Genome-wide identification, characterization, and expression analysis of the NAC transcription factor family in orchardgrass (*Dactylis glomerata* L.)

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Abstract

**Background:** Orchardgrass (*Dactylis glomerata* L.) is one of the most important cool-season perennial forage grasses that is widely cultivated in the world and is highly tolerant to stressful conditions. However, little is known about the mechanisms underlying this tolerance. The NAC (*NAM*, *ATAF1/2*, and *CUC2*) transcription factor family is a large plant-specific gene family that actively participates in plant growth, development, and response to abiotic stress. At present, owing to the absence of genomic information, NAC genes have not been systematically studied in orchardgrass. The recent release of the complete genome sequence of orchardgrass provided a basic platform for the investigation of DgNAC proteins.

**Results:** Using the recently released orchardgrass genome database, a total of 108 NAC (DgNAC) genes were identified in the orchardgrass genome database and named based on their chromosomal location. Phylogenetic analysis showed that the DgNAC proteins were distributed in 14 subgroups based on homology with NAC proteins in *Arabidopsis*, including the orchardgrass-specific subgroup Dg_NAC. Gene structure analysis suggested that the number of exons varied from 1 to 15, and multitudinous DgNAC genes contained three exons. Chromosomal mapping analysis found that the DgNAC genes were unevenly distributed on seven orchardgrass chromosomes. For the gene expression analysis, the expression levels of DgNAC genes in different tissues and floral bud developmental stages were quite different. Quantitative real-time PCR analysis showed distinct expression patterns of 12 DgNAC genes in response to different abiotic stresses. The results from the RNA-seq data revealed that orchardgrass-specific NAC exhibited expression preference or specificity in diverse abiotic stress responses, and the results indicated that these genes may play an important role in the adaptation of orchardgrass under different environments.

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Conclusions: In the current study, a comprehensive and systematic genome-wide analysis of the NAC gene family in orchardgrass was first performed. A total of 108 NAC genes were identified in orchardgrass, and the expression of NAC genes during plant growth and floral bud development and response to various abiotic stresses were investigated. These results will be helpful for further functional characteristic descriptions of DgNAC genes and the improvement of orchardgrass in breeding programs.

Keywords: Orchardgrass, NAC genes, Gene expression, Floral bud development, Stress response, Phylogenetics

Background

Transcription factors (TFs) are deemed to govern cellular processes in plants, such as signal transduction, cellular morphogenesis, and resistance to environmental stress [1, 2]. Generally, TFs regulate gene expression by binding to specific cis-acting promoters to activate or inhibit the transcription level of target genes [3, 4]. Among them, NAC is one of the largest and most plant-specific TF families and is named according to three proteins: petunia no apical meristem (NAM), Arabidopsis thaliana ATAF1/2 and cup-shaped cotyledon (CUC) [5, 6]. Typical NAC proteins include a highly conserved N-terminal region (NAC domain), which comprises five subdomains (A–E), whereas the C-terminal region contains a transcriptional activation/repression region (TAR or TRR) that is relatively divergent [5, 7, 8]. The subdomains of NAC domains are relevant to DNA binding, dimer formation and localization [8–11]. In addition, compared with subdomains B and E, subdomains A, C, and D are highly conserved [12–15]. The C-terminal regions might also be involved in protein-protein interactions and contribute to their regulation specificities [16].

NAC transcription factors play a critical role in the regulation of plant growth and development. In Arabidopsis thaliana, AtNAC1 and AtNAC2 are involved in lateral root development by downregulating auxin signals [17], while NAP is related to leaf senescence [18] and floral morphogenesis [19]. In addition, NTL8 controls seed germination by regulating gibberellin acid-mediated salt signaling [20] and regulates trichome formation by activating target genes (TRY and TCLI) in Arabidopsis [21]. In a previous study, it was reported that ORE1 could positively regulate aging-induced cell death in Arabidopsis leaves [22]. The NAC TFs of ONAC020/023/026 were associated with seed size/weight in rice (Oryza sativa) [23]. In cotton (Gossypium hirsutum), GhFSN1 participates in fiber development by activating its downstream secondary cell wall-related genes [24]. The NAC domain transcription factors NST1 and NST3 are involved in secondary wall biosynthesis, including the production of xylary and interfascicular fibers and pod shattering [25–27]. In Medicago truncatula, loss of MtNST1 function resulted in reduced lignin content associated with reduced expression of most lignin biosynthetic genes [28].

