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Research Article
Investigation of the Effect of AtWIN1/SHN1 Overexpression on Poplar Trees

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Abstract

Background: Interactions between plants and the environment occur primarily at the leaf level. The plant cuticle consists of a menagerie of lipids, waxes and polymers merging to form an insoluble membrane to protect plant leaves from contamination. In *Arabidopsis*, wax Inducer1/shine1 (WIN1/SHN1) and its family members have demonstrated roles in wax biosynthesis and cutin formation, the primary component of the cuticle layer composition. Constitutive overexpression of the *Arabidopsis* WIN1/SHN1 (wax inducer1, shine1, *At*WIN1/SHN1) gene has led to improved water stress tolerance, altered stomatal densities and morphological changes in leaf and flower development in *Arabidopsis*. Other expression studies using the WIN1/SHN1 gene have shown heightened defense responses and malformations of the cuticle. **Materials and Methods:** Constructs of *At*WIN1/SHN1 were developed and used to genetically transform poplar trees that were later analyzed to verify presence of the construct. **Results:** A total of five transgenic lines with 100 ramets each were generated for water-use efficiency testing. All lines displayed glossy leaves, decreased stomatal densities and improved water-use efficiencies. Several lines presented similar phenotypes and water-use efficiencies but others were unique. **Conclusion:** This study used an inventive method to generate ornamental trees with improved water-use efficiencies as a proactive method for protecting water resources and resisting drought.

Key words: *Arabidopsis*, DREB, morphology, poplar, stomatal density, water-use efficiency, wax, WIN1/SHN1

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

In plants, the leaf cuticle and all other aerial surfaces are covered with a waxy coating consisting of two main polymers cutin and cutan. Cutin, cutan and other waxes form a water-repelling barrier to help protect plants against additional water loss during transpiration. Variation in epicuticular wax composition and its inherent properties are seen in many plant species and has been shown to be involved in drought tolerance, disease resistance, light reflection and use as an insect deterrent[1-8]. Environmental conditions such as water availability, light intensity and humidity may also influence wax development[9-12]. Not all environmental factors were shown to increase epicuticular wax levels as UV-B radiation resulted in decreased overall wax content and altered composition in tobacco (*Nicotania tabacum* L.)[13]. Plants with thicker cuticle layers have improved drought tolerance capabilities as extraneous water is prevented from transpiring through leaves. Studies of *Arabidopsis* and maize (*Zea mays*) models have revealed a number of genes involved in the wax biosynthesis pathway[14-17].

In *Arabidopsis* wax inducer1/shine1 (WIN1/SHN1) is a transcription factor containing a single AP2 (Apetala2) domain in Ethylene Response Factor (ERF) subfamily B-6 of ERF/AP2 transcription factors (EREBP). Many of the 141 *Arabidopsis* EREBP family genes have been linked to developmental (flower formation), metabolic (jasmonate, ethylene) and abiotic stress (drought, cold) response pathways[18-20]. The WIN1/SHN1 mutant was isolated by activation-tagging and is a gain-of-function mutant. The WIN1/SHN1, a small protein with 199 amino acid residues has been reported to be involved in several developmental processes including cutin biosynthesis, DNA binding, ethylene mediated signaling and wax metabolism. There are 12 members of this subfamily however not all display the glossy leaf phenotype, increased stem wax production and drought tolerance abilities shown in *Arabidopsis*. The SHN1 and 2 other gene family members, shine2 (AT5G25390), with 189 amino acid residues and shine3 (AT5G11190), with 186 amino acid residues do exhibit increased glossiness when overexpressed in *Arabidopsis*[15].

The WIN1/SHN1 gene is involved in many developmental processes including cutin biosynthesis, DNA binding, ethylene mediated signaling and wax metabolism. The WIN1/SHN1 has been shown to induce wax formation in overexpressing plants up to 4.5-fold greater than control plants[21] and resulted in up regulation of other wax biosynthesis genes, an indication that WIN1/SHN1 expression influenced other wax biosynthesis pathway members[15,22]. Recent study by Ambavaram *et al.*[23] and Shi *et al.*[24] indicated that WIN1/SHN1 transcription factors are responsible for the regulation and accumulation of cell wall biosynthesis genes, such as cutin, lignin and cellulose. Induced WIN1/SHN1 gene expression can alter cuticular wax composition and enhance drought tolerance, resulting from decreasing stomatal density in *Arabidopsis*[17,25]. Recent study by Sela *et al.*[8] indicated that overexpression of AtWIN1/SHN1 also leads to unique defensive reactions in response to pathogens. The study presented here was an attempt to visualize the impact and initial effects of the overexpression of AtWIN1/SHN1 in poplar when compared to wild-type (WT).

