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Robust reference gene selection in Norway spruce: essential for real-time quantitative PCR across different tissue, stress and developmental conditions

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Accurate gene expression analysis in Norway spruce (*Picea abies*) under diverse stress conditions requires the identification of stable reference genes for normalization. Notably, the literature lacks reports on suitable reference genes in Norway spruce. Here, we aimed to address this gap by identifying suitable reference genes for quantitative real-time PCR in Norway spruce across various stress conditions (drought, heat, pathogen infection) in seedlings, tissues (needle, phloem, root), and developmental stages (seedlings, mature trees). We evaluated the stability of 15 candidate reference genes and assessed their expression stability using five statistical algorithms (ΔCt, geNorm, NormFinder, BestKeeper, and RefFinder). Our results highlight *ubiquitin-protein ligase* (*SP1*), *conserved oligomeric Golgi complex* (*COG7*), and *tubby-like F-box protein* (*TULP6*) as the most stable reference genes, while *succinate dehydrogenase* (*SDH5*) and *heat shock protein 90 (HSP90)* were the least stable under various experimental conditions. *COG7* and *TULP6* are novel candidate reference genes reported for the first time. The expression stability of the identified reference genes was further validated using dehydrin-like protein 5 (*PaDhn5*) under drought conditions in Norway spruce. Pairwise variation analysis suggests that two reference genes were sufficient to normalize gene expression across all sample sets. This study provides a comprehensive analysis of reference gene stability under different experimental conditions and a catalog of genes for each condition, facilitating future functional genomic research in Norway spruce and related conifers.

KEYWORDS

RT–qPCR, housekeeping genes/reference genes, gene expression, developmental stages and tissues, abiotic and biotic stress

1 Introduction

Norway spruce [*Picea abies* (L.) Karst.] is one of the most ecologically and economically important coniferous tree species in Europe. Adapted to cool and wet conditions, its distribution extends from central and eastern Norway across Fennoscandia, the Baltic states, Belarus, Russia, and Central and Southeastern Europe (Danielsen et al., 2021). Over the past several decades, Norway spruce has been extensively planted in areas beyond its natural

distribution range due to its excellent growth performance and desirable wood properties for commercial forestry (Caudullo et al., 2016; Hlásny et al., 2019). However, planting it outside its niche has led to health and vitality issues, increasing its susceptibility to biotic and abiotic stresses. For instance, Norway spruce forests in the Czech Republic experienced a dramatic increase in mortality in 2019, losing an average of 118 million m^3 due to droughts, heatwaves, and bark beetle outbreaks (Ebner, 2020). Traditional management strategies, such as pheromone-based mass trapping, salvage logging, insecticides, and anti-attractants, have been largely ineffective in controlling mass bark beetle attacks (Dobor et al., 2020; Singh et al., 2024a). Consequently, there has been rapid exploration of functional genomics-based tools to develop effective management strategies. Notably, sequencing of the Norway spruce genome, the first among conifers (Nystedt et al., 2013), has significantly advanced gene expression studies aimed at understanding gene functions and molecular regulation under various stresses. However, the appropriate reference genes required for gene expression normalization in Norway spruce have not yet been comprehensively established. Therefore, identifying reliable reference genes is crucial for improving studies on gene expression profiling, providing novel insights into biological processes, and deepening the understanding of regulatory gene networks that contribute to spruce resistance, resilience, and survival (Wise et al., 2007).

The significance of differential gene expression analysis in understanding gene function and molecular regulation in response to various environmental factors is crucial. Real-time quantitative polymerase chain reaction (RT–qPCR) is a widely used technique for analyzing the expression of target genes owing to its high sensitivity, accuracy, specificity, reproducibility, and rapidity (Bustin, 2002; Gachon et al., 2004; Bustin et al., 2005; Takamori et al., 2017). However, the precision of RT–qPCR is highly dependent on using a reference gene as an internal control. Reference genes, also referred to as housekeeping genes, are consistently expressed in cells and are vital for fundamental cellular functions. These genes encode proteins that play crucial roles in essential cellular activities, including cell cycle regulation, DNA replication, and metabolism. An ideal reference gene maintains consistent expression levels across different experimental conditions and tissues and remains unaffected by developmental stages or the organism's genotype (Han et al., 2012; Lu et al., 2018; Sen et al., 2021).

Several studies have identified and validated reference genes in crops, commercially valuable tree species, and herbs, including *Solanum tuberosum* L. (Nicot et al., 2005), *Arabidopsis thaliana* (L.) Heynh. (Czechowski et al., 2005), *Oryza sativa* L. (Jain et al., 2006), *Triticum aestivum* L. (Paolacci et al., 2009), *Linum usitatissimum* L. (Huis et al., 2010), *Eucalyptus robusta* Sm. (de Oliveira et al., 2012), *Populus euphratica* Oliv. (Wang et al., 2014), *Solanum melongena* L. (Mogilicherla et al., 2016), *Bromus sterilis* L. (Sen et al., 2021), *Melissa officinalis* L. (Bharati et al., 2023), and *Populus tremula* L. (Pastierovič et al., 2024). Recently, efforts have been made to identify and validate reference genes in coniferous species such as Chinese fir (*Cunninghamia lanceolata* (Lamb.) Hook.) and Masson pine (*Pinus massoniana* Lamb.) (Bao et al., 2016; Mo et al., 2019; Chen et al., 2019). However, these studies were limited to specific tissues and treatment conditions. Previous research on internal control genes in *P. abies* was limited to embryogenic cell lines (e.g., Vestman et al., 2011; de Vega-Bartol et al., 2013). In maritime pine (*Pinus pinaster*), genes such as *GADPH, 18S rRNA, UBQ,* and *eIF4AII* were found unsuitable as internal controls during embryo development due to the high variability in gene expression during embryogenesis and the involvement of these genes in both basal metabolism and other functions (Gonçalves et al., 2005). More recently, a study identified *elongation factor 1-gamma, histone H1, GAPDH,* and *α-tubulin* as stable reference genes for somatic embryogenesis tissues in *Liriodendron* hybrids, while *elongation factor 1-gamma* and *actin* were most reliable for germinative organ tissues (Li et al., 2021). These findings suggest that results from embryogenic cell lines may not accurately represent seedlings or mature trees under varying stress conditions. Therefore, establishing a set of stable reference genes for Norway spruce across different developmental stages and stress conditions is crucial for improving the accuracy and reliability of gene expression analysis, enhancing our understanding of its physiological processes and stress responses.

Here, we analyzed the expression profiles of 15 candidate reference genes in different conifer species based on published research and our in-house transcriptome data (de Vega-Bartol et al., 2013; Bao et al., 2016; Mo et al., 2019; Chen et al., 2019). These genes are known for performing conserved cellular functions in gymnosperms and, therefore, are expected to show stable expression across various conditions, developmental stages, and tissues. We evaluated the expression stability of *ribosomal protein L26 (RPL26), serine/threonine-protein phosphatase (PP2A2), ribosomal protein L7Ae (RPL7Ae), ubiquitin-like domain-containing CTD phosphatase (UBCP), F-box protein (SKIP22), heat shock protein (HSP90), vacuolar fusion protein (CCZ1), succinate dehydrogenase (SDH5), 18S rRNA (RID2), putative lysine-specific demethylase (JMJ16), conserved oligomeric Golgi complex (COG7), ribosomal protein S10 (RSP10), ubiquitin-protein ligase (SP1), tubby-like F-box protein (TULP6),* and *actin-related protein (ARP9)* across different tissues (needle, phloem and root), developmental stages (seedlings and mature trees), and under various biotic and abiotic stress conditions (drought, heat stress, and pathogen infection) in Norway spruce seedlings. The present study will enhance the accuracy of future gene expression studies and contribute to a more reliable evaluation of reference gene stability in Norway spruce. These reference genes are also expected to serve as templates for other related conifer species and should be further investigated.