In addition, NAC genes also play an important role in the response to abiotic stresses. In Arabidopsis thaliana, AtNAP is a negative regulator that represses AREB1 under salt stress [29]. ANAC069 recognizes the DNA sequence of C[A/G]CG[T/G], which negatively regulates tolerance to salt and osmotic stress by reducing ROS scavenging capability and proline biosynthesis [30]. In wheat (Triticum aestivum), the overexpression of TaRNAC1 enhances drought tolerance [31]. The overexpression of TaNAC69 results in enhanced dehydration tolerance and the transcript levels of stress-induced genes in wheat [32]. The overexpression of TaNAC29 increased salt tolerance by enhancing the antioxidant system to reduce H2O2 accumulation and membrane damage [33]. Overexpression of OsNAC6/SNAC2 could also improve the drought, salt and cold tolerance of rice seedlings [34, 35]. In rice, ONAC022 enhanced drought and salt tolerance by regulating an ABA-mediated pathway [36]. Furthermore, the NAC transcription factor JÜNGBRÜNNEN 1 enhances tomato tolerance to drought stress [37]. In Arabidopsis, the heteroexpression of the Miscanthus NAC protein MINAC12 was found to result in activation of ROS scavenging enzymes to improve drought and salt tolerance [38]. A previous study illustrated that NAC genes are related to vernalization and flowering in orchardgrass by transcriptome analysis [39].

Orchardgrass (Dactylis glomerata L.) is one of the most important cool-season perennial grasses and is native to Europe and North Africa [40]. Orchardgrass is grown widely across the world due to its high biomass and nutritional quality, good shade, drought and barren tolerance, and high feed quality [41]. In addition, orchardgrass is also an important species in rocky desertification control in southwestern China. Therefore, orchardgrass has great economic and ecological value, and identification of functional genes is required to improve orchardgrass productivity. NAC genes have been widely studied in various plant species, such as Arabidopsis thaliana [13], Oryza sativa [7], Zea mays [42],
Glycine max [43], Solanum tuberosum [44], Pyrus bretschneideri [45], Fagopyrum tataricum [46], and Panicum miliaceum [47]. However, the NAC gene family in orchardgrass has not been systematically studied. With the completion of Dactylis glomerata L. genome sequencing, a systematic analysis of the NAC family during orchardgrass is expected to accelerate molecular breeding in orchardgrass [48]. In this study, we identified 108 orchardgrass NAC genes and classified them into 14 subgroups, including the orchardgrass-specific subgroup Dg_NAC. Comprehensive and systematic characteristics, including gene structure, conserved motif compositions, chromosomal distribution, gene duplications and phylogenetic characteristics, and homologous relationships were further investigated. In addition, the expression of DgNAC genes during plant growth and floral bud development and the response to various abiotic stresses were analyzed. The present results will be useful for illustrating the molecular mechanisms of orchardgrass adaptability under various environmental conditions, further analysis of the functional characteristics of candidate DgNAC genes and providing valuable clues for molecular assisted breeding in orchardgrass.

Results

Identification of the DgNAC genes in orchardgrass

Members of the NAC family were identified in the orchardgrass genome using the Hidden Markov Model (HMM) search with the HMM profile (PF02365) of the NAM domain. A total of 108 candidate gene models were matched across the whole genome and designated DgNAC001 to DgNAC108 based on their order on the chromosomes (Additional file 1). The basic information of 108 DgNAC genes was analyzed in this study, including the CDS length, protein sequence length, relative molecular weight (MW), and isoelectric point (pI) (Additional file 1). The protein sequence length of all DgNAC proteins ranged from 134 (DgNAC031) to 938 (DgNAC094) amino acids. The MW of the proteins varied from 14.70 to 181.91 kDa. The pI ranged from 4.28 (DgNAC042) to 10.25 (DgNAC012), with an average of 6.79, suggesting that most DgNAC proteins were weakly acidic.

