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ORIGINAL RESEARCH

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Carbohydrate dynamics in *Populus* trees under drought: An expression atlas of genes related to sensing, translocation, and metabolism across organs

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Abstract

In trees, nonstructural carbohydrates (NSCs) serve as long-term carbon storage and long-distance carbon transport from source to sink. NSC management in response to drought stress is key to our understanding of drought acclimation. However, the molecular mechanisms underlying these processes remain unclear. By combining a transcriptomic approach with NSC quantification in the leaves, stems, and roots of *Populus alba* under drought stress, we analyzed genes from 29 gene families related to NSC signaling, translocation, and metabolism. We found starch depletion across organs and accumulation of soluble sugars (SS) in the leaves. Activation of the trehalose-6-phosphate/SNF1-related protein kinase (SnRK1) signaling pathway across organs via the suppression of class I *TREHALOSE-PHOSPHATE SYNTHASE* (*TPS*) and the expression of class II *TPS* genes suggested an active response to drought. The expression of *SnRK1*α and β subunits, and *SUCROSE SYNTHASE6* supported SS accumulation in leaves. The upregulation of active transporters and the downregulation of most passive transporters implied a shift toward active sugar transport and enhanced regulation over partitioning. SS accumulation in vacuoles supports osmoregulation in leaves. The increased expression of sucrose synthesis genes and reduced expression of sucrose degradation genes in the roots did not coincide with sucrose levels, implying local sucrose production for energy. Moreover, the downregulation of invertases in the roots suggests limited sucrose allocation from the aboveground organs. This study provides an expression atlas of NSC-related genes that respond to drought in poplar trees, and can be tested in tree improvement programs for adaptation to drought conditions.

1 | INTRODUCTION

Drought-driven tree mortality has triggered renewed interest in nonstructural carbohydrates (NSCs) as important factors in tree adaptation to climate change. NSC regulation ensures carbon and energy balance throughout the life of a tree, particularly under various stresses. NSC are mostly composed of two components: (1) soluble sugars (SS), which function directly in metabolism, energy, and

protection, but can also serve as storage in the vacuole; and (2) starch, a stationary, osmotically inactive carbon polymer that acts as a sink and a source of energy (MacNeill et al., 2017). Plants allocate SS, mainly as sucrose, from the photosynthetic source organs to the non-photosynthetic sink organs, where they are used as is, stored in the vacuole, or converted to starch in amyloplasts for energy reserves. Starch storage is important for plant survival, especially in long-lived plants, as it buffers the asynchrony of photosynthetic-carbohydrate supply and demand, and provides a crucial energy source under conditions that impair carbon intake. These include nighttime, seasonal dormancy, and stresses such as drought, in which CO_2 intake and transpiration are reduced following stomatal closure (MacNeill et al., 2017; Thalmann & Santelia, 2017). The process of carbohydrate distribution at the whole-plant level is complex, highly regulated, and diverse among plant species and age. It is tightly regulated by molecular components, although they are not fully understood (Paul, 2008; Rolland et al., 2006; Yoon et al., 2021).

Drought stress causes a decrease in cell turgor pressure, stomatal closure, and a reduction in photosynthesis rate and growth (Muller et al., 2011). Accordingly, the source-sink balance is altered and stress metabolic reprogramming is triggered, activating starch depletion. Consequently, SS accumulate and function as a fast and accessible energy pool, as reactive oxygen species scavengers, and also increase osmotic pressure to maintain water flux (Kaur et al., 2021). Such metabolic reprogramming requires a shift in major molecular regulations (Belda-Palazón et al., 2020; Dong & Beckles, 2019).

Among the sugar-specific signal transduction pathways that exist in plants, the trehalose-6-phosphate (Tre-6P)/SUCROSE-NONFERMENTING1-RELATED KINASE1 (SnRK1) pathway is known to be a key regulator of carbon sensing and partitioning, activated in response to drought stress (Fichtner & Lunn, 2021; Lunn, 2007a; Paul, 2008). Tre-6P, an important signaling molecule for sucrose levels, is synthesized by TREHALOSE-PHOSPHATE SYNTHASE (TPS) and phosphorylated to trehalose by TREHALOSE-PHOSPHATE PHOSPHATASE (TPP). SnRK1 proteins regulate low nutrient stress responses, and function in heterotrimeric complexes, composed of catalytic subunit α and regulatory subunits β and βγ (Baena-González & Lunn, 2020; Janse van Rensburg et al., 2019). In the leaves, an increase in Tre-6P concentration signals for starch synthesis and photosynthesis inhibition in an SnRK1-independent manner (Yoon et al., 2021; Zhang et al., 2009). Whereas in sink organs, specifically in growing young tissues, Tre-6P promotes sucrose consumption and starch biosynthesis by inhibiting the SnRK1 catalytic subunits (Dong & Beckles, 2019; Fichtner & Lunn, 2021; Figueroa & Lunn, 2016). The Tre-6P/ SnRK1 potential in altering drought adaptation was shown in several plant species. Increased expression of *TPP* in maize ears improves yield under drought (Nuccio et al., 2015), affects source-sink relations, and increases the photosynthesis rate under stress (Oszvald et al., 2018). In Arabidopsis, *TPS1* overexpression confers drought tolerance with glucose and ABA insensitivity (Avonce et al., 2004).

Sugar transport from source to sink is supported by various sugar transporters, such as SUGARS WILL EVENTUALLY BE EXPORTED TRANSPORTERS (SWEET) and SUCROSE TRANSPORTERS (SUTs; Julius et al., 2017). These transporters play a critical role in controlling sugar levels, facilitating both subcellular and long-distance transport, specifically under drought conditions (Saddhe et al., 2021). There are four clades of the *SWEET* gene family: clade I (SWEET1-3) and clade II (SWEET4-8) are hexose transporters, clade III (SWEET9-15) are sucrose transporters and clade IV (SWEET16-17) are fructose transporters (Klemens et al., 2014; Zhang et al., 2021). SWEET transporters are bidirectional and are mainly involved in apoplastic loading and vacuolar hexose export along a concentration gradient (Chen et al., 2012). In comparison, SUT transporters maintain active sucrose transport, either from the apoplast into cells or from the vacuole to the cytosol (Gottwald et al., 2000; Julius et al., 2017).

Sucrose is typically synthesized in the cytosol by SUCROSE-PHOSPHATE SYNTHASE (SPS) and SUCROSE-PHOSPHATE PHOSPHATASE (SPP). After sucrose is loaded into the phloem and unloaded into the sink tissues, it is degraded into hexoses or their derivatives by either INVERTASE (INV) or SUCROSE SYNTHASE (SUS). SUS is mostly cytosolic and is associated with sucrose metabolic utilization and sink strength (Stein & Granot, 2019). INV can be localized in the cytosol, or in different cell components. In Arabidopsis, it has been found to be related to growth and cell elongation via osmoregulation, specifically in the roots (Barratt et al., 2009; Sergeeva et al., 2006). Cell-wall INVs (cwINVs) are generally associated with reproductive sinks governing phloem unloading, whereas vacuole INVs (vINVs) contribute to sugar accumulation and cell expansion (Hussain et al., 2020; Li et al., 2021). Less stable, more numerous cytosolic INV are less known and are localized in multiple subcellular compartments (Braun et al., 2014; Ruan et al., 2010; Wan et al., 2018).

The role of NSCs in the response of trees to drought stress has been debated. Although NSCs have been suggested to function as a backup energy reserve during the inhibition of carbohydrate production in response to drought, other studies have indicated that NSCs may play a pivotal role in osmoregulation. A recent analysis of a large database showed that higher concentrations of total NSCs are not necessarily associated with site aridity and suggested that NSCs likely function in osmoregulation rather than in energy reserves (Blumstein et al., 2023). Poplar trees respond to drought by reducing total NSC content and increasing the concentration of sucrose, glucose, and fructose, which are the main sugars that operate during the dry season (Regier et al., 2009; Regier et al., 2010).

The dynamics of NSCs in trees have been reviewed broadly, emphasizing their importance and highlighting knowledge gaps, especially regarding the molecular regulatory processes governing NSC reprogramming in response to abiotic stresses (Dietze et al., 2014; Hartmann & Trumbore, 2016; Kozlowski & Pallardy, 2002). The relatively large and growing molecular resources for the model tree *Populus* provide a great opportunity to uncover this gap (Jansson & Douglas, 2007; Sundell et al., 2015). Although the drought transcriptome of poplar species has been studied extensively (Hamanishi et al., 2015; Shuai et al., 2014; Street et al., 2006; Tang et al., 2013; Yan et al., 2012), molecular research focusing on NSC-related genes is still scarce. Molecular studies on NSCs have mainly focused on wood formation processes rather than stress adaptation (Dominguez et al., 2021; Geisler-Lee et al., 2006; Mahboubi & Niittylä, 2018; Wei et al., 2015).

