Expression profiling of the Dof gene family under abiotic stresses in spinach

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DNA-binding with one finger (Dof) are plant-specific transcription factors involved in numerous pathways of plant development, such as abiotic stresses responses. Although genome-wide analysis of Dof genes has been performed in many species, these genes in spinach have not been analyzed yet. We performed a genome-wide analysis and characterization of Dof gene family in spinach (Spinacia oleracea L.). Twenty-two Dof genes were identified and classified into four groups with nine subgroups, which was further corroborated by gene structure and motif analyses. Ka/Ks analysis revealed that SoDofs were subjected to purifying selection. Using cis-acting elements analysis, SoDofs were involved in plant growth and development, plant hormones, and stress responses. Expression profiling demonstrated that SoDofs expressed in leaf and inflorescence, and responded to cold, heat, and drought stresses. SoDof22 expressed the highest level in male flowers and under cold stress. These results provided a genome-wide analysis of SoDof genes, their gender- and tissue-specific expression, and response to abiotic stresses. The knowledge and resources gained from these analyses will benefit spinach improvement.

Spinach (Spinacia oleracea L.) is an annual or biennial diploid species, belong to the Amaranthaceae family in the order Caryophyllales1. Its annual worldwide gross production in 2016 was about 26 million tonnes (FAOSTAT; http://faostat3.fao.org). Spinach is a dietary source of Ca, Ca, Fe, K, Mg, Mn, P, Zn, folate, vitamins, and dietary fiber4, providing its great potential for medical economy. However, like many other crops, its development and production is hampered by biotic stresses (diseases, pests and weed infestations,) and abiotic stresses (salinity, drought, and heat). Climate change causes elevated temperature and a network of events triggering the response of plants and animals. Although it seems that organisms on earth gradually developed local thermal adaptation to impact their healthy condition8. Spinach is cold tolerant but having heat-sensitive characteristics that influencing its growth and significantly decrease yield and quality under high temperature9. Winter sweet treatment (WST), termed the cold enrichment technique, has been established for cultivating high-quality leafy spinach during winter10. At that time (early December), the average daily temperature is generally below 5 °C. But staying at a low temperature for a long time would also damage spinach by reactive oxygen species (ROS)11. Although drought stress has no direct effects on the leaf nutrition quality, some physiological indicators could be decreased, such as leaf area, fresh and dry weight, leaf relative water content, and specific leaf area, which might change the shape of plant12.

Dof domain proteins are plant-specific transcription factors that contain a highly conserved 52 amino acid DNA-binding domain at the N-terminal including a single Cys2/Cys2 zinc finger structure13. It was projected that Cys2/Cys2 zinc finger specifically binds to a conserved sequence with 5′-(T/A)AAAG-3′ in gene promoters14. At the C-terminal of the Dof proteins, there is a transcription regulation domain with diverse functions involving interaction with a variety of regulatory proteins and activating the gene expression15. Indeed, previous studies corroborated its functional role in plant growth and development, such as in flowering control16,17, maturation18, seed development19, and germination20,21. Specifically, mutant dag1 (encoding a Dof transcription factor in Arabidopsis) seeds are induced to germinate by much lower red light fluence rates22; the COG1 gene (encoding a Dof protein in Arabidopsis) functions as a negative regulator in phytochrome signaling pathways23; CDFs (CYCLING DOF FACTORS, Dof-type transcriptional repressors) that directly suppresses the expression of CONSTANS (CO), which could prevent the expression of photoperiodic gene, the perception of day-length and...
the floral transition in Arabidopsis\textsuperscript{23}. Moreover, Dof transcription factors also participated in phytohormone and stress responses, such as the TDDF1 (encoding a Dof protein in tomato) which could improve drought, salt, various hormones stress as well as resistance to late blight\textsuperscript{26}; ThZFP1 and ThDof1.4 improve salt and osmotic stress tolerance by increase the proline level and ROS scavenging capability\textsuperscript{26}. Therefore, Dof gene family plays an essential role in the life cycle of plants.

