Comprehensive Identification and Functional Analysis of Stress-Associated Protein (SAP) Genes in Osmotic Stress in Maize

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Abstract: Stress-associated proteins (SAPs) are a kind of zinc finger protein with an A20/AN1 domain and contribute to plants’ adaption to various abiotic and biological stimuli. However, little is known about the SAP genes in maize (Zea mays L.). In the present study, the SAP genes were identified from the maize genome. Subsequently, the protein properties, gene structure and duplication, chromosomal location, and cis-acting elements were analyzed by bioinformatic methods. Finally, their expression profiles under osmotic stresses, including drought and salinity, as well as ABA, and overexpression in Saccharomyces cerevisiae cells, were performed to uncover the potential function. The results showed that a total of 10 SAP genes were identified and named ZmSAP1 to ZmSAP10 in maize, which was unevenly distributed on six of the ten maize chromosomes. The ZmSAP1, ZmSAP4, ZmSAP5, ZmSAP6, ZmSAP7, ZmSAP8 and ZmSAP10 had an A20 domain at N terminus and AN1 domain at C terminus, respectively. Only ZmSAP2 possessed a single AN1 domain at the N terminus. ZmSAP3 and ZmSAP9 both contained two AN1 domains without an A20 domain. Most ZmSAP genes lost introns and had abundant stress- and hormone-responsive cis-elements in their promoter region. The results of quantitative real-time PCR showed that all ZmSAP genes were regulated by drought and saline stresses, as well as ABA induction. Moreover, heterologous expression of ZmSAP2 and ZmSAP7 significantly improved the saline tolerance of yeast cells. The study provides insights into further underlying the function of ZmSAPs in regulating stress response in maize.

Keywords: A20/AN1 zinc finger; stress associated proteins; expression pattern; maize

1. Introduction

Plants constantly encounter biotic and abiotic stresses from their surroundings. Consequently, plant growth, development and production are restricted by these stimuli, such as drought and salt stress [1,2]. To avoid adverse conditions, plants have evolved multifaceted strategies at morphology, physiology, and molecular levels to perceive, transfer and activate signal transduction to response stresses [3,4]. Among them, stress-related genes play pivotal roles in stress response. The stress-associated proteins (SAPs), a family of zinc-finger proteins with A20/AN1 domains, were first discovered in humans and Xenopus laevis and played key roles in innate immunity and cell death [5–7]. Most SAPs possess a typical SAP domain containing both A20 and AN1 domains presented in the N-terminal and C-terminal, respectively, and were separated by a variable stretch of amino acids [8]. Subsequently, the SAPs have been identified in all eukaryotes and confirmed as novel regulators in plant abiotic stress response [9–11].

In plants, OSISAP1 was first identified as A20/AN1 zinc-finger protein from rice and induced by multiple stresses, including cold, desiccation, salt, submergence and heavy
metals, as well as injury [10]. Meanwhile, transgenic tobacco with an OSISAP1 gene showed enhancements in the tolerance of cold, dehydration and salt [10]. Thereafter, using the OsSAP1 sequence as a reference, 18 and 14 SAP genes (named OsSAP and AtSAP) were identified from the genome of rice and Arabidopsis, respectively, through a sequence similarity approach blast [11]. Most OsSAPs and AtSAPs have been confirmed to regulate abiotic stress responses such as drought, salt, and temperature [12–18]. Previous studies also showed that SAPs from wheat (TaSAP5), banana (MusaSAP1), Populus trichocarpa (PtSAP13), Aeluropus littoralis (AlSAP) and Leymus chinensis (LeSAP) positively regulate drought and salt tolerance [19–23].

