

CRISPR knockout of winter-biased *SUT4* alters phenology, biomass accrual, and fertility of field-grown hybrid poplar

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Abstract

Woody perennial trees annually transition between photoautotrophic and heterotrophic states. As carbohydrate accumulation and utilization patterns shift, so does the expression of genes encoding various sucrose-proton symporters (SUTs). The poplar genome contains five *SUT* gene family members involved in intracellular and long-distance sucrose transport. Among these, only the single-copy *SUT4* and the *SUT5/SUT6* genome duplicates are expressed year-round, with *SUT4* strongly expressed in winter. Using CRISPR mutagenesis, we investigated the role of winter-expressed *SUTs* in carbohydrate allocation, biomass accrual, seasonal phenology, and sexual reproduction in *Populus tremula* × *P. alba* under field conditions. In *sut4* mutants, fall leaf senescence occurred earlier and spring bud flush was delayed compared to controls, resulting in reduced diameter and overall height growth. These mutants also exhibited altered sugar partitioning and reduced levels of raffinose family oligosaccharides, which are typically associated with cold and desiccation tolerance. Knockouts of *SUT5* and *SUT6*, whether single or double, did not affect seasonal phenology or overall tree growth. All genotypes flowered precociously after two years in the field, but *sut4* catkins accumulated abnormally high levels of sucrose, galactinol, and raffinose. Transcriptome profiling showed down-regulation of genes associated with proanthocyanidin biosynthesis and vacuolar transport, and up-regulation of genes involved in protein oligomerization, misfolding, and refolding responses. Development of *sut4* ovules was compromised, and no seeds were produced in *sut4* catkins following controlled pollination. Our findings suggest that the single-copy tonoplast SUT4 may be more critical than other SUTs for mitigating stress associated with winter fitness and growth transitions during cool seasons.

Introduction

Woody trees annually cycle through periods of active growth, leaf senescence and abscission, dormant bud quiescence, and bud break in accordance with temperature and day length cues (Rohde and Bhalerao, 2007; Cooke et al., 2012; Savage and Chuine, 2021). The timing of such environmental cues influences plant phenology, which ultimately determines perennial biomass growth in temperate and boreal regions with freezing winter temperatures (Keunen et al., 2013; Kuster et al., 2014; Zohner et al., 2021). In sink organs that remain active after fall senescence, reserves of nonstructural carbohydrates (NSC) are utilized to sustain winter survival (Kozłowski, 1992). During the winter, NSC reserves are converted to sugar and oligosaccharide (e.g., galactinol, raffinose, stachyose) pools for basal metabolism, respiration, membrane stabilization, reactive oxygen species (ROS) scavenging, and protection against frost and freezing damage (Ameglio et al., 2004; Regier et al., 2010). With the arrival of spring, NSC reserves are remobilized to fuel the emergence of new organs, including flowers, leaves, and vasculature, before resumption of photosynthesis (Loescher et al., 1990; Tixier et al., 2019). Not surprisingly, seasonal variation in NSC dynamics is observed across various phylogenetic groups (e.g., gymnosperms, angiosperms), life habits (e.g., evergreen, deciduous), and climate biomes (e.g., boreal, temperate, Mediterranean, tropical) (Martínez-Vilalta et al., 2016; Palacio et al., 2018; Furze et al., 2019).

Although studied to a lesser extent, seasonal changes in the expression of genes involved in NSC synthesis, utilization, and transport have been reported (Ko et al., 2011; Dobbelsstein et al., 2018; Jokipii-Lukkari et al., 2018). Genes associated with starch hydrolysis and the biosynthesis of

raffinose family oligosaccharides (RFOs) are most strongly expressed during winter dormancy when starch is depleted and RFOs accumulate in the stems of poplar (*Populus trichocarpa*) and Norway spruce (*Picea abies*) (Kozłowski, 1992; Schrader et al., 2004; Regier et al., 2010; Ko et al., 2011; Jokipii-Lukkari et al., 2018). Various carbohydrate transporters also exhibit seasonal expression patterns. In poplar, the vascular-specific Type I *SUT* (the *SUT1* and *SUT3* genome duplicates) transcripts are only detected in the summer, whereas the broadly expressed Type II (the *SUT5* and *SUT6* genome duplicates) and Type III (single-copy *SUT4*) transcripts are detected year-round, with *SUT4* being strongly winter-biased (Ko et al., 2011; Sreedasyam et al., 2023; Tuma et al., 2024). The divergent seasonal expression patterns observed amongst members of the *SUT* gene family have also been reported in Norway spruce (*Picea abies*), beech (*Fagus sylvatica*), and oak (*Quercus robur*) (Dobbelstein et al., 2018), hinting at season-specific roles in the utilization of NSC throughout the year.

The summer-biased expression of poplar *SUT1* and *SUT3* is consistent with the involvement of these Type I plasma membrane symporters in lateral sucrose partitioning during stem secondary growth (Mahboubi et al., 2013). The physiological function of the Type II plasma membrane SUTs remains unspecified in any species (Peng et al., 2014). The tissue distribution of poplar *SUT5* and *SUT6* transcripts is distinct from that of summer-biased plasma membrane isoforms, but more similar to that of the winter-biased tonoplast isoform (Payyavula et al., 2011). The poplar tonoplast *SUT4* is the predominant SUT isoform in source leaves, modulating sucrose efflux from the vacuole and impacting the transport of sucrose from source to sink organs (Payyavula et al., 2011). The effects of *SUT4*-knockdown (KD) or knockout (KO) on plantwide sucrose homeostasis, starch utilization, turgor control, osmotic adjustment, and sensitivity to

drought and defoliation treatments have been reported (Payyavula et al., 2011; Xue et al., 2016; Harding et al., 2020; Harding et al., 2022). Recently, we extended our investigation to uncover an effect of *SUT4* on the dynamics of NSC reserves in sink organs during heterotrophic growth. Following stem coppicing, *SUT4*-KO poplars showed delayed epicormic bud emergence and reduced biomass in winter but not summer glasshouse conditions (Tuma et al., 2024). The results contrasted those of previous reports showing subtle growth differences between *SUT4*-KD and wild type (WT) plants when grown under relatively stable and controlled glasshouse conditions (Payyavula et al., 2011; Frost et al., 2012), suggesting that *SUT4* plays a critical role in supporting growth under cooler temperatures.

