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Testing the chilling- before drought-tolerance hypothesis in Pooideae grasses

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Abstract

Temperate Pooideae are a large clade of economically important grasses distributed in some of the Earth's coldest and driest terrestrial environments. Previous studies have inferred that Pooideae diversified from their tropical ancestors in a cold montane habitat, suggesting that above-freezing cold (chilling) tolerance evolved early in the subfamily. By contrast, drought tolerance is hypothesized to have evolved multiple times independently in response to global aridification that occurred after the split of Pooideae tribes. To independently test predictions of the chilling-before-drought hypothesis in Pooideae, we assessed conservation of whole plant and gene expression traits in response to chilling vs. drought. We demonstrated that both trait responses are more similar across tribes in cold as compared to drought, suggesting that chilling responses evolved before, and drought responses after, tribe diversification. Moreover, we found significantly more overlap between drought and chilling responsive genes within a species than between drought responsive genes across species, providing evidence that chilling tolerance genes acted as precursors for the novel acquisition of increased drought tolerance multiple times independently, partially through the cooption of chilling responsive genes.

KEYWORDS
chilling stress, climate change, differential gene expression, drought stress, grasses, pre-adaptation

1 | INTRODUCTION

With the rise of atmospheric CO_2, current global average temperatures have increased by about 1°C compared to pre-industrial times (IPCC, 2021). As a result, we are experiencing increasingly severe drought and extreme weather events, the latter including unseasonal bouts of low and high temperatures that can adversely affect agricultural yield and lead to food insecurity (Cook et al., 2018; Craufurd & Wheeler, 2009; Moore & Lauenroth, 2017; Raza et al., 2019). The fact that plants have experienced periods of global warming and cooling in the past provides an opportunity for us both to understand how they might adapt to abiotic stress in the future and to utilize mechanistic insights across diverse clades for targeted crop improvement.

Grasses such as wheat (*Triticum* L. sp.) and barley (*Hordeum vulgare* L.) (Pooideae), and maize (*Zea mays* L.) (Panicoideae) have gained particular attention in respect to morpho-physiological characters contributing to drought tolerance, including leaf-related traits, root characteristics, water use efficiency, abscisic acid (ABA) levels, evapotranspiration efficiency and membrane stability

(Campos et al., 2004; Denčić et al., 2000; Sebastian et al., 2016; Tondelli et al., 2006). Similarly, work on rice (*Oryza sativa* L.) and maize has demonstrated that several traits, such as reduced photosynthesis, increased chlorophyll accumulation and improved germination, are associated with above freezing cold (chilling) stress tolerance (Sanghera et al., 2011). These physiological changes are induced at the transcriptional level by numerous genes (e.g., cold-responsive element/dehydration-responsive element [*CBF/DREB*] genes) to increase fitness (Agarwal et al., 2006; James et al., 2008; VanWallendael et al., 2019).

The evolution of tolerance to one environmental stressor can result in pre-adaptation to another stressor, particularly when the stressors put similar strains on metabolism and physiology (Edwards et al., 2017; Pembleton & Sathish, 2014). For example, both freezing and drought can cause cellular dehydration, and as such, acclimation to one of these stresses often results in acclimation to the other (Hussain et al., 2018; Medeiros & Pockman, 2011). Despite this knowledge, the extent of physiological and genetic crosstalk particularly between chilling and drought stress responses is limited to a few studies. In *Dianthus spiculifolius* Schur. (Caryophyllaceae), tomato (*Solanum lycopersicum* L.; Solanaceae) and cassava (*Manihot esculenta* Crantz; Euphorbiaceae), a common mechanism exists for drought and chilling stress responses involving signalling cascades such as the salt overly sensitive (SOS) pathway, calcium signalling, mitogen-activated protein kinase (MAPK), reactive oxygen species (ROS) signalling and dehydrins (Li et al., 2017; Zhou et al., 2017, 2019). Furthermore, constitutive expression of a rice U-box E3 ubiquitin ligase (*CaPUB1*) increases both chilling stress tolerance and reduces drought stress tolerance, acting as a novel regulatory module for drought and chilling stress (Min et al., 2016).

The grass subfamily Pooideae comprises around 4000 species and are the dominant grasses of the cold temperate northern hemisphere (Edwards & Smith, 2010). The subfamily as a whole is strongly adapted to seasonal cold and drought, but it is unknown whether these adaptations evolved once or multiple times, and/or if tolerance to one these stressors acted as a precursor to the other (Kemp & Culvenor, 1994; Monneveux et al., 2012; Pardo et al., 2020; Schubert, Grønvold, et al., 2019). In a recent study, Schubert, Marcussen, et al. (2019) hypothesized that the last common ancestor (LCA) of Pooideae originated around 68.7 million years ago (Ma) in a cool montane micro-niche (Figure 1a). Moreover, the requirement of an extended period of chilling (vernalization) to promote floral competency maps to a single origin at the base of Pooideae, suggesting that vernalization responsiveness originally evolved as an adaptation to above-freezing cold, rather than freezing per se (McKeown et al., 2016).

During the last 65–44 million years, Pooideae diversified into 10 tribes (Kellogg, 2015; Soreng et al., 2015), after which time global aridification peaked (~36 Ma), and the LCAs of each tribe are hypothesized to have independently increased their drought resistance

