Background: Apomixis is a naturally occurring asexual mode of seed reproduction resulting in offspring genetically identical to the maternal plant. Identifying differential gene expression patterns between apomictic and sexual plants is valuable to help deconstruct the trait. Quantitative RT-PCR (qRT-PCR) is a popular method for analyzing gene expression. Normalizing gene expression data using proper reference genes which show stable expression under investigated conditions is critical in qRT-PCR analysis. We used qRT-PCR to validate expression and stability of six potential reference genes (EF1alpha, EIF4A, UBCE, GAPDH, ACT2 and TUBA) in vegetative and reproductive tissues of B-2S and B-12-9 accessions of *C. ciliaris*.

Findings: Among tissue types evaluated, EF1alpha showed the highest level of expression while TUBA showed the lowest. When all tissue types were evaluated and compared between genotypes, EIF4A was the most stable reference gene. Gene expression stability for specific ovary stages of B-2S and B-12-9 was also determined. Except for TUBA, all other tested reference genes could be used for any stage-specific ovary tissue normalization, irrespective of the mode of reproduction.

Conclusion: Our gene expression stability assay using six reference genes, in sexual and apomictic accessions of *C. ciliaris*, suggests that EIF4A is the most stable gene across all tissue types analyzed. All other tested reference genes, with the exception of TUBA, could be used for gene expression comparison studies between sexual and apomictic ovaries over multiple developmental stages. This reference gene validation data in *C. ciliaris* will serve as an important base for future apomixis-related transcriptome data validation.

Keywords: Apomixis, Apospory, Reference genes, qRT-PCR, Buffelgrass, *Cenchrus ciliaris*
necessity of differential gene expression studies between sexual and apomictic plants enables comparison between apomictic and sexual tissues, young inflorescences from B-12-9 and B-2S grown in the greenhouse were collected at 80°C prior to total RNA extraction. To collect reproductive tissues, young inflorescences from B-12-9 and B-2S grown in the greenhouse were covered with pollination bags to prevent cross-pollination during the flowering season. Four developmental stages for ovary and three developmental stages for anthers.

**Methods**

**Plant materials and sample collection for qRT-PCR**

Two genotypes of *C. ciliaris* (buffelgrass syn. *Pennisetum ciliare*) used in this study (obligate sexual, B-2S, and obligate aposporous, B-12-9) were maintained in the greenhouse with a temperature ranging from 24°C to 30°C. The plants were maintained by vegetative propagation as described in Roche et al. [14]. Leaves and roots were collected from greenhouse-grown B-2S and B-12-9 plants, frozen in liquid nitrogen and stored at −80°C prior to total RNA extraction. To collect reproductive tissues, young inflorescences from B-12-9 and B-2S grown in the greenhouse were covered with pollination bags to prevent cross-pollination during the flowering season. Four developmental stages were collected for ovary
samples based on anther developmental stages: stage I, early pro-metiotic; stage II, tetrad; stage III, DOP (the head contained one or two florets with anthers emerged, although tissue was collected from florets in which anthers had not yet emerged); and stage IV, DOP + 5 days (pollinated and seed were developing). Three different stages were collected for anthers: stage I, early pro-metiotic; stage II, tetrad; stage III, DOP (tissue was collected from anthers that had not yet emerged). The developmental stages I and II were determined by carbol fuchsin staining of anther squashes [41]. Staged inflorescence segments were initially stored in RNA-Later (Ambion) at −20°C and later ovary and anther samples were dissected and stored in RLT lysis buffer (Qiagen) at −80°C. At least two biological replicates were collected for all samples.

**Total RNA extraction and cDNA synthesis**

Total RNA was extracted using the RNeasy plant mini kit (Qiagen) and then subjected to DNase treatment (Turbo DNA free DNase, Ambion). The DNase treated RNA was concentrated using RNeasy mini elute columns (Qiagen). The final total RNA samples were checked by a Nanodrop spectrophotometer (260/280, 260/230 ratios). RNA with 260/280 in the range of 1.9-2.0 and 260/230 > 2 were checked for RNA integrity using an Agilent Bioanalyzer. Only RNA with a RIN score > 7 was used for the experiment. First strand cDNA synthesis used 5 μg of total RNA with oligo-dT and superscript II enzyme (Invitrogen), according to the manufacturer's instructions. The generated cDNAs were quantitated in triplicate using ribogreen [42], diluted to 3 ng/μl and stored in aliquots to avoid freeze thaw cycles.