In the present study, we sought to investigate the effects of *SUT4*-KO on poplar seasonal growth through a multi-year field trial. For comparison, we also generated single- and double-KO lines of the *SUT5/SUT6* paralog (hereafter *SUT56*-KO or *sut56* for the double mutants). *SUT4*-KO but not *SUT56*-KO showed accelerated leaf senescence in the fall and delayed bud flush in the spring, culminating in reduced biomass growth. Moreover, *SUT4*-KO altered female catkin transcriptomes in the spring which led to sterility following controlled pollination. Our data show the greater importance than previously recognized of *SUT4* function in tree response to environmental cues of daylength and temperature.

Materials and Methods

Production of *sut* KO lines

The generation of the *sut4* mutants and Cas9-only vector controls in the *Populus tremula* × *alba* INRA 717-1B4 background were reported previously (Harding et al., 2022; Zhou et al., 2015). To generate the *sut5* and *sut6* single and *sut56* double mutants, gRNAs targeting individual or paralogous genes were assembled as a pair of oligos (Supplemental Table S1) into p201N-Cas9 vector behind the *Medicago truncatula* U6.6 promoter, following the approach of Bewg et al. (2022). *Agrobacterium*-mediated transformation and plant regeneration was performed as described (Bewg et al., 2022).

Amplicon sequencing to determine mutation patterns

Leaves from independent events of tissue cultured plants were collected for genomic DNA extraction and amplicon sequencing library preparation (Bewg et al. 2022) using consensus primers (Supplemental Table S1). Samples were then barcoded with Illumina amplicon indexing primers, pooled, and sequenced on an Illumina MiSeq at the Georgia Genomics and Bioinformatics Core at the University of Georgia to obtain PE150 reads. Sequencing reads were processed and indel patterns identified using the Analysis of Genome Editing by Sequencing (AGEseq) program (Xue & Tsai, 2015) with a mismatch allowance set at 1%, followed by manual curation. Tetra allelic mutations were confirmed in all lines (Supplemental Table S2).

Plant propagation and field establishment

Multiple independent lines each for *sut4* (8), *sut5* (3), *sut6* (2), and *sut56* (3) mutants along with wild-type (WT) and Cas9 controls were propagated from single-internode stem cuttings (Frost et al., 2012). Cuttings were transferred to 1-gallon pots containing commercial soil mixture (Fafard 3B) supplemented with Osmocote (15-9-12 NPK, 3-month slow release) and placed in a walk-in growth chamber for ~3 weeks (Tuma et al., 2024). Plants were moved into an adjacent greenhouse for 2 weeks before transfer to a lath house within a fenced nursery at the University of Georgia's Whitehall Forest for an additional 2 weeks. For logistical reasons having to do with the timing of cutting establishment, *sut4* plants were slightly shorter than control plants at the time of out-planting. Plants were transferred to 20-gallon pots containing a mix of Jolly Gardener premium soil (75% aged pine bark, 25% peat moss) and Fafard 3B with additional Osmocote (15-9-12 NPK, 3-month slow release) and planted at the nursery in summer 2021. Genotypes were completely randomized throughout the site and spaced 1.5 meters apart from one another (Supplemental Figure 1). The first planting occurred in early June 2021 for *sut4* mutants and controls (WT and *Cas9*), and the second planting occurred in mid-August 2021 for *sut4*, *sut5*, *sut6*, *sut56* and controls. Plants were watered using an overhead irrigation system. The site was watered between 2-4 hours daily from early April to late October. The field trial was conducted with approval from the Animal and Plant Health Inspection Service (APHIS) of the U.S. Department of Agriculture.

Field growth and phenology monitoring

The diameter of the main stem ~1m above the soil surface was measured monthly or bimonthly from August 2021 - April 2023. Plant height was measured from the base of the stem at soil level to shoot tip in April 2022 and 2023. Trees were monitored throughout the fall to compare onset

and rate of leaf abscission between genotypes. Leaf color was compared between genotypes as an indicator of leaf senescence on October 11th, 2022. In the spring, the appearance of bud flush on the dominant stem and flowering were recorded. No floral tissues were detected in the first growth season, but catkins were observed during spring of the second (late February 2023) and third (early March 2024) growing seasons. The total number of trees that developed catkins was recorded prior to their removal from the field site per the APHIS regulation. Further information regarding the growth cycles, phenotyping, and sampling are reported in the Supplemental Figures (Supplemental Figure 1).

Tissue sampling

Approximately 12 – 17 months after out planting, increment cores (10 mm in length and 2 mm in diameter) were collected from the main stem ~1.3 meter above soil level using a micro-coring device (Costruzioni Meccaniche Carabin C, San Vito di Cadore, Italy) according to Rossi and colleagues (2006). Stems ($n = 10-12$) were approximately 2–6 cm in diameter at the time of sampling. Increment cores were collected from the same stems during active growth in August 2022 (summer) and during dormancy in January 2023 (winter). Xylem and bark were separated from the cores in the field, snap-frozen in liquid nitrogen before being transported back to laboratory facilities where they were stored at -80°C until sample processing occurred. Temperatures ranged from a night-time low of 22°C to a daytime high of 31°C (daily average 25°C) on the day of summer sampling, and from a low of 3°C to a high of 18°C (daily average 11°C) on the day of winter sampling (Supplemental Figure 2). Catkins were observed in February 2023 after the second winter in the field. Three developmental stages of catkins were collected on March 1, 2023 for metabolite and transcript profiling. All tissue sampling occurred

under sunny conditions between 11AM – 2PM. Tissues were snap frozen, ground under liquid nitrogen with a mortar and pestle, and stored at -80°C.