MATERIALS AND METHODS

Plant materials: Transgenic trees were generated *in vitro* from the transformation of wild-type poplar *Populustremula* x *P. alba* (717-1B4) leaf pieces under a 16:8 h photoperiod at a temperature of 23$\pm$2°C. Light levels remained steady at 300 µmol m^{-2} sec^{-1} with fluorescent bulbs. The *At*WIN1/SHN1 construct was generated from the pBI121 backbone vector under control of the cauliflower mosaic virus (CaMV) 35S promoter. The GUS (GUS: Beta-glucuronidase) was removed from the vector before insertion of AtWIN1/SHN1. Poplar plants were transformed using *Agrobacterium*-mediated genetic transformation using a protocol was adapted from Noel *et al.*[26], Ma *et al.*[27] and Cseke *et al.*[28]. All 717-1B4 shoot cultures were grown on callus and shoot induction media formulated according to Ma *et al.*[27]. Only leaves were used rather than inclusion of stems and petioles for transformation. Transformation efficiencies for this experiment were 2% and produced 5 transgenic poplar lines (35S::*At*WIN1/SHN1-12, line 12, 35S::*At*WIN1/SHN1-18, line 18, 35S::*At*WIN1/SHN1-19, line 19, 35S::*At*WIN1/SHN1-24, line 24 and 35S::*At*WIN1/SHN1-25, line 25). Only apical meristems were used as explants material for propagation to ensure an abundance of leaf material for later experiments. Wild-type materials were grown in Magenta GA-7 culture vessels (Magenta Corp., Chicago, IL) containing 60 mL rooting medium composed of (1 mM 2-[N-morpholino]ethanesulfonic acid (MES), 0.5 mM 1,2,3,5/4,6-hexahydroxycyclohexane (myo-inositol), 1 mM L-glutamine, 20 g L^{-1} sucrose, 4.3 g L^{-1} Murashige and Skoog Basal salts (MS, M499, PhytoTechnology Laboratories, Shawnee Mission, KS), liquid organics and 7.6 g L^{-1} Difco-Bacto agar. To control *Agrobacterium* contamination 1.6 mM timentin was added to callus and shoot induction media.

Growth conditions: The five transgenic lines obtained were propagated every 3 weeks until 100 plantlets per line were generated. Seventy-five plantlets from each line were rooted and established in tissue culture. Twenty-five additional plantlets from each line were chosen for acclimatization and moved to a cleared greenhouse zone using sealed transport containers to prevent contamination from outdoor flora. Greenhouse plants were grown under a 16:8 h photoperiod at a temperature of $23\pm2°C$. Light levels ranged from 300-400 μmol m^{-2} sec^{-1} in natural light supplemented with fluorescent bulbs. Relative humidity ranged from 55-65%. Nighttime temperatures ranged between $18.3\pm2.1°C$. Plants were grown in 20 cm round pots with soil mix as a 4:1 mixture of sun GroRedi earth plug and seedling mix (Sun Gro Horticulture). Plants were distributed in a complete random block design, watered every third day and acclimatized to greenhouse conditions for 3 weeks prior to the start of data collection.

DNA extraction and amplification: Genomic DNAs from wild-type (ornon-transgenic) and transgenic plants were isolated using the DNeasy plant Maxi kit (Qiagen). Original *Arabidopsis* gene amplification was done via polymerase chain reaction (PCR) analysis with gene-specific sense (5'-AAGAATGGTACAGACGAAGAA G-3') and anti-sense (5'-TTAGTTACAAACACCAATACTTTATTA-3') primers followed by nucleic acid sequencing (http://www.genomics.purdue.edu/~core/) for additional confirmation. The NCBI BLAST database (http://blast.ncbi.nlm.nih.gov/) was used to obtain homology information for *Arabidopsis* and poplar. Nucleic acid alignments were generated using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The poplar putative *Pta*WIN1/SHN1 sequence was isolated and amplified using the consensus sequence identified from ClustalW2 and COBALT alignment data (http://www.ncbi.nlm.nih.gov/tools/cobalt/cobalt.cgi).

Physiological measurements: Chlorophyll values were recorded using a SPAD-502 meter (Konica-Minolta, Japan) and are hereafter referred to as SPAD values. Five groups of 15 transgenic (3 per line) and 15 wild-type plants of uniform height were selected per group to establish both biological and scientific replicates. Three independent measurements were taken per plant.

A portable photosynthesis system, the LI6400X (LI-COR., Lincoln, NE, USA) was used to obtain instantaneous transpiration data. All plants were watered to capacity and allowed to drain before water was withheld. Measurements were taken at 11 am for three consecutive days. All transpiration data were obtained from three groups at 4 increments (24 h (0 day), 5, 10 and 15 days). Plants were watered to capacity and allowed to drain after 10 days measurements were obtained. Gravimetric water-loss measurements were taken from five groups of 15 transgenics (3 per line) and 5 wild-type plants of uniform height. All plants were watered to capacity and allowed to drain before water was withheld. Plants were placed in a growth chamber programmed with the same conditions as the greenhouse. Openings in plant pots were covered with plastic wrap and sealed with packing tape. Plastic wrap was also wrapped around the base of the plant and the edges were sealed to prevent evaporation of water from the soil. Water-loss data were collected 24, 48, 72 and 96 h after withholding water. Carbon isotope data were collected from oven dried leaf samples after pulverization in liquid nitrogen. Samples were analyzed for a ratio of $\delta^{13}C/\delta^{12}C$ (expressed relative to the PDB standard) by the Idaho State Stable Isotopes Lab (http://www.uidaho.edu/cnr/research/facilities/stableisotopeslab). All data were analyzed using SAS9.3 (http://www.SAS.com)[29].

Chlorophyll leaching: Chlorophyll was leached from a single mature leaf from both WT and transgenic plants into tubes containing 30 mL room temperature 80% ethanol for 2 h from transgenic and wild-type leaves according to the protocol of Aharoni *et al.*[15]. Micromolar concentration data was obtained using the formula[30]:

$$\text{Chlorophyll } (\mu\text{mol}) = \frac{7.93\,(\text{A664}) + 19.53\,(\text{A647})}{\text{Gram tissue fresh weight}}$$

Experiment performed in triplicate.