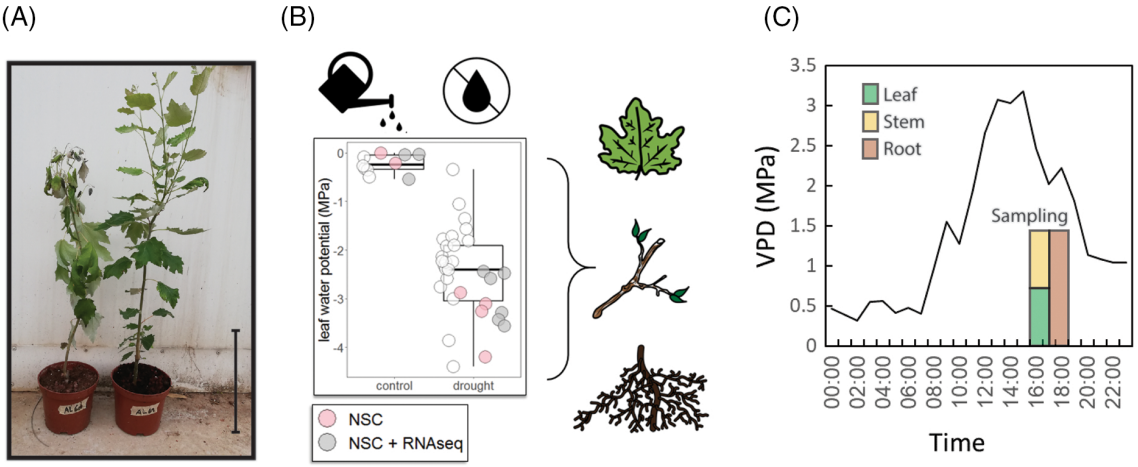

FIGURE 1 Drought stress experiment layout for five well-watered (control) and 10 drought-treated *Populus alba* saplings. (A) Watered (right) and drought-stressed (left) trees before sampling. Scale bar—24 cm. (B) Leaf water potential (MPa) was measured immediately before sampling. Colored points denote trees selected for analysis from among all the trees in the experiment (white). (C) Vapor pressure deficit (VPD) in the greenhouse on the sampling day. Colored boxes represent the time frame of organ sampling. Leaves and stems were sampled together, and roots were sampled immediately thereafter. The green, yellow, and brown colors represent the leaves, stems, and roots, respectively.

Here, we hypothesized that gene expression analysis can assist in determining the trajectory of NSCs in poplar trees in response to drought. To this end, we performed transcriptome analysis along with NSC quantification in the leaves, stems, and roots of *Populus alba* under well-watered and drought conditions. We analyzed the expression levels of genes related to NSC metabolism, sensing, and translocation to identify the genes related to NSC reprogramming which are affected by drought stress in each organ. We show that gene expression profiles imply utilization of NSCs for osmoregulation in leaves and stems and for energy reserves in roots.

2 | MATERIALS AND METHODS

2.1 | Plant material and drought experiments

Populus alba L. cuttings were grown in 1 l pots for four weeks in a greenhouse with semicontrolled conditions at the Weizmann Institute, Rehovot, Israel. The tree's mean stem diameter and height were 6.6 ± 0.2 mm and 61.2 ± 3.6 cm, respectively. Drought treatment was applied in late July 2019 via the cessation of irrigation for seven days. The temperature, relative humidity (RH), and vapor pressure deficit (VPD) in the greenhouse measured during the experiment were: 27.9 ± 0.1°C, 48.6 ± 0.3%, and 2 ± 0.03 kPa, respectively. After seven days of dehydration, when the wilting phenotype and water potential (measured by a pressure bomb) indicated a response to drought, ten selected trees were sampled for NSCs and gene expression between 3 and 4 p.m. and placed immediately in liquid nitrogen (Figure 1). Samples included fully expanded leaves of similar positions, 1-cm-long segments of the main stem including all tissues, and represented portions of the central root system, including fine roots. The roots of the control plants seemed to be less lignified and whiter in color than those of drought-treated plants. All samples were later ground in liquid nitrogen into a fine powder using a mortar and pestle and a Retsch Mixer Mill grinder (MM301; Retsch GmbH). Each sample was divided into two for paired NSC and gene expression analyses.

2.2 | NSC quantification

The first part of the sample (30 ± 1 mg) devoted to NSC quantification (five control and ten drought-treated plants) was lyophilized for 48 h (Gamma 2-16 LSCplus). NSCs were extracted, quantified, and calculated according to the Landhäusser et al. protocol (2018). SSs were first extracted from the plant tissue in 1 ml ethanol (80%) and heated to 90°C for 15 min. Ethanol was then fully evaporated (60°C), replaced with 1 ml of HPLC grade water, heated to 90°C for 10 min, and vortexed for homogenization. Simultaneously, starch from the same tissue was fully dried (60°C) after three cycles of ethanol wash (1.5 ml, 80%) and heat (90°C, 15 min). Starch was later homogenized with α-amylase (600 units in 1 ml water) for 60 min at 85°C (*Bacillus licheniformis*, Sigma A4551), of which 0.1 ml were transferred to an amyloglucosidase solution (six units in 0.5 ml sodium acetate buffer, pH 4.6) for 30 min at 55°C (*Aspergillus niger*, Roche #11202367001, ROAMYGLL). Both hydrolyzed starch and SS samples were filtered using an Acrodisc Minispike Syringe Filter, PTFE 13 mm, 0.2 µm (Pall Corporation) before running them in an analytical ultra-high-speed liquid chromatography (UFLC) system (Prominence UFLC, Shimadzu Scientific Instruments), equipped with an SIL-20AC autosampler, a CTO-20AC column oven, and a RID-20A detector (Shimadzu). The system was fitted with an Aminex HPX-87C Column (300 × 7.8 mm, 9 µm particle) and a guard column with micro-guard IG carbo C cartridges (Bio-Rad). Sugar separation was performed under the following conditions: 84°C column temperature, water as mobile phase, 0.6 ml/

min flow rate (according to manufacturer recommendations). The sugar retention times were determined using a standard carbohydrate mix (Bio-Rad, Hercules). Glucose, sucrose, and fructose standard curves were used to quantify the extracted and hydrolyzed NSC samples (Sigma cat. 47829, 47289, and F2793, respectively).

2.3 | RNA extraction, library preparation, and sequencing

The second portion of each sample used for gene expression analysis was subjected to RNA extraction. These included three controls and six drought-treated plants that were used for NSC quantification (Figure 1B). RNA was extracted according to the method described by Gambino et al. (2008) with minor modifications: incubation in CTAB at 65°C for 60 min. RNA quality and quantity were accessed using a 2200 TapeStation System (Agilent Technologies). A total of 24 RNA samples (2000 ng each with an RNA Integrity Number > 6.5) were sent for library preparation and sequencing by Macrogen. Libraries were constructed using random fragmentation followed by 5′ and 3′ adapter ligation (Illumina TruSeq Stranded mRNA; PolyA enrichment). Paired-end sequencing was conducted using Illumina NovaSeq6000.

2.4 | Transcriptome assembly, functional annotation, and differential expression analysis

Raw reads were filtered and cleaned using Trimmomatic (Bolger et al., 2014). The FASTX-Toolkit version 0.0.13.2 used for (1) trimming read-end nucleotides with quality scores <30 using fastq_quality_trimmer and (2) removing reads with <70% base pairs with a quality score ≤30 using fastq_quality_filter. TopHat2 (Kim et al., 2013) was used to map clean reads onto the *P. alba* reference genome (ASM523922v1, https://www.ncbi.nlm.nih.gov/bioproject/PRJNA491245/). Gene abundance estimation was performed using Cufflinks v2.2 (Trapnell et al., 2010) combined with gene annotations from the NCBI database (https://ftp.ncbi.nlm.nih.gov/genomes/all/GCF/005/239/225/GCF_005239225.1_ASM523922v1/GCF_005239225.1_ASM523922v1_genomic.gff.gz). Gene expression values were computed using the fragments per kilobase of mapped reads technique. Heatmap visualization was performed using the R Bioconductor software (Gentleman et al., 2004). Differential expression analysis was performed using the DESeq2 R package (Love et al., 2014). Genes ≥1-fold differentially expressed with a false discovery-corrected statistical significance of at most 0.05 were considered differentially expressed (Benjamini & Hochberg, 1995). Differentially expressed genes are listed in Table S1, and the normalized raw count reads of all biological replicates are presented in Table S2. Because we used an unbalanced design with a larger number of shoots and roots than leaves, we conducted ten tests with three different drought-treated root samples each, showing that significance was canceled for only one gene owing to uneven sampling (Table S3). The *P. alba* proteins were used as a query term to search the NCBI nonredundant (nr) protein database using the DIAMOND program (Buchfink et al., 2015). The search results were imported into the Blast2GO version 4.0 (Conesa et al., 2005) for gene ontology (GO) assignments.

