Sucrose metabolism and candidate genes during catkin fibers development in poplar

Running title: Sucrose metabolism in poplar catkin fiber.

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Abstract

Poplar is an important tree species for ecological protection, wood production, landscaping and urban greening; it has been widely planted worldwide. However, the catkin fibers produced by female poplars can cause environmental pollution, human health risks and safety hazards during spring. This study focused on *Populus tomentosa* Carr., and reveals the sucrose metabolism regulatory mechanism of catkin fibers development from morphological, physiological and molecular aspects. And two key genes were selected to demonstrate the exact role. Sucrose degradation plays the dominant role during poplar catkin fibers development. Thereinto, invertase has a function during the early stage and sucrose synthase has a close correlation with the elongation of catkin fibers. The expression patterns revealed that sucrose metabolism-related and *cellulose synthase* (*CesA*) genes play important roles during catkin fiber development. Expression profiles and co-expression networks indicated that the mechanism underlying poplar catkin fiber development is similar, but not identical, to that of cotton fibers. Finally, two key genes, *sucrose synthase 2 (PtoSUS2)* and *vacuolar invertase 3 (PtoVIN3)*, had roles in *Arabidopsis thaliana* trichome density, indicating that sucrose metabolism is important in poplar catkin fibers development. This study is not only helpful for clarifying the mechanism of sucrose regulation during trichome development in perennial woody plants, but also establishes a foundation to solve poplar catkin fiber pollution through biotechnology and genetic engineering methods.

Key words: *Populus tomentosa*, Catkin fibers development, Sucrose metabolism, Gene expression, Regulatory network.

Introduction

Poplar is an ecologically important species and one of the three main tree species used for afforestation in China. The poplar plantation area has reached 7,570,700 hm^2, occupying 13.25% of the total plantation area in 2019. Poplar is not only major contributor to wood production, but also provides resource for eco-environment construction, biomass energy, landscape and urban greening (Sannigrahi et al., 2010). Furthermore, poplar is a crucial model system for research on perennial woody plants due to its small genome, easy propagation and rapid growth (An et al., 2022). In female poplars, the capsules contain seeds and catkin fibers (Fig. 1a), and begin to open as the seeds become mature, usually in early April in northern China (Fig. 1b). Finally, catkin fibers cover the whole catkins (Fig. 1c, d), which is a common phenomenon in poplar (Fig. 1e, f), and the fibers can be carried over long distances with seeds on the wind (Fig. 1g, h). Poplar catkin fibers can survive for > 40 days in the environment depending on the various poplar species and temperatures. The long period during which poplar fibers are suspended in the air can cause serious environmental pollution (Ye et al., 2014), although it can be used as raw materials for bioethanol (Zhang et al., 2018), oil super absorbents (Likon et al., 2013) and textile bulk insulators (Chen and Cluver, 2010). Almost 185,000 female poplar trees that can produce catkins are located in the urban areas of Beijing, the capital of China, and the catkin fibers produced by these trees have extremely harmful effects on human health through inhalation exposure, and they may also affect metal/metalloid concentrations

Fig. 1 Morphological characteristics of capsules and catkin fibers in poplar. a-d Characteristics of *Populus tomentosa*.. **e, f** Other poplars. **g-h** Environmental pollution caused by poplar catkin fibers. Scale bar: **a** = 1 cm, **b** = 2 cm.

69 in the environment (Wan et al., 2020). Ădditionally, the catkin fibers may pose a

safety hazard due to their inflammability. In recent years, studies of sex differentiation and female flower development in poplar have revealed some key genes such as *type-A cytokinin response regulator* (Yang et al., 2021), *FERR-R* and *MSL* (Xue et al., 2020), *ARABIDOPSIS RESPONSE REGULATOR 17* (Müller et al., 2020), *AGAMOUS* and *SEEDSTICK* (Lu et al., 2019), and *LEAFY* (Klocko et al., 2016). However, the molecular basis of catkin fibers formation and development in poplar is still unclear. Therefore, analyzing the development process of catkin fibers is helpful not only for understanding this progress in Salicaceae plants, but also for improving the urban environment.

Trichomes, which originate from epidermal cells, are distributed on the surfaces of plants and protect them from biotic and abiotic stresses (Pattanaik et al., 2014). Many previous studies of trichomes have been conducted in *Arabidopsis thaliana* and cotton, focusing on epidermal hairs and cotton fibers, respectively (Grebe, 2012; Naoumkina, 2018). According to morphological characters, trichome development can be divided into three stages: initiation, extension and maturation. An additional stage, secondary cell wall (SCW) synthesis, is presumed to occur between extension and maturation in cotton fibers (Grebe, 2012; Naoumkina, 2018). Similar regulatory mechanisms were observed during the initiation stage in *A. thaliana* and cotton (Bui et al., 2019). *GLABROUS1* (*GL1*, a MYB transcription factor [TF] gene), *GLABROUS3* (*GL3*, a bHLH TF gene), *ENHANCER OF GLABRA3* (*EGL3*, a bHLH TF gene) and *TRANSPARENT TESTA GLABRA1* (*TTG1*, a WD40 TF gene) have been suggested to play regulatory roles in the trichome development through the induction

of *GLABRA2* (*GL2*, a HD-ZIP IV TF gene) (Rerie et al., 1994; Walker et al., 1999; Bernhardt et al., 2005; Kirik et al., 2005; Bloomer et al., 2012). In addition, *GhMYB25*, another type of *MYB* gene, was found to play a major role in cotton fiber initiation and extension, and similar functions were found for its homologues, *GhMYB25-LIKE, GhMML3_A12* and *GhMML4_D12* (Wu et al., 2018). *GbML1*, another HD-ZIP IV TF, was found to have a similar expression pattern to *GhMYB25* and to interact with *GhMYB25* to control fiber development (Zhang et al., 2010). As a model of rapid single-cell growth, sucrose metabolism (SM)-related proteins also play important roles in the process (Ruan, 2014).

Sucrose is an important form of transportable sugar in plants. Plants convert CO_2 into organic carbon through photosynthesis in leaves, and then transport it to heterotrophic sinks, such as the stem, root and fruit, for energy storage and utilization (Ruan, 2014). Thus, SM plays a critical role in plant growth and development, including trichome development. Sucrose can be synthesized by sucrose phosphate synthase (SPS) and sucrose phosphate phosphatase (SPP), and degraded by sucrose synthase (SUS) or invertase (INV) in plants (Ruan, 2014). Sugar signals produced by INV likely influence the cotton fiber initiation through MYB TFs and auxin signals (Wang et al., 2014). High turgor pressure induced by sucrose degradation via INV and SUS promotes the rapid elongation of cotton fibers (Wang et al., 2010; Ahmed et al., 2018). In contrast to INV, uridine diphosphate (UDP)-glucose produced by SUS is a vital substrate for cellulose synthesis, and it influences cell wall synthesis, especially of the SCW (Fujii et al., 2010; Ahmed et al., 2018). Additionally, two overexpression

studies showed that SPS has an effect on fiber development (Haigler et al., 2007; Park et al., 2008),which presumably because SPS can recycle fructose released by SUS to obtain more sucrose (Martin and Haigler, 2004). Similar to cotton fibers and *Arabidopsis* epidermal hairs, in essence, poplar catkin fibers also belong to plant trichomes. However, compared with the numerous important advances in *Arabidopsis* and cotton, the research foundation of catkin fibers development in poplar is quite weak, the relationship between SM and poplar catkin development, and the real role of many key homologues in poplar are almost unknown.

Chinese white poplar (*Populus tomentosa* Carr.) is a representative species of poplar, widely planted in northern China. We previously conducted transcriptome analysis of catkin fibers development in this plant, and found that many Gene Ontology (GO) terms related to sucrose metabolism were significantly enriched during this process (Ye et al., 2014). Therefore, in this study, we made histological observations of catkin fibers development in *P. tomentosa*, and measured the sugar content and activities of key SM enzymes at various developmental stages. Using the genomic data of *P. tomentosa* compiled by our group (An et al., 2022), we searched for SM-related genes and performed detailed fluorescence-based real-time quantitative polymerase chain reaction (RT-qPCR) analysis. Additionally, based on a thorough review of the literatures, we summarized the key genes involved in trichome development in cotton and *A. thaliana*, and then identified their homologues in the poplar genome database (Table S1). The expression patterns of these potentially SM-related genes were also analyzed, and a gene co-expression network was

constructed. The relationships between SM-related genes and TFs were also analyzed. Finally, two key genes, *PtoSUS2* and *PtoVIN3*, were selected to investigate the function of SM in catkin fibers formation using the model plant *A. thaliana*.