Safranin-O staining: Leaves from transgenic poplar overexpressing *At*WIN1/SHN1 were destained in 9:1 ethanol/acetic acid before being rinsed with water and stained with 1 ug mL^{-1} safranin-O for 1 h. After staining, the leaves were rinsed with water again and water-mounted onto coverslips. Differential Interference Contrast (DIC) images were captured with a Zeiss LSM710 microscope (Carl Zeiss, Inc.). Images were all taken from a consistent location three-fifths of the way down the leaf from tip-stem and close to the mid-vein (about 1 cm to the right). Images were obtained from 3 individual plants from each of the 5 lines.

Cryogenic-SEM: Tissues samples for cryogenic scanning electron microscopy surface examination were prepared and imaged at the life sciences microscope facility (Purdue University, West Lafayette, IN.). All leaf samples were cut into 1.0×0.4 cm pieces. Tissue sections were affixed to the slide

using Tissue Tek (Sakura Finetek). Samples were frozen in slush liquid nitrogen to -160°C and then transferred to a GATAN CT2500 (Abingdon, Great Britain) pre-chamber, which was cooled to -160°C. The samples were sublimed for 3 min, sputter coated with platinum and transferred to the FEI NOVA nano-SEM field emission SEM microscope (http://www.fei.com), cryostage. Images were recorded using the through-the-lens (TLD) or Everhart Thornley (ET) detector operating at 5 kV accelerating voltage and ~4.8-5.0 mm working distance. Cryo-SEM does not require critical point dehydration common to non-cryogenic SEM[31]. All images were acquired from plants grown side by side under the same growth conditions.

RNA extraction, RT-PCR and qRT-PCR: *Arabidopsis* flowers, leaves and stems were harvested from five control plants while leaves and stems were harvested from 5 poplar plants per transgenic line and a 717-1B4 control line. Tissues were immediately frozen in liquid nitrogen and stored overnight at-80°C. Total RNA from each of the six lines was extracted using the RNeasy Plant Mini kit (Qiagen) and treated with RNase-free DNase I (Invitrogen). The RNA quality and quantity was assessed using a NanoDrop 8000 spectrophotometer (Thermo Scientific) followed by gel electrophoresis on a 1% RNase-free agarose gel. First strand cDNA was synthesized from 5 µg total RNA per sample using the iScript cDNA synthesis kit (Bio-Rad) following the supplied protocols. Reverse transcriptase PCR was performed in 8-tube strips Quantitative RT-PCR (qRT-PCR) was then performed in 96-well plates with a Bio-Rad iQ5 real-time PCR detection system using Power SYBR Green PCR Master Mix (Applied Biosystems). The qRT-PCR reaction was assembled and optimized following manufacturer's guidelines. Each reaction consisted of 2 µL of the cDNA sample (equivalent to 20 ng total RNA), 0.2 µM of each primer and 7.5 µL of the PCR SYBR master mix added to a 15 µL reaction mixture. Reaction conditions for all primers were: 95°C for 5 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Melting curve analyses were performed at 95°C for 15 sec, 60°C for 1 min, 95°C for 15 sec, 60°C for 15 sec and presence of a single peak confirmed primer specificity. Each qRT-PCR reaction was performed in triplicate and each line was replicated in triplicate. Gene specific primers were designed for the poplar WIN1/SHN1 gene and the internal controls (Table 1S). The β-actin gene was used to detect DNA while 18S rRNA and ubiquitin were used as standard housekeeping genes. Real-time PCR data were analyzed using the $2^{-\Delta\Delta CT}$ method[32].

Statistical analyses: Statistically significant differences were computed using statistical applications software (SAS 9.3.1, SAS Institute Inc.)[29]. Average readings are represented. Data are the Means±SEM. Each study was repeated in triplicate. Means represented with the same letter were not significantly different at $p<0.05$.

RESULTS

There are a number of known wax biosynthesis genes within the *Arabidopsis* genome (Table S2) and use of ClustalWand COBALT (http://www.ncbi.nlm.nih.gov/) revealed that several of these genes are closely related (Fig. 1a). Comparison of the AtWIN1/SHN1(*Arabidopsis* wax inducer1) and potential poplar 717-1B4orthologs revealed several homologous transcripts related to the *Arabidopsis* gene (Fig. 1b, c).

Poplar overexpressing AtWIN1/SHN1 produced leaves with significantly lower stomatal densities and greater luster and gloss than those of control plants (Table 1). This study

Table 1S: Primers used for RT-PCR and qRT-PCR amplification

Name	Forward primer (5'-3')	Reverse primer (5'-3')	Purpose
*At*WIN1/SHN1	ACAGACGAAGAAGTTCAGAGGT	ATTTTGGCTGTAAATCAGCAATGA	RT-PCR
*Pta*WIN1/SHN1	ATGGTACAATCAAAGAAGTTCA	CCTGTTAAGTAGCTCCTCTATCAT	RT-PCR
*At*WIN1/SHN1	AGCTCCTCCCAAGAGACTAC	TTTCAACTTCAGTGGTCGGAGCAA	qRT-PCR
β-actin	ACCCTCCAATCCAGACACTG	TTGCTGACCGTATGAGCAAG	qRT-PCR
18S rRNA	GGTAACGGGGAATCAGGGT	TGCCTTCCTTGGATGTGGT	qRT-PCR
Ubiquitin	CGTGGAGGAATGCAGATTTT	GATCTTGGCCTTCACGTTGT	qRT-PCR

Table 1: Effect of overexpression of *At*WIN1/SHN1 in poplar on basic leaf parameters. Means with same letter were not significantly different at the p<0.05 level of probability