In recent years, with the sequencing of genome, the identification of Dof genes was widely researched in various plant species, such as Arabidopsis, rice\textsuperscript{27}, soybean\textsuperscript{28}, sorghum\textsuperscript{29}, sugarcane\textsuperscript{30}, and so on. The spinach draft genome was reported in 2017\textsuperscript{1}, however, few gene families were analyzed for the genome. The functions of members of Dof genes remain unknown in spinach. As previously reported, plants different sex types show different responses to abiotic stress\textsuperscript{32}. The reproductive potential of male, female, and monoecious spinach differ under water-limited condition\textsuperscript{33}. But the expression of Dof genes in different sex types of spinach under abiotic stresses is still unknown. In this study, we identified 22 Dof genes, showed the structure and motifs, and classified the group of Dof genes in spinach. In addition, duplication events and cis-element on their promoters were predicted. Functional prediction was performed based on gene expression analysis in different tissues and in responses to different abiotic stresses. The results will provide a foundation for gene cloning and functional characterization of Dofs in spinach.

Materials and methods
Identification of SoDof gene family members in the spinach genome. To identify the Dof gene family members in Spinacia oleracea L., all proteins from the spinach genome were scanned by HMMER-3.2\textsuperscript{24} using the Hidden Markov Model (HMM) corresponding to the Dof domain (PF02701). The spinach genome data was downloaded from SpinachBase (http://www.spinachbase.org/?q=download). The predicted proteins were confirmed for the presence of the conserved Dof domain by NCBI Conserved Domain Database (CDD)\textsuperscript{35}, Pfam\textsuperscript{36} and SMART\textsuperscript{37} tools. Similarly, Arabidopsis and sugarbeet (Beta vulgaris L.) Dof genes were identified by scanning Arabidopsis database (ftp://ftp.ensemblgenomes.org/pub/plants/release-42/fasta/arabidopsis_thaliana/) and sugarbeet database (ftp://ftp.ensemblgenomes.org/pub/plants/release-42/fasta/beta_vulgaris/) using HMM and CDD. We performed the ExPASy server\textsuperscript{38} to detect the theoretical pl and molecular weight of candidate SoDof genes.

Multiple sequences alignment and phylogenetic characterization. For phylogenetic analysis of the Dof gene family, multiple sequence alignments were conducted on the amino acid sequences of Dof protein from spinach, Arabidopsis, and sugarbeet with MUSCLE with default settings. After that, MEGA-X-10.0.4 software was used to construct phylogenetic tree among these three species with the Neighbour-Joining (NJ) method and 1000 bootstraps. Alignment of multiple SoDofs was performed by DNAMAN-6.0.

Chromosomal locations and duplication time. The distribution information for each SoDof gene on chromosome was obtained from their annotation file. MG2C (http://mg2c.iask.in/mg2c_v2.1/) was used to map the chromosomal locations for each SoDof gene with default settings. To estimate the synonymous and non-synonymous substitution, Ka and Ks values were calculated. ClustalW was used to align the nucleotide sequence of SoDof genes. Ka and Ks values were used to estimate by DnaSp-5.10. The time (million years ago, Mya) of segmental duplication events for each SoDof gene was estimated using a formula, T = Ks/2Ka which assumed A of 7.0e\textsuperscript{-9} synonymous/substitution site/year for spinach\textsuperscript{1}.

Gene structure analysis and conserved motif identification. The exon–intron organizations of the genes with phylogenetic tree and Dof motifs were determined using the Gene Structure Display Server (http://gsds.cbi.pku.edu.cn/). The motifs distribution of the Dof protein in spinach, Arabidopsis, and sugarbeet were statistically identified by the MEME program (http://meme-suite.org/) with the motif length set to 6–100 and the maximum number of motifs was set to 15. Then TBtools-1.082\textsuperscript{29} was employed to create the motif structure with phylogenetic tree.

Cis-elements identification in promoter regions of SoDofs. To investigate cis-elements in promoter sequences of Dof coding genes in spinach, the upstream sequences (2000 bp) of each SoDof gene were extracted from spinach genome according to the GFF3 (general feature format) file. Then the retrieved sequences were submitted to a search by the PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/)\textsuperscript{40} for predicting the cis-elements which might be involved in regulation of SoDof genes expression.