Results

Morphological characteristics of poplar catkin fibers

The morphology of catkin fibers changed rapidly during hydroponic culture (Fig. 2). Histological analysis revealed that no catkin fibers appeared when the branches had been cultured for 0-48 h (Fig. 2a-d). The epidermal cells of funicles initially showed larger nuclei (Fig. 2e, f), in accordance with the endoreduplication stage in *A. thaliana*, indicating the initial stage of development of catkin fibers. Subsequently, the epidermal cells exhibited protrusion and extension from 58 to 120 h, until the catkin fibers finally full filled the ovary (Fig. 2g-j). Thus, according to the morphological characteristics of catkin fibers, we divided the development process into five stages: stage 1 (preparatory stage, cultured for 48 h, Fig. 2d), stage 2 (initial stage, cultured for 58 h, Fig. 2e-f), stage 3 (apophysis stage, cultured for 72 h, Fig. 2g-h), stage 4 (extension stage, cultured for 96 h, Fig. 2i), stage 5 (maturation stage, cultured for 120 h, Fig. 2j). Furthermore, as shown in Fig. 2, catkin fibers were produced from the epidermal cells of funicle and placenta, independent from seeds. Samples from the five stages were used for analyses of enzyme activities and gene expression patterns.

Changes in sugar content and key enzyme activities in poplar catkin fibers

The sucrose content of the catkin of *P. tomentosa* showed a downward trend, indicating that sucrose is gradually consumed during the development of catkin (Fig. 3a). In particular, during the stage 3-4, sucrose was consumed at a dramatic rate when the catkin fibers extended rapidly. Glucose and fructose showed similar trends, which both had higher contents during stage 2-4. The ratio of hexose to sucrose was also

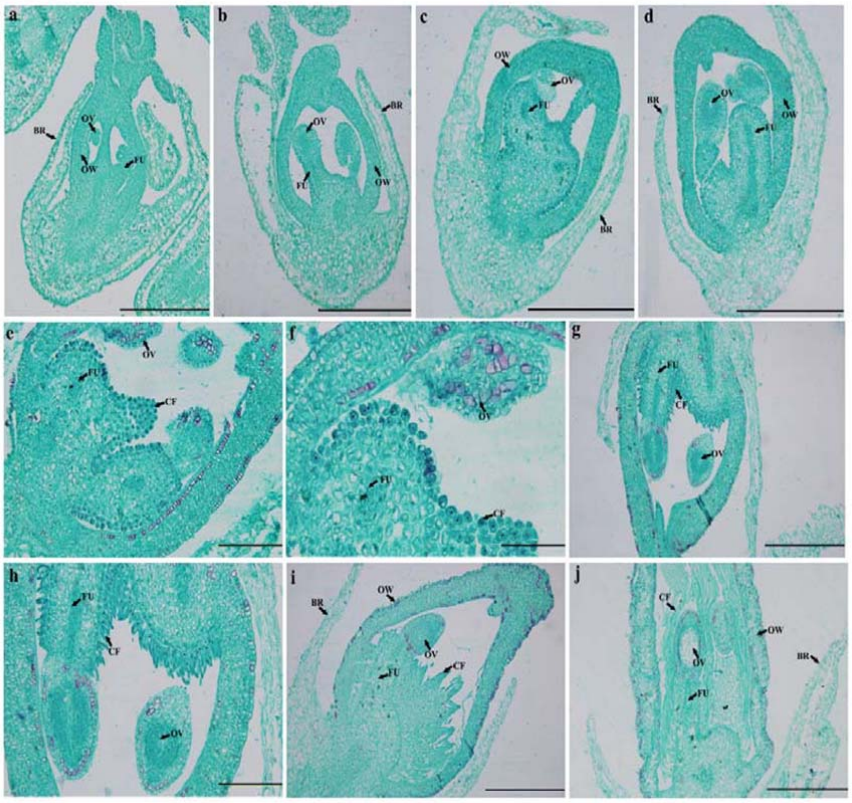

Fig. 2 Histological characteristics of female buds in *P. tomentosa.* a-d Female buds on branches that were hydroponically cultured for 0, 24, 34, 48 h, respectively. No catkin fibers were observed. **e, f** Female buds on branches that were hydroponically cultures for 58 h. **g, h** Female buds on branches that were hydroponically cultured for 72 h. **i** Female buds on branches that were hydroponically cultured for 96 h. **j** Female buds on branches that were hydroponically cultured for 120 h. OW, ovary wall, OV, ovule, FU, funicle, BR, bract, CF, catkin fibers. Scale bar: **a-d, g, i, j** = 500 μm, **e, h** = 200 μm, **f** = 100 μm.

162 analyzed. As shown in Fig. 3b, the ratio increased sharply between the apophysis

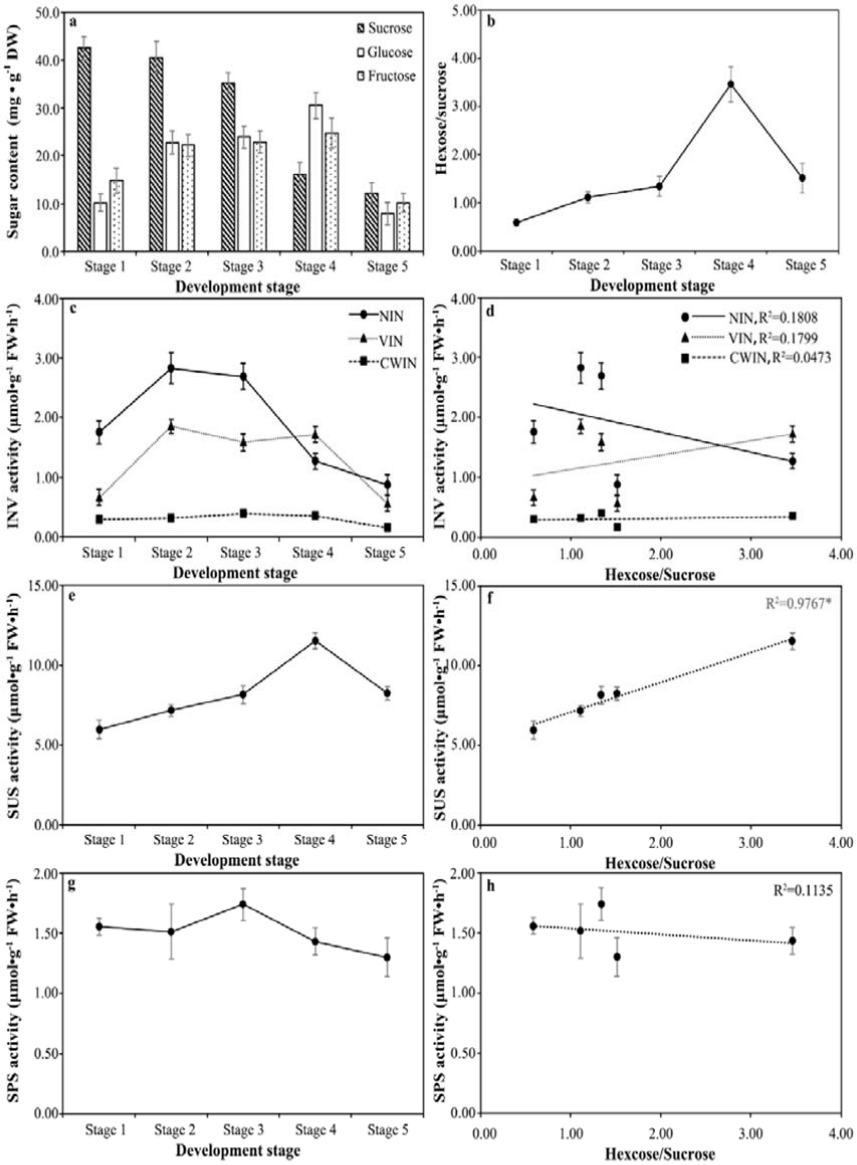

Fig. 3 Changes in sugar contents and key enzymes involved in SM during poplar catkin fibers development. Bars represent the standard deviation (SD, n = 3). * $P \leq 0.05$.

163 stage (stage 3) and extension stage (stage 4), and decreased between the extension

164 stage (stage 4) and maturation stage (stage 5). Changes in the ratio indicated that the
165 high hexose concentration may play a role in catkin fibers extension. When the catkin
166 fibers began to extend, most of the sucrose present was degraded into glucose and
167 fructose, which likely provided greater turgor pressure, thus promoting catkin fibers
168 elongation. To further explore the role of sucrose in catkin fibers development, we
169 also determined the activities of key enzymes in SM. Consistent with the results of
170 sugar content analysis, sucrose degradation was the dominant process.