### Sequencing of conserved reference gene domains from B-25 and B-12-9

EST sequences from conserved regions of each reference gene were used to design the first set of primers (Table 1) using Primer3 (v. 0.4.0) software (http://frodo.wi.mit.edu/, [43]). Using the conserved region primers from Table 1, fragments specific to each reference gene were amplified from both B-25 and B-12-9 leaf cDNA, cloned into pCR*-Blunt II-TOPO* vector (Zero Blunt*TOPO* PCR Cloning Kit, Invitrogen) and sequenced at the Georgia Genomics Facility (Athens, GA). At least 17 sequences per genotype from each reference gene were aligned using clustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

### Primer design and real-time PCR assay

A set of real-time primers was designed from the aligned region of each of the six reference genes (EF1alpha, EIF4A, UBCE, GAPDH, ACT2 and TUBA) using Primer3, (v. 0.4.0) software [43] and oligo analysis was performed using IDT oligoanalyzer tool (www.idtdna.com) with care taken to ensure that the reference gene real-time primer sequences were from the conserved regions of B-25 and B-12-9 and did not encompass any SNPs (single nucleotide polymorphisms). Real-time RT-PCR amplification reactions were performed using SYBR Green detection chemistry and run on 384-well plates with the Light Cycler 4.8 (Roche Applied Science). To estimate PCR efficiency of each reference gene primer pair, standard curves were generated using cDNA samples derived from different organs (leaf, root, anther and ovary) for both B-25 and B-12-9 genotypes. Samples were run in a 2-fold serial dilution range across 5 points with initial concentration of template starting at 0.6 ng. The corresponding PCR efficiencies and the error values were determined by the Light

### Table 1 Candidate reference gene description and details of the first set of primer sequences designed from the conserved regions of the specific reference genes

| Gene name/description | Gene function | NCBI accession1 | Primers-(S5′-3′) forward/reverse | Amplicon size (bp) |
|-----------------------|--------------|-----------------|----------------------------------|-------------------|
| EF1alpha/elongation Factor 1alpha | Translation | EB672663.1 | AGGAACCTTGCGCTCTTCTC /TCTCAACGCTGGTTATGGG | 225 |
| EIF4A/eukaryotic Initiation factor 4A | Translation | EB659100.1 | CTGCGAGCTCCTCAATCATC /TTTGCCACTCACGGTGACAT | 301 |
| ACT2/actin2 | Cytoskeleton | EB670295.1 | ACTACGAGAAGAGCTTGGG /TGCCTACCTACGTTGACAT | 425 |
| UBCE/ubiquitin Conjugating enzyme | Protein | EB661936.1 | GGCTGCTGACTGTAATTT /CTGGGAAGAGGATGTCAT | 433 |
| GAPDH/glyceraldehyde-3-phosphate dehydrogenase | Glycolysis | EB653168.1 | ATCACTGCCACCAAGAGCA/ATCGATGATTGTCAT | 404 |
| TUBA/tubulin alpha | Microtubules | EB655254.1 | TTGATGTTGCTATCAACGTG /TTGATGTTGCTATCAACGTG | 560 |

1 NCBI accession id for the C. ciliaris ESTs that were used in the primer design.
Cycler 4.8 software. All samples to be analyzed were run in the same plate in triplicate. Reactions were prepared in a total volume of 10 μl containing: 2 μl of template, 1 μl of each reference gene primer pair (optimized concentration: 300 nM), 5 μl of 2x FastStart SYBR Green Master (Roche Applied Science) and 2 μl of sterile water. Each PCR

