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ORIGINAL ARTICLE

Genome-wide identification and expression analysis of the GRAS family proteins in *Medicago truncatula*

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Abstract The GRAS gene family performs a variety of functions in plant growth and development processes, and they also play essential roles in plant response to environmental stresses. *Medicago truncatula* is a diploid plant with a small genome used as a model organism. Despite the vital role of *GRAS* genes in plant growth regulation, few studies on these genes in *M. truncatula* have been conducted to date. Using the *M. truncatula* reference genome data, we identified 68 *MtGRAS* genes, which were classified into 16 groups by phylogenetic analysis, located on eight chromosomes. The structure analysis indicated that *MtGRAS* genes retained a relatively constant exon–intron composition during the evolution of the *M. truncatula* genome. Most of the closely related members in the phylogenetic tree had similar motif compositions. Different motifs distributed in different groups of the *MtGRAS* genes were the sources of their functional divergence. Twenty-eight *MtGRAS* genes were expressed in six tissues, namely root, bud, blade, seedpod, nodule, and flower tissues, suggesting their putative function in many aspects of plant growth and development. Nine *MtGRAS* genes were upregulated under cold, freezing, drought, ABA, and salt stress treatments, indicating that they play vital roles in the response to abiotic stress in *M. truncatula*. Our study provides valuable information that can be utilized to improve the quality and agronomic benefits of *M. truncatula* and other plants.

Keywords GRAS transcription factor · Exon–intron composition · Phylogenetic analysis · Bioinformatics · Gene expression

Abbreviations

GA	Gibberellic acid
GAI	Gibberellic acid insensitive
HAM	Hairy meristem
Ls	Lateral suppressor
NSP1	Nodulation signaling pathway 1
NSP2	Nodulation signaling pathway 2
PAT	Phytochrome A signal transduction
RGA	Repressor of GAI
SCR	Scarecrow
SCL	Scarecrow-like
SHR	Short root
TF	Transcription factor

Introduction

Transcription factors (TFs) are a vital part of functional genomics, which participate in various physiological processes and regulatory networks (Sun et al. 2012). The first TF was discovered in maize, and then a large number of TFs in vascular plants have been identified (Huang et al. 2015). GRAS family proteins are plant-specific TFs, which are named after the three functional members, GIBBERELLIC ACID INSENSITIVE (GAI), REPRESSOR OF GAI (RGA), and SCARECROW (SCR) (Sun et al. 2011). GRAS

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TFs typically consist of 400–770 amino acid residues and possess conserved motifs in their C-terminal region, including LHR I, VHIID, LHR II, PFYRE, and SAW (Bolle 2004). Other GRAS proteins are highly variable in N-terminal region, except that the DELLA subfamily contains conserved N-terminal domains of DELLA and TVHYNP (Tian et al. 2004; Lu et al. 2015). Based on the functional similarity and sequence homology, the GRAS protein family is divided into DELLA, SCR, PAT1, HAM, LS, SHR, and SCL9 protein subfamilies; each subfamily has distinct conserved domains and functions (Bolle 2004).

The GRAS family of putative transcriptional regulators has been identified in vascular plants including *Arabidopsis*, tomato, *Prunus mume*, rice, and *Populus* (Mayrose et al. 2006; Lee et al. 2008; Lu et al. 2015; Liu and Widmer 2014). These proteins play important role in plant growth and development, such as root and shoot development, phytochrome A signal transduction (PAT), gibberellic acid (GA) signal transduction, and disease resistance (Hirsch and Oldroyd 2009; Lu et al. 2015). PAT1 protein in *Arabidopsis thaliana* participates in phytochrome A signal transduction (Bolle et al. 2000), and *Lateral suppressor* (*Ls*) gene from tomato plays a significant role in the formation of lateral branches (Schumacher and Theres 1999). SHORT ROOT (SHR) is a TF essential for endodermis specification in the *Arabidopsis* root. SCR is a member of the GRAS protein family associated with radial patterning of both roots and shoots. Recently, it has been shown that BnSCL1, a SCARECROW-LIKE (SCL) protein from *Brassica napus*, is expressed predominantly not only in roots but also in shoots, suggesting its mode of action in the plant's auxin response (Gao et al. 2004). SCL from *Lilium longiflorum* plays a transcriptional regulation role during microsporogenesis within the lily anther (Morohashi et al. 2003). Arbuscular Mycorrhizal 18 (OsAM18) is a GRAS protein in rice, which likely affects the colonization process and functionality of arbuscular mycorrhiza, and the systemic arbuscular mycorrhizal symbiosis (Fiorilli et al. 2015). A class of GRAS proteins, the DELLA proteins, regulates plant growth through hormone signaling. DELLA mutants are partially insensitive to gene induction in methyl-jasmonate treatment, whereas the constitutively active dominant DELLA mutant *gai* is sensitized for jasmonate; these results indicated that DELLA proteins are involved in the perception of jasmonate signaling (Navarro et al. 2008). *Helianthus annuus* GRAS-like gene (*Ha-GRASL*) lacking the DELLA motif belongs to the SCL4/7 subfamily. The metabolic flow of gibberellins was reduced in overexpressed *Ha-GRASL Arabidopsis*, and this modification could be relevant in axillary meristems development (Fambrini et al. 2015). In a recent study, the Jumonji-C domain-containing gene *JMJ524* was not only upregulated by GA treatment in tomato, but it also responded to circadian rhythms. Moreover, *JMJ524* altered GA responses by stem elongation or by regulating SlGLD1, a GRAS protein lacking DELLA domain, at least partially (Li et al. 2015). The GRAS proteins NODULATION SIGNALING PATHWAY 1 (NSP1) and NSP2 from *Medicago truncatula* directly bind to the promoter of a nodulation-associated gene, highlighting the importance of GRAS proteins for efficient nodulation in legume plants (Hirsch et al. 2009; Kaló et al. 2005). In *Lotus japonicus*, the NSP1 protein is involved in bacterial release, infection, and normal bacteroid formation in nodule cells (Heckmann et al. 2006).

Plant-specific GRAS TFs also play diverse roles in response to biotic and abiotic stress conditions. In a recent study, *PeSCL7* was highly induced by high-salt and drought treatment but repressed by GA treatment in poplar leaves. Overexpressing *PeSCL7* from poplar improved drought and salt tolerance in *Arabidopsis* (Ma 2010). Overexpressed *OsGRAS23* in rice enhanced drought tolerance by reducing H_2O_2 accumulation in cells (Xu et al. 2015). *Triticum aestivum TaSCL14* was expressed in various wheat organs by high light stress, with high levels detected in stems and roots. The study on silencing *TaSCL14* in wheat revealed that *TaSCL14* may act as a regulator involved in plant growth, photosynthesis, and tolerance to oxidative stress (Chen et al. 2015). The increased expression of the *GRAS* gene family in tomato (*Solanum lycopersicum*), which responds to disease stress, in part depends on jasmonic acid signaling. Expression of *SlGRAS6* is suppressed by virus-induced gene silencing, impairing tomato resistance to *Pseudomonas syringae* (Mayrose et al. 2006).

Medicago truncatula is a diploid plant with a small genome and undetermined growth of nodules (López et al. 2008). The high-efficiency genetic transformation makes it an excellent model organism in studies on legume plants. Characterization of GRAS proteins, which has been conducted in other plant species, in *M. truncatula* is focused on plant signal transduction, growth, and nodulation signaling of these proteins. In this study, 68 members from the *MtGRAS* gene family were analyzed comprehensively, and the analysis included phylogenetic analysis, gene structure, chromosomal location, and expression profiles under different abiotic stresses. The investigation of *GRAS* genes in the whole genome of *M. truncatula* in response to abiotic stresses is essential, because it will provide an essential foundation for functional study on GRAS proteins in *M. truncatula*.

