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Phylogenetic and transcriptional analysis of chrysanthemum *GRAS* transcription factors

T.W. GAO, W.W. ZHANG, A.P. SONG, C. AN, J.J. XIN, J.F. JIANG, Z.Y. GUAN, F.D. CHEN, and S.M. CHEN*

College of Horticulture, Nanjing Agricultural University, Nanjing 210095, P.R. China

Abstract

The *GRAS* transcription factors encoding proteins ranging from 400 to 700 residues are recognized by their conserved C terminus. Here, a set of 23 *CmGRAS* genes was identified from a scan of the chrysanthemum (*Chrysanthemum morifolium*) transcriptome. A phylogenetic analysis implied that nine of these genes could be assigned orthologs to the *GRAS* gene family, and that four of them formed two pairs of paralogs. A phylogenetic analysis of the GRAS protein family based on the chrysanthemum and recent study of eight representative species of angiosperms showed that most of the *CmGRAS* genes belong to a recognized sub-group. *CmGRAS4* and *CmGRAS10* were strongly transcribed in flowers and roots, respectively. The DELLA subfamily transcript abundance of the *CmGRAS19* and *CmGRAS20* was high in the reproductive tissues, and they were responsive to phytohormones and stresses. Establishment of the orthology relationships between the known representative *GRAS* genes and *CmGRAS*, and transcriptional profiles of *CmGRASs* after phytohormone treatments or stresses will facilitate subsequent functional analyses in the *GRAS* gene family.

Additional key words: abscisic acid, *Chrysanthemum morifolium*, cold, heat, methyl jasmonate, osmotic stress, salicylic acid, salinity, wounding.

Introduction

Transcription factors play important roles in gene expression regulation. One of the most important groups of plant transcription factors is the *GRAS* family (Bolle 2004). GRAS proteins were named by its first three identified members: gibberellic acid insensitive (GAI), repressor of GA1 (RGA), and scarecrow (SCR) (Pysh *et al*. 1999, Zhang *et al*. 2012). The GRAS proteins range in size from 400 to 700 residues and have several motifs including two leucine heptad repeats (LHR I and II), VHIID with the conserved residues of proline - asparagine - histidine - aspartic acid - glutamine (P-N-H-D-Q), PFYRE characterized by three units: proline (P), aromatic phenylalanine, and tyrosine (FY), and arginine, and glutamic acid residues (RE), RVER designated after the respective conserved amino acids and SAW motif which contains three pairs of conserved residues: R-E, W-G, W–W.(Hirsch and Oldroyd 2009, Liu and Widmer 2014). Only 9 GRAS proteins were found in sunflower but 69 in rice (Zhang *et al*. 2012). The 37 members of the *GRAS* family in *Arabidopsis thaliana* have been classified into eight clades, namely DELLA, hairy meristem (HAM), scarecrow-like9 (SCL9) also named LISCL, phytochrome A signal transduction (PAT), lateral suppressor (LS), SCR, short root (SHR), and scarecrow-like4/7 (SCL4/7) (Bolle 2004), whereas a cross-species comparative analysis has suggested that at least 13 sub-families can be identified (Liu and Widmer 2014). Recently, 29 orthologous groups (OGs) from the *GRAS* gene family have been recognized, based on a comprehensive phylogenetic analysis in eight

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Abbreviations: ABA - abscisic acid; DLT - dwarf and low-tillering; GA - gibberellin; HAM - hairy meristem; LS - lateral suppressor; MeJA - methyl jasmonate; NSP - nodulation signaling pathway; OG - orthologous group; PAT - phytochrome A signal transduction; PEG - polyethylene glycol, RAD1 - required for arbuscule development 1; RAM - reduced arbuscular mycorrhization; SA - salicylic acid; SCL - scarecrow-like; SCR - scarecrow; SHR- short root.

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* Corresponding author; e-mail: chensm@njau.edu.cn

representative species of angiosperms. The OGs were regrouped into 17 subfamilies, and 5 new subfamilies dwarf and low-tillering (DLT), required for arbuscule development 1 (RAD1), reduced arbuscular mycorrhization 1(RAM1), SCLA, and SCLB were described (Cenci and Rouard 2017). Interestingly, no species contains all the orthologous groups. Therefore, the identification of *GRAS* members in additional species will improve our understanding of the *GRAS* gene family.

GRAS proteins are important for a variety of processes, ranging from signal transduction to plant growth and development (Bolle 2004, Lee *et al.* 2008, Hirsch *et al*. 2009). The *PAT1* gene was shown to be a positive regulator in transduction of phytochrome A signal (PAT) in *A. thaliana* (Bolle 2004). The DELLA proteins named by the conserved DELLA domain in the sequence of the N-terminal region are responsible for the negative regulation of GA signal transduction (Ubeda-Tomás *et al*. 2008, Heo *et al*. 2011) and underpin the "Green Revolution" semi-dwarf phenotype which increases grain yield of rice and wheat (Hedden and Peter 2003). Nodulation signaling pathway 1 (NSP1) and nodulation signaling pathway 2 (NSP2) participate in the legume/*Rhizobium* nodulation signal transduction (Kaló *et al*. 2005, Smit *et al*. 2005). In *A. thaliana*, PAT1, SCL21, and SCL13 act in light signal transduction pathway (Torres-Galea *et al*. 2006, 2013). The SCR and SHR act as positive regulators of root architecture and are responsible for the maintenance of the root meristem (Koizumi *et al*. 2012). Two genes in lateral suppressor (LS) OG were functionally characterized, where the rice *MOC1* controls tillering (Li *et al*. 2003), and *A. thaliana LAS* is involved in the formation of lateral shoots (Greb *et al*. 2003). HAM subfamily is responsible for maintaining the shoot apical meristem (Stuurman *et al*. 2002). Moreover, genes in *GRAS* family are involved in responses to abiotic stresses, *e.g.*, *PeSCL7* is up-regulated by severe salt stress, while it is down-regulated by exogenous gibberellins (GAs). The overexpression of this gene increases the tolerance of *A. thaliana* to salt and drought (Ma *et al*. 2010). Silencing of *TaSCL14* results in a reduced tolerance of wheat to oxidative stress (Chen *et al*. 2015).

