Chapter 8

Water Use and Drought Response in Cultivated and Wild Apples

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Additional information is available at the end of the chapter

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1. Introduction

Water availability is the single most important factor determining plant survival. Many environments experience water-limited periods of various degrees and duration. The issue of water availability for agricultural crops where maintenance of high yields over variable growing seasons is desirable is particular critical. For plants, the strategy choices for survival can be summarized as dehydration avoidance (e.g., deep rooting), dehydration tolerance (e.g., accumulation of osmoprotectants) and drought escape (e.g., reproductive completion before the dry season). Drought adaptation in most plants is controlled by complicated interactions between anatomy, physiology and biochemistry, all of which are directly or indirectly under genetic control [1-3].

2. Water use efficiency and drought resistance

All living organisms have evolved mechanisms for adapting to changes in their environment, whether biotic such as pest related, or abiotic such as physically effected. For plants this is especially challenging, since they are unable to relocate to avoid adverse conditions. As a result, numerous strategies employed by plants leading to successful acclimation have been identified. Broadly speaking, these adaptive mechanisms can be divided into two major categories, namely morphological modifications and physiological adjustments. Through combinations of these basic strategies, plants can respond quickly to environmental cues, often maintaining the response for relatively long periods [4,5].

The term drought resistance is sometimes confused with water use efficiency (WUE). Drought resistance is determined primarily by 'drought avoidance' (high plant water status maintained

under water deficit) and/or 'drought tolerance' (capacity to sustain plant function in a dehydrated state) [6,7]. Drought resistance in a genetic/physiological context refers to the ability of one genotype to yield 'better' than another during severe drought stress. On the other hand WUE is defined as the ratio between diffusion of CO_2 into the leaf (photosynthesis) and loss of H_2O through transpiration, indicated as WUE = A/E, where A is carbon assimilation and E is transpiration. It is positively correlated with carbon isotope discrimination ($\Delta^{13}C$) based on the stable carbon isotope ratio, $\delta^{13}C$ ($^{12}C/^{13}C$ relative to a standard, i.e. PeeDee Belemnite), in the plant tissue relative to the atmospheric ratio and is calculated as: $\Delta^{13}C = \delta^{13}C$ in air - $\delta^{13}C$ of the plant/1- $\delta^{13}C$ of the plant. Since most gas exchange occurs via the stomata, it is expected that guard cell function would be closely associated with WUE. Indeed, the size and density of stomates correlates well with water use efficiency [8-10]. For drought resistance, yield is not necessarily adversely affected by resistance, whereas for WUE reduced transpiration through stomatal closure is often accompanied by reduced yield potential through reduced carbon assimilation, particularly in herbaceous C_3 plants (however, see below). Other parameters, such as root depth, leaf size, and trichome size and density have also been linked to water use efficiency [2], but they have also been linked to drought resistance as well [6].

Different methods have been used to measure drought resistance and WUE [11]. These methods measure the energy status of water in plant tissues and the trans-port processes into and out of the soil-plant-atmosphere continuum. In general, these methods isolate specific plant tissues at instantaneous moments in time, whereas $\Delta^{13}C$ represents a time-integrated value of the ratio of C_i (intercellular CO_2 concentration) to ambient CO_2 which, as previously indicated, reflects the plant's capacity for gas exchange via stomata [12] and discrimination of rubisco against ^{13}C. The use of carbon isotope discrimination to select in-dividuals with higher WUE has been applied successfully to cereal breeding programs (13). The extent of $\delta^{13}C$ varies substantially among wheat genotypes, and heritability is high because genotype X environment interactions are relatively low [14, 15]. Rebetzke et al. [16] reported on the selection of plants with greater biomass, harvest index and kernel weight using results from contrasting high and low $\Delta^{13}C$ groups in combination with a backcrossing program. There were significant correlations between $\Delta^{13}C$ and yield and between $\Delta^{13}C$ and biomass. The resulting high yielding strain, 'Drysdale', produces around 10% more grain under drought conditions than other dry-area wheat varieties.

3. Adaptation and the relationship of $\delta^{13}c$ to yield

Adaptive changes in populations growing in different environments have been amply demonstrated in a variety of plants [17]. Divergence among populations associated with different environments provides the raw material for speciation and differentiation among closely related species. Higher fitness of genotypes in their native environment compared to genotypes transplanted from contrasting environments provides evidence of local adaptation [9]. For example, when two populations of *Boechera holboellii* growing in xeric and wet environments were grown in reciprocal transplant experiments, significantly higher survival was observed with plants growing in their native habitat [18]. Furthermore, genes identified

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by cDNA-AFLP analysis showed genotype-specific expression patterns related to the indigenous environment (population). In another study populations of *Encelia farinosa* growing over a broad rainfall gradient in the deserts of southwestern North America, were evaluated for leaf characteristics. Leaf pubescence declined as mean water availability increased [19]. Variations among populations for both pubescence and carbon isotope discrimination persisted when the plants were grown in common environments differing in water availability, indicating a genetic basis for variation in these traits [20].