Materials and methods

Identification and characteristics of the GRAS family

The whole genome sequence of *M. truncatula* and the summary of gene annotation were downloaded from the Phytozome database (http://phytozome.jgi.doe.gov/

medicago.php) (Goodstein et al. 2012). The hidden Markov model (HMM) profiles of the GRAS domain PF03514 were downloaded from Pfam database (Punta et al. 2012). HMM search of the GRAS domain PF03514 profiles from *M. truncatula* protein database were conducted with a cutoff *E* value of 1.0 (Johnson et al. 2010). After determining the GRAS domain profile, the integrity of the GRAS domain was evaluated with the online program SMART with an *E* value <0.1 (http://smart.embl-heidelberg.de/) (Letunic et al. 2012).

Phylogenetic analysis and conserved motifs and intron/exon structure identification

To investigate the phylogenetic relationship of the GRAS protein families in *M. truncatula*, full-length GRAS protein sequences were retrieved from Phytozome, and all the candidate GRAS proteins were aligned to *Arabidopsis* GRAS proteins (Goodstein et al. 2012). GRAS proteins sequence information of *A. thaliana* previously reported was retrieved from the *Arabidopsis* Information Resource (Grimplet et al. 2016). GRAS TFs were aligned using BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html), and phylogenetic trees were constructed using neighbor-joining method implemented in MEGA5.1 program with 1000 replicates (Tamura et al. 2011). The resulting tree file was visualized by Tree View1.6 (Shen et al. 2015). Branches corresponding to partitions that received less than 50% bootstrap support were collapsed. Genes were classified according to the distance homology with *Arabidopsis* genes (Lee et al. 2008).

To identify the unknown conserved motifs using the online MEME (http://meme.ebi.edu.au/meme/intro.html) analysis (Bailey and Elkan 1994), the parameters were as following: 1) optimum motif width was set to ≥6 and ≤50 (inclusive); 2) identification of the maximum motif number was set to 25; 3) the distribution of a single motif occurrences among sequences was set to 0 or 1 occurrence per sequence (-mod zoops). The MEME motifs were annotated using the Pfam database (Bailey et al. 2015). Based on the *M. truncatula* genome, the DNA and cDNA sequences corresponding to each predicted gene and the information about *MtGRAS* intron distribution pattern were obtained from Phytozome (Goodstein et al. 2012).

Chromosomal location and gene duplication of *GRAS* genes

Based on gene annotation information, sequences of putative *GRAS* genes were extracted from the whole genome sequence using a Perl script. *M. truncatula GRAS* gene structures was displayed using Gene Structure Display Server program (http://gsds.cbi.pku.edu.cn/index.php) (Hu et al. 2015). *GRAS* family genes were subjected to a BLASTN search to explore gene duplication. If two genes shared more than 70% similarity, they were identified as tandem duplications or segmental duplications (Li et al. 2016). The chromosomal locations of *GRAS* genes in *M. truncatula* were plotted by Circos software (Krzywinski et al. 2009).

In silico expression analysis of *GRAS* genes from *M. truncatula*

Genome-wide transcriptome data from different development tissues in *M. truncatula* were downloaded from the NCBI database (Accession No.: SRX099057–SRX099062) (Liu et al. 2015). The transcriptome data were obtained from six tissue types: root, bud, nodule, seedpod, blade, and flower tissues. TopHat and Cufflinks were used to analyze per million mapped reads (Trapnell et al. 2009, 2010). The expression profiles of the *MtGRAS* genes were retrieved from these expression data, analyzed, clustered, and displayed by the ggplot2 package in R software (Version 3.1.0) (Wickham 2009).

Expression of GRAS protein under abiotic stress in *M. truncatula*

According to the *M. truncatula* transcriptome data downloaded from the NCBI database (Accession numbers: SRX1056987–SRX1056992), the molecular functions of *MtGRAS* genes were investigated under abiotic stress. All of the 8-week-old seedlings of *M. truncatula* 'Jemalong A17' were randomly divided into six groups for stress treatments, namely control (untreated) and treatment with cold (4 °C), freeze (−8 °C), salt (200 mM NaCl solution), drought (300 mM mannitol solution), and abscisic acid (ABA) solution (100 μM ABA). All the seedlings were harvested 3 h after the treatment, and five whole seedlings per group were bulked separately as described in Shu et al. (2016). The expression levels of GRAS TF genes were detected in transcriptome data, which included five abiotic stresses treatments (cold, freezing, salt, drought, and ABA stresses).

Quantitative real-time PCR analysis

The genes identified in the transcriptome analysis were validated and quantified by quantitative real-time PCR (qPCR). The primers were designed using Primer3plus 1.0.0 (Untergasser et al. 2012) based on transcriptome sequencing data; the primer pairs are listed in Table S1. According to manufacturer's instructions, the total RNA from six groups alfalfa mentioned above was extracted using an RNAprep Pure Plant Kit (Tiangen, Beijing,

China), and cDNA synthesized and qPCR analyzed were using a ReverTra Ace kit and SYBR Premix Ex TaqTM II (Toyobo, Shanghai, China), respectively. qPCR was performed in a Roche LightCycler 96 System (Roche, China). *MtActin* was used as an internal control to normalize the expression levels (Zhang et al. 2011). The details of qPCR analysis referenced to Song et al. (2016).

Results

Identification and phylogenetic analysis of GRAS proteins in *M. truncatula*

To identify the full complements of *GRAS* genes in *M. truncatula*, BLASTP searches were performed using the *GRAS* genes from *Arabidopsis* and the *M. truncatula* genome. Sixty-eight proteins were identified as predicted by *GRAS* genes in total. The length of amino acid sequences encoded by MtGRAS varied from 69 (MtGRAS26) to 805 (MtGRAS62) amino acids, the molecular mass ranged from 7939.1 (MtGRAS26) to 89030.2 (MtGRAS62) kDa, and the pI values of MtGRAS proteins varied from 4.72 (MtGRAS61) to 9.8 (MtGRAS48) (Table 1).

The evolutionary relationship between *M. truncatula* and *Arabidopsis* was assessed with respect to GRAS TFs and visualized with a neighbor-joining phylogenetic tree. As shown in Fig. 1, 16 groups were obtained according to the clade support values, topology of the tree, and classification of *Arabidopsis*. Among the GRAS proteins in *M. truncatula*, eight subfamilies, SCL3, HAM, SCL, SHR, PAT, SCR, DELLA, and LAS, were previously described in other plant species, and two newly detected subfamilies, GRAS8 and SCL26, were also found in *Vitis vinifera*. The remaining six subfamilies, labeled GRASM1–GRASM6, formed a clade with SCL3, SCL26, and SCL. The smallest subfamily was GRAS8, with only one member, and the largest subfamily was PAT, which contained 12 members. Regarding the PAT subfamily, 12 *MtGRAS* genes were clustered and homologous with genes from *Arabidopsis* and *V. vinifera*, indicating that they might be derived from gene duplications of the same gene locus during *M. truncatula* genome evolution. These results suggested that these paralogous genes may perform the same function in the life cycle of *M. truncatula*.