Sample collection and preparation. Spinach II9A0073 seeds were obtained from CAAS (China Academy of Agricultural Sciences). Seeds were sown in plots, and seedlings grew in an artificial climate chamber with a photoperiod of 16 h light/8 h dark, temperature at 24 °C and humidity at about 60%. After three weeks, spinach seedlings with consistent growth were selected and prepared for environmental stress treatment. Abiotic stresses were performed by adding 20% (mass fraction) PEG 4000 to simulate the drought condition and adjusting the temperature of the artificial climate box to simulate high-temperature stress (40 °C) and low-temperature stress (4 °C). Under stress conditions, the spinach leaves were sampled at 0, 2, 4, 7, 12, 24 h after treatment. The plants with non-treatment were collected for their roots, leaves, and stems in vegetative growth stage, as well as their male flowers and female flowers. All samples were immediately frozen in liquid nitrogen and stored at −80 °C.
RNA extraction and quantitative real-time PCR analysis. Total RNA from different samples was extracted using the Trizol reagent. The quality and concentration of RNA were tested on 1.0% agar gel electrophoresis and the NanoDrop 2000 (Thermo Fisher Scientific, USA). The total RNA was reverse transcribed into cDNA with its 200 ng per microliter final work concentration using Evo M-MLV RT Kit with gDNA Clean for qPCR (Accurate Biotechnology, China) following the manufacturer's instruction. For qRT-PCR, Actin11 gene was used as a reference gene. The specific primers were designed by IDT (https://sg.idtdna.com/pages) and the sequences of all primers are listed in Supplementary Table S3. The qRT-PCR was conducted with SYBR Green qPCR (Accurate Biotechnology, China) according to the manufacturer’s instruction. For qRT-PCR, the relative gene expression level was calculated by the 2 − ΔΔCT method. Graphpad Prism8 (Graphpad Software Inc., La Jolla, CA) was performed to calculate the p-value. Expression values were calculated as the arithmetic mean and then presented as the heatmap by R package.

Result

Identification and classification of SoDofs genes. To identify the Dof gene family members in spinach, all proteins from the spinach genome were scanned by using HMMER-3.2 and 22 genes were predicted as Dof gene family members in spinach. These Dof candidate genes in spinach were named as SoDof1–SoDof22 (Table 1). The predicted proteins were further confirmed to contain the conserved Dof domain. Similarly, 36 Dof genes had been identified in Arabidopsis and 22 gene family members in spinach. These candidate genes in spinach were named as SoDof1–SoDof22 (Table S1). The full length of the coding sequence (CDS) ranged from 492 (SoDof12) bp to 1485 (SoDof13) bp with an average length of 1060 bp. The quantity of aa (amino acids) for SoDof varied from 4.6 (SoDof20) to 8.92 (SoDof9) (Table 1). Multiple sequence alignment showed a Dof conserved motif of 52 amino acids located in 22 SoDof genes, with a single Cys2/Cys2 zinc-finger structure at the N-terminal (Fig. 1A). Phylogenetic tree was constructed between