2 Materials and methods

2.1 Plant materials and experimental conditions

2.1.1 Greenhouse experiment

Three-year-old containerized Norway spruce seedlings, approximately 40 cm high, were procured from a local nursery and planted in a moist peat/perlite mixture in May 2022. The seedlings were grown using seed material originating from the Bohemian-Moravian highlands (Czech Republic), characterized by 600–750 mm of annual rainfall and an altitude of 500–600 m above sea level. The seedlings were transferred to a greenhouse with a temperature range of 23 ± 2°C and 16-h/8-h light/dark photoperiod cycles at the Czech University of Life Sciences, Prague. Before any treatment, the plants were irrigated regularly with tap water for acclimation to the greenhouse conditions. A total of 16 plants were used for each treatment. The plants were

grouped into sets of four, which were pooled to form one biological replicate. Consequently, each treatment comprised four biological replicates, with each replicate consisting of four plants. ***Control treatment:*** This group was irrigated regularly and was not subjected to any abiotic or biotic treatment. ***Water deficit treatment:*** One group was subjected to drought stress by gradually interrupting the water supply until the mean plant water potential decreased below −2.1 MPa. A water potential value of −2.1 MPa in Norway spruce is considered low, indicating water stress that may lead to embolism formation and a loss of hydraulic conductivity (Rosner et al., 2019). The degree of drought stress was quantified by measuring the predawn water potential using a Scholander pressure chamber (PMS Instruments, Corvallis, Oregon, United States). ***Heat stress:*** To induce heat stress, the seedlings were placed in a climate chamber (FytoScope FS-SI 3400, PSI, Drásov, Czech Republic) at 35°C for 10 days with a constant relative humidity of 80% and regular irrigation. ***Biotic stress:*** The seedlings were inoculated with the Ophiostomatoid fungus *Ophiostoma flexuosum,* and incubated for 1 month in a controlled climate chamber mentioned above. Throughout the treatment period, the plants were carefully maintained under optimal humidity, light, and nutrient conditions.

Samples were collected from three different tissues (needle, phloem, and roots) from all treatments, including abiotic stress (drought and heat), biotic stress (pathogen infection), and control (well-watered) treatments. Tissue samples were snap-frozen in liquid nitrogen immediately after collection and stored at −80°C for further processing.

2.1.2 Field experiment

In addition to the seedlings, samples from mature trees (90–100 years) were collected from research plots of the Czech University of Life Sciences, managed by the School Forest Enterprise (ŠLP) in Kostelec nad Černými lesy (49.9940° N, 14.8592° E) in the eastern district of the Central Bohemian region of the Czech Republic (Singh et al., 2023). The climate in this region is dry and warm in summer, with a vegetation season lasting 150–160 days and an average annual temperature and precipitation of 7–7.5°C and 600 mm, respectively. Norway spruce tissue samples (needle, phloem, and root) were collected from 16 trees in August 2022. The needle samples were collected by shooting branches with a shotgun (Burnett et al., 2021), while phloem and root samples were obtained using a 5 mm cork borer. The phloem samples were collected from the trunk at a height of 2 m. The cork borer was used to extract tissue cores that included both the phloem and cambium layers (Mao et al., 2019). For the root samples, lateral roots were selected from the same trees approximately 1 m from the trunk base (Kalyniukova et al., 2024). After carefully removing layers of soil and moss, the roots were exposed and sampled at 15 cm depth using the same 5 mm cork borer. All tissue samples were snap-frozen in liquid nitrogen immediately after collection and stored at −80°C for further processing. Similar to the greenhouse experiment, four biological replicates were used for each tissue type, with each replicate composed of four individual samples pooled together to reduce individual heterogenicity. The experimental design is schematically represented in Figure 1.

2.2 Selection of candidate reference genes for evaluation

A total of 15 candidate internal control genes, *RPL26, PP2A2, RPL7Ae, UBCP, SKIP22, HSP90, CCZ1, SDH5, RID2, JMJ16, COG7, RPS10, SP1, TULP6,* and *ARP9* were selected for identifying the most suitable reference genes in Norway spruce (Table 1). The selected candidate genes were previously identified in other conifer species (de Vega-Bartol et al., 2013; Bao et al., 2016; Chen et al., 2019; Mo et al., 2019) and showed consistent expression in our in-house transcriptome data (*Unpublished data*). Primers for selected genes were designed through the PrimerQuest™ Tool (Integrated DNA Technologies, United States), and their primer efficiency and correlation coefficients (R^2) were calculated (Table 1). The sequence used to design primers is provided in Supplementary material S1.

2.3 Total RNA extraction, cDNA synthesis, primer design, and quantitative RT–qPCR analysis

Total RNA from all three tissues (needle, phloem, and root) was isolated using a modified cetyltrimethylammonium bromide (CTAB) protocol described by Singh et al. (2024b). Briefly, 100 mg of homogenized tissue was added to the CTAB extraction buffer, followed by the addition of an equal amount of Chloroform:isoamyl alcohol (24:1) and subsequent centrifugation. The top aqueous layer was transferred to a new tube, and 0.5 volume of 5 M lithium chloride was added and incubated for 1 h at −20°C. Then, the mixture was centrifuged at 12,000 rpm for 10 min at 4°C, and the supernatant was discarded. The resulting RNA pellet was washed twice with 70% ethanol. The dried RNA pellet was eluted in 50 µL RNase-free water. The isolated RNA was further treated with the TURBO DNase Kit (Invitrogen, United States) to remove any genomic DNA contamination. RNA integrity and purity (260/280 and 260/230 nm absorbance ratios) were verified by electrophoresis on a 1.2% agarose gel and NanoDrop spectrophotometer (Thermo Scientific™, USA), respectively. Complementary DNA (cDNA) was synthesized from 1 µg of RNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) according to the manufacturer's protocol and stored at −20°C.

Before performing RT–qPCR, the cDNA samples were diluted by a factor of five. The RT–qPCR reaction mixture consisted of 5.0 µL of SYBR® Green PCR Master Mix (Applied Biosystems, United States), 1.0 µL of cDNA from fivefold dilutions, 10 µM of forward and reverse primers, and 3.0 µL of RNase-free water (Invitrogen, USA) to make up a total volume of 10.0 µL. Amplification was conducted with initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The reactions were carried out using an Applied Biosystems™ StepOne™ Real-Time PCR System (Applied Biosystems, United States). To verify primer specificity, melting curve analysis was performed, ensuring gene-specific amplification with a gradual increase in temperature from 60 to 95°C. All RT–qPCR assays were conducted with four biological replicates per treatment and tissue, each including four technical replicates.