Phylogenetic analyses and classification of DgNAC genes

To explore the evolutionary relationship of the NAC gene family in orchardgrass, an unrooted phylogenetic tree was constructed by using the amino acid sequences of DgNACs and AtNACs (Fig. 1). The results showed that 108 DgNAC genes could be divided into 14 subgroups, including an orchardgrass-specific subgroup named Dg_NAC. As shown in Fig. 1, the NAC proteins of orchardgrass were distributed in the ONAC003, ANAC063, AtNAC3, NAP, ATAF, ONAC022, TERN, TIP, ANAC011, OsNAC7, NAC1, NAC2, and NAM subgroups and orchardgrass-specific subgroup DgNAC. However, in orchardgrass, no NAC members were identified from the OsNAC8, SENU5, and ANAC001 subgroups. Among the 108 DgNAC proteins, only one DgNAC protein belonged to NAC1, the subgroups NAP, ANAC011 and NAC2 contained five DgNAC proteins each, and the orchardgrass-specific subgroup Dg_NAC included 15 DgNAC proteins, whereas the NAM subgroup contained the most DgNAC proteins (16).

Gene structure and protein motif analysis of DgNAC genes

To obtain more insights into the evolution of the NAC family in orchardgrass, the structural features of all the identified DgNAC genes were analyzed. As shown in Fig. 2b, among the DgNAC genes, 17 (approximately 15.74%) were intronless, 20 (12.96%) had one exon, nearly half (50, 46.30%) had three exons, and only 2 genes (DgNAC011 and DgNAC094, with 15 and 11 exons, respectively) had more than ten exons. Among the 15 orchardgrass-specific NAC genes, more than half (10, 66.67%) had only one exon.

To reveal the protein structural diversification of DgNAC proteins, 10 conserved motifs were identified by MEME (Fig. 2c). The amino acid sequences of each motif are listed in Additional file 2. The lengths of these conserved motifs varied from 10 to 55 amino acids. Motifs-1, 2, 3, and 5 were the most conserved parts (Fig. 2c). The orchardgrass-specific NACs DgNAC068 and DgNAC078 contain one type of motif, whereas DgNAC035 contains the highest number of motifs (8 types). The motifs of DgNAC members within the same subgroups display similar patterns, indicating that the same subgroup of genes have similar functions. However, the specific biological function of most of these motifs is unclassified and remains to be further investigated.

Chromosomal locations and synten analysis of DgNAC genes

To clarify the distribution of DgNAC genes on 7 chromosomes of orchardgrass, the MG2C program was used to map DgNAC genes on the chromosome (Fig. 3). A total of 108 DgNACs were randomly designated onto 7 chromosomes. Chromosome 2 had the highest number of DgNAC genes (20, 18.5%), and chromosome 7 harbored the lowest number (7, 6.5%). The orchardgrass-specific NAC genes are distributed on chromosomes 1, 3, 4, 5 and 6, and one-third of them are on chromosome 5. The duplication events of DgNAC genes were also examined in this study. The results showed that only 5 pairs of genes of tandem duplicates in the DgNAC gene family were identified, including DgNAC14/15,
DgNAC15/16, DgNAC21/22, DgNAC31/32, and DgNAC42/43, and they were linked with the red line, (Fig. 3). The tandem duplicated genes were present on chromosomes 1, 2, and 3, and only one pair of genes was common on chromosome 3.