2.5 | Gene classification, identification, and nomenclature

Following a literature survey of genes regulating NSC dynamics in plants, we identified 29 families involved in the sensing, transport, synthesis, and degradation of starch and sucrose (Table S4). The corresponding genes from the *P. alba* genome were identified and classified into families according to genome annotation and GO affiliation. Then, 9 of the 29 families were chosen for manual validation and nomenclature. Based on previous studies on other poplar species, gene names were assigned to specific members of *TPS* (*P. trichocarpa*; Yang et al., 2012), *SUT* (*P. tremula* X *P. alba*; Payyavula et al., 2011), *SWEET* (*P. trichocarpa*; Zhang et al., 2021), *INV* (*P. trichocarpa*; Chen, Gao, et al., 2015), and *SUS* (*P. trichocarpa*; An et al., 2014). *Early response to dehydration protein 6* (*ESL* or *ERD6*) and *SNF1-related protein kinase* (*SnRK1*) gene names were assigned according to Arabidopsis (Ramon et al., 2013; Slawinski et al., 2021). *TPP* genes were named based on Arabidopsis genes and phylogenetic clades instead of the chromosomal positions published by Gao et al. (2021). All but *SnRK1* family members were identified via BLASTP searches for genes previously defined in any poplar species (Altschul et al., 1997). To identify missing family members, searches (TBlastN) were also conducted against the *P. alba* genome in NCBI. *Arabidopsis thaliana*, other poplar species, and *Medicago truncatula* were added to the collection of sequences for a particular family to aid naming. For the *SnRK1* subunits, the sequences were validated to contain the proper domains with CDD (Marchler-Bauer & Bryant, 2004). Multiple alignments were performed with Muscle 3.8.31 (Edgar, 2004), and phylogenetic trees were built using neighbor-joining in ClustalW 2.1 (Larkin et al., 2007) and maximum-likelihood in PhyML3, an automatic model selection (Guindon et al., 2010). PhyML trees were visualized using iTol (Letunic & Bork, 2021; https://itol.embl.de/).

2.6 | Statistical analysis

Statistical tests were conducted using the R software (v.3.1; R Foundation for Statistical Computing). Significant differences in NSCs between treatments and organs were determined using a *t* test and *var.test* functions ("stats" package). An equal variance *t* test was used for all but starch, in all organs, total SSs in the leaves, and total NSCs in the stems and roots, where an unequal variance *t*-test was used. Correlation between gene expression and NSC measurements was performed using the test of no correlation, the default method for the cor.test function from the STATS core package.

TABLE 1 Concentrations of NSCs across organs of control and drought-treated poplar trees. Values are means (mg g^{-1} dry weight) ± standard errors of starch, sucrose, fructose, and glucose. Total SS included sucrose, fructose, and glucose, whereas total NSCs included also starch. *P*-values of significant differences between the control and drought-treated plants are indicated.

	Leaf (mg g^{-1} DW)			Stem (mg g^{-1} DW)			Root (mg g^{-1} DW)		
	Control $n = 5$	Drought $n = 9$	*t* Test *p*	Control $n = 5$	Drought $n = 10$	*t* Test *p*	Control $n = 5$	Drought $n = 9$	*t* Test *p*
Starch	183.1 ± 15.3	57.2 ± 14.8	<0.001	32.5 ± 9.1	3.8 ± 2.9	<0.05	13.2 ± 9.3	3.1 ± 1.6	0.342
Sucrose	48.5 ± 5.0	66.6 ± 3.9	<0.05	33.1 ± 3.8	39.4 ± 1.8	0.110	17.3 ± 2.0	19.9 ± 1.6	0.343
Glucose	2.6 ± 1.0	13.2 ± 1.9	<0.01	4.8 ± 1.5	6.6 ± 1.1	0.345	2.9 ± 1.8	6.6 ± 2.1	0.285
Fructose	8.0 ± 2.7	17.3 ± 1.3	<0.01	7.6 ± 2.8	7.4 ± 0.9	0.934	1.0 ± 1.0	2.3 ± 1.0	0.422
Total SS	59.1 ± 8.5	97.1 ± 2.4	<0.01	45.5 ± 5.2	53.1 ± 2.8	0.17	21.2 ± 4.5	28.8 ± 3.4	0.21
Total NSC	242.3 ± 11.4	148.6 ± 15.1	<0.01	78.0 ± 10.2	57.2 ± 3.0	0.11	34.3 ± 8.0	31.9 ± 4.4	0.776

Abbreviations: NSCs, nonstructural carbohydrates; SS, soluble sugars.

3 | RESULTS

3.1 | Drought effects on saplings

Drought caused leaf wilting in *P. alba* saplings (Figure 1A) and a decrease in leaf water potential (−3.1 ± 0.17 MPa) compared to control seedlings (−0.6 ± 0.1 MPa; water potential × treatment, $t_{13} = 11.347$, $p < 0.001$; Figure 1B). Over the drought period, the mean daytime (7 a.m.–6 p.m.) temperature was 27.9 ± 0.1°C, the RH was 48.6 ± 0.3%, and the VPD was 1.9 ± 0.03 kPa (Figure 1C).

3.2 | NSC concentrations

The control plants had the highest NSC concentration in the leaves and the lowest in the roots (Table 1). Analysis of variance for total NSCs showed a significant effect to treatment, $F(1, 41) = 13.96$, $p < 0.001$. Under drought stress, starch pools decreased across organs, followed by a significant accumulation of SSs in the leaves. The leaves showed the largest effect, where starch concentration dropped from 183.1 ± 15 mg g^{-1} in the control to 57.2 ± 15 mg g^{-1} dry weight (DW) in the treated saplings (*p*-value <0.001; Table 1). Starch depletion was significant in the stem (*p*-value <.05), but not in the roots (*p*-value = 0.342), decreasing from 32.5 ± 9 to 3.8 ± 3 and 13.2 ± 9 to 3.1 ± 2 mg g^{-1} DW, respectively. SS in drought-treated leaves increased from 59.1 to 97.1 mg g^{-1} DW (*p*-value <0.01), whereas in stems and roots, SS accumulation was mild but insignificant (*p*-value = 0.17 and 0.21, respectively). Of the three quantified SS, sucrose was the most abundant across organs and treatments (Table 1; Figure S4).

3.3 | Gene families related to NSC sensing, transport, and metabolism

The comprehensive identification, classification, nomenclature, and the phylogenetic trees of genes related to carbohydrates in *P. alba* are presented in Tables S4 and S5, Figure S1, and Figure S2. The results revealed same-sized gene families compared to Arabidopsis for the *TPP* (10) and *SnRK1* α-subunits (3). In contrast, smaller gene families compared to Arabidopsis, were found for *ESL* (11) and *SUT* (five). The results for the *SUT* gene family were in agreement with those reported by Payyavula et al. (2011). Larger gene families have been found in *TPS* (12), the *SnRK1* β subunit (6), the *SnRK1* γ subunit (4), the *SnRK1* βγ subunit (4), *SWEET* (25), *SUS* (7), acid INV (11), and neutral/alkaline INV (11). Phylogenetic analysis revealed that *P. alba TPS1* and *TPS2* were grouped with Arabidopsis Class I *TPS* genes together with *TPS13*, a newly discovered *Populus TPS* gene, whereas *TPS4* was found to be a pseudogene. The *P. alba TPS5-11* genes clustered with the Arabidopsis Class II *TPS* together with *TPS12*, which is not present in Arabidopsis (Figure S1). These results were in agreement with those of Yang et al. (2012), except for *TPS13*, which was not included in their study. The 10 SnRK1 subunits including subunit γ, which was not described in *Populus* before the current study, were named according to their homologs from Arabidopsis and Medicago (Table S5, Figure S1). The *SnRK1* genes included three α subunits, six β subunits, four βγ subunits, and four γ subunits (Figure S2). As demonstrated below, particular genes from each family responded to drought stress, with several cases of tissue-specific differential expression in relation to NSC changes in leaves, stems, and roots.

