst others[28], may not necessarily show a stable constitutive expression under particular experimental conditions. As such, the identification of specific stable reference genes is required for each plant genotype, tissue type and experimental treatment.

Several mathematical algorithms are available for assessing the stability of potential reference genes, with three programs widely employed, namely geNorm[25], NormFinder[26] and BestKeeper[31]. Each offers distinct statistical parameters for the prediction of gene stability and the optimal number of reference genes for accurate normalization of RT-qPCR data.

Here, 12 candidate reference genes were compared for gene expression normalization in root tissues in *Musa* genotypes contrasting in resistance to the vascular wilt pathogen *F. oxysporum* f. sp. *cubense* STR4, namely *Musa acuminata* subsp. *burmannicoides* var. Calcutta 4 (C4) (resistant) and *Musa* sp. cultivar Prata-anã (PA) (susceptible). The stability of the candidate reference genes was validated through examination of the relative expression of a Thaumatin-like protein 1 (*TL1*) target gene, encoding a Pathogenesis-related protein 5 (PR5). The reference gene sets developed in this study will enable accurate analysis of expression of target genes involved in defence responses to this important pathogen.

Results

Analysis of expression levels and Cq values of candidate reference genes

Information regarding primer sequences and PCR amplification conditions for the 12 candidate reference genes is summarized in Table 1. Dissociation curve analysis confirmed amplification specificity for all the tested primers, with single peaks observed in melting curves with cDNA material from inoculated and non-inoculated treatments in C4 and PA (Fig. 1). Variations in cycle quantification (Cq) values across treatments were depicted in a Box-plot representation (Fig. 2), with intervals, means, medians, and outliers presented for the tested reference genes across the four treatments. Overall, Cq values were generally lower in C4 when compared to PA, indicating greater expression. The lowest Cq values were observed for the genes *ACTA1* and *bTUB3* in C4, and for *GAPDH* and *bTUB3* in PA. Highest Cq values, by contrast, were observed for the genes *APT*, *RAN* and *Ubiq1* in both genotypes, indicating lowest expression levels. In the case of C4, greater variation in outlier Cq values was observed in the genes *bTUB1* and *Ubiq1*. No such variation was observed in the case of PA.

Determination of candidate reference genes stability

Stability analysis and rankings for the candidate reference genes were conducted utilizing the mathematical algorithms geNorm, NormFinder and BestKeeper. Data was analysed separately for both genotypes, with gene expression data originating from root tissue samples collected at 1, 2, and 4 days after inoculation (DAI) and similarly from mock-inoculated controls. The combined analysis of gene expression stability was based on the total datasets generated for the two genotypes, combining data for biotic stress treatments and non-stressed controls.

When considering all C4 samples, *L2* and *BTUB3* were ranked as the most stable genes according to analyses conducted with geNorm and NormFinder, exhibiting the lowest M (average expression stability) and SV (stability value). Based on BestKeeper, *UBIQ2* and *UIBIQ1* genes were the most highly ranked in terms of stability, with the lowest SD (standard deviation) observed in expression values (Table 2). In the case of all PA samples, *GAPDH* and *ACTIN* 1 were identified as the genes with the lowest M values according to analysis with geNorm. By contrast, NormFinder identified *TUBa* and *APT* as the most stable genes based on SV values, with BestKeeper indicating the greatest stability in *bTUB3* and *L2*, with these genes displaying the lowest SD. For both genotypes, following the $M \leq 0.5$ cutoff set by geNorm, the first 7 genes would qualify as stable and suitable for use (Table 2).

In analysis of the Global datasets, geNorm identified only *TUBa* and *ACTIN1* as stable genes, with M values ≤ 0.5. NormFinder identified *TUBa* and *bTUB3*, based on SV values, while BestKeeper indicated *L2* and *bTUB3* as the most stable genes, based on SD values (Table 2).

Determination of the optimal number of reference genes for normalizing the expression of target genes was assessed using the algorithm geNorm (Fig. 3). Based on a $V \leq 0.15$ cutoff, two genes were considered sufficient for normalization in each of C4 and PA, with V values of 0.141 and 0.092, respectively. However, when considering the global sample, two genes would be insufficient ($V = 0.175$), with three genes required for data normalization ($V = 0.134$).

Gene	Description	Primer name	Primer sequence (5'-3')	Tm (°C)	Amplicon size (bp)
ACTIN1	Actin-3	Macu_Act1pp1-Fw1	CTGCGACAATGGTACTGGAAT	81.95	146
		Macu_Act1pp1-Rv1	CCTCGTCACCAACATAAGCAT		
APT	Adenine phosphoribosyltransferase	Macu_APT-Fw	TTGAACTGCCAGAATTGAAGG	80.47	125
		Macu_APT-Rv	TTGGGAAGAACAGAGAAGCAG		
EF1a	Tr-type G domain-containing protein	Macu_EF1a-Fw	GCTACAACCCAGAGAAGATACCCTT	82.25	80
		Macu_EF1a-Rv	CAGGTTGGTAGACCTCTCAATCATG		
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase 2, cytosolic	Macu_GAPDH-Fw	CATCAAGCAAGGACTGGAGAG	80.91	99
		Macu_GAPDH-Rv	AAGCAGGGAGAACTTTTCCAA		
TUBa	Tubulin alpha-3 chain	Macu_TubA-Fw	GGAAGAAGTCGAAGCTTGGTT	75.53	95
		Macu_TubA-Rv	GGAATGGGTGGATAGGACACT		
RAN	Translation factor GUF1, mitochondrial	Macu_RAN-Fw	AGCTGCAATTGGATCGAAAGT	78.82	90
		Macu_RAN-Rv	GTAACATCGCCACCATAGCAT		
UBIQ1	Ubiquitin 1	Macu_Ubq1-Fw	GGCAGGAGTAACGAACAACAA	81.8	142
		Macu_Ubq1-Rv	CATTTCTCGTAGCTGGGTCAG		
UBIQ2	Ubiquitin domain-containing protein DSK2a	Macu_Ubq2-Fw	AGAGAGATGCTGCAAAATCCA	78.82	140
		Macu_Ubq2-Rv	CCAGCTGTCTGCTCTTGTTCT		
bTUB3	Tubulin beta-3 chain	bTUB-F	TGTTGCATCCTGGTACTGCT	81.36	150
		bTUB-R	GGCTTTCTTGCACTGGTACAC		
L2	Ribosomal protein L2	L2-F	AGGGTTCATAGCCACACCAC	81.06	100
		L2-R	CCGAACTGAGAAGCCCCTAC		
bTUB1	Tubulin beta-1 chain	bTUB1-F	TGTTGGACAACGAAGCTCTCT	81.21	192
		BTUB1-R	GTGGCCGAAATAAGATGGTTC		
ACTA1	Actin-2	ACTA1-F	AGGAGCATCATCTCCAGCAAAG	78.37	78
		ACTA1-R	ACACTGACGACATTCAGCCTCTT		

