DOI: 10.1007/s10535-015-0498-x BIOLOGIA PLANTARUM 59 (2): 253-265, 2015

Genome-wide identification and characterization of the DREB transcription factor gene family in mulberry

X.Q. LIU, J.J. ZHU, C.J. WEI, Q. GUO, C.K. BIAN, Z.H. XIANG, and A.C. ZHAO*

State Key Laboratory of Silkworm Genome Biology, Key Laboratory for Sericulture Functional Genomics and Biotechnology of Agricultural Ministry, Southwest University, Chongqing 400716, P.R. China

Abstract

The dehydration responsive element binding (DREB) transcription factor (TF) family comprises unique and important proteins involved in abiotic stress responses and tolerance in plants. Although DREB TFs have been well identified and characterized in a few model plants, there is no detailed information available for mulberry. In this study, 110 AP2/ERF family genes were identified based on a genome-wide analysis of the *Morus* genome database. Among them, 30 *Morus notabilis* DREB family genes (*MnDREB*s) were identified. A comparative analysis with *DREB* gene families from other plants suggests that *MnDREB*s could be divided into six subgroups (A-1 to A-6) and could have similar functions in response to abiotic stresses since they have similar conserved domains/motifs within each subgroup. The expression patterns of *MnDREB*s were analyzed using transcriptome data of different organs from *M. notabilis* and the quantitative real-time polymerase chain reaction. The expression of most *MnDREB*s was detected in different organs and induced by various abiotic stresses, which suggest their vital roles in abiotic stress tolerance.

Additional key words: abiotic stress, cold, expression profile, heat, *Morus notabilis*, salinity, water stress.

Introduction

Plants are exposed to different kinds of biotic and abiotic stresses (Boyer 1982) having serious adverse effects on plant growth and productivity. In response and adaption to these stresses, plants have evolved a variety of biochemical and physiological alterations (Hasegawa *et al.* 2000). An array of genes can be induced to produce the functional and regulatory proteins and to alter metabolic pathways of the stressed plant (Seki *et al.* 2001, Zhu *et al.* 2001, Huang *et al.* 2012). Of these proteins, transcription factors (TFs) are among the most significant. Furthermore, TFs are considered as one of the most appropriate candidate for genetic engineering (Agarwal and Jha 2010, Hussain *et al.* 2011).

Members of the AP2/ERF family are plant-specific TFs well known for their APETALA2 (AP2) domain. As a repeated motif involved in flower development, the AP2 domain was first identified in *Arabidopsis thaliana* (Jofuku *et al.* 1994). This domain consists of 60 - 70 amino acids and is involved in DNA binding indispensable for the AP2/ERF family to carry

Submitted 9 June 2014, *last revision* 27 October 2014, *accepted* 29 October 2014.

Abbreviations: ABRE - element involved in the abscisic acid responsiveness; AP2 - APETALA2; ARE - element for the anaerobic induction; CBF1 - CRT/DRE-binding factor 1; CDS - corresponding coding sequence; DRE - dehydration-responsive element; DRTF - database of rice transcription factors; DREB - dehydration responsive element binding; ERE - ethylene-responsive element; ERF - ethylene responsive element binding factors; GARE - gibberellin-responsive element; Glu - glutamine; GWD - genome-wide duplications; HSE - element involved in heat stress responsiveness; LTR - element involved in low-temperature responsiveness; MBS - MYB binding site; MBSI:MYB - binding site involved in flavonoid biosynthetic genes regulation; NCBI - National Center for Biotechnology Information; NJ - neighbor-joining; NLS - nuclear localization sequence; ORF - open reading frame; pI - isoelectric point; plant TFDB - plant transcription factor database; RAV - related to ABI3/VP1; RPL15 - ribosomal protein L15; RT-qPCR - real-time quantitative polymerase chain reaction; SMART - simple modular architecture research tool; TAIR - *Arabidopsis* information resource; TF - transcription factor; Val - valine, WUN - wound-responsive element.

Acknowledgements: This work was supported by grants from the Chongqing Science & Technology Commission (No. cstc2012jjA80039), the China Agriculture Research System (No. CARS-22-ZJ0102), and the National Hi-Tech Research and Development Program of China (No. 2013AA100605-3). The first two authors contributed equally to this paper.

* Corresponding author; fax: (+86) 23 68251128, e-mail: zhaoaichun@hotmail.com or zhaoaichun@swu.edu.cn

out its functions. Based on the number of AP2 domains and their gene function, this family is divided into five subfamilies: AP2, related to ABI3/VP1 (RAV), ethylene responsive element binding factors (ERF), dehydration responsive element binding (DREB), and others (Nakano *et al.* 2006). The proteins from the AP2 subfamily are characterized by two repeats of the conserved AP2 domain. Proteins from the RAV subfamily possess a single AP2 domain and a B3 domain, another conserved DNA-binding domain in plant TFs. Both the ERF and DREB subfamily proteins contain only a single AP2 domain, but have slight but important differences in specific amino acid sites. Alanine at position-14 and aspartic acid at position-19 are conserved in the ERF subfamily, whereas the conserved amino acids in the DREB subfamily are valine (Val) at position-14 and glutamine (Glu) at position-19 (Sharma *et al.* 2010).

The DREB TF family is one of the most promising regulons in genetic engineering to improve abiotic stress tolerance in plants (Zhao *et al.* 2012). The first identified DREB was CRT/DRE-binding factor 1 (CBF1) in *Arabidopsis* which is induced by a low temperature and a water deficit (Stockinger *et al.* 1997). Since then, a number of *DREB* homologous genes have been successfully identified and characterized from different plants (Agarwal *et al.* 2006), including *Arabidopsis* (Hwang *et al.* 2012), wheat (Shen *et al.* 2003, Egawa *et al.* 2006, Lucas *et al.* 2011), barley (Xue and Loveridge 2004), maize (Kizis and Pages 2002, Qin *et al.* 2007), pearl millet (Agarwal *et al*. 2007), potato (Bouaziz *et al.* 2012), rice (Dubouzet *et al.* 2003, Matsukura *et al.* 2010, Cui *et al.* 2011), and soybean (Chen *et al.* 2007, Marcolino-Gomes *et al.* 2013). Based on their structural characteristics, DREB proteins can be further divided into six subgroups: A-1 to A-6 (Sakuma *et al.* 2002). DREB members in different subgroups play diverse roles in plants. In rice, expressions of *OsDREB1A* and *OsDREB1B* are induced by cold, whereas *OsDREB2A* is induced by drought and salt stresses (Dubouzet *et al.* 2003). Transgenic *Arabidopsis* overexpressing *AtDREB2C* has a greater oxidative stress tolerance than non-transgenic plants (Hwang *et al.* 2012).

Up to now, most studies have focused on *DREB* genes of subgroups A-1 and A-2 of herbaceous plants, whereas little attention has been paid to the functions of *DREBs* of the A-3 to A-6 subgroups and to woody plants. Recently, increasing attention has been paid to genes of the A-3 to A-6 subgroups (Islam and Wang 2009, Dong and Liu 2010) and woody plants such as *Populus* and *Malus*, with their genome sequence completion in recent years (Tuskan *et al*. 2006, Velasco *et al*. 2010). In 2008, 200 *AP2/ERF* genes were identified from the *Populus* genome database (Zhuang *et al.* 2008); and 68 *DREB* genes in *Malus domestica* were also identified after a thorough genome-wide analysis (Zhao *et al.* 2012). To date, a few *DREBs* from woody plants have been characterized: *FaDREB1* (Li *et al.* 2011a) and *FaDREB2* (Li *et al.* 2011b) in mulberry, *JcDREB* in *Jatropha curcas* (Tang *et al.* 2011), *PeDREB2a* in *Populus euphratica* (Zhou *et al.* 2012), and *MdCBF1* in *Malus domestica* (Xue *et al.* 2014). Similarly, only a few *DREBs* from the A-3 to A-6 subgroups, such as *JcDREB* (of the A-6 subgroup) in *J. curcas*, have been identified and characterized. Therefore, a greater effort remains to be made to comprehensively understand the various functions of *DREBs* from woody plants and the A-3 to A-6 subgroups.

Mulberry trees are ecologically and economically important perennial woody plants (Ramachandra *et al.* 2004, Singhal *et al.* 2010) which can adapt to many different stresses, including cold, waterlogging, drought, and salinity (Checker and Khurana 2013), but there has been little research on their physiology, biochemistry, and molecular biology (Wei *et al.* 2014). There is also very little information on mulberry *DREBs* in public databases (*e.g*., *NCBI* and *EMBL*). After the *Morus notabilis* genome sequence became available (He *et al.* 2013), it is possible to analyze defense genes, such as *DREB*s, involved in abiotic responses in mulberry, which will promote the understanding of mulberry extensive strong adaptability.

