A Stress-Associated Protein, PtSAP13, From Populus trichocarpa Provides Tolerance to Salt Stress

Jianbo Li 1,2,*, Pei Sun 2,3,†, Yongxiu Xia 1, Guangshun Zheng 1, Jingshuang Sun 1 and Huixia Jia 2,3,‡

1 Experimental Center of Forestry in North China, Chinese Academy of Forestry, Beijing 102300, China; yxxia@caf.ac.cn (Y.X.); guangshunzheng@caf.ac.cn (G.Z.); sjshuang@caf.ac.cn (J.S.)
2 State Key Laboratory of Tree Genetics and Breeding, Chinese Academy of Forestry, Beijing 100091, China; Sunpei@caf.ac.cn
3 Research Institute of Forestry, Chinese Academy of Forestry, Beijing 100091, China
* Correspondence: Lijb2017@caf.ac.cn (J.L.); Jiahx@caf.ac.cn (H.J.)
† These authors have contributed equally to this work.

Received: 31 October 2019; Accepted: 15 November 2019; Published: 17 November 2019

Abstract: The growth and production of poplars are usually affected by unfavorable environmental conditions such as soil salinization. Thus, enhancing salt tolerance of poplars will promote their better adaptation to environmental stresses and improve their biomass production. Stress-associated proteins (SAPs) are a novel class of A20/AN1 zinc finger proteins that have been shown to confer plants’ tolerance to multiple abiotic stresses. However, the precise functions of SAP genes in poplars are still largely unknown. Here, the expression profiles of Populus trichocarpa SAPs in response to salt stress revealed that PtSAP13 with two AN1 domains was up-regulated dramatically during salt treatment. The β-glucuronidase (GUS) staining showed that PtSAP13 was accumulated dominantly in leaf and root, and the GUS signal was increased under salt condition. The Arabidopsis transgenic plants overexpressing PtSAP13 exhibited higher seed germination and better growth than wild-type (WT) plants under salt stress, demonstrating that overexpression of PtSAP13 increased salt tolerance. Higher activities of antioxidant enzymes were found in PtSAP13-overexpressing plants than in WT plants under salt stress. Transcriptome analysis revealed that some stress-related genes, including Glutathione peroxidase 8, NADP-malic enzyme 2, Response to ABA and Salt 1, WRKYs, Glutathione S-Transferase, and MYBs, were induced by salt in transgenic plants. Moreover, the pathways of flavonoid biosynthesis and metabolic processes, regulation of response to stress, response to ethylene, dioxygenase activity, glucosyltransferase activity, monooxygenase activity, and oxidoreductase activity were specially enriched in transgenic plants under salt condition. Taken together, our results demonstrate that PtSAP13 enhances salt tolerance through up-regulating the expression of stress-related genes and mediating multiple biological pathways.

Keywords: stress-associated protein; expression analysis; transgene; salt tolerance; transcriptome; Populus trichocarpa

1. Introduction

Soil salinization is a major environmental constraint on plant growth, development, and production [1]. High salinization limits the ability of plants to extract water and disrupt ion distribution, and usually causes oxidative stresses [2,3]. Worldwide, there are at least 800 million hectares of land subjected to salinity, accounting for ~6% of the land surface [3]. Due to intensive land use, lack of freshwater resources, and irrational irrigation, the salinized area is increasing. To overcome the detrimental effects of salt stress, plants have developed multiple complex strategies, including
ion sequestration and/or exclusion, osmotic adjustment, metabolic adjustment, and antioxidative defense under salt stress. Several salt tolerance-related genes, such as Salt-Overly-Sensitive [4], Na+/H+ Exchanger [5,6], High-affinity K+ transporters [7], NAC [8], WRKY [9,10], and AP2/ERF [11,12], have been characterized to improve salt tolerance in some plant species. However, salt response and tolerance are complex regulatory networks involving the coordinated action of multiple genes [13]. Thus, more efficient genes need to be identified to facilitate the cultivation of varieties with high salinity tolerance.

The stress-associated protein (SAP) genes encode a novel class of zinc finger proteins and widely present in eukaryotes. Since the OsSAP1 was firstly isolated in plants, their homologs genes have been identified in Arabidopsis thaliana [14], Oryza sativa [14], Populus euphratica [15], Medicago truncatula [16], and Brassica napus [17]. The essential characteristic of SAP protein is the presence of two special zinc finger domains (A20 domain and/or AN1 domain). The A20 zinc finger domain has Cx2-Cx13-Cx2C consensus sequence at the N-terminus [18]. The AN1 zinc finger domain has two types, including Cx2-Cx9-12-Cx12-Cx4-Cx2-Hx5-HxC consensus sequence and Cx4-Cx9-12-Cx12-Cx4-Cx2-Hx5-HxC consensus sequence at the C-terminus [19].

To date, several SAP genes with one A20 domain and one AN1 domain from different species have been confirmed to be involved in tolerance to multiple abiotic stresses. In rice, the expression of OsSAP1 is induced by different stresses (cold, drought, salt, heavy metals, ABA, and injury), and overexpression of OsSAP1 improves the tolerance to drought, cold, and salt stresses, and enhances its immunity to pathogen in transgenic plants [20–22]. The transgenic plants overexpressing OsSAP8, OsSAP9, and OsSAP11 also showed high tolerance to salt, drought, and cold stresses [23–25]. In Arabidopsis, the expression of AtSAP5 is induced by salt, osmotic, drought, and cold stresses. Constitutively, overexpression of AtSAP5 significantly increases tolerance to salt, drought, and heat stresses by up-regulating the expression of the stress tolerance genes [26,27]. Overexpressing Aeluropus littoralis AtSAP enhances the tolerance to cold, drought, and salt stresses through maintenance of photosynthesis in transgenic rice [28], and increases rice grain yield under drought condition [29]. In addition, SbSAP14 from Sorghum bicolor [30] and MusaSAP1 from banana cv. Karibale Monthan [31] also confer tolerance to different stresses. Except for the function investigations of SAPs containing A20 and AN1 domains, AtSAP13 with two AN1 domains and one C2H2 domain is up-regulated under ABA, salt, and Cd stresses, and AtSAP13-overexpressing plants exhibit high tolerance to drought, salt, and toxic metals [32]. Wheat TaSAP17-D with two AN1 domains and two C2H2 domains is induced by salt, drought, and cold stresses, and it can enhance tolerance to salt stress in transgenic Arabidopsis [33]. Despite these advances, these studies mainly concentrated on herbaceous plants, and the biological functions of SAP genes in woody plants is far from complete.

During their long life spans, perennial woody plants have evolved sophisticated systems to cope with salt stress. In view of the key roles of SAP genes in plant stress responses, deciphering the SAP functions in woody plants will be helpful for better understanding their salt response mechanisms. As a model woody plant, poplar has great economic and ecology benefits. Due to its rapid growth and high biomass yield, poplar is used widely to generate wood, fiber, feedstock for biofuels, and other bioproducts [34,35]; and it also plays important roles in soil and water conservation, sand break, carbon fixation, phytoremediation, and so on [34,35]. To our knowledge, only stress-associated protein 1 (PagSAPI1) from Populus alba × P. glandulosa has been investigated, which showed down-regulation of PagSAPI1 increases salt stress tolerance in poplar [36]. In this study, the expression profiles of P. trichocarpa PtSAPs were surveyed to screen candidate genes involved in salt stress tolerance, and we found that PtSAP13 with two AN1 domains was up-regulated dramatically under salt treatment. Thus, we generated the pPtSAP13::GUS and p35S::PtSAP13 transgenic Arabidopsis plants to investigate the PtSAP13 function. Moreover, RNA-seq was performed to explore the molecular mechanisms of PtSAP13 in salt tolerance. Our research indicates that PtSAP13 plays a positive role in salt tolerance.
2. Results