2.4 Statistical analyses

Five different algorithms and tools, i.e., geNorm, NormFinder, BestKeeper, ΔCt, and RefFinder were used to evaluate the gene expression stability of 15 candidate reference genes in Norway spruce. The comprehensive expression stability of candidate reference genes was evaluated using RefFinder, a web-based tool that combines the

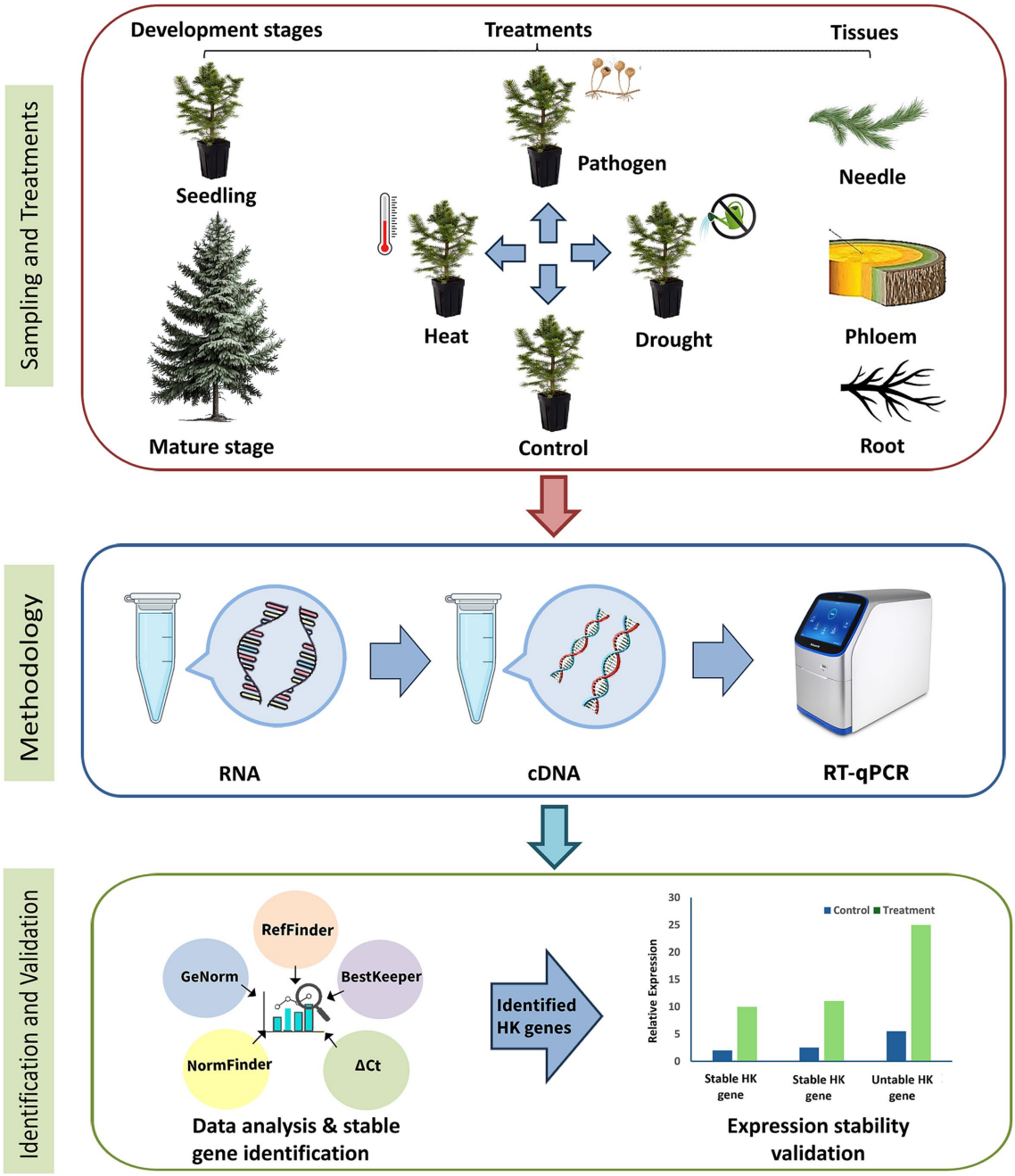

FIGURE 1
Schematic representation of the experimental design used to identify suitable reference genes in Norway spruce.

outputs of the former four algorithms. The geNorm algorithm calculates the expression stability value (M) and conducts pairwise variation (V) comparisons, where the lowest M value suggests the most stable expression (Vandesompele et al., 2002). NormFinder ranks reference genes based on their expression stability within a given set of samples (Andersen et al., 2004). BestKeeper, another freely available algorithm, assesses the standard deviation and correlation coefficient using the Cq (quantification cycle) values of all reference genes (Pfaffl et al., 2004). The ΔCt method directly evaluates the relative expression of gene pairs within each sample (Silver et al., 2006). The mean Cq values of each reference gene from each experiment served as input data and were processed using RefFinder, which combines widely used reference gene screening programs (geNorm, NormFinder, BestKeeper, and the ΔCt method) to calculate the geometric mean of their ranking values to provide an overall comprehensive ranking.

The optimum number of reference genes required under each experimental condition was determined by pairwise variation (V) analysis by calculating the variable value Vn/n + 1 based on the average expression stability (M) with a cutoff of 0.15 using the geNorm algorithm. The Vn/Vn + 1 value indicates the pairwise variation between two consecutive normalization factors. When the value falls below this threshold, adding the n + 1 reference gene becomes redundant.

TABLE 1 Primer information of the reference genes and target genes used in the study.

Gene symbol	Gene name	Primer sequence (5′-3′)		Amplicon length (bp)	PCR efficiency	Regression coefficient
RPL26	Ribosomal protein L26	F: R:	CATCTAGTGTGCGGCGTATT CTCGTACCACCTGAACTTCATC	114	96.72	0.999
PP2A2	Serine/threonine-protein phosphatase PP2A-2	F: R:	GGCCTATGTGTGATCTACTGTG GTGATTGAATTGGGCTGCTATG	113	96.99	0.997
RPL7Ae	Ribosomal protein L7Ae	F: R:	CCACTGTTGGCTGAGGATAAG CCCTCATTGGTTGTCACAGAA	119	100.47	0.996
UBCP	Ubiquitin-like domain-containing CTD phosphatase	F: R:	CCACAGAATGGGCTTGTGATA GTGGCTCAGATCATCCAGTTC	126	96.96	0.999
SKIP22	F-box protein	F: R:	TTCCCACAGAGCTCAAACTG CTCAGCCGCATACTTCTTCTT	134	91.19	0.995
HSP90	Heat shock protein	F: R:	GGCGATCAAGATGAAGCAAAG AAGCACACATGGCGAAGA	138	111.92	0.991
CCZ1	Vacuolar fusion protein	F: R:	CAAACAGCAATAGCAGTGAAGG ACAGAGAGCTGGCTAGTAAGA	133	93.88	0.999
SDH5	Succinate dehydrogenase	F: R:	GCTCTACGGGCTGCATATAAA ACCAACAGCGTCACTAACC	119	106.87	0.879
RID2	18S rRNA	F: R:	GAGGAGCACGAGCTGTATTG CACAACCAGACCACCTGAAA	104	93.72	0.987
JMJ16	Putative lysine-specific demethylase	F: R:	AGCAGATGTGGAGACTAGGA GGAGCCGTGCAATGTTATTTAG	122	93.88	0.993
COG7	Conserved oligomeric Golgi complex	F: R:	CCTCGGCTGAAGAAGACAAT TTGTCCCAGCACCCAATAC	135	103.63	0.997
RPS10	ribosomal protein S10	F: R:	CCTCGATTTGGTGACAGAGATG GCCCTAAATTGAGGCTGGTATT	110	96.72	0.999
SP1	ubiquitin-protein ligase SP1	F: R:	GGCACTACTCTGACTGTTGT GGCAGATCCTAGCCGTTTC	117	114.86	0.999
TULP6	Tubby-like F-box protein	F: R:	CCTGAGCCTAATCCATCAGTTT AGTGGGTAGCGATAATCCATTG	104	94.79	0.999
ARP9	Actin-related protein 9	F: R:	CCTGGAAAGGTGGTGCTATT AATCTCTGTACTTTCGACCACTC	120	93.65	0.999
Target gene						
PaDhn5	Dehydrin like protein	F: R:	ATCAATGTGCGGGTGAAG ACTCCCACACTGATCTGAA	122	120.48	0.951

2.5 Reference gene validation

Dehydrins are a part of the group 2 late embryogenesis abundant (LEA) protein family that protects conifers against osmotic stress in response to cold and drought conditions (Stival Sena et al., 2018; Čepl et al., 2020; Krokene et al., 2023). To validate the selected reference genes, the relative expression levels of the dehydrin gene *PaDhn5* were analyzed according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) using the most and the least stable reference genes. The data was checked for normality using the Kolmogorov–Smirnov test, and mRNA expression levels of target genes were assessed via single factor ANOVA using XLSTAT cloud (ver. 1.0), and a significance level of $\alpha = 0.05$ was used as the cutoff to indicate significant differences among the control and treatment groups.