Table 2 Primer sequences used for qRT-PCR analysis

| Gene name | Primers-(5'→3') forward/reverse | Amplicon size (bp) | PCR Efficiency ± SD |
|-----------|----------------------------------|--------------------|---------------------|
| EF1alpha  | GTGGTTCATGATGATGACCTGGGA/         | 82                 | 1.90 ± 0.03         |
|           | TGGTTATGGGCTCAACTCCAA            |                    |                     |
|           | TGGTATGACACACCGGAGTAA/           |                    |                     |
| EIF4A     | TCACGGTGACATGAGCAACACC          | 80                 | 1.90 ± 0.05         |
| ACT2      | CTCCTCTGATACCCATCACA/           | 103                | 1.85 ± 0.03         |
|           | CTTAGGTTCTCTCTGACACC            |                    |                     |
| UBCE      | TGTCAATGGCAAGCCTATCCT/          | 106                | 1.87 ± 0.03         |
|           | TGCCCAATTGAAACCATTGCTCCGAC      |                    |                     |
| GAPDH     | TGTCACCAGTGAAGTCCGTGAAA/         | 94                 | 1.95 ± 0.04         |
|           | AAGAAGGCTATCAAGGCTCGTCT         |                    |                     |
| TUBA      | CGACAGAATTCTAGGTTGATGTTGC/       | 80                 | 2.05 ± 0.05         |
|           | GTACGCGGGTAAGAAACAAGGTTGG        |                    |                     |

1SD, standard deviation; efficiency values for genes were calculated using the standard curve method of Light Cycler 4.8 software (absolute quantitation via second derivative method). An efficiency of 2 denotes 100%. All the error values for standard curves were below 0.02.

Figure 1 Specificity of PCR amplification. Dissociation curves for all six reference genes with a single product peak were obtained using three technical replicates of 18 cDNA samples. The arrow indicates no template control.
analysis also included reverse transcription negative control (RT minus, without reverse transcriptase) to confirm the absence of genomic DNA and a no-template negative control to check for primer-dimer and contamination. Uracil-N-Glycosylase (1 μl per 100 μl of reaction mixture) was also included in the reaction mixture in order to avoid PCR product carryover contamination. The PCR cycling conditions were set as follows: pre-incubation at 37°C for 10 min to activate Uracil-N-Glycosylase, an initial denaturation step of 95°C for 10 min to inactivate Uracil-N-Glycosylase and activate the FastStart Taq DNA polymerase, followed by 45 cycles of denaturation at 95°C for 10s, annealing at 60°C for 10s and extension at 72°C for 10s. The amplification process was followed by a melting curve analysis, ranging from 65°C to 90°C, increasing temperature in steps of 0.2°C every 10s. Cp values were automatically determined using the Light Cycler 4.8 software (absolute quantitation via second-derivative method). For every cDNA sample, the mean expression level and standard deviation for each set replicate was calculated (cut-off value for standard deviation was kept as 0.3; only samples below this cut-off were considered). To confirm

Figure 2 Cp distribution graph. The plot shows the Cp distribution of each candidate reference gene for the different samples (Vegetative-leaves and roots, Ovary-all stages and Anther-all stages) as generated by GenEx software. The x-axis shows genes, whereas the y-axis shows Cp values. a. B-2S and b. B-12-9.
the reproducibility of the assay and to reconfirm reference gene stability, the experiment was repeated for B-2S and B-12-9 ovary samples. Two biological and three technical replicates were included.

Gene expression stability analysis
For ranking reference genes based on their stability, GenEx (version 4.1.7, MultiD Analyses) which includes both GeNorm and NormFinder software was used. The Microsoft Excel file with raw expression Cp values for each tested gene in the 18 different samples generated with the Light Cycler 4.8 software was transferred into GenEx software.