In addition to abiotic stress response, plant SAPs are found to serve as an important hub to mediate disease resistance and development. For instance, Pha13 containing A20/AN1 zinc finger domains and its homologs AtSAP5, AtSAP9 and OsSAP1 are also involved in virus resistance, basal resistance against pathogen infection and compromising innate immunity [24–26]. The AtSAP9, PagSAP11 of poplar, and PpSAP1 of Prunus persica control flowering time, branching of lateral shoots, and cell expansion [25,27,28]. The Os-DOG and ZFP185 are A20/AN1 zinc-finger proteins, negatively regulating cell elongation and size in rice [29,30]. Abscisic acid (ABA) is one of the most important phytohormones and plays key roles in plant growth, development and stress response [31,32]. Available reports showed that SAPs likewise mediate ABA signaling. Rice OsiSAP7 negatively regulates ABA signaling to impart sensitivity to water-deficit stress [33]. However, ZFP185 modulates the expression of ABA biosynthesis-related genes and alters ABA content in plants to negatively regulate stress tolerance [30]. AtSAP9 is involved in the ABA-dependent regulation of downstream ABA-responsive genes and confers hypersensitivity to ABA of overexpressing plants [25].

Previous studies indicate that SAPs have emerged as promising candidates for improving stress tolerance and growth during unfavorable conditions in plants. As one of the most important crops, maize is a key factor in developing the national economy and maintaining food security [34]. However, the SAPs of maize remain poorly understood. In this study, the ZmSAPs were first identified in the maize genome and comprehensively characterized for protein properties, gene structure and duplication, chromosomal locations, cis-acting regulatory elements and tissue expression profiles. Additionally, the expression profiles of ZmSAPs under different abiotic stress and hormone induction were investigated by RT-qPCR. The function of ZmSAPs was validated in yeast. The study provides insights into the further underlying function of ZmSAPs and helps in understanding the known modes of SAPs action in plants.

2. Results

2.1. The ZmSAP Members in Maize

To identify ZmSAPs in maize, the amino acid sequences of 14 and 18 SAPs of Arabidopsis and rice were used as queries in local BLAST searches, respectively. Totally, 10 SAP genes were identified from the maize genome and named ZmSAP1~ZmSAP10 (Table 1). The encoding sequences of ZmSAPs were 459 to 873 bp in length, encoding 152 to 290 amino acids (aa), with a molecular weight of 16.00 to 32.04 kDa, respectively. All ZmSAP proteins were predicted to be basic proteins with high theoretical isoelectric points ranging from 8.28 to 9.53. The grand average of hydropathicity (GRAVY) and instability indices of all ZmSAPs was <0 and >40, respectively, suggesting they were hydrophilic and instable proteins. Meanwhile, ZmSAPs showed no signal peptides and transmembrane region but were predicted to be nuclear localization.
Table 1. Characteristics of ZmSAP genes in Zea mays.

| Gene ID | Gene Name | Number of Amino Acids | Molecular Weight (KDa) | pI | CDS (bp) | GC (%) | Grand Average Hydrophy | Subcellular Locations | Instability Index |
|---------|-----------|-----------------------|------------------------|----|----------|--------|------------------------|----------------------|-------------------|
| Zm00001eb034760 | ZmSAP1 | 176 | 18.75 | 9.12 | 531 | 73.07 | -0.572 | N | 68.93 |
| Zm00001eb060270 | ZmSAP2 | 161 | 16.68 | 9.53 | 486 | 65.02 | -0.515 | N | 54.33 |
| Zm00001eb099020 | ZmSAP3 | 204 | 22.15 | 9.39 | 615 | 71.22 | -0.713 | N | 48.66 |
| Zm00001eb101840 | ZmSAP4 | 161 | 16.78 | 9.19 | 486 | 73.25 | -0.276 | N | 50.93 |
| Zm00001eb181400 | ZmSAP5 | 152 | 16.00 | 9.01 | 459 | 70.81 | -0.387 | N | 51.56 |
| Zm00001eb205990 | ZmSAP6 | 171 | 18.31 | 8.28 | 516 | 54.84 | -0.323 | N | 31.15 |
| Zm00001eb293630 | ZmSAP7 | 171 | 18.29 | 8.28 | 516 | 56.59 | -0.235 | N | 30.92 |
| Zm00001eb316600 | ZmSAP8 | 163 | 17.20 | 9.45 | 492 | 73.37 | -0.458 | N | 62.8 |
| Zm00001eb324750 | ZmSAP9 | 290 | 32.04 | 8.58 | 873 | 49.37 | -0.59 | N | 38.37 |
| Zm00001eb388350 | ZmSAP10 | 174 | 18.41 | 8.48 | 525 | 59.62 | -0.198 | N | 26.45 |

Note: N stands for nucleus.

