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Genome-wide identification and characterization of stress-associated protein (SAP) gene family encoding A20/AN1 zinc-finger proteins in *Medicago truncatula*

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Abstract: Stress associated proteins (SAPs) play important roles in developmental processes, responses to various stresses and hormone stimulation in plants. However, little is known about the SAP gene family in *Medicago truncatula*. In this study, a total of 17 MtSAP genes encoding A20/AN1 zinc-finger proteins were characterized. Out of these 17 genes, 15 were distributed over all 8 chromosomes at different densities, and two segmental duplication events were detected. The phylogenetic analysis of these proteins and their orthologs from *Arabidopsis* and rice suggested that they could be classified into five out of the seven groups of SAP family genes, with genes in the same group showing similar structures and conserved domains. The *cis*-elements of the *MtSAP* promoters were studied, and many *cis*-elements related to stress and plant hormone responses were identified. We also investigated the stress-responsive expression patterns of the MtSAP genes under various stresses, including drought, exposure to NaCl and cold. The qRT-PCR results showed that numerous MtSAP genes exhibited transcriptional responses to multiple abiotic stresses. These results lay the foundation for further functional characterization of SAP genes. To the best of our knowledge, this is the first report of a genome-wide analysis of the SAP gene family in *M. truncatula*.

Key words: *Medicago truncatula*; stress associated protein (SAP); gene family; expression analysis; abiotic stress

INTRODUCTION

Various biotic and abiotic stresses, including drought, salinity, high/low temperature and light intensity, have huge impacts on the growth and productivity of crop plants. Plants have evolved to resist these stresses by developing morphological, biochemical and physiological changes governed by genetic regulation at the transcriptional level [1]. In recent years, a zinc-finger protein family that contains an N-terminal A20 and a C-terminal AN1 zinc-finger domain and is collectively called stress associated proteins (SAPs) has been identified as an important gene family for the protection of plants against environmental stresses.

The A20/AN1 zinc-finger proteins are characterized by the presence of two specific zinc-finger domains, namely A20 and/or AN1 [2]. The A20 zinc-finger domain, which is characterized by multiple Cys_2/Cys_2 finger motifs, was first identified in a TNFα-inducible protein of human endothelial cells and plays an important role in regulating the immune response by inhibition of NFκB activity [3,4]. The AN1 zinc-finger domain was first identified as a putative zinc-finger domain in an ubiquitin-like fusion protein encoded by the *Xenopus laevis* animal hemisphere 1 (AN1) maternal RNA [5]. The AN1 zinc-finger domain is usually considered to be associated with the A20 zinc-finger [6], and they can interact with each other as determined by yeast two-hybrid analysis of OsSAP8 [7].

The A20/AN1 zinc-finger proteins are known to be involved in responses to various environmental stresses in both animals and plants. In animals, some A20/AN1 zinc-finger proteins play important roles

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in regulating the immune response, such as ZNF216 and AWP1 in humans [8-10], and ZNF216 in mice [11]. In plants, it has been well documented that A20/AN1 zinc-finger proteins play a central role in stress response and management. A genome-wide survey revealed that A20/AN1 zinc-finger proteins are present in many plant species, including rice (18 members), *Arabidopsis* (14 members), maize (11 members), sorghum (18 members), poplar (19 members) and grape (10 members) [12,13]. The Indica rice *OsiSAP1* was identified as the first plant protein having A20/AN1 zinc-fingers, and its transcription is induced by various stresses, including cold, desiccation, salt, submergence, heavy metals, wounding and abscisic acid (ABA). Overexpression of *OsiSAP1* could confer water-deficit stress tolerance to tobacco and rice [14,15]. Further studies indicated that the majority of OsSAP genes are inducible by one or more abiotic stresses in rice [13,16], pointing to their roles in the abiotic stress response. As an ortholog of *OsiSAP1*, *OsiSAP8* is induced by various abiotic stress treatments, such as *OsSAP1*, and overexpression of *OsiSAP8* in rice and tobacco also confers tolerance to cold, drought and salt stresses [7]. Moreover, overexpression of *OsSAP9/ZFP177* in tobacco enhanced tolerance to both low and high temperature stresses [17]. *AtSAP5*, induced by various abiotic stressors and plant growth regulators, such as cold, mannitol and ABA, encodes an E3 ubiquitin ligase to confer tolerance to dehydration stress [18]. In addition, AtSAP5 contributes to plant adaptability under high temperature by influencing heat-responsive gene regulation together with MBF1c [19]. Overexpression of *AtSAP10* in *Arabidopsis* results in a strong tolerance to several toxic metals and high temperature stress [20]. Besides rice and *Arabidopsis*, the A20/AN1 zinc-finger-containing proteins are also conserved in other plants and represent components of the stress response. For example, in *Sorghum bicolor*, *SbSAP14* is specifically induced in response to dehydration, salt and oxidative stress, and is involved in the induction of plant antioxidant systems to confer tolerance to salt stress [21]. In banana, one member of the *SAP* family, *MusaSAP1*, functions as a positive regulator in different stress responses [22].

Overexpression of *SAPs* has been shown to confer abiotic stress tolerance in tobacco, rice and *Arabidopsis*, suggesting that they are positive regulators of stress signaling. However, recent studies showed that some OsSAP genes play a negative role in stress tolerance. The expression of *OsiSAP7* was downregulated under ABA and water-deficit stress in rice, and OsiSAP7 acts as a negative regulator of ABA and water-deficit stress signaling by acting as an E3 ubiquitin ligase [23]. Another A20/AN1-type zinc-finger protein, *ZFP185*, increases the sensitivity to drought, cold and salt stresses, and regulates plant growth and stress responses by affecting GA and ABA biosynthesis in rice [24]. Additionally, overexpression of *ZmAN13* in *Arabidopsis* resulted in an increase of cold tolerance, but increased plant sensitivity to salt and drought at seed germination and seedling stage [25].