3 Results

3.1 Amplification efficiency and specificity of candidate reference genes

Each primer pair produced a single amplicon with an expected amplicon length, as shown by agarose gel electrophoresis (Supplementary material S2). Melting curve analysis revealed a single peak, indicating no primer dimer was present (Supplementary material S3). The amplification efficiency of each primer pair ranged from 91.19 to 114.86%, and the correlation coefficient (R^2) values were greater than or equal to 0.879 (Table 1 and Supplementary material S4). The average cycle threshold (Ct) value was 28.14 (Supplementary material S5), while *SDH5, SP1, RID2,* and *ARP9* showed the highest transcript abundance across most

experimental conditions. In contrast, *RPL7Ae* and *RPL26* were the least expressed reference genes. The remaining nine reference genes exhibited moderate expression levels.

3.2 Expression stability of candidate reference genes

3.2.1 Tissuewise stability

In needle tissues, the three most stable genes were *SP1*, *JMJ16,* and *RID2* according to the comprehensive ranking by RefFinder and by ΔCt, while *UBCP, HSP90,* and *SDH5* were the least stable genes (Table 2). The BestKeeper and NormFinder rankings were inconsistent with those of RefFinder and ΔCt, suggesting that *PP2A2* and *UBCP* are the most stable genes, respectively. The geNorm ranking suggested a combination of *RID2/ TUPL6* as the most stable gene (Supplementary material S6).

For phloem tissues, *RID2, SP1,* and *PP2A2* were the most stable genes according to RefFinder, while the least stable genes were *SKIP22, HSP90,* and *SDH5*. This ranking was consistent with that of the ΔCt method, except that ΔCt ranked *COG7* as the third most stable gene. The BestKeeper and NormFinder algorithms ranked *PP2A2* as the most stable gene in phloem tissues. According to geNorm, the combination of *RPL26/RPS10* was the most stable gene in phloem tissues (Supplementary material S6).

In root tissues, the BestKeeper, ΔCt, and NormFinder rankings for the three most stable genes were not consistent with those of RefFinder. *UBCP, RPL7Ae,* and *SP1* were suggested to be the three most stable genes based on the comprehensive rankings of RefFinder (Table 2). A combination of *COG7/TULP6* was the most stable reference gene in root tissues according to geNorm (Supplementary material S6). The least stable reference genes by the RefFinder comprehensive ranking were *SDH5, HSP90,* and *RID2.*

3.2.2 Treatmentwise stability

3.2.2.1 Control conditions

According to the comprehensive rankings of RefFinder, the three most stable genes under control conditions were *RPS10, RPL26,* and *SP1,* while *CCZ1, SDH5,* and *HSP90* were the least stable (Table 3). BestKeeper ranked *RID2* as the most stable gene, whereas NormFinder ranked *COG7* as the most stable gene. The ΔCt value indicated that *RPS10* was the most stable gene, which is consistent with the RefFinder ranking. The best combination of reference genes according to geNorm was *PP2A2/ TULP6* under control conditions (Supplementary material S7). The *HSP90* gene was the least stable, as determined by geNorm analysis and the other four algorithms.

3.2.2.2 Drought stress conditions

Under drought conditions, *RID2, SP1,* and *COG7* were identified as the three most stable reference genes by the RefFinder algorithm, while a combination of *UBCP/SP1* was the most stable as per geNorm (Supplementary material S7). BestKeeper and NormFinder identified *PP2A2* and *RPL26* as the most stable genes, respectively. Both geNorm and other algorithms (ΔCt, BestKeeper, NormFinder, and RefFinder) identified *SDH5* as the least stable reference gene under drought conditions.

3.2.2.3 Heat stress conditions

For studies under heat stress conditions, *RID2, SP1*, and *RPL26* were identified as the most stable genes by comprehensive ranking by the RefFinder program, whereas BestKeeper and NormFinder ranked *PP2A2* and *RPL26* as the most stable genes, respectively. A combination of *RPL26/ARP9* was ranked as the most stable gene according to geNorm (Supplementary material S7). Unanimously, *SDH5* was suggested as the least stable gene under heat stress conditions by geNorm and other algorithms.

3.2.2.4 Pathogen infection conditions

The topmost three stable genes after Ophiostomatoid fungus inoculation were *SP1, COG7,* and *RPL26* based on the rankings of the RefFinder algorithm, whereas the combination of *PP2A2/ RPL7Ae* was the most stable according to geNorm (Table 2; Supplementary material S7). BestKeeper and NormFinder ranked *PP2A2* and *RPL26* as the most stable genes, respectively, which was not consistent with ΔCt and RefFinder rankings. The gene *SDH5* was the least stable reference gene after fungal inoculation as per four algorithms, and geNorm ranked *RID2* as the least stable reference gene.

3.2.2.5 Mature tree

In mature tree samples, the comprehensive ranking algorithm RefFinder identified *SP1, COG7,* and *TULP6* as the most stable reference genes and *SDH5* as the least stable gene. Similar to previous observations, the BestKeeper and NormFinder rankings were not consistent with that of RefFinder. BestKeeper and NormFinder ranked *PP2A2* and *HSP90*, respectively, as the most stable genes. In contrast, a combination of *RPL26/ARP9* was suggested to be the most stable gene and *COG7* as the least stable reference gene by geNorm analysis (Supplementary material S7).

3.3 Optimal reference gene selection for normalization

To achieve more accurate and reliable gene expression results, it is often recommended to use multiple reference genes. According to Vandesompele et al. (2002), if the Vn/Vn + 1 value is below 0.15, the addition of an additional reference gene (n + 1) is deemed unnecessary, suggesting that the initial reference gene is sufficient for normalizing target gene expression. Using the geNorm algorithm, we determined the optimal number of reference genes for each condition. Our analysis revealed that at least two reference genes were necessary for accurate normalization in (mature trees and combined conditions), as indicated by the pairwise comparison values (Figure 2).

3.4 Validation of reference gene selection

Based on the RefFinder comprehensive analysis, *SP1* and *COG7* were identified as the most stable genes, whereas *SDH5* was identified as the least stable gene for gene expression studies in Norway spruce samples under drought conditions. To validate the reliability of the candidate reference genes, the relative expression of the target gene *PaDhn5* under drought stress was normalized with the most stable reference genes (*SP1* and *COG7*) and the least stable reference gene

TABLE 2 Tissue-wise rankings of candidate reference genes based on stability value calculated by ΔCt, BestKeeper, NormFinder, and RefFinder. Each tissue group represent 4 biological replicates, each individually consisting of 4 plants pooled together.