Line	Stomatal density (mm^{-2})	Stomatal Density (% ↓ vs WT)	TD (vs WT)	SPAD (cm^2)	WUE_{ti} ($\delta^{13}C/\delta^{12}C$)	WUE_{ti} (% ↑ vs WT)
12	314.9 ± 12.1^{bc}	14.5	↓	46.0 ± 1.3^{a}	-15.86±0.1	20.4
18	297.6 ± 9.5^{d}	19.7	↓	45.0 ± 1.0^{b}	-15.64±0.2	21.0
19	304.3 ± 8.1^{cd}	18.3	↓	41.3 ± 1.0^{d}	-16.37±0.2	17.7
24	317.8 ± 7.1^{bc}	15.0	↓	41.6 ± 1.6^{d}	-18.16±0.4	7.4
25	324.9 ± 9.6^{b}	12.5	↓	43.1 ± 1.6^{c}	-18.42±0.1	7.6
WT	371.1 ± 11.2^{a}	–	–	37.2 ± 1.8^{e}	-19.74±0.3	–

↓: Decreased, ↑: Increased, TD: Trichome density, WUE_{ti}: Time-integrated water-use efficiency (based on leaf carbon isotope analysis), WT: Wild-type

Table 2S: Summary of the most often studied wax biosynthesis genes and their respective phenotypes

			Expression profile			Leaf Phenotype					
Name	Locus ID	Functional activity (or enzyme encoded)	Location	SHN1ox	WL	SD	SI	TN	TB	MCW	References
CER1	*At*1g02205	Aldehyde decrbonylase leaves, petals	Stem, flower,	↑↑	n/a	↑		n/a	↓	n/a	Aarts *et al.*[40] and Hansen *et al.*[41]
CER2	*At*4g24510	Co-A acyltransferase	Stem, flower, leaves, GC	↑	n/a	n/a	n/a	n/a	n/a	↓	Xia *et al.* 1996[42]
CER[a]	*At*5g57800	Sterol desaturase/SC dehydrogenase-RL	Stem, flower leaves, root	n/a	n/a		↓	*	n/a	↓	Hannoufa *et al.*[43] and Chen *et al.*[44]
CER5	*At*1g51500	ABC transporter	Stem, flower, leaves, root	n/a	n/a	n/a	n/a	n/a	n/a	n/a	Pighin*et al.*[45]
CER6[b]	*At*1g68530	Ketoacyl-CoA synthase6 Biosynthesis of VLCFA	Trichome, GC stem, root	n/a	n/a		↑	n/a	n/a	↓	Fiebig *et al.*[46]
FDH[c]	*At*2g26250	Epidermis-specific Keto-acyl-CoA synthase10	Stem, flower, leaves, GC	n/a	n/a	Δ	Δ	Δ	n/a	↓	Yephremov *et al.*[47] and Pruitt *et al.*[48]
GL1	*AA*B87597	Transports wax biosyn-thesis products, mediates membrane fusion	Silk, anther leaves, root	n/a	n/a	Δ	Δ	*	n/a	Δ	Hansen *et al.*[41] and Sturaro *et al.*[16]
GL2	*At*1g79840	Affects epidermal cell identity, root hair growth	Trichome, root flower	n/a	n/a			↓	n/a	n/a	Szymanski *et al.*[49] and Ohashi *et al.*[50]
GL8	NP_001105406	3-ketoacyl reductase Biosynthesis of VLCFA	Stem, fruit leaves, root	n/a	n/a				n/a	n/a	Xu *et al.*[51] and Dietrich *et al.*[52]
GL15	NP_001105890	Controls leaf transition from juvenile to adult	Leaves	n/a	n/a	n/a	n/a	n/a	n/a	n/a	Moose and Sisco[53,54]
HIC[d]	*At*2g46720	Ketoacyl-CoA synthase13 Biosynthesis of VLCFA	Rosette, flower, cauline leaves	n/a	n/a		Δ	n/a	Δ	n/a	Gray *et al.*[55]
KCS1	*At*1g01120	Ketoacyl-CoA synthase1 critical fatty acid elongase	Leaves, root stem, flower	↑	n/a	Δ	Δ	Δ	n/a	n/a	Todd *et al.*[56]
SHN1	*At*1g15360	Ethylene response factor B-6 subfamily member	Petals, leaves	↑	↑			↓	Δ	↑	Aharoni *et al.*[15] and Broun *et al.*[21]
SHN2	*At*5g25390	Ethylene response factor B-6 subfamily member	Anther, silique	n/a	↑			↓	Δ	↑	Aharoni *et al.*[15]
SHN3	*At*5g11190	Ethylene response factor B-6 subfamily member	Root cap, anther	n/a	↑			↓	Δ	↑	Aharoni *et al.*[15]

[a]WAX2, [b]CUT1/KCS6, [c]KCS10, [d]KCS13, *↓Trichome size, Δ: Changed, WL: Water loss, RL: Reductase-like, SC: Short-chain, SD: Stomatal density, SI: Stomatal index, TN: Trichome number, TB: Trichome branching, MCW: Mutant cuticular wax, VLCFA: Very long-chain fatty acids, n/a: Not available

showed decreased stomatal density in all lines however the greatest reduction was in line18 (19.7%) while line 25 was least affected (12.5%). Examination of transgenic poplar chlorophyll content showed a significant increase in greenness when compared to control plants. The greatest increase in greenness was seen in line 18 (46.0±1.3) when compared to WT (37.2±1.8) (Table 1). A chlorophyll leaching assay indicated that chlorophyll leached more readily from transgenics than WT plants over time (Fig. 2).