Although the *SAP* family has been extensively investigated in different plants, little is known about the *SAP* members in *M. truncatula*. *MtSAP1* is the first identified gene encoding a stress-associated protein in *M. truncatula* [26]. Overexpression of *MtSAP1* leads to nitric oxide accumulation, confers tolerance to abiotic stress and affects proline accumulation in transgenic tobacco [27,28]. In this study, we characterized the SAP gene family that consists of 17 members in *M. truncatula*. The phylogenetic relationship and domain organization of the *SAP* family proteins were identified and characterized in response to various stresses. *M. truncatula* is a model legume species that contributes a lot to the understanding of mutualistic interactions between *M. truncatula* and arbuscular mycorrhizal fungi. The great importance of *SAPs* in stress responses indicates that a systematic investigation of the *SAPs* in *M. truncatula* would be highly necessary, and would in turn enable the clarification of the possible roles of *MtSAPs* in the stress response pathways of *M. truncatula*.

MATERIALS AND METHODS

Identification of MtSAP

The zf-A20 (PF01754) and zf-AN1 (PF01428) domains were downloaded from the Pfam database (http://www.sanger.ac.uk/Software/Pfam/). Putative MtSAP were searched from BLAST program (Hmmer 3.0; http://hmmer.janelia.org/ provided by the *M. truncatula* genome database (http://www.medicagohapmap.org/tools/Blastform). The proteins identified by the BLAST program were used for a domain

search with the Pfam and SMART (http://smart.embl-heidelberg.de/) databases. The theoretical molecular weight (MW) and isoelectric point (pI) values were predicted using the ProtParam tool (http://web.expasy.org/protparam).

Phylogenetic analysis

Multiple sequence alignments were carried out using the Clustal X (Version 2.0; http://www.clustal.org/) program with all predicted motifs of MtSAPs. The neighbor-joining (NJ) tree was constructed by MEGA 5.1 [29], using the p-distance method with gaps treated by pairwise deletion and a 1000 bootstrap replicate.

Chromosomal location, gene duplication and structure analysis

The data of chromosomal location, genomic DNA sequences, full-length cDNA sequences and open reading frame (ORF) sequences for each *MtSAP* were collected from the Phytozome database (https://phytozome.jgi.doe.gov). Chromosomal maps were generated using the GenomePixelizer, and the duplicated genes were analyzed according to the criteria previously described [30]. Exon and intron structures of *MtSAPs* were determined by the Gene Structure Display Server (GSDS) (http://gsds.cbi.pku.edu.cn/).

Cis-elements analysis of MtSAP genes

To investigate *cis*-elements in the promoter sequences of MtSAP genes, 1500 bp of genomic DNA sequence upstream of the transcriptional start site was obtained from the Phytozome database (https://phytozome.jgi.doe.gov). The PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) was used to identify the *cis*-elements in the promoter regions of each *MtSAP*.

Microarray expression analysis

The raw microarray data of MtSAP genes in different tissues were downloaded from *M. truncatula* Gene Expression Atlas (MtGEA) Web Server (http://mtgea.noble.org/v2/) and normalized using the RMA algorithm with Expression Console software (Affymetrix Technologies) [31]. The identifiers were updated based on the file "AffyMap-Mt4.0v1 Genes Spliced Transcript Seq" (http://www.medicagohapmap.org/?genome). Differentially expressed *MtSAPs* were identified through fold change as well as *P*-value calculated using *t*-test with a fold change ≥2.0 or ≤0.05, as previously described [32].

Plant materials and treatments

Seeds of *M. truncatula* (cv. Jemlog) were germinated and grown for 4 weeks under the conditions described previously [32]. For drought treatment, the seedlings were transferred to dry Whatman 3 MM paper in a sterile Petri dish for 0, 2, 6 and 12 h. For cold treatment, the seedlings were transferred to 4°C for 0, 2, 6 and 12 h. For salt treatment, the seedlings were transferred to solutions containing 300 mM NaCl for 0, 2, 6 and 12 h. After the treatments, the seedlings were harvested, frozen in liquid nitrogen immediately and stored at -80°C until further analysis.

Quantitative RT-PCR analysis

Quantitative RT-PCR (qRT-PCR) was carried out on the CFX96 Real-Time PCR Detection System (Bio-Rad), and the data were analyzed using CFX Data Analysis Manager Software as previously described [32]. The relative expression level was normalized to that of *MtACTIN (MTR_2g008050)*, which was used as the internal control, with the $2^{-\Delta\Delta CT}$ method representing the relative quantification of gene expression [33]. The primers used for qRT-PCR are presented in Supplementary Table S1.

RESULTS

Genome-wide identification of MtSAP gene family in *M. truncatula*

To identify *MtSAP* family members from *M. truncatula*, the SA*P* proteins from *Arabidopsis* and rice were used to perform a BLASTp search against the *M. truncatula* genome. As a result, 17 proteins were identified. To further confirm the reliability of these candidate sequences, the sequences were analyzed by the Pfam

Table 1. Distribution of *MtSAP* family encoding A20/AN1 zinc finger proteins in the *Medicago* genome.