To further explore the evolutionary relationship of the NAC gene family in orchardgrass, five comparative syntenic maps were constructed, which consisted of a dicotyledonous plant (Arabidopsis thaliana) and five monocotyledonous plants (Oryza sativa, Brachypodium distachyon, Hordeum vulgare, Sorghum bicolor and Setaria viridis) (Fig. 4). Seventy-seven DgNAC genes showed a syntenic relationship with Brachypodium distachyon, Setaria viridis (69), Oryza sativa (69), Hordeum vulgare (68), Sorghum bicolor (64) and Arabidopsis thaliana (6) (Additional file 3). The number of homologous
pairs between the other six species (Sorghum bicolor, Setaria viridis, Oryza sativa, Brachypodium distachyon, Hordeum vulgare and Arabidopsis thaliana) was 145, 114, 107, 98, 84 and 8, respectively.

Expression profiling of DgNAC genes in different tissues based on RNA-seq data
To better understand the function of DgNAC genes in orchardgrass, the transcript levels of DgNAC genes in different tissues were examined via the transcriptome data of different orchardgrass tissues derived from the orchardgrass genome database (Fig. 5, Additional file 5). Among the 108 DgNAC genes, eight DgNACs (DgNAC007/031/070/074/083/084/085/095) were not expressed in all detected samples, which may be pseudogenes or have special spatiotemporal expression patterns. Forty-two genes in roots, 3 genes in stems, 3 genes in leaves, 8 genes in spikes, and 17 genes in flowers presented high transcript abundances and may play a critical role in tissue development.

Expression profiling of DgNAC genes in different floral bud development stages with RNA-seq data
To further analyze the role of NAC genes in orchardgrass flowering, we used RNA-seq data to analyze the transcript levels of all 108 DgNAC genes in different floral bud development stages. The DgNAC genes exhibited different expression profiles with floral bud development. Several DgNAC genes presented similar expression patterns from the before vernalization (BV) stage to the heading (H) stage, such as DgNAC087 and DgNAC107, with gradually increased expression levels (Fig. 6, Additional file 6). Some genes showed preferential expression during the floral bud development of orchardgrass. Among them, eleven genes in the vernalization stage, four genes (DgNAC048/049/056/090) in the after vernalization stage, and twenty genes in the heading stage showed high transcript abundances. These DgNAC genes may play a critical role in the different floral development stages. In addition, the special temporal expression patterns of DgNAC genes may be related to changes in environmental conditions. For example, DgNAC genes respond to low temperatures in vernalization and long days in the heading stage.

Expression patterns of DgNAC genes in response to different abiotic stress
Gene expression patterns can provide crucial information for determining gene function. To investigate the role of NAC genes in orchardgrass under various abiotic stresses, 12 DgNAC members were selected for quantitative expression analysis in response to ABA, PEG, heat, and salt treatment durations (Fig. 7). Some DgNAC genes were induced/repressed by multiple treatments, such as DgNAC092 was inhibited by ABA, PEG, heat, and salt treatments, and DgNAC023 was induced by salt and ABA treatment after 3 h. In contrast, multiple DgNAC genes can be induced simultaneously by the same treatment. For instance, four DgNAC genes (DgNAC034/050/054/061/066/084) were induced by salt treatment. Interestingly, the expression level of DgNAC034 was higher than that of other selected genes under salt and heat treatment.
expression levels of many DgNAC genes, such as DgNAC008, DgNAC023, DgNAC079 and DgNAC092, were reduced by heat treatment. Furthermore, some genes showed opposing expression patterns under different treatments; for example, DgNAC023 was induced by ABA and salt but repressed by heat treatment.

To understand the potential function of orchardgrass-specific NAC genes in resisting environmental stress, we also analyzed the transcriptional levels of DgNAC genes from the Dg_NAC subgroup. The results showed that Dg_NACs are differentially expressed under submergence and heat tolerance (Fig. 8). In the submergence-tolerant cultivar ‘Dianbei’, DgNAC045, DgNAC094 and DgNAC085 were significantly upregulated after submergence treatment for 8 h (Fig. 8a). For drought stress treatment (18 d), the expression of DgNAC043, DgNAC010, and DgNAC095 was significantly upregulated in the roots of the tolerant variety ‘Baoxing’ (Fig. 8b). Under heat conditions, DgNAC062 and DgNAC077 were significantly upregulated in the heat-resistant variety ‘Baoxing’, while these two genes were downregulated in the heat-susceptible variety ‘01998’ (Fig. 8c).

