2023

The YUCCA Auxin Biosynthetic Genes and Drought: How Does Gene Expression Change in Water Stressed Leaves?

Samantha Pelletier
Connecticut College, sampelletier19@gmail.com

The YUCCA auxin biosynthetic genes and drought: How does gene expression change in water-stressed leaves?

An honors thesis presented by
Samantha Pelletier

To the Department of Botany
In partial fulfillment of the requirements for
Honors

Connecticut College
New London, Connecticut

May 3, 2023

Abstract

As drought becomes more pronounced due to climate change, it is important to characterize the responses of plants to water stress. One area of interest includes how auxin, the hormone most associated with growth and development, is affected by drought. In particular, the final size of an expanding leaf is often reduced in response to drought and auxin is known to play a key role in leaf expansion. The YUCCA proteins catalyze the conversion of indole-3-pyruvic acid to indole-3-acetic acid (IAA), the dominant form of auxin in most plants, and most of this IAA is produced in rapidly expanding leaves. However, in addition to synthesizing IAA, the YUCCA proteins are also associated with thiol reductase activity. This enzymatic function decreases the amount of reactive oxygen species (ROS), which can accumulate in plant tissue under stress such as drought. The YUCCA gene family in *Populus* includes 12 YUCCAs, but they are not well described. This experiment was aimed to assess whether YUCCA genes in *Populus* leaves at different stages of development are differentially expressed in response to drought. Multi-shoot, one-year-old hybrid poplar plants (*Populus tremula* x *alba*; INRA 717-1B4) were either watered to field capacity daily (control; n = 5) or deprived of water (drought; n = 5) for nine days. Over that period, stomatal conductance, and stem water potential both decreased significantly in the droughted trees relative to the control trees. The shoot apex, leaf 8, and leaf 16 (i.e., the eighth and sixteenth leaves counted down from the apex) were harvested for gene expression analysis, with the apex representing rapid growth, leaf 8 representing a leaf approximately one half the size of the average fully mature leaf, and leaf 16 representing a fully expanded leaf. Leaf relative water content (RWC) measured on leaf disks at the time of harvest and showed that the RWC of leaf 8 was significantly reduced under drought compared to controls, but the RWC of leaf 16 was similar between treatments. qRT-PCR was used to determine expression levels of five different YUCCA genes that had previously been shown to be expressed in *Populus* leaves: YUC1, YUC2, YUC4, YUC6, and YUC12. These results were normalized using actin, ubiquitin, and tubulin, genes that were found to significantly decrease in their expression in the droughted plants when their Cq values were used to take a geometric mean. Results were then analyzed by normalizing with this geometric mean as well as with the gene least affect by drought, ubiquitin. It was shown that YUC12 did not significantly change between treatments in the apices in either method of normalization. However, YUC1, YUC4, and YUC6 had decreased expression in drought in the apices, as well as in some of the leaf samples when normalized with ubiquitin. YUC2 was initially normalized to have significantly increased expression in the droughted plants, however this result was made insignificant by normalization with ubiquitin. Future work should find stable normalizer genes to draw more reliable conclusions from as well as measure IAA concentrations in leaf tissue under drought to better understand these hormonal cues.

Acknowledgements

I would like to thank Dr. Rachel Spicer, who has had a lasting impact on me, both as a scientist and as a person. It was due to Dr. Spicer's guidance that this thesis work was both completed and communicated effectively. Dr. Spicer's mentorship to me as a student is something I will always cite as one of the most important parts of my time here at Connecticut College. Thank you.

I would like to thank my parents Keith and Cynthia Pelletier for their support throughout this process, despite not knowing what a SAM is other than their daughter. I would like to thank my two lab mates throughout my time in the Spicer lab, Seth Stadheim and Brian Speers, whose time and collaboration helped me become the scientist I am today. Seth also helped me harvest tissue for this work, for which I am eternally grateful. I would also like to thank Dr. T. Page Owen, whose comments on an earlier draft allowed for a more polished final copy. I would like to thank my friends here at Conn for being supportive and learning to plan lunch with me around the times that my samples would be done centrifuging. Finally, I would also like to thank my partner Anna Myers, who has heard enough about this project to present its findings herself.

Contents

Introduction

Climate change has created more frequent and severe episodes of drought across the globe such as the megadrought in North America (Williams et al., 2020). Drought can affect forests by changing the makeup of species that are able to tolerate limited water availability (Clark et al., 2016). In one study, it was shown that 70% of 226 studied species have very little tolerance to drought (Choat et al., 2012). As drought intensifies with our warming planet, the consequences are likely to be devastating.

Drought and the plant

Leaves are major players when it comes to drought; water loss is almost exclusively due to the opening and closing of stomata, which are the tiny pores in the leaves that facilitate gas exchange (Chaves et al., 2003). Through the release and uptake of oxygen and carbon dioxide, respectively, plants lose high amounts of water: on average 400 molecules of water are lost for every one molecule of carbon dioxide (McElrone et al., 2019). Thus, water loss is a major concern when a plant is experiencing drought, despite the need to build carbohydrates through photosynthesis. Instead, water loss needs to be mitigated, which is accomplished by closing stomata (Abbate et al., 2004; Buckley, 2019). Additionally, drought can cause younger leaves to expand less and mature leaves to be senesced (Chaves et al., 2002; Farooq et al., 2008; Skirycz and Inzé, 2010; Anjum et al., 2011). This is likely done to reduce transpiration area on a plant that is experiencing water stress.

Water is brought to leaves through the inner systems of the plant. In angiosperms, the internal structure for water transport is especially vulnerable to damage during a drought, which can lead to the death of the plant. This structure consists of xylem, which is made up primarily of fibers and vessels. Vessels contain long columns of cells called vessel elements that are stacked end-on-end. They can span from a few centimeters to few meters in length. Vessels transport water over longer distances by connecting to one another via small pits, which allows the water stream to span the entire plant. As described above, the evaporation of water through the stomata is the driving force for water being pulled up a stem. This pull on the water column from evaporation creates tension (i.e., negative pressure) in the xylem, not unlike the tension of sucking on a straw when one enjoys a beverage. However, in the case of the plant, this "straw" can be under too much tension and air can enter this system. There can be a certain threshold of tension in a stem that causes the water column within one vessel to snap, which causes air to enter the stream. Air bubbles can fill entire vessels, which is referred to as embolism, and renders them useless. This can lead to an increase in tension in the remaining vessels, which can increase the rate at which embolism happens and can lead to hydraulic failure (Figure 1).

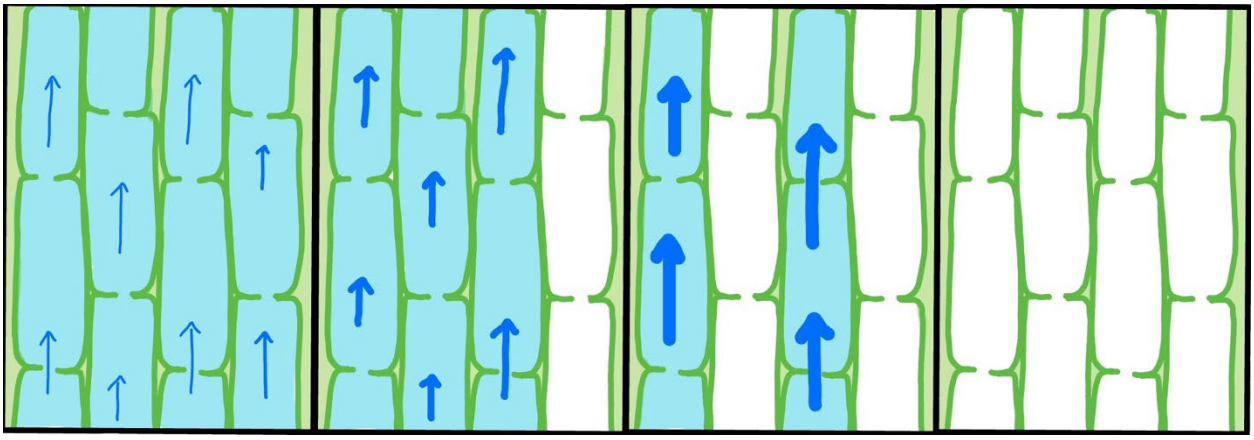

Figure 1. Water (shown in light blue) flows through the vessels, which make up the xylem of the plant (Panel A). If one vessel embolizes due to increased tension on the water column (air shown in white), it puts the other vessels under increased tension which is indicated by increased arrow width (Panel B). This increase in tension can cause further embolism (Panel C), which can lead to total plant failure if the rate of water loss is unchanged (Panel D). This rate of water loss can be slowed by the closure of stomata.

Wood is essentially an extensive network of xylem, so the structure of wood with respect to vessel size and arrangement may determine how trees cope with drought. However, it is important to note that every species of plant has an internal system of xylem that is a little different structurally, due to the selective pressures that plants have in different environments. Moreover, drought also affects wood development in different ways that reduce the risk of future embolism. In the species *Vitis vinifera* (grapevine), droughted plants produce a greater density of vessels that are smaller in diameter, introducing the idea that plants may decrease vessel size to minimize risk of embolism but produce more vessels to conserve flow rate (Lovisolo and Schubert, 1997). This is also consistent in trees within the genera *Acer, Betula*, and *Populus* (Zhang et al., 2004; Arend et al., 2007; Fichot et al. 2009; Jupa et al., 2021). Although these plants tend to produce more dense areas of vessels, these smaller vessels are unable to maintain the same flow rates as previous wood. This is because the flow rate through an individual vessel is controlled by the radius of the vessel to the fourth power (Dixon and Joly, 1895). These modifications in wood, therefore, are likely to decrease a plant's ability to transport water – while simultaneously making them more resistant to embolism. These physical changes are governed by molecular mechanisms that are not yet known.

Auxin and plant development: leaves and wood

Plant hormones (phytohormones) are vital to the functioning of a healthy plant; plants must adapt to constant changes in their environment without the ability to physically move to another location, which requires the use of signaling molecules. There are many hormones that are key to plant growth, the most important of which is auxin. The most well described and abundant auxin in plants is indole-3-acetic acid (IAA). IAA is a key hormone throughout a plant's development,

playing an important role in growth of stems, leaf expansion, apical dominance, and root formation (Teale et al., 2006; Vanneste and Friml, 2009).

IAA is produced primarily via the YUC pathway, which involves the conversion of tryptophan (Trp) into IAA (Figure 2) (Mashiguchi et al., 2011). The first step involves a tryptophan aminotransferase (TAA) as a catalyst to convert Trp into indole-3-pyruvic acid, which is then converted into IAA by the YUCCA enzyme.

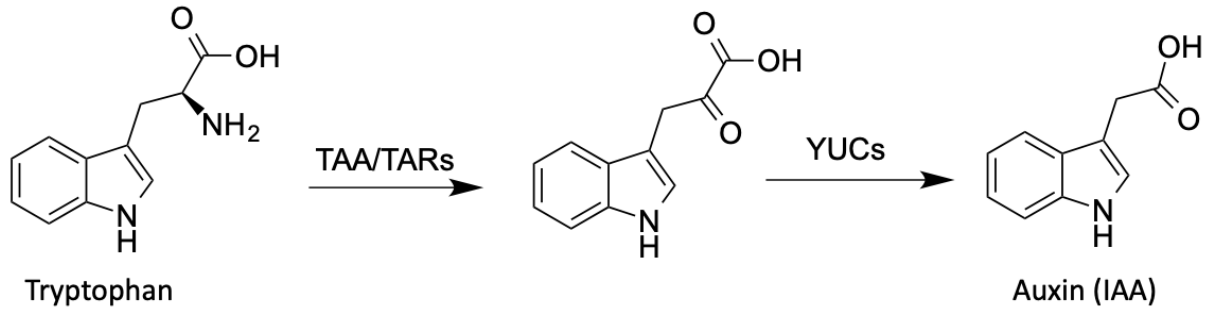

Figure 2. Auxin is made through the conversion of tryptophan using the YUCCA (YUCs) protein.

There are two main routes by which auxin regulates growth processes. The first is driving the actual expansion of cells that are needed to grow. This expansion is termed auxin-mediated growth, which is described by the acid growth hypothesis, in which the presence of auxin helps to expand cells (Nakayama et al. 2012). First proposed in the 1970s, scientists have proven that in localized points in a plant, growth is stimulated by osmotic uptake of water in a cell due to the acidification of the cell wall (Rayle and Cleland, 1992; Cosgrove, 1999). This acidification is triggered by auxin which increases the activity of hydrogen pumps in the cell. This acidification leads to increased activity of expansins, which are enzymes that loosen the cell wall. This expansion results in a reduction of the cell's water potential, leading to an influx of water and thereby expansion of the cell (McQueen-Mason, 1992).

Auxin also regulates the expression of genes that are related to growth and differentiation. Genes are often turned “on” and “off”, depending on the cell that they are in and the signals around them. One thing that can turn cells on and off is the presence (and absence) of a hormone. Auxin is an important regulator of gene expression, with many known auxin responsive genes. These genes are expressed when auxin is present because auxin binds to repressors that inhibit gene expression and designates them for degradation. The fact that auxin turns on specific genes makes this hormone important to very local processes (Woodword and Bartel, 2004).

Cellular differentiation is thought to be controlled, in part, by auxin as well. Plants have zones of meristematic cells, commonly known as stem cells, that can differentiate into any cell type. Areas that contain many stem cells in the plant, meristematic zones, include the shoot apical meristem (SAM), the root apical meristem (RAM), and the vascular cambium. Auxin is made in the SAM as well as in the rapidly expanding leaves and channeled down into the stem (Ljung et al., 2001; Woodword and Bartel, 2004; Evanich, 2015). It is thought that the coordination of organ formation, prompted by the differentiation of cells in these areas, is caused by local concentrations of auxin (Benková et al., 2003).

Auxin plays several distinct roles in the development of leaves. The establishment of where cells divide to form a leaf is completely controlled by the development of an auxin maxima at the SAM (Kalve et al., 2014; Xiong et al., 2019; Perico et al., 2022). The formation of vasculature (i.e., the veins) in the leaf is also under the control of auxin (Scarpella et al., 2006; Perico et al., 2022). The blockage in the movement of auxin in the developing leaves by the application of an

auxin movement inhibitor causes them to cease growth, indicating its importance in the growth of a leaf (Ljung et al., 2001).

Auxin is important in processes in the plant beyond leaf development. Auxin is made primarily in the leaves and SAM, from where it moves downwards (basipetally) through the plant to regulate other key processes (Cheng et al., 2006). One zone of importance is the vascular cambium, which is a single ring of meristematic cells found in the stem of a woody plant (Figure 3). It is here that cells divide and differentiate into xylem or phloem cells. Auxin is involved in the expression of many genes that control the differentiation of vascular cambium derivatives to xylem cells, which deems IAA an important component in wood formation (Nilsson et al., 2007). In fact, it has been found that by inhibiting the transport of auxin within the cambium of *Populus* saplings, vessels become smaller and shorter (Johnson et al., 2018), suggesting concentrations of auxin may affect vessel size and shape in wood (Hacke et al., 2016).

