Understanding Mechanisms of Adaptation of Potato to Long-term Heat Stress: Physiological and Molecular Aspects

by

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General and Chapter Abstracts

General Abstract

Potatoes are known to grow well in cool climates. Most studies have documented the impact of heat stress on potato under short-term high temperatures (days). However, very little has been reported on long-term adaptation (weeks) of potato plants to heat stress. The three projects in this study were conducted to investigate strategies employed by potato plants to physiologically adapt to heat stress, investigate the genes and gene pathways that may be important to heat stress adaptation, and to create a methodology based on membrane thermostability to calculate an estimate of heat tolerance (HT) and heat tolerance ability (HAA). These studies used commercial cultivars, diverse varieties, or wild species derived from shoot culture plantlets or seed tubers that were grown in a controlled environment room under cool (20/15ºC, day/night) and heat stress (35/ 25ºC, day/night) conditions. The leaflets produced from the same plant under both conditions were compared and the heat-adapted leaves had higher rates of photosynthesis and stomatal conductance (SC) and lower leaf temperatures compared to control leaflets. These results show that heat-adapted leaflets can maintain lower leaf temperatures by increasing transpiration (higher SC) thereby allowing higher photosynthetic rates and shows a potential strategy used by potato to mitigate high temperature stress. RNA expression analysis between Atlantic and wild *Solanum microdontum* showed genes and gene pathways that were differentially expressed between these cool and heat-adapted leaflets. The most enriched pathways and most highly regulated genes among both genotypes were involved with heat shock proteins, lipid saturation, oxidative stress mitigation, ABA, and photosynthesis. These results suggest the involvement

of genes and pathways that may be important in HT in potato. A HT assay, based on membrane thermostability, was developed to calculate HT estimates in control and heat-adapted leaflets that were used to calculate an estimate of HAA in diverse plant materials. This method was able to differentiate significant variability between diverse genotypes using all three estimates. This work contributes knowledge to possible traits, genes, and gene pathways that may contribute to HT in potato and developed a methodology to screen for potato plant material that may demonstrate improved HT and HAA.

Chapter 2: Adaptation of photosynthesis in potato leaves to long-term heat stress: Physiological explanations

Abstract

Potatoes are known to grow well in cool climates, while higher temperatures generally reduce foliage growth, photosynthetic rates, and partitioning to tubers. Most studies to date have documented the adverse impact of heat stress on potato plant growth and development. However, very little has been reported on long-term adaptation of potato plants to heat stress. Our recent studies have shown that under long-term heat stress (4-6 weeks) the newly developed leaves have reduced size and dramatically higher stomatal density. The present study was conducted to investigate strategies employed by potato plant leaves to adapt to long-term heat stress. These adapted leaves can maintain chlorophyll and survive under heat stress as opposed to the leaves produced under non-stress conditions on the same plant. This experiment used shoot culture plantlets of *Solanum tuberosum* L. cvs. Atlantic (ATL) and Dark Red Norland (DRN) that were grown in 12.7 L pots, using an artificial soil mix (Metro Mix) and subjected to a 14-hour photoperiod with a 290 $\mu Mol \cdot m^{-2} \cdot s^{-1}$ average PAR light intensity and a 60% relative humidity. Plants were irrigated every day more than saturation

with ¼ strength Hoagland's solution. After growing in cool (20ºC/ 15ºC, day/night) temperatures for six weeks the plants were transferred to a controlled environment room for heat stress treatment (35ºC/ 25ºC, day/night). Measurements (photosynthesis, stomatal conductance, leaf temperature) were taken within the first two days of heat stress and weekly thereafter for 2 weeks in the same leaves (unadapted). After 4 weeks the measurements were made on the newly developed (adapted) leaves. Photosynthetic rates were 30-40% lower in the unadapted leaves as compared to control (prior to heat stress) after two weeks of heat stress. Photosynthetic rates were significantly higher in adapted leaves as compared to unadapted leaves under heat stress in both cultivars. In DRN the adapted leaves had similar photosynthetic rates under heat stress as the control leaves under non-stress conditions. As compared to control (non-stress), the stomatal conductance (SC) increased initially (1 day of heat stress) under heat stress but declined again to control level in DRN and to a significantly lower level as compared to the control in ATL. The heat adapted leaves in both varieties showed higher SC as compared to unadapted leaves under heat stress. Leaf temperatures were lower in the adapted leaves as compared to unadapted leaves under heat stress suggesting that higher SC in the adapted leaves lowered the leaf temperatures and mitigated the heat stress. Scatter plots demonstrated that as SC increased the photosynthetic rates increased to almost four-fold. Furthermore, photosynthetic rates decreased dramatically as leaf temperatures increased from 32-35°C under heat stress. These results suggest that leaflets produced under heat stress (adapted) can maintain lower leaf temperatures by increasing the transpiration (higher SC) and lowered leaf temperatures allowed these adapted

