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CHARACTERIZING DROUGHT ADAPTATIONS, PHENOTYPIC PLASTICITY, AND FIXED GENE EXPRESSION PATTERNS WITHIN QUERCUS

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CHARACTERIZING DROUGHT ADAPTATIONS, PHENOTYPIC PLASTICITY, AND FIXED GENE EXPRESSION PATTERNS WITHIN *QUERCUS*

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Abstract

This dissertation was written on topics related to the genus *Quercus* with a primary focus on *Quercus ellipsoidalis* (northern pin oak) and *Quercus rubra* (northern red oak). Within this dissertation are chapters related to the setup of experimental common gardens within the Ford and Kellogg experimental forest, a literature review describing drought adaptations of *Quercus* sect. *Lobatae* (red oak group), identification of transcription factors within the *Q. robur* (English oak) and *Q. rubra* genomes, a study comparing leaf trait phenotypic plasticity of *Q. ellipsoidalis* and *Q. rubra*, and an RNA-seq experiment studying ecological speciation between *Q. ellipsoidalis* and *Q. rubra*. Within these studies, I found that *Q. ellipsoidalis* and *Q. rubra* have similar leaf trait phenotypic plasticity, and unique molecular phenotypes related to upregulation of genes related to photosynthesis and innate immune response, respectively. Within the *Q. rubra* genome, I identified multiple regions of transcription factor gene clusters that could have a significant role related to drought adaptation for this species.

1 Chapter 1: dissertation introduction

1.1 Background

The *Quercus* genus (oaks) is a biologically diverse hardwood taxa within the *Fagaceae* family; the genus is estimated to include 500 distinct species (Denk et al., 2017; Nixon, 2002, 2006). Oaks are found throughout the northern hemisphere in Europe, Africa, Asia, and the Americas. Oaks live in a range of habitats including tropical, subtropical, temperate, Mediterranean, and arid climates. Drought adaptations are common to the *Quercus* genus, and, as a result, there is a great diversity and distribution of oak species in areas of marginal precipitation and/or high elevation (Cavender-Bares et al., 2000; Fallon et al., 2018; Valencia, 2010). *Quercus* is an ecologically important taxa too, providing structural habitat and contributing prominently to nutrient cycling (Anderson et al., 2007; Hansen, 2000; Stoler et al., 2011; Thompson et al., 2012). Mixed oak forests are a prominent part of North America, constituting more than half of all forested land area, and oaks themselves account for nearly one fifth of all forest biomass within the North American range (Cavender-Bares, 2019; Little, 1979; Smith et al., 2009).

Most oaks in North America are assigned to either the *Lobatae* (red oak) or *Quercus* (white oak) sections. While section *Quercus* is found throughout the Northern hemisphere, the *Lobatae* section is exclusive to the western hemisphere from Canada to Columbia (Johnson et al., 2019). There are four major distinctions between red and white oaks. (1) The lobes of a of red oak leaf are usually pointed with bristle tips; the lobes of a white oak are rounded. (2) Red oaks need two seasons for acorn maturation; white oaks require one season (3). White oak vessel elements have tyloses, a cellular outgrowth that plugs the vessel during drought and infection slowing the rate of spread (Cochard et al., 1990); most

red oaks do not have tyloses. (4) Inner surfaces of acorn caps are tomentose in red oaks, and glabrous in white oaks (Johnson et al., 2019; Tucker, 1980).

Most of my thesis describes experiments using two species from the red oak section: *Quercus ellipsoidalis* E. J. Hill (northern pin oak, upland pin oak, or Hill's oak) and *Quercus rubra* Linnaeus (northern red oak). The native ranges of both species differ in that *Q. ellipsoidalis* grows in xeric and dry-mesic habitats within the northern hardwood forest in Michigan, Wisconsin, Minnesota, Iowa, Illinois, and Indiana, while *Q. rubra* grows in mesic environment that span from the northern hardwood forest into the northern sections of the southern mixed forest (Figure 1) (Bonner et al., 2008; Little, 1979).

These species have unique morphology and anatomy, and in some cases, these traits confer an adaptation to drought. In form, *Q. ellipsoidalis* is a small to medium sized tree (12-18 m height, 30-50 cm diameter) with many horizontal branches forming a cylindrical crown; *Q. rubra* can grow to be a very large tree (20-28 m height, 50-100 cm diameter) with a rounded crown formed by a few heavy branches with a wide spread. As the tree ages, lower branches senesce in both species, but persist in *Q. ellipsoidalis* and self-prune in *Q. rubra* (Barnes, 2004). Leaves of both species have been discussed at length by Gailing et al., 2012. To summarize, *Q. ellipsoidalis* leaves are smaller (7-13 cm length and width), with narrow lobes (between 5 and 7) and deep rounded sinuses, while *Q. rubra* leaves are larger (13-23 cm length, 9-15 cm wide), with wide base lobes (between 5 and 11) and rounded sinuses (Figure 2b) (Barnes, 2004; Gailing et al., 2012; Hipp, 2010). Anatomical differences between the leaves of both species have also been noted: *Q. ellipsoidalis* leaves have a relatively high leaf thickness, high stomatal density, and small stomatal aperture,

and *Q. rubra* leaves have a longer stomatal pore (Abrams, 1990). These adaptations may provide a greater water use efficiency in *Q. ellipsoidalis*, and better temperature regulations through increased transpiration rates in *Q. rubra* (Abrams, 1990). Like many other *Lobatae* species, *Q. ellipsoidalis* and *Q. rubra* have a ring-porous xylem anatomy, meaning that early wood vessel elements have a larger diameter then those found in late wood tissues (Barnes, 2004; Robert et al., 2017). Like many other *Lobatae* species, *Q. ellipsoidalis* and *Q. rubra* have a ring-porous xylem anatomy, meaning that early wood vessel elements have a larger diameter then those found in late wood tissues (Abrams, 1990; Barnes, 2004). It is possible these difference in root structure explain the soil preferences of both species (Bonner et al., 2008).

These species have interesting taxonomic history. Jensen et al., 1984 hypothesized that *Q. ellipsoidalis* is a product of a hybrid swarm between *Q. coccinea, Q. palustris, Q. rubr*a and *Q. velutina*. Hybridization and introgression have been observed between each of these species (Cavender-Bares, 2019; Owusu et al., 2015). Genetic STRUCTURE analysis reveals that hybridization and introgression occurs at very low rates between Q. *rubra* and *Q. ellipsoidalis* (Cavender-Bares, 2019; Owusu et al., 2015). This suggests that the species identity is maintained by an unknown biological or environmental barriers (Gailing et al., 2012; Lind-Riehl et al., 2013). These barriers are commonly observed within the *Quercus* genus (Oney-Birol et al., 2018) and many consider *Quercus* to be a model taxa for studying the interactions between ecology and evolution (Cavender-Bares, 2019).

The regional and international importance of *Quercus* sets a precedent for understanding how the genus and its taxa will respond to rapid climate change. Since the industrial revolution, mankind's increasing use and reliance on fossil fuels has drastically increased greenhouse gas emissions and the planet's capacity for retaining solar energy (Prather et al., 2001; Prentice et al., 2001; Schimel et al., 1996). Since the 1880's globally averaged temperatures have risen more than 1℃. Initially global temperatures increased by 0.08 ℃ per decade, but have more than doubled since the 1980's, and currently reside at 0.18 ℃ increase per decade (Blunden et al., 2020; NOAA, 2020; Stocker, 2013). If these trends continue, by the year 2100 global average temperatures are expected to rise somewhere within the range of 2.6 and 4.8 ℃ (Collins et al., 2013).

To quote Seddon (2010), "climate change changes everything." Regarding the biosphere and water cycle, we have seen a greater occurrence in extreme weather events, including (but not limited to) severe and protracted droughts (Dai, 2011; Jentsch et al., 2007; Min et al., 2011; Pachauri et al., 2014). Many forest scientists consider severe drought events to be a major threat to tree and forest health (Frelich et al., 2010). From the perspective of an individual tree, droughts lower plant water content, which can arrest or diminish photosynthesis, and negatively impact a tree's physiology and biochemistry (Anjum et al., 2011; Chaves et al., 2003). From the forest perspective, drought cause stands to becomes more susceptible to insect pests, disease, and wildfire (Choat et al., 2018; Kolb et al., 2016; Stephens et al., 2018).

Since selective pressures are increasing with climate change, the suitable habitats for many forest species are now in a dynamic state of flux. These shifts in suitable habitat have

been well modeled for the eastern US forests by Prasad et al. (2008). Looking at the modeled data for all tree species, we can make two general statements: (1) suitable habitat is generally shifting to more northern latitudes; and (2) an average tree species requires a migration rate of 1km/year to keep up with changes in suitable habitat (Iverson et al., 2002; Iverson et al., 2004). This second point is rather alarming: because of their sessile nature, limited seed dispersal range, and long juvenile periods, generally trees have very slow migration rates (Krutovsky et al., 2012). Without some form of human intervention, some species face extirpation or extinction (McLachlan et al., 2007). Maintaining forest biodiversity requires a complex mitigation strategy that assists migration into the leading edge of suitable habitat, and migrates climate change resilient individuals into pre-existing populations. Adding more complexity to this issue, these mitigation strategies must be species specific (Vitt et al., 2010).

The models by Prasad et al., 2008 show an interesting trend for the species in genus *Quercus*. While suitable habitat normally decreased for tree species of the eastern US, the suitable habitat for all tested red oak species (16 in total; this list includes *Q. ellipsoidalis* and *Q. rubra*) is projected to increase by at least 50% (Peters et al., 2019a, 2019b; Prasad et al., 2008). These results are very interesting and maybe related to the drought adaptations that are common to oaks.

Selecting the appropriate populations for migration is best informed with provenance trials. In provenance experiments, an assortment of seed sources are planted together across several common gardens. Measurements related to growth and physiology are regularly taken from each common garden to assess the performance of a provenance or population

performance in relation to the environment (Risk et al., 2021). Although provenance experiments have a large time investment, the results of these experiments can show a populations climate change resilience, phenotypic plasticity, and the adaptation (González-Martínez et al., 2006; Pedlar et al., 2012). This data is best used for understanding how seed sources can be used within assisted migration (Pedlar et al., 2012; Risk et al., 2021; Ste-Marie et al., 2011).

For my PhD project, I designed a *Q. ellipsoidalis* and *Q. rubra* provenance experiment. This experiment has two common garden sites located in the Ford and Kellogg experimental forest. The methods for the design of this experiment are detailed here.

1.2 Methods

1.2.1 Population Sampling

Q. ellipsoidalis and *Q. rubra* populations were sampled by selecting forested stands within the native distribution ranges of both species (Figure 1). We sampled between 3 and 10 families within each population range (Table 1). The word family here refers to a collection of half-siblings, meaning that acorns within a family have the same maternal and assumed differing paternal linages. Sampling the families, acorns were collected directly off the branch of and/or from the immediate area underneath the mother tree's canopy. In the first described method, pole-saws were used to access out-of-reach branches. We only conducted collection by the second method if another oak canopy was not directly overlapping the mother tree's canopy. With these sampling methods, we collected twenty or more acorns from each family. We sampled a total of 28 populations; 22 *Q. rubra*, 4 *Q.*

ellipsoidalis, and 2 *Q. alba* (a white oak species that I had misidentified as *Q. rubra*) (Table 1).

After collecting acorns from each family, we subjected the acorns to a float test to remove dehydrated and infected acorns from our collection (Gribko et al., 1995). Dehydration and infection cause space to from between the acorn's cotyledon and pericarp: the air within this space cause the "bad" acorns to float in a bucket of water. The viable acorns can be collected from the bottom of the bucket. The viable seeds were then stored at 4 °C for 3 to 6 months (time length depended on the date the population was sampled). This cold storage provided a stratification period required for *Q. ellipsoidalis* and *Q. rubra* germination (Bonner et al., 2008).

1.2.2 Greenhouse propagation

Acorns were planted in the Michigan Technological University's College of Forest Resources and Environmental Sciences greenhouse facilities on April 3rd and 4th 2019. Growth within the greenhouses lasted for approximately 3 months. We intended to plant 20 acorns from each of the collected families: in a few situations, because of the float test, this was impossible. After recording the acorns mass, each seed was planted into a half-liter plug containing a loamy soil, a mixture of 1 part sand and 3 parts garden soil. This was the best soil type for growing both species (Bonner et al., 2008). Greenhouse conditions were maintained at 16-hour light and 8-hour dark periods. Day and night temperatures were maintained at 23 °C and 18 °C, respectively. Every three days, the plugs were watered until soil saturation. Dates of seedling germinations were recorded. A

germination success rate can be calculated from these observations, but these values will be artificially high because the dehydrated/infected acorns were removed by the float test.

Seedlings were moved outside two weeks prior to their common garden planting dates to ease the transition. To reduce photodamage from sun exposure, a mesh screen was placed over the seedlings during the first week. Watering was continued at the same frequency.

1.2.3 Naming system

On the date of planting, each seedling was assigned a name that would provide a detailed description of each plant. These names consisted of three numbers separated by periods. The first number (01-30) indicates which population the seedling belongs to; the second number (001-155) indicates which family the seedling belongs to; the third number (1-20) represents the individual within the family.

1.2.4 Common garden description and design

The two common garden sites are located at the Michigan Technological University's Ford Experimental Forest (Alberta, Michigan; 46.64 N, -88.48 W) and Michigan State University's W.K. Kellogg Experimental Forest (Augusta, Michigan; 42.47 N, -85.36 W). The Kellogg site is considerably warmer and wetter than the Ford sites. Mean annual temperatures at the Ford and Kellogg are 4.9 and 9.9 ℃, respectively, and annual precipitation is 879 and 1027mm, respectively (Fick et al., 2017). The soil type at the Ford and Kellogg sites are a coarse sandy loam. The loam at the Ford Site is more gravelly then the Kellogg (Post et al., 2007).

The Kellogg and Ford common gardens were respectively established on June 12th and 22nd, 2019. Each common garden was designed to contain 810 experimental plants. The plants were arranged into 27 blocks, where each block has 30 plants arranged in 10 rows and 3 columns that are spaced 1.5 m and 0.76 m apart, respectively. A single row of buffer plants was placed outside of each block. The block design served two purposes. First each block should be able to contain at least one plant from each population, and second, the blocks could be used to establish treatments for future experimentation. A plant's location within the common garden was set using a semi-random block design (Ariel et al., 2010). Tables 2 and 3 show the layout of each common garden site.

To prevent herbivory from deer, the common garden perimeters are surrounded by fences 3 m tall. To reduce resource competition, the weeds at the Ford and Kellogg sites were regularly removed from the surrounding area of each tree by herbicide application and lawn mowing, respectively. Because of the drier site conditions, plants at the Ford site were regularly watered during the 2019 and 2020 growing seasons. Our watering methods used a bucket with holes at the bottom to simulate rain. The watering schedule was as follows: June 17 to July 14, 2019, every three days; July 15 to September 2, 2019 and June 15 to July 12, 2020, every seven days; July 13 to July 26, 2020 every ten days; and July 22 to August 23, 2020 every two weeks. The plants were not watered during the 2021 growing season. To support root nutrient uptake, iron chelate was administered to all trees at the Ford common garden on August 12th, 2019 and again on June 13th, 2020.

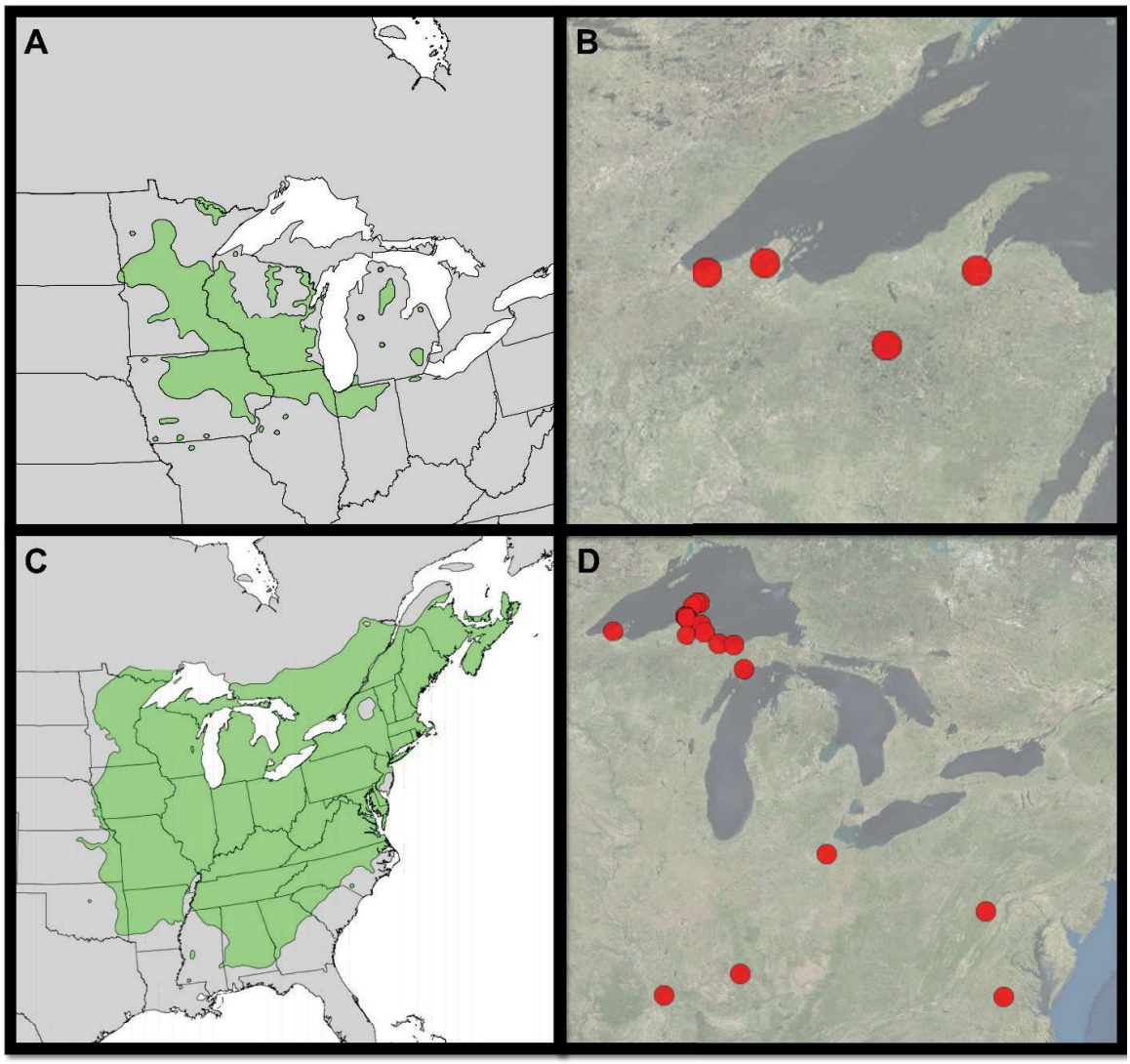
1.2.5 Water exclusion treatments

My initial intention was to use water exclusion treatments to characterize differences in phenotypic plasticity and genetic response between each species (*Q. ellipsoidalis* and *Q. rubra*), and among their populations. To do this, 13 of the 27 blocks at each common garden site were selected as water restriction treatments. The water restriction treatments can be described as follows: plastic tarps were cut to the size of the blocks, and holes were made where the trees would be. 15 cm diameter Plastic drainage pipes were cut to 7 cm width and secured to the plastic sheet using a plastic adhesive glue and duct tape. The water restriction apparatus was placed over the block and secured using rocks and woody debris. The soil moisture (volumetric water content: VWC%) was measured at in each treatment at one-week intervals using a Campbells data logger attached to Vegetronix VH400 soil moisture probes. Soil moisture measurements were collected every 5 minutes and an average was taken from the measurements each hour.

A two-way ANOVA was run on our data to determine if the treatments and/or the measurement timeframe had a significant effect on VWC%. Both variables had a significant independent and combined effect on VWC%. However, upon examination of the data, we found that when compared to the controls, the water restriction treatments greatly increased VWC% (Tables 4 and 5). Although the water restriction apparatus may have lowered the amount of water entering the blocks, the water within the treatments was trapped by the plastic sheet and unable to leave by evaporation. The experiments that were initially planned were adjusted accordingly, and only measurements from the control treatments were used from the 2021 growing season.

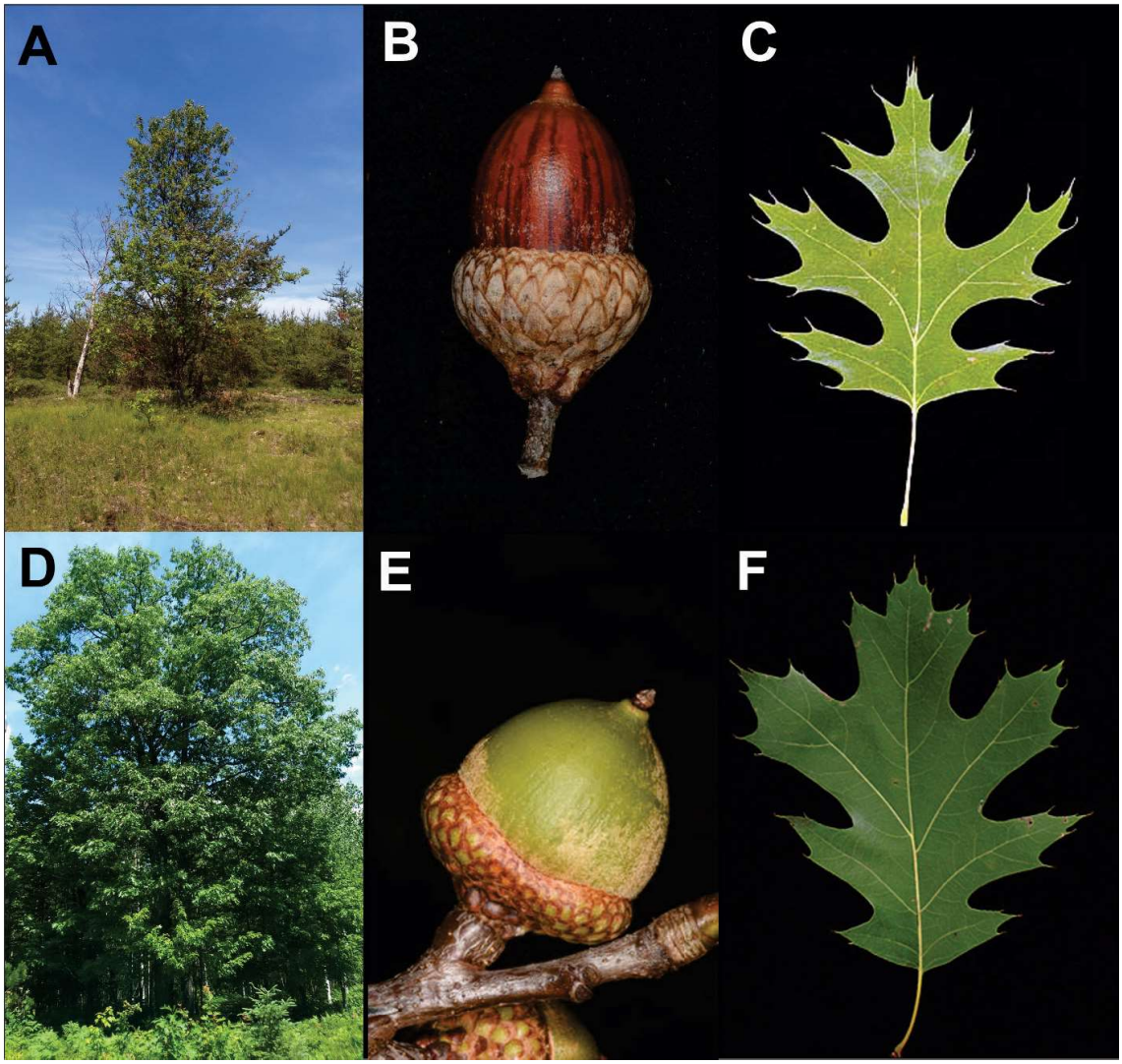
1.3 Chapter 1 figures

1.3.1 Figure 1



Q. ellipsoidalis (QE) and *Q. rubra* (QR) ranges and population locations. Natural range of *QE* (A). Sampling locations of the 4 *QE* populations (B). Natural range of *QR* (C). Sampling locations of the 22 *QR* populations (D). Maps of species ranges were taken from Little (1979). Maps of the sampling locations were made using gpsvisualizer.com.

1.3.2 Figure 2



Images of *Q. ellipsoidalis* (QE) and *Q. rubra* (QR). QE tree (A). QE acorn (B). QE leaf (UWGB, 2004) (C). QR tree (D). QR acorn (E). QR leaf (F) (Seiberling et al., 2005). Acorn images are from Kew (2021).

1.4 Chapter 1 tables

1.4.1 Table 1

Information on oak populations planted at the Ford and Kellogg common gardens. The species of each population is listed in the "species" column: Qa – *Quercus alba*, Qe –

Quercus ellipsoidalis, and Qr – *Quercus rubra*. MAT is short of mean annual temperature for the populations seed source. Pop is short for population.

Population Name	Species	Lat.	Long.	Pop	Families	MAT
Dow forest, MI	Qr	47.425	-88.056	1	1,4,5,6	4.2
Copper Harbor Sand dunes, MI	Qr	47.440	-88.219	2	7,8,9,10,11,12	4.5
Cliff Drive, MI	Qr	47.354	-88.350	3	13,14,15,16	4.3
Maasto Hiihto (1), MI	Qr	47.138	-88.610	4	17,18,19,20,21,22	4.9
Maasto Hiihto (2), MI	Qr	47.140	-88.612	30	153,154,155	4.9
Nara Trails (1), MI	Qr	47.103	-88.520	5	24,25	4.9
Nara Trails (2), MI	Qr	47.107	-88.551	6	26,27,28,29,30,31	4.9
Nara Trails (3), MI	Qr	47.105	-88.529	7	32,33,34,35,36	4.9
Nara Trails (4), MI	Qr	47.102	-88.554	8	37,38,40,41,42	4.9
Nara Trails (5), MI	Qr	47.106	-88.555	9	43,44,45,46,47	4.9
Baraga Plains Red Oak Stand, MI	Qr	46.673	-88.529	11	58,60,61	4.4
Mouth of the Huron River, MI	Qr	46.909	-88.036	12	62,63,64,65,66,67	5.4
Eagle Mine, MI	Qr	46.749	-87.891	13	68,69,70,71,72,73	4.1
Blue Berry Ridge Trails, MI	Qr	46.456	-87.424	14	74,75,76,77,78,79	5.0
Au Train, MI	Qr	46.438	-86.878	15	80,81,82,83,84,85	5.4
Big Bay de Noc, MI	Qr	45.868	-86.518	16	86,87,88	5.5
Hoosier, IN	Qr	38.060	-86.661	18	95,96,97,98	12.9
Trail of Tears, IN	Qr	37.480	-89.360	21	109,110,111,112,113,114	13.6
Russell, WI	Qr	46.768	-91.105	23	121,122,123	4.6
Warfordsberg, PA	Qr	39.737	-78.180	27	140,141,142,143	11.3
Cygnet, OH	Qr	41.238	-83.681	28	144,145,146,147,148	9.8
Rockwood Park, VA	Qr	37.451	-77.580	29	149,150,151,152	14.2
Baraga Plains Pin Oak Stand, MI	Qe	46.658	-88.576	10	50,51,52,53,54,55,56,57	4.4
Nebish, WI	Qe	46.057	-89.623	24	124,125,126,127	4.0
Valhalla, WI	Qe	46.721	-91.045	25	128,129,131,133	4.4
Brule, WI	Qe	46.649	-91.742	26	134,135,136,137,138,139	4.6
Grayling, MI	Qa	44.624	-84.761	17	89,90,91,92,93	6.2
Steven's Point, WI	Qa	44.541	-89.274	22	115	6.2

Species	Total Populations (#)	Total Families (#)
Q. rubra	22	102
Q. ellipsoidalis	4	21
Q. alba	2	6

1.4.2 Table 2

Ford Forest common garden map consisting of 27 blocks (A-ZZ). Blocks colored in blue are control treatments. Blocks colored in orange are drought treatments. Blocks A- O.

A	B	C

01.005.17	15.083.18	06.026.1	21.114.12	16.088.11	26.136.19	13.071.1	21.110.7	12.064.8
14.078.15	07.035.9	23.122.3	25.129.14	18.095.5	03.015.6	26.138.7	21.114.15	27.142.6
03.014.6	10.053.2	15.081.9	14.077.12	10.057.11	21.111.2	22.115.14	01.004.18	14.076.4
16.086.10	26.136.8	27.142.15	02.011.5	07.035.2	12.064.7	02.008.3	28.146.15	26.134.18
26.139.17	10.056.11	04.019.19	03.015.16	16.088.5	30.154.13	13.068.3	26.135.16	10.054.19
12.067.17	21.112.20	30.154.4	26.134.5	03.016.7	06.028.11	01.005.16	06.031.10	14.077.11
14.076.8	13.069.7	26.137.1	18.097.8	09.045.1	13.068.7	28.145.17	26.137.17	12.062.17
04.021.19	05.025.16	21.113.12	10.051.5	29.150.14	26.139.14	06.026.15	26.136.7	17.093.7
06.031.6	15.083.16	28.145.6	15.081.5	03.013.9	16.086.6	21.111.14	13.068.13	24.124.6
28.145.3	16.087.8	04.020.19	08.042.19	02.012.11	25.133.3	30.154.18	11.058.9	30.154.6
D			E			F		
11.060.14	21.112.19	29.150.9	04.020.15	26.135.4	06.026.12	03.013.2	17.090.8	30.155.5
16.088.19	10.056.15	27.141.15	26.138.15	27.142.17	13.069.6	02.012.18	12.064.3	30.155.16
13.070.7	01.006.17	12.063.12	15.085.8	16.087.5	02.008.15	28.146.19	21.114.7	10.056.13
29.151.7	16.087.14	22.115.1	13.072.13	26.139.15	15.083.9	02.012.7	11.058.8	18.095.7
28.148.19	17.090.19	08.042.4	04.017.10	13.073.2	28.145.13	30.153.6	23.121.13	13.070.4
26.136.6	02.012.8	28.144.16	28.144.9	02.011.3	09.043.20	26.138.17	21.114.2	27.140.17
26.139.9	13.072.17	02.010.20	14.076.19	09.046.5	26.137.12	03.015.7	9.047.8	13.070.12
09.046.9	27.143.1	29.152.10	04.022.17	29.151.16	09.045.13	10.050.15	15.084.3	26.134.1
15.081.13	02.009.12	04.019.10	30.153.18	08.038.7	04.018.14	26.136.1	17.092.9	15.080.12
10.054.8	18.097.1	21.111.1	18.096.7	18.095.15	10.053.15	29.151.12	13.070.14	09.047.18
G			H			I		
23.121.7	14.076.13	12.064.11	26.135.15	09.047.19	09.045.7	02.008.6	04.017.8	26.138.13
15.085.11	03.013.5	07.035.8	15.081.7	04.020.6	17.093.17	21.112.14	02.007.1	14.079.7
28.148.14	21.110.2	01.001.4	01.004.7	09.043.8	01.004.3	02.010.6	02.007.3	08.037.6
10.057.8	03.015.1	16.086.13	21.110.14	21.111.4	12.062.13	28.145.7	08.042.3	18.098.15
26.137.11	02.010.11	07.035.15	04.019.8	06.026.5	27.142.7	15.080.14	14.078.10	28.146.2
03.015.4	04.017.4	21.113.5	16.088.14	16.087.2	02.011.20	01.006.7	02.012.19	27.143.8
30.155.19	08.042.8	02.008.8	29.151.2	12.065.13	04.022.11	08.042.16	25.129.10	01.005.1
02.010.10	03.013.7	12.065.12	28.148.12	04.021.8	04.019.1	03.013.13	14.078.17	14.078.20
12.062.19	12.067.10	14.078.8	09.043.5	29.150.7	10.055.4	10.056.9	14.077.9	27.141.2
02.007.5	29.150.6	14.077.3	03.016.11	29.149.9	01.006.10	21.111.10	Buffer	26.138.6
J			K			L		
16.088.4	21.111.11	16.088.6	14.078.3	28.146.6	15.083.19	02.012.15	10.055.2	28.146.4
04.019.20	04.021.2	8.041.10	07.034.16	22.115.8	09.045.2	26.138.9	10.054.12	02.009.1
29.150.11	18.096.8	15.085.2	28.147.8	13.072.6	21.112.15	26.137.15	21.113.2	27.143.3
02.011.7	17.090.10	14.079.6	10.054.10	12.062.10	13.072.20	04.017.14	04.019.15	12.065.18
01.004.5	13.069.8	15.081.10	10.053.11	07.035.14	24.124.2	12.064.4	18.098.5	02.010.8
18.095.18	18.096.2	08.042.14	22.115.13	26.137.4	07.032.14	29.149.17	15.082.15	29.152.19
04.020.18	07.034.19	15.084.6	12.066.5	16.086.4	15.084.8	03.015.17	13.070.15	15.082.11
30.153.1	23.122.12	04.017.15	08.040.6	23.121.3	29.149.8	30.155.11	02.007.6	15.082.18
14.078.9	28.148.17	16.087.15	12.067.1	13.071.10	04.017.12	03.015.15	14.074.6	01.004.14

09.044.1	17.092.7	23.122.1	25.133.8	26.135.17	25.129.7	14.076.5	15.080.9	14.074.8
M			N			O		
06.031.4	30.154.12	28.146.11	17.093.18	15.080.5	13.068.18	26.137.10	03.015.13	30.153.15
01.004.8	13.073.19	18.097.17	26.139.1	10.052.6	06.028.5	10.057.14	10.053.16	02.011.11
03.013.10	26.136.11	17.090.20	03.013.4	06.028.4	15.080.6	21.114.4	30.153.13	02.008.9
21.113.11	04.019.4	04.018.20	30.155.18	02.011.15	13.073.15	09.045.8	29.151.20	09.043.15
30.155.8	30.155.20	26.135.18	07.035.6	11.061.9	12.067.13	26.135.5	13.071.8	13.069.12
12.066.1	05.025.2	12.063.2	15.083.17	28.147.17	29.151.15	13.068.1	27.142.20	16.087.9
10.054.20	27.140.9	18.097.11	09.045.11	28.147.18	02.010.19	23.121.2	4.019.16	12.062.8
03.016.5	26.135.13	15.083.4	26.139.16	13.070.11	15.082.8	28.145.14	06.026.7	03.014.2
04.021.11	27.143.5	02.007.9	09.045.20	26.138.20	10.057.13	26.134.4	04.019.18	13.072.14
Buffer	14.075.3	24.124.3	08.038.8	04.021.1	06.031.2	18.096.4	14.076.20	07.035.18
P			Q			R		
09.047.3	26.138.18	02.008.2	18.097.6	26.139.20	09.044.5	29.150.4	12.067.14	28.148.4
01.006.18	13.070.19	10.054.5	02.011.18	09.047.14	30.153.11	27.140.4	08.042.15	18.098.7
18.095.16	08.042.20	28.145.4	28.146.20	13.071.6	13.068.4	26.134.14	21.113.19	21.110.17
21.113.3	04.022.15	12.065.9	03.013.11	02.010.15	14.075.1	28.148.11	08.037.10	28.147.15
26.134.13	06.026.4	26.137.18	26.136.13	30.154.7	04.018.10	26.135.3	15.084.16	28.146.3
07.034.7	03.014.1	13.073.7	13.071.14	13.069.3	10.052.5	16.086.7	28.144.8	18.097.13
15.084.13	27.141.6	06.026.14	26.136.15	23.121.15	30.154.11	14.076.11	30.155.17	13.068.9
25.133.10	02.007.10	28.144.1	07.032.12	11.058.20	10.056.8	13.071.19	01.004.9	13.068.12
13.069.15	28.144.20	Buffer	30.155.14	04.018.8	06.028.15	12.065.4	17.090.1	13.073.9
12.062.4	30.153.14	15.082.16	09.046.12	04.020.17	Buffer	13.073.20	29.151.18	09.044.9
S			T			U		
10.055.17	28.145.10	04.022.13	28.146.10	04.020.20	14.077.7	23.123.11	Buffer	Buffer
28.148.16	13.070.8	13.072.15	17.091.11	15.082.9	12.065.3	28.147.4	21.109.2	06.029.2
09.046.8	16.086.14	02.012.16	Buffer	23.121.19	09.043.14	Buffer	29.150.12	Buffer
15.080.2	18.096.6	25.129.2	26.138.4	16.086.11	23.122.11	07.036.1	Buffer	08.037.1
05.025.17	13.069.9	15.085.14	03.014.3	01.006.12	28.146.8	24.125.3	Buffer	10.054.16
02.011.13	15.082.10	13.073.14	26.136.9	03.015.12	29.151.1	05.024.4	11.060.13	Buffer
29.149.7	14.078.1	21.113.7	28.144.6	04.017.11	11.058.15	Buffer	Buffer	24.126.2
15.081.11	13.071.3	12.063.18	21.110.1	23.121.8	12.065.5	06.027.13	24.127.4	Buffer
30.154.8	14.078.6	27.142.5	04.017.6	27.140.14	09.045.16	30.155.12	17.089.8	18.096.17
29.150.5	28.146.18	07.034.20	Buffer	01.004.10	09.046.10	21.113.17	07.033.2	25.128.6
V			W			X		
02.007.20	26.135.1	26.139.8	04.022.9	01.006.3	15.085.16	13.071.7	18.097.9	26.139.3
26.134.6	15.082.6	10.050.7	26.134.7	13.068.2	02.011.4	28.147.9	04.018.11	14.074.3
21.112.11	07.032.17	07.032.7	16.088.3	28.144.13	10.057.12	21.112.4	21.110.8	16.087.19
04.020.16	01.004.20	15.080.20	02.007.15	29.151.6	13.070.16	27.141.13	10.051.6	27.140.7
03.016.1	02.012.13	15.083.13	11.061.10	03.016.2	01.006.5	12.067.16	13.073.5	29.152.13
16.086.16	29.151.10	08.037.13	25.133.9	02.008.20	10.054.7	03.013.16	17.090.3	02.011.14
09.047.12	04.021.9	02.008.10	29.149.12	15.080.3	28.144.10	13.070.1	15.081.6	18.098.3

15.083.1	28.146.7	27.142.1	16.088.10	17.090.7	26.139.6	25.129.1	04.018.2	02.012.1
13.068.17	13.072.3	30.154.16	23.121.11	26.137.7	03.014.13	15.083.3	16.088.1	10.050.1
21.114.8	18.095.11	10.051.10	12.063.6	21.110.4	04.022.1	10.056.10	25.133.6	27.140.1
Y			Z			ZZ		
06.028.12	29.152.6	13.072.10	01.005.9	18.098.4	10.057.18	11.061.7	02.009.14	13.073.17
03.014.5	15.085.19	12.062.14	14.078.7	21.112.12	03.016.10	04.021.10	15.083.11	12.065.10
21.114.11	16.086.1	02.010.4	04.017.5	14.076.3	10.053.10	12.063.14	27.141.4	17.093.19
02.010.7	23.122.5	06.031.8	08.037.19	13.069.5	10.056.6	13.073.4	04.017.2	28.147.6
01.004.16	21.113.9	17.089.2	04.022.10	26.136.18	02.007.11	27.140.6	18.096.10	15.082.13
03.014.11	12.062.2	21.112.7	10.055.7	21.111.3	16.088.7	13.072.11	15.080.8	28.145.16
26.135.2	2.012.17	18.096.14	13.072.12	21.114.10	02.009.17	04.021.16	17.090.4	12.063.8
10.055.14	04.018.7	13.069.18	03.015.9	02.012.14	15.080.11	30.153.10	13.069.10	02.008.16
21.110.6	03.013.8	10.056.3	08.040.14	09.043.13	12.063.9	06.026.3	18.097.4	09.045.4
02.008.13	15.081.2	04.021.18	30.153.7	10.056.19	11.061.1	05.025.13	13.071.15	28.144.15

1.4.3 Table 3

Kellogg Forest common garden map consisting of 27 blocks (A-ZZ). Blocks colored in blue are control treatments. Blocks colored in orange are drought treatments.