WUE in trees adapted to different environments has been documented in several forest species [21-24]. For example, a study of four birch (*Betula pendula* Roth) clones from environments with different rainfall amounts indicated strong clonal differences in a number of water use and photosynthetic traits, including leaf $\delta^{13}C$ values [21]. A follow-up study correlated differences in leaf and root morphological parameters and carbon partitioning with clones from the drier environments [25]. Although individual leaf areas were smaller in drought-treated clones, regardless of their region of origin, total leaf and specific leaf areas (leaf area/leaf weight) were actually higher for the drought-treated clones from the drier environment. This is in contrast to the often observed reduction in leaf surface area seen in plants exposed to water deficit (WD) [26].

Poplar species are differentially adapted to a variety of environments, and because poplar is a rapidly growing tree with heavy water use, there is growing interest in developing lines that are drought resistant. Links between productivity and $\Delta^{13}C$ varied in a study comparing different poplar genotypes, suggesting that genotypes displaying simultaneous high productivity and improved water use efficiency could be selected [27]. To obtain more practical information regarding productivity and WUE, a field study was conducted on the same genotypes analyzed in the previous study. Significant clonal diversity was observed for several traits related to productivity and for Δ which showed high heritability ($H2 = 0.71$) [28]. A lack of correlation between above ground biomass and Δ was reflected in several clones where high productivity was combined with improved WUE. This observation supports previous studies with cereals indicating that WUE and yield can be inherited as separate traits.

Yield of deciduous tree fruit crops is not measured as total biomass yield in commercial production as are agronomic crops such as corn, wheat and rice or forest trees. In commercial orchards it is common practice to reduce yield potential of fruit trees by as much as 50% to insure large fruit size and high fruit quality [29]. Consequently, the paradigm that increased WUE is tied to reduced yield potential is not necessarily valid for tree fruit production. For example, Glenn et al. [30] have demonstrated the practicality of identifying peach cultivars with high WUE without compromising productivity. This study, taken together with those cited previously, demonstrates the feasibility of selecting for improved WUE without loss in productivity.

4. Adaptation, WUE and $\delta^{18}O$

Transpiration rate (E) affects water loss to the atmosphere and is negatively correlated with WUE. Despite the fact that atmospheric ^{18}O is low (ca. 0.2% of total oxygen), plants tend to accumulate ^{18}O and ^{2}H in leaf water due to the difference in vapor pressure between heavy

water and 'normal' water and to differences in diffusivity with air. However, the relationship between E and isotopic enrichment is complex. For example, variation in E can be caused by changes in evaporative demand and/or changes in stomatal conductance, gs [31]. If the source of variation is evaporative demand, then as E increases, ^{18}O enrichment increases. On the other hand, if stomates are the source of variation, then as E decreases (stomates close), leaf water enrichment increases. How does this relate to ^{18}O enrichment of plant organic matter? Plants accumulate ^{18}O in their tissues as a result of the exchange of oxygen between water and carbonyl oxygens in triose phosphates. An enrichment of about 27 parts per thousand (ppt) has been observed in the organic material of several different plants relative to leaf water [32]. This suggests that differences in ^{18}O enrichment can be used to distinguish genotypes with favorable yields and stomatal function. In fact Barbour et al. [33] recently demonstrated a reliably negative correlation between yield and $\delta^{18}O$ in wheat which was used to identify water use efficient varieties for breeding.

5. Specific genes associated with WUE and/or drought responses in apple and other plants

The recent advent of global gene expression methodology has spawned a number of studies of abiotic stress responses, including drought, in several plant species [34-38]. In Arabidopsis, a compilation study of microarray analyses on plants subjected to a variety of stress treatments highlighted overlap among genes up-regulated in the early stages of all the stress responses [39]. Studies on WD stress in cereals and dicots have cataloged a large number of genes up-regulated during treatment [35-37, 40, 43]. Comparisons among these studies reveal that a number of genes are reproducibly up-regulated in response to WD regardless of how the stress was imposed or what plant system was involved, including apple (Table 1) [44].

6. Genes associated with wue

Recent reports of genes associated with regulation of transpiration demonstrate the complexity of water use and transport, as well as the overlap in gene response to other stresses. *ESKIMO1*, which was originally associated with cold response, has recently been shown to affect both drought and salt responses in Arabidopsis. Insertional mutation lines inactivating *ESK1* had reduced transpiration rates and were only slightly less drought tolerant than wild type [45]. Furthermore, there was a reduction in biomass when mutant plants were grown without stress, suggesting that alterations in WUE were reducing both transpiration and CO_2 exchange; biomass differences between WT and *esk1* lines were negligible under stress. Another example of pleotrophic gene effects on plant physiology was reported in a study by Masle et al. [46]. A leucine-rich repeat receptor kinase (*ERECTA*) previously associated with inflorescence development was also shown to regulate transpiration in different races of Arabidopsis. The gene is implicated in epidermal cell expansion, cell-cell contact and mesophyll cell proliferation, but its relationship to reduced transpiration may be linked to differences in stomatal

Genes Up-regulated in 'Royal Gala' Roots	Other Plants	Tissue	Citation
HMW HSP	*Arabidopsis*	various	[40][2]
	Poplar proteome	white roots	[41]
aquaporin	*Arabidopsis*	various	[40]
	barley	leaves and roots	[35]
	maize	leaves and roots	[43]
	Poplar proteome	white roots	[41]
protease inhibitor	*Arabidopsis*	various	[40,42]
	chickpea	whole seedlings	
Histone H2	*Arabidopsis*	various	[40]
	chickpea	whole seedlings	[42]
	maize	leaves and roots	[43]

[1] Apple ('Royal Gala') genes are from two SSH root libraries of water deficit-treated plants (manuscript submitted)

[2] This citation is a review of several different studies of water deficit-response in *Arabidopsis*.

