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Identification of *TPS* and *TPP* gene families in *Cannabis sativa* and their expression under abiotic stresses

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Abstract

Trehalose is a nonreducing disaccharide that is involved in the regulation of plant responses to a variety of environmental stresses. Trehalose 6-phosphate synthase (TPS) and trehalose 6-phosphate phosphatase (TPP) are two key enzymes in trehalose synthesis and they are widely distributed in higher plants. At present, *TPS* family genes have been systematically identified and analyzed in many plant species, but the *TPP* family genes have been rarely studied. In this study, ten *TPS* and six *TPP* genes in cannabis (*Cannabis sativa* L.) were identified at the genomic level. The phylogenetic tree of *TPS* and *TPP* family members in cannabis, *Arabidopsis*, and rice was constructed, and all the genes were divided into three subgroups: Class I, Class II, and Class III. The number of exons and motif types among Class I members was exactly the same, as were Class II members, but the gene structure and motif types of Class III members were slightly different. There were four pairs of *CsTPSs* and *CsTPPs* that had gene duplication, indicating that gene duplication events played an important role in the amplification of *TPS* and *TPP* families in cannabis. The results of expression analysis under abiotic stresses showed that 68.75 % of *CsTPS* and *CsTPP* genes were significantly induced by at least one abiotic stress. Among these genes, the expression of *CsTPS1*, *CsTPS9*, and *CsTPPA* was highest under at least one abiotic stress. These three genes may play a key role in abiotic stress responses. Most of the *CsTPS* and *CsTPP* genes that are closely located in the evolutionary tree have the same or similar functions. To our knowledge, this is the first paper that systematically reports the *TPS* and *TPP* gene families in cannabis.

Keywords: *Cannabis sativa*, cold, drought, phylogenetic tree, salt stress, *TPP* and *TPS* gene families, trehalose.

Introduction

Trehalose is a nonreducing disaccharide that is widely distributed in organisms and has different biological functions in different species. In plants, trehalose is involved in the regulation of the response to a variety of environmental stresses (Paul *et al*. 2008). Trehalose has a stronger ability to bind water than other sugars (Lerbret *et al*. 2005). Trehalose can maintain the biological structure and function of biomolecules by replacing water, concentrating water around biomolecules or in the form of a vitrification agent under the conditions of water shortage or freezing (Sundaramurthi *et al*. 2010, Hackel *et al*. 2012). Because trehalose has a strong anti dehydration effect,

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Abbreviations: CBD - cannabidiol; G6P - glucose-6-phosphate; Ka - nonsynonymous substitution rate, Ks - synonymous substitution rate; Mr - relative molecular mass; pI - isoelectric point; T6P - trehalose 6-phosphate; TPP - trehalose 6-phosphate phosphatase; TPS - trehalose 6-phosphate synthase; UDPG - UDP glucose.
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it can protect biofilms and proteins from damage under drought, cold, high salinity, and other stress conditions.

The pathway of trehalose synthesis in higher plants is relatively clear and includes two main steps of enzymatic reactions. First, trehalose 6-phosphate synthase (TPS) catalyzes UDP glucose (UDPG) and glucose-6-phosphate (G6P) to produce trehalose 6-phosphate (T6P), and then trehalose 6-phosphate phosphatase (TPP) catalyzes the dephosphorylation of T6P to produce trehalose (Avonce *et al*. 2006). In the above synthetic pathway, two key enzymes, the TPS enzyme encoded by *TPS* genes, catalyze the biosynthesis of T6P, and the TPP enzyme encoded by *TPP* genes catalyzes the biosynthesis of trehalose. TPS and TPP enzymes are widely distributed in higher plants. To date, systematic identification and analysis of *TPS* family genes have been performed in rice (Ge *et al*. 2008), *Arabidopsis* (Yang *et al*. 2012), *Populus* (Yang *et al*. 2012), wheat (Xie *et al*. 2015), lotus (Jin *et al*. 2016), cotton (Mu *et al*. 2016), cassava (Han *et al*. 2016), potato (Xu *et al*. 2017), apple (Du *et al*. 2017), drumstick tree (Lin *et al*. 2018), *Brachypodium distachyon* (Wang *et al*. 2019), sugarcane (Hu *et al*. 2020), *Prunus mume* (Yang *et al*. 2020), and grapevine (Morabito *et al*. 2021). Some genes show potential functions under stress conditions, but their expression patterns are also diverse. In the field of the *TPP* family, genes have been identified in rice (Ge *et al*. 2008), *Arabidopsis* (Vandesteene *et al*. 2012), cassava (Han *et al*. 2016), and *Brachypodium distachyon* (Wang *et al*. 2019). The *TPP* gene family in plants has been studied less than the TPS gene family.

Cannabis (*Cannabis sativa* L.) has been an economically important crop since ancient times (Skoglund *et al*. 2013). Its rich phytochemicals can be used in the field of medicine, and its high-quality fibre is widely used in textiles, building materials, chemicals, and energy. In addition, cannabis also has good drought resistance and insect resistance. Its developed roots can fix the soil and prevent soil erosion, and compared with other crops, its water demand is also rather low (Andre *et al*. 2016). The above shows that cannabis has good stress resistance and adaptability, and is easy to be cultivated so that its various functions can be widely used. In recent years, cannabidiol (CBD) has been proven to play an important role in the treatment of schizophrenia, epilepsy, neurodegenerative diseases, multiple sclerosis, emotional disorders and other nervous system diseases, which has caused worldwide attention, with the demand for cannabis increasing dramatically (Pretzsch *et al*. 2019).

Abiotic stresses such as drought, low temperature, and soil salinity are the main factors affecting crop growth and yield reduction (Vij *et al*. 2010). Drought and high concentrations of NaCl can reduce the soil water potential, thus reducing the water absorption by the roots. Salt stress inhibits crop growth by causing ion imbalance and osmotic stress (Dong *et al*. 2020). Low temperature and chilling injury can delay the growth period of crops, resulting in yield decline (Zhang *et al*. 2014). Abiotic stresses are major threats to global agriculture and an important reason leading to the reduction of cannabis production (Mahajan *et al*. 2005, Hu *et al*. 2019). In this study, evolution, gene structure, and gene duplication analyses of the *TPS* and *TPP* gene families in cannabis were conducted, and the expression of selected genes under abiotic stress was analyzed by RT-qPCR. The purpose of this study was to identify the *TPS* and *TPP* gene families of cannabis and understand their functions under abiotic stresses to lay a foundation for future understanding of stress resistance mechanisms in cannabis.