171 The activity of CWIN showed no significant fluctuations except at stage 5, when
172 it decreased as the catkin fibers matured (Fig. 3c). The activities of NIN and VIN
173 were higher than that of CWIN. VIN activity increased during catkin fibers initiation
174 and decreased during the catkin fibers maturation, indicating that VIN may play a role
175 in catkin fibers development (Fig. 3c). Meanwhile, the activity of NIN was high only
176 during the initial (stage 2) and apophysis stages (stage 3), and appeared to play a role
177 in the early development of catkin fibers (Fig. 3c). Compared with INV, SUS showed
178 higher activity during the development of catkin fibers, up to the extension stage
179 (stage 4); it then decreased during the maturation stage (stage 5), showing an inverse
180 correlation with sucrose content (Fig. 3e). Similar results were obtained though fitting
181 analysis. SUS activity showed a significant correlation with the ratio of hexose to
182 sucrose, indicating that SUS plays a major role in sucrose degradation during the
183 development of catkin fibers (Fig. 3f). Furthermore, SPS activity was analyzed and
184 used to represent the sucrose synthesis pathway. As shown in Fig. 3g, SPS exhibited
185 steady activity throughout catkin fibers development, except during the apophysis

stage (stage 3).

Gene expression patterns related to SM in poplar catkin fibers

The expression of *CWIN* showed a similar trend to enzyme activity. We analyzed three members of the gene family, *PtoCWIN3*, *PtoCWIN4* and *PtoCWIN5*, during catkin fibers development. *PtoCWIN3* appeared to play a major role in catkin fibers development based on its high and variable expression level (Fig. 4a). However, Pearson correlation analysis showed that *PtoCWIN4* rather than *PtoCWIN3* was positively correlated with sucrose content, which may be due to the dramatic decreased in *PtoCWIN3* at the initial stage (Table S4). Two members of the *VIN* gene family were observed, and both had elevated expression at stage 2-4, coinciding with the enzyme activity results (Fig. 4b). And both members of the *VIN* gene family had positive correlations with the concentrations of fructose and glucose (Table S4). For *NIN*, nine members were detected during catkin fibers development, including two low-expression members, *PtoNIN2* and *PtoNIN11* (Fig. 4d, f). According to their expression patterns, the other members can be divided into two groups. *PtoNIN3* and *PtoNIN4* had higher expression at the apophysis stage (Fig. 4e), while the other five members peaked at the initial stage (Fig. 4c-d, f-g).

There are seven members in the *SUS* gene family, all of which were detected during poplar catkin fibers development. Their expression pattern showed a clear correlation with the phylogenetic tree (Fig. S1c). The first cluster, comprising *PtoSUS1* and *PtoSUS2*, had high expression during catkin fibers development. Compared with *PtoSUS1*, *PtoSUS2* had higher expression, and both had their highest

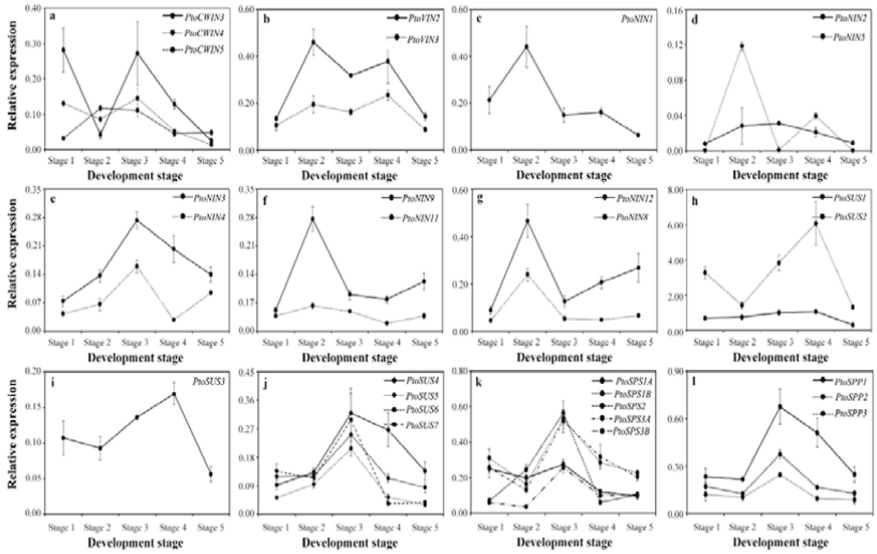

Fig. 4 Analysis of SM related gene families in the context of poplar catkin fibers development. a Expression analysis obtained by RT-qPCR for *CWIN* gene family. **b** Expression analysis obtained by RT-qPCR for *VIN* gene family **c-g** Expression analysis obtained by RT-qPCR for the *NIN* gene family. **h-j** Expression analysis obtained by RT-qPCR for the *SUS* gene family. **k, l** Expression analysis obtained by RT-qPCR for *SPS* and *SPP* gene family, respectively. Bars represent the ± SD.

208 levels during the extension stage (Fig. 4h). The second cluster only contains one

member, *PtoSUS3*, which had low expression but a similar pattern to the first cluster (Fig. 4i). The other four members belonged to the third cluster, and had high expression at the apophysis stage (Fig. 4j). The expression levels of most *SUS* gene members had significant positive correlations with the concentration of hexose (Table S4).

The expression patterns of *SPS* and *SPP* gene families were analyzed. Five of the six members of the *SPS* gene family were analyzed, and they all had high expression at the apophysis stage, in accordance with the results for SPS activity (Fig. 3g and 4k). All three *SPP* members (*PtoSPP1*, *PtoSPP2* and *PtoSPP3*) were detected, and all had elevated expression during the apophysis stage (Fig. 4l). The results of both the enzyme activity and gene expression analyses showed that the sucrose synthesis pathway might only have a slight effect at the apophysis stage.

CesA gene family and other potential genes in poplar catkin fibers development

Poplar catkin fibers follows a similar development process to cotton fiber, and cellulose is the main component of cotton fiber (Kim and Triplett, 2007). Thus, we also checked the expression pattern of the *cellulose synthase* (*CesA*) gene family in poplar catkin fibers. Thirteen members of the *CesA* gene family were analyzed in this study, which belonged to five of six groups on the phylogenetic tree, i.e., all except cluster P2 (Fig. S1d). Among them, *PtoCesA17*, belonging to clade P1, had high expression at the apophysis stage, while its homologue *PtoCesA7* had low expression (Fig. 5d). Homologous pairs in clade P3 had similar expression patterns, except *PtoCesA2* and *PtoCesA5* (Fig. 5e-g). *PtoCesA6* and *PtoCesA9* had high expression

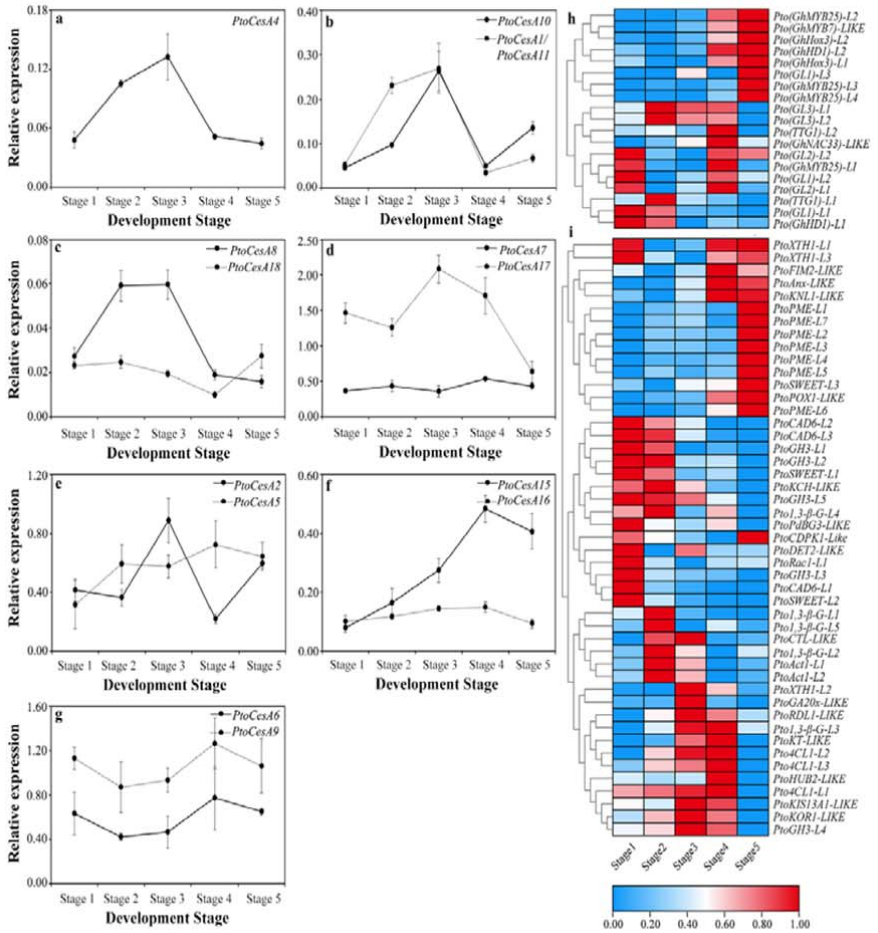

Fig. 5 Analysis of the *CesA* gene family and other potential genes in the context of poplar catkin fibers development. a-g Expression analysis based on RT-qPCR for *CesA* gene family. **h, i** Heat map of gene expression obtained by RT-qPCR for TFs-related and other genes, respectively. Bars represent the ± SD.