Table 1. Genes up-regulated in water deficit-treated apple roots vs other plants responding to water deficit[1]

density between lines. Interestingly, there was no compensation in biomass for the reduction in E, indicating that as with WUE and $\Delta^{13}C$, there are workable strategies for increasing WUE without sacrificing carbon assimilation under normal growth conditions.

Using a suppression subtractive hybridization (SSH) approach to drought-responsive gene isolation in a commercial apple, 'Royal Gala', we identified numerous genes commonly found to be WD responsive in other plants. We also identified several up-regulated genes unique to apple roots (Table 2; manuscript submitted). Some of these genes may reflect the role of roots in nutrient transport during stress, including a copper chaperone and a high affinity nitrate transporter that is a putative Arabidopsis NRT2.4 homolog.

Genes Up-regulated in 'Royal Gala'		
Roots	**Bark**	**Leaves**
BYPASS1	Anthocyanin reductase	Auxin/Aluminum-induced protein
Serine acetyltransferase	GAST1-like gene	Proteasome maturation factor
High affinity nitrate transporter	Asparagine synthetase	Asparagine synthetase
NPR1	Chloroplast membrane protein Tic40	Glyoxylate aminotransferase

[1] These genes have not yet been reported as up-regulated in response to WD in these tissues of other plants.

Table 2. Genes uniquely up-regulated in WD-treated apple tissues[1]

7. Genes associated with drought avoidance and escape

An example of the rapid evolution of a drought escape mechanism (early flowering) was demonstrated in a population of *Brassica napa* subjected to a multiyear drought [47]. Comparison of seeds collected from individual plants before the drought with those obtained from the drought-affected population indicated a significant earlier onset of flowering in the latter population. This observation was further expanded in a study of quantitave trait loci (QTLs) in maize associated with flowering time (*Vgt1*) where a *cis*-acting region upstream of a transcription factor was shown to be the link between a QTL and the early flowering trait [48]. The authors speculate that natural genetic variations in flowering time enabled the selection of maize lines adapted to a range of latitudes and growing seasons, including drought tolerance. Another study of natural variation in ecotypes of Arabidopsis [49] found a strong positive genetic correlation (rG = 0.98) between $\delta^{13}C$ (drought avoidance) and flowering time (drought escape). They also observed compelling evidence for pleiotropy in lines varying in FLOWERING LOCUS C, suggesting that correlated evolution of $\delta^{13}C$ and flowering time could be partly explained by coordinated allele fixation altering both traits.

In alfalfa a gene encoding a zinc-finger motif is expressed in roots [50, 51]. The protein encoded by *Alfin1* binds DNA at a specific *cis*-elemtent and is proposed to be a root growth regulator, as transgenic lines overexpressing the gene show enhanced root growth under both normal and high salt conditions [52]. These same transgenic lines were significantly more salt tolerant, and presumably more drought tolerant (high salt concentrations in the media decrease water uptake), althought drought tolerance per se was not measured.

8. Genes associated with drought tolerance and resistance

Studies of specific genes associated with dehydration responses have been conducted in a number of plants, and roles for many of these genes have been correlated with specific morphological or physiological traits known to be involved in drought resistance. Abscisic acid (ABA) signaling and stomatal function are correlated with WUE and drought resistance, so it is not surprising that several genes involved in ABA perception and stomata opening/closing respond to severe dehydration. Two calcium-dependent protein kinases from Arabidopsis have been implicated in slow-type anion channel activation [53]. In the double mutants, ABA and Ca^{2+}-induced stomatal closing were impaired, but not completely. These genes may contribute to a rapid Ca^{2+}-reactive response resulting in stomatal closure, as opposed to the slower Ca^{2+}-programmed response which maintains long-term stomatal closure. Similar studies have also implicated a G protein-coupled receptor, GCR1, in ABA signaling perception in guard cells and during seed germination [54]. To examine stomatal function in more detail, Klein et al. [55] used a T-DNA insertion disrupting At*MRP5*, an ABC transporter in Arabidopsis. The mutants had reduced transpiration rates and showed increased water use efficiency. In a similar study using T-DNA insertion disruption of At*MRP4* (another type of ABC transporter), Klein et al. [56] found the mutant lines to be more drought susceptible. In a

recent study of the effects of overexpressing or silencing early response to dehydration (*ERD15*) in Arabidopsis, Kariola et al. [57] observed decreased drought tolerance in the overexpressing lines, whereas the silenced lines were hypersensitive to ABA and showed enhanced tolerance to drought. This study also suggests a negative role for *ERD15* in mediating stress-related ABA signaling.

The cuticle is an important barrier to moisture loss in plants, therefore genes associated with cuticle synthesis and turnover are expected to contribute to plant water status. A recent study in alfalfa demonstrated that increased wax production activated by a putative TF (transcription factor) [*WXP1*] also enhanced drought tolerance in transgenic plants [58]. Similar studies with other transcription associated factors have also demonstrated a correlation between increased wax synthesis and drought tolerance [59, 60].