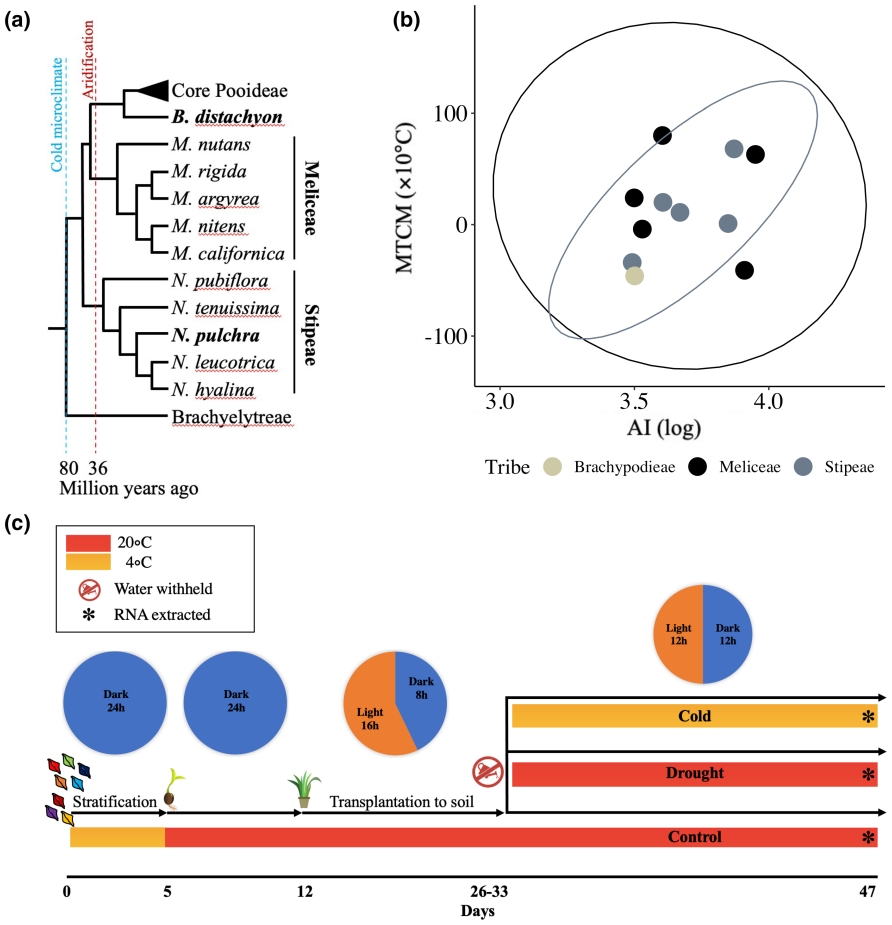

FIGURE 1 Pooideae sampling and climate space, and experimental design. (a) Simplified phylogeny of Pooideae showing the estimated timing of selective climates (blue dotted line, cold; red dotted line, aridification) relative to tribal diversification based on Schubert, Grønvold, et al. (2019a) and Schubert, Marcussen, et al. (2019b). Focal accessions are shown, with those used for differential gene expression in bold. (b) Overlapping niche space for focal Pooideae accessions from three Pooideae tribes based on a biplot of aridity index (AI) and minimum temperature of the coldest month (MTCM). Each tribe is represented by a different colour. Ellipses represent a bivariate equivalent of the 95% confidence interval for each tribe where sampling allows. Data are from Das et al. (2021). (c) Experimental design for control, drought and chilling treatments.

(Kellogg, 2001; Mannion et al., 2014; Zachos et al., 2001). If chilling tolerance evolved prior to drought tolerance in Pooideae, this sets up the hypothesis that chilling tolerance pre-adapted Pooideae grasses to evolve higher drought tolerance (Lamers et al., 2020). Similar hypothesized pre-adaptations in grasses include increased above-ground biomass allocation in the origin of Pooideae annuality (Lindberg et al., 2020) and increased vascular bundle sheath tissue in the origin of grass C4 photosynthesis (Christin et al., 2013).

To test the chilling- before drought-tolerance hypothesis, we here use a combination of morphological, physiological and gene expression data to compare across different Pooideae tribes. We first test the prediction that Pooideae tribes are more similar in their chilling vs. drought responses, the former being shared from their LCA and the latter having independent origins. We then test the prediction that chilling and drought stress responses within species will be more similar than drought stress responses across tribes. Overall, our study provides insight into the evolutionary relationship between Pooideae chilling and drought adaptation strategies.

2 | MATERIALS AND METHODS

2.1 | Plant material and experimental conditions

Eleven accessions from the U.S. Department of Agriculture National Plant Germplasm System's Germplasm Resources Information Network (USDA NPGS GRIN), representing the Pooideae tribes Brachypodieae, Meliceae and Stipeae (Table S1), were chosen based on a number of criteria. At the tribe level, we aimed to capture a wide phylogenetic diversity (~57 million years) within Pooideae, and to sample taxa that diversified from each other after the global aridification event ~36 Ma (Figure 1a). Although there are three Pooideae tribes successively sister to the clade containing our focal tribes (Schubert, Marcussen, et al., 2019), they are relatively small, and have been notoriously difficult to work with in the context of growth chamber experiments, based on low germination, high mortality and unpredictable flowering (e.g., McKeown et al., 2016). Within each tribe, we chose species that collectively spanned broad climatic regions such that there was higher niche variation within rather than between tribes, and thus no conflation between tribe and climate of origin (Figure 1b; Das et al., 2021). The model species *Brachypodium distachyon* (L.) P. Beauv (accession Bd1-1; Brachypodieae) and *Nassella pulchra* (A. Hitchc.) Barkworth (Stipeae) were further selected for transcriptome analyses as they are both diploid and the former has a fully sequenced genome (Gordon et al., 2014; Love, 1954).

Sixty to 80 seeds from each species were surface sterilized with 70% ethanol followed by 20% bleach/0.05% Triton X-100. Each seed was then plated on 1% agar and stratified for 5 days at 4°C in the dark (McKeown et al., 2016). To warm acclimate seeds, agar plates were moved to room temperature in the dark for 7 days before planting in Pro-mix BX potting soil. Germinating seeds in soil were initially incubated in a Conviron model A1000 Growth Chamber (Conviron) at 20°C long days (16 hr light: 8 hr dark) with maximum light intensity (325 μmol photons·m^{-2} s^{-1}), 50% relative humidity, and ample water for 2–3 weeks depending on their growth (Figure 1c). Seedlings were then thinned out to one individual per pot, and 20 pots per species were randomly assigned to control, drought and chilling treatments under neutral day (12 h light: 12 h dark) conditions with low light intensity (125 μmol photons·m^{-2} s^{-1}) for 1 week to allow conditions that would mimic winter for the chilling treatment (Figure 1c).

Individuals assigned to two replicate control and two replicate drought treatments were maintained in a common 20°C chamber with neutral days, 50% relative humidity and the lowest light intensity, whereas the chilling treatment was conducted under the same conditions at 4°C in two replicate chambers for 2 weeks (Figure 1c). To control for water availability, soil moisture was assessed three times a week in the afternoon using a Fieldscout Soil Sensor Reader and Waterscout SMEC 300 Soil Moisture Temperature Sensor (Spectrum Technologies). Control and chilling treatment soils were kept around 25%–30% volumetric water content (VWC), whereas drought treatment soils were allowed to dry down to around 4%–10% VWC, hereafter the "drought zone" (Figure S1). After 2 weeks of all treatments, which translated to approximately 9 days in the drought zone for the drought treatment (Figure S1), individuals were transferred to a common 18–24°C long day (16 h light: 8 h dark) climate-controlled greenhouse with regular watering until flowering, death or termination of the experiment (Figure 1c). Drought stress was confirmed by the fact that mortality rates were 9%–13% higher in all droughted species relative to the control treatment, as reported in Das et al. (2021), and expression of the drought-responsive C4-methylsterol oxidase gene *SMO-1* (Bradi4g23800) in *B. distachyon* (Figure S2) (Verelst et al., 2013). Furthermore, the timing of treatments was estimated to occur during the vegetative phase of development for all accessions based on the fact that all but two taxa—*Melica argyrea* Hack. and *Nassella tenuissima* (Trin.)—have a vernalization requirement to attain floral competency, and the two nonvernalization responsive taxa take 129 and 82 days on average to flower without cold, respectively (Das et al., 2022b).