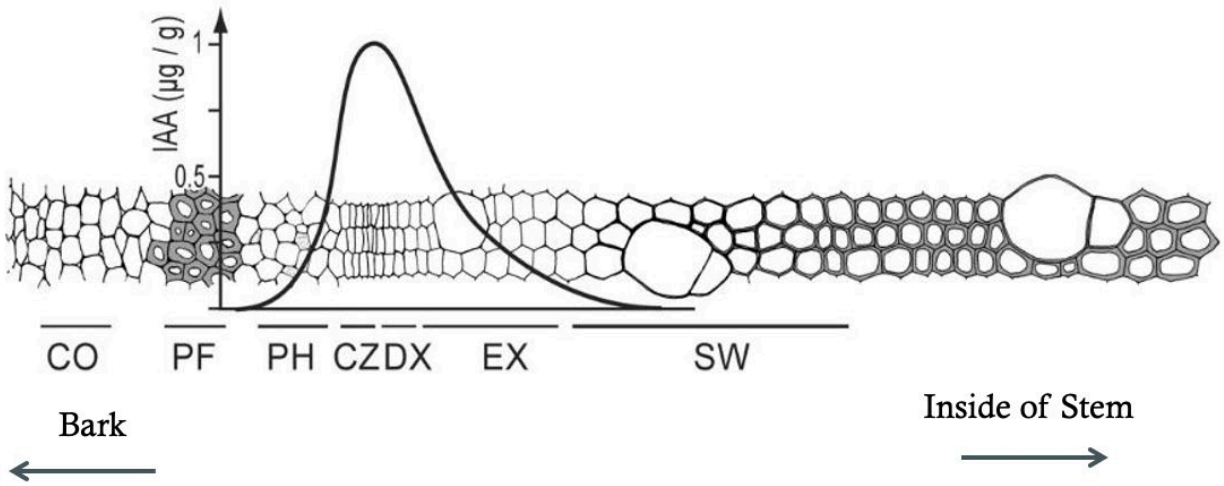

Figure 3. Auxin (IAA) is an important hormone for wood formation. The concentration of this hormone is highest in the vascular cambium, from which cells differentiate into xylem cells. Figure modified from Schrader et al., 2003.

Auxin and drought

IAA has been looked at in drought responses in small herbaceous plants as well as commercially important crops. The research that has been done to understand the links between auxin and drought usually involve modifying concentrations of auxin as opposed to measuring their natural concentrations; these results show that increasing auxin concentrations aids in drought tolerance (Kim et al., 2013; Ljung, 2013; Iqbal et al., 2022). Little work has been done to understand natural IAA biosynthesis in droughted conditions, which leaves many questions unanswered about the connection between drought leaf and wood developmental processes. This idea stems from the fact that during drought, smaller and shorter vessels are produced, which seems to be under the control of auxin concentrations. The inhibition of auxin transport also decreases the size of leaves, confirming its importance in controlling leaf size, which may connect this physiological response to auxin (Ljung, 2001). This is a big deal, because the leaves are the source of auxin made in the plant.

The lack of research that relates IAA and drought creates a question like that of the chicken and the egg: is plant tissue modified due to constraints on the plant in the absence of water or does the plant undergo regulation of these processes to create a water distribution system that is better adapted to drought? Are leaves reduced during drought because there's just no water available to expand their cells, or is this controlled by changes in gene expression regulated by auxin?

It appears the YUCCA proteins might be more described than IAA in terms of drought. In both Arabidopsis and potato, it was found that overexpression of specific YUCCA genes increased drought tolerance (Lee et al 2012; Kim et al., 2013; Cha et al., 2015). Additionally, in droughted

treatments of rice, YUCCA expression increased dramatically (Sharma et al., 2018). However, this tolerance may have been because of the YUCCA protein's ability to decrease the amount of reactive oxygen species (ROS), which contribute to the death of a plant during drought (Park et al., 2013; Mantova et al., 2021). This is because of the thiol reductase function that is present in the YUCCAs, which allows for the proteins to play a role in oxidizing dangerous oxygen species when they are high in concentration (Cha et al., 2015). This link between YUCCAs and drought does not describe the levels at which auxin is being produced in a droughted plant. The findings do, however, emphasize the complex nuances of plant biology that must be considered when accessing any physiological changes on the molecular level.

Current work on YUCCAs in Populus

Auxin is less studied in trees than it is in smaller herbaceous plants because studying large scale impacts of hormones can be difficult as organism size increases. To study molecular signaling in trees, it is important to use a plant with a published and publicly available genome in order to target the expression of specific genes. The genus *Populus* is a model genus used in the scientific community to study tree biology. It was the first tree genome published and is by far the best understood tree genetically, with a whole community of scientists working on it (Tuskan et al., 2006). Species in this genus have fast reproductive cycles which make them practical for experiments (Taylor, 2002; Jansson and Douglas, 2007). The U.S. Department of Energy has also taken special interest in hybrid *Populus* trees due to their fast growth rates, noting that they might become a key bioenergy crop (Takemura, 2021). Understanding how drought might affect *Populus* is important as water shortages could affect entire crops of trees. Thus, the scientific community has taken an interest in how *Populus* behaves in droughted conditions, not only for

the knowledge of molecular signaling, but also to better understand how to avoid large scale tree mortality.

In the genus *Populus*, there are 12 known YUCCA genes with similar function (Ye et al., 2009). Previous work determined the expression of different YUCCA proteins within different tissues of a leaf (Stadheim, et al 2023, Figure 4). For instance, the blade tissue predominantly express YUC2 along with YUC12, whereas the petiole and midvein have only high expression of YUC12.

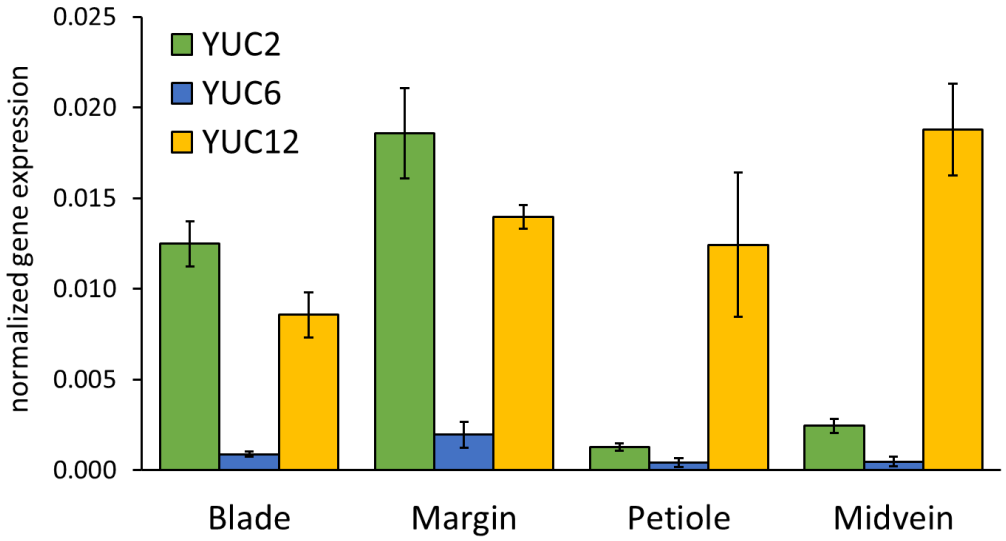

Figure 4. Previous work in the Spicer lab has shown the dominant YUCCA genes in the blade and margin are YUC2 and YUC12, whereas in the petiole and midvein the only dominant YUCCA is YUC12.

It has also been shown that YUC12 has prominent expression in the rapidly differentiating and dividing cells of the shoot apex, with expression steadily decreasing as leaf age increases (Spicer, unpublished data; Figure 5). Understanding the dominant YUCCA genes expressed throughout different tissues of the plant is vital to measuring potential expression changes in the plant.

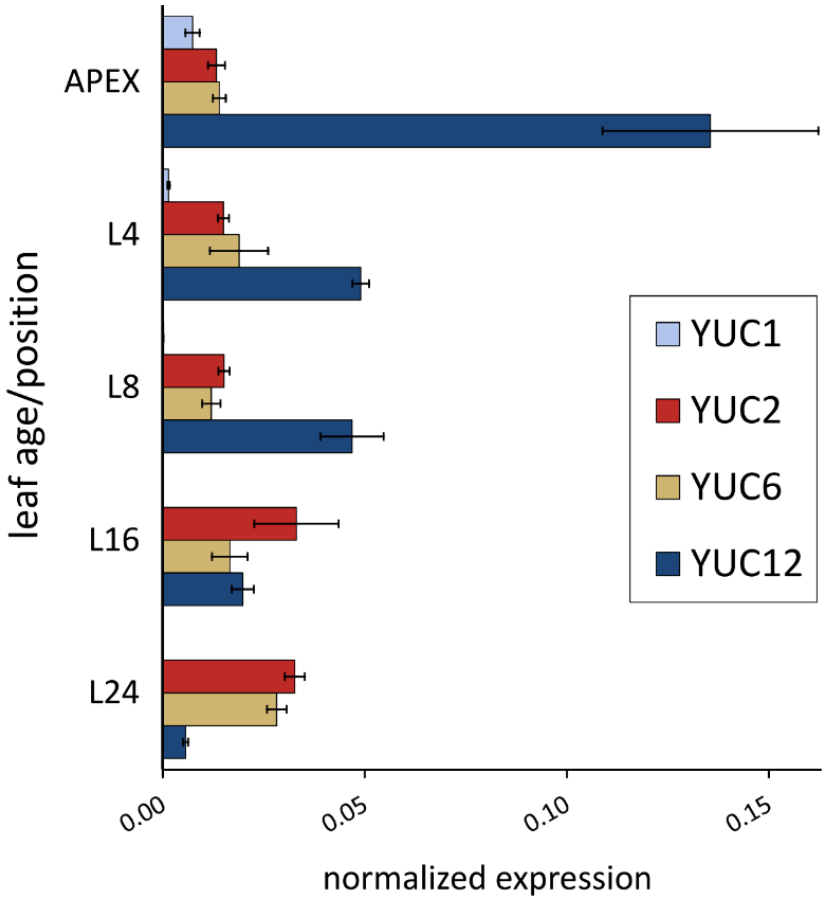

Figure 5. Work in the Spicer lab has been done to understand the general trends of the YUCCA genes in leaves of different ages down the stem, as counted from the shoot apical meristem.

Aim of this work

For my senior thesis, I proposed using *Populus* as a model species to understand the hormonal cues associated with drought and the physiological changes in apex and leaf auxin biosynthesis due to drought. I conducted a drought experiment to understand the general trends of specific YUCCA genes in a short-term water stress event. I also wanted to explore the idea that YUCCAs could be upregulated, despite decreased growth, due to their function of reducing ROS. Understanding these processes has become important as we move towards projected droughts with increased intensity and duration

Materials & Methods

Plant material

Genetic clones of *Populus tremula* x *alba* 717-1B4 were subcloned in sterile tissue culture and grown in an incubator during the spring of 2021. They were transferred to soil pots in May and June of 2021, where they grew in a growth chamber inside of Ziploc™ bags for several weeks before acclimating to the chamber's conditions, with gradual opening of the bags. These plants were then moved to the Connecticut College greenhouse in September 2021, where they were trimmed to their base and allowed to resprout several times, with the most recent cutback having occurred May 31, 2022. The resulting plants used for this experiment had above ground vegetation that ranged from two to three shoots that were about one-month-old with an extensive root system. They had also received nutritional supplements both through Osmocote Plus™ treatment of 1 tbsp to the topsoil as well as in 800mL of Miracle Grow solution in two doses (0.25tbsp/1L).

Experimental drought treatments

Ten plants were selected for the experiment. The plants varied in size as well as number of dominate shoots, so they were divided evenly into two groups so that the size and morphology of the plants in the two groups were similar (Table 1). The plants were then organized on a greenhouse bench randomly, with the remaining plants that were not included in the experiment forming a periphery around them to minimize any edge effect. The two experimental groups were designated as control and drought treatments.

The control treatment plants were watered daily to the point of bottom draining of excess water. The drought plants were completely deprived of water for 9 days with the following exceptions: on day 3, one plant was watered using 600mL, and on day 5 a second plant was watered using 200mL. These instances of watering were due to the larger plants exhibiting drought-like symptoms (wilted leaves, petiole depression) before the others, therefore allowing the treatment duration to be lengthened and have comparable replicates.

Table 1. Plants were divided into two groups, which were not significantly different from one another in height or stem diameter (mean ± se, n = 5, two-tailed t-test)

Parameter	Control group (cm)	Drought group (cm)	p value
Height	94.2 ± 17.3	84.6 ± 12.8	0.346
Stem diameter at base	1.16 ± 0.4	1.0 ± 0.2	0.421

Stomatal conductance

Measurements to quantify stomatal conductance were made several times in the days leading up the harvest to characterize the drought treatments. Using a porometer (SC-1 model), leaves between leaf 8 and leaf 16 (counting down from the shoot tip) on the experimental shoot had measurements taken in between any major veins around the perimeter of the leaf. After two days, the readings were not significantly different between the groups. However, the day before harvest, the groups had a significant difference in evaporative water loss.

Stem water potential

Stem water potential readings were made two days (day three and day six) leading up to the harvest as well as the date of harvest. Each time, a single leaf attached to a non-experimental shoot, located between leaf 8 and leaf 16, was fully enclosed in an opaque bag the evening before the measurement (between 7-9pm). The following day, the bag was removed, and the leaf

was cut from the stem around 9 am and put into a PMS pressure chamber (Model 1505D-EXP), where balance pressure was measured for each experimental shoot. This method is often used to measure water potential within a stem. When a leaf is bagged, it is presumed to not be losing water through evaporation due to the lack of a light signal, therefore it is not transpiring. When a leaf is not transpiring, it comes into equilibrium with the water potential in the xylem because there is no pull from evaporation. This to use the water potential of a bagged leaf to estimate the amount of tension (i.e., negative pressure) that the xylem is under.

Tissue collection & leaf relative water content

The apex was defined as containing the shoot apical meristem as well as the smallest differentiating leaves on the experimental shoot of the plant. This contained any leaf primordia that were less than 2/3 uncurled along the margin. The first leaf that was at least 2/3 uncurled was considered leaf 1 and leaves were numbered consecutively from this leaf. The apex from each experimental shoot was harvested as well as leaf 8 and 16. Leaf 8, a half-expanded leaf, represents the leaves in which there is a transition from sink to source of carbohydrates (Larson et al., 1980; Turgeon, 2006). This export of photosynthate indicates the ability for this leaf to be a source of auxin production for other parts of the plant through phloem-mediated transport of conjugated auxin (Bajguz and Piotrowska, 2009). Leaf 16 represents a fully expanded leaf. The cessation in growth associated with a mature leaf is likely caused by a decrease in the production of auxin in the plant. Each leaf was sliced in half using a razor blade along either side of the midrib (i.e., the midrib was excluded from leaf tissue samples) (Figure 6). One half was used for relative water content measurements (RWC), and the other half was plunged in LN_2 for use in qRT-PCR.