Chrysanthemum (*Chrysanthemum morifolium*) is one of the leading cut flower species. Though *GRAS* genes are essential for development and abiotic stresses responses, they have not been sufficiently characterized in this species. Since the genome sequence of chrysanthemum is not available yet, we exploited chrysanthemum *GRAS* genes using transcriptomic data (Xia *et al*. 2014, Zhang *et al*. 2014, Song *et al*. 2016). The transcription of *GRAS* genes in response to various phytohormone treatments and abiotic stresses have been characterized. Establishment of the orthology relationships between the known representative *GRAS* genes and genes in chrysanthemum will facilitate subsequent functional analyses of the *GRAS* gene family.

Materials and methods

Plants and cultivation: Cuttings of the chrysanthemum (*Chrysanthemum morifolium* Ramat.) cv. Jinba were obtained from the Chrysanthemum Germplasm Resource Preserving Center (Nanjing Agricultural University, China). Cuttings were potted into a 1:1 (v/v) mixture of soil and *Vermiculite*, and cultivated in a greenhouse at a day/night temperature of 25/18 ºC, a 16-h photoperiod with an irradiance of 50 μmol m^{-2} s^{-1}, and a relative humidity of 70 %. Seedlings at 6 - 8 leaf stage were subjected to different stresses and phytohormone treatments. Roots, stems, leaves (the second leaves counted from the apex), tubular and ray florets at bud stage, and pollen grains were sampled for transcription analysis of *CmGRASs*. The experiment included three replicates, three plants were included in each replicate.

Isolation and sequencing full-length *CmGRAS* cDNAs: *GRAS* sequences downloaded from *TAIR* (https://www.arabidopsis.org/) were used to query the chrysanthemum transcriptome (Xia *et al*. 2014, Zhang *et al*. 2014, Song *et al*. 2016). The hits were subjected to multiple alignment using *MEGA 7.0* to identify redundancies, and full length cDNAs of the unique sequences were amplified *via* 5'- and 3'-RACE PCR. For the 5'-RACE PCR reactions, primers AAP and AUAP supplied with 5' RACE system kit *v2.0* by the *Invitrogen* (Carlsbad, CA, USA), and the gene-specific primer pairs CmGRASx-5-F1/F2/F3 were used (Table 1 Suppl). The resulting PCR products were gel-purified using an *AxyPrep* DNA gel extraction kit (*Axygen*, Hangzhou, China) and inserted into pMD19-T (*TaKaRa*, Dalian, China) for sequencing. Totally, 23 pairs of gene-specific primers (CmGRASx-F/R) (Table 1 Suppl.) were designed to amplify each gene open reading frame (ORF).

Phylogenetic analysis: The set of known representative *GRAS* genes from three monocots: *Musa acuminata*, *Phoenix dactylifera*, and *Oryza sativa* and five dicots: *Arabidopsis thaliana*, *Vitis vinifera*, *Theobroma caca*, *Coffea canephora, Amborella trichopoda*, and *Chrysanthemum morifolium* were initially aligned using *ClustalW* software (Larkin *et al*. 2007), the sequences were downloaded from the supplementary data of Cenci and Rouard (2017). A maximum likelihood phylogenetic analysis supported by a bootstrap analysis (1 000 iterations) was carried out. The output was graphically

represented using *MEGA7* software (Kumar *et al*. 2016) and *iTOL* (http://itol.embl.de/) (Letunic and Bork 2016). The *MEME v.4.8.1* program (Bailey *et al*. 2009) was used to identify known GRAS motifs in the CmGRAS proteins, using the parameter settings suggested by Huang *et al*. (2012) and only motifs associated with an E value < e^{-5} were retained.

Stress and phytohormone treatments: Seedlings at the 6 - 8 leaf stage were exposed to the following abiotic stresses: 200 mM NaCl (salt stress), 20 % (m/v) PEG 6000 (drought) (Song *et al*. 2012), low temperature (4 °C), high temperature (40 °C,) and wounding. For the NaCl and PEG 6000 treatments, seedlings were transferred to a hydroponic culture, and the second leaf (counting from the apex) was sampled at 0, 1, 4, 12, 24 h post the imposition of stress (Song *et al*. 2016a,b). For the low or high temperature treatments, seedlings were exposed to 4 °C or 40 °C at a 16-h photoperiod (an irradiance of 50 μmol m^{-2} s^{-1}), and the second leaf from shoot apex was sampled (Song *et al*. 2014). The second leaves from shoot apex were subjected to wounding treatment following the previous description (Moran and Thompson 2001). Seedlings at the 6 - 8 leaf stage were also exposed to phytohormone treatments, where 50 μM abscisic acid (ABA) (Ricachenevsky *et al*. 2010), 1 mM methyl jasmonate (MeJA) (War *et al*. 2013), or 200 μM salicylic acid (SA) (Alaey *et al*. 2011) were sprayed on the seedlings. Leaves were collected prior to the stress treatment and 1, 4, 12, and 24 h after being imposed to phytohormone treatments. Six plants are included at each time point for each treatment.