Materials and methods

Identification and basic information for cannabis *TPS* and *TPP* gene family members: The TPS and TPP protein sequences of *Arabidopsis thaliana* were used as query sequences to search and screen the genome of cannabis (https://www.ncbi.nlm.nih.gov/assembly/GCF_900626175.2/) by *BLASTP* applying default parameters. The candidate protein sequences were submitted to *Pfam* (http://pfam.xfam.org/) for verification, and the sequences exhibiting incomplete domains were deleted. Finally, all genes of the *TPS* and *TPP* families in cannabis were obtained. The nucleic acid sequence, coding sequence (CDS) and protein sequences were obtained from the cannabis genome. The relative molecular mass (Mr) and isoelectric point (pI) of the proteins were predicted in *ExPasy* (https://web.expasy.org/protparam). The subcellular localization of each member of the *CsTPS* and *CsTPP* families was predicted by the *CELLO* server (http://cello.life.nctu.edu.tw).

Phylogenetic analysis: All *TPS* and *TPP* genes of *Arabidopsis* and rice were obtained from the *Arabidopsis* genome database (https://www.arabidopsis.org/) and rice genome database (http://rice.plantbiology.msu.edu/). The TPS and TPP proteins of cannabis, *Arabidopsis*, and rice were aligned by *Clustal W* software. The phylogenetic tree was constructed by the neighbor-joining (NJ) method of *MEGA 7.0* software, and bootstrap analysis was carried out. The repeat value was set to 1 000, and other parameters were set to default values.

Gene structure and conserved motifs: In *GSDS 2.0* online software (http://gsds.cbi.pku.edu.cn/), the gene and CDSs were input to analyze and map the gene structure of *CsTPS* and *CsTPP* family members. The online software *MEME* (https://meme-suite.org/meme/tools/meme) was used to predict and analyze the motifs of the *CsTPS* and *CsTPP* genes, and the number of motifs was set to 20.

Gene duplication and substitution rate analysis: The plant genome duplication database (https://popgenie.org/node/42) was used to analyze the duplication of each gene, and the maximum distance between the duplicated genes was 500 kb. The length of each chromosome and the position of *CsTPS* and *CsTPP* genes on the chromosome were obtained from the cannabis genome. *TBtools* software (Chen *et al*. 2020) was used to construct the collinearity analysis map of *CsTPS* and *CsTPP* genes and chromosomes.

According to the CDS of the duplicated gene pairs, the nonsynonymous substitution rate (Ka), synonymous substitution rate (Ks), and Ka/Ks were calculated by *DnaSP 5.0* software (Librado and Rozas 2009). The formula T = Ks/2r was used to estimate the divergence time of duplicated gene pairs. The r value was 1.5×10^{-8} for dicots (Yang *et al*. 2020).

Plants and stress treatments: The seeds of cannabis (*Cannabis sativa* L.) cv. DMG245 were sown in a seedling tray. The seedling tray was 26 cm in length, 26 cm in width, and 10 cm in height with 25 holes. Three seeds were sown in each hole, and two seedlings were pulled out after emergence to ensure one seedling in each hole. The seedlings were cultured in an artificial climate room - the day/night temperatures were 24 °C/16 °C, a 16-h photoperiod, an irradiance of 300 µmol m^{-2} s^{-1}, and relative humidity of 65 %. Abiotic stress treatments were carried out when the seedling heights were approximately 20 ± 0.5 cm. A total of four seedling trays, each containing 25 seedlings, were used for salt, drought, low temperature, and control treatment. NaCl solution at a concentration of 3.0 % was placed in the seedling tray for salt stress treatment, and polyethylene glycol (PEG)-6000 at a 20 % concentration was used to mimic drought stress. The seedlings were transferred to an incubator for a 4 °C low-temperature treatment, and the seedlings under normal growth conditions were used as controls. One leaf was cut at 0, 8, 16, and 24 h after the beginning of treatment, respectively. Three seedlings were taken from each treatment as three repetitions. The cut seedlings were quickly put into liquid nitrogen and then stored at -80 °C for total RNA extraction.

Quantitative real-time PCR: *TRIzol* (*Invitrogen*, Carlsbad, CA, USA) was used to extract the total RNA from cannabis, and a *NanoDrop 2000* (*Thermo Scientific*, Wilmington, USA) was used to detect the quality, purity, and integrity of RNA. The RNA of each sample was reverse transcribed into cDNA by using the *Prime HiFi-MMLV* cDNA kit (*CWBIO*, Beijing, China).

RT-qPCR primers for the *CsTPS* and *CsTPP* genes were designed by *Primer Premier 5.0* software (Table 1 Suppl.). The RT-qPCR system (20 mm^3) consisted of 0.5 mm^3 *UltraSYBR One Step EnzymeMix* (*CWBIO*), 10 mm^3 of buffer, 0.5 mm^3 of upstream and downstream specific primers, 1 mm^3 of cDNA template, and 7.5 mm^3 of ddH_2O. The reaction procedure was 95 °C for 5 min, 45 cycles of 94 °C for 10 s, 60 °C for 20 s, and 72 °C for 20 s. *ACTIN* was used as an internal reference gene (Hu *et al*. 2019). The relative expression of each gene was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Statistical analyses: In the RT-qPCR experiment, mean values and standard deviations (SD) were obtained from three replicates. Statistical significance was performed using a Student's *t*-test at $P \leq 0.05$ and $P \leq 0.01$ (*SPSS 21.0*, Chicago, IL, USA) to evaluate the difference of relative gene expressions among at 0 h and other time points.

Results

The *Arabidopsis* TPS and TPP protein sequences were used as query sequences to blast the genome of cannabis in *NCBI*. The aligned cannabis *TPS* and *TPP* genes were submitted to the *Pfam* database. After eliminating the redundant sequences without typical TPS and TPP domains, ten cannabis *TPS* and six *TPP* genes were obtained. The ten *CsTPSs* contained the Glyco_transf_20 (PF00982) domain and contained the trehalose_PPase (PF02358) domain. The six *CsTPPs* only contained the Trehalose_PPase

Table 1. Basic information about *CsTPSs* and *CsTPPs* in cannabis. Chr - chromosome, aa - amino acids, Mr - molecular mass, pI - isoelectric point, SL - subcellular localization.