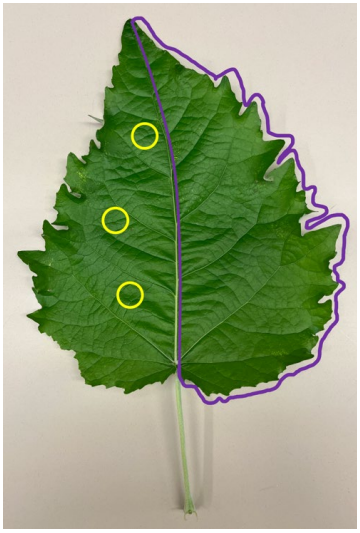

Figure 6. Leaf 8 and 16 were collected from each plant. Half of the blade was sectioned to take disks for RWC measurements (yellow sections). The other half of the blade (excluding the midrib) was weighed and immediately plunged into LN_2 (purple section).

A cork borer was used on the tissues designated for RWC to make 3 disks with a diameter of 0.90 cm each. These disks were weighed on an analytic balance for their fresh weight (FW), enclosed in tissue embedding cassettes, and immediately submerged in deionized water and placed on ice in a 4°C refrigerator for 24 hours. After 24 hours the disks were removed from the cassette, dabbed surface dry with a Kimwipe™, and weighed for the saturated weight (SW). The disks were then folded in paper and dried at 70°C for 48 hours. The disks were then weighed one final time for a dry weight (DW). RWC was calculated using the equation $\frac{(FW-DW)}{(SW-DW)} * 100$.

Quantitative real-time PCR

Tissues collected for quantitative real time polymerase chain reactions (qRT-PCR) were ground in LN_2 with a mortar and pestle and never allowed to thaw and stored in a -80°C freezer. To generate RNA, a the three-day CTAB extraction procedure was used (Porebski et al., 1997; Box 1).

The resulting RNA concentration was read on a Nanodrop (Thermo Scientific NanoDrop Lite Spectrophotometer). Samples contained 300-1200 ug/mL RNA. The samples were then treated with DNase using the Invitrogen TURBO DNA-*free*™ kit following the routine DNase treatment instructions. Reactions used about 150-200 ug/mL RNA. cDNA generation followed by using the BIO-RAD iScript™ Advanced cDNA Synthesis Kit for qRT-PCR. The resulting cDNA concentration was read on a Qubit™ (Q32857). For each tissue, resulting cDNA concentrations ranged from 5 ug/mL to 15 ug/mL.

Box 1: CTAB extraction protocol for RNA from tough tissues

Day one: Frozen ground tissue is submerged by 800 microliters of 65°C CTAB buffer with 2% by volume beta-mercaptoethanol added. The solution is warmed to 65°C and incubated for 15 minutes before 800uL of the solution CHISAM (24:1 chloroform: isoamyl alcohol) is added. This is then thoroughly mixed by flicking and centrifuged to produce three layers, of which the top elution is obtained. 800uL CHISAM is once again added to the original sample, which is mixed and centrifuged down. The final top layer obtained, ¼ volume of 10MLiCl is added, and the sample is left overnight at 4°C to precipitate.

Day two: Samples are centrifuged at 4°C for 20 minutes and the supernatant is discarded. 300uL of 60°C SSTE buffer is then added to dissolve the pellet while sample are kept at 60°C. 300uL of CHISAM is also added. The samples are then centrifuged for 10 minutes at max speed, and the upper phase of this tube is extracted. This upper phase then receives 300 uL of CHISAM, then is mixed and centrifuged for 10 minutes at maximum speed. The elution from this tube is then extracted. 100uL of SSTE buffer is then added to the original samples, both are centrifuged at top speed for 10 minutes, and both top layers are added to the final sample. 2 volumes of cold 99.5% RNase free ethanol are added to tube C, and it is stored at -20°C overnight.

Day three: The sample is removed from the -20°C freezer and centrifuged at 4°C and maximum speed for 20 minutes. The supernatant is poured off, and 1 mL of 70% ethanol is added and gently mixed. Sample is once again centrifuged at 4°C and maximum speed for 20 minutes. The liquid is removed by pipetting and weak vacuum suctioning and hand drying, and the final pellet is suspended in 50 uL of RNase free water.

qRT-PCR was performed on the cDNA generated from the apex, leaf 8, and leaf 16 with target genes YUC1, YUC2, YUC4, YUC6, and YUC12 (Table 2). These reactions took place in a CFX Connect™ RealTime system using iTAQ™ Universal SYBR ® Green Supermix (Biorad, Hercules, CA, USA). Each plant sample run on the plate had three "normalizer" genes. These genes – actin, ubiquitin, and tubulin - are expressed at high levels in all tissues and allow us to relate the level of expression that we get from a target gene to a baseline in order to easily compare each individual experimental gene's expression.

Table 2. Information about the primers used for qRT-PCR.

Gene	Type of primer	Sequence	Melting temperature °C	Amplicon (bp)
Actin	Forward	CCATCATGAAGTGTGATGTG	60.0	114
Actin	Reverse	CAGTGATTTCCTTGCTCATAC	60.1	114
Tubulin	Forward	CCTACTGTAGTACCTGGGGGTG	58.2	230
Tubulin	Reverse	CCAACTTCCTCGTAATCCTTCTCA	56.2	230
Ubiquitin	Forward	CAGCTTGAAGATGGGAGGAC	55.4	154
Ubiquitin	Reverse	CAATGGTGTCTGAGCTCTCG	55.5	154
YUCCA1	Forward	GGAAAGACACCAGTGTTAGAT	61.0	86
YUCCA1	Reverse	GTTACCTCCTTCACACCTTC	61.0	86
YUCCA2	Forward	AGGAGAGTGTGGGCTATATG	61.7	100
YUCCA2	Reverse	CCTACACCGTTCAATGTCTTC	61.8	100
YUCCA4	Forward	CCCATTGAGCTAAAGAATGTC	60.2	109
YUCCA4	Reverse	TTATCTCCTTCACACCTTCC	60.1	109
YUCCA6	Forward	CTTTCCAAGATGACACCAAC	60.2	99
YUCCA6	Reverse	TGTACATGATATAGCTGAGGTC	60.2	99
YUCCA12	Forward	GTTTCCCACTTCAAGATCAG	59.7	102
YUCCA12	Reverse	GCTCACATTTCTAGCCTTAAC	60.1	102

Three technical replicates were run for each gene on every plate. The SYBR Green Supermix protocol was followed for a 20 uL reaction, and the reactions took place in a 96 well plate. This supermix contains a fluorescent marker that binds to genetic material, allowing for the machine to visualize increasing levels of genetic material. Strands were denatured at 95 °C for 30 seconds, then amplified over the course of 37 cycles of denaturation (95 °C for 30 seconds)

followed by annealing and extension (30 seconds at 53 °C). The software on the RealTime system records the number of cycles at which the fluorescence level crosses a threshold that is just above what the software determines as "background", which is represented by a Cq value. Following the amplification cycles, a melt curve analysis was conducted. In a melt curve analysis, the sample is heated up in 5°C intervals from 65°C to 95°C to show the temperature at which the double-stranded PCR product falls apart, or denatures, which is a unique temperature for each gene and termed the "melt temperature".

There were measures taken to ensure the data produced was accurate. Each plate was then analyzed for inconsistencies between the three technical replicates; each of the three samples should be close to identical as they contain the exact same DNA and reagents. This is done to ensure that the numbers are accurate and not inflated or deflated because of a technical error, such as accuracy in pipetting. Plates are also analyzed for inconsistencies in melt temperatures within genes. As previously mentioned, each amplified section has one, specific melt temperature. The data captured by the qRT-PCR machine includes at what temperature the sample denatures should be consistent, with only one peak. The presence of more than one peak indicates that there is more than one amplified section.

Relative gene expression was calculated by comparing the target YUCCA genes against the normalizer genes. $Relative\ expression = \frac{2^{Cq\ norm}}{2^{Cq\ GOI}}$ where norm is the geometric mean of the three normalizer genes and GOI is the "gene of interest", in this case, the specific YUCCA gene.

Statistics

Statistics were run using the software R version 4.2.0 (2022-04-22). A lmer mixed model was run on the complete set of data, using treatment and tissue as fixed effects and plant as a random effect. The mixed model included treatment x tissue, treatment x gene, tissue x gene, and treatment x tissue x gene interaction terms. The mixed models were then analyzed using an Analysis of Variance (ANOVA). For comparisons between treatments, two-tailed t-tests were run separately for each gene within a single tissue using Excel.

Results

Results that characterize the effects of the drought treatment are described in terms of days into the treatment, with day 1 being the start day of the experiment, day 2 being 24 hours after the start, etc.

Stomatal conductance

To understand how the plants were affected by the drought treatment, stomatal conductance readings were taken throughout the experiment (Figure 7). The measurements were not significantly different between the treatments two days into the drought ($n=5$, $p<0.1$). After three days of water deprivation, the measurements were significantly different ($n=5$, $p<0.01$). Measurements confirmed that the effect of drought was still significant on the sixth day into the treatment ($n=5$, $p<0.001$).

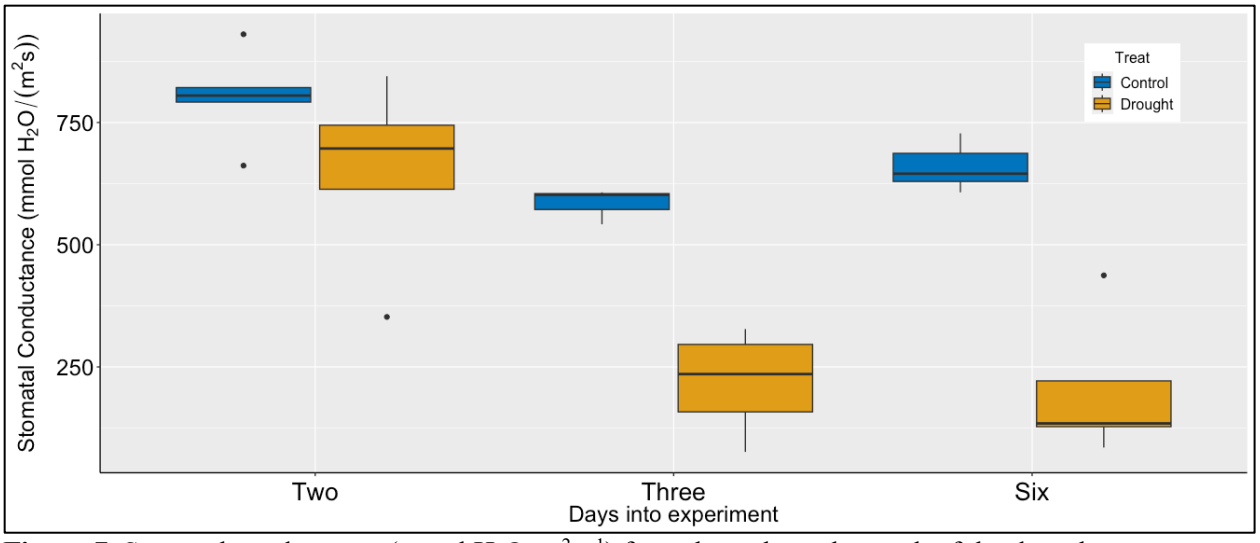

Figure 7. Stomatal conductance (mmol H_2O m^{-2} s^{-1}) from throughout the week of the drought treatment.

Stem water potential

Stem water potential readings were also taken to better understand the effects of water stress on the trees. Stem water potential was taken 3 days into the treatment, 6 days into the treatment, and 8 days into the treatment (harvest day) (Figure 8). Each measurement date showed a significant difference between treatments (n=5, $p<0.01$).

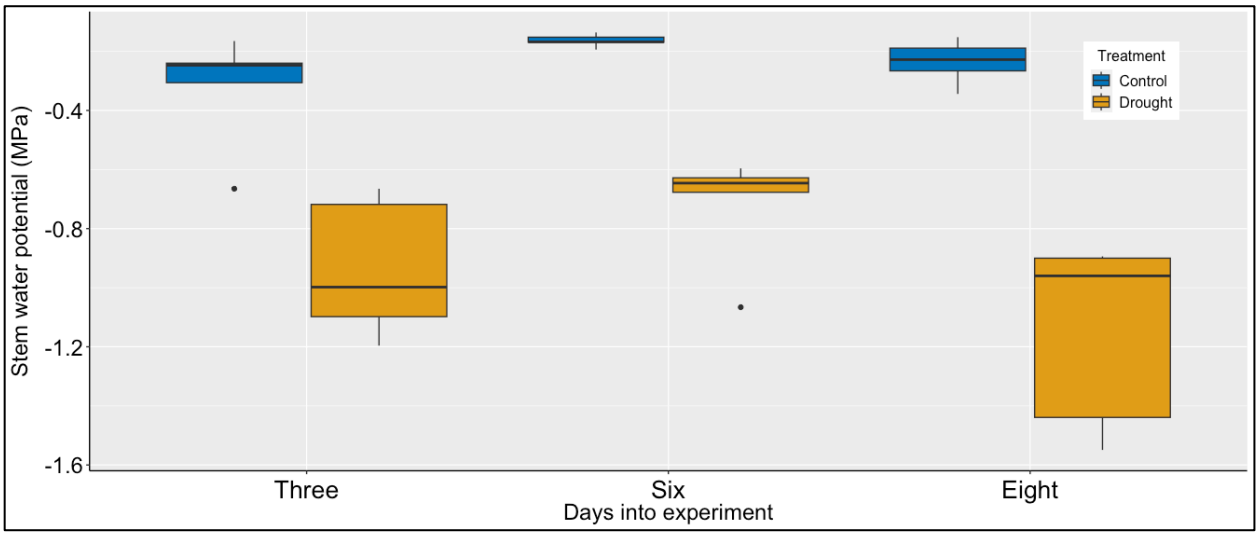

Figure 8. Stem water potential (MPa) was taken from each plant.

Relative water content

The relative water content (RWC) of leaf 8 and leaf 16 was measured on the day of the harvest (Figure 9). Due to the limited material for qRT-PCR in the apices, which are very small, RWC was not measured for this tissue. There was a highly significant difference between treatments for leaf 8, with the droughted plants having lower RWC than control plants (n=5, $p<0.001$). There was no significant difference in leaf RWC between treatments for leaf 16 (n=5, $p=0.6$).