### Table 1. Spinach Dof genes and their related information.

| Gene name  | Gene ID   | Chromosome | Location                  | Gene DNA (bp) | CDS (bp) | Protein length (aa) | Molecular weight | Theoretical pI | Dof domain | Intron | Subgroup |
|------------|-----------|------------|---------------------------|---------------|----------|---------------------|------------------|----------------|-----------|--------|----------|
| SoDof1     | Spo01218  | chr2       | 58115820..58118612 forward | 2793          | 1104     | 367                 | 40,642.53        | 8.52           | 57–114    | 1       | C2.1     |
| SoDof2     | Spo26525  | chr4       | 115910084..115910743 reverse | 660           | 660      | 219                 | 23,339.72        | 8.47           | 23–79     | 0       | A        |
| SoDof3     | Spo14528  | chr3       | 51468026..51469123 forward | 1098          | 1098     | 365                 | 39,514.46        | 7.32           | 41–96     | 0       | B2       |
| SoDof4     | Spo15329  | chr5       | 13015823..13016842 forward | 1020          | 1020     | 339                 | 37,310.74        | 5.59           | 52–108    | 0       | A        |
| SoDof5     | Spo26037  | chr6       | 40210301..40212950 forward | 2630          | 1197     | 398                 | 44,408.07        | 6.25           | 38–115    | 1       | C2.1     |
| SoDof6     | Spo19252  | chr5       | 33891..39454 reverse      | 2055          | 1287     | 428                 | 46,606.00        | 8.80           | 90–146    | 1       | B2       |
| SoDof7     | Spo26525  | chr6       | 115910084..115910743 reverse | 1381          | 1110     | 369                 | 39,234.09        | 6.93           | 47–104    | 1       | C1       |
| SoDof8     | Spo14528  | chr3       | 51468026..51469123 forward | 762           | 762      | 253                 | 25,482.15        | 8.12           | 28–83     | 0       | D2       |
| SoDof9     | Spo13386  | chr5       | 110099..110860 reverse    | 1164          | 1165     | 387                 | 41,004.88        | 8.92           | 79–135    | 0       | B2       |
| SoDof10    | Spo20892  | Super_scaf- | 124549..1248131 reverse   | 2638          | 1326     | 441                 | 46,968.23        | 8.21           | 95–150    | 1       | B1       |
| SoDof11    | Spo28108  | chr5       | 10912822..10916291 forward | 3410          | 1344     | 447                 | 49,445.56        | 5.39           | 108–164   | 1       | D1       |
| SoDof12    | Spo04353  | chr5       | 92311..92802 forward      | 492           | 492      | 163                 | 18,468.93        | 8.87           | 44–99     | 0       | D1       |
| SoDof13    | Spo05430  | chr5       | 340472..34569 forward     | 4988          | 1485     | 494                 | 54,499.48        | 5.63           | 154–210   | 1       | D1       |
| SoDof14    | Spo16539  | chr5       | 13249..16754 forward      | 3506          | 1059     | 352                 | 38,506.78        | 6.46           | 99–155    | 1       | D1       |
| SoDof15    | Spo26832  | chr6       | 26503975..26505054 reverse | 1080          | 1080     | 359                 | 40,449.07        | 6.23           | 28–82     | 0       | C2.2     |
| SoDof16    | Spo22365  | chr1       | 19149992..19153942 reverse | 1951          | 1098     | 365                 | 39,747.75        | 8.50           | 84–138    | 1       | B1       |
| SoDof17    | Spo22229  | chr1       | 149590..151164 forward    | 1575          | 1101     | 366                 | 40,015.00        | 8.51           | 87–141    | 1       | B1       |
| SoDof18    | Spo07164  | chr5       | 1203..2777 forward        | 1575          | 1101     | 366                 | 40,027.05        | 8.51           | 87–141    | 1       | B1       |
| SoDof19    | Spo25703  | Super_scaf- | 553984..554928 reverse    | 945           | 945      | 314                 | 35,306.63        | 8.53           | 58–111    | 0       | B2       |
| SoDof20    | Spo00332  | chr4       | 8389644..83900468 reverse | 825           | 825      | 274                 | 30,538.30        | 4.60           | 34–88     | 0       | C2.2     |
| SoDof21    | Spo10686  | chr1       | 4163045..41632583 forward | 2169          | 1305     | 434                 | 47,592.39        | 5.74           | 149–205   | 1       | D1       |
| SoDof22    |Spo16511   | Spo0982    | 142499..143254 forward    | 756           | 756      | 251                 | 27,368.16        | 7.60           | 44–98     | 0       | C3       |
22 SoDof genes, 22 BvDof genes, and 36 Dofs in Arabidopsis (Fig. 2). A total of 22 SoDof TFs from spinach were classified into four main groups (Groups A–D), which could be divided into multiple subgroups, A, B1, B2, C1, C2.1, C2.2, C3, D1, and D2. The number of SoDofs in Group B, C, and D was similar with a total number of 20. Specifically, Group B (contained the most number among all groups) could be divided into subgroup B1 and subgroup B2 with SoDof10, SoDof16, SoDof17, SoDof18 in subgroup B1 and SoDof3, SoDof6, SoDof9, SoDof19 in subgroup B2 (Fig. 2). Subgroup D1 had the largest number of SoDofs (SoDof11, SoDof12, SoDof13, SoDof14, SoDof21) in subgroups. SoDof2 and SoDof4 belonged to Group A (Fig. 2). Over half SoDofs were alkaline which contained all members in Group B, and subgroup D1 (Table 1).

Mapping SoDof genes in spinach chromosomes and Ka/Ks analysis. The spinach genome consists of only 6 chromosomes. The 22 putative SoDof genes were found to be distributed in 6 chromosomes, and unplaced contigs (Fig. 3). Only 50% SoDof genes were anchored in chromosomes. The largest number of SoDof members was located in chromosome 5, which contains SoDof7, 11, and 4. Compared with the gap of SoDof in other chromosomes, these three genes were closer to each other, especially SoDof11 and SoDof4. There were 2 SoDof genes in chromosomes 1, 4, and 6, respectively. SoDof1 and SoDof3 were located in chromosomes 2 and 3, respectively. Ka and Ks value calculation aims to identify duplication events for each SoDof gene. The duplication of SoDof genes originated from about 5.66 Mya (Ks = 0.793) to 41.27 Mya (Ks = 5.778) with an average of
Figure 2. Phylogenetic tree of Dof proteins among spinach, Arabidopsis and sugarbeet. Figure was made by MEGA-X-10.0.4.