2.1. Phylogenetic Analysis of SAPs in P. trichocarpa and Other Species

A total of 19 SAP genes were identified from *P. trichocarpa* genome, which is in keeping with previous research [19]. Based on their locations in the genome, we sequentially named them from PtSAP1 to PtSAP19 (Table S1). Conserved domain analysis revealed that two PtSAPs (-14 and -17) included one AN1 domain (AN1); PtSAP3 contained one A20 domain (A20); PtSAP13 contained two AN1 domains (AN1–AN1); PtSAP8 contained two AN1 domain, and two CH2–CH2 domains (AN1–AN1–CH2–CH2); and the remaining 14 SAP members included one A20 domain and one AN1 domain (A20–AN1) (Table S1, Figure 1 and Figure S1). To reveal the evolutionary pattern and phylogenetic relationships of SAP, an unrooted phylogenetic tree was constructed using the protein sequences of SAPs from *P. trichocarpa* and another eight species (*Eucalyptus grandis*, *Manihot esculenta*, *Marchantia polymorpha*, *Oryza sativa*, *Selaginella moellendorfii*, *Zea mays*, *Arabidopsis thaliana*, and Volvox carteri) (Figure 1A). The result shows that the SAPs were classified into seven classes (Class A–Class G). The SAPs containing A20–AN1 were present in Class A–Class E, and almost all of SAPs in Class F and Class G contained AN1 and/or CH2 domains, including AN1, AN1–AN1, AN1–AN1–CH2, or AN1–AN1–CH2–CH2 (Figure 1A). Except Class E, the 19 PtSAPs were unevenly distributed in the other six classes. The SAP members of each species were counted (Figure 1B). The lower algae *V. carteri* had the minimum number of SAPs, with only two members, while *P. trichocarpa* had the maximum number of SAPs (19), followed by *O. sativa* (18), *M. esculenta* (16), *A. thaliana* (14), *E. grandis* (11), and *Z. mays* (11) (Figure 1B).
Figure 1. The SAP gene family underwent gene expansion in evolution history. (A) Phylogenetic tree and evolutionary relationship of the SAPs from *Populus trichocarpa* (Pt), *Eucalyptus grandis* (Eg), *Manihot esculenta* (Me), *Marchantia polymorpha* (Mp), *Oryza sativa* (Os), *Selaginella moellendorffii* (Sm), *Zea mays* (Zm), *Arabidopsis thaliana* (At), and *Volvox carteri* (Vc). (B) The number of SAP genes and zinc finger domains in different plants. The detailed SAP gene information and protein sequences are listed in Table S1.

2.2. Expression Profiles of PtSAP Genes

Revealing the expression profiles in various tissues under stress conditions will provide clues for gene potential functions; thus, we firstly analyzed the expression patterns of PtSAPs. Based on the public database of *Populus*, the expression profiles of PtSAPs in different tissues and developmental processes, including leaf expansion, germination of bud, male/female development, and different nitrogen nutrition treatment in root and stem, were investigated (Figure S2). Three PtSAPs (-2, -10, and -11) were more highly expressed in root than other tissues, and three PtSAPs (-4, -11, and -13) were induced under ammonia, nitrate, or urea condition in root (Figure S2). Only five PtSAPs (-4, -10, -11, -12, and -13) were highly expressed in the middle of male catkin development. In the process of
pre-dormant bud I to fully open bud, the expression of three PtSAPs (-2, -5, and -13) increased early and then declined, which indicates that these genes might be involved in dormant bud formation (Figure S2).

In addition, we detected the expression patterns of PtSAPs in response to salt stress using qRT-PCR. Except PtSAP16, the expressions of other PtSAPs were increased under salt treatment. Seven PtSAPs (-1, -2, -3, -4, -9, -11, and -13) exhibited significant up-regulation (Figure 2). Notably, PtSAP13 was rapidly induced (~8-fold) at 1 h and reached its maximal level (~12-fold) at 6 h, after which, it began to decline slowly at 24 h and 48 h (Figure 2).

![Figure 2.](image)

**Figure 2.** Expression analysis of PtSAP genes under salt stress. The gene expression levels under salt stress were determined using qRT-PCR. Error bars represent the standard deviations of three biological replicates and four technical replicates.

2.3. Expression Patterns of PtSAP13

According to the expression level of PtSAP13 under salt treatment, we chose PtSAP13 for further function analysis. The full length CDS of PtSAP13 was 450 bp and it encoded a 149 amino acid protein with two AN1 domains (Table S1). The molecular weight of PtSAP13 was 16.97 kDa and its predicted isoelectric point was 9.02, respectively (Table S1). In order to further determine the expression patterns of PtSAP13, the promoter fragment was amplified and constructed into the pMDC164 vector to fuse with the reporter gene GUS (namely, pPtSAP13::GUS), and transgenic Arabidopsis was generated. GUS staining in Arabidopsis revealed that GUS signal was detected in roots in 1-day-old seedlings (Figure 3A). Subsequently, 3-day-old seedlings showed high GUS signal in cotyledons and roots (Figure 3B), as well as detected in 19-day-old seedlings (Figure 3C) and 1-month-old seedlings (Figure 3D). In addition, GUS signaling was also detected in stigma, stamen, and siliques (Figure 3E,F). When a 10-day-old pPtSAP13::GUS transgenic plant was treated with salt for 12 h, strong GUS activity was detected (Figure 3H), which confirms the results of the expression profile of PtSAP13.
2.4. Effect of Salt Stress on Seed Germination in Transgenic PtSAP13 Arabidopsis Lines

To gain insight into the function of PtSAP13 in salt stress tolerance, transgenic Arabidopsis lines with overexpressing PtSAP13 were generated. Three transgenic lines (Line 6, Line 15, and Line 30) with high expression of PtSAP13 were used for its stress tolerance investigation (Figure 4A). On the normal 1/2 MS medium, there was no difference in germination rates between the WT and PtSAP13-overexpressing plants, and the germination rate was nearly 100% (Figure 4E). After being cultured on 200 mM NaCl medium for 7 days, the germination rates of the three transgenic lines (Line 6, Line 15, and Line 30) were 74.5%, 80.7%, and 84.0%, respectively; whereas the WT exhibited a lower germination rate, with nearly 45.4%, than transgenic lines (Figure 4D). This result indicates that overexpression of PtSAP13 increased the seed germination under salt stress.
Figure 4. Salt tolerance analysis in panel growth condition. (A) qRT-PCR analysis of PtSAP13 expression in wild-type (WT) and transgenic plants. (B) Seed germination of WT and transgenic lines under normal and salt medium. (C) Photograph of WT and transgenic seedlings under normal and salt treatment medium. (D–F) Measurement of the seed germination (D), fresh weight (E), and root length (F) of WT and PtSAP13-overexpressing plants under normal condition and stress treatments. Error bars indicate the standard deviations, and * indicate significant differences compared with WT at the \( p < 0.05 \) level.

2.5. PtSAP13 Transgenic Arabidopsis Seedling Resistance to Salt Stress

One-week-old WT and PtSAP13-overexpressing plants were transferred to 1/2 MS solid medium containing 0 mM (normal) or 150 mM NaCl (salt stress). No significant phenotypic difference was observed between the WT and transgenic plants under normal medium (Figure 4C). Under salt condition, the growth of the WT and transgenic plants was significantly inhibited, but the growth situation of the transgenic plants was better than that of the WT plants (Figure 4C). Both average fresh weight and root length of the transgenic plants were more than the WT plants (Figure 4E,F). Furthermore, similar results were also obtained in the soil-grown WT and transgenic plants (Figure 5A,B). The relative electric conductivity in the transgenic plants was lower than in the WT plants (Figure 5C), whereas the proline content was higher in the transgenic plants than in the WT plants under salt condition (Figure 5D). The peroxidase (POD), Superoxide dismutase (SOD), and catalase (CAT) activities of the transgenic plants were higher than that of the WT plants after salt treatment (Figure 5E–G), indicating...
that scavenging of reactive oxygen species (ROS) was enhanced by PtSAP13 in transgenic plants. All of these results indicate that overexpression of the PtSAP13 improved salt tolerance.

**Figure 5.** Salt stress tolerance analysis in soil growth condition. (A, B) Photograph of WT and transgenic seedlings under normal condition (A) and salt treatment (B). (C–G) Measurement of the Relative electric conductivity (REC) (C), proline content (D), POD activity (E), SOD activity (F), and CAT activity (G). Error bars indicate the standard deviations, and * indicates significant differences compared with WT at the \( p < 0.05 \) level.