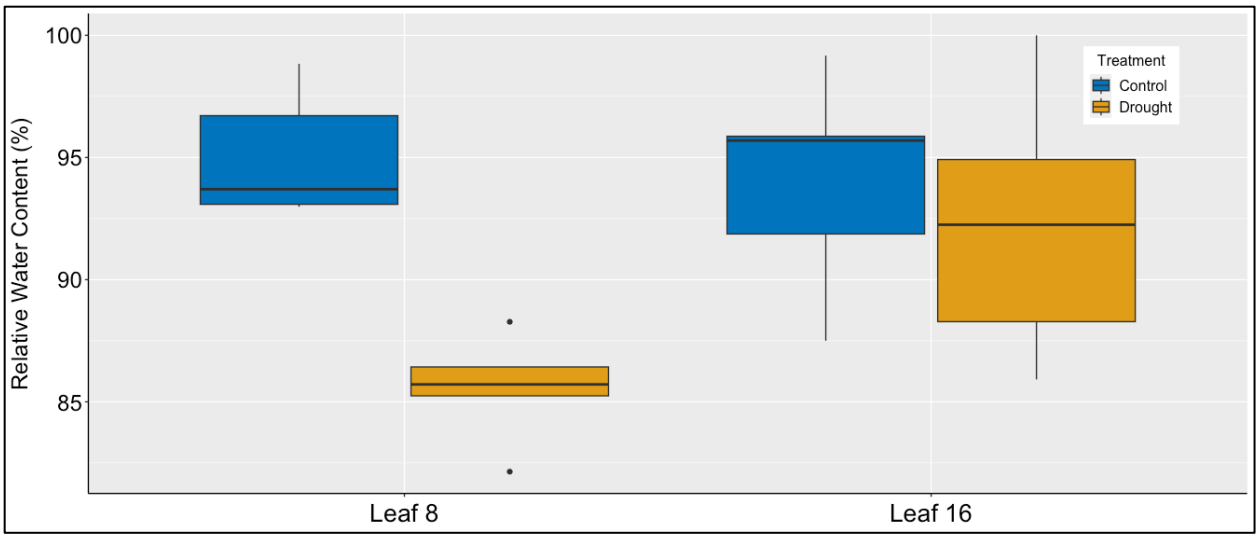

Figure 9. The relative water content (%) of leaf 8 and leaf 16 was measured eight days into the treatment, the day of harvest.

Quantifying gene expression: the normalizer genes

The results were first normalized by the normalizer genes actin, tubulin, and ubiquitin. This normalization uses a geometric mean of the three genes as a reference point for expression. This means that the average of the three genes affects the expression values for each gene of interest, because the genes are discussed *in reference* to this expression. However, in times of stress, genetic expression can change holistically as a plant is adapting to its circumstances. For the geometric mean of our three normalizer genes, we found higher Cq values for the plants receiving the drought treatment compared to the controls (Figure 10). These Cq values were significantly higher in the drought treatment across each tissue, which means that expression of these normalizer genes was lower in the drought plants than the control plants. This is because it took more amplification cycles in the qRT-PCR process for there to be an increase of fluorescence detected by the machine.

The Cq values of the normalizer genes used here increasing in drought is very important, because it means that all of the results that were calculated using the three normalizer genes are an

overestimation of expression. This is because relative expression is calculated by dividing the calculated expression of the normalizer genes by the gene of interest, which would be the YUCCA genes in this study. Therefore, all the results normalized by these three genes are an overestimation of YUCCA expression of the droughted tissues. These results, normalized by all three normalizer genes, will be described as G3 results henceforth for the ease of discussion.

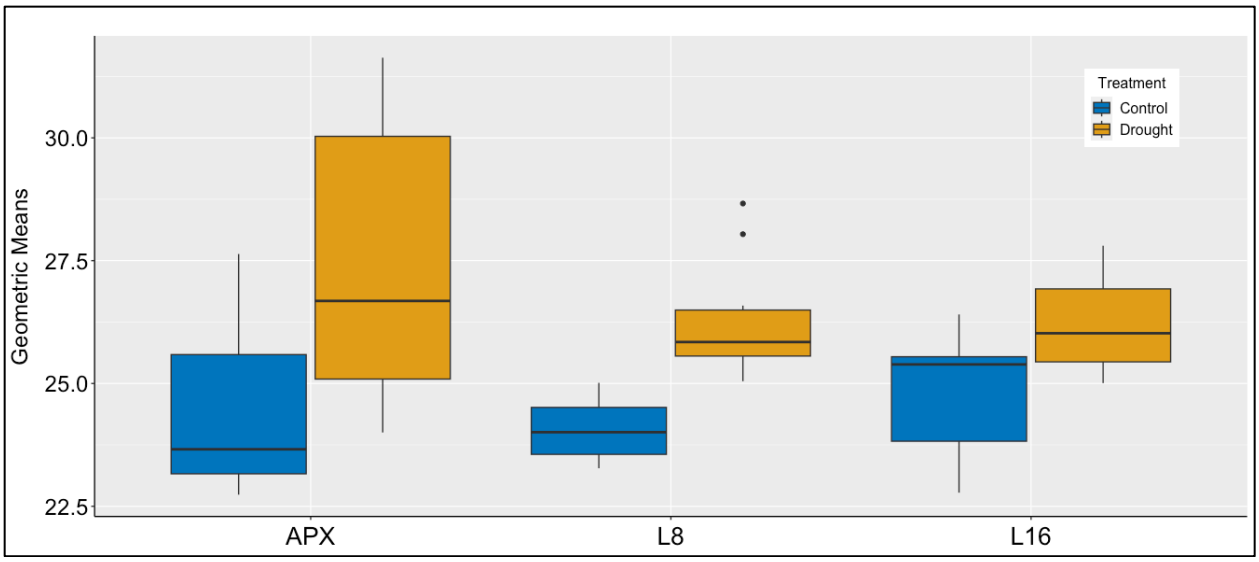

Figure 10. The geometric means of the three normalizer genes- ACT, TUB, and UBQ- were plotted across each tissue for the control and drought plants.

Using three normalizer genes is the best practice for calculating expression; it allows for the minute variations in individual tissues and plants in a gene's expression to have less of an effect on the calculated expression. However, the increase in Cq value (and therefore decrease in expression) indicates that normalizing by these genes is not representative of what is *actually* happening in these tissues under water stress.

When the normalizer gene Cq values were compared individually within each tissue, it was determined that ubiquitin (UBQ) changed the least between the two treatments (Figure 11). For the apex and leaf 16, UBQ did not have a significant change in expression between control and

drought (p=0.08, p=0.12, respectively). However, in leaf 8, the Cq values were significantly increased in drought (p=0.02). This statistical significance, or lack thereof, is trivial. In other words, although ubiquitin is the most stable normalizer gene, even the significant differences are not meaningful enough to draw any major conclusions from. Despite this, using just UBQ to normalize the expression of the YUCCA genes may give us insight into what the plant is *actually* doing. Additionally, it is useful to compare the results from the two methods side by side to predict which conclusions are completely unable to be supported by the data, especially if any conclusions from the G3 method show higher expression in drought (again, these results are an inflation of what is truly happening). Using just UBQ to normalize the expression of the YUCCA genes will henceforth be referred to as G1 normalization.

Expression of the YUCCAs

Five YUCCA genes were analyzed across three different tissues and were normalized in two different ways as described above (Figure 12). The apex had the highest expression of the YUCCA genes, with both YUC2 and YUC12 having the highest values of normalized expression across all tissues (Figure 12A, 12B). The expression of the other YUCCAs was low in the apices. Expression of all YUCCAs, excluding YUC2, decreases in leaf 8 and leaf 16 in relation to the apex (Figures 12C-F).

The G3 normalized results were analyzed using a mixed model that used treatment and tissue as fixed effects and plant as a random effect to see if there were interactions between treatment and tissue in a way that might affect expression (Table 3). It was found that there were significant interactions between YUCCA gene expression and tissue type, meaning that gene expression varied across tissue. The treatment also had a significant impact on the expression of these genes,

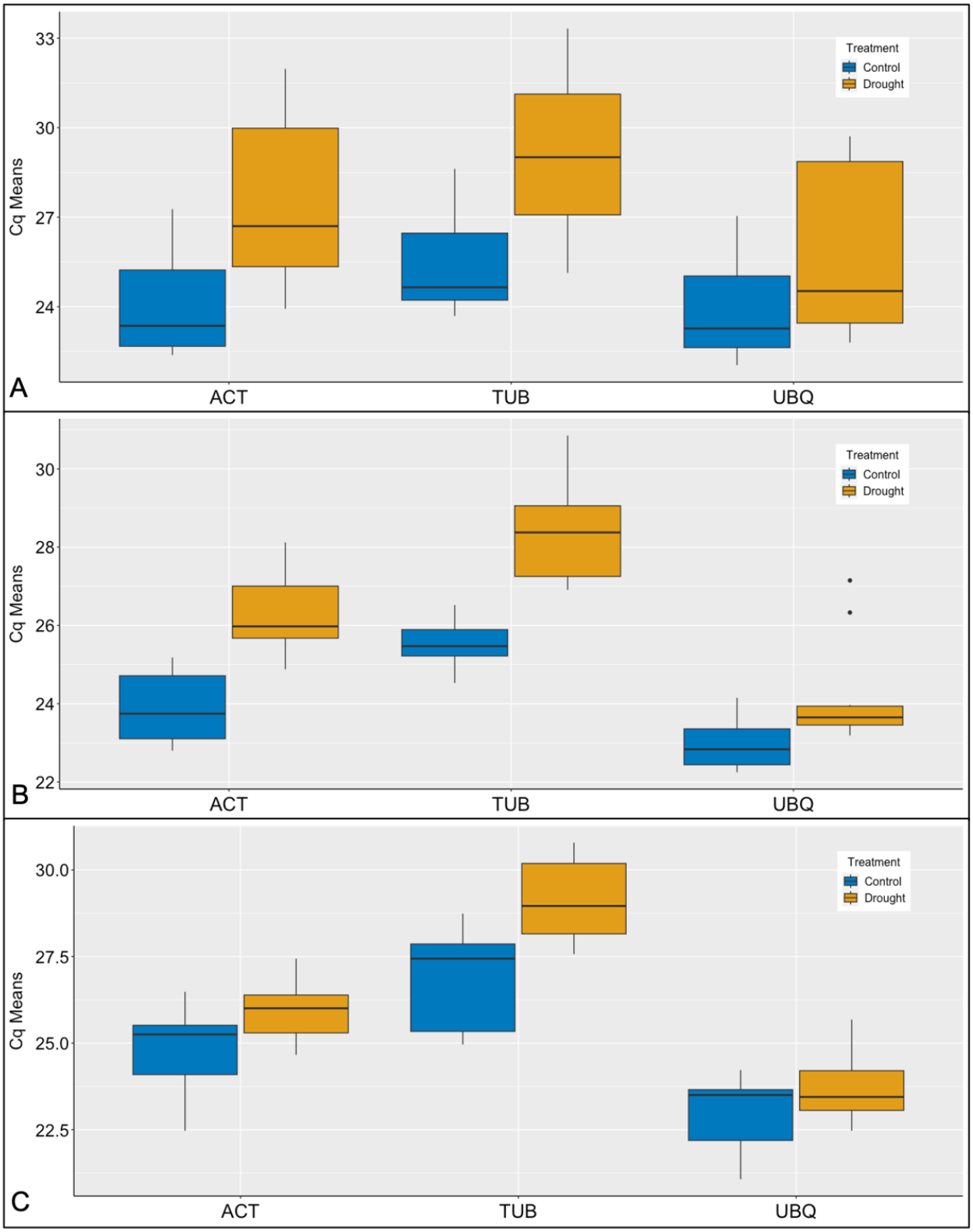

Figure 11. A comparison of the effects of drought on each normalizer gene Cq value between each tissue, for apices (A), leaf 8 (B), and leaf 16 (C).

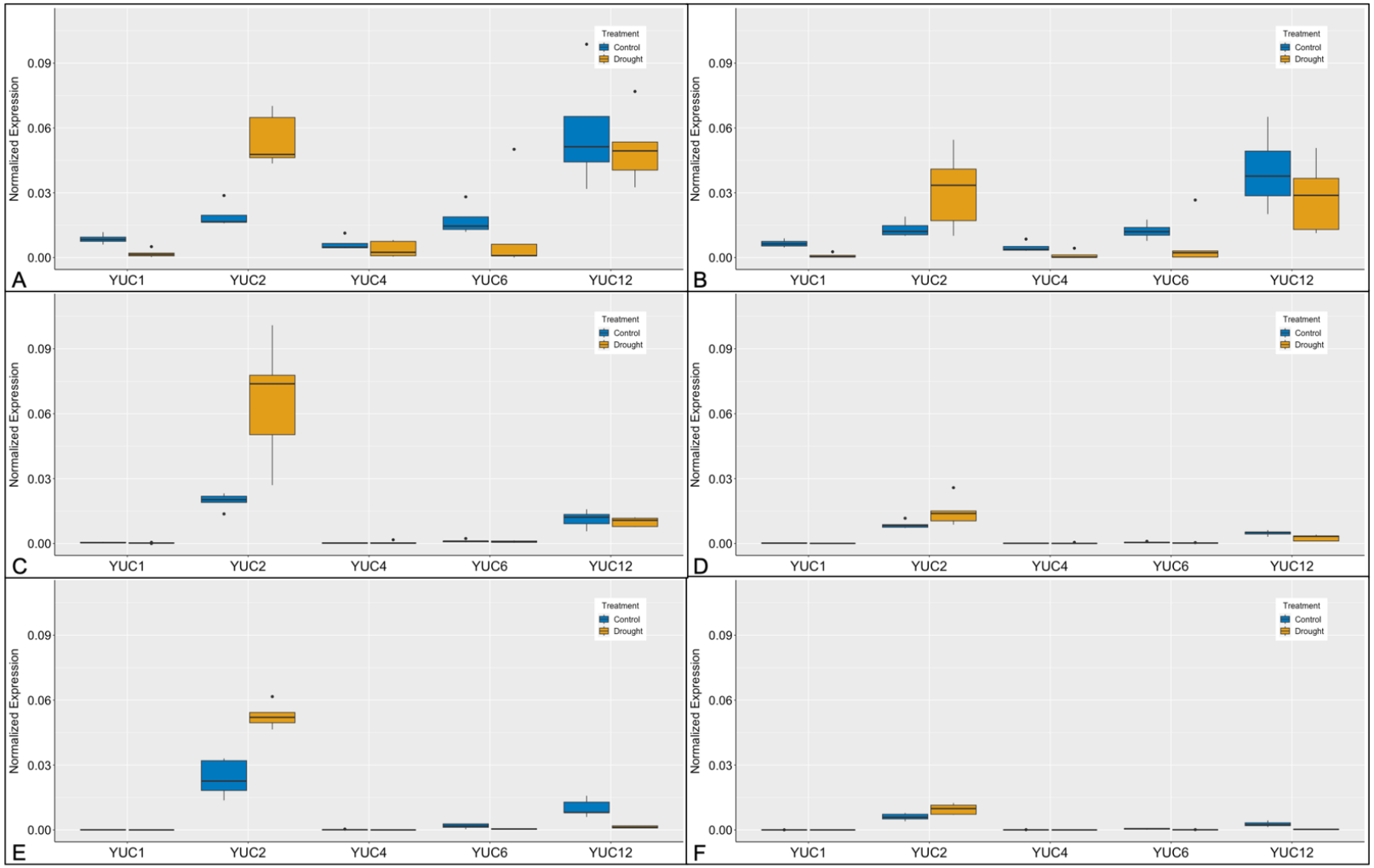

Figure 12. The genes of interest, YUC1, YUC2, YUC4, YUC6, and YUC12, were normalized by three normalizer genes (Panels A, C, and E) and by just ubiquitin (Panels B, D, and F). The genes were plotted for the apex (A & B), leaf 8 (C & D), and leaf 16 (E & F). The normalization values were plotted on the same axis.

meaning that there were genes affected by the water deprivation. However, the tissue was not significantly impacted by the treatment. This means that the different tissues did not behave differently from one another due to drought.