2.2. Conserved Domains and Phylogenetic Analysis of ZmSAPs

The CDD analysis and sequence alignments showed that seven ZmSAPs, including ZmSAP1, ZmSAP4, ZmSAP5, ZmSAP6, ZmSAP7, ZmSAP8 and ZmSAP10, had an A20 domain at the N terminus and an AN1 domain at the C terminus, respectively. Only ZmSAP2 possessed a single AN1 domain at the N terminus. ZmSAP3 and ZmSAP9 both contained two AN1 domains without an A20 domain. Moreover, ZmSAP9 had two C2H2 domains (Figures 1A and S1). Meanwhile, to explore the conserved motifs of ZmSAPs, these amino acid sequences were analyzed using the MEME tool. The results showed that ZmSAPs exhibited similar motif composition (Figure 1B). Among them, motif 1 and motif 3 were highly conserved and contributed to the A20 and AN1 domains of ZmSAP1, ZmSAP2, ZmSAP4, ZmSAP5, ZmSAP6, ZmSAP7, ZmSAP8 and ZmSAP10, respectively. In addition, there was a conserved motif 2 at the N terminus of these eight ZmSAPs behind the AN1 domain. Motif 4 and motif 5 were composed of two AN1 domains of ZmSAP3 and ZmSAP9, respectively.

Figure 1. The schematic diagram of the conserved domain and motif composition of ZmSAP members. (A) The domain composition. (B) The motif composition.

To analyze the phylogenetic relationship between ZmSAPs and SAPs of Arabidopsis and rice, the amino acid sequences of 10 ZmSAPs, 14 AtSAPs and 18 OsSAPs were multi-aligned and used for phylogenetic tree construction. As shown in Figure 2, a total of 42 SAPs were divided into five branches. However, ZmSAPs were distributed in four branches besides group II (Figures 2 and S2). The ZmSAP5 was branched in group I. The ZmSAP6, ZmSAP7 and ZmSAP10 were located in subgroup III. The ZmSAP1, ZmSAP2, ZmSAP4 and ZmSAP8 were located in subgroup IV. The ZmSAP3 and ZmSAP9 were branched in group V.
ZmSAP4 and ZmSAP8 were located in subgroup IV. The ZmSAP3 and ZmSAP9 were branched in group V.

Figure 2. Phylogenetic evolutionary tree of SAP family in maize, *Arabidopsis* and rice. The red dot indicates the ZmSAP, the blue triangle indicates the OsSAPs, and the green diamond represents the AtSAPs.

2.3. Chromosome Localization and Synteny Analysis

According to the information on the physical positions of ZmSAPs in maizeGDB, their chromosomal locations were visualized to ten maize chromosomes (Figure 3). ZmSAPs were located on six chromosomes. There were two ZmSAP genes on chromosome 1 (ZmSAP1 and ZmSAP2), chromosome 2 (ZmSAP3 and ZmSAP4), chromosome 4 (ZmSAP5 and ZmSAP6) and chromosome 7 (ZmSAP8 and ZmSAP9), respectively. The ZmSAP7 and ZmSAP10 were located on chromosome 5 and chromosome 9, respectively (Figure 3).

In addition, gene duplication analysis showed that ZmSAP6 and ZmSAP7 were identified as segmental replication, which belonged to the paralogous pair. Likewise, multi-species collinearity analysis spectra were constructed with *Arabidopsis* and rice. The results showed no orthologous pairs between maize and *Arabidopsis*, while fourteen orthologous pairs between maize and rice were identified as orthologs (Figure 3 and Table S1), indicating that there was a high frequency of gene duplication between rice and maize in the process of evolution.
2.4. Gene Structure and Cis-Acting Elements of ZmSAPs

The exon–intron structure analysis showed that the genomic DNA (gDNA) sequence of ZmSAP9 contained one intron. Other ZmSAPs had no intron and one exon. Among them, ZmSAP2 and ZmSAP4 had only one exon and no un-translation region (UTR), while others possessed a 5’-UTR and 3’-UTR, respectively (Figure 4).