2.2 | Physiological trait measurements

As an indirect measure of photosynthetic activity, leaf chlorophyll fluorescence was determined at noon using a portable chlorophyll fluorimeter (SPAD 502 Plus, Minolta) (Netto et al., 2005). Readings were taken every 2 days from 6-mm^2 leaf discs in the centre of the longest leaves from at least six individuals per species, replicate and treatment. The indexed chlorophyll content readings ranged from 0 to 20, with ~>8 indicating a uniform and ~<8 nonuniform, chlorophyll content distribution across the leaf surface (Uddling et al., 2007). An integrated measure of stomatal conductance (gw; also known as gs) data on the final day of stress were taken from Das et al. (2021) using the modified equation of Franks and Beerling (2009):

$$gw = \frac{d}{v}.D.a_w / \left(l + \frac{\pi}{2}\sqrt{\frac{a_w}{\pi}}\right).$$

where d is the diffusivity of water vapour in air (24.9×10^{-6} m^2s^{-1} at 25°C), v is the molar volume of air (24.4×10^{-3} m^3 mol^{-1} at 25°C, 101.3 kPa) (Franks & Farquhar, 2001), l is stomatal pore length (μm), D is the mean number of stomata visible in a 0.45-mm^2 field area and pore area after drought or cold treatments (a_w, μm^2). All stomatal traits were measured manually based on 1000× stomata images using an Olympus BX60 Upright Compound Microscope, imported into Fiji using the "freehand selection" tool and "measure" functions (Schindelin et al., 2012).

Relative growth rate (RGR) was calculated using four to five plants per species, replicate and treatment by measuring the ratio of the difference of the natural logs for above-ground height from day 1 to 14 of each treatment, according to the equation [ln(final height)−ln(initial height)]/(final time−initial time) (Kramer-Walter et al., 2016). Fourteen days after each treatment, four to five plants per species, replicate and treatment were removed from their pots, washed thoroughly to remove soil, and weighed to obtain fresh total biomass and fresh root biomass. Shoots and roots were then dried in an oven for 24 h at 105°C (Kramer-Walter et al., 2016; Zhao et al., 2016), after which total dry biomass, shoot dry biomass, root dry biomass and root length were measured. Specific root length (SRL) was calculated by dividing root length (m) by dry root biomass (kg) (Valverde-Barrantes & Blackwood, 2016), whereas above-ground water content (AWC) was defined as (fresh shoot biomass−dry shoot biomass)×100/(fresh shoot biomass), and shoot to root ratio (S:R) as (dry shoot biomass [mg])/(dry root biomass [mg]) (Zhao et al., 2016). For the remaining four to five individuals per species, replicate and treatment, days to flowering was determined as the number of days between seed germination and appearance of the first inflorescence.

2.3 | RNA extraction, cDNA synthesis, quantitative PCR and RNA sequencing

The youngest newly expanded leaves from three individuals for drought-treated *B. distachyon* and *N. pulchra* were collected on the final day of the treatment period, 3 h after dawn. Newly expanded leaves were also harvested from control plants in the drought experiment (hereafter drought control) on the same day as the drought plants, developmentally matching control and drought plants based on total leaf number. Total RNA was extracted using TriReagent (Ambion), and samples were cleared of contaminating DNA using Turbo DNase (Invitrogen). RNA was stabilized with the addition of Superase RNase Inhibitor (Invitrogen) and assessed for quantity using a Quantus Fluorometer E6150 (Promega) and quality using an Agilent 2100 Bioanalyzer (Agilent). To confirm that the drought treatment was long enough to elicit a gene expression response prior to leaf transcriptome sequencing, three replicates of *B. distachyon* total leaf RNA from the control and drought treatments were converted into cDNA using an iScript cDNA synthesis kit (BioRad). Primers were designed for *SMO-1* using PRIMER3 version 4.1.0 (Koressaar & Remm, 2007) that was previously found to be strongly upregulated with drought (Verelst et al., 2013): SMO-332F 5′-CAACATCGCCATCACATTGGTCGTCT-3′ and SMO-757R 5′-ATGAGCATCTCAGCCCAGTGT-3′. Primer efficiencies were tested using a dilution series on a StepOne Real-Time PCR System (ThermoFisher) with iTaq Universal SYBR Green Supermix (BioRad), and quantification for three technical and three biological replicates was done relative to the housekeeping gene *UBIQUITIN CONJUGATING ENZYME18* (Figure S2) (Schwartz et al., 2010).

Triplicate samples from the final day of treatment for drought-control and drought-treated *B. distachyon* and *N. pulchra* were submitted for 200-bp single-end sequencing on an Illumina HiSeq 1000 (Illumina Inc.) at the University of Vermont. To minimize lane effects, all 12 libraries were run with equimolar concentrations across two lanes. Raw sequence data were also downloaded from a previously published chilling experiment conducted in the same two species (Zhong et al., 2018). Growth conditions and sampling stages/times for the previous experiment were the same as for the drought plants with a few exceptions. Specifically, for the chilling experiment, plants were grown at 16-h long days instead of 12-h neutral days during the stress treatment period, and the youngest newly expanded leaves were harvested after 6 weeks of 4°C chilling compared to the 15 days of dry-down (Zhong et al., 2018). Control plants for the chilling experiment (hereafter chilling control) were collected prior to the chilling treatment and had the same leaf numbers as plants for which chilling-treated tissues were harvested (Zhong et al., 2018).

2.4 | Transcriptome assembly, differential gene expression analysis and functional annotation

For the drought data set, raw reads from *B. distachyon* and *N. pulchra* were trimmed to remove adapters in TRIMMOMATIC-0.36 (Bolger et al., 2014), and the resulting sequences comprised input for species-specific concatenation and de novo assembly using the TRINITY version 2.1.1 package (Haas et al., 2013). A similar pipeline was utilized for the separate analysis of the chilling data set, except that the available sequences were previously trimmed in the same manner (NCBI SRA project PRJNA412137; Zhong et al., 2018). Lowly expressed transcripts (fragments per kb of transcript per million mapped reads [fpkm] < 1) (Bourgon et al., 2010) were filtered out and aligned to post-filtered contigs using BOWTIE1.01 and TRINITY scripts (Langmead et al., 2009) for each treatment of each species. Transcript abundances of the assembled reads were quantified using the RSEM method (Li & Dewey, 2011). TRANSDECODER-3.0.1 was used to identify protein coding genes from assembled transcripts and BLASTP and HMMSCAN searches were carried out using the UniProt database (Haas et al., 2013; The UniProt Consortium, 2019).

To identify and analyse differentially expressed genes (DEGs), the DESEQ2 package was used to perform pairwise comparisons between drought control and drought, and between chilling control and chilling, replicates (Love et al., 2014). The DESEQ2 output was normalized and prefiltered by discarding genes with less than one read, and a cut-off of log 2-fold change and $adjp < .05$ was applied. The overlap

between DEGs of different species and treatments in the same direction (i.e., excluding upregulated genes that were downregulated in the other species and vice versa) was visualized as a Venn diagram using the R package "VennDiagram." To transform the count data to the $\log_2$ scale, rlog transformation was applied. Transformed data were then used to generate heatmaps with the "heatmap.2" function in R version 3.6.2. The variance stabilizing transformation function was used to normalize count data to generate principal component analysis (PCA) plots. Conserved gene lists were derived by overlapping the DESEQ2 output of DEGs across drought and cold experiments and/or species, followed by orthology verification by visualizing gene tree outputs generated in ORTHOFINDER-0.4 (Emms & Kelly, 2015). To identify classes of genes that were over-represented for specific biological processes, we performed a gene ontology (GO) enrichment analysis using an over-representation Fisher test with false discovery rate (FDR) correction in PANTHER (Ashburner et al., 2000; Carbon et al., 2021). The Kyoto Encyclopedia of Genes and Genomes (KEGG) database was then used to determine the involvement of DEGs in different signalling pathways (Kanehisa et al., 2007).