### 2.6. Overexpression of PtSAP13 Induced the Expression of Stress-Related Genes

To further investigate the regulatory mechanism of PtSAP13, RNA-seq analysis was performed for the WT and PtSAP13-overexpressing plants under normal and salt conditions. A total of 79 differentially expressed genes (DEGs), including 54 up-regulated genes and 25 down-regulated genes, were identified in the PtSAP13-overexpressing plants compared to the WT plants under normal condition (SAP13-Normal/WT-Normal) (Figure 6). Compared with the normal condition, 3641 DEGs (2184 up-regulated genes and 1457 down-regulated genes) were detected in WT plants under salt treatment (WT-Salt/WT-Normal), and 2879 DEGs (2035 up-regulated genes and 844 down-regulated genes) in the PtSAP13-overexpressing plants under salt treatment (SAP13-Salt/SAP13-Normal) (Figure 6). In addition, 510 DEGs (219 up-regulated genes and 291 down-regulated genes) were found in the PtSAP13-overexpressing plants compared to the WT plants under salt treatment (WT-Salt/SAP13-Salt) (Figure 6).
Comparative analysis revealed that 2145 DEGs were overlapped between SAP13-Salt/SAP13-Normal and WT-Salt/WT-Normal (Figure 6). Notably, 464 DEGs were specific in the PtSAP13-overexpressing plants under salt stress (PtSAP13-Salt/PtSAP13-Normal) but not in the WT plants under salt stress (WT-Salt/WT-Normal) (Figure 6). Among these DEGs, some stress-related genes, including BZIP60, DII9-4, Glutathione peroxidase (GPX8), NADP-malic enzyme 2 (NADP-ME2), Response to ABA and Salt 1 (RAS1), WRKYs (-14, -24, -35, and -65), Glutathione S-Transferase (GSTUs -20 and GSTU22), and MYBs (-29, -67, -69, -76, and -93), were up-regulated in the transgenic plants under salt condition.

To gain insight into the major functional categories represented by the DEGs, GO enrichment analysis was performed in four comparison sets (WT-Salt/WT-Normal, SAP13-Normal/WT-Normal, SAP13-Salt/SAP13-Normal, and SAP13-Salt/WT-Salt). The results show that DEGs were enriched in 83 BP GO terms (Figure 7). A considerable proportion of the GO terms, including multiple responses to abiotic or biotic stresses, responses to various hormones, glutathione metabolic process, oxidation-reduction process, phenylpropanoid biosynthesis and metabolic process, and toxin catabolic and metabolic process, were mainly enriched in WT-Salt/WT-Normal and SAP13-Salt/SAP13-Normal, implying that these pathways were universal and common response mechanisms under salt stress in both the WT and PtSAP13-overexpressing plants (Figure 7). The GO terms of flavonoid biosynthesis and metabolic process, regulation of response to stress, response to ethylene, and cellular response to hypoxia were uniquely enriched only in SAP13-Salt/SAP13-Normal. In addition, DEGs were enriched in 29 MF GO terms (Figure 7). The dioxygenase activity, glucosyltransferase activity, monooxygenase activity, and oxidoreductase activities acting on CH–OH group of donors and NAD or NADP as acceptor were uniquely enriched only in SAP13-Salt/SAP13-Normal (Figure 8). These results suggested
that PtSAP13-overexpressing plants might possess specific regulatory pathways to enhance their salt tolerance (Figure 8).

### Table 7

| Term ID | Go Terms | WT-Soil / WT-Normal | SAP3-Soil / SAP13-Soil | SAP3-Soil / WT-Normal | SAP13-Soil / WT-Soil |
|---------|----------|---------------------|------------------------|----------------------|----------------------|
| GO:0036202 | Defense response | 1.87 | 0.27E-19 | 1.87 | 1.21E-19 |
| GO:0042220 | Defense response to biotic stimuli | 1.89 | 2.10E-26 | 2.92 | 2.07E-26 |
| GO:0042222 | Response to benzoate | 2.63 | 1.24E-19 | 2.63 | 7.54E-19 |
| GO:0042224 | Response to fatty acids | 2.66 | 4.48E-21 | 2.66 | 1.71E-21 |
| GO:0042226 | Response to iron deficiency | 1.81 | 2.43E-23 | 1.81 | 1.71E-23 |
| GO:0042228 | Response to light stimulus | 1.79 | 1.54E-20 | 1.79 | 9.41E-20 |
| GO:0042230 | Response to nitrogen stimulus | 1.72 | 1.22E-15 | 1.72 | 6.45E-15 |
| GO:0042232 | Response to osmotic stress | 2.18 | 3.39E-20 | 2.18 | 1.46E-20 |
| GO:0042234 | Response to phosphate stimulus | 2.14 | 3.94E-19 | 2.14 | 1.39E-19 |
| GO:0042236 | Response to potassium stimulus | 2.14 | 3.94E-19 | 2.14 | 1.39E-19 |
| GO:0042238 | Response to sodium stimulus | 2.14 | 3.94E-19 | 2.14 | 1.39E-19 |
| GO:0042240 | Response to temperature change | 2.14 | 3.94E-19 | 2.14 | 1.39E-19 |
| GO:0042242 | Response to various responses | 2.14 | 3.94E-19 | 2.14 | 1.39E-19 |
| GO:0042244 | Responses to auxin stimulus | 1.77 | 3.49E-14 | 1.77 | 2.15E-14 |
| GO:0042246 | Responses to biotic stimulus | 2.20 | 2.98E-17 | 2.20 | 6.31E-17 |
| GO:0042248 | Responses to chemical stimulus | 2.20 | 2.98E-17 | 2.20 | 6.31E-17 |
| GO:0042250 | Responses to physical stimulus | 2.20 | 2.98E-17 | 2.20 | 6.31E-17 |

Figure 7. Biological processes in gene ontology (GO) enrichment analysis of all DEGs in four types of comparisons in WT and PtSAP13-overexpressing plants. Only significantly enriched terms with corrected p-value < 0.05 are indicated.
### Table

| Term ID | Go Terms | WT-Salt / WT-Normal | SAP13-Salt / SAP13-Normal | SAP13-Normal / WT-Normal | SAP13-Salt / WT-Salt |
|---------|-----------|---------------------|---------------------------|--------------------------|----------------------|
| A       | GO:0003824 Catalytic activity | 1.22 9.06E-14 | 1.23 1.65E-11 | 1.30 1.18E-02 |                     |
|         | GO:0003614 Molecular function   | 1.08 3.19E-12 | 1.07 5.81E-09 | 1.11 7.66E-04 |                     |
|         | GO:0016891 Oxidoreductase activity | 1.91 3.22E-19 | 1.94 2.68E-20 | 1.99 4.14E-03 |                     |
|         | GO:0016864 Oxidoreductase activity, acting on peroxide as acceptor | 3.08 3.12E-07 | 3.29 1.96E-07 | 4.95 2.05E-02 |                     |
|         | GO:004601 Peroxidase activity   | 3.17 2.13E-07 | 3.47 1.64E-07 | 5.18 1.33E-02 |                     |
| B       | GO:0008059 Anion transmembrane transporter activity | 1.79 4.49E-02 | 1.95 1.60E-02 |                     |                     |
|         | GO:0016209 Antioxidant activity  | 2.29 2.19E-06 | 3.22 6.48E-08 |                     |                     |
|         | GO:0034169 Cation binding       | 1.24 2.83E-02 | 1.31 2.10E-03 |                     |                     |
|         | GO:0066663 Coenzyme binding     | 2.67 1.72E-05 | 2.69 3.10E-04 |                     |                     |
|         | GO:0408373 Cofactor binding     | 1.98 1.26E-12 | 1.62 1.41E-12 |                     |                     |
|         | GO:0071949 FAD binding          | 3.17 1.35E-02 | 3.37 2.19E-02 |                     |                     |
|         | GO:0066665 Flavin adenine dinucleotide binding | 2.68 4.33E-03 | 2.73 6.72E-03 |                     |                     |
|         | GO:004354 Glutathione transferase activity | 3.29 1.67E-02 | 4.05 3.85E-04 |                     |                     |
|         | GO:0202037 Heme binding         | 2.08 9.19E-04 | 2.13 3.98E-03 |                     |                     |
|         | GO:0015103 Inorganic ion transmembrane transporter activity | 2.40 1.95E-02 | 2.63 1.26E-02 |                     |                     |
|         | GO:0041617 Ion binding          | 1.11 5.54E-04 | 1.22 2.79E-03 |                     |                     |
|         | GO:046873 Metal ion binding     | 1.25 3.05E-02 | 1.31 1.95E-03 |                     |                     |
|         | GO:0016705 Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen | 1.75 2.88E-02 | 2.08 1.32E-04 |                     |                     |
|         | GO:0489556 Tetrapyrrole binding | 1.99 1.26E-03 | 1.96 1.64E-02 |                     |                     |
| C       | GO:0007300 DNA-binding transcription factor activity | 1.42 3.20E-05 |                     |                     |                     |
|         | GO:0015138 Inorganic molecular entity transporter activity | 1.49 3.39E-02 |                     |                     |                     |
|         | GO:0140198 Transcription regulator activity | 1.34 2.81E-03 |                     |                     |                     |
| D       | GO:0051213 Dioxigenase activity  |                     | 2.26 4.30E-02 |                     |                     |
|         | GO:045827 Glucoamylase activity  |                     | 2.09 4.91E-02 |                     |                     |
|         | GO:000497 Monooxygenase activity |                     | 1.97 2.53E-02 |                     |                     |
|         | GO:0016614 Oxidoreductase activity, acting on CH-OH group of donors |                     | 2.41 9.01E-04 |                     |                     |
|         | GO:0016616 Oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor |                     | 2.30 7.21E-03 |                     |                     |
|         | GO:0088504 Quinol:cytochrome c oxidoreductase activity |                     | 2.71 2.79E-02 |                     |                     |
|         | GO:0088504 Quinone:cytochrome c oxidoreductase activity |                     | 2.76 4.11E-02 |                     |                     |