Figure 3. Chromosomal location of SoDof genes. The size of a chromosome is indicated by its relative length. Figure was made by MG2C (http://mg2c.iask.in/mg2c_v2.1/).
16.12 Mya (Supplementary Table S2). All values of Ka/Ks were lower than 1 and some SoDofs were even lower than 0.1 (Table 2).

**Gene structure and motif analysis of SoDof genes.** Candidate SoDof genes were analyzed using Gene Structure Display Server to investigate the characterization of exon–intron structure. There was no more than two introns in each SoDof (Fig. 4). To further reveal the diversification of SoDof genes, we performed the MEME program to detect motif patterns, and 15 distinct motifs were identified (Fig. 5). It was predicted that motif1 could be considered as the Dof region (Fig. 1B). The schematic distribution of the 15 motifs showed that motif1 (Fig. 1B) and motif2 (Fig. 1C) were highly conserved in all SoDof proteins. Notably, SoDofs shared similar conserved motif compositions in some subgroups. Motif 7 in front of the Dof region were highly conserved in subgroup B1. And members of subgroup C2.2 contained motif13. Interestingly, motif5 was prominently conserved in subgroup D1 (contained the most SoDof members among all subgroups). Specifically, motif5 presented at the N-terminal in all subgroup D1 members, and motif4 appeared at the C-terminal in majority of subgroup D1 members.

**Cis-regulatory element analysis.** PlantCARE was used to analyze the cis-regulatory element for each SoDof gene by retrieving the 2 kb upstream sequence of each candidate, except for SoDof18 because of lack of 2 kb upstream sequence on its scaffold location (Supplementary Data). Dof gene family in spinach had TATA-box and CAAT-box. SoDof genes may also be controlled by many phytohormones, such as methyl jasmonate (MeJA), gibberellins (GA), ethylene, auxin, and salicylic acid (SA). We also detected many other important cis-elements on Dof gene family that involve in plant growth and development. For example, there were a large number of elements associated with physiological processes, such as light responsiveness, circadian control, endosperm expression, meristem and flower meristem expression, root-specific and seed-specific regulation.

Table 2. The Ka/Ks value of SoDof genes (lower than 0.1). The details Ka/Ks information are shown in Supplementary Table S2.

| Seq1  | Seq2  | Ks    | Ka    | Time (mya) | Ka/Ks  |
|-------|-------|-------|-------|------------|--------|
| SpdDof2 | SpdDof3 | 5.2612 | 0.3741 | 37.58       | 0.071105451 |
| SpdDof4 | SpdDof7 | 5.2531 | 0.5222 | 37.52214286 | 0.099407969 |
| SpdDof5 | SpdDof15 | 4.1515 | 0.3321 | 29.65357143 | 0.079995182 |
| SpdDof12 | SpdDof21 | 3.7472 | 0.2989 | 26.76571429 | 0.079766225 |
| SpdDof20 | SpdDof22 | 5.7779 | 0.4813 | 11.68785714 | 0.083300161 |

**Figure 4.** The exon–intron structure of Dof genes in Spinach: CDS (yellow), UTR (blue), Intron (black line) and zf-Dof region (pink). SoDof6 contains one intron which is too short to recognize in this figure resolution. Figure was made by the Gene Structure Display Server (http://gsds.cbi.pku.edu.cn/).
The sum of cis-elements of subgroup D1 was greatest in plant growth and development. The sum of cis-elements of subgroup D1 was also greatest in phytohormones class. The greatest mean of cis-elements in phytohormones class was subgroup C3. The greatest mean of cis-elements in light responsiveness and physiological process were in subgroup C2.2 and C1 respectively (Table 3). In physiological process, some elements, participated in some small molecule pathway, were also found, such as zein metabolism regulation and flavonoid biosynthetic genes regulation (Supplementary Data). Moreover, nine cis-elements (WUN-motif, STRE, TC-rich repeats e.g.) were also predicted, which were related to defense and stress responsiveness. The sum and mean of cis-elements of subgroup A were greatest in stress response.