Real-time quantitative PCR (qPCR): Total RNA was extracted using the *RNAiso* reagent (*TaKaRa*), following the manufacturer's instructions, then digested with *RNase-free DNase I* (*TaKaRa*) to remove any contaminating genomic DNA. Reverse transcription was performed using *M-MLV* reverse transcriptase (*TaKaRa*), the qPCR using *SYBR Premix Ex Taq TMII* was employed to analyse the expression of *CmGRAS* using specific primer pairs for *CmGRASs* (Table 2 Suppl.), and *CmEF1α* was chosen as the reference gene (Song *et al*. 2014). Each 20 mm^3 of qPCR reaction solution contained 10 mm^3 of *SYBR® Premix Ex Taq™ II* (*TaKaRa*), 1 mm^3 of 10 μM solution of each primer, 4 mm^3 of H_2O and 5 mm^3 of cDNA (50 ng mm^{-3}). The qPCR regime consisted of an initial denaturation at 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 s, 55 °C for 15 s, 72 °C for20 s. A melting curve was generated for each reaction to verify the specificity of the amplification. Relative transcript abundances were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Data analysis: The experiments included three biological replicates. Each biological replicate included three technical replicates. Relative transcript abundances were log_2 transformed, and the profiles were compared by *Cluster v3.0 s*oftware (De Hoon *et al*. 2004), and visualized using *Treeview* software (Eisen *et al*. 1998). Statistical analyses were performed using *SPSS v. 17.0* package (*SPSS Inc*., Chicago, IL, USA).

Results

A total of 25 distinct *GRAS* sequences was retrieved from the chrysanthemum transcriptome. The 25 GRASs have been designated as *CmGRAS1* through *CmGRAS25*, however, *CmGRAS7* lacking of conservative motifs and *CmGRAS14* with incomplete ORF were not included. The length of remaining 23 *CmGRASs* cDNA ranged from 1 482 to 2 848 bp. Their predicted proteins were in a length of 414 - 810 residues (46.86 - 91.24 kDa) with a pI of 4.87 - 8.45 (Table 1). The proteins were predicted to be localized in the nucleus. An alignment of the deduced polypeptide sequences of 23 chrysanthemum GRASs with those of eight representative species of angiosperms (Cenci and Rouard 2017) were performed, *CmGRASs* subjected to nine recognized clades, namely PAT, SHR, SCL32, SCL4/7, SCL3, DELLA, HAM, LISCL, and SCR (Fig. 1 Suppl.). Nine CmGRAS proteins were identified as orthologs of GRAS, for example, *CmGRAS5* is the OG of *CcPAT3*, *CmGRAS6* is the OG of *AtSCL8*, *CmGRAS9* is OG of *CcSHR*; *CmGRAS11* is the OG of *AtGRAS*; *CmGRAS21* is OG of *TcSCL3*; and *CmGRAS25* is the OG of *CcLISCL*. Two pair of paralogous *CmGRAS* were identified, *CmGRAS19/GRAS20* and *CmGRAS12/GRAS16*.

The carboxy termini of the GRAS proteins were characterized by the motifs LHR I and II, VHIID, PFYRE, RVER, and SAW (Fig. 1, Fig. 2 Suppl.). Though all the *CmGRASs* harbored VHIID and SAW motifs, LHR I and II, VHIID, PFYRE, and RVER motifs were only detected in the PAT, LISCL, SCL3, SCL4/7, and SCR-type chrysanthemum *GRASs*. The motif 7 (harboring relatively conserved DELLA residues) was detected in *CmGRAS 9* of SHR subfamily and *CmGRAS17*, *18*, *19*, *20* in DELLA subfamily; motif 8 (with a conserved glutamic acid residue) was shared by *A. thaliana* SCL9 subfamily and *CmGRAS15*, *23*, *24*, and *25* (Fig. 1, Fig. 1 Suppl.). The motif 9 (although only the aspartic acid and alanine were absolutely conserved) was presented in *CmGRAS17*, *18*, and *20* of DELLA subfamily (Fig. 1, Fig. 2 Suppl.).

The transcriptional profiles of the *CmGRAS* genes were characterized in the root, stem, leaf, pollen, and tubular and ray florets at the bud stage. Generally, the abundance of transcript was lowest in the root with the

exception of *CmGRAS10* and *CmGRAS16*. The transcription abundance of *CmGRAS11, 12, 23*, and *18* was highest in the stem compared with other tissues. The expressions of *CmGRAS16* and *20* were highest in ray florets in comparison to other *CmGRASs*. The expressions of *CmGRAS2*, *CmGRAS4*, and *CmGRAS19* were highest in pollens. All the *CmGRASs*, with exception of *CmGRAS2*, *3*, *18*, *19*, and *20*, showed higher expressions in leaves than in the remaining tissues. The transcription profiles of *CmGRAS19* and its paralog *CmGRAS20* were very similar, but this was not the case for the other paralogous pair *CmGRAS12* (expressed mainly in stem) and *CmGRAS16* (mainly in ray florets). *CmGRAS5* and *CmGRAS21* were primarily expressed in tubular florets and leaf, respectively (Fig. 2).