The cis-acting elements analysis exhibited that seven kinds of cis-elements associated with stress response were identified in ZmSAP promoters (Figure 5). Among these
cis-elements, ARE (anaerobic response element) was the most abundant cis-element. There were six and five AREs in the ZmSAP3 and ZmSAP6 gene promoters. Meanwhile, except ZmSAP7 and ZmSAP9, the other eight ZmSAP genes contained at least one MBS (Myb binding site) element. In addition, eight kinds of hormone-responsive cis-elements were observed in ZmSAP promoters and associated with different hormones, including ABA (ABRE), ethylene (ERE), MeJA (CGTCA-Motif), salicylic acid (TCA element), auxin (TGA element) and gibberellin (P-box, GARE-motif and TATC-box) response elements. This suggests that the ZmSAP genes may play different roles in stress and hormone response.

![Figure 5](image_url)

**Figure 5.** The cis-acting elements ZmSAP promoter region. The numbers in the blocks represent the number of cis-acting elements. LTR—low-temperature response; GC-motif—enhancer-like element involved in anoxic specific inducibility; MBS—(Myb binding site) drought response; ARE—(anaerobic response element) anaerobic induction; TC-rich repeats and W-box—defense and stress response; WUN-motif—wounding response; ABRE (ABA response element)—ABA response; ERE (ethylene response element)—ethylene response; CGTCA-Motif—MeJA response; TC-element—salicylic acid response; TGA-element—auxin response; P-box—GARE-motif and TATC-box—gibberellin response.

### 2.5. Expression Patterns of ZmSAPs

The expression profile of ZmSAPs in different development stages of maize was analyzed using RNA-seq data. We found that ZmSAPs showed no tissue specificity in the transcription level in maize, and ZmSAP6 exhibited a high expression level in all tissues. (Figure S3). To investigate the response of ZmSAPs to external stimuli, the expression profiles of 10 ZmSAP genes under osmotic stresses, including drought and salt, and hormone induction (ABA) were studied by RT-qPCR. Under drought stress mimicked by PEG treatment, the transcription levels of ZmSAP2, ZmSAP3, ZmSAP5 and ZmSAP8 were significantly up-regulated at 6, 12, 12 and 12 h of treatment, respectively. While ZmSAP1, ZmSAP4, ZmSAP6, ZmSAP7 and ZmSAP9 were significantly down-regulated by drought stress (Figure 6). In the process of salt stress, the transcription level of all ZmSAPs was inhibited by NaCl stress, and ZmSAP1, ZmSAP2, ZmSAP5, ZmSAP7, ZmSAP8, ZmSAP9 and ZmSAP10 showed a down-regulated during the treatment process (Figure 7). Under the induction of exogenous ABA, the expression of ZmSAP3, ZmSAP4, ZmSAP6 and ZmSAP8 was significantly induced by ABA at 12, 24, 24 and 24 h of treatment, respectively. However, they reached the lowest transcription level at 9 h of treatment. The transcription levels of ZmSAP1, ZmSAP2, ZmSAP5, ZmSAP7, ZmSAP9 and ZmSAP10 were significantly inhibited by ABA, especially ZmSAP9 (Figure 8). These results suggest that ZmSAPs may play an important role in osmotic stress response.
Figure 6. The expression of ZmSAPs under drought stress mimicked by 16% PEG-6000 treatment. * and **, indicates significant differences at $p < 0.05$ and $p < 0.01$, respectively.