Constitutive expression of a barley Group III LEA (late embryogenesis abundant) protein placed in wheat under control of the maize *ubi1* promoter resulted in improved water use efficiency and higher total dry mass in the majority of transgenic lines [61]. Overexpressing a small molecular weight heat shock protein (*HSP17.6*) conferred drought tolerance in Arabidopsis transgenic lines [62]. The authors also demonstrated that *HSP17.6* had chaperone-like activity and could protect citrate synthase from chemical denaturation. Taken together these studies emphasize the different mechanisms so far discovered that affect water use and drought resistance in plants and indicate that different genes in the same pathway may be used by plants to control WD responses.

9. Regulation of pathways/signaling networks associated with dehydration

Most studies of dehydration responsive signaling pathways implicate ABA directly in altering specific gene expression [63]. In fact genes that respond to ABA usually have multiple copies of an ABA response element (ABRE) or a combination of an ABRE with other motifs such as Myb, Myc or coupling elements [for example, 64]. A second pathway involves drought response element binding (DREB) proteins, particularly *DREB2*-encoding genes, and may also involve ABA indirectly [65]. A recent report on the isolation of a fourth CBF (*CBF4*) from Arabidopsis suggests that this TF only responds to drought, in contrast to observations reported for CBFs1-3 which respond to both cold and drought stress [66].

Compelling evidence indicates that the ABA pathway likely involves Ca^{2+} signal transduction as an early step and important relay system for dehydration responses. ABA can also increase reactive oxygen species through higher levels of H_2O_2 [67, reviewed in 68]. Other studies have suggested stress-responsive pathways that operate through osmotic sensing independently of ABA [69]. An osmotic sensor similar to bacterial two-component receptors has been identified in Arabidopsis [70]. The gene was able to complement several mutations in yeast osmosensors and activated the *HOG1* response pathway through a mitogen-activated protein kinase. No doubt other signaling components, both ABA-dependent and independent, will be identified in the near future.

Numerous studies support the existence of extensive cross-talk between plant hormone signaling pathways [71-74]. It is therefore expected that both the salicylic acid and Jasmonic acid (JA)/ethylene pathways indirectly influence the expression of genes that respond to drought. Along these lines an Arabidopsis mutant (*rcd1*) belonging to the ADP-(ribosyl)transferase domain-containing subfamily of the WWE family exhibits reduced sensitivity to ABA, ethylene and Me-JA, suggesting that it acts at an integrative node in hormonal signaling regulating different stress-responsive genes [75]. *rcd1* is just one example of many where one gene participates in multiple pathways.

10. Studies in *Malus sieversii*

Genetic polymorphisms from twenty populations of *M. sieversii* in Xinjiang, China were analyzed with RAPD markers to assess genetic diversity [76]. Based on the bands generated with 42 randomly chosen primers, variation within a population (83.1%) was higher than among populations (16.9%). The authors conclude that *M. sieversii* is a rich source of genetic diversity.

Evaluation of six *Malus* species using a variety of morphological and physiological traits led to the conclusion that *M. toringoides* and *M. sieversii* were the most drought tolerant of those analyszed [77]. Measurement of root parameters indicated that *M. sieversii* root surface area decreased in response to drought to ~25% of the well-watered control in contrast to *M. toringoides* roots which decreased to ~ 43% of the control. On the other hand, root surface activity (absorption) declined the least in *M. toringoides* (17%) compared to *seiversii* (33%). When data from all the measurements were taken into consideration, *M. toringoides* and *M. seiversii* were the top two most drought resistant species.

A study of the contribution of rootstock source to drought resistance was conducted using 'Gale Gala' apple scions grafted onto *Malus sieversii* or *Malus hupehensis* roots [78]. Differential responses of the grafted material to drought stress were determined by a number of physiological and morphological traits. Typical reductions in biomass, growth rate and leaf area are observed under drought conditions, but *M. sieversii* showed smaller reductions in these traits during drought treatment than *M. hupehensis*. Furthermore, a larger increase in whole plant WUE was measured in grafts on *M. sieversii* rootstocks compared to *M. hupehensis*.

Problem Statement: Many of our agronomically important fruit trees are derived from a rather narrow genetic base. To provide methods for enhancing apple germplasm resistance to drought and other dehydrative abiotic stresses it is imperative that we identify those genes that contribute to drought survival. Once these genes are identified and characterized they can be used in marker assisted selection strategies or altered by genetic engineering.

Application Area: The origin of the domesticated apple is believed to be in Central Asia via the silk route through Kazakhstan [79]. The predominant species contributing to the domestication of the modern apple is believed to be *Malus sieversii* which is thought to be the progenitor of *M.* × *domestica* and a possible source of resistance alleles lost during domestication

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[80]. A significant secondary contributor to the genetics of current apple varieties is the European crabapple, *Malus sylvestris* [79] which may also possess important resistant genes lost in the modern varieties.

Several studies of *M. sieversii* material collected from geographically and climatologically different sites in Kazakhstan have concluded that significant genetic diversity can be captured in small-sized sub-populations of these site collections [81, 82]. In a subsequent study of the Kazakhstan collection, Richards et al. [83] concluded that differentiation in genetic diversity was greater among individual families than among sites and that gene diversity and allelic richness varied significantly among collection sites. The use of this material to study drought responses in apple at the morphological and genetic levels without the complication of grafted rootstocks provides the cornerstone of our approach to identifying novel drought resistant mechanisms or factors contributing to drought susceptibility.

Figure 1. *Malus sieversii* collection sites in Kazakhstan [after 80].