Table 1. Supporting information for candidate reference genes, primers and amplification parameters for reverse transcription-quantitative PCR (RT-qPCR) normalization of gene expression data for root tissues in *Musa* spp. in the presence and absence of *Fusarium oxysporum* f. sp. *cubense* STR4.

Following analysis of stability values of gene pairs with the lowest SV values generated by NormFinder (Fig. 4), *L2-RAN*, *bTUB3-EF1a*, and *L2-EF1a* were identified as pairs with SV values ≤ 0.15 in the case of C4, and *APT-TUBa* and *GAPDH-TUBa* in the case of PA. No pairs were observed with SV ≤ 0.15 in the case of the global dataset.

Expression analysis of the *TL1* gene (PR5) using selected reference genes

To verify the stability of any potential reference gene, analysis of normalization of expression of a target marker gene for a specific experimental condition is required. Here, the relative expression of a *TL1* gene, which is a *PR5* gene involved in plant host resistance to various pathogens, including *Foc*[32,33], was evaluated in cDNA samples for the *Foc* STR4-resistant genotype C4, inoculated at 1, 2, and 4 DAI, as well as for corresponding non-inoculated controls. Evaluations were also conducted in the susceptible *Musa* genotype PA, under the same conditions.

The four most stable genes for relative expression normalization, based on rankings through geNorm and NormFinder analyses, were tested for each *Musa* genotype. In the case of C4, the most stable genes employed comprised *L2*, *bTUB3*, *EF1a*, and *TUBa*, while for PA, these comprised *GAPDH*, *ACTIN1*, *APT*, and *TUBa*. *Ubiq1* and *bTUB1* were also included in relative expression normalization analysis of the target gene *TL1* to represent the least stable genes for employment in normalization for both genotypes.

In C4, differential expression of the *TL1* gene between inoculated and non-inoculated controls was observed when normalizing using the two pairs formed by the four most stable genes. This contrasted with the relative expression data normalized using the least stable genes, where no differential expression of the *TL1* gene between inoculated and non-inoculated controls was observed. In PA, where there was no differential expression of *TL1* between inoculated and non-inoculated controls, expression levels following normalization using the most or least stable genes did significantly differ, with the same expression trends observed (Fig. 5).

Discussion

The interaction between different *Musa* genotypes and *Foc* has been the subject of several transcriptome analyses, with a focus on host gene characterization during both the compatible and incompatible response[28]. To verify in silico-derived gene expression data, RT-qPCR is widely employed to quantify the expression of a specific gene in cDNA samples originating from different treatments[17,27,29,30,34–37]. Accurate analysis of gene expression, however, requires normalization to reference genes that display stable expression under the investigated experimental conditions. Such normalization will then correct for variation inherent between cDNA samples. Screening of potential reference genes for normalization thus requires consideration of specific experimental samples[30], whether originating from different organisms, tissue types, biotic or abiotic stresses or cDNA sample.