To determine the relative number of drought and chilling responsive DEGs in our two species that are also known to be drought or chilling responsive outside Pooideae, we searched the literature (references in Tables S2–S5) for drought and chilling responsive genes in rice and *Arabidopsis thaliana* (L.) Heynh. The same was also done for chilling–drought DEGs of *B. distachyon* or *N. pulchra* (references in Tables S2–S5). In addition to the previously published literature, we also used Oryzabase (https://shigen.nig.ac.jp/rice/oryzabase/) and Ensembl Plants (https://plants.ensembl.org/index.html) for the rice comparison, and the TAIR database (https://www.arabidopsis.org/) for the *A. thaliana* comparison. For this analysis we considered genes previously observed to be drought or chilling responsive, as well as genes functionally characterized to affect drought or chilling tolerance.

2.5 | Statistical analyses and comparative methods

Principal component analysis describing variation in leaf chlorophyll fluorescence, *gw*, RGR, SRL, AWC, S:R and days to flowering was applied to visualize the untransformed responses of different Pooideae tribes to control, drought and chilling, or to the difference between drought vs. drought control (hereafter drought response) and chilling vs. chilling control (hereafter chilling response), using the *prcomp* function of the *stats* package in R version 3.6.2 (Marschner et al., 2018; Vu, 2011). Ellipses around each tribe were estimated using the R package "ellipse" based on the 95% pairwise confidence region. The relative influence of tribal affiliation vs. growing conditions on trait variation was then determined using factorial analysis of variance (ANOVA) with tribe and treatment as independent variables and either principal component (PC) 1 or PC2 as dependent variables. To test the first prediction that tribes show a more conserved chilling relative to drought response, consistent with the chilling- before drought-tolerance hypothesis, multivariate ANOVA (MANOVA) was performed for the chilling and drought responses separately, designating tribe as the independent variable and the combination of chlorophyll fluorescence, *gw*, RGR, AWC, SRL, S:R and days to flowering as dependent variables (O'Brien & Kaiser, 1985). Bayes factor analysis was performed to quantify the difference in response to drought and cold stress across tribes, with or without inclusion of the single Brachypodieae species (Makowski et al., 2019).

To test the second prediction that chilling DEGs are more conserved across tribes than drought DEGs, the proportion of DEGs shared across representative Stipeae and Brachypodieae species was determined by dividing the number of shared DEGs by the total number of genes (i.e., DEGs plus non-DEGs for drought or chilling) detected that have orthologues in both *N. pulchra* and *B. distachyon*. The difference in proportions for the drought and cold treatments was determined using an estimate of conservative confidence intervals for a proportion test, setting the confidence intervals to 95% (Meeker et al., 2017; Preston et al., 2022), followed by a two-sample test for equality of proportions with continuity correction (Dolman, 2003). Statistical significance of overlapping genes between drought and cold in each species was tested with the R package *GeneOverlap* that uses a Fisher's exact test, where $p < .001$ means the overlap is highly significant and an odds ratio (OR) > 1 means the association is strong (Shen & Sinai, 2013). For qRT-PCR (quantitative real-time polymerase chain reaction) analyses, differences between treatments were determined using a Student *t* test in R version 4.0.3. All plots were created using the "ggplot2" R package (Wickham & Wickham, 2007).

3 | RESULTS

3.1 | Tribal affiliation explains more variation in multivariate trait space than growing conditions

To determine the relative importance of trait responses vs. tribe on growth trait means in multivariate space, we inspected the first two PCs of untransformed species trait means measured under control, chilling and drought conditions, and observed tighter clustering among points based on tribe vs. treatment affiliation (Figure 2a). ANOVA on the first PC showed a significant effect for tribe ($p < .001$), but not for treatment ($p = .203$), whereas ANOVA on the second PC showed a marginal effect for treatment ($p = .067$) and again no effect for tribe ($p = .278$) (Table S6a). Taken together, these data suggest that tribe has a larger effect on growth and development traits across species than response to the environment (Figure 2a).

3.2 | Growth trait responses across tribes are consistent with predictions of the chilling-before-drought hypothesis

Based on the chilling- before drought-tolerance hypothesis, we reasoned that Pooideae evolved mechanisms to resist cold stress

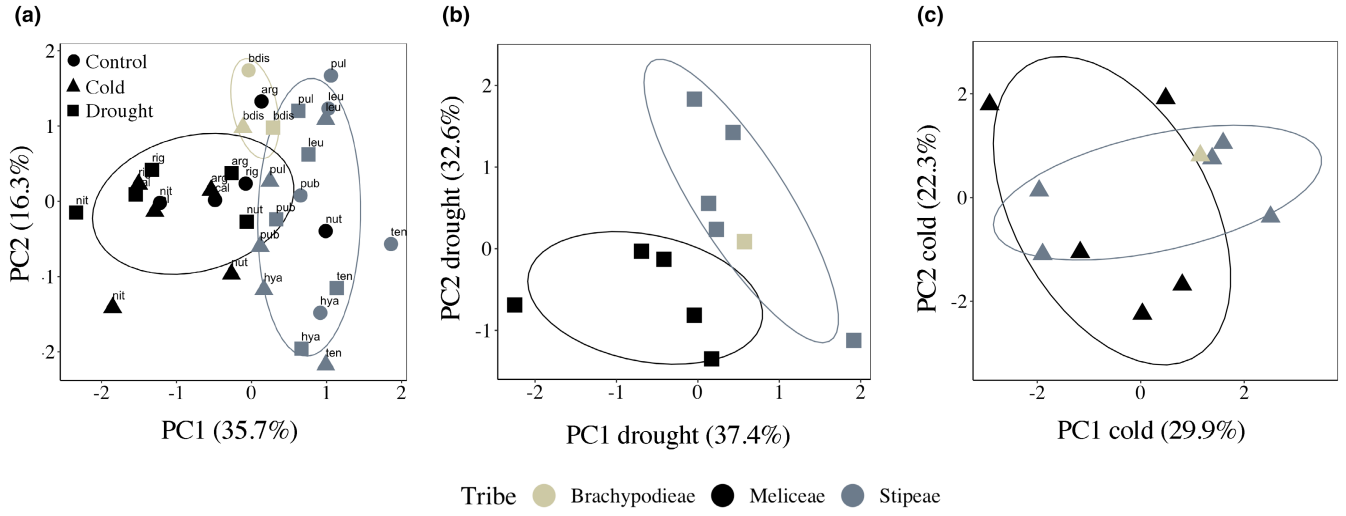

FIGURE 2 PCA displaying growth trait-based responses of Pooideae tribes under abiotic stress. (a) PCA plot showing that tribe explains more variation in multivariate growth responses than treatment ($p < .001$ based on ANOVA). (b) PCA showing that drought responses of Meliceae and Stipeae are distinct along PC1 and PC2 ($p = .027$ based on MANOVA). (c) PCA showing that cold responses of Meliceae and Stipeae are indistinct along PC1 and PC2 ($p = .714$ based on MANOVA). Each tribe and treatment are represented by a different colour and symbol, respectively. Ellipses represent a bivariate equivalent of the 95% confidence interval for each tribe where sampling allows.