Table 3. The sum and mean of cis-elements for each subgroup.

| Subgroup | Growth and development | Phytohormone pathways | Light responsiveness | Physiological pathways | Stress response |
|----------|------------------------|-----------------------|----------------------|------------------------|-----------------|
|          | Sum | Mean | Sum | Mean | Sum | Mean | Sum | Mean | Sum | Mean |
| A        | 33  | 16.5 | 19  | 9.5  | 33  | 16.5 | 49  | 24.5 |
| B1       | 56  | 18.67| 6   | 2    | 38  | 12.67| 34  | 11.33|
| B2       | 86  | 21.5 | 19  | 4.75 | 65  | 15.75| 49  | 12.25|
| C1       | 19  | 19   | 13  | 13   | 10  | 10   | 7   | 7    |
| C2.1     | 43  | 21.5 | 16  | 8    | 35  | 17.5 | 14  | 14   |
| C2.2     | 52  | 26   | 9   | 4.5  | 32  | 16   | 13  | 13   |
| C3       | 14  | 14   | 4   | 4    | 30  | 30   | 5   | 5    |
| D1       | 99  | 19.8 | 33  | 6.6  | 96  | 19.2 | 43  | 8.6  |
| D2       | 17  | 17   | 6   | 6    | 23  | 23   | 15  | 15   |
Tissue-specific expression analysis of SoDof genes. We isolated RNA samples from roots, stems, leaves, male flowers, and female flowers, and detected expression of all SoDof genes in spinach using qRT-PCR. Expression profile of the SoDof genes revealed that nine SoDofs exhibited their highest transcript level in reproductive organs and eight SoDofs in leaves (Fig. 6A). Only two SoDofs (SoDof1 and SoDof5) were expressed in roots and stems, respectively. Notably, SoDof10 and SoDof15 had extremely high expression in leaves; SoDof22 showed high expression in male flowers (Fig. 6B). Comparing with leaves or inflorescences, the transcript level of these three genes in other tissues was negligible, indicating that their expression was tissue-specific. There were three homologous genes (SoDof16, SoDof17, and SoDof18) with same mRNA sequence, and their expression pattern was not analyzed.

Expression patterns of SoDof genes under abiotic stresses. To investigate the stress responsiveness and expression pattern of SoDof gene between different sex-types, we treated female male plants, and plants at vegetative stage under three types of abiotic stress (low-temperature 4 °C, high-temperature 40 °C, and drought 20%PEG4000). Spinach leaves were collected at 0 h, 2 h, 4 h, 7 h, 12 h, and 24 h after treatment and detected by qRT-PCR.

The majority of SoDof genes in female plants were up regulated under low temperature (Fig. 7A). The greatest increase in expression occurred in SoDof22 (up to the top at 24 h after treatment) in female plants (Supplementary Fig. S2A). SoDof14 experienced the same trend, but the expression level was much lower than that in SoDof22. Compared with other SoDofs, the SoDof22 expressed the most in plants at vegetative stage, and its extreme expression reached the top at 7 h and then went down (Supplementary Fig. S2B). However, in male plants, the expression pattern of SoDof3 and SoDof5 was similar. The expression of SoDof3 reached the highest level at 4 h and the expression of SoDof5 reached the highest level at 7 h (Supplementary Fig. S2C). In vegetative plants, 95% SoDof genes (more than those in male or female plants) were up-regulated and almost all of their highest expression
Figure 7. The expression pattern of SoDof genes under stresses. (A) The expression pattern of all SoDof genes under cold stress, heat stress and drought stress. The color scheme used to present expression level is sky-blue/red: light-yellow boxes indicate low variation in gene expression, sky-blue indicate a fold decrease, and red boxes indicate a fold increase in relation to mean value. The Y-axis indicates each SoDof gene and the X-axis indicated the time after treatment. The expression value were calculated as the arithmetic mean. (B) The expression level of down-regulated SoDofs. F-SoDof means the SoDof gene in female plants; V-SoDof means the SoDof gene in vegetative plants; M-SoDof means the SoDof gene in male plants. The Y-axis indicates relative expression level and the X-axis indicated the time after treatment: 0 h (gray); 2 h (light brown); 4 h (orange); 7 h (green); 12 h (purple); 24 h (pink). Asterisk indicates a significant difference from 0 h (p < 0.05). Error bars indicate standard error of independent technological replicates. Figure (A) and (B) were made by Graphpad Prism8.
appeared at 7 h (Fig. 7A). Among them, SoDof3, SoDof4, SoDof8 and SoDof9 were down-regulated at 2 h and 4 h. After that, they expressed the highest level at 7 h and then went down. The trends of six SoDofs (SoDof11, SoDof12, SoDof13, SoDof19, SoDof20, and SoDof21) were similar. Their expression went up slightly at 2 h and 4 h and reached the highest at 7 h, and then went down (Supplementary Fig. S2B). But there were difference between female and male plants. In male plants, there were the most number of SoDofs (SoDof6, SoDof8, and SoDof9) down-regulated, indicating that SoDof genes in males showed more negative response under 4 °C (Fig. 7B).