Figure 7. The expression of ZmSAPs under salt stress by 250 mM NaCl treatment. * and **, indicates significant differences at $p < 0.05$ and $p < 0.01$, respectively.
2.6. Overexpression of ZmSAP2 and ZmSAP7 Enhanced the Saline Tolerance in Yeast

To validate the function of ZmSAPs in osmotic stresses, each ZmSAP gene was heterologously expressed in Saccharomyces cerevisiae W303a cells to phenotype on plates supplemented with mannitol or NaCl. The results showed no significant difference between yeast cells carrying pYES2-ZmSAPs and pYES2 (control) plasmid on the plates with mannitol (Figure S4). As shown in Figure 9A, under the plates without NaCl for control, 0.5 and 1.0 M NaCl, the yeast strain with every ZmSAP gene showed no difference compared to the yeast strain with empty vector pYES2, although the growth of all yeast was slightly inhibited by 1.0 M NaCl. On the plates with 1.5 M NaCl, the growth of yeast was severely inhibited. However, the yeast strains expressing ZmSAP2 and ZmSAP7 showed preferential growth vigor than that of pYES2 and other ZmSAPs. Subsequently, the yeast cells harboring ZmSAP2 and ZmSAP7 were cultured in liquid YNB-Ura-Gal 2% medium supplemented with 1.5 M NaCl and used to measure the growth curves. The results showed that the yeast strains with pYES2-ZmSAP2 and pYES2-ZmSAP7 exhibited a higher growth speed than that of pYES2 after 12 h to 72 h. The OD\textsubscript{600} of them was significantly higher than the control. (Figure 9B). These results confirmed that expression of the ZmSAP2 and ZmSAP7 genes provide the yeast with the ability to tolerate saline stress.
ZmSAPs from Glycine max with 27 potential to establish a more quick and accurate response to stimuli by reducing the number of introns per gene. For instance, most ZmSAP genes had a trait of being intron-less. In maize, there were nine ZmSAPs without intron, and only ZmSAP9 was identified by a single intron and two exons in gDNA (Figure 4). It has been confirmed that intronless genes (no introns) and intron-poor genes (three or fewer introns per gene) were more likely to play roles in osmotic stress response, including drought and salt stress, compared with intron-rich genes [43]. The genes with fewer introns could be rapidly regulated during stress and well confer the potential to establish a more quick and accurate response to stimuli by reducing the number of introns in gDNA.

**Discussion**

SAPs, a kind of zinc-finger protein, have been reported to be involved in multiple stress responses in plants and the immune system in humans [5–7,9]. Hence, the SAP genes are identified through genome-wide analyses from a few monocot and dicot plants such as Arabidopsis, rice, soybean, tomato, apple, Brassica napus, cucumber, castor bean [11,35–41]. However, the SAP genes in maize were rarely reported. In the study, 10 ZmSAP genes were identified in maize (Table 1). The number of ZmSAPs shows a great deal of variation with SAPs in other plants, such as Brassica napus with 57 BnSAPs, Glycine max with 27 GmSAPs and Populus trichocarpa with 19 PtSAPs [20,35,39]. Likewise, it’s reported that there were at least 11 ZmSAPs in maize [42]. This should be due to the updated genome used in the present study and gene duplications resulting in variation of SAP numbers [38–40]. We also found that one pair of paralogous ZmSAPs and fourteen orthologous pairs between maize and rice were identified as orthologs (Figure 3 and Table S1).

Previous studies showed that a significant majority of the SAP genes found in various plants had a trait of being intron-less. For instance, most SAP genes in rice, soybean, tomato, cucumber and castor bean possessed no intron, and only a small number of SAP genes contain a few introns in their gDNA [11,35,36,40,41]. Similarly, in maize, there were nine ZmSAPs without intron, and only ZmSAP9 was identified by a single intron and two exons in gDNA (Figure 4). It has been confirmed that intronless genes (no introns) and intron-poor genes (three or fewer introns per gene) were more likely to play roles in osmotic stress response, including drought and salt stress, compared with intron-rich genes [43]. The genes with fewer introns could be rapidly regulated during stress and well confer the potential to establish a more quick and accurate response to stimuli by reducing the number of introns in gDNA.