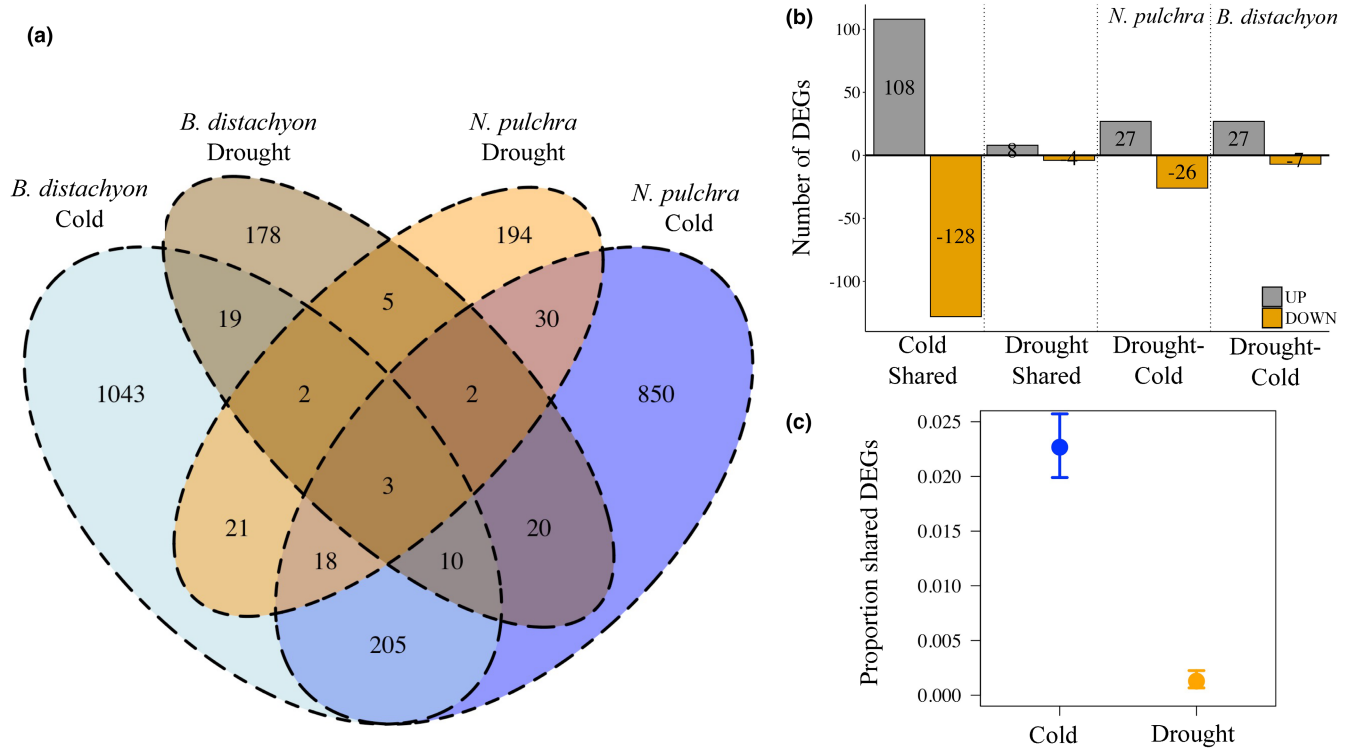

FIGURE 3 Chilling (cold) stress DEGs are more conserved between tribes than drought stress DEGs. (a) Venn diagram showing the numbers of DEGs in *Nassella pulchra* and *Brachypodium distachyon* in response to drought, cold or both, based on a cut-off of log 2-fold change and $adjp < .05$. (b) Bar plot showing the number of upregulated (grey bars) and downregulated (orange yellow bars) genes shared across species or treatments. (c) Point plot showing estimates of 95% conservative confidence intervals based on the proportion of DEGs shared across species for drought or cold relative to all orthologous genes detected in the drought or cold transcriptomes.

early in their history and that drought resistance evolved later after the diversification of its tribes (Figure 1a). Following this, we predicted that tribal differences in growth responses would be more pronounced under drought relative to chilling conditions. Inspection of the drought PC plot showed that PC1 and PC2 together explained 70% of trait variation (Figure 2b), with Meliceae showing a lower decrease in chlorophyll fluorescence, and a lower increase in SRL and AWC relative to control than Stipeae

(Figures S3a and S4–S7). In contrast, PC1 and PC2 for the chilling plot together explained 52%, and showed little discrimination in trait space between tribes based on overlap of the 95% confidence regions (Figure 2c; Figures S3b and S4–S7). A larger difference in drought relative to chilling response between tribes was supported by a MANOVA, whereby a significant difference between Meliceae and Stipeae was found for drought ($p = .027$) but not chilling ($p = .714$) response (Table S6b). Moreover, Bayes factor analysis revealed that the difference in response of Meliceae vs. Stipeae to drought stress (PC1: $1.574 \pm 0.02\%$; PC2: $1.527 \pm 0.01\%$) was three times higher than that for chilling stress (PC1: $0.587 \pm 0\%$; PC2: $0.513 \pm 0\%$) (Table S6c). Adding the single Brachypodieae species to the MANOVA resulted in no significant difference between tribes for drought ($p = .359$) or chilling ($p = .533$), although the difference in drought response between tribes was still higher for drought vs. chilling (Table S6d).

3.3 | Chilling stress DEGs are more conserved between tribes than drought stress DEGs

A total of 705×10^8 cleaned reads were obtained across species and treatments after de novo assembly and after downstream processing of the assembled transcripts, 9306 being defined as orthologous for drought and 10,174 for chilling between *Nassella pulchra* and *Brachypodium distachyon*, and 41.94% being functionally annotated in the Uniprot database beyond "uncharacterized." A PCA plot of filtered transcripts showed clustering between replicates for the drought experiment (Figure S4a,b), similar to previously published results for the chilling data set (Figure S8c,d) (Zhong et al., 2018). A heat map also grouped replicates together, with the exception of the third *B. distachyon* drought replicate that grouped sister to the control group (Figure S9).

Analyses comparing control and drought samples revealed 239 (162 upregulated and 77 downregulated) DEGs in *B. distachyon* (Figure 3a; Figure S10a), and 275 (152 upregulated and 123 downregulated) DEGs in *N. pulchra* (Figure 3a; Figure S10b) with a *padj* < .05 and 2-fold change cut-off. Likewise, 1321 genes (754 upregulated and 567 downregulated) in *B. distachyon* (Figure 3a; Figure S10c), and 1138 (517 upregulated and 621 downregulated) in *N. pulchra* were differentially expressed for control vs. chilling stress samples (Figure 3a; Figure S10d). Of these genes, only 2.39% (eight upregulated and four downregulated) drought DEGs were found to overlap between species, whereas this number was 10.61% (108 upregulated and 128 downregulated) for chilling DEGs (Figure 3a,b).