Under high temperature, most SoDofs were up-regulated and all SoDof genes were up-regulated in female plants. Compared with other SoDof genes, the expression of SoDof3 (up to the top at 24 h) was the highest in females, males, and vegetative plants (Supplementary Fig. S3). SoDof12, SoDof13, SoDof14, SoDof15, and SoDof22 also exhibited the highest expression at 24 h in female plants. The expression of some genes (SoDof1, SoDof2, SoDof5, SoDof6, SoDof11, SoDof19, and SoDof20) went up to the highest at 4 h which means they responded earlier than others did. In plants at vegetative stage, there was only one down-regulated SoDof gene (SoDof1) (Fig. 7B). Additionally, the expression of SoDof6, SoDof8, and SoDof9 were suppressed in male plants (Fig. 7B). 68% SoDofs showed the highest transcript level at 24 h in plant at vegetative stage, and 84% SoDofs showed the highest transcript level at 7 h or before 7 h in male plants (Supplementary Fig. S3).

To investigate the expression profile for each SoDofs under drought condition. All SoDof genes were up-regulated in female plants. Compared to other SoDof genes, the expression of SoDof15 was highest in females, males, and vegetative plants (Supplementary Fig. S4). But it was up to the top at 24 h in females, at 12 h in vegetative plants, and at 2 h in males. SoDof3 and SoDof7 were down-regulated in plants at vegetative stage (Fig. 7B). In male plants, six SoDof genes (SoDof1, SoDof3, SoDof5, SoDof9 SoDof14, and SoDof20) exhibited suppressed expression, and the expression of all SoDofs was lower than in female and vegetative plants (Supplementary Fig. S4).

Discussion
Identification and characteristics of SoDof genes. The Dof gene family is a plant-specific family of transcription factors. Since the discovery of the first Dof gene in maize41, its members in other species have been uncovered and its function in the growth and development has been characterized. We identified 22 SoDof genes in spinach genome and constructed a phylogenetic tree to divide them into four categories (A, B, C, and D) (Fig. 2). The quantity of SoDofs is lower than that of Arabidopsis (36)27, tomato (34)42, wheat (96)43, rice (30)27, potato (35)44, soybean (78)45, and sugarcane (29)31, but it is same to that of sugarbeet. This is because spinach separated with Arabidopsis just after the ancient whole-genome triplication and there was no whole-genome duplication in spinach genome1. The theoretical isoelectric points (pI) of Dof proteins ranged from 4.6 to 8.92.
Only two Dof proteins have an isoelectric point between 6.5 and 7.5, and over half Dof proteins were alkaline. All values of Ka/Ks were lower than 1 (Supplementary Table S2), indicating that SoDof genes were subjected to purifying selection45.

Structural conservation and chromosome location of SoDof genes. From our analysis of the spinach genome, only half of the Dof genes were assembled in chromosomes. Their distribution was relatively even, but three Dof genes clustered on one end of the chromosome 5 (Fig. 3). Although the spinach genome has no recent whole-genome duplication, partial gene duplications may lead to the formation of specific Dof genes clustered in specific parts of chromosomes. It is the main effect on gene family expansion46. The exon–intron divergence is supporting evidence to determine the evolutionary relationship of plants47. The intron–exon analysis showed that there were no more than two introns in each Dof gene (Fig. 4). The distribution of motifs is indicative of an evolutionary relationship48. The protein sequence analysis of the 80 Dof genes (22 SoDof, 22 BvDof, and 36 Dof in Arabidopsis) revealed that only Dof motifs of these 80 protein sequences are conserved (Fig. 5). The Dof proteins in the same subgroup contain relatively conserved motif structures. Motif 7 is in subgroup B1 and motif13 is in subgroup C2.2. Motif5 were prominently conserved in the subgroup D1. Specifically, motif5, motif3, and motif14 are only conserved in subgroup D1.