In this study, we identified 110 proteins containing the AP2 domain and provided a list of DREB family members (30 *DREB* genes) from *M. notabilis*. These *MnDREB*s were also classified into six subgroups: A-1 to A-6. The expression patterns of *MnDREB*s from different subgroups were analyzed using the transcriptome data of different tissues from *M. notabilis* and the real-time quantitative polymerase chain reaction (RT-qPCR) technique. This research is basis for further character-rization of the physiological functions of mulberry DREBs.

Materials and methods

Plants and stress treatments: Winter buds, roots, male flowers, bark, and leaves of *Morus notabilis* C.K. Schneid. were collected from the Yingjing county of Ya'An city, Sichuan Province, China. All of the five organs were collected from only one big mulberry tree locating in 53.878E, 45.278N, and 1 460 m asl. The winter buds were collected on 27th September 2010 when a temperature was about 22 °C. The roots, male flowers, bark, and leaves were collected on 2nd May 2011 when a temperature was about 18 °C. Seeds from *Morus atropurpurea* Roxb. cv. Yuesang No. 69851 were planted in pots with about 300 g of a nutritive substrate (*Klasmann-Deilmann*, Germany) mixed with *Vermiculite* and *Perlite* (2:1:1). Then, they were cultured in a *PQX*

type plant incubator (*Ningbo Southeast Instrument Corporation*, Ningbo, China) under a 16-h photoperiod, an irradiance of 150 μmol m^{-2} s^{-1}, day/night temperatures of 26/22 °C, and a relative humidity of 75 %. After about two months, approximately 20 cm-tall mulberry seedlings were subjected to various abiotic stresses under the same irradiance and photoperiod: a high temperature (40 °C for 24 h), a low temperature (4 °C for 24 h), salinity (250 mM NaCl for 24 h), and dehydration [30 % (m/v) PEG 6000 for 24 h]. The mulberry seedlings were irrigated once with 100 cm^3 of NaCl or PEG solutions, whereas control (CK) seedlings were irrigated once with a clean water. Each treatment had three biological replicates. Leaves and roots of the CK and treated seedlings were harvested and stored at -80 °C before RNA extraction.

Cloning and sequence identification of *DREBs* from mulberry: All of the logged DREB amino acid sequences for several sequenced species were downloaded from the following websites: the National Center for Biotechnology Information (*NCBI*, http://www.ncbi.nlm.nih.gov/); the *Arabidopsis* information resource (*TAIR*, http://www.arabidopsis.org/); *EnsemblPlants* (http://plants.ensembl.org/index.html); the database of rice transcription factors (*DRTF*, http://drtf.cbi.pku.edu.cn/index.php); and the plant transcription factor database (*Plant TFDB*, http://planttfdb.cbi.pku.edu.cn/). *Arabidopsis* AP2 domain amino acid sequences were modelled to obtain putative DREB proteins from the *Morus* database (http://morus.swu.edu.cn/morusdb/). In addition, the simple modular architecture research tool (*SMART*, http://smart.embl-heidelberg.de/) was used to verify the presence of the AP2 domain in the resulting sequences. Cloning primers of the identified *MnDREB*s (Table 1 Suppl.) were designed by *Primer Premier 5.0* (Premier Biosoft International, CA, USA) to amplify the corresponding coding sequence (CDS) using the total cDNA of *M. notabilis* leaves as PCR templates. The amplified sequences were cloned into the PMD®19-T vector (*TaKaRa*, Dalian, China) and sequenced by *Invitrogen*™ from *Life Technologies* (Shanghai, China). The exon/intron organization for *MnDREB* genes were ascertained with the online tool gene structure display server (*GSDS*: http://gsds.cbi.pku.edu.cn) by identifying gaps through the alignment of full-length CDS with genomic sequences (Guo *et al.* 2007). In addition, the analysis of conserved *cis*-elements in about 1 500-bp upstream of the open reading frame (ORF) of *MnDREB*s was conducted using the plant *cis*-acting regulatory element (*PlantCARE*; http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

Conserved domain and phylogenetic analysis: The full-length and AP2 domain of the obtained *MnDREB*s were multi-aligned using the *ClustalX 2.0* program (Thompson *et al.* 1997). Some specific conserved sites were displayed with the help of the *GeneDoc* program. Afterwards, a combined un-rooted neighbor-joining (NJ) tree (Saitou and Nei 1987) along with *M. notabilis*, *A. thaliana* (Nakano *et al.* 2006), *Malus domestica* (Zhao *et al.* 2012), *Populus trichocarpa* (Chen *et al.* 2013), and *Vitis vinifera* (Zhao *et al.* 2014) was generated in *MEGA 5.0* (Tamura *et al.* 2011) with 1 000 resampling replicates.

Analysis of MnDREB expression profiles: To further investigate a *MnDREB* expression in different organs, we downloaded transcriptome data from the *Morus* database (http://morus.swu.edu.cn/morusdb/). RPKM (reads per kilobase of exon model per million mapped reads) was used as criterion to measure the expression of each *MnDREB*. These data were presented as heat maps in red/green coding with the *MeV 4.9.0* software (*Dana-Farber Cancer Institute*, USA).

To confirm an *MnDREB* expression in response to different abiotic stresses, the *MnDREB* expression profiles in roots and leaves under different treatments were analyzed by RT-qPCR with three replicates. RNA was isolated from leaves and roots stored at -80 °C using *RNAiso Plus* (*TaKaRa*) as the instructions described. To eliminate any genomic DNA contamination and synthesize first-strand cDNA, 1 μg of total RNA was extensively treated with the perfect real time version of a *PrimerScript*™ RT reagent kit with a gDNA eraser (*TaKaRa*). The amplification specificities of designed RT-qPCR primer pairs were confirmed by semiquantitative PCR using the cDNA from *M. atropurpurea* Roxb cv. Yuesang No. 69851 as template. The RT-qPCR analysis of *MnDREB*s under several stresses was performed with the corresponding cDNA as template primers (Table 2 Suppl.) using a *StepOne Plus* real-time PCR system (*Applied Biosystems*). The mulberry ribosomal protein L15 (*RPL15*) gene was used as control for expression normalization. Primers used in this step were designed by *Primer Premier 5.0* (*Premier Biosoft International*) and selected according to the principles of RT-qPCR. The reaction procedures were described in our previous study (Wei *et al.* 2014). Results were organized and analyzed with the *Excel* and *SPSS Statistics 17.0* (*SPSS Inc*., Chicago, USA) and *Origin 7.0* (*OriginLab*, USA) software.

Results

Based on the *Morus* database, 110 proteins with AP2 domain were obtained and the corresponding nucleic acid sequences were downloaded. The presence and number of AP2 domain(s) were confirmed using the *SMART*

online tool. Based on the number of AP2 domains, the obtained proteins were classified into five subfamilies (Table 3 Suppl.) (Nakano *et al.* 2006, Lata and Prasad 2011). Seventeen proteins containing two repeats of the conserved AP2 domain were classified into the AP2 subfamily. Three proteins with an AP2 domain and a B3 domain were assigned to the RAV subfamily. The remaining 90 proteins all contained a single AP2 domain: six were assigned to the 'others' subfamily since they did not have a WLG motif (Nakano *et al.* 2006); 84 were divided into the ERF and DREB subfamilies according to the amino acids in the specific sites mentioned above. Finally, 30 *MnDREB*s were identified from the *Morus* database (Table 1), and 27 were successfully cloned and sequenced with the primers (Table 1 Suppl.). Gene data of 30 *MnDREB*s were deposited in GenBank and given

Table 1. Description information about *M. notabilis DREB* genes. Gene IDs are from the *Morus* database.