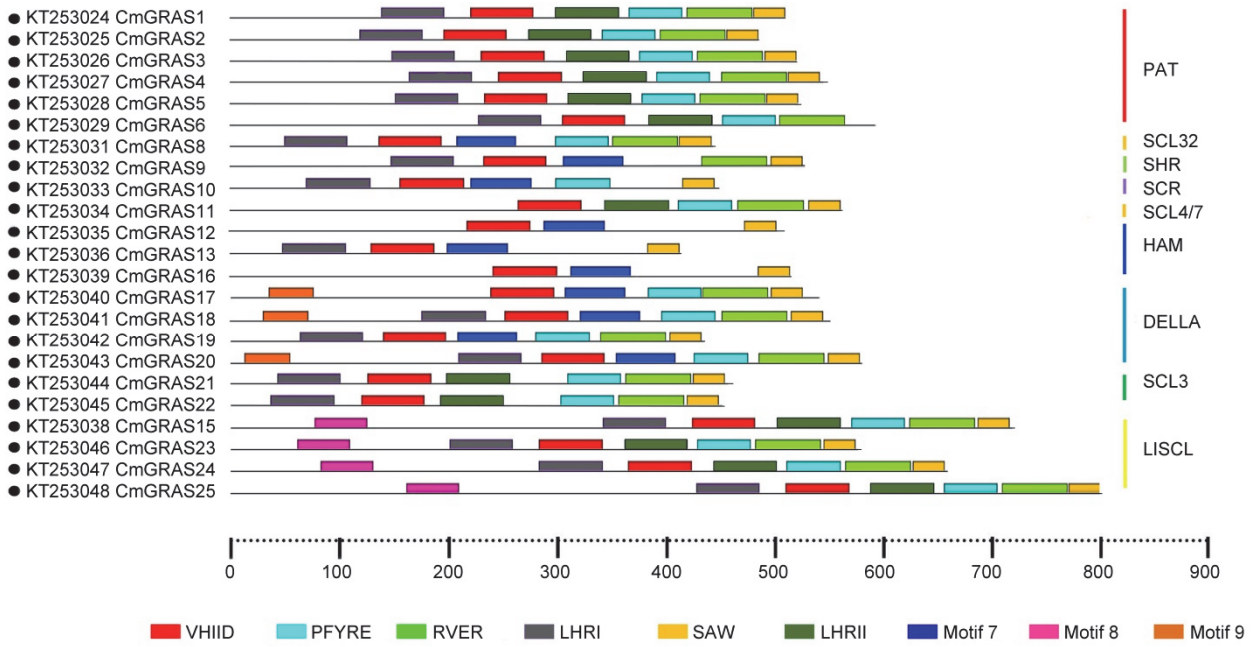

Fig. 1. The content of conserved motifs in different subclades of *CmGRAS*. Motifs 1 through 6 refer to VHIID, PFYRE, RVER, LHRI, SAW, and LHRII, respectively; motifs 7 through 9 are not known outside of chrysanthemum.

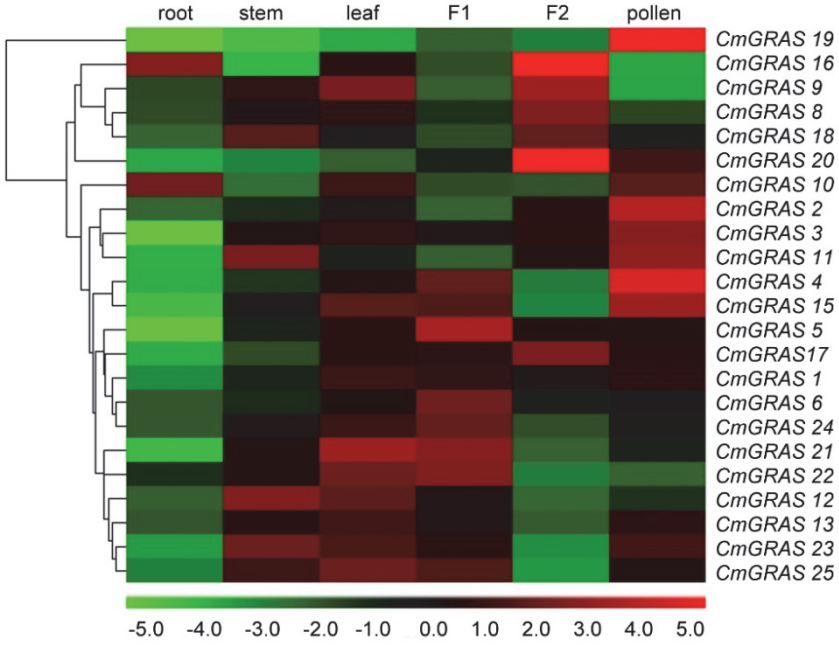

Fig. 2. *CmGRAS* transcription profiling in roots, stems, leaves, pollen, and tubular (F1) and ray florets (F2) sampled at the budding stage. Up-regulated genes are shown in *red* and down-regulated ones in *green*.

When exposed to exogenous phytohormones, expression of *CmGRAS10* and *23* were induced by ABA within 1 h, *CmGRAS8* within 4 h, and *CmGRAS2, 9, 11*, and *13* within 24 h; while the remaining sixteen genes (*1*, *3-6*, *12*, *15-18*, *19-22*, *24* and *25*) were all down-regulated by ABA (Fig. 3*A*). Transcriptions of *CmGRAS11* and *24* were induced after a 1 h exposure to MeJA, additional 12 *CmGRASs* (*1-4*, *11-13*, *16*, *18*, *20*, *24*, and *25*) were enhanced after a 4 h exposure to Me-JA, and *CmGRAS1-4* remained up-regulated until 24 h after MeJA treatment. The MeJA treatment suppressed the transcription of *CmGRAS19* and *CmGRAS21*, while nine