231 throughout the process of catkin fibers growth (Fig. 5g). *PtoCesA15* appeared to play

232 a more important role than *PtoCesA16*, as it had dramatically higher expression
233 during catkin fibers development (Fig. 5f). Other genes belonging to clades S1, S2
234 and S3, had low expression in catkin fibers, although their expression increased at the
235 initial and apophysis stages with the exception of *PtoCesA18* (Fig. 5a-c).

236 TFs, such as *MYB*, *WD40* and *bHLH*, have been shown to play important roles in
237 the development of cotton fibers (Bui et al., 2019). Thus, homologues of the factors
238 that have been identified in cotton were examined in poplar catkin fibers (Table S1).
239 According to their expression patterns, these homologues can be divided into three
240 subgroups (Fig. 5h). Among bHLH TFs, *Pto(GL3)-L1* and *2* had highest expression at
241 the initiation stage. In the *WD40* gene family, *Pto(TTG1)-L1* had high expression at
242 the initiation stage, while the another WD40 TF *Pto(TTG1)-L2* had high expression at
243 the extension stage. Members of the MYB gene family may have divergent functions
244 during catkin fibers development. *Pto(GL1)-L1* and *2* had high expression during
245 early development of catkin fibers, and *Pto(GL1)-L2* had an additional role in the
246 extension stage. In contrast, *Pto(GL1)-L3* showed increased expression at the
247 maturation stage. The homologues of *GhMYB25* all had increased expression at the
248 maturation stage, except *Pto(GhMYB25)-L1*. *Pto(GhMYB7)-LIKE*, the cotton
249 homologue of which was speculated to be a lipid transfer protein-related gene, also
250 had high expression at the maturation stage. Another important TF gene family,
251 HD-ZIP IV, was also analyzed during poplar catkin fibers development. *Pto(GL2)-L1*
252 and *2* had high expression at the preparatory and extension stages, and the
253 homologues of *GhHox3* had functions at the maturation stage. Intriguingly, opposing

results were found between *Pto(GhHD1)-L1* and *Pto(GhHD1)-L2*.

We also examined the expression of other poplar homologues of genes that are important to the composition of cotton fibers (Table S1). As shown in Fig. 5i, three subgroups played different roles in the early, middle and late development stages. In cluster I, *PtoPME-related* genes played an important role in catkin fibers maturation, which indicted dramatic changes in the composition of cell wall. In cluster II, *PtoCAD6-related* and *PtoGH3-related* appeared to be important to the early development of catkin fibers. During the middle development stage (cluster III), many genes were involved. Thereinto, *PtoAct1-L1* and *2* and *Pto1,3-β-G-L1* and *5* had high expression at the initial stage. *PtoGA20x-LIKE, PtoKIS13A1-LIKE, PtoKOR1-LIKE* and *PtoXTH1-L2* functioned mainly at the apophysis stage, while *PtoHUB2-LIKE, Pto1,3-β-G-L3, Pto4CL1-L2, Pto4CL1-L3* and *PtoKT-LIKE* had high expression at the extension stage.

Co-expression network of genes involved in poplar catkin fibers development

To further explore the relationship between SM and poplar catkin fibers development, co-expression network analysis was performed using genes related to SM and homologues of genes that have effects on cotton fiber development. As shown in Fig. 6a, there were four divergent gene clusters, and SM related genes mainly occurred in cluster I-III. Thereinto, cluster II was mainly composed of sucrose synthesis-related genes, with *PtoSPS2* playing a leading role. Notably, *PtoSUS5-7*, which is a homologue according to the phylogenetic tree, and *PtoGA20x-LIKE* had close relationships with *SPS* and *SPP*. Different members of the *CesA* gene family

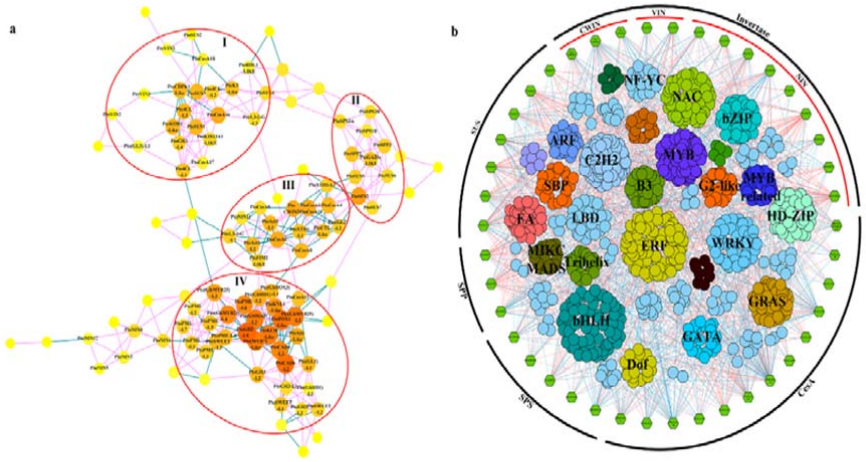

Fig. 6 Co-expression network analysis. a Co-expression network of 108 candidate genes associated with catkin fibers development in *P. tomentosa*. The threshold for significance of the Pearson correlation coefficient was set to ≥ 0.8 (or ≤ − 0.8), p-value ≤ 0.05. Larger and redder the circles, indicate higher connectivity. **b** Co-expression network of SM-related genes and TFs, the threshold for significance of the Pearson correlation coefficient was set to ≥ 0.8 (or ≤ − 0.8), p-value ≤ 0.01.

276 play specific roles in clusters I and III, respectively. Cluster IV represents the group of

genes that function in the late stages of catkin fibers development. Additionally, we performed network analysis between SM-related genes and TFs using data from a previous study (Ye et al., 2014). As shown in Fig. 6b, many TF gene families were involved in poplar catkin fibers development, including *bHLH*, *ERF*, *WRKY*, *MYB* and *HD-ZIP*. *Cis*-acting elements were also predicted for genes related to SM in this study. As shown in Fig. S2, light-responsive elements were the most important environmental factors, while abscisic acid-, methyl jasmonate (MeJA)- and salicylic acid- responsive elements were the most common motifs in plant hormone factors.

Overexpression of *PtoSUS2* and *PtoVIN3* increases trichome density in wild-type *A. thaliana* and *tl1/2* mutant

The results for SUS and VIN indicate roles for SM in poplar catkin fibers development; two key genes, *PtoSUS2* and *PtoVIN3*, were selected to demonstrate the exact role. Thereinto, *PtoSUS2* had a stable expression level except in root, while *PtoVIN3* had higher expression levels in stem and root, and the two genes all played roles during the early development of female and male floral bud (Fig. S3). Because of the feasibility and long period involved in generating transgenic poplars and investigating the phenotype of poplar catkins, the *A. thaliana* trichome system was used to analyze gene function. We introduced *PtoSUS2* and *PtoVIN3* constructs driven by the 35S promoter into wild-type *A. thaliana* and used T3-generation plants to analyze the phenotype. Thirteen and twenty-two independent transgenic lines were finally obtained for *PtoSUS2* and *PtoVIN3*, respectively. Three lines of each transgenic type were extensively evaluated. In wild-type *A. thaliana*, trichomes are