Research Course: In order to isolate and characterize genes responding to drought from a commercially important cultivar as a standard for comparison, we used SSH on cDNA prepared from 'Royal Gala' subjected to a moderate-severe drought. Genes identified by this method were further characterized for their expression in various tissues under drought treatment or in fully watered controls.

To begin analyzing *M. sieversii* lines for drought responsiveness, we first surveyed core populations of individuals collected from xeric site 6 and later, xeric site 9 for WUE using stable carbon isotope composition ($\delta\ ^{13}C$) to identify individuals with better WUE. Morphological features, e.g. leaf area, leaf length, stomatal density and number of leaves per current year's branch length were evaluated [84]. Individuals showing $\delta\ ^{13}C$ extreme values were then

Individual Seedling ID	Site[a]	Rainfall[b]	$\delta^{13}C$[c]	Fire Blight	Scab[d]	Juiciness	Surface Russet
GMAL3975.k	6	250 mm	-27.09 ppt	R	R	dry	0
GMAL3685.e	6	250 mm	-29.30 ppt	R	S	dry	10%
GMAL3623.f	9	450 mm	-26.33 ppt	S	S	moderate	1%
GMAL4455	4	800 mm	-26.30 ppt	R	R	medium	20%

[a] Geographical location of populations in Kazakhstan.

[b] Annual rainfall; source Forsline et al. (2003)

[c] ppt = parts per thousand; differences of 0.5 ppt are significant.

[d] R = resistant, S = susceptible

Table 3. Example of phenotypic diversity of select lines from *M. sieversii* Kazakhstan populations

clonally propagated for simulated drought experiments where photosynthesis and stomatal conductance were determined and roots, bark and leaves were collected for gene expression analysis. We duplicated these studies using 'Royal Gala', a relatively drought tolerant variety for comparison [85].

Methods Used: We used standard methods for the morphological and physiological measurements. For the drought experiments, young trees (~1 m tall) of 'Royal Gala' propagated by shoot proliferation were grown for several weeks in a controlled environment with standard light and temperature conditions [44]. A simulated moderate-severe drought were imposed by withholding water until the pots reached 40% of full saturation and maintained for 2 weeks at this level after which the trees were sampled. A parallel control group was grown under the same conditions, but watered to full capacity every other day. Samples from roots, bark and leaves (fully expanded) were taken and quickly immersed in liquid N_2. Roots were washed for 5 min in room temperature tap water, blotted dry and placed in liquid N_2. Bark was removed by scraping the outer layers (down to the xylem) directly into liquid nitrogen. All samples were stored at -80°C until use. Bark was lyophilized prior to storage at -80°C.

Total RNA was isolated, cDNA prepared and SSH performed using the protocol reported by Bassett et al. [86] for peach. For gene analysis, we designed primers for several genes shown to be associated with dehydration responsiveness in apple [44; manuscript submitted]. Each primer pair was quality tested and used to prime RT-qPCR reactions in order to quantitate gene expression in different tissues. The qPCR reactions were conducted using a kit containing all reagents (Life Technologies, Applied Biosystems, Grand Island, NY) and the reaction parameters were as follows: 95°C 5 min, followed by 35 cycles of 95°C 1 min, 60-65°C 1 min, 72°C 1 min and a final extension of 72°C for 10 min. Primers for a translation elongation factor (TEF2) was used as an internal control for the qPCR experiments [87]. The relative standard curve method was used to analyze the data.

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10.1. Status and results

Analysis of 'Royal Gala' response to a simulated moderate-severe drought: Clonally replicated individuals growing on their own roots were generated for the suppression subtractive hybridization experiments. We identified several hundred different genes up-regulated or down-regulated in roots, bark and leaves when DNA from drought-treated tissues served as 'tester' and DNA from well watered controls acted as 'driver' (10-fold higher amount). Some genes unique to our experiments that increased in response to drought are shown in Table 2. A number of drought-responsive genes were common to genes isolated from other plants systems, both dicot and monocot. Figures 2-4 show the relative expression of some of the common and unique genes in apple roots, bark and leaves.

Most of the genes examined regardless of tissue origin showed around a two-fold difference between watered and water-deficit treaments. A few genes were substanitally up-regulated in response to drought. including the auxin-induced gene from leaves (8-fold increase) and asparagine synthase from bark (4-fold increase). A few genes in roots were also significantly up-regulated in response to drought treatment, one of which was NPR1 (3-fold increase; manuscript submitted). From this information, including the expression of genes not shown here, we have generated a list of potential up-regulated genes responding to a relatively long term drought that can be used to determine if there is any correlation of expression in *M. sieversii* lines with high and low WUE values.

10.2. Promoter comparison of NRT2.4 from apple, peach and Arabidopsis

A high affinity nitrate transporter gene from the 'Royal Gala' root SSH library (see Table 2) was shown by RT-qPCR to be elevated in roots and bark in response to drought treatment (manuscript submitted). Approximately 700 bp upstream of the ATG start codon was obtained from the genomic sequence of 'Golden Delicious' (Genome Database for Rosaceae; http://www.rosaceae.org/). Several *cis*-elements associated with tissue specificity or stress response were identified. To identify elements preserved during evolution, promoter regions from peach NRT2.4 and an Arabidopsis AtNRT2.4 gene were analyzed for comparison (Figure 5). All three genes contained consensus, well defined TATA boxes within 80-100 bp of the translation start. A number of MYB and MYC binding sites were identified in similar positions in all three promoters. The peach promoter was missing a root-specific element seen in both MdNRT2.4 and AtNRT2.4. Interestingly, both the apple and peach promoters contained a drought responsive element on the reverse strand. Overall the elements identified in the apple NRT2.4 promoter are consistent with the expression analysis results.