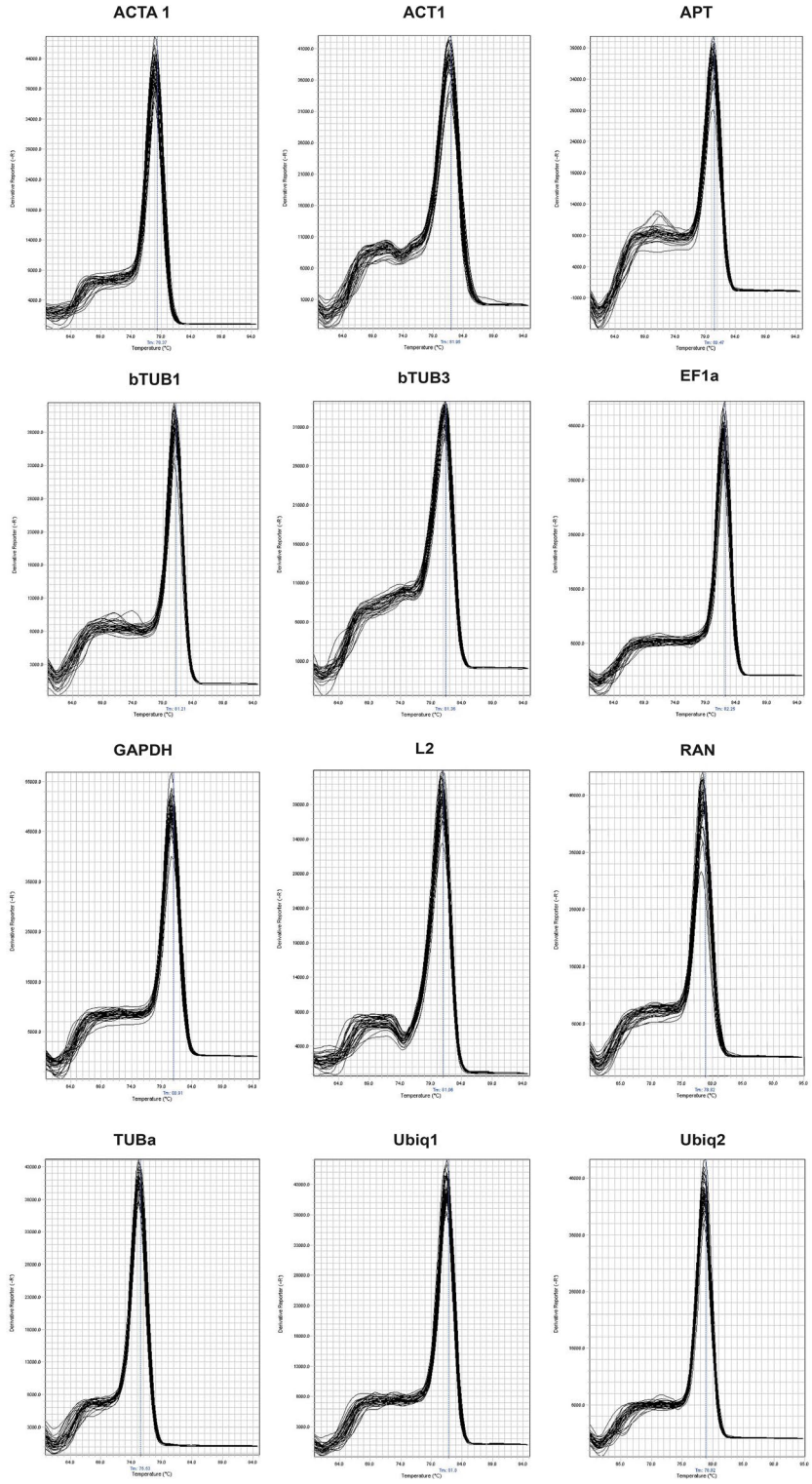

Figure 1. Dissociation curves for the 12 candidate reference genes evaluated in *Musa acuminata* Calcutta 4.

Previous reference genes developed for *Musa* have included those encoding essential and constitutive proteins in plants. These have included the ribosomal protein 2 (*RPS2*), ribosomal protein L2 (*L2*), and actin (*Actin*), which constitutes an essential component of the cell cytoskeleton. Proteins responsible for translation and formation of other proteins, such as the elongation factor 1α (*EF1α*) and ubiquitin (*UBIQ1* and *UBIQ2*), have also been employed, as well as proteins responsible for cellular metabolism, such as glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), among others[27–30]. In all these cases, however, gene expression, even when considered