S.No	Gene_ID	Accession number	Zinc Wngers	Predicted protein (aa)	Mol wt (kDa)	pI	Chromosome
1	*MtSAP1*	Medtr1g060380.1	A20-AN1	156	16.93	8.79	1
2	*MtSAP2*	Medtr1g100773.1	A20-AN1	141	15.99	7.77	1
3	*MtSAP3*	Medtr2g086190.1	A20-AN1	150	16.64	8.42	2
4	*MtSAP4*	Medtr2g098160.1	A20-AN1	172	18.39	6.85	2
5	*MtSAP5*	Medtr3g028010.1	A20-AN1	163	18.20	8.26	3
6	*MtSAP6*	Medtr4g053440.1	A20-AN1	170	18.42	8.78	4
7	*MtSAP7*	Medtr7g092400.1	A20-AN1	260	29.74	8.64	7
8	*MtSAP8*	Medtr7g104320.1	A20-AN1	168	18.14	8.21	7
9	*MtSAP9*	Medtr7g114920.1	A20-AN1	134	14.94	8.71	7
10	*MtSAP10*	Medtr0249s0070.1	A20-AN1	169	18.35	6.71	-
11	*MtSAP11*	Medtr2g054650.1	AN1	128	14.13	7.99	2
12	*MtSAP12*	Medtr3g025570.1	AN1	111	12.79	6.73	3
13	*MtSAP13*	Medtr4g065570.1	AN1	137	14.93	8.21	4
14	*MtSAP14*	Medtr8g036980.1	AN1	151	16.85	9.39	8
15	*MtSAP15*	Medtr6g008210.1	AN1-AN1	193	21.19	8.79	6
16	*MtSAP16*	Medtr7g091810.1	AN1-AN1-C2H2-C2H2	268	29.43	8.21	7
17	*MtSAP17*	Medtr0100s0160.1	AN1-AN1-C2H2-C2H2	287	31.75	8.49	-

database for the presence of the A20 domain (Pfam: 01754) and/or AN1 domain (Pfam: 01428). Each of the 17 members was given a generic name (*MtSAP1-MtSAP17*) based on the A20/AN1 domains and its location on the chromosomes (Table 1). Most of the 17 members were distributed on 8 chromosomes, except for two members (*MtSAP16* and *MtSAP17*), which were located on unassembled scaffolds. Physicochemical analysis of MtSAPs revealed that the 17 predicted MtSAPs ranged from 111 to 287 amino acids (aa) in length, their relative molecular weight (MW) varied from 12.79 kDa to 31.75 kDa, and their pIs ranged from 6.71 to 9.39 (Table 1).

Phylogenetic analysis of MtSAPs

In order to evaluate the evolutionary relationships among the MtSAPs and facilitate their classification, an unrooted NJ tree was generated based on multiple alignments of the predicted amino acid sequences from *M. truncatula*, *Arabidopsis* and rice. As shown in Fig. 1, all the identified MtSAPs were classified into 7 different groups (G1-G7) together with their orthologs in *Arabidopsis* and rice. Group 1 contained 4 MtSAPs, group 2 contained 3 MtSAPs, group 3 contained 2 MtSAPs and groups 4 and 7 contained 4 MtSAPs each. No MtSAP and AtSAP were found in two groups (G5 and G6), which only contained OsSAPs, and group 5 only included *Arabidopsis thaliana*

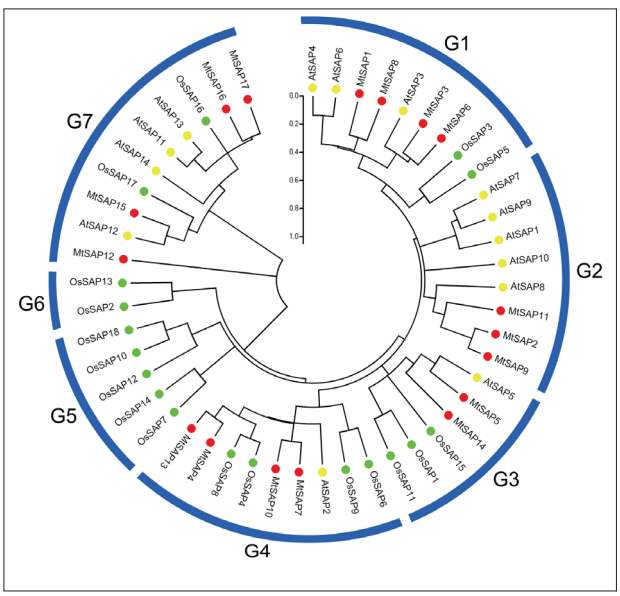

Fig. 1. Phylogenetic analysis of SAPs in *M. truncatula*, *Arabidopsis* and rice.

and *M. truncatula* SAPs. These findings implied that the expansion of these groups had occurred after the separation of mono- and dicot plants. In addition, there were several closely related orthologous MtSAPs between *M. truncatula* and *Arabidopsis*, such as MtSAP3/MtSAP6 and AtSAP3, MtSAP5 and AtSAP5, MtSAP15 and AtSAP12, indicating that they may have similar functions.

Functional domain analysis of MtSAPs

To further understand the functions of MtSAPs in *M. truncatula*, their functional domains were predicted by the SMART and InterPro databases. Based on the domain analysis, both the A20 and the AN1 domain were present in 10 MtSAPs, all of which contained one A20 domain and one AN1 domain (Table 1; Fig. 2). The remaining 7 MtSAPs only contained the AN1 domain, with 4 possessing only one single AN1 domain and the other 3 containing two AN1 domains. Two members, MtSAP16 and MtSAP17, had the additional C2H2 domain (Pfam: 13894). Interestingly, no MtSAPs with only one single A20 domain were present in *M. truncatula*, and the same phenomenon was also found in *Arabidopsis* [13], tomato [2] and cotton [34]. The differences in motif distribution among different groups of MtSAPs might lead to the functional divergence of *MtSAPs*.