Screening the *M. sieversii* population at site 6 in Kazakhstan: A core diversity population of *M. sieversii* trees (34 individuals representing 14 sibling groups) collected from Kazakhstan site 6 and maintained as seedlings at the Geneva, NY ARS Plant Genetic Resources Unit, was sampled for stable carbon isotope discrimination to select individuals with extreme values compared to 'Royal Gala'. The results are shown in Figure 6. Two individuals from each end of the WUE spectrum were chosen for further characterization.

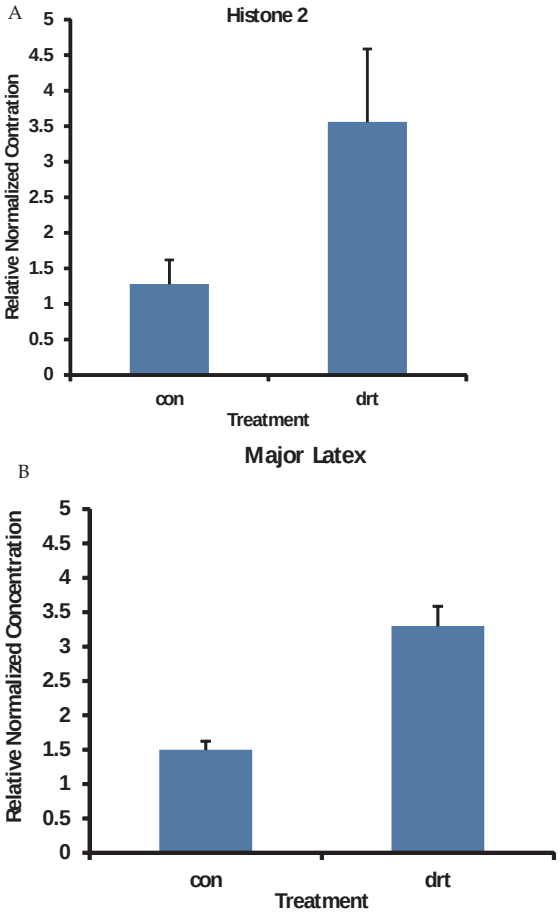

Figure 2. Relative expression of root genes up-regulated by drought treatment. Experiments were conducted as described in Methods. Test transcripts were normalized against TEF2. con: well watered controls; drt: treated to 40% saturation for two weeks. A: relative expression of Histone H2b gene; B: relative expression of the Major Latex protein gene.

M. sieversii lines GMAL4002.e and GMAL3975.k were propagated by shoot proliferation techniques to obtain a number of clonal individuals on their own roots. At the same time, 'Royal Gala' was propagated as a standard for comparison. Individuals from each line (4-6 trees per treatment) were acclimated under controlled conditions of light, water, fertilizer and temperature. Water was withheld from half of each group, while the other half received sufficient water to saturate the pot. The water-deficit trees reached 40% of pot saturation in about 7-10 days, at which time the individual plant and a comparable control were tagged. At the end of two weeks of treatment, the plants were individually sampled. Out of six

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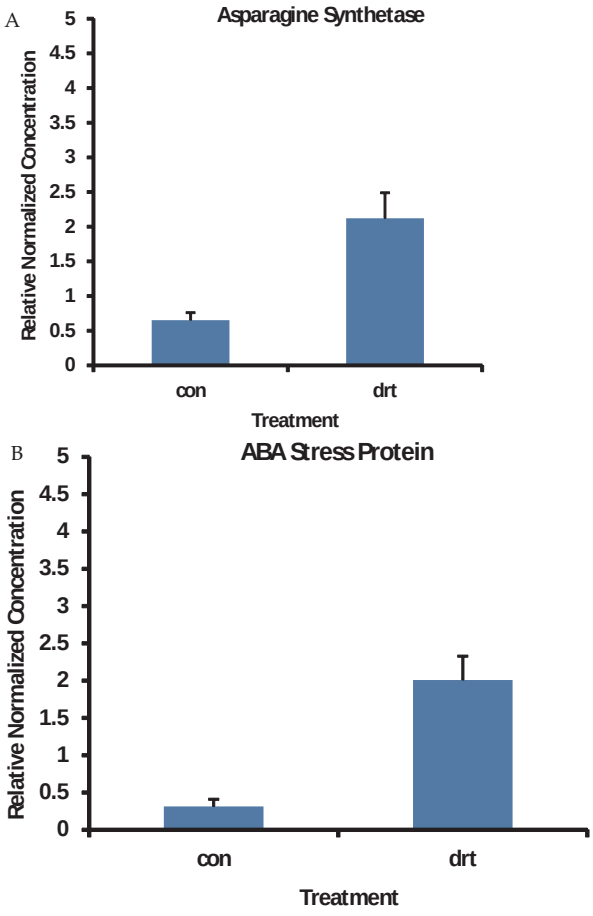

Figure 3. Relative expression of bark genes up-regulated by drought treatment. Experiments were conducted as described in Methods. Test transcripts were normalized against Actin. con: well watered controls; drt: treated to 40% saturation for two weeks. A: relative expression of asparagine synthetase gene; B: relative expression of an ABA stress protein gene.