3.4 | Differentially expressed genes related to starch metabolism

Following drought treatment, starch degradation-related genes were generally upregulated across organs or downregulated in the aboveground organs (Figure 2A). The transcripts of *β-amylase 1a* (*BAM*.1a), *BAM*.1c, α-amylase b (*AMY*.b), and *AMY*.c were upregulated across organs, whereas those of *BAM*.a, *BAM*.b, and *disproportionating enzymes* (*DPE*) a and b were remarkably downregulated in the leaves (Figure 2A). Most of the genes involved in starch degradation (*LSF*, *DSP*, *PHS*, *LDA*, *GWD*, and *DPE*) were downregulated in the leaves and

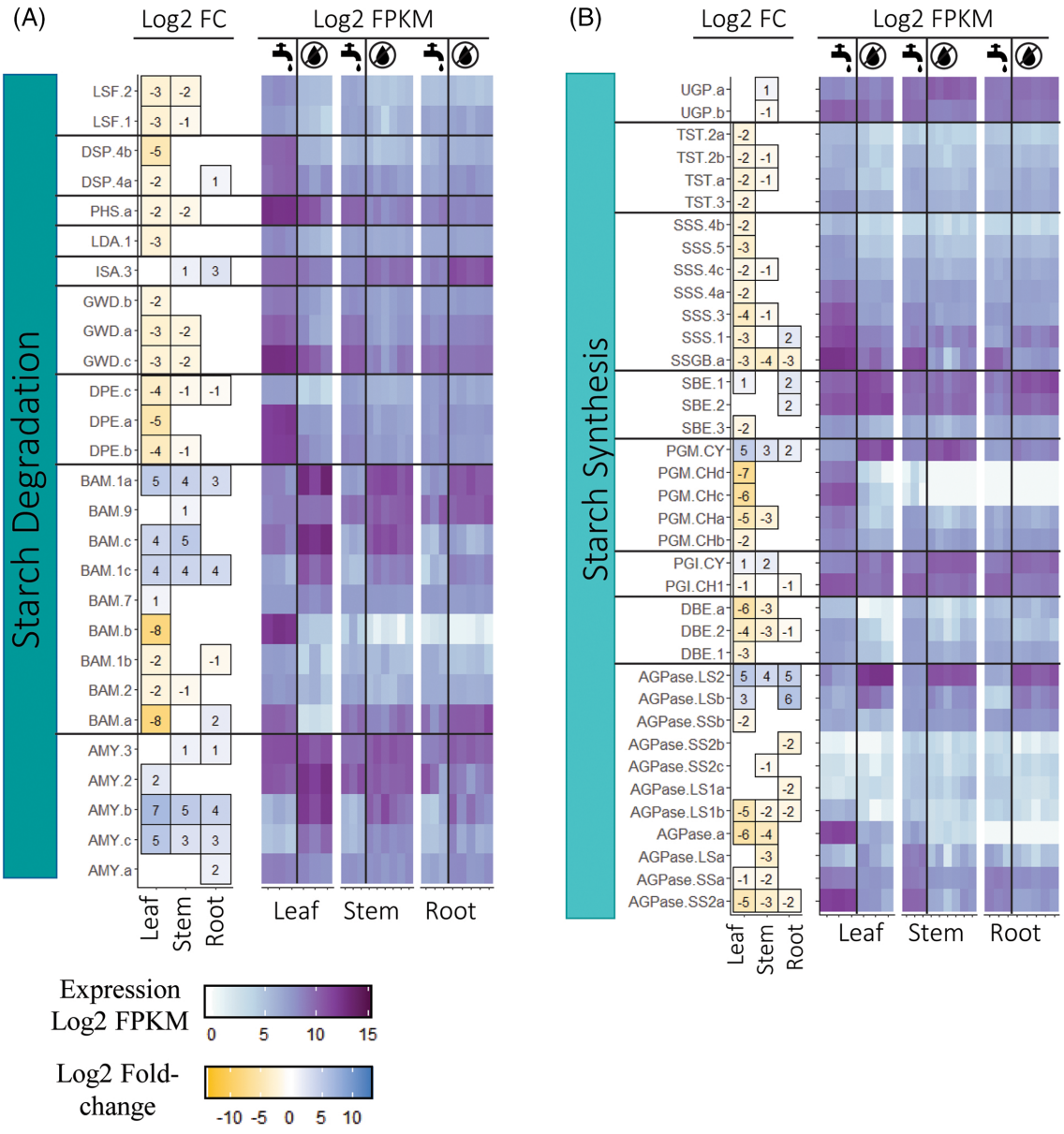

FIGURE 2 Expression patterns of gene families grouped according to their known functions in starch degradation (A) and synthesis (B). Log2 FC indicates fold-change between watered and drought-treated trees across organs and is denoted by text and color. Missing values indicate nonsignificant differences. Fragments per kilobase per million mapped fragments (FPKM) indicate normalized log2 transcript abundance for each watered or drought-treated individual tree (columns). Complete gene names are listed in Table S1.

some in the stem. *Isoamylase 3* (*ISA.3*) was upregulated only in the stems and roots (Figure 2A).

Starch synthesis-related genes were highly expressed in watered trees, notably in leaves and stems, and were mostly downregulated under drought conditions (Figure 2B). *Granule-bound starch synthase* (*SSGB*), *ADP-glucose pyrophosphorylase small-subunit 2a* (*AGPase SS2a*), and *AGPase.a* were highly expressed in the leaves, whereas *AGPase large-subunit a* (*LSa*) was expressed specifically in the stem (Figure 2B). *SSGB* was downregulated across organs, along with various other genes in this pathway. Pronounced downregulation was evident for the chloroplast isoforms (CH) *PGMc* and *PGMd*, which were highly and dominantly expressed in the leaves of watered plants. Drought stress induced the expression of *AGPase LS2* and cytosolic *PGM* across the organs (Figure 2B).

3.5 | Differentially expressed genes related to sucrose metabolism

Six *SUS* and 11 *INV* transcripts were differentially expressed following drought, most were upregulated in aboveground organs and downregulated in the roots (Figure 3). While *SUS3* was steadily expressed across organs and upregulated upon drought, *SUS2* was dominant in stems and roots and downregulated across organs (Figure 3). Drought

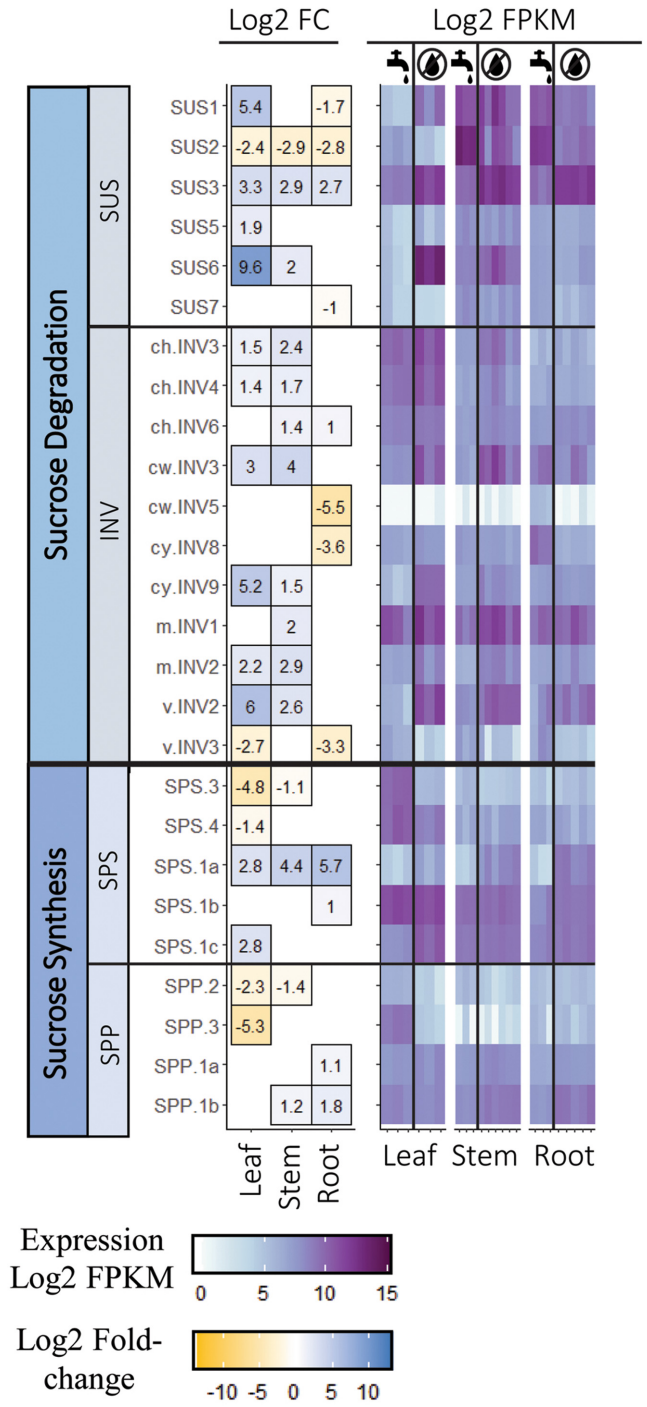

FIGURE 3 Expression patterns of gene families involved in sucrose metabolism. Log2 FC indicates fold-change between watered and drought-treated trees across organs. The fold-change is denoted by text and color. Missing values indicate nonsignificant differences. Fragments per kilobase per million mapped fragments (FPKM) indicate normalized log2 transcript abundance for each watered or drought-treated individual tree (columns). Ch, chloroplast; cw, cell wall; cy, cytosolic; m, mitochondria; v, vacuole. Complete gene names are indicated in Table S1.