Structure and conserved motifs of GRAS family in *M. truncatula*

Exon–intron structural diversity is an important part of gene families' evolution, and provided additional evidence to support phylogenetic groupings (Wei et al. 2016). To elucidate phylogenetic relationship and classification of MtGRAS, we employed the GRAS domain (PF03514) to search against these predicted *GRAS* genes. All predicted *GRAS* genes containing conserved motifs in the C-terminal and N-terminal region of their corresponding GRAS proteins were highly variable. The number of introns varied from one to seven, which was consistent with *GRAS* genes in *M. truncatula* (Fig. 2). The exon and intron number in *M. truncatula GRAS* genes ranged from 1 to 8 and 0 to 7, respectively. Among the 68 *MtGRAS* genes, 41 genes had introns, 34 genes had only one intron, and 27 genes had no introns. Group GRASM3 contained 0–7 introns, group GRASM6 contained 0–5 introns, group GRASM1 contained 1–4 introns, group PAT contained 0–2 introns, group SCL26 contained 0–4 introns, groups GRASM2, GRASM5, SCL, SCR, LAS, SCL26, and SHR contained 0–1 intron, groups GRASM4 and HAM contained only one intron, and groups GRAS8 and DELLA had no introns. Generally, *MtGRAS* genes within the same group showed similar exon–intron structure in the phylogenetic tree.

The MEME analysis of GRAS family proteins in *M. truncatula* revealed 25 conserved motifs (designated motifs 1–25) (Fig. 3, Fig. S1; Table S2). Among those, motif 1 encoding the GRAS domain was found in all *MtGRAS* genes and was the most conserved motif in all MtGRAS proteins. A combination of motif 3 and other motifs (motif 2, 4, 13, 15, 17, and 19) was found in the C terminus, while the N terminus contained various motifs. The results indicated that most of the closely related members had similar motif composition in the phylogenetic tree, while among different groups or subgroups the motifs were divergent. For example, groups SHR, SCR, and DELLA had eight similar conserved motifs (motifs 9, 6, 5, 1, 8, 4, 14, and 3), groups SCL3, SCL26, and SCL had only one similar motif (motif 5), group HAM had four conserved motifs (motifs 12, 8, 4, and 3), and five motifs (motifs 3, 4, 1, 2, and 5) were present in groups GRASM2–5. While motif 19 was specific to group GRASM6, motif 25 was specific to groups SCL3 and GRASM1, and motifs 11, 15, 18, and 20 were specific to groups GRASM2–4. Based on the results, it indicated that different motifs distributed in different groups of the *MtGRAS* genes were the sources of their functional divergence.

Chromosomal locations and gene duplication analysis of *MtGRAS* Genes

The physical locations of 68 *GRAS* TF genes were distributed on eight chromosomes of *M. truncatula* shown in Fig. 4. Each chromosome in *M. truncatula* contained between 1 and 16 *GRAS* genes. Chromosome 4 had the highest number of *GRAS* genes (16 genes) distributed throughout the chromosome. Chromosomes 2 and 3

Table 1 The characteristic of *GRAS* genes in *Medicago truncatula* genome

Gene name	Gene locus	Chromosome location	Length (aa)	Molecular weight (kDa)	PI	Subgroup
MtGRAS30	Medtr3g065980	chr3:29799202–29797559	547	60,002.6	5.01	DELLA
MtGRAS48	Medtr4g122240	chr4:50482784–50480334	117	13,469.8	9.8	DELLA
MtGRAS46	Medtr4g102790	chr4:42605026–42601807	677	75,349	5.51	GRAS8
MtGRAS22	Medtr3g021320	chr3:6233722–6234191	130	14667	7.7	GRASM1
MtGRAS23	Medtr3g022005	chr3:6413225–6412663	128	15,457.9	8.45	GRASM1
MtGRAS24	Medtr3g022580	chr3:6727556–6726015	186	21,812.2	8.27	GRASM1
MtGRAS25	Medtr3g022830	chr3:6862026–6858778	438	49,706.3	6.61	GRASM1
MtGRAS27	Medtr3g027430	chr3:8573822–8572567	333	38,417.9	9.51	GRASM1
MtGRAS17	Medtr2g097410	chr2:41596394–41599413	743	84,140.6	5.42	GRASM2
MtGRAS34	Medtr4g064120	chr4:23903648–23905939	628	71,758.9	5.86	GRASM2
MtGRAS35	Medtr4g064150	chr4:23914035–23910953	735	83,871.8	5.19	GRASM2
MtGRAS37	Medtr4g064180	chr4:23923635–23921018	628	72,032.3	5.86	GRASM2
MtGRAS13	Medtr2g097310	chr2:41556096–41558077	640	73,256	5.71	GRASM3
MtGRAS14	Medtr2g097350	chr2:41569861–41571797	642	73,409.6	5.4	GRASM3
MtGRAS15	Medtr2g097380	chr2:41581189–41584207	563	64,537.9	7.66	GRASM3
MtGRAS16	Medtr2g097390	chr2:41588763–41590819	643	73,552.5	5.16	GRASM3
MtGRAS18	Medtr2g097463	chr2:41619642–41622827	657	74,536.9	5.16	GRASM4
MtGRAS19	Medtr2g097467	chr2:41622958–41625456	656	74,704.1	5.77	GRASM4
MtGRAS20	Medtr2g097473	chr2:41625860–41628478	656	74,496.9	5.63	GRASM4
MtGRAS38	Medtr4g064200	chr4:23928886–23931600	652	73,442.7	5.84	GRASM4
MtGRAS47	Medtr4g104020	chr4:43029167–43030858	521	58,794.2	6.43	GRASM5
MtGRAS58	Medtr7g027190	chr7:9074523–9072111	674	74,869.2	5.65	GRASM5
MtGRAS68	Medtr8g442410	chr8:16023753–16022101	536	60,329.3	4.84	GRASM5
MtGRAS4	Medtr1g086970	chr1:38927951–38926509	480	54,823.4	6.84	GRASM6
MtGRAS8	Medtr2g034250	chr2:13084065–13082144	587	67,184.1	5.23	GRASM6
MtGRAS9	Medtr2g034260	chr2:13088374–13086374	586	67,296.7	5.17	GRASM6
MtGRAS10	Medtr2g034280	chr2:13094204–13092471	577	65,847.8	5.14	GRASM6
MtGRAS63	Medtr7g104380	chr7:42300897–42299395	228	26,040.8	6.32	GRASM6
MtGRAS64	Medtr7g109580	chr7:44838783–44836902	556	64,507.5	5.61	GRASM6
MtGRAS1	Medtr0092s0100	scaffold0092:52124–49118	729	81,433.8	5.61	HAM
MtGRAS33	Medtr4g026485	chr4:9111815–9114789	625	70,120.8	5.44	HAM
MtGRAS53	Medtr5g019750	chr5:7489471–7487775	295	33,956.5	8.61	HAM
MtGRAS66	Medtr8g077940	chr8:33202769–33205096	542	60,492.4	5.58	HAM
MtGRAS5	Medtr1g096030	chr1:43289642–43291027	461	52,766.1	5.73	LAS
MtGRAS43	Medtr4g077760	chr4:29814298–29811772	555	61,509.9	5.09	LAS
MtGRAS2	Medtr1g029420	chr1:10095890–10092148	592	65,944.8	4.79	PAT
MtGRAS7	Medtr2g026250	chr2:9568804–9567008	598	67,703.5	5.39	PAT
MtGRAS11	Medtr2g082090	chr2:34499615–34496389	579	64,449.5	5.88	PAT
MtGRAS29	Medtr3g056110	chr3:22303144–22299859	542	61,852.5	5.12	PAT
MtGRAS32	Medtr3g089055	chr3:40767318–40769015	565	63,676.5	4.96	PAT
MtGRAS39	Medtr4g074310	chr4:28295694–28296517	225	25,671.4	4.9	PAT
MtGRAS40	Medtr4g074320	chr4:28297237–28296671	188	21,186.3	7.72	PAT
MtGRAS49	Medtr4g133660	chr4:55915463–55912594	544	61,196.1	5.6	PAT
MtGRAS55	Medtr5g094450	chr5:41280345–41277425	532	60,268.2	5.1	PAT
MtGRAS56	Medtr5g097480	chr5:42699470–42703065	544	61,006.9	5.96	PAT
MtGRAS57	Medtr6g047750	chr6:17217691–17220135	662	73,400.2	6.28	PAT
MtGRAS59	Medtr7g057230	chr7:20532599–20530626	657	73,671	6.12	PAT
MtGRAS36	Medtr4g064160	chr4:23918205–23915589	686	78,061	5.72	SCL