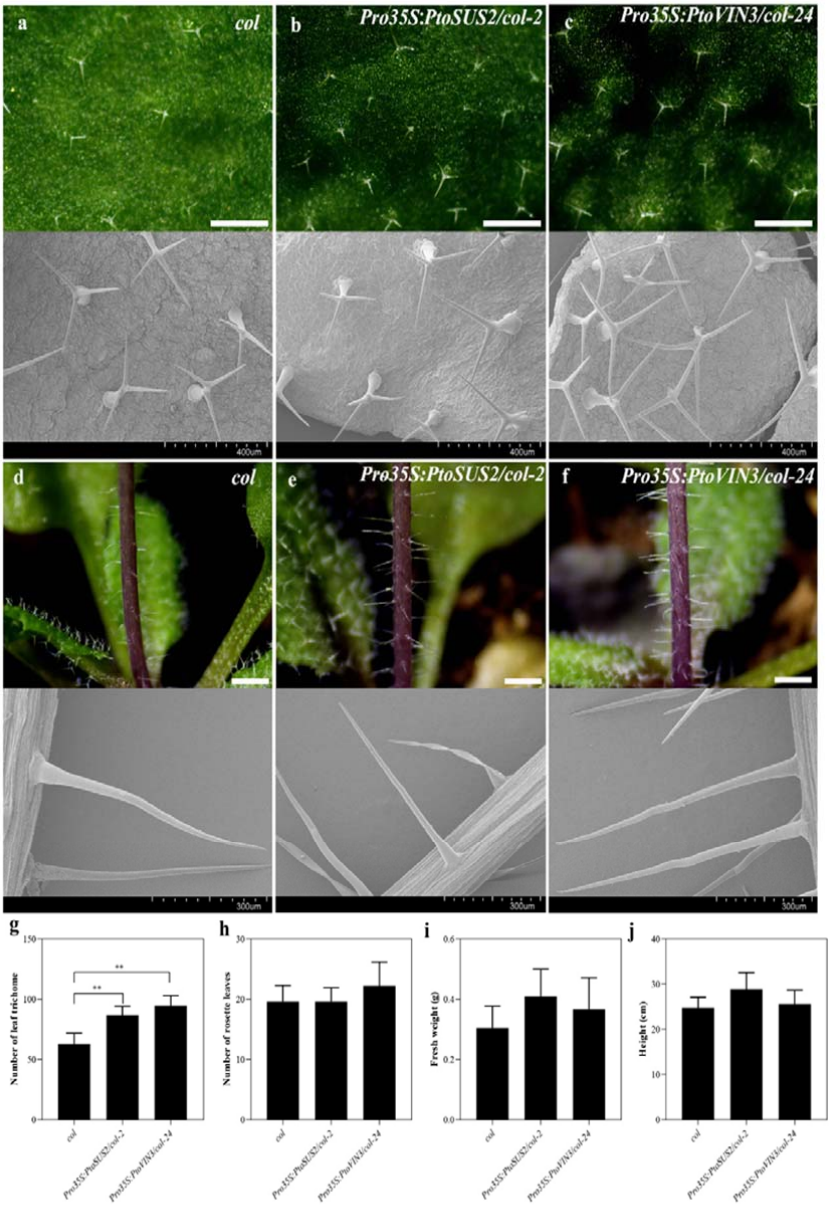

Fig. 7 Leaf trichome morphology and number, rosette leaf number, fresh weight, and height in col and its transgenic lines. a-f Upper panel, trichome morphology under a stereomicroscope; scale bars = 1 mm. Lower panel, trichome morphology under a scanning electron microscope. **g-j** Phenotypic characteristics of wild-type and transgenic plants; bars represent ± SD.

widely distributed on the adaxial and abaxial surfaces of rosette leaves and cauline

300 leaves, as well as stems. Compared with the wild-type, the *PtoSUS2* and *PtoVIN3*
301 overexpression lines had higher leaf trichome densities on the adaxial surface of
302 rosette leaves and stems at 30 DAG, and trichome shape was unaffected (Fig. 7a-f;
303 Fig. S4). The number of sixth-rosette leaf trichome in the wild-type was 67 ± 8.80,
304 significantly fewer than in the transgenic plants, *Pro35S:PtoSUS2/col-2* (87 ± 6.89)
305 and *Pro35S:PtoVIN3/col-24* (94 ± 8.41) (Fig. 7g). However, the rosette leaf number,
306 fresh weight and height did not significantly differ between wild-type and
307 overexpression plants (Fig. 7h-j). Similar results were obtained in terms of root length
308 (10 DAG), rosette leaf shape (20 DAG) and whole plants (40 DAG) (Fig. S5).
309 Additionally, the trichome density of transgenic plants on the abaxial surface of
310 rosette leaves slightly increased, but the numbers of trichomes on the adaxial and
311 abaxial surfaces of cauline leaves had no significant difference, and these results were
312 not affected by incubation time (Fig. S6).

313 Overexpression of *PtoSUS2* and *PtoVIN3* also led to greater trichome density in
314 mutants *tl1* and *tl2*. Three transgenic lines of each transgenic type were selected for
315 detailed comparison. In *tl1*, eleven and thirteen independent transgenic plants were
316 obtained for *PtoSUS2* and *PtoVIN3*, respectively. Compared with the glabrous
317 phenotype (lacking trichomes) in *tl1*, overexpression of *PtoSUS2* or *PtoVIN3* resulted
318 in normal trichomes on the adaxial surface of rosette leaves; trichomes were observed
319 in the stems of some transgenic plants (Fig. 8a-c; Fig. S7). In *tl2*, twelve and eleven
320 independent transgenic plants were obtained for *PtoSUS2* and *PtoVIN3*, respectively.
321 In *tl2*, trichomes on the adaxial surface of rosette leaves were malformed; few

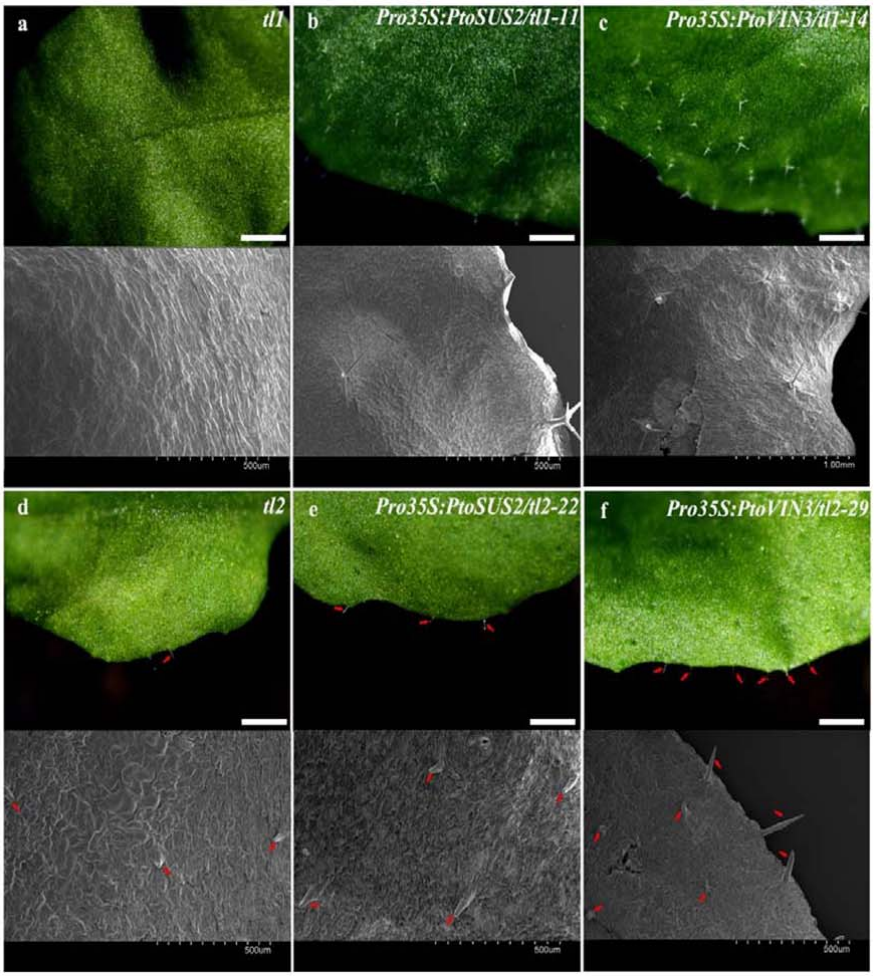

Fig. 8 Morphological characteristics of leaf trichome in *tl1/2* and their transgenic lines. Upper panel, trichome morphology under a stereomicroscope; scale bars = 1 mm. Lower panel, trichome morphology under a scanning electron microscope.

trichomes could be distinguished on the leaf edges. Overexpression of *PtoSUS2* or

PtoVIN3 led to greater malformed trichome density in rosette leaves, especially along the leaf margins. However, the glabrous phenotype of stems was unchanged in *tl2* transgenic plants (Fig. 8d-f; Fig. S8).