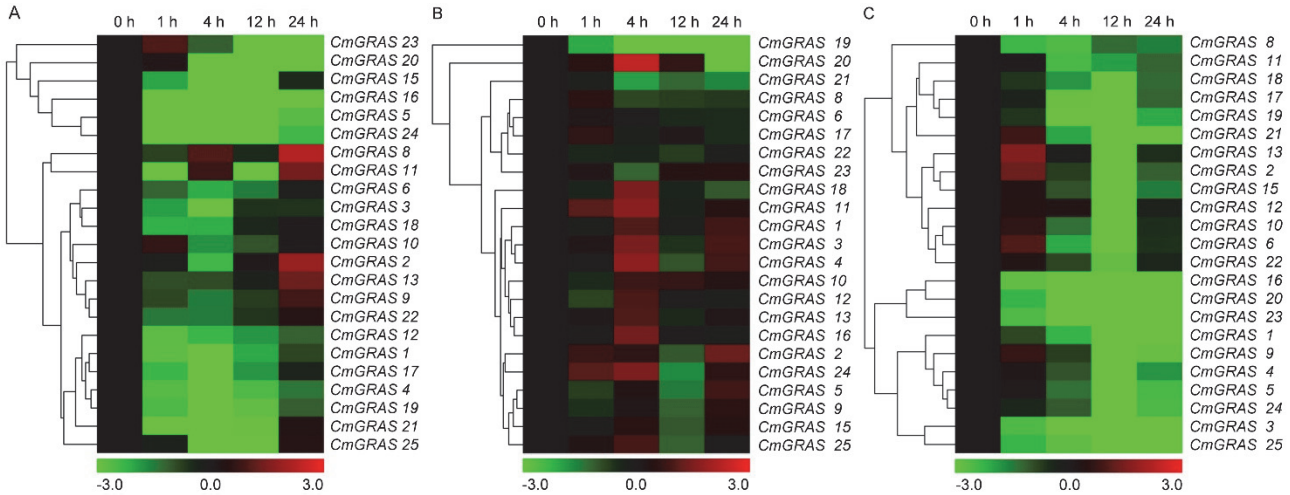

Fig. 3. The transcriptional response of the *CmGRAS* genes in leaf tissue following spraying with 50 μM ABA (*A*), 1 mM MeJA (*B*), and 200 μM SA (*C*). Up-regulated genes are shown in *red* and down-regulated ones in *green*.

Table 1. Twenty-three *CmGRAS* genes identified in *Chrysanthemum* transcriptome and homolog genes. Subcellular location in the nucleus (N) was predicted by *Softberry* programs *ProtComp 9.0 Prediction* (the numbers represent the reliability of the results, the total score is 10), the molecular mass (Mr) and the theoretical isoelectric point (PI) were evaluated by *ExPASy*.

Gene	GenBank acc. No.	Amino acids	Subclades	GRAS homologs	Locus name	PI	Mr	Subcellular localization
CmGRAS1	KT253024	516	PAT	*OsCIGR2*	Os07g39470	5.46	57917.33	N(5.33)
CmGRAS2	KT253025	491	PAT	*AtPAT1*	AT5G48150	5.54	55030.63	N(5.22)
CmGRAS3	KT253026	526	PAT	*CcPAT2*	Cc05_g13080	6.33	59309.37	N(5.59)
CmGRAS4	KT253027	551	PAT	*CcPAT1*	Cc09_g00640	5.67	61714.48	N(5.38)
CmGRAS5	KT253028	528	PAT	*CcPAT3*	Cc10_g13150	5.06	59707.32	N(5.38)
CmGRAS6	KT253029	600	PAT	*AtSCL8*	AT5G52510.1	6.36	66123.43	N(7.06)
CmGRAS8	KT253031	449	SCL32	*CcSCL32*	Cc07_g06890	5.68	50187.47	N(8.72)
CmGRAS9	KT253032	534	SHR	*CcSHR*	Cc07_g07090	5.75	61020.84	N(7.36)
CmGRAS10	KT253033	450	SCR	*TcSCR*	Tc01_g017050	5.11	49721.73	N(8.83)
CmGRAS11	KT253034	561	SCL4/7	*AtGRAS*	AT3G50650	4.87	62556.49	N(8.51)
CmGRAS12	KT253035	504	HAM	*CcHAM-I*	Cc07_g09180	5.18	56614.22	N(8.46)
CmGRAS13	KT253036	414	HAM	*TcHAM-II*	Tc01_g038130	7.62	46866.62	N(8.78)
CmGRAS15	KT253038	725	LISCL	*CcLISCL*	Cc01_g13060	5.28	82584.45	N(8.81)
CmGRAS16	KT253039	527	HAM	*CcHAM-I*	Cc07_g09180	5.67	58659.64	N(8.20)
CmGRAS17	KT253040	547	DELLA	*CcDELLA*	Cc06_g09110	5.51	60022.95	N(8.80)
CmGRAS18	KT253041	551	DELLA	*CcDELLA*	Cc07_g13590	5.04	60315.39	N(9.07)
CmGRAS19	KT253042	439	DELLA	*CcDELLA*	Cc11_g08290	5.15	48181.74	N(8.95)
CmGRAS20	KT253043	586	DELLA	*CcDELLA*	Cc11_g08290	4.94	63682.97	N(8.83)
CmGRAS21	KT253044	462	SCL3	*TcSCL3*	Tc10_g000490	5.79	51892.84	N(5.41)
CmGRAS22	KT253045	456	SCL3	*CcSCL3*	*Cc01_g05900*	6.21	51325.13	N(5.55)
CmGRAS23	KT253046	582	LISCL	*CcLISCL*	Cc01_g13070	6.02	66737.39	N(8.62)
CmGRAS24	KT253047	666	LISCL	*CcLISCL*	Cc02_g07650	6.15	75364.85	N(8.82)
CmGRAS25	KT253048	810	LISCL	*CcLISCL*	Cc06_g22670	8.45	91249.09	N(8.81)

genes (*5*, *6*, *8-10*, *15*, *17*, *22*, and *23*) were unaffected by the treatment (Fig. 3*B*). In response to exposure to SA, six of the genes (*2*, *6*, *9, 10*, *13*, and *21*) were induced within 1 h but were down-regulated thereafter; the remaining 17 genes were down-regulated by the whole treatment (Fig. 3*C*).