Cis-elements of SoDof genes. Cis-elements play significant roles during the life cycle of plants, such as phytohormone and stress response. In SoDof gene family, most cis-elements we identified were those related to light response, revealing that light signals may influence the regulation of SoDofs expression. Moreover, we identified cis-elements associated with the development of plant tissues in the promoter region of SoDofs, such as AP-148. Cis-elements associated with hormones and stress response were also identified in the promoter region of SoDofs. These results suggested that SoDof genes may participate in plant development and response to hormone and stress.

Potential Role of SoDof genes in different tissues. To figure out the potential roles of SoDofs, we analyzed the expression profiles of 19 SoDof genes in different spinach tissues. The other three genes, SoDof16, SoDof17, and SoDof18, were excluded from the analyses because they shared the mRNA sequences that are not distinguishable from each other. Among the 19 SoDofs expressed in spinach, 42% SoDofs showed a dominant expression in leaves and 47% in reproductive organs (Fig. 6A). In grapevine, eleven of twenty-five Dof gene expressed in inflorescence (similar to the number of SoDofs). Over half of Dof genes were expressed in vascular system in spinach, as in Arabidopsis49. Among them, there were six SoDof (SoDof4, SoDof11, SoDof19, SoDof20, SoDof21, and SoDof22) that expressed at a high level in flowers, indicating that they might be involved in the development of reproductive organs, especially for SoDof22 (Fig. 6B). SoDof22 is orthologous to AT4G21050, which is involved in regenerated shoot numbers51. Comparing with the number of cis-elements of SoDofs, SoDof22 contained the most cis-elements associated with plant hormone. One-third of them were ERE52, which are ethylene-responsive elements. This gene also contained the most auxin-responsive cis-elements, such as AuxRR-core53 and TGA-box54. These Dof genes might involve in the growth and development of spinach reproductive organs.

Potential role of SoDof genes in response to abiotic stress. In the expression profile for abiotic stress, the expression of SoDofs in male plants was lower than that in female plants and the plants at vegetative stage (Supplementary Figs. S2–S4). The trend of expression in each subgroup under each condition is different. SoDof22, SoDof3, and SoDof15 showed the highest level in expression after treatment under cold, heat, and drought stress, respectively (Fig. 7B). As previous studies have shown, Dof genes participate in responding to various stresses. In tomato, SCD1-5 genes were induced in response to osmotic, salt, heat, and low-temperature stresses. Over-expressing SICDF1 or SICDF3 in Arabidopsis showed an increasing drought and salt tolerance55. In brassica, the BnCDF1 gene was induced in response to low temperatures, and overexpressing BnCDF1 in Arabidopsis could increase freezing tolerance56. In watermelon, nine selected Dof genes showed differential expression under salt stress and ABA treatments57. In Chinese cabbage, most Dof genes were up-regulated quickly under salt, drought, heat and cold stresses58. Higher expression level of SoDof22, SoDof3, and SoDof15 were detected after abiotic stress treatment, indicating that these genes might have an important role in responding to heat, cold and drought stresses. Over-expressing BnCDF1 in Arabidopsis also delayed flowering time by reducing the expression of CO and FT59. SoDof22 showed high expression level both in inflorescence and under cold stress, suggesting that the role of SoDof22 might be similar to BnCDF1 within the interplay between environmental conditions and flowering time.

The promoter of BnCDF1 contains an LTR cis-element responding to low temperature and the promoter of SoDof15 contains an MBS cis-element that participated in drought inducibility59 (Supplementary Data). The response of its cis-element leads to an increased expression under low temperature or PEG4000. According to the expression profile of each stress, there was an expression difference between each sex type in spinach. Under cold stress, SoDof4 was down-regulated in female plants and SoDof7 was down-regulated in female and vegetative plants. While, in male plants, they showed expression increase at 2 h after treatment. Under heat stress, SoDof genes in female plants were all up-regulated, while, vegetative plants and male plants contained down-regulated SoDof genes. Under drought stress, the quantity of down-regulated SoDof genes in male plants was much more than that in others. Female plants are more sensitive to drought than male plants, similar to the response in Populus yunnanensis60.
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Author contributions
R.M. and H.Y. conceived the project and designed experiments. H.Y., Y.M. and Y.L. performed the qRT-PCR experiments. H.Y. and Y.M. draw the figures. H.Y. and J.Y. discussed the results. H.Y. wrote the manuscript and R.M. revised it.

Competing interests
The authors declare no competing interests.

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