Table 1 continued

Gene name	Gene locus	Chromosome location	Length (aa)	Molecular weight (kDa)	PI	Subgroup
MtGRAS60	Medtr7g062120	chr7:22543834–22546912	742	84,092.2	6.18	SCL
MtGRAS31	Medtr3g072710	chr3:32727063–32725078	508	56,336.9	5.71	SCL26
MtGRAS54	Medtr5g058860	chr5:24257848–24256328	506	56,739.5	4.8	SCL26
MtGRAS61	Medtr7g069740	chr7:25713741–25715739	585	67,022.3	4.72	SCL26
MtGRAS67	Medtr8g093070	chr8:38898984–38900682	507	58,326.4	4.76	SCL26
MtGRAS6	Medtr1g106590	chr1:48194978–48191881	342	38,704.3	5.63	SCL3
MtGRAS26	Medtr3g025340	chr3:7746409–7746689	69	7939.1	6.7	SCL3
MtGRAS42	Medtr4g076140	chr4:29138995–29141367	472	53,482.5	6.64	SCL3
MtGRAS50	Medtr5g009080	chr5:2087595–2085054	481	54,037.8	5.52	SCL3
MtGRAS3	Medtr1g069725	chr1:30384606–30383115	472	53,482.5	6.64	SCR
MtGRAS41	Medtr4g076020	chr4:29063677–29061945	438	48,640.8	5.04	SCR
MtGRAS62	Medtr7g074650	chr7:27914302–27910369	805	89,030.2	6.1	SCR
MtGRAS12	Medtr2g089100	chr2:37602592–37604640	458	51,691.2	5.58	SHR
MtGRAS21	Medtr2g099110	chr2:42486505–42484922	451	51,774.4	5.63	SHR
MtGRAS28	Medtr3g053270	chr3:21161852–21163198	448	50,729.9	6.22	SHR
MtGRAS44	Medtr4g095500	chr4:39825882–39823303	470	52,854.8	5.78	SHR
MtGRAS45	Medtr4g097080	chr4:39986688–39988664	504	57,826.3	5.09	SHR
MtGRAS51	Medtr5g015490	chr5:5366627–5364696	491	55,787.5	5.34	SHR
MtGRAS52	Medtr5g015950	chr5:5616565–5617911	448	50,729.9	6.22	SHR
MtGRAS65	Medtr8g020840	chr8:7352443–7354694	554	61,826.6	5.76	SHR

contained 11 genes, distributed on both ends of the chromosome 2 or evenly distributed on chromosome 3. Moreover, chromosome 6 contained only one gene located in the middle of the chromosome. The *GRAS* genes were not randomly distributed on each chromosome, as is the case with some gene clusters on chromosomes forming "hot regions." For example, chromosome 4 contained five *GRAS* genes (*MtGRAS34–38*) in a short chromosome region; similar gene clusters were observed in chromosomes 2, 3, and 5.

MtGRAS gene clusters or hot regions were distributed in different chromosomes, such as clusters *MtGRAS 34–38*, *MtGRAS 39–40*, *MtGRAS 41–42*, *MtGRAS 44–45*, and *MtGRAS 46–47* on chromosome 4; clusters *MtGRAS 8–10* and *MtGRAS 13–16* on chromosome 2; cluster *MtGRAS 22–24* on chromosome 3; cluster *MtGRAS 51–52* on chromosome 5. Many homologous *GRAS* genes on different chromosomes, which were produced by segmental duplication, will expand the number of *MtGRAS* genes from the same group. For example, as products of genome segmental duplication, *MtGRAS4* and *MtGRAS64* from group GRASM6 were distributed on chromosome 1 and chromosome 7, respectively, and *MtGRAS6* and *MtGRAS50* from group SCL3 were distributed on chromosome 1 and 5, respectively. In addition, 14 pairs of gene duplications that arose from tandem duplications and segmental duplications were identified in this study. *MtGRAS* gene clusters or hot regions were produced by tandem duplications, such as the cluster *MtGRAS13–16* on chromosome 2 and cluster *MtGRAS44–45* on chromosome 4.

Expression patterns analysis of *GRAS* genes in *M. truncatula* tissues

The *MtGRAS* gene expression profiles in six tissues (nodule, root, bud, flower, blade, and seedpod tissues) were investigated. The number of expressed genes in the six tissues was 51, 51, 45, 44, 42, and 31, respectively. The highest expression of *MtGRAS* genes was detected in nodule and root tissue (51/68, 75%), followed by bud (45/68, 66%), flower (44/68, 64.7%), blade (42/68, 61.8%), and seedpod (31/68, 45.6%) tissue. Of the 28 *MtGRAS* genes expressed in all tissues, 24 were upregulated, suggesting that the function of *MtGRAS* genes may affect plant growth and development, such as root system regulation, embryonic development, apical dominance, and shoot initiation and growth. The transcription patterns of *MtGRAS* genes were clustered across the six tissues (Fig. 5). All *MtGRAS* genes, except *GRAS53*, that were clustered in groups HAM, SCL, SCR, GRAS8, and DELLA were highly expressed across all the six tissues. A number of genes were expressed only in one tissue; for example, *GRAS3* and *GRAS67* were specifically expressed in the nodule, and *GRAS10*, *GRAS22*, and *GRAS24* were specifically