A			B			C		
02.011.19	12.063.4	17.090.2	02.011.9	03.014.10	03.016.8	26.137.16	Buffer	28.145.18
26.135.9	15.081.15	13.071.9	07.032.18	Buffer	Buffer	30.153.4	03.015.8	26.138.10
13.071.13	06.026.8	Buffer	21.112.3	02.007.18	15.080.13	26.136.4	02.007.17	18.097.12
12.062.5	26.134.17	01.004.1	13.072.1	02.009.11	26.138.11	28.147.13	10.053.6	18.095.9
Buffer	26.136.17	28.148.15	28.148.9	17.090.6	26.135.6	26.139.10	21.110.15	21.112.8
02.008.5	03.014.9	13.070.13	28.144.2	27.142.12	13.072.9	16.086.2	12.067.20	02.007.4
05.025.10	13.073.16	21.114.9	17.090.14	Buffer	09.047.7	Buffer	27.142.13	03.013.3
27.143.4	02.012.2	18.098.6	Buffer	12.065.16	04.020.9	30.155.6	23.121.14	12.067.19
13.070.17	26.134.15	04.018.5	13.073.11	01.004.15	04.021.15	01.004.17	09.045.17	15.081.1
15.082.7	29.151.9	Buffer	16.086.8	10.056.16	29.151.14	29.149.4	25.129.4	Buffer
D			E			F		
04.022.16	14.078.11	14.076.12	21.109.1	Buffer	01.001.10	Buffer	10.054.2	27.141.1
Buffer	15.080.15	Buffer	23.123.8	Buffer	Buffer	14.075.6	09.047.10	14.077.8
15.082.3	18.097.2	28.148.13	24.126.3	15.083.14	07.033.3	10.053.12	10.054.6	12.063.15
12.065.1	08.042.13	03.013.15	Buffer	15.084.7	Buffer	26.139.4	11.058.5	29.150.3
22.115.18	28.147.2	04.021.6	25.128.7	Buffer	08.038.2	02.010.5	15.084.4	Buffer
13.069.11	28.147.12	04.017.9	25.131.3	17.089.6	08.040.9	04.022.14	18.096.5	01.005.19
26.135.20	06.031.7	11.061.3	Buffer	15.085.17	08.041.19	10.056.1	30.155.9	06.031.3
14.077.1	16.088.16	12.062.16	27.143.7	17.091.19	10.055.15	29.151.5	01.006.9	27.141.9

10.054.1	04.020.14	14.078.14	28.144.5	18.096.11	10.056.5	30.154.3	03.015.2	04.017.17
13.068.16	10.054.15	10.057.9	Buffer	Buffer	14.079.8	28.146.5	16.087.1	30.154.17
G			H			I		
18.098.9	Buffer	Buffer	28.145.9	15.083.10	18.097.7	13.068.8	03.013.6	02.011.2
18.095.6	26.137.2	09.047.8	26.139.5	04.020.8	03.013.19	15.084.15	Buffer	01.005.6
Buffer	13.071.18	Buffer	10.055.10	10.056.14	18.098.11	08.042.10	04.021.5	Buffer
12.062.3	27.141.10	02.011.12	09.043.6	30.154.5	01.006.2	26.139.11	16.086.17	21.114.13
27.141.7	05.025.6	03.016.9	02.012.9	23.121.4	25.129.5	16.087.18	02.010.1	02.008.12
25.133.5	26.136.16	02.012.4	13.069.4	16.088.15	10.053.14	17.090.11	04.020.5	04.018.13
21.114.5	12.067.4	04.021.20	15.082.12	08.042.6	06.028.7	26.138.16	02.011.8	21.112.2
09.043.7	30.155.1	12.062.6	30.154.1	Buffer	Buffer	26.137.3	15.083.12	14.076.15
30.154.9	25.133.1	16.088.8	16.086.15	26.138.14	21.110.18	13.073.13	26.136.14	02.012.5
Buffer	04.021.12	26.136.2	Buffer	13.072.8	26.135.10	28.144.7	Buffer	21.114.18
J			K			L		
28.145.11	14.076.1	10.057.6	06.026.10	26.139.19	21.110.19	02.008.1	11.058.18	08.037.17
13.068.20	25.133.7	12.062.15	04.017.13	02.010.9	Buffer	13.073.12	08.042.17	15.083.6
03.013.18	03.016.6	04.019.6	21.113.15	15.082.20	16.088.18	13.068.14	Buffer	15.084.9
Buffer	18.097.10	03.013.1	10.057.17	28.147.3	18.096.1	09.046.7	04.021.17	13.070.6
13.068.5	Buffer	14.075.5	21.113.16	17.089.14	02.007.7	12.062.20	14.078.12	05.025.19
02.007.19	15.084.1	02.011.17	01.004.12	16.088.13	26.137.9	04.017.20	02.012.10	16.088.20
02.008.19	21.112.9	Buffer	02.011.1	15.085.6	13.068.11	10.057.1	06.026.2	12.064.6
14.076.17	03.015.20	29.149.19	04.020.10	23.121.17	Buffer	02.010.3	17.090.18	10.055.13
28.147.16	29.151.11	16.087.17	23.122.6	14.077.4	15.083.20	Buffer	26.134.16	25.129.3
01.006.11	10.056.4	15.085.15	08.042.7	Buffer	28.146.13	26.135.11	13.070.5	02.007.13
M			N			O		
29.150.2	06.028.6	10.054.14	18.096.19	15.080.16	03.014.8	28.144.14	11.058.12	26.136.20
13.071.2	13.070.3	04.018.6	29.150.16	29.152.16	02.008.7	09.045.12	21.110.12	12.067.3
23.121.5	03.015.11	29.151.19	17.093.11	02.010.16	07.035.12	27.142.3	16.086.18	14.077.13
08.042.9	Buffer	29.152.12	30.155.2	16.086.12	16.088.2	13.069.17	01.004.13	Buffer
27.140.2	16.087.12	22.115.16	Buffer	21.111.15	02.011.16	28.144.18	10.055.1	03.015.18
25.129.8	04.022.7	30.153.9	14.078.2	21.112.17	Buffer	Buffer	28.145.5	04.022.5
21.111.8	30.155.10	14.078.4	10.052.1	29.152.8	13.073.3	10.054.11	23.122.8	06.027.8
02.008.14	21.110.10	28.144.4	21.113.4	18.097.15	28.145.1	28.146.12	21.111.17	10.050.12
10.054.3	Buffer	17.093.5	06.026.11	15.082.17	14.074.7	02.008.4	01.005.11	21.114.16
15.084.2	30.155.15	03.013.14	13.071.4	10.051.8	02.009.20	10.053.9	02.007.8	28.147.14
P			Q			R		
18.098.10	23.122.2	09.045.3	01.006.20	21.111.9	Buffer	07.035.20	06.026.9	13.068.19
Buffer	12.063.5	02.007.14	26.138.5	28.144.19	29.151.3	Buffer	10.052.4	01.006.14
13.070.20	15.082.2	18.098.2	28.146.1	08.037.5	14.074.4	15.081.4	Buffer	18.096.13
04.017.16	15.080.7	13.071.17	Buffer	29.149.14	21.113.20	17.092.10	30.155.4	15.080.10
26.138.3	26.138.19	29.151.13	26.137.19	21.111.7	13.071.12	06.026.16	Buffer	12.063.17
18.095.8	13.070.10	15.083.15	13.072.16	23.122.7	28.148.7	09.046.6	Buffer	26.135.14

26.139.18	Buffer	13.073.8	21.110.11	04.018.16	12.065.2	01.005.12	07.032.16	13.073.6
03.013.17	26.135.8	Buffer	30.153.5	28.145.8	04.022.19	04.019.5	29.150.10	13.073.10
28.147.10	27.140.18	03.015.19	01.004.19	15.081.12	13.068.6	26.137.13	02.012.3	02.010.12
12.062.9	09.043.16	07.032.8	10.053.5	29.151.17	13.071.20	21.112.5	21.114.17	21.113.13
S			T			U		
12.066.9	15.081.14	29.150.17	18.097.3	Buffer	10.056.18	Buffer	03.016.4	12.065.17
12.062.18	26.139.7	22.115.11	30.154.10	Buffer	08.042.5	02.010.18	Buffer	02.008.17
04.019.11	26.136.3	02.008.11	26.137.8	13.072.7	07.034.19	09.045.19	10.056.12	26.137.5
26.136.12	28.148.2	Buffer	17.093.9	12.066.2	15.080.1	15.082.14	16.087.13	21.114.19
30.154.15	16.086.3	03.015.10	15.080.19	15.083.7	Buffer	01.006.15	30.155.7	03.014.12
01.006.19	16.087.7	07.032.11	18.096.12	01.004.6	18.095.19	15.085.18	30.154.19	15.081.17
15.080.4	23.121.12	27.140.16	02.007.12	18.096.9	28.145.15	03.015.3	26.134.10	04.021.14
13.072.5	07.035.4	02.011.10	09.046.2	09.045.5	12.067.9	Buffer	13.072.2	28.146.16
13.069.20	Buffer	02.012.17	27.142.8	08.037.18	26.139.2	13.068.15	21.113.18	06.028.10
17.092.13	09.043.19	03.013.12	30.153.20	09.043.2	04.018.12	Buffer	18.097.14	28.144.3
V			W			X		
14.076.7	07.035.1	11.058.16	02.011.6	06.026.6	03.014.7	01.004.2	09.047.20	04.017.3
13.071.5	10.051.2	03.015.5	10.057.5	18.095.2	28.144.12	04.020.2	26.134.2	26.136.10
13.069.2	12.065.15	15.081.8	04.019.2	10.050.5	03.016.13	16.088.12	27.142.18	27.140.20
13.069.14	04.019.9	13.070.2	12.063.16	Buffer	04.019.17	Buffer	10.057.2	14.076.16
29.150.13	04.019.12	04.017.7	15.080.17	21.114.1	Buffer	12.063.11	10.056.20	13.069.16
12.065.20	15.084.12	Buffer	Buffer	29.149.18	13.070.18	21.113.8	02.009.2	09.044.2
26.134.8	08.037.4	12.064.1	Buffer	17.090.17	10.056.17	25.133.4	09.045.9	12.065.14
03.015.14	10.054.17	02.009.3	27.142.16	04.018.17	28.148.5	Buffer	Buffer	28.145.12
Buffer	29.149.1	02.012.20	27.141.12	Buffer	Buffer	14.076.14	06.028.2	09.046.4
02.008.18	16.087.6	04.021.13	26.134.9	30.153.12	05.025.11	21.110.16	25.133.2	17.089.5
Y			Z			ZZ		
10.057.15	04.017.18	26.135.7	15.083.8	27.143.9	13.072.19	Buffer	12.065.19	06.028.1
26.139.12	26.134.11	24.124.7	Buffer	13.071.11	10.054.4	29.149.5	27.141.11	16.088.9
15.082.4	13.069.13	12.063.13	28.146.9	13.073.1	Buffer	02.010.17	15.082.5	13.068.10
13.073.18	07.035.19	09.044.8	14.078.5	18.096.15	03.016.3	Buffer	26.137.6	27.140.13
23.121.16	06.031.1	04.017.1	24.124.4	Buffer	21.112.6	14.078.19	26.138.8	04.022.18
21.112.13	15.083.5	16.086.5	27.140.11	01.004.4	23.122.10	14.078.13	15.080.18	29.151.8
Buffer	14.076.2	03.014.4	23.121.20	09.043.11	10.055.3	09.047.1	17.090.16	21.111.13
13.072.4	14.074.9	30.153.17	26.135.12	07.035.17	30.155.3	Buffer	28.146.17	11.061.5
26.139.13	09.045.18	Buffer	13.069.1	04.020.11	08.042.12	06.031.9	02.010.2	13.070.9
09.045.10	21.113.6	28.146.14	15.085.4	29.149.16	10.050.14	04.017.19	14.078.16	02.010.13

1.4.4 Table 4

Results of the two-way ANOVA for testing if water restriction treatments had significant effect. DFn – degree freedom numerator; DFd – degrees of freedom denominator.

Effect	DFn	DFd	P
Treatment	1	3752	1.58E-93
Week	1	3752	8.65E-04
Treatment * Week	1	3752	4.25E-04

1.4.5 Table 5

Weekly VWC% averages for each block. R – water restriction treatments. C – control treatments.

Block	Mean VWC%	Treatment	Week
B	15.75	C	1
W	9.75	C	1
L	14.65	C	2
Q	10.56	C	2
A	21.65	C	3
E	21.01	C	3
H	13.44	C	4
K	22.75	C	4
Y	12.44	C	5
ZZ	8.7	C	5
F	15.78	C	6
I	13.21	C	6
R	13.4	C	6
C	19.89	R	1
V	13.31	R	1
M	15.77	R	2
O	17.86	R	2
D	29.09	R	3
G	34.59	R	3
J	25.7	R	4
N	30.43	R	4
X	27.86	R	5
ZZ	14.17	R	5
P	31.64	R	6

S	20.03	R	6
T	16	R	6

2 Chapter 2: strategies to mitigate shifts in red oak (*Quercus* sect. *Lobatae*) distribution under a changing climate

2.1 Author contribution statement

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2.2 Abstract

Red oaks (*Quercus* sect. *Lobatae*) are a taxonomic group of hardwood trees, which occur in swamp forests, subtropical chaparral, and savannahs from Columbia to Canada. They cover a wide range of ecological niches, and many species are thought to be able to cope with current trends towards a warmer and drier climate. Genus *Quercus* encompasses ca. 500 species, of which ca. 80 make up sect. *Lobatae*. Species diversity is greatest within the southeastern USA and within the northern and eastern regions of Mexico. This review discusses the weak reproductive barriers between species of red oaks and the effects this has on speciation and niche range. Distribution and diversity have been shaped by drought adaptations common to the species of sect. *Lobatae*, which enable them to fill various xeric niches across the continent. Drought adaptive traits of this taxonomic group include deciduousness, deep tap roots, ring-porous xylem, regenerative stump sprouting, greater leaf thickness, and smaller stomata. The complex interplay between these anatomical and morphological traits has given red oaks features of drought tolerance and avoidance. Here, we discuss physiological and genetic components of these adaptations to address how many species of sect. *Lobatae* reside within xeric sites and/or sustain normal metabolic function during drought. Although extensive drought adaptation appears to give sect. *Lobatae* a resilience to climate change, aging tree stands, oak life history traits and the current genetic structures place many red oak species at risk. Furthermore, oak decline, a complex interaction between abiotic and biotic agents, has severe effects on red oaks and is likely to accelerate species decline and fragmentation. We suggest that assisted migration can be used to avoid species fragmentation and increase climate change resilience of sect.

Lobatae. This review was originally being written to synthesize a comprehensive understanding of the genetic, morphological, and physiological components of drought adaptations for *Quercus* sect. *Lobatae*. After completing this research, we found that information relating to *Lobatae* drought adaptation has been characterized by morphology and physiology. After reading this publication it is our hope that more work related to the genetics of *Lobatae* drought adaptation will be conducted in the future.

2.3 Introduction

The genus *Quercus* (oak, *Fagaceae*) is a species-rich taxon with extensive and prominent distribution throughout the northern hemisphere (Hipp et al., 2020). As a result of anthropogenic climate change, these regions have been perturbed by an increasing frequency of droughts (Otu-Larbi et al., 2020). Climate change has increased mean annual temperature and aridity (IPCC, 2014), which has altered seasonal precipitation patterns from historical norms (Dai, 2011; Jentsch et al., 2007; Pachauri et al., 2014). Forest ecosystems now experience heavy precipitation events (Min et al., 2011), heatwaves and protracted droughts at greater frequency (Dai, 2011; Jentsch et al., 2007; Pachauri et al., 2014). Droughts can either directly damage trees, altering carbon balance and stunting growth, or, more commonly, droughts reduce the trees' ability to defend against pests and pathogens (Choat et al., 2012; Frelich et al., 2010; IPCC, 2014; Kolb et al., 2016). It is hence important to investigate how oaks fare under acute drought stress and extended drought conditions, particularly considering anthropogenic climate change. This review focuses on the physiological effects protracted droughts have on *Quercus* section *Lobatae*. We also investigate if the genetic potential restricts the ability of red oaks to persist in a changing climate.

Droughts can lower plant water potential, decrease plant water content, arrest or diminish photosynthetic capacity, and negatively impact physiological and biochemical processes (Anjum et al., 2011; Chaves et al., 2003; Chaves et al., 2002). Under ideal environmental conditions, the water demands of a tree are met through extraordinary means outlined by the cohesion tension theory. In essence, a continuous water column spans the trees' length. Stomatal gas exchange generates lowered water potential within leaves capable of pulling groundwater from soil, across root and xylem, to sites of active photosynthesis (Bohrer et al., 2005). Assuming a normal rate of metabolic activity is maintained when water availability is restricted, tree water potentials are lowered. This indicates that the cohesive/adhesive hydrogen bonds of the water column are being stretched. The increased tension on the water column creates a risk of xylem conduit embolism that intensifies with drought severity and ultimately may lead to tree dieback after starving the canopy of water (Choat et al., 2018). At the ecosystem level, drought-stressed forests have already seen severe tree mortality (Park et al., 2013). Moreover, mass tree mortality events have not been restricted to arid ecosystems and are cause for global concern (Anderegg et al., 2012; Asner et al., 2016; Carnicer et al., 2011; Duke et al., 2017). Various long-lived tree taxa have evolved mechanisms to cope with drought conditions, including oaks (*Quercus*) (Ramirez-Valiente et al., 2009; Villar-Salvador et al., 2004).

Quercus contains ca. 500 distinctive species (Denk et al., 2017; Nixon, 2002, 2006) that are found throughout Europe, northern Africa, Asia, and the Americas (Denk et al., 2017). Oak species can be found from tropical to continental climates (Köppen groups A–D) and in regions of moderate to low annual precipitation (Köppen subcategories w, f, s). Oaks commonly show characteristics of drought avoidance and drought tolerance, which

has contributed to species diversity and distribution in areas of marginal precipitation and/or high elevation (Cavender-Bares et al., 2000; Fallon et al., 2018; Valencia, 2010). Oaks are typical foundation species, providing structural habitat and food for an assortment of flora and fauna (Anderson et al., 2007; Tallamy et al., 2009; Thompson et al., 2012). In addition, oaks prominently contribute to ecosystem nutrient cycles and carbon storage (Hansen, 2000; Stoler et al., 2011). The genus, *Quercus*, is currently divided into two subgenera, *Cerris* (ca. 150 sp. in three sections) and *Quercus* (ca. 350 sp.). The subgenus *Quercus* has been divided further into five sections: *Lobatae* (red oaks), *Protobalanus* (golden cup oak), *Quercus* (white oak), *Virentes* (southern live oaks) and *Ponticae* (Denk et al., 2017; Hipp et al., 2020).

Here, we focus on section *Lobatae*, a monophyletic clade native to the Americas. We investigate aspects of climate adaptation, evolution, speciation, and physiology of *Lobatae*. When no studies or examples of *Lobatae* were available in the literature, we sought examples from other *Quercus* taxonomic groups or from model species such as poplar and *Arabidopsis*. Oak species of sections *Lobatae* and *Quercus* inhabit a substantial portion of forested land within North and Central America (Figure 1). Oak-type forests make up more than half of all forested land within North America. In this range, oaks account for nearly a fifth of all forest biomass (Cavender-Bares, 2019; Little, 1979; Oswalt et al., 2014; Smith et al., 2009). Mexico is a hotspot of *Quercus* diversity and is home to an estimated 160 constituent species (Figure 1). Within Mexico's geographical regions, between 16 species (Baha California Peninsula) and 56 species (Sierra Madre Oriental) are found (Valencia, 2010).

Ancestors of the American oak clades arrived within the higher latitudes of North America sometime between the Early and Middle Eocene (Grímsson et al., 2016; McIntyre et al., 1991). Geological records from the Eocene/Oligocene boundary show a progression of cooling and drying of the global climate (Krause et al., 2010; Zachos et al., 2001). Ranges of tropical taxa shifted south, and many underwent localized extinctions (Prothero, 2009). These conditions provided an evolutionary opportunity for rapid colonization and speciation of oaks, and sections *Quercus* and *Lobatae* radiated in parallel sympatry from North America into Central America (Cavender-Bares, 2019; Hipp et al., 2018). Analysis based on phylogenetic inference suggests that oaks colonized Mexico and Central America sometime between 20 and 10 million years ago (Cavender-Bares, 2019; Hipp et al., 2018). Deciduousness, where leaves are shed either in winter or during dry periods, is an adaptive trait common in oaks, but drought deciduousness is not typical for sect. *Lobatae*. Oaks' ability to shed leaves during dry seasons or drought is a critical factor in the evolution of the American oak clades. This characteristic provides an adaptive, plastic solution to the formative selective pressures of the changing Early-Middle Eocene climate: drought and wintering (Cavender-Bares, 2019; Firmat et al., 2017; Hernández-Calderón et al., 2013; Ramírez-Valiente et al., 2017). The heterogenous montane regions of Mexico also provided ecological opportunity for niche diversification of oaks, which explains the higher speciation rates (Figure 2) and a great species diversity of the region (Figure 1) (Hipp et al., 2018; Torres-Miranda et al., 2011; Valencia, 2010).

At present, many species of sect. *Lobatae* have large, overlapping distributions within eastern North America (Figure 1). This was caused by a combination of oaks adaptive traits like winter deciduousness in addition to human-mediated migration after the

last glacial maximum (Abrams, 2002; Dey, 2014; Leroy et al., 2020). Initially, land management practices of European settlers and their descendants increased frequency of which forests were subjected to burns, clear-cuts, selective timber harvests, livestock browse and wildlife overexploitation (e.g., deer, turkey) by unregulated hunts (Dey, 2014; Schulte et al., 2007; Williams, 1992). Unlike other hardwoods, oaks persisted within the disturbed sites. After perturbation, oak root stocks deployed new shoots that regenerated into the canopy (Dey, 2014; Johnson et al., 2019). By the 1930s, land management practices of the eastern USA changed considerably, and forest fire suppression was common practice. By the 1950s, this management strategy allowed forest regeneration, and the once open stands of the eastern USA became dominated by oak (Dey, 2014; Williams, 1992). The practice of forest fire suppression continues to this day and has yielded unintended consequences. Within the eastern and central USA, lack of fire disturbance has generated heavily shaded and aging forest stands (Crow, 1992; Dey, 2014; Lhotka et al., 2009), and although the canopies are oak dominated, lower irradiance sites favor regeneration of shade-tolerant and fast-growing competitors such as *Acer* and *Populus*, respectively (Clark, 1993; Dey, 2002; Fei et al., 2007).

2.4 Hybridization and introgression in section *Lobatae*

Hybridization and introgression are processes that drive plant evolution and speciation (Coyne et al., 2004; Rieseberg, 1997). Interspecific gene flow introduces a large amount of genetic diversity and may increase adaptive genetic variation (Arnold et al., 2010; Rieseberg, 1997; Tovar-Sánchez et al., 2004). Hybrid zones, where two or more related sympatric species occur, are an important source of genetic recombination and diversity in plant evolution (Rieseberg, 1997). The occurrence and frequency of

hybridization could be explained by pre- and post-zygotic barriers. Pre-zygotic barriers limit the opportunity for interspecific mating and include species abundance, pollen production, flowering time, and pollen compatibility. Post-zygotic barriers reduce the fitness of hybrids through environmentally dependent selection on introgressed alleles and habitat availability (Ortiz-Barrientos et al., 2014) In oaks, the latter is supported by a decreasing frequency of hybrids from acorns to seedlings to adult trees (Curtu et al., 2009; Sullivan et al., 2016). Moran et al. (2012) found over 20% of seedlings from four species in sect. *Lobatae* at two old mixed stands in North Carolina, USA, were of hybrid origin. This is in contrast to studies at the mature tree level, where hybrids were typically found at frequencies of 1–5 % (P. Aldrich et al., 2003; Lind-Riehl et al., 2013), though both Sullivan et al. (2016) and Pérez-Pedraza et al. (2021) found higher frequencies of hybridization and introgression in mature red oak populations with 25 and 16%, respectively. Populations of *Q. ellipsoidalis* were particularly prone to introgression, with <50% being 'pure' (Sullivan et al., 2016). This could indicate variable post-zygotic barriers that depend on the species investigated.

Reproductive barriers between sympatric species of oak are often weak (Abadie et al., 2012; Lagache et al., 2013). Species complexes occur naturally and frequently between two or more species (Curtu et al., 2009; Peñaloza-Ramírez et al., 2010; Valencia-Cuevas et al., 2015). Oaks in general, and sect. *Lobatae* in particular, frequently form hybrids (Figure 3) and show genetic signs of introgression between two or more species (González-Rodríguez et al., 2004; Peñaloza-Ramírez et al., 2010; Pérez-Pedraza et al., 2021; Ramos-Ortiz et al., 2016; Sullivan et al., 2016; Tovar-Sánchez et al., 2004). Sympatric sect. *Lobatae* species typically hybridize within their major clades (having more recent common

ancestors), with few notable exceptions such as *Q. hypoleucoides* and *Q. shumardii* (Figure 3). Oak hybrids can often reproduce with each other as well as with both parents (Sork, Riordan, et al., 2016; Tovar-Sánchez et al., 2004). Despite the frequent occurrence of hybrids and introgressed forms, genetic and adaptive distinctness is well maintained in species of sect. *Lobatae*. This could be due to natural selection operating against the exchange of genes that constitute the basis for functional diversity between species (González-Rodríguez et al., 2004), and local environmental selection for climate or soil (McCauley et al., 2019; Pérez-Pedraza et al., 2021; Sullivan et al., 2016).

The history of species in sect. *Lobatae* greatly differs between geographic regions (Cavender-Bares, 2019). Central American and south-west North American species have maintained relatively larger effective population sizes and more stable distribution ranges over time, compared with north-eastern North American species, which experienced range and population contractions and expansions during glacial cycles (Hipp et al., 2018). With more stable species ranges, it should be possible to investigate longer term introgression signatures in Central America and south-west North America. This was the case in *Quercus acutifolia* and *Quercus grahamii*, two species which have different climate niches and show leaf morphological adaptations to lower minimum temperature in colder months for *Q. grahamii* (Pérez-Pedraza et al., 2021). Few of the investigated individuals were assessed as F1 hybrids; however, 16% of individuals showed evidence of introgression, based on 10 microsatellite markers (Pérez-Pedraza et al., 2021).

A study by McCauley et al. (2019) found that soil types were a significant selective force governing hybridization and introgression among Mexican sect. *Lobatae* species

Quercus conzattii, Quercus radiata, Quercus urbanii and *Quercus tarahumara*. Soil specialist species showed lower introgression, whereas the soil generalists had greater genetic admixture. Furthermore, species hybrids were more commonly observed within the non-specific soil type location (McCauley et al., 2019). In eastern North America, when studying interspecific gene flow between *Quercus coccinea, Quercus falcata, Quercus rubra* and *Quercus velutina*, Moran et al. (2012) found that while hybridization occurred between the sympatric species, there was little genetic evidence to show that introgression was occurring. These findings could suggest that selection pressures limit introgression between these widely distributed species. Two studies by Owusu et al. (2015) and Sullivan et al. (2016) investigated hybridization between four sect. *Lobatae* species *Q. ellipsoidalis, Q. coccinea, Q. velutina* and *Q. rubra*, across sites with varied water accessibility. Xeric and mesic sites are the preferred habitat of *Q. ellipsoidalis* and *Q. rubra*, respectively, whereas the other two species inhabit intermediate environments (Sullivan et al., 2016). In their study, hybrids between the species were common, while signatures of introgression were rare, indicating lower fitness of hybrids, possibly due to selection for the species' respective water availability environment. Selection for environment is therefore one of the major forces maintaining species identity in sect. *Lobatae*. All these examples of genetic assessments used Random Amplified Polymorphic DNA (RAPDs) or microsatellites and may not have had enough resolution to identify ancient introgressions (McCauley et al., 2019; Moran et al., 2012; Owusu et al., 2015; Sullivan et al., 2016). Using high-throughput genotyping or sequencing will allow scientists to simultaneously genotype thousands of loci in a larger number of individuals and thereby provide better evidence of

introgression and hybridization as well as allowing the identification of loci responsible for species cohesion.

In light of current and future anthropogenic climate change, three aspects of hybridization and introgression will be imperative to understand if we are to help future species and population persistence of sect. *Lobatae*. First, the amount of adaptive potential found in a species' or populations' genome will be a main factor for survival and past introgression events will strongly influence the amount of genetic adaptive potential. Second, introgression may be the most important long-distance dispersal mechanism in species of limited seed dispersal as is the case for species in sect. *Lobatae*. Species which are pressed to the limit of their climatic range may be saved through hybridization. Third, climate change causes geographical displacement of suitable conditions for the species, which may move lines of sympatric populations and cause selection for or against one of the species, thereby leading to a reduced potential to produce hybrids and further lower genetic adaptive potentials.

2.5 Climate change and sect. Lobatae drought tolerance adaptations: physiology, anatomy, and morphology

Species of genus *Quercus* commonly demonstrate capacity for drought tolerance and/or drought avoidance. Section *Lobatae* is no exception, as evidenced by multiple species inhabiting xeric sites (e.g., *Q. coccinea, Q. ellipsoidalis, Q. falcata, Q. ilicifolia, Q. laevis, Q. marilandica, Q. myrtifolia, Q. texana, Q. velutina* and *Q. wislizeni*) (Abrams, 1990). Moreover, climate change will likely increase suitable habitat ranges of multiple North American *Lobatae* species (Prasad et al., 2008). Thus, it is imperative to understand

drought adaptations of sect. *Lobatae*, and how these traits are suited for the changing climate.

Drought-tolerant adaptations allow drought stress to exert increasing tension onto the continuous water column without negating function (Claeys et al., 2013; Kozlowski et al., 2002). Although the decreasing water potentials risk embolism, tolerant plants maintain normal rates of photosynthesis during water stress (Brunner et al., 2015). (Abrams, 1990) reviewed *Quercus* drought adaptations, including relationships between drought tolerance and photosynthesis, with detailed data for species from sect. *Lobatae*. Results of the review can be summarized by three statements. First, compared to co-occurring hardwoods (e.g., *Acer saccharum, Cornus florida, Juglans nigra, Fraxinus pennsylvanica*), species from section *Lobatae* (e.g., *Q. rubra, Q. velutina, Q. coccinea, Q. ellipsoidalis*) have greater photosynthesis and leaf conductance at lowered osmotic potential (Abrams, 1990; Bahari et al., 1985; Hinckley et al., 1978; Wuenscher et al., 1971). Second, multiple *Lobatae* species have higher water use efficiency (WUE) than co-occurring hardwoods (Wuenscher et al., 1971). This argument was strengthened by Turnbull et al. (2002), who found both WUE and photosynthetic rates to be greater in *Q. rubra* compared with *Acer rubrum*. Finally, drought-tolerant traits common to species from sect. *Lobatae* help explain their adaptation to xeric sites (Abrams, 1990; Wuenscher et al., 1971). In a study that investigated leaf morphological traits of central Wisconsin hardwoods, species from sect. *Lobatae* (*Q. ellipsoidalis, Q. rubra* and *Q. velutina*), compared with 21 non-*Quercus* hardwoods, had significantly greater leaf thickness and smaller stomatal guard cell lengths (Abrams et al., 1990). These adaptations increase WUE and are common xeric site

adaptations. In comparison with species from sect. *Quercus*, stomatal guard cell length was notably smaller for species from sect. *Lobatae* (Abrams et al., 1990).

Leaf turgor loss point (ψ_{TLP}, MPa) can measure a plant's degree of drought tolerance. This physiological trait is the lowest pressure potential where leaves remain turgid and functional. This trait is a proximal measurement of the degree of drought stress that can be sustained before stomatal closure (Kramer et al., 1995; Skelton et al., 2018). Plants found to have lower ψ_{TLP} (more negative pressures) can maintain stomatal conductance, hydraulic conductivity and photosynthesis gas exchange during arid conditions (Abrams et al., 1990; Sack et al., 2003). Compared with other trees, low ψ_{TLP} pressure potentials are commonly associated with species from sect. *Lobatae* (Abrams, 1990). As a reference, ψ_{TLP} pressure potentials for species of *Populus, Acer* and *Betula* range between −1.5 and −1.7 MPa, whereas ψ_{TLP} pressure potentials for *Q. rubra, Q. coccinea, Q. velutina* and *Q. marilandica* were reported to be −1.85, −2.1, −2.45 and −3.7 MPa, respectively (Chambers et al., 1985; Federer, 1977; Hinckley et al., 1978; Phelps et al., 1976; Reich et al., 1980). This demonstrated that many species in sect. *Lobatae* were more drought tolerant compared with other genera of hardwood trees found in similar environments.

Ranges in hydraulic conductance have also been used to characterize drought tolerance within the *Lobatae* section. Drought stress reduces hydraulic conductance by increasing pressure potential (decreasing Ψ_{leaf}) (Bohrer et al., 2005). Changes in hydraulic conductance can be monitored through optical vulnerability experimentation. The percentage of embolized tissue is attributed to continually monitored leaf measurements.

Xylem embolism onset (P_e) and embolism vulnerability (Ψ_{50}) are the physiological markers denoting 5 and 50% reductions in hydraulic conductivity (Brodribb et al., 2016; Petruzzellis et al., 2020). These measurements establish a 'timeline' illustrating the functional range of hydraulic conductance (lower P_e and Ψ_{50} indicated higher drought tolerance), and relative speed of hydraulic failure (range between P_e and Ψ_{50} dictate how rapidly hydraulic conductance is lost during drought) (Brodribb et al., 2016; Petruzzellis et al., 2020; Skelton et al., 2018). In a survey of eight Californian *Quercus* species, Skelton et al. (2018) found that six species showed no significant difference among tissues for Pe, which suggests that for these six species, leaves do not buffer stem tissue from embolism formation (Cochard et al., 1992; Skelton et al., 2018). Interestingly, this trend was observed in all measured *Lobatae* species (*Quercus agrifolia, Q. wislizenii* and *Q. kelloggii*) (Skelton et al., 2018). This observation could potentially explain why drought-induced leaf abscission is not commonly observed in sect. *Lobatae* (Abrams, 1990). Worth noting are the low P_e and Ψ_{50} pressure potential values observed for the *Lobatae* species. The lowest was observed in *Q. kelloggii* and ranged from −3.28 to −4.73 MPa for P_e and Ψ_{50}, respectively (Skelton et al., 2018). These values reflect a range of drought tolerance and xeric site adaptations characteristic of sect. *Lobatae*. The study by Skelton et al. (2018) also observed a mechanism of drought avoidance within sect. *Lobatae* by noting the relatively close association between ψ_{TLP} and P_e. With regard to lack of hydraulic segmentation between tissue types discussed earlier, the close association of turgor loss point and embolism onset suggests that xylematic embolism triggers stomatal closure within sect. *Lobatae*, which is a unique mechanism of drought avoidance (Martin-StPaul et al., 2017; Skelton et al., 2018; Tyree et al., 1993). Relatively few species and populations

were tested by Skelton et al. (2018), making this an exciting topic for future studies. Links between this unique mechanism and the absence of drought-related leaf abscission also need further investigation.

Drought avoidance has been widely observed in species of sect. *Lobatae* (Fallon et al., 2018; Hinckley et al., 1979). In contrast to drought tolerance, drought avoidance adaptations minimize plant water loss by reducing stomatal conductivity and photon capture, while maximizing water absorption (Basu et al., 2016; Reich, 2014). During droughts, these adaptations preserve a plants' cellular turgidity and water potential (Chaves et al., 2003; Ehleringer et al., 1992; Jackson et al., 2000). In an experiment investigating differences in age class response for *Q. rubra*, mature trees increased WUE, thereby minimizing diminishment on C uptake (Cavender-Bares et al., 2000). Furthermore, Cavender-Bares et al. (2000) found rain events during droughts had little effect on mature *Q. rubra*, a conclusion evident by the relatively small change in pre-dawn and midday ψ_{leaf} measurements, which were opposite to the observations with seedling and juvenile *Q. rubra*. These results demonstrated that mature trees have greater rooting depth that provides better access to water and allows older trees to avoid drought stress. Rooting depth for *Lobatae* species *Q. rubra* and *Q. ellipsoidalis* has been recorded at ~ 0.7 and >2.5 m in depth, respectively (T. T. Kozlowski, 1971; Lyford, 1980). Observations for rooting depth of co-occurring trees from genus *Acer* and *Pinus* were considerably shallower, which may restrict the amount of water they can access during drought conditions (T. T. Kozlowski, 1971; Lyford, 1980). T. T. Kozlowski (1971) also noted that *Q. ellipsoidalis*, which are commonly found in xeric sites within Wisconsin, had less extensive rooting system within its shallower rooting depth compared with deeper soil regions. These results suggest that

deeper rooting is critical for drought avoidance within sect. *Lobatae* (Abrams, 1990). Higher pre-dawn ψleaf, a physiological observation commonly observed for trees with greater rooting depth, is commonly observed for species within sect. *Lobatae*. Compared with other co-occurring species, *Q. rubra, Q. velutina* and *Q. texana*, all showed higher pre-dawn water potential measurements (Bahari et al., 1985; Fonteyn et al., 1985; Ginter-Whitehouse et al., 1983; Hinckley et al., 1979).

The xylem anatomy of sect. *Lobatae* (and more generally, genus *Quercus*) explains how drought tolerance and avoidance are used simultaneously as a unique adaptation to xeric sites. Species in sect. *Lobatae* have ring porous xylem, wherein the early-wood vessel elements have considerably larger diameter than late-wood vessel elements (Robert et al., 2017). Although larger vessels efficiently transport sap (quicker rates of flow), these conduits are less hydraulically safe and have greater chance for embolism (Jacobsen et al., 2012; Lens et al., 2011). In a comparative study between *Q. rubra* and *A. saccharum* conducted in Michigan, *Q. rubra* showed anisohydric activity (Meinzer et al., 2013; Thomsen et al., 2013). Diurnally, during the day, the smaller vessels of the late-wood bypass emboli formed within the early-wood xylem. At night, hydraulic conductance is restored within early-wood vessels and assists in rapid water absorption by the deep tap roots. Both the deep rooting system and ring-porous xylem anatomy can be attributed to *Q. rubra* being comparatively better adapted to xeric conditions than *A. saccharum* (Meinzer et al., 2013; Thomsen et al., 2013).

When compared with other species within genus *Quercus*, average diameter of early-wood vessels are considerably smaller for species of sect. *Lobatae*. Using a dataset

compiled by (Robert et al., 2017), the mean vessel diameter for genus *Quercus* (minus sect. *Lobatae*) and sect. *Lobatae* were ~62 ± 5 μm (N = 42 species) and ~47 ± 2 μm (N = 7 species), respectively. This suggests that smaller vessel diameter could be a useful adaptation for xeric sites within sect. *Lobatae*, and additional experiments should investigate this area further.

2.6 Oak drought stress genetic adaptation

Plant transcriptomes are highly specific; gene expression is variable and dependent on environment conditions and tissue type (Padovan et al., 2013; Seki et al., 2002). Gene regulation in response to drought has been well documented with the model organism *Arabidopsis*. Enriched expression has been reported for genes involved in abscisic acid (ABA) signaling, osmotic stress response, reactive oxygen species (ROS) tolerance, salt stress and cell wall modifications (Clauw et al., 2015; Matsui et al., 2008). ROS have various functions in plants, often leading to changes in gene expression, which play a significant role in drought adaptation. The accumulation of ROS in response to environmental stressors triggers a signalosome that provides adaptive acclimation and defense (Davletova et al., 2005; Miller et al., 2008; Miller et al., 2010). ROS levels increase in plants when environmental stresses disrupt cell homeostasis. During droughts, this occurs when water potentials decrease and limited water is available for metabolic activities (Mittler et al., 2004; Torres et al., 2005) Photosynthesis is particularly vulnerable to drought, and ROS production occurs in chloroplasts when CO_2 fixation and electron transport chain reduction exceed water availability (Chaves et al., 2003; Miller et al., 2010). Within chloroplasts, ROS are continually produced and scavenged by antioxidants (glutathione, tocopherol, flavonoids, alkaloids, carotenoids) and enzymes (superoxide

dismutase, catalase, ascorbate peroxidase, glutathione peroxidase) (Apel et al., 2004). During drought stress, ROS levels are elevated by respiratory burst oxidase homologs (Rboh) genes: the increased levels of ROS serve as a signal, initiating stomatal closure and transcriptome modifications (Miller et al., 2010; Torres et al., 2005).

There have been no studies of sect. *Lobatae* transcriptomes in relation to oxidative stress published yet. Madritsch et al. (2019) compared gene expression for ROS scavenging genes between three European oak species: *Q. robur, Q. pubescens* (both section *Quercus*) and *Q. ilex* (section *Ilex*). Compared with the other species, the drought transcriptome of *Q. robur* showed a more moderate increase in gene expression for ROS scavenging genes. The authors further concluded that ROS response contributed to the greater degree of drought tolerance of *Q. ilex* and *Q. pubescens*, and allows these species to grow in the arid, Mediterranean environment (Madritsch et al., 2019). This conclusion is bolstered by the finding of another study that investigated the variations in transcriptome response to drought between independent populations of *Quercus lobata* (sect. *Quercus*). Gugger et al. (2016) found substantial differences between populations, including induced expression of a gene ortholog of WRKY51, a transcription factor mediating ROS response. Their study concludes that ROS responsive genes confer local adaptation and contribute to the dynamic population structure of *Q. lobata* (Gugger et al., 2016).

Two separate groups of cis-acting genetic mechanisms are employed by plants in their response to drought stress, the ABA-dependent and ABA-independent pathways (Chaves et al., 2003; Yamaguchi-Shinozaki et al., 2006). The phytohormone abscisic acid is synthesized in roots in response to soil dehydration. It serves as a long-distance signaling

molecule that induces stomatal closure (Finkelstein et al., 2002; Seo et al., 2011; Xiong et al., 2002) and initiates a cascade of kinases and transcriptional regulators (Yoshida et al., 2014), which leads to the induction of drought responsive genes (Busk et al., 1997; Hattori et al., 1995; Leung et al., 1998). The ABA-independent pathway also mediates plant response to drought stress. Extensive crosstalk occurs between both ABA-dependent and -independent regulatory mechanisms and both induce transcription of similar gene families such as the Late Embryogenesis Abundant (LEA) family, glucoside and glucosinolate biosynthesis, amino acid derivative metabolism and oxidation reduction (Matsui et al., 2008).

In a greenhouse study, root tissue from *Quercus suber* (sect. *Cerris*) grown under different drought treatments were sampled for transcriptome analysis. Drought-induced expression of transcripts containing Abscisic acid-responsive element (ABRE) promoter sequences, orthologs of ABA-dependent transcription factors and multiple effector gene orthologs that include LEA proteins were reported (Magalhães et al., 2016). This study concluded that the ABA-dependent pathway is an important part of *Q. suber* drought adaptation. The previously mentioned study by Madritsch et al. (2019) also investigated the role of gene expression in drought adaptation within *Quercus*. Their findings demonstrate that ABA-dependent pathway gene response is a drought stress adaptation employed by *Q. robur* and *Q. ilex*. Gugger et al. (2016) reported that increased expression of ABA-dependent transcription factors in *Q. lobata* did not appear to be specific to individual populations and did not significantly contribute to local adaptation or population demography. In addition, they reported similar results for ABA-independent transcription factors and LEA proteins (Gugger et al., 2016).

The effects of drought climate variables on population demography and local adaptation have been investigated in *Q. lobata* (Gugger et al., 2016; Sork, Squire, et al., 2016). These studies used the *Q. lobata* published transcriptome (Cokus et al., 2015), and a list of drought-related candidate genes based partially on the work of Porth et al. (2005) to observe genome-wide associations to drought. Both observed a significant effect of drought climate variables on genetic structure between *Q. lobata* populations. Both studies reported that precipitation levels of a site are a significant environmental factor for determining demography of *Q. lobata* SNPs associated with temperature (Sork, Squire, et al., 2016), and osmotic- and drought-stress (Gugger et al., 2016).

Oney-Birol et al. (2018) explored how climate variables explain patterns of introgression within multiple species of *Quercus*. Effects of drought on three oak species were assessed in a transcriptome analysis. A total of 24 individuals were selected from nine sites of *Quercus engelmannii*, *Quercus berberidifolia*, *Quercus cornelius-mulleri* (all sect. *Quercus*). Their experimentation identified 11 genes with species-specific association and demonstrated that environmental adaptation is a determinant of species identity (Oney-Birol et al., 2018). Although these findings are all specific to species from section *Quercus*, their significance could be used to direct future studies in sect. *Lobatae*. Despite interest in drought ecophysiology in *Quercus* section *Lobatae*, genomes specific to this section are yet to be published. Presently, sequenced *Quercus* genomes include *Q. robur* (Plomion et al., 2018), *Q. lobata* (both section *Quercus*) (Sork, Fitz-Gibbon, et al., 2016) and *Q. suber* (section *Cerris*) (Ramos et al., 2018). Several other genomes are sequenced and about to be published including *Q. rubra* (Phytozome, 2021) *and Q. ellipsoidalis* (K. Carlson et al., in preparation).

2.7 Habitable range and an arid future: effects of climate change on section *Lobatae*

Anthropogenic climate change has altered water cycles, created protracted droughts and increased aridity across most regions of naturally occurring red oaks in America. These shifts pose great threats to forest ecosystems and tree species (Dai, 2011; Frelich et al., 2010; Jump et al., 2005; Rosenzweig et al., 2008). However, based on the information presented thus far and assuming species migration rates keep up with changing suitable habitat, one might project that sect. *Lobatae* will escape the negative impacts of increasingly severe droughts. There are three ways this thinking could be reinforced by interpretations of eastern US species distribution modeling: (1) both oak–hickory and oak–pine forest types are projected to increase substantially under low and high greenhouse gas emission scenarios, expanding north into current maple–beech– birch forest types in the north-eastern region of the USA (General Circulation Models (GCM) modeling) (Iverson et al., 2008); (2) all the modeled sect. *Lobatae* species are predicted to have similar expansions in habitat suitability. Notably, regions of suitability are projected to shift northward and increase in area by >50% under all emission scenarios (Figure 4) (Peters et al., 2019a, 2019b; Prasad et al., 2008); (3) these models predict that many shade-tolerant, mesophytic competitors of red oaks will have significantly decreased suitable habitat (Peters et al., 2019a, 2019b; Prasad et al., 2008). Since sect. *Lobatae* are characteristically shade intolerant (Morrissey et al., 2010), reduction in suitable species habitat for non-oak species could allow for mixed stands to become red oak dominated, and adaptive radiation of red oaks into open stands.

These assumptions do not account for the difficulties associated with oak regeneration. As discussed earlier, present day fire suppression trends, albeit originally a cause for oak dominance within eastern US forests, are a major constraint on oak regeneration (Dey, 2014; Williams, 1992). The aging oak stands of eastern and central USA forests have lowered irradiance and promote regeneration of shade-tolerant competitors (Clark, 1993; Dey, 2002; Fei et al., 2007).

Biotic stresses limit red oak regeneration and migration potential. Sect. *Lobatae* regeneration approaches must also address herbivory from an increasing deer population. Within the eastern USA, deer browse significantly diminishes a stands potential for sect. *Lobatae* regeneration (Dey, 2014; Laurent et al., 2021). Thus, despite increasing suitable habitat range (Peters et al., 2019a, 2019b; Prasad et al., 2008), the current state of *Lobatae* stands does not support future regeneration (McEwan et al., 2006). Sudden oak death caused by *Phytophthora ramorum*, a fungal pathogen originally relegated to western North America, is now present in eastern US forests. In addition, gypsy moth (*Lymantria dispar*) defoliation presents a significant biotic stress for sect. *Lobatae* in the eastern USA (Fajvan et al., 1996; Grünwald et al., 2012; Lovett et al., 2006; McPherson et al., 2005). Drought both impacts red oak regeneration and further increases their susceptibility to biotic stresses. Oak decline, a phenomenon wherein mature oaks die due to complex interactions of disease–insect–drought stress is especially detrimental to species of sect. *Lobatae* (Greenberg et al., 2011; Haavik et al., 2015; Kabrick et al., 2008; Voelker et al., 2008).