GMAL4002.e plants, four began to show signs of severe wilting within a few days after reaching 40% as illustrated in Figure 7. This is consistent with WUE measurements which indicated that this particular line was not adept at maintaining healthy water status under the water deficit regime. Well watered GMAL4002.e controls showed no signs of wilting throughout the experiment. In contrast, GMAL3975.k with a WUE close to that of 'Royal Gala' showed no signs of wilting under the well-watered regime or water deficit stress (Figure 6). These results indicate that WUE can be used to identify apple lines that are drought sensitive, as well as drought tolerant.

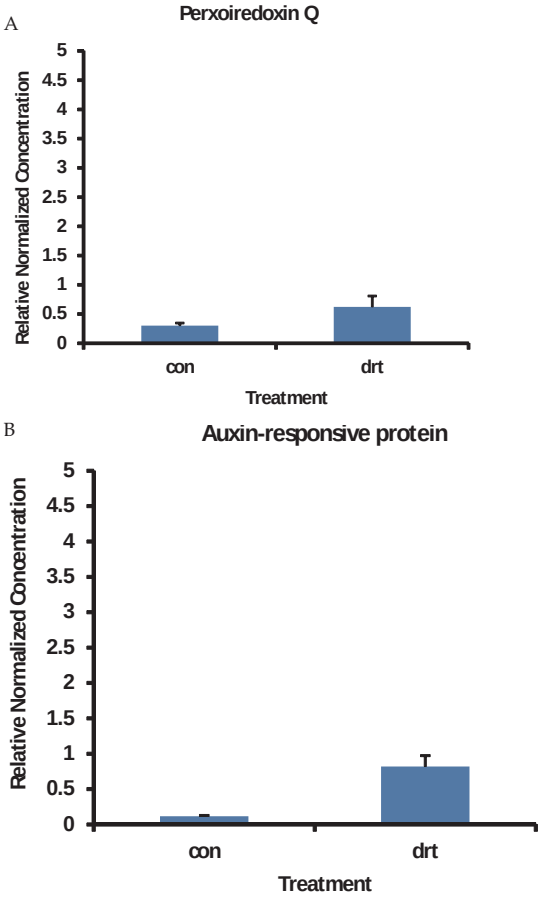

Figure 4. Relative expression of leaf genes up-regulated by drought treatment. Experiments were conducted as described in Methods. Test transcripts were normalized against Actin. con: well watered controls; drt: treated to 40% saturation for two weeks. A: relative expression of a peroxiredoxin gene; B: relative expression of an auxin-responsive/ripening protein gene.

Leaf size and number have been shown to respond negatively to drought, resulting in longer intervals between newly initiated leaves and smaller sizes, all features designed to reduce transpiration to conserve water. Leaf morphological features and stomatal characteristics were examined in the site 6 subpopulation [84]. GMAL3683.o had the smallest leaves by all traits measured, whereas GMAL3687.d and GMAL3989.f had the largest leaves by area. Within the GMAL3683 sibling group, GMAL3683.o leaf area (8.3 cm^2) clearly segregated from the other three members (average = 20.3 cm^2).

Stomatal density has also been linked to drought tolerance and sensitivity. Therefore, we also examined stomata size and density in the site 6 *M. sieversii* population. GMAL3691.m had the

Figure 5. Figure 5. Comparison of NRT2.4 promoter regions from apple and Arabidopsis. Only the first 700 bases upstream of the translation start (**atg**) are shown. *Cis*-elements were identified by PLACE [88] and PLANTCare [89]. A: Promoter region from apple MdNRT2.4; B: Promoter region from peach genome; C: Promoter region from Arabidopsis AtNRT2.4. **AAACAAA:** anaerobic induction [90]; TGACG: WRKY stress responsive binding element [91]; **A^{A}/$_{C}$GTCA** and**^{G}/$_{A}$GACTTTTC:** bZIP and NF-B binding sites, respectively [92]; **CAAGCATGCTTCTTGC:** consensus root-spscific element [93]; **TATA box:** PolII binding; **TCATTACTAA:** wound-inducible element [94]; ACGTG/AT: ABRE core element [95]. An element of unknown function in the Arabidopsis NRT2.4 gene promoter (**GTCAACT**) is also present in the MdNRT2.4 and peach promoters. A *cis*-element for hypoosmolarity-responsiveness [96] is identified by an oval. Dashed underlines indicate a MYB (WAACCA) binding site [97] and MYC core sequences (CANNTG); fuschia-underlined MYC element on the negative strand in A and B (CAACTG) is associated with drought response [73]. A CBF/DREB element [98, 99] is boxed in A and B.

largest stomates, whereas GMAL3684.a had the smallest. GMAL3689.n had the highest density of stomates (58 per 0.09 mm^2); GMAL3685.e had the lowest density (23 per 0.09 mm^2). There was no correlation between leaf and stomate features in the *M. sieversii* site 6 population, nor was there a correlation between the leaf and stomate extreme values and the ^{13}C extreme values.