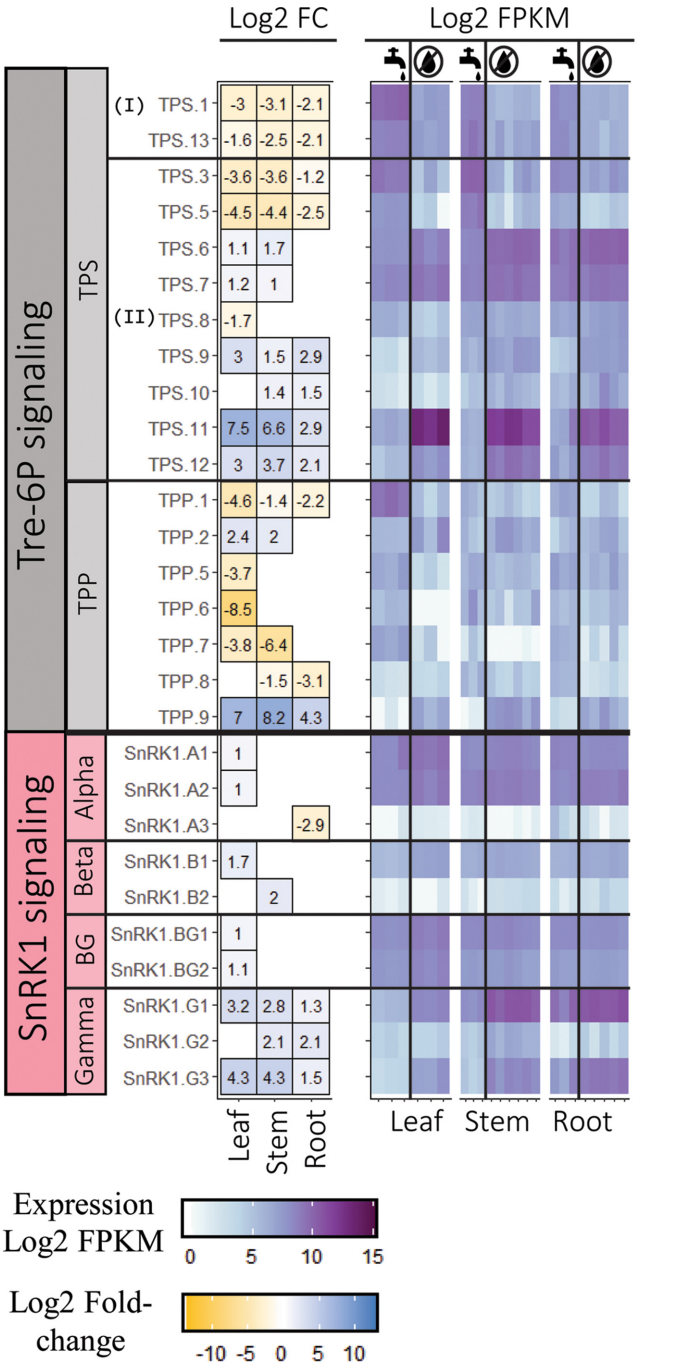

FIGURE 4 Expression pattern of gene families of trehalose-6-phospate (Tre-6P) and sucrose-non-fermenting1-related kinase1 (SnRK1). Log2 FC indicates fold-change between watered and drought-treated trees across organs. Fold-change is denoted by text and color. Missing values indicate nonsignificant differences. Fragments per kilobase per million mapped fragments (FPKM) indicate normalized log2 transcript abundance for each watered or drought-treated individual tree (columns). Class I and II TPSs were marked. A, alpha; B, beta; BG, beta-gamma; G, gamma. The complete gene names are listed in Table S1.

dramatically induced the expression of *SUCROSE SYNTHASE6* (*SUS6*) in the leaves and moderately in the stems, in agreement with the pattern of SS accumulation under drought conditions (Table 1). Interestingly, *SUS1* was upregulated in the leaves but downregulated in the roots. Differentially expressed genes of the INV family were mainly upregulated in leaves and stems and did not change in the

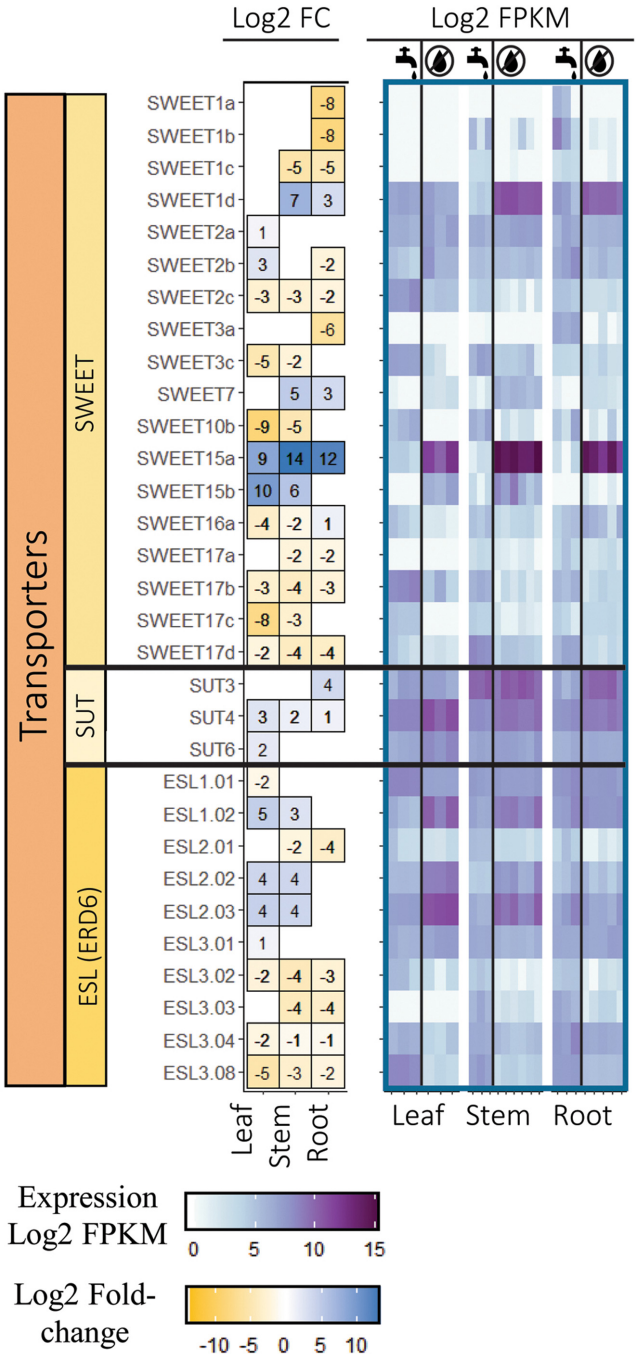

FIGURE 5 Expression patterns of sugar transporter gene families. Log2 FC indicates fold-change between watered and drought-treated trees across organs. Fold-change is denoted by text and color. Missing values indicate nonsignificant change. Fragments per kilobase per million mapped fragments (FPKM) indicate normalized log2 transcript abundance for each watered or drought-treated individual tree (columns). Complete gene names are indicated in Table S1.

roots. However, two of the three genes that were downregulated, the cell-wall acid *INV5* and cytosolic-neutral *INV8*, were specific to the roots (Figure 3).

The differentially expressed transcripts of the sucrose biosynthesis pathway consisted of five *SPS* and four *SPP* (Figure 3). *SPS1* genes were upregulated during drought: *SPS1a* was upregulated across organs, *SPS1b* was upregulated in roots, and *SPS1c* was upregulated in leaves. *SPS3* and *SPS4* were downregulated in the leaves. *SPP1a* and *SPP1b* were upregulated in the stems and roots, whereas *SPP2* and *SPP3* were downregulated in leaves and stems following drought stress (Figure 3).

3.6 | Drought suppresses class I TPS and induces class II TPS and SnRK1 genes

Differentially expressed transcripts involved in Tre-6P signaling included 11 *TPSs* and seven *TPPs* (Figure 4). Two class I *TPS* genes, *TPS1* and *TPS13*, were differentially expressed and were downregulated across organs. Similarly, *TPP1* and the Class II genes *TPS3* and *TPS5* were downregulated across organs. In contrast, Class II *TPSs* (TPS9, 11, and 12) and *TPP9* were upregulated in all organs, of which *TPS11* was most remarkably upregulated (Figure 4). Leaf-specific downregulation was observed for *TPS8*, *TPP5*, and *TPP6*.