**Figure 8.** Molecular function in GO enrichment analysis of all DEGs in four types of comparisons of WT and PtSAP13-overexpressing plants. Only significantly enriched terms with corrected p-value < 0.05 are indicated.

### 3. Discussion

The number of SAP genes increased from lower algae (two SAPs in *V. carteri*) through early land plants (from three SAPs in *M. polymorpha* to six SAPs in *S. moellendorffii*), to higher plants (from 11 SAPs in *O. sativa* to 19 SAPs in *P. trichocarpa*). *P. trichocarpa* had the maximum number of SAPs, suggesting that PtSAP genes underwent expansion through whole genome duplication. Previous studies have found that the HTK1 (*high affinity K*⁺ *transporter 1*) family in *P. euphratica* [37] and Hsfs (*Heat shock transcription factors*) family in *P. trichocarpa* [38] are also expanded to defense against abiotic stresses. *P. trichocarpa* is widely distributed across western North America, with wide geographical and environmental gradients [39]. In addition, compared with herbaceous plants, *P. trichocarpa* has a long life span, thus it might have evolved sophisticated adaptive systems that need the participation of more stress-related genes to ensure it survives in prolonged and repeated environmental stresses.

The expression patterns of PtSAPs under salt stress were analyzed. We found that most of PtSAPs were induced under salt stress, and similar results have been found in SAPs from other species. For example, *Gossypium hirsutum* GhSAPs (-7A, -7D, -8A, -11D, and -16D) and *P. euphratica* PeuSAPs (-1, -2, -8/14, and -16/17) were significantly induced under salt treatment [15]. Among these PtSAPs, PtSAP13 exhibited more significant up-regulation than other PtSAPs under salt stress, suggesting that PtSAP13 might be involved in salt tolerance in *P. trichocarpa*. Structural analysis found that PtSAP13 protein contained two AN1 domains without AN20. The specific role of AN1 domain is still poorly...
known. Two SAP proteins, including Arabidopsis AtSAP13 and T. aestivum TaSAP17-D, that contain two AN1 domains have been reported to enhance the salt tolerance in transgenic plants [33]. In our study, overexpression of PISAP13 also improved salt tolerance of transgenic plants, as evidenced by the better growth in fresh weight and root length, as well as increased activities of antioxidative enzymes than WT plants under salt treatment. These results further demonstrate that SAPs containing AN1–AN1 had conserved function and played essential roles in salt tolerance.

Salt stress can trigger two types of stresses, including osmotic stress and ion toxicity. Early effects of salt stress are to prevent plants from absorbing water, and quickly cause decrease of growth rate [40]. During this process, many physiological and transcriptional responses are identical to those caused by water stress [13,40,41]. After excessive amounts of Na⁺ and Cl⁻ enter the plants, ion toxicity will eventually give rise to premature senescence of leaves and limit plant growth and development [13]. To adapt to salt stress, plant have evolved a complex regulatory network that mediates multiple processes were uniquely enriched under salt stress, which might help mediate the salt tolerance of transgenic plants.

In our study, the activities of these enzymatic antioxidants were higher in transgenic plants, and these DEGs were enriched in multiple biological pathways. These results confirm that plant response and tolerance to salt stress is a complex system. Transcriptome data revealed that some stress-related genes, such as BZIP60, DI19-4, GPX8, NADP-ME2, RAS1, WRKYs, GSTUs, and MYBs, were up-regulated in the PisAP13-overexpressing plants, but did not change in the WT plants under salt stress. BZIP60, a member of basic leucine zipper transcription factor, is involved in plant immunity and abiotic stress responses [42]. Overexpression of TabZIP60 from T. aestivum can enhance the resistance of transgenic Arabidopsis to abiotic stresses and enhance ABA sensitivity [43]. DI19-4 is a member of the Drought-Induced gene family, its expression in Arabidopsis is induced by salt and dehydration [44,45]. Overexpression of OsDI19-4 from O. sativa increases stress tolerance by enhancing ROS-scavenging activity [44,45]. GPX8 participates to withstand oxidative damage and/or to modulate oxidative signaling caused by reactive oxygen species. In the mutant of gpx8, the plant exhibits increased sensitivity to oxidative damage by enhancing the oxidized proteins, while overexpression of Arabidopsis AtGPX8 increases the oxidative tolerance [46]. The expression of O. sativa NADP-ME2 is induced by NaHCO₃, NaCl, and PEG stresses. Overexpression from O. sativa and SbNADP-ME from Sorghum bicolor enhances tolerance of transgenic plants’ salt stress [47,48]. RAS1 is associated with salt tolerance and ABA sensitivity using QTL mapping in Arabidopsis [49]. Although there are no reports of WRKYs (-11, -17, -20, and -25) and G. hirsutum GhWRKYs (-17, -34, -39-1, and -41), have been confirmed to enhance plant salt tolerance [50–55]. The expression levels of these genes increased in the PisAP13-overexpressing plants under salt stress, suggesting that PisAP13 might enhance plant salt tolerance by collaborating with these stress-related genes.

Salt stress causes excessive generation of ROS, which gives rise to oxidative damage to DNA, proteins, and lipids [56]. To cope with the oxidative damage, plants activate defense systems mediated by enzymatic and non-enzymatic antioxidants to maintain ROS homeostasis [56]. Enzymatic antioxidants, such as POD, SOD, and CAT, play important roles in scavenging ROS and maintaining redox equilibrium [57]. In our study, the activities of these enzymatic antioxidants were higher in PisAP13-overexpressing plants than in the WT plants under salt stress, indicating that the ability of scavenging ROS was enhanced by PisAP13 in transgenic plants. Flavonoids have been identified as non-enzymatic antioxidants to scavenge ROS and protect plants from oxidative damage [58]. Overexpression of Vitis amurensis NAC26 and Antirrhinum AmDEL significantly increases the accumulation of flavonoids and enhances salt tolerance in transgenic plants [59,60]. In PisAP13-overexpressing plants, flavonoid biosynthesis and metabolic processes were uniquely enriched under salt stress, which might help mediate the salt tolerance of transgenic plants.