Conditions	Gene names	Methods										Recommended gene
		geNorm		ΔCt		BestKeeper		NormFinder		RefFinder		
		Stability value	Ranking	Avg. of STDEV	Ranking	Stability value	Ranking	Stability value	Ranking	Stability value	Ranking	
Needle	*SP1*	0.265	2	0.71	1	0.77	3	0.150	2	1.32	1	*SP1, JMJ16, RID2*
	JMJ16	0.328	4	0.71	2	0.85	6	0.234	10	2.21	2	
	RID2	0.231	1	0.74	3	0.77	4	0.208	5	3.66	3	
	TULP6	0.231	1	0.76	4	0.94	9	0.225	9	4.56	4	
	RPL7Ae	0.443	8	0.77	6	0.72	2	0.236	12	4.92	5	
	PP2A2	0.516	12	0.94	10	0.59	1	0.316	13	5.62	6	
	COG7	0.370	6	0.77	5	0.97	11	0.210	6	5.76	7	
	CCZ1	0.349	5	0.78	7	0.86	7	0.190	4	6.48	8	
	ARP9	0.486	11	0.82	8	0.79	5	0.224	8	7.11	9	
	RPS10	0.418	7	0.83	9	0.94	8	0.215	7	8.74	10	
	RPL26	0.477	10	1.03	11	0.97	10	0.163	3	10.98	11	
	SKIP22	0.300	3	1.03	12	1.17	13	0.236	11	11.98	12	
	UBCP	0.463	9	1.12	13	1.02	12	0.146	1	12.74	13	
	HSP90	0.567	13	1.60	14	1.54	15	0.318	14	14.24	14	
	SDH5	0.684	14	1.89	15	1.31	14	0.475	15	14.74	15	
Phloem	*RID2*	0.633	8	0.85	1	0.61	7	0.164	9	1.63	1	*RID2, SP1, PP2A2*
	SP1	0.710	10	0.86	2	0.62	8	0.180	10	2.38	2	
	PP2A2	0.478	3	0.94	6	0.42	1	0.075	1	3.22	3	
	RPS10	0.316	1	0.91	4	0.46	2	0.137	6	3.56	4	
	COG7	0.796	12	0.91	3	0.64	9	0.226	12	4.82	5	
	TULP6	0.601	7	0.92	5	0.56	6	0.149	8	5.33	6	
	ARP9	0.504	4	0.98	7	0.46	3	0.137	7	5.86	7	
	RPL26	0.316	1	0.98	8	0.46	4	0.097	2	6.51	8	
	RPL7Ae	0.560	6	1.00	10	0.51	5	0.127	3	7.21	9	
	JMJ16	0670	9	0.98	9	0.78	10	0.199	11	9.74	10	
	CCZ1	0.887	14	1.13	11	0.79	11	0.246	13	11.47	11	
	UBCP	0.532	5	1.17	13	0.88	12	0.129	4	12.22	12	
	SKIP22	0.841	13	1.17	12	0.98	13	0.265	15	12.24	13	
	HSP90	0.753	11	2.28	14	1.51	14	0.249	14	14	14	
	SDH5	0.413	2	2.70	15	2.09	15	0.135	5	15	15	

(Continued)

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TABLE 2 (Continued)

Conditions	Gene names	Methods										Recommended gene
		geNorm		ΔCt		BestKeeper		NormFinder		RefFinder		
		Stability value	Ranking	Avg. of STDEV	Ranking	Stability value	Ranking	Stability value	Ranking	Stability value	Ranking	
Root	*UBCP*	0.449	4	1.06	3	1.30	6	0.197	1	3.22	1	*UBCP, RPL7Ae, SP1*
	RPL7Ae	0.529	6	1.12	5	1.20	5	0.321	10	3.34	2	
	SP1	0.343	2	1.01	1	1.43	9	0.215	2	3.46	3	
	PP2A2	0.562	7	1.13	6	1.02	1	0.321	9	3.46	4	
	RPS10	0.492	5	1.14	7	1.18	4	0.304	7	3.74	5	
	COG7	0.330	1	1.04	2	1.38	8	0.218	3	4.56	6	
	RPL26	0.583	8	1.21	9	1.08	3	0.266	5	5.05	7	
	TULP6	0.330	1	1.07	4	1.30	7	0.242	4	5.29	8	
	ARP9	0.593	9	1.28	11	1.05	2	0.311	8	5.76	9	
	SKIP22	0.622	10	1.20	8	1.50	10	0.387	12	9.21	10	
	JMJ16	0.639	11	1.26	10	1.62	13	0.372	11	11.20	11	
	CCZ1	0.722	13	1.51	12	1.60	12	0.525	14	12.00	12	
	RID2	0.871	14	1.65	13	1.51	11	0.683	15	12.47	13	
	HSP90	0.391	3	1.92	14	1.93	14	0.270	6	14.00	14	
	SDH5	0.677	12	2.75	15	2.18	15	0.410	13	15.00	15	

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TABLE 3 Expression stability rankings of the reference genes, calculated by ΔCt, BestKeeper, NormFinder, and RefFinder across various treatment conditions in seedlings and untreated mature trees. Each treatment group represents 4 biological replicates, each individually consisting of 4 plants pooled together.

Conditions	Gene names	Methods										Recommended gene
		geNorm		ΔCt		BestKeeper		NormFinder		RefFinder		
		Stability value	Ranking	Avg. of STDEV	Ranking	Stability value	Ranking	Stability value	Ranking	Stability value	Ranking	
Control	*RPS10*	0.158	2	0.45	1	0.31	5	0.100	3	1.78	1	*RPS10*, *RPL26*, *SP1*
	RPL26	0.209	8	0.45	2	0.27	4	0.112	5	2.38	2	
	SP1	0.168	3	0.46	4	0.35	8	0.121	6	3.56	3	
	PP2A2	0.136	1	0.48	6	0.23	2	0.129	7	3.83	4	
	JMJ16	0.195	6	0.46	3	0.31	7	0.167	10	4.21	5	
	RID2	0.183	4	0.50	7	0.23	1	0.176	12	4.30	6	
	COG7	0.189	5	0.48	5	0.31	6	0.089	1	5.18	7	
	TULP6	0.136	1	0.56	9	0.26	3	0.145	8	6.98	8	
	RPL7Ae	0.258	11	0.56	8	0.54	12	0.175	11	9.12	9	
	SKIP22	0.240	10	0.57	10	0.49	10	0.163	9	10.00	10	
	ARP9	0.201	7	0.60	11	0.59	13	0.094	2	10.91	11	
	UBCP	0.217	9	0.68	13	0.40	9	0.101	4	11.62	12	
	CCZ1	0.309	12	0.67	12	0.51	11	0.202	13	11.98	13	
	SDH5	0.389	13	0.97	14	0.72	14	0.358	14	14.00	14	
	HSP90	0.471	14	1.32	15	1.04	15	0.361	15	15.00	15	
Drought	*RID2*	0.274	7	0.85	1	0.79	5	0.102	9	2.11	1	*RID2*, *SP1*, *COG7*
	SP1	0.169	1	0.86	2	0.80	7	0.080	4	2.30	2	
	COG7	0.211	2	0.89	3	0.88	9	0.081	5	4.49	3	
	ARP9	0.305	9	0.95	7	0.74	3	0.097	8	4.74	4	
	RPL26	0.221	3	0.93	5	0.77	4	0.063	1	4.86	5	
	JMJ16	0.322	10	0.95	8	0.96	10	0.130	11	5.32	6	
	PP2A2	0.265	6	1.02	10	0.55	1	0.092	7	5.48	7	
	RPL7Ae	0.357	12	0.97	9	0.70	2	0.137	13	5.58	8	
	RPS10	0.342	11	0.93	4	0.79	6	0.138	14	5.63	9	
	TULP6	0.232	4	0.93	6	0.85	8	0.077	2	6.62	10	
	SKIP22	0.293	8	1.05	11	1.06	13	0.120	10	11.47	11	
	UBCP	0.169	1	1.13	13	1.00	11	0.084	6	12.22	12	
	CCZ1	0.253	5	1.13	12	1.01	12	0.080	3	12.24	13	
	HSP90	0.370	13	1.94	14	1.46	14	0.137	12	14.00	14	
	SDH5	0.384	14	3.07	15	2.46	15	0.150	15	15.00	15	