Discussion

DgNAC gene identification and evolutionary analysis in orchardgrass

The NAC gene family is an important transcription factor in plants that plays roles in the regulation of growth, development, and stress responses [49–51]. Genome-wide identification of NAC genes has been studied in many plant species, while little is known about this gene family in the high-quality forge D. glomerata. In this study, a total of 108 NAC genes were identified based on the D. glomerata genome database [48], which was higher than the 104 NAC genes identified in Arabidopsis thaliana [13], 151 NAC genes identified in Capsicum annuum [52], 82 NAC genes identified in Cucumis melo [53], 80 NAC genes identified in Fagopyrum tataricum [46], and 96 NAC genes identified in Manihot esculenta [54] but lower than the 115 NAC genes identified in Arabidopsis thaliana [13], 151 NAC genes identified in...
Oryza sativa [55], 152 NAC genes identified in Zea mays [42], 152 NAC genes identified in Glycine max [43], 110 NAC genes identified in Solanum tuberosum [44], and 204 NAC genes identified in Chinese cabbage [56]. Evidence from physical and chemical parameters and gene structure and protein motifs confirms that genes originating from progenitors can gradually evolve and expand. Duplication events are important in the rapid expansion and evolution of gene families, and the size difference might be due to the more duplication events that occurred in other species after differentiation from their earliest ancestors. For example, the orchardgrass genome experienced one genome duplication event [17], while the Arabidopsis genome went through five such events [57]. A collinearity analysis demonstrated that there were 5 pairs of tandem replications without segmental duplication events (Fig. 3). Tandem replication of NAC genes has been observed in many species, such as Arabidopsis thaliana, Oryza sativa, Solanum tuberosum, and Panicum virgatum. However, the duplication event of orchardgrass increases the genome size rather than increasing many NAC gene members, which may be related to the expansion of long terminal repeat retrotransposons (LTR-RTs) [48].

The unrooted tree was constructed using NAC protein sequences from orchardgrass and Arabidopsis to explain the phylogenetic relationship. According to the sequence homology with Arabidopsis, all 108 DgNAC genes were divided into 13 subgroups [13]. The results were inconsistent with other species, such as Fagopyrum tataricum (15 subgroups) [46], Capsicum annum (14 subgroups) [52] and Capsicum annum (12 subgroups) [47], suggesting that NAC proteins exhibit diversity in various species. The results of conserved motif analysis of orchardgrass NAC proteins further confirm the classification of the DgNAC family. Only 8 pairs of homologous genes were found in orchardgrass and Arabidopsis by collinearity analysis, whereas more homologous gene pairs were identified in the five monocotyledons, including those of Oryza sativa, Brachypodium distachyon, and Hordeum vulgare (Fig. 4). The results indicated that the NAC genes are more homologous and conserved in monocotyledons.
Expression patterns and functional prediction of the DgNAC genes