Based on the chilling- before drought-tolerance hypothesis prediction (Figure 1a), and results of our growth trait data (Figure 2b,c; Table S7), we expected a proportionally higher number of shared DEGs between representatives of our two tribes for chilling relative to drought stress. Estimates of conservative confidence intervals based on the proportion of DEGs shared across species for drought or chilling relative to all orthologous genes detected in the drought or chilling transcriptomes, respectively, showed a much lower proportion for drought vs. chilling (Figure 3c). The combination of nonoverlapping 95% confidence intervals, and a $p < .001$ for the two-sample test for equality of proportions with continuity correction, supported our prediction, again suggesting that the Pooideae chilling response is more conserved than its drought response. Reanalysis of these data excluding the *B. distachyon* drought replicate that clustered with control replicates had no significant effect on the results based on a 95% confidence intervals test. Furthermore, reduction of the drought fold-change cut-off from 2 to 1.5, resulting in 318 and 390 DEGs in *B. distachyon* and *N. pulchra*, respectively, similarly found only 2.75% overlap between species. This number was again significantly lower ($p < .001$) than the species overlap for cold DEGs based on a two-sample test (Figure S11).

Gene enrichment analysis assigned 31 GO categories for drought (Figure 4a; Table S8) and 117 for chilling DEGs in *B. distachyon* (Table S8), with 24 GO categories shared between treatments (Figure 4a). For *N. pulchra*, 25 GO categories for drought (Figure S12; Table S8) and 97 for chilling DEGs (Table S8) were enriched, with 20 GO categories overlapping between treatments (Figure S12). To determine the relative number of drought responsive DEGs in our two species that are also known to be drought responsive outside Pooideae, we searched the literature for drought responsive genes in rice and *Arabidopsis thaliana*. Only 38 (15.89%) *B. distachyon* (Table S2) and 34 (12.36%) *N. pulchra* (Table S3) drought responsive DEGs have previously been characterized as drought responsive or involved in drought tolerance in rice and/or *A. thaliana*. This tentatively suggests that the remaining genes were recruited for drought tolerance specifically in Pooideae.

3.4 | Drought DEGs are more similar to cold DEGs within species than they are to drought DEGs in other tribes

A second prediction of the chilling- before drought-tolerance hypothesis is that Pooideae-specific drought genes will be more similar to chilling responsive genes within species than to drought responsive genes across species from different tribes, and that the former overlap would be more than expected by chance. This prediction specifically gets to the idea that the chilling tolerance pathway acted as a precursor to facilitate independent origins of drought tolerance. Supporting this prediction, a Fisher's exact test revealed that the number of overlapping genes between chilling and drought was larger than expected by chance both within *B. distachyon* (27 upregulated and seven downregulated; $p < .001$, odds ratio [OR] = 25.9) and within *N. pulchra* (27 upregulated and 26 downregulated; $p < .001$, OR = 25.8) (Figure 3b). Moreover, comparison of these genes with the drought DEGs shared across species revealed that the shared drought-chilling genes were significantly more numerous than the drought genes shared across tribes, both according to nonoverlapping 95% confidence intervals, and a $p < .001$ for the two-sample test for equality of proportions with continuity correction (Figure 4b). We also found

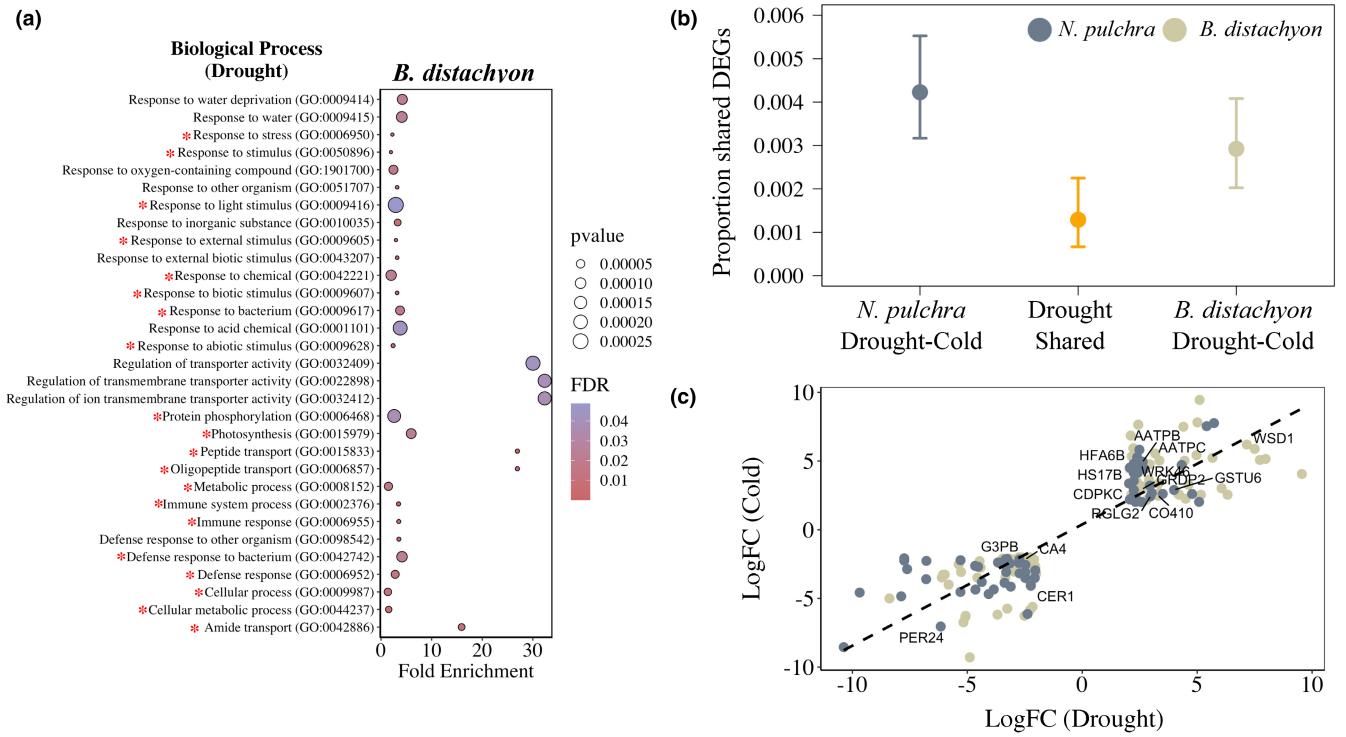

FIGURE 4 Drought and chilling (cold)-responsive genes are more conserved within species, than drought-responsive genes are conserved across species from different tribes. (a) Enrichment analysis of *Brachypodium distachyon* based on drought stress genes. Size of the circles are scaled based on *p*-value, and the colour palate is scaled based on false discovery rate (FDR). A red asterisk (*) indicates chilling–drought shared GO categories in *B. distachyon*. A blue asterisk (*) indicates chilling–drought shared GO categories with *Nassella pulchra*. (b) Point plot with estimates of 95% conservative confidence intervals based on the proportion of DEGs showing that Pooideae-specific drought and chilling responsive genes are more conserved within species than drought responsive genes are across species from different tribes. (c) Scatter plot showing a positive correlation between log-fold change (FC) of drought and chilling stress in *N. pulchra* and *B. distachyon*. The black dotted line is based on a linear regression model. Protein names are shown for known stress responsive protein coding genes.

a strong positive correlation between overlapping chilling and drought responsive genes based on their expression levels for both species (Figure 4c; $R^2_{adj} = .765$, $p < .001$).