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TABLE 3 (Continued)

Conditions	Gene names	Methods										Recommended gene
		geNorm		ΔCt		BestKeeper		NormFinder		RefFinder		
		Stability value	Ranking	Avg. of STDEV	Ranking	Stability value	Ranking	Stability value	Ranking	Stability value	Ranking	
Heat	*RID2*	0.816	7	0.92	1	1.06	6	0.249	6	1.57	1	*RID2, SP1, RPL26*
	SP1	0.978	11	0.94	2	1.07	7	0.264	8	3.60	2	
	RPL26	0.320	1	1.00	4	0.98	4	0.181	1	3.72	3	
	TULP6	0.767	6	1.00	5	1.17	8	0.205	2	4.23	4	
	COG7	0.891	9	0.96	3	1.19	9	0.290	9	4.24	5	
	ARP9	0.320	1	1.03	7	0.94	3	0.206	4	5.38	6	
	PP2A2	0.679	5	1.14	10	0.65	1	0.326	10	5.61	7	
	RPL7Ae	0.582	4	1.06	9	0.82	2	0.261	7	5.80	8	
	RPS10	0.370	2	1.02	6	1.03	5	0.244	5	5.96	9	
	JMJ16	0.853	8	1.04	8	1.26	10	0.339	11	7.95	10	
	SKIP22	0.936	10	1.16	11	1.40	12	0.384	13	10.98	11	
	UBCP	0.484	3	1.27	12	1.29	11	0.206	3	11.98	12	
	CCZ1	1.107	13	1.29	13	1.41	13	0.495	14	12.74	13	
	HSP90	1.022	12	2.29	14	2.01	14	0.384	12	14.00	14	
	SDH5	1.216	14	3.01	15	2.73	15	0.496	15	15.00	15	
Pathogen infection	*SP1*	0.430	4	0.89	1	0.87	5	0.258	2	1.50	1	*SP1, COG7, RPL26*
	COG7	0.583	9	0.93	2	0.93	9	0.293	6	3.22	2	
	RPL26	0.616	12	0.97	4	0.85	3	0.311	7	3.83	3	
	PP2A2	0.320	1	1.01	7	0.66	1	0.260	4	4.14	4	
	JMJ16	0.479	5	0.98	5	0.95	10	0.389	9	4.47	5	
	RPL7Ae	0.320	1	1.01	8	0.79	2	0.087	1	5.03	6	
	TULP6	0.583	10	0.96	3	0.90	8	0.436	12	5.09	7	
	RPS10	0.512	6	0.98	6	0.89	7	0.262	5	5.38	8	
	ARP9	0.645	13	1.05	9	0.85	4	0.455	14	7.35	9	
	RID2	0.850	14	1.23	13	0.89	6	0.963	15	10.72	10	
	SKIP22	0.596	11	1.07	10	1.07	13	0.417	11	10.94	11	
	UBCP	0.364	2	1.11	11	1.02	12	0.258	3	10.98	12	
	CCZ1	0.536	7	1.15	12	0.95	11	0.407	10	11.74	13	
	HSP90	0.563	8	2.13	14	1.79	14	0.442	13	14.00	14	
	SDH5	0.368	3	2.71	15	2.08	15	0.328	8	15.00	15	

(Continued)

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TABLE 3 (Continued)

Conditions	Gene names	Methods										Recommended gene
		geNorm		ΔCt		BestKeeper		NormFinder		RefFinder		
		Stability value	Ranking	Avg. of STDEV	Ranking	Stability value	Ranking	Stability value	Ranking	Stability value	Ranking	
Mature trees	*SP1*	0.688	9	0.91	1	0.96	7	0.192	3	1.63	1	*SP1*, *COG7*, *TULP6*
	COG7	0.851	14	0.94	2	1.02	9	0.355	14	2.45	2	
	TULP6	0.638	7	0.98	3	0.96	6	0.189	2	3.57	3	
	PP2A2	0.443	2	1.03	6	0.67	1	0.312	10	3.66	4	
	RPL7Ae	0.531	4	1.02	5	0.83	3	0.334	11	4.36	5	
	RPS10	0.473	3	1.02	4	0.89	5	0.278	7	4.47	6	
	ARP9	0.333	1	1.13	9	0.83	2	0.344	12	6.00	7	
	RPL26	0.333	1	1.10	8	0.86	4	0.245	6	6.51	8	
	JMJ16	0.754	11	1.07	7	1.13	12	0.312	9	8.53	9	
	UBCP	0.552	5	1.16	11	1.08	10	0.230	5	10.24	10	
	SKIP22	0.816	13	1.15	10	1.20	13	0.355	15	11.20	11	
	RID2	0.728	10	1.21	13	0.97	8	0.296	8	11.29	12	
	CCZ1	0.780	12	1.20	12	1.12	11	0.347	13	11.98	13	
	HSP90	0.603	6	1.95	14	1.63	14	0.168	1	14.00	14	
	SDH5	0.670	8	2.45	15	1.87	15	0.203	4	15.00	15	

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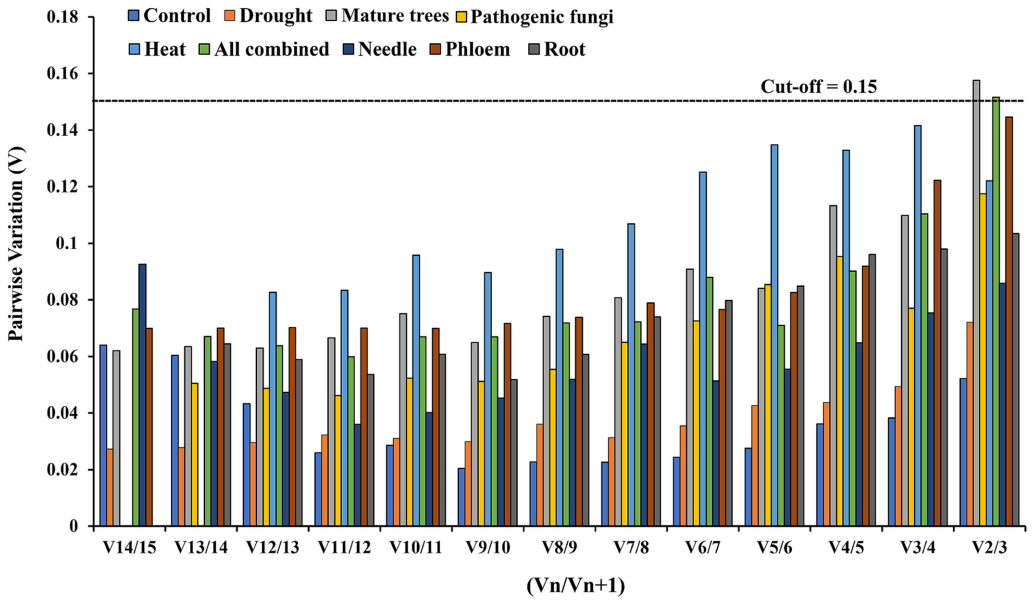

FIGURE 2
Pairwise variation analysis to determine the optimal number of reference genes required for normalization. The recommended cutoff value is 0.15, under which another gene is not required for normalization.