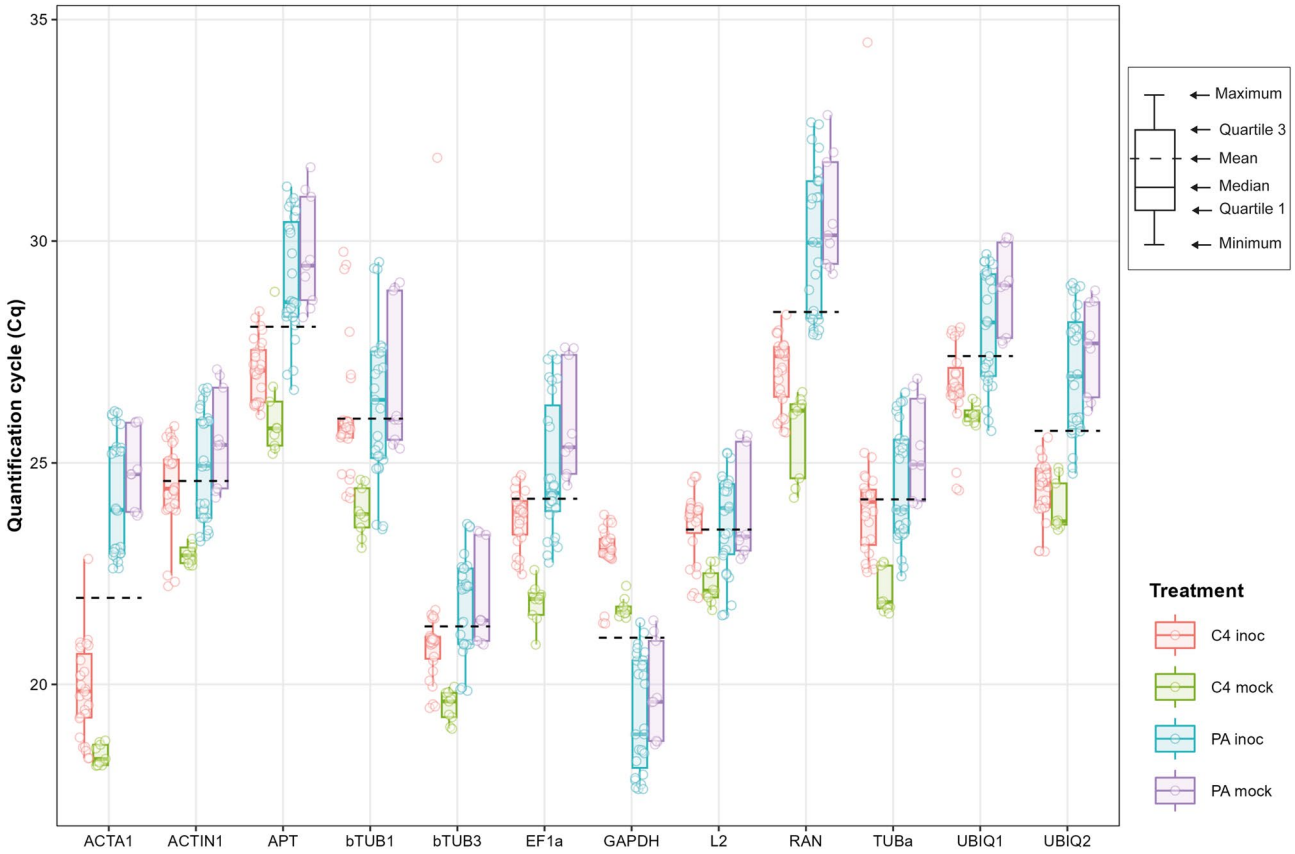

Figure 2. Representative boxplot of quantification cycle (Cq) values for 12 candidate reference genes tested in root material in *Musa acuminata* subsp. *burmannicoides* Calcutta 4 (C4) and *Musa* sp. cultivar Prata-anã (PA), both inoculated (inoc) with *Fusarium oxysporum* f. sp. *cubense* STR4 and non-inoculated (mock).

basal, is often variable, such that different genotypes, tissues, developmental stages, and responses to biotic and abiotic stresses must all be considered in the examination of gene expression stability.

Previously, Chen and colleagues[27] evaluated expression stability of 20 candidate genes in *M. acuminata* Cavendish subgroup fruit tissues, during different developmental stages, including post-harvest, different abiotic stresses (chilling and high temperature), biotic stress (*Colletotrichum musae*), and during ethylene hormone treatment. Stability ranking varied among all treatments, with only the *APT* gene being less stable in four out of the six tested conditions. Rego and coworkers[29] also characterized the expression of eight candidate genes in leaves of *M. acuminata* Cavendish Grand Naine and Calcutta 4 under biotic stress (*Pseudocercospora musae*). Data revealed *APT* and *UBQ2* as the most stable genes for normalization in Calcutta 4, whilst *RAN* and α-*TUB* were more stable in Cavendish Grand Naine. Podevin and coworkers[30] similarly investigated the expression of six candidate reference genes in various tissues of *Musa* genotypes from the AAA, AA, and ABB groups, and under various experimental treatments that included physiological growth conditions, biotic (*Pseudocercospora fijiensis*) and abiotic (osmotic) stress conditions. Data demonstrated that for each experimental condition, an investigation of appropriate reference genes is necessary.

In our study on the stability of 12 candidate reference genes in *Musa* C4 and PA during the interaction with *Foc* STR4, data based on analyses of stability using geNorm were supported by NormFinder-based analysis. BestKeeper-derived rankings were not considered for determining genetic stability, with different stability rankings to the former algorithms based on high coefficient of variation values. We demonstrate that the employment of a set of distinct most stable genes is necessary for each genotype: *L2* and *bTUB3* for C4 and *GAPDH* and *ACTIN1* for PA. The least stable reference genes comprised *UBIQ1* and *bTUB1* for both C4 and PA. Rankings were also corroborated based on Cq value outlier numbers, as observed in Fig. 1. Whilst more outliers were apparent for *UBIQ1* and *bTUB1*, the opposite was observed for the most stable genes, with a low amplitude of Cq for *bTUB3* and *L2* between the inoculated and mock treatments in C4, and similarly for *GAPDH* and *ACTIN1* in the inoculated and mock treatments in PA. Previously, Zhang and colleagues[28] reported candidate reference genes for normalization of target gene expression in the *Musa* genotypes Guangfen No.1 (ABB) and Cavendish Brazilian (AAA) following inoculation with *Foc* TR4 and *Foc* R1, with *L2* and *TUB* most stable in Guangfen No. 1, and *ACT1* and *TUB* most stable in Brazilian. Whilst *L2* and *TUB* were also found to be the most stable genes for C4 in our study, indicating that certain genotypes from different groups (AA and ABB, respectively) may share stable reference genes appropriate for studies with *Foc*, comparisons also revealed differences between specific genotypes. Such data highlights that for each experimental condition and genotype examined, specific