As concerns the response to salinity stress, *CmGRAS1*, *3*, *6*, *15*, *17, 23,* and *25* were mildly suppressed by NaCl; whereas expressions of *CmGRAS5*, *8*, *10*, *12*, *13*, *16*, *18*, *20-22* were initially mildly inductive up to 12 h, but all these genes except *CmGRAS8* and *20* were suppressed after 12 h. *CmGRAS8* was down-regulated after 12 h, while *CmGRAS20* remained up-regulated till 24 h. Expression of *CmGRAS2*, *9*, and *11* were induced within 1 h, and expression of *CmGRAS4* was not affected by salinity (Fig. 4A). When challenged with PEG6000, *CmGRAS12* and *25* were up-regulated within 1 h, but were repressed after 4 and 24 h. *CmGRAS21*, *23*, and *24* were both up-regulated within 1, 24, and 12 h, respectively. The remaining 19 genes were all down-regulated by the PEG treatment (Fig. 4*B*). Under low temperature, *CmGRAS2-4*, *8*, *12*, *15*, and *18* were strongly repressed within 1 h, and *CmGRAS6*, *9*, *10*, *19*, and *25* were also decreased under low temperature. However, *CmGRAS1*, *3*, *5*, *8*, *12*, *13*, *16-18*, and *20-23* were all up-regulated after a 4 h exposure to 4 °C, while *CmGRAS8* was strongly induced at both the 4 and 24 h time points (Fig. 4*C*). Under high temperature, *CmGRAS6*, *9*, *10*, *11*, *13*, *16-20* were rapidly down-regulated, in contrast to the up-regulation of *CmGRAS3*, *5*, *8*, *21-23*. The *CmGRAS1*, *4*, *15*, and *25* were all initially repressed, but later moderately induced. Neither *CmGRAS12* nor *24* was affected by the treatment (Fig. 4*D*). *CmGRAS18-20*, *24* and *25* were strongly up-regulated by wounding, while *CmGRAS1*, *2*, *5*, *6*, *8-10*, *13*, *16*, *17*, *21*, and *23* were all down-regulated. The abundance of *CmGRAS3*, *4*, and *15* transcripts was initially increased, but later fell below the pre-treatment level, while *CmGRAS11* and *12* were both up-regulated after 4 h (Fig. 4*E*).