Findings
Six reference gene sequences (EF1alpha, EIF4A, UBCE, ACT2, GAPDH and TUBA) from other plant species were used in BlastN analysis against the *C. ciliaris* pistil EST library at NCBI where *C. ciliaris* EST sequences with high similarity to all six selected reference genes (E-value = 0.0) were retrieved. High sequence similarity with high similarity to all six selected reference genes were used in BlastN analysis against the non-redundant database at NCBI. The amplicon length for the first set of primers used to generate B-2S and B-12-9 clones ranged between 225–560 bp whereas amplicon length with real-time RT-PCR primers ranged between 80–106 bp. Primer Tm ranged between 59-60°C, and primer lengths varied between 20–26 bp. All real-time RT-PCR primers specific for the reference genes showed acceptable amplification efficiencies (Table 2), and dissociation curves showed a single PCR product for each (Figure 1).

Table 3: Tissue specific reference gene stability analyses of B-2S (sex) and B-12-9 (apo) using GeNorm and NormFinder

| Genotype_Tissue type | Samples | GeNorm - M values | NormFinder_Gene ranking (SD values) |
|----------------------|---------|-------------------|-------------------------------------|
|                      |         | ACT2  | EF1alpha | EIF4A | GAPDH | TUBA | UBCE |
| Ovary (early/tetrad/DOP) | Sex     | 0.23  | 0.30     | 0.19  | 0.19  | 0.47  | 0.37 | 1 (0.09) 4 (0.34) 3 (0.25) 2 (0.13) 6 (0.68) 5 (0.50) |
|                      | Apo     | 0.15  | 0.15     | 0.37  | 0.30  | Oc    | 0.24 | 1 (0.07) 1 (0.07) 4 (0.48) 2 (0.22) 5 (0.87) 3 (0.38) |
| Ovary (early/tetrad/DOP + 5) | Sex     | 0.44  | Oc       | 0.16  | 0.16  | Oc    | 0.39 | 4 (0.53) 6 (1.06) 1 (0.07) 2 (0.08) 5 (0.64) 3 (0.39) |
|                      | Apo     | 0.20  | 0.20     | 0.32  | 0.27  | Oc    | 0.38 | 2 (0.14) 1 (0.10) 4 (0.40) 3 (0.18) 6 (0.74) 5 (0.48) |
| Anther (early/tetrad/DOP) | Sex     | Oc1   | Oc       | 0.47  | 0.47  | Oc    | Oc   | 5 (1.20) 3 (0.53) 2 (0.45) 1 (0.10) 5 (1.20) 4 (0.85) |
|                      | Apo     | Oc    | 0.43     | 0.27  | 0.36  | Oc    | 0.27 | 6 (0.82) 4 (0.64) 2 (0.19) 3 (0.63) 5 (0.72) 1 (0.13) |
| All tissues          | Sex     | Oc    | Oc       | 0.38  | 0.38  | Oc    | Oc   | 4 (0.80) 5 (0.97) 1 (0.20) 2 (0.40) 6 (1.10) 3 (0.60) |
|                      | Apo     | Oc    | 0.46     | Oc    | Oc    | Oc    | Oc   | 3 (0.84) 4 (0.86) 1 (0.50) 2 (0.60) 6 (0.95) 5 (0.90) |

GeNorm stability value is represented by M value (the selected cut off for M value is ≤0.5). NormFinder stability value is represented by SD (standard deviation). There is no cut-off for SD, hence the ranking of genes based on the SD value is used as a measure to evaluate stability (1 stands for most stable, 6 is least stable).
The ovary and anther stages are as follows: early stands for early premeiosis, tetrad, DOP for day of pollination, and DOP + 5 is 5 days after day of pollination.

For each B-2S and B-12-9 sample, specific cDNA (leaf, root, anther and ovary) standard curves were generated to measure primer efficiency. The amplification efficiency for primers, calculated by the Light Cycler 4.8 software, ranged from 1.85 ± 0.03 (92.5%) to 2.05 ± 0.05 (102.5%) (Table 2) and were considered appropriate for qRT-PCR [44]. An optimized primer concentration of 300 nM for all genes produced the lowest Cq value (calculated using absolute quantitation via second-derivative method). No-RT and no-template controls either did not generate any Cq or the difference in Cq between test and control samples was more than 10 to 13 cycles apart, values considered to be negligible [45]. The six candidate reference genes displayed a relatively wide range of mean Cq values from 17.79 (EF1alpha) to 24.9 (TUBA). The Cq distribution data for B-2S and B-12-9, for all samples (vegetative, ovary-all stages and anther-all stages) are shown in Figure 2a and 2b, respectively. In both B-2S and B-12-9, EF1alpha showed the highest expression whereas TUBA showed the lowest expression level. Studying expression levels of candidate reference genes will allow us to choose reference genes with similar expression patterns to that of the tested genes for future work.