Modeled effects of climate change on the suitable habitat for sect. *Lobatae* species have also been examined within Mexico (Gómez-Mendoza et al., 2007). In both severe and

conservative climate forecasts (HHGGA50Mex and HHGSDX50Mex, respectively), suitable habitat is projected to diminish for all tested sect. *Lobatae* species by the year 2050. It is troublesome that the severe scenario model predicts that suitable habitat could diminish by >30% for sect. *Lobatae* species of both prominent and restricted niche range (Gómez-Mendoza et al., 2007). Reports from recent years have also shown that oak decline, perpetuated by *Phytophthora cinnamomi*, is an issue within central Mexico (Alvarado-Rosales et al., 2008; Tainter et al., 2000). Although *Quercus salicifolia* (sect. *Lobatae*) can survive *P. cinnamomi* inoculation for multiple years, the disease ultimately results in mortality (Tainter et al., 2000). It is possible that increasing aridity will accelerate disease mortality for Central American sect. *Lobatae* species, particularly due to the complex interaction between drought stress and insects/disease.

2.8 Assisted migration and sect. Lobatae: climate change preparations

Because climate change is rapidly increasing selective pressures, many species are moving closer towards extinction (McLachlan et al., 2007). Within the Americas, suitable tree habitat is shifting (Gómez-Mendoza et al., 2007; Iverson et al., 2008; Peters et al., 2019a). The survival of these species depends on their capacity for migration within and beyond their range border (Vitt et al., 2010). Climate projections of North America suggest that required migration rates could be as great as 1 km year−1 for some tree species (Iverson et al., 2002; Iverson et al., 2004). Given their long juvenile periods, mechanisms for seed dispersal, and sessile nature, most species will require some form of human intervention to survive the climate change era (Krutovsky et al., 2012). To paraphrase Seddon (2010), ‘climate change changes everything’ and assisted migration (AM) is an option to adapt or

rescue species from climate change. Using AM, individuals are intentionally moved outside of their natural population and/or species range into appropriate habitats (Hannah et al., 2002; Hunter, 2007; Minteer et al., 2010; Pedlar et al., 2012; Shirey et al., 2010; Ste-Marie et al., 2011; Vitt et al., 2010). The use of AM for trees is a matter of hot debate. Proponents suggest that AM could be used to rescue species from extinction or increase species adaptation by introducing genetic variation (Aitken et al., 2008; Krutovsky et al., 2012; Minteer et al., 2010; Vitt et al., 2010). Their opponents point to environmental disasters resulting from human-mediated species movement (Ricciardi et al., 2009; Simler et al., 2019), and cost of seed/cone collection and plantation development (Bansal, Harrington, et al., 2015; Bansal, St. Clair, et al., 2015; Handler et al., 2018). Assuming AM follows changes in suitable habitat, these points are moot for two reasons: (1) provenance trials uncover the differences between populations, which reduces the chance of selecting an incorrect seed stock for AM (Bansal, Harrington, et al., 2015; Bansal, St. Clair, et al., 2015; Handler et al., 2018); (2) seeds selected for AM are climate change adapted; therefore, the cost of AM will be similar to the existing cost of forest regeneration (Handler et al., 2018; Pedlar et al., 2012). For the remainder of this section, we discuss whether and when AM should be used with species in sect. *Lobatae*.

Many species of sect. *Lobatae*, such as *Q. rubra, Q. velutina* (Figure 4) and *Q. mexicana*, have large geographic ranges and are a significant component of North and Central American forest landscapes (Iverson et al., 2008). Despite showing a considerable degree of drought adaptation, red oak decline is a significant threat to forest health (Iverson et al., 2002). Without human intervention, increasing selection through climate factors could shrink forested land area by fragmenting species ranges. Although fragmented sect.

Lobatae populations can maintain relatively stable rates of gene flow through wind pollination, acorn dispersal range is limited to the confines of a population range (Fernández-M et al., 2007; Gerwein et al., 2006; Oyama et al., 2017). Fragmentation of geographic ranges of widely distributed sect. *Lobatae* species threatens both forest ecosystems and, under high selection pressures, could lead to local extinction following genetic bottlenecks (Fernández-M et al., 2007).

Within widely distributed sect. Lobatae species, AM is a tool that adds adaptive potential to a species and can reduce the negative effects of population fragmentation. The migrated populations are grown in plantations and populations are selected based on their potential to cope with a sites' future climate condition. A populations' potential is determined using provenance trials. These experiments should reveal a populations' phenotypic plasticity, environmental tolerance and genetic basis of climate change adaptations, including tree phenology (González-Martínez et al., 2006; Pedlar et al., 2012). Provenance trials are time and resource intensive and could be done with representative species within a clade or ecological niche. Because AM reinforces current populations and moves with climate change, risk of introducing invasive species, new diseases or novel hybridization is minimal (Pedlar et al., 2012; Ste-Marie et al., 2011).

Etterson et al. (2020) explored feasibility and effectiveness of AM in *Q. rubra*. They established plantations within Minnesota, along the northern edge of the *Q. rubra* species range, using populations from northern and southern seed zones. Under these conditions, the southern populations showed both greater survival and growth compared with their northern counterparts (Etterson et al., 2020). Previous work suggested that

provenance data should be used to regulate AM seed zone transferring (Jump et al., 2005; Krutovsky et al., 2012; Mátyás, 2010), and the results of Etterson et al. (2020) strongly support this idea.

In contrast to widely distributed species, sect. Lobatae also includes many species inhabiting considerably narrower ranges. The endangered maple-leaf oak, *Q. acerifolia*, is just one example (Wenzell et al., 2016). Conservation of this and similar species could be achieved through species rescue AM, a practice where populations are moved into new sites with environmental conditions suited for the species (Pedlar et al., 2012). Although species rescue AM is intended to prevent extinction events, this approach has considerable risks for introducing diseases, turning the migrated species into an invasive, or creating novel hybrids (Pedlar et al., 2012). The risk for novel hybrids is particularly great with sect. *Lobatae* (Figure 3) (Peñaloza-Ramírez et al., 2010; Whittemore et al., 1991), and introgression could allow an introduced species to replace the local species within a short timeframe (Petit et al., 2004). In our opinion, species rescue AM should not be used to preserve rare species with unknown hybridization and introgression patterns. Although species like *Q. acerifolia* face extinction, further structure analysis is required before any decision can be made (Backs et al., 2021; Beckman, 2019).

Nevertheless, there are many *Quercus* sect. *Lobatae* species on the decline, particularly in Central America and those with small ecological niches. Without any intervention these species may be gone before the end of the 21st century. Other species may have more time, but due to the time and cost of testing for appropriate source and target populations and regions through provenance trials, it is important to start the

discussion now and invest in basic research that can guide policy for AM. Moving populations without proper assessment may be more harmful than the status quo.

2.9 Conclusion

Climate change models predict increasing aridification of *Quercus*, sect. *Lobatae* habitat. At first glance, this appears to be beneficial for sect. *Lobatae*, since many species from this taxon have evolved mechanisms of drought avoidance, drought tolerance or both. Suitable habitat is projected to increase for eastern US *Lobatae* species (Figure 4), but this does not mean that species distribution will actually increase, and the issues outlined above (aging stands with decreased regeneration; fire suppression; deer herbivory on seedlings and saplings; sudden oak death; gypsy moth defoliation; and drought, also combined in oak decline) will likely decrease *Lobatae* population sizes in the USA. Projections are worse for Mexico, where suitable habitat of most species of *Lobatae* is predicted to shrink.

Quercus sect. *Lobatae* is a species-rich, recently radiated, taxonomic group distributed in the Americas (Figures 1 and 2). *Lobatae* has low levels of interspecific reproductive barriers, and hybridizations occur frequently within their major clades (Figures 2 and 3). Species identity is maintained through selection against hybrids and likely species-identity relevant introgressed loci. *Lobatae* are ecologically and economically important and yet understudied. Here, we synthesized current knowledge of *Lobatae* drought adaptation and highlight important areas of future studies. We hope to increase the focus of research groups and funding agencies towards this incredible taxonomic group. With genomic data being produced at ever higher frequencies, studies of past and ancient introgression, as well as molecular mechanisms of drought adaptation can

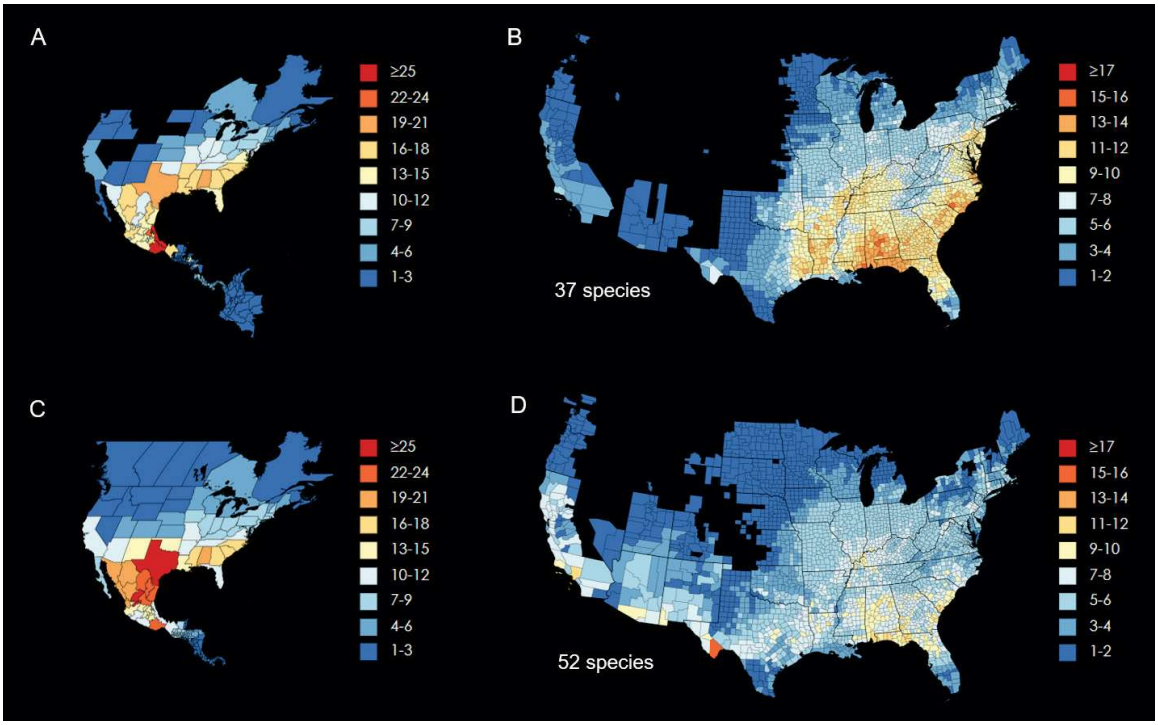
now be studied. Finding molecular signatures for environmental adaptations may enable interspecies transfer of knowledge and mitigate the need for provenance trials for each Lobatae species. Combining knowledge from genomic and physiological studies can direct provenance trials and inform assisted migration in sect. *Lobatae*.

2.10 Acknowledgments

The authors would like to thank Dr Andrew Hipp for providing the phylogeny data used in this study. The authors would like to thank Dr Erik Lilleskov for commenting on and improving Figure 3 and like to thank Matthew Peters for assistance with Figure 4.

2.11 Chapter 2 figures

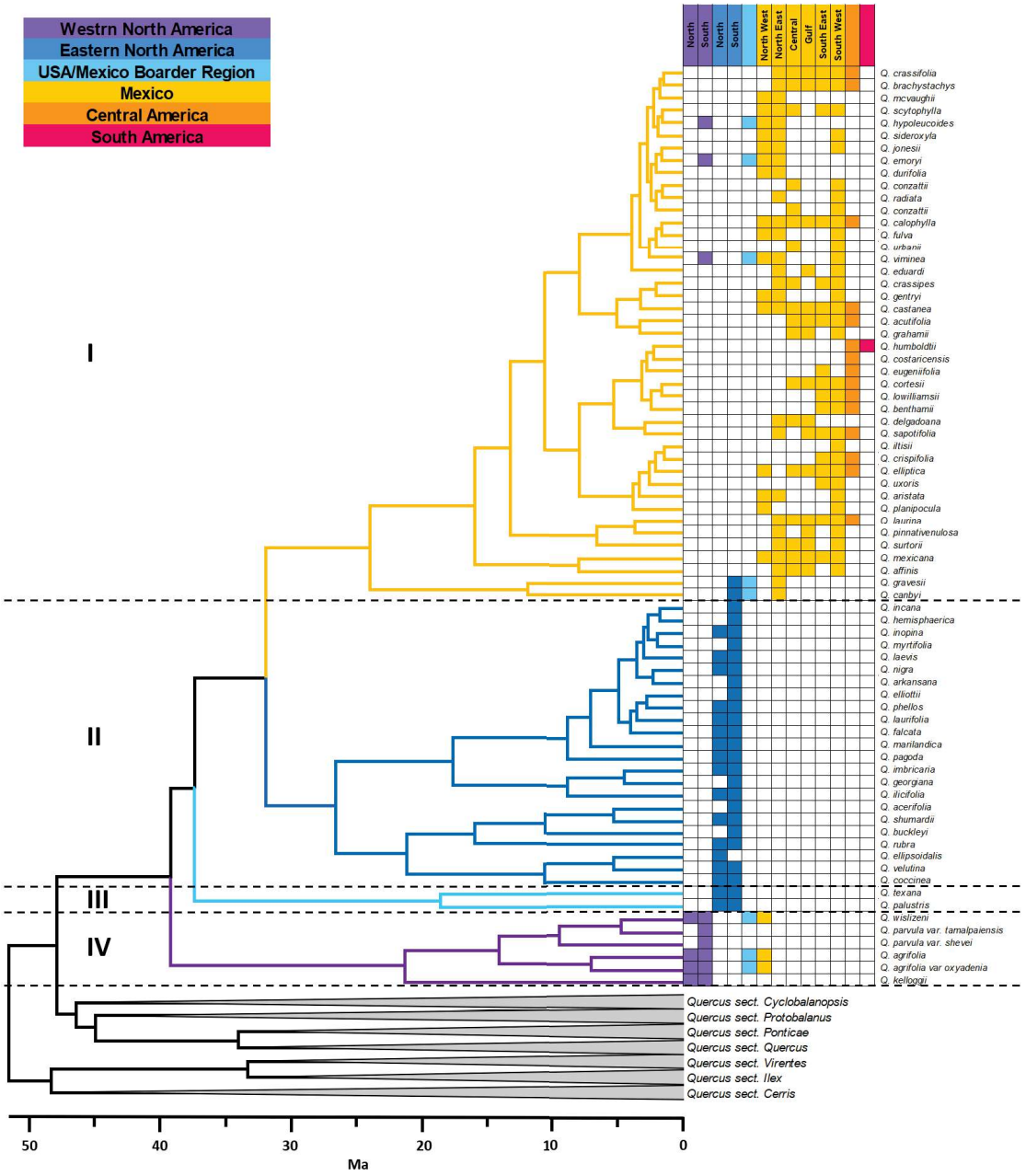
2.11.1 Figure 1



Species density within *Quercus* sect. *Lobatae* and *Quercus* sect. *Quercus* taxa in the Americas. *Quercus* sect. *Lobatae* distribution and density within Northern, Central and

South America (A), and the lower 48 United States (B). *Quercus* sect. *Quercus* distribution and density within Northern, Central and South America (C), and the lower 48 United States (D). The number of species described in each geographic region is shown. Sources for species range distributions are detailed in the chapter 2 supplementary materials sections A.1 and A.2.

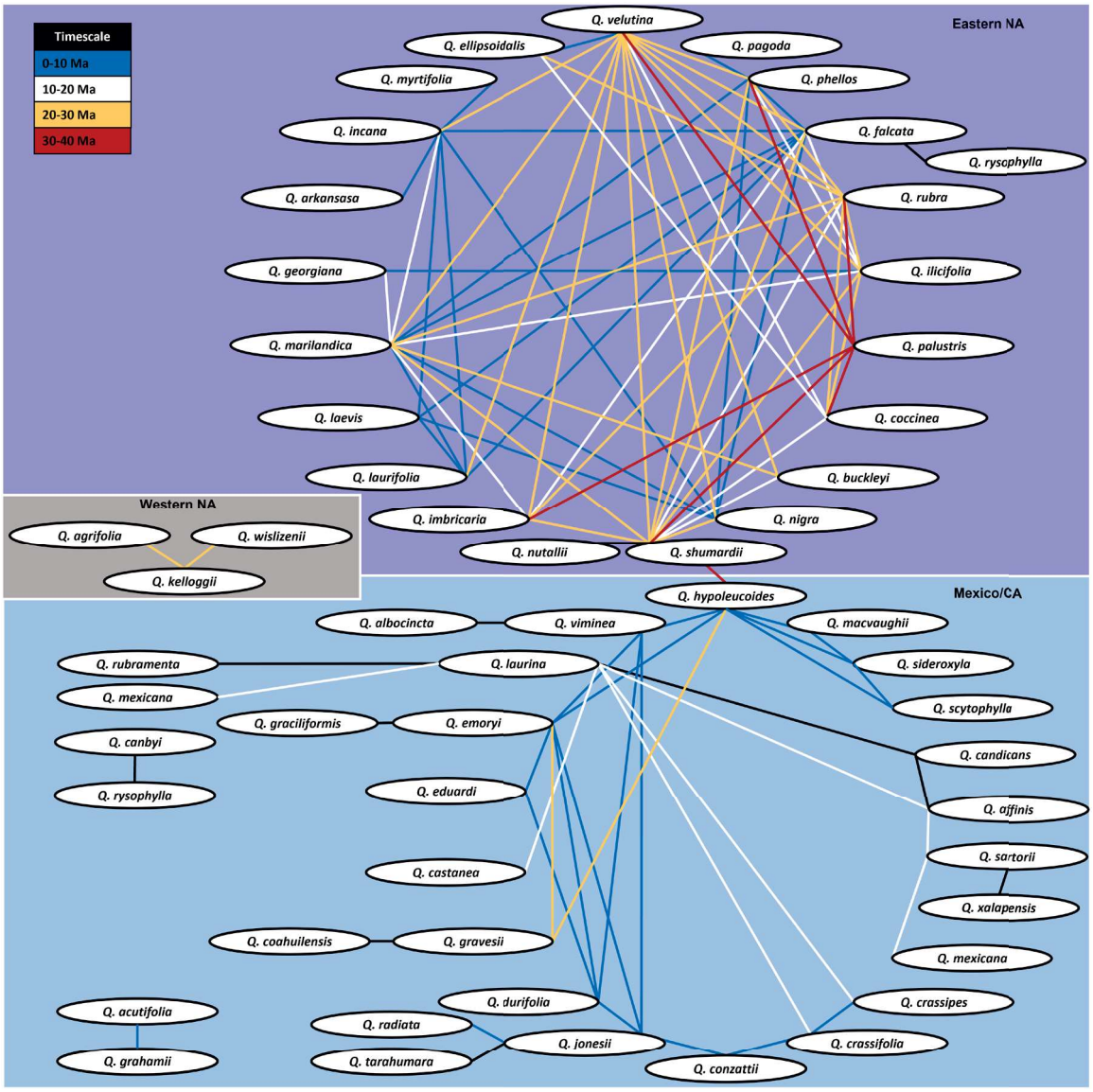
2.11.2 Figure 2



Crown-calibrated maximum likelihood phylogeny and geographic relations of the *Quercus* sect. *Lobatae* taxa reveals four distinctive clades: Mexico and Central American clade I; eastern North American clade II; and western North American clade IV. Clade III is a monophyletic eastern North American clade sister to clades I and II. Divergence time

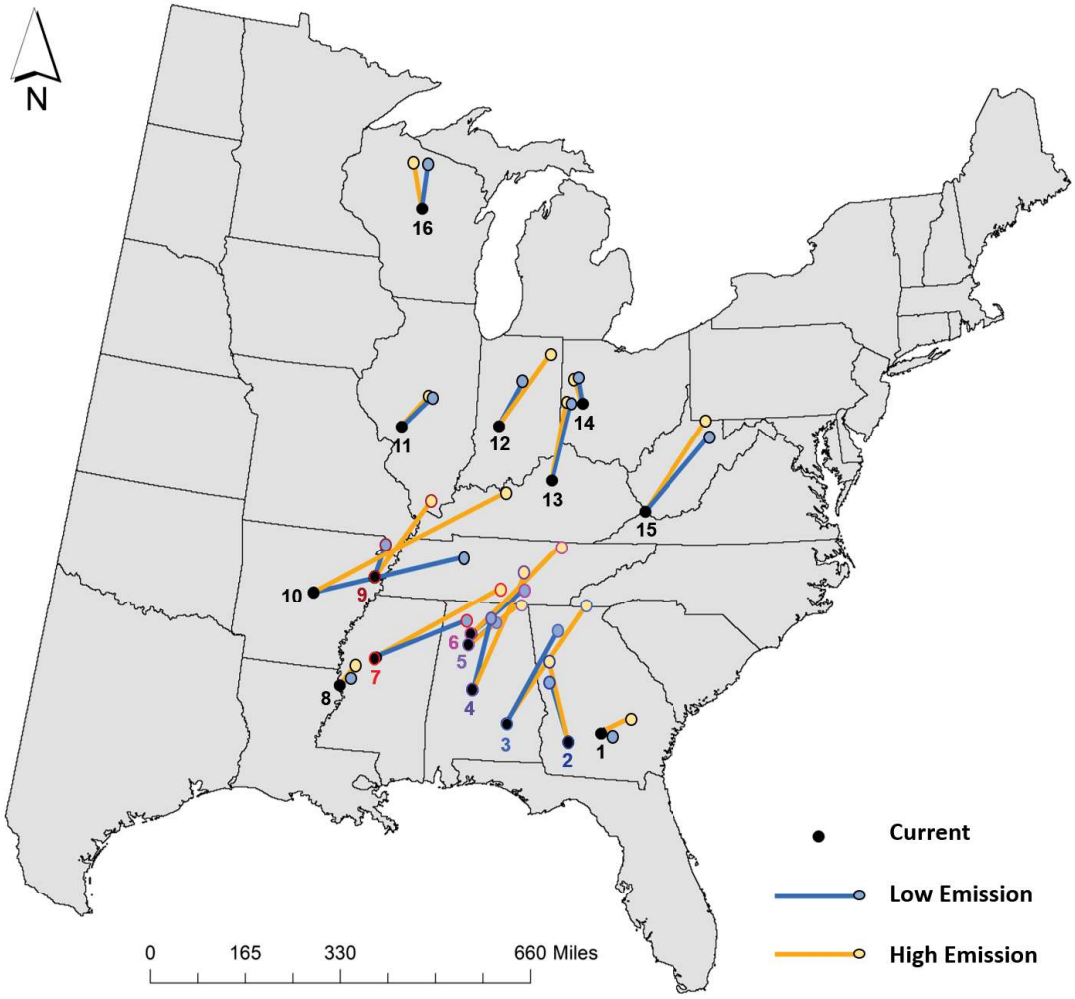
estimates are shown in million-year intervals (MA). Species biogeographic region is shown to the right. Clade assignment was informed by phylogeny, spatial geography and species hybridization patterns. ML phylogenetic data provided by Andrew Hipp and based on Hipp et al. (2020). Terminals from all sections other than Lobatae were collapsed to provide more details of the taxa and their geographic distribution.

2.11.3 Figure 3



The dynamics of naturally occurring hybridization within the *Quercus* sect. *Lobatae* taxa. Except in the case of *Quercus shumardii* × *Quercus hypoleucoides*, and any listed hybridization with *Quercus palustris*, naturally occurring hybridizations appear to occur within clades (Figure 2). Colored lines connecting species indicate the age of their last common ancestor based on Figure 2. Timescale increments are million-year intervals (MA). The species have been subdivided into their respective clades outlined in Figure 2: Eastern North American (Eastern NA), Western North American (Western NA) and Mexico/Central American (Mexico/CA) clades. Sources for species range distributions are detailed in the chapter 2 supplementary materials section A.3.

2.11.4 Figure 4



Species			Range Center (Lat, Long)			Current range	Range Increase (%)	
Scientific	Common	#	Current	LE	HE	(Km^2)	LE	HE
Q. laevis	turkey oak	1	31.78, -83.32	31.71, -82.93	32.21, -82.42	53800	150	162
Q. laurifolia	swamp laurel oak	2	31.55, -84.27	33.16, -84.87	33.71, -84.97	379500	153	207
Q. incana	bluejack oak	3	31.97, -86.10	34.38, -84.65	35.05, -83.75	30800	528	742
Q. nigra	water oak	4	32.76, -87.15	34.66, -86.68	35.88, -85.66	893300	109	177
Q. phellos	willow oak	5	33.91, -87.35	34.45, -86.66	35.05, -85.77	298500	197	262
Q. falcata	southern red oak	6	34.18, -87.28	35.45, -85.68	36.54, -84.62	780800	126	180
Q. pagoda	cherrybark oak	7	33.41, -90.09	34.57, -87.38	35.44, -86.35	289900	231	328
Q. texana	Texas red oak	8	32.61, -91.08	32.94, -90.79	33.26, -90.59	65900	73	123
Q. shumardii	shumard oak	9	35.43, -90.36	36.34, -90.07	37.53, -88.74	95500	378	803
Q. marilandica	blackjack oak	10	34.89, -92.16	36.11, -87.54	37.88, -86.29	281700	580	895
Q. imbricaria	shingle oak	11	39.27, -89.93	40.10, -88.99	40.11, -89.11	156800	80	57
Q. palustris	pin oak	12	39.45, -86.79	40.67, -85.95	41.47, -85.12	139400	228	416
Q. velutina	black oak	13	38.16, -85.01	40.22, -84.42	40.27, -84.54	1135300	113	120
Q. rubra	northern red oak	14	40.11, -84.08	40.84, -84.26	40.85, -84.44	1529500	50	51
Q. coccinea	scarlet oak	15	37.41, -82.03	39.32, -79.94	39.80, -79.96	536000	71	74
Q. ellipsoidalis	northern pin oak	16	44.82, -89.90	46.10, -89.82	46.10, -90.39	115200	161	151

Current and future predicted species distribution centroids of 16 *Lobatae* species under two emission scenarios. The black, blue, and yellow dots represent current center (centroid) of species distribution range, and the projected center of the species habitable range distributions under low and high emission scenarios, respectively. The numbers on the map correspond to a specific species and are detailed in the table below the map. LE and HE are low and high emission scenarios by the year 2100, respectively. The latitude (lat) and longitude (long) coordinates for each centroid are listed below the map, as is the current habitable range (current range) and change in range under low and high emission scenarios (range increase (%) with LE and HE). Data for this analysis were provided by Prasad et al. (2008) and Peters et al. (2019a, 2019b). Data specific to species range distributions are detailed in the chapter 2 supplementary materials section A.4.

3 Chapter 3: *Populus trichocarpa* transcription factors related to drought stress response have homologous sequences in *Quercus robur* and *Quercus rubra*

3.1 Abstract

Because of climate change, forest ecosystems and trees will be subjected to new and/or more extreme selection pressures. Droughts are just one of the abiotic stresses that are expected to become increasingly severe, but many consider this stress type to be the greatest threat to forest communities. This concern is based on recent history where we have observed massive tree mortality events and extreme wildfires. In part, these events are linked to increasingly severe droughts, which are products of multiple abiotic components that include water, temperature, and salinity abiotic stress. Through extensive characterization by morphology and physiology, the forest tree genus *Quercus* is known for extensive drought adaptations, but we presently lack an understanding of genes related to these adaptations. Using a list of poplar transcription factors responsive to drought, salinity, temperature, and water stress, I identified homologous genetic sequences within *Q. robur* and *Q. rubra*. Additionally, transcription factors responsive to jasmonates, a phytohormone known to regulate drought response (in addition to other environmental stresses), were also investigated here. Homologous sequences were found across ten transcription factor families. Within *Q. rubra*, transcription factor clusters were observed within the bHLH, C2H2, ERF, GRAS, and MYB gene families. The *Q. rubra* genetic sequences within clusters are typically within single monophyletic clades. This study has identified candidate genes within Quercus that might have a transcriptional regulation related to drought adaptation. Determining the function of these candidate genes will require additional experiments. Such experiments may include an RNA-seq

analysis comparing transcription of these gene in response to drought treatments and/or the development transgenic knockout and overexpression lines specific to these candidate genes. This research is a first step towards developing/identifying trees with greater drought adaptation.

3.2 Introduction

Compared to animals, a plants' life is relatively stationary. Although simple, this distinction has profound effects in the evolution of stress adaptation. While animals use movement and sensory cues to mitigate stress, plants either tolerate or perish (Bradshaw, 1972; Huey et al., 2002). In their seminal work, Bradshaw (1972) hypothesized that this distinction causes plants to suffer stronger selection pressures, and as a result, plant evolution favors increased physiological tolerance and phenotypic plasticity. This make physiomics, a field bringing together genomics, transcriptomics, and biochemistry/molecular analysis with physiology, an exciting field of study (Tripathi et al., 2014; Weckwerth, 2011). With the aid of transcriptomes and gene models, tree genomes serve as tools for developing hypothesis regarding molecular networks behind physiological/adaptive response. This study uses the genomes of *Quercus robur* and *Q. rubra* to identify potential genetic mechanisms related to hardwood drought adaptation.

A plants' transcriptome, referring to the sum of RNA at a given time, is highly specific and can be tailored according to the types of stress occurring, developmental stage, and tissue types. Additional complexity is added when considering epigenetic effects, gene interactions, hormone cross talk, and metabolic dynamics (Bustos-Segura et al., 2017; Hsieh et al., 2018; Kitano, 2002; Külheim et al., 2015; Padovan et al., 2013; Seki et al.,

2002; Tripathi et al., 2014). A transcriptomes' novel genes, ones with differential expression, can be lumped into two distinctive groups: effector proteins, genes that help to maintain cellular homeostasis, and regulatory proteins, genes involved in modifying gene expression. Within the latter group are transcription factors (TF), a class of proteins that can either promote or repress transcription of targeted gene sequences (Liu et al., 1999). Activity of TFs are often tightly associated with the detection of environmental cues and/or phytohormone signaling. This manner of gene regulations allows plants to alter their biochemistry and physiology in ways best suited for dealing with certain stresses (Aguan et al., 1993; Baldoni et al., 2015; Tripathi et al., 2014; Umezawa et al., 2006).

Within their distinctive gene families, we observe structural and functional similarities between TFs that can be used to predict function within phylogenetic clades and among species (Liu et al., 1999). This is in part due to a unique evolutionary pattern common to TFs: at specific chromosomal localities are clusters of highly homologous TF sequences. TF clusters are unlike metabolic gene clusters. While metabolic gene clusters include genes encoding various types of enzymes with functions specific to a certain biological process (Nützmann et al., 2020; H.-W. Nützmann et al., 2016), TF clusters are chromosomal segments containing multiple genes specific to one TF family (Shoji et al., 2021). TF clusters are the products of local gene duplication events and often have some conserved function that can quantitatively add to a plants' range of adaptation to specific stress (Kerstetter et al., 1994; Liu et al., 1999; Tague et al., 1995). Gene duplications have the potential to increase range the of adaptations because after duplication, one gene copy is released from the constraints of purifying selection (Hurles, 2004). Purifying selection is a prevalent form of natural selection that preserves fitness by eliminating the deleterious

mutations that naturally arise each generation (Monteiro et al., 2010). Once duplicated, a homolog can accumulate mutations because the other copy preserves the gene's original function (Hurles, 2004; Moore et al., 2014). In response to the accumulation of mutations, pseudogenes, non-functional genetic sequences, are most likely to arise (Balakirev et al., 2003), but homologs can also undergo subfunctionalization (Lynch et al., 2000) and neofunctionalization (Ober, 2010). Under subfunctionalization, proteins retain only part of their ancestral function and can possibly become more optimized for a specific biological process. Under neofunctionalization, one homolog retains its ancestral function and the other develops a completely new function (Espinosa-Cantú et al., 2015).

Regarding climate change, drought stress is identified as a major threat to forest health. At a global scale, we are witnessing extreme changes within our water cycle that have caused protracted droughts and increasing aridity (Dai, 2011; Frelich et al., 2010; Jump et al., 2005; Rosenzweig et al., 2008). In addition to extreme weather events, there have been massive tree mortality events and severe reductions in plant productivity that become worse over time (Choat et al., 2018; Gustafson et al., 2013; Leister et al., 2015; Stephens et al., 2018). It is also worth noting that droughts inhibit initial growth phases which makes establishment for new generations increasingly difficult (Rochefort et al., 1992; Shao et al., 2009). In response to drought, plants reduce stomatal aperture, diminish rates of transpiration, lower water potentials, reduce/arrest photosynthesis, and accumulate secondary metabolites, effector proteins and ROS scavengers (Reddy et al., 2004; Yildiz-Aktas et al., 2009; Yordanov et al., 2003). These effects have been extensively studied by molecular biologists, mostly by the application of water restriction treatments with various plant species (Harb et al., 2010; Plomion et al., 2006). Although these studies are quite

useful, they don't address the fact that within the natural environment, droughts are complex events that often include – but are not limited to – extreme temperature and increased salinity in addition to reduced water availability (Kitano, 2002; Tripathi et al., 2014).

Phytohormones are also important for coordinating a trees' response to environmental stress. These biochemicals facilitate a measured response to the environment by altering plant physiology and regulating TF activity. Jasmonates (JAs) are a phytohormone class that, among other things, can help regulate drought responses (Munné-Bosch et al., 2007; Reinbothe et al., 2009; Yun-xia et al., 2010). Studies with *Arabidopsis* note that endogenous concentrations of JAs rapidly increase in response to drought and can trigger stomatal closure (Savchenko et al., 2014). Others note that increasing JA concentrations can also induce leaf senescence in some plant species (Reinbothe et al., 2009; Yun-xia et al., 2010). These responses help prevent excessive decline in leaf water potential and runaway cavitation (Munné-Bosch et al., 2004). Moreover, activity of certain TFs can be directly altered through JA interactions with JAZ (JASMONATE ZIM DOMAIN) proteins, which can tailor plant response to drought severity (Fu et al., 2017; Niu et al., 2011). By no means is this the only phytohormone involved in drought response, however it is the one selected for the scope of this investigation.

Genus *Quercus* (oak) is a prominent group of hardwoods extensively distributed throughout the northern hemisphere. Great diversity is found within the genus, wherein estimations suggest the genus includes 500 distinct species (Denk et al., 2017; Nixon, 2002,

2006). Oak distribution ranges span Europe, Africa, Asia, and the Americas; individual species can be found in tropical, subtropical, temperate, Mediterranean, and arid climates. Ecologically, oaks are incredibly valuable at global and regional scales, fostering structural habitats (Anderson et al., 2007; Tallamy et al., 2009; Thompson et al., 2012) and contributing to ecosystem nutrient cycling (Hansen, 2000; Stoler et al., 2011). These species are often found in areas of marginal precipitation and/or high elevation. Because of this, the evolution within *Quercus* has been largely shaped by drought adaptation (Fallon et al., 2018; Valencia, 2010).

Although the morphological and physiological components of Quercus drought adaptation have been extensively characterized, we presently lack an understanding of the genetic components of these adaptations (Rauschendorfer et al., 2022). In this study, we address this issue by identifying candidate genes within *Quercus* that are homologous to drought responsive TFs from *P. trichocarpa*. Homologous sequences were identified within two *Quercus* genomes, *Q. robur* (English oak) (Plomion et al., 2018) and *Q. rubra* (northern red oak) (Phytozome, 2021). The sequenced genome of *Q. robur* has not been well assembled, and there are many fragmented, non-contiguous sequences. Because of the issues associated with this genome, only annotated homologous sequences from Q. robur have been reported by this study.

3.3 Methods

3.3.1 Identifying poplar TFs responsive to environmental stresses related to drought

A list of *P. trichocarpa* TFs were downloaded from the Database of Poplar Transcription Factors (DPTF) (Zhu et al., 2007). Within the *P. trichocarpa* TFs list, genes

with differential expression in response to drought, heat, JA, salinity, salt, temperature, and water to identified using the Expression Atlas online resource (Papatheodorou et al., 2020).

P. trichocarpa genes responsive to these cues are grouped into ten gene families: bHLH, C2H2, DBB, EIL, ERF, GATA, GRAS, HSF, MYB, and WRKY. The bHLH gene family describes a class of zinc finger TFs that have a basic helix loop helix protein secondary structure. Within plants, the bHLH gene family has been found to regulate transcriptional responses to salinity and drought stress responses (Sun et al., 2018). The C2H2 gene family is a class of zinc finger TFs defined by repeated Cys_2His_2 amino acid repeats within DNA-binding domains. Within plants, the C2H2 gene family has been found to regulate transcriptional responses to temperature, salinity, and drought (Wang et al., 2019). The DBB (double B-box) gene family is a class of zinc finger TFs which have conserved B-box protein domains, which are thought to mediate protein – protein interactions (Khanna et al., 2009). Within plants, the DBB gene family has been found to regulate transcriptional responses to temperature, salinity, and drought (Kumagai et al., 2008). The EIL (ETHYLENE INSENSITIVE3-LIKE) gene family is a class of TFs with affinity for the GCC-box DNA-binding domain (Yamasaki et al., 2005). The EIL gene family tailors plant transcriptional responses to ethylene for specific stresses (Salih et al., 2020). The ERF (ethylene response factor) gene family is also a class of TFs that are known mediate crosstalk between ethylene and JA transcriptional regulation (Shoji et al., 2021). Protein sequences for all members of the ERF gene family contain at least one copy of the AP2/ERF DNA-binding domain which consists of three β-strand proceeded by an α-helix. This DNA-binding region has been found to interact with GCC-box as well as other cis-regulatory elements (Allen et al., 1998; Magnani et al., 2004; Shoji et al., 2021). The GATA

gene family is a class of TFs that are known to interact with T/AGATAG/A DNA promoter sequences (Ko et al., 1993). These proteins contain the conserved type-IV zinc finger domain which is defined as $CysXaa_2CysXaa_{17\text{-}20}CysXaa_2Cys$ (Xaa is the three letter code for any amino acid) (Gupta et al., 2017). The GRAS gene family is a class of TFs that get their name from the first three discovered members: GIBBERELLIC-ACID INSENSITIVE (GAI), REPRESSOR OF GAI (RGA), and SCARECROW (SCR) (Hirsch et al., 2009). GRAS proteins have a GRAS protein domain (also called the "cap" domain) which contains five conserved protein subdomains, which are stretches of amino acids with very specific sequence patterns essential to the proteins function. The five protein subdomains within the GRAS cap are LRI, VHIID, LRII, PFYRE, and SAW, each of the letters written here indicates which conserved amino acids are present (Hofmann, 2016). The GRAS gene family has been seen to regulate transcriptional response to phytohormone crosstalk (Hirsch et al., 2009). The HSF (heat shock transcription factor) gene family is a class of TFs with activity regulated at the post-translational level. Under heat stress, HSF proteins from trimers which bind to DNA-promoter regions called HSE. This activity induces the transcription for heat shock proteins (HSP), a chaperone protein that ensures protein folding occurs in the correct manner (Zhou et al., 2021). The MYB gene family are TF defined by conserved MYB repeats located at the N-terminus (Roy, 2016). The MYB gene family has been found to regulate transcriptional responses to temperature, salinity, drought and JA (Ambawat et al., 2013). The WRKY gene family have a conserved N-terminal WRKY DNA-binding domain defined by a sequence of 70 amino acids and a C-terminal zinc finger domain. The WRKY domain has an affinity for the W-box DNA

sequence. In plants, this gene family has been found to regulate plant transcriptional response to different phytohormones and stresses (Jiang et al., 2017).

3.3.2 Identifying homologous sequences in *Quercus robur* and *Quercus rubra* genomes

The DNA sequences of the identified *P. trichocarpa* TFs with differential expression in response to the cues of interest were used to identify homologous sequences within *Q. robur* (Plomion et al., 2018) and *Q. rubra* (Konar et al., 2017) genomes. Annotated and unannotated homologous genetic sequences were identified in each species using a BLAST (Basic Local Alignment Search Tool, tblastn search) (Gertz et al., 2006). All results with an expect e-value of less than 1e-10 were recorded. Genomic coordinates from the BLAST searches were used to identify redundancies within our results, which were removed. Amino acid sequences were retrieved for the annotated TFs. In cases where there were no gene models, the nucleotide sequence was downloaded, and 6-way translated using Expasy (https://web.expasy.org/translate/). Translated open reading frames were added to the *Quercus* abiotic TF DEG list. Putative functional genes smaller than 100 amino acids were also removed. Protein sequences for the poplar TFs were downloaded from Phytozome (Goodstein et al., 2012), an online resource for plant genetic information.

3.3.3 Making Phylogenetic trees for TF gene families

Amino acid sequences from each gene family were aligned in a muscle multiple alignment with 10,000 iterations. This was completed using the Geneious Prime software (version 2020.0.5; Biomatters Ltd.). Each of the family alignments were manually adjusted according to the conserved genetic regions. The total number of sequences aligned for each TF gene family are listed in table 1. The alignments were truncated to ensure that the sites

being compared were homologous. Some *Quercus* sequences were removed from the analysis when sequence overlap was insufficient (Table 1). A complete list of the genetic sequences used for the phylogenetic analysis is found in Supplemental Table 1.

To create the phylogeny for each gene family, we first tested which amino acid substitution model had the best Akaike's information criterion (AIC) value using Phyml's Smart Model Selection (Lefort et al., 2017). The models with the best AIC value was Jones-Taylor-Thornton (JTT) with gamma distribution estimated and proportion of invariable sites fixed. The phylogeny for each TF gene family was determined using 100 bootstrap replicates with the best substitution model. The phylogeny was visualized in FigTree v1.4.4.

3.3.4 Identifying TF clusters in *Q. rubra* genome

TF clusters for each gene family were identified in *Q. rubra* by comparing each genetic sequences' chromosomal proximity. For this study, TF clusters were defined as containing three or more homologous TF sequences that were within 100 kilo-base pairs (Kbp) of another TF genetic sequences from the same family (as in Shoji et al., 2021). Their figure shows TF clusters contain three or more homologous TF sequences, and the genetic sequences in each cluster are within 100 kilo-base pairs (Kbp) of another TF genetic sequence from the same gene family. The potential Q. rubra TF clusters are described by Table 2, Figure 1, and Supplemental Table 2. Names of each cluster have at least two and sometimes a third part: first is the name of TF family, second is the chromosome number, and third is a letter indicating when there are more than one cluster for a given family on a specific chromosome.

These search parameters also identified pairs of homologous TF sequences that had close chromosomal proximity. The rules used to name the homologous pairs were similar to the rules used to name the clusters. The difference between the cluster and homolog names is the phrase “hom” which comes after the TF name and before the chromosome number.

3.4 Results and discussion

3.4.1 bHLH genes responsive to JA have homologous sequences within *Quercus*

Six poplar bHLH genes were used as queries for a BLAST search of *Q. robur* and *Q. rubra* genomes: four and two of these poplar sequences were JA and temperature responsive, respectively. The BLAST searches identified 10 and 16 bHLH homologous sequences within *Q. robur* and *Q. rubra* genomes, respectively (Table 1). Most of these Quercus sequences were homologous to JA responsive bHLH genes (Supplemental Table 1).

The JA signaling gene model identifies bHLH genes as having a significant role in mediating JA response (Brkljacic et al., 2017; Chini et al., 2016; Goossens et al., 2017; Qi et al., 2015). Certain classes of bHLH factors have JIDs (JAZ-interacting domains) which bind to JAZ (JASMONATE ZIM DOMAIN) proteins and inhibit TF activity. When JA concentrations increase the JAZ proteins are degraded and the unbound bHLH factors can form complexes regulating gene expression in response to increasing JA concentrations (Goossens et al., 2017; Wasternack et al., 2013). There are two amino acid motifs commonly found in this type of bHLH factor: the Myc-type and MYC/MYB domains (InterPro identifiers IPR011598 and IPR025610) (Goossens et al., 2017). These motifs are

present in all the poplar bHLH TFs used for *Quercus* genome query and are also found within all the homologous *Quercus* sequences. Although we do not know if *Quercus* bHLH homologous sequences are functional genes, their abundance within the *Quercus* genomes could suggest that JA signaling is mediated by bHLH gene in both *Q. robur* and *Q. rubra*.