SnRK1 subunits $\alpha 1$, $\alpha 2$, $\beta 1$, $\beta\gamma 1$, and $\beta\gamma 2$ (Figure 4) showed high constitutive expression across organs and only slightly elevated expressions in leaves under drought conditions (fold-change <1 for $\alpha 1$ and $\alpha 2$; Table S1). Relatively low expression was found for $\beta 2$, which was significantly upregulated in the stem, whereas $\alpha 3$, expressed predominantly in the roots, was the only *SnRK1* subunit downregulated following drought. *SnRK1* $\gamma 2$ was upregulated only in sink tissues, while *SnRK1* $\gamma 1$ and $\gamma 3$ subunits were highly upregulated across organs (Figure 4).

3.7 | Differentially expressed genes related to SS transport

Most of the Clade I *SWEET* genes were downregulated in the stem or roots (or both), except for *SWEET1d*, *SWEET2a*, and *SWEET2b*, which demonstrated tissue-specific upregulation (Figure 5). The only clade II *SWEET* gene that responded to drought was *SWEET7*, which was upregulated in stems and roots. Only three clade III genes responded to the drought stress. *SWEET10b* was downregulated in leaves and stems, whereas *SWEET15a* was strongly upregulated across organs, and *SWEET15b* was upregulated in leaves and stems. Clade IV genes *SWEET16* and *SWEET17* were mostly downregulated across organs (Figure 5).

All three differentially expressed *SUT* genes were upregulated under drought conditions. *SUT3* was predominantly expressed in the stems of the control plants but was upregulated only in the roots. The tonoplast isoform *SUT4* was upregulated across organs, whereas the plasma membrane expressed *SUT6* was upregulated only in leaves (Figure 5). Ten *ESL* transporters were differentially expressed under drought conditions. Of these, *ESL1.02*, *ESL2.02*, and *ESL2.03* were upregulated in leaves and the stem. Aside from *ESL3.01*, the rest of the *ESL* genes, *ESL3.02*, *ESL3.04*, and *ESL3.08*, were downregulated in response to drought across organs (Figure 5).

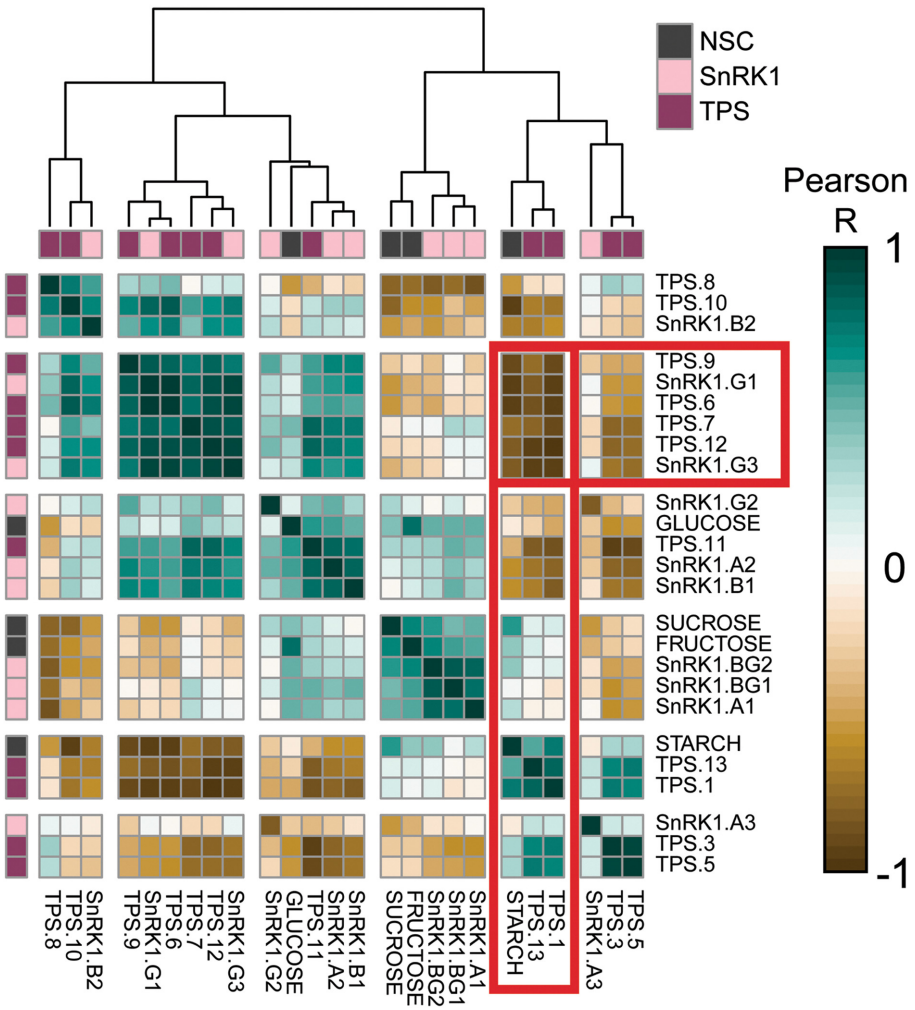

FIGURE 6 Clustered correlation heat map of starch and soluble sugars (% dry weight) and normalized gene expression (log2 count reads) involved in sugar sensing. R is denoted by color intensity, green—positive correlation and purple-negative correlation.

3.8 | Correlations

Pearson's correlation analysis revealed that fructose concentrations were strongly correlated with those of glucose (Figure 6). Class II *TPS* genes (except for *TPS3* and *5*) were positively correlated with *SnRK1 γ* subunits, and both negatively correlated with starch concentrations and class I *TPSs* (Figure 6, Figure S4A, and Table S6).

4 | DISCUSSION

The present study offers a comprehensive analysis of genes involved in sensing and signaling, transportation, and metabolism of NSCs in response to drought stress in poplar. We have identified specific genes that likely play a crucial role in utilizing NSCs for osmoregulation in leaves and for growth in roots in response to drought.

The control poplar saplings employed in our study had NSC concentrations comparable to those found in poplar hybrids (Regier et al., 2010) and poplar stems (Pagliarani et al., 2019). As expected, lower NSC concentrations were observed in roots than in leaves (He et al., 2020; Regier et al., 2010). The low starch concentrations in the roots of our plants, regardless of treatment, are typical of young plants, which invest their root energy in growth rather than storage. Consistent with previous studies on various tree species (He et al., 2020), we found that leaves responded to drought by reducing starch and increasing SS concentrations (Table 1). The accumulated SS is likely to be used for osmoregulation to maintain high turgor pressure (Bartlett et al., 2012). The current study focused on sucrose, glucose, and fructose as SS; however, it is important to note that the raffinose family oligosaccharides might also be involved in the response to drought, as suggested by Frost et al. (2012). The variations in NSC concentrations among source (leaf) and sink (stem and root) organs found in our study suggest that different sets of genes are activated in each organ to regulate NSCs in response to drought. The discussion below highlights the common and organ-specific NSC-related genes that are activated or deactivated in response to drought compared with well-watered conditions.

Starch remobilization during drought or osmotic stress is thought to be mediated mainly by BAM1 and AMY3 (Thalmann & Santelia, 2017). In agreement with a previous study on poplar (Pagliarani et al., 2019), our results suggest that *BAM1a* and *BAM1c*, but not *BAM1b*, facilitate drought-induced starch degradation across organs (Figure 2A). Instead of *AMY3*, the genes *AMYb* and *AMYc* appear to dominate starch degradation across organs. Although most