In order to minimize bias introduced by the reference gene validation approach, two programs with different algorithms, GeNorm and NormFinder, were used for measuring gene expression stability. GeNorm software assumes that none of the tested genes being analyzed are co-regulated [46]. The stability measure provided by GeNorm (M-value) is the mean pairwise variation between a gene and all other tested candidates, and hence a pair of highly co-regulated genes could be eliminated during the selection if they show high inter-sample variability [28]. Genes with the lowest M-value are considered most stable. M-values below 0.5 indicate good measure of
stability [39,46]. We also tested the NormFinder software since it is less sensitive to co-regulation [28]. NormFinder relies on a ’model-based approach’; it determines expression stability of candidate reference genes by comparing the expression variation between and within groups and then combines both results in a stability value for each tested gene [47]. For NormFinder, the gene with the lowest standard deviation (SD) will be top ranked [28,32]. NormFinder software was found to be more robust with larger sample sizes [48]. Differences in gene ranking between different stability software have been reported [20].

In order to measure expression stability of genes across organs and stages, the Cp data in the Microsoft Excel file was transported into GenEx software and analysis was performed.

Table 4 Ovary (second independent assay) reference gene stability analyses of B-2S (sex) and B-12-9 (apo) using GeNorm and NormFinder

| Genotype_Tissue type | Samples | GeNorm - M values | NormFinder_Gene ranking (SD values) |
|----------------------|---------|-------------------|------------------------------------|
|                      |         | ACT2  | EF1alpha | EIF4A | GAPDH | TUBA | UBCE |             | ACT2  | EF1alpha | EIF4A | GAPDH | TUBA | UBCE |
| Ovary (early/tetrad/DOP) | Sex     | 0.19  | 0.10    | 0.09  | 0.03  | 0.16 | 0.03 | 5 (0.24) | 1 (0.01) | 1 (0.01) | 2 (0.13) | 4 (0.23) | 3 (0.15) |
|                      | Apo     | 0.30  | 0.26    | 0.33  | 0.39  | 0.26 | 0.45 | 4 (0.32) | 2 (0.25) | 1 (0.12) | 3 (0.30) | 5 (0.44) | 6 (0.54) |
| Ovary (early/tetrad/DOP/ DOP + 5) | Sex     | 0.24  | 0.16    | 0.09  | 0.06  | 0.29 | 0.06 | 4 (0.29) | 5 (0.31) | 1 (0.05) | 2 (0.07) | 6 (0.35) | 3 (0.12) |
|                      | Apo     | 0.30  | 0.30    | 0.32  | 0.35  | Oc1 | 0.40 | 3 (0.23) | 2 (0.16) | 1 (0.10) | 4 (0.28) | 6 (0.81) | 5 (0.56) |

GeNorm stability value is represented by M value (the selected cut off for M value is ≤0.5). NormFinder stability value is represented by SD (standard deviation). There is no cut-off for SD, hence the ranking of genes based on the SD value is used as a measure to evaluate stability (1 stands for most stable, 6 is least stable).

The ovary stages are as follows: early stands for early premeiosis, tetrad, DOP for day of pollination, and DOP + 5 is 5 days after day of pollination.