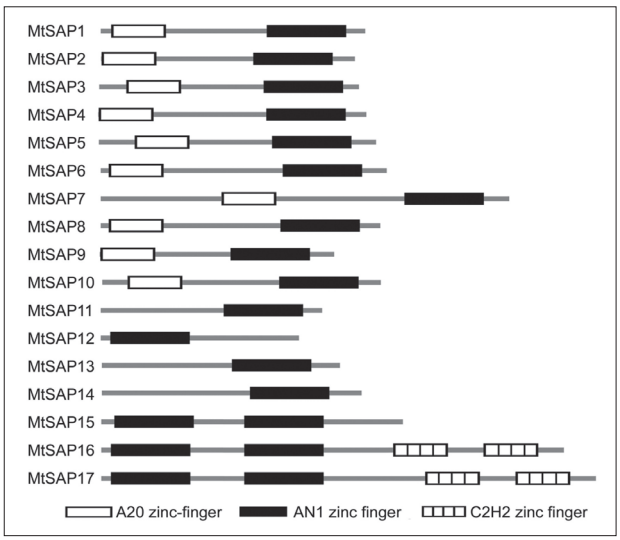

Fig. 2. Conserved domain analysis in *MtSAP*s. Lengths of the domains and proteins are in scale.

Structural divergence of MtSAP genes

To further understand the structural divergence of *MtSAPs*, we performed a comparison between the full-length cDNA sequences and the corresponding genomic DNA sequences for each *MtSAP*. Out of the 17 MtSAP genes, 7 intron-free genes were distributed in G1, G2 and G3 according to the phylogenetic relationship (Fig. 3). Among them, none of the members in G2 and G3 had an intron. Additionally, 7 out of the 17 *MtSAPs* contained only one intron and fell into G1, G4 and G7. All of the four MtSAP genes in G7 possessed one intron each. Two members, *MtSAP4* and *MtSAP7*, contained 3 exons and 2 introns in G4. Four introns were present in *MtSAP13*, which is rarely observed in *M. truncatula* and other plant species. Most MtSAP genes within the same group of the phylogenetic tree exhibited similar exon-intron organization patterns, which further supports the classification of the MtSAP genes in this study.

Chromosomal distribution and gene duplication of MtSAP genes

To determine the genomic distribution of the MtSAP genes, we analyzed the chromosomal distributions of

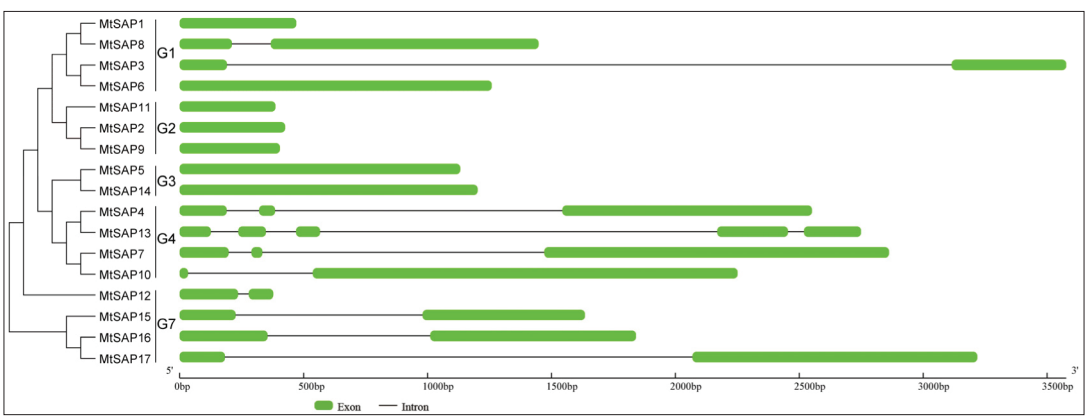

Fig. 3. Exon-intron structure analyses of *MtSAP* family according to the phylogenetic relationship. The green line represents the exon and the black line represents the intron. Lengths of exons and introns of each *MtSAP* are exhibited proportionally. G1, G2, G3, G4 and G7 indicate the classification of MtSAPs according to the phylogenetic relationship.

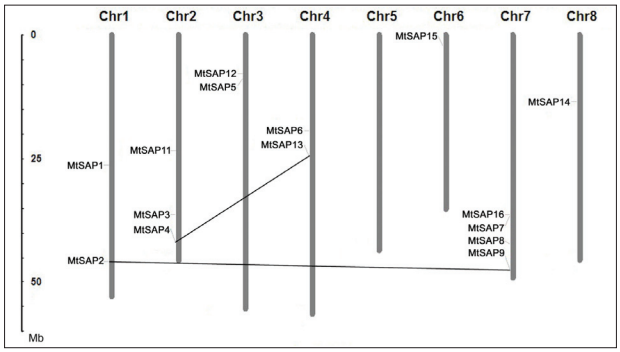

Fig. 4. Distribution of the MtSAP genes on *M. truncatula* chromosomes. Segmental duplication between different chromosomes is linked with black lines. The scale is in megabases (Mb).

the 17 *MtSAPs*. Out of these 17 genes, 15 were distributed across 7 chromosomes excluding chromosome 5, while 2 genes could not be conclusively mapped to any chromosome (Table 1; Fig. 4). Among these 7 chromosomes, chromosome 7 contained the largest number of *MtSAPs* (4), whereas chromosomes 6 and 8 contained the smallest number (1). Chromosomes 1, 3 and 4 each contained two *MtSAPs* and chromosome 2 had three MtSAP genes, respectively (Fig. 4). In addition, a cluster with a relatively high density of MtSAP genes was observed on chromosome 7. Based on gene duplication analysis, two segmental duplication events were identified between *MtSAP2* and *MtSAP9*, *MtSAP4* and *MtSAP13*, respectively (Fig. 4), implying that they may have similar or divergent expression patterns.