Implementation of a water-withholding experiment revealed that no leaf abscission or leaf rolling phenotypes, often indicative of water stress, were observed in the transgenic poplar lines before, during or after the water-withholding treatment. Transpiration measurements indicated that transgenics lost smaller volumes of water each day than control plants when subjected to artificial drought (water-withholding) conditions (Fig. 3). When all plants were rehydrated to capacity at the conclusion of the experiment, transgenic plants showed no ill effects but control plants were slower to recover and abscised numerous leaves. Verification of increased time-integrated water-use efficiency (WUE_{ti}) using stable carbon isotopes revealed that the poplar transgenics all had greater WUE_{ti} than control plants. The greatest improvement was seen in line 18 (21%) and these plants were the only line that exhibited marginally (not significant) slower development and stature when compared to controls (data not shown).

Use of RT-PCR analysis showed that *At*WIN1/SHN1 transcript levels in transgenics varied by tissue. Comparison of *At*WIN1/SHN1 expression within *Arabidopsis* and transgenic poplar tissues showed levels were highest in flowers rather than stems or leaves for *Arabidopsis*. Leaves, as opposed to stems, had higher levels of expression in both *Arabidopsis* and poplar (Fig. 4).

Normal wax phenotype and trichome density were observed in WT (Fig. 5a-c) for comparison to transgenics. Cryogenic Scanning Electron Microscopy (CryoSEM) showed trichome densities were lower in transgenic lines overexpressing *At*WIN1/SHN1 than control plants (Fig. 5b-e) and numerous arrested stomata were visualized (Fig. 5f, g) in

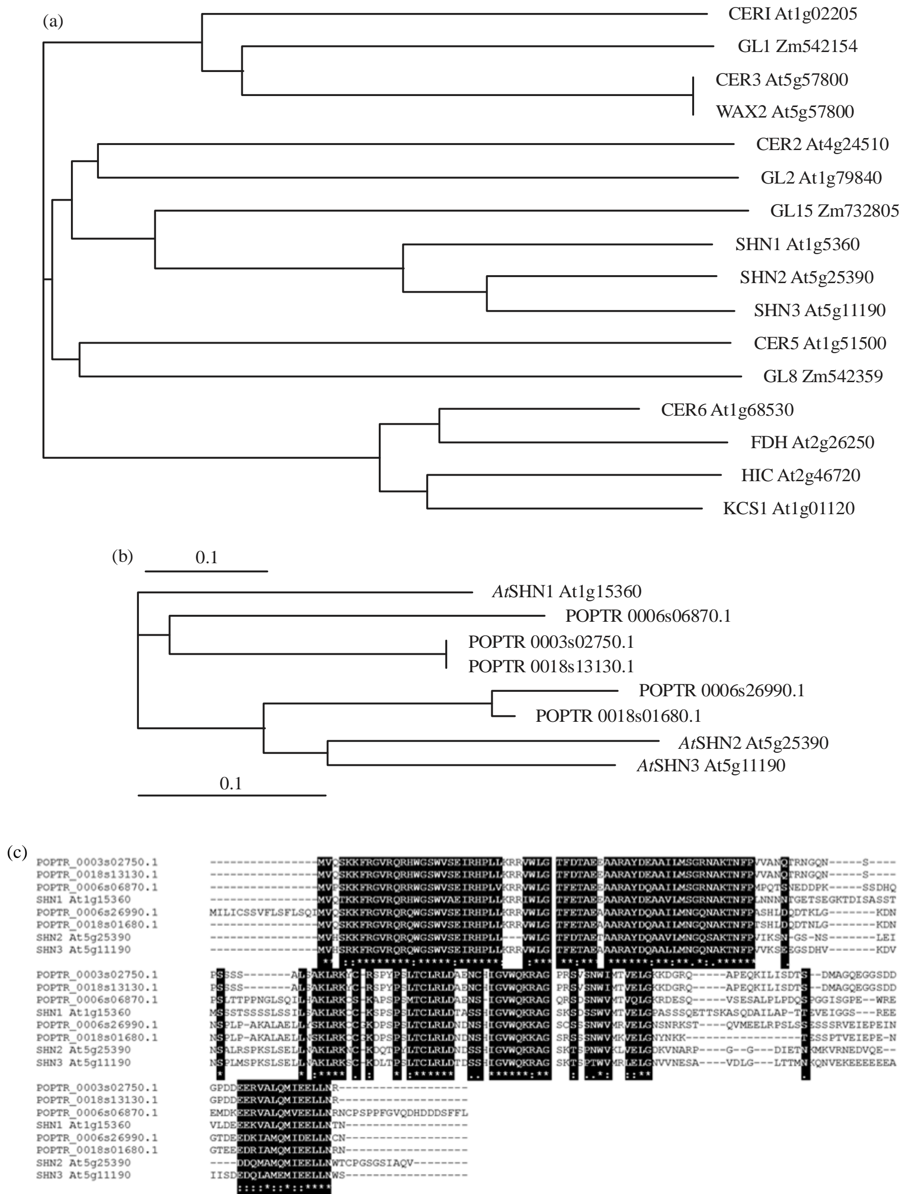

Fig. 1(a-c): Analysis of homology, (a) Phylogenetic analysis of WIN1/SHN1 (At1g15360) with the most well-studied wax biosynthesis genes in the *Arabidopsis* genome, (B) Clade representation of *At*WIN1/SHN1 with similar genes within the poplar genome. Scale bars represent mean number of mutational changes per residue and indicate divergence of these genes from each other and WIN1/SHN1, (c) Sequence comparison of the *Arabidopsis* WIN1/SHN1 gene with homologous poplar genes