Using the genome coordinates of the *Q. rubra* bHLH homologous sequences, I identified a potential TF cluster located on chromosome 11 between 4.02 and 4.03 Mbp (one million base pairs) (Table 2). This cluster is comprised of three bHLH homologous sequences: one sequence is annotated, two are unannotated (Figure 1, Table 2, and Supplemental Table 2). Based on the BLAST analysis, the *Q. rubra* sequences within the cluster were homologous to poplar JA responsive bHLH genes. Within the phylogenetic analysis, two of the sequences within this cluster had similar sequence identity, which suggests these sequences are closely related homologs (Figure 2 highlighted region).

3.4.2 C2H2 TF cluster identified within a small region of *Q. rubra* chromosome 7

Five poplar C2H2 genes were used in BLAST queries of the *Q. robur* and *Q. rubra* genomes. Each of these sequences had differential expression in response to temperature stress. BLAST results from *Q. robur* and *Q. rubra* identified six and sixteen annotated homologs, respectively (Table 1 and Supplemental Table 1). Using the genome coordinates of the *Q. rubra* C2H2 homologs, I identified a potential TF cluster consisting of 4 genetic sequences located on 19.8 kbp segment of chromosome 7 (Figure 1, Table 2 and Supplemental Table 2). By standards of metabolic and TF clusters, this chromosomal segment is relatively small (H.-W. Nützmann et al., 2016; Shoji et al., 2021). Based on the phylogeny of these four sequences – all are found within a single phylogenetic clade

highlighted in Figure 3 – these *Q. rubra* sequences could potentially be homologous C2H2 genes. When reflecting on the importance of homologous genes in the evolution of TF, this cluster is rather interesting, as the homologous sequences have the potential for sub and neofunctionalization (Lynch et al., 2000; Ober, 2010), which could expand the adaptive response of *Q. rubra*. Future experimentation could investigate if these sequences encode functional genes that are temperature responsive. If significant results are discovered, variant alleles for genes within this cluster should be identified in *Q. rubra* populations as these results might reflect on population specific adaptations.

The nucleotide binding sites of the C2H2 gene family are not well understood. Protein domains that are important for C2H2 DNA binding in plants include the Q-type C2H2 motif, QALGGH, and multiple zinc finger domain (Brayer et al., 2008; Klug et al., 1995; Kubo et al., 1998; Lyu et al., 2018). Although targets are not understood, we do know that C2H2 factors activity is responsive to salt, drought, and temperature stress. Moreover, these genes have can act as master regulators within the direct and indirect ABA responsive pathway, and thus have a substantial role in the regulation of plant drought response (Huang et al., 2007). In some instances, C2H2 genes have been observed to circuitously increase concentrations of ABA, proline, and soluble sugars, helping to limit water loss during a drought (Luo et al., 2012).

3.4.3 A set of homologous DBB sequences discovered on *Q. rubra* chromosome 10

Two poplar DBB TFs responsive to salt stress were used as BLAST queries against the *Q. robur* and Q. rubra genomes. This uncovered 4 and 11 homologous sequences in the *Q. robur* and *Q. rubra* genomes (Table 1, Supplemental Table 1). Although previous work

has observed differential expression of DBB factors in response to alkali-salt treatments, the mechanistic function of these genes is largely unknown and in some instance tied to circadian rhythms (Kiełbowicz-Matuk et al., 2014). When examining the homologs from *Q. rubra* we identified a set of paralogs (Qurub.10G050000 and Qurub.10G050100) on chromosome 10 (Supplemental Table 3). The protein sequences for these genes were very similar (Supplemental Table 1), and within our phylogenetic analysis of DBB homologs, these genes were found within the same clade as Porti.017G028300 (Figure 4 highlighted branch). Further experimentation is required to determine if the salinity responsive function of the Porti.017G028300 is conserved in Qurub.10G050000 and Qurub.10G050100.

3.4.4 *Quercus* EIL and ERF homologs suggest conserved genetic mechanisms for regulating response to ethylene and environmental stress

Using five poplar EIL gene that were responsive to JA, three and eight homologous sequences were found within the *Q. robur* and *Q. rubra* genomes, respectively (Table 1 and Supplementary Table 1). No EIL clusters were found within the Q. rubra genome. The phylogenetic relationships for these sequences are detailed in Figure 5.

Twenty poplar ERF genes were used in BLAST queries of the *Q. robur* and *Q. rubra* genomes: thirteen are related to JA response, six are related to temperature stress, and one is related to water stress. Within *Q. robur* and *Q. rubra* genomes, 30 and 135 homologous sequences were identified, respectively. Most of these homologous sequences were identified by BLAST queries by multiple poplar ERFs that were responsive to different stress types (Table 1 and Supplementary Table 1). This demonstrates that the poplar ERFs have highly conserved protein sequences and are closely related. Using the

135 *Q. rubra* ERF homologs, I identified nine ERF clusters. Four of these clusters had homologs specific to poplar temperature responsive ERFs: these clusters are ERF.1, ERF.6.a, ERF.8.a, and ERF.8.b (Figure 1, Table 2 and Supplemental Table 2). Three of nine ERF clusters had homologs specific to poplar ERFs responsive to temperature and JA: these clusters are ERF.4, ERF.6.c, and ERF.9.b (Figure 1, Table 2 and Supplemental Table 2). The remaining two clusters, ERF.6.b and ERF.9.a, contained homologs for poplar ERFs responsive to a myriad of stimuli (Figure 1, Table 2 and Supplemental Table 2). In addition to these ERF clusters, 10 sets of homologs were also identified (Supplemental Table 3).

Looking at the phylogenetic relationships between the ERF clusters and homolog sets, we see evidence for multiple large duplication events within *Q. rubra* (Table 2 and Figure 6). Within Figure 6 are four distinctive clades demonstrating this. Within phylogenetic clade A are all the homologs from ERF.6.b (Supplemental Table 2). Within clade B are all the homologs from sets ERF.hom.9.c and ERF.hom.12.a (Supplemental Table 3). Within clade C are all the *Q. rubra* homologs from ERF.4 (Supplemental Table 2). Within clade D are a series of subclade that are populated by *Q. rubra* homologs from ERF.1, ERF.6.a, ERF.8.a, ERF.8.b and ERF.9.a (Supplemental Table 2).

EIL and ERF results are being discussed together because these TFs have common interactions outlined by the ethylene response pathways. This genetic model can be summarized as follows: various abiotic stress, including temperature, water, and/or light, increase a plants' ethylene concentration. Plant cells detect extracellular ethylene with ethylene receptors, triggering a kinase cascade that activates TF activity of EIL genes. In

turn, EILs initiate transcription of multiple ERF genes, which initiates an ethylene transcription cascade that is tailored to respond to specific stresses (Müller et al., 2015; Poór et al., 2021). The results from this experiment provide initial evidence that EIL factors are the master regulatory TF of ERF. First, using a small number of poplar EILs, a small number of EIL homologs were found within the two *Quercus* genomes. For context, using a small number of MYB and WRKY poplar TF, I found many homologous sequences within *Quercus* (Table 1). Finding a low number of EIL factors in *Quercus* suggests that these genes have strong and conservative regulatory role within this genus too. Because EILs regulate the transcription of other TFs, sub and neofunctionalization for genes within this family would be selected against (Lynch et al., 2000; Ober, 2010). If duplication events of EIL factors did occur, the duplicated sequence would likely become a pseudogene. Second, many ERF homologs were identified in *Quercus* (Table 1), and many TF clusters and homolog sets were observed among the *Q. rubra* ERF homologs (Figure 1, Table 2, Supplemental Table 2, and Supplemental Table 3). The ERF TF clusters have been found across various plant species, and moreover, transcription of ERF genes within clusters is often responsive to JA and specific TFs (Shoji et al., 2021). These types of ERF clusters have been observed in *Nicotiana tabacum* (two clusters of ten and five ERF genes) (Kajikawa et al., 2017), *Solanum tuberosum* (a cluster of eight ERF genes) (Cárdenas et al., 2016), *Solanum lycopersicum* (a cluster of five ERF genes) (Thagun et al., 2016), *Vinca minor* (a cluster of five ERF genes), giant milkweed (a cluster of four ERF genes), *Gelseminum sempervirens* (a cluster of four ERF genes) (Singh et al., 2020), *Daucus carota* (a cluster of six ERF genes) and *Glycine max* (a cluster of 5 ERF genes) (Shoji et al., 2021). These TF clusters have specific metabolic functions too. For example, the

Nicotiana tabacum and *Solanum lycopersicum* ERF cluster induces biosynthesis of nicotine and steroidal glycoalkaloids, respectively. Both these secondary metabolites aid plant herbivory defenses (Hayashi et al., 2020; Nakayasu et al., 2018; Shoji et al., 2021). Discovering nine ERF clusters within *Q. rubra* is rather exciting and suggests that the ERF TFs have opportunities accumulate mutations and become sub and neofunctionalized (Lynch et al., 2000; Ober, 2010). Future experimentation should investigate if these clusters increase adaptive capacity of *Q. rubra* and if there are population specific allelic variants for these genes.

3.4.5 Sets of homologous GATA TFs discovered on *Q. rubra* chromosomes 9 and 11

Nucleotide sequences from three poplar genes were used to find GATA homologs within *Quercus*. These genes have three things in common: (1) a GATA-type zinc finger domain (InterPro identifier IPR000679), (2) a TIFY domain (InterPro identifier IPR010399: previously known as a ZIM domain), and (3) each gene's expression is responsive to changes in JA concentrations. Based on the domains present, these GATA genes are all within the TIFY group 1 subfamily (Vanholme et al., 2007). The TIFY motif is also found in JAZ proteins, and although function of TIFY proteins is still being determined, TIFY proteins are often responsive to JA (Bai et al., 2011; Ye et al., 2009). The methods of this study identified one and five annotated homologs within *Q. robur* and *Q. rubra*, respectively (Table 1 and Supplemental Table 1). Two sets of GATA homologs were observed in the *Q. rubra* genome at chromosomes 9 and 11 (Supplemental Table 3). A phylogenetic tree for all GATA genes is found in Figure 7. Both the presence of the

TIFY domain and the JA responsive poplar homologs could suggest that some GATA genes within *Quercus* regulate JA mediate responses to abiotic stress (Bai et al., 2011).

3.4.6 Multiple GRAS TF clusters identified in *Q. rubra* genome within distinctive phylogenetic clades

Nine poplar GRAS genes responsive to salinity and/or JA were identified in poplar (Supplemental Table 1). The interactions between JA and salinity signaling pathways are not unusual. Studies from numerous plant species have observed that salinity stress treatments increase JA concentrations and the transcription of JA biosynthesis genes (Moons et al., 1997; Walia et al., 2006). In some cases, preemptive application of JA can improve plant's tolerance of salinity stress (Walia et al., 2007). GRAS genes can also act as nodes for hormonal crosstalk. Work in chrysanthemums has been demonstrated that JA and salicylic acid have been observed to induce and suppress expression of certain GRAS genes, respectively (Gao et al., 2018).

The methods of this study identified 13 and 61 homologs within *Q. robur* and *Q. rubra*, respectively. The homology of these sequences and the poplar genes suggests that all *Quercus* homologs are related to salinity and JA response (Supplemental Table 1). A total of four GRAS clusters were identified within the *Q. rubra* genome (Figure 1, Table 2 and Supplemental Table 2). The GRAS.1 cluster, which spans a 245.5 Kbp segment of *Q. rubra* chromosome 1, contained 12 annotated homologs, and is the TF cluster with the greatest number of duplication events identified by this study (Figure 1, Table 2). The genes within cluster GRAS.1 have similar phylogeny to genes within the GRAS.8, a four gene cluster spanning a 44.6 Kbp segment of chromosome 8 (Figure 1, Table 2). All of these genes from GRAS.1 and GRAS.8 TF clusters are found in their own clade (Figure 8,

clade A). The remaining TF clusters, GRAS.4.a and GRAS.4.b, are made up of four and three GRAS homologs, respectively. These clusters span 26.2 and 64.1 Kbp segments within chromosome 4 (Figure 1, Table 2). The constituents of clusters GRAS.4.a and GRAS.4.b are each found in distinctive phylogenetic clades too (Figure 8, clades B and C).

3.4.7 Lowered number of HSF homologs identified in *Quercus* genomes

Initially identified were 30 HSF poplar genes that were responsive to temperature stress. Through BLAST, 10 and 20 annotated HSF homologs were identified in *Q. robur* and *Q. rubra*, respectively (Table 1 and Supplementary Table 1). No HSF clusters were identified within *Q. rubra* (Figure 1 and Table 2). Phylogenetic analysis did not reveal unique *Quercus* clades but does suggest that the high number of HSF poplar genes are potentially related to recent duplication events within poplar (Figure 9).

Given the relatively large number of HSFs used in our BLAST query, I was rather surprised by the lower number of homologs within *Quercus*. These results may indicate that *Quercus* is less reliant on transcriptional memory when responding to heat stress. When under heat stress, a plant's ABA concentration increases causing activation of the SWI/SNF chromatin remodeling complex (CRC). This protein complex is a central regulator of both the ABA and the chaperone signaling pathways. Subsequently, transcription of HSFs is initiated both by the ABA-induced response and the CRC. Activity of the CRC and HSFs confer transcriptional memory, which, at the cost of plant growth increases plant tolerance to heat stress (Figure 10) (Bezhani et al., 2007; Bulgakov et al., 2019; Efroni et al., 2013; Han et al., 2014; Lämke et al., 2017; Sarnowska et al., 2016). These results could demonstrate how poplar and *Quercus* differ in their adaptations to

temperature stress. The fewer HSF homologs may indicate that evolution of *Quercus* drought adaptation favored anatomical adaptations (e.g. deep taproots and ring-porous xylem) (Abrams, 1990; Robert et al., 2017; Skelton et al., 2018) and plant growth (Efroni et al., 2013) over transcriptional memory.

3.4.8 Many homologous MYB sequences discovered within the *Q. rubra* genome

Four MYB genes regulating temperature response in poplar were found to have homology with 17 and 128 genetic sequences in *Q. robur* and *Q. rubra*, respectively (Table 1, Supplementary Table 1). The huge difference between the number of homologs within *Q. robur* and *Q. rubra* may be a result of the approaches used to annotate each genome. Five MYB clusters are found in *Q. rubra* chromosomes 2, 8 and 10, and all homologs within these clusters are annotated sequences (Figure 1, Table 2 and Supplementary Table 2). Moreover, 10 sets of MYB homologs were also identified, and all of the homologs within these sets are also annotated (Supplemental Table 3). When looking at the at the phylogenetic relationships between these *Q. rubra* MYB clusters and homologs, I observed distinct clades populated by their constituents (Figure 11). Within the phylogenetic clade A are the members of MYB.hom.5.a, MYB.hom.8, MYB.8.a, and MYB.8.b: the similar sequence identity between the homologous MYB sequences of chromosome 8 are interesting and suggest a possibility for multiple duplication events within one linkage group within *Q. rubra*.

The MYB gene family is quite large and can be separated into three distinctive sub-families. These are the 3R-MYB, R2R3-MYB and the MYB-related classes (Baldoni et al., 2015). When discussing activity of the MYB family, functions should be viewed as

flexible. There are many instances where MYBs characterized for development, biotic defense, or metabolism are later found to be drought responsive (Baldoni et al., 2015). Thus, there is room to discover more about drought adaptation response among the characterized MYB genes. Capturing the MYB family's role in drought adaptation are these facts: 65% of rice MYB genes were differentially regulated under drought stress; 51% and 41% of *Arabidopsis* genes were upregulated and downregulated by drought stress, respectively (Katiyar et al., 2012). Although members of each sub-family include factors that regulate drought response, the classes most associated with this trait are found in the R2R3 and MYB-related classes (Dubos et al., 2010). Paraphrasing Baldoni et al. (2015), R2R3 genes have roles in primary and secondary metabolism, cell identity/development, and/or stress response. MYB-related genes generally regulate cellular morphogenesis, metabolism, circadian rhythm, and/or drought response (Baldoni et al., 2015; Cai et al., 2015; Dubos et al., 2010). Within plants, roles of MYBs in drought response are known to include cuticular wax synthesis (Baldoni et al., 2015; Lee et al., 2015), stomatal aperture (Cominelli et al., 2010), and lateral root development (Zhao et al., 2014). The latter is responsive to ABA/Auxin concentrations and is just one example of MYBs facilitating pathway crosstalk in response to drought. Other examples of this type of MYB activity can be found in Ding et al. (2009); Jung et al. (2008); and Jaradat et al. (2013). In trees, drought responsive MYB factors are also being investigated. Transgenic overexpression of MdMYB10, MdMYB121, and MdSIMYB1, genes from the common apple (*Malus x domestica*), can confer significant drought responsive phenotypes (Espley et al., 2007). QsMYB1, an R2R3-MYB from *Q. suber* (cork oak), has also been seen to have increased expression under drought and heat treatments. It is thought that increased QsMYB1

expression provides thermotolerance (increased proline concentration) and maintains healthy (although diminished) photosynthesis rates (Almeida, Menéndez, et al., 2013; Almeida, Pinto, et al., 2013).

3.4.9 A set of WRKY homologs found on *Q. rubra* chromosome 12

A defining characteristic of all WRKY factors are two sequence domains. On the N-terminus is the WRKY domain, a sequence of 60 amino acid residues containing the WRKY motif, which binds to W-box promoters (Yamasaki et al., 2013). The other domain is a zinc finger located on the C-terminus, which is used to classify WRKY factors into sub-families (Eulgem et al., 2000). Member of the WRKY family have a wide array of regulatory roles that range from biotic stress response, which have been examined extensively, to abiotic stress response, which are not as well understood (Rushton et al., 2010). Our BLAST search began with three poplar WRKY factors: Potri.001G361600, Potri.006G224100, and Potri.013G153400. These genes are responsive to drought, JA, and temperature/salt, respectively. Transcriptome regulation in response to these stimuli is common. Overexpression of certain WRKY genes confers increased drought, temperature, and salinity tolerance in *Arabidopsis*, *Oryza sativa, Hordeum vulgare*, and *Boea hygrometrica* (Gómez-Mendoza et al., 2007; Straub et al., 1994). We have also observed that WRKY factors response to JA can elicit drought adaptive responses and act as a form of crosstalk with the ABA response pathways (Gómez-Porras et al., 2007; Rabara et al., 2013).

Using BLAST, we found 10 and 21 homologs within *Q. robur* and *Q. rubra*: most of the sequences shared homology with at least two of the poplar WRKY factors; all

homologs within *Q. rubra* were previously annotated (Table 1, Supplementary Table 1). No TF clusters were identified within *Q. rubra* (Supplemental Table 2). One set of WRKY homologs was found on chromosome 12: the homologs within this set were homologous to poplar WRKYs responsive to salt, drought, and temperature stress, and JA. In the phylogenetic analysis of these homologous sequences, I did not observe unique phylogenetic clades (Figure 12).

3.5 Conclusion

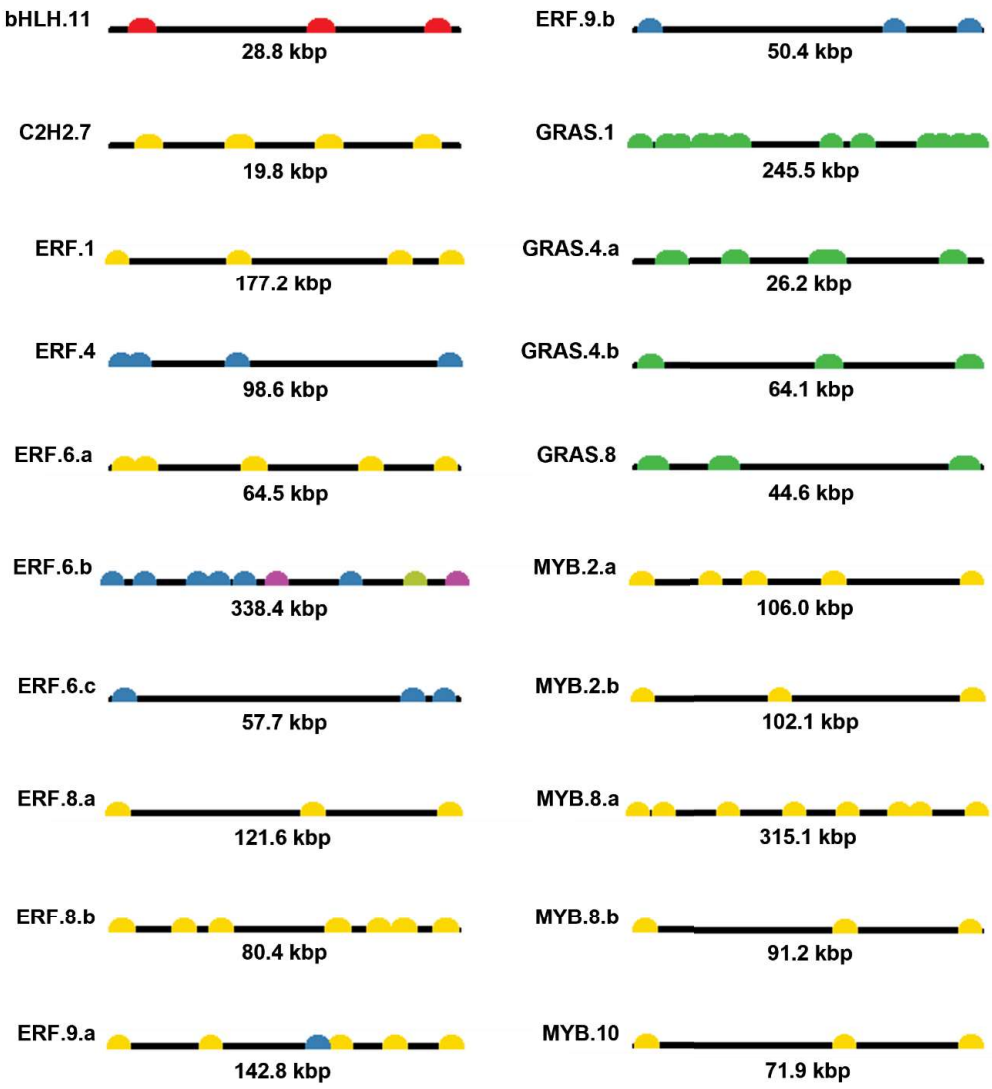
The study was conducted to identify candidate drought regulatory genes within *Q. robur* and *Q. rubra*. Our methods initially found transcription factors from 10 different gene families. Based on the chromosomal proximity of the *Q. rubra* homologs that were within the same gene families, I identified twenty unique TF clusters specific to the four families: bHLH, ERF, GRAS, and MYB (Figure 1). The gene family with the greatest number of clusters, nine of the twenty, was ERF. Previous experiments across multiple plant species have demonstrated that ERF clusters are important in metabolic responses to JA: ERFs within TF clusters have been found to be under the regulation of specific TFs, and often have complementary functions (Shoji et al., 2021). Gene duplications are known to reduce the purifying selection on homologous sequences (Hurles, 2004). This is important within in the evolution of TFs because the sub and neofunctionalization of these genes can increase the adaptive capacity of a species (Lynch et al., 2000; Ober, 2010).

Presently, the TF homologs identified in this study are all candidate genes. Next steps in experimentation will involve differentiation between functional genes and pseudogenes. For Q. rubra, this process should be rather simple and can be tested with

using j-browse in phytozome by identifying which sequences are transcribed. Determining function roles for each of these genes will require more elaborate experimentation, this could include RNA-seq analysis comparing transcription levels for these different homologs in response to drought and non-drought treatments. Function for these homologs can also be determined by generating transgenic knockout and/or overexpression lines and identifying how these mutants responds to drought stress. This is an excellent direction for future research to move towards because it will aid in the development and identification of trees with greater drought adaptation.

3.6 Chapter 3 Figures

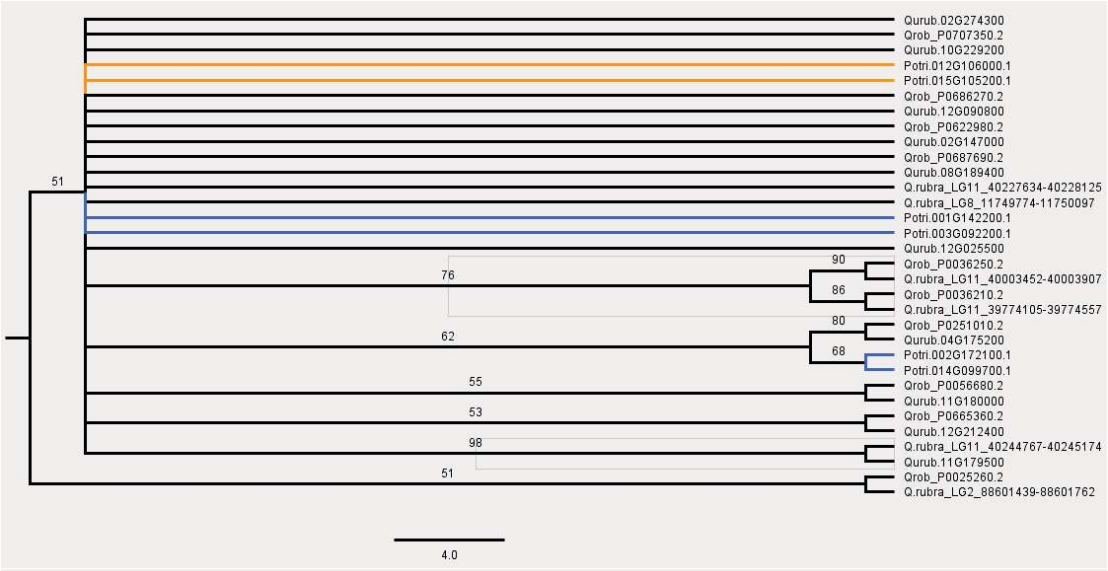
3.6.1 Figure 1



Chromosomal maps of *Q. rubra* TF clusters. The names of each TF cluster are listed to the left of each map. TF cluster names can have three parts: TF gene family, a number indicating *Q. rubra* chromosome, and a letter indicating if there more than one TF cluster within a given chromosome (when necessary). The black lines represent chromosomal regions, the lengths of each are listed underneath each map. Dots on each chromosomal map indicate the locations of TF *Q. rubra* homologous sequence, the color of each indicates

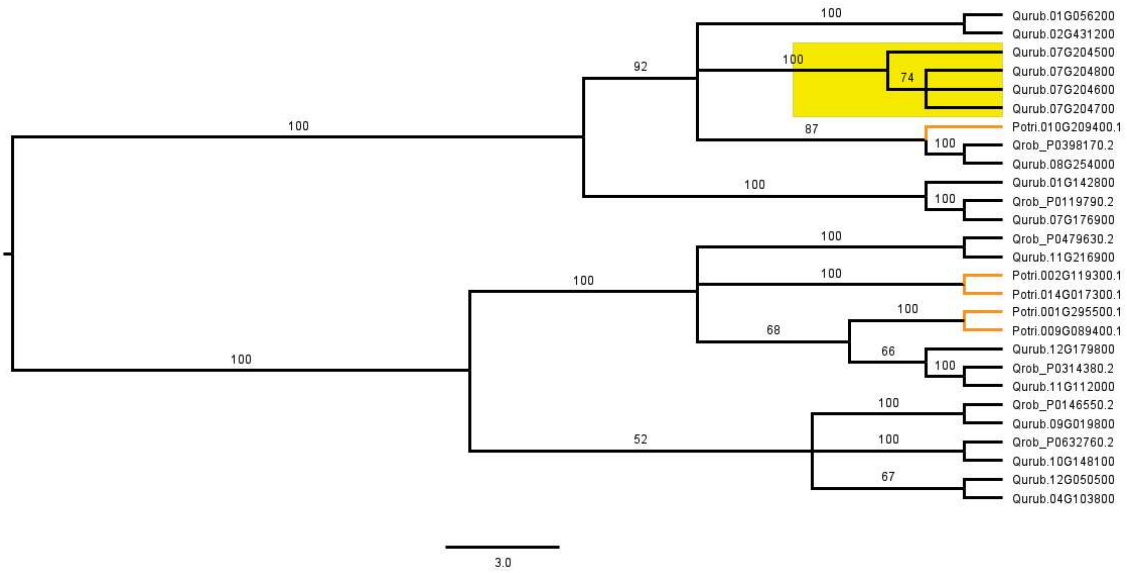
the stress responses of homologous *P. trichocarpa* genes. Red – JA; yellow – temp; blue – temp and JA; pink – temp, JA and water; green/yellow – temp and water; green – salt and JA. More details on each cluster are found in Table 1 and Supplemental Table 1.

3.6.2 Figure 2



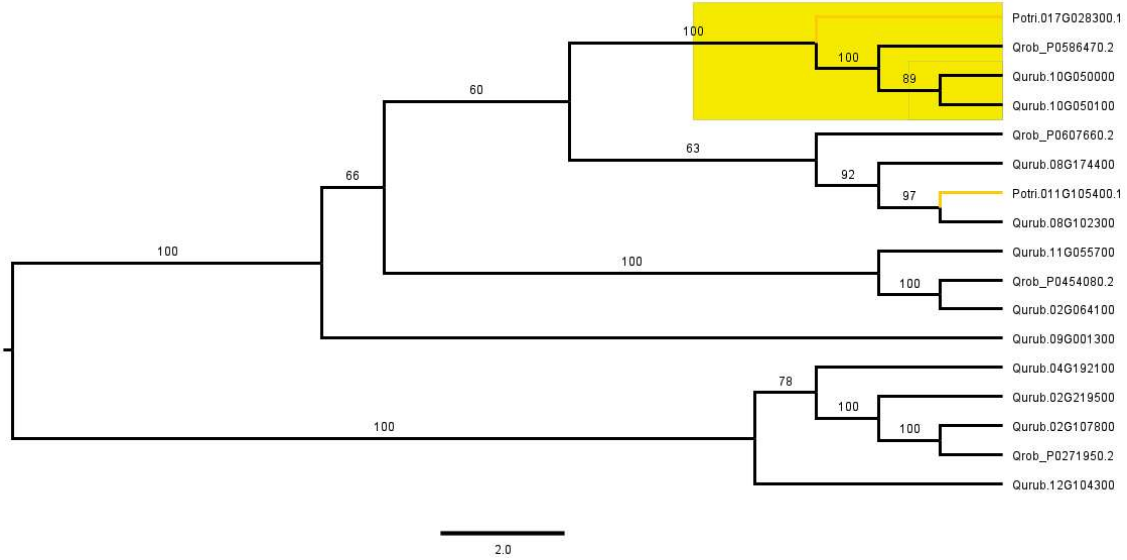
A phylogenetic tree of bHLH genes from *Q. rubra*, *Q. robur* and *P. trichocarpa*. Poplar bHLH gene have been labeled to indicate what they are responsive to: blue – JA, orange – temperature. Bootstrap values are written above each branch. Highlighted branch discussed in results and discussion.

3.6.3 Figure 3



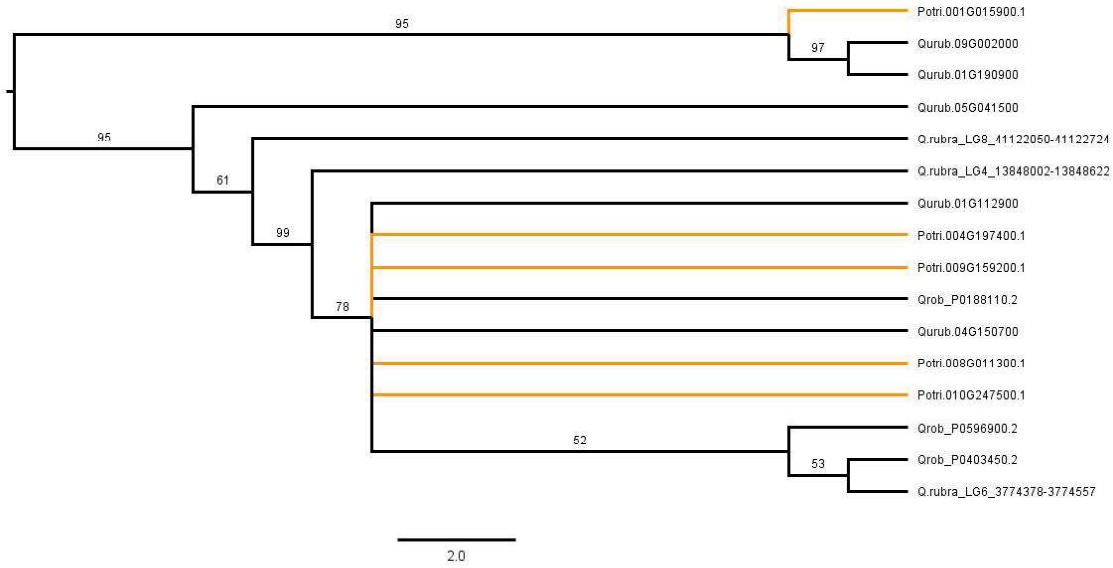
A phylogenetic tree of C2H2 genes from *Q. rubra*, *Q. robur* and *P. trichocarpa*. All *Quercus* genes were found using the temperature responsive C2H2 genes of *P. trichocarpa* (labeled orange). Bootstrap values are written above each branch. The highlighted branch within this tree contains all the homologs from the *Q. rubra* cluster C2H2.7.

3.6.4 Figure 4



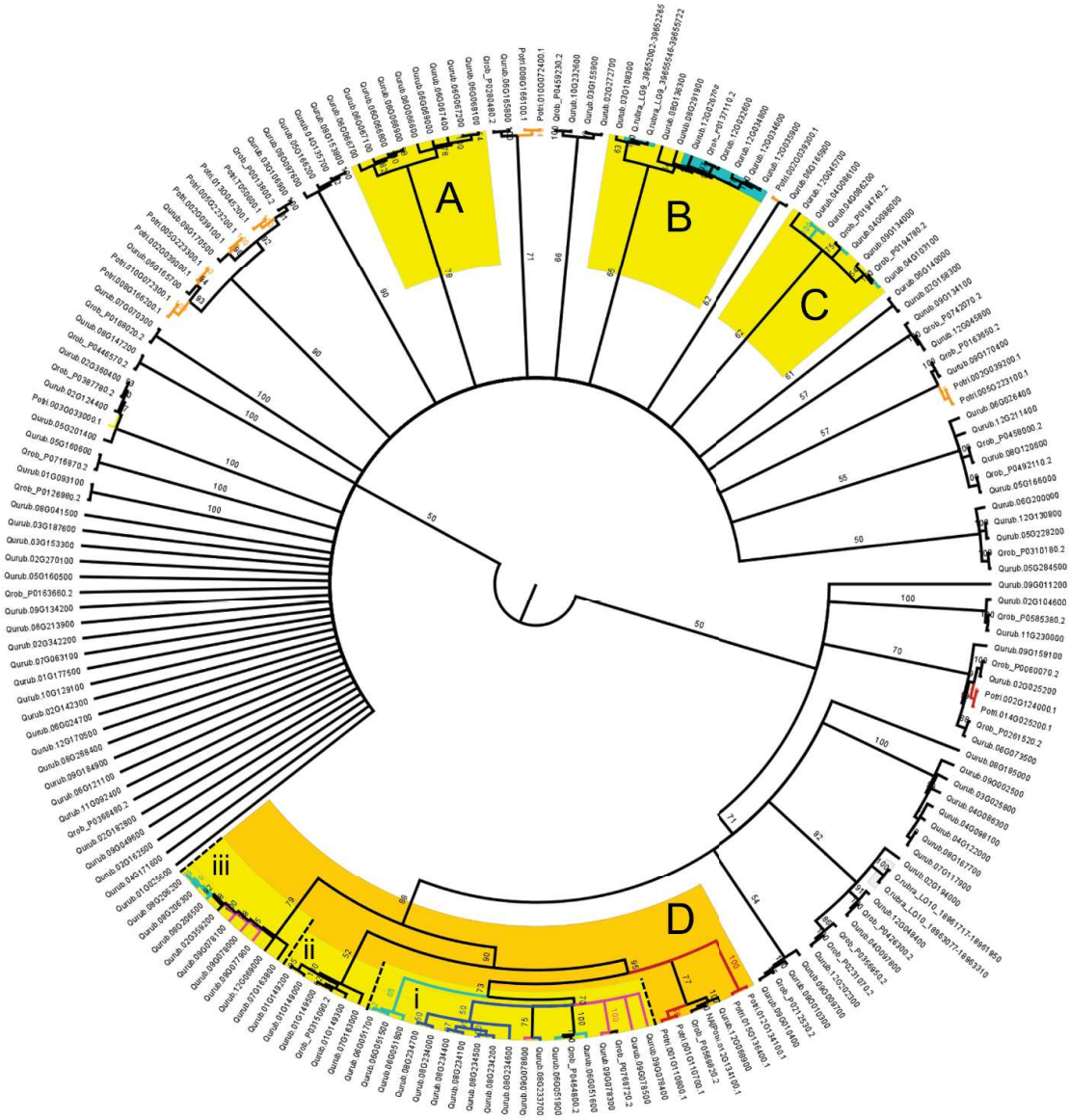
A phylogenetic tree of DBB genes from *Q. rubra*, *Q. robur* and *P. trichocarpa*. All *Quercus* genes were found using the salt responsive DBB genes of *P. trichocarpa* (labeled orange). Bootstrap values are written above each branch. The highlighted branch within this tree contains all the homologs from the *Q. rubra* paralog set DBB.par.10.

3.6.5 Figure 5



A phylogenetic tree of EIL genes from *Q. rubra*, *Q. robur* and *P. trichocarpa*. All *Quercus* genes were found using the temperature responsive EIL genes of *P. trichocarpa* (labeled orange). Bootstrap values are written above each branch.

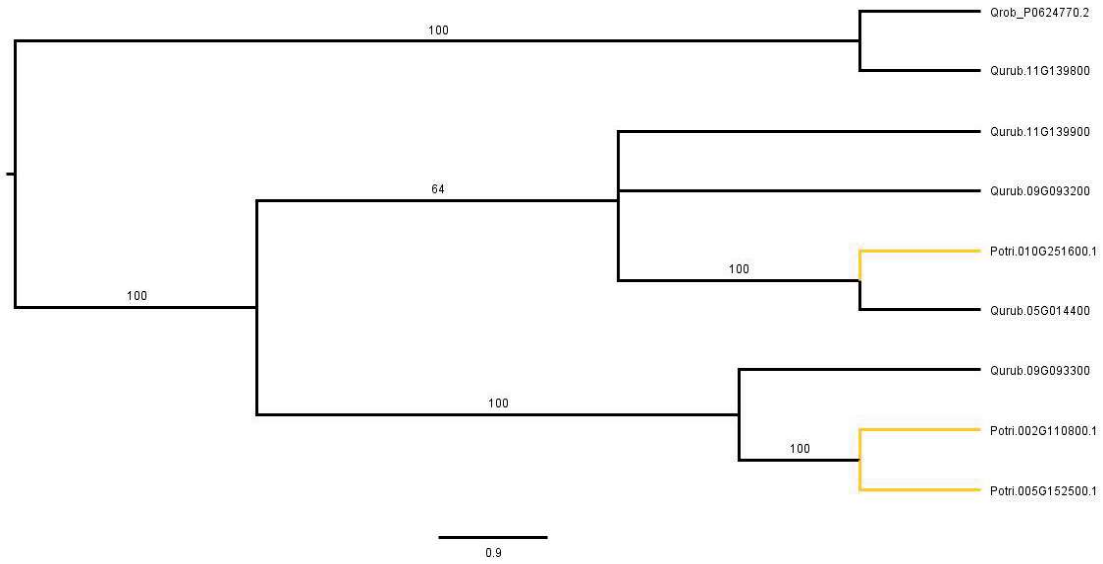
3.6.6 Figure 6



A phylogenetic tree of ERF gene from *Q. rubra*, *Q. robur* and *P. trichocarpa*. All *Quercus* genes were found using the temperature, JA, and water responsive ERF genes of *P. trichocarpa*: their branches are red, orange and yellow colors, respectively. Four clades (A, B, C, and D) are mostly populated by ERF homolog clusters from *Q. rubra* (see Table 2): these have been highlighted yellow and orange, respectively. Clade A contains all constituents of the ERF.6.b cluster. Clade B contains all constituents of the ERF.hom.9.c

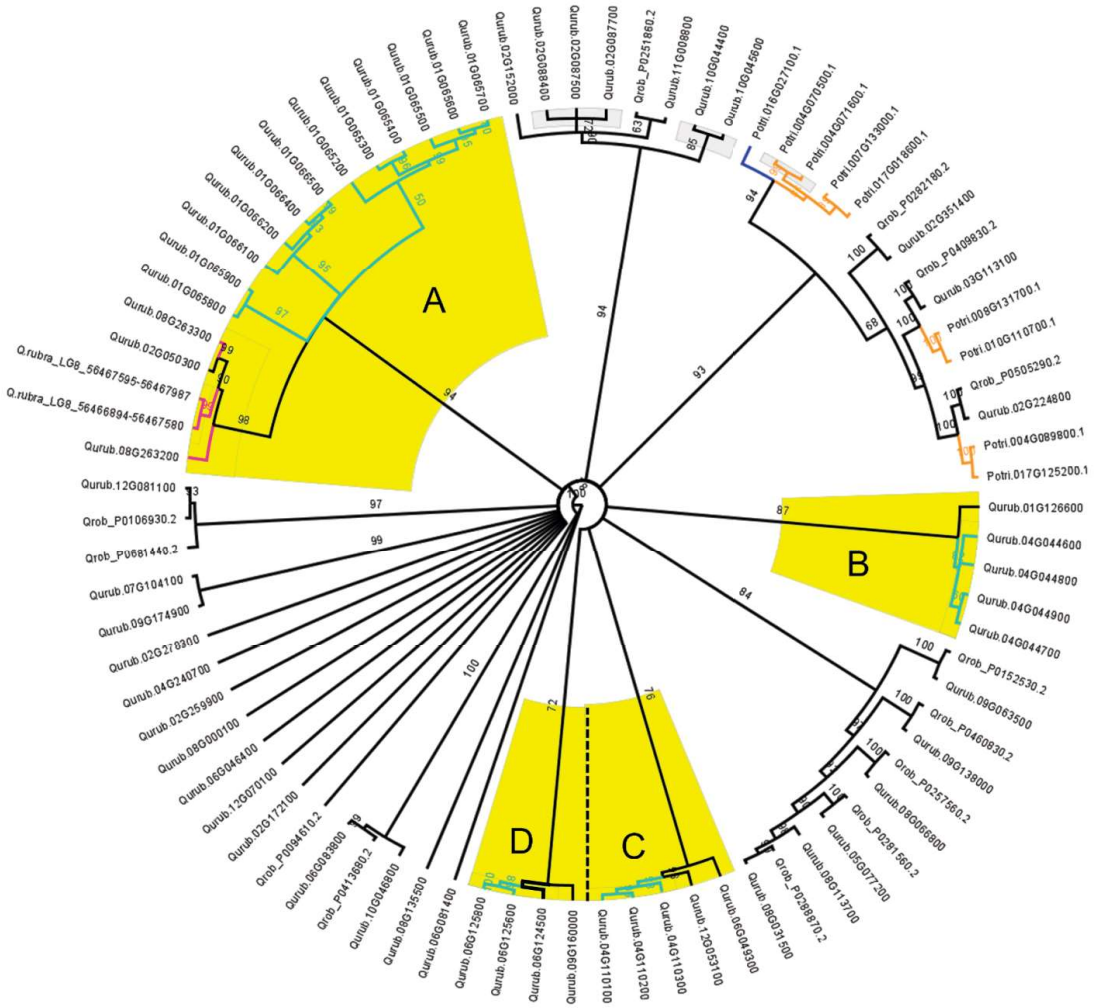
and ERF.hom.12.a homologs: since both clades are on separate branches, these homologous groups are both highlighted teal. Clade C contains all constituents of the ERF.4 cluster: their branches are teal color. Clade D is highlighted orange and contains three sub clades (i, ii, and iii) that are highlighted yellow. Subclade i contains all constituents of the ERF.6.a and ERF.8.b clusters and 4 of the 7 constituents of ERF.9.a: their branches are colored teal, dark blue, and pink, respectively. Subclade ii contains all the constituents of the ERF.1. cluster. Subclade iii contains all the constituents of the ERF.8.a cluster and the 3 of the 7 constituents of ERF.9.a: their branches are colored teal and pink, respectively. Bootstrap values are written next to each branch. Highlighted branches are discussed in results and discussion.

3.6.7 Figure 7



A phylogenetic tree of GATA genes from *Q. rubra*, *Q. robur* and *P. trichocarpa*. All *Quercus* genes were found using the temperature responsive GATA genes of *P. trichocarpa* (labeled orange). Bootstrap values are written next to each branch.