Changes in expression of trichome-related genes in *A. thaliana* overexpressing *PtoSUS2* and *PtoVIN3*

RT-qPCR was performed to evaluate the expression levels of the exogenous *PtoSUS2* and *PtoVIN3* genes. The two exogenous genes were strongly expressed in the transgenic lines but were undetectable in wild-type *A. thaliana* (Fig. 9). Endogenous genes associated with trichome development were also influenced by *PtoSUS2* and *PtoVIN3*. Overexpression of *PtoSUS2* or *PtoVIN3* in the wild-type increased the expression levels of *AtGL1*, *AtGL2*, *AtGL3*, *AtTTG1*, and *AtMYB23*. The expression of *AtGL1* had a trend similar to the trends of exogenous *PtoSUS2* and *PtoVIN3*; it played a major role in the trichome phenotype of transgenic lines. Similar results were obtained in mutants *tl1* and *tl2*. The phenotype of transgenic lines was closely related to gene expression. For individuals with the most pronounced phenotype in *tl1*, the expression levels of *AtGL2*, *AtGL3*, and *AtMYB23* were increased in *Pro35S:PtoSUS2/tl1-11* and *Pro35S:PtoVIN3/tl1-14*. In *tl2*, elevated expression levels of *PtoSUS2* and *PtoVIN3* led to increased expression of *ATGL1*, *AtGL3*, *AtMYB23*, and *AtTTG1*, especially the results in *Pro35S:PtoSUS2/tl2-22* and *Pro35S:PtoVIN3/tl2-29*. In different transgenic lines, the expression patterns of *AtGL1*, *AtGL3*, and *AtMYB23* showed trends similar to *PtoSUS2* and *PtoVIN3;* they were significantly increased, compared with *tl2*.

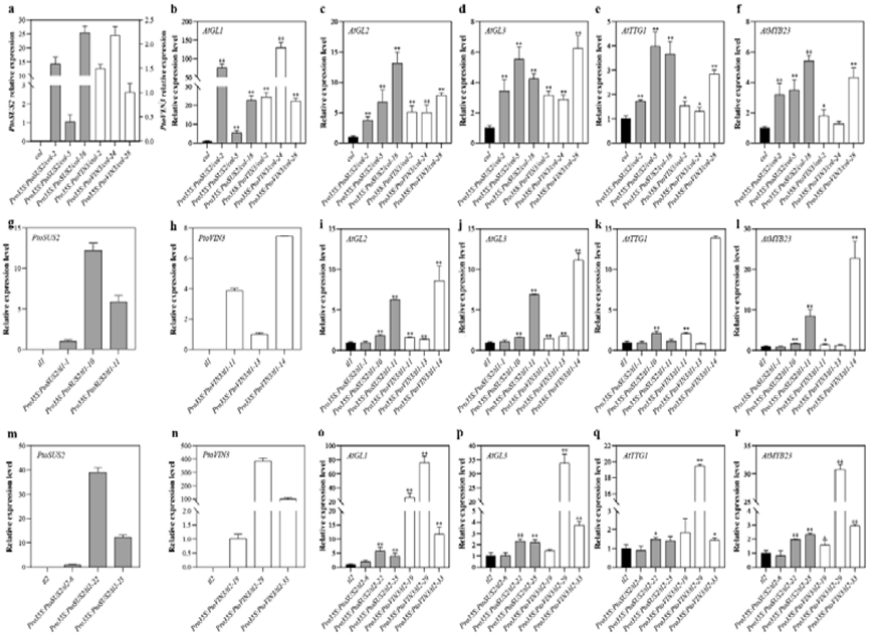

Fig. 9 Gene expression patterns in col, tl1/2, and their transgenic lines, as determined RT-qPCR. Bars represent ± SD, ** significant difference at $P < 0.01$, and * significant difference at $P < 0.05$.

Discussion

The catkin fibers are widely presented in poplar, and are similar to *A. thaliana* trichomes and cotton fibers. Numerous studies have indicated that sucrose is important for cotton fiber development (described in detailed in a follow-up), but similar research has not been carried out on poplar catkin fibers. Thus, in this study, SM was thoroughly analyzed during poplar catkin fibers development. And as we performed, sucrose degradation was the primary metabolic factor in catkin fibers.

Invertase has a function during the early development of poplar catkin fibers

According to the pH and subcellular localization, INVs can be divided into CWIN, VIN and NIN, all of which have different functions in plants (Ruan, 2014). Although CWIN and VIN both belong to the acid INV, they can be divided into two subgroups based on evolutionary relationships (Fig. S1a). In cotton fibers, *CWIN* appears to have no regulatory effect in cotton fibers, based on its low and steady expression level (Ruan and Chourey, 1998; Wang et al., 2010). Similarly, low CWIN activity was observed in poplar catkin fibers (Fig. 3c). In contrast with CWIN, VIN has been proven to participate in both initiation and elongation process in cotton fibers. The hexose produced by *GhVIN1* can be sensed by hexokinase, and then influences cotton fiber initiation though MYB TFs and auxin signaling (Wang et al., 2014). Meanwhile, hexose production can promote fiber elongation by increasing osmotic potential (Wang et al., 2010). Two other members of *VIN* were shown to be associated with cotton fiber development (Taliercio et al., 2010). Among them, *GhVIN2*, probably an allele of *GhVIN1*, was expressed primarily in elongating fibers, and

368 decreased during fiber maturation. Compared with results in cotton fibers, VIN
369 activity had a similar pattern during poplar catkin fibers development, indicating that
370 it probably plays the similar role in poplar catkin fibers. Thereinto, *PtoVIN3* has a
371 closest relationship with *GhVIN1* and *GhVIN2*. Surprisingly, NIN appeared to have a
372 function during the early development of poplar catkin fibers, differing from cotton
373 fibers. In contrast with their low expression levels in cotton fiber (Wang et al., 2010),
374 the NIN high activity in poplar catkin fibers led us to infer that NIN function differs
375 between poplar and cotton.

376 **Sucrose synthase has a close correlation with the elongation of poplar catkin**
377 **fibers**

378 In this study, SUS showed greater activity than INV (Fig. 3e, f), indicating a
379 potential role in poplar catkin fibers development. In cotton, SUS has been proposed
380 to play direct role in fiber yield and quality (Ahmed et al., 2018). *GhSUS3* was firstly
381 found to have high expression during cotton fiber development, and was thus thought
382 to have a role in that process (Ruan et al., 1997). Subsequently, fiberless mutant and
383 SUS-suppression transgenic plant confirmed the function of *GhSUS3* (Ruan and
384 Chourey, 1998; Ruan et al., 2003). In the fiberless mutant and transgenic plant, the
385 activity of SUS had a close relationship with fiber quality. Furthermore, Elizabeth et
386 al. (Elizabeth et al., 2011) found four isoforms of SUS that are expressed during
387 cotton fiber development. Three of these four members were similar to *GhSUS3*, with
388 high expression in fibers at the extension stage (at 8 days post-anthesis, dpa). Another
389 isoform, *GhSUSC*, was highly expressed in fibers during SCW synthesis (at 20 dpa)

390 (Elizabeth et al., 2011). Different results were obtained in *Gossypium arboretum*
391 (Chen et al., 2012), with four homologous genes having high expression during fiber
392 development (at 15 dpa), similar to our results in poplar. Two homologous members,
393 *PtoSUS1* and *PtoSUS2*, had high expression during catkin fibers development, while
394 the five members in other two clades had consistently low expression, similar to the
395 pattern in xylem (Zhang et al., 2011). High SUS activity was accompanied by a high
396 hexose to sucrose ratio, which was also observed in cotton fibers (Ruan et al., 1997).
397 High turgor pressure, caused by high hexose content, is required for rapid cell
398 extension (Ruan and Chourey, 1998). Therefore, SUS probably promotes catkin fibers
399 elongation by breaking down sucrose to increase turgor pressure. Although VIN
400 activity was also high during catkin fibers extension, fitting analysis suggested that
401 SUS plays the dominant role (Fig. 3).

402 **The cellulose synthase gene family and other important genes play roles during**
403 **poplar catkin fibers development**

404 The *CesA* gene family has been found to play roles during cotton fiber
405 development (Haigler et al., 2009; Kim et al., 2011; Kim et al., 2012). To elaborate,
406 the global genome analysis of the *CesA* gene family in cotton was performed (Li et al.,
407 2013). Members of the S1, S2 and S3 clades showed preferential expression during
408 SCW synthesis, while other members appeared to play a role in cotton fiber initiation
409 and extension. However, in poplar catkin fibers, members of S1-S3, which are
410 involved in SCW synthesis, all had low expression levels, although their levels
411 increased sharply during early development of catkin fibers except for *PtoCesA18*

412 (Fig. 5a-c). Members of P1-P3, which are involved in primary cell wall (PCW)
413 synthesis, played a more critical role in catkin fibers development as their high
414 expression. Thereinto, *PtoCesA17*, *PtoCesA2* and *PtoCesA15* are notable due to their
415 unusual expression pattern. Compared with cotton, less changes of *CesAs* expression
416 were observed in poplar catkin fibers, and we speculate that this difference underlies
417 the different qualities of catkin fibers in the two plants.