Gene name	NCBI accession	Chr	Location	Number of aa	Mr [kDa]	pI	SL
CsTPS1	XP_030490413	1	88260818..88281140	928	104.61	6.37	cytoplasm
CsTPS2	XP_030480449	7	68900724..68907646	949	106.91	6.15	cytoplasm
CsTPS3	XP_030508575	9	177011..184079	817	93.32	6.97	cytoplasm
CsTPS4	XP_030484585	8	49637121..49641495	836	95.38	5.63	nucleus
CsTPS5	XP_030500602	5	83521789..83526035	861	97.34	5.40	cytoplasm
CsTPS6	XP_030506745	9	57346939..57350893	856	96.57	5.86	cytoplasm
CsTPS7	XP_030496147	10	52857530..52864078	854	96.69	5.93	plasma membrane
CsTPS8	XP_030493634	3	13012251..13017444	858	96.89	6.22	cytoplasm
CsTPS9	XP_030510183	6	4517436..4521020	868	97.55	5.87	cytoplasm
CsTPS10	XP_030499885	4	3333932..3338557	862	96.49	5.75	cytoplasm
CsTPPA	XP_030489124	1	3557033..3562147	385	43.25	8.35	chloroplast
CsTPPB	XP_030501902	5	74809140..74814109	392	44.85	7.77	mitochondrion
CsTPPC	XP_030500530	5	4669812..4678812	347	38.97	7.70	nucleus
CsTPPD	XP_030481171	10	94256116..94258867	379	42.46	9.11	nucleus
CsTPPE	XP_030499315	4	49532570..49535876	374	41.96	9.26	nucleus
CsTPPF	XP_030488733	1	69650913..69655396	330	37.33	7.61	cytoplasm

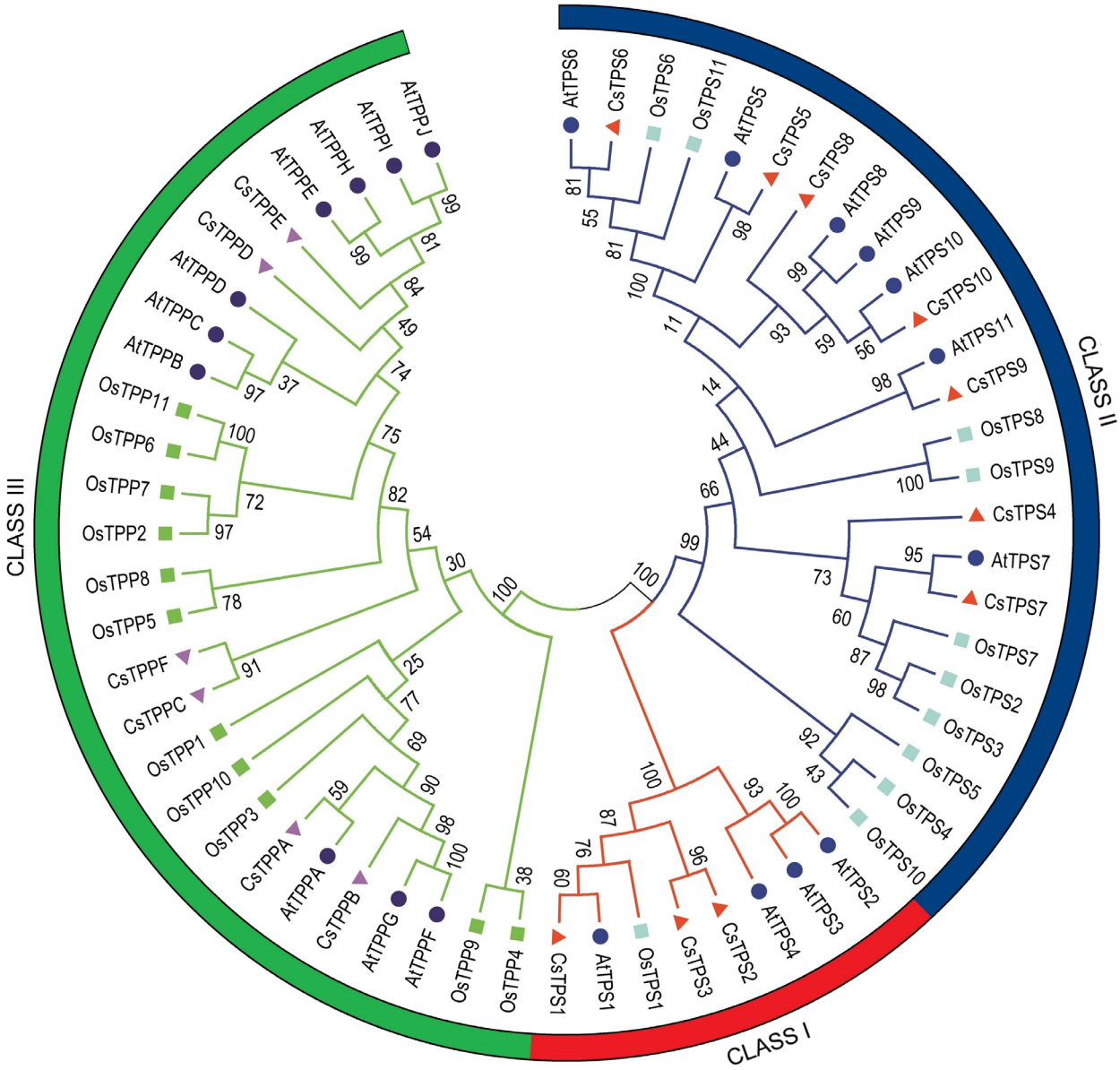

Fig. 1. Phylogenetic tree of *TPS* and *TPP* family genes in cannabis, *Arabidopsis*, and rice. *MEGA 7.0* (bootstrap value = 1 000) was used to create a maximum likelihood tree and display the bootstrap value of each branch. *Red*, *blue*, and *green* represent Class I, Class II, and Class III, respectively. *Triangle* represent cannabis, *circles* represent *Arabidopsis*, and squares represent rice.

domain. The *TPS* and *TPP* genes of cannabis were named *CsTPS1-10* and *CsTPPA-F* (Table 1), respectively, according to the homologous relationship with the *TPS* and *TPP* genes of *Arabidopsis* in the phylogenetic tree (Fig. 1).

ExPASy was used to predict the basic physical and chemical properties of *TPS* and *TPP* family members in cannabis (Table 1). The amino acid length of *CsTPSs* was 817 - 949 aa and that of *CsTPPs* was 330 - 392 aa. The Mr of the *CsTPS* protein was 93.32 - 106.91 kDa and that of the *CsTPP* protein was 37.33 - 44.85 kDa. *CsTPS* protein was significantly larger than *CsTPP* protein. The pIs of *CsTPS* and *CsTPP* were 5.40 - 6.97 and 7.61 - 9.26, respectively. The results of subcellular localization prediction showed that *CsTPS1*, *CsTPS2*, *CsTPS3*, *CsTPS5*, *CsTPS6*, *CsTPS8*, *CsTPS9*, *CsTPS10*, *CsTPPF* were located in cytoplasm, *CsTPS4*, *CsTPPC*, *CsTPPD*, *CsTPPE* in the nucleus, *CsTPS7* in the plasma membrane, *CsTPPA* in chloroplast, and *CsTPPB* in mitochondrion.