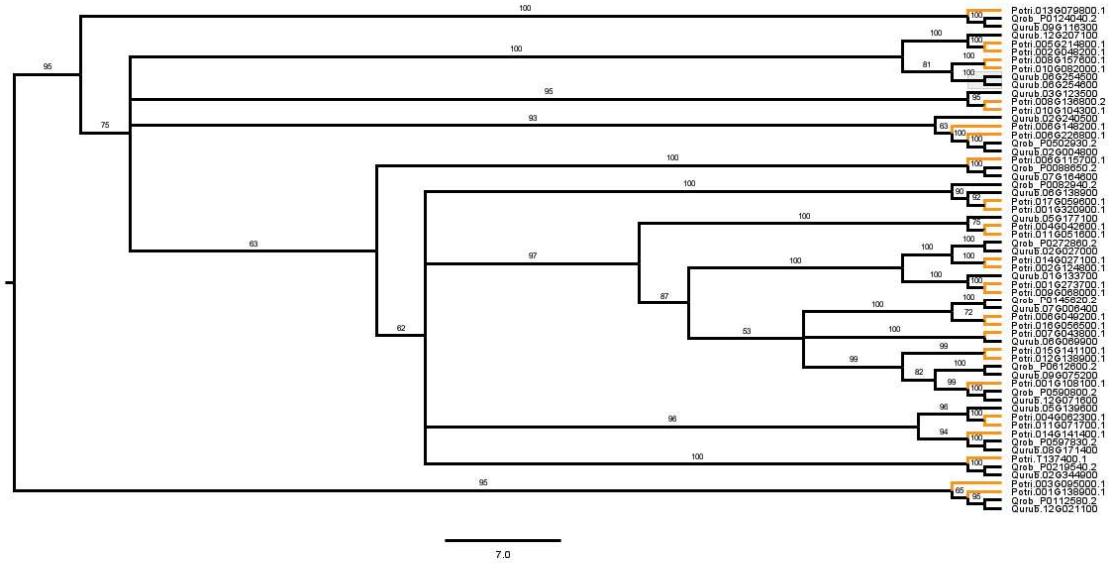
3.6.8 Figure 8



A phylogenetic tree of GRAS genes from *Q. rubra*, *Q. robur* and *P. trichocarpa*. All *Quercus* genes were found using the salinity and JA/salinity responsive GRAS genes of *P. trichocarpa* (labeled blue and orange, respectively). Four clades (A, B, C, and D) are constituted predominantly by GRAS homolog clusters or homolog sets from *Q. rubra* (see Table 7): these have been highlighted yellow. Clade A contains all constituents of the GRAS.1 and GRAS.8 clusters: the branches to these homologs have been colored teal and pink, respectively. Clade B contains all constituents of the GRAS.4.a cluster: these

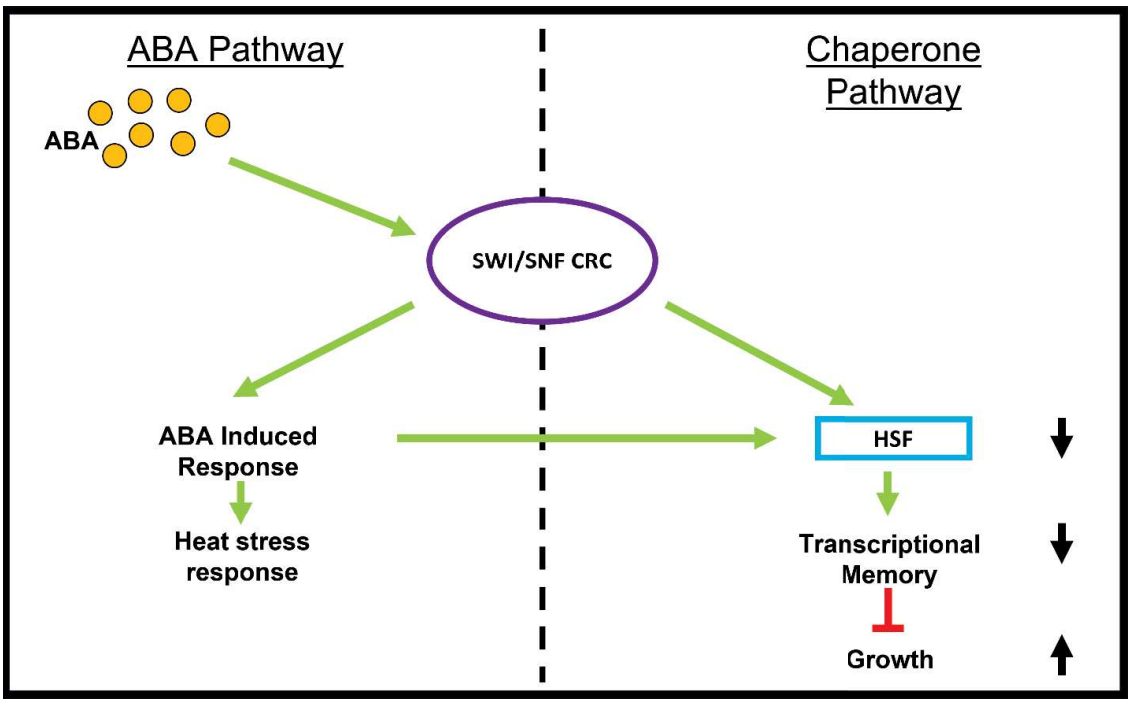
homologs have been colored teal. Clade C contains all the constituents of GRAS.4.b: these homologs have been colored teal. Clade D contains all constituents of GRAS.hom.6.a: these homologs have been colored teal.

3.6.9 Figure 9



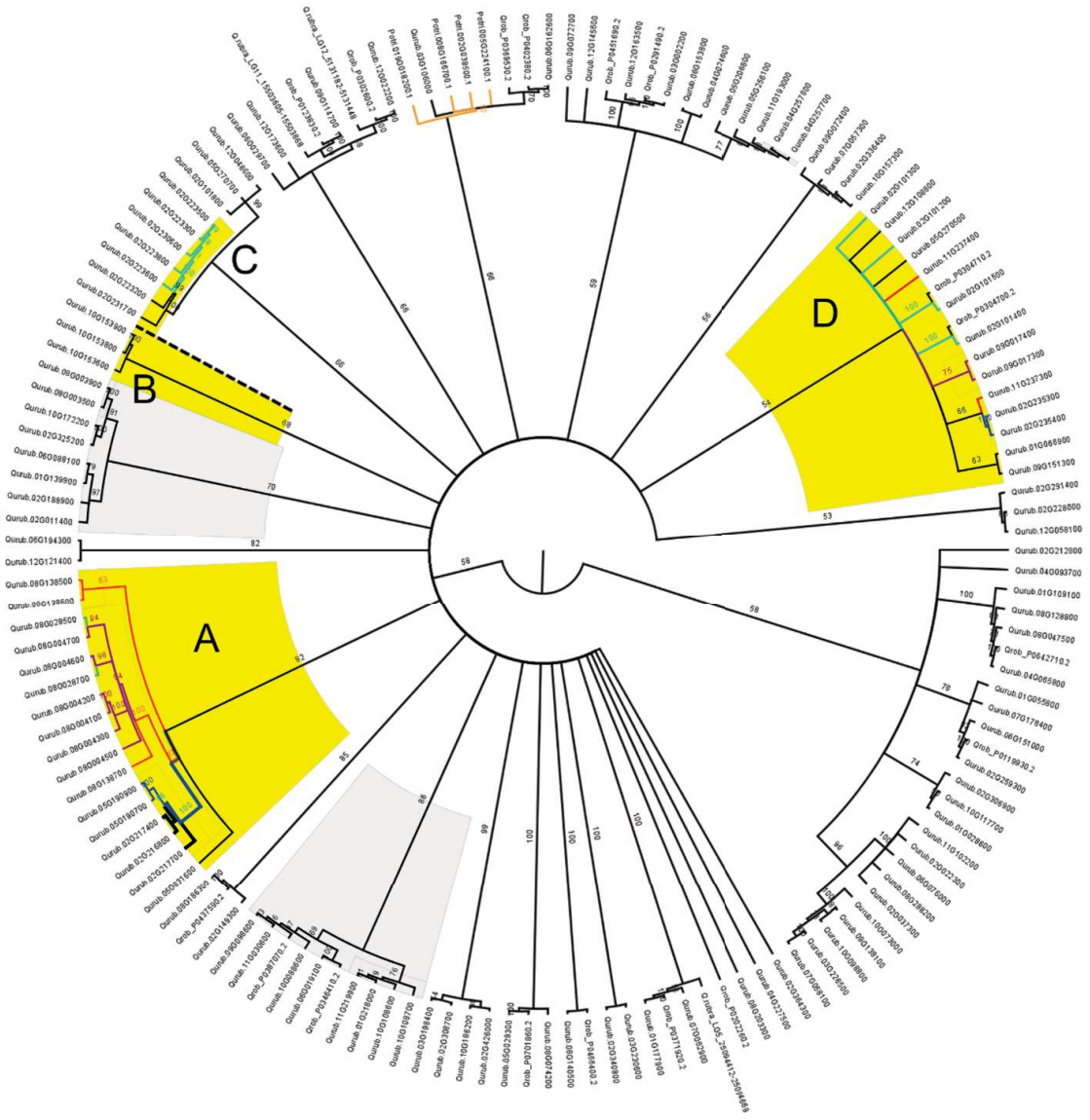
A phylogenetic tree of HSF genes from *Q. rubra*, *Q. robur* and *P. trichocarpa*. All *Quercus* genes were found using the salinity and temperature responsive HSF genes of *P. trichocarpa* (labeled orange). Bootstrap values are written next to each branch.

3.6.10 Figure 10



The ABA and chaperone pathways have been seen to elicit plant responses to temperature stresses. The ABA pathway ultimately induces genes that elicit an instant response. This is quite different from the effect of the chaperone pathway: here transcription of HSF genes facilitates a transcriptional memory response that ultimately reduces growth (Bulgakov et al., 2019) Intriguingly we found a large reduction in HSF homologs in both *Q. robur* and *Q. rubra*. This result could indicate that *Quercus* temperature response is less reliant on transcriptional memory and favors an ABA induced response. Abbreviations: ABA – abscisic acid, HSF – heat shock factor, SWI/SNF CRC - chromatin remodeling complex (CRC).

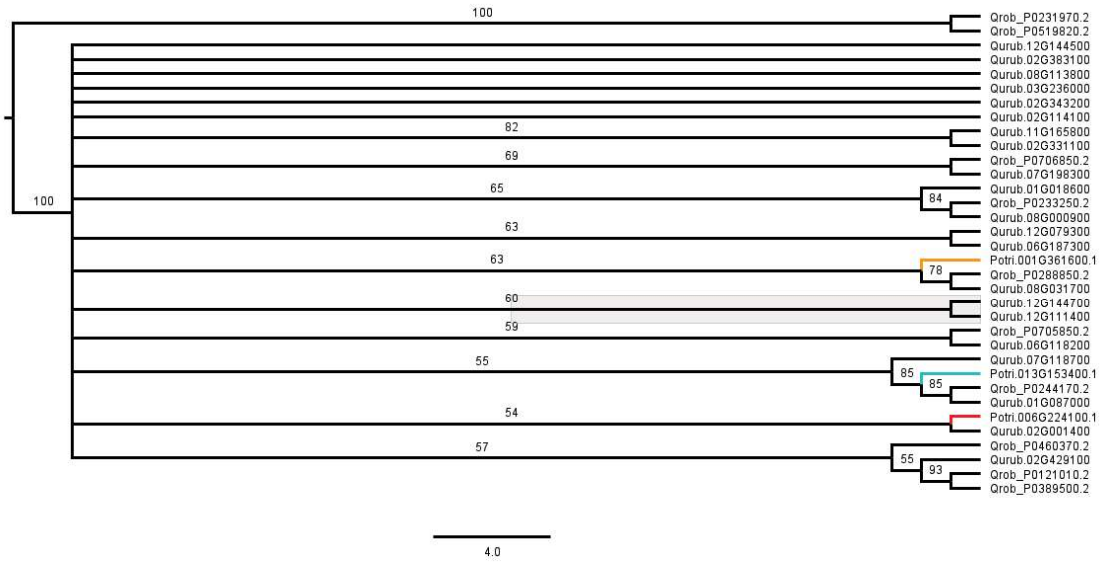
3.6.11 Figure 11



A phylogenetic tree of MYB genes from *Q. rubra*, *Q. robur* and *P. trichocarpa*. All *Quercus* genes were found using the temperature responsive MYB genes of *P. trichocarpa* (these have been labeled orange). Four clades (A, B, C, and D) are constituted predominantly by MYB homolog clusters from *Q. rubra* (see table 2): these have been highlighted yellow. Clade A contains all constituents of the MYB.8.b cluster and the

MYB.hom.5.a and MYB.hom.8.a homologs: the branches of these homologs have been labeled red, blue and green. Clade A also contains 6 of the 8 constituents of MYB.8.a cluster: the branches of these homologs have been labeled purple. Clade B contains all constituents of MYB.10. Clade C contains all constituents of the MYB.2.b cluster: the branches of these homologs have been labeled teal. Clade D contains all constituents of the MYB.hom.2.b, MYB.hom.9.a, MYB.hom.11 homologs: the branches of these homologs have been colored blue, purple, and red, respectively. Clade A also contains 4 of the 5 constituents of the MYB.2.a cluster: these branches have been colored teal. Bootstrap values are written next to each branch.

3.6.12 Figure 12



A phylogenetic tree of WRKY genes from *Q. rubra*, *Q. robur* and *P. trichocarpa*. All *Quercus* genes were found using the drought, salinity/temperature, and JA responsive WRKY gene of *P. trichocarpa* (these have been respectively labeled orange, blue, and red). Bootstrap values are written next to each branch.

3.7 Chapter 3 tables

3.7.1 Table 1

BLAST search results for drought, temperature, water, salinity, and JA response TF within *Quercus*. Our query was conducted using *P. trichocarpa* gene with differential expression in response to the listed stimuli. The search was conducted in the *Q. robur* and *Q. rubra* genomes. The initial alignment row indicates how many peptide sequences were aligned in the phylogenetic analysis. In this analysis, we found that some of the *Quercus* peptide sequences were too short or did not have a matching alignment, these sequences were removed from the analysis. The final alignment row indicates how many peptide sequences were kept for the phylogenetic analysis. The last two column indicate the number of annotated and unannotated peptide sequences from *Q. rubra* that were used for the phylogenetic analysis. Only annotated gene were used for *Q. robur*.

	bHLH	C2H2	DBB	EIL	ERF	GATA	GRAS	HSF	MYB	WRKY
Initial alignment	**35**	**30**	**19**	**17**	**213**	**9**	**94**	**79**	**150**	**38**
P. trichocarpa	6	5	2	5	20	3	9	30	4	3
Q. robur	12	7	6	3	35	1	19	25	17	13
Q. rubra	17	18	11	9	158	5	66	3	129	22
Final alignment	**32**	**27**	**17**	**16**	**185**	**9**	**83**	**69**	**149**	**34**
P. trichocarpa	6	5	2	5	20	3	9	30	4	3
Q. robur	10	6	4	3	30	1	13	18	17	10
Q. rubra	16	16	11	8	135	5	61	21	128	21
Q. rubra annotated	10	16	11	5	131	5	59	21	125	21
Q. rubra unannotated	6	0	0	3	4	0	2	0	3	0

3.7.2 Table 2

Q. rubra bHLH, C2H2, ERF, GRAS, and MYB TF clusters. TF cluster names can have three parts: TF gene family, a number indicating *Q. rubra* chromosome, and a letter

indicating if there is more than one TF cluster within a given chromosome (when necessary). The "start" and "stop" columns indicate the range in nuceleotide base pairs (bp) that each TF cluster spans. The length of each cluster is listed in kilo-base pairs (Kbp). The average distance between *Q. rubra* TF homologs within the cluster is listed in the "mean spacer distance" column, these distances are measured in Kbp. The stress responses of the homologous *P. trichocarpa* genes are listed in the corresponding column. The homologous sequence column indicates how many *Q. rubra* homologous TF genetic sequences are within the TF cluster.

Cluster names	Start (bp)	Stop (bp)	Length (kbp)	Mean spacer distance (kbp)	Stress response	Homologous sequences
bHLH.11	40227634	40256399	28.8	13.7	JA	3
C2H2.7	41656389	41676161	19.8	6.1	Temp	4
ERF.1	37320558	37497770	177.2	58.3	Temp	4
ERF.4	18617475	18716081	98.6	32.5	Temp|JA	4
ERF.6.a	8614465	8678930	64.5	15.7	Temp	5
ERF.6.b	11431432	11769790	338.4	42.1	Temp|JA|Water	2 of 9
					Temp|JA	6 of 9
					Temp|Water	1 of 9
ERF.6.c	34740235	34797904	57.7	28.6	Temp|JA	3
ERF.8.a	47620085	47741684	121.6	60.1	Temp	3
ERF.8.b	52311183	52391617	80.4	12.7	Temp	7
ERF.9.a	13102640	13245423	142.8	27.9	Temp	5 of 6
					Temp|JA	1 of 6
ERF.9.b	25802106	25852536	50.4	24.9	Temp|JA	3
GRAS.1	20899111	21144655	245.5	21.2	Salt|JA	12
GRAS.4.a	8894716	8920937	26.2	7.7	Salt|JA	4
GRAS.4.b	25146497	25210551	64.1	30.8	Salt|JA	3
GRAS.8	56456902	56501458	44.6	13.7	Salt|JA	4
MYB.2.a	16536876	16642867	106.0	26.2	Temp	5
MYB.2.b	48465893	48568010	102.1	50.8	Temp	3
MYB.8.a	549898	865021	315.1	44.8	Temp	8
MYB.8.b	34180500	34271689	91.2	45.1	Temp	3
MYB.10	41825296	41897185	71.9	35.7	Temp	3

4 Chapter 4: *Quercus ellipsoidalis* and *Quercus rubra* have similar leaf trait phenotypic plasticity

4.1 Abstract

Species within genus *Quercus* have been found to have a high degree of variability across leaf traits. Previous experiments have shown that leaf trait variability is a related to species identity and hybridization, in addition to phenotypic plasticity. *Quercus ellipsoidalis* (northern pin oak) and *Q. rubra* (northern red oak) are recently diverged, closely related species, that have overlapping geographic ranges within the northern Midwest region of America. Both species have unique environmental preferences – xeric and mesic sites, respectively – as well as unique leaf morphologies. In this experiment, phenotypic plasticity for physiological and morphological leaf traits was quantified to determine if plasticity differs between the species *Q. ellipsoidalis* and *Q. rubra*. For this experiment, I developed two working hypotheses. H1: independent variables species, site, and interactive effects between these independents would have significant effect on leaf trait variability. H2: *Q. rubra*, the species with greater geographical range that preferentially grows within mesic sites, would have greater leaf trait phenotypic plasticity. For each of these species, three populations from similar seed source climates were planted in common gardens within the Ford and Kellogg experimental forests: these common gardens are found in Michigan's upper and lower peninsula, respectively. Both hypotheses were not supported by the results of the analyses: an interactive effect between species and site was not observed to have a significant effect on leaf trait variation, and both species had similar leaf trait phenotypic plasticities that were not statistically significant. Within this experiment, physiological leaf traits and LMA were seen to vary significantly in response to the different common garden sites. Our results also found that morphological

leaf traits of both species can be used to distinguish between both species when planted in different common gardens. This study demonstrates that morphological leaf trait variation between *Q. ellipsoidalis* and *Q. rubra* is related to species genotype, while physiological leaf trait variation for these species is responsive to environmental settings. Moreover, the phenotypic plasticity for physiological leaf traits appears to be equal for both species. To our knowledge, our study is the first to make these associations between leaf physiology and morphology for *Q. ellipsoidalis* and *Q. rubra*.

4.2 Introduction

Early taxonomists working with species in *Quercus* section *Lobatae* (red oaks) frequently encountered a contradiction: leaves are simultaneously helpful and misleading for species identification (Hickey et al., 1975; Jensen, 1977a; Jensen et al., 1984). Palmer (1942) stated that red oak leaf traits were "extremely variable" but "hold good for typical" leaf material, while Palmer's contemporary described leaf size as "too variable" but leaf shape as "trustworthy" for red oak species identification (Muller, 1942). More recently, Kirchoff et al. (2011) notes that creating dichotomous keys for *Quercus* species in the United States is an incredibly difficult task, and others have expressed skepticism that morphological leaf character based on landmarks can even be used for studying species in *Quercus* (Graham et al., 2010). Within the literature addressing *Quercus* leaf variability, two alternative hypotheses emerge: (1) leaf variability is primarily caused by hybridization and introgression among a complex of closely related red oak species (Abadie et al., 2012; Curtu et al., 2007; Gailing et al., 2012; Owusu et al., 2015; Valencia-Cuevas et al., 2015), and (2) leaf variability primarily reflects the phenotypic plasticity of a species in response to environment (Bresson et al., 2011; Kusi et al., 2020; Viscosi, 2015; Vitasse et al., 2010).

The first hypothesis, that oak variability is driven by genetics, has been tested extensively using a set of red oaks native to the northeastern and Midwest regions of North America: the species in question included *Q. coccinea* (scarlet oak), *Q. velutina* (black oak), *Q. palustris* (pin oak), *Q. rubra* (northern red oak, abbreviation Qr), and *Q. ellipsoidalis* (northern pin oak, abbreviation Qe). The interest in this set of species can be traced back to a hypothesis regarding the species status of Qe. Before the advent of genetic experimentation, Overlease (1964) hypothesized that Qe was a northern variant of *Q. coccinea* that emerged through hybridization and backcrossing between *Q. coccinea* with *Q. velutina* and *Q. palustris*. Their argument is based on leaf trait similarities: Qe, *Q. coccinea* and *Q. palustris* have deep c shaped leaf sinuses, and similar patterns in leaf lobing are observed in Qe, *Q. velutina* and *Q. palustris* (Stein, 2003). This went against the historical classification of Qe (Trelease, 1924) and subsequent publications tested the Overlease (1964) hypothesis (Jensen, 1977a). By measuring leaf traits, Jensen developed a hypothesis wherein Qe is a unique species taxon that originated from a hybrid swarm among species *Q. coccinea*, *Q. velutina*, *Q. palustris*, and Qr (Jensen, 1977a, 1977b; Jensen et al., 1984). Subsequent publications tested these hypothesis using genetic microsatellite markers; their results largely support the Jensen et al. (1984) hypothesis. Using amplified fragment length polymorphism genetic markers Hipp et al. (2008) found strong evidence to support the classification of Qe as a distinctive species from *Q. coccinea*. Moreover, this publication found strong evidence that demonstrates introgression between Qe and other red oak species (Hipp et al., 2008). These results are reiterated by other genetic molecular marker studies too. Qe is found to have high interspecific gene flow with *Q. velutina* and Qr, moderate gene flow with *Q. palustris*, and low gene flow with *Q. coccinea* (Hipp et al.,

2008; Owusu et al., 2015; Sullivan et al., 2013). Within this complex of *Lobatae* species, scientists observed a trend common within the genus *Quercus*; species level variation within leaf traits is in part caused by weakened boundaries between closely related species (Curtu et al., 2007, 2009; Owusu et al., 2015; Valencia, 2010). Moreover, variation in leaf morphology for species in *Quercus* is related introgression and hybridization with closely related species (Jensen et al., 1993; Owusu et al., 2015; Sullivan et al., 2016).

Leaf trait variability for species of *Quercus* is also explained by phenotypic plasticity. Phenotypic plasticity is the capacity of a single genotype to display multiple phenotypes in response to different environmental inputs (West-Eberhard, 2008). In plants, phenotypic plasticity is described by reaction norms for morphological and physiological traits in response to a range of environmental settings (Schlichting, 1986; West-Eberhard, 2008). For plants, phenotypic plasticity is important for an individual's survival. Unlike animals, which can migrate or seek shelter in response to selective pressures, plants are sessile and must endure stress to survive (Bradshaw, 1965, 1972; Schlichting, 1986; Viscosi, 2015). In *Quercus*, leaf traits have been observed to be plastic in response to site irradiance (Balaguer et al., 2001; Batos et al., 2010; Kusi, 2013; Sack et al., 2006), temperature (Vitasse et al., 2010; Vitasse et al., 2009), water availability (Bresson et al., 2011; Dickson et al., 1996), and nutrient availability (Valladares, Martinez-Ferri, et al., 2000). The importance of phenotypic plasticity to *Quercus* leaf variability is also supported by a study measuring genetic variation between Qe and Qr populations from northern Michigan. Despite observing variability in leaf morphologies within species and populations, Gailing et al. (2012) found that hybridization and introgression between Qe and Qr occurred at a low frequency. They concluded that variation of leaf morphology for

both species was largely caused by variations in environmental conditions at each site, which demonstrate the importance of phenotypic plasticity (Gailing et al., 2012).

The topic of phenotypic plasticity is important to understand within the context of climate change. Across the world, climate change has increased annual temperature and aridity (IPCC, 2014), and this has altered seasonal precipitation patterns considerably (Dai, 2011; Jentsch et al., 2007; Pachauri et al., 2014). Forested ecosystems now experience events of heavy precipitation, heatwaves, and periods of protracted drought at greater frequency (Dai, 2011; Jentsch et al., 2007; Min et al., 2011; Pachauri et al., 2014). As the selection pressures associated with climate change continue to increase, scientists have started considering the benefits and drawbacks of phenotypic plasticity. While an individual within a population can benefit from adaptive plasticity (Vitasse et al., 2010) – a form of phenotypic plasticity where a trait becomes optimized to the environment – adaptive plasticity also diminishes the selection pressures on populations (Fox et al., 2019). In this situation, the current populations' allelic frequencies remain fixed, and the populations' future generations have the same fitness level under growing selective pressure (Fox et al., 2019).

In response to climate change, systems can exhibit resilience, resistance, and/or stability. A resilient system is one that maintains its original function after absorbing a disturbance (Holling, 1973; Walker et al., 2012). Resistant and stable systems are ones that after absorbing a disturbance remain unchanged and persist, respectively (Holling, 1973). Although adaptive plasticity makes current populations stable and resistant to climate

change, future generations are less resilient because allelic frequencies have not changed in response to natural selection (Fox et al., 2019).

But population persistence buys time for artificially increasing biodiversity with assisted migration. In population assisted migration, individuals from southern ecotypes are often intentionally planted into northern populations (Etterson et al., 2020; Hannah et al., 2002; Minteer et al., 2010). Those that favor population assisted migration argue that the southern genotypes are under selective pressures that promote adaptations for dealing with more extreme climate conditions (ex. increased temperature and water restriction) (Pedlar et al., 2012; Vitt et al., 2010), and their introduction into northern populations can increase climate change resilience (Reusch et al., 2005). Thus, identifying which traits have adaptive phenotypic plasticity can inform assisted migration to select individuals that will promote the population's resilience to climate change.

The present study was conducted to investigate phenotypic plasticity specific to Qe and Qr leaf traits. Northern populations of both species were planted in two common gardens located in Michigan's upper and lower peninsulas. The northern common garden has ecological conditions like the Qe/Qr population's seed source; in contrast, conditions at the southern common garden are warmer and drier. The *Quercus* taxon is both environmentally and economically significant within North American forests, and it is important to understand how these species will respond to the warming climate (McShea et al., 2007; Rauschendorfer et al., 2022). Although there is commonality between the geographical ranges of Qe and Qr, these species preferentially grow in xeric and mesic environments, respectively (Abrams, 1990). Moreover, Qr has an extensively larger range

than Qe. The northern and southern geographical range of Qe span from northern Minnesota into central Indiana, whereas the Qr geographical range spans from Quebec into central Louisiana. The eastern and western geographical range of Qe span from Michigan into eastern North Dakota, whereas the Qr geographical range spans from the East coast into central Kansas (Little, 1979; Stein, 2003). We measured leaf traits of Qe and Qr from common gardens to test the following hypotheses: (1) leaf trait phenotypes differ in mean and variance in response to common garden site, species, and between these independent variables. Evidence for this hypothesis is based on changes in leaf traits linked to environmental effects (Balaguer et al., 2001; Valladares, Martinez-Ferri, et al., 2000), and quantifiable differences in Qe and Qr leaf traits (Gailing et al., 2012; Jensen et al., 1993). (2) I expected Qr, a mesic species, to be more phenotypically plastic than Qe, a species that preferentially grows in xeric site. This hypothesis is based on the specialization hypothesis, which argues that plants from harsher environments become adapted to environmental extremes by selection pressure (Lortie et al., 1996). Moreover, plants from less adverse environments are more plastic because selective pressure is lowered (Lortie et al., 1996; Sultan, 1995; Valladares, Martinez-Ferri, et al., 2000; Valladares, Wright, et al., 2000).

4.3 Methods

4.3.1 Population selection and common garden study sites

In the Fall of 2018, I identified three populations each of Qe and Qr with similar latitudinal ranges and mean annual temperatures (Table 1). In this experiment, a population was defined as a stand of oak trees dominated by a single species. Acorns were collected from the area beneath isolated maternal tree's crowns. Maternal trees were considered "isolated" when their crowns did not overlap with the crown of another oak tree. The

collected acorns were cold-stratified at 4 ℃ for 4 months, and propagated in the MTU greenhouse. The seedlings from these populations were planted in a common garden site located at two common garden sites. The Kellogg common garden site is in Lower Michigan, located at Augusta, Mi (42.37 N, -85.36 W); the Ford common garden site is in Upper Peninsula Michigan, in Alberta (46.64 N, -88.48 W). In this experiment, the selected populations have seed source climate conditions that are similar to the Ford site. Each garden consists of 27 blocks each housing 30 experimental plants from 4 and 22 Qe and Qr populations, respectively. A seedlings placement within the common gardens was selected using a semi-randomized block design (Ariel et al., 2010).

Leaf trait measurements were collected from oak saplings residing in the Ford and Kellogg *Lobatae* common gardens on August 11-12 and 16-17, 2021, respectively. During the 2021 growing season, in the months leading up to the leaf trait sampling (May-August), the environmental conditions of the Ford and Kellogg site were quite unique. Using data available from the TerraClim database (Abatzoglou et al., 2018), average measurements for site irradiance, maximum temperature, precipitation and palmer drought severity index (PSDI) were collected for months May-August. Average measurements for irradiance, max temperature and PSDI for the Ford site were 230.4 W m-2, 23.2 ℃, and 0.45 (normal/wet site conditions). The Ford site received 407 mm of precipitation in this time. Average measurements for irradiance, max temperature and PSDI for the Kellogg site were 231.7 W m^{-2}, 26.7 ℃, and -2.82 (moderate/severe drought site conditions). The Kellogg site received 401 mm of precipitation in this time. According to the NRCS soil survey for 2021, the soil profiles for the Ford and Kellogg site are loam and coarse sandy loam, respectively (Post et al., 2007). Differences in canopy openness at each site are also observed. The Ford

site is also located within an open field outside of the range of forest canopy, here the trees at this site receive direct sunlight. The east and west boarders of the Kellogg site common garden are shaded by mature forest trees, at this site the trees have greater restrictions to direct sunlight.

4.3.2 Leaf sampling and trait measurements

In this experiment, a set of 22 traits were measured using a single leaf from each of the selected seedlings. The minimum sample number for each population at each site was 5. More information on each populations sample size is listed in Table 1. At the Ford site, Qe and Qr sample sizes were 23 and 27 seedlings, respectively. At the Kellogg site, Qe and Qr sample sizes were 30 and 33, respectively. Qe and Qr sample sizes are 53 and 60 seedlings, respectively (Table 1).

The leaves selected for measurement were the youngest, fully-expanded leaf possessing the lease amount of environmental damage (e.g. insect herbivory, disease, sun damage). The measured traits can be characterized as morphological and physiological leaf traits. A brief description of each trait, including trait type, short definitions, units, and the abbreviations used throughout the text is found in Table 2.

Of the 22 traits, 19 are morphological. These traits include leaf area, leaf blade length (LBL), interval between center vein intervals (center), interval between apical vein intervals (apex), leaf blade width basal lobe (LBWB), leaf blade width middle lobe (LBWM), leaf blade width apical lobe (LBWA), interval between basal sinus pair (INTB), interval between middle sinus pair (INTM), interval between apical sinus pair (INTA), basal lobe leaf angle (angle 1, ang1), middle lobe leaf angle (angle 2, ang2), apical lobe

leaf angle (angle 3, ang3), number of bristle tips (NBT), ratio between INTB and INTM (INTB/INTM), ratio between LBL and INTM (LBL/INTM), ratio between LBL and apex (LBL/apex), ratio between LBWM and INTM (LBWM/INTM), and leaf mass per unit area (LMA) (Table 2).

Traits 1-13 were measured using tools in ImageJ (Schneider et al., 2012). Traits 1-10 required setting a distance ratio between a known distance (set using a ruler) and number of pixels in a photograph. Area (trait 1) is a measurement of the leaf blades surface area. Traits 2-13 have been traditionally used to characterize differences between oak species Qe and Qr (Gailing et al., 2012; Jensen et al., 1993). The leaf landmarks used to measure traits 2-13 are shown in Figure 1. NBT (trait 14) is a count of the number of bristle tips on a single leaf. Bristle tips are defined as stiff hairs at the ends of leaf tips. This trait has also been traditionally used to characterize differences between red oak species (Gailing et al., 2012; Jensen et al., 1993). Traits describing ratios between two morphological trait measurements (traits 15-18) were calculated after the direct measurements were taken, These traits were shown by Gailing et al. (2012) to be effective for characterizing Qe and Qr.

Leaf mass per area (LMA, g m^{-2}, trait 19) is a morphological trait strongly associated with photosynthesis, respiration, site conditions, and many physiological traits (de la Riva et al., 2016). LMA is associated with many traits because its measurement is dependent on leaf thickness and density (Coble et al., 2017), or leaf surface area and dry weigh (Reich et al., 1992) and these variables are independently responsive to different environmental factors (Coble et al., 2017; Niinemets, 1999). Dry mass was measured after

the leaf had been oven dried for 48 hours at 70 ℃ using a scientific scale. Leaf area was measured as described above.

The physiological leaf traits included photosynthetic capacity (A_{max}), chlorophyll content (CCI) and water content (WC%). Both A_{max} and CCI (traits 20 and 21) were measured while the leaf was still attached to the plant's stem. A_{max} was measured using an LI-6800 infared gas analyser (LI-COR, Inc, Lincoln, NE). Chamber settings were 400 ppm CO_2, 400 µmol m^{-2} s^{-1} flow rate, 25 °C block temperature, a saturating rate of photosynthetic photon flux density of 800 µmol m^{-2} s^{-1}, and vapor pressure deficit between 1-2 kPa when possible (max 3 kPa). The trait chlorophyll content index (CCI) was measured for each leaf using the CCM200-Plus Leaf Chlorophyll Meter (Opti-Sciences, Inc, Hudson, NH). CCI is a measurement of the ratio of the percent of light transmission at 931 nm and 653 nm (Parry et al., 2014). Percent water content (WC%, trait 22) was measured using the following formula:

$$WC\% = 100 \times \left(\frac{FW-DW}{FW}\right) \quad \text{Eq.1}$$

where FW is leaf fresh weight and DW is leaf dry weight. To preserve the leaf's water after harvesting, leaves were stored in a moist plastic bag and kept in the dark immediately after harvesting. Fresh mass was measured using a scientific scale within 12 hours of harvesting. Dry mass was measured as described above.

4.3.3 Phenotypic plasticity

For both species, the phenotypic plasticity for a given leaf trait was calculated as a coefficient of variation percentage (CV%) (Valladares et al., 2006). CV% was calculated using the following formula:

$$CV\% = 100 \times \left(\frac{\sigma pooled}{\bar{x}pooled}\right) \quad \text{Eq.2}$$

where σ_{pooled} and $\bar{x}_{pooled}$ are the pooled standard deviation and pooled mean for a specific trait of a single species, respectively. In this experiment, the term "pooled" means that trait measurements from the Kellogg and Ford sites for a species were grouped together. To determine the overall leaf trait phenotypic plasticity for a species, the mean phenotypic plasticity across all traits was calculated ($\bar{x}_{CV\%}$).

4.3.4 Statistical tests

Principal component analysis (PCA) (Holland, 2008) was conducted using the prcomp function within R. All trait measurements were used for this PCA. PCA was conducted to determine how samples clustered in relation to common garden site and species. The contributions of each trait to principal components one and two, the loading variable components, were calculated to determine which traits had the greatest influence on the PCA data distribution.

Two-way ANOVA was conducted for all measured traits to determine if variation in trait was in response to independent variables site, species or an interactive effect between site and species (Fujikoshi, 1993) P-values of less than 0.05 were considered statistically significant (Gallagher, 1999). Two-way ANOVAs were conducted in R.

To identify which traits were significantly different between species, pooled means ($\bar{x}_{pooled}$) for each leaf trait for both species were compared using an appropriate t-test. Since sample sizes were unequal, a Welch's or Student's t-test was used (Sakai, 2016). If variance was unequal between pooled samples, Welch's t-test was used (Ruxton, 2006). If variance was equal between pooled samples, Student's t-test was used (Owen, 1965). Variances for each sample set were assessed using an F-test (Tiku, 1967). P-values were considered statistically significant when below a 0.05 critical value (Gallagher, 1999). Statistically significant p-values signify that a leaf trait can be used to distinguish between Qe and Qr. F-tests and T-tests were conducted using R software.

For Qe and Qr, the trait means at both the Ford and Kellogg site were compared using an appropriate T-test. This comparison allowed us to identify the traits that changed significantly in response to environmental conditions for both species. Qe and Qr sample size at the Ford site were 23 and 27, respectively. Qe and Qr sample sizes at the Kellogg site were 30 and 33, respectively (Table 1). Depending on the variance between Ford and Kellogg samples, a Welch's or Student's test was used in the manner described above. Statistically significant p-values signify that a leaf trait is phenotypically plastic within a species.

Overall leaf trait phenotypic plasticity, $\bar{x}_{CV\%}$, for Qe and Qr was compared to determine if one species was more phenotypically plastic. The $\bar{x}_{CV\%}$ for Qe and Qr was compared using a paired t-test (Xu et al., 2017). Results from the t-test were considered statistically significant when p-values were less than 0.05 (Gallagher, 1999).

4.4 Results

4.4.1 Morphological leaf traits distinguish Qe and Qr

Morphological leaf traits can be used to distinguish between Qe and Qr. Using the pooled means for both species leaf traits in t-test comparisons, 11 of the 19 morphological traits were found to be statistically significant between Qe and Qr (Table 4). These 11 statistically significant traits include area, LBL, LBWB, INTB, INTM, INTA, ang2, NBT, INTB/INTM, LBL/INTM, and LBWM/INTM. Pooled means for the significant traits related to leaf size – area, LBL, LBWB, INTB, INTM and INTA – were greater in Qr. The INTB/INTM ratio was also greater in Qr. The other significant leaf trait ratios – LBL/INTM and LBWM/INTM – were greater in Qe. The NBT count was also higher in Qe (Table 3 and Table 4). Pooled means for the physiological leaf traits were not statistically significant between Qe and Qr (Table 3 and Table 4).

4.4.2 Phenotypic plasticity related to physiological leaf traits in both Qe and Qr

To identify the growing site effect on traits, I compared the Ford and Kellogg sites means using an appropriate statistical t-test. For both species most leaf traits did not have a statistically significant difference between the Ford and Kellogg. Within Qe, I observed a statistically significant difference between leaf traits ang1, LMA, and CCI. For Qe, each of the traits was greatest at the Ford site (Table 3 and Table 4). Within Qr, statistically significant differences were observed between leaf traits center, LMA, CCI and WC%. Both center and WC% were greatest at the Kellogg site. Traits LMA and CCI were greatest at the Ford site (Table 3 and Table 4).

Although not always statistically significant, interesting trends between the Ford and Kellogg sites were observed for LMA and the physiological leaf traits for both species. LMA and CCI were greatest at the Ford site. A_{max} and WC% were greatest at the Kellogg site. Site means for LMA, CCI and WC% were very similar for both species (Table 3). Moreover, both species had similar degrees of phenotypic plasticity for the LMA, A_{max}, CCI and WC% leaf traits. In Qe, phenotypic plasticity (expressed as CV%) for these traits was 22.40 %, 39.07 %, 34.65 % and 6.19 %, respectively (Table 5). In Qr, phenotypic plasticity for these traits was 21.91 %, 38.48 %, 34.71 % and 6.51 %, respectively (Table 5).

4.4.3 Mean phenotypic plasticity for leaf traits is similar between Qe and Qr

The mean CV% for Qe and Qr was used to determine the overall phenotypic plasticity. Both species had similar CV% means. The values for Qe and Qr were reported at 29.60 % and 29.03 %, respectively (Table 5). Using a paired t-test, these results were not observed to be statistically significant (Table 5).

4.4.4 Species and site do not have significant interactive effects influencing leaf trait variability

Two-way ANOVA was used to compare differences between leaf trait means in response to species, site, and interactive effects between these independents. The independent variable “species” did have a statistically significant effect on 10 of the 19 morphological leaf traits. These ten traits include area, LBL, LBWB, INTB, INTM, INTA, NBT, INTB/INTM, LBL/INTM and LBWM/INTM (Table 6). The independent variable

"site" had a statistically significant effect on center, LMA and all the physiological leaf traits (Table 6). A statistically significant interactive effect between species and site was not observed for any leaf trait (Table 6).

4.4.5 PCA differentiated samples grouped by species but not samples grouped by site

Using measurement data for the 22 leaf traits, the PCA showed separation between samples grouped by species (Qe and Qr) but showed no separation for samples grouped by site (Ford and Kellogg). This PCA generated 22 principal components (PCs), the associated variance of each is shown by the Figure 2 scree plot. For this PCA, a variance threshold of 12.5% was used to identify the PCs used within the biplot. This threshold was arbitrarily chosen. PC1 and PC2 were above the threshold, accounting for 33.76% and 14.46% of variance, respectively (Figure 2). These PCs were used as axes of a PCA biplot shown in Figure 3. The loading contributions of the 22 leaf traits for PC1 and PC2 are shown in Table 7 and Figure 3a. For this experiment a variable loading contribution within a PC of greater than 0.3 was considered large, this value had been arbitrarily chosen (Table 3). For PC1, significant variables included area, LBL, LBWM and INTM: all loading contributions were positive, and each variable contributed more than 8.5% to PC1 (Table 7 and Figure 3a). For PC2, significant variables included NBT, LBL/INTM and LBWM/INTM: all loading contributions were negative, and each variable contributed more than 11.0% to PC2 (Table 7 and Figure 3a).

The seven leaf traits with significant PC loading contributions are all morphological (Table 7 and Table 2), and excluding LBWM, the variation within the traits had a

statistically significant relation to species identity (see results from two-way ANOVA, table 6). Within the PCA, we observed separation for samples grouped by species (Figure 3b) but no separation for samples grouped by site (Figure 3c).

4.5 Discussion

4.5.1 Qe and Qr can be differentiated using morphological traits

The results from this study demonstrate that morphological leaf traits can be used to distinguish between Qe and Qr (Table 4, Table 6, Table 7, Figure 3a, and Figure 3b). When using leaf tissues, multiple factors make *Quercus* species identification difficult. This is in part due to leaf response to environmental factors like sunlight (Kusi, 2013; Kusi et al., 2020), temperature (Vitasse et al., 2010; Vitasse et al., 2009) and water availability (Bresson et al., 2011). This is also linked to interspecific gene flow and relaxed boundaries between species taxa (Curtu et al., 2007, 2009; Hipp et al., 2008; Rauschendorfer et al., 2022). Although these factors complicate field identification, numerous studies have demonstrated that morphological leaf characters can be used for species identification in *Quercus* (Gailing et al., 2012; Jensen et al., 1984; Kremer et al., 2002; Tovar-Sánchez et al., 2004).

In this study, we found that morphological leaf traits can be used to differentiated between Qe and Qr, regardless of planting site. When comparing leaf trait means using T-test and two-way ANOVA, our results showed that ten of the nineteen morphological leaf traits – area, LBL, LBWB, INTB, INTM, INTA, NBT, INTB/INTM, LBL/INTM and LBWM/INTM – differed by species (Table 4 and Table 6). Many of these traits had large loading contributions within the PCA (Table 7 and Figure 3a) and within

the PCA biplot, samples separated by species (Figure 3b). Collectively, these results demonstrate that leaf trait morphology for Qe and Qr is related to the differences between each species genotype.