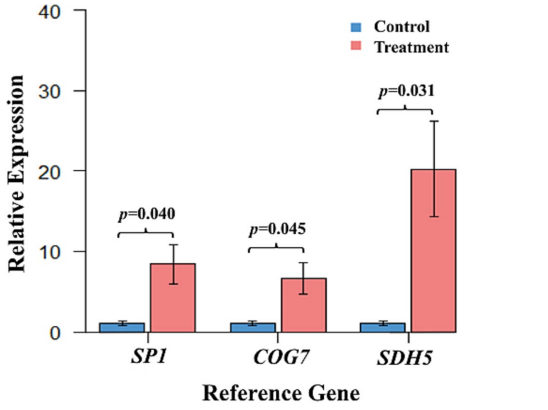

FIGURE 3
Relative expression levels of the target gene *PaDhn5* in drought-treated and control phloem samples after normalization with the most stable and the least stable gene. Normalization was performed using the most stable genes, *SP1* and *COG7*, and the least stable gene, *SDH5*, under drought conditions. The data represent the mean ± SD values of four biological replicates. Single-factor ANOVA was performed to compare the control and treatment groups of each gene to statistically analyze the relative expression of *PaDhn5*. *p*-values for each were generated as 0.04, 0.045, and 0.031 for the expression of *PaDhn5* when calculated against reference genes *SP1*, *COG7*, and *SDH5*, respectively.

(*SDH5*). The results indicated that the expression levels of *PaDhn5* in drought-treated samples increased by 8-fold and 6-fold (*p*-values = 0.040 and 0.045), respectively, when normalized with the stable reference genes *SP1* and *COG7*, compared to the control. In contrast, normalization with the least stable reference gene *SDH5* resulted in an almost 20-fold increase ($p = 0.031$) in expression (Figure 3).

4 Discussion

In recent years, forests worldwide have experienced climate change-driven droughts, storms, windthrows, and high-temperature disturbances, leading to extensive tree mortality. In Europe, one of the most economically important forest tree species is Norway spruce, which was widely planted outside its natural distribution area during the eighteenth and nineteenth centuries. Nevertheless, recent climatic conditions have led to a decline in its vitality, increasing susceptibility to stress and rendering the species particularly vulnerable to the tree-killing spruce bark beetle, *Ips typographus* and other *Ips* beetles (Biedermann et al., 2019; Singh et al., 2024a). These factors underscore the importance of understanding the molecular responses of Norway spruce to both abiotic and biotic stresses.

RT–qPCR is a highly effective and dependable technique for quantitatively studying the relative abundance of target genes. This method is widely employed to investigate plant-pathogen interactions and uncover underlying molecular mechanisms (Vijayakumar and Sakuntala, 2024). RT–qPCR is a relatively less expensive and faster method for exploring target genes under specific conditions. Despite its frequent use, there are concerns that researchers may not always adhere to recommended protocols, which can lead to misleading results (Bustin et al., 2009). Housekeeping genes, commonly referred to as reference genes, are a group of genes that are consistently expressed in cells and are crucial for fundamental cellular functions. They are stable throughout a species irrespective of treatments but can be specific to a particular tissue or developmental stage (Lin and Lai, 2010; Li et al., 2021). For accurate measurement of relative gene expression, it is crucial to use multiple reference genes with stable expression under specific biological conditions (Dai et al., 2018; Wang et al., 2021). While RT–qPCR is effective and reliable

for quantifying mRNA levels across different experimental conditions, several factors, such as RNA extraction, cDNA synthesis, primer design, and material handling, can impact the results (Schmittgen and Zakrajsek, 2000; Bustin et al., 2005; Huggett et al., 2005). Reliable reference genes help mitigate these variations, necessitating the evaluation of the stability of reference genes for each experimental condition to ensure precise and reliable data interpretation. Recent studies have evaluated reference genes in various coniferous species (e.g., Bao et al., 2016; Mo et al., 2019; Chen et al., 2019); however still, such studies on Norway spruce are limited to the embryogenic developmental stages (e.g., Vestman et al., 2011; de Vega-Bartol et al., 2013).

Among the 15 evaluated candidates, *SP1, COG7,* and *TULP6* were the most stable reference genes, making them suitable for gene expression normalization in Norway spruce. The *SP1* ubiquitin-protein ligase plays a crucial role in regulating chloroplast protein import, essential for plant development (Ling and Jarvis, 2015). It interacts with the translocon at the outer envelope membrane of chloroplasts (TOC) to recognize and import client proteins. *SP1* ubiquitin-protein ligase associates with TOC complexes and mediates the ubiquitination of TOC components, leading to their degradation through the ubiquitin-proteasome system (UPS) and reorganization of the TOC machinery. Ubiquitin-protein ligase (ubiquitin-conjugating enzyme) genes have been identified as suitable reference genes in various tissues of Brazilian rubber trees (*Hevea brasiliensis*) (Li et al., 2011), in alfalfa (*Medicago sativa*) cultivars under different stress conditions (Castonguay et al., 2015), in *Arabidopsis thaliana* (Czechowski et al., 2005), *Brachypodium distachyton* (Hong et al., 2008), *Vernicia fordii* (Han et al., 2012), *Capsicum annuum* (Wan et al., 2011) and *Brachiaria brizantha* (Silveira et al., 2009). Our findings are in accordance with these studies, showing stable expression of *SP1* in various tissue types and under various environmental conditions (Tables 2–4). Nevertheless, not all ubiquitin conjugating enzymes can be used as internal controls for normalization (Wan et al., 2011).

Conserved oligomeric Golgi complex subunit 7 (*COG7*) is a crucial component of the COG complex and is essential for maintaining the normal morphology and function of the Golgi apparatus (Klink et al., 2022). Specifically, *COG7* is part of lobe B of the COG complex, which contains COGs5-8 (Ungar et al., 2002; Blackburn et al., 2019). This protein plays a vital role in various developmental processes, including embryo development, pigmentation, cell and organ expansion, and the formation of the organized shoot apical meristem (Ishikawa et al., 2008; Vukašinović et al., 2017; Rui et al., 2020, 2021). Despite its unstable expression across different tissue types, *COG7* was ranked second in overall expression stability after *SP1,* according to RefFinder analysis. This implies that *COG7* could be a viable reference gene for gene expression normalization, particularly in experiments investigating mechanisms of resistance to drought and pathogens (Tables 3, 4). To our knowledge, no previous studies have considered *COG7* as a candidate reference gene.

Tubby-like F-box proteins (TULPs) were first discovered in mice and are highly conserved across a wide range of organisms. In plants, these Tubby-like proteins play a role in stress signaling pathways (Lai et al., 2004; Reitz et al., 2013). To date, 11 Tubby genes have been identified in *Arabidopsis*, 14 in rice, and 11 in poplar (Yang et al., 2008; Hong et al., 2015). Most plant TULPs possess a conserved F-box domain at the N-terminus and a Tubby domain at the C-terminus (Yang et al., 2008). Although TULPs are believed to function as transcription factors, their exact mechanisms of action remain largely unknown (Yulong et al., 2016). TULP6, while exhibiting variable expression across treatments, was the third most stable gene after *SP1* and *COG7* according to RefFinder analysis, indicating its potential as a reference gene for normalizing gene expression, especially in studies involving developmental stages (Tables 3, 4). Notably, TULPs have not been previously considered as reference genes.