There were 11 *TPS* and 10 *TPP* genes in *Arabidopsis* and 11 *TPS* and 11 *TPP* genes in rice. A phylogenetic tree of *TPS* and *TPP* family members in cannabis, *Arabidopsis* and rice was constructed by the neighbor-joining (NJ) method of *MEGA 7.0* software (Fig. 1). The figure shows that all genes are divided into three subgroups (Class I, Class II, and Class III). Class I includes cannabis *CsTPS1-3*, *Arabidopsis AtTPS1-4*, and rice *OsTPS1*. Class II includes cannabis *CsTPS4-10*, *Arabidopsis AtTPS5-11*, and rice *OsTPS2-11*. All Class III genes are *TPP* genes, including cannabis *CsTPPA-F*, *Arabidopsis AtTPPA-J*, and rice

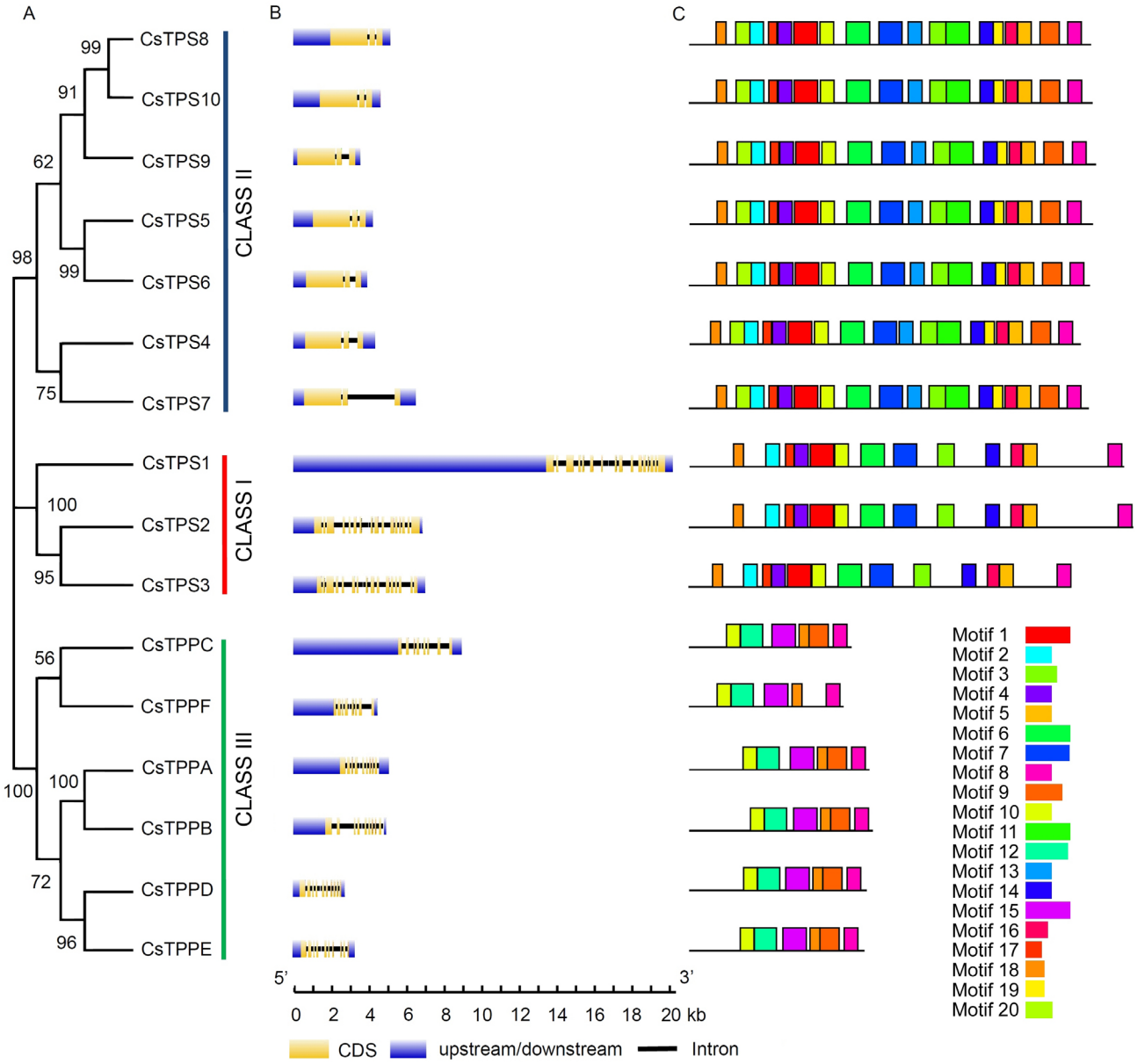

Fig. 2. Gene structure and motif distribution of the *TPS* and *TPP* families in cannabis. *A* - Phylogenetic tree of *CsTPSs* and *CsTPPs*; *B* - the gene structure corresponding to *CsTPSs* and *CsTPPs* in the phylogenetic tree; *C* - Conservative motifs corresponding to *CsTPSs* and *CsTPPs* in the phylogenetic tree.

OsTPP1-11.

Compared with *Arabidopsis*, which is a dicotyledonous plant, the cannabis *TPS* gene is one less than the *Arabidopsis TPS* gene, which occurs in the Class I subgroup, and there are also four fewer *TPP* genes in cannabis than in *Arabidopsis*.

Based on the gene structure analysis by *GSDS 2.0* (Fig. 2), both *TPS* and *TPP* genes of cannabis contain exons and introns, but the number of exons in different *CsTPSs* and *CsTPPs* is very different. The exons of Class I, Class II, and Class III were 11, 3, and 8 - 10, respectively, suggesting that the differences in gene structure between *CsTPSs* and *CsTPPs* may lead to their different functions, especially in Class I and Class II subfamilies. Although they are all *CsTPS* genes, there are huge differences in gene structure. Therefore, it is necessary to study the function of these genes further.

The motif types and permutations of *TPS* and *TPP* gene family members were analyzed by the online software *MEME*. As shown in Fig. 2, the number and species of the 20 predicted motifs were different between *TPS* and *TPP* family members. Each member of the Class I subfamily contains 13 motifs, each member of the Class II subfamily contains 18 motifs, and each member of the Class III subfamily contains 6 motifs except *CsTPPF*. Only three motifs were found in all genes: motif 8, motif 10, and motif 18.