Results from my experiment were similar to the findings of (Gailing et al., 2012). When measuring leaf traits for mature trees within native Qe and Qr populations within Michigan's Upper Peninsula, their study also found that LBL, LBWB, INTB, INTM, and INTA measurements were greatest in Qr. Similarly, the LBWM/INTM and LBL/INTM ratios were greater in Qe (Table 3) (Gailing et al., 2012). However, their study found that NBT and INTB/INTM were greater in Qr and Qe, respectively (Gailing et al., 2012). The opposite is observed here (Table 3). The differences between our findings might reflect differences between our experiments. One possibility is that the environmental conditions of the common gardens elicited unexpected differences between Qe and Qr in the NBT and INTB/INTM leaf traits. As mentioned earlier, Qe and Qr have environmental preferences for xeric and mesic site conditions, respectively (Abrams, 1990). Since both the common gardens are within a closer proximity to native Qr populations, it is possible that the phenotype of Qe reflects the changes associated with being in an alternate type of environment. The differences between our studies may also be related to the age class of our samples. While the trees sampled by (Gailing et al., 2012) were mature age class, the trees sampled in this study were juveniles. With age, many tree species shift resource allocation away from growth to promote defense and maintenance (Martínez-Vilalta et al., 2007; Orwig et al., 2001; Weiner et al., 2001). Changes to morphological leaf traits are common across different age classes (England et al., 2006; Steppe et al., 2011).

4.5.2 Leaf traits related to physiology have similar phenotypic plasticity in Qe and Qr

Qe and Qr physiological leaf traits and LMA had similar responses to the Ford and Kellogg site (Table 3, Table 4). While LMA is a morphological trait, this trait is strongly associated with site conditions and are has been correlated with leaf physiology (Coble et al., 2017). Results from the two-way ANOVA reiterated these findings: LMA, Amax, CCI, and WC% were all found to have statistically significant variation in response to site (Table 6). Phenotypic plasticity for these traits was also very similar for both species. Comparing the phenotypic plasticity (CV%) for these traits in Qe and Qr, we observe a difference of less than 1 % (Table 5). Qe and Qr are closely related species with interspecific gene flow (Gailing et al., 2012; Owusu et al., 2015). The result for this study indicates that foliar physiological responses to site are similar for Qe and Qr. Moreover, the changes to these traits might demonstrate how both species protect carbon assimilation in response to increasing temperature and drought conditions of the Kellogg site (Ackerly et al., 2000).

4.5.3 Site and species do not have a significant effect on leaf trait variation for Qe and Qr

In my first hypothesis, I predicted that independent variables site and species would have a significant interactive effect on leaf trait variation for Qe and Qr. This hypothesis was based on leaf trait plasticity measured across multiple Qe and Qr populations (Gailing et al., 2012), the variability within *Quercus* leaf traits in response to site conditions (Balaguer et al., 2001; Valladares, Martinez-Ferri, et al., 2000), and the quantified differences between Qe and Qr (Jensen et al., 1993). However, the results from a two-way ANOVA do not support this hypothesis. In this analysis, ten of the nineteen morphological traits were significant by species, all physiological traits and LMA were significant by site,

and an interactive effect between species and site was not observed for any leaf trait (Table 6). These results demonstrated that variation morphological leaf traits for these species is related to species genotype (Qe or Qr), and variation in physiological leaf trait is related environmental conditions.

4.5.4 Average leaf trait phenotypic plasticity is similar for Qe and Qr

In my second hypothesis, I predicted that Qr would have a greater phenotypic plasticity across leaf traits. My logic for this hypothesis was related to the much greater geographical range of Qr (Abrams, 1990; Stein, 2003). Species with greater environmental range, like Qr, are thought to have greater phenotypic plasticity because these species are generalist (Sultan, 1995; Valladares, Wright, et al., 2000). Furthermore, species from harsher environments, like Qe within xeric sites, are thought to have lowered phenotypic plasticity. This is because specialization within less favorable environments causes ecotypic differentiation, wherein increased selection pressure on genotypes decreases the phenotypic plasticity of a species (Lortie et al., 1996; Ramírez-Valiente et al., 2010; Valladares, Wright, et al., 2000).

The cumulative phenotypic plasticity ($\bar{x}_{CV}$) for Qe and Qr leaf traits were nearly equal and not statistically significant (Table 5). One possible explanation for the similarity in leaf trait phenotypic plasticity could be related to the populations sampled for this experiment. The populations used in this experiment were collected from the northern geographical ranges of both species (Table 1), therefore these populations are northern ecotypes, which may have developed similar adaptations and phenotypic plasticity. Another possible explanation is that Qe specialization for xeric environments did not select

for leaf trait adaptations. Based on previous studies comparing Qe and Qr, it is more likely that adaptations to xeric environments for Qe are related to root structure: while both Qe and Qr have deep tap roots, lateral roots of Qe are exclusive to deeper soil horizons whereas Qr lateral roots have more uniform distribution (Abrams, 1990). Future experimentation should investigate if traits associated with roots have different degrees of phenotypic plasticity. Based on what was reported by Abrams (1990), I would predict that differences between Qe and Qr roots are related to specialization of Qe to xeric environments. Furthermore, I would expect to observe greater root trait phenotypic plasticity for Qr.

Although the average phenotypic plasticity was greatest in Qe, Qr had greater phenotypic plasticity across more individual traits. Of the nineteen morphological leaf traits, ten were more plastic in Qr; of the three physiological leaf traits, two were more plastic in Qr (Table 5). While phenotypic plasticity for many of these traits were within one percent for Qe and Qr, this finding does support the initial hypothesis. These results may suggest that the specialist environmental preferences of Qe caused the northern Qe ecotypes to be less phenotypically plastic than the northern ecotypes of Qr. Furthermore, it could be possible to imagine that southern ecotypes for both species have greater phenotypic plasticity than their northern counterparts.

4.6 Conclusion

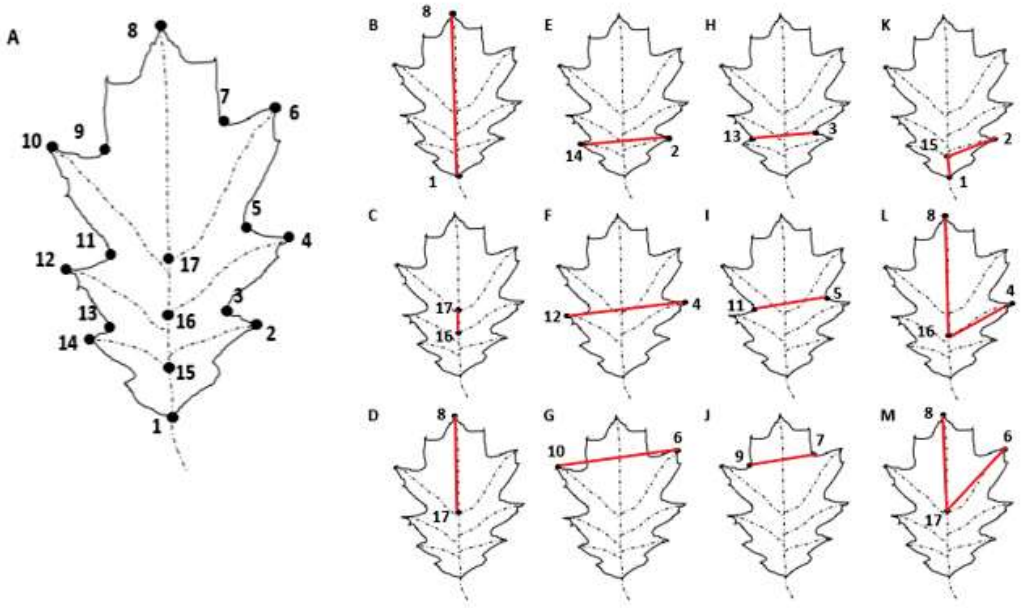
This study was initially conducted to determine if Qe and Qr showed measurable differences between leaf trait phenotypic plasticity. Based on the specialist hypothesis, an initial hypothesis predicted that Qe leaves would be adapted for xeric environments and would be less phenotypically plastic than Qr leaves. In a statistical hypothesis, I also

predicted that leaf trait variation would be related to environmental site, species genotypes, and an interactive effect between these two independents. The results from my experiments did not conclusively support either hypothesis. Both species had similar leaf trait phenotypic plasticity and a significant interactive effect between species and site was not observed for any trait.

This study did find interesting results specific to morphological and physiological leaf traits for Qe and Qr. In T-test, two-way ANOVA, and PCA we observed that differences in leaf trait morphology between the two species is completely dependent on species genotype. Moreover, the leaf shapes of Qe and Qr do not differ in response to the common garden environmental setting. We also found that all the measured physiological leaf traits and LMA, a morphological trait that is strongly associated to leaf physiology and environmental conditions (Coble et al., 2017), responded to environmental differences at the common garden sites and had very similar degrees of phenotypic plasticity. From this study, it is possible to conclude that (1) differences between species genomes accounts for Qe and Qr leaf morphology, and (2) leaf trait physiology is responsive to environmental stimuli for these closely related oak species.

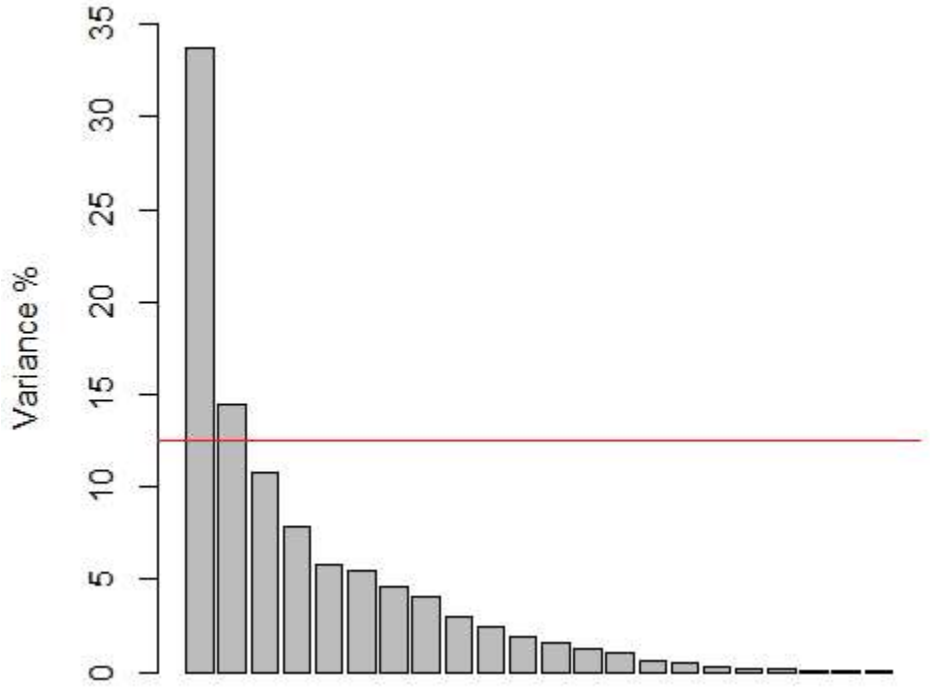
4.7 Chapter 4 figures

4.7.1 Figure 1



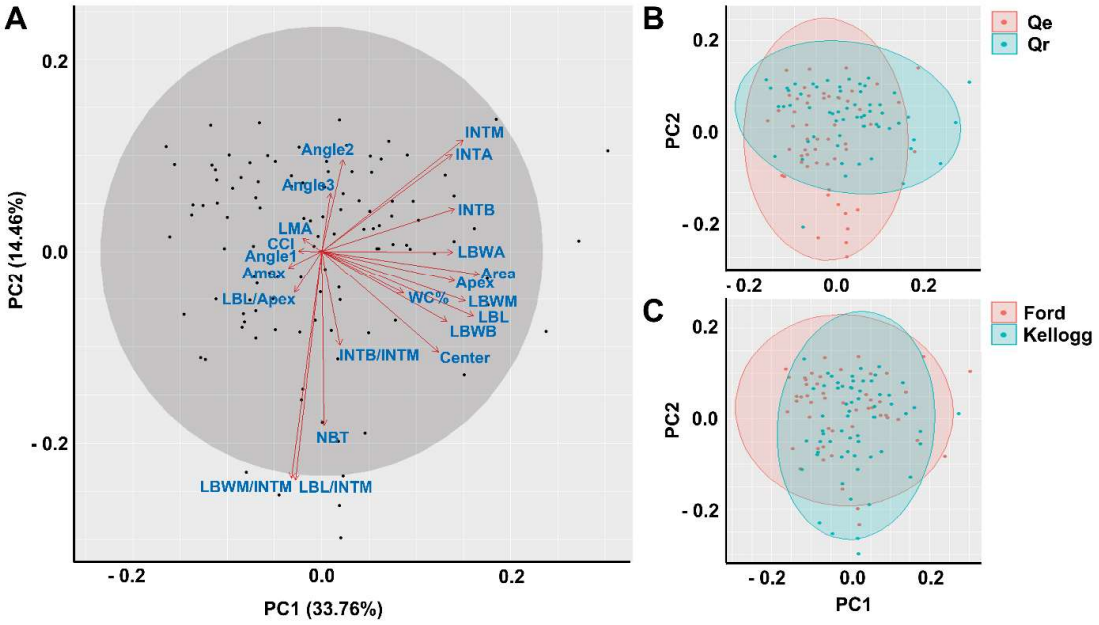
Leaf landmarks used to measure morphological leaf traits 2-13 (table 2). Leaf landmarks (A); LBL (B); center (C); apex (D); LBWB (E); LBWM (F); LBWA (G); INTB (H); INTM (I); INTA (J); Angle1 (K); Angle2 (L); Angle3 (M).

4.7.2 Figure 2



Principal component scree plot. Vertical bars correspond to the amount of variance each PC contributes. The red line indicates 12.5% variance contribution.

4.7.3 Figure 3



Principal component analysis biplot results. Loading contributions of each measured leaf traits (A). Results grouped by species (B). Results grouped by site (C).

4.8 Chapter 4 tables

4.8.1 Table 1

Descriptions of populations used in experimentation. Columns Ford and Kellogg list the number of saplings sampled for each population and common garden. Abbreviations: pop. ID – population ID; lat. – latitude; long. – longitude; MAT – mean annual temperature.

Species	Pop. ID	Provenance (abb.)	Lat.	Long.	MAT ℃	Ford (N)	Kellogg (N)
Qe	10	Baraga Plains, Mi	46.66	-88.58	4.4	8	11
Qe	25	Valhalla, Wi	46.72	-91.05	4.4	6	5
Qe	26	Brule, Wi	46.65	-91.74	4.6	9	14
Qr	2	Copper Harbor, Mi	47.44	-88.22	4.5	9	9
Qr	4	Maasto Hiihto, Mi	47.14	-88.61	4.9	9	15
Qr	14	Blueberry Ridge Trails, Mi	46.46	-87.42	5	9	9

4.8.2 Table 2

Measured leaf traits. Numbers within the trait column are trait ID numbers that are used in the methods section. Abbreviations for each trait are listed in the "abb." Column. The abbreviated terms are used throughout the text. The landmarks column indicates how particular morphological traits were measured across leaves; the location of these landmarks is shown in Figure 1.

Trait	Unit	Abb.	Trait type	Landmarks
Area (1)	cm^2	Area	Morphological	
Leaf blade length (2)	cm	LBL	Morphological	1-8
Interval between center vein intervals (3)	cm	Center	Morphological	16-17
Interval between apical vein intervals (4)	cm	Apex	Morphological	17-8

Leaf blade width basal lobe (5)	cm	LBWB	Morphological	2-14
Leaf blade width middle lobe (6)	cm	LBWM	Morphological	4-12
Leaf blade width apical lobe (7)	cm	LBWA	Morphological	6-10
Interval between basal sinus pair (8)	cm	INTB	Morphological	3-13
Interval between middle sinus pair (9)	cm	INTM	Morphological	5-11
Interval between apical sinus pair (10)	cm	INTA	Morphological	7-9
Angle 1 (11)	degree	Ang1	Morphological	1-15-2
Angle 2 (12)	degree	Ang2	Morphological	8-16-4
Angle 3 (13)	degree	Ang3	Morphological	8-17-6
Number of bristle tips (14)	number	NBT	Morphological	
Ratio of INTB and INTM (15)	unitless	INTB/INTM	Morphological	
Ratio of LBL and INTM (16)	unitless	LBL/INTM	Morphological	
Ratio of LBL and APEX (17)	unitless	LBL/Apex	Morphological	
Ratio of LBWM and INTM (18)	unitless	LBWM/INTM	Morphological	
Leaf mass per unit area (19)	g m^{-2}	LMA	Morphological	
Max rate photosynthesis (20)	µmol m^{-2} sec^{-1}	Amax	Physiological	
Chlorophyll content index (21)	unitless	CCI	Physiological	
Percent water content (22)	%	WC%	Physiological	

4.8.3 Table 3

Mean ($\bar{x}$) and standard error means (SEM) for Qe and Qr leaf traits. Measurements at the Ford and Kellogg sites are subscripted with an f or k, respectively. Pooled measurements are subscripted with a p.

Q. ellipsoidalis

Trait	$\bar{x}_f$	SEM_f	$\bar{x}_k$	SEM_k	$\bar{x}_p$	SEM_p
Area	39.58	4.34	41.01	3.44	40.38	2.68
LBL	10.81	0.63	11.2	0.49	11.03	0.38

Center	2.19	0.12	2.43	0.13	2.33	0.09
Apex	4.76	0.35	4.74	0.27	4.74	0.21
LBWB	4.2	0.32	4.75	0.34	4.51	0.24
LBWM	6.62	0.42	6.56	0.27	6.58	0.24
LBWA	5.28	0.49	4.92	0.28	5.07	0.26
INTB	2.79	0.28	2.84	0.21	2.82	0.17
INTM	3.68	0.35	3.32	0.19	3.48	0.18
INTA	2.91	0.27	2.38	0.13	2.61	0.14
Ang1	121.15	2.37	114.68	1.15	117.48	1.28
Ang2	54.04	2.01	55.09	1.39	54.64	1.17
Ang3	41.15	1.96	41.93	1.12	41.59	1.05
NBT	17.09	1.24	18	1.04	17.6	0.79
INTB/INTM	0.86	0.04	0.77	0.04	0.82	0.03
LBL/INTM	3.62	0.24	3.27	0.24	3.46	0.17
LBL/Apex	2.47	0.1	2.33	0.07	2.41	0.06
LBWM/INTM	2.11	0.13	1.96	0.13	2.05	0.09
LMA	113.67	3.22	79.45	1.77	94.3	2.9
Amax	10.12	0.86	12.27	0.82	11.32	0.61
CCI	15.43	1.14	12.32	0.67	13.67	0.66
WC%	51.83	0.57	53.26	0.65	52.64	0.45

Q. rubra

Trait	$\bar{x}_f$	SEM_f	$\bar{x}_k$	SEM_k	$\bar{x}_p$	SEM_p
Area	48.05	6.83	58.92	4.49	54.02	3.97
LBL	11.51	0.77	13.16	0.51	12.42	0.45
Center	2.23	0.16	2.75	0.14	2.51	0.11
Apex	4.84	0.41	5.66	0.31	5.29	0.26
LBWB	4.98	0.4	5.67	0.29	5.36	0.24
LBWM	6.36	0.46	7.42	0.29	6.94	0.27
LBWA	4.91	0.47	5.47	0.27	5.22	0.26
INTB	3.75	0.31	4.37	0.22	4.09	0.19
INTM	4.33	0.32	4.82	0.23	4.6	0.19
INTA	3.27	0.29	3.38	0.19	3.33	0.17
Ang1	119.63	1.72	119.34	1.94	119.47	1.31
Ang2	51.89	1.3	51.34	1.52	51.58	1.01
Ang3	42.72	2.1	40.58	1.28	41.51	1.18
NBT	15.07	0.91	13.12	0.81	14	0.62
INTB/INTM	0.93	0.03	0.89	0.05	0.91	0.04
LBL/INTM	2.87	0.14	2.73	0.09	2.8	0.09
LBL/Apex	2.44	0.09	2.52	0.11	2.47	0.07
LBWM/INTM	1.6	0.06	1.49	0.04	1.55	0.04
LMA	109.75	3.02	81.66	2.59	94.3	2.67

Amax	8.98	0.73	10.52	0.64	9.83	0.49
CCI	16.56	1.02	11.84	0.56	13.96	0.63
WC%	51.82	0.76	54.87	0.4	53.5	0.45

4.8.4 Table 4

T-test comparisons of leaf trait means. Results for t-test comparisons between species site means are described by the Qe and Qr columns. Results for t-test comparisons between trait pooled means of both species are described by the Qe|Qr column. The t-test columns indicate the type of t-test performed. Abbreviations within t-test columns: w – Welch's t-test; s – Student's t-test. The p-value column indicates if there are significant differences between the tested means (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$). Means with the greatest values are described in the trend columns. Abbreviations within trend columns: F – Ford mean; K – Kellogg mean; Qe – Qe mean; Qr – Qr mean; NC – no change between means. Values of each mean are listed in table 3.

	Qe			Qr			Qe|Qr		
Trait	**t-test**	**p-value**	**Trend**	**t-test**	**p-value**	**Trend**	**t-test**	**p-value**	**Trend**
Area	s	-	K	w	-	K	w	***	Qr
LBL	s	-	K	w	-	K	s	*	Qr
Center	s	-	K	s	*	K	w	-	Qr
Apex	s	-	F	s	-	K	w	-	Qr
LBWB	s	-	K	s	-	K	s	*	Qr
LBWM	s	-	F	w	-	K	s	-	Qr
LBWA	w	-	F	w	-	K	s	-	Qr
INTB	s	-	K	s	-	K	s	***	Qr
INTM	w	-	F	s	-	K	s	***	Qr
INTA	w	-	F	s	-	K	s	***	Qr
Ang1	w	*	F	s	-	F	s	-	Qr

Ang2	s	-	K	s	-	F	s	*	Qe
Ang3	w	-	K	w	-	F	s	-	Qe
NBT	s	-	K	s	-	F	s	***	Qe
INTB/INTM	s	-	F	w	-	F	s	*	Qr
LBL/INTM	s	-	F	w	-	F	w	***	Qe
LBL/Apex	s	-	F	s	-	K	s	-	Qr
LBWM/INTM	s	-	F	w	-	F	w	***	Qe
LMA	w	***	F	w	***	F	s	-	NC
Amax	s	-	K	s	-	K	s	-	Qe
CCI	w	*	F	w	***	F	s	-	Qr
WC%	s	-	K	w	***	K	s	-	Qr

4.8.5 Table 5

Leaf trait phenotypic plasticity. Phenotypic plasticity is expressed as a coefficient of variation percentage (CV%). CV% is the ratio between the pooled mean ($\bar{x}_p$) and pooled standard deviation (σ_p). The mean of each species CV% ($\bar{x}_{CV}$) was used to assess overall phenotypic plasticity of leaf traits for a species. Statistical significance of these means was assessed using a paired t-test.

	Qe			**Qr**		
	$\bar{x}_p$	**σ_p**	**CV%**	**$\bar{x}_p$**	**σ_p**	**CV%**
Area	40.38	19.53	48.36	**54.02**	30.74	56.90
LBL	11.03	2.79	25.32	**12.42**	3.52	28.37
Center	2.33	0.66	28.54	2.51	0.83	33.14
Apex	4.74	1.55	32.66	5.29	1.98	37.48
LBWB	4.51	1.74	38.54	**5.36**	1.87	34.95
LBWM	6.58	1.72	26.08	6.94	2.07	29.80
LBWA	5.07	1.92	37.88	5.22	2.01	38.43
INTB	2.82	1.24	43.86	**4.09**	1.45	35.49
INTM	3.48	1.33	38.17	**4.60**	1.51	32.84
INTA	2.61	1.05	40.11	**3.33**	1.30	39.09
Ang1	117.48	9.32	7.94	119.47	10.13	8.48
Ang2	**54.64**	8.49	15.55	51.58	7.84	15.21
Ang3	41.59	7.68	18.40	41.51	9.12	21.95
NBT	**17.60**	5.75	32.68	14.00	4.77	34.04
INTB/INTM	0.82	0.22	26.74	**0.91**	0.29	25.30

LBL/INTM	**3.46**	1.24	35.82	2.80	0.67	23.73
LBL/Apex	2.41	0.47	19.73	2.47	0.54	21.70
LBWM/INTM	**2.05**	0.66	32.44	1.55	0.31	20.11
LMA	94.30	21.13	22.40	94.30	20.67	21.91
Amax	11.32	4.42	39.07	9.83	3.78	38.48
CCI	13.67	4.77	34.65	13.96	4.85	34.71
WC%	52.64	3.26	6.19	53.50	3.48	6.51
$\bar{x}_{CV}$			**29.60**			**29.03**
p-value			**0.36**			

4.8.6 Table 6

Results from two-way ANOVA testing effects of independent variables species, site and their interaction effects on measured leaf traits. Significant interactions are indicated (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$).

Trait	Species	Site	Species x Site
Area	**	-	-
LBL	*	-	-
Center	-	**	-
Apex	-	-	-
LBWB	*	-	-
LBWM	-	-	-
LBWA	-	-	-
INTB	***	-	-
INTM	***	-	-
INTA	***	-	-
Ang1	-	-	-
Ang2	-	-	-
Ang3	-	-	-
NBT	***	-	-
INTB/INTM	*	-	-
LBL/INTM	***	-	-
LBL/Apex	-	-	-
LBWM/INTM	***	-	-
LMA	-	***	-
Amax	-	*	-
CCI	-	***	-
WC%	-	***	-

4.8.7 Table 7

Loading contributions of leaf trait variables and the % variance explained by PCs one and two. Graphical representation of variable loading contributions is shown in figure 2a. Variable loading contributions with an absolute value greater than 0.3 are bold within the table. This value was arbitrarily assigned.

	PC1		PC2	
	Loading	**%**	**Loading**	**%**
Area	**0.354**	9.53	-0.051	1.46
LBL	**0.341**	9.18	-0.144	4.13
Center	0.262	7.04	-0.223	6.39
Apex	0.297	8.01	-0.065	1.88
LBWB	0.281	7.56	-0.155	4.45
LBWM	**0.321**	8.64	-0.109	3.13
LBWA	0.294	7.92	-0.001	0.03
INTB	0.298	8.02	0.094	2.69
INTM	**0.316**	8.50	0.246	7.06
INTA	0.292	7.86	0.214	6.14
Ang1	0.004	0.10	0.004	0.11
Ang2	0.048	1.30	0.202	5.81
Ang3	0.019	0.52	0.129	3.70
NBT	0.005	0.14	**-0.384**	11.02
INTB/INTM	-0.042	1.12	-0.207	5.95
LBL/INTM	-0.059	1.59	**-0.504**	14.48
LBL/Apex	-0.061	1.65	-0.091	2.61
LBWM/INTM	-0.068	1.84	**-0.500**	14.37
LMA	-0.042	1.13	0.029	0.84
Amax	-0.075	2.01	-0.036	1.03
CCI	-0.052	1.39	0.002	0.05
WC%	0.183	4.94	-0.092	2.65
Variance (%)	33.76		14.46	

5 Chapter 5: using RNA-seq to study ecological speciation between *Quercus ellipsoidalis* and *Quercus rubra*

5.1 Abstract

Ecological speciation occurs when environmental factors limit gene flow between populations and species. In direct ecological speciation, variant alleles and certain gene expression patterns become fixed in response to environmental selective pressure. *Quercus ellipsoidalis* (northern pin oak) and *Quercus rubra* (northern red oak) are red oak sister species that have been traditionally used to study ecological speciation. Although distinguished by unique forms, morphology, physiology, and site preferences (xeric and mesic, respectively), both species share interspecific gene flow. In previous experiments, ecological speciation within *Q. ellipsoidalis* and *Q. rubra* has been studied using genetic molecular markers, which helped identify fixed alleles for both species. In this study, ecological speciation between *Q. ellipsoidalis* and *Q. rubra* is measured using RNA-seq. This method identified fixed patterns of differential gene expression between both species. Additionally, annotation of the differentially expressed genes identified species specific molecular phenotypes. Because *Q. ellipsoidalis* prefers xeric environments, the initial hypothesis of this experiment predicted that *Q. ellipsoidalis* would have an enriched molecular phenotype specific to osmotic stress tolerance (H1). In addition to the RNA-seq experiment, a hydraulic trait pilot study was also performed to assess how *Q. ellipsoidalis* and *Q. rubra* differed in response to increasing drought severity. In this pilot study, the two species were compared in optical vulnerability and pressure volume curves. Like the hypothesis for molecular phenotypes, the hypothesis here anticipated that *Q. ellipsoidalis* would have greater tolerance for increasing water deprivation (H2). The first hypotheses

were not validated by our results. Within the *Q. rubra* transcriptome was an enrichment of genes related to pest defense, while the *Q. ellipsoidalis* transcriptome had an enrichment for genes related to photosynthesis. Our results did find support our second hypothesis. The mean measurements for all hydraulic traits measured in this study were lower in *Q. ellipsoidalis*, indicating that this species has a greater adaptation to drought stress than *Q. rubra*. This experiment demonstrates RNA-seq can be used for identifying species-specific molecular phenotypes, and moreover, is an appropriate tool for studying the effects of ecological speciation.

5.2 Introduction

Red oaks (Genus *Quercus*, section *Lobatae*) constitute a taxa of hardwood trees unique and prominent within North America. Red oaks originated within the northern latitudes of North America. During a period of global warming spanning from the early-Eocene through the mid-Oligocene, this section diversified and moved into the continent's southern range (Cavender-Bares, 2019; Hipp et al., 2018; Zachos et al., 2001). Oaks have great ecological importance within North American forests too. In both the USA and Mexico, oaks have a species richness and biomass greater than any other hardwood taxa (Cavender-Bares, 2016). Within the Americas, red oak species diversity is estimated to include more than 80 species that inhabit ecosystems ranging from swamp forests to arid highland mountains (Hipp et al., 2020; Rauschendorfer et al., 2022; Rodríguez-Correa et al., 2015).

This study focuses on two red oak species: *Quercus ellipsoidalis* (northern pin oak, Qe) and *Q. rubra* (northern red oak, Qr). Both species have overlapping native ranges within the northeastern-central region of the United States mainland (Stein, 2003).

However, the range of Qe is much smaller than that of Qr. Although both species are found in northern latitudes, Qe is found in excessively-drained xeric sites, and Qr is found across mesic environments (Abrams, 1990; Lind-Riehl et al., 2013). The ecological preferences of these species can be partly explained by their leaf morphologies. Although the leaves of both species have adaptations for arid conditions – smaller stomata and greater leaf thickness than co-occurring hardwoods (Abrams et al., 1990) – Qe leaves have a significantly reduced surface area (Gailing et al., 2012). This characteristic makes Qe better adapted for xeric environments with higher irradiance (Schuepp, 1993). Rooting structures of Qe are better adapted for xeric sites too. While both species have deep taproots, Qe lateral roots are exclusive to the lower soil horizons (Abrams, 1990; T. Kozlowski, 1971; Lyford, 1980).

Although distinct in form and site preference, Qe and Qr share interspecific gene flow (Jensen et al., 1993; Owusu et al., 2015). This isn't particularly unique, in *Quercus*, introgressive gene flow between species is frequently observed (Rauschendorfer et al., 2022; Tovar-Sánchez et al., 2004; Valencia, 2010), and genetic barriers appear to be maintained by ecological selective pressures (Scotti-Saintagne et al., 2004). This makes *Quercus* an ideal taxon for studying ecological speciation (ES) (Goicoechea et al., 2012; Owusu et al., 2015; Scotti-Saintagne et al., 2004). ES is a type of speciation that occurs when ecological factors limit gene flow between populations of a species. ES is promoted by indirect and direct mechanisms. Indirect ES occurs when a colonizer persists within a new environment by adjusting gene expression and phenotypes in response to the environment (Pavey et al., 2010; Price et al., 2003). Direct ES occurs over generations as certain alleles and patterns of gene expression become fixed within a population in

response to environmental pressures (Aubin-Horth et al., 2009; Pavey et al., 2010; Price et al., 2003; Richard Svanbäck et al., 2009; Schluter et al., 2009; Via et al., 1985).

Previous work has used genetic molecular markers to understand ES between cohabitating *Quercus* species. These studies often find high rates of interspecific gene flow and fixation of a few key alleles (Owusu et al., 2015). Higher rates of gene flow are generally observed among *Quercus* species with similar ecological preferences (Owusu et al., 2015; Scotti-Saintagne et al., 2004). Some studies have been greatly interested in the species identity and ES of Qe. Using genetic molecular markers, degrees of interspecific gene flow has been measured between *Q. velutina, Q. coccinea, Q. palustris*, Qe and Qr. For many, these results are evidence that the emergence of Qe is a result of a hybrid swarm between *Q. velutina, Q. coccinea, Q. palustris* and Qr (Hipp et al., 2010; Hipp et al., 2008; Lind-Riehl et al., 2013; Lind-Riehl et al., 2014; Sullivan et al., 2013). These studies also demonstrate that *Quercus* species identity can be accurately assigned using genetic molecular markers. This accomplishment is incredibly important, since the use of morphological traits for species identification in *Quercus* is made difficult by fluid species boundaries and phenotypic plasticity (P. R. Aldrich et al., 2011; Bolstad et al., 2003; Gailing et al., 2012; Muir et al., 2005; Quero et al., 2006). Moreover, these studies show that morphological species identification with *Quercus* is prone to error, and genetic assignment of species is particularly helpful for determining an individual's introgression status (Owusu et al., 2015).

When using genetic molecular markers, scientists are often restricted to working with visible phenotypes that are measured by traditional approaches. To paraphrase Pavey et al. (2010), use of visible phenotypes ignores the importance of molecular phenotypes

and limits our understanding of evolution by ES. Molecular phenotypes are important to ES because they are environmentally responsive (Grishkevich et al., 2013; Lasky et al., 2014) and heritable (Falconer et al., 1996; Gibson et al., 2005). Molecular phenotypes in plants can be related to accumulation of certain secondary metabolites that provide protection from abiotic and biotic stresses (Külheim et al., 2011), and since plants are sessile, these phenotypes are incredibly important to fitness and survival (Bradshaw, 1972). Unfortunately, without proper tests and prior knowledge, molecular phenotypes are difficult to study (Pavey et al., 2010). Moreover, differences between molecular phenotypes of ecologically significant species are understudied because these species are typically not genetic model organisms, and there is a lack of common garden facilities to conduct these experiment in (Pavey et al., 2010).

RNA-seq, a type of next generation sequencing experiment, can be used for discovering molecular phenotypes related to ES (Pavey et al., 2010). In these experiments, RNA-sequences are quantified and annotated with bioinformatic tools. By quantifying the RNAs we find a gene's transcription level. By annotating the RNAs we can identify molecular phenotypes specific to the transcription patterns between groups. Thus, RNA-seq can be used to characterize the molecular phenotypes that are relevant to ES and species identity. Additionally, RNA-seq was used to identify expression quantitative trait loci (eQTLs) for molecular phenotypes that are related to ES (Gilad et al., 2008; Mackay et al., 2009; Pavey et al., 2010). In this experiment, RNA-seq is used to identify molecular phenotypes and study ES related to Qe and Qr. Based on the environmental preferences of both species, we expected to find a Qe molecular phenotype related to increased drought

tolerance (i.e., an enrichment of upregulated genes related to osmotic stress response and/or cell wall biosynthesis – OS/CW).

In addition to the RNA-seq experiment, I also conducted a hydraulic trait experiment to understand how Qe and Qr respond to increasing water deprivation. Hydraulic traits linked to leaf turgor loss and decreasing hydraulic conductivity are measured using pressure volume curves (PVC) and optical vulnerability (OV) experiments, respectively (Bartlett et al., 2012; Brodribb et al., 2016; Skelton et al., 2018). PVCs are used to determine the leaf turgor loss point (ψ_{TLP}, Mpa), the point at which the leaf remains turgid and functional. This trait is a proximal measurement of how much drought stress a plant will tolerate before stomatal closure (Skelton et al., 2018). Plants with lower ψ_{TLP} are better adapted for maintaining stomatal conductance, hydraulic conductivity, and photosynthesis in arid conditions (Sack et al., 2003). Compared to other hardwoods, low ψ_{TLP} is commonly associated with *Quercus* (Abrams, 1990). OV measures leaf pressure potentials (ψ_{leaf}, Mpa) across the range of hydraulic conductance (xylem embolism percent, %) (Brodribb et al., 2016; Petruzzellis et al., 2020). Embolism onset (P_e), embolism vulnerability (ψ_{50}), and critical embolism (ψ_{88}) are measurements of ψ_{leaf} at 5%, 50%, and 88% loss in hydraulic conductance (Brodribb et al., 2016; Petruzzellis et al., 2020; Skelton et al., 2018). Using both PVC and OV, Skelton et al. (2018) found interesting mechanism of drought avoidance for *Lobatae* species endemic to California. They observed a close association between stomatal closure (ψ_{TLP}) and the onset of embolism (P_e) suggesting that xylematic embolism triggers stomatal closure (Skelton et al., 2018). To determine if this mechanism of drought avoidance is common to *Lobatae*, I piloted a study measuring hydraulic traits of one population each of Qe and Qr. I expected to find that both species would have close

association between ψ_{TLP} and P_e. Since Qe is found in xeric sites, I expected that this species would have a greater tolerance to increasing water deprivation than Qr. I hypothesized that increased drought tolerance of Qe would be partially explained by the molecular phenotypes specific to OS/CW gene expression.

5.3 Methods

5.3.1 Site description and populations selected for experiments

In Fall 2018, I identified four Qe and four Qr populations with similar latitudinal ranges and mean annual temperatures (Table 1). For this experiment, a population was defined as a stand of oak trees dominated by a single species. Acorns were collected from the area beneath isolated maternal trees. The maternal trees were considered "isolated" when their crowns did not overlap the crowns of another oak tree. The collected acorns were cold-stratified and propagated in the MTU greenhouse. The seedlings from these populations were planted in a common garden site located at the Ford Experimental Forest (Alberta, Michigan: 46.64 N, -88.48 W). Within this common garden, the trees were arranged across 27 blocks, consisting of 30 individuals, spaced in rows 1.5 m by 0.76 m. A seedlings placement in the common garden was assigned using a semi-random block design (Ariel et al., 2010). The garden's construction was completed on June 22nd, 2019. According to the NRCS soil survey 2021 the garden has a gravelly coarse sandy loam soil profile (Post et al., 2007). Using WorldClim 2020 data, the garden's mean annual temperature and annual precipitation were 4.9 °C and 879 mm, respectively. Because the garden is situated outside of the forest canopy, the common garden receives direct sunlight.

5.3.2 RNA-seq experimental design

Initially, the RNA-seq experiment was designed to include four samples from each population. All samples within each population had different maternal lineages. Unfortunately, we encountered difficulty extracting RNA from multiple samples within Qe populations 24, 25, and 26. These three Northern Wisconsin populations were grouped into a single population (QeNW). Information on each population is listed in Table 1.

5.3.3 RNA isolation, library prep and sequencing

The youngest, fully-expanded leaf with minimal environmental damage was harvested from each sample on September 10th, 2021. The samples were immediately frozen in liquid nitrogen and stored at -80°C until RNA isolation. RNA was extracted from the leaf tissues using the TakaRA Bio Nucleospin® plant and fungi RNA isolation kit. To optimize the extracted RNA yields, the samples were ground to a fine powder using a mortar and pestle. Liquid nitrogen was used to chill the mortar, pestle, and leaf tissue during the entire process. During trial RNA extractions, I found that some modifications to the TakaRA extraction protocol helped to increase RNA yields. These included the use of 50 mg and 100 mg leaf tissue from Qe and Qr, respectively, using 75 µl PFR and 750 µl buffer PFL buffers during lysis step, and the use of 750 µl PFB buffer when adjusting RNA binding conditions. Samples that passed the quality standards were shipped to Novogene for mRNA library preparation and sequencing. Novogene checked RNA sample quality, isolated fully synthesized mRNAs (poly A enrichment), and used next generation sequencing technology to sequence the mRNA transcripts.

5.3.4 RNA-seq library cleaning and filtering

The forward and reverse sequences for each sample were quality checked using the FastQC software (Külahoglu et al., 2014). Results from the FastQC were aggregated to single file using the MultiQC software (Ewels et al., 2016). These outputs can be viewed in the Supplemental File 1. Using the BBTools package, the feature BBDuk was used to clean and filter the RNA sequence library (Bushnell, 2020). Libraries were cleaned for sequence adapters and overrepresented sequences identified with FastQC. Library sequence reads were trimmed to a quality trim cutoff of Q30 and kmer-processing proceeded in the right-hand direction. A hamming distance was set to 1 and sequences were trimmed by overlap between paired reads. The Quality of the cleaned and filtered libraries was assessed using FastQC and MultiQC software (Ewels et al., 2016). These outputs can be viewed in Supplemental File 2.

5.3.5 Genome indexing, transcriptome assembly, and RNA sequence library mapping with STAR

Using the genome-indexing feature within the STAR software package, the *Q. rubra* genome was indexed (Dobin et al., 2012). The *Q. rubra* genome file was downloaded from Phytozome (2021). The cleaned and filtered RNA sequence libraries from the Qe and Qr samples were mapped to the indexed *Q. rubra* genome using read mapper feature within STAR (Dobin et al., 2012): default read length was set to 100, and sorted bam files were the output file type. The quality of the library mapping was assessed using Qualimap software (García-Alcalde et al., 2012). Outputs from Qualimap can be viewed in Supplemental Table 1, and Supplemental Table 2.

5.3.6 Mapped RNA library indexing and RNA sequence counting with samtools

The mapped RNA sequence libraries were indexed using the "index" command from the samtools software package. Using the *Q. rubra* gene annotation file and the indexed RNA sequence libraries, the "htseq-count" command within the samtools software package was used to create sequence counts matrix (Zhang et al., 2021). The *Q. rubra* gene annotation file was retrieved from Phytozome (2021).

5.3.7 Principal component analysis, and differential gene expression analysis of RNA sequence counts

DESeq2, an R software package, was used to conduct principal component analysis (PCA) on the RNA sequence counts (Love et al., 2014). PCA is a statistical approach unbiased by prior knowledge that can be used to find patterns in transcriptomics (Lever et al., 2017). The PCA was conducted using genes that had a read count sum (a total across all samples) of greater than 10. In total 1,923 genes were used for this analysis.

Differentially expressed genes (DEGs) between Qe and Qr were identified using the DESeq2 software package. DESeq2 uses a negative binomial distribution model to identify DEGs between sample groups (Love et al., 2014). In this experiment, Qe samples were used as the control group. Differential gene expression is reported as L2FC (log2 fold-change) between the two species (Love et al., 2014). Positive L2FC indicated that a gene had greater level of expression in Qr: in this experiment, these genes are upregulated in Qr and/or downregulated in Qe. Negative L2FC indicated that a gene had lower level of expression in Qr: in this experiment these genes are upregulated in Qe and downregulated in Qr. For the remainder of this chapter, the statistically significant DEGs with negative

L2FC values are collectively called Qe upregulated DEGs. To reduce the likelihood of recording a false positive result, the DEGs with significant p-value of less than 0.01 were sorted using a Benjamini-Hochberg procedure (Thissen et al., 2002). The Qe upregulated DEG library is shown in Supplemental Table 3: within this table, the absolute value of each gene's L2FC value is reported. The Qr upregulated DEG library is shown in Supplemental Table 4.

5.3.8 RNA sequence annotation and gene ontology enrichment analysis

Translated peptide sequences from the *Q. rubra* transcriptome were downloaded from Phytozome (2021). This file contained 47,780 peptide sequences. The *Q. rubra* peptide sequences were annotated using the bioinformatic software Blast2Go (Conesa et al., 2005). Annotation of the *Q. rubra* peptides was accomplished using BLAST (basic local alignment sequence tool) to compare the *Q. rubra* sequences against *Arabidopsis* peptides in the Swiss-Prot database (Boutet et al., 2007). Due to file size of the *Q. rubra* peptide sequence file, the BLAST word size was set to 3 amino acid residues.