To validate the identified reference genes, we evaluated the expression of dehydrin-like protein 5 (*PaDhn5*), a stress-responsive protein, across various Norway spruce tissues, developmental stages, and stress conditions. Dehydrins, part of the late embryogenesis abundant (LEA) family, accumulate in response to abiotic stresses and play a role in cryoprotection and membrane protection from reactive oxygen species (Close, 1997; Nylander et al., 2001; Rorat et al., 2006; Sun et al., 2021). Our results revealed that using the least stable reference gene (*SDH5*) led to erroneous data, showing a 20-fold increase in *PaDhn5* expression under stress conditions. In contrast, the most stable reference genes, *SP1* and *COG7*, resulted in more consistent *PaDhn5* expression levels, with an 8-fold and 6-fold increase, respectively (Figure 3). The significant discrepancy between the most and least stable reference genes highlights the importance of using stable reference genes to prevent bias in RT-qPCR normalization. Based on our findings, we recommend using the identified stable reference genes and their combinations (Figure 4) for accurate normalization in RT-qPCR analyses of gene expression in Norway spruce, which will improve the sensitivity and reproducibility of the results.

5 Conclusion

This study provides a catalog of genes that have been reported in various conifer species and validated for Norway spruce for the first time. We evaluated 15 candidate reference genes in various tissues and developmental stages under different environmental conditions using RT–qPCR and systematically assessed their expression stability to identify the most suitable reference gene for each condition. The study identified *ubiquitin-protein ligase (SP1), conserved oligomeric Golgi complex (COG7),* and *tubby-like F-box protein (TULP6)* as the most suitable candidate reference genes, while *succinate dehydrogenase (SDH5)* was found to be the least stable. Notably, this is the first report testing *COG7* and *TULP6* as candidates for reference genes and confirming their stable expression. In addition, normalizing RT–qPCR data with both stably and unstably expressed genes showed that *PaDhn5* expression aligns with the current knowledge of Norway spruce physiology. The fluctuations in gene expression between stable and unstable gene normalizations emphasize the need for validating reference genes for reliable RT–qPCR results. In summary, the identified and recommended reference genes and their combinations for normalizing gene expression in Norway

TABLE 4 Overall expression stability rankings of the reference genes throughout the tissues across all stress treatments, tissue types, and developmental stages as calculated by geNorm, ΔCt, BestKeeper, NormFinder, and RefFinder.

Conditions	Gene names	Methods										Recommended gene
		geNorm		ΔCt		BestKeeper		NormFinder		RefFinder		
		Stability value	Ranking	Avg. of STDEV	Ranking	Stability value	Ranking	Stability value	Ranking	Stability value	Ranking	
Overall	*SP1*	0.567	6	0.91	1	0.96	7	0.195	2	1.63	1	*SP1*, *COG7*, and *TULP6*
	COG7	0.633	8	0.94	2	1.02	9	0.243	5	2.45	2	
	TULP6	0.597	7	0.98	3	0.96	6	0.215	4	3.57	3	
	PP2A2	0.487	3	1.03	6	0.67	1	0.244	7	3.66	4	
	RPL7Ae	0.507	4	1.02	5	0.83	3	0.265	8	4.36	5	
	RPS10	0.459	2	1.02	4	0.89	5	0.243	6	4.47	6	
	ARP9	0.389	1	1.13	9	0.83	2	0.265	9	6.00	7	
	RPL26	0.389	1	1.10	8	0.86	4	0.192	1	6.51	8	
	JMJ16	0.663	9	1.07	7	1.13	12	0.285	10	8.53	9	
	UBCP	0.515	5	1.16	11	1.08	10	0.202	3	10.24	10	
	SKIP22	0.698	10	1.15	10	1.20	13	0.322	12	11.20	11	
	RID2	0.869	14	1.21	13	0.97	8	0.347	15	11.29	12	
	CCZ1	0.762	12	1.20	12	1.12	11	0.330	13	11.98	13	
	HSP90	0.725	11	1.95	14	1.63	14	0.307	11	14.00	14	
	SDH5	0.806	13	2.45	15	1.87	15	0.340	14	15.00	15	

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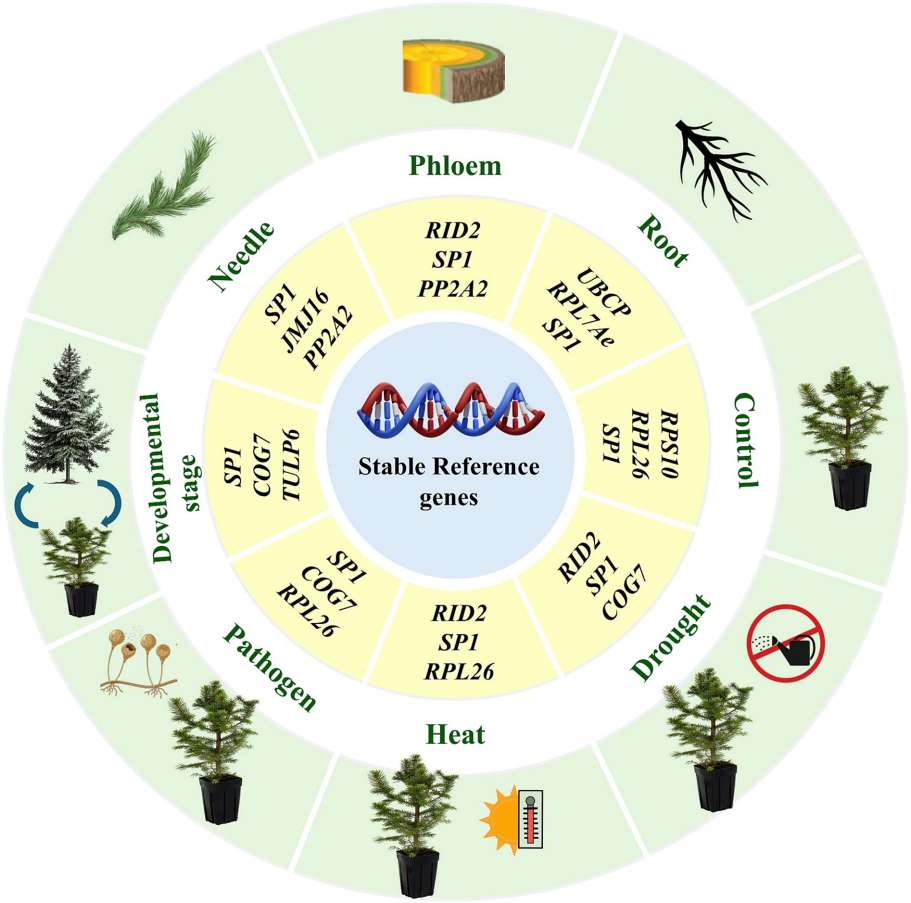

FIGURE 4
Recommended reference genes under different experimental conditions, tissues, and developmental stages.

spruce under various experimental conditions will benefit future gene expression and functional genomics studies in Norway spruce and related conifer species.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

VVS: Conceptualization, Methodology, Data curation, Formal analysis, Visualization, Resources, Project administration, Funding acquisition, Writing – original draft, Writing – review & editing. AN: Methodology, Data curation, Formal analysis, and Writing – review & editing. GS: Methodology, Data curation, Formal analysis, Writing – review & editing. KM: Methodology, Writing – review & editing. RG: Methodology, Writing – review & editing. AR: Methodology, Resources, Writing – review & editing. RJ: Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/ffgc.2024.1458554/full#supplementary-material

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