the transgenic lines. Further inspection of transgenics showed rounded or stippled wax clumps, wax sheeting, or strands of wax on transgenic leaves not found on any of the 5 control plants analyzed. The most obvious wax phenotype was seen in line 18 where the adaxial and abaxial surfaces showed a thick wax layer with numerous wax clumps that made pavement cells difficult to differentiate when compared to WT (Fig. 5a, h). The adaxial surfaces of lines 12, -19 and -24 leaves showed a thin coating of wax that formed irregular stippling and strands (Fig. 5i, j). Line 25 presented a similar phenotype to line 25 but to a lesser extent and was the line that most closely resembled WT. Quantitative RT-PCR analysis

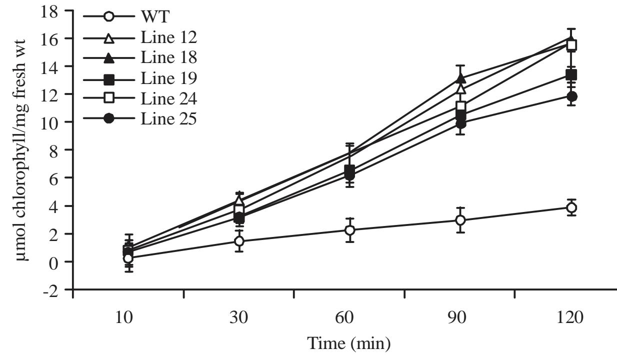

Fig. 2: Chlorophyll leaching. Assay with 80% ethanol indicated that transgenics leached considerably more chlorophyll than WT plants over a 2 h period, Error±SEM

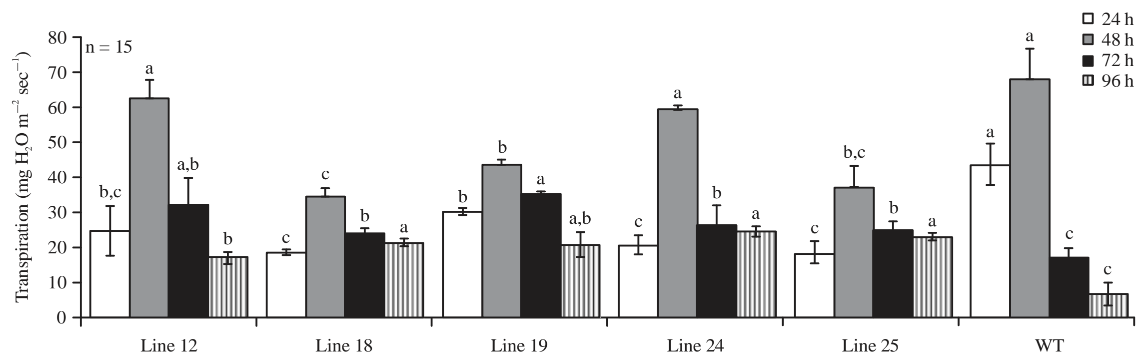

Fig. 3: Transpiration over time. Transpiration over the course of 96 h was measured for transgenics and WT (717-1B4) lines. Means represented with the same letter were not significantly different at $p<0.05$. Error bars (±SEM)

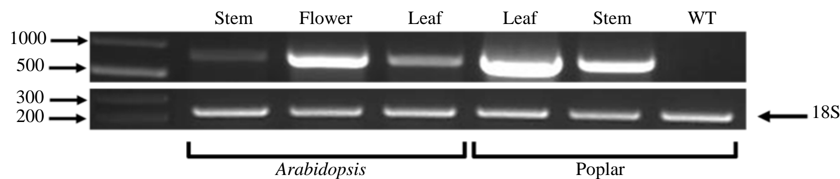

Fig. 4: RT-PCR of *At*WIN1/SHN1 in *Arabidopsis* and poplar. *At*WIN1/SHN1 expression levels were visualized in wild-type Col-0 *Arabidopsis* stems, flowers and leaves alongside pooled leaf and stem RNA from all five transgenic poplar lines. Wild-type refers to an untransformed poplar plant

indicated that levels of AtWIN1/SHN1 in transgenics was directly reflected in the severity of the observed wax phenotypes (Fig. 5k).

DISCUSSION

Decreased stomatal and trichome densities, sluggish growth and increased leaf glossiness were the primary phenotypes seen in *Arabidopsis* overexpressing WIN1/SHN1. In *Arabidopsis*, sluggish growth was previously reported to lead to delayed flowering times and high incidences of infertility[21]. Flowering data could not be collected for poplar as maturation and flowering occur after 6 and 10 years. The increased chlorophyll leaching seen in *Arabidopsis* plants overexpressing WIN1/SHN1 was also observed in the five transgenic lines created here. The intensity of leaf shine or "Glossiness" was proposed by Broun *et al.*[21] to be attributed to changes in light reflectance and refraction patterns because of alterations in epicuticular wax makeup. Enhanced wax production resulting from constitutive overexpression of WIN1/SHN1 is not thought to be a side effect and argues favorably for WIN1/SHN1 serving a vital role in wax