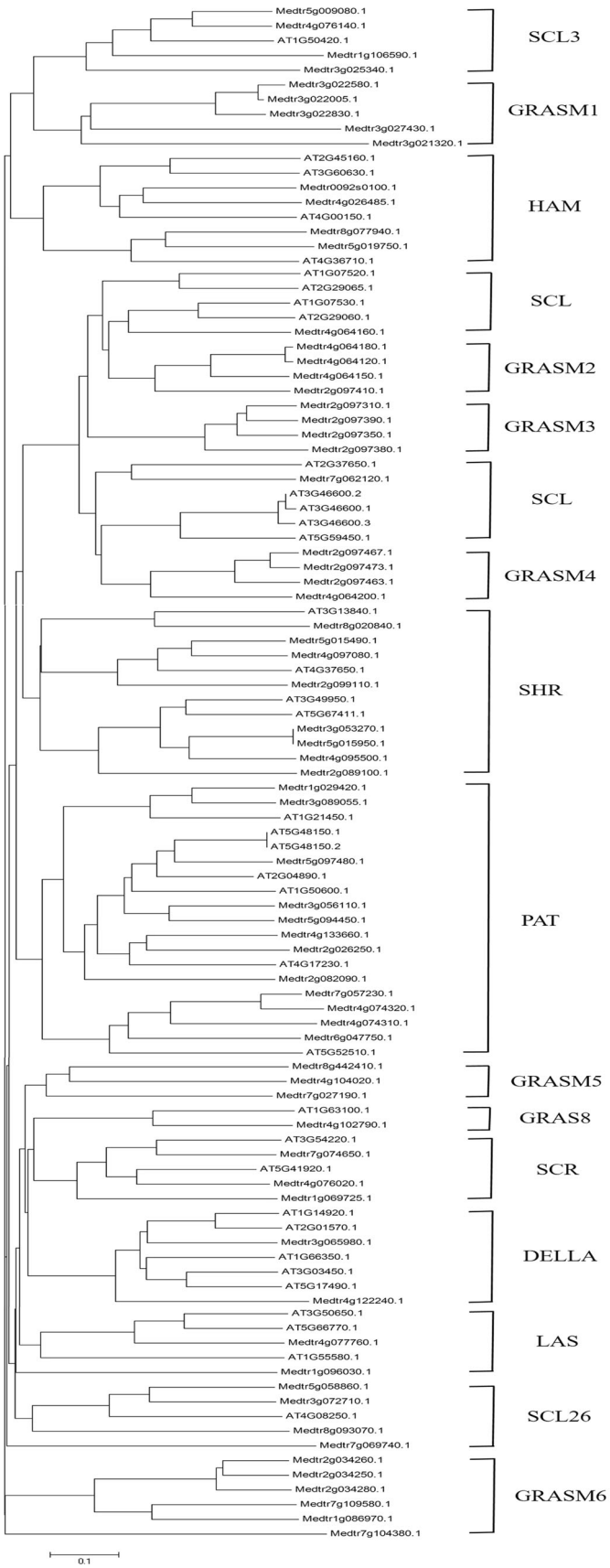

Fig. 1 Phylogenetic analysis of *Medicago truncatula* and *Arabidopsis GRAS* genes. Sixteen subfamilies were identified in *M. truncatula*: the known SCL, SCL3, SCL26, SHR, SCR, HAM, PAT, LAS, DELLA, GRAS8, and six new subfamilies GRASM1-6

Fig. 2 Exon–intron structure of *GRAS* genes in *Medicago truncatula*. *Green boxes* indicate exons, and *black lines* indicate introns. The *scale* represents 2500 bp. *Thin lines* indicated the length by the scale

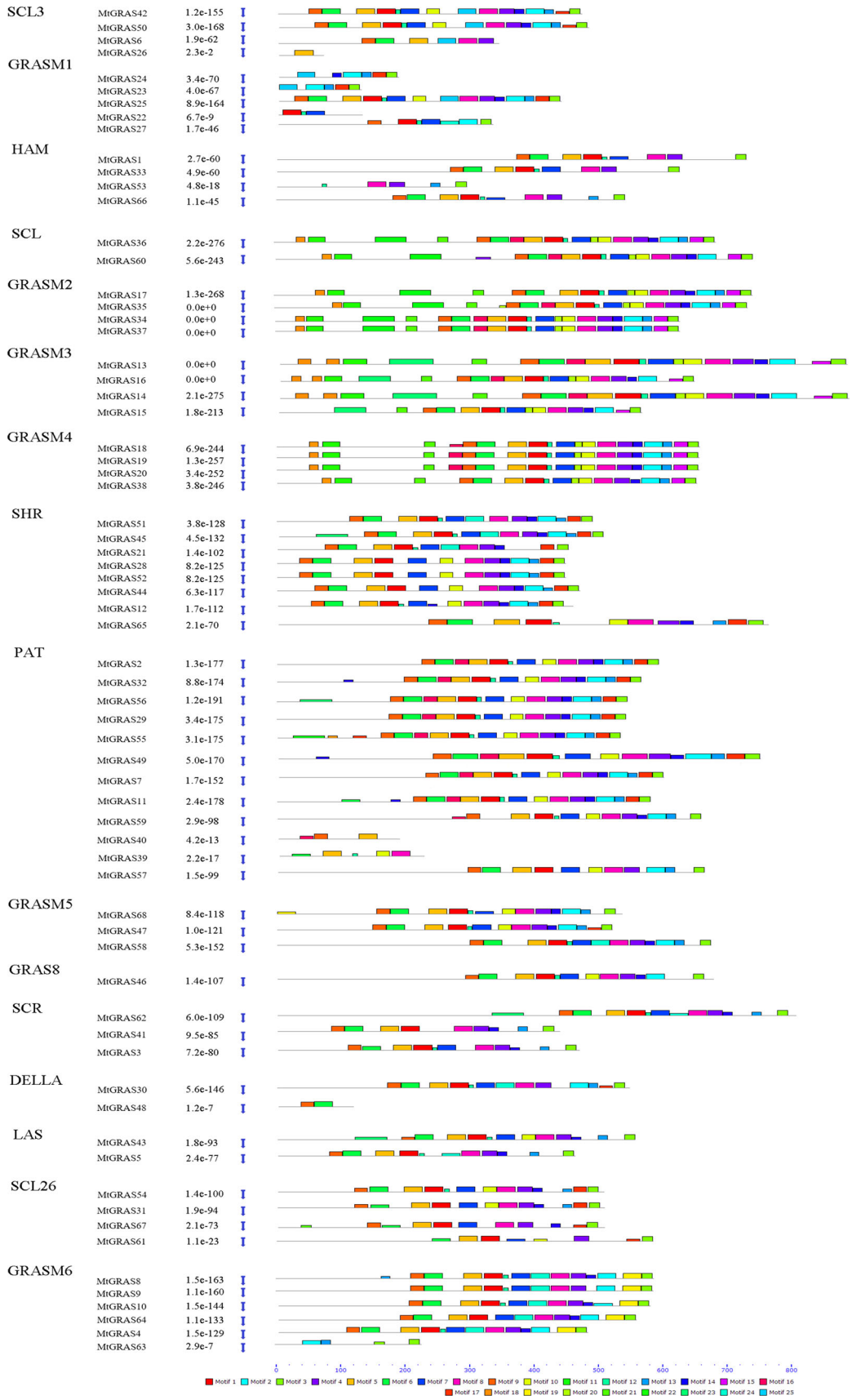

Fig. 3 Distribution of conserved motifs within *GRAS* genes family in *Medicago truncatula*