**Figure 9.** The phenotype of yeast cells carrying ZmSAP genes under salt stress by NaCl treatment. (A) The phenotype of all yeast strains on solid YNB-Ura-Gal 2% medium without (control) or with 0.5, 1.0 and 1.5 M NaCl. Photographs were taken after four days of incubation at 28 °C. (B) The growth curve of yeast cells expressing pYES2, pYES2-ZmSAP2 and pYES2-ZmSAP7 plasmid in YNB-Ura-Gal 2% liquid medium supplemented with 1.5 M NaCl for three days at 28 °C with an initial OD600 = 0.2, respectively. **, indicates significant differences at p < 0.01.
of steps required for post-transcriptional processing [43,44]. SAP proteins are characterized by containing A20 or AN1 domains. In the study, ZmSAP2 contained one A20 domain, ZmSAP3 and ZmSAP9 richen two AN1 domains, and other ZmSAPs possessed one A20 and AN1 domain, respectively (Figure 1). These findings suggest that ZmSAP genes may function in the quick response to abiotic stress.

Stress-related genes can be regulated by environmental stimuli and require cis-elements in promoter regions to drive their transcription [45]. Herein, the composition of cis-elements affects gene expression and is crucial for the transcriptional control of plant growth, development, and various stress responses [46,47]. In the ZmSAPs promoter, different cis-acting elements, such as MBS, ABRE, CGTCA-motif, TGA-element, and ARE, were found and linked to responses to abiotic stimuli and hormone response (Figure 5). The MBS and ABRE elements are involved in drought and ABA response [46]. As well known, ABA acts as a crucial phytohormone and regulates plant development and stress responses [32,48]. Available reports have demonstrated that ABA can induce or inhibit the expression of several SAP genes, including AtSAP9, AtSAP13, OsSAP1 and GmSAP16, which influence the expression of stress-related genes to respond to stress [10,18,25,35]. Here, we found that the transcription level of ZmSAP genes was significantly changed under osmotic stress, including drought and salt and ABA induction (Figures 6–8). These findings further imply the potential roles of ZmSAPs in stress response.

In yeast cells, the heterologous expression of ZmSAPs did not confer yeast tolerance to drought mimicking by mannitol (Figure S4). Only ZmSAP2 and ZmSAP7 significantly improved yeast tolerance to high salt (Figure 9). The functional differentiation of ZmSAPs can be explained by their evolutionary diversification with uneven distribution and duplication on chromosomes and different composition of the domain (Figures 1 and 2) [49]. Interestingly, the expression of ZmSAP2 and ZmSAP7 was inhibited by salt in maize (Figure 7). Similarly, the phenomenon is found in previous studies. The expressions of CaSAPs are up-regulated by low temperature and dehydration stress, but CaSAPs-silenced pepper plants show tolerance to low temperature and drought [50]. Likewise, the maize ZmBES1/BZR1-5 is inhibited by drought, but its overexpression improves drought tolerance in transgenic Arabidopsis [51]. It can be explained by its unknown upstream regulators and elucidated in further study.

Moreover, it has been proven that heterologous expression of the SAP gene from Aeluropus littoralis (AlSAP), Lobularia maritima (LmSAP), Leymus chinensis (LcSAP) also enhanced cell tolerance to salt, ionic and osmotic stresses in yeast [21,52,53]. More importantly, SAPs have exhibited functional diversity in osmotic stress, including drought and salt. For instance, OSISAP1, OsiSAP1, OsiSAP8 and AtSAP5, as positive regulators, improved drought, salt, and osmotic tolerance in transgenic tobacco, rice and Arabidopsis, respectively [10,16,17,54]. However, some SAP genes play a negative role in stress tolerance. For example, rice OsiSAP7 and ZFP185 negatively regulate ABA stress signaling and impart sensitivity to drought and salt stress in transgenic Arabidopsis and rice [30,33]. Likewise, the downregulation of PagSAPI in poplar significantly enhances tolerance to salt stress, increases the K+/Na+ ratio in roots, and alters gene expression related to cellular ion homeostasis [55].