Subgroup	Gene number	Gene ID	Gene name	Acc. No.	Exon number	Gene length [bp]	Protein length [aa]	Molecular mass [kDa]	pI
A-1	2	Morus013794	*MnDREB1A*	KF678375	1	762	239	26.63	5.02
		Morus001049	*MnDREB1B*	KF678397	1	720	253	27.46	6.84
A-2	6	Morus012269	*MnDREB2A*	KF678373	1	1101	366	40.42	4.78
		Morus008736	*MnDREB2B*	KF678400	2	1029	402	44.26	8.52
		Morus009488	*MnDREB2C*	KF678399	5	2232	743	82.44	6.87
		Morus024469	*MnDREB2D*	KF678390	1	600	199	22.06	5.44
		Morus019405	*MnDREB2E*	KF678401	11	2349	782	85.03	8.36
		Morus002388	*MnDREB2F*	KF678378	1	858	285	32.04	6.07
A-3	1	Morus009903	*MnDREB3A*	KF678395	1	1077	358	38.73	6.48
A-4	8	Morus023492	*MnDREB4A*	KF678389	1	627	208	22.57	4.78
		Morus025497	*MnDREB4B*	KF678393	1	633	210	23.26	5.79
		Morus024074	*MnDREB4C*	KF678394	1	816	271	30.00	5.31
		Morus010702	*MnDREB4D*	KF678391	1	750	249	26.60	5.97
		Morus010902	*MnDREB4E*	KF678382	1	804	267	29.14	5.11
		Morus026252	*MnDREB4F*	KF678384	1	804	267	28.51	5.39
		Morus001767	*MnDREB4G*	KF678374	1	786	261	27.70	4.93
		Morus013800	*MnDREB4H*	KF678376	1	639	212	22.48	4.91
A-5	8	Morus007221	*MnDREB5A*	KF678379	1	534	177	19.75	7.81
		Morus008873	*MnDREB5B*	KF678380	1	540	179	20.54	7.91
		Morus020177	*MnDREB5C*	KF678377	1	777	258	28.38	5.59
		Morus014282	*MnDREB5D*	KF678402	1	477	158	16.97	6.13
		Morus003928	*MnDREB5E*	KF678381	1	567	188	20.45	5.29
		Morus002531	*MnDREB5F*	KF678387	1	516	171	18.56	6.09
		Morus018629	*MnDREB5G*	KF678385	1	843	280	31.47	4.91
		Morus002530	*MnDREB5H*	KF678386	1	636	211	23.73	4.80
A-6	5	Morus003964	*MnDREB6A*	KF678392	1	1008	335	37.10	5.78
		Morus021553	*MnDREB6B*	KF678396	1	1065	354	38.72	7.18
		Morus003883	*MnDREB6C*	KF678383	1	963	320	35.17	8.93
		Morus011587	*MnDREB6D*	KF678388	1	1323	440	49.26	5.73
		Morus021618	*MnDREB6E*	KF678398	1	1173	390	43.62	7.78

Table 2. The comparison of *DREB* gene numbers in *M. notabilis* with other plants. Classification by [a] - Sakuma *et al.* (2002), [b] - Sharoni *et al.* (2011), [c,d] - Zhuang *et al.* (2008, 2009), [e] - Zhao *et al.* (2012), [f] - Mayer *et al.* (2000), [g] - Yu *et al.* (2002), [h] - Tuskan *et al.* (2006), [i] - Jaillon *et al.* (2007), [j] - Velasco *et al.* (2010), [k] - He *et al.* (2013). GWD - genome-wide duplications, Y - yes, N - no.

Subgroup	*A. thaliana* [a]	*O. sativa* [b]	*P. trichocarpa* [c]	*V. vinifera* [d]	*M. domestica* [e]	*M. notabilis*
A-1	6	10	6	7	3	2
A-2	8	4	18	4	16	6
A-3	1	1	2	0	2	1
A-4	16	15	26	13	17	8
A-5	16	13	14	7	19	8
A-6	10	9	11	5	11	5
Total	57	52	77	36	68	30
GWD	Y[f]	N[g]	Y[h]	Y[i]	Y[j]	N[k]

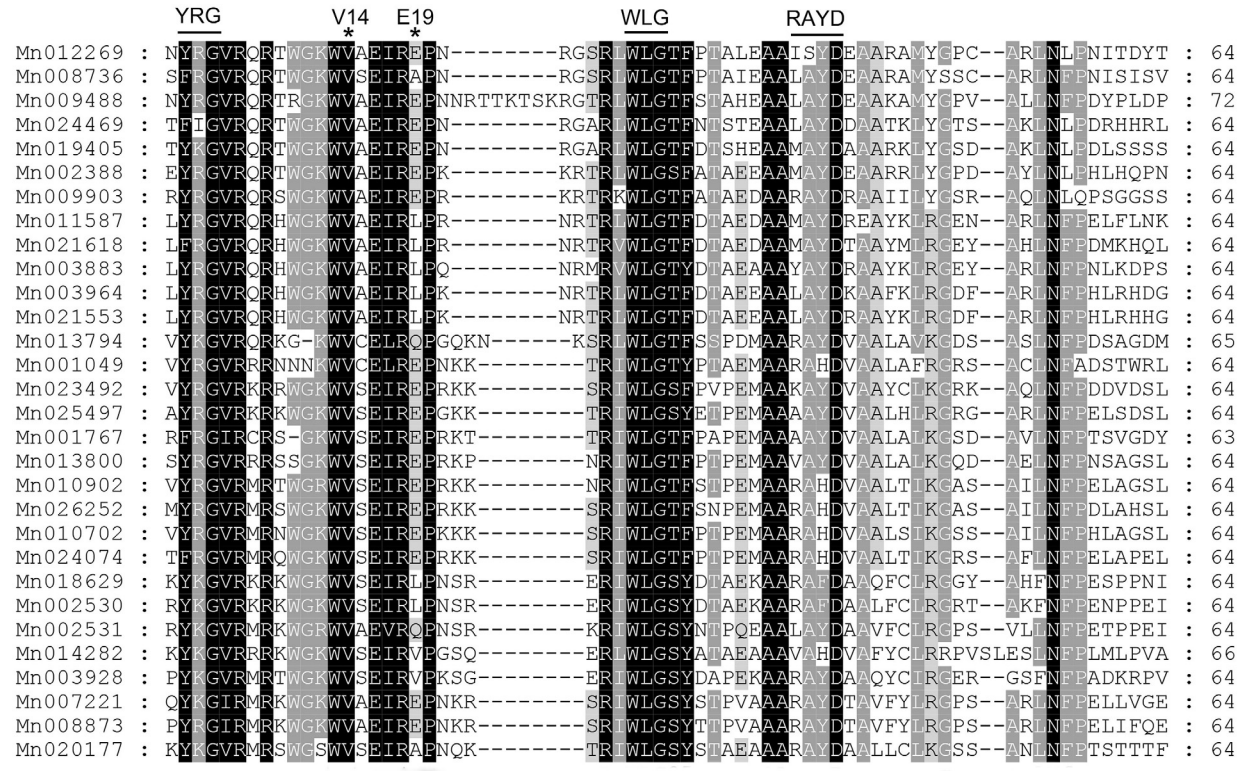

Fig. 1. The protein sequence multi-alignment of AP2 domains of DREBs from *M. notabilis*. The alignment was performed using the *GeneDoc* program. Conserved V14, E19, YRG, RAYD, and WLG motifs are highlighted by the *asterisks* and *lines*. Conservative sequences are highlighted by *black* and *grey shading*.

accession numbers (Table 1). The information analysis of *MnDREB*s shows that their CDS sizes were in the range of 477 - 2 352 bp and encoded proteins of 158 - 782 amino acids. Deduced protein molecular masses were 16.97 - 85.03 kDa and isoelectric points (pIs) 4.78 - 8.93 (Table 1). To gain a further insight into the structural features of *MnDREB*s, the exon/intron organization of each *MnDREB* was predicted with *GSDS* (Table 1). The results show that 27 *MnDREB*s possessed a single exon, and only three *MnDREB*s from subgroup A-2 contained two or more exons (Table 1) similarly as in previous reports (Sakuma *et al.* 2002, Egawa *et al.* 2006, Agarwal *et al.* 2007).

To estimate the possible response pattern of different *MnDREB*s to abiotic stresses, the conservative *cis*-acting regulatory elements within 1 500 bp upstream of ORFs of 28 *MnDREB*s (except for *MnDREB1B* and *MnDREB3A* without sufficiently long upstream sequences) were analyzed by *PlantCARE* and results were predicted (Table 4 Suppl.). The results show that each of the genes possessed many different kinds of *cis*-acting regulatory elements related to different abiotic and biotic stresses and phytohormones, which indicates that the expression of *MnDREB*s could be modulated by many different factors.

Multiple alignments with the AP2 domain of MnDREBs indicate that the 30 MnDREBs had a highly conserved domain and exhibited the typical features of DREBs (Fig. 1). The AP2 domain of DREBs were found to contain conserved Val at position-14 and Glu at position-19 (not as important as Val-14) (Sharma *et al.* 2010). In the present study, all the 30 MnDREBs had conserved Val at position-14, and 17 MnDREBs possessed Glu at position-19. Except for the conserved amino acids in the specific sites, all of them also contained the conserved YRG, WLG, and RAYD motifs (Zhao *et al.* 2012).