Using Blast2Go (Conesa et al., 2005), the annotated *Q. rubra* peptide sequences were used to annotate the Qe and Qr upregulated DEG libraries. Enriched GO (gene ontological) terms for each species were found by comparing the annotated, upregulated DEG libraries against the *Q. rubra* annotated transcriptome using a Fisher's exact test (Upton, 1992). Blast2Go default parameters were used in this Fisher's exact test. Significantly enriched GO terms had a false discovery rate (FDR) of less than 0.05. These significant enriched GO terms were used to identify molecular phenotypes for each species.

5.3.9 Identification of gene expression markers unique to both species

Gene expression for all the identified Qe and Qr upregulated DEGs across the 27 sample libraries (11 Qe, 16 Qr) was standardized using the RPKM (Reads Per Kilobase Million) approach (Starmer, 2015). For each DEG, we compared the RPKM means of both species using a Student's t-test: DEGs with $p < 0.05$ were statistically significant and kept for further analysis (Boneau, 1960). Next, we counted the number of samples with statistically significant RPKM values in both species categories. Here, a statistically significant sample had and RPKM valued greater than the 97.5% confidence interval specific to the species with "non-differential" gene expression (Hazra, 2017): for example, if a DEG was specific to Qe, the 97.5% confidence interval was calculated using Qr RPKM mean. Significance of these counts was assessed using a chi-square test (Tallarida et al., 1987): the expected value for each species was based on the number of species samples and the probability of 0.5. Expected values for Qe and Qr were 5.5 and 8, respectively. A significance threshold in the chi-square test was set to $p < 0.05$.

5.3.10 Expression quantitative trait loci (eQTL) hotspot identification

Collectively, DEGs related to molecular phenotypes identified by GO enrichment are all linked to quantitative traits and are referred to eQTLs (expression quantitative trait loci). In some instances, eQTLs are in close genetic proximity to one another and these chromosomal regions are referred to as eQTL hotspots (Pavey et al., 2010). In this study, we reported eQTL hotspots consisting of DEGs within 50 kbp of one another annotated by one or more enriched GO terms.

5.3.11 Hydraulic trait experimental design and sampling

Hydraulic traits of both species were measured using three replicates from QeBP and QrHr populations (see Table 1 for more information). Before daybreak, branches were destructively harvested from each of the selected replicates using pruning shears. The severed branch was placed in a water bucket so that the cut was submerged in water. This process ensures that the dry-down process is halted. The bucket was then covered with a black plastic bag ensuring that transpiration did not occur. Branches were removed from water once the optical vulnerability experiment started.

5.3.12 Pressure volume curves

From the collected branches, a single leaf was removed to create a pressure volume curve (PVC). This experiment required multiple measurements conducted during a drying period with a single leaf. Between 10 and 15 measurements were taken for a single leaf from an individual sample. Regular measurement of the leaf's water potential (Ψ_{leaf}, MPa) was taken by matching the leaf's pressure potential in a pressure bomb (Model 670, PMS Instrument Company, Albany, OR) using pressurized nitrogen gas. After each Ψleaf measurement, the mass of the leaf was taken so that the relative water content (R, %) of the leaf could be calculated. R was calculated as follows:

R = 100 * (Leaf Wet Weight – Leaf Dry Weight) / Leaf Dry Weight Eq.1

From this experiment, we found π_0 (ψ_{leaf} at full turgor), RWC at TLP (relative water content at the turgor loss point), and π_{TLP} (ψ_{leaf} at turgor loss point). These traits were determined using the protocol outline in Bartlett et al. (2012). Statistical significance for these

measurements was assessed using a Student's t-test (Boneau, 1960). P-value significance was set at less than 0.05.

5.3.13 Measuring optical vulnerability and embolism safety margins

The remaining leaves and the harvested branches were used in our optical vulnerability (OV) experiment. The harvested branches were removed from the water, and a single leaf, attached to the branch, was fixed within the viewing field of an Epson Perfection V600 scanner. A section of the fixed leaf was photographed every 10 minutes to track the progression of embolism across the leaf venation. As the photos were being collected, leaves adjacent to the fixed leaf were periodically excised from the branch, and used to measure Ψ_{leaf} during the branch's desiccation. Pressure potentials at embolism onset (P_e, 5% embolism), embolism vulnerability (Ψ_{50}, 50% embolism), and critical embolism (Ψ_{88}, 88% embolism) were determined using the method outlined in Brodribb et al. (2016). The safety margin (MS, Mpa) was calculated using a method outlined in Skelton et al. (2018). The formula for MS is as follows:

$$MS = \pi_{TLP} - P_e \quad \text{Eq.2}$$

Statistical significance for these measurements was assessed using a Student's t-test (Boneau, 1960). P-value significance was set at less than 0.05.

5.4 Results

5.4.1 Transcriptome assembly and annotation

The RNA transcriptome profiles of the Qe and Qr samples were sequenced with next-generation sequencing technology by Novogene. In total, 1,225,347,257 reads were

sequenced from the 27 samples: 510,479,168 reads were sequenced from the 11 Qe samples, and 714,868,089 reads were sequenced from the 16 Qr samples. On average, a sample had 45,383,089 sequenced reads: mean sequence read counts for Qe and Qe were 46,407,197 and 44,679,256, respectively (Supplemental Table 1).

For each sample, the sequenced reads were used for transcriptome assembly. In this experiment, the transcriptome assembly was a *Q. rubra* genome reference-based assembly (Behera et al., 2021; Dobin et al., 2012). Transcriptomes were assembled using STAR software. In total, 738,940,703 transcripts were assembled across all samples. Mean number of assembled transcripts for a sample was 27,368,175: mean number of assembled transcripts for Qe and Qr were 28,432,438 and 26,636,493, respectively (Supplemental Table 1). On average, an assembled transcriptome for a sample mapped to 19,612 genes, with 1,400 transcripts per gene. Averages for the Qr samples were 19,243 mapped genes with 1,481 transcripts per gene. Average for the Qe samples were 19,864 mapped genes with 1,344 transcripts per gene (Supplemental Table 1). Read mapping ratios, the percentage of each samples sequences that can be aligned to the Q. rubra reference genome, were calculated for each sample using the Qualimap software. Read mapping ratio for each sample was 100%. The samples had an average mean mapping quality score (MMQ) of 30.48. Average MMQ for Qe and Qe were 30.57 and 30.42, respectively (Supplemental Table 1). One-way ANOVAs were performed to determine if independent variables "population" and "species" had a significant effect on a samples MMQ score. Statistical significance was not observed for either independent variable (Supplemental Table 2).

5.4.2 Principal component analysis

In a PCA, the sample transcriptome assemblies were not found to have unique clustering patterns based on population or species assignment (Figure 1). Eigenvectors 1 and 2 accounted for 44 and 31 percent of the data's variance. When grouped by population and species, unique clusters were not observed within the PCA biplot (Figure 1). This result is likely related to only finding a small number of genes that had significant differential expression between the two species.

Because most genes did not have significant differential expression between Qe and Qr, most samples plotted to the centers of the PCA biplots. Along the first principal component, most samples had eigenvalues between 1 and -1. Three Qr samples had positive eigenvalues outside of this range: these samples were from the QrBR, QrHR and QrMH populations. One Qe sample from the QeBP population had a negative eigenvalue outside of this range (Figure 1b). Along the second principal component, most samples had eigenvalues between 2 and -2. Two Qe samples had positive eigenvalues outside of this range: these samples were from the QeNW and QeBP populations. Three Qr samples had negative eigenvalues outside of this range: these samples were from the QrBP, QrHR, and QrMH populations (Figure 1b). The QrHR and QrMH samples were the same individuals described in the above paragraph: these samples had positive eigenvalues along the first principal component.

5.4.3 Overrepresented differentially expressed gene libraries for Qe and Qr

This experiment used transcriptomes from Qe and Qr to understand if ecological speciation these species to have differentially expressed genes (DEGs). Since the

experiment compared transcriptomes for Qe and Qr, overrepresentation in the context of this experiment means that expression is statistically higher within one of the two species. To avoid confusion when talking about DEGs, I describe these DEG libraries as overrepresented within Qe or Qr.

Between Qe and Qr, a small number of DEGs were discovered. Using the 27 assembled transcriptomes, 782 DEGs were identified: of these DEGs, 304 and 478 were upregulated in Qe and Qr, respectively (Supplemental Tables 3 and 4). Compared to the Qr upregulated DEG library, average differential expression was lower for genes within the Qe upregulated DEG library. Mean differential expression for the Qe and Qr upregulated DEGs was 1.36 ± 0.09 and 2.01 ± 0.10 L2FC, respectively. Distribution of differential expression across each dataset gives more details about these results. Within the datasets of Qe and Qr upregulated DEGs, 66.78 and 32.64 percent had differential expression of less than 1.00 L2FC, respectively (Supplemental Tables 3 and 4).

5.4.4 Enriched GO terms identify molecular phenotypes for Qe and Qr

Annotation of the overrepresented DEGs for both species was completed using the Blast2Go bioinformatic software. The upregulated Qe and Qr DEG libraries were annotated by 1,282 and 1,782 unique GO terms, respectively (Table 2). In this experiment, the term “enriched” indicates that the frequency of a GO term is statistically significant and greater than what is observed in the annotated *Q. rubra* peptide sequence database. The enriched GO terms summarize differences between the molecular phenotypes of both species based on significant gene overrepersentation. Because we are comparing transcriptomes of two species, enriched means that one species has a greater

overrepresentation for a certain category of genes when compared to the other.. The Qe and Qr DEG libraries had 17 (1.3 %) and 159 (8.9 %) enriched GO categories, respectively (Table 2). A list of all enriched GO terms is found in Supplemental Tables 5 and 6.

GO terms are organized into three groups: (1) biological process, (2) molecular function, and (3) cellular components. Qe enriched GO terms are split between the three groups 23.5 %, 70.6 % and 5.9 %, respectively. Qr enriched GO terms are split between the three groups 69.8 %, 16.4 % and 13.8 %, respectively (Table 2).

Within the list of enriched GOs pertaining to upregulated Qr genes, we found many GO terms that were related to defense response. Within Supplemental Table 6, terms directly associated defense response – GO phrases that contained the words immune, defense, hypersensitive, bacterial, bacterium, fungus, and oomycetes – have been bolded. Terms indirectly associated with defense response – GO phrases relating to salicylic acid (SA), jasmonic acid (JA), ethylene (ET), abscisic acid (ABA) and sugar modifications – have been underlined. Of the 159 enriched Qr GO terms, 56 (35.2%) have these descriptions: 37 GO terms were directly related defense response; 13 GO terms are potentially related to sugar signaling and sugar modification; 6 are related to hormone signaling (SA, ABA and CK, specifically).

The ten most significantly enriched GO categories for each species are shown in Figure 2. The word “category” is used here because multiple lower-level GO terms are included within a single category. These ten GO categories help to broadly define the molecular phenotypes attributed to upregulation within a species. The 10 most enriched GO categories for Qe are shown in Figure 2a. The 10 most enriched GO categories for Qr

are shown in Figure 2b. The lower-level GO terms within each category are shown in Supplemental Tables 7 and 8.

Enriched GO terms related to osmotic stress response and cell wall (OS/CW) annotate the overrepresented Qr DEGs. Enriched OS/CW GO terms for Qr include GO:0000302 (response to reactive oxygen species), GO:0006950 (response to stress), GO:0006979 (response to oxidative stress), GO:0008219 (cell death), GO:0009725 (response to hormone), GO:0009737 (response to abscisic acid), GO:0009751 (response to salicylic acid), GO:0010941 (regulation of cell death), GO:0012501 (programmed cell death), GO:0071554 (cell wall organization of biogenesis), GO:0071555 (cell wall organization), and GO:0080134 (regulation of response to stress) (Supplemental Table 8). Enriched GO terms related of OS/CW did not annotate upregulated Qe DEGs.

5.4.5 Significant DEGs and their relation to molecular phenotype

To summarize significant findings for Qe/Qr upregulated DEG libraries and their relationship to molecular phenotypes, I created four DEG groups. These groups independently examine gene upregulation for both species. Each group has ten upregulated DEGs. Genes in group I have the highest differential expression. Genes in group II are DEG with the highest differential expression that are annotated by at least one enriched GO category. Genes in group III are DEGs with the greatest differential expression that are annotated by at least two GO terms related to OS/CW: the OS/CW terms used for this group did not have to be enriched. Genes within group IV are species-specific gene expression markers (GEMs). Genes in group IV are the 10 most statistically significant

GEMs that are also annotated by at least one GO terms from either the enriched or OS/CV categories. This group is discussed in the next section of the results.

Group I genes for Qe and Qr are accordingly listed in Tables 3 and 5. Gene expression for this group of DEGs is correspondingly shown in Figures 3a and 3b. Within group I, average differential expression for Qe and Qr was 6.9 ± 0.4 and 11.5 ± 2.4 L2FC, respectively. Three Qr DEGs within group I had upregulation greater than 10 L2FC: Qurub.08G158900.1, Qurub.08G158800.1, Qurub.04G207300.1 (Figure 3b, Table 5).

Group II genes for QE and Qr are correspondingly listed in Tables 3 and 5. The gene expression for this group of DEGs is shown in Figures 3c and 3d, respectively. Within group II, average differential expression for Qe and Qr was 1.5 ± 0.6 and 7.8 ± 1.6 L2FC, accordingly. Enriched GO terms specific to DEGs from group II are listed in Tables 4 and 6. Overlap between groups I and II were observed for both species. Overlap between groups for Qe and Qr are listed in Tables 3 and 5.

All genes within Qe group II are annotated by GO terms from enriched categories plastid (GO:0009536) and chloroplast (GO:0009507): nine of these ten genes were directly annotated with the chloroplast GO term (Table 4). Other Qe enriched GO categories had little representations within this group. None of these genes were annotated by go terms from the enriched category plastid organization (GO:0009657) (Table 4).

Genes within Qr group II were commonly annotated by GO terms from the enriched categories response to stimulus (GO:0050896) and response to stress (GO:0006950): GO terms from these categories annotated seven of the group II genes (Table 6). GO terms

from the enriched categories membrane (GO:0016020), response to chemical (GO:004221), cellular response to stimulus (GO:0051716), and response to organic substance (GO:0010033) were the least common to annotate Qr group II: GO terms from these categories annotate four of these genes (Table 6). Qurub.04G207300.1, a Qr upregulated DEG with differential expression greater than 10 L2FC, is within group II: this gene is annotated by GO terms from all Qr enriched categories except GO:0051716 (cellular response to stimulus) (Table 6).

Within this experiment, group III DEGs was a category for genes with the greatest expression and annotated by OS/CW GO terms. Qe and Qr DEGs within group III are correspondingly listed in Tables 3 and 5. Expression levels are shown in Figure 3e and 3f, accordingly. Within group III, average differential expression for Qe and Qr was found to be 4.2 ± 0.3 and 5.2 ± 0.3 L2FC, respectively. Overlap between groups III and I were seen in both species for a single gene: Qurub.07G185300.1, and Qurub.09G222900.1 for Qe and Qr, respectively (Tables 3 and 5). Because Qe enriched GO terms were not related to OS/CW, we did not find any overlap between gene in these groups for Qe. Overlap between Qr groups III and II was observed for a single gene, Qurub.04G165800.1: this gene was annotated by the OS/CV terms GO:0006979 (response to oxidative stress), GO:0009693 (ethylene biosynthetic process), GO:0009733 (response to auxin), GO:0009753 (response to jasmonic acid), and GO:0010087 (phloem or xylem histogenesis) (Tables 3 and 5).

5.4.6 Species specific gene expression markers and molecular phenotyping

In this study, gene expression markers (GEMs) were identified to understand if differentially expressed genes that could be used to assign species identity. By identifying

(GEMs) for both species, I was attempting to characterize direct ES between Qe and Qr. In this experiment, GEMs are based on the assignment of DEGs as with greater representation within the Qe or Qr transcriptomes, these genes consistantly have a greater expression for either Qe or Qr.

51 and 78 potential GEMs were identified for Qe and Qr, respectively. Among the Qe GEMs, 17 (33.3 %) and 9 (17.6 %) were annotated by Qe enriched category or OS/CW GO terms(Supplemental Table 9). Among the Qr GEMs, 44 (56.4%) and 28 (34.6%) were annotated by Qr enriched category or OS/CW GO terms(Supplemental Table 10).

Genes within DEG group IV are the 10 most statistically significant GEMs that are also annotated by at least one GO terms from either the enriched or OS/CV categories. GEMs for Qe and Qr are listed in Tables 3 and 5, respectively. The GO terms associated with each GEM are listed in Tables 4 and 6, respectively. For Qe, genes within groups II and III overlapped with group IV. Within Qe group II are five GEMs: Qurub.01G026900.1, Qurub.02G360100.1, Qurub.06G228100.1, Qurub.09G019200.1 and Qurub.09G098000.1. Within Qe group III is a single GEM Qurub.05G283400.1 (Table 3). The Qr GEMs did not have overlap with any genes in Qr DEG groups (Table 5).

5.4.7 Qr upregulated DEGs and GEMs are annotated by defense response GO terms

GO terms related to defense response annotate genes from Qr groups I-IV. Of the 33 genes listed in Qr DEG groups I-IV, fifteen genes (11 DEGs and 4 GEMs) were annotated by seventeen different GO terms related to defense response (Table 6). Twelve of these seventeen GO terms are directly related to defense response. GO terms directly

related to defense response contain words like immune, defense, hypersensitive, bacterial, bacterium, fungus, or oomycetes. These terms annotate six DEGs and three GEMs (Table 6). The other five defense response GO terms were indirectly related. GO terms that are indirectly related to defense response contain words or phrases related to salicylic acid (SA), jasmonic acid (JA), ethylene (ET), abscisic acid (ABA), sugar modifications, and sugar signaling. Four of these GO terms related to phytohormone signaling: these terms annotated seven DEGs and one GEM. The other GO term (Glucuronosyltransferase activity: GO:0015020) is related to sugar modification and sugar signaling. Two DEGs are annotated by this term (Table 6).

Looking at the annotation specific to Qr GEMs, I found that 50 % of these genes (39 out of 78) were annotated by a GO term directly or indirectly related to defense response (seen as bolded gene ID in Supplemental Table 10). 25 of these 39 genes were annotated by GO terms directly associated with defense response. 14 of these 39 genes were annotated by GO terms indirectly associated with defense response (Supplemental Table 10).

5.4.8 Qe and Qr eQTL hotspot distribution and descriptions

In this experiment, eQTL hotspots identify chromosomal regions that are related to enriched GO categories for both species. These eQTLs identify genomic regions related to molecular phenotypes observed in both species. Two Qe eQTL hotspots were identified within chromosomes 10 and 11. Each hotspot contained two upregulated Qe DEGs. These genes within these hotspots, Qe10 and Qe11 are annotated by a single enriched GO term: GO:0009535 (chloroplast thylakoid membrane) and GO:0009507 (chloroplast),

respectively (Table 7). None of the Qe hotspot genes are found within the Qe DEG groups (Tables 4 and 7).

For Qr fourteen eQTL hotspots were identified. These Qr hotspots were found in ten of the twelve Qr chromosomes. Of the fourteen hotspots, thirteen were defined by two upregulated Qr DEGs. The other Qr hotspot, Qr5, had three upregulated Qr DEGs (Table 8). The most common Qr enriched GO category annotating these hotspots were GO:0050896 (response to stimulus), GO:0006950 (response to stress) and GO:0006952 (defense response): these categories annotated ten of the fourteen hotspots. The least common Qr enriched GO category for these hotspots was GO:0010033 (response to organic substance): these terms annotated three of the fourteen hotspots (Table 8). The genes Qurub.05G085300.1 and Qurub.02G400800.1, which are respectively within the hotspots Qr5 and Qr2, are also within DEG groups II and III (Table 8 and 3).

The majority of the eQTL hotspots from Qr were annotated by enriched GO terms that were directly/indirectly associated with defense response. The directly associated GO terms annotating Qr hotspots are GO:0002237 (response to molecule of bacterial origin), GO:0002764 (immune response-regulating signaling pathway), GO:0006952 (defense response), GO:0009626 (plant-type hypersensitive response), GO:0016045 (detection of bacterium), GO:0042742 (defense response to bacterium), GO:0045089 (positive regulation of innate immune response), GO:0050829 (defense response to Gram-negative bacterium), GO:0050832 (defense response to fungus), and GO:0098542 (defense response to other organism). The indirectly associated GO term annotating the Qr hotspots was GO:0009737 (response to abscisic acid). A total of 9 hotspots – Qr1, Qr3.1, Qr5, Qr6,

Qr8.2, Qr8.3, Qr9.2 and Qr10 – are annotated by these terms. Genes within in these hotspots are homologous to disease resistance proteins, WRKY transcription factors, hormone signaling genes (Table 8).

5.4.9 Pressure volume curves

The results from our pressure volume curve (PVC) experiment found significantly lowered π_0 and π_{TLP} measurement within the Qe provenance compared to Qr. Mean π_0 for Qe and Qr were found to be -1.49Mpa and -0.95Mpa, respectively, and mean π_{TLP} for Qe and Qr were found to be -2.48Mpa and -1.58Mpa, respectively (Figure 4 and Table 9). Although RWC at TLP was greater in QE, a statistically significant difference was not observed between species (Table 9).

5.4.10 Optical vulnerability

Statistical significance was not observed between Qe and Qr for P_e, Ψ_{50}, Ψ_{88}, and MS measurements (Table 9). This result may be related to not gathering results for one of the Qr samples (12.065.13). Mean measurements for P_e, Ψ_{50}, and Ψ_{88} were lower in Qe, but as tissue embolism increased, differences between Qe and Qr measurements became smaller (Table 9, Figure 5). From the OV composite images in Figure 6, we observed that most Qe embolisms occurred at lower pressure potentials then Qr (-3.5Mpa and -2.3Mpa, respectively). Both species had similar negative MS; this demonstrates that stomatal closure occurs after 5% of tissue has embolized (Table 9).

5.5 Discussion and conclusions

5.5.1 Similar transcriptomic gene expression observed between Qe and Qr leaves

When conducting this experiment, we initially expected that there would be many DEGs between Qe and Qr. This expectation was based is based on a previous RNA-seq experiment that compared the transcriptomes from two branches from the same individual tree. For this experiment, one branch was disease resistant and the other was disease susceptible, and although these branches belonged to the same individual thousands of DEGs could be assigned to each branch (Padovan et al., 2013). The experiment here was conducted across two species, so we initially thought that there would be thousands of DEGs that would distinguish these two species from each other. It was very surprising to find that Qe and Qr only had 782 unique DEGs between both transcriptomes. This result demonstrates that ES does always cause substantial changes between gene expression of different species.

In the case of transcriptomic data, PCAs are conducted without prior knowledge specific to a sample library's origin or a gene's function (Lever et al., 2017). Using PCA for initial analysis of Qe and Qr leaf transcriptomes, we did not observe unique clusters related to species or provenance (Figure 1). Studies using genetic molecular markers to study ES among species within section Lobatae – including Qe and Qr – have demonstrated that species identity can often result from the fixation of a handful of alleles (Gailing et al., 2012; Hipp et al., 2010; Owusu et al., 2015). Gene expression is a heritable trait that is responsive to environmental pressures (Falconer et al., 1996; Gibson et al., 2005; Grishkevich et al., 2013; Lasky et al., 2014). Thus, these results suggest, that under the

environmental conditions within a common garden location, Qe and Qr have similar gene expression profiles, potentially due to high interspecific gene flow.

5.5.2 Qe molecular phenotype related to increased photosynthetic capacity

Within the Qe transcriptomes, the majority of genes with increased differential expression were related to photosynthesis The 304 Qe upregulated genes are annotated by 1,284 unique GO terms, but only 17 (1.3 %) were found to be enriched (Table 2). For reference, among the Qr upregulated genes,1,782 unique GO terms were observed of which 159 (8.9 %) were enriched (Table 2). Furthermore, among the enriched Qe GO terms, there was low diversity: 16 of the 17 enriched Qe GO terms were directly related to photosynthesis/chloroplasts (Supplemental Table 5). This result may demonstrate that ES between Qe and Qr favors increased differential gene expression in Qe related to photosynthesis.

This Qe molecular phenotype might help to explain the site preferences of Qe and Qr. Previously work reports that Qr inhabits mesic environments, while Qe preferentially resides in xeric sites with greater irradiance (Abrams, 1990). Although sunlight is a primary requirement for photosynthesis, photosynthetic machinery can be paradoxically damaged by overexposure and, moreover, lower water availability exacerbates the stress experienced by the Qe photosynthetic machinery (Woo et al., 2008). Survival within these abiotically stressful environments may require Qe to synthesize photosynthesis related genes at faster rates than what is observed in Qr. This molecular phenotype may have become fixed within Qe and now serves as a distinguishable feature of the two species. This result could explain higher rates of A_{max}, the maximum capacity rate of photosynthesis, within Qe (see results

from Chapter 4, Table 2). By increasing transcription of genes related to photosynthesis, Qe has potential for greater rates of photosynthesis than Qr and is better adapted to sites with greater irradiance than Qr.

5.5.3 Qr molecular phenotype related to defense response

When comparing the Qe and Qr transcriptomes, I observed increased differential expression for genes related to defense response in Qr. Plant defense and immunity is a system of surveillance that is responsive to pathogen activity and physical injury (Boller et al., 2009; Jones et al., 2006; Reimer-Michalski et al., 2016). For plants, pathogenic organisms include bacteria, fungi, and oomycetes. Based on their specific interactions with the plant host, these pathogens can be categorized as biotrophic or necrotrophic pathogens (Boller et al., 2009; Jones et al., 2006). Biotrophs are microorganisms that use living host cells to acquire nutrients (Andolfo et al., 2015). To detect this class of pathogen, plants primarily rely on of salicylic acid (SA) signaling (Glazebrook, 2005). The other type of pathogen, necrotrophs, are microorganisms that use various enzymes and toxins to kill tissues of the host for the purposes of feeding (Andolfo et al., 2015). To detect this pathogen type, plants primarily rely on jasmonic acid (JA), ethylene (ET), and sugar signaling pathways (Glazebrook, 2005). In response to physical injury, which can be caused by insect feeding and egg deposition, plant innate immunity responds by wound-induced resistance (WIR) (Reimer-Michalski et al., 2016; Ryan, 1990). The WIR response triggers the accumulation of protease inhibitors, a class of genes that inhibits digestion of plant material by insect-gut proteases (Ryan, 1990). Initiation of WIR primarily relies on JA signaling (Howe, 2004).

Although there are primary signaling pathways associated with aspects of the innate immune response, plant innate immunity is moderated by extensive crosstalk from many phytohormone signals (Kazan et al., 2014; Mur et al., 2006; Reymond et al., 2000; Robert-Seilaniantz et al., 2011; Scala et al., 2013; Scharte et al., 2005; Trouvelot et al., 2014). Abscisic acid (ABA) signaling has been found to have extensive crosstalk related to pathogen detection and WIR. In addition to crosstalk with multiple sugar signaling pathways, ABA is found to antagonize and synergize with innate immune response related to SA and JA, respectively (Andolfo et al., 2015; Asselbergh et al., 2008; Pieterse et al., 2009; Reymond et al., 2000; Scala et al., 2013). In addition to ABA, the innate immune system can also be influenced by crosstalk from auxin, cytokinins, brassinosteroids, gibberellins, and strigolactones signaling (Andolfo et al., 2015; Asselbergh et al., 2008).

The importance of defense and immune responses as a Qr specific molecular phenotype was observed regularly within our results. When examining the 159 enriched GO terms specific to the unregulated Qr genes, we found that 35.2 % (56/159) could be linked to this phenotype. Among these defense response terms, 23.3 % (37/159) were directly affiliated. The remaining 11.9% (19/159) were indirectly affiliated with defense response: these GO terms were associated with relevant phytohormones and sugar signaling/modification (Supplemental Table 6). Annotation for defense response molecular phenotype was common among the significant Qr DEGs too: 19 of the 33 (57.6 %) significant Qr upregulated genes have defense response annotation (Table 6). This annotation is also commonly observed for the other upregulated Qr genes (Supplemental Table 4), and these results identify eQTLs specific to the Qr defenseresponse molecular phenotype. Significance of the defense response molecular phenotype is also observed

across multiple Qr eQTL hotspots. The Qr eQTL hotspots are genomic regions where multiple upregulated gene are found in close chromosomal proximity to one another: all genes within these hotspots share common annotation. Because these genes share close proximity and annotation, their transcription is possibly linked to a specific environmental cue (H. W. Nützmann et al., 2016; Shoji et al., 2021). For the Qr hotspots, 64.3 % (9/14) are annotated with GO terms directly related to defense response (Table 8). Many of the genes found in these eQTLs are significant too, particularly interesting are the homologs to WRKY transcription factors 55 and 70 and their annotation by the "Defense response to fungus" GO term (Table 8). Within *Arabidopsis*, expression of the WRKY55 transcription factor increases in response to fungal infection (Brotman et al., 2013), and the WRKY70 transcription factor has been found to increase resistance to fungal pathogens by enhancing and diminishing SA and JA mediated responses, respectively (Li et al., 2006; Li et al., 2004). If these functions are conserved by the Qr homologous genes, this eQTL hotspot may have a significant role in regulating response to biotrophic fungal pathogens.

SA and ABA signaling pathways can activate and diminish response to biotrophic pathogens, respectively (Andolfo et al., 2015). Among the upregulated Qr gene, GO terms for response to ABA and SA (GO:0009737 and GO:0009751) were frequently observed. Of the 478 upregulated Qr genes, 55 (11.5 %) had this annotation. Future experimentation could investigate the role that ABA and SA have in Qr defense response.

Despite observing an increased defense response molecular phenotype, observations by our lab have found that Qr suffers more biotic stress than Qe (data not presented). These results may be related to the differences in native ranges of both species.

Since Qr has a greater natural range (Stein, 2003), it is likely the host for a wider range of parasites, herbivores, and pathogens than Qe. Moreover, within the common gardens, Qe is not within its native habitat, and species outside of their traditional range are often seen to have a reduction in pests and pathogens (Torchin et al., 2004). Since plant transcriptomes are dynamic in response to the environment (Padovan et al., 2013; Seki et al., 2002), the defense response specific to Qr might also be related to the lack of herbivory and infection in Qe (Nobori et al., 2018). Additionally, the increased biotic stress exerted on Qr might also be related to the differential gene expression. When comparing the Qr and Qe transcriptome, we find that the most upregulated genes for Qr are homologs for chalcone synthase (Qurub.08G158900.1 and Qurub.08G158800.1) (see Qr DEG group I, Table 5), while some of the most downregulated genes are a homologs for sesquiterpene synthase (Qurub.08G062400.1 and Qurub.08G158700.1) (see Qe DEG group I, Table 3). Both chalcone and sesquiterpene are plant secondary metabolites (Külheim et al., 2011). Chalcone is a molecular precursor for flavonols and isoflavonoids, which are molecular compounds with anti-fungal properties (Moore et al., 2014). Sesquiterpenes are molecules that attract the predators of insect herbivores (Degenhardt et al., 2003; Moore et al., 2014). Although we cannot say with certainty, increased expression of chalcone synthase within Qr might be increasing secondary metabolites for fungal defense. It is also possible that the decrease in sesquiterpene synthase makes Qr an easier target for herbivory compared to Qe.

The defense response molecular phenotype associated with Qr may also partially explain why Qe has greater transcription in gene related to photosynthesis. Processes related to the defense response molecular phenotype are associated with repression of

genes related to photosynthesis; this occurs to diminish the hosts support of biotroph and necrotroph pathogens (Rolland et al., 2006; Scharte et al., 2005). Thus, it is possible that the enriched GO terms specific to the upregulated Qe DEGs are a result related to a down-regulation of photosynthesis related gene within Qr.

5.5.4 Fixed patterns of gene expression related to species

In addition to molecular phenotypes, I also identified GEMs, which provide additional information on ES between Qe and Qr that is related to fixed gene expression. The GEMs in this study are species specific overrepresented genes that have common gene expression patterns across population and species. Future studies should investigate if these patterns of fixed gene expression are maintained within southern ecotypes for Qe and Qr. It is possible that these patterns of gene expression would not be maintained across different ecotypes. Furthermore, transcriptomes are dynamic in their responses to environmental stimuli (Matsui et al., 2008; Seki et al., 2002), therefore it is possible that the GEMs discovered by this study might not be observed in an RNA-seq experiment conducted at different times of the growing season and/or different times of the day.

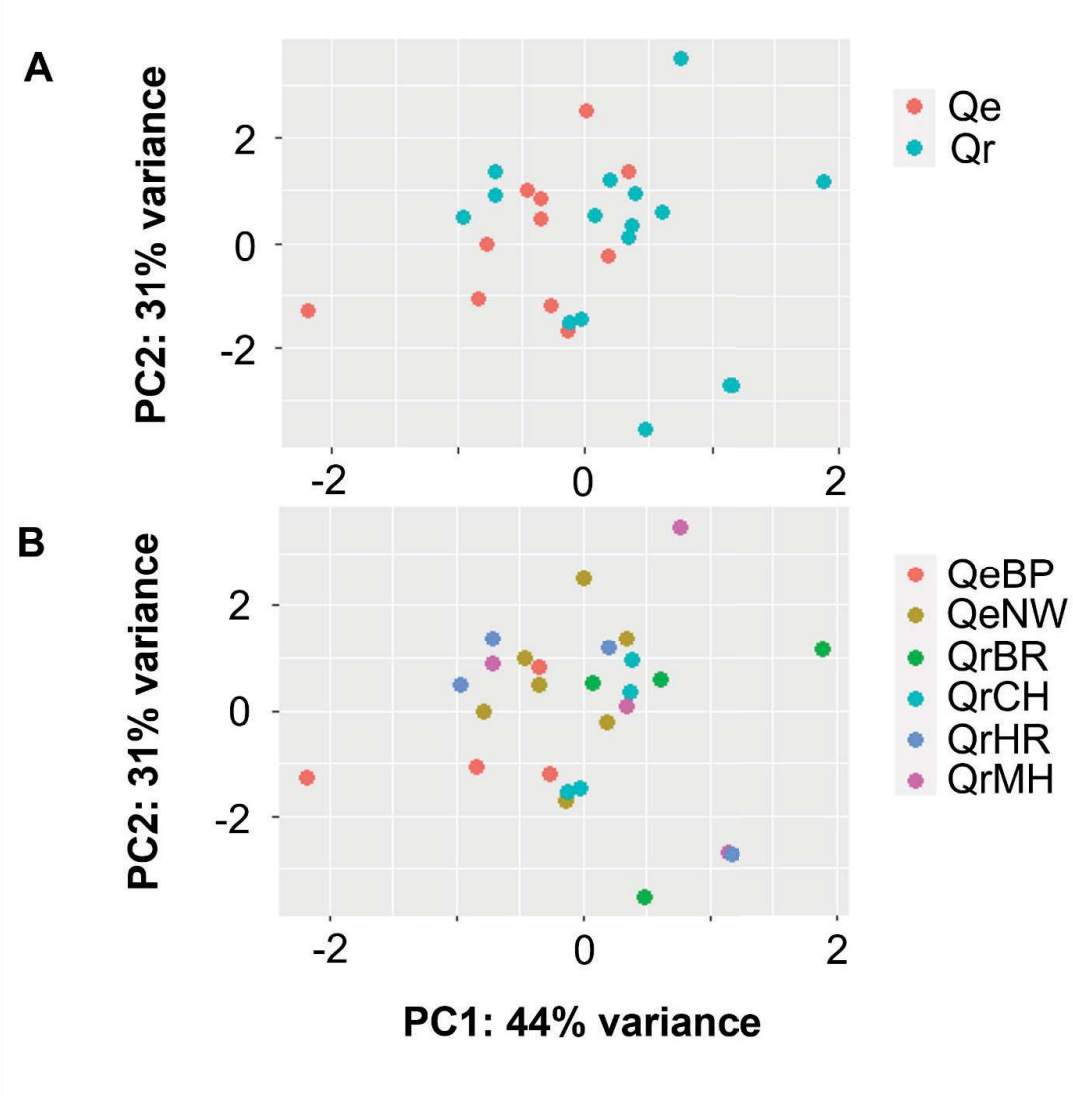
5.5.5 Hydraulic traits and their relation to molecular phenotype

Results observed in our PVC and OV experimentation were aligned with our initial hypothesis. In both experiments we found that Ψ_{leaf} measurements were lower within Qe, and although the results from the OV experimentation were not significant – possibly due to low sample number in Qr – the mean values for P_e, Ψ_{50}, Ψ_{88} and the hydraulic SM were lower in Qe (Table 9). These results possibly demonstrate that Qe is better adapted than Qr to increasing drought severity.

Contrary to our initial hypothesis, we observed multiple enriched GO terms specific to OS/CW within Qr, while no OS/CW enriched GO terms were observed Qe. Since Qe has preference for xeric habitats, this result was initially surprising. It is possible that environmental conditions at the Ford Forest did not elicit drought adaptive molecular phenotype within Qe. Future experimentation should attempt to investigate this, perhaps by sampling differences between Qe and Qr transcriptomes near the P_e, Ψ_{50} and Ψ_{88} timepoints. This may indicate drought conditions are required for this Qe trait to be measurable.

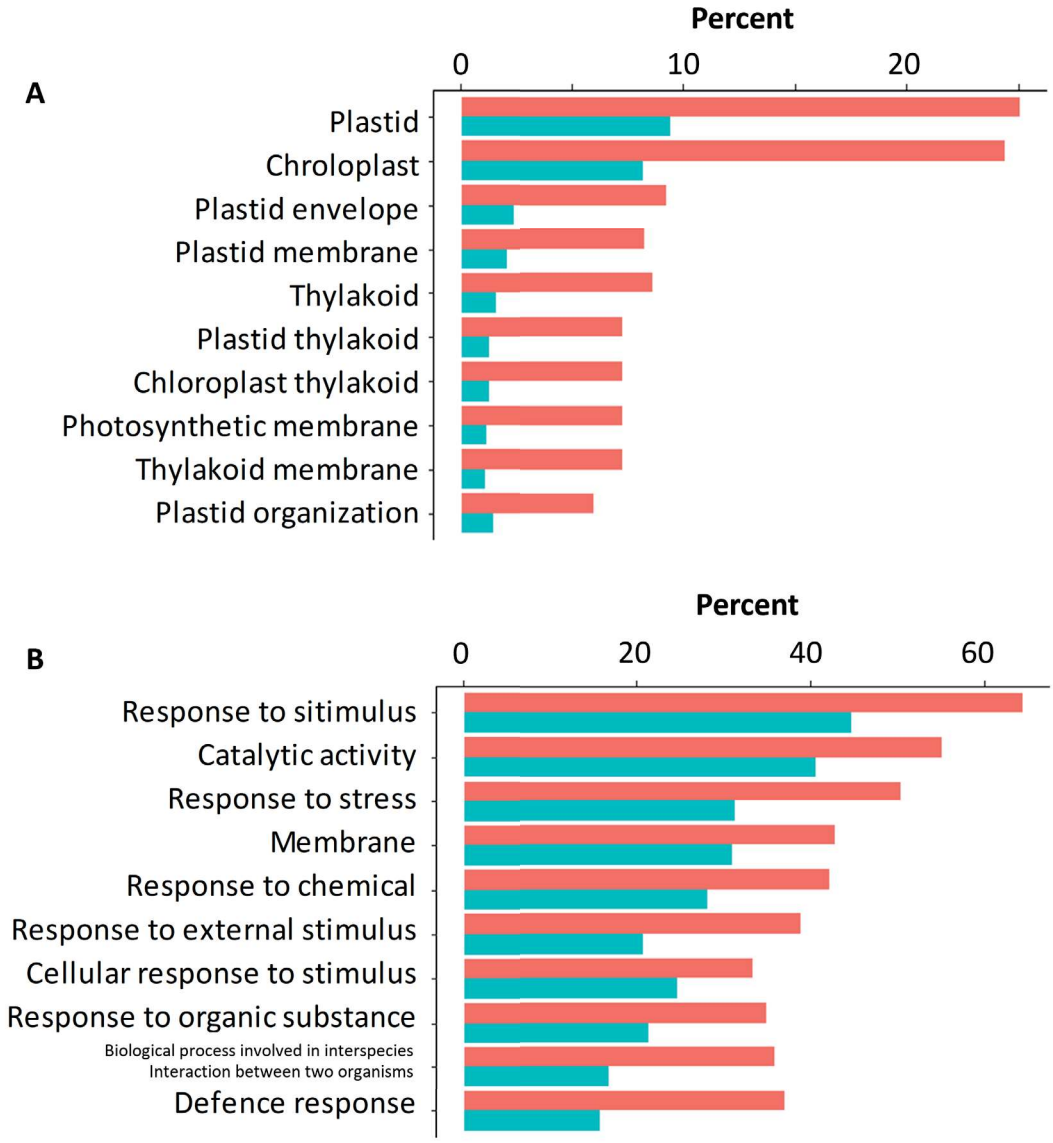
5.6 Chapter 5 figures

5.6.1 Figure 1



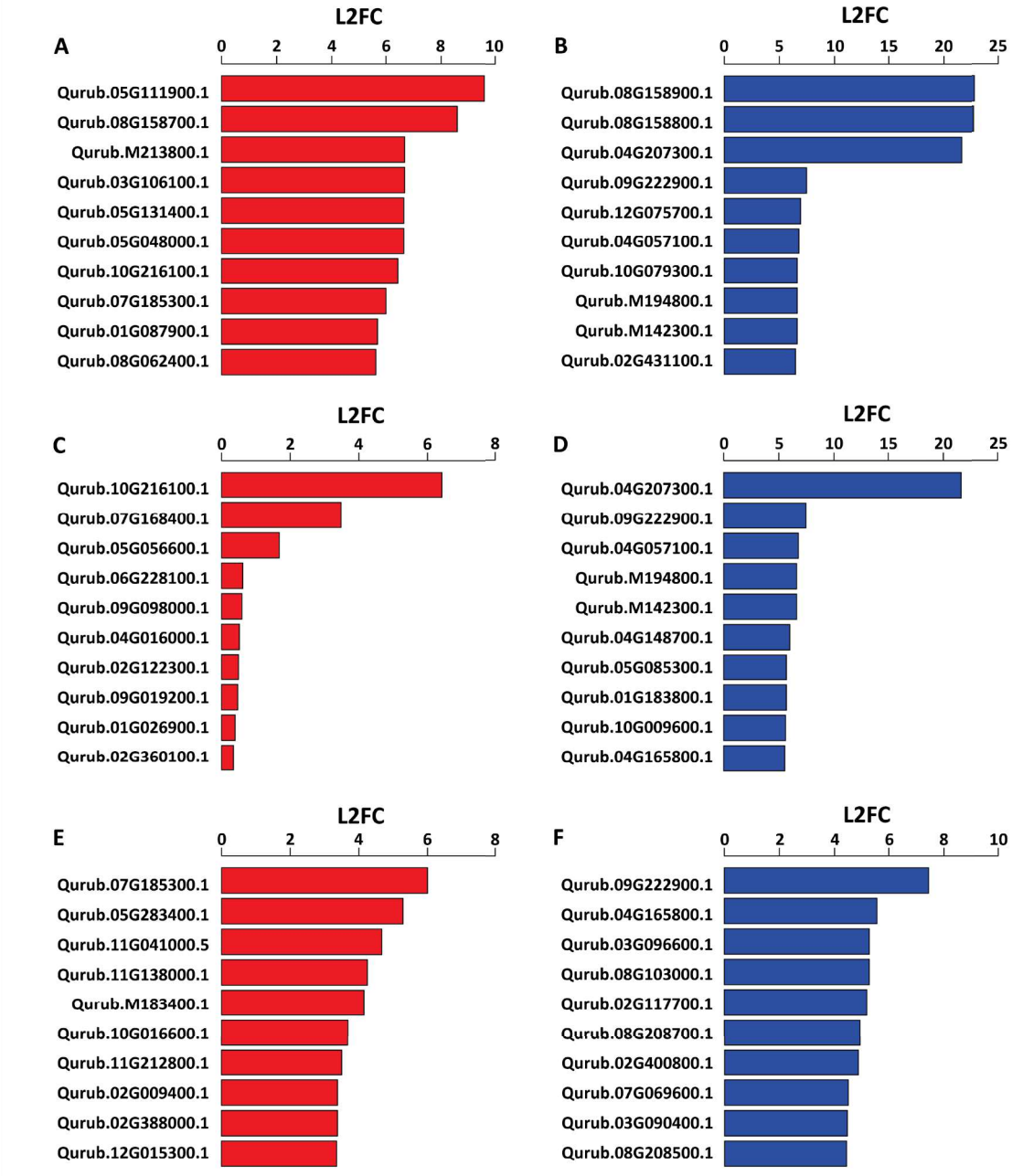
PCA biplots with (**A**) species and (**B**) provenances labels. The eigenvectors, PC1 and PC2, are plotted on the x and y axis and explain 44% and 31% of the variance, respectively. Abbreviations: QeBP – Baraga Plains; QeNW – Northern Wisconsin; QrBR – Blueberry Ridge; QrCH – Copper Harbor; QrHR – Huron River; QrMH – Maasto Hiihto.