Sample	Rank	geNorm		NormFinder		BestKeeper		
		Gene	M	Gene	SV	Gene	SD	CV
Calcutta 4	1	*L2*	0.28	*L2*	0.19	*UBIQ2*	0.54	2.22
	2	*bTUB3*	0.28	*bTUB3*	0.23	*UBIQ1*	0.61	2.31
	3	*EF1a*	0.39	*EF1a*	0.25	*APT*	0.67	2.50
	4	*TUBa*	0.42	*RAN*	0.26	*GAPDH*	0.70	3.11
	5	*GAPDH*	0.46	*ACTIN1*	0.27	*bTUB3*	0.71	3.48
	6	*ACTIN1*	0.48	*TUBa*	0.28	*RAN*	0.81	3.03
	7	*APT*	0.50	*GAPDH*	0.31	*L2*	0.85	3.67
	8	*ACTA1*	0.54	*APT*	0.32	*EF1a*	0.86	3.68
	9	*UBIQ2*	0.58	*ACTA1*	0.34	*Actin1*	0.91	3.78
	10	*RAN*	0.61	*UBIQ2*	0.55	*ACTA1*	0.94	4.80
	11	*UBIQ1*	0.65	*bTUB1*	0.56	*TUBa*	0.96	4.12
	12	*bTUB1*	0.74	*UBIQ1*	0.66	*bTUB1*	1.15	4.49
Prata-Anã	1	*GAPDH*	0.29	*TUBa*	0.15	*bTUB3*	0.95	4.34
	2	*ACTIN1*	0.29	*APT*	0.17	*L2*	0.97	4.07
	3	*ACTA1*	0.31	*GAPDH*	0.24	*ACTIN1*	1.09	4.34
	4	*TUBa*	0.36	*ACTIN1*	0.27	*UBIQ1*	1.10	3.88
	5	*APT*	0.40	*RAN*	0.29	*GAPDH*	1.11	5.71
	6	*bTUB3*	0.46	*ACTA1*	0.32	*APT*	1.13	3.86
	7	*L2*	0.50	*bTUB3*	0.34	*ACTA1*	1.16	4.75
	8	*RAN*	0.54	*L2*	0.35	*TUBa*	1.18	4.77
	9	*UBIQ1*	0.58	*UBIQ2*	0.35	*RAN*	1.22	4.50
	10	*UBIQ2*	0.63	*EF1a*	0.49	*EF1a*	1.28	5.09
	11	*EF1a*	0.70	*UBIQ1*	0.51	*bTUB1*	1.40	5.29
	12	*bTUB1*	0.81	*bTUB1*	0.56	*UBIQ2*	1.41	4.69
Global	1	*TUBa*	0.44	*TUBa*	0.30	*L2*	0.90	3.81
	2	*ACTIN1*	0.44	*bTUB3*	0.32	*bTUB3*	0.91	4.28
	3	*bTUB3*	0.54	*ACTIN1*	0.39	*RAN*	1.02	3.79
	4	*L2*	0.57	*EF1a*	0.48	*ACTIN1*	1.07	4.35
	5	*RAN*	0.66	*APT*	0.60	*TUBa*	1.09	4.55
	6	*EF1a*	0.73	*L2*	0.67	*EF1a*	1.15	4.74
	7	*UBIQ1*	0.78	*UBIQ1*	0.68	*UBIQ1*	1.16	4.23
	8	*APT*	0.83	*RAN*	0.78	*bTUB1*	1.31	5.03
	9	*bTUB1*	0.92	*bTUB1*	0.83	*APT*	1.37	4.88
	10	*ACTA1*	11.34	*ACTA1*	1.81	*GAPDH*	1.64	7.78
	11	*UBIQ2*	13.46	*UBIQ2*	2.28	*ACTA1*	2.48	11.28
	12	*GAPDH*	15.77	*GAPDH*	2.49	*UBIQ2*	2.89	10.65

Table 2. Analysis of expression stability of 12 candidate genes for root tissues of *Musa acuminata* subsp. *burmannicoides* Calcutta 4 and *Musa* sp. cultivar Prata-anã during interaction with *Fusarium oxysporum* f. sp. *cubense* STR4 and non-inoculated controls. Analyses were conducted using the geNorm, NormFinder and BestKeeper algorithms. M: average expression stability, SV: stability value, Std: standard deviation, CV: variation coefficient. Lowest M and V values indicate greater gene stability. Standard deviation values with BestKeeper greater than 1 indicate gene stability inconsistency.

reference genes are essential and that disregarding biological differences is not suitable for correct normalization, potentially interfering with accurate analysis of target gene expression via RT-qPCR.

Validation of the stability of the reference genes was conducted through analysis of the relative expression of the *TL1* gene during the interaction of each genotype with *Foc* STR4. This gene encodes a thaumatin-like protein 1, which is a PR-5 protein involved in the defence response to biotic stress. Previously, Mahdavi and coworkers[33] demonstrated that insertion of a thaumatin-like protein *(TLP)* gene in susceptible Pisang Nangka bananas (AAB) conferred resistance to *Foc* TR4. Here, expression analysis of the *TL1* gene in C4 when performed with the four most highly ranked stable reference genes based on geNorm (Table 2) demonstrated that all four genes were appropriate for normalization of target gene expression when there was differential expression of the gene in response to *Foc* STR4. As expected, the *TL1* gene did not show differential expression in response to infection by *Foc* STR4 in the susceptible genotype PA, with low relative expression even after infection. No difference in the normalization pattern was observed between the most and least stable reference genes in this genotype.

In summary, given the importance of reference genes for accurate normalization of target gene expression in the *Musa-Foc* SRT4 pathosystem, this study developed reference gene sets for application in candidate gene