TPS and *TPP* genes were distributed on all nine

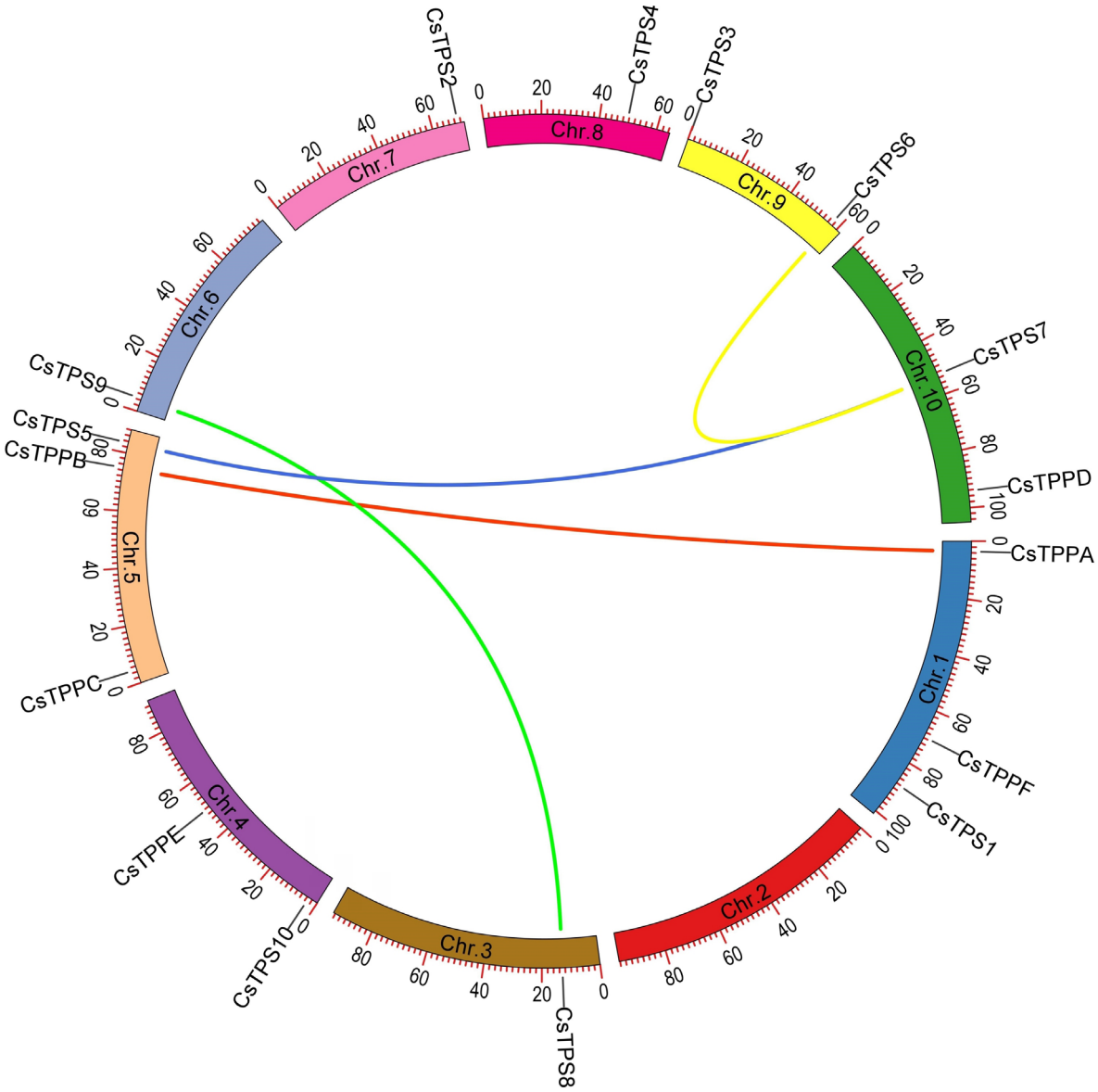

Fig. 3. Chromosomal location and gene replication relationship of *TPS* and *TPP* family genes in cannabis. The *coloured lines* in the circle represent the duplicated gene pairs.

chromosomes except chromosome 2 (Fig. 3). Among these genes, there were three on chromosomes 1 and 5, two on chromosomes 4, 9, and 10, and one on chromosomes 3, 6, 7, and 8. *CsTPSs* and *CsTPPs* were randomly distributed on chromosomes, and there was no single distribution of *CsTPSs* or *CsTPPs* on one chromosome. For example, there are *CsTPS1*, *CsTPPA*, and *CsTPPF* on chromosome 1 and *CsTPS5*, *CsTPPB*, and *CsTPPC* on chromosome 5. The distribution of genes on chromosomes is not necessarily related to their position in the evolutionary tree.

Plant Genome Duplication Database (PGDD) was used for gene duplication analysis. The results showed that there were four pairs of genes that had gene duplication (Fig. 3, Table 2), and the duplication type was segmental duplication. Duplicated genes accounted for 43.75 % of the total genes, indicating that gene duplication events played an important role in the amplification of *TPS* and *TPP* families in cannabis, especially *CsTPSs*, which added three members through gene duplication. The number of *TPP* genes in cannabis is much less than the number of *TPP* genes in *Arabidopsis*, which may also be due to the loss caused by genome duplication.

In genetics, Ka/Ks represents the ratio of the nonsynonymous substitution rate to the synonymous substitution rate of two protein-coding genes, which can determine whether there is selective pressure on genes. Ka/Ks < 1 was considered purification selection, which indicates that natural selection eliminates harmful mutations and keeps the protein unchanged. Ka/Ks > 1 is positive selection, which indicates that natural selection acts on the change of protein, makes the mutation site quickly fixed in the population and accelerates gene

Table 2. Nonsynonymous substitution rate (Ka), synonymous substitution rate (Ks), Ka/Ks analysis, and divergence times of duplicated *CsTPSs* and *CsTPPs* in cannabis.

Duplicated pair	Duplicate type	Ka	Ks	Ka/Ks	Divergence time [Mya]
CsTPS7/*CsTPS6*	segmental duplication	0.4429	0.5208	0.8504	17.36
CsTPS7/*CsTPS5*	segmental duplication	0.3692	0.5904	0.6253	19.68
CsTPS9/*CsTPS8*	segmental duplication	0.4035	0.4948	0.8155	16.49
CsTPPB/*CsTPPA*	segmental duplication	0.2624	0.6689	0.3923	22.30

evolution. Ka/Ks = 1 is neutral selection, which indicates that natural selection has no effect on mutation (Swanson *et al*. 2001, Hurst *et al*. 2002). To explore the selection mode of *TPS* and *TPP* genes in cannabis after duplication, Ka, Ks, and Ka/Ks ratios were calculated by using the CDSs of duplicated gene pairs (Table 2). The Ka/Ks ratios of the four pairs of duplicated genes in the *TPS* and *TPP* gene families were all less than 1, which indicated that they carried out purification selection during duplication. The divergence time of duplicated gene pairs was calculated by the T = Ks/2k equation. The divergence time of the four pairs of genes was 16.49 - 22.30 million years ago (Mya), which was after the divergence of monocotyledons and dicotyledons (approximately 200 Mya) (Wolfe *et al*. 1989) and after the divergence of cannabis and *T. orientale* (approximately 52 Mya) (Gao *et al*. 2020).