Metabolite profiling

Tissues were lyophilized for 48 hours (FreezeZone 2.5, Labconco), ground through a 40-mesh sieve using a Wiley Mill (Thomas Scientific), and further ball-milled to a fine powder in a S1600 Mini G Bead-beater (Spex SamplePrep; Metuchen, New Jersey) for metabolite profiling by Gas Chromatography-Mass Spectrometry (GC-MS) as detailed previously (Harding et al., 2022; Tuma et al., 2024). Peak identities were assigned based on the NIST08, FiehnLib metabolomics library (Agilent) (Kind et al., 2009) and in-house spectral libraries from authentic standards (Xue et al., 2013; Chen et al., 2014; Harding et al., 2022). A standard mix of succinic acid, sucrose, glutamic acid, fructose, glucose, and ascorbic acid was loaded at the beginning and end of each sample sequence to monitor derivatization and instrument performance. Peak intensities were normalized by the internal standard and reported on a sample dry weight basis (normalized peak area). Sucrose and hexose abundance (sum of fructose and glucose) was reported as µg/mg dry weight.

Starch

Starch content was determined by hydrolyzing starch to glucose using enzymatic digestion with α-amylase and amyloglucosidase (Chow and Landhausser, 2004) using 10 mg of lyophilized, wiley- and ball-milled tissue powder as previously described (Tuma et al., 2024). Starch abundance was reported as µg/mg dry weight.

Wood chemical composition and physical property analyses

Fifteen-cm long pieces of the main stem ($n = 6$) were destructively harvested following bud flush in March 2023. For wood chemistry analysis, stem segments were debarked, dried at 55°C for 24 hours, and ground to pass through a 40-mesh screen using a Wiley mill. Samples were then run through 99 cycles of hot ethanol extraction using a Soxhlet extraction unit (Buchi) and air dried. Klason lignin content was determined as described (Swamy et al., 2015). The acid hydrolysates were used for structural carbohydrate analysis by high-performance anion exchange according to (Huntley et al., 2003). The ratio of lignin monomers in the stems (syringyl/guaiacyl, S/G ratio) was determined by pyrolysis-molecular beam mass spectrometry according to Harman-Ware et al. (2022). Three ~7.5 mm longitudinal wood sections were obtained from the main stem approximately ~1 m above soil level for analysis of specific gravity, acoustic velocity, and modulus of elasticity as described (Vieilledent et al., 2018; Tsai et al., 2020; Dahlen et al., 2023).

RNA-seq analysis

Snap-frozen catkin tissues ($n = 3-5$) were ground to a fine powder under liquid nitrogen and an aliquot was used for RNA extraction using the Direct-zol RNA Miniprep kit (Zymo) with PureLink Plant RNA Reagent (Life Technologies) as described (Harding et al., 2018). Illumina low input RNA (with PolyA selection) library preparation and NovaSeq sequencing (NovaSeq XP V1.5 reagent kits, 10B flowcell, 2 ×151 run length) were performed at the Joint Genome Institute (proposal: 10.46936/10.25585/6000884). RNA-seq reads were aligned to the *Populus tremula* haplotype of the 717 reference genome (Zhou et al., 2023) using STAR-2.7.10 (Dobin and Gingeras, 2015) with the following parameters: --alignMatesGapMax 20000, --alignIntronMax 10000, --outFilterScoreMinOverLread 0.1, and --outFilterMatchNminOverLread

0.1. Expression levels were estimated using featureCounts from the Subread package v2.0.6 with the options -Q 2, -s 0, -T 12, -p, and --countReadPairs -C (Liao et al., 2014) and reported as FPKM (fragments per kilobase of transcript per million mapped reads). Differential gene expression analysis were performed using DESeq2 v1.34.0 (Love et al., 2014). Thresholds for significant differential expression were based on minimum FPKM ≥ 5 in all samples of at least one group, $Q \leq 0.005$, and fold change ≥ 1.5 . Gene ontology (GO) enrichment analysis of significantly up- or down-regulated genes was performed using ShinyGO v0.77 (Ge et al., 2020) available at <http://aspen.db.uga.edu/ShinyGO/>. Expression responses of selected genes were visualized using the HeatMapperPlus tool (http://bar.utoronto.ca/ntools/cgi-bin/ntools_heatmapper_plus.cgi).

Controlled pollination

The branches containing flower buds were collected from WT and *sut4* trees and incubated in $\frac{1}{4}$ MS macro salt media at room temperature in a confined laboratory to force flower. After catkin flowers opened, wild *P. tremuloides* pollen sourced from Minnesota 2022 (with assistance of Kathy Haiby, Poplar Innovations Inc.) was brushed onto individual WT and *sut4* catkins which were then covered by glassine bags. After 7-10 days, the capsules opened, and the cottony fuzz was collected for observation under a dissecting microscope. The length of catkins, seed size and number, and ovule size were recorded. The controlled pollination experiments were carried out in both 2023 and 2024 with reproducible results.

Statistics

Repeated measures analysis of variance (ANOVA) was used to determine significant differences in growth over time, genotypic effects, and their interactions using *R* v4.1.0 (R Core Team, 2016) in Rstudio v.2022.12.353). All other reported genotypic contrasts were assessed with Student's *t*-test ($\alpha = 0.05$).

Results

Altered phenology and reduced biomass accrual of field-grown sut4 poplar

We established a field trial in summer 2021 to investigate the extent to which CRISPR-KO of winter-expressed *SUT* genes would alter phenology and/or seasonal growth. Over 200 trees were planted in 20-gal pots in early June (cohort 1: control and *sut4*) and mid-August (cohort 2: control, *sut4*, *sut5*, *sut6*, and *sut56*). Following the first partial (2021) growth season, *sut4* mutants showed signs of leaf yellowing in late October when leaves of the other genotypes remained green regardless of cohort (Figure 1A). By mid-November nearly 90% of *sut4* trees had lost all of their leaves while the majority of the controls and *sut5*, *sut6*, and *sut56* mutants still retained the majority of their foliage (Figure 1B-C). In early December, the majority of the trees had lost nearly all of their foliage (Figure 1C). Bud flush the following spring (March 2022) was delayed by an average of 7 days for the *sut4* trees compared to the controls and *sut5/sut6/sut56* mutants (Figure 1D). Similar trends were also observed in two subsequent years, suggesting a reduced leaf lifespan in *sut4* trees.