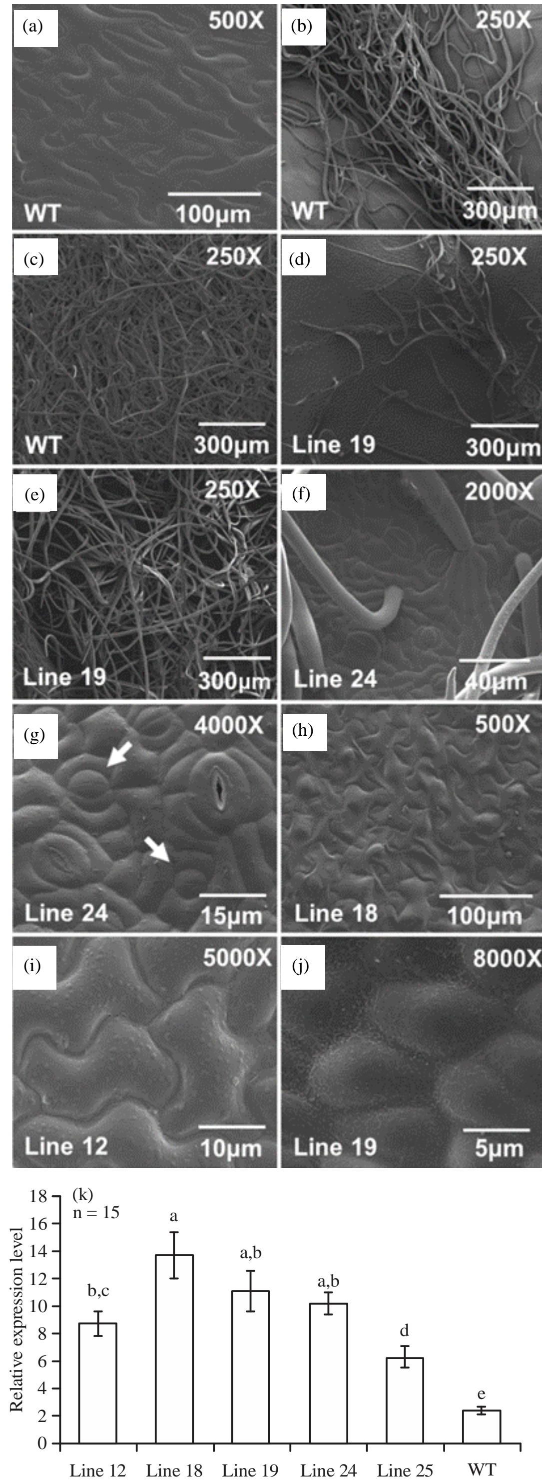

Fig. 5(a-k): Cryo-SEM and qRT-PCR analysis. Poplar WT (a) Adaxial wax, (b) Adaxial leaf vein trichome, (c) Abaxial trichome density phenotypes, (d) Transgenic adaxial leaf vein, (e) Abaxial trichome density example (f, g) (arrows)), Transgenics also possessed arrested stomata, (H) Wax clumps, (i) Microsheets, (j) Strands were observed in transgenic lines and (k) Relative expression analysis of the five transgenic poplar lines expressing *At*WIN1/SHN1 and normalized to the expression of ubiquitin. Means represented with the same letter were not significantly different at $p<0.05$. Bars and magnifications as indicated

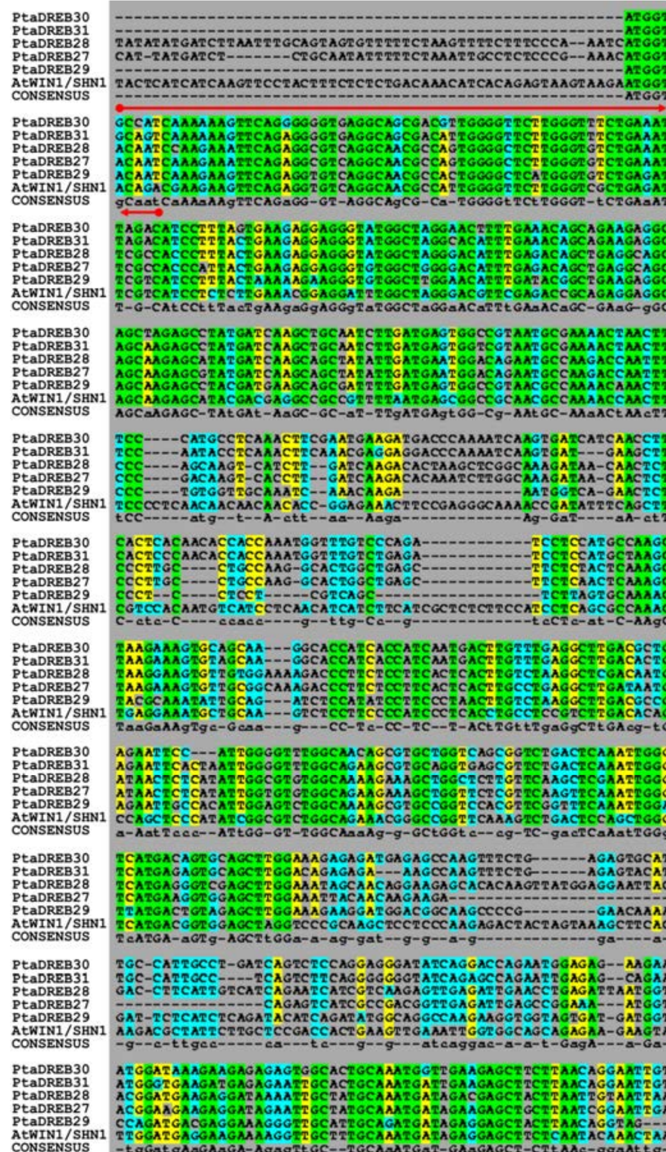

Fig. 1S: Homology between *At*WIN1/SHN1 and *PtaDREB* genes. ClustalW2 alignments indicated a considerable degree of homology between *Arabidopsis* and poplar nucleotide sequences. The conserved AP2/ERF binding domain is 61 bp long and is found at the beginning of the sequence (arrow)