In addition, water and nutrients were transported in conductive tissues to maintain plant growth. When plants are subjected to salt stress, their nutrient homeostasis is disrupted [61]. Improvement of the ability to absorb nutrients and increase amounts of essential nutrients will help plants resist the salt
stress [61,62]. In our study, PtSAP13 was strongly expressed in the conductive tissues (Figure 3), and its expression was induced under nitrate and urea condition (Figure S2). Thus, we speculated that PtSAP13 might also be involved in uptake and/or transport of nutrients. We also found that the nutrient-related biological processes, including the pathways of cellular response to nutrient levels and response to starvation, were enriched in SAP13-Normal/WT-Normal and SAP13-Salt/WT-Salt. Thus, these results further indicate that PtSAP13 might enhance salt tolerance by regulating nutrient-related processes.

4. Materials and Methods

4.1. Plant Materials and Salt Treatments

Seeds of A. thaliana (ecotype Columbia) which conserved in Chinese Academy of Forestry were surface-sterilized with 75% (v/v) ethanol twice, followed by 100% (v/v) ethanol once in clean bench. After being dried on filter paper, the seeds were sowed on 1/2 Murashige-Skoog (MS) solid medium containing 0.8% agar. After being vernalized at 4 °C for 3 days in the dark, the seeds were grown at 20–22 °C under long-day conditions (16/8 h light/dark) in medium or soil.

Two-month-old clones of P. trichocarpa were water-cultured using Hoagland solution with 150 mM NaCl for salt treatment. Fully matured leaves from six clones were immediately harvested after 0, 1, 6, 12, and 48 h of salt treatment and stored at −80 °C for further expression analysis. The dosages of the salt treatment were determined based on previous study [63]. This experiment included three biological replicates.

4.2. Gene Expression Analysis of PtSAPs

Expression data of PtSAPs were obtained from Populus Gene Atlas database (https://phytozome.jgi.doe.gov/pz/portal.html), including leaf: Leaf immature standard (1), leaf young standard (2), and leaf first fully expanded standard (3); root: Root standard (4), root tip standard (5), root ammonia (6), root nitrate (7), and root urea (8); stem: Stem node standard (9), stem inode standard (10), stem ammonia (11), stem nitrate (12), and stem urea (13); bud-related: Pre-dormant bud I (14), pre-dormant bud II (15), early dormant bud (16), late dormant bud (17), and fully open bud (18); male catkin-related: Male early GW9592.ZK 10 (19), GW9840.ZE 30 (20), and male mid GW9911.ZK 51 (21); female catkin-related: Female early BESC423.ZL 7 (22), female late BESC842.ZI 22 (23), and female receptive BESC443.ZG 43 (24). The expression levels of PtSAPs across the tissues, developmental processes, or treatments were compared with the mid-value. The fold changes were log2 transformed for the heatmap.

4.3. RNA Isolation and Quantitative Real-Time PCR (qRT-PCR) Analysis

An RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) was used for extracting RNA from plant samples, according to the manufacturer’s instructions. During the extraction process, DNase I (Qiagen, Hilden, Germany) was used to degrade genomic DNA. The SuperScript III first-strand synthesis system (Life technologies, Carlsbad, CA, USA) was used to synthesize the first-strand cDNA with 3 μg mRNA. The qRT-PCR was performed on the Roche LightCycler 480 (Roche Applied Science, Penzberg, Germany) using SYBR Premix Ex Taq (Takara, Kusatsu, Japan). Each sample was performed in three biological replicates and four technical replicates. AtActin2 (AT3g18780) and PtActin (Potri.001G309500) were used as reference genes in Arabidopsis and P. trichocarpa, respectively. The $2^{-\Delta\Delta CT}$ method was used to calculate the relative expression levels of each gene [64]. The primers used for qRT-PCR in this study are listed in Table S2.

4.4. Generation of Transgenic A. thaliana Plants

The coding sequence of PtrSAP13 was amplified from the cDNA of P. trichocarpa using gene-specific primers (Table S2). The amplified fragment was cloned into the pDONR222 vector (Life technologies, USA) to produce pENTR for sequencing. The correct sequence was sub-cloned into pMDC32 to construct the plasmid p35S::PtSAP13, which was used to generate overexpressing transgenic plants.
The promoter sequence of PtSAP13 was amplified from the genomic DNA of *P. trichocarpa* and cloned into the pMDC164 vector to construct the plasmid pPtSAP13::GUS, which was used to study the expression pattern of PtSAP13. The correct constructs described above were transformed into *Agrobacterium GV3101* by electroporation and transform *A. thaliana* using the floral dip method [65]. The positive transgenic plants were selected on 1/2 MS plates containing 25 mg/L hygromycin and PCR identification. More than 30 independent transgenic lines of pPtSAP13::GUS and 31 independent transgenic lines of p35S::PtSAP13 were obtained, respectively. Finally, three transgenic lines of pPtSAP13::GUS were used for GUS staining, according to previous research [66]. Three transgenic lines with high abundance of PtSAP13 were used for further salt tolerance analysis and RNA-Seq analysis.

4.5. Analysis of Salt Tolerance

For the seed germination assays, T3 homozygous seeds (100–150 seeds) of transgenic lines and wild-type (WT) were sown on 1/2 MS medium containing 0 mM (normal) or 200 mM NaCl (salt stress). After being vernalized at 4 °C for 3 days in the dark, seeds were transferred under normal conditions, and seed germination was counted after 1 week. Germination was scored as rupture of the seed coat.

For the salt tolerance experiments of seedlings, 1-week-old PtSAP13-overexpressing and WT plants were cultured on 1/2 MS medium containing 0 mM or 150 mM NaCl for 2 weeks, and the fresh weight and root length were measured. In addition, after growth on 1/2 MS normal medium for 2 weeks, the transgenic and WT plants were transferred on soil for 4 weeks, and then irrigated with 30 mL of 200 mM NaCl for 2 weeks, and followed with pure water for 7 days. The relative electric conductivity, proline content, and enzyme activities were measured after salt tolerance experiments. The relative electric conductivity was determined by conductivity meter (DDS-307). The proline content was analyzed according to Dubois et al. [67]. The activities of SOD, POD, and CAT were measured according to Li et al. [63]. All experiments were performed in three biological replicates. The data were analyzed with a t-test to detect significant differences.

4.6. RNA-Seq Analysis of Transgenic Arabidopsis

The seeds from WT and transgenic plants were grown under normal condition for 1 week. Subsequently, the seedlings were transferred into 1/2 MS medium containing 0 mM or 150 mM NaCl for 2 weeks. Finally, the whole plants of WT under normal and salt conditions were collected and marked as WT-Normal and WT-Salt, respectively; three independent transgenic lines under normal and salt conditions were equally pooled and marked as SAP13-Normal and SAP13-Salt, respectively.

Total RNA was also extracted using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and treated with DNase I (Qiagen, Hilden, Germany). The RNA purity and integrity were detected by the NanoPhotometer spectrophotometer and Agilent2100 Bioanalyzer, and the RNA quantity was measured by Qubit2.0 Fluorometer (Life Technologies, New York, USA). The cDNA libraries were constructed and sequenced on the Illumina HiSeq2500 sequencing platform by Gene Denovo Biotechnology Co. (Guangzhou, China), with paired-end sequencing and read lengths of 150 bp. Each sample was performed for three biological replicates. Quality-control procedure was performed to filter adapter sequences and low-quality reads using fastp (version 0.18.0) ([https://github.com/OpenGene/fastp](https://github.com/OpenGene/fastp)) [68]. The clean data were obtained for subsequent analyses. Clean reads were mapped to the reference genome using HISAT2 (version 2.4) ([http://ccb.jhu.edu/software/hisat/index.shtml](http://ccb.jhu.edu/software/hisat/index.shtml)) [69]. Gene expression levels were calculated as reads per kilobase of transcript sequence per million base pairs sequenced (FPKM) using StringTie (version 1.3.1) ([https://ccb.jhu.edu/software/stringtie/](https://ccb.jhu.edu/software/stringtie/)) [70]. The differentially expressed genes (DEGs) were identified by DESeq2 ([https://bioconductor.org/packages/release/bioc/html/DESeq2.html](https://bioconductor.org/packages/release/bioc/html/DESeq2.html)) [71]. Genes with \log_{2}FoldChange \text{ > } 1.0 \text{ and false discover rate (FDR)} < 0.05 were considered differentially expressed. To study the biological function of DEGs, gene set enrichment with gene ontology (GO) terms was performed using Blast2GO (version 5.2.5) ([https://www.blast2go.com/](https://www.blast2go.com/)) [72]. Fischer’s exact test was used to assess the significance of GO categories. *p*-values were corrected by Benjamini–Hochberg FDR and the corrected *p*-value cut-off
was 0.05. RNA-Seq data have been deposited in the NCBI Sequence Read Archive under accession number PRJNA579744.