Analysis of *cis*-elements in promoters of MtSAP genes

To further understand the gene function and regulation patterns, *cis*-elements in *MtSAP* promoter sequences were determined. As a result, 1500 bp of genomic DNA sequence upstream of the transcrip-

Table 2. Types and numbers of known stress-related and hormone-related *cis*-acting elements in the promoters of each *MtSAP*.

	HSE	TC-rich repeat	LTR	MBS	ARE	Box W1	TGA element	AuxRR core	ABRE	ERE	P Box	GARE motif	TCA element	CGTCA motif	TGACG motif
	Heat	Defense and stress	Low temperature	Drought	Anaerobic	Fungal	Auxin	ABA	Ethylene	Gibberellin	Salicylic acid	MeJA			
MtSAP1	1	2		5	7										
MtSAP2	1			1	2	2				2			1	1	1
MtSAP3	1	1		1				1			1				1
MtSAP4	1	1		1	1				1		2	1	2		
MtSAP5	1	1		1			2			1			2		2
MtSAP6	2	2	1		2						1		2		
MtSAP7	3	5	2		1								1	1	1
MtSAP8	1				1	1	1						1	1	1
MtSAP9	3	2		2	1	1				2					
MtSAP10	2	2		1	2									2	2
MtSAP11	3			1	1	1							2	2	2
MtSAP12	2			1		2				2					
MtSAP13	4	2			1	1							1		
MtSAP14	3	1					2					1	1	3	3
MtSAP15	1	1	1		3				1				1	3	3
MtSAP16				1	1							1			
MtSAP17	4	1			1	1									1

tional start site was obtained from the *M. truncatula* genome database in Phytozome and was analyzed by PlantCARE. As shown in Table 2, 15 known stress- and hormone-related elements were identified in the *MtSAP* promoters. Among them, 6 stress-responsive *cis*-elements were determined, such as the heat shock element (HSE), the defense and stress-responsive element (TC-rich repeat), low temperature-responsive element (LTR), the MYB binding site involved in drought inducibility (MBS), ARE and Box-W1, which are involved in plant responses to heat, defense stresses, low temperature, drought, anaerobic and fungal induction, respectively (Table 2). All of the *MtSAP* promoter sequences contained at least two types of *cis*-elements involved in stress response, suggesting important roles of MtSAP genes in various stresses. Interestingly, HSE, which is involved in heat response, was present in nearly all the *MtSAP* promoters except for *MtSAP16*, suggesting that *MtSAPs* may be involved in the response to heat stress. In addition, all 17 MtSAP genes contained 1-5 types of *cis*-elements related to hormone response in their promoter regions, including auxin-responsive elements (TGA element and AuxRR core), abscisic acid (ABA) response element (ABRE), ethylene-responsive element (ERE), gibberellin-responsive elements (P Box and GARE motif), salicylic acid (SA)-responsive element (TCA element), and the methyl jasmonate (MeJA) responsive elements (CGTCA motif and TGACG motif), implying that MtSAP genes may play key roles in responses to hormones.

Expression of MtSAP genes in different tissues

The tissue-specific expression of MtSAP genes was determined according to the *M. truncatula* Gene Expression Atlas (MtGEA) Web Server (http://mtgea.noble.org/v2/). The $\log_2$-based fluorescence intensity values of several MtSAP genes during *M. truncatula* panicle development and root development, as well as under salt stress, were used to create a heat-map of *MtSAP* expression. As shown in Fig. 5A, nearly all of the detected *MtSAPs* exhibited a broad expression pattern in all the tested tissues, such as flower, leaf, petiole, pod, stem, vegetative bud, root (0, 1, and 3 mm tip) and 10, 12, 16, 20, 24 and 36 day-after-pollination (DAP) seed, as well as 16-24 DAP seed coat, except for *MtSAP6*, whose expression was much lower in the pod. In addition, three genes (*MtSAP5*, *MtSAP6* and *MtSAP8*) displayed relatively lower expression during early seed development (Fig. 5A). Similar expression patterns of the MtSAP genes were also observed during root development, with the exception of *MtSAP7*, which was downregulated at 28 dpi (Fig. 5B). We also examined the expression data of the MtSAP genes during root development under salt stress, and all of the detected *MtSAPs* exhibited a constitutive expression pattern (Fig. 5C). These results suggested that the MtSAP genes have a constitutive expression pattern in the tested tissues and developmental stages.