Oc refers to value outside the cut-off range.
performed via GeNorm (as described by Vandesompele et al. [31]) and NormFinder (as described by Anderson et al. [32]). As analysis of gene expression in apomictic development requires the comparison between sexual and apomictic plants, which are both genetically and developmentally different, we analyzed B-2S and B-12-9 tissue types separately. In order to monitor if the developmental stages of ovaries and anthers influence the reference gene stability, organ and/or developmental stage specific analysis was performed. Each sample set was first subjected to GeNorm analysis and all genes with an M-value ≤ 0.5 are reported (Table 3). The GeNorm results were further confirmed using NormFinder. Since there is no cut-off value for standard deviation, SD (stability value of NormFinder), the criterion for genes to be considered stable was that the genes confirmed as stable by GeNorm should be among the top four ranking in NormFinder. The result of NormFinder analysis is shown in Table 3. Although results in Table 3 show EIF4A as the most stable gene across all tissues of B-2S and B-12-9, there were tissue specific changes with respect to gene stability, using both GeNorm and NormFinder (Table 3).

Given our specific interest in ovary development and comparison of gene expression between sexual and apomictic genotypes (in our case B-2S and B-12-9), a detailed analysis of ovary samples of B-2S and B-12-9, delineating groupings of developmental stages, showed very similar results between GeNorm and NormFinder (Table 3). Except for TUBA, all tested reference genes (M-value below 0.5) could be used for developmental grouping up to DOP for sexual vs. apomictic gene comparisons. The addition of the developmental stage DOP + 5, excluded EF1alpha and TUBA as good candidate reference genes. As genes involved in the apomeiosis pathway should be triggered towards early ovary developmental stages (early premeiosis and tetrad), a detailed analysis of early-stage B-2S and B-12-9 ovaries using GeNorm graph, as plotted by GenEx (Figure 3) shows that all genes had an M-value below 0.5. Both GeNorm and NormFinder results for B-2S and B-12-9 showed that the combination of EF1alpha, ACT2, UBCE, and GAPDH occupied the most stable positions in the graph.

A second independent assay of B-2S and B-12-9 ovary samples was performed using two independent biological replicates, with samples collected at the same developmental stages (Table 4). Unlike the first analysis, TUBA qualified up to the DOP stage as stable in GeNorm (M < 0.5) but was still among the lowest rankings in NormFinder. In the second analysis, EF1alpha was considered stable at DOP + 5. Both analyses of ovaries including DOP + 5 identified TUBA to be unstable in at least one genotype (as per GeNorm). The reference genes EIF4A, GAPDH, UBCE and ACT2 were found to be stable in both first (Table 3) and second (Table 4) analyses of DOP as well as DOP + 5 ovary stages. All tested reference genes qualified for GeNorm stability for early + tetrad ovary tissue in the second analysis (data not shown).

### Conclusion
Our data present the first in-depth analysis of reference gene stability validation in *C. ciliaris*, a tetraploid and important model for understanding apomixis in grasses. Using both apomictic and sexual accessions of *C. ciliaris* and different organs/developmental stages, we were able to understand the reference gene stability specific to each organ, stage, or mode of reproduction. Our analysis used six reference genes based on their previous use in other plant species. These genes were analyzed in 18 samples, from both apomictic and sexual plants. All-tissue analysis using GeNorm and NormFinder found EIF4A as the most stable gene. There was a tight correlation between analyses with GeNorm and NormFinder, as genes detected stable by GeNorm occupied top gene rankings in NormFinder analysis.

Detailed analyses of ovary tissue in two independent assays, confirmed stability of genes in specific ovary stages that could be used in both B-2S and B-12-9, irrespective of the mode of reproduction. Based on our results, all tested reference genes were found to be stable (as per GeNorm, M-value below 0.5) for early to tetrad stage ovary development between the two species, while ACT2, UBCE, GAPDH and/or EIF4A would be most suitable for ovary stages up to DOP and DOP + 5. These reference gene expression and stability analyses will provide an important guideline for our future study involving apomixis-related gene expression studies via qRT-PCR in *C. ciliaris* or other related grasses.

### Competing interests
The authors declare that they have no competing interests.

### Authors’ contributions
BS led the experiments and drafted the manuscript. JAC and POA conceived the experiments, participated in experimental design, and finalized the manuscript. All authors read and approved the final manuscript.

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### Author details
1Department of Horticulture, The University of Georgia Tifton Campus, Tifton, GA 31793, USA. 2Current Address: Burnett School of Biomedical Sciences, University of Central Florida, Florida 32826, USA.

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