Diameter of the main stems was monitored monthly for cohort 1 and bimonthly for cohort 2. For cohort 1, diameter increases during the 2022 growth season were significantly smaller in *sut4* than control trees (Figure 1E). The differential was evident by March 2022 when 88% of control trees had increased diameters versus only 31% of *sut4* trees (Figure 1F). To ascertain whether the slower spring growth was associated with slightly smaller *sut4* trees at the start of the experiment, a subset of initial size-matched 13 control and 13 *sut4* trees was examined. The same pattern was observed, with the first measurable diameter increases occurring 2-3 months later in *sut4* than in control trees (Figure 1E cohort 1 inset). Interestingly for cohort 2 that was released two months behind cohort 1, *sut4* diameter growth was reduced by a much lesser extent, whereas *sut5*, *sut6*, and *sut56* were indistinguishable from controls (Figure 1E). Closer examination confirmed a slower spring growth of *sut4* trees in cohort 2 as well. Diameter growth of *sut4* trees between February 5, 2022 (before bud break) and June 1, 2022 was half of that in control trees (Figure 1E cohort 2 inset). However, diameter growth rates were similar among genotypes through the remainder of the growth season (June to October, 2022). The 2022-2023 winter was unseasonably warm (Supplemental Figure S2) and the diameter differentials between *sut4* and the other genotypes remained constant through April 2023 before the field trial was terminated (Figure 1E). A subset of cohort 2 trees was permitted to continue, and diameter growth was monitored monthly during spring 2024. We recorded first diameter increases by April for the vast majority of control and *sut56* trees (92% for both), but only 58% of *sut4* trees (Figure 1F).

Tree height was slightly reduced, by ~10%, in *sut4* compared to the controls and *sut56* trees in April of 2022 (Figure 1G-H). At the end of the experiment, *sut4* trees were ~ 20% shorter than control and *sut56* trees (Figure 1G). Together, these results highlight altered phenology, including

shorter leaf lifespans and shorter cambial growth periods, as an underlying cause of the growth reduction in *sut4* trees and this penalty was particularly severe for cohort 1.

Altered NSC homeostasis in sut4 stems

We compared levels of monosaccharides and oligosaccharides in the bark and xylem sampled during summer and winter seasons. Because growth was unaltered in single- and double-KO of *SUT5/SUT6*, only *sut56* trees were included in subsequent analyses. Sucrose was more abundant in bark and xylem of *sut4* mutants than control regardless of season (Figure 2A), consistent with earlier work on *SUT4-KD* and *sut4* poplars in the greenhouse (Payyavula et al., 2011; Harding et al., 2022; Tuma et al., 2024). Sucrose levels changed little in *sut56* xylem or bark relative to the control (Figure 2A).

Unlike sucrose, hexose (fructose and glucose) levels increased sharply between summer and winter in bark and xylem of all lines (Figure 2B). Hexoses decreased in *sut4* bark in the summer but increased in the winter in both bark and xylem. Hexose levels also increased in the bark of *sut56* in both summer and winter (Figure 2B). As a result, the hexose-to-sucrose ratio was much lower in bark of *sut4* than other lines during summer (Figure 2A-B). Bark and xylem starch abundance differed little between genotypes in summer or winter and exhibited large declines of similar magnitude in all genotypes during the transition to winter growth (Figure 2C).

The RFOs galactinol and raffinose and their precursor myo-inositol offer protection during cold or drought (Kozlowski, 1992; ElSayed et al., 2014). Myo-inositol levels increased significantly in both bark and xylem of *sut4* in summer, but not in winter when its overall abundance

decreased (Figure 3A). Stem galactinol and raffinose abundances were at marginally detectable levels in summer but increased sharply in all genotypes during the winter (Figure 3B-C). Both galactinol and raffinose levels were lower in winter *sut4* bark and xylem but changed little in *sut56* relative to the controls (Figure 3B-C). The attenuated winter increases of RFOs in *sut4* are consistent with a previous report of compromised induction of RFOs in drought-stressed *SUT4*-KD plants (Frost et al., 2012).

Wood physicochemical properties were altered in sut4 trees

Neither lignin content nor syringyl-to-guaiacyl (S/G) monolignol ratio was altered in *sut4* or *sut56* (Figure 4A-B). Acoustic velocity did not differ between genotypes (Figure 4C). However, the specific gravity and the modulus of elasticity of the stem wood was reduced in both *sut4* and *sut56* mutants relative to the controls (Figure 4D-E).

Altered female catkin transcriptomes and reproductive competence in sut4

Field-grown female 717 trees usually flower after 5-8 years (Mohamed et al., 2010). Surprisingly, catkins were observed on many of the trees in late February through early March 2023 (Figure 5A) and removed immediately to comply with APHIS regulations. In total, 76% of the controls and 59% of the *sut4* in cohort 1 and 30% of the controls, 45% of the *sut4*, and 22% of the *sut56* in cohort 2 flowered in early March 2023 (Figure 5B). In the extended trial with a subset of cohort 2 trees, 75% of the controls, 75% of the *sut4*, and 77% of the *sut56* trees flowered in late February 2024 (Figure 5B). Thus, no apparent differences in flowering frequency were detected between genotypes.

SUT4 is the predominant *SUT* gene expressed in poplar flowers (Payyavula et al., 2011). To assess the effects of *SUT4*-KO on catkin development, we performed GC-MS analysis on unopened floral buds, newly emerged catkins, and elongating catkins (Figure 5A inset). Sucrose levels remained constant during WT catkin development and changed little between genotypes in dormant floral buds (Figure 5C, stage I). However, sucrose increased significantly in newly emerged (stage II) and elongating catkins (stage III) of *sut4* relative to WT (Figure 5C). Hexose levels increased steadily during catkin development in both genotypes and were significantly higher in newly emerged catkins of *sut4* than WT (Figure 5C). Both galactinol and raffinose levels were highest in the dormant buds of WT and declined to similar levels in newly emerged and elongating catkins (Figure 5C). However, the RFOs remained high in *sut4* catkins. As a result, galactinol levels were significantly higher in newly emerged and elongating catkins of *sut4* than WT (Figure 5C). Raffinose exhibited a similar trend but was only significant in elongating catkins (Figure 5C).