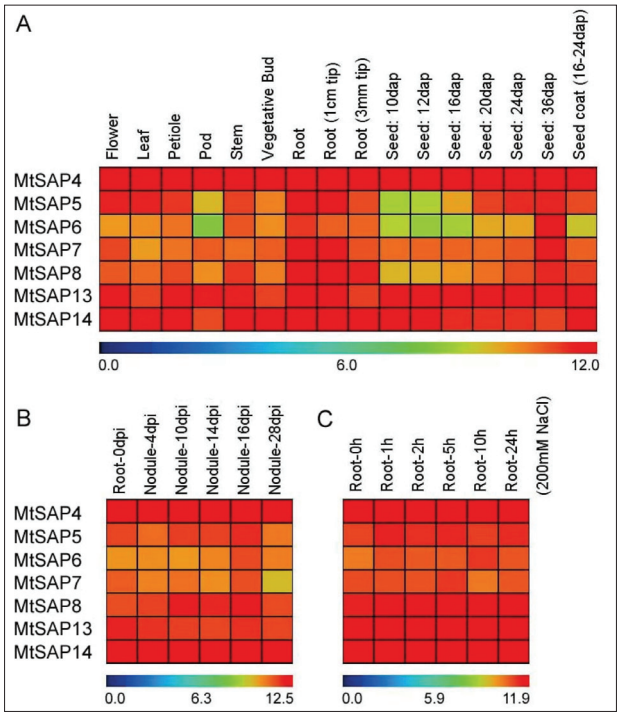

Fig. 5. Expression profiles of MtSAP genes differentially expressed during panicle development (**A**), root development (**B**) and under salt stress condition (**C**). Expression values are $\log_2$-transformed.

Expression patterns of MtSAP genes in response to abiotic treatments

To identify the potential functions of the SAP genes in response to abiotic stresses in *M. truncatula*, we investigated the expression levels of the 17 *SAPs* in 4-week-old seedlings under various abiotic stresses using qRT-PCR. Unfortunately, *MtSAP1*, *-3*, and *-17* were not expressed in 4-week-old seedlings. Perhaps these three genes are specifically expressed in other

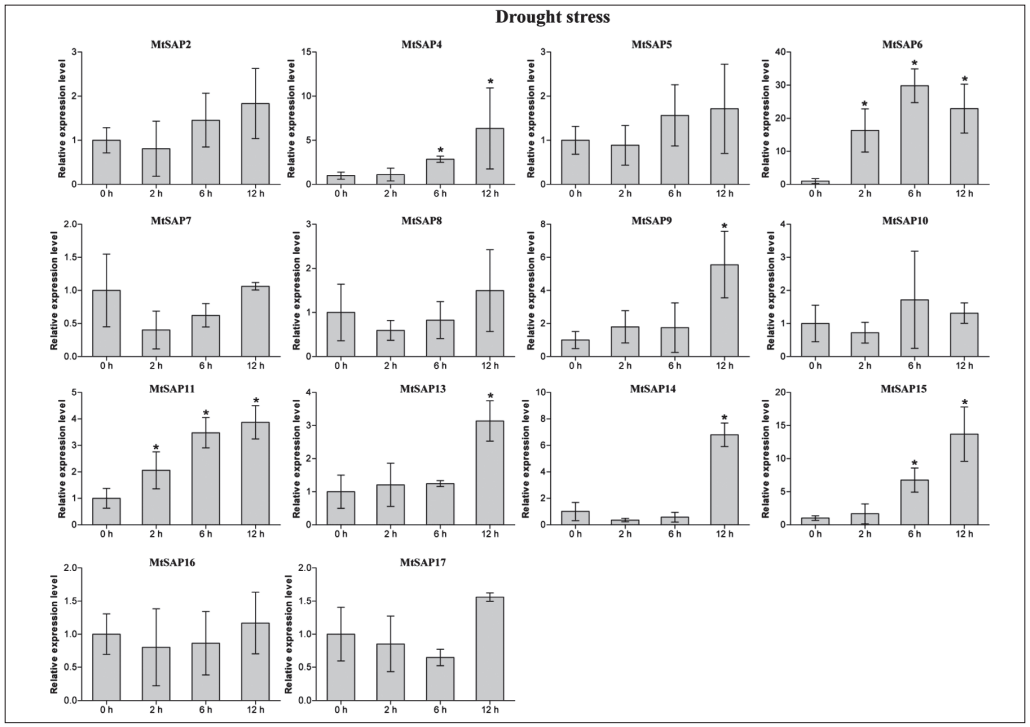

Fig. 6. qRT-PCR analysis of the expression of MtSAP genes under drought stress. The whole experiment was repeated three times. Data are the mean±SD of three independent experiments. The 2, 6, and 12 h values are plotted relative to the expression value at 0 h time point. Significant differences are indicated by *asterisk* ($P<0.05$) as determined by Student's *t*-test compared with 0 h.