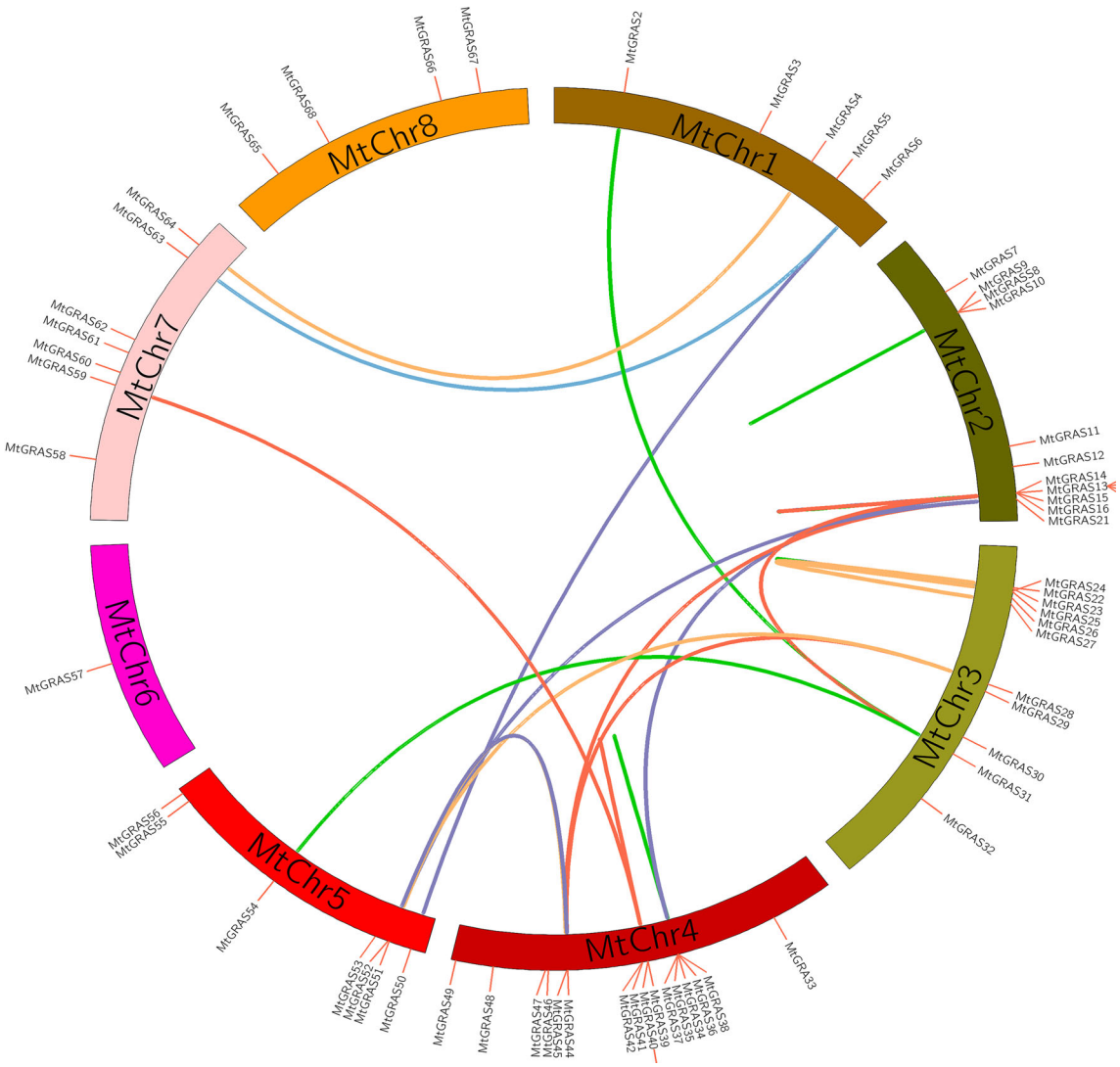

Fig. 4 Chromosomal location of *Medicago truncatula* GRAS proteins. Similarity of segmental duplicate genes is linked by *different lines*. The *red lines* represent less than 95% similarity, the *orange lines* represent 90–95% similarity, the *purple lines* represent 85–90% similarity, the *blue lines* represent 80–85% similarity, and the *green lines* represent 75–80% similarity

expressed in the root, suggesting that the expression of these genes is organ specific. Eleven *GRAS* genes (4, 23, 26, 27, 28, 39, 40, 52, 53, 58, and 67) were not expressed in any tissues. These results indicated that *MtGRAS* genes were expressed in a particular tissue or at certain developmental stages and thus were involved in the development of *M. truncatula*.

Expression analysis of *MtGRAS* genes responses to abiotic stress

The expression levels of *GRAS* genes under stress treatments (cold, freezing, salt, drought, and ABA) were detected to explore the molecular functions of *MtGRAS* genes under abiotic stress (Accession numbers: SRX1056987–SRX1056992). Compared to the control library, of the 68 expressed *MtGRAS* genes, we identified 44 *MtGRAS* genes that were differentially expressed under five stress conditions (Fig. 6). In total, 48 *MtGRAS* genes were differentially expressed under freezing and drought stress, 47 *MtGRAS* genes were expressed under salt and cold treatment, and 45 *MtGRAS* genes were differentially expressed in ABA treatment. The expression of 22, 24, 25, 28, and 34 *MtGRAS* genes was downregulated under freezing, cold stress, drought, salt, and ABA stress, respectively. The other genes exhibited group-specific profiles; for example, the expression of *MtGRAS* genes clustered in groups GRASM4 and DELLA was highly upregulated under cold and freezing stress, and the expression of those clustered in groups GRASM2, GRASM4, and SCL was highly upregulated under drought stress. Notably, nine *MtGRAS* genes (*MtGRAS11*, *MtGRAS13*, *MtGRAS18*, *MtGRAS19*,

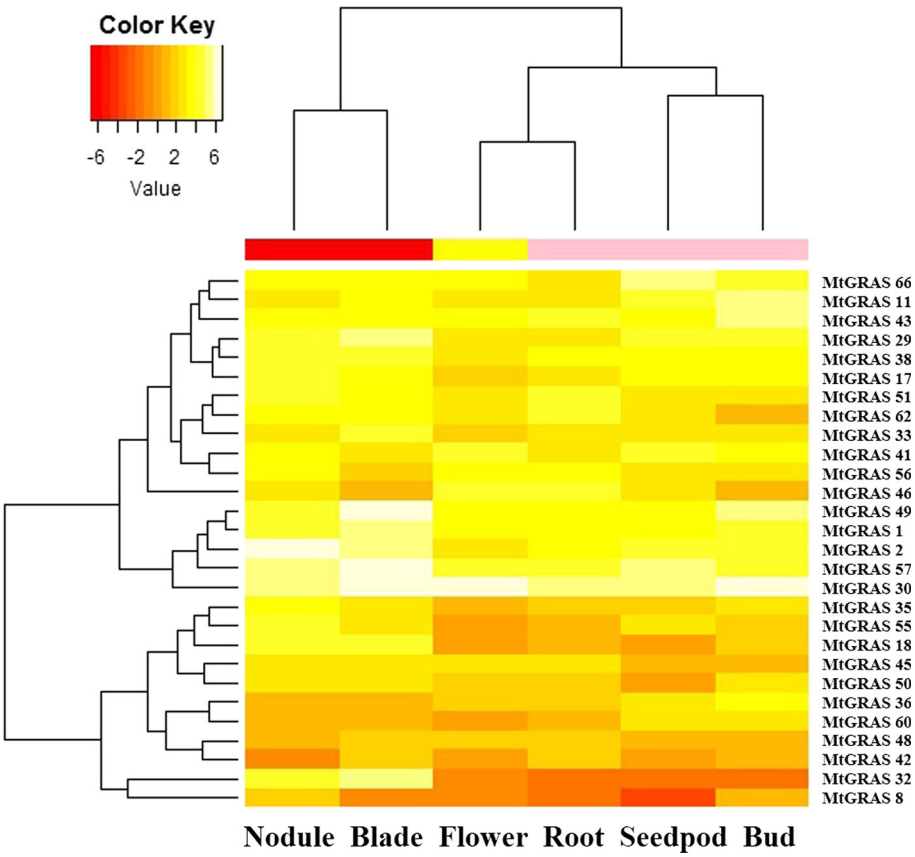

Fig. 5 Expression profile of *GRAS* genes in tissues of *Medicago truncatula*. In the heat map, *rows* represent genes, *while columns* represent different tissues, including nodule, blade, flower, root, seedpod and bud. *Red and light yellow color* gradients indicate a decrease and increase in transcript abundance, respectively

MtGRAS29, *MtGRAS35*, *MtGRAS37*, *MtGRAS38*, and *MtGRAS49*) were upregulated under all stress conditions. In summary, the expression level of these genes under different stress conditions was as follows. Under cold stress: 24 up-, 26 downregulated; freeze stress: 26 up-, 24 downregulated; drought stress: 23 up-, 28 downregulated; salt stress: 20 up-, 31 downregulated; ABA: 16 up-, 33 downregulated.

To further validate the reliability of the RNA-sequencing analysis, the expression of 16 subfamilies of GRAS proteins that were annotated by the NCBI Nr database was monitored by qPCR. Compared with the results of qPCR and RNA-Seq analysis, most of the *MtGRAS* gene expression patterns were basically the same, but the magnitude of the fold-changes was different between qPCR experiments and RNA-seq (Fig. 7). All of the above results indicated that *MtGRAS* genes play vital roles in response to abiotic stress, especially cold, freezing, and water deficit.