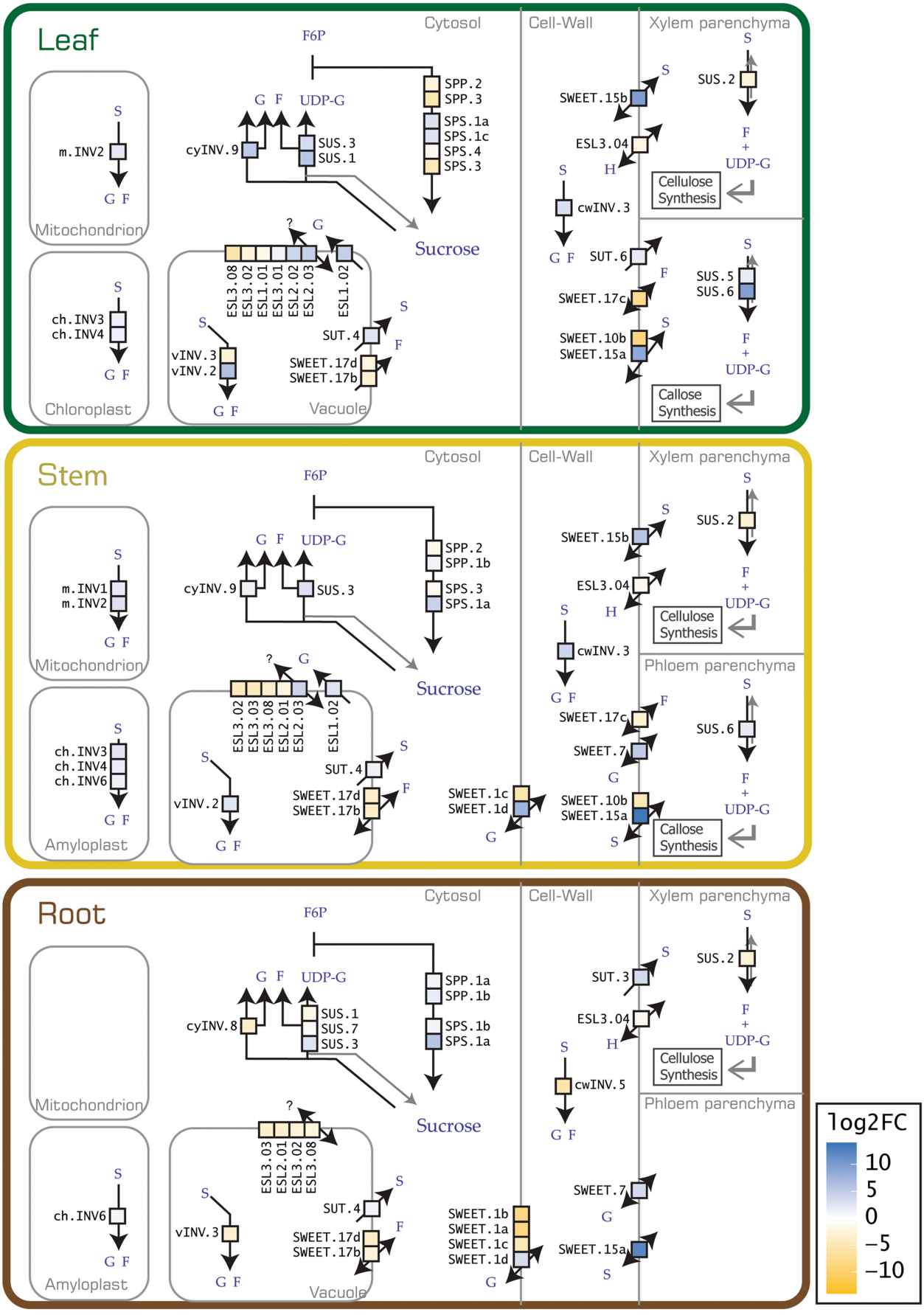

FIGURE 7 Legend on next page.

of the starch synthesis genes were downregulated, it is likely that starch synthesis was still functioning to some extent across the plant, as suggested by the upregulation of a few starch synthesis genes (Figure 2B). Interestingly, the opposite response of the starch degradation genes *DSP4a* and *BAMa* and the starch synthesis gene *SSS1* in source versus sink tissues implies opposite roles for starch in these organs (MacNeill et al., 2017). These indications for starch synthesis suggest a possible acclimation process in response to drought conditions. However, further research is required to test this hypothesis. Starch degradation leads to the accumulation of hexoses, which play a crucial role in various metabolic processes including sucrose synthesis. The accumulation of SS was accompanied by either upregulated or downregulated genes related to sucrose synthesis in the leaves and stems, while most genes related to sucrose degradation were upregulated in these tissues (Figures 3 and 7, supported by references listed in Table S7). Although the degradation and resynthesis of sucrose might seem energetically wasteful, they allow for the precise control over carbohydrate partitioning (Ruan, 2014). Hexose accumulation in the leaves was supported by intense upregulation of *SUS6*. The upregulation of *SUS6*, along with the aid of *SUS5*, may imply phloem-specific expression and callose synthesis in sieve elements, similar to that observed in Arabidopsis (Barratt et al., 2009). Callose deposition in the phloem parenchyma and plasmodesmata has been shown to limit solute movement in sieve elements (Maeda et al., 2006). The hindered water movement due to drought, combined with callose deposition, may inhibit solute allocation across plants. However, the expression pattern and specific role of *SUS6* in poplar trees remain unknown. The high expression of *SUS2* in the stem and root in the control plants correlated with its role in active growth (Wei et al., 2015), as was also demonstrated via RNAi in *Populus* (Dominguez et al., 2021; Gerber et al., 2014). As expected, it was downregulated in all organs following drought stress. As opposed to *SUS2*, *SUS3* was upregulated across organs, which is in agreement with Tang et al. (2013), who studied the drought response in *Populus euphratica*. Interestingly, *SUS3* expression has been found specific to stomata in Arabidopsis (Bieniawska et al., 2007), whereas its localization in poplar is yet unknown. Similar to our results, *vINV2* was upregulated in *Populus* in response to salt and cold conditions (Chen, Gao, et al., 2015), suggesting a significant role in sucrose cleavage in the vacuole upon abiotic stresses, as part of the osmoregulation trajectory (Hussain et al., 2020). The organ-specific upregulation of the *chINV*s and *mINV*s further supports osmoregulation in leaves and stems, but not in the root, and suggests that hexose accumulation in the plastids and mitochondria protects these sensitive organelles from oxidative damage (Dahro et al., 2016). As cwINV are generally associated with phloem unloading in reproductive sinks (Li et al., 2021), the root-specific downregulation of *cwINV5* further supports the lack of sucrose translocation to the root. In addition, as vINV increases sugar accumulation (Hussain et al., 2020), its downregulation in the root supports the lack of osmoregulation in the roots.

The expression pattern of genes in the roots revealed an intriguing picture where all the differentially expressed genes related to sucrose degradation were primarily downregulated, whereas genes related to sucrose synthesis were upregulated (Figure 3). Nevertheless, there was only an insignificant increase in SS concentrations (Table 1). Therefore, we conclude that some sucrose synthesis took place in the roots by *SPS1a* and *SPS1b* for local energy use. Downregulation of genes related to sucrose degradation suggests that there is limited or no sucrose allocation to the roots (Figure 7). As mentioned earlier, this may have resulted from hindered solute translocation due to callose deposition in the leaves.

Metabolic and allocation changes in NSCs are governed by the Tre-6P/SnRK1 signaling pathway, a key regulator of carbon sensing in response to drought (Fichtner & Lunn, 2021; Lunn, 2007b; Paul, 2008). While Tre-6P signaling has been extensively studied in poplar trees (Gao et al., 2021; Lunn, 2007b; Yang et al., 2012), our study is the first to characterize *SnRK1* complex-related genes in poplar. One proposed function of class II TPS is to regulate class I TPSs (Fichtner & Lunn, 2021). Consistent with this proposed function, our study showed downregulation of the two differentially expressed Class I *TPS*, while most of the Class II *TPS*s were upregulated, suggesting their potential antagonistic role (Figure 4 and Figure 6). This expression trend is in line with a previous study on poplar trees, which showed high induction of class II *TPS11* (*PtTPS13*) and *TPS12* (*PtTPS6*) under drought stress (Gao et al., 2021; Tang et al., 2013). The function of *TPS11*, like all other class II *TPS*s, remains unknown, although its involvement in regulating NSCs and abiotic stress tolerance has been demonstrated in wheat and Arabidopsis (Liu et al., 2019; Wang et al., 2016).