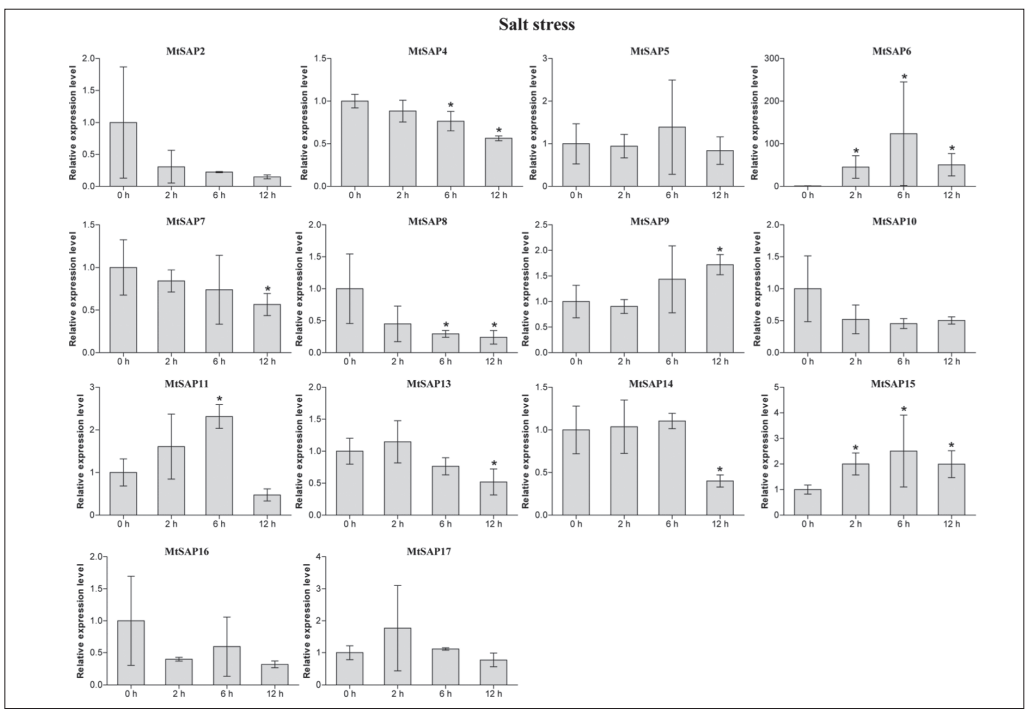

Fig. 7. qRT-PCR analysis of the expression of MtSAP genes under salt stress. The experiment was repeated three times. Data are the mean±SD of three independent experiments. The 2, 6, and 12 h values are plotted relative to the expression value at 0 h time point. Significant differences are indicated by an asterisk ($P<0.05$), as determined by Student's *t*-test compared with 0 h.

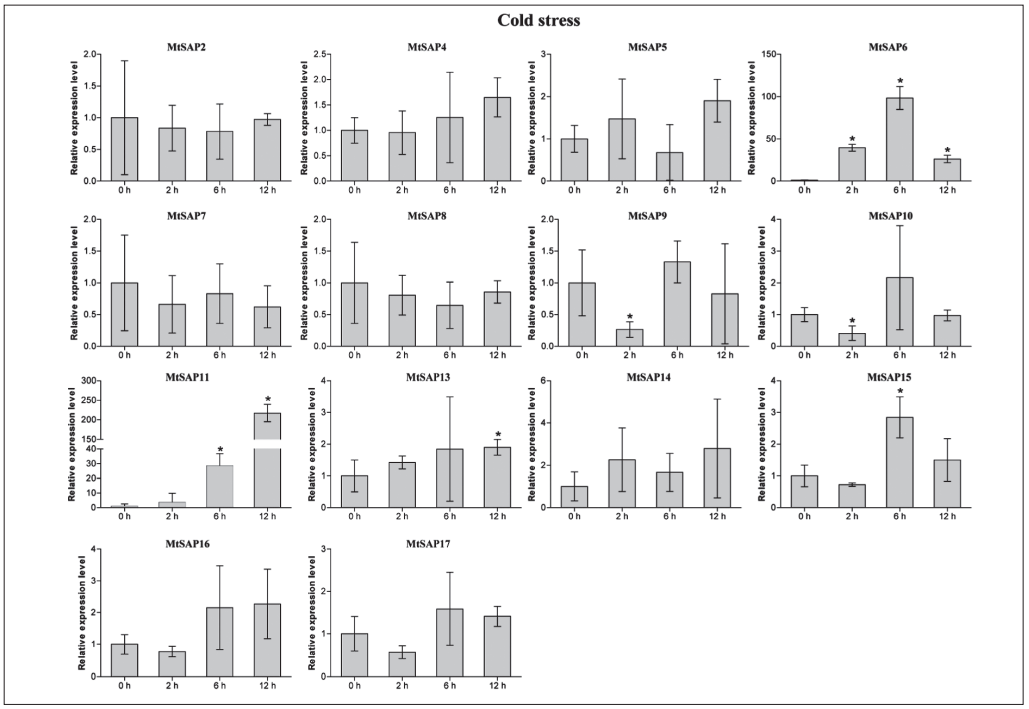

Fig. 8. qRT-PCR analysis of the expression of *MtSAP* genes under cold stress. The experiment was repeated three times. Data are the mean±SD of three independent experiments. The 2, 6, and 12 h values are plotted relative to the expression value at the 0 h time point. Significant differences are indicated by an asterisk ($P<0.05$), as determined by Student's *t*-test compared with 0 h.

tissues. For drought treatment, some of the detected genes (*MtSAP4*, *-6*, *-9*, *-11*, *-13*, *-14*, and *-15*) were induced at some time points after treatment (Fig. 6). Among them, *MtSAP4*, *MtSAP11* and *MtSAP15* showed continually upregulated transcripts that peaked at 12 h.

For NaCl treatment, the expression of *MtSAP6*, *-11*, and *-15* showed an increasing tendency at first, and was then followed by a decrease (Fig. 7). It is worth noting that *MtSAP6* showed a relatively high expression level after NaCl treatment. The remaining MtSAP genes, including *MtSAP4*, *-7*, *-8*, *-13*, and *-14*, were downregulated at some time points after treatment, indicating their possible negative regulatory roles.

For cold treatment, only *MtSAP6*, *-11*, *-13* and *-15* were upregulated at some time points after treatment, and *MtSAP6* and *MtSAP11* showed significantly higher expression at 6 and 12 h, respectively (Fig. 8). The expression of *MtSAP9* and *MtSAP10* was relatively low at 2 h, and was upregulated thereafter. The remaining SAP genes were not affected substantially.