Discussion

With the development of bioinformatics analysis, various genomes with important information can be investigated to clarify the mechanisms about plant growth and development. Based on the *M. truncatula* genome database, 68 *MtGRAS* TF genes were identified and characterized in this study. The number of identified *GRAS* genes in *M. truncatula* is much higher than that in *Arabidopsis* (33), *Carica papaya* (42), *Vitis vinifera* (43), *Prunus mume* (46), and *Oryza sativa* (60), but lower than that in *Musa acuminata* (73), *Populus trichocarpa* (102), and *Malus* × *domestica* (127). Recent research on gene duplication events plays an essential role in the rapid expansion and evolution of gene families (Cannon et al. 2004; Lu et al. 2015). The considerably large *GRAS* gene family in *Malus domestica* is likely the result of a whole genome duplication event (Shu et al. 2016), whereas the *GRAS* gene family in *Medicago truncatula* has different characteristics and patterns of evolution.

Expansion of GRAS family in *M. truncatula*

The junction pattern analysis of the exon–intron can provide additional insights into gene families' evolution (Huang et al. 2015). The exon–intron structures of *MtGRAS* genes vary significantly among the 16 groups. The number of introns and exons in all groups varied from 0 to 7 and from 1 to 8, respectively. Among the 68 identified *MtGRAS* genes, 24 genes had no introns. Meanwhile, the exon–

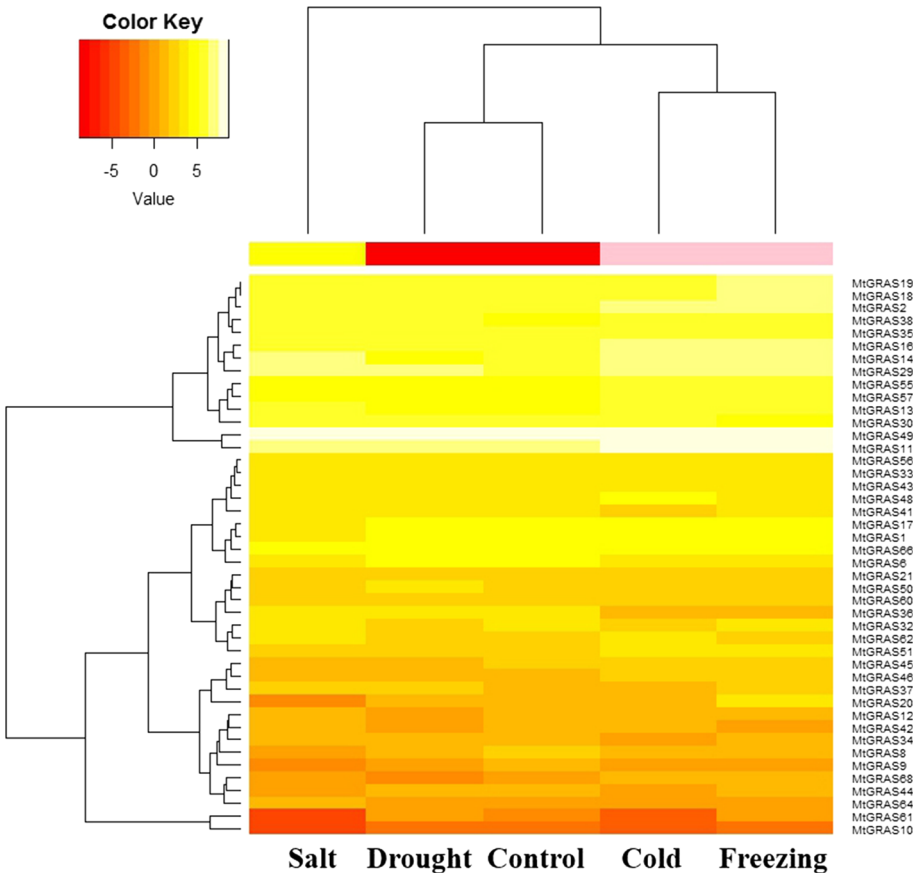

Fig. 6 Expression analysis of *GRAS* genes' response to abiotic stress in *Medicago truncatula*. In the heat map, rows represent genes, while columns represent different abiotic stress, namely cold, freezing, salt, ABA and drought stress. *Red and light yellow color* gradients indicate a decrease and increase in transcript abundance, respectively

intron analysis showed that 64.7% of *GRAS* genes lacked introns in *M. truncatula* (Fig. 2), compared to 82.2, 77.4, 67.6, 55, and 54.7% of genes that lacked introns in *Prunus mume*, tomato, *Arabidopsis*, rice, and *Populus*, respectively (Xin et al. 2016; Huang et al. 2015). This suggested that the structure of *GRAS* genes is species specific. Although, the groups had different intron structures, they had similar exon–intron structure, suggesting that the *GRAS* genes family retained a relatively constant exon–intron composition during the evolution of the *M. truncatula* genome. The different motifs were distributed in different groups of the *MtGRAS* genes and, therefore, were the source of their functional divergence.

Transcription regulators belonging to the same taxonomic group, exhibited a common evolutionary origins, and have conserved motifs associated with their molecular functions (Lu et al. 2015). A comparison of *GRAS* genes' structure enhances our understanding of the roles of these TFs in *M. truncatula*. The results form phylogenetic analysis indicated that *GRAS* genes, which are involved in plant development, were clustered in 16 groups in *M. truncatula*. The *MtGRAS* genes were distributed on eight chromosomes, but most of them were present on chromosome 4. The distribution of *MtGRAS* genes showed that recent duplication events have occurred in this gene family. Segmental duplication, which expanded the number of *MtGRAS* genes from different groups, produced many homologous *GRAS* genes on different chromosomes. For example, *MtGRAS28*, *44*, *45*, *51*, and *52* from group SHR are distributed on different chromosomes (*MtGRAS28* on chromosome 3, *MtGRAS44* and *45* on chromosome 4, *MtGRAS51* and *52* on chromosome 5) and are products of genome segmental duplication. These results suggested that the expansion of *MtGRAS* gene family is the result of gene duplication events during the evolutionary process.

Variety in the expression patterns of GRAS family members in different tissues

Recent studies on functional and structural genomics in vascular plant model species revealed that GRAS TFs are involved in plant development in processes including GA signal transduction (DELLA), phytochrome signal transduction (PAT1, SCL21, and SCL13), axillary shoot meristem formation (HAM and MOC1), cell maintenance and proliferation (SCR and SHR), root radial pattering, and male gametogenesis (LlSCL) (Song et al. 2014). With tissue-specific expression in various plant species, the *GRAS* gene family may play essential roles in plant tissue development. The tissue expression analysis showed that