Table 3. An ANOVA was run on the mixed model results analyzing the effects that tissue, gene, and treatment have on one another from G3 gene expression calculations. Significance is designated with ☆, with "☆" < 0.05, "☆☆" < 0.01, and "☆☆☆" < 0.001.

	Sum of Squares	Mean of Squares	Degrees of Freedom	F value	P value (>F)	Significance
Tissue	0.0054	0.00271	2	31.34	$1.74x10^{-11}$	☆☆☆
Gene	0.0317	0.00793	4	91.63	$2.2x10^{-16}$	☆☆☆
Treatment	0.0006	0.00063	1	7.287	0.0262	☆
Tissue x Gene	0.0094	0.00118	8	13.58	$1.93x10^{-13}$	☆☆☆
Tissue x Treatment	0.00029	0.00014	2	1.67	0.1929	
Gene x Treatment	0.00916	0.00229	4	26.45	$2.8x10^{-15}$	☆☆☆
Tissue x Gene x Treatment	0.0003	0.00004	8	0.437	0.8962	

The results from G1 normalization were also analyzed in a mixed model that used treatment and tissue as fixed effects and plant as a random effect to see if there were interactions between treatment and tissue in a way that might affect expression (Table 4). The interactions were similar of those in the G3 expression between tissue and gene as well as gene and treatment. Therefore, the effects will be analyzed by gene, and not by tissue for each means of normalization for ease of discussion.

Table 4. An ANOVA was run on the mixed model results analyzing the effects that tissue, gene, and treatment have on one another from G1 gene expression calculations. Significance is designated with ☆, with "☆" < 0.05, "☆☆" < 0.01, and "☆☆☆" < 0.001.

	Sum of Squares	Mean of Squares	Degrees of Freedom	F value	P value (>F)	Significance
Tissue	0.0015	0.00076	2	27.35	$8.31x10^{-7}$	☆☆☆
Gene	0.0048	0.0012	4	43.128	$2.2x10^{-16}$	☆☆☆
Treatment	0.000001	0.000001	1	0.0513	0.8228	
Tissue x Gene	0.0034	0.0004	8	15.38	$3.98x10^{-14}$	☆☆☆
Tissue x Treatment	0.000014	0.000007	2	0.258	0.77481	
Gene x Treatment	0.0009	0.0002	4	8.149	$1.1x10^{-5}$	☆☆☆
Tissue x Gene x Treatment	0.0005	0.00006	8	2.220	0.03279	☆

Expression of YUC12 is the highest of all the YUCCAs and is also the highest in the apices (Figure 13). Using the G3 method of normalization, YUC12 is not significantly different between the treatments in the apices or leaf 8 (Figure 13A), but it is significantly decreased in the droughted leaf 16 samples ($p<0.01$). In the G1 method, both leaf 8 and leaf 16 have significantly decreased expression of YUC12 in droughted plants ($p<0.05$, $p<0.01$, respectively; Figure 13B).

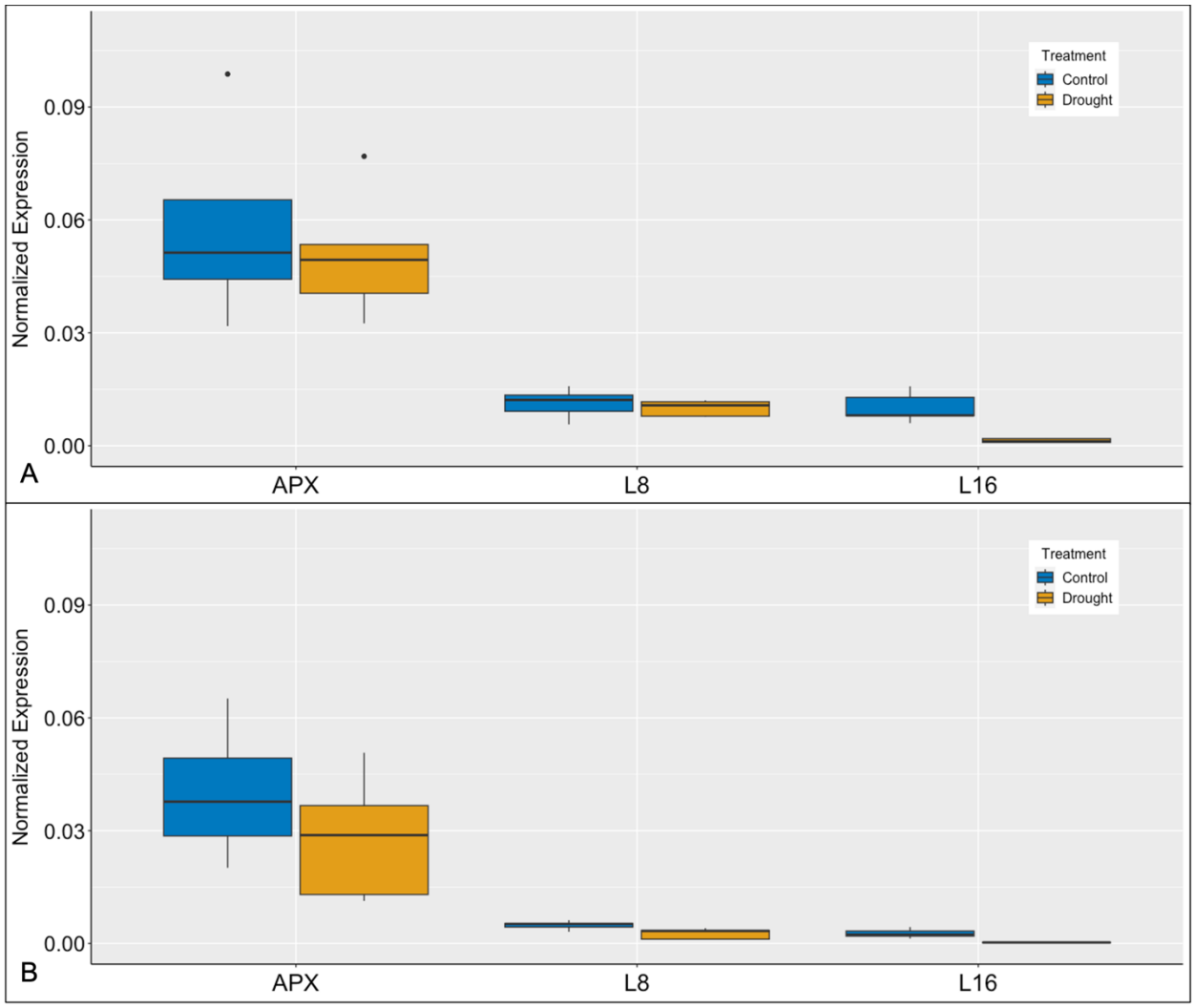

Figure 13. Expression of YUC12 was normalized across all three tissues for each treatment using the three reference genes (A) and just UBQ (B). The results of both normalization methods are on the same axis for ease of comparison.

The change between the two different normalization methods is most dramatic in YUC2 (Figure 14). In the G3 method of normalization, YUC2 is expressed at a significantly higher level in the

droughted plants in the apex, leaf 8, and leaf 16 ($p<0.01$, $p<0.01$, $p<0.001$ respectively; Figure 14A). However, in the G1 method, there is only a significant increase of YUC2 expression in droughted plants in leaf 16 ($p<0.05$; Figure 14B).

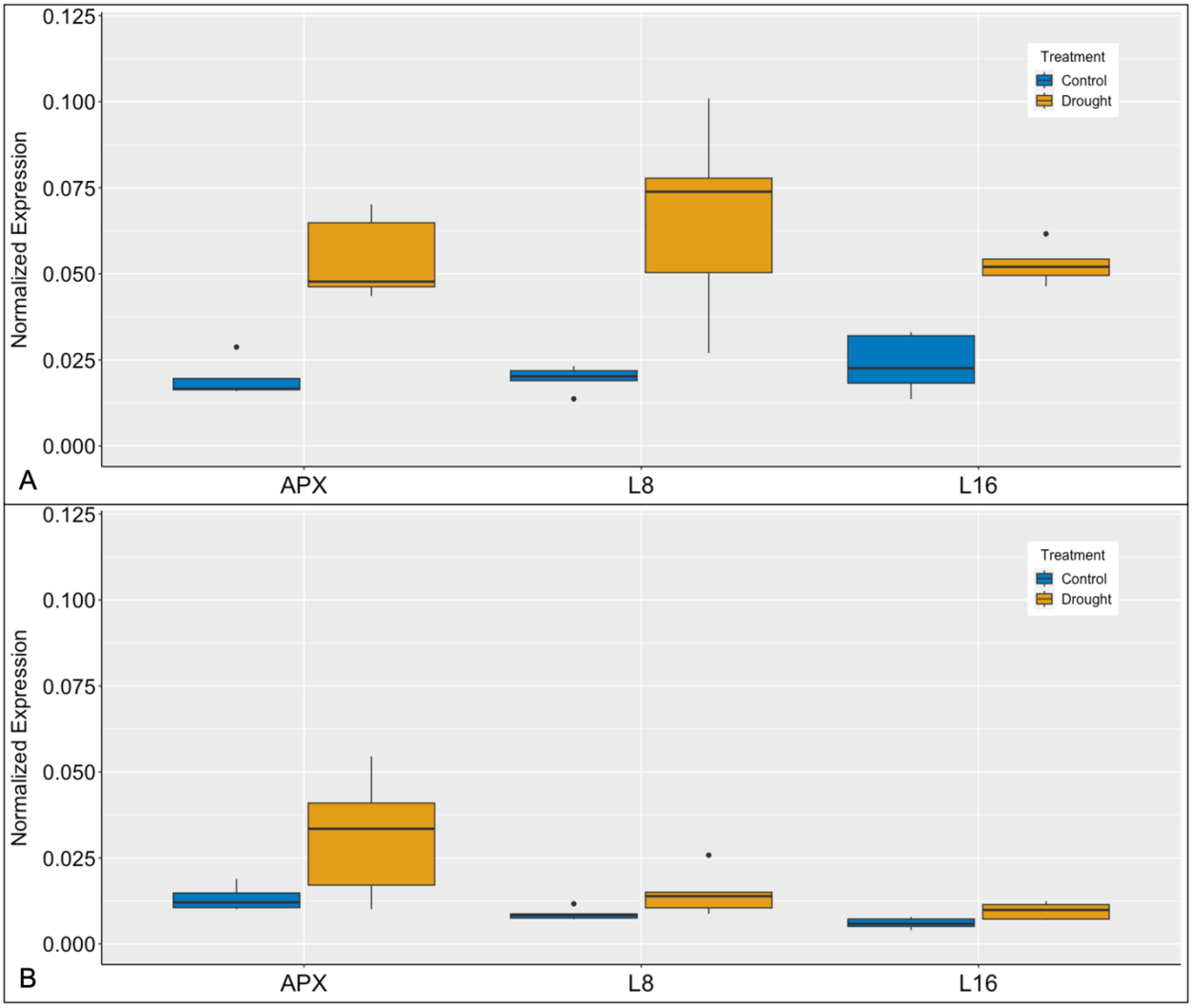

Figure 14. Expression of YUC2 was normalized across all three tissues for each treatment using the three normalizer genes (A) and just UBQ (B). The results of both normalization methods are on the same axis for ease of comparison.

YUC1 seems to have similar trends between the two different normalizations (Figure 15). Using G3 normalization, the apex and leaf 16 have significantly less expression of YUC1 in the droughted trees ($p<0.01$, $p<0.05$ respectively; Figure 15A). With G1 normalization, the apex and leaf 16 were again calculated to have significantly less expression of YUC1 ($p<0.001$, $p<0.05$

respectively; Figure 15B). It is important to note that this YUCCA, as well as YUC4 and YUC6 are expressed at much *lower* levels than the previous two YUCCAs.

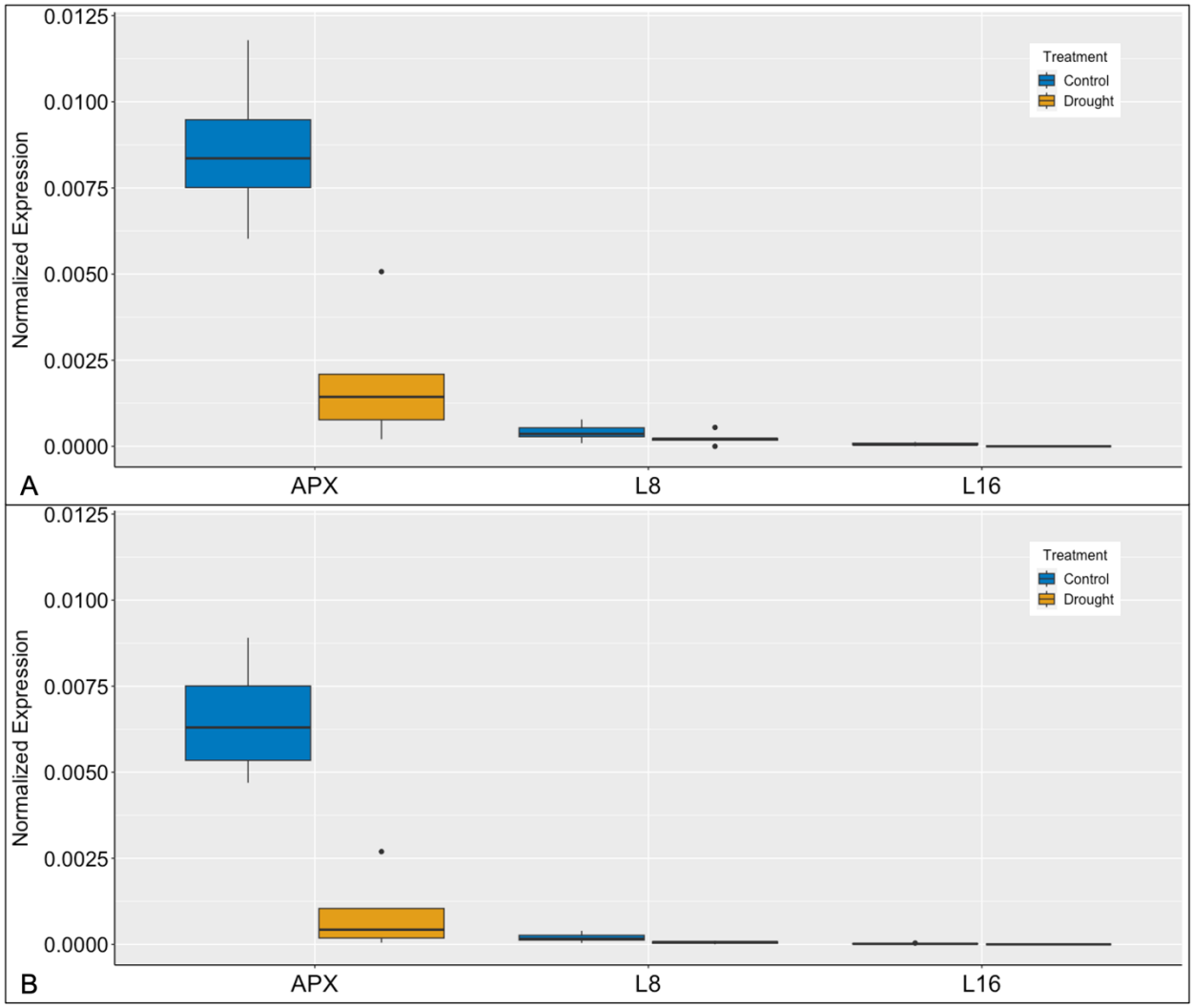

Figure 15. Expression of YUC1 was normalized across all three tissues for each treatment using the three normalizer genes (A) and just UBQ (B). The results of both normalization methods are on the same axis for ease of comparison.