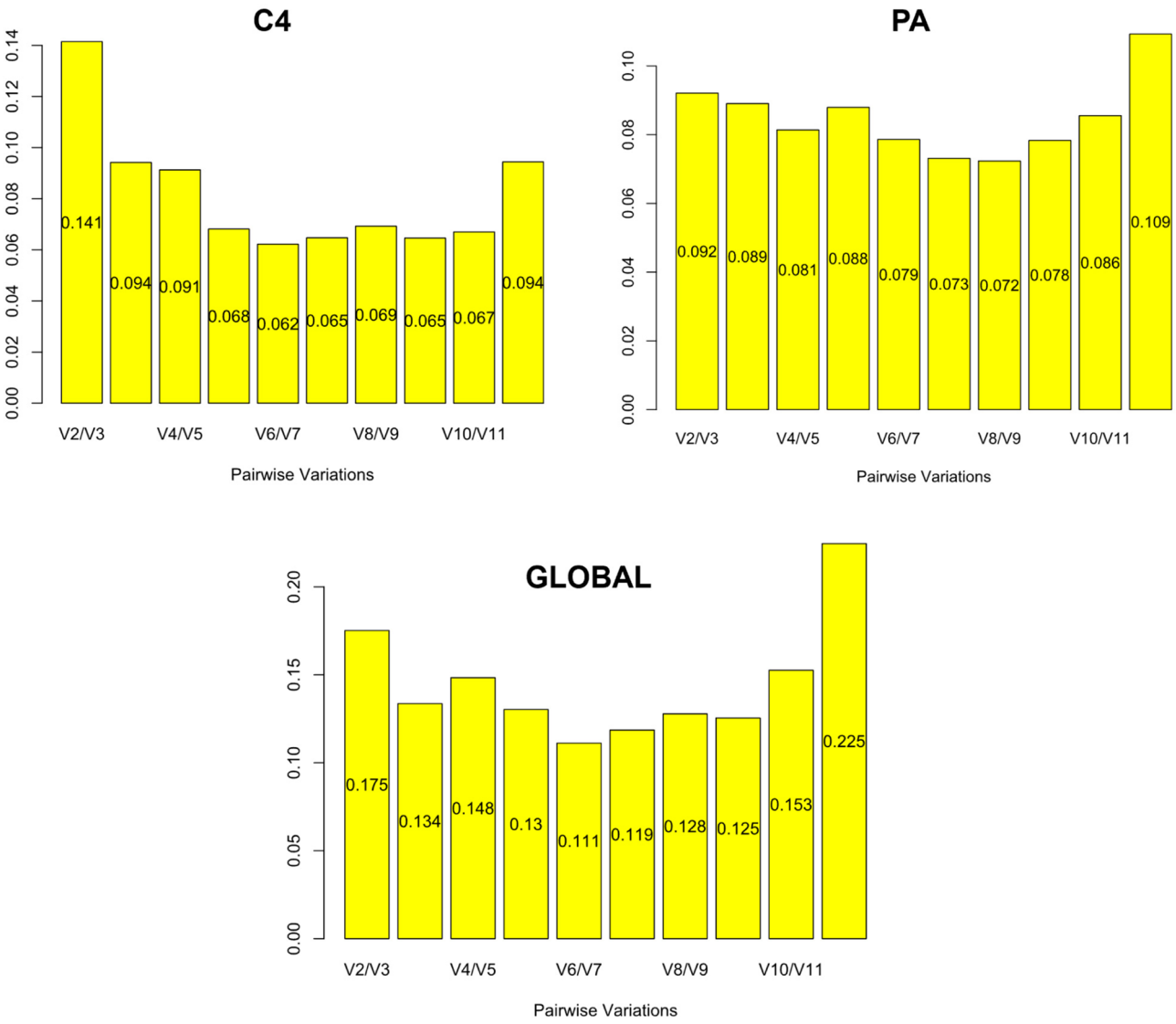

Figure 3. Pairwise variation values obtained using geNorm to determine the optimal number of reference genes for accurate RT-qPCR normalization, for both genotypes *Musa acuminata* subsp. *burmannicoides* Calcutta 4 (C4) and *Musa* sp. cultivar Prata-anã (PA), conditions, and the global analysis. When values are below the cutoff of 0.15, the inclusion of an additional reference gene is not necessary.

discovery in genotypes contrasting in resistance to this important pathogen. Based on algorithm ranking, *L2* and *bTUB3* should be prioritized for use in C4 root materials both inoculated with *Foc* STR4 or non-inoculated, and similarly with *GAPDH* and *ACTIN1* in PA. These reference genes will contribute to advancing understanding of the host defence responses to this important pathogen.

Methods

Plant material

In vitro propagated plantlets of the susceptible *Musa* sp. cultivar 'Prata-anã' (subgroup 'Pome' AAB) and the resistant wild genotype 'Calcutta 4' (*Musa acuminata* subsp. *burmannicoides*, subgroup AA) were provided by Embrapa Cassava and Tropical Fruits. Plantlets were acclimatized and subsequently grown in pots containing coconut fibre substrate. Eighteen plants of each genotype were maintained in a greenhouse at an average temperature of 24 °C, with watering at 48-h intervals for 60 days prior to inoculation.

Root inoculation with *Foc* STR4

Plants of each genotype were inoculated with *F. oxysporum* f. sp. *cubense* STR4 (isolate CNPMF 218A from Embrapa Cassava and Tropical Fruits). Inoculation was conducted according to Rocha and colleagues[38]. Inoculum comprised 50 g of rice, previously infested with the pathogen at a concentration of 10^6 CFU/g of rice, added directly to the substrate. Controls comprised non-infested rice, with application to the substrate as above. Following a complete randomized design, with three distinct biological replicates per treatment, root samples