Transcriptional adjustments to flavonoid biosynthesis in sut4 catkins

We then performed RNA-Seq of elongating catkins and identified 821 significantly up-regulated and 660 down-regulated genes in *sut4* relative to the control catkins. Gene Ontology (GO) categories of ‘protein oligomerization’, ‘cellular response to unfolded protein’, ‘misfolded protein binding’, ‘protein refolding’, and cellular responses to heat, hypoxia, and hydrogen peroxide were over-represented among up-regulated genes (Figure 5D). Specifically, over 22% of the ~380 717 genes annotated to encode various heat shock proteins (HSPs) (Yer et al., 2018) were significantly up-regulated in *sut4* catkins. Also up-regulated were genes encoding several heat shock factors (HSFs) and late embryogenesis abundant (LEA) proteins. Three *GolS*

(galactinol synthase) genes were detected in elongating catkins and all three were sharply induced in *sut4* catkins, including ortholog of the *Populus alba* × *grandidentata* *Pa* × *gGolSII* previously shown to exhibit cool season-biased expression (Unda et al., 2012). Expression of raffinose synthase genes was largely unchanged. The results are consistent with significantly elevated levels of galactinol, but not raffinose, in the elongating *sut4* catkins (Figure 5C).

The smaller group of down-regulated genes were enriched with GO terms associated with ‘proanthocyanidin biosynthetic process’, ‘flavonoid biosynthetic process’, ‘response to UV-B’, ‘regulation of systemic acquired resistance’, ‘seed coat development’, and ‘mitotic cell cycle phase transition’ (Figure 5D). Essentially all flavonoid pathway genes and several upstream regulators (Tsai et al., 2006) that were detected in catkins were significantly down-regulated in *sut4* flowers (Figure 5E). All four genes encoding orthologs of *Arabidopsis* TRANSPARENT TESTA12 (TT12), a multidrug and toxin extrusion (MATE)-type proton antiporter involved in the vacuolar deposition of proanthocyanidin precursors (Debeaujon et al., 2001; Marinova et al., 2007), were repressed in *sut4* catkins (Figure 5E). The *Arabidopsis* *TT12* is specifically expressed in ovules and developing seed, and *tt12*, like other *tt* mutants, show impaired proanthocyanidin accumulation in the seed coat (Appelhagen et al., 2014).

We next performed controlled pollination to test whether *sut4* catkins are fertile. Of the 13 WT and 14 *sut4* catkins pollinated, nine of each genotype elongated and produced capsules with an abundance of seed trichomes (Figure 5F). There was no significant difference in the catkin length between genotypes. While WT flowers produced seed as expected, no seed was obtained from any of the *sut4* capsules examined under a dissecting microscope (Figure 5G). Unfertilized

ovules can undergo limited development in aspens (Fechner, 1972) and they were indeed observed in both WT and *sut4* samples, but they were much smaller in *sut4* than in WT (Figure 5G). Together with the transcriptomic changes, our results reveal a critical role of SUT4 during ovule and seed development in poplar.

Discussion

Seasonal alternation between carbohydrate storage during photoautotrophic growth and remobilization during heterotrophic subsistence is a hallmark of the perennial lifestyle (Kozłowski, 1992; Regier et al., 2010; Tixier et al., 2019). However, disruptive effects of global climate change on the timing of seasonal phenology, such as fall leaf senescence and spring bud flush, are increasingly evident (Estiarte and Peñuelas, 2015; Calinger and Curtis, 2023). Here we report on evidence which points toward a conditioning effect of SUT4 on the ability of poplar to adapt to climate change. Our main findings were that under field conditions, CRISPR-KO of the winter-biased *SUT4*, but not the other winter-expressed *SUT5/SUT6*, had detrimental effects on tree growth, phenology, and reproduction.

A role of SUT4 in seasonal phenology

Several lines of evidence suggest that altered plant phenology contributed to the growth penalty we observed in the *sut4* mutants. While the mechanism is not yet clear, the *sut4* trees consistently exhibited premature leaf senescence, delayed spring budbreak, and delayed spring diameter growth relative to control and *sut56* trees (Figure 1). This translated into a shortened growth

season for *sut4* trees. However, soluble sugar and starch levels were not limiting in *sut4* stems during the summer and winter depletion of bark or xylem starch reserves was not impaired in *sut4* mutants (Figure 2). In addition, diameter growth rates were similar between *sut4* and controls during summer of 2022, 2023, and 2024 for the second cohort (Figure 1E). This is consistent with previous reports of no apparent growth differences of *SUT4*-KD or KO trees when compared to the control under greenhouse conditions (Payyavula et al., 2011; Frost et al., 2012; Tuma et al., 2024). Nevertheless, KO effects on sucrose partitioning and sprouting performance under cool temperature, short day growth conditions in coppiced stems was observed (Tuma et al., 2024). Elsewhere, effects of altered carbohydrate partitioning on phenology have been reported for hybrid poplar (*Populus alba* × *Populus grandidentata* Michx.) by Park et al. (2009). However, growth was not negatively impacted, presumably because leaf lifespan was increased following sucrose phosphate synthase overexpression in that study (Park et al., 2009). These results support the idea that *sut4* effects on growth were due at least in part to negative impacts of altered sucrose partitioning in stems as well as in leaves under cooler and/or short-day conditions. Notably, such changes to seasonal growth were not found in the *sut5*, *sut6*, or *sut56* trees.

Soluble sugars including RFOs act as antioxidants and osmoprotectants during periods of dehydration stress such as water deficit and low temperatures (Ahmad et al., 2020). For instance, raffinose and galactinol are usually detected during the dormant season or under stress conditions (Regier et al., 2010; Frost et al., 2012; Richardson et al., 2013) and genes associated with their biosynthesis are up-regulated in the stems of poplar and Norway spruce in the winter (Ko et al., 2011; Unda et al., 2012; Jokipii-Lukkari et al., 2018). The winter increases of raffinose and

galactinol were smaller in the stems of *sut4* than the control and *sut56* trees (Figure 3). Thus, vacuolar sucrose sequestration in *sut4* appeared to affect the accrual of RFOs associated with cold tolerance and perhaps the preparation for growth resumption in the spring.