418 To further elucidate the role of sucrose in poplar catkin fibers, we investigated
419 TFs and other genes that may be involved in the development process (Fig. 5h, i,
420 Table S1). Similar expression patterns of *TF* gene family members were observed in
421 poplar catkin fiber and cotton fibers. *GhMYB109* (Suo et al., 2003), *GhDEL65*
422 (Shangguan et al., 2016) and *GhTTG1/3* (Humphries et al., 2005) are homologues of
423 *GL1* (MYB), *GL3/EGL3* (bHLH) and *TTG1* (WD40), respectively, in cotton and are
424 thought to exert effects on fiber initiation. *Pto(GL1)-L1*, *Pto(GL3)-L1* and *2* and
425 *Pto(TTG1)-L1*, which are the representatives in poplar, all showed increased
426 expression at the initiation stage of catkin fibers development. *GhMYB25* (MYB) and
427 *GhHox3* (HD-ZIP IV) were found to play regulatory roles in fiber extension
428 (Machado et al., 2009; Shan et al., 2014), however their homologues had delayed
429 expression in poplar catkin fibers, aside from *Pto(GhMYB25)-L1* (Fig. 5h). Notably,
430 *MYB186*, the homologue of *Pto(GhMYB25)-L4*, is vital in poplar leaf trichome
431 development (Plett et al., 2010; Patrick Bewg et al., 2022).

432 ***PtoSUS2* and *PtoVIN3* increased trichome density in *A. thaliana***

433 In poplar, *PtoSUS2* is widely expressed in roots, vegetative buds, and floral

434 catkins; its homologue *PtrSUS2* is highly expressed in xylem tissues and moderately
435 expressed in leaves and roots (Zhang et al., 2011; An et al., 2014). In this study,
436 *PtoSUS2* had higher expression than other members of the sucrose synthase gene
437 family during poplar catkin fibers development; it was significantly correlated with
438 SUS activity (Fig. 4, Table S4). Therefore, *PtoSUS2* may play a major role in poplar
439 catkin fibers development; this hypothesis was supported by the results of *PtoSUS2*
440 overexpression in *A. thaliana*. Overexpression wild-type plants had increased
441 trichome density on rosette leaves and stems, and transgenic plants of the glabrous
442 mutants produced more trichomes on rosette leaves (Fig. 7, Fig. 8). Another candidate
443 gene, *PtoVIN3*, was constitutively expressed in roots and leaves; it is also regulated by
444 abiotic and biotic stresses (Chen et al., 2015). In this study, overexpression of
445 *PtoVIN3* increased trichome density on rosette leaves and stems, indicating that it
446 functions with *PtoSUS2* to control catkin fibers development in poplar. Notably, the
447 increased density of trichomes is not due to enhanced vegetative growth in *A. thaliana*
448 transgenic lines (Fig. 7 h-j, Fig. S5). Trichome development in *A. thaliana* is
449 controlled by the *AtGL1-AtGL3-AtTTG1* complex (Walker et al., 1999; Bernhardt et
450 al., 2005; Bloomer et al., 2012; Pesch et al., 2015); *AtGL2* acts downstream of this
451 complex to control normal trichome development (Rerie et al., 1994). *AtMYB23*
452 controls trichome branching and initiation at leaf edges; it is functionally redundant
453 with *AtGL1* (Kirik et al., 2005). In wild-type plants overexpressing *PtoSUS2* and
454 *PtoVIN3*, the expression levels of all above-mentioned genes were increased. This
455 demonstrates the effect of *PtoSUS2* and *PtoVIN3* on trichome development in *A.*

456 *thaliana* at the molecular level. In different transgenic lines, the expression patterns of
457 *AtGL1* are closely like the expression of *PtoSUS2* or *PtoVIN3*. *GhSUS3*, which has a
458 close relationship with *PtoSUS2* in cotton, is implicated in cotton fiber cell initiation
459 and elongation (Ruan et al., 2003). Overexpression of *SUS* increases cotton fiber
460 length, while decreased expression of *GhSUS3* induces the fiberless mutation (Ruan
461 and Chourey, 1998; Ahmed et al., 2018). Additionally, high vacuolar invertase activity
462 is important for cotton fiber development; the homologue of *PtoVIN3*, *GhVIN1*, is a
463 key factor in that process (Wang et al., 2010). *GhVIN1* regulates fiber initiation from
464 the ovule epidermis, probably via sugar signaling with *cis*-acting elements of the
465 sugar-response element in *GhMYB25* and *GhMYB25like* (Wang et al., 2014), which is
466 similar to poplar catkin fibers development. The promoter regions of *Pto(GL1)-L1/2/3*,
467 the homologues of *AtGL1*, and *Pto(GhMYB25)-L1/2/3/4*, the homologues of
468 *GhMYB25*, have also been analyzed in poplar. SURE motifs (i.e., sugar-responsive
469 elements) (Grierson et al., 1994), were found in the promoter region of
470 *Pto(GhMYB25)-L1/2/4* (Fig. S9). Additionally, the SP8B-like motif (TACT(X)TT),
471 which is similar to another sugar-responsive element (Ishiguro and Nakamura, 1992),
472 was found in the promoter regions of *Pto(GL1)-L2* and *Pto(GhMYB25)-L3/4*.
473 Therefore, we speculate that *PtoSUS2* and *PtoVIN3* probably regulate catkin fibers
474 development via sugar signaling. This result was confirmed by wild-type and *tl2*
475 transgenic plants because *AtGL1* showed similar trends to *PtoSUS2* and *PtoVIN3*.
476 In this study, SM was analyzed in detail in the context of poplar catkin fibers
477 development, and sucrose degradation was found to play the dominant role in the

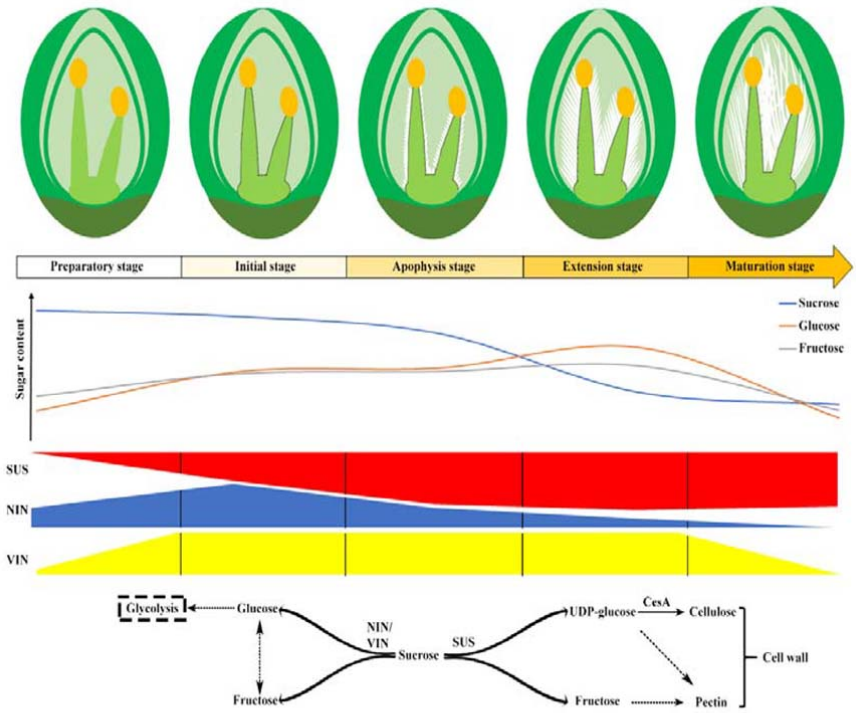

Fig. 10 Potential regulatory model of poplar catkin fibers development

process. As shown in Fig. 10, sucrose is consumed rapidly as poplar catkin fibers

479 mature, especially at the extension stage. VIN and SUS, probably derived by *PtoVIN3*
480 and *PtoSUS2*, were thought to perform the function. Furthermore, the PCW
481 synthesis-related *PtoCesAs* may play a major role in cellulose synthesis. As an
482 important metabolic process in poplar catkin fibers, analyzing the regulatory
483 mechanism of SM is not only helpful for us to understand the regulatory mechanism
484 in woody plants, and for identifying key candidate genes with high breeding value in
485 *P. tomentosa*, but also provides a foundation for solving environmental problems
486 caused by poplar catkins though genetic engineering.

487 ## Materials and Methods

488 **Plant materials and histological analysis**

489 Live branches of adult female *P. tomentosa* were collected in February 2018, at
490 which time the flower buds were undergoing megasporogenesis and embryo sac
491 formation (An, 2010). The branches were placed in a greenhouse (25 ± 2°C, 70%
492 humidity) for hydroponic culture, and samples were collected as described previously
493 (Ye et al., 2014). Capsules were collected after the branches had been cultured for 0,
494 24, 34, 48, 58, 72, 96 and 120 h. Samples collected at these eight time points were
495 subjected to histological analysis; according to the analysis results, the samples from
496 the last five time points, which were related to catkin fibers development, were
497 subjected to RNA extraction as well as sugar content and enzyme activity analyses.
498 RNA extraction and histological analysis were also performed as described previously
499 (Ye et al., 2014).