To clarify phylogenetic relationships among DREBs, a bootstrap phylogenetic tree was constructed by the multi-alignment of the sequences from the AP2 domains of the reported DREBs of *A. thaliana*, *P. trichocarpa*, *V. vinifera*, *M. domestica*, and *M. notabilis* (Fig. 2). This shows that *MnDREB*s were clustered into six subgroups consistent with results of Sakuma *et al*. (2002). There were 10 *MnDREB*s clustered firstly with those from *P. trichocarpa*, seven with genes from *V. vinifera*, three with genes from *M. domestica*, and seven with genes from *A. thaliana* (Fig. 2). This revealed that most *MnDREBs* are close to genes from woody plants. The names of the 30 *MnDREB*s were designated (Table 1) according to the results of the phylogenetic analysis of *MnDREB*s and the nomenclature of *Arabidopsis DREBs*. A-4 and A-5 with eight *MnDREB*s each are the largest, and A-3 with one *MnDREB* the smallest, of the six

subgroups (Table 1). In addition, the number of *MnDREB*s was compared with that of reported *DREB*s of *A. thaliana*, *Oryza sativa*, *P. trichocarpa*, *V. vinifera*, and *M. domestica* (Table 2) – the A-3 was the smallest subgroup in all these species (Table 2). Considering the genome-wide duplications (GWD), the number distribution pattern of *MnDREB*s in each subgroup was similar to those of *A. thaliana*, *P. trichocarpa*, and *M. domestica*.

Furthermore, multiple alignments of each DREB subgroup from different plants were conducted to reveal the features of primary structure of each subgroup (Fig. 3). DREBs in each subgroup had high sequence similarities, especially in the A-3 subgroup. All DREBs from the subgroups A-1 to A-3 had a conserved nuclear localization sequence (NLS) in their N-termini. In addition, DREBs in the subgroup A-1 all possessed DSAW and LWSY conserved-motifs in their C-termini. However, DREBs from the A-4 to A-6 subgroups did not show any other conserved motif except for a single AP2 domain, which indicates that DREBs from A-4 to A-6 may function in different organelles compared to those from A-1 to A-3.

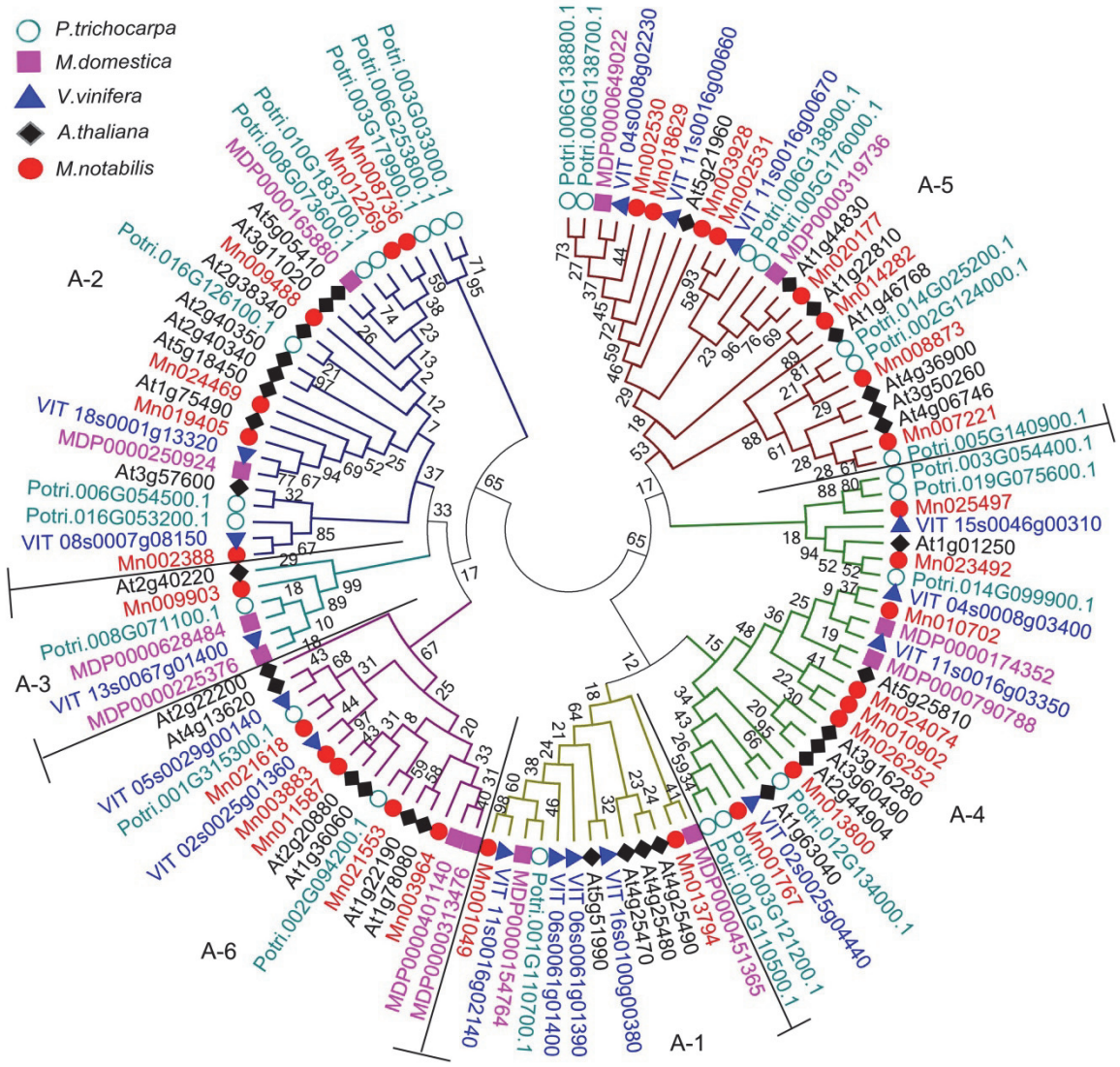

Fig. 2. Phylogenetic relationships between *DREB* gene families from different plants. *DREB* gene families are from *A. thaliana*, *P. trichocarpa*, *V. vinifera*, *M. domestica*, and *M. notabilis*. Amino acid sequences of the AP2 domains of DREBs from different plants were aligned using the *Clustal-X* computer software and subjected to a phylogenetic analysis using the NJ method with 1 000 resampling replicates. MnDREBs are highlighted by the *red dots* and the other DREBs from *A. thaliana*, *P. trichocarpa*, *V. vinifera*, *M. domestica*, and *M. notabilis* are indicated by the different *colored shapes* as shown in the legend. The amino acid sequences of DREBs from *A. thaliana*, *P. trichocarpa*, *V. vinifera*, *M. domestica*, and *M. notabilis* were downloaded from databases shown in Materials and methods. Different groups are separated by the *black lines*. Bootstrap values from 1 000 replicates are indicated at each node.

Fig. 3. The protein sequence multi-alignment of each DREB subgroup from different plants. All DREBs in subgroups A-1 to A-3 contained NLS in N-termini (indicated with the *lines*). All DREBs in a subgroup A-1 have conserved DSAW and LWSY motifs in C-termini (indicated with the *asterisks*). AP2 domains are highlight by the *lines*. Conservative sequences are highlighted by *black* and *grey shading*. The source and Gene Bank acc. No. of each DREB used in this figure are shown in Table 5 Suppl.

To investigate the expression patterns of *MnDREB*s in various organs, the transcriptome data of 27 *MnDREBs* (except for *MnDREB3A*, *MnDREB4H*, and *MnDREB6C*) were obtained from the transcriptome database. The expression profiles of these genes were visualized in heat maps (Fig. 4). The *MnDREB*s transcriptome data show

that *MnDREB*s had different expression patterns in different organs or even in the same organ (Fig. 4). However, based on the heat map, 27 *MnDREB*s were grouped into six clusters. Seven showed a preferentially high expression in winter buds (Fig. 4, CLUSTER 1), nine in roots (CLUSTER 2), six in leaves (CLUSTER 3), two in bark and leaves (CLUSTER 4), two in bark and male flowers (CLUSTER 5), and one only in male flowers (CLUSTER 6).