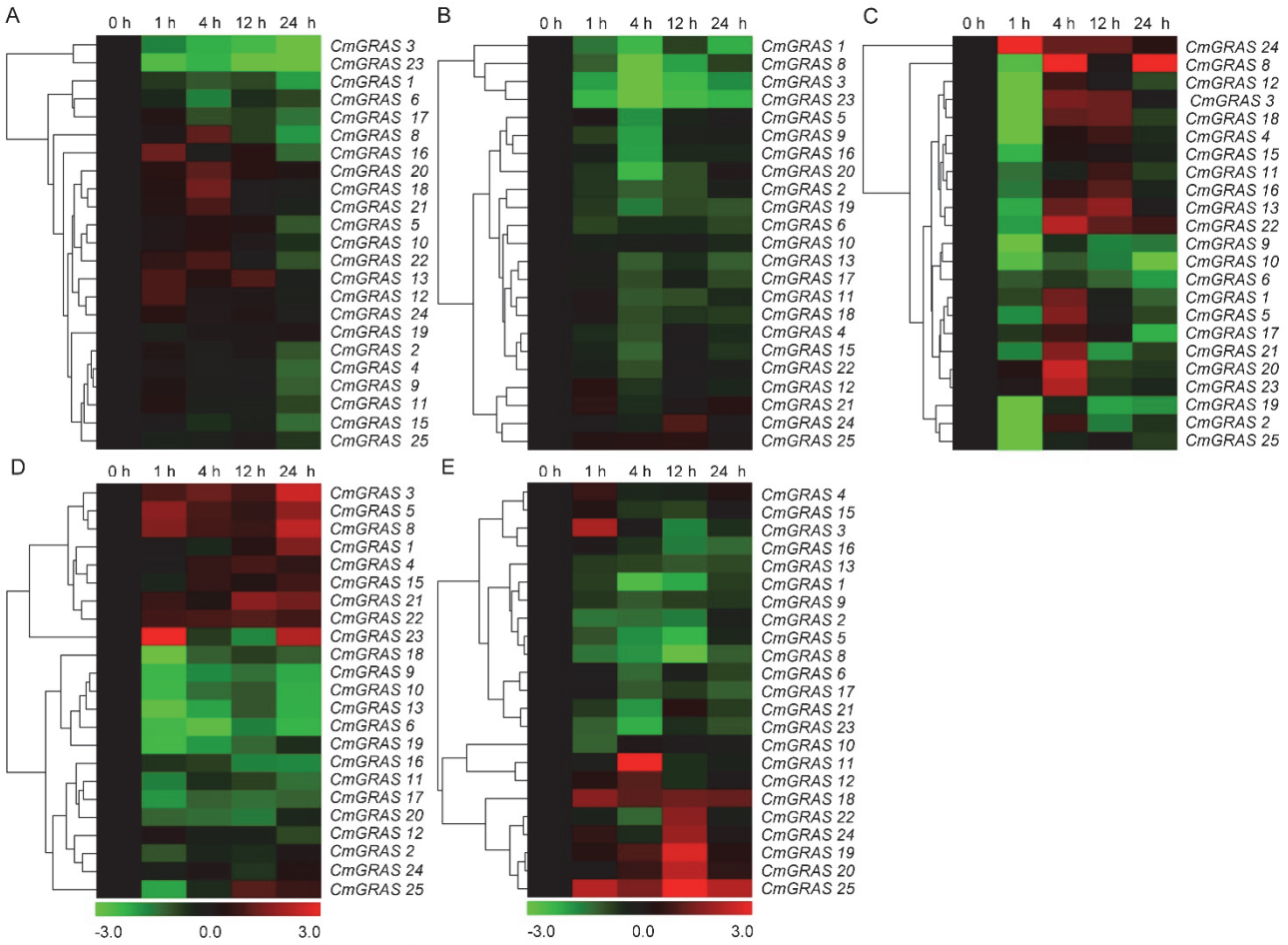

Fig. 4. Transcriptions of *CmGRAS* genes in leaf tissue following exposure to 200 mM NaCl (*A*), 20 % PEG 6000 (*B*), low temperature of 4 °C (*C*), high temperature of 40 °C (*D*), and wounding (*E*). Up-regulated genes are shown in *red* and down-regulated ones in *green*.

Discussion

A phylogenetic analysis demonstrated that the chrysanthemum *GRAS* genes fell into nine of the 17 sub-families represented in Fig. 1 Suppl. The chrysanthemum *GRAS* sequences harbored most of the conserved *GRAS* motifs, implying that their function may be inferred from that of relevant orthologs. The DELLA proteins, well-known members of the *GRAS* family, negatively regulate the gibberellin response. Functional mutations in the DELLA domain produce gibberellin insensitivity, manifested as a dwarf phenotype in *A. thaliana*, barley, maize, wheat, and rice (Peng *et al*. 1999, Banno *et al*. 2001, Chandler *et al*. 2002, Willige *et al*. 2007). Four of the chrysanthemum genes (*CmGRAS17-20*) belong to the DELLA clade, which suggests that one or more could be targets for inducing a semi-dwarf stature. In *Brassica napus*, the VHIID motif interacts with a histone deacetylase, which has been suggested to regulate genes expression (Gao *et al*. 2004). The VHIID motif and the SAW motif were both harbored by all of the *CmGRAS* members, but this was not the case for any of the other motifs: the implication is that SAW and VHIID are fundamental for function and so have been well conserved during evolution.

Several *CmGRASs* were transcribed preferentially in reproductive tissue. For example, *CmGRAS4*, *19*, and *20* were all strongly transcribed in the pollen and the ray florets, and *CmGRAS5* and *6* were strongly transcribed in the tubular florets (Fig. 2). Transcription of the *A. thaliana* ortholog of *CmGRAS4* (*AtSCL13*) was similarly abundant in the inflorescences, and its knock-out resulted in precocious flowering (Torres-Galea *et al*. 2006); the implication is that *CmGRAS4* might play a significant role in flowering and the development of flower. *CmGRAS12* expressed highly in stem and was included in HAM subfamily, hairy meristem mutation of *Petunia hybrid* is essential and specific for maintaining the shoot apical meristem (Stuurman *et al*. 2002). SCL3 proteins were involved in the elongation of the root and shoot organs, respectively. Expression of *CmGRAS21*, member of SCL3 subfamily, was highest in leaves, so it might be involved in the leaf development of chrysanthemum. In the root, the elongation is regulated by GA signaling pathway, whereas in the meristematic tissue, the combination of SHR/SCR regulate timing of periclinal division for endodermis and additional cortex at later stages *via* the GA signaling pathway (Heo *et al*. 2011).The expression of *CmGRAS10*, member of SCR subfamily, was highest in the root, and its *A. thaliana* ortholog *AtSCL23* was known to be involved in the determination of cell fate in the root endodermis (Long *et al*. 2015, Yoon *et al*. 2016); the result suggests that *CmGRAS10* possibly regulated the development of root in the chrysanthemum.

Hormones and hormonal cross-talk play an important role in plant development and stresses response. Abscisic acid is the principal hormone that regulates plant responses to abiotic stresses. However, it is becoming increasingly evident that ABA interacts with JA and SA signaling pathway (Santino *et al*. 2013). Jasmonic acid increases trichome density and number, while SA suppresses them (Traw and Bergelson 2003). MeJA and SA interacted with ABA to promote stomatal closure, which helped to minimize water loss in osmotically stressed plants (Hossain *et al*. 2011, Miura *et al*. 2013). GRAS proteins are known to regulate a number of phytohormone-responsive genes, thereby mediating the response to a variety of abiotic stresses (Fode *et al*. 2008). The expression of *CmGRAS1*, *3*, *4*, *11*, *12*, *16*, *18*, *20*, *24*, and *25* were induced by MeJA while suppressed by the SA (Fig. 3*B*,*C*), suggesting that these *CmGRASs* might be members of JA and SA signaling crosstalk. Noteworthy, *CmGRAS1*, *3*, *4*, *12*, *16*, *18*, *20*, *24*, and *25* induced by MeJA were down regulated by SA and ABA (Fig. 3*A*), suggesting that these *CmGRASs* might play roles in ABA mediated regulation of JA and SA signaling, which was important for the crosstalk between these three phytohormones. In addition, the expression of several *GRASs* in the chrysanthemum in response to phyto-hormones are different from *A. thaliana*. For example, the abundance of *AtSCL15*, also named as *AtHAM4* based on its genetic interaction with two *Petunia* HAM homologs *AtHAM1* and *AtHAM2* (Engstrom *et al*. 2011), responded positively to the exogenous ABA (Gao *et al*. 2015). However, its homologs *CmGRAS12* and *CmGRAS16* were suppressed by ABA application (Fig. 3*A*), suggesting functional diversification of members of HAM subfamily.