In conclusion, a total of 10 ZmSAP genes were identified in the maize genome. All ZmSAPs belong to the family of zinc-finger proteins with the A20/AN1 domain. The expression of ZmSAP genes was regulated by osmotic stresses, including drought and salt, as well as ABA. Furthermore, heterologous expression of ZmSAP2 and ZmSAP7 significantly improved the saline tolerance in yeast cells. The study suggests that ZmSAPs may play important roles in response to abiotic stresses and provides insights into further underlying the regulatory mechanisms of ZmSAPs in regulating stress response in maize.
4. Materials and Methods

4.1. Identification of ZmSAPs in Maize

The genome and protein sequence data (reference 5.0) of maize B73 were retrieved from the MaizeGDB database (https://download.maizegdb.org/Zm-B73-REFERENCE-NAM-5.0/, accessed on 15 October 2021) and used for ZmSAPs search. Subsequently, the 14 and 18 SAP protein sequences of Arabidopsis and rice were downloaded from the Arabidopsis Information Resource (http://www.arabidopsis.org/, accessed on 15 October 2021) and the Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/index.shtml, accessed on 15 October 2021) and used as queries to perform local BLASTp search against above maize database by using the BLAST + suite with an E-value $1e^{-10}$, respectively [11]. Meanwhile, the A20 domain (Pfam ID: PF01754) and AN1 domain (Pfam ID: PF01428) were downloaded from the Pfam database (https://pfam-legacy.xfam.org/, accessed on 15 October 2021) and used to further identify ZmSAP candidates using HMMER3.0. Then, the redundant sequences were removed manually to acquire ZmSAPs.

According to the method described by Yu et al. [49], the amino acid sequences of ZmSAPs were used to analyze physical and chemical properties and secondary structure by using ProtParam (http://web.expasy.org/protparam/, accessed on 20 October 2021), GOR IV (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_gor4.html, accessed on 20 October 2021), TMHMM Server v. 2.0 (https://services.healthtech.dtu.dk/service.php?TMHMM-2.0/, accessed on 20 October 2021) and SignalP 4.1 (https://services.healthtech.dtu.dk/service.php?SignalP-5.0/, accessed on 20 October 2021), respectively. The subcellular location was predicted by using WoLF PSORT (http://www.genscript.com/wolf-psort.html, accessed on 20 October 2021). The conserved domains and motifs of ZmSAPs were further analyzed via searching the Conserved Domain Search Database (CDD, http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml, accessed on 20 October 2021) and using Multiple Em for Motif Elicitation (MEME) online program (https://meme-suite.org/meme/doc/meme.html, accessed on 20 October 2021) [56], respectively.

The amino acid sequence of AtSAPs and OsSAPs were used for phylogenetic analysis against with ZmSAPs of maize. The phylogenetic tree was constructed using MEGA7.0 with the neighbor-joining (NJ) method (http://www.megasoftware.net, accessed on 28 October 2021) with 1000 bootstrap replications. The phylogenetic tree among ZmSAP members was also constructed using the same method.

4.2. Chromosomal Location, Gene Replication and Structure analysis

The physical location of ZmSAPs was obtained from position information in the MaizeGDB database. Subsequently, ZmSAPs were mapped to maize chromosomes by using Circos [37]. The gene replication events among ZmSAP genes were analyzed using the Multiple collarinear scanning toolkits (MCScanX) with the default parameters [57]. The synteny relationship among SAP genes of maize, Arabidopsis and rice was analyzed using Dual Synteny Plotter software (https://github.com/CJ-Chen/TBtools, accessed on 10 November 2021) [58]. The cDNA and genomic sequences of ZmSAPs were obtained from MaizeGDB and then used to analyze the exon–intron organizations and intron type by using Gene Structure Display Server (GSDS) (http://gsds.gao-lab.org/, accessed on 10 November 2021).

The 2000 bp upstream of translation start site (TSS) of ZmSAPs were downloaded from MaizeGDB and used for cis-elements analysis by using PlantCARE online software (available online: http://bioinformatics.psb.ugent.be/webtools/plantcare/html/, accessed on 15 November 2021).

4.3. Tissue Expression Analysis of ZmSAPs

To examine the potential role of ZmSAPs in maize development, the expression feature of ZmSAPs in different development stages was analyzed. The RNA-seq data of ZmSAPs in different tissues were obtained from MaizeGDB qTeller center (https://qteller.maizegdb.org/, accessed on 5 November 2022), displayed as fragments per kilobase of the
exon model per million mapped fragments (FPKM) values and visualized as a heatmap using TBtools [56].