The expression of YUC4 was affected by the different normalization methods (Figure 16). In the G3 method of normalization, YUC4 is not significantly different between the treatments in any of the tissues (Figure 16A). In the G1 method, the apices have significantly decreased expression of YUC4 ($p<0.05$, Figure 16B).

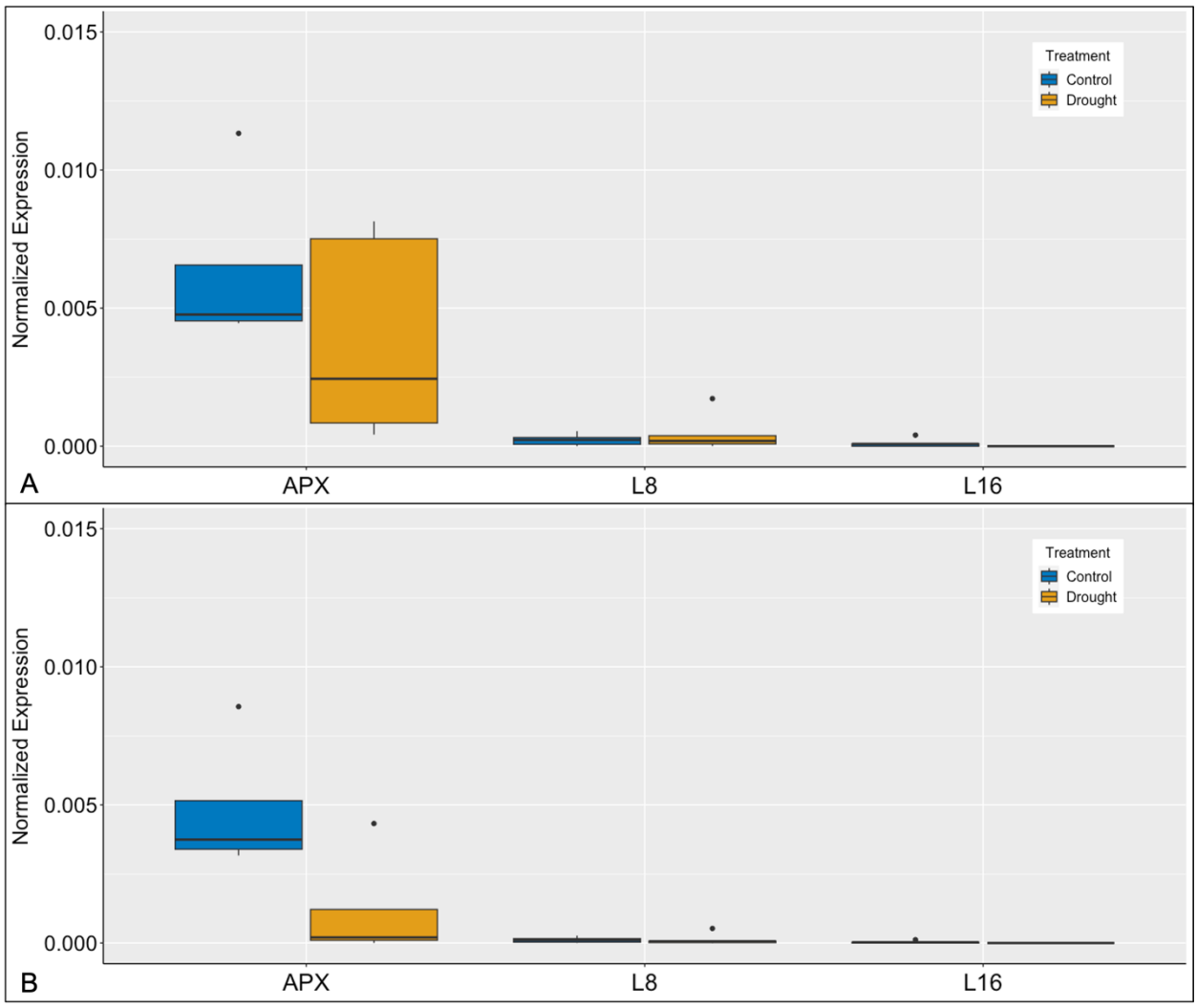

Figure 16. Expression of YUC4 was normalized across all three tissues for each treatment using the three normalizer genes (A) and just UBQ (B). The results of both normalization methods are on the same axis for ease of comparison.

The two normalization methods result in different conclusions being drawn about the expression of YUC6, however there is an outlier in both datasets that likely prevent significant results as well (Figure 17). In the G3 method, there were no significant differences across the different tissues (Figure 17A). There seems to be a decrease in expression in the apex, however there is a significant outlier that prevents this difference from being significant. When the G1 method was used, leaf 16 had a significant difference between treatments for YUC6 ($p<0.01$; Figure 17B).

Again, the lack of significance between treatments in the apices is likely due to the presence of an outlier in that data, as most of the tissues have decreased expression of YUC6 in the apex.

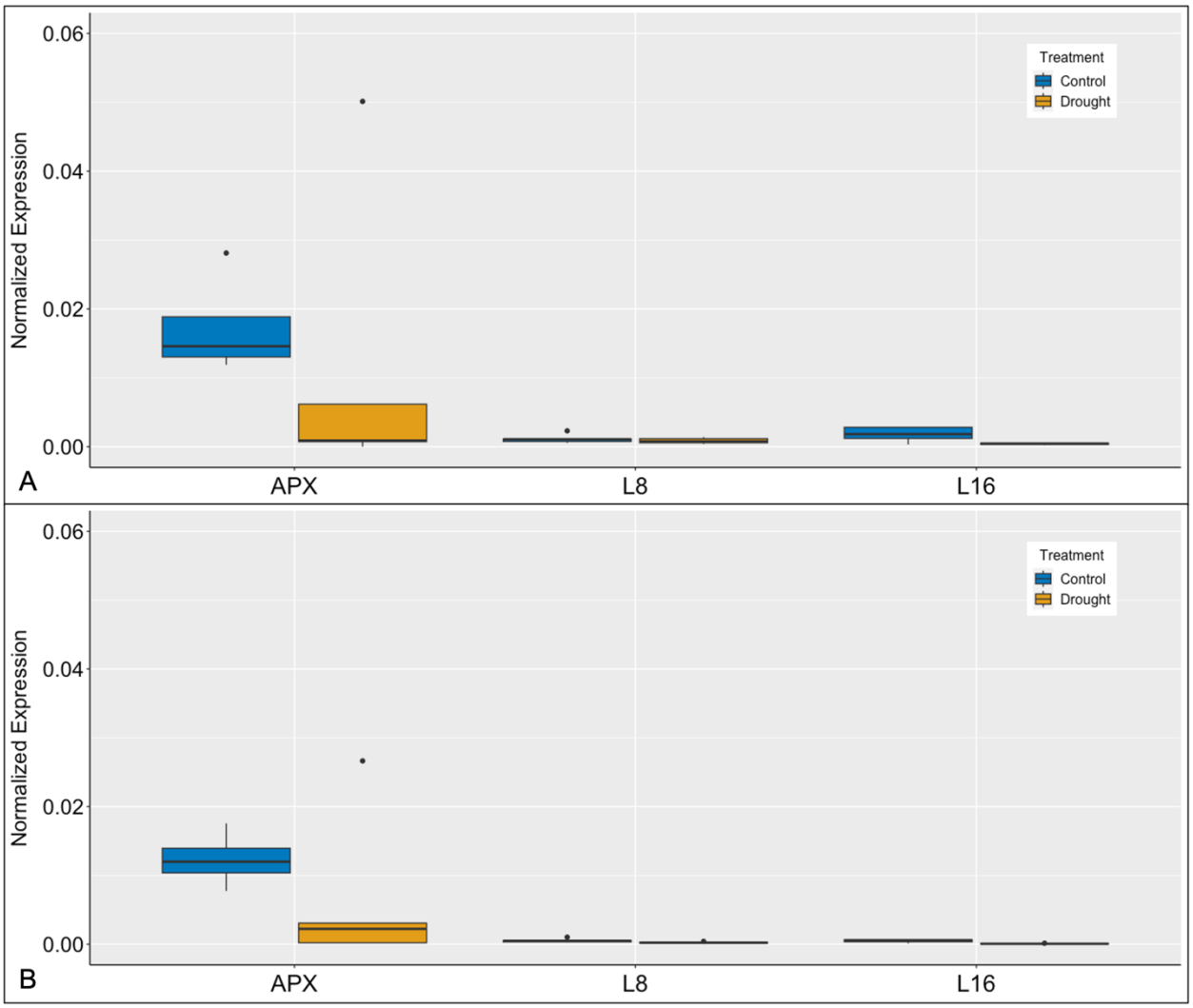

Figure 17. Expression of YUC6 was normalized across all three tissues for each treatment using the three normalizer genes (A) and just UBQ (B). The results of both normalization methods are on the same axis for ease of comparison.

Discussion

Effects of water stress on physiology

The plants in the experiment had statistically significant decrease in stomatal conductance in the drought treatments in the days leading up to the harvest, which was measured on leaves halfway between leaf 8 and leaf 16 (typically leaf 10) (Figure 7). Leaves are the main source of water loss in plants due to the opening and closing of stomata. Therefore, it is unsurprising that the rates of stomatal conductance would decrease significantly during drought; the plant is likely preserving the water available. In fact, the increased loss of water impacts the turgor pressure of cells, which in turn signals stomata to close (Chaves et al., 2009). Some studies have shown that species of *Populus* are likely to maintain moderate stomatal conductance until drought heightens in severity (Tang et al., 2013), though it seems that the point at which stomata are signaled to close is highly species dependent (Almeida-Rodriguez et al., 2010; Yang, 2010; Rosso et al., 2023).

The water in the xylem is under negative pressure, otherwise termed tension. The trees in the drought treatment experienced significantly more tension in the xylem, as shown in the more negative MPa values (Figure 8). This pressure decreasing (becoming more negative) means the xylem is under more stress, which could eventually lead to cavitation. In fact, many researchers describe the point at which 50% of conductivity is lost. This point of reference is helpful because plants at this stage are impaired in their ability to transport water and are considerably more likely to have long term consequences (Choat et al., 2008). In most hybrid species of *Populus*, the stem water potential at which 50% of the xylem is compromised is about −1.55 MPa (Rosso, 2023). The xylem water potential of the trees in the drought treatment here ranged from -0.9 MPa to -1.55 MPa after eight days, suggesting that some may have lost conductivity due to

embolism. The fact that stomatal conductance was significantly reduced after three days of water deprivation, coupled with a significant reduction in stem water potential, indicates that the trees were experiencing severe water stress.

The relative water content was not significant in leaf 16 across the two treatments, however leaf 8 in the control trees had significantly higher relative water content compared to the droughted trees (Figure 9). This seems to represent a shift in where drought might be felt in the plant. Another study on *Populus* leaves has shown that the stomatal conductance of mature leaves has a regulatory effect on the stomatal development of expanding leaves: when mature leaves were exposed to environmental cues such as increasing CO_2 concentrations, expanding leaves produced a lower density of stomata (Miyazawa et al., 2006). This survey introduces the idea that mature leaves, when faced with changed conditions, may be able to signal a developmental change to younger leaves. The relative water contents displayed that older leaves maintained their water content whereas younger leaves did not. Perhaps the more mature leaves, due to their inability to developmentally change their stomatal density, are likely to cease gas exchange and thus maintain their water content. These leaves, in turn, could be signaling to younger leaves to change their stomatal patterning in response to this changed condition (lack of access to water), which could allow these leaves to maintain their gas exchange. This idea is purely speculative, but interesting when trying to connect the differing results. When thinking about drought as a full plant experience, understanding how leaves might be experiencing water stress differently across different ages might provide insight into the pathways that plants take to mitigate the impact of drought.

Normalizer genes

"Housekeeping" genes are typically chosen for use as normalizer genes in qRT-PCR experiments due to their high and consistent expression levels in various tissues. The genes chosen for this survey were no exception to this rule; actin, tubulin, and ubiquitin are all imperative to the basic function of cells. In fact, the most abundant protein in all eukaryotic cells is actin; it is the key component in the formation of the cytoskeleton in cells (Zhang et al., 2010). Tubulin is also important to structure as it is the protein that make up the microtubules that are associated with the structuring of cell walls in plants (Oakley et al., 2007). Ubiquitin is important in regulating many major processes by controlling protein degradation (Moon et al., 2004). These three genes are often used as normalizer genes, and whether their usage in drought surveys is appropriate has come to the forefront of research (Zhao et al., 2015; Zhang et al., 2022). In two different species of *Populus*, the effect that drought has on these genes seems to be completely different, highlighting the likelihood that the effects of stress on these fundamental genes could be species specific (Chen et al., 2013; Wang et al., 2014; Tang et al., 2019). Our results displaying significant differences between treatments also highlight this concern and point towards the need to select different normalizer genes for surveys such as this where the target gene might not be the only thing affected by the treatment.

This change highlights the importance of understand how genes might respond to environmental stress. These particular genes are important in growth and development, and drought fundamentally affects these processes. In this way, it is likely that finding genes that are unaffected by water deprivation is difficult in general; drought affects the whole plant, and each tissue reacts differently. One way of identifying stably expressed genes is by doing the novel

technique where all of the mRNA in a tissue is sequenced to determine what genes are being expressed and at what concentrations. A logical next step for this type of survey is to perform this technique, known as RNA-Seq, to find genes that are still stably expressed in a stressed plant.

YUCCAs and drought - Normalization by all normalizer genes

Because the normalizer genes were affected by the treatment, it is important to keep in mind that all YUCCA expression levels in the drought treatment are an overestimation when normalized by all three genes (G3). Therefore, any comparison that shows drought plants with lower YUCCA expression than control plants is only strengthened, as the actual expression in the drought plants is likely lower than calculated. Due to the inflation of expression caused by the effect of drought on the normalizers, the discussion of this decrease in expression of YUCCA genes will be explored here. In contrast, any comparison that shows drought plants with higher YUCCA expression must called into question. This is also discussed below.