5. Conclusions

In our study, we isolated PtSAP13 from *P. trichocarpa* and characterized its function in salt tolerance. *PtSAP13* was highly induced by salt stress and constitutive expression of *PtSAP13* increased the tolerance to salt stress. The *PtSAP13*-overexpressing plants enhanced salt tolerance by improving ROS-scavenging ability. Moreover, RNA-seq data showed that some stress-related genes were significantly induced in *PtSAP13*-overexpressing plants, suggesting that *PtSAP13* may play a role in plant salt tolerance by regulating the expression of these genes, although detailed work will be needed to identify the exact regulatory mechanism of *PtSAP13* in stress tolerance in poplar.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/20/22/5782/s1. Figure S1: Multiple sequence alignment of the PtSAP proteins. The A20 domain, AN1 domain, and CH2–CH2 domain are marked with red, black, and rule boxes, respectively. Figure S2: The expression patterns of PtSAP genes across different tissues. (1) Leaf immature standard, (2) leaf young standard, (3) leaf first fully expanded standard, (4) root standard, (5) root tip standard, (6) root ammonia, (7) root nitrate, (8) root urea, (9) stem node standard, (10) stem node standard, (11) stem ammonia, (12) stem nitrate, (13) stem urea, (14) pre-dormant bud I, (15) pre-dormant bud II, (16) early dormant bud, (17) late dormant bud, (18) fully open bud, (19) GW9952.ZK 10 male early, (20) GW9840.ZE 30 male early, (21) GW9911.ZK 51 male mid, (22) BESC423.ZL 7 female early, (23) BESC842.ZI 22 female late, (24) BESC443.ZG 43 female. All the expression data was log2 transformed. Table S1: The detailed SAP gene information and protein sequences in different species. Table S2: The primers sequence used in the study.

Author Contributions: H.J. and J.L. conceived and designed the research. J.L. performed the experiments. P.S., Y.X., G.Z., and J.S. collected the data. J.L. wrote the manuscript. H.J. modified the manuscript. All authors read and approved the final manuscript.

Funding: This work was supported by the National Nonprofit Institute Research Grant of Chinese Academy of Forestry (CASYBB2019ZY003), the National Natural Science Foundation of China (31800569 and 31800570), China Postdoctoral Science Foundation (2018M631625), and the National Key Program on Transgenic Research (2018ZX08020002).

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Yamaguchi, T.; Blumwald, E. Developing salt-tolerant crop plants: Challenges and opportunities. *Trends Plant Sci.* 2005, 10, 615–620. [CrossRef]
2. Munns, R.; Tester, M. Mechanisms of salinity tolerance. *Annu. Rev. Plant Biol.* 2008, 59, 651–681. [CrossRef]
3. Bekturganov, Z.; Tussupova, K.; Berndtsson, R.; Sharapatova, N.; Aryngazin, K.; Zhanasova, M. Water related health problems in central Asia-A review. *Water* 2016, 8, 219. [CrossRef]
4. Quintero, F.J.; Ohta, M.; Shi, H.; Zhu, J.; Pardo, J.M. Reconstitution in yeast of the Arabidopsis SOS signaling pathway for Na⁺ homeostasis. *Proc. Natl. Acad. Sci. USA* 2002, 99, 9061–9066. [CrossRef]
5. Apse, M.P.; Aharon, G.S.; Snedden, W.A.; Blumwald, E. Salt tolerance conferred by overexpression of a vacuolar Na⁺/H⁺ antiport in *Arabidopsis*. *Science* 1999, 285, 1256–1258. [CrossRef] [PubMed]
6. Yokoi, S.; Quintero, F.J.; Cubero, B.; Ruiz, M.T.; Bressan, R.A.; Hasegawa, P.M.; Pardo, J.M. Differential expression and function of *Arabidopsis thaliana* NHX Na⁺/H⁺ antiporters in the salt stress response. *Plant J.* 2002, 30, 529–539. [CrossRef] [PubMed]
7. Liu, W.; Schachtman, D.P.; Zhang, W. Partial deletion of a loop region in the high affinity K⁺ transporter HKT1 changes ionic permeability leading to increased salt tolerance. *J. Biol. Chem.* 2000, 275, 27924. [PubMed]
8. Zhang, X.; Cheng, Z.; Zhao, K.; Yao, W.; Sun, X.; Jiang, T.; Zhou, B. Functional characterization of poplar NAC13 gene in salt tolerance. *Plant Sci.* 2019, 281, 1–8. [CrossRef] [PubMed]
9. Zhao, K.; Zhang, D.; Lv, K.; Zhang, X.; Cheng, Z.; Li, R.; Zhou, B.; Jiang, T. Functional characterization of poplar WRKY75 in salt and osmotic tolerance. *Plant Sci.* 2019, 289, 110259. [CrossRef] [PubMed]
10. Zhang, L.; Cheng, J.; Sun, X.; Zhao, T.; Li, M.; Wang, Q.; Li, S.; Xin, H. Overexpression of VaWRKY14 increases drought tolerance in *Arabidopsis* by modulating the expression of stress-related genes. *Plant Cell Rep.* 2018, 37, 1159–1172. [CrossRef]