As previously shown, a decrease in Tre6-P removes inhibition from the catalytic site (α subunit) of the SnRK1 complex (Baena-González & Lunn, 2020). Belda-Palazón et al. (2020) demonstrated that the heterotrimeric SnRK1 complex is activated under drought conditions by an inhibitory removal as part of the abscisic acid (ABA) signaling pathway. This rapid regulation requires a constant supply of SnRK1 subunits, which explains the high expression levels observed for most canonical *PaSnRK1* subunits across organs, irrespective of treatment (Figure 4). Notably, the γ subunits, which were not previously identified as physical components of the SnRK1

FIGURE 7 Proposed scheme for selected transporter and sucrose metabolism related genes across tree organs under drought conditions. Each rectangle represents the change in the expression of a single gene transcript. Genes were grouped according to their family and subcellular localization. SUCROSE SYNTHASE (SUS) bidirectional activity is denoted with emphasis toward more common activity of Suc cleavage. SWEET and ESL are broadly bidirectional, except for potentially unidirectional tonoplast transporters: ESL1.02 and leaf-SWEET17. Question marks indicate uncertain subcellular localization, specificity, or directionality. Inhibitors and unmentioned subcellular localizations were excluded. Color intensity of rectangle denotes the log2 fold-change value between watered and drought-treated trees. Blue: upregulation; yellow: downregulation. Note the uniform color index for all organs. 2DG, 2-deoxyglucose; F6P, fructose-6-posphete; F, fructose; G, glucose; H, hexose; S, sucrose; UDP-G, UDP-glucose. Tables S4 and S7 provide the complete gene names and reference tables.

complex (Ramon et al., 2013), were upregulated across organs in our study, indicating their possible role in response to drought, potentially via Tre6-P signaling. Alternatively, they may be involved in binding SnRK1 to the SnRK2 complex, which has been shown to inhibit SnRK1 activity (Punkkinen et al., 2019), or in binding to the glucose sensor HEXOKINASE1 (HXK1; Van Dingenen et al., 2019). We inferred that the expression of class II *TPS*s inhibited the expression of class I *TPS*s, potentially aided by the *SnRK1* γ subunits. The upregulation (although not dramatic) of *SnRK1* $\alpha 1$, $\alpha 2$, and $\beta 1$ subunits, as well as the two $\beta\gamma$ subunits, specifically in the leaves (Figure 4), implies positive regulation over SS accumulation, further supporting osmoregulation in the leaves. While the downregulation of $\alpha 3$ specifically in the root suggests activation of growth via Tre-6P which limits SnRK1 activity (Figueroa & Lunn, 2016). SnRK1 is known to be involved in the activation of the low-energy stress response, stimulating catabolic processes. It acts by the direct phosphorylation of enzymes and extensive transcriptional regulation (>1000 genes), including some central genes involved in carbon metabolism. Among the SnRK1 targets, some are covered here: SPS, SUS, class II TPS, INV, starch synthase, SEX4, AGPase, and α-amylase (Figures 2–5; Peixoto & Baena-González, 2022). However, the functional relevance of this phosphorylation remains unknown. Others, such as bZIP transcription factors of class C/S1 and their targets (Dröge-Laser & Weiste, 2018), fructose-2,6-bisphosphatase, and nitrate reductase, are beyond the scope of this research. However, they were included in our database (Bioproject PRJNA805084) and supplementary table (Table S1) for further investigation.

Following stress-induced changes, sugar transporters redistribute SS across the organs and cell organelles. Poplar trees passively load their phloem, utilizing extensive plasmodesmata connections and sugar concentration gradients (Zhang et al., 2014), precluding phloem-specific plasma membrane SUT transporters (Frost et al., 2012). Nevertheless, the expression of the *PtSUT3* gene, primarily found in the stem, has been shown to support active radial sucrose transport for the growth of secondary cell wall-forming wood fibers (Mahboubi et al., 2013). In our study, we observed dominant *SUT3* expression in the stems of control plants, but upregulation in roots of drought-treated plants, suggesting a potential role in supporting root growth under drought stress (Figure 5). *SUT4*, which was upregulated across organs, is a tonoplast transporter that facilitates the export of sucrose from vacuoles to the cytosol (Payyavula et al., 2011). It has been implicated in regulating sucrose allocation across the plant, as well as coupling carbon use with changes in plant water status via osmotic adjustments and ABA signaling (Frost et al., 2012; Payyavula et al., 2011; Xue et al., 2016). Sucrose-responsive leaf plasma membrane SUTs have also been found to play a role in controlling sucrose exportation rates in active loading species (Yoon et al., 2021). Therefore, *SUT*s that are differentially expressed under drought conditions may play a role in sugar sensing to control sucrose redistribution. The upregulation of *PaSUT4* and *PaSUT6* in leaves may indicate a similar role for these genes.

In contrast to active SUT transporters, SWEETs are passive. In our study, expression of *SWEET* genes in the control plants is consistent with previous research by Zhang et al. (2021). Our results suggest that under drought, the downregulation of most clade I *SWEET*s in sink tissues limits the allocation of hexoses, and that this process is primarily facilitated by *SWEET1d* and *SWEET7*, the only clade II gene that responds to drought (Figure 5). In leaves, the only upregulated clade I gene that responds to drought is *SWEET2d*. Additionally, the downregulation of clade IV *SWEET*s (*SWEET16* and *SWEET17*), which transport fructose across the tonoplast, suggests that the intracellular and intercellular translocations of hexoses are limited and tightly regulated by a few specific *SWEET* genes.

SWEET15a demonstrated minor expression across tissues in control plants, however it was dramatically upregulated due to drought in all tissues. In fact, it demonstrated the 6th highest expression change among all differentially expressed genes in the current study (Table S1). *SWEET15a* is a sucrose transporter primarily expressed in male poplar flowers (Zhang et al., 2021). Previous studies have shown that *SWEET15* is involved in sugar transport from the phloem to the reproductive sinks in Arabidopsis (Chen, Cheung, et al., 2015), in grapes (Ren et al., 2020), and soybean (Wang et al., 2019). Moreover, *SWEET15* has been reported to be highly expressed in response to drought stress in Arabidopsis (Durand et al., 2016), olive branches (Tsamir-Rimon et al., 2021) and rice plants (Mathan et al., 2021). Taken together, *SWEET15* genes appear to be central sucrose transporters that are activated in response to drought, with *SWEET15a* being the dominant gene in poplars.

Overexpressing *SWEET7* in poplar increased SS content and wood formation (Zhang et al., 2021). Therefore, we propose that increased expressions of phloem transporters under drought facilitate sugar transport from companion cells to sink cell walls, likely restricting sugar mobility. The upregulation of xylem-associated *SWEET15b* in aboveground organs is unclear, yet it could facilitate the redistribution of sucrose under low transpiration demand by loading sugars into the xylem sap, as observed in almond trees diurnally (Tixier et al., 2018). In poplar, only *PagSWEET17b* and *PagSWEET17d* are localized to the tonoplast (Zhang et al., 2021). Our finding indicate that they are widely downregulated alongside most *SWEET* isoforms (Table S6).

ESL genes belong to a large family of antiporters that are involved in glucose efflux across the tonoplast (Wormit et al., 2006). With only a few functionally characterized members, it is considered the least investigated subgroup of sugar transporters in Arabidopsis (Desrut et al., 2020; Niño-González et al., 2019). *AtESL1.02*, a tonoplast proton-driven stress-induced transporter (Klemens et al., 2014; Slawinski et al., 2021; Yamada et al., 2010), was found to export glucose from the vacuole into the cytosol (Poschet et al., 2011). In our study, *ESL1.02*, which was upregulated in aboveground organs (Figure 5), showed positive correlation with *INV2* levels (Figure S4D). These results suggest an increased glucose concentration level in the vacuole, and its release to the cytosol, perhaps to fit metabolic needs and assist with osmoregulation. The scheme in Figure 7 summarizes the sucrose degradation and synthesis genes, as well as the transporters that were studied, presenting an estimated picture of the gene map that drives the differences in SS content in the three organs.

In conclusion, our main findings suggest that poplar saplings activate *BAM1a*, *BAM1c*, *AMYb*, and *AMYc* for starch degradation across

organs. They downregulate *SUS2* across organs to reduce growth and upregulate *SUS6* in leaves to support osmoregulation and restrict sugar mobility. We found that class II *TPS* and *SnRK1* subunits were expressed, whereas class I *TPS* genes were suppressed across organs, promoting drought response via the Tre-6P/SnRK1 signaling pathway. The downregulation of sucrose degradation genes and the upregulation of sucrose synthesis genes in the roots suggest reduced sucrose allocation to the roots and local sucrose production for maintenance and growth, implying use of energy sources. The upregulation of *INV* genes in leaves and stems, further implies osmoregulation in leaves and stems, but not in roots, whereas the downregulation of cw*INV5* in the roots supports limited sucrose translocation. The diminishing expression of passive, gradient-dependent transporters, such as *SWEET* and *ESL*, and the upregulation of active transporters, such as *SUT*, likely allow better control over sugar partitioning when carbon is limited. Overall, our study provides significant insights into the molecular components involved in the response of NSCs to drought stress in poplars, highlighting the potential for targeted genetic modifications to improve drought tolerance in tree species.

AUTHOR CONTRIBUTIONS

Hagar Fox performed the study guided by Tamir Klein and Rakefet David-Schwartz. Adi Doron-Faigenboim performed the transcriptome assembly, functional annotation and differential expression analysis. Shifra Ben-Dor conducted phylogenetic analysis and gene naming and annotation to selected families. Moshe Goldsmith assisted with sugar quantification via HPLC. Hagar Fox wrote the manuscript together with Tamir Klein and Rakefet David-Schwartz.

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DATA AVAILABILITY STATEMENT

The data related to this research are provided in the Supporting Information. Bioproject Id is PRJNA805084 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA805084).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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