Gene expression patterns can provide an important basis for research on gene function. Cannabis seedlings were treated with NaCl, PEG-6000, and 4 °C, and the expression patterns of the *CsTPS* and *CsTPP* gene families under salt, drought, and low-temperature stress were analyzed by RT-qPCR. The results showed that some *CsTPS* and *CsTPP* genes were induced to be expressed under abiotic stress, but there were differences among different genes (Fig. 1 Suppl.). *CsTPS1*, *CsTPS6*, *CsTPS9*, *CsTPS10*, *CsTPPA*, and *CsTPPC* were significantly upregulated under the three stresses, indicating that they may be important abiotic stresses regulatory genes. Among these genes, *CsTPS1* was more upregulated under salt stress and low temperature, *CsTPS9* was more upregulated under salinity and drought, and *CsTPPA* was more upregulated under salt stress than in other conditions, suggesting that the above three genes may play a key role in these stresses.

Some genes were significantly activated by one or two stresses; for example, *CsTPS2* was significantly upregulated under salt and drought stress, *CsTPS8* was significantly upregulated under salt stress, and *CsTPS3*, *CsTPS5*, and *CsTPPB* were significantly upregulated under low temperature. All differentially expressed genes were upregulated, and only *CsTPS5* was downregulated by salt and drought stress, but there was no significant difference compared with 0 h at most time points. Although the expression of *CsTPPF* was upregulated at several time points under the three stresses, the expression of *CsTPPF* was also upregulated at 16 and 24 h in control, suggesting that *CsTPPF* may be related to growth and development or time rhythm, and its relationship with abiotic stress needs to be studied further. The expression of *CsTPS4*, *CsTPS7*, *CsTPPD*, and *CsTPPE* was not significantly induced, indicating that they had little relationship with abiotic stresses.

Discussion

In recent years, studying the classification, sequence characteristics, evolutionary characteristics, and functional prediction of plant gene families at the whole genome level has become an important means to fully understand gene families and carry out gene function research. The *TPS* and *TPP* families are gene families with important biological functions in plants. These genes are confirmed to be widely involved in the regulation of plant growth, development and metabolism, especially in improving stress resistance (Satoh-Nagasawa *et al*. 2006, Kosar *et al*. 2019).

In this study, 10 *CsTPSs* and 6 *CsTPPs* were identified from the genome of cannabis. *CsTPSs* contained complete Glyco_transf_20 and Trehalose_PPase domains, and *CsTPPs* contained only Trehalose_PPase domains. The number of ten *CsTPSs* was less than the number of *Arabidopsis* (11) (Yang *et al*. 2012), rice (11) (Ge *et al*. 2008), and cotton (14) (Mu *et al*. 2016) and more than the number of loti (9) (Jin *et al*. 2016), *Brachypodium distachyon* (9) (Wang *et al*. 2019), and potato (8) (Xu *et al*. 2017). Six *CsTPPs* were less abundant than those of *Arabidopsis* (10) (Vandesteene *et al*. 2012), rice (11) (Ge *et al*. 2008), and *Brachypodium distachyon* (10) (Wang *et al*. 2019).

In this study, *CsTPSs* and *CsTPPs* were clustered into three subfamilies. *CsTPSs* were divided into Class I and Class II subfamilies, and all class III subfamilies were *CsTPPs*. The clustering result was consistent with the clustering result of other plants. In terms of gene structure, Class I and Class III contained more exons, indicating that they have more complex structural characteristics. However, class II, which is the same as *CsTPSs*, contains few exons, which is consistent with the gene structure of *TPS* and *TPP* in other plants. The gene structure and motif of Class I and Class II members are basically the same (Fig. 2), which is shown in the exact same number of exons and motif types of each class. There were slight differences in gene structure and motifs between Class III members. The *TPS* and *TPP* gene families are highly conserved in evolution and structure, which can provide some reference for the study of *TPS* and *TPP* gene function in cannabis.

In the evolutionary process, with the different

duplication selection events experienced by different species, homologous genes have different degrees of differentiation. The common types are neofunctionalization, subfunctionalization, gene functional redundancy, and gene loss (Force *et al*. 1999). The number of *TPS* genes in cannabis was more or less similar than that in other plants. However, the number of *TPP* genes was 4, 5, and 4 less than the number of *TPS* genes of *Arabidopsis*, rice, and *Brachypodium distachyon*, respectively, which may be due to the loss of some *TPP* genes after gene duplication, because a newly duplicated gene can be either lost or fixed in the chromosome by genetic drift or natural selection (Lynch *et al*. 2001).

There were four gene duplications in *CsTPS* and *CsTPP* genes: *CsTPS5* and *CsTPS7*, *CsTPS6* and *CsTPS7*, *CsTPS8* and *CsTPS9*, and *CsTPPA* and *CsTPPB*, suggesting that gene duplication events played an important role in the amplification of *TPS* and *TPP* families in cannabis. The results of expression analysis under abiotic stress showed that *CsTPS7* was not induced to be expressed under the three stresses, while its duplicated genes *CsTPS5* and *CsTPS6* were expressed under all three stresses, indicating that gene duplication led to the divergence of some cannabis *TPS* genes into new functions.

TPS and *TPP* are recognized as genes that can improve plant stress resistance (Delorge *et al*. 2014, Kosar *et al*. 2019). With the development of molecular biology, the stress-resistant functions of many *TPS* and *TPP* family members in *Arabidopsis* have been elucidated. Overexpression of *AtTPS1* results in increased trehalose content, and transgenic plants show strong drought tolerance (Avonce *et al*. 2004). *AtTPPD*-deficient mutants are sensitive to salt stress, while *AtTPPD* overexpressing plants are more tolerant to salt stress (Krasensky *et al*. 2014). *AtTPPF* deletion mutation results in a drought-sensitive phenotype of *Arabidopsis*, while its overexpression lines show significant drought tolerance and trehalose accumulation (Lin *et al*. 2019). *AtTPPI* can enhance the drought resistance of *Arabidopsis* by regulating the stomatal opening and root structure (Lin *et al*. 2020). These results indicate that genes in the different subgroups of *TPS* and *TPP* have similar or different functions.

In this study, 11 cannabis *TPS* and *TPP* genes, *CsTPS1*, *CsTPS2*, *CsTPS3*, *CsTPS5*, *CsTPS6*, *CsTPS8*, *CsTPS9*, *CsTPS10*, *CsTPPA*, *CsTPPB* and *CsTPPC*, were significantly induced by at least one abiotic stress, accounting for 68.75 % of the total gene family. *CsTPS1* was significantly upregulated under three abiotic stresses, which is consistent with the obvious function of its *Arabidopsis* and rice homologous genes *AtTPS1* and *OsTPS1* (Ge *et al*. 2008) under abiotic stresses. *CsTPPA* and *OsTPP1* have similar positions in the evolutionary tree, and both exhibit certain abiotic stress regulation functions (Zhang *et al*. 2017), suggesting that the *TPS1* and *TPP1* genes have similar functions in different species.