YUC12 seems to be unchanged in the apex and leaf 8 in droughted trees. In *Populus tremula* x *alba*, YUC12 is the predominant gene from the YUCCA family expressed in the apex, whereas it decreases down the stem (see introduction, Figure 5). Interestingly, it seems that this expression is mirrored in the droughted apices, where the highest expression was found of YUC12 in both control and drought plants. The trend of expression decreasing in the leaves is also confirmed with our experiment. These findings underscore the importance of this dominant YUCCA in the apex (Spicer unpublished data; Stadheim et al., 2023).

In both methods of normalization, YUC12 is significantly decreased in the drought treatment in leaf 16. This is interesting because leaf 16 displayed a relative water content that was not significantly different between treatments, which indicates that relative water content is likely not signaling a change in the expression of YUCCAs, and therefore the production of auxin. This drop in YUC12 production could, however, be connected to leaf abscission. In some plants, such as *Populus spp.*, it is common for the lower leaves to be shed in times of water stress (Barigah et al., 2007). This is due to the idea that the younger leaves are higher on the plant, likely receiving more sunlight and therefore able to photosynthesize more effectively with the limited water source. Moreover, when water is scarce, plants tend to prioritize the tissues underground to assimilate water and increase the root to shoot ratio. This ratio can also be maintained by shedding leaves. Leaf abscission is a process that is also governed by auxin, where the absence of auxin is a signal to the plant that the leaf should be senesced, or shed (Jin et al., 2015; Meir et al., 2015; Ma et al., 2021). In fact, it has been shown that drought disrupts the auxin responsible for delaying flower abscission, thereby increasing the rate at which flowers senesce under drought (Florkiewicz et al., 2020). The connection between auxin and leaf senescence could be indicative of why this important YUCCA has decreased expression in drought plants but only in the lower leaves.

Although YUC12 is not reduced in the apex for droughted plants, there are other indications that the production of auxin might be affected by water stress. YUC1 and YUC6 were shown to have decreased expression in the droughted plants' apices. Additionally, when YUC4 is normalized with just ubiquitin, it is significantly reduced in the apex. Because the apex is the source for most of the auxin for the plant, this reduction in YUCCA expression could indicate that auxin levels

are lower in the plant in times of water stress due to these YUCCAs. This decrease fits with the ideas that auxin is a major factor in plant growth, plants grow less under water stress, therefore there is likely less auxin being biosynthesized, by the YUCCA proteins. However, it has been shown that in *Populus tremula* x *alba*, the dominant YUCCA in the apex is YUC12, which is not significantly affected by drought. YUC1, YUC4, and YUC6 have much lower expression than YUC12. Therefore, total auxin production may not be severely impeded by these genes having less expression.

YUC1, YUC4, and YUC6 could be significantly decreased in drought plants because of the minute differences in the apex tissue. The apex, as we defined it, contained the shoot apical meristem (SAM) as well as any leaf primordia that were less than 2/3 uncurled along the margin. As known through previous experiments in the Spicer lab, an expanding leaf has YUCCA genes expressed at different levels within different tissues (Stadheim et al., 2023). Within the complex structure of the apex, there are likely many YUCCA proteins doing many different things as it relates to the SAM and the young developing leaves. The SAM is coordinated by auxin, which controls where new leaf primordium will emerge and the cells that will divide to form these cells (Kalve et al., 2014; Xiong et al., 2019; Perico et al., 2022). The developing leaves are home to many different sites of auxin maximum and minimum, which control the establishment of veins and the division of cells (Scarpella et al., 2006; Perico et al., 2022). The fact that the apex is home to so many different processes that involve auxin means that changes among individual YUCCA gene expression is linked to the fact that some processes are going to be affected by drought, such as leaves expanding, whereas total plant auxin production by the SAM might be preserved.

The influence of ROS

YUC2 was the only gene to show increased expression in drought, and this increase was seen across each tissue with G3 normalization. However, this increased expression is known to be over-estimated due to the normalizers' decreased expression, and therefore it is difficult to draw any real conclusion from these data. In fact, the differences between the two treatments in YUC2 expression become insignificant in the apex and leaf 8 when the G1 method is used to normalize expression.

However, other surveys have found YUCCA genes to be upregulated in times of water stress due to the thiol-reductase activities of YUCCA proteins, an activity that is thought to prevent damage due to reactive oxygen species, or ROS (Park et al., 2013; Cha et al., 2015; Mantova et al., 2021). The production of ROS increases in stress events such as drought. This is because when stomata close during drought, photosynthesis is unable to regenerate the compounds needed to accept electrons, ultimately leading to the reduction of oxygen to reactive species such as hydrogen peroxide and super oxide (Cruz de Carvalho, 2008; Chaves, 2009). ROS are then able to cause damage to different parts of a plant, including the DNA, which can lead to cell death (Abel and Hirt, 2004). Interestingly, ROS signal downstream effects in the plant that allow for stress tolerance, such as the upregulation in genes that help plants cope with stress. There are also ways for the plant to cope with increased production of ROS, such as the increase in ROS scavenging proteins (Mittler 2004; Cruz de Carvalho, 2008). Proteins with thiol reductase activity, like the YUCCAs, are known to help with this scavenging by providing the electrons needed to reduce the ROS to non-reactive oxygen species (Groot et al., 2022).

What is particularly interesting is the fact that each YUCCA protein in *Populus tremula* x *alba* has the cysteine residue that are deemed imperative for this function as described in Cha 2015. The fact that none of the YUCCAs have significantly increased gene expression in this experiment is surprising, due to this preserved area. Although we cannot say with certainty that any YUCCA measured in this experiment is a contender for contributing to ROS protection, it is certainly interesting that our results do not align with other publications, which have speculated a role for the YUCCA proteins in this stress response. Due to this, we can suggest that YUC2 might be gene that could be expressed at a higher level in drought, and therefore may be participating in ROS protection. This idea stems from the initial increase in expression that was calculated for YUC2 by the G3 method, which disappeared in the G1 method. While this significant difference does disappear between the two methods of normalization, it provides motive to look at this YUCCA specifically. This speculation serves for a starting point for a future experiment to truly assess the changes in YUCCA gene expression with stable normalizer genes.

Conclusions

It seems that the expression of the normalizer genes actin, tubulin, and ubiquitin varies in abiotic stress such as drought. This finding calls to attention the need to use normalizer genes that are stable in the specific treatment applied in an experiment while still having high expression to normalize against. One way of finding stable genes in a specific treatment is to perform the technique RNA-Seq on a sample to quantify highly expressed genes in a tissue that has been droughted.

YUC12 is a highly expressed YUCCA in the apex and its expression seems to be maintained in abiotic stress conditions. YUC12 expression was unaffected by the drought treatment in the apex, which supports previous research in *Populus tremula* x *alba* that has shown the importance of this gene. However, in the older leaves, there was a significant decrease in YUC12 expression in droughted tissues when expression is normalized by just ubiquitin. This is an interesting trend due to the lack of significant difference in relative water content results from these leaves, and this finding could be confirmed by more stable normalizer genes.

It seems that many of the YUCCA genes have decreased expressed overall in times of water stress. When normalized using all three normalizer genes and just ubiquitin, YUC1 is decreased in the apex and in leaf sixteen in the droughted plants. YUC4 is significantly decreased in the droughted apices using just ubiquitin as a normalizer. And YUC6 expression is likely decreased in the apices in droughted apices and leaf sixteen, though an outlier in the apex data set prevented any significance from being confirmed. Although these differences were reevaluated via normalization with just ubiquitin, these results should be verified further by using stable

normalizer genes. Moreover, these results represent trivial decreases in expression due to the low levels of expression compared to other YUCCAs. Thus, measuring auxin may provide a better idea for what this decreased expression represents in terms of total auxin biosynthesis in the plant.

YUC2 potentially is responsible for thiol-reductase activities. Some drought surveys in the past have found YUCCA expression to increase in response to increased ROS production. While the increase of YUC2 is inconclusive due to the issues with normalizer genes, this YUCCA seems to be the best candidate for future studies on YUCCA connection to thiol-reductase activity. Further experiments could supplement better normalizer genes with quantitation of ROS to connect any increased expression with an increase in ROS.

Future work in the scientific community should aim to use new techniques to characterize the expression of the YUCCAs in drought. One such way is through the technique RNA-Seq, where an individual can identify all RNA in a sample and the specific levels expressed in each tissue. We also will aim to quantify the auxin hormone itself in plant tissue between treatments to see if there is a difference in hormone concentrations in drought.

References

Abbate PE, Dardanelli JL, Cantarero MG, Maturano M, Melchiori RJM, Suero EE. 2004. Climatic and water availability effects on water-use efficiency in wheat. Crop Science 44(2):474-83. https://www.researchgate.net/publication/237470347_Climatic_and_Water_Availability_Effects_on_Water-Use_Efficiency_in_Wheat. DOI:10.2135/cropsci2004.0474.

Almeida-Rodriguez AM, Cooke JEK, Yeh F, Zwiazek JJ. 2010. Functional characterization of drought-responsive aquaporins in *Populus balsamifera* and *Populus simonii×balsamifera* clones with different drought resistance strategies. Physiologia Plantarum 140(4):321-33. https://www.academia.edu/3305279/Functional_characterization_of_drought_responsive_aquaporins_in_Populus_balsamifera_and_Populus_simonii_balsamifera_clones_with_different_drought_resistance_.

Anjum S, Xie X, Wang L, Saleem M, Man C, Lei W. 2011. Morphological, physiological and biochemical responses of plants to drought stress. African Journal of Agricultural Research 6(9):2026-32. https://academicjournals.org/article/article1380900919_Anjum%2520et%2520al.pdf. DOI: 10.5897/AJAR10.027.

Arend M and Fromm J. 2007. Seasonal change in the drought response of wood cell development in poplar. Tree Physiology 27(7):985-92. https://pubmed.ncbi.nlm.nih.gov/17403651/. DOI: 10.1093/treephys/27.7.985.

Bajguz A and Piotrowska A. 2009. Conjugates of auxin and cytokinin. Phytochemistry (Oxford) 70(8):957-69. https://pubmed.ncbi.nlm.nih.gov/19524990/. DOI: 10.1016/j.phytochem.2009.05.006.

Barigah TS, Charrier O, Douris M, Bonhomme M, Herbette S, Améglio T, Fichot R, Brignolas F, Cochard H. 2013. Water stress-induced xylem hydraulic failure is a causal factor of tree mortality in beech and poplar. Annals of Botany 112(7):1431-7. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3806533/. DOI:10.1093/aob/mct204.

Benková E, et al. 2003. Local, efflux-dependent auxin gradients as a common module for plant organ formation. Cell 115(5):591. https://pubmed.ncbi.nlm.nih.gov/14651850/. DOI: 10.1016/s0092-8674(03)00924-3.

Buckley TN. 2019. How do stomata respond to water status? The New Phytologist 224(1):21-36. https://nph.onlinelibrary.wiley.com/doi/10.1111/nph.15899.

Cha J, et al. 2015. A novel thiol-reductase activity of *Arabidopsis* YUC6 confers drought tolerance independently of auxin biosynthesis. Nat Commun 6(1): 1-13. https://www.nature.com/articles/ncomms9041.

Chaves MM, Flexas J, Pinheiro C. 2009. Photosynthesis under drought and salt stress: Regulation mechanisms from whole plant to cell. Annals of Botany 103(4):551-60. https://academic.oup.com/aob/article/103/4/551/164096.

Chaves MM, Pereira JS, Maroco J, Rodrigues ML, Ricardo CPP, Osorio ML, Carvalho I, Faria T, Pinheiro C. 2002. How plants cope with water stress in the field. photosynthesis and growth. Annals of Botany 89(7):907-16. https://academic.oup.com/aob/article/89/7/907/151103.

Chaves MM, Maroco JP, Pereira JS. 2003. Understanding plant responses to drought — from genes to the whole plant. Functional Plant Biology : FPB 30(3):239-64. https://pubmed.ncbi.nlm.nih.gov/32689007/. DOI: 10.1071/FP02076.

Chen J, Song Y, Zhang H, Zhang D. 2013. Genome-wide analysis of gene expression in response to drought stress in *Populus simonii*. Plant Mol Biol Rep 31(4):946-62. https://www.researchgate.net/publication/257639628_Genome-Wide_Analysis_of_Gene_Expression_in_Response_to_Drought_Stress_in_Populus_simonii. DOI:10.1007/s11105-013-0563-6.

Cheng Y, Dai X, Zhao Y. 2007. Auxin synthesized by the YUCCA flavin monooxygenases is essential for embryogenesis and leaf formation in *Arabidopsis*. The Plant Cell 19(8):2430-9. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2002601/. DOI: 10.1105/tpc.107.053009.

Cheol Park H, Cha J, Yun D. 2013. Roles of YUCCAs in auxin biosynthesis and drought stress responses in plants. Plant Signaling & Behavior 8(6):e24495(1)-e24495(3). https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3907447/. DOI: 10.4161/psb.24495.

Choat B, Jansen S, Broibb TJ, Cochard H, Delzon S, Bhaskar R, Bucci SJ, Feild TS, Gleason SM, Hacke UG, et al. 2012. Global convergence in the vulnerability of forests to drought. Nature (London) 491(7426):752-5. https://pubmed.ncbi.nlm.nih.gov/23172141/. DOI: 10.1038/nature11688.

Clark JS, Iverson L, Woodall CW, Allen CD, Bell DM, Bragg DC, D'Amato AW, Davis FW, Hersh MH, Ibanez I, et al. 2016. The impacts of increasing drought on forest dynamics, structure, and biodiversity in the United States. Glob Change Biol 22(7):2329-52. https://pubmed.ncbi.nlm.nih.gov/26898361/. DOI: 10.1111/gcb.13160.

Cleland RE, Buckley G, Nowbar S, Lew NM, Stinemetz C, Evans ML, Rayle DL. 1991. The pH profile for acid-induced elongation of coleoptile and epicotyl sections is consistent with the acid-growth theory. Planta 186(1):70-4. https://pubmed.ncbi.nlm.nih.gov/24186576/. DOI: 10.1007/BF00201499.

Cosgrove D. 2014. Plant cell growth and elongation. eLife. https://www.osti.gov/biblio/1385275.

Cruz de Carvalho MH. 2008. Drought stress and reactive oxygen species. Plant Signaling & Behavior 3(3):156-65. https://pubmed.ncbi.nlm.nih.gov/19513210/. DOI: 10.4161/psb.3.3.5536.

Evanich, Daniel, "A Whole-plant Approach to Identifying Sites of Auxin Biosynthesis in *Populus*" (2015). *Botany Honors Papers*. 5. http://digitalcommons.conncoll.edu/botanyhp/5.

Farooq M, Wahid A, Kobayashi N, Fujita D, Basra SMA. 2009. Plant drought stress: Effects, mechanisms and management. Agron Sustain Dev 29(1):185-212. https://link.springer.com/article/10.1051/agro:2008021.