To understand the extent to which chilling–drought DEGs of *B. distachyon* (Table S4) and *N. pulchra* (Table S5) are also chilling or drought responsive outside Pooideae, we compared these DEGs with known cold or drought responsive rice and *A. thaliana* orthologues. Only six (17.54%) of *B. distachyon* (Table S4) and 14 (26.41%) of *N. pulchra* (Table S5) chilling–drought DEGs have previously been characterized as chilling responsive in rice and/or *A. thaliana*. This again tentatively suggests that most of the cross-regulated genes were recruited for chilling and drought tolerance within Pooideae.

3.5 | Crosstalk between chilling and drought response pathways

To determine the biological nature of the crosstalk between chilling and drought responsive genes within our two focal species, we conducted GO enrichment analyses. In the case of *B. distachyon*, several GO categories were overrepresented for conserved chilling and drought stress genes, such as the categories plant-type hypersensitive response (GO:0009626), amino acid transport (GO:0006865), recognition of pollen (GO:0048544), response to abiotic stimulus (GO:0009628), cellular response to stimulus (GO:0051716) and response to stimulus (GO:0050896) (highlighted by red asterisks in Figure 4a). KEGG analysis also revealed that some of these genes participate in pathways involved in plant–pathogen interactions, photosynthesis, and cysteine and methionine metabolism (Figure S13a). By contrast, conserved chilling–drought stress genes in *N. pulchra* were enriched for categories such as photosynthetic electron transport chain (GO:0009767), fatty acid derivative metabolic process (GO:1901568), cellular response to external stimulus (GO:0071496) and regulation of secondary metabolic process (GO:0043455) (highlighted by red asterisks in Figure S12). KEGG pathway analysis suggested that the *N. pulchra* drought–chilling genes participate in pathways related to biosynthesis of secondary metabolites, and the metabolic mitogen-activated protein kinase (MAPK) signalling pathway (Figure S13b).

Despite several differences in GO categories for drought–chilling responsive genes, eight were shared across species (highlighted by blue asterisks in Figure 4a). Furthermore, we found 15 KEGG pathways conserved between *N. pulchra* and *B. distachyon* for shared chilling–drought genes, including plant hormone signal transduction, MAPK signalling, and cysteine and

methionine metabolism pathways (highlighted by purple asterisks in Figure S13).

4 | DISCUSSION

Worldwide changes in precipitation regimes and extreme weather events are impacting the performance and persistence of many species (Berdugo et al., 2020; Csilléry et al., 2020; IPCC, 2021). The ability of local populations to endure in the face of climate change will depend on several factors, not least of which is the inheritance of adaptive stress responses that have been shaped by previous geological cycles of warming, cooling and drying (Bansal et al., 2016). Of particular concern are the innate stress responses of agriculturally important plants, such as several members of the temperate grass subfamily Pooideae. Previous studies have hypothesized that the Pooideae LCA evolved in a cold highland microclimate of the tropical Eocene, resulting in descendants being pre-adapted to freezing seasonal conditions that were to emerge with dramatic cooling during the Eocene–Oligocene (E–O) boundary (Schubert, Marcussen, et al., 2019). Concomitant aridification at the E–O boundaryprobably also selected for increased drought responses independently in the already-established Pooideae tribes, positing a model of chilling-tolerance as a precursor of drought-tolerance. We tested predictions of this model, first that Pooideae from different tribes would show more conserved chilling than drought responses, and second that there would be higher overlap between differentially expressed chilling–drought genes within species compared to drought genes across tribes. Both of these predictions were supported based on physiological trait responses and DEGs, consistent with chilling tolerance pathway genes acting as precursors for the novel acquisition of increased drought tolerance multiple times independently.

4.1 | Evidence that chilling-tolerance evolved before drought-tolerance mechanisms in Pooideae

Both Pooideae and Danthonioideae stand out among the largely tropical grasses for their widespread distribution in cold temperate and boreal regions (Linder & Bouchenak-Khelladi, 2017; Preston & Fjellheim, 2020). As such, previous studies have suggested that the early acquisition of cold adaptations in these grasses, and smaller grass clades such as temperate bamboos (Bambusoideae), acted as key innovations to promote diversification through niche expansion (Edwards & Smith, 2010; Hoffmann et al., 2013). Ancestral state reconstructions of contemporary ranges also imply early origins of cold tolerance in Pooideae and Danthonioideae (Humphreys & Linder, 2013; Schubert, Grønvold, et al., 2019; Schubert, Marcussen, et al., 2019). However, despite some evidence that cold tolerance does not limit the distribution of Danthoniodieae grasses (Humphreys & Linder, 2013), data suggest that cold adaptation has been ongoing in these subfamilies as species have moved into even colder habitats (Das et al., 2021; Humphreys & Linder, 2013; Schubert, Grønvold, et al., 2019; Zhong et al., 2018).

Relative to cold tolerance, grass drought and desiccation tolerance is known to be quite labile (Pardo et al., 2020), with many crops being categorized as drought sensitive (Bowles et al., 2021; Dietz, 2011). The evolution of C4 photosynthesis in predominantly tropical panicoideae-arundinoideae-chloridoideae-micraioideae-aristidoideae-danthonioideae grasses is posited as one of the key traits conferring drought tolerance to grasses (Pardo & VanBuren, 2021; Pau et al., 2013; Taylor et al., 2014). Nonetheless, many C3 Pooideae grasses are also drought tolerant (Knapp et al., 2020; Linder & Bouchenak-Khelladi, 2017). Compared to tropical subfamilies, the number of origins and genetic basis for Pooideae drought tolerance are poorly understood (Bouchenak-Khelladi et al., 2010; Bowles et al., 2021; Estep et al., 2014). Pronounced global aridification starting at the E-O boundary and peaking in the late Miocene, after the diversification of Pooideae tribes, provides one reference point for increased selection for drought tolerance (Estep et al., 2014; Li et al., 2018), and sets up the scenario that significant increases in drought tolerance evolved after an early origin of chilling tolerance. To distinguish this from other scenarios, we reasoned that standard ancestral state reconstruction of chilling and drought tolerance alone might be misleading due to the high lability of either trait (Bromham, 2015). Instead, we decided to investigate tribal similarities at two mechanistic levels that would be less likely to show signals of convergence: multiple growth and development traits and differential gene expression. Results of both analyses showed higher overlap for chilling vs. drought responses across tribes, supporting an earlier origin of chilling vs. drought tolerance in Pooideae. Future work expanding these analyses to other taxa and tribes will be useful to further support or refute these findings within Pooideae and to determine if a similar pattern is found for Danthoniodieae.

Beyond the overlap in trait space across tribes per se, analysis of individual chilling and drought responsive DEGs within a broader comparative framework can provide information as to what elements are uniquely stress-responsive in Pooideae relative to other plants. Previous analyses of chilling DEGs shared by *B. distachyon* and *N. pulchra* plus *Melica nutans* L. (Meliceae) found that a large percentage were annotated as chilling inducible outside Pooideae, suggesting retention of ancient chilling response pathways, some of which (e.g., photosynthesis, general metabolism, dehydrins and *CBF/DREB* genes) are known to provide tolerance to multiple stressors (Agarwal et al., 2006; James et al., 2008; Schubert, Marcussen, et al., 2019; VanWallendael et al., 2019; Zhong et al., 2018). However, an even greater number were found to be Pooideae-specific, including *VERNALIZATION 1* (*VRN1*) involved in chilling responsive flowering, the 14-3-3 gene *GF14h*, and a D-mannose binding lectin family gene (McKeown et al., 2016; Zhong et al., 2018). Taking all the DEGs into account, the vast majority were either tribe-specific or potentially suffered from inaccurate orthology assessment. A similar pattern was found for a larger comparative study of Pooideae grasses, together supporting a model of stepwise evolution of cold tolerance

mechanisms, one step being at the base of the subfamily (Schubert, Grønvold, et al., 2019).