Generally, the expression level of a gene determines its function, while the functions of genes are related to their expression patterns [58]. Transcription factors usually play a key role in controlling the expression of tissue-specific genes [59–61]. In this study, the tissue-specific expression pattern showed that more than 40 DgNAC genes exhibited higher expression in roots than other detected orchardgrass tissues, such as DgNAC008/052/026/023/034/061/045. Similar results were also found in other plants, such as Fagopyrum tataricum [46], Panicum miliaceum [47] and Triticum aestivum [62]. DgNAC046, DgNAC087, and DgNAC103 exhibited higher expression than the other genes in the stems of orchardgrass, and they may play an important role in stem development. In addition, previous studies have demonstrated that the development of tissues could be promoted by overexpression of tissue-specifically expressed NAC genes, such as NAC15 from poplar, which enhanced wood formation [63], and the NAC domain transcription factor PdWND3A affected lignin biosynthesis and composition in populus [64]. In general, genes in one branch of the phylogenetic tree often have the same function and similar expression profiles. Although DgNAC021 and DgNAC022 are duplicated genes within the same subgroup, the expression pattern of DgNAC021 was different from that of DgNAC022, which might be caused by variation in gene regulation after duplication events, and the differential expression patterns of duplicated DgNAC genes indicated that they might have experienced functionalization during the evolutionary process [65, 66]. The NAM subgroups may regulate cell division and leaf development [67–72], and the gene DgNAC090 is most highly expressed in the leaf followed by the root, indicating that DgNAC090 may function in leaf development and cell division through expression in both the leaf and root (Fig. 5). These results demonstrated that DgNAC genes are widely involved in the tissue development of orchardgrass.
Fig. 7 Expression profiles of 12 selected DgNAC genes in response to various abiotic stress treatments. Data were normalized to GAPDH gene and vertical bars indicate standard deviation.
Orchardgrass is a high-quality perennial forage grass, and flowering time is a critical factor affecting forage quality and utilization. In the current study, the potential role of NAC genes in the regulation of orchardgrass flowering time was investigated by using transcriptome data. The DgNAC genes were most highly expressed in different floral bud development stages (Fig. 6). Among them, DgNAC033 had a special expression pattern during the vernalization and after vernalization stages in orchardgrass, suggesting that it has an important function in the induction of flower primordia. A previous study indicated that the CUC1 gene regulates shoot apical meristem formation in Arabidopsis [72]. After vernalization of orchardgrass, three DgNAC genes (DgNAC034/050/082) showed high expression in vegetative growth and before the heading stage, indicating that these genes may play an important role in young inflorescence development and regulation of flowering time. The overexpression of the BnNAC485 gene in Brassica napus alters flowering time [73]. In Arabidopsis, NAC050 and NAC052 are involved in transcriptional repression and flowering time control by associating with the histone demethylase JMJ14 [74]. Overall, the expression of DgNAC genes varies in different floral bud development stages, which potentially regulates orchardgrass flowering time.

Orchardgrass is a widely adapted perennial forage grown on all continents. Orchardgrass is more tolerant of shade, drought, and heat than other cool-season perennial grasses. In plants, most of the NAC genes involved in the response to abiotic stress, such as drought, salinity, and heat, have been studied. However, there are few reports of NAC genes involved in the abiotic stress response in orchardgrass. Therefore, one of the goals of this study was to obtain more insights into the expression patterns and putative functions of DgNAC genes in response to various abiotic stresses. The expression levels of 12 DgNAC genes under four stress treatments (ABA, PEG, salt, and heat) were calculated (Fig. 7). All 12 DgNAC genes were induced by these treatments; in particular, DgNAC034 and DgNAC050 were significantly upregulated after PEG treatment for 12 h, salt treatment for 6 h, and heat treatment for 3 h, and DgNAC092 was repressed by all treatments. The expression pattern of orchardgrass-specific NAC genes under submergence, drought, and heat stress showed that NAC may play an important role in orchardgrass adaptation and resistance to various environmental stresses. These results provide new insight into how the accumulation of DgNAC effectively reduces abiotic stress damage.

Fig. 8 Expression profiles of orchardgrass-specific NAC genes in response to submergence stress, drought stress, and heat stress. a The heat map of DgNAC genes in submergence treatment for 0, 8, and 24 h, ‘Dianbei’ is submergence tolerant and ‘Anba’ is submergence sensitive. b The heat map of DgNAC genes in leaf and root of highly drought-resistant variety ‘Baoxing’ under drought treatment for 0 and 18d. c The heat map of part of orchardgrass-specific NAC genes under heat treatment for 0, 10, and 26d, ‘Baoxing’ is heat resistant, ‘01998’ is heat sensitive.