4.4. Plant Materials and Stress Treatments

The seeds of the maize inbred line B73 were surface-sterilized and germinated in filter paper for 48 h. The seedlings were transplanted into a Hoagland’s solution for a hydroponic culture under 16 h light at 28 °C/8 h dark at 25 °C periods. At the three-leaf stage, the seedlings with the same size were divided into four groups. Each of the three groups of seedlings was treated with 16% PEG-6000, 250 mM NaCl and 100 µM ABA solution, respectively. One group of seedlings was used as a control without treatment. At 0, 3, 6, 9, 12, and 24 h of treatment, the leaves were sampled, frozen and ground in liquid nitrogen, and stored at −80 °C for an RNA extraction, with three replicates.

4.5. QRT-PCR Analysis

The total RNA of every sample was extracted by using RNAiso plus kit (TaKaRa, Dalian, China) according to the manufacturer’s instruction, then treated with RNase-free DNase, and quantified using NanoDrop™ OneC (ThermoScientific, Waltham, MA, USA). Subsequently, the 100 ng RNA of every sample was reverse transcribed into cDNA using the PrimeScript™ reagent kit (TaKaRa, Dalian, China). The cDNA samples were stored at −20 °C and used for quantitative real-time PCR (qRT-PCR).

The specific primer pairs of ZmSAPs and ZmEF-1a for internal control were designed by using the Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast, accessed on 5 January 2022) and synthesized at TSINGKE (China) (Table S2). As described by Sun et al. [52], the qRT-PCR was conducted in CFX96™ Real-Time System (BioRad, Hercules, CA, USA) using SYBR PremixEx Taq™ II (TaKaRa, Dalian, China) with three technical replicates. The expression of ZmSAP genes was analyzed and normalized by using the 2^−ΔΔCT method of the CFX Manager™ software version 2.0 (Bio-Rad, Hercules, CA, USA) [59]. The data were shown as mean value ± standard deviation (SD). The statistical significance among three biological replicates was tested by the Student’s t-test.

4.6. Stress Tolerance Test of ZmSAPs in Yeast Cells

The specific primer pairs of ZmSAPs were designed using Primer 5, synthesized at TSINGKE (China) (Table S2), and used to amplify the open reading frame (ORF) of ZmSAPs from cDNA of maize B73 inbred line by PCR amplification. The purified PCR product of every ZmSAP was inserted into the BamHI/Xhol site of the pYES2 vector (INVITROGEN, Waltham, MA, USA). The recombinant plasmid and empty vector pYES2 were transformed into Saccharomyces cerevisiae W303a (MATa ade2 ura3 leu2 his3 trp1) by standard PEG lithium acetate method, respectively [60]. The transformed yeast solution was spread on the yeast nitrogen plates lacking uracil (YNB-Ura) and cultured at 28 °C in an incubator for 2–3 days. The positive colony transformed by every ZmSAP was identified by PCR and incubated overnight in liquid YNB-Ura medium to OD600 to 1.0.

According to the methods of Ben et al. [52,53], with minor modification, the yeast cultures were diluted to successive gradient dilutions (10^−1, 10^−2, 10^−3, 10^−4) using liquid YNB-Ura medium. Then, 8 µL of each diluent was placed on YNB-Ura-Gal 2% solid medium supplemented with 0.5, 1.0, 1.5 M NaCl or 2.0, 2.5, 3.0 M Mannitol, respectively, and cultured at 28 °C in an incubator for 2–3 days for phenotyping. Subsequently, the candidates showing preferential growth vigor on solid medium were selected for dissecting the growth curve. The OD600 of yeast line with candidate genes was adjusted to 0.2, then 1 mL of them was added into 20 mL YNB-Ura-Gal 2% liquid medium, and cultured at 28 °C. At 0, 12, 24, 36, 48 and 72 h, the OD600 of every culture was monitored with three replicates. The yeast transformed by the pYES2 vector was used as a control. The 2% galactose (Gal) was added into the YNB-Ura medium to induce the expression of ZmSAPs under the control of the Gal promoter.
Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms232214010/s1.

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