DISCUSSION

The *SAP* family has been previously studied in various plant species, but not in *M. truncatula*. In this study, we identified 17 SAP genes in the genome of *M. truncatula* in the Phytozome database. The number of *MtSAPs* was similar to that in *Arabidopsis* (14) [13], rice (18) [13], tomato (13) [2], maize (11) [35], *Populus euphratica* (18) [36], *P. trichocarpa* (19) [35], *Salix purpurea* (19) [36] and *S. suchowensis* (15) [36], but was much smaller than that in cotton (37) [34]. Among the 17 *MtSAPs*, 15 were mapped to 7 out of 8 chromosomes and no *MtSAP* was found on chromosome 5. In addition, 2 genes could not be conclusively mapped to any chromosome. Gene duplication events, which consist of tandem and segmental duplications, can be a crucial factor for plant genome evolution [30,37]. In our study, two segmental duplication events were identified between *MtSAP2* and *MtSAP9*, *MtSAP4* and *MtSAP13*, respectively (Fig. 4), which may contribute to the restriction of genome expansion and evolution.

Analysis of the phylogenetic relationships among *Arabidopsis*, rice and *M. truncatula* SAP genes re-

vealed that these genes could be classified into 7 different groups. All of the 17 *MtSAPs* were phylogenetically clustered with at least one member of the *Arabidopsis MtSAP* family and were distributed in most groups, with the exception G5 and G6, suggesting that orthologous genes between dicots have a closer relationship than those between monocots. In addition, the MtSAP genes within the same group shared similar highly conserved domains, indicating that they may share similar functions.

Variation in exon-intron structure plays a significant role in the evolution of gene families, and can provide additional proof for phylogenetic analysis. In rice, 11 *OsSAPs* have no introns, 6 have one intron and only *OsSAP8* has two introns [13]. In *Arabidopsis*, 9 *AtSAPs* do not have any intron, 4 have one intron, and only *AtSAP14* has three introns. In *Populus euphratica*, 15 PeuSAP genes are intron-free, 2 have one intron, and only *PeuSAP18* has two introns [36]. In our study, 7 *MtSAPs* were intron-free, 7 contained one intron, 2 contained two introns, and *MtSAP13* had 4 introns, which is rarely observed in other plant species. Most of the *SAPs* are intron-free, revealing that plant *SAP* families are highly structurally conserved. One important characteristic of the *SAP* family is the prevalent lack of introns [36]. These intron-free SAP genes could reduce posttranscriptional processing and be rapidly transcribed and translated under abiotic stresses [38]. Interestingly, the *MtSAPs* belonging to the same groups always exhibited similar gene structures, suggesting that these genes may have similar functions. For example, members in G2 and G2 have no introns, while members in G7 contain one intron. In cotton, nearly all A20-AN1-type *SAPs* are intron-free, whereas AN1-AN1-type SAPs contain one intron [34]. However, *MtSAPs* in G4 are characterized by a relatively large number of introns (ranging from 1 to 4). These results reflect the diverse functions of MtSAP genes and will be helpful in future research into their functions. According to our heat-map results, most of the *MtSAPs* were widely expressed in different tissues, including flower, leaf, petiole, pod, stem, vegetative bud, root, seed and seed coat, indicating that they may be involved in diverse physiological functions and confirming the functional divergence of MtSAP genes.

There is increasing evidence that *SAP* genes can function as a positive regulator in different stress responses, such as *OsiSAP1* [14], *OsiSAP8* [7], *AtSAP5* [18], *AtSAP10* [20], *SbSAP14* [21] and *MusaSAP1* [22]. In this study, we first found that all the detected MtSAP genes exhibited a constitutive expression pattern during root development under salt stress, which is consistent with a previous report [34] suggesting that the MtSAP genes may be involved in salt stress response. Moreover, many stress-related *cis*-elements were detected in the promoters of MtSAP genes, indicating that the expression of *MtSAPs* can be induced by different stresses. To further investigate *MtSAPs* that are potentially associated with responses to abiotic stresses, their expression profiles under multiple abiotic stresses (drought, NaCl and cold) were analyzed by qRT-PCR. The qRT-PCR results demonstrated that most of the MtSAP genes could transcriptionally respond to the three types of stress, further suggesting that they may participate in the response to various stresses. Furthermore, *MtSAP6* was the most significantly induced gene by all three types of stress, implying that it may be used as a candidate to confer abiotic stress tolerance.

Interestingly, five SAP genes (*MtSAP4*, *-7*, *-8*, *-13*, and *-14*) were downregulated at some time point under salt stress, indicating their possible negative regulatory roles. Several studies have shown that some *OsSAPs* play negative roles in plant responses to abiotic stress [23,24]. In addition, the expression levels of *GhSAP7A/D*, *GhSAP8A/D* and *GhSAP11A/D* were significantly downregulated by PEG in cotton [34]. These data suggest that *SAP* family genes might be positively or negatively involved in stress responses.

Many studies have shown that SAPs can function in regulating phytohormone synthesis and signal transduction [24,39]. Many stress-induced SAP genes, such as *GhSAP7A/D*, *GhSAP12A/D*, *GhSAP16A/D* and *GhSAP17A/D*, were also upregulated under defense-related phytohormone treatments [34]. In this study, a series of *cis*-elements involved in phytohormone responses was identified, indicating that MtSAP genes might be involved in hormone signaling transduction.

CONCLUSIONS

In this study, we performed genome-wide identification and comprehensive analysis of the SAP genes in *M. truncatula*. A total of 17 *SAP* members containing

the characteristic A20/AN1 zinc-finger domains were identified. The gene structures, evolution, expression profiles and the promoters of these *MtSAPs* were investigated, and the results show that the MtSAP genes have functional roles in plant growth and development. This study expands the knowledge about plant SAP genes and lays a solid foundation for their future functional characterization. More functional analysis will be needed to further characterize MtSAP genes to unravel their biological roles.

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