Furthermore, both sugars and RFOs serve as signaling molecules during growth, development, and stress responses (Rolland et al., 2006; ElSayed et al., 2014). Sugars integrate multiple environmental cues such as light, temperature, and photoperiod to modulate ROS homeostasis, hormonal signaling, and circadian rhythms (Rolland et al., 2006; Román et al., 2021). Specifically, accumulation of sugars has been associated with the initiation and/or acceleration of leaf senescence (Wingler et al., 2005). This is consistent with hastened leaf senescence in *sut4* trees with elevated sugar levels. A signaling role has also been suggested for galactinol in poplar where hyper-accumulation of galactinol via overexpression of *Arabidopsis thaliana* *AtGolS3* resulted in stunted growth and altered xylem anatomy and wood chemistry, including smaller- yet-more-numerous vessels, gelatinous fibers, reduced lignin, and increased cellulose accrual (Unda et al., 2017). Reduced RFO levels in the *sut4* stems could have contributed to the delay in bud break and resumption of growth we observed in the spring. An alternative explanation is that the nutrient recapture from senescing leaves in the *sut4* mutants was compromised and as a result this delayed the spring bud flush. Together, our findings implicate a role for SUT4 in modulating sugar allocation and signaling, with consequences in seasonal phenology and overall plant growth.

SUT4 modulation of proanthocyanidins and RFOs in reproductive competence

High carbohydrate levels usually promote flavonoid biosynthesis, especially at low temperatures (Araguirang and Richter, 2022). Given sucrose levels increased in the *sut4* catkins like many other organs (Harding et al., 2022; Tuma et al., 2024), the widespread suppression of flavonoid biosynthesis pathway gene expression is counterintuitive. It is possible that vacuolar sucrose sequestration in *sut4* mutants led to a false “low sucrose” cue perceived by other subcellular compartments. Alternatively, impaired SUT4 symport activity for vacuolar efflux of sucrose might have disrupted the membrane potential and affected other proton-dependent tonoplast transport systems (Martinoia, 2017). In support of this idea, we detected in *sut4* catkins down-regulation of the single-copy ortholog (PtXaTreH.01G136800, Figure 5E) of *Arabidopsis* *TT13* encoding a P-type ATPase proton pump that generates the driving force for TT12-mediated proanthocyanidin delivery to the vacuole (Appelhagen et al., 2015). The coordinated down-regulation of genes involved in proanthocyanidin biosynthesis and vacuolar transport machinery along with widespread up-regulation of heat shock protein chaperons associated with protein oligomerization, misfolding, and refolding responses provides molecular support for a potential signaling role of SUT4 during catkin development.

Galactinol and raffinose are known to accumulate in dormant buds (Cox and Stushnoff, 2001; Falavigna et al., 2018) as well as developing embryos and seeds to confer desiccation and/or cold tolerance (Peterbauer and Richter, 2001). Their levels were highest in unopened floral buds of WT poplar trees and depleted as catkins emerged and elongated. However, this developmental trajectory was altered in *sut4* trees, as galactinol and raffinose levels remained high in developing catkins, consistent with up-regulation of *GolS* genes in *sut4* relative to WT catkins. Elevated RFOs may signify a stress status of *sut4* catkins due to altered sucrose partitioning and/or

impaired vacuolar proanthocyanidin deposition, or stress signaling as discussed above. The overrepresentation of GO terms associated with responses to heat, hypoxia, and hydrogen peroxide, and with various protein misfolding and refolding processes among genes induced in *sut4* catkins is consistent with a heightened cellular stress status. Thus, multiple developmental, metabolic, signaling, and stress responses were altered in developing *sut4* catkins, resulting in aborted ovules and reproductive infertility.

There is precedence for impaired vacuolar SUT function that impacts plant growth. Rice (*Oryza sativa*) mutant deficient in tonoplast *OsSUT2* showed impaired growth, reduced tiller number, and reduced 1000-grain weight (Eom et al., 2011). Silencing of potato (*Solanum tuberosum*) *StSUT4* affected the onset of flowering, tuberization, and circadian regulation via a proposed signaling role (Chincinska et al., 2008; Chincinska et al., 2013). In *Arabidopsis*, suppression of cell wall invertase genes impeded ovule development via sugar signaling not associated with carbon provision (Liao et al., 2020). The present work highlights the value of field trials in revealing previously underappreciated effects of SUT4 on seasonal phenology, overall biomass growth, and reproduction of poplar. Amongst the winter-expressed *SUT* genes, the winter-dominant *SUT4* has larger roles than *SUT5* and *SUT6* in protective metabolism during dormant season for both vegetative and reproductive growth. The complex interplay between sucrose partitioning, RFOs, proanthocyanidins, and phenology suggests a signaling role for SUT4 that is sensitive to environmental cues.

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Authors' Contributions: T.T.T, S.A.H, & C-J.T. conceived the study and designed all experiments. T.T.T. performed all experiments and analyzed the data, with assistance from H.A.M., H.P., & W.P.B. for transgenic plant production and amplicon sequencing, S.A.H for metabolic analyses, B.L. for RNA extraction, A.L., K.W.B., & R.Z., for RNA-seq analysis, D.L.W.W. for diameter and flower monitoring, M.D. & R.Z. for controlled pollination, J.D. for wood property analysis, and S.S. & A.E.H.-W. for wood chemistry analysis. T.T.T. prepared the manuscript which was revised by C.J.T. with contributions from S.A.H.

Legends to Figures

FIGURE 1: Biomass yield and growth characteristics of *sut* and control poplars.

(A) Representative adaxial views of leaf plastochron index (LPI)-4, LPI-6, LPI-8, LPI-10, and LPI-12 prior to leaf abscission.

(B) Representative photos of field-grown trees taken in November 2021. Arrows denote *sut4* trees.