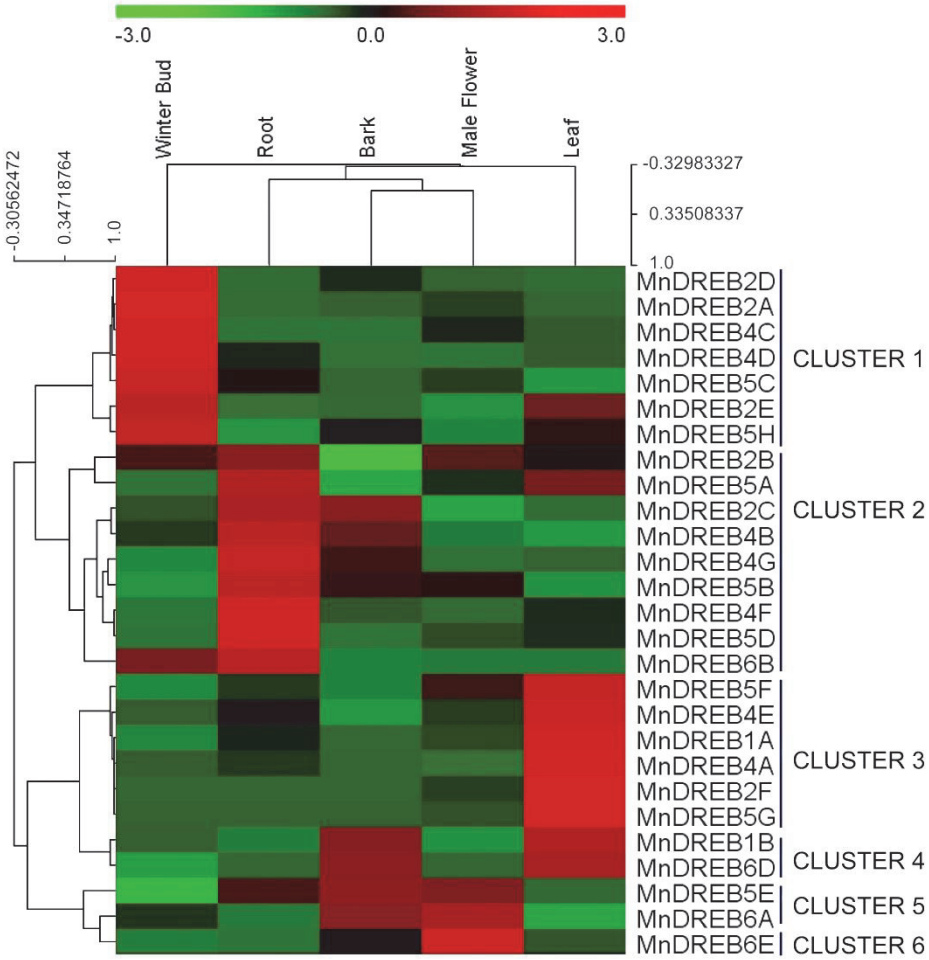

Fig. 4. The expression analysis of the *DREB* genes based on the *RPKM* profiles of five tissues (roots, bark, winter buds, male flowers, and leaves) downloaded from http://morus.swu.edu.cn/morusdb/transcriptomes. The *Mev* software was used to normalize the expression of all *DREB* genes from RNA sequencing data. Genes with similar expression profiles were grouped into six clusters by a hierarchical clustering method. Rows and columns in heat maps represent genes and samples. Sample names are shown above the heat maps. The *colour bar* at the top indicates the degree of expression: *green* - a low expression; *red* - a high expression.

The expression profiles of part of *MnDREB*s under different abiotic stresses were analyzed by RT-qPCR. There were 55 qRT-PCR primer pairs designed based on the *MnDREB* sequences, but only 16 suitable primer pairs were finally selected for *MnDREB*s from the different subgroups by confirming the amplification specificities of the designed RT-qPCR primer pairs *via* a semi-quantitative PCR using the cDNA from *M. atropurpurea* as template (Table 2 Suppl.). Because seeds of *M. notabilis* cannot be germinated to produce seedlings in laboratory, we chose *M. atropurpurea* instead of *M. notabilis* to analyze expression profiles. The RT-qPCR analysis shows that *MnDREB1B* was significantly induced by the heat and cold treatments in leaves and roots (Table 3). *MnDREB2A*, *MnDREB2B*, *MnDREB2C*, and *MnDREB2F* were all highly upregulated in response to a high temperature (Table 3) and exhibited changes under salinity (Table 3). The expression profiles of *MnDREB*s in the subgroup A-4 were similar to each other when exposed to different abiotic stresses. *MnDREB4A*, *MnDREB4B*, *MnDREB4D*, and *MnDREB4F* all displayed significant changes in response to a high temperature, cold, NaCl, and PEG in leaves or in roots (Table 3). It is noteworthy that the

MnDREB4A expression was lower in roots than in leaves and was down-regulated in response to a high temperature, cold and dehydration in roots, but up-regulated by a high temperature and cold in leaves (Table 3, Fig. 1 Suppl.). Genes in the subgroup A-5 seem to have different expression profiles under these treatments. *MnDREB5B* and *MnDREB5C* responded to high and low temperatures (Table 3); however, *MnDREB5D* exhibited significant changes after heat, cold, NaCl, and PEG (Table 3). All genes in the subgroup A-6 were significantly induced by heat and cold (Table 3). Otherwise, the expressions of genes in A-6 were higher than of genes from the other subgroups, consistent with the results from the transcriptome data (Fig. 1 Suppl.). Some genes in one subgroup showed different expression patterns in roots and leaves, but the patterns in roots and leaves were complementary (Fig. 1 Suppl.), such as *MnDREB2A*/*MnDREB2C* and *MnDREB2B*, *MnDREB4A* and *MnDREB4F*, and *MnDREB5B* and *MnDREB5C*. This indicates that genes in one subgroup played roles in different organs. In addition, all genes responded to the temperature treatments, especially a high temperature. This may lie in the HSE element upstream of ORF; it is a *cis*-acting element involved in heat stress responsiveness (Table 4). *MnDREB2B*, *MnDREB2C*, *MnDREB4A*, *MnDREB4B*, *MnDREB4D*, *MnDREB5C*, *MnDREB5D*, *MnDREB6A,* and *MnDREB6D* which contained an MBS element showed changes in expression when treated with 30 % PEG (Table 4). However, some genes that did not contain corresponding elements could also respond to abiotic stresses. For instance, *MnDREB4F* did not have elements for abiotic stresses, but its expression changed when exposed to heat, cold, NaCl, or PEG (Table 4). In conclusion, our results indicate that different genes exhibited different expression profiles in response to abiotic stresses. Further research is required to reveal detailed regulatory mechanisms and functions of these genes.

Table 3. *MnDREB* genes expression under different abiotic stress treatments (40 °C, 4 °C, 250 mM NaCl, and 30 % PEG for 24 h) were analyzed by qRT-PCR. Statistically significant differences between the amount of transcripts of the treated samples and the control samples are indicated with the *arrows* and *asterisks* ($n = 3$). The *up* and *down arrows* represent an increase and decrease in expression, respectively. The *arrow without an asterisk* represents a significant difference at $P < 0.05$, the *arrow with an asterisk* represents a significant difference at $P < 0.001$. The *line* indicates a non-significant difference.

Gene name	Expression profiles in leaves				Expression profiles in roots			
	40 °C	4 °C	NaCl	PEG	40 °C	4 °C	NaCl	PEG
MnDREB1B	↑*	↑	—	—	↑*	↑*	↑*	↓
MnDREB2A	↑*	↑*	—	—	↑*	↑*	↑*	—
MnDREB2B	↑*	—	↑*	↑*	↑*	↓*	↓*	↓*
MnDREB2C	↑*	—	—	↑*	↑*	—	↓*	↓*
MnDREB2F	↑*	↑*	↓*	↓*	—	↑	↓*	↓*
MnDREB4A	—	↑*	↓*	↓*	↓*	↓*	—	↓*
MnDREB4B	↑*	↑*	—	—	↑*	—	↓*	↓*
MnDREB4D	—	↑*	—	—	↓*	↓*	↓*	↓*
MnDREB4F	↑*	↑*	—	↑*	↑*	↑*	↓*	↓*
MnDREB5B	↑*	—	—	—	↑*	↑*	—	—
MnDREB5C	↑*	↑*	—	—	↑*	—	—	↓*
MnDREB5D	↑*	—	↑*	↑*	↑*	↓	↑*	↑*
MnDREB6A	↑*	—	↓	↓	↑*	↑*	—	—
MnDREB6D	↑*	—	↑	—	↑*	↑*	—	↓*

Discussion

The DREB TF family is part of the AP2/ERF gene family which is well known for its AP2 domain. It has been reported that the constitutive expression of *DREBs* in plants can make the transgenic plants resistant to cold, heat, drought, and salinity by inducing the expression of numerous stress-related genes even under non-stress conditions (Lata and Prasad 2011). To date, a large number of DREB TFs have been identified and functionally characterized in a few model plants. The number and distribution of *DREBs* in monocotyledons, *e.g.*, in *Oryza sativa*, were obviously different to those in dicotyledons (Sharoni *et al.* 2011). Only a few woody plant species *DREB* families have been identified at the genome-wide level, including *P. trichocarpa* (Zhuang *et al.* 2008), *V. vinifera* (Zhuang *et al.* 2009), and *M. domestica* (Zhao *et al.* 2012). However, detailed information about mulberry DREB TFs is not available. In this report, 30 *MnDREB*s were identified from mulberry. Interestingly, this number of *MnDREB*s was almost half of that from other species, including

Table 4. The comparison of promoter elements of each *MnDREB* with its pattern in response to abiotic stresses. Y - responses corresponded.