500 The Columbia ecotype (Col) and two trichome mutants, *trichome less 1* (*tl1*,

501 SALK_133117C) and *trichome less 2* (*tl2*, SALK_039825C), were used in this study.
502 The seeds were sterilized in 5% sodium hypochlorite solution for 15 min, and then
503 placed in half-strength Murashige-Skoog (MS) medium at 4℃ for 72 h. Subsequently,
504 the seeds were cultivated in an incubator at 22℃ under LD (16 h light/8 h dark)
505 condition for 7 days, then transplanted into nutrient soil with added perlite.

506 **Quantitation of sugar content and enzyme activities**

507 Samples of 1 g (dry weight, DW) were added to 5 mL distilled water or 90%
508 alcohol, and the mixture was extracted through ultrasonication for 10 min.
509 Subsequently the mixture was centrifuged at 4℃ and 12,000 rpm for 10 min. The
510 mixture was filtered through a 0.45-μm hydrophilic filter prior to analysis. The
511 concentrations of sucrose, fructose and glucose were analyzed by high-performance
512 liquid chromatography (HPLC) using an Agilent 1260 HPLC system (Agilent
513 Technologies, Santa Clara, CA, USA), and a carbohydrate column (4.6 × 150 mm, 5
514 μm, Agilent Technologies) was maintained at 35 ℃. The sugar concentrations were
515 quantified according to a standard solution (Sigma, St. Louis, MO, USA), and defined
516 in terms of $mg \cdot g^{-1}$ DW.

517 The activities of SUS and SPS were analyzed according to the method of Yang et
518 al. (Yang et al., 2013) with some modifications. Sample of 5 g (fresh weight, FW) was
519 weighed and ground in liquid nitrogen for 5 min, and then extraction buffer (see
520 Methods S1) was added, followed by mixing and centrifuging at 20,000 rpm for 30
521 min. Subsequently, the supernatant (1 mL) was desalted using a Sephadex G25 PD-10
522 column with desalination buffer (see Methods S1). For SUS analysis, 1 mL of reaction

523 mixture I (see Methods S1) was incubated at 37℃ for 30 min. Subsequently, the
524 mixture was placing in boiling water for 5 min to stop the reaction. The optical
525 density (OD) of the mixture was measured at 540 nm using an ultraviolet
526 spectrophotometer (Cary 5000, Agilent). For SPS activity, 200 μL reaction mixture II
527 (see Methods S1) was incubated at 30℃ for 30 min. Subsequently, 200 μL NaOH (5
528 mol/L) was added, and the mixture was placing in boiling water for 10 min to stop the
529 reaction. After cooling, 3.5 mL of anthrone (0.15%, dissolved in 80% H_2SO_4) was
530 added, and the mixture was placed in a 40 ℃ water bath for 20 min. The OD of the
531 mixture was measured at 620 nm using the Cary 5000 instrument. The activities of
532 neutral/alkaline, vacuolar, and cell wall INVs (NIN, VIN and CWIN, respectively)
533 were analyzed according to previously described methods (Chen et al., 2015), and the
534 enzyme activities were recorded as $\mu mol \bullet g^{-1}$ $FW \bullet h^{-1}$.

535 **Candidate gene identification and RT-qPCR**

536 To determine the expression levels of key genes during the development of
537 catkin fibers, primers were designed to amplify fragments specific to each gene.
538 Genes from *A. thaliana* and cotton were used as queries for a Basic Local Alignment
539 Search Tool for Proteins (BLASTP) search against the protein sequences from the *P.*
540 *tomentosa* genomic data (An et al., 2022). The sequences of the primer pairs used for
541 each gene are listed in Table S2.

542 Total RNA was used for first-strand cDNA synthesis with the Reverse
543 Transcription System I (Promega, Madison, WI, USA), then diluted 1:50 in water. The
544 diluted cDNA was used as a template, and PCR amplification was performed with the

545 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using
546 the following program: 94℃ for 5 min (one cycle); 94℃ for 30 s, 60℃ for 20 s, 72℃
547 for 30 s (35 cycles); 72℃ for 5 min. The *PtActin* gene was used as an internal
548 reference gene, as described previously (Chen et al., 2018), and the SYBR Premix Ex
549 Taq kit (Takara, Shiga, Japan) was used as the reaction solution. All analyses were
550 performed with three technical and three biological replicates.

551 **Phylogenetic tree and co-expression network**

552 To confirm the evolutionary relationships in SM related-gene families, all genes
553 were compared with their homologues from poplar, *A. thaliana* and cotton through
554 multiple sequence alignment (Table S3) using the ClustalW program within MEGA
555 7.0 software. Phylogenetic trees were draw with MEGA 7.0 using the
556 neighbor-joining method with complete deletion, and 1,000 replications were used for
557 bootstrap analysis. Gene co-expression network analysis was performed to identify
558 correlated genes using R software (v3.6.3), and the networks were visualized using
559 Cytoscape (v.3.7.1).

560 **Transformation and gene expression analysis in *A. thaliana***

561 The open reading frame sequences of *PtoSUS2* and *PtoVIN3* were amplified and
562 cloned into the binary vector pCAMBIA 1301 to generate overexpression vectors.
563 Transgenic lines were obtained by the *Aarobacterium tumefaciens*-mediated method
564 established by (Zhang et al., 2006). Transgenic plants were selected on half-strength
565 MS medium containing 25 mg/L hygromycin. Gene expression analysis was
566 performed as above, except that relative expression levels were calculated using the

567 $2^{-\Delta\Delta Ct}$ method. The sequences of the primer pairs used for trichome-related genes in *A.*
568 *thaliana* and exogenous genes are listed in Table S2.

569 **Morphological analysis and scanning electron microscopy (SEM)**

570 The sixth rosette leaves, stems (morphological bottom), and bottom-most cauline
571 leaves were used for trichome comparison with a stereomicroscope (Stemi 305,
572 ZEISS). The number of trichomes in rosette leaves was determined in plants that had
573 been cultured for 30 days after germination (DAG). Five biological replicates were
574 performed. For SEM, the samples were cut into 1 × 1-cm pieces, and dehydrated in a
575 vacuum freeze dryer (FreeZone 4.5L, Labconco) for 48 h. Images were obtained using
576 a scanning electron microscope (S-3400N, Hitachi, Japan).

577 **Statistical Analysis**

578 Sugar content, enzyme activity and gene expression data were analyzed through
579 one-way analysis of variance (ANOVA) using SPSS software (version 22.0 for
580 Windows; SPSS Inc., Chicago, IL, USA). The correlations between genes expression
581 patterns in SM and sugar contents was analyzed by Pearson correlation using the same
582 software. Two-tail test was used to test for significance level as $P < 0.05$ and $P < 0.01$.

583 **Accession numbers**

584 The gene sequences can be found under the accession numbers: *PtoSUS2*
585 (ON534005), *PtoVIN3* (ON534006).

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Authors contributions

Z.C. and X.Y. designed the work; X.Y., T.Z. and N.Y. performed experiments and analyzed data; Co-expression network analysis was carried out by P.R.; X.Y. and T. Z. drafted the manuscript; and Z.C., G.L., L. J. and X.A. revised the manuscript. X. Y. and T. Z. contributed equally to this work.

Supporting information

Fig. S1 Phylogenetic tree of the SM-related gene families.

Fig. S2 Analysis of cis-acting elements for SM-related genes in *P. tomentosa*.

Fig. S3 Tissue-specific expression analysis of *PtoSUS2* and *PtoVIN3*.

Fig. S4 Morphological characteristics of trichome on rosette leaves and stems in *A. thaliana* wild-type and transgenic lines.

Fig. S5 Characteristics of root, rosette leaves, and whole plant in *A. thaliana* wild-type and transgenic lines.

Fig. S6 Changed in trichome density on leaves during leaf development in *A. thaliana* wild-type and transgenic lines.

Fig. S7 Morphological characteristics of trichome on leaves and stems in transgenic lines of *tl1* and *tl1*.

Fig. S8 Morphological characteristics of trichome on leaves and stems in transgenic lines of *tl2* and *tl2*.

611 **Fig. S9 Analysis of sugar signal-related cis-acting elements for *Pto(GL1)-L1/2/3***

612 **and *Pto(GhMYB25)-L1/2/3/4*.**

613 **Table S1 Comparison of potential TFs and other genes in poplar and cotton**

614 **Table S2 Primers used for RT-qPCR**

615 **Table S3 Accession numbers of genes used for phylogenetic tree construction**

616 **Table S4 Correlations between the expression patterns of genes involved in SM**

617 **and sugar contents in poplar catkin fibers development**

618 **Methods S1: reagent formulation for enzyme activities quantitation**

619

620

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