(C) Progression of leaf abscission from the main stem in Fall 2021. Data represent the percentage of trees per genotype that had abscised or desiccated leaves using pooled transgenic events and biological replicates ($n = 69$ for control, $n = 78$ for *sut4*, $n = 13$ for *sut5*, $n = 11$ for *sut6*, and $n = 19$ for *sut56*).

(D) Bud flush was evaluated in the spring of 2022 based on the epicormic buds on the main stem. Data represent the percentage of trees per genotype that had flushed buds using pooled

transgenic events and biological replicates ($n = 69$ for control, $n = 78$ for *sut4*, $n = 13$ for *sut5*, $n = 11$ for *sut6*, and $n = 19$ for *sut56*).

(E) Stem diameter of trees from Cohort 1 and Cohort 2 measured 1 m above root collar monthly or bi-monthly. Data are mean $\pm$ standard deviation of pooled transgenic events and biological replicates ($n = 40$ for control or 29 for *sut4* in cohort 1, or $n = 23$ for control, 40 for *sut4*, 13 for *sut5*, 11 for *sut6*, and 18 for *sut56* in cohort 2). The effects of time (t), genotype (g), and their interaction ($t \times g$) were assessed with a repeated measures two-way ANOVA. ***, $P \leq 0.001$.

Inset in cohort 1 was from size-matched control or *sut4* trees at the start of the monitoring ($n = 13$). Inset in cohort 2 represent diameter increments during spring and summer of 2022.

(F) Cambial growth reactivation in the spring of 2022 (cohort 1) and 2024 (cohort 2). Data represent the percentage of trees per genotype that had the first measurable diameter increases, with months indicated by numbers ($n = 29$ -40 trees for cohort 1 or $n = 13$ -24 trees for cohort 2).

(G) Height of dominant stem measured at the beginning of the growing season in 2022 and 2023. Data are mean $\pm$ standard deviation of $n = 15 - 22$ trees per genotype using pooled transgenic events and biological replicates. Significance was conducted using 2-sample t -test. *, $P < 0.05$.

(H) Representative image of control, *sut4*, and *sut56* trees 14 months after field outplanting.

FIGURE 2: Nonstructural carbohydrate abundance in stem organs of different genotypes.

(A) Sucrose, (B), hexose (sum of fructose + glucose), and (C) starch abundance in bark and xylem during summer and winter. Data are mean $\pm$ standard error of $n = 8$ -12 biological replicates. Significance was determined by Student's t -test against control. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

FIGURE 3: Oligosaccharide abundance dynamics in stem organs of different genotypes.

(A) Myo-inositol, (B), galactinol, and (C) raffinose abundance in bark and xylem during summer and winter. Data are mean $\pm$ standard error of $n = 8-12$ biological replicates. Significance against control was determined by Student's t -test. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

FIGURE 4: Wood characteristics and chemical properties.

(A) total lignin level, (B) syringyl-to-guaiacyl lignin (S/G) ratio, (C) acoustic velocity, (D) specific gravity, and (E) modulus of elasticity. Data are mean $\pm$ standard deviation of $n = 6-12$ biological replicates. Significance was determined against control trees by Student's t -test; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

FIGURE 5: Flowering phenotypes in control and *sut* trees.

(A) First sign of axillary inflorescence bud flushing on early spring 2023. Inset shows representative catkin developmental stages used in metabolite analysis (I, unopened floral bud; II, newly emerged catkin; and III, elongating catkin). Bar = 1 cm.

(B) Total percentage of trees per genotype from Cohort 1 and Cohort 2 that developed catkins during the spring of the 2023 and 2024.

(C) Sucrose, hexose (sum of fructose + glucose), galactinol, and raffinose abundance in unopened floral bud, newly emerged catkins, and elongating catkins. Data are mean $\pm$ standard error of $n = 6-8$ biological replicates. Significance was determined by Student's t -test. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

(D) GO enrichment of differentially up- or down-regulated genes in the elongating catkins of the *sut4* mutants relative to the control. Representative GO terms with fold-enrichment >6 and gene number >5 are shown.

(E) Significant expression response heatmaps of genes associated with flavonoid and proanthocyanidin biosynthesis and regulation. Log₂FC, log₂-transformed fold-change (*sut4*/control) of *n* = 7 (pooled from 5 WT and 3 *Cas9*) and 5 (*sut4*) biological replicates. ANR, anthocyanidin reductase; ANS, anthocyanidin synthase; CHS, chalcone synthases; CHI, chalcone isomerase; DFR, dihydroflavonol reductase; F3H, flavanone 3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; F3'5'H, flavonoid 3',5'-hydroxylase; LAR, leucoanthocyanidin reductase; TT, transparent testa.

(F) Representative *sut4* capsules with seed trichomes after controlled pollination.

(G) Visual representation of seeds and ovules from control and *sut4* catkins. Scale bar = 1 mm.