Inducible expression of *CmGRAS12*, *21*, *24*, and *25* by PEG treatment suggested their potential roles in the drought stress response, while these four members were overall suppressed by ABA, inferred that their roles in drought response is independent of ABA. In contrast, the expression of *CmGRAS1*, *3*, *4*, *15-20*, *22*, and *23* were down-regulated by PEG and ABA treatment, which suggesting that they probably regulated the drought response *via* ABA, since it is is a well-known that ABA is involved in drought or osmotic stresses regulation (Zhang *et al*. 2006). Induction of *CmGRAS5*, *8*, *10*, *12*, *13*, *16*, *18*, *20*, *21*, and *22* transcriptions by NaCl implied their positive contribution to salt tolerance, the expression of *CmGRAS12* and *21* were induced by both PEG and NaCl, suggested that these two members might be involved in the osmotic stress response. For the temperature stress, expressions of *CmGRAS3*, *5*, *8*, and *21-23* were induced, whereas, those of *CmGRAS6*, *9*, and *10* were inhibited by low and high temperatures, suggesting their participation in the response to extreme temperatures. In contrast, the expressions of *CmGRAS13*,

and *16-18* were induced by low temperature while suppressed by high temperature. Abundance of *CmGRAS11*, *3*, *4*, *24*, *18*, *20*, and *25* were increased by wounding and JA, while decreased by SA, suggesting antagonistic roles of JA and SA in regulating those *CmGRASs* in response to wounding. An induction of the *CmGRASs* by wounding and JA suggested their roles in wounding *via* JA (Fig. 4E). DELLA subfamily functions as negative regulators in gibberellin signal transduction (Heo *et al*. 2011, Zhang *et al*. 2011). *Arabidopsis* DELLAs maintained low ROS content under salt stress, thus delaying cell death and promoting tolerance, and loss the function DELLA mutants are sensitive to cold (Achard *et al*.2008). The expression of *CmGRAS18*, *20*, members of DELLA family, were induced by NaCl and cold temperature, implicated their positive roles in salt and low temperature tolerance, probably *via* maintaining ROS content under the stresses. *A. thaliana* plants overexpressing SCL4/7 subfamily genes showed increased tolerance to salt and drought stresses (Ma *et al*. 2010). Expression of *Populus euphratica* ortholog *PeSCL7* was induced during the early stage of severe salt-stress (Wang *et al*. 2011). Similarly, expression of *CmGRAS11*, member of SCL4/7 subfamily, was induced at early stage by NaCl, suggested a conserved function in salt tolerance of *CmGRAS11* of SCL4/7 members. In *A. thaliana*, the abundance of *SCL13* transcript, member of PAT subfamily, was unaffected when the plants were treated with cytokinin, auxin, ethylene, gibberellins, or brassinosteroids, but it did respond to treatment with either MeJA, ABA, salinity, low temperature, osmotic stress, or wounding (Torres-Galea 2006). By comparison, the transcription of its chrysanthemum homolog *CmGRAS4* was lowered by an ABA or a SA treatment, by exposure to PEG6000 or low temperature stress; however, it was elevated by MeJA treatment, by exposure to salinity, high temperature, or wounding (Fig. 4*B*,*D*,*E*). Thus, the roles of these two homologs only partially overlapped.

Conclusions

This study provides a first transcriptome-wide analysis of the *GRAS* gene family in *Chrysanthemum*, where *CmGRAS4*, *5*, *6*, *19*, and *20* were strongly transcribed in the flowers, *CmGRAS12* in the stem, *CmGRAS21* in leaf, and *CmGRAS10* in the root, suggesting their potential roles in corresponding tissue development. *CmGRAS1*, *3*, *4*, *12*, *16*, *18*, *20*, *24*, and *25* were induced by MeJA, while down regulated by SA and ABA, inferring that these *CmGRASs* might play roles in ABA mediated balance of JA and SA signaling. *CmGRAS1*, *3*, *4*, *15-20*, *22*, *23* facilitated responses to PEG could be ABA dependent, in contrast, responses of *CmGRAS12*, *21*, *24,* and *25* to drought stress might be independent of ABA. *CmGRAS5*, *8*, *10*, *12*, *13*, *16*, *18*, *20*, *21*, and *22* positively responded to salt stress. *CmGRAS12* and *21* might be involved in the salt derived osmotic stress response. *CmGRAS3*, *5*, *8*, *21-23*, and *CmGRAS6*, *9*, *10* showed similar expression profiles upon low or high temperature. Abundance of *CmGRAS11*, *3*, *4*, *24*, *18*, *20,* and *25* was increased by wounding and JA, while decreased by SA. Taken together, present study should facilitate the elucidation of the functional significance of members in this transcription factor family.

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