In the phylogenetic tree, *CsTPS1*, *CsTPS2*, and *CsTPS3* were in the same subfamily and are clustered together. The results of expression analysis showed that these three genes were upregulated to varying degrees under the three abiotic stresses, and the relative expression at most time points was significantly different from the relative expression at 0 h, indicating that some genes with similar positions in the phylogenetic tree may have similar functions. The gene pairs *CsTPS4* and *CsTPS7*, *CsTPS8* and *CsTPS10*, *CsTPPD* and *CsTPPE* were very close in the phylogenetic tree. Similarly, the two genes of these gene pairs have similar functions. The gene pair *CsTPS5* and *CsTPS6* was clustered together in the phylogenetic tree. Although they were expressed under three abiotic stresses, their expression trend was opposite under salt stress and drought stress. The expression patterns of *CsTPPA* and *CsTPPB* were the same only under cold stress, but they were significantly different under salt and drought stresses. These results indicate that the two groups of genes with similar positions in the phylogenetic tree have partial functional divergence, which can be used as references for each other in the study of gene function.

Conclusions

In this study, systematic analysis of *TPS* and *TPP* family genes have been performed in cannabis, and ten *CsTPS* and six *CsTPP* genes were identified at the genomic level. The *TPS* and *TPP* family members of cannabis, *Arabidopsis*, and rice were classified as three subgroups based on an evolutionary tree. There were four pairs of *CsTPSs* and *CsTPPs* that had gene duplication, indicating that gene duplication events played an important role in the amplification of *TPS* and *TPP* families in cannabis. The expression patterns under various abiotic stresses showed that most of the *TPS* and *TPP* genes may be involved in stress tolerance. In particular, *CsTPS1*, *CsTPS9*, and *CsTPPA* might be more induced by at least one abiotic stress, indicating that these three genes may play key roles in abiotic stresses. This study lays a foundation for further study on the biological functions of *CsTPSs* and *CsTPPs*.

References

Andre, C.M., Hausman, J.F., Guerriero. G.: *Cannabis sativa*: the plant of the thousand and one molecules. - Front. Plant. Sci. **7**: 19, 2016.

Avonce, N., Leyman, B., Mascorro-Gallardo, J., Van Dijck, P., Thevelein, J.M., Iturriaga, G.: The *Arabidopsis* trehalose-6-P synthase *AtTPS1* gene is a regulator of glucose, abscisic acid, and stress signaling. - Plant Physiol. **136**: 3649-3659, 2004.

Avonce, N., Mendoza-Vargas, A., Morett, E., Iturriaga, G.: Insights on the evolution of trehalose biosynthesis. - BMC Evol. Biol. **6**: 109, 2006.

Chen, C.J., Chen, H., Zhang, Y., Thomas, H.R., Frank, M.H., He, Y.H., Xia, R.: TBtools: an integrative toolkit developed for interactive analyses of big biological data. - Mol. Plants **13**: 1194-202, 2020.

Delorge, I., Janiak, M., Carpentier, S., Dijck, P.V.: Fine tuning of trehalose biosynthesis and hydrolysis as novel tools for the generation of abiotic stress tolerant plants. - Front. Plant Sci. **5**: 147, 2014.

Dong, Y.J., Zhang, Q., Dai, X.L., He, M.R.: Effects of potassium chloride and nitric oxide on growth and physiological

characteristics of winter wheat under salt stress. - Biol. Plant. **64**: 258-265, 2020.

Du, L., Qi, S., Ma, J., Xing, L., Fan, S., Zhang, S., Li, Y., Shen, Y., Zhang, D., Han, M.: Identification of *TPS* family members in apple (*Malus* × *domestica* Borkh.) and the effect of sucrose sprays on *TPS* expression and floral induction. - Plant Physiol. Biochem. **120**: 10-23, 2017.

Force, A., Lynch, M., Pickett, F.B., Amores, A., Yan, Y., Postlethwait, J.: Preservation of duplicate genes by complementary, degenerative mutations. - Genetics **151**: 1531-1531, 1999.

Gao, S., Wang, B., Xie, S., Xu, X., Zhang, J., Pei, L., Yu, Y., Yang, W., Zhang, Y.: A high-quality reference genome of wild *Cannabis sativa*. - Hort. Res. **7**: 73, 2020.

Ge, L.F., Chao, D.Y., Shi, M., Zhu, M.Z., Gao, J.P., Lin, H.X.: Overexpression of the trehalose-6-phosphate phosphatase gene *OsTPP1* confers stress tolerance in rice and results in the activation of stress responsive genes. - Planta **228**: 191-201, 2008.

Hackel, C., Zinkevich, T., Belton, P., Achilles, A., Reichert, D., Krushelnitsky, A.: The trehalose coating effect on the internal protein dynamics. - Phys. Chem. Chem. Phys. **14**: 2727-2734, 2012.

Han, B., Fu, Li., Zhang, D., He, X., Chen, Q., Peng, M., Zhang, J.: Interspecies and intraspecies analysis of trehalose contents and the biosynthesis pathway gene family reveals crucial roles of trehalose in osmotic-stress tolerance in cassava. - Int. J. mol. Sci. **17**: 1077, 2016.

Hu, H., Liu, H., Du, G., Yang, F., Deng, G., Yang, Y., Liu, F.: Fiber and seed type of hemp (*Cannabis sativa* L.) responded differently to salt-alkali stress in seedling growth and physiological indices. - Indust. Crop. Prod. **129**: 624-630, 2019.

Hu, X., Wu, Z.D., Luo, Z.Y., Burner, D.M., Pan, Y.B., Wu, C.W.: Genome-wide analysis of the trehalose-6-phosphate synthase (TPS) gene family and expression profiling of *ScTPS* genes in sugarcane. - Agronomy **10**: 969, 2020.

Hurst, L.D.: The Ka/Ks ratio: diagnosing the form of sequence evolution. - Trends Genet. **18**: 486-487, 2002.

Jin, Q., Hu, X., Li, X., Wang, B., Wang, Y., Jiang, H., Mattson, N., Xu, Y.: Genome-wide identification and evolution analysis of trehalose-6-phosphate synthase gene family in *Nelumbo nucifera*. - Front. Plant Sci. **7**: 1445, 2016.

Kosar, F., Akram, N.A., Sadiq, M., Al-Qurainy, F., Ashraf, M.: Trehalose: a key organic osmolyte effectively involved in plant abiotic stress tolerance. - J. Plant Growth Regul. **38**: 606-618, 2019.

Krasensky, J., Broyart, C., Rabanal, F.A., Jonak, C.: The redox-sensitive chloroplast trehalose-6-phosphate phosphatase *AtTPPD* regulates salt stress tolerance. - Antioxid. Redox Sign. **21**: 1289-1304, 2014.