Gene name	The conserved promoter elements				Response to abiotic stresses			
	HSE	MBS	LTR	C-repeat/DRE	40 °C	PEG	4 °C	NaCl
MnDREB2A	2	1	1		Y		Y	Y
MnDREB2B	3	2		1	Y	Y	Y	Y
MnDREB2C	1	1	1		Y	Y		Y
MnDREB4A	1	1	1		Y	Y	Y	Y
MnDREB4B	1	1	2		Y	Y	Y	Y
MnDREB4D	1	2	1		Y	Y	Y	Y
MnDREB4F					Y	Y	Y	Y
MnDREB5B	2	1		1	Y		Y	
MnDREB5C	2	3			Y	Y	Y	
MnDREB5D		1			Y	Y	Y	Y
MnDREB6A	3	1			Y	Y	Y	Y
MnDREB6D	2	3			Y	Y	Y	Y

A. thaliana (Sakuma *et al.* 2002), *P. trichocarpa* (Zhuang *et al.* 2008), and *M. domestica* (Zhao *et al.* 2012) (Table 2). This may be because sequenced *M. notabilis* is diploid and has not undergone any genome-wide duplications (He *et al.* 2013).

According to the bootstrap phylogenetic tree constructed with the AP2 domains of DREBs from *A. thaliana*, *P. trichocarpa*, *V. vinifera*, *M. domestica,* and *M. notabilis*, *MnDREB*s were classified into six groups: A-1 to A-6 (Fig. 2) as suggested by Sakuma *et al*. (2002). The analysis of the gene structure of *MnDREB*s shows that only three genes in the A-2 subgroup contained two or more exons (Table 1) as found in previous reports (Sakuma *et al.* 2002, Egawa *et al.* 2006, Agarwal *et al*. 2007). Genes in the A-1 and A-2 subgroups are mainly involved in cold and osmotic stress-responsive gene expressions, respectively, and there is crosstalk between them (Akhtar *et al.* 2012). *MnDREB1B* of the subgroup A-1 was significantly induced by the heat and cold treatments in leaves and roots (Table 3). *MnDREB2A*, *MnDREB2B*, *MnDREB2C*, and *MnDREB2F* of the subgroup A-2 exhibited changes induced by salinity (Table 3). However, these genes can also respond to other stresses in this study indicating that they may have other functions. The characteristics and functions of members of the A-3, A-4, and A-5 subgroups remain to be studied (Akhtar *et al.* 2012). The expression patterns of some genes in roots and leaves were complementary (Fig. 1 Suppl.). This result was also verified by the transcriptome data of various organs which show that the genes of one subgroup were distributed in different clusters (Fig. 4). In the present study, we found that the *cis*-elements on the promoters could help to a certain extent in gaining an insight into gene functions. Therefore, we speculate that some genes might be regulated by other MnDREB factors because they contained a C-repeat/DRE upstream element (Table 4 Suppl.). In addition, some *MnDREBs* might respond to biotic stresses because they contained elements related to biotic stresses (Table 4 Suppl.). However, the expression patterns of some genes were not consistent with the *cis*-element within the upstream sequences when exposed to the abiotic stresses. A recent research showed that the DREB1A/CBF3, DREB2A, and DREB2C proteins interact physically with AREB/ABF proteins (Lee *et al.* 2010). These data suggest crosstalk between elements of the ABA-dependent and ABA-independent response pathways. So, we speculate that some proteins for regulating the mulberry *DREB* gene expression, such as *MnDREB4F*, can also interact with other proteins in response to related stresses. Taken together, *MnDREB*s respond to environmental conditions and may improve stress resistance, but future studies are necessary to understand the regulatory function of the *MnDREB* members.

In conclusion, we performed the first genome-wide analysis of the DREB TFs family in mulberry and conducted a detailed investigation of their structure characterization and expression profiles under various abiotic stresses. To the best of our knowledge, this report is the first genome-wide analysis of *DREB* genes in mulberry, and our data provide some insights into potential functions of mulberry DREBs. The results will provide a useful basis for the further understanding of the structure-function relationship of these gene family members. This will help in obtaining one or more excellent candidate genes for genetic engineering to improve stress tolerance of mulberry and other valuable plants.

References

Agarwal, P., Agarwal, P.K., Nair, S., Sopory, S.K., Reddy, M.K.: Stress-inducible DREB2A transcription factor from *Pennisetum glaucum* is a phosphoprotein and its phosphorylation negatively regulates its DNA-binding activity. - Mol. Genet. Genomics **277**: 189-198, 2007.

Agarwal, P.K., Agarwal, P., Reddy, M.K., Sopory, S.K.: Role of DREB transcription factors in abiotic and biotic stress tolerance in plant. - Plant Cell Rep. **25**: 1263-1274, 2006.

Agarwal, P.K., Jha, B.: Transcription factors in plants and ABA dependent and independent abiotic stress signaling. - Biol. Plant. **54**: 201-212, 2010.

Akhtar, M., Jaiswal, A., Taj, G., Jaiswal, J.P., Qureshi, M.I., Singh, N.K.: DREB1/CBF transcription factors: their structure, function and role in abiotic stress tolerance in plants. - J. Genet. **91**: 385-395, 2012.

Bouaziz, D., Pirrello, J., Ben Amor, H., Hammami, A., Charfeddine, M., Dhieb, A., Bouzayen, M., Gargouri-Bouzid, R.: Ectopic expression of dehydration responsive element binding proteins (StDREB2) confers higher tolerance to salt stress in potato. - Plant Physiol. Biochem. **60**: 98-108, 2012.

Boyer, J.S.: Plant Productivity and Environment. - Science **218**: 443-448, 1982.

Checker, V.G., Khurana, P.: Molecular and functional characterization of mulberry EST encoding remorin (MiREM) involved in abiotic stress. - Plant Cell Rep. **32**: 1729-1741, 2013.

Chen, M., Wang, Q.Y., Cheng, X.G., Xu, Z.S., Li, L.C., Ye, X.G., Xia, L.Q., Ma, Y.Z.: GmDREB2, a soybean DRE-binding transcription factor, conferred drought and high-salt tolerance in transgenic plants. - Biochem. biophys. Res. Commun. **353**: 299-305, 2007.

Chen, Y., Yang. J., Wang, Z., Zhang, H., Mao, X., Li, C.: Gene structures, classification, and expression models of the DREB transcription factor subfamily in *Populus trichocarpa*. - Sci. World J. doi.org/10.1155/2013/954640, 2013.

Cui, M., Zhang, W., Zhang, Q., Xu, Z., Zhu, Z., Duan, F., Wu, R.: Induced over-expression of the transcription factor OsDREB2A improves drought tolerance in rice. - Plant Physiol. Biochem. **49**: 1384-1391, 2011.

Dong, C.J., Liu, J.Y.: The *Arabidopsis* EAR-motif-containing protein RAP2.1 functions as an active transcriptional repressor to keep stress responses under tight control. - BMC Plant Biol. **10**: 47, 2010.

Dubouzet, J.G., Sakuma, Y., Ito, Y., Kasuga, M., Dubouzet, E.G., Miura, S., Seki, M., Shinozaki, K., Yamaguchi-Shinozaki, K.: *OsDREB* genes in rice, *Oryza sativa L.*, encode transcription activators that function in drought, high salt and cold responsive gene expression. - Plant J. **33**: 751-763, 2003.

Egawa, C., Kobayashi, F., Ishibashi M., Nakamura, T., Nakamura, C., Takumi, S.: Differential regulation of transcript accumulation and alternative splicing of a *DREB2* homolog under abiotic stress conditions in common wheat. - Genes. Genet. Syst. **81**: 77-91, 2006.

Guo, A.Y., Zhu, Q.H., Chen, X., Luo, J.C.: GSDS: a gene structure display server. - Yi Chuan **29**: 1023-1026, 2007.

Hasegawa, P.M., Bressan, R.A., Zhu, J.K., Bohnert, H.J.: Plant cellular and molecular responses to high salinity. - Annu. Rev. Plant Physiol. Plant mol. Biol. **51**: 463-499, 2000.

He, N., Zhang, C., Qi, X., Zhao, S., Tao, Y., Yang, G., Lee, T.H., Wang, X., Cai, L., Li, D., Lu, M., Liao, S., Luo, G., He, R., Tan, H., Xu, Y., Li, T., Zhao, A., Jia, L., Fu, Q., Zeng, Q., Gao, C., Ma, B., Liang, J., Wang, X., Shang, J., Song, P., Wu, H., Fan, L., Wang, Q., Shuai, Q., Zhu, J., Wei, C., Zhu-Salzman, K., Jin, D., Wang, J., Liu, T., Yu, M., Tang, C., Wang, Z., Dai, F., Chen, J., Liu, Y., Zhao, S., Lin, T., Zhang, S., Wang, J., Wang, J., Yang, H., Yang, G., Wang, J., Paterson, A.H., Xia, Q., Ji, D.: Draft genome sequence of the mulberry tree *Morus notabilis*. - Nat. Commun. **4**: 2445, 2013.