Fichot R, Laurans F, Monclus R, Moreau A, Pilate G, Brignolas F. 2009. Xylem anatomy correlates with gas exchange, water-use efficiency and growth performance under contrasting water regimes: Evidence from *Populus deltoides* x *Populus nigra* hybrids. Tree Physiology 29(12):1537-49. https://pubmed.ncbi.nlm.nih.gov/19825869/. DOI: 10.1093/treephys/tpp087.

Florkiewicz AB, Kućko A, Kapusta M, Burchardt S, Przywieczerski T, Czeszewska-Rosiak G, Wilmowicz E. 2020. Drought disrupts auxin localization in abscission zone and modifies cell wall structure leading to flower separation in yellow lupine. International Journal of Molecular Sciences 21(18):6848. https://pubmed.ncbi.nlm.nih.gov/32961941/. DOI: 10.3390/ijms21186848.

de Groot A, Blanchard L, Rouhier N, Rey P. 2022. Thiol reductases in *Deinococcus* bacteria and roles in stress tolerance. Antioxidants 11(3):561. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8945050/. DOI: 10.3390/antiox11030561.
Hacke UG, et al. 2016. An ecophysiological and developmental perspective on variation in vessel diameter. Plant, Cell & Environment 40(6):831-45. https://pubmed.ncbi.nlm.nih.gov/27304704/. DOI: 10.1111/pce.12777.
Iqbal S, et al. 2022. Phytohormones trigger drought tolerance in crop plants: Outlook and future perspectives. Front Plant Sci 12(1):1-14. https://www.frontiersin.org/articles/10.3389/fpls.2021.799318/full.
Jansson S and Douglas CJ. 2007. *Populus* : A model system for plant biology. Annual Review of Plant Biology 58(1):435-58. https://pubmed.ncbi.nlm.nih.gov/17280524/.
Jin X, Zimmermann J, Polle A, Fischer U. 2015. Auxin is a long-range signal that acts independently of ethylene signaling on leaf abscission in *Populus*. Frontiers in Plant Science 6:634. https://www.frontiersin.org/articles/10.3389/fpls.2015.00634/full.
Johnson D, Eckart P, Alsamadisi N, Noble H, Martin C, Spicer R. 2018. Polar auxin transport is implicated in vessel differentiation and spatial patterning during secondary growth in *Populus*. American Journal of Botany 105(2):186-96. https://pubmed.ncbi.nlm.nih.gov/29578291/. DOI: 10.1002/ajb2.1035.
Jupa R, et al. 2021. Do angiosperm tree species adjust intervessel lateral contact in response to soil drought? Physiologia Plantarum 172(4):2048-58. https://onlinelibrary.wiley.com/doi/10.1111/ppl.13435.
Kalve S, De Vos D, Beemster GTS. 2014. Leaf development: A cellular perspective. Frontiers in Plant Science 5:362. https://www.frontiersin.org/articles/10.3389/fpls.2014.00362/full
Kim JI, Baek D, Park HC, Chun HJ, Oh D, Lee MK, Cha J, Kim W, Kim MC, Chung WS, et al. 2013. Overexpression of *Arabidopsis* YUCCA6 in potato results in high-auxin developmental phenotypes and enhanced resistance to water deficit. Molecular Plant 6(2):337-49. https://pubmed.ncbi.nlm.nih.gov/22986790/. DOI: 10.1093/mp/sss100.
Larson PR, Isebrands JG, Dickson RE. 1980. Sink to source transition of *Populus* leaves. 93:79-90. https://onlinelibrary.wiley.com/doi/10.1111/j.1438-8677.1980.tb03322.x.
Lee M, Jung J, Han D, Seo PJ, Park WJ, Park C. 2012. Activation of a flavin monooxygenase gene YUCCA7 enhances drought resistance in *Arabidopsis*. Planta 235(5):923-38.https://pubmed.ncbi.nlm.nih.gov/22109847/. DOI: 10.1007/s00425-011-1552-3.
Ljung K. 2013. Auxin metabolism and homeostasis during plant development. Development (Cambridge) 140(5):943-50. https://pubmed.ncbi.nlm.nih.gov/23404103/. DOI: 10.1242/dev.086363.
Ljung K, Bhalerao RP, Sandberg G. 2001. Sites and homeostatic control of auxin biosynthesis in *Arabidopsis* during vegetative growth. The Plant Journal : For Cell and Molecular Biology 28(4):465-74. https://pubmed.ncbi.nlm.nih.gov/11737783/. DOI: 10.1046/j.1365-313x.2001.01173.x.
Lovisolo C and Schubert A. 1998. Effects of water stress on vessel size and xylem hydraulic conductivity in *Vitis vinifera* L. Journal of Experimental Botany 49(321):693-700. https://academic.oup.com/jxb/article/49/321/693/450349.
Ma C, Jiang C, Gao J. 2021. Regulatory mechanisms underlying activation of organ abscission. Chichester, UK: John Wiley & Sons, Ltd. Annual Plant Reviews Online: 27-56 https://www.researchgate.net/publication/349773163_Regulatory_Mechanisms_Underlying_Activation_of_Organ_Abscission.

Mantova M, Herbette S, Cochard H, Torres-Ruiz JM. 2022. Hydraulic failure and tree mortality: From correlation to causation. Trends in Plant Science 27(4):335-45. https://www.sciencedirect.com/science/article/abs/pii/S1360138521002788.
Mashiguchi K, Tanaka K, Sakai T, Sugawara S, Kawaide H, Natsume M, Hanada A, Yaeno T, Shirasu K, Yao H, et al. 2011. Main auxin biosynthesis pathway in *Arabidopsis*. Proceedings of the National Academy of Sciences - PNAS 108(45):18512-7. https://www.pnas.org/doi/10.1073/pnas.1108434108.
Mcelrone AJ, et al. 2013. Water uptake and transport in vascular plants. Nature Education Knowledge 4(5):6. https://www.nature.com/scitable/knowledge/library/water-uptake-and-transport-in-vascular-plants-103016037/.
McQueen-Mason S, Durachko DM, Cosgrove DJ. 1992. Two endogenous proteins that induce cell wall extension in plants. The Plant Cell 4(11):1425. https://pubmed.ncbi.nlm.nih.gov/11538167/.
Meir S, Sundaresan S, Riov J, Agarwal I, Philosoph-Hadas S. 2015. Role of auxin depletion in abscission control. Stewart Postharvest Review 11(2):1-15. https://access.portico.org/Portico/auView?auId=ark:%2F27927%2Fphx64r3z8gq. DOI: 10.2212/spr.2015.2.2.
Mittler R, Vanderauwera S, Gollery M, Van Breusegem F. 2004. Reactive oxygen gene network of plants. Trends in Plant Science 9(10):490-8. https://pubmed.ncbi.nlm.nih.gov/15465684/. DOI: 10.1016/j.tplants.2004.08.009.
Miyazawa S, Livingston NJ, Turpin DH. 2006. Stomatal development in new leaves is related to the stomatal conductance of mature leaves in poplar (*Populus trichocarpa* x *P. deltoides*). Journal of Experimental Botany 57(2):373-80. https://pubmed.ncbi.nlm.nih.gov/16172139/. DOI: 10.1093/jxb/eri278.
Moon J, Parry G, Estelle M. 2004. The ubiquitin-proteasome pathway and plant development. The Plant Cell 16(12):3181-95. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC535867/.
Nakayama N, Smith R, Mandel T, Robinson S, Kimura S, Boudaoud A, Kuhlemeier C. 2012. Mechanical regulation of auxin-mediated growth. Current Biology 22(16):1468-76. https://pubmed.ncbi.nlm.nih.gov/22818916/. DOI: 10.1016/j.cub.2012.06.050.
Nilsson J, Karlberg A, Antti H, Lopez-Vernaza M, Mellerowicz E, Perrot-Rechenmann C, Sandberg G, Bhalerao RP. 2008. Dissecting the molecular basis of the regulation of wood formation by auxin in hybrid aspen. The Plant Cell 20(4):843-55. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2390731/. DOI: 10.1105/tpc.107.055798.
Oakley RV, Wang Y, Ramakrishna W, Harding SA, Tsai C. 2007. Differential expansion and expression of α- and β-tubulin gene families in *Populus*. Plant Physiology (Bethesda) 145(3):961-73. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2048781/. DOI: 10.1104/pp.107.107086.
Perico C, Tan S, Langdale JA. 2022. Developmental regulation of leaf venation patterns: Monocot versus eudicots and the role of auxin. The New Phytologist 234(3):783-803. https://nph.onlinelibrary.wiley.com/doi/full/10.1111/nph.17955.
Rosso L, Cantamessa S, Bergante S, Biselli C, Fricano A, Chiarabaglio PM, Gennaro M, Nervo G, Secchi F, Carra A. 2023. Responses to drought stress in poplar: What do we know and what can we learn? Life (Basel, Switzerland) 13(2):533. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9962866/. DOI: 10.3390/life13020533.

Scarpella E, Marcos D, Friml J, Berleth T. 2006. Control of leaf vascular patterning by polar auxin transport. Genes & Development 20(8):1015-27. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1472298/. DOI: 10.1101/gad.1402406.
Schrader J, Baba K, May ST, Palme K, Bennett M, Bhalerao RP, Sandberg G. 2003. Polar auxin transport in the wood-forming tissues of hybrid aspen is under simultaneous control of developmental and environmental signals. Proceedings of the National Academy of Sciences - PNAS 100(17):10096-101. https://www.pnas.org/doi/10.1073/pnas.1633693100.
Sharma L, Dalal M, Verma RK, Kumar SVV, Yadav SK, Pushkar S, Kushwaha SR, Bhowmik A, Chinnusamy V. 2018. Auxin protects spikelet fertility and grain yield under drought and heat stresses in rice. Environmental and Experimental Botany 150:9-24. https://www.cabdirect.org/cabdirect/abstract/20183157125.
Skirycz A and Inzé D. 2010. More from less: Plant growth under limited water. Current Opinion in Biotechnology 21(2):197-203. https://pubmed.ncbi.nlm.nih.gov/20363612/. DOI: 10.1016/j.copbio.2010.03.002.
Stadheim S, Pelletier S, Spicer R. 2023. YUCCA gene expression in expanding leaves of hybrid poplar. Abstract book North East American Society of Plant Biology Northeastern Section's 86th annual meeting:56. https://northeast.aspb.org/wp-content/uploads/2023/04/NE-2023-Abstract-book.pdf.
Tang F, et al. 2019. Selection and validation of reference genes for quantitative expression analysis of mRNAs and mRNAs in Poplar. Plant Methods 15(1):35(1)-35(15). https://plantmethods.biomedcentral.com/articles/10.1186/s13007-019-0420-1.
Tang S, Liang H, Yan D, Zhao Y, Han X, Carlson JE, Xia X, Yin W. 2013. Populus euphratica: The transcriptomic response to drought stress. Plant Mol Biol 83(6):539-57. https://pubmed.ncbi.nlm.nih.gov/23857471/. DOI: 10.1007/s11103-013-0107-3.
Taylor G. 2002. *Populus*: *Arabidopsis* for forestry. do we need a model tree? Annals of Botany 90(6):681-9. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4240366/. DOI: 10.1093/aob/mcf255.
Teale WD, Paponov IA, Palme K. 2006. Auxin in action: Signalling, transport and the control of plant growth and development. Nature Reviews. Molecular Cell Biology 7(11):847-59. https://www.nature.com/articles/nrm2020.
Turgeon R. 2006. Phloem loading: How leaves gain their independence. 56(1):15-24. https://academic.oup.com/bioscience/article/56/1/15/224791.
Tuskan GA, DiFazio S, Jansson S, Bohlmann J, Hellsten U, Putnam N, Ralph S, Rombauts S, Salamov A, Schein J, et al. 2006. Genome of black cottonwood, *Populus trichocarpa* (Torr. & Gray). Science 313(5793):1596-604. https://pubmed.ncbi.nlm.nih.gov/16973872/. DOI: 10.1126/science.1128691.
Vanneste S and Friml J. 2009. Auxin: A trigger for change in plant development. Cell 136(6):1005-16. https://pubmed.ncbi.nlm.nih.gov/19303845/. DOI: 10.1016/j.cell.2009.03.001.
Wang H, Chen J, Tian Q, Wang S, Xia X, Yin W. 2014. Identification and validation of reference genes for *Populus euphratica* gene expression analysis during abiotic stresses by quantitative real-time PCR. Physiologia Plantarum 152(3):529-45. https://pubmed.ncbi.nlm.nih.gov/24720378/. DOI: 10.1111/ppl.12206.
Williams AP, Cook ER, Smerdon JE, Cook BI, Abatzoglou JT, Bolles K, Baek SH, Badger AM, Livneh B. 2020. Large contribution from anthropogenic warming to an emerging North

American megadrought. Science (American Association for the Advancement of Science) 368(6488):314-8. https://pubs.giss.nasa.gov/abs/wi08400w.html.
Woodward AW and Bartel B. 2005. Auxin: Regulation, action, and interaction. Annals of Botany 95(5):707-35. https://pubmed.ncbi.nlm.nih.gov/15749753/. DOI: 10.1093/aob/mci083.
Xiong Y and Jiao Y. 2019. The diverse roles of auxin in regulating leaf development. Plants 8(7):243. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6681310/. DOI: 10.3390/plants8070243.
Yang F and Miao L. 2010. Adaptive responses to progressive drought stress in two Poplar species originating from different altitudes. Silva Fennica 44(1):23-37. https://www.researchgate.net/publication/265294194_Adaptive_Responses_to_Progressive_Drought_Stress_in_Two_Poplar_Species_Originating_from_Different_Altitudes.
Ye X, Kang B, Osburn LD, Li Y, Zong-Ming (Max) C. 2009. Identification of the flavin-dependent monooxygenase-encoding *YUCCA* gene family in *Populus trichocarpa* and their expression in vegetative tissues and in response to hormone and environmental stresses. Plant Cell, Tissue and Organ Culture 97(3):271-83. https://www.bioenergycenter.org/besc/publications/ye_yucca.pdf.
Zhang D, Du Q, Xu B, Zhang Z, Li B. 2010. Actin multigene family in Populus: Organization, expression and phylogenetic analysis. Mol Genet Genomics 284(2):105-19. https://pubmed.ncbi.nlm.nih.gov/20577761/. DOI: 10.1007/s00438-010-0552-5.
Zhang L, et al. 2022. Selection and evaluation of candidate reference genes for quantitative real-time PCR in aboveground tissues and drought conditions in rhododendron delavayi. Front Genet 13(1):876482 https://www.frontiersin.org/articles/10.3389/fgene.2022.876482/full.
Zhang X, Zang R, Li C. 2004. Population differences in physiological and morphological adaptations of *Populus davidiana* seedlings in response to progressive drought stress. Plant Science (Limerick) 166(3):791-7. https://www.sciencedirect.com/science/article/abs/pii/S0168945203004989.
Zhao H, Wang S, Chen S, Jiang J, Liu G. 2015. Phylogenetic and stress-responsive expression analysis of 20 WRKY genes in *Populus simonii*×*Populus nigra*. Gene 565(1):130-9. https://pubmed.ncbi.nlm.nih.gov/25843624/. DOI: 10.1016/j.gene.2015.04.002.