Lerbret, A., Bordat, P., Affouard, F., Guinet, Y., Hedoux, A., Paccou, L., Prevost, D., Descamps, M.: Influence of homologous disaccharides on the hydrogen-bond network of water: complementary Raman scattering experiments and molecular dynamics simulations. - Carbohydrate Res. **340**: 881-887, 2005.

Librado, P., Rozas, J.: DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. - Bioinformatics **25**: 1451-1452, 2009.

Lin, M., Jia, R., Li. J., Zhang, M., Chen, H., Zhang, D., Zhang, J., Chen, X.: Evolution and expression patterns of the trehalose-6-phosphate synthase gene family in drumstick tree (*Moringa oleifera* Lam.). - Planta **248**: 999-1015, 2018.

Lin, Q., Wang, S., Dao, Y., Wang, J., Wang, K.: *Arabidopsis thaliana* trehalose-6-phosphate phosphatase gene *TPPI* enhances drought tolerance by regulating stomatal apertures. - J. exp. Bot. **71**: 4285-4297, 2020.

Lin, Q., Yang, J., Wang, Q., Zhu, H., Chen, Z., Dao, Y., Wang, K.: Overexpression of the trehalose-6-phosphate phosphatase family gene *AtTPPF* improves the drought tolerance of *Arabidopsis thaliana*. - BMC Plant Biol. **19**: 381, 2019.

Livak, K.J., Schmittgen, T.D.: Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. - Methods **25**: 402-408, 2001.

Lynch, M., O'Hely, M., Walsh, B., Force, A.: The probability of preservation of a newly arisen gene duplicate. - Genetics **159**:1789-1804, 2001.

Mahajan, S., Tuteja, N.: Cold, salinity and drought stresses: an overview. - Arch. Biochem. Biophys. **444**: 139-158, 2005.

Morabito, C., Secchi, F., Schubert, A.: Grapevine TPS (trehalose-6-phosphate synthase) family genes are differentially regulated during development, upon sugar treatment and drought stress. - Plant Physiol. Biochem. **164**: 54-62, 2021.

Mu, M., Lu, X.K., Wang, J.J., Wang, D.L., Yin, Z.J., Wang, S., Fan, W.L., Ye, W.W.: Genome-wide identification and analysis of the stress-resistance function of the TPS (Trehalose-6-Phosphate Synthase) gene family in cotton. - BMC Genet. **17**: 54, 2016.

Paul, M.J., Primavesi, L.F., Jhurreea, D., Zhang, Y.: Trehalose metabolism and signaling. - Annu. Rev. Plant Biol. **59**: 417-441, 2008.

Pretzsch, C.M., Freyberg, J., Voinescu, B., Lythgoe, D., Horder, J., Mendez, M.A., Wichers, R., Ajram, L., Ivin, G., Heasman, M., Edden, R.A.E., Williams, T., Murphy, D.G.M., Daly, E., McAlonan, G.M.: Effects of cannabidiol on brain excitation and inhibition systems; a randomised placebo-controlled single dose trial during magnetic resonance spectroscopy in adults with and without autism spectrum disorder. - Neuropsychopharmacology **44**: 1398-1405, 2019.

Satoh-Nagasawa, N., Nagasawa, N., Malcomber, S., Sakai, H., Jackson, D.: A trehalose metabolic enzyme controls inflorescence architecture in maize. - Nature **441**: 227-230, 2006.

Skoglund, G., Nockert, M., Holst, B.: Viking and early middle ages northern Scandinavian textiles proven to be made with hemp. - Sci. Rep. **3**: 2686, 2013.

Sundaramurthi, P., Patapoff, T.W., Suryanarayanan, R.: Crystallization of trehalose in frozen solutions and its phase behavior during drying. - Pharm. Res. **27**: 2374-2383, 2010.

Swanson, W.J., Yang, Z., Wolfner, M.F., Aquadro, C.F.: Positive Darwinian selection drives the evolution of several female reproductive proteins in mammals. - Proc. nat. Acad. Sci. USA **98**: 2509-2514, 2001.

Vandesteene, L., Lopez-Galvis, L., Vanneste, K., Feil, R., Maere, S., Lammens, W., Rolland, F., Lunn, J.E., Avonce, N., Beeckman, T., Van Dijck, P.: Expansive evolution of the trehalose-6-phosphate phosphatase gene family in *Arabidopsis*. - Plant Physiol. **160**: 884, 2012.

Vij, S., Tyagi, A.K.: Emerging trends in the functional genomics of the abiotic stress response in crop plants. - Plant Biotechnol. J. **5**: 361-380, 2010.

Wang, S., Ouyang, K., Wang, K.: Genome-wide identification, evolution, and expression analysis of TPS and TPP gene families in *Brachypodium distachyon*. - Plants **8**: 362, 2019.

Wolfe, K.H., Gouy, M., Yang, Y.W., Sharp, P.M., Li, W.H.: Date of the monocot-dicot divergence estimated from chloroplast DNA sequence data. - Proc. nat. Acad. Sci. USA **86**: 6201-6205, 1989.

Xie, D.W., Wang, X.N., Fu, L.S., Sun, J., Zheng, W., Li, Z.F.: Identification of the trehalose-6-phosphate synthase gene family in winter wheat and expression analysis under

conditions of freezing stress. - J. Genet. **94**: 55-65, 2015.
Xu, Y., Wang, Y., Mattson, N., Yang, L., Jin, Q.: Genome-wide analysis of the *Solanum tuberosum* (potato) trehalose-6-phosphate synthase (TPS) gene family: evolution and differential expression during development and stress. - BMC Genomics **18**: 926, 2017.
Yang, H.L., Liu, Y.J., Wang, C.L., Zeng, Q.Y.: Molecular evolution of trehalose-6-phosphate synthase (TPS) gene family in *Populus*, *Arabidopsis* and rice. - PLoS ONE **7**: e42438, 2012.
Yang, Y., Ma, K., Zhang, T., Li, L., Wang, J., Cheng, T., Zhang, Q.: Characteristics and expression analyses of trehalose-6-phosphate synthase family in *Prunus mume* reveal genes involved in trehalose biosynthesis and drought response. - Biomolecules **10**: 1358, 2020.
Zhang, Q., Chen, Q., Wang, S., Hong, Y., Wang, Z.: Rice and cold stress: methods for its evaluation and summary of cold tolerance-related quantitative trait loci. - Rice **7**: 24, 2014.
Zhang, Z., Li, J., Li, F., Liu, H., Yang, W., Chong, K., Xu, Y.: OsMAPK3 phosphorylates OsbHLH002/OsICE1 and inhibits its ubiquitination to activate *OsTPP1* and enhances rice chilling tolerance. - Dev. Cell **43**: 731-743, 2017.