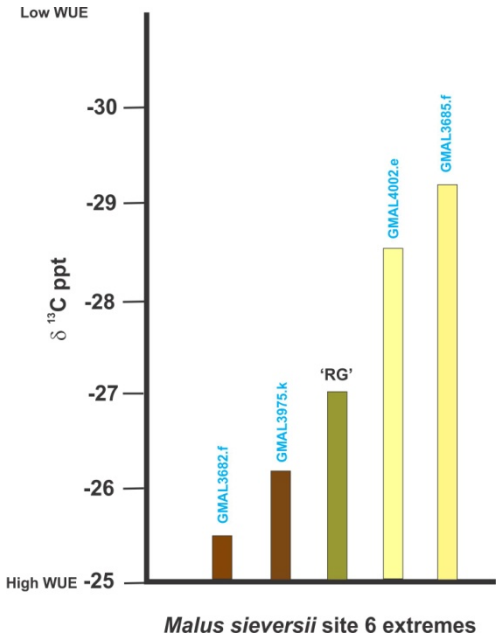

Figure 6. Stable carbon isotope analysis of select *M. sieversii* individuals from the site 6 population Measurements were made from branches representing current year's growth. Collection of samples was made from dormant trees for three years and averaged. RG: 'Royal Gala' standard.

In other words, although leaf and stomate physical characteristics might contribute to the drought response of *M. sieversii* individuals from site 6, another mechanism(s) appears to better explain the WUE data.

Further Research: The overall goal of this project is to link drought responsiveness and/or WUE to specific apple genes. To this end we are interested in candidate genes that are either up-regulated in response to drought (drought 'defensive' genes) or down-regulated (drought survival 'assisting' genes). The latter can be used to identify genetic alterations that could hamper the physiological state attained by up-regulated genes and therefore to be avoided in breeding programs. Up-regulated genes are of interest because of their obvious association with drought resistance. We have developed primers for many of the drought up-regulated genes identified in 'Royal Gala' and have tested them against individual lines of *M. sieversii*. Experiments to quantify their expression in the roots, bark and leaves of the site 6 lines representing WUE extremes are currently underway. We are also replicating additional *M. sieversii* lines on their own roots for simulated drought experiments like the ones shown in Figure 7 to provide physiological, morphological and molecular biological data to detect associations to drought resistance or susceptibility. Since the *M. sieversii* site 6 and 9 populations have undergone rapid adaptation to the xerophytic environments they currently occupy, it seems likely that alterations in patterns of expression could account for their survival. To

http://dx.doi.org/10.5772/55537

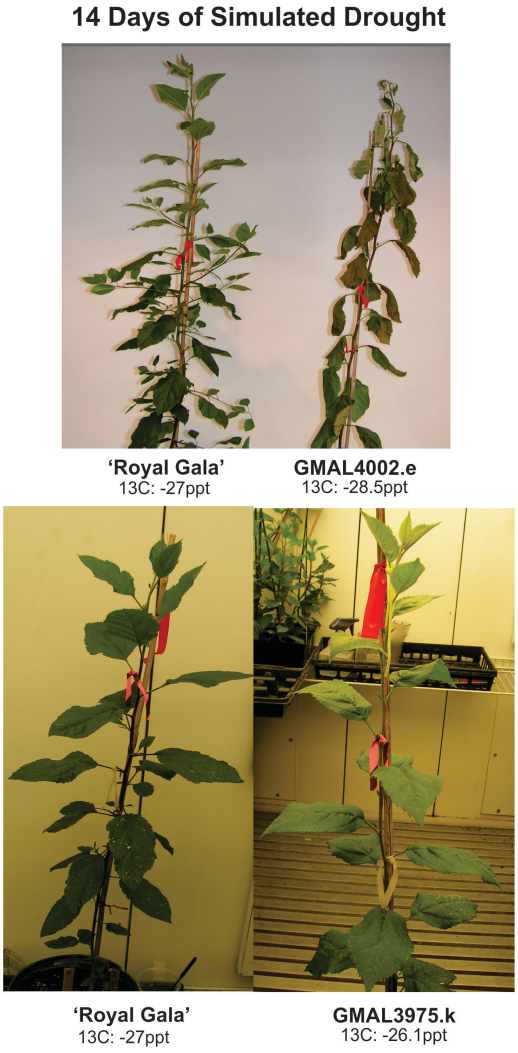

Figure 7. 'Royal Gala' and different *M. sieversii* genotypes under severe water deficit conditions. Delta ^{13}C values are included under the cultivar/genotype name. A difference of 0.50 ppt is considered significant. Note the wilting observed with GMAL4002.e compared to 'Royal Gala' under identical SD conditions. Also note GMAL3975.k and 'Royal Gala' respond similarly to the SD. 13C = $\delta 13C$

this end we are planning experiments to determine the kinetics of expression of candidate genes over a broader time period in select *M. sieversii* lines. Finally, we plan to examine promoter elements for single nucleotide polymorphisms or other alterations that might influence expression and to identify changes in methylation that might contribute to differences in expression between the more drought resistant lines and those that are more sensitive.

The information generated from these experiments can be used in breeding programs to select drought resistant progeny using marker assisted selection. In addition the use of genetic engineering of select genes is another potentially successful approach to obtaining new varieites with improved drought resistance or enhanced WUE. With increasing competition between agriculture and urban populations for fresh water and with climate change which predicts increasing episodes of intense drought periods worldwide, there is a critical need for the development of crop varieties with more efficient use of water and the ability to survive longer drought periods. Since apples are a good source of nutrition and can be conveniently stored and shipped, they are a logical target for improvement.

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