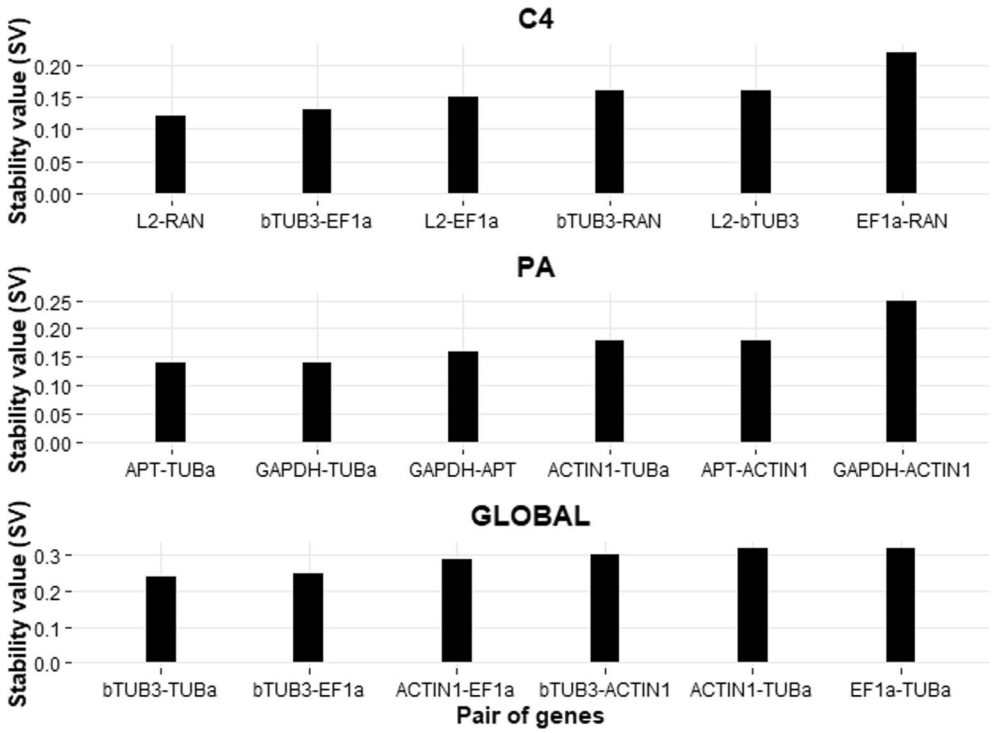

Figure 4. NormFinder-based analysis of stability values of combinations of reference gene pairs for normalizing RT-qPCR gene expression data. Lower SV values for each gene combination indicate greater expression stability.

were collected from Calcutta 4 (C4) and Prata-Anã (PA) at 1, 2, and 4 DAI and similarly from mock-inoculated controls. Samples were immediately flash-frozen in liquid nitrogen and stored at—80^0 C.

RNA extraction and cDNA synthesis

Total RNA was extracted from frozen 1 g root samples using the Plant RNA Reagent (Invitrogen ThermoFisher Scientific, MA, USA) and purified using the Directzol Plant RNA Purification kit (Zymo Research, CA, USA) following the manufacturer's protocol. RNA quality was verified using 1% agarose gels and quantified using a NanoDrop One spectrophotometer (Thermo Fisher Scientific, MA, USA). A 1 µg aliquot of RNA from each treatment and replicate was used for cDNA (complementary DNA) synthesis via the Super Script IV Reverse Transcriptase kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's protocol.

Selection of candidate reference genes

A total of 12 candidate genes were selected for testing as potential reference genes based on recent literature for genes with stable expression in *Musa spp.* and involvement in basal cell activities. Actin-3 (*ACTIN1*), *APT*, *EF1α*, *GAPDH*, α-TUB, *RAN*, *β-TUB* (*bTUB1*), *Ubiq1*, and *Ubiq2* were selected from Rego et al. (2019), *L2* and *β-TUB* (*bTUB3*) from Zhang and coworkers[28], and Actin-2 (*ACTA1*) from Castañeda and coworkers[39] (Table 1).

Real-time quantitative PCR

Analysis of gene expression was conducted using the iTaq™ Universal SYBR® Green kit (Bio-Rad, Hercules, CA, USA). Each qPCR reaction was conducted in a final volume of 10 µL, containing 2 µL of a 1:20 dilution of each cDNA stock, 0.2 µM of each primer, and 5 µL of the iTaq™ Universal SYBR® Green kit. PCR amplifications were carried out on an ABI StepOne™ Real-Time PCR thermocycler (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) according to the following parameters: an initial two-step phase of 50 °C for 2 min and 95 °C for 10 min, 40 cycles of denaturation at 95 °C for 15 s, and primer annealing and extension at 60 °C for 60 s. Three biological and three technical replicates were included for each gene. Melting temperatures (Tm) were determined using the StepOne Software v2.3 (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA).

Determination of candidate reference gene stability

Expression analysis of each of the 12 candidate genes was measured for all C4 and PA replicate cDNA samples based on quantification cycle (Cq) values. Gene expression stability was determined using the statistical algorithms geNorm[25], NormFinder[26] and BestKeeper[31], according to default parameters. Analyses were conducted for expression data from each individual genotype, as well as based on a global analysis of data for both genotypes, both inoculated and non-inoculated. In the case of geNorm, the lower the average M value, below a threshold of ≤ 0.5, the greater the stability for a candidate reference gene. This algorithm also estimates a V value (Vn/n + 1)