*Int. J. Mol. Sci.* 2019, 20, 5782
11. Wang, S.; Zhou, B.; Yao, W.; Jiang, T. PsnERF75 transcription factor from Populus simonii x P. nigra confers salt tolerance in transgenic Arabidopsis. *J. Plant Biol.* **2018**, *61*, 61–71. [CrossRef]
12. An, J.P.; Zhang, X.W.; Xu, R.R.; You, C.X.; Wang, X.F.; Hao, Y.J. Apple MdERF4 negatively regulates salt tolerance by inhibiting MdERF3 transcription. *Plant Sci.* **2018**, *276*, 181–188. [CrossRef] [PubMed]
13. Garg, R.; Verma, M.; Agrawal, S.; Shankar, R.; Majee, M.; Jain, M. Deep transcriptome sequencing of wild halophyte rice, *Porteresia coarctata*, provides novel insights into the salinity and submergence tolerance factors. *DNA Res.* **2013**, *21*, 69–84. [CrossRef] [PubMed]
14. Vij, S.; Tyagi, A.K. Genome-wide analysis of the stress associated protein (SAP) gene family containing A20/AN1 zinc-finger(s) in rice and their phylogenetic relationship with *Arabidopsis*. *Mol. Genet. Genom.* **2006**, *276*, 565–577. [CrossRef] [PubMed]
15. Jia, H.; Li, J.; Zhang, J.; Ren, Y.; Hu, J.; Lu, M. Genome-wide survey and expression analysis of the stress-associated protein gene family in desert poplar, *Populus euphratica*. *Tree Genet. Genomes* **2016**, *12*, 78. [CrossRef]
16. Zhou, Y.; Zeng, L.; Chen, R.; Wang, Y.; Song, J. Genome-wide identification and characterization of stress-associated protein (SAP) gene family encoding A20/AN1 zinc-finger proteins in *Medicago truncatula*. *Arch. Biol. Sci.* **2017**, *70*, 87–98. [CrossRef]
17. He, X.; Xie, S.; Xie, P.; Yao, M.; Liu, W.; Qin, L.; Liu, Z.; Zheng, M.; Liu, H.; Guan, M. Genome-wide identification of stress-associated proteins (SAP) with A20/AN1 zinc finger domains associated with abiotic stresses responses in *Brassica napus*. *Environ. Exp. Bot.* **2019**, *165*, 108–119. [CrossRef]
18. Opivari, A.; Boguski, M.; Dixit, V. The A20 cDNA induced by tumor necrosis factor alpha encodes a novel type of zinc finger protein. *J. Biol. Chem.* **1990**, *265*, 14705–14708.
19. Jin, Y.; Wang, M.; Fu, J.; Xuan, N.; Zhu, Y.; Lian, Y.; Jia, Z.; Zheng, J.; Wang, G. Phylogenetic and expression analysis of ZnF-AN1 genes in plants. *Genomics* **2007**, *90*, 265–275. [CrossRef]
20. Dansana, P.K.; Kothari, K.S.; Vij, S.; Tyagi, A.K. OsSAP1 overexpression improves water-deficit stress tolerance in transgenic rice by affecting expression of endogenous stress-related genes. *Plant Cell Rep.* **2014**, *33*, 1425–1440. [CrossRef]
21. Arnab, M.; Shubha, V.; Akhilesh, K.T. Overexpression of a zinc-finger protein gene from rice confers tolerance to cold, dehydration, and salt stress in transgenic tobacco. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 6309–6314.
22. Tyagi, H.; Jha, S.; Sharma, M.; Giri, J.; Tyagi, A.K. Rice SAPs are responsive to multiple biotic stresses and overexpression of OsSAP1, an A20/AN1 zinc-finger protein, enhances the basal resistance against pathogen infection in tobacco. *Plant Sci.* **2014**, *225*, 68–76. [CrossRef] [PubMed]
23. Kanneganti, V.; Gupta, A.K. Overexpression of OsSAP8, a member of stress associated protein (SAP) gene family of rice confers tolerance to salt, drought and cold stress in transgenic tobacco and rice. *Plant Mol. Biol.* **2008**, *66*, 445. [CrossRef] [PubMed]
24. Huang, J.; Wang, M.M.; Jiang, Y.; Bao, Y.M.; Huang, X.; Sun, H.; Xu, D.Q.; Lan, H.X.; Zhang, H.S. Expression analysis of rice A20/AN1-type zinc finger genes and characterization of ZFP177 that contributes to temperature stress tolerance. *Genes* **2008**, *420*, 135–144. [CrossRef]
25. Giri, J.; Vij, S.; Dansana, P.K.; Tyagi, A.K. Rice A20/AN1 zinc-finger containing stress-associated proteins (SAP1/11) and a receptor-like cytoplasmic kinase (OsRLCK253) interact via A20 zinc-finger and confer abiotic stress tolerance in transgenic Arabidopsis plants. *New Phytol.* **2011**, *191*, 721–732. [CrossRef]
26. Hozain, M.D.; Abdelmageed, H.; Lee, J.; Kang, M.; Fokar, M.; Allen, R.D.; Holaday, A.S. Expression of A1sSAP5 in cotton up-regulates putative stress-responsive genes and improves the tolerance to rapidly developing water deficit and moderate heat stress. *J. Plant Physiol.* **2012**, *169*, 1261–1270. [CrossRef]
27. Kang, M.; Fokar, M.; Abdelmageed, H.; Allen, R.D. *Arabidopsis* SAP5 functions as a positive regulator of stress responses and exhibits E3 ubiquitin ligase activity. *Plant Mol. Biol.* **2011**, *75*, 451–466. [CrossRef]
28. Ben, S.R.; Fabre, D.; Mieulet, D.; Meynard, D.; Dingkuhn, M.; Al-Doss, A.; Guiderdoni, E.; Hassairi, A. Expression of the *Aeluropus littoralis* A1SAP gene in rice confers broad tolerance to abiotic stresses through maintenance of photosynthesis. *Plant Cell Environ.* **2012**, *35*, 626–643.
29. Ghneimbarrera, T.; Selvaraj, M.G.; Meynard, D.; Fabre, D.; Peña, A.; Ben, R.W.; Ben, S.R.; Ogawa, S.; Rebollode, M.C.; Ishitani, M. Expression of the *Aeluropus littoralis* A1SAP gene enhances rice yield under field drought at the reproductive stage. *Front. Plant Sci.* **2017**, *8*, 994. [CrossRef]
30. Wang, Y.; Zhang, L.; Zhang, L.; Xing, T.; Peng, J.; Sun, S.; Chen, G.; Wang, X. A novel stress-associated protein SbSAP14 from *Sorghum bicolor* confers tolerance to salt stress in transgenic rice. *Mol. Breeding* **2013**, *32*, 437–449. [CrossRef]
31. Sreedharan, S.; Shekhawat, U.K.S.; Ganapathi, T.R. MutSaPI, a A20/AN1 zinc finger gene from banana functions as a positive regulator in different stress responses. Plant Mol. Biol. 2012, 80, 503–517. [CrossRef] [PubMed]

32. Dixit, A.; Tomar, P.; Vaine, E.; Abdullah, H.; Hazen, S.; Dhandker, O.P. A stress–associated protein, AtSAP13, from Arabidopsis thaliana provides tolerance to multiple abiotic stresses. Plant Cell Environ. 2018, 41, 1171–1185. [CrossRef] [PubMed]

33. Xu, Q.F.; Mao, X.G.; Wang, Y.X.; Wang, J.Y.; Xi, Y.J.; Jing, R.L. A wheat gene TaSAP17-D encoding an AN1/AN1 zinc finger protein improves salt stress tolerance in transgenic Arabidopsis. J. Integr. Agric. 2018, 17, 507–516. [CrossRef]

34. Gordon, J.C. Poplars: Trees of the people, trees of the future. For. Chron. 2001, 77, 217–219. [CrossRef]

35. Sannigrahi, P.; Ragauskas, A.J.; Tuskan, G.A. Poplar as a feedstock for biofuels: A review of compositional characteristics. Biofuels Bioprod. Biorefin. 2010, 4, 209–226. [CrossRef]

36. Yoon, S.; Bae, E.; Lee, H.; Choi, Y.; Han, M.; Choi, H.; Kang, K.; Park, E. Downregulation of stress-associated protein 1 (PagSAP1) increases salt stress tolerance in poplar (Populus alba × P. glandulosa). Trees-Struct. Funct. 2018, 32, 823–833. [CrossRef]

37. Ma, T.; Wang, J.; Zhou, G.; Yue, Z.; Hu, Q.; Chen, Y.; Liu, B.; Qiu, Q.; Wang, Z.; Zhang, J. Genomic insights into salt adaptation in a desert poplar. Nat. Commun. 2013, 4, 2797. [CrossRef]

38. Liu, B.; Hu, J.; Zhang, J. Evolutionary divergence of duplicated Hsf genes in Populus. Cells 2019, 8, 438. [CrossRef]

39. McKown, A.D.; Guy, R.D.; Klášťé, J.; Geraldens, A.; Friedmann, M.; Cronk, Q.C.; El–Kassaby, Y.A.; Mansfield, S.D.; Douglas, C.J. Geographical and environmental gradients shape phenotypic trait variation and genetic structure in Populus trichocarpa. New Phytol. 2014, 201, 1263–1276. [CrossRef]

40. Munns, R. Comparative physiology of salt and water stress. Plant Cell Environ. 2002, 25, 239–250. [CrossRef]

41. Denby, K.; Gehring, C. Engineering drought and salinity tolerance in plants: Lessons from genome-wide expression profiling in Arabidopsis. Trends Biotechnol. 2005, 23, 547–552. [CrossRef] [PubMed]

42. Moreno, A.A.; Mukhtar, M.S.; Blanco, F.; Boatwright, J.L.; Moreno, I.; Jordan, M.R.; Chen, Y.; Brandizzi, F.; Dong, X.; Orellana, A. IRE1/bZIP60-mediated unfolded protein response plays distinct roles in plant immunity and abiotic stress responses. PLoS ONE 2012, 7, e31944. [CrossRef] [PubMed]

43. Zhang, L.; Zhang, L.; Xia, C.; Zhao, G.; Liu, J.; Jia, J.; Kong, X. A novel wheat bZIP transcription factor, TabZIP60, confers multiple abiotic stress tolerances in transgenic Arabidopsis. Physiol. Plantrarum 2015, 153, 538–554. [CrossRef] [PubMed]