In the case of our novel drought responsive gene data set, only 12 genes were shared across species. Three of these also showed a shared chilling response (*GPAT1*, Bradi2g11450; *PP2C06*, Bradi1g17430; and *WAK3*, Bradi4g32830), orthologues of two were previously found to be drought responsive in rice and foxtail millet (*Setaria italica*) (*PMEI8*, Bradi3g45080; and *LEA14-A*, Bradi2g07480) (Wang et al., 2014), and two have close drought responsive paralogues in rice (*Os01g0656200*, Bradi1g17430; *CML10*, Bradi2g60660). This pattern suggests that at least half of these shared genes were already drought responsive at the base of the bambusoideae-oryzoideae-pooideae clade, again supporting the scenario that, within the context of Pooideae, drought tolerance increased relatively late in the subfamilies' history.

4.2 | Evidence that chilling-tolerance acted as a precursor of drought-tolerance in Pooideae

Precursor or enabler traits are those that act as positive constraints for the subsequent evolution of other traits, either allowing organisms to tolerate novel conditions that will select for further adaptation, or facilitating more rapid adaptation when novel conditions are encountered (Bromham & Bennett, 2014; Christin et al., 2013). The former can be interpreted as a pre-adaptation if the precursor trait allows for a non-negative population growth rate in the novel environment (Sibly & Hone, 2002). In grasses, vascular bundle sheath cell size and arrangement, C4 photosynthesis, and above- to below-ground resource allocation have all been posited as enablers of C4 photosynthesis, salinity and drought tolerance, and annuality, respectively (Bromham & Bennett, 2014; Lindberg et al., 2020; Osborne & Freckleton, 2009; Strömberg, 2011). Evidence for precursors is usually based on the ancestral reconstruction of two traits to infer when, where and how often each occurred relative to the other. Some drawbacks of this approach are false impressions of causality when one trait is more labile than the other, or where environmental extremes that select for these traits are correlated across a specialist clade's range (Bromham, 2015).

Both drought and cold can affect water balance and carbon metabolism, and in severe cases can cause tissue damage and hydraulic failure due to plasmolysis and embolism, respectively (Charrier et al., 2021). Responses to both stresses have been shown to involve ABA pathway-regulated solute accumulation, synthesis of dehydrins and sucrose:fructan 6-fructosyltransferases that work collectively to stabilize membranes and associated macromolecules (He et al., 2015; He et al., 2017; Li et al., 2007; Tamura et al., 2014). Furthermore, successive drought and cold can provide passive "legacy" or active "priming" changes in physiology to defend against future stress, such as long-term carbohydrate reallocation from growth to resistance or delayed phase transitions (Charrier et al., 2021; McDowell et al., 2008; Savvides et al., 2016; Van Mantgem & Stephenson, 2007). These observations, combined with our evidence that chilling responses are more conserved across Pooideae tribes than drought responses, are necessary but not sufficient to support the chilling-tolerance as a precursor of drought-tolerance hypothesis. However, our finding that there is significantly more overlap between drought and chilling responsive genes within a species than between drought responsive genes across species provides an extra piece of evidence that drought tolerance evolved independently in at least two tribes (Brachypodieae and Stipeae), partially through the cooption of chilling responsive genes.

Gene ontology enrichment and KEGG analyses suggest some functional overlap between the *B. distachyon* and *N. pulchra* shared chilling–drought DEGs in relation to abiotic and biotic stress response, transmembrane transport, MAPK signalling and metabolism. However, the majority of DEG functional categories are tribe-specific, and have not been previously characterized as stress responsive outside Pooideae. These 67 tribe-specific genes are primarily annotated as being involved in metabolism, and as such represent good candidates for engineering chilling–drought cross-tolerance in temperate grass crops (Dong et al., 2020; Priest et al., 2014). Characterization of drought responsive genes alone also revealed genes that are known to be involved in oxidative and salt stress tolerance pathways outside of Pooideae (Abbasi-Vineh et al., 2021; Chen, Jiang, et al., 2012; Chen, Lv, et al., 2012; Liu et al., 2014; Tasseva et al., 2004; Wang et al., 2017). These include *DIRIGENT PROTEIN 11* (Bradi4g41320), *EARLY-RESPONSIVE TO DEHYDRATION STRESS PROTEIN* (Bradi2g60810), *IRON–SULFUR CLUSTER ASSEMBLY PROTEIN 1* (Bradi2g45740), *AMINO ACID PERMEASE 2* (*AAP2*; Bradi2g24910) and *CHOLINE KINASE 1* (Bradi2g48260) in *B. distachyon*; and *NITRATE TRANSPORTER 1* (*NRT1*; Bradi1g43970 ortholog), *ACID PHOSPHATASE 27* (Bradi4g03787 ortholog), *CHITINASE 8* (Bradi3g32340 orthologue) and *GLUTATHIONE S-TRANSFERASE U6* (*GSTU6*; Bradi3g31880 ortholog) in *N. pulchra*. In addition to chilling tolerance pathways, this result suggests that ancient oxidative and salt stress pathways might have facilitated drought tolerance in Pooideae, and perhaps other lineages.

5 | CONCLUSIONS

Our results, combined with previous studies, suggest that Pooideae increased chilling tolerance early in their evolution, potentially facilitating faster or more numerous innovations in drought tolerance as species from different tribes expanded into more open and arid habitats. Since then, fine-tuning of both cold and drought tolerance traits has continued, but whether the same traits are targets of selection across taxa is unclear. Identification of Pooideae-wide DEGs shared for chilling and drought provides a framework for targeted breeding to multiple stresses. Furthermore, the identification of clade-specific chilling and drought genes broadens avenues for crop improvement through the manipulation of a wide suite of traits. These insights are particularly relevant in temperate regions of the world where warming at the end of winter is becoming more rapid, leading to increased evapotranspiration when water uptake

is minimal (i.e., winter drought), and droughts that are extending from summer into the decreasing temperatures of autumn (Charrier et al., 2021).

AUTHOR CONTRIBUTIONS

A.D., S.F. and J.C.P designed the research; A.D., N.D. and N.E. performed the research; A.D. and J.C.P. analysed the data; and A.D., S.F. and J.C.P. wrote the paper with final approval from all authors.

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CONFLICT OF INTEREST

The authors confirm that they have no conflicts of interest.

DATA AVAILABILITY AND BENEFIT-SHARING STATEMENT

Raw sequences have been deposited in SRA project PRJNA899871 (Das et al., 2022a). Data and R scripts for analyses have been uploaded to Dryad at https://doi.org/10.5061/dryad.0k6djhb3r (Das et al., 2022b).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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