Fig. 2S: Protein homology between *At*WIN1/SHN1 and *PtaDREB* genes. COBALT was used to convert poplar and *Arabidopsis* nucleotide sequences into proteins. The high degree of similarity is illustrated with bold lettering

biosynthesis. Recent data from Jager *et al.*[33] reported expression of a WIN1/SHN1-type regulator from wheat triggered the disorganized proliferation of *Arabidopsis* pavement cuticle cells. This could indicate involvement of WIN1/SHN1 in wheat cuticle formation in addition its other, already implicated, functions.

The isolation and identification of potential poplar orthologs to *At*WIN1/SHN1 revealed that a number of homologous genes have been identified and annotated as Dehydration Responsive Element Binding (DREB) genes, which most often play roles in stress signaling pathways[34], (Fig. 1S, 2S). In addition, drought induced decreases in chlorophyll content or "Greenness" have been observed for decades[35]. However, increases in chlorophyll content have recently been recorded in almond (*Prunusdulcis*) undergoing severe water stress[36]. This unusual occurrence was attributed to the concentration of leaf pigmentation. Adjustments in water loss have been seen in other wax biosynthesis mutants (*cer*) however a link to a particular cause was not determined[9]. These studies indicated that higher wax levels led to increased WUE_{ti}. Transpiration experiments indicated that transgenics lost water at a slower rate than WT trees during the five day experimental period. It is possible that the increased accumulation of wax resulted in a more extensive boundary layer around the leaves and therefore decreased evaporation. This observation could indicate another explanation for the improved WUE of transgenics. There were no significant growth defects in any of the ramets produced from the five overexpression lines generated here though line 18 exhibited marginally slower development and stature when compared to controls (data not shown). It may be discovered in later experiments that increased concentrations of wax could hinder proper stomatal functioning thus allowing excess water loss or preventing CO_2 uptake. If monitored for a longer period of time, significantly decreased carbon assimilation may be seen.

As with *Arabidopsis* and studies using other model species, overexpression analyses of *At*WIN1/SHN1 in poplar transgenics showed similar morphological and physiological alterations. Study by Aharoni *et al.*[15] indicated overexpression of SHN2 and SHN3 resulted in analogous phenotypes therefore it is possible that the two additional sequences elucidated in our homology search may also bestow the same phenotypic expression to poplar leaves. Aharoni *et al.*[15] also described identification of *Os*SHN1 (BAD15859), a potential rice homolog to the *Arabidopsis* SHN clade of proteins. Zhou *et al.*[37] noted the role of OsGL1-3 in wax biosynthesis and its subsequent involvement in abiotic stress tolerance. The potential for identification of a poplar ortholog to WIN1/SHN1 lies in the similarity between sequence motifs as was seen for the putative rice WIN1/SHN1 (*Os*SHN1) ortholog. Marques *et al.*[38] indicated that, as was seen in this study, *At*WIN1/SHN1 gene sequences have greater homology to *PtaDREB* genes rather than *AtDREB* genes. Sequence homology searches and phylogenetic analysis within the poplar genome have revealed the existence of several additional putative protein sequences with a great degree of similarity to the *Arabidopsis* WIN1/SHN1 protein family however greater study is needed to determine what role they play, if any in wax development in poplar species. Most recently, a study by Kumar *et al.*[39] noted that wheat with greater expression levels of WIN1/SHN1 and several other cuticle genes were also most resistant to pathogens. Future research may involve further identification of all putative poplar orthologs and generation of knockouts to examine epicuticular wax responses, transpiration and stomatal density effects along with potential resistance to pathogens. These data will help support research into the poplar wax and cutinbiosynthesis pathways and may help provide specific locations within the poplar genome for instrumental wax genes.

CONCLUSION

- Altogether, this study presented data that confirmed over expression of AtWIN1/SHN1 in poplar decreased stomatal and trichome density, increased presence of leaf wax and increased leaf greenness
- Use of stable isotopes confirmed transgenic also had a greater WUE_{ti} than WT plants
- Results shown here are consistent with that seen in the literature for other species however; negligible negative growth effects were observed in poplar as compared to the much more unfavorable effects seen in other species
- Thus, it is proposed here that genes with high homology to *DREB* genes be examined as future targets for manipulation to improveWUE
- Transgenics with improved greenness may beboth visually appealing and environmentally conscious in regions with limited water resources

SIGNIFICANT STATEMENTS

Water is a major component for the growth, development and survival of trees. Adelicate balance exists between water used for metabolism and water lost through transpiration. To maximize water usage during periods of drought, tree leaves can be genetically modified to have lower stomatal numbers

and greater wax deposition. Thus far, this is the only study that used the *At*WIN1/SHN1 gene to introduce a prized ornamental trait (glossy leaves) and water-use efficiency simultaneously into poplar. These enhancements mean that poplar can now be used as ornamentals, functional windbreaks and be planted in regions where water is often scarce without the negative effects observed in other species. Use of inventive ways to generate and use ornamentals with improved water-use efficiencies is a proactive method to protect dwindling water supplies, shade dwellings to lower summer utility cooling costs and allow greater quantities of water free to be used by other ornamental plants or for the household if they draw their water from a well.

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