Huang, G.T., Ma, S.L., Bai, L.P., Zhang, L., Ma, H., Jia, P., Liu, J., Zhong, M., Guo, Z.F.: Signal transduction during cold, salt, and drought stresses in plants. - Mol. Biol. Rep. **39**: 969-987, 2012.

Hussain, S.S., Kayani, M.A., Amjad, M.: Transcription factors as tools to engineer enhanced drought stress tolerance in plants. - Biotechnol. Progr. **27**: 297-306, 2011.

Hwang, J.E., Lim, C.J., Chen, H., Je, J., Song, C., Lim, C.O.: Overexpression of *Arabidopsis* dehydration-responsive element-binding protein 2C confers tolerance to oxidative stress. - Mol. Cells **33**: 135-140, 2012.

Islam, M.S., Wang, M.H.: Expression of dehydration responsive element-binding protein-3 (DREB3) under different abiotic stresses in tomato. - BMB Rep. **42**: 611-616, 2009.

Jaillon, O., Aury, J.M., Noel, B., Policriti, A., Clepet, C., Casagrande, A., Choisne, N., Aubourg, S., Vitulo, N., Jubin, C., Vezzi, A., Legeai, F., Hugueney, P., Dasilva, C., Horner, D., Mica, E., Jublot, D., Poulain, J., Bruyère, C., Billault, A., Segurens, B., Gouyvenoux, M., Ugarte, E., Cattonaro, F., Anthouard, V., Vico, V., Fabbro, C.D., Alaux, M., Gaspero, G.D., Dumas, V., Felice, N., Paillard, S., Juman, I., Moroldo, M., Scalabrin, S., Canaguier, A., Clainche, I.L., Malacrida, G., Durand, E., Pesole, G., Laucou, V., Chatelet, P., Merdinoglu, D., Delledonne, M., Pezzotti, M., Lecharny, A., Scarpelli, C., Artiguenave, F., Pè, M.E., Valle, G., Morgante, M., Caboche, M., Adam-Blondon, A.F., Weissenbach, J., Quétier, F., Wincker, P.: The grapevine genome sequence suggests ancestral hexaploidization in major angiosperm phyla. - Nature **449**: 463-467, 2007.

Jofuku, K.D., Den Boer, B.G., Van Montagu, M., Okamuro, J.K.: Control of *Arabidopsis* flower and seed development by the homeotic gene *APETALA2*. - Plant Cell **6**: 1211-1225, 1994.

Kizis, D., Pages, M.: Maize DRE-binding proteins DBF1 and DBF2 are involved in rab17 regulation through the drought responsive element in an ABA-dependent pathway. - Plant J. **30**: 679-689, 2002.

Lata, C., Prasad, M.: Role of DREBs in regulation of abiotic stress responses in plants. - J. exp. Bot. **62**: 4731-4748, 2011.

Lee, S.J., Kang, J.Y., Park, H.J., Kim, H.D., Bae, M.S., Choi, Choi, H.I., Kim, S.Y.: DREB2C interacts with ABF2, a bZIP protein regulating abscisic acid-responsivegene expression, and its overexpression affects abscisic acid sensitivity. - Plant Physiol. **153**: 716-727, 2010.

Li, M., Li, Y., Li, H., Wu, G.: Improvement of paper mulberry tolerance to abiotic stresses by ectopic expression of tall fescue *FaDREB1*. - Tree Physiol. **32**: 104-113, 2011a.

Li, M.R., Li, Y., Li, H.Q., Wu, G.J.: Ectopic expression of

FaDREB2 enhances osmotic tolerance in paper mulberry. - J. Integr. Plant Biol. **53**: 951-960, 2011b.

Lucas, S., Durmaz, E., Akpınar, B.A., Budak, H.: The drought response displayed by a DRE-binding protein from *Triticum dicoccoides*. - Plant Physiol. Biochem. **49**, 346-351, 2011.

Marcolino-Gomes, J., Rodrigues, F.A., Oliveira, M.C., Farias, J.R., Neumaier, N., Abdelnoor, R.V., Marcelino-Guimarães, F.C., Nepomuceno, A.L.: Expression patterns of GmAP2/EREB-like transcription factors involved in soybean responses to water deficit. - PLOS ONE. **8**: e62294, 2013.

Matsukura, S., Mizoi, J., Yoshida, T., Todaka, D., Ito, Y., Maruyama, K., Shinozaki, K., Yamaguchi-Shinozaki, K.: Comprehensive analysis of rice DREB2-type genes that encode transcription factors involved in the expression of abiotic stress-responsive genes. - Mol. Genet. Genomics **283**: 185-196, 2010.

Mayer, K., White, O., Bevan, M., Lemcke, K., Creasy, T.H., Bielke, C., Haas, B., Haase, D., Maiti, R., Rudd, S., Peterson, J., Schoof, H., Frishman, D., Morgenstern, B., Zaccaria, P., Ermolaeva, M., Pertea, M., Quackenbush, J., Volfovsky, N., Wu, D., Lowe, T.M., Salzberg, S.L., Mewes, H.W.: Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana.* - Nature **408**: 796-815, 2000.

Nakano, T., Suzuki, K., Fujimura, T., Shinshi, H.: Genome-wide analysis of the *ERF* gene family in *Arabidopsis* and rice. - Plant Physiol. **140**: 411-432, 2006.

Qin, F., Kakimoto, M., Sakuma, Y., Maruyama, K., Osakabe, Y., Tran, L.S., Shinozaki, K., Yamaguchi-Shinozaki, K.: Regulation and functional analysis of ZmDREB2A in response to drought and heat stresses in *Zea mays* L. - Plant J. **50**: 54-69, 2007.

Ramachandra Reddy, A., Chaitanya, K.V., Jutur, P.P., Sumithra, K.: Differential antioxidative responses to water stress among five mulberry (*Morus alba L.*) cultivars. - Environ. exp. Bot. **52**: 33-42, 2004.

Saitou, N., Nei, M.: The neighbor-joining method: a new method for reconstructing phylogenetic trees. - Mol. Biol. Evol. **4**: 406-425, 1987.

Sakuma, Y., Liu, Q., Dubouzet, J.G., Abe, H., Shinozaki, K., Yamaguchi-Shinozaki, K.: DNA-binding specificity of the ERF/AP2 domain of *Arabidopsis* DREBs, transcription factors involved in dehydrationand cold-inducible gene expression. - Biochem. biophys. Res. Commun. **290**: 998-1009, 2002.

Seki, M., Narusaka, M., Abe, H., Kasuga, M., Yamaguchi-Shinozaki, K., Carninci, P., Hayashizaki, Y., Shinozaki, K.: Monitoring the expression pattern of 1300 *Arabidopsis* genes under drought and cold stresses by using a full-length cDNA microarray. - Plant Cell **13**: 61-72, 2001.

Sharma, M.K., Kumar, R., Solanke, A.U., Sharma, R., Tyagi, A.K., Sharma, A.K.: Identification, phylogeny, and transcript profiling of *ERF* family genes during development and abiotic stress treatments in tomato. - Mol. Genet. Genomics **284**: 455-475, 2010.

Sharoni, A.M.1., Nuruzzaman, M., Satoh, K., Shimizu, T., Kondoh, H., Sasaya, T., Choi, I.R., Omura, T., Kikuchi, S.: Gene structures, classification and expression models of the AP2/ERFBP transcripttion factor family in rice. - Plant Cell Physiol. **52**: 344-360, 2011.

Shen, Y.G., Zhang, W.K., He, S.J., Zhang, J.S., Liu, Q., Chen, S.Y.: An EREBP/AP2-type protein in *Triticum aestivum* was a DRE-binding transcription factor induced by cold, dehydration and ABA stress. - Theor. appl. Genet. **106**: 923-930, 2003.

Singhal, B.K., Khan, M.A., Dhar, A., Baqual, F.M., Bindroo, B.B.: Approaches to industrial exploitation of mulberry (*Mulberry sp*.) fruits. - J. Fruit Ornam. Plant Res. **18**: 83-99, 2010.

Stockinger, E.J., Gilmour, S.J., Thomashow, M.F.: *Arabidopsis thaliana CBF1* encodes an AP2 domain-containing transcriptional activator that binds to the C-repeat/DRE, a cis-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit. - Proc. nat. Acad. Sci. USA **94**: 1035-104, 1997.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S.: *MEGA5*: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. - Mol. Biol. Evol. **28**: 2731-2739, 2011.

Tang, M., Liu, X., Deng, H., Shen, S.: Over-expression of *JcDREB*, a putative AP2/EREBP domain-containing transcription factor gene in woody biodiesel plant *Jatropha curcas*, enhances salt and freezing tolerance in transgenic *Arabidopsis thaliana*. - Plant Sci. **181**: 623-631, 2011.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G.: The *CLUSTAL_X* windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. - Nucl. Acids Res. **25**: 4876-4882, 1997.