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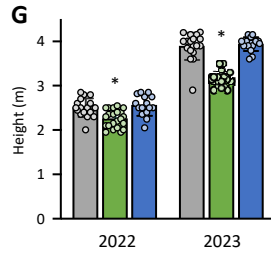
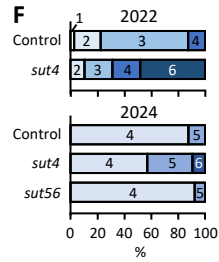
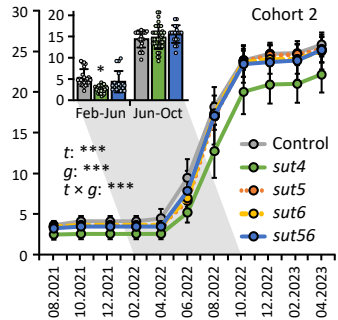
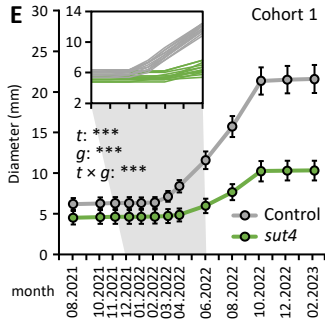
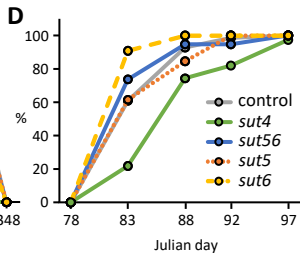
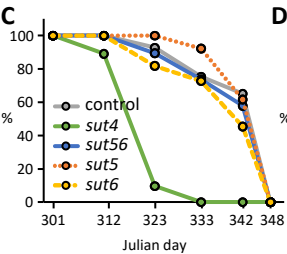
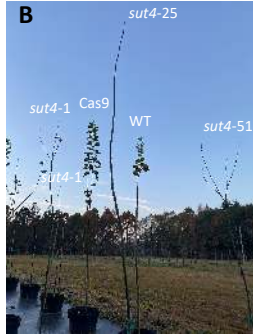
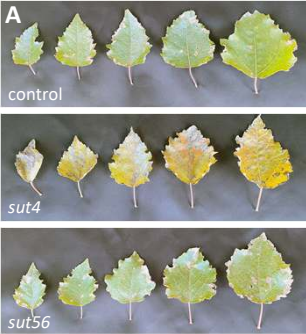
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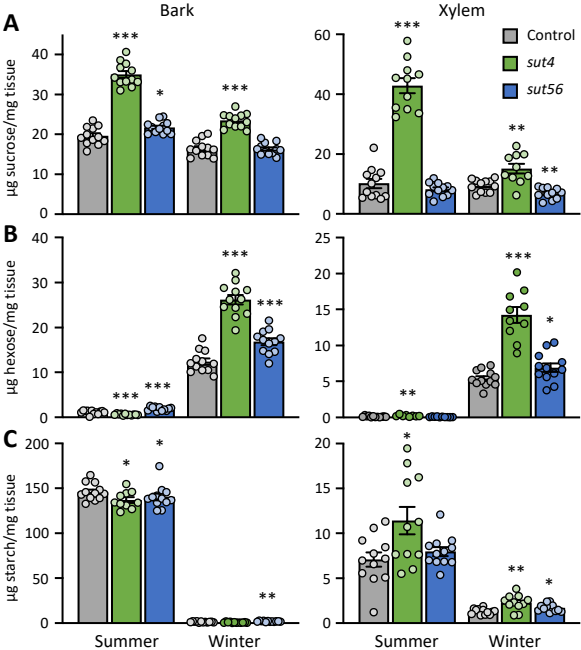
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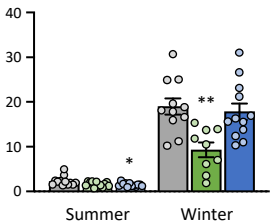
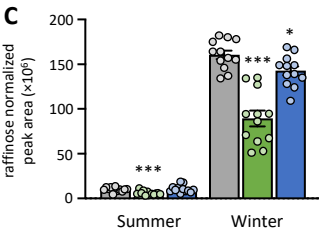
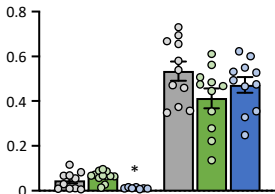
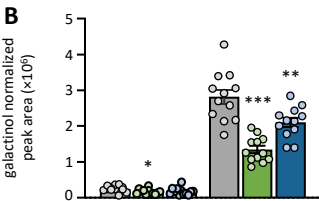
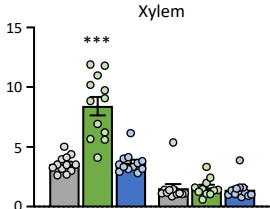
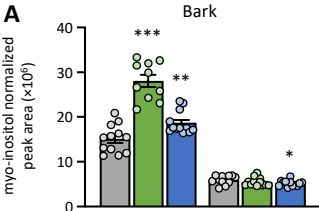
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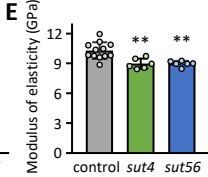
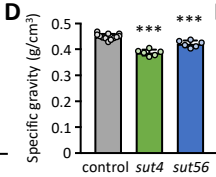
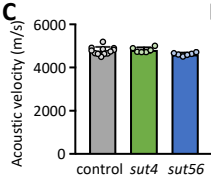
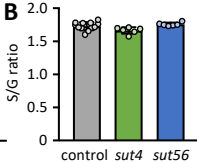
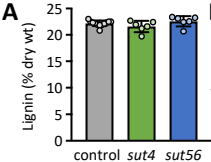
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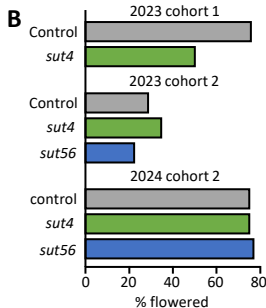
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E

Gene model	Log ₂ FC	name
PtXaTreH.14G116200	-0.9	CHS
PtXaTreH.01G043200	-1.7	CHS
PtXaTreH.01G043300	-1.6	CHS
PtXaTreH.10G178000	-1	CHI
PtXaTreH.05G089200	-1.3	F3H
PtXaTreH.05G089100	-1.3	F3H
PtXaTreH.13G063700	-0.7	F3'H
PtXaTreH.09G055400	-1.6	F3'5'H
PtXaTreH.02G029100	-1.4	DFR
PtXaTreH.05G177900	-1.1	DFR
PtXaTreH.03G097500	-1.6	ANS
PtXaTreH.01G094900	-1.2	ANS
PtXaTreH.04G021600	-1.4	ANR
PtXaTreH.08G095200	-1.1	LAR
PtXaTreH.10G109000	-1.3	LAR
PtXaTreH.15G039500	-1.8	LAR
PtXaTreH.06G183200	-0.7	TT2
PtXaTreH.02G046300	-0.9	TT8
PtXaTreH.05G157900	-1.7	TT12
PtXaTreH.05G157600	-1.6	TT12
PtXaTreH.02G047100	-1.5	TT12
PtXaTreH.01G136800	-0.8	TT13