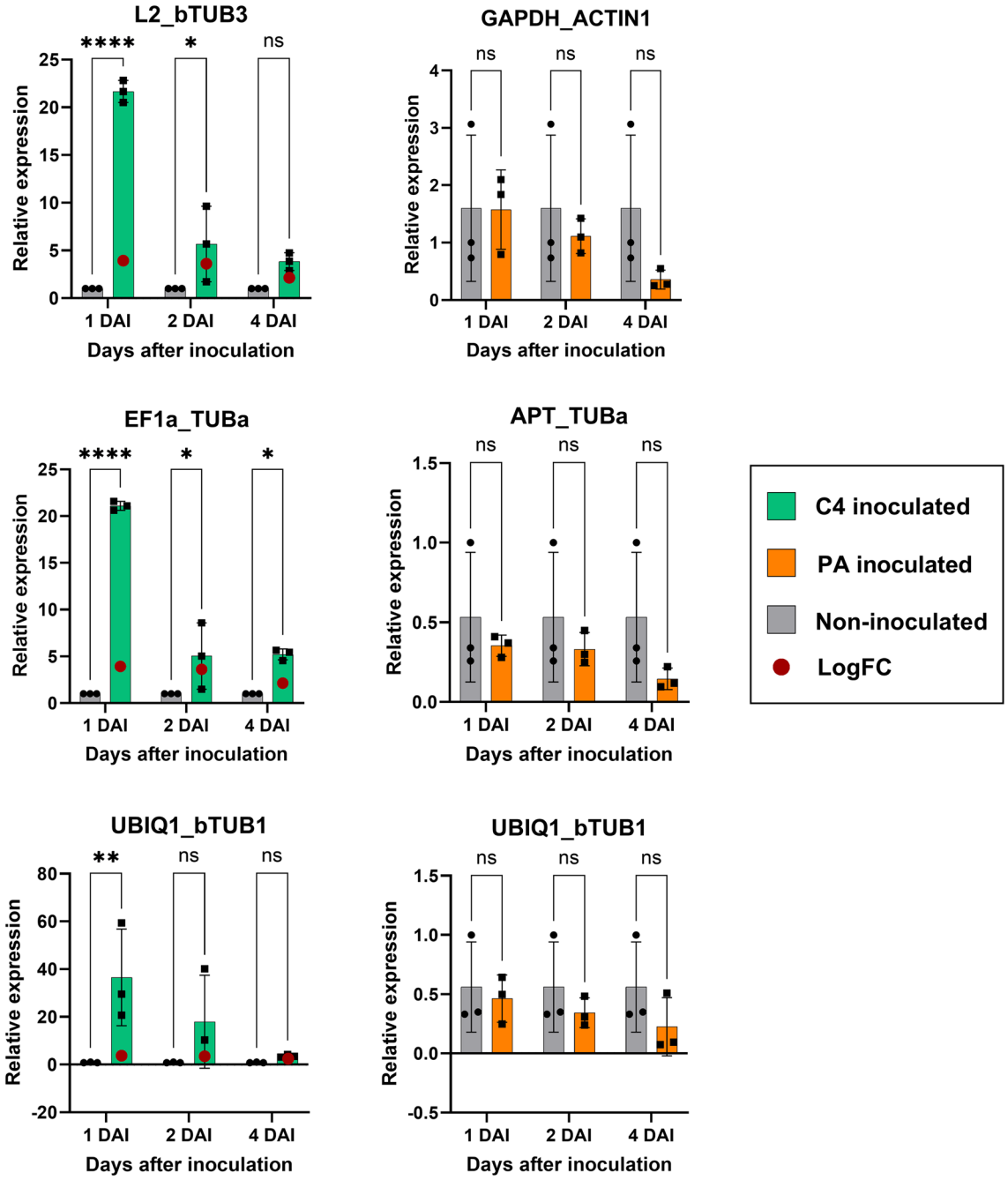

Figure 5. Relative expression levels of Thaumatin-like protein 1 (TL1) in *Musa acuminata* subsp. *burmannicoides* var. Calcutta 4 (C4) and *Musa* sp. cultivar Prata-anã (PA), under infection with *Fusarium oxysporum* f. sp. *cubense* STR4 isolate 218A 1, 2 and 4 days after inoculation. Normalization was examined using the two most stable gene pairs for C4 (*L2* and *bTUB3*; *EF1a* and *TUBa*), and for PA (*GAPDH* and *ACTIN1*; *APT* and *TUBa*), with each compared with the least stable pair for C4 and PA (*UBIQ1* and *bTUB1*). **** indicated significance at $p < 0.0001$, ** indicated significance at $p < 0.001$ and * indicated significance at $p < 0.5$.

to determine the optimal number of reference genes for use in normalization. NormFinder calculates expression stability based on the stability value (SV), where lower values indicate higher gene stability. This algorithm also identifies the most appropriate combinations of pairs of stable genes. In the case of BestKeeper, lower coefficient of variation (CV) and standard deviation (SD) values indicate greater expression stability. R software[40] was employed for analysis of data generated with all algorithms and for preparation of figures.

Expression analysis of a PR-5-encoding gene

Combinations of the most stable genes in C4 (*L2* and *bTUB3*; *EF1a* and *TUBa*) and PA (*GAPDH* and *ACTIN1*; *APT* and *TUBa*), together with the least stable genes in both C4 and PA (*Ubiq1* and *bTUB1*), were all compared in terms of efficiency in normalization of expression of a gene in C4 encoding a Thaumatin-like protein 1 (*TLP1*)

(Macma4_05_g17130), which is member of the Pathogenesis-related-5 protein family (PR-5). In a previous transcriptome study by the group (data not shown), the Log2 fold-change (LogFC) expression of this gene in C4 in inoculated root materials compared to non-inoculated controls was 3.86, 3.46 and 2.37 for 1, 2 and 4 DAI, respectively. Specific primers designed for this gene comprised TL1-F (GATGCGACGCTGATGAAA) and TL1-R (AGACCGGCCATAAGATACA). RNA extraction, cDNA synthesis and RT-qPCR were conducted as described above.

Data availability

All data generated and analysed during the study is included in the published article.

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Author contributions

R.N.G.M. conceived and designed the study. E.C.C. conducted the biotic assays and RT-qPCR analysis. E.C.C., L.S.B. and T.G.G. analysed the data. R.N.G.M., E.C.C. and T.G.G. provided intellectual input. E.C.C. and R.N.G.M. wrote and revised the manuscript. All authors have read and approved the final manuscript.

Competing interests

The authors declare that they have no competing financial and/or non-financial interests in relation to the work described.

Additional information

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