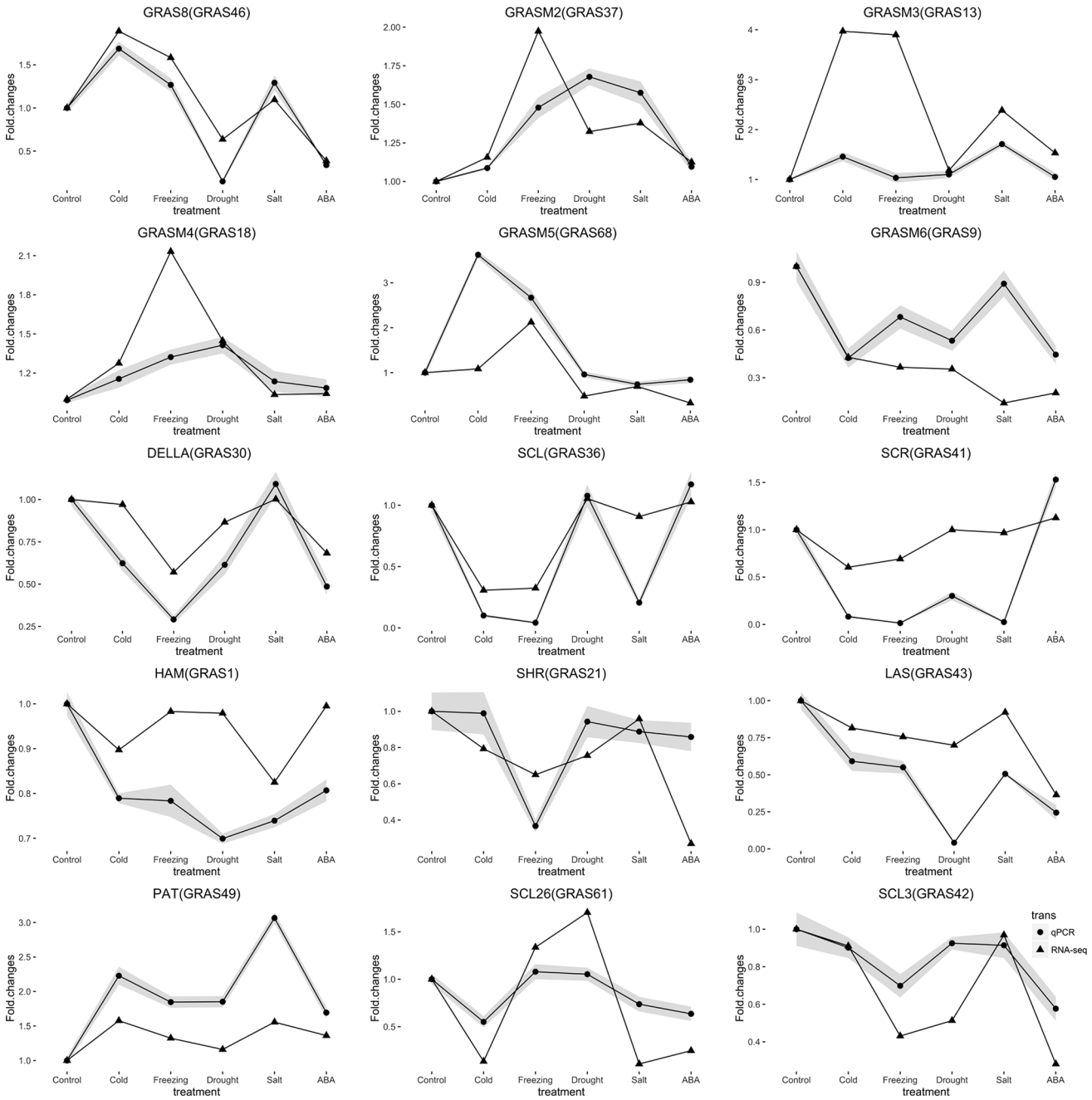

Fig. 7 Validation of transcriptome sequencing data by qPCR analysis. Expression analysis of 15 *GRAS* genes from different subfamilies' response to cold, freezing, salt, ABA and drought stress by qPCR. *MtActin* gene was used as internal control. The expression of the *MtActin* gene in non-treated controls was set to 1.0. The *y*-axis represents the relative expression, and the *x*-axis depicts the different abiotic stress. The mean values are calculated from three biological replicates. The *error bars* represented the standard deviation of the mean expression values

11 *MtGRAS* genes had no discernible expression level in any tissues/organs. These results suggest that those genes may have degenerated after gene duplication or undergone loss of function during the evolution of the gene family. Based on high-throughput sequencing data analysis, 58 *MtGRAS* genes were expressed in at least one tissue, and 28 *MtGRAS* genes were expressed in various tissues, root, blade, nodule, seedpod, bud, and flower tissues. These results suggested that these highly expressed genes may regulate plant growth and development. The groups HAM, SCL, SCR, GRAS8, and DELLA had the highest gene expression across all the six tissues during development. Notably, *MtGRAS3* (homologous to At5g41920.1, At3g54220.1) and *MtGRAS67* (homologous to

At4g08250.1) were specifically expressed in nodule tissue, whereas *MtGRAS10*, *22*, and *24* (not homologous to *Arabidopsis*) were expressed only in the roots. In the present study, the *MtGRAS* genes from SCR, SCL, and SHR groups were highly expressed in the root. SCR-regulated root meristem in *Arabidopsis* is located downstream of SHR. SCL13 regulates the expression of SIN1, which plays an essential role in lateral root elongation and root symbiosis and thereby nodulation in common bean (Mbe et al. 2014). These results suggested that the members from GRAS family in *M. truncatula* may have similar functions to those in *Arabidopsis* and grapevine. Many *PAT* genes, which regulate the phytochrome signal transduction pathway, might be involved in several developmental processes in *Arabidopsis*. A large number of genes from PAT subfamily in *M. truncatula* were highly expressed in the photosynthetic tissue, which was inconsistent with PAT expression in grapevine. These results indicated that PAT subfamily may regulate plant developmental processes, since various MtGRAS proteins were involved in diverse morphological features of plant development. In summary, the expression patterns of *MtGRAS* genes in different tissues may lay a foundation for further investigation of alfalfa development.

GRAS family putatively involved in biotic and abiotic stress

TF families are involved in the regulation of defense responses to environmental stresses in plants. Because of the functional diversity of *GRAS* genes, the GRAS TF family in *M. truncatula* need to be further studied. The expression patterns analysis of *MtGRAS* genes could be useful in assessing their possible functions under various abiotic stress conditions. Compared to control samples, there were 44 *MtGRAS* genes differentially expressed under abiotic stress treatments (shown in Fig. 6). Among these, *MtGRAS* genes from groups GRASM2–4 and PAT were induced in response to all the tested abiotic stress conditions. Overexpression of *VaPAT1*, which belongs to the GRAS family, increases the cold, high-salinity, and drought tolerance in transgenic *Arabidopsis* (Yuan et al. 2016). Meanwhile, we found that 54 genes were responsive to freezing or cold treatment. Interestingly, 22 *MtGRAS* genes were highly upregulated under both freezing and cold stresses, including the members from the groups PAT, GRAS8, and GRASM2–4. *MtGRAS* genes that belong to the SCL subfamily respond to drought stress, and overexpression of the poplar GRAS protein SCL7 enhances the tolerance of drought in *Arabidopsis* (Ma 2010). Two poplar homologs of SCL were putatively associated with salt tolerance according to the single nucleotide polymorphism (SNP) method (Galovic et al. 2015). Overall, the above results suggested that not only different *MtGRAS* gene but also a single *GRAS* gene may participate in multiple signaling and stress processes. Furthermore, most of the *MtGRAS* genes can be induced quickly and significantly by cold, freezing, and drought stresses, indicating that *GRAS* genes play vital roles in the regulation of *M. truncatula* response to abiotic stress.

Conclusions

In summary, 68 *GRAS* genes were identified from the *M. truncatula* genome sequence and their classification, structure, evolution, and tissue-specific expression were investigated. The results in this study revealed that *MtGRAS* genes are broadly involved in the regulation of plant tissue development, in which a large number of tissue-specific expression. Meanwhile, we identified a number of candidate *MtGRAS* genes that participate in nodulation process of alfalfa; these were also differentially expressed by freezing and/or cold treatment. In addition, the results of the qPCR analysis were corroborated by the transcriptome analysis. These results indicated that nodulation might critically contribute to freezing tolerance of alfalfa. Furthermore, the expression levels of *GRAS* genes under abiotic stresses indicated that they had a comprehensive response to salt, freezing, cold, ABA, and drought stresses, thereby implying that GRAS may represent convergence points among different signaling pathways. In summary, this study would provide a solid foundation for further functional analysis of *GRAS* genes to be used for transgenic applications.

Author contribution statement CG and LS conceived and designed the experiments; CG helped perform the analysis with constructive discussions. LS performed the experiments and wrote the manuscript. LT and HC contributed to treatment the materials. LL helped perform the analysis with the structure and chromosomal location of GRAS genes, and the expression of GRAS genes in different tissues. All authors read and approved the manuscript.

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