Conclusions

In the current study, a comprehensive and systematic genome-wide analysis of the NAC gene family in orchardgrass was first performed. A total of 108 DgNAC genes were identified and classified into 14 subgroups, including the orchardgrass-specific subgroup Dg_NAC. Comprehensive and systematic characteristics, including gene structure, conserved motif compositions, chromosomal distribution, gene duplications and phylogenetic characteristics, and homologous relationships were further investigated. In addition, the expression of DgNAC genes in various tissues, developmental stages of floral bud development, and responses to various abiotic
stresses implied that DgNAC may participate in the development and stress tolerance of orchardgrass. These results are useful for revealing the adaptability of orchardgrass under various environmental stresses. This comprehensive analysis of the NAC gene in orchardgrass is a valuable resource for further studying the functional characteristics of DgNAC genes and cultivating high-quality orchardgrass varieties.

Methods
Identification of NAC genes in orchardgrass
The orchardgrass genome resources were downloaded from the orchardgrass genomics database (http://orchardgrassgenome.sicau.edu.cn/) [48]. For the identification of NAC proteins, the hidden Markov model (HMM) file of the NAM domain (PF02365) was downloaded from the Pfam database (http://pfam.sanger.ac.uk/) as the query [75]. HMMER 3.0 was used to scan the annotated protein with the NAM HMM file. The proteins acquired through the NAM HMM were aligned to construct Neighbor-Joining (NJ) trees with the following parameters: Blosum62 cost matrix, Jukes-Cantor global alignment and bootstrap value of 1000.

Phylogenetic analysis and classification of the DgNAC gene family
The NAC protein sequences of Arabidopsis were downloaded from the Arabidopsis genome TAIR 11 (https://www.arabidopsis.org/) [77]. All the identified DgNAC genes were assigned into different groups based on the classification of AtNACs [13]. Geneious 2020 was used to construct neighbor-joining (NJ) trees with the following parameters: Blosum62 cost matrix, Jukes-Cantor model, global alignment and bootstrap value of 1000.

Gene structure and motif analysis
The exon-intron display was constructed according to the Gene Structure Display Server (GSDS, http://gsds.gao-lab.org/) program [78] according to the available CDS and genomic information of the DgNACs. The Multiple Expectation Maximization for Motif Elicitation (MEME, http://meme-suite.org/tools/meme) program [79] was used to identify the conserved motifs in DgNAC protein sequences with parameters that maximize 10 motifs and range of motif width 6 to 200.

Chromosomal mapping and gene duplication analysis
The chromosomal positions of the DgNAC genes were acquired from the orchardgrass genome annotations. The chromosomal map of DgNAC genes was drafted by MapGene2Chrome (MG2C, http://mg2c.iask.in/mg2c_v2.0/). DgNAC gene duplication was examined by using MCScanX software with default parameters. The Dual Synteny Plotter of TBtools (https://github.com/CJ-Chen/TBtools) [80] was used to analyze the homology of the NAC gene between orchardgrass and the other plants (including Arabidopsis thaliana, Oryza sativa, Brachypodium distachyon, Hordeum vulgare, Sorghum bicolor and Setaria viridis).

Plant material, growth condition and stress treatments
The Dactylis glomerata cv. DONATA (Registered No. 398) seeds were provided by DLF (Beijing, China). The seeds were sown in pots (18.5 cm length, 13.5 cm width, and 5 cm deep) filled with sterilized quartz and ddH2O in growth chambers. The parameters of the growth chamber were set as a 22 °C 14 h photoperiod and a 20 °C 10 h dark period. After 1 week of germination, seedlings were irrigated with Hoagland’s solution for another 60 days. Then, the seedlings were separately subjected to various stress treatments, including drought, ABA, salt, and heat. For salt, ABA, and drought treatments, the plants were exposed to 250 mmol NaCl, 100 μmol ABA, and 20% PEG 6000 (W/V) Hoagland’s solution, respectively. For heat treatment, the plants were exposed to high temperature at 40 °C/35 °C (day/night). Several DgNAC genes were selected to analyze the expression profile under various stresses by qRT-PCR analysis. The samples were collected at 0, 3, 6, 12 and 24 h after treatments. All materials harvested from each treatment were immediately frozen in liquid nitrogen and stored at −80 °C before RNA isolation. All experiments were conducted three times with three biological replicates for qRT-PCR analysis.