Tuskan, G.A., Difazio, S., Jansson, S., Bohlmann, J., Grigorive, I., Hellsten, U., Putnam, N., Ralph, S., Rombauts, S., Salamov, A., Schein, J., Sterck, L., Aerts, A., Bhalerao, R. R., Bhalerao, R. P., Blaudez, D., Boerjan, W., Brun, A., Brunner, A., Busov, V., Campbell, M., Carlson, J., Chalot, M., Chapman, J., Chen, G.L., Cooper, D., Coutinho, P. M., Couturier, J., Covert, S., Cronk, Q., Cunningham, R., Davis, J., Degroeve, S., Déjardin, A., De Pamphilis, C., Detter, J., Dirks, B., Dubchak, I., Duplessis, S., Ehlting, J., Ellis, B., Gendler, K., Goodstein, D., Gribskov, M., Grimwood, J., Groover, A., Gunter, L., Hamberger, B., Heinze, B., Helariutta, Y., Henrissat, B., Holligan, D., Holt, R., Huang, W., Islam-Faridi, N., Jones, S., Jones-Rhoades, M., Jorgensen, R., Joshi, C., Kangasjärvi, J., Karlsson, J., Kelleher, C., Kirkpatrick, R., Kirst, M., Kohler, A., Kalluri, U., Larimer, F., Leebens-Mack, J., Leplé, J.C., Locascio, P., Lou, Y., Lucas, S., Martin, F., Montanini, B., Napoli, C., Nelson, D. R., Nelson, C., Nieminen, K., Nilsson, O., Pereda, V., Peter, G., Philippe, R., Pilate, G., Poliakov, A., Razumovskaya, J., Richardson, P., Rinaldi, C., Ritland, K., Rouzé, P., Ryaboy, D., Schmutz, J., Schrader, J., Segerman, B., Shin, H., Siddiqui, A., Sterky, F., Terry, A., Tsai, C.J., Uberbacher, E., Unneberg, P., Vahala, J., Wall, K., Wessler, S., Yang, G., Yin, T., Douglas, C., Marra, M., Sandberg, G., Van de Peer, Y., Rokhsar, D.: The Genome of black cottonwood, *Populus trichocarpa* (Torr. & Gray). - Science **313**: 1596-1604, 2006.

Velasco, R., Zharkikh, A., Affourtit, J., Dhingra, A., Cestaro, A., Kalyanaraman, A., Fontana, P., Bhatnagar, S.K., Troggio, M., Pruss, D., Salvi, S., Pindo, M., Baldi, P., Castelletti, S., Cavaiuolo, M., Coppola, G., Costa,F., Cova, V., Ri, A.D., Goremykin, V., Komjanc, M., Longhi, S., Magnago, P., Malacarne, G., Malnoy, M., Micheletti, D., Moretto, M., Perazzolli, M., Si-Ammour, A., Vezzulli, S., Zini, E., Eldredge, G., Fitzgerald, L.M., Gutin, N., Lanchbury, J., Macalma, T., Mitchell, J.T., Reid, J.,

Wardell, B., Kodira, C., Chen, Z., Desany, B., Niazi, F., Palmer, M., Koepke, T., Jiwan, D., Schaeffer, S., Krishnan, V., Wu, C., Chu, V.T., King, S.T., Vick, J., Tao, Q., Mraz, A., Stormo, A., Stormo, K., Bogden, R., Ederle, D., Stella, A., Vecchietti, A., Kater, M.M., Masiero, S., Lasserre, P., Lespinasse, Y., Allan, A.C., Bus, V., Chagné, D., Crowhurst, R.N., Gleave, A.P., Lavezzo, E., Fawcett, J.A., Proost, S., Rouzé, P., Sterck, L., Toppo, S., Lazzari, B., Hellens, R.P., Durel, C.E., Gutin, A., Bumgarner, R.E., Gardiner, S.E., Skolnick, M., Egholm, M., Van de Peer, Y., Salamini, F., Viola, R.: The genome of the domesticated apple (*Malus* × *domestica* Borkh.). - Nat. Genet. **42**: 833-839, 2010.

Wei, C., Liu, X., Long, D., Guo, Q., Fang, Y., Bian, C., Zhang, D., Zeng, Q., Xiang, Z., Zhao, A.: Molecular cloning and expression analysis of mulberry MAPK gene family. - Plant Physiol. Biochem. **77C**: 108-116, 2014.

Xue, G.P., Loveridge, C.W.: *HvDRF1* is involved in abscisic acid mediated gene regulation in barley and produces two forms of AP2 transcriptional activators, interacting preferably with a CT-rich element. - Plant J. **37**: 326-339, 2004.

Xue, Y., Wang, Y.Y., Peng, R.H., Zhen, J.L., Zhu, B., Gao, J.J., Zhao, W., Han, H.J., Yao, Q.H.: Transcription factor *MdCBF1* gene increases freezing stress tolerance in transgenic *Arabidopsis thaliana*. - Biol. Plant. **58**: 499-506, 2014.

Yu, J., Hu, S., Wang, J., Wong, G.K., Li, S., Liu, B., Deng, Y., Dai, L., Zhou, Y., Zhang, X., Cao, M., Liu, J., Sun, J., Tang, J., Chen, Y., Huang, X., Lin, W., Ye, C., Tong, W., Cong, L., Geng, J., Han, Y., Li, L., Li, W., Hu, G., Huang, X., Li, W., Li, J., Liu, Z., Li, L., Liu, J., Qi, Q., Liu, J., Li, L., Li, T., Wang, X., Lu, H., Wu, T., Zhu, M., Ni, P., Han, H., Dong, W., Ren, X., Feng, X., Cui, P., Li, X., Wang, H., Xu, X., Zhai, W., Xu, Z., Zhang, J., He, S., Zhang, J., Xu, J., Zhang, K., Zheng, X., Dong, J., Zeng, W., Tao, L., Ye, J., Tan, J., Ren, X., Chen, X., He, J., Liu, D., Tian, W., Tian, C., Xia, H., Bao, Q., Li, G., Gao, H., Cao, T., Wang, J., Zhao, W., Li, P., Chen, W., Wang, X., Zhang, Y., Hu, J., Wang, J., Liu, S., Yang, J., Zhang, G., Xiong, Y., Li, Z., Mao, L., Zhou, C., Zhu, Z., Chen, R., Hao, B., Zheng, W., Chen, S., Guo, W., Li, G., Liu, S., Tao, M., Wang, J., Zhu, L., Yuan, L., Yang, H.: A draft sequence of the rice genome (*Oryza sativa* L. ssp.*indica*). - Science **296**: 79-92, 2002.

Zhao, T., Liang, D., Wang, P., Liu, J., Ma, F.: Genome-wide analysis and expression profiling of the DREB transcription factor gene family in *Malus* under abiotic stress. - Mol. Genet. Genomics **287**: 423-436, 2012.

Zhao, T., Xia, H., Liu, G., Ma, F.: The gene family of dehydration responsive element-binding transcription factors in grape (*Vitis vinifera*): genome-wide identification and analysis, expression profiles, and involvement in abiotic stress resistance. - Mol. Biol. Rep. **41**: 1577-1590, 2014.

Zhou, M.L., Ma, J.T., Zhao, Y.M., Wei, Y.H., Tang, Y.X., Wu, Y.M.: Improvement of drought and salt tolerance in *Arabidopsis* and *Lotus corniculatus* by overexpression of a novel DREB transcription factor from *Populus euphratica*. - Gene **506**: 10-17, 2012.

Zhu, T., Budworth, P., Han, B., Brown, D., Chang, H.S., Zou, G.Z., Wang, X.: Toward elucidating the global expression patterns of developing *Arabidopsis*: parallel analysis of 8 300 genes by a high-density oligonucleotide probe array. - Plant Physiol. Biochem. **39**: 221-242, 2001.

Zhuang, J., Cai, B., Peng, R.H., Zhu, B., Jin, X.F., Xue, Y., Gao, F., Fu, X.Y., Tian, Y.S., Zhao, W., Qiao, Y.S., Zhang, Z., Xiong, A.S., Yao, Q.H.: Genome-wide analysis of the AP2/ERF gene family in *Populus trichocarpa*. - Biochem. biophys. Res. Commun. **371**: 468-474, 2008.

Zhuang, J., Peng, R.H., Cheng, Z.M., Zhang, J., Cai, B., Zhang, Z., Gao, F., Zhu, B., Fu, X.Y., Jin, X.F., Chen, J.M., Qiao, Y.S., Xiong, A.S., Yao, Q.H.: Genome-wide analysis of the putative AP2/ERF family genes in *Vitis vinifera*. - Scientia Hort. **123**: 73-81, 2009.