44. Milla, M.A.R.; Townsend, J.; Chang, I.F.; Cushman, J.C. The Arabidopsis AtDi19 gene family encodes a novel type of Cys2/His2 Zinc-finger protein implicated in ABA-independent dehydration, high-salinity stress and light signaling pathways. Plant Mol. Biol. 2006, 61, 13–30. [CrossRef] [PubMed]

45. Wang, L.; Yu, C.; Chen, C.; He, C.; Zhu, Y.; Huang, W. Identification of rice Dvi19 family reveals OsDvi19-4 involved in drought resistance. Plant Cell Rep. 2014, 33, 2047–2062. [CrossRef] [PubMed]

46. Gaber, A.; Ogata, T.; Maruta, T.; Yoshimura, K.; Tamo, M.; Shigeoka, S. The involvement of Arabidopsis Glutathione Peroxidase 8 in the suppression of oxidative damage in the nucleus and cytosol. Plant Cell Physiol. 2012, 53, 1596–1606. [CrossRef] [PubMed]

47. Liu, S.; Cheng, Y.; Zhang, X.; Guan, Q.; Nishiuchi, S.; Hase, K.; Takano, T. Expression of an NADP-malic enzyme gene in rice (Oryza sativa. L) is induced by environmental stresses; over-expression of the gene in Arabidopsis confers salt and osmotic stress tolerance. Plant Mol. Biol. 2007, 64, 49. [CrossRef]

48. Guo, Y.; Song, Y.; Zheng, H.; Zhang, Y.; Guo, J.; Sui, N. NADP-malate dehydrogenase of sweet sorghum improves salt tolerance of Arabidopsis thaliana. J. Agric. Food Chem. 2018, 66, 5992–6002. [CrossRef]

49. Ren, Z.; Zheng, Z.; Chinnusamy, V.; Zhu, J.; Cui, X.; Iida, K.; Zhu, J.K. RAS1, a quantitative trait locus for salt tolerance and ABA sensitivity in Arabidopsis. Proc. Natl. Acad. Sci. USA 2010, 107, 5669–5674. [CrossRef]

50. Jiang, Y.Q.; Deyholos, M.K. Functional characterization of Arabidopsis NaCl-inducible WRKY25 and WRKY33 transcription factors in abiotic stresses. Plant Mol. Biol. 2009, 69, 91–105. [CrossRef] [PubMed]

51. Ali, M.A.; Azeem, F.; Nawaz, M.A.; Acet, T.; Abbas, A.; Imran, Q.M.; Shah, K.H.; Rehman, H.M.; Chung, G.; Yang, S.H. Transcription factors WRKY11 and WRKY17 are involved in abiotic stress responses in Arabidopsis. J. Plant Physiol. 2018, 226, 12–21. [CrossRef] [PubMed]
52. Zhou, L.; Wang, N.N.; Gong, S.Y.; Lu, R.; Li, Y.; Li, X.B. Overexpression of a cotton (*Gossypium hirsutum*) WRKY gene, *GhWRKY34*, in *Arabidopsis* enhances salt-tolerance of the transgenic plants. *Plant Physiol. Biochem.* 2015, 96, 311–320. [CrossRef] [PubMed]
53. Chu, X.; Wang, C.; Chen, X.; Lu, W.; Li, H.; Wang, X.; Hao, L.; Guo, X. The cotton WRKY gene *GhWRKY41* positively regulates salt and drought stress tolerance in transgenic *Nicotiana benthamiana*. *PLoS ONE* 2015, 10, e0143022. [CrossRef] [PubMed]
54. Yan, H.; Jia, H.; Chen, X.; Hao, L.; An, H.; Guo, X. The cotton WRKY transcription factor *GhWRKY17* functions in drought and salt stress in transgenic *Nicotiana benthamiana* through ABA signaling and the modulation of reactive oxygen species production. *Plant Cell Physiol.* 2014, 55, 2060–2076. [CrossRef] [PubMed]
55. Shi, W.; Hao, L.; Li, J.; Liu, D.; Guo, X.; Li, H. The *Gossypium hirsutum* WRKY gene *GhWRKY39* promotes pathogen infection defense responses and mediates salt stress tolerance in transgenic *Nicotiana benthamiana*. *Plant Cell Rep.* 2014, 33, 483–498. [CrossRef]

56. Miller, G.; Suzuki, N.; Ciftci-Yilmaz, S.; Mittler, R. Reactive oxygen species homeostasis and signalling during drought and salinity stresses. *Plant Cell Environ.* 2010, 33, 453–467. [CrossRef]
57. Ahmad, P.; Sarwat, M.; Sharma, S. Reactive oxygen species, antioxidants and signaling in plants. *J. Plant Biol.* 2008, 51, 167–173. [CrossRef]
58. Agati, G.; Azzarello, E.; Pollastri, S.; Tattinic, M. Flavonoids as antioxidants in plants: Location and functional significance. *Plant Sci.* 2012, 196, 67–76. [CrossRef]
59. Wang, F.; Zhu, H.; Kong, W.; Peng, R.; Liu, Q.; Yao, Q. The *Antirrhinum* AntDEL gene enhances flavonoids accumulation and salt and drought tolerance in transgenic *Arabidopsis*. *Planta* 2016, 244, 59–73. [CrossRef]
60. Fang, L.; Su, L.; Sun, X.; Li, X.; Sun, M.; Karungo, S.K.; Fang, S.; Chu, J.; Li, S.; Xin, H. Expression of *Vitis amurensis* NAC26 in *Arabidopsis* enhances drought tolerance by modulating jasmonic acid synthesis. *J. Exp. Bot.* 2016, 67, 2829–2845. [CrossRef]
61. Nahar, K.; Hasanuzzaman, M.; Rahman, A.; Alam, M.; Mahmud, J.; Suzuki, T.; Fujita, M. Polyamines confer salt tolerance in mung bean (*Vigna radiata* L.) by reducing sodium uptake, improving nutrient homeostasis, antioxidant defense, and methylglyoxal detoxification systems. *Front. Plant Sci.* 2016, 7, 1104. [CrossRef] [PubMed]
62. Ravikovitch, S.; Porath, A. The effect of nutrients on the salt tolerance of crops. *Plant Soil* 1967, 26, 49–71. [CrossRef]
63. Li, J.; Zhang, J.; Jia, H.; Li, Y.; Xu, X.; Wang, L.; Lu, M. The *Populus trichocarpa* PtHSP17.8 involved in heat and salt stress tolerances. *Plant Cell Rep.* 2016, 35, 1587–1599. [CrossRef] [PubMed]
64. Livak, K.J.; Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the 2−∆∆CT Method. *Methods* 2001, 25, 402–408. [CrossRef]
65. Clough, S.J.; Bent, A.F. Floral dip: A simplified method for *Agrobacterium* -mediated transformation of *Arabidopsis thaliana*. *Plant J.* 1998, 16, 735–743. [CrossRef]
66. Li, J.; Zhang, J.; Jia, H.; Liu, B.; Sun, P.; Hu, J.; Wang, L.; Lu, M. The *WUSCHEL*-related homeobox 5a (*PtoWOX5a*) is involved in adventitious root development in poplar. *Tree Physiol.* 2018, 38, 139–153. [CrossRef]
67. Dubois, M.; Gilles, K.A.; Hamilton, J.K.; Rebers, P.A.; Smith, P. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* 1956, 28, 350–356. [CrossRef]
68. Chen, S.; Zhou, Y.; Chen, Y.; Gu, J. Fastp: An ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics* 2018, 34, 1884–1890. [CrossRef]
69. Kim, D.; Langmead, B.; Salzberg, S.L. HISAT: A fast spliced aligner with low memory requirements. *Nat. Methods* 2015, 12, 357. [CrossRef]
70. Pertea, M.; Pertea, G.M.; Antonescu, C.M.; Chang, T.C.; Mendell, J.T.; Salzberg, S.L. StringTie enables improved reconstruction of a transcriptome from RNA-seq reads. *Nat. Biotechnol.* 2015, 33, 290. [CrossRef]
71. Love, M.I.; Huber, W.; Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 2014, 15, 550. [CrossRef] [PubMed]
72. Conesa, A. Blast2GO: A comprehensive suite for functional analysis in plant genomics. *Int. J. Genom.* 2008, 2008, 619832. [CrossRef] [PubMed]