RNA isolation, cDNA synthesis, and qRT-PCR
The Hipure HP plant RNA mini kit (Magen, R4165–02) was used to extract total RNA. DNA-free RNA was used for the synthesis of cDNA by using Rever’Tra Ace qPCR RT Master Mix (TOYOBO, FSQ-301) according to the manufacturer’s recommendations. qRT-PCR was performed with a Bio-Rad CFX96 instrument using SYBR® green real-time PCR master Mix (TOYOBO, QPK-201). Primers used for qPCR were designed with primer 6.0, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was selected as the reference gene (Additional file 4) [81]. The detailed methods of reaction and relative
quantitative calculations have been described in a previous study [39]. The transcriptome data of various orchardgrass tissues were obtained from the orchardgrass genome database (Additional file 5) [48], and the transcriptome data of vernalization and floral bud development of orchardgrass were obtained from Feng et al. (Additional file 6) [39]. The RNA-seq data of orchardgrass-specific NAC genes (Additional file 7) under submergence, drought and heat stress were obtained from Zeng et al. [82], Ji et al. [83], and Huang et al. [84], respectively.

Abbreviations
ABA: Abscisic acid; CDS: Coding sequence; CUC: Cup-shaped cotyledon; HMIM: Hidden Markov model; MW: Molecular weight; NAM: No apical meristem; TFS: Transcription factors; PEG: Polyethylene glycol

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s12864-021-07485-6.

Additional file 1 List of the 108 DgNAC genes identified in orchardgrass.
Additional file 2 Analysis and distribution of conserved motifs in orchardgrass NAC proteins.
Additional file 3 One-to-one orthologous relationships between orchardgrass and other plants.
Additional file 4 Sequences of the primers used in this study.
Additional file 5 Expression (FPKM) of DgNAC genes in different tissues.
Additional file 6 Expression (FPKM) of DgNAC genes in different floral bud development stages.
Additional file 7 RNA-seq data of the orchardgrass-specific NAC genes that were used in this study.

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Authors’ contributions
ZY, LH and XZ conceived and designed the experiments. ZY and JH performed the experiments. ZY analyzed the data. ZY and GN contributed equally. All authors read and approved the final manuscript.

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Availability of data and materials
All data generated or analyzed during this study are included in this article and its additional files. The orchardgrass genome resources were downloaded from http://orchardgrassgenome.sicau.edu.cn/ [48]; the genome data used for comparative syntenic analysis were obtained from open database, the genome data of Arabidopsis thaliana was downloaded from TAIR (https://www.arabidopsis.org/download/index-auto.jsp?dir=428fdownload_files%2FSequences%2FArabidopsis%2FArabidopsis%2Fns谢set%3A1|blastset%3A77); the genome data of Oryza sativa was downloaded from Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/pub/data/Eukaryotic_Projects/o_sativa/annotation_dbs/pseudomolecules/version_7.0/all/dlriv/ [85], the genome data of Hordeum vulgare (variety Barke) was downloaded from https://webblast.ipk-gatersleben.de/downloads/barley_pangenome/Barke/ [86], the genome data of Brachypodium distachyon (v3.1, https://genome.jgi.doe.gov/portal/pages/dynamicOrganismDownload.js?organism=Bdistachyon), Sorghum bicolor (v3.1.1, https://genome.jgi.doe.gov/portal/pages/dynamicOrganismDownload.js?organism=Sbicolor) and Setaria viridis (v2.1, https://genome.jgi.doe.gov/portal/pages/dynamicOrganismDownload.js?organism=Sviridis) were downloaded; the transcriptome data of DgNACs in different tissues also be obtained on the orchardgrass genome website (http://orchardgrassgenome.sicau.edu.cn/ [48], the raw RNA-seq reads of vernalization and floral bud development [39], submergence [82], drought [83] and heat [84] stress of orchardgrass were obtained from NCBI database (accession SRR5341102, PRJNA565626 and PRJNA554779, SRP158919, SRP049351, respectively).

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

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

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