5.6.2 Figure 2



Enriched GO terms specific to (A) Qe and (B) Qr. On the y axis are the GO term names. On the x-axis are the percent of sequences in each species DEG set and the Qr genome (reference set). In each figure panel, the species DEG sets, and the reference set are respectively labeled red and blue.

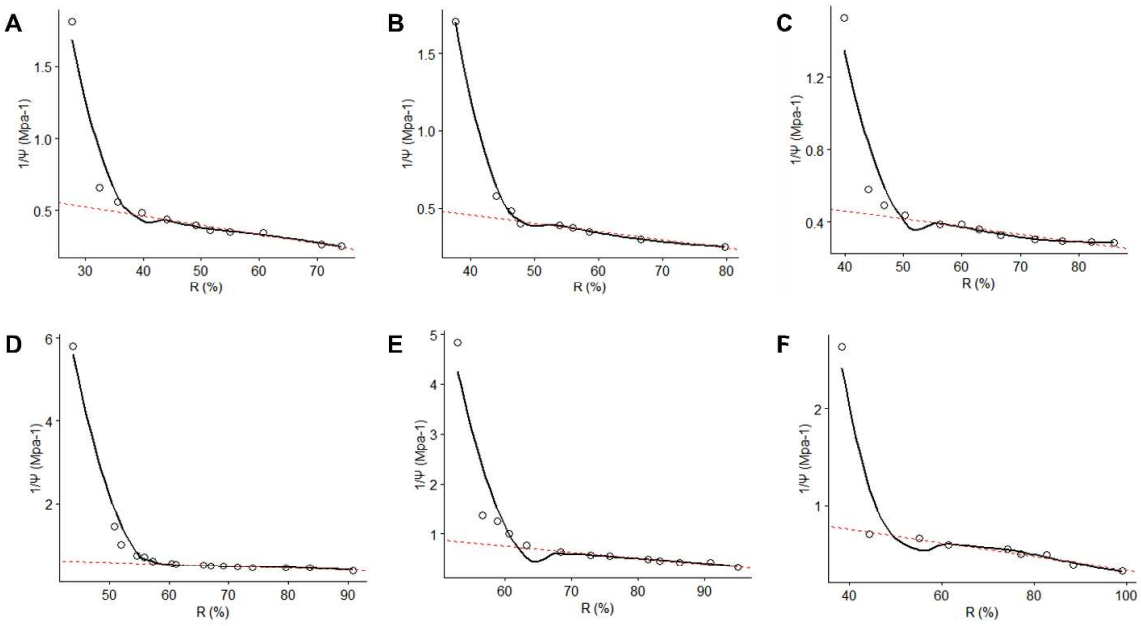
5.6.3 Figure 3



Expression levels for significant DEGs from Qe (red) and Qr (blue). Expression levels are count values expressed as log2 fold-change. (A, B) The 10 DEGs with highest differential expression: more information is found in Tables 3 and 4, respectively. (C, D) The 10 most statistically significant DEGs with GO terms related to the 10 most enriched categories

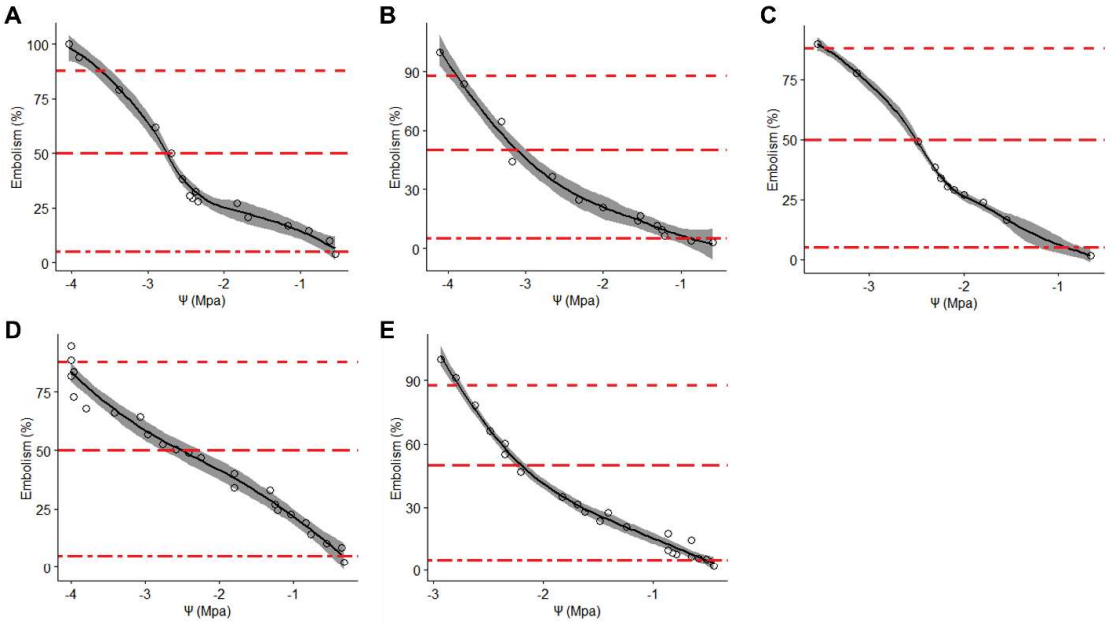
from their respective species: more information is found in Tables 5 and 6, respectively. (E, F) The 10 DEGs with highest differential expression and at least 2 GO terms related to plant osmotic stress response or cell wall: more information is found in Tables 7 and 8, respectively.

5.6.4 Figure 4



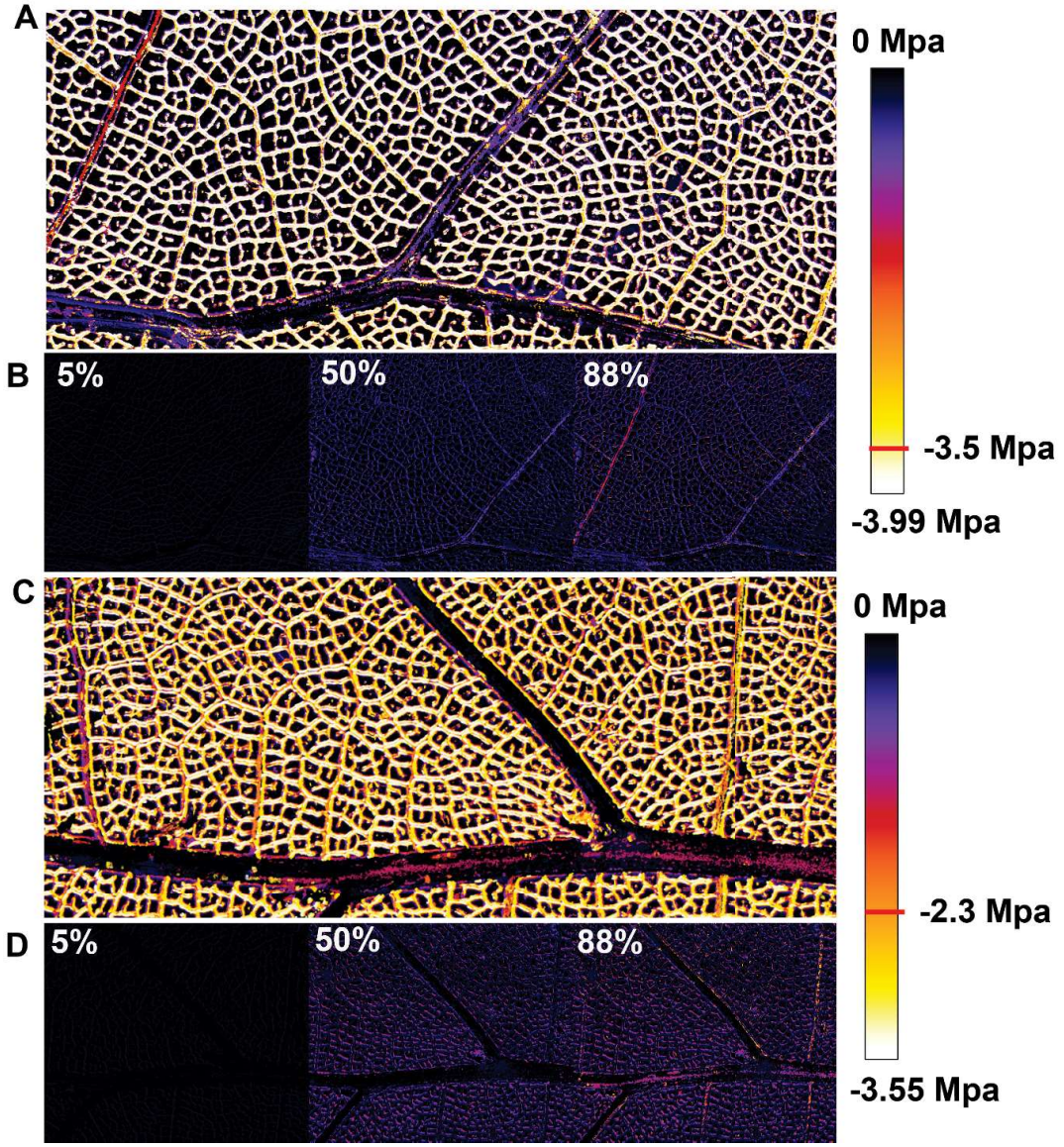
Pressure volume curves from different families within provenances QeBP (A-C) and QrHR (D-F). On the x axis is R (100-RWC; %). On the Y axis is 1/ψ (Mpa^{-1}), the reciprocal of all ψleaf measurements from the experiment. Dashed red lines show the 1/ψs linear relationship.

5.6.5 Figure 5



Optical vulnerability curves from different families within provenances QeBP (A-C) and QrHR (D-E). On the x axis is ψ (ψ_{leaf}, Mpa). On the Y axis is Percent embolism (%). Intersection of the dot-dash red line and the optical vulnerability curve show ψ_{leaf} at 5% embolism. Intersection of the long-dashed red line and the optical vulnerability curve show ψl_{eaf} at 50% embolism. Intersection of the short-dashed red line and the optical vulnerability curve show ψ_{leaf} at 88% embolism.

5.6.6 Figure 6



Optical vulnerability results for selected samples for Qe (QeBP) (A and B) and Qr leaf (QrHR) (C and D). The leaf venation has been colored to indicate the pressure potential when embolism occurred. (A and C) Z project stacked image for the leaf section (final image is at the top of the stack). (B and D) Cumulative Z project montage for the leaf section, showing embolism progression at 5%, 50%, and 88% embolism (first images are at the top of the stacks). Pressure potential scales are shown next to their respective images.

5.7 Chapter 5 tables

5.7.1 Table 1

Information on the Qe and Qr provenances. Initially we intended to have 4 samples per population. Unfortunately, we had difficulties isolating RNA from 5 Qe samples from populations 24, 25, and 26 and had to combine these populations to a single provenance (QeNW). Abbreviations: ID – identification number; Lat. – latitude; Long. – longitude; MAT – mean annual temperature; Pro. ID – provenance identity.

Species	ID	Lat.	Long.	MAT °C	Pro. ID (abbreviation)	Samples
Qe	10	46.66	-88.58	4.4	Baraga Plains (QeBP)	4
Qe	24	46.06	-89.62	4	Northern Wisconsin (QeNW)	7
Qe	25	46.72	-91.05	4.4	Northern Wisconsin (QeNW)	
Qe	26	46.65	-91.74	4.6	Northern Wisconsin (QeNW)	
Qr	2	47.44	-88.22	4.5	Copper Harbor (QrCH)	4
Qr	4	47.14	-88.61	4.9	Maasto Hiihto (QrMH)	4
Qr	12	46.91	-88.04	5.4	Huron River (QrHR)	4
Qr	14	46.46	-87.42	5	Blueberry Ridge Trails (QrBR)	4

5.7.2 Table 2

GO term summary. The "total" category refers to all unique GO terms from the species DEG list. The "significant" category refers to all unique, enriched GO terms for the species DEG list. For each species, the GO terms were binned into biological process, molecular function, and cellular component Go categories; count and percentages are listed here. The final column indicates the percent of the total GO terms that were significant for each species. Abbreviations: DEG – differentially expressed genes; GO – gene ontology.

	Total		**Significant**		**%**
	Count	**%**	**Count**	**%**	
Qe GO terms	**1282**		**17**		**1.33**
Biological Process	746	58.19	4	23.53	0.54

Molecular function	340	26.52	12	70.59	3.53
Cellular Component	196	15.29	1	5.88	0.51
Qr GO terms	**1782**		**159**		**8.92**
Biological Process	1145	64.25	111	69.81	9.69
Molecular function	407	22.84	26	16.35	6.39
Cellular Component	230	12.91	22	13.84	9.57

5.7.3 Table 3

Descriptions of significant Qe DEGs. Gene ID numbers, descriptions, and differential gene expression (L2FC) are used to describe each DEG. Each DEG listed here is relevant to at least one group; each group is populated by 10 DEGs. Group I: DEGs with highest differential gene expression. Group II: DEGs annotated by Qe enriched GO categories with the highest differential expression. Group III: DEGs annotated by at least 2 OS/CW GO terms with highest differential expression. Group IV: statistically significant gene expression markers with annotation specific to the Qe enriched and OS/CW GO terms. Statistical information specific to category 4 on the right of the table: the "> 97.5% CI" column indicates how many samples from each species had an RPKM above the 97.5% threshold based on Qr sample mean; 11 and 16 samples were assigned as Qe and Qr, respectively. Statistical significance of each gene expression marker is described in the $\chi 2$ column: one, two, and three asterisks indicates that p-values were less than 0.05, 0.01, and 0.005, respectively.

			Categories				> 97.5% CI		
Gene ID	**Homolog/Description**	**L2FC**	**I**	**II**	**III**	**IV**	**Qe**	**Qr**	**$\chi 2$**
Qurub.05G111900.1	Flotillin-like protein 2	9.6	X				5	2	NA
Qurub.08G158700.1	(-)-germacrene D synthase	8.6	X				5	1	NA
Qurub.M213800.1	LRR receptor-like serine/threonine-protein kinase	6.7	X				5	1	NA
Qurub.03G106100.1	Ankyrin-3	6.7	X				4	3	NA

Qurub.05G131400.1	14-3-3-like	6.6	X				6	2	NA
Qurub.05G048000.1	NA	6.6	X				2	1	NA
Qurub.10G216100.1	Inactive purple acid phosphatase 27	6.4	X	X			7	3	NA
Qurub.07G185300.1	Nucleoredoxin 1-1	6.0	X		X		5	2	NA
Qurub.01G087900.1	LURP-one-related 7	5.7	X				5	2	NA
Qurub.08G062400.1	Sesquiterpene synthase 2	5.6	X				4	2	NA
Qurub.07G168400.1	Spermidine hydroxycinnamoyl transferase	3.5		X			4	3	NA
Qurub.05G056600.1	Uncharacterized protein	1.7		X			8	4	NA
Qurub.06G228100.1	Guanylate kinase	0.6		X		X	10	3	**
Qurub.09G098000.1	Protein low PSII accumulation 2	0.6		X		X	11	5	*
Qurub.04G016000.1	Chloroplastic small ribosomal subunit protein cS22	0.5		X			10	7	NA
Qurub.02G122300.1	ATP synthase subunit b'	0.5		X			10	5	*
Qurub.09G019200.1	Chaperone protein DnaJ	0.5		X		X	11	3	***
Qurub.01G026900.1	50S ribosomal protein L15	0.4		X		X	11	6	*
Qurub.02G360100.1	Heme oxygenase	0.3		X		X	10	4	*
Qurub.05G283400.1	GDSL esterase/lipase 5	5.3			X	X	8	2	*
Qurub.11G041000.5	Regulator of nonsense transcripts 1 homolog	4.7			X		7	3	NA
Qurub.11G138000.1	L-type lectin-domain containing receptor kinase IX.1	4.3			X		2	1	NA
Qurub.M183400.1	Polygalacturonase-1 non-catalytic subunit beta	4.2			X		2	2	NA
Qurub.10G016600.1	Salicylic acid glucosyltransferase 1	3.7			X		6	7	NA
Qurub.11G212800.1	Spermidine hydroxycinnamoyl transferase	3.5			X		5	6	NA
Qurub.02G009400.1	Vacuolar-processing enzyme	3.4			X		7	2	NA
Qurub.02G388000.1	Receptor kinase-like protein Xa21	3.4			X		5	3	NA
Qurub.12G015300.1	AAA-ATPase 1	3.4			X		8	4	NA
Qurub.05G182800.1	Transcription factor EGL1	2.7				X	9	2	**
Qurub.09G216900.1	ETHYLENE-DEPENDENT GRAVITROPISM-DEFICIENT AND YELLOW-GREEN 2	1.1				X	11	4	**
Qurub.10G154600.1	Chlorophyll a-b binding protein P4	0.5				X	11	4	**

Qurub.10G174600.1	70 kDa ribosomal protein S6 kinase 1	0.5				X	10	4	*

5.7.4 Table 4

GO annotation specific to Qe enriched and OS/CW terms for significant Qe DEGs. GO terms with superscripted numbers are from the 10 most enriched Qe GO categories. Significance of the GO terms are organized in a descending order: (1) plastid; (2) chloroplast; (3) plastid envelope; (4) plastid membrane; (5) thylakoid; (6) plastid thylakoid; (7) chloroplast thylakoid; (8) photosynthetic membrane; (9) thylakoid membrane; (10) plastid organization. Each DEG listed here is relevant to at least one group; each group is populated by 10 DEGs. Group I: DEGs with highest differential gene expression. Group II: DEGs annotated by Qe Enriched GO terms with the highest differential expression. Category III: DEGs annotated by at least 2 OS/CW GO terms with highest differential expression. Category IV: statistically significant gene expression markers with annotation specific to the Qe enriched and/or OS/CW GO terms. A complete list of all GO terms annotation for each Qe DEG is found in Supplementary Table 3.

Gene ID	Category	GO terms
Qurub.05G111900.1	I	NA
Qurub.08G158700.1	I	NA
Qurub.M213800.1	I	NA
Qurub.03G106100.1	I	NA
Qurub.05G131400.1	I	NA
Qurub.05G048000.1	I	No terms
Qurub.10G216100.1	I,II	Chloroplast[1,2]
Qurub.07G185300.1	I,III	Cellular oxidant detoxification; Thioredoxin-disulfide reductase activity; Oxidoreductase activity
Qurub.01G087900.1	I	NA
Qurub.08G062400.1	I	NA
Qurub.07G168400.1	II	Chloroplast[1,2]

Qurub.05G056600.1	II	Chloroplast[1,2]
Qurub.06G228100.1	II,IV	Chloroplast[1,2]
Qurub.09G098000.1	II,IV	Chloroplast[1,2]; Chloroplast thylakoid[1,2,5,6,7]; Plastid[1]
Qurub.04G016000.1	II	Chloroplast[1,2]
Qurub.02G122300.1	II	Chloroplast thylakoid membrane[1,2,3,4,5,6,7,8,9]
Qurub.09G019200.1	II,IV	Chloroplast[1,2]
Qurub.01G026900.1	II,IV	Chloroplast[1,2]
Qurub.02G360100.1	II,IV	Regulation of stomatal movement; Chloroplast[1,2]
Qurub.05G283400.1	III,IV	Response to salicylic acid; Response to jasmonic acid; Induced systemic resistance, ethylene mediated signaling pathway; Jasmonic acid and ethylene-dependent systemic resistance, ethylene mediated signaling pathway; Negative regulation of auxin mediated signaling pathway
Qurub.11G041000.5	III	Salicylic acid mediated signaling pathway; Jasmonic acid mediated signaling pathway
Qurub.11G138000.1	III	Systemic acquired resistance, salicylic acid mediated signaling pathway; Positive regulation of hydrogen peroxide metabolic process; Positive regulation of cell death
Qurub.M183400.1	III	Plant-type cell wall modification; Cell wall modification involved in multidimensional cell growth; Plant-type cell wall
Qurub.10G016600.1	III	Salicylic acid metabolic process; Cellular response to water deprivation; Cellular response to hydrogen peroxide; Cellular response to abscisic acid stimulus; Cellular hyperosmotic salinity response; Salicylic acid glucosyltransferase (ester-forming) activity; Salicylic acid glucosyltransferase (glucoside-forming) activity
Qurub.11G212800.1	III	Lignin biosynthetic process; Auxin homeostasis
Qurub.02G009400.1	III	Response to ethylene; Response to salicylic acid; Response to jasmonic acid; Leaf senescence
Qurub.02G388000.1	III	Cellular response to stress; Cell wall organization; Response to oxygen-containing compound
Qurub.12G015300.1	III	Response to singlet oxygen; Response to cold; Response to water deprivation; Response to salt stress; Response to abscisic acid
Qurub.05G182800.1	IV	Jasmonic acid mediated signaling pathway
Qurub.09G216900.1	IV	Response to salt stress
Qurub.10G154600.1	IV	Chloroplast thylakoid membrane[1,2,3,4,5,6,7,8,9]
Qurub.10G174600.1	IV	Response to heat; Response to cold; Response to salt stress

5.7.5 Table 5

Descriptions of significant Qr DEGs. Gene ID numbers, descriptions, and differential gene expression (L2FC) are used to describe each DEG. Each DEG listed here is relevant to at least one group; each group is populated by 10 DEGs. Group I: DEGs with highest

differential gene expression. Group II: DEGs annotated by Qr enriched GO categories with the highest differential expression. Group III: DEGs annotated by at least 2 OS/CW GO terms with highest differential expression. Group IV: statistically significant gene expression markers with annotation specific to the Qr enriched and OS/CW GO terms. Statistical information specific to category 4 on the right of the table: the "> 97.5% CI" column indicates how many samples from each species had an RPKM above the 97.5% threshold based on Qr sample mean; 11 and 16 samples were assigned as Qe and Qr, respectively. Statistical significance of each gene expression marker is described in the $\chi 2$ column: one, two, and three asterisks indicates that p-values were less than 0.05, 0.01, and 0.005, respectively. Bold gene IDs indicate an enriched GO term directly related to defense response; underlined gene IDs indicate an enriched GO term indirectly associated with defense response; italicized gene IDs indicate an OS/CW GO terms that is potentially associated defense response.

Gene ID	Description	L2FC	Categories				> 97.5% CI		$\chi 2$
			I	II	III	IV	Qe	Qr	
Qurub.08G158900.1	Chalcone synthase	22.9	X				0	6	NA
Qurub.08G158800.1	Chalcone synthase	22.8	X				0	6	NA
Qurub.04G207300.1	Wall-associated receptor kinase-like 8	21.6	X	X			0	4	NA
Qurub.09G222900.1	Ankyrin repeat-containing protein BDA1	7.4	X	X	X		1	1	NA
Qurub.12G075700.1	RNA-binding protein NOB1	6.9	X				2	7	NA
Qurub.04G057100.1	Receptor kinase-like protein Xa21	6.7	X	X			1	4	NA
Qurub.10G079300.1	Pentatricopeptide repeat-containing protein	6.6	X				1	8	NA
Qurub.M194800.1	UDP-glucose glucosyltransferase 2	6.6	X	X			3	12	NA
Qurub.M142300.1	7-deoxyloganetin glucosyltransferase	6.6	X	X			3	11	NA
Qurub.02G431100.1	DNA (cytosine-5)-methyltransferase 1A	6.5	X				1	5	NA
Qurub.04G148700.1	Receptor kinase-like protein Xa21	5.98		X			1	6	NA

Qurub.05G085300.1	Disease resistance protein Pik-1	5.71		X			1	7	NA
Qurub.01G183800.1	Expansin-A4	5.67		X			0	7	NA
Qurub.10G009600.1	Prostaglandin reductase 1	5.61		X			0	10	*
Qurub.04G165800.1	1-aminocyclopropane-1-carboxylate synthase 1	5.56		X	X		1	6	NA
Qurub.03G096600.1	Ankyrin-3	5.27			X		1	11	*
Qurub.08G103000.1	PTI-COMPROMISED RECEPTOR-LIKE CYTOPLASMIC KINASE 1	5.27			X		0	9	*
Qurub.02G117700.1	Caskin-1	5.17			X		2	3	NA
Qurub.08G208700.1	Cytochrome P450 87A3	4.94			X		2	6	NA
Qurub.02G400800.1	Receptor kinase-like protein Xa21	4.87			X		2	14	**
Qurub.07G069600.1	Low temperature-induced protein 6B	4.51			X		2	4	NA
Qurub.03G090400.1	Endochitinase 1	4.46			X		1	6	NA
Qurub.08G208500.1	Cytochrome P450 87A3	4.45			X		3	5	NA
Qurub.07G211700.1	Receptor-like cytoplasmic kinase 176	0.45				X	2	16	***
Qurub.10G227800.1	Serine palmitoyltransferase 2	0.52				X	3	16	***
Qurub.02G157900.1	F-box/LRR-repeat protein 14	0.59				X	2	15	***
Qurub.04G174700.1	Polyamine oxidase 2	0.5				X	2	15	***
Qurub.06G251600.1	Serine/threonine/tyrosine-protein kinase 13	0.86				X	2	15	***
Qurub.08G160400.1	Glucosidase 2 subunit beta	0.4				X	2	15	***
Qurub.10G193400.1	Transitional endoplasmic reticulum ATPase	0.63				X	2	15	***
Qurub.11G236300.1	Germin-like protein 1	1.52				X	2	15	***
Qurub.02G029800.1	WEAK CHLOROPLAST MOVEMENT UNDER BLUE LIGHT 1	0.42				X	3	15	**
Qurub.02G047400.1	REDUCED RESIDUAL ARABINOSE 3	0.44				X	3	15	**

5.7.6 Table 6

GO annotation specific to Qr enriched and OS/CW terms for significant Qr DEGs. GO terms with superscripted numbers are from the 10 most enriched Qr GO categories. Significance of the GO terms are organized in a descending order: (1) response to stimulus; (2) catalytic activity; (3) response to stress; (4) membrane; (5) response to chemical; (6) response to external stimulus; (7) cellular response to stimulus; (8) response to organic

substance; (9) biological process involved in interspecies interaction between organisms; (10) defense response. Each DEG listed here is relevant to at least one group; each group is populated by 10 DEGs. Group I: DEGs with highest differential gene expression. Group II: DEGs annotated by Qe Enriched GO terms with the highest differential expression. Category III: DEGs annotated by at least 2 OS/CW GO terms with highest differential expression. Category IV: statistically significant gene expression markers with annotation specific to the Qe enriched and/or OS/CW GO terms. A complete list of all GO terms annotation for each Qe DEG is found in Supplementary Table 4.

Gene ID	Category	GO terms
Qurub.08G158900.1	I	NA
Qurub.08G158800.1	I	NA
Qurub.04G207300.1	I,II	**Defense response[1,3,10]; Response to bacterium[1,6,9]; Response to fungus[1,6,9];** Protein kinase activity[2]; Membrane[4]; Response to salicylic acid[1,5,8]
Qurub.09G222900.1	I,II,III	**Defense response to bacterium[1,3,6,9,10]; Innate immune response[1,3,6,9,10];** Cellular response to stimulus[1,7]; Response to salicylic acid[1,5,8]; Cellular response to stimulus[1,7]
Qurub.12G075700.1	I	NA
Qurub.04G057100.1	I,II	**Response to molecule of bacterial origin[1,5,6,8,9]; Plant-type hypersensitive response[1,3,6,7,9,10]; Detection of bacterium[1,6,9];** Protein serine/threonine kinase activity[2]; Transmembrane receptor protein kinase activity[2]; Endoplasmic reticulum membrane[4]; Perinuclear endoplasmic reticulum membrane[4]; **Immune response-regulating signaling pathway[1,3,7,10]**
Qurub.10G079300.1	I	NA
Qurub.M194800.1	I,II	Glucuronosyltransferase activity[2]; Trans-zeatin O-beta-D-glucosyltransferase activity[2]; Cis-zeatin O-beta-D-glucosyltransferase activity[2]
Qurub.M142300.1	I,II	Glucuronosyltransferase activity[2]; Trans-zeatin O-beta-D-glucosyltransferase activity[2]; Cis-zeatin O-beta-D-glucosyltransferase activity[2]
Qurub.02G431100.1	I	NA
Qurub.04G148700.1	II	**Response to molecule of bacterial origin[1,5,6,8,9]; Immune response-regulating signaling pathway[1,3,7,10]; Plant-type hypersensitive response[1,3,6,7,9,10]; Detection of bacterium[1,6,9];** Protein serine/threonine kinase activity[2]; Transmembrane receptor protein kinase activity[2]; Endoplasmic reticulum membrane[4]; Perinuclear endoplasmic reticulum membrane[4]

Qurub.05G085300.1	II	**Innate immune response-activating signal transduction[1,3,7,10]; Plant-type hypersensitive response[1,3,6,7,9,10]; Defense response to bacterium[1,3,6,9,10]**
Qurub.01G183800.1	II	Membrane[4]
Qurub.10G009600.1	II	Response to oxidative stress[1,3]
Qurub.04G165800.1	II,III	**Defense response[1,3,10]**; Response to oxidative stress[1,3]; *Ethylene biosynthetic process; Response to auxin; Response to jasmonic acid*; Phloem or xylem histogenesis
Qurub.03G096600.1	III	Response to abiotic stimulus; Response to salicylic acid[1,5,8]
Qurub.08G103000.1	III	*Regulation of salicylic acid biosynthetic process*; Positive regulation of stomatal opening
Qurub.02G117700.1	III	Response to abiotic stimulus; Response to salicylic acid[1,5,8]
Qurub.08G208700.1	III	*Response to jasmonic acid; Brassinosteroid homeostasis; Brassinosteroid biosynthetic process*
Qurub.02G400800.1	III	Cellular response to stress; Cell wall organization
Qurub.07G069600.1	III	Response to cold; Response to water deprivation; Response to abscisic acid[1,5,8]; Hyperosmotic salinity response
Qurub.03G090400.1	III	Response to heat; Response to water deprivation; Response to salt stress; *Response to cytokinin; Regulation of salicylic acid metabolic process*
Qurub.08G208500.1	III	*Response to jasmonic acid; Brassinosteroid homeostasis; Brassinosteroid biosynthetic process*
Qurub.07G211700.1	IV	**Response to fungus[1,6,9]; Innate immune response[1,3,6,9,10]**; *Regulation of jasmonic acid biosynthetic process; Regulation of salicylic acid biosynthetic process*; Positive regulation of stomatal opening; Protein serine/threonine kinase activity[2]
Qurub.10G227800.1	IV	Regulation of programmed cell death
Qurub.02G157900.1	IV	Membrane[4]
Qurub.04G174700.1	IV	Peroxisome
Qurub.06G251600.1	IV	Response to stress[1,3]; Signal transduction[1,3,7,10]; Response to external stimulus[1,6]**; Response to abscisic acid[1,5,8]**; Regulation of stomatal movement; Protein serine/threonine kinase activity[2]; Membrane[4]
Qurub.08G160400.1	IV	**Defense response to bacterium[1,3,6,9,10]**
Qurub.10G193400.1	IV	Cellular response to heat
Qurub.11G236300.1	IV	*Auxin-activated signaling pathway*; **Defense response to Gram-negative bacterium[1,3,6,9,10]; Defense response to fungus[1,3,6,9,10]**
Qurub.02G029800.1	IV	Response to stimulus[1]
Qurub.02G047400.1	IV	Cell wall organization

5.7.7 Table 7

Qe eQTL hotspots for molecular phenotypes related to enriched GO terms. Only genes within 50 kbp of each other are reported here. Significance of the GO terms are organized in a descending order: (1) plastid; (2) chloroplast; (3) plastid envelope; (4) plastid

membrane; (5) thylakoid; (6) plastid thylakoid; (7) chloroplast thylakoid; (8) photosynthetic membrane; (9) thylakoid membrane; (10) plastid organization.

Hotspot ID	Gene IDs	Homolog/Description	GO terms
Qe10	Qurub.10G154400.1 Qurub.10G154600.1	Photosynthetic NDH subunit of subcomplex B4 Chlorophyll a-b binding protein P4	Chloroplast thylakoid membrane[1,2,3,4,5,6,7,8,9]
Qe11	Qurub.11G119700.1 Qurub.11G119800.1	Aspartate--tRNA(Asp/Asn) ligase F-box/kelch-repeat protein	Chloroplast[1,2]

5.7.8 Table 8

Qr eQTL hotspots for molecular phenotypes related to enriched GO terms. Only genes within 50 kbp of each other are reported here. Significance of the GO terms are organized in a descending order: (1) response to stimulus; (2) catalytic activity; (3) response to stress; (4) membrane; (5) response to chemical; (6) response to external stimulus; (7) cellular response to stimulus; (8) response to organic substance; (9) biological process involved in interspecies interaction between organisms; (10) defense response.

Hotspot ID	Gene IDs		GO terms
Qr:1	**Qurub.01G052400.1** **Qurub.01G052500.1**	Disease resistance protein Disease resistance protein	**Defense response to other organism[1,3,6,9,10]**
QR:2	Qurub.02G400800.1 Qurub.02G401100.1	Receptor kinase-like protein Xa21 Receptor kinase-like protein Xa21	Protein serine/threonine kinase activity[2]
QR:3.1	**Qurub.03G054300.1** **Qurub.03G054400.1**	Linoleate 9S-lipoxygenase 5 Linoleate 9S-lipoxygenase 5	**Defense response[1,3,10]**; Response to abscisic acid[1,5,8]; Linoleate 13S-lipoxygenase activity[2]; Linoleate 9S-lipoxygenase activity[2]
QR:3.2	Qurub.03G225000.1 Qurub.03G225100.1	Glutathione S-transferase U10 Glutathione S-transferase U10	Toxin catabolic process[1,3,5,10]
QR:4	Qurub.04G140400.1 Qurub.04G140800.1	Ferroptosis suppressor protein 1 Ferroptosis suppressor protein 1	Membrane[4]
QR:5	**Qurub.05G085200.1** **Qurub.05G085300.1**	Disease resistance protein Pik-1 Disease resistance protein Pik-1	**Defense response to bacterium[1,3,6,9,10]**

	Qurub.05G085600.1	Disease resistance protein RGA5	
QR:6	**Qurub.06G060000.1** **Qurub.06G060100.1**	Disease resistance protein Disease resistance protein	**Defense response to bacterium**[1,3,6,9,10]
QR:8.1	Qurub.08G069800.1 Qurub.08G070000.1	G-type lectin S-receptor-like serine/threonine-protein kinase G-type lectin S-receptor-like serine/threonine-protein kinase	Protein serine/threonine kinase activity[2]; Membrane[4]
QR:8.2	**Qurub.08G264800.1** **Qurub.08G264900.1**	Receptor kinase-like protein Xa21 Receptor kinase-like protein Xa21	**Response to molecule of bacterial origin**[1,5,6,8,9]**; Immune response-regulating signaling pathway**[1,3,7,10]**; Plant-type hypersensitive response**[1,3,6,7,9,10]**; Detection of bacterium**[1,6,9]**;** Protein serine/threonine kinase activity[2]; Transmembrane receptor protein kinase activity[2]; Endoplasmic reticulum membrane[4]; Perinuclear endoplasmic reticulum membrane[4]
QR:8.3	**Qurub.08G302300.1** **Qurub.08G302400.1**	WRKY transcription factor 55 WRKY transcription factor 70	**Defense response to fungus**[1,3,6,9,10]
QR:9.1	Qurub.09G053700.1 Qurub.09G053800.1	Receptor-like protein EIX2 Receptor-like protein EIX1	Transferase activity[2]
QR:9.2	**Qurub.09G213500.1** **Qurub.09G213600.1**	Receptor kinase-like protein Xa21 Receptor kinase-like protein Xa21	**Response to molecule of bacterial origin**[1,5,6,8,9]**; Immune response-regulating signaling pathway**[1,3,7,10]**; Plant-type hypersensitive response**[1,3,6,7,9,10]**; Detection of bacterium**[1,6,9]; Protein serine/threonine kinase activity[2]; Transmembrane receptor protein kinase activity[2]; Endoplasmic reticulum membrane[4]; Perinuclear endoplasmic reticulum membrane[4]
QR:10	**Qurub.10G176000.1** **Qurub.10G176200.1**	**Enhanced disease susceptibility 1** **Enhanced disease susceptibility 1 protein B**	**Plant-type hypersensitive response**[1,3,6,7,9,10]**; Defense response to Gram-negative bacterium**[1,3,6,9,10]
QR:11	**Qurub.11G164400.1** **Qurub.11G164500.1**	**BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1**	**Positive regulation of innate immune response**[1,3,7,9,10]

		BRASSINOSTEROID INSENSITIVE 1-associated receptor kinase 1	

5.7.9 Table 9

Mean and standard error for Qe and Qr hydraulic traits. Results from a student t test, used for finding if there was statical significance between species, are recorded in the third column. Bolded p-values are less than 0.05 and are statistically significant. Abbreviations: Π0 - ψ_{leaf} at full turgor; RWC at TLP – relative water content at the turgor loss point; π_{TLP} - ψleaf at turgor loss point; P_e – ψ_{leaf} at embolism onset (5% embolism); MS – margin of safety; ψ_{50} - ψ_{leaf} at 50% embolism; and ψ_{88} - ψ_{leaf} at 88% embolism.

	Mean ± SEM		
	Qe	**Qr**	**p-value**
π_0 (Mpa)	-1.49 ± 0.06	-0.95 ± 0.16	**0.034**
RWC at TLP (%)	54.24 ± 5.88	43.11 ± 6.35	0.268
π_{TLP} (Mpa)	-2.48 ± 0.03	-1.58 ± 0.18	**0.007**
P_e (Mpa)	-1.00 ± 0.25	-0.42 ± 0.09	0.169
MS (Mpa)	-1.48 ± 0.25	-1.26 ± 0.35	0.635
Ψ_{50} (Mpa)	-2.80 ± 0.21	-2.39 ± 0.13	0.252
Ψ_{88} (Mpa)	-3.68 ± 0.11	-3.37 ± 0.62	0.57

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7 Supplementary Material

A link to all supplemental materials is found here.

https://JRauschendorfer.github.io/James_Rauschendorfer_PhD_thesis_supplemental_information/

A Chapter 2

A.1 Supplementary Table 1

Sources for *Quercus* sect. *Lobatae* species distribution map in figure 1a. Numbers under primary and secondary sources correspond to the citations used. Sources: 1 - Kew (2021), 2 - Stein (2003), 3 - USDA (2021), 4 - BONAP (2021), 5 -Ramírez-Toro et al. (2017), and 6 - Torres-Miranda et al. (2011).

https://JRauschendorfer.github.io/James_Rauschendorfer_PhD_thesis_supplemental_information/Ch2_Table_1.pdf

A.2 Supplementary Table 2

Sources for *Quercus* sect. *Quercus* species distribution map in figure 1a. Numbers under primary and secondary sources correspond to the citations used. Sources: 1 - Kew (2021), 2 - Stein (2003), 3 - USDA (2021), 4 - BONAP (2021), 5 -Ramírez-Toro et al. (2017), and 6 - Torres-Miranda et al. (2011).

https://JRauschendorfer.github.io/James_Rauschendorfer_PhD_thesis_supplemental_information/Ch2_Table_2.pdf

A.3 Supplementary Table 3

Sources for *Quercus* sect. *Lobatae* hybridization. Numbers under sources correspond to the citations used. Sources: 1 - Torres-Miranda et al. (2011), 2 - USDA (2021), 3 - Arboretum-Wespelaar (2020), and 4 - Burns et al. (1990).

https://JRauschendorfer.github.io/James_Rauschendorfer_PhD_thesis_supplemental_information/Ch2_Table_3.pdf

A.4 Supplementary Table 4

Sources for *Quercus* sect. *Quercus* hybridization. Numbers under sources correspond to the citations used. Sources: 1 - Torres-Miranda et al. (2011), 2 - USDA (2021), 3 - Arboretum-Wespelaar (2020), and 4 - Burns et al. (1990).

https://JRauschendorfer.github.io/James_Rauschendorfer_PhD_thesis_supplemental_information/Ch2_Table_4.pdf

B Chapter 3

B.1 Supplementary Table 1

Raw data for *P. trichocarpa* TF and homologous sequences within the *Q. robur* and *Q. rubra* genome.

https://JRauschendorfer.github.io/James_Rauschendorfer_PhD_thesis_supplemental_information/Chapter_3/Ch3_Table_1.html

B.2 Supplementary Table 2

Q. rubra transcription factor gene clusters.

https://JRauschendorfer.github.io/James_Rauschendorfer_PhD_thesis_supplemental_information/Chapter_3/Ch3_Table_2.html

B.3 Supplementary Table 3

Q. rubra transcription factor homolog sets.

https://JRauschendorfer.github.io/James_Rauschendorfer_PhD_thesis_supplemental_information/Chapter_3/Ch3_Table_3.html

C Chapter 5

C.1 Supplementary File 1

MultiQC report for RNA-sequencing raw data.

https://JRauschendorfer.github.io/James_Rauschendorfer_PhD_thesis_supplemental_information/Chapter_5/Ch5_File_1.html

C.2 Supplementary File 2

MultiQC report for RNA-sequencing raw cleaned data.

https://JRauschendorfer.github.io/James_Rauschendorfer_PhD_thesis_supplemental_information/Chapter_5/Ch5_File_2.html

C.3 Supplementary Table 1

Qualimap output summary.

https://JRauschendorfer.github.io/James_Rauschendorfer_PhD_thesis_supplemental_information/Chapter_5/Ch5_Table_1.html

C.4 Supplementary Table 2

Mean mapping quality ANOVA results.

https://JRauschendorfer.github.io/James_Rauschendorfer_PhD_thesis_supplemental_information/Chapter_5/Ch5_Table_2.html

C.5 Supplementary Table 3

Upregulated *Quercus ellipsoidalis* differentially expressed genes (DEGs).

https://JRauschendorfer.github.io/James_Rauschendorfer_PhD_thesis_supplemental_information/Chapter_5/Ch5_Table_3.html

C.6 Supplementary Table 4

Upregulated *Quercus rubra* differentially expressed genes (DEGs).

https://JRauschendorfer.github.io/James_Rauschendorfer_PhD_thesis_supplemental_information/Chapter_5/Ch5_Table_4.html

C.7 Supplementary Table 5

Enriched gene ontology (GO) terms in *Quercus ellipsoidalis*.

https://JRauschendorfer.github.io/James_Rauschendorfer_PhD_thesis_supplemental_information/Chapter_5/Ch5_Table_5.html

C.8 Supplementary Table 6

Enriched gene ontology (GO) terms in *Quercus rubra*.

https://JRauschendorfer.github.io/James_Rauschendorfer_PhD_thesis_supplemental_information/Chapter_5/Ch5_Table_6.html

C.9 Supplementary Table 7

The ten gene ontology (GO) categories with greatest enrichment in *Quercus ellipsoidalis*.

https://JRauschendorfer.github.io/James_Rauschendorfer_PhD_thesis_supplemental_information/Ch5_Table_7.pdf

C.10 Supplementary Table 8

The ten gene ontology (GO) categories with greatest enrichment in *Quercus rubra*.

https://JRauschendorfer.github.io/James_Rauschendorfer_PhD_thesis_supplemental_information/Ch5_Table_8.pdf

C.11 Supplementary Table 9

Gene expression markers (GEMs) identified in *Quercus ellipsoidalis*.

https://JRauschendorfer.github.io/James_Rauschendorfer_PhD_thesis_supplemental_information/Chapter_5/Ch5_Table_9.html

C.12 Supplementary Table 10

Gene expression markers (GEMs) identified in *Quercus rubra*.

https://JRauschendorfer.github.io/James_Rauschendorfer_PhD_thesis_supplemental_information/Chapter_5/Ch5_Table_10.html