leaves to maintain higher photosynthetic rates. These adaptations appear to help potato plants tolerate the effects of long-term heat stress.

Chapter 3: Potential Genes Involved in Heat Stress Adaptation in Potato: Analysis of Differential Gene Expression between Control and Heat-adapted Plant Leaves of the Cultivated Solanum tuberosum L. cv. 'Atlantic' and wild *Solanum microdontum* Bitter

Abstract

The genus *Solanum* represents approximately 1,500 species, is genotypically diverse, and widely adaptive to environmental conditions. However, *S. tuberosum* performs best when temperatures are near 20/15 °C (day/night). High temperature stress has been identified as one of the most significant uncontrollable abiotic factors that affect potato plant growth, development, and tuber yield. With the potential of climate change, in addition to the expansion of potato production to areas with higher average daily temperatures, the importance of the effects of heat stress on the growth and productivity of potato has become an important issue. The identification of genes that are important in enhanced heat tolerance in potato are needed by breeders to develop plant materials that can remain productive under supra-optimal temperatures (>25°C). The purpose of our study was to evaluate the differential expression of genes, in the leaves of potato plants grown at optimal (20/15 °C, day/night) and supra-optimal conditions (35/25°C, day/night, for 4.5 weeks), that may contribute knowledge to the pathways and potential genes that are involved in the adaptation to prolonged high temperatures in two potato genotypes. *Solanum tuberosum* L. cv. 'Atlantic' (ATL) and *Solanum microdontum* Bitter (MCD), used in this study were previously observed in our experiments to show contrasting tolerance to high temperature using ion leakage analysis of leaves adapted to long-term heat stress, where ATL was found

to be relatively sensitive to heat stress as compared to MCD. Plants were derived from tissue culture and were subsequently grown in a controlled environment room and were irrigated to excess using a modified Hoagland's solution under a 14-hour photoperiod. Our previous studies focused on anatomical and physiological adaptations of potato leaves after growth under long-term high temperature (~3 weeks) that resulted in anatomical, physiological, and morphological adaptation. These leaflets were smaller in size, as compared to the control, and showed (i) increased stomatal index, stomatal density, and cell density (ii) altered ground cell and stomatal complex size (iii) greater stomatal % area of the leaf surface (iv) altered leaflet chlorophyll concentration and content (v) increased photosynthetic rates (vi) increased stomatal conductance and (vii) reduced leaf temperatures. The current study found several significantly up and down-regulated pathways and genes that were differentially expressed between conditions that overwhelmingly included the production of heat shock proteins (HSP's) in addition to pathways and genes involved in oxidative stress, lipid metabolism, isoprenoid synthesis (ABA and terpene), and photosynthesis. These data offer insight into potential pathways and genes involved in long-term adaptation to high temperature in potato.

Chapter 4: Standardization of an in vitro assay to estimate relative heat sensitivity/tolerance and heat acclimation ability (HAA) of potato germplasm using excised whole leaves

Abstract

Breeding for enhanced temperature or abiotic stress tolerance in agricultural production has become an important consideration in recent decades with interest in the expansion in the range of production, concerns over climate change, and an ever-increasing world population. The screening, identification, and breeding of heat-tolerant potato

genotypes in this regard would serve to increase the possible range of agricultural production of potato and expand food production to areas with higher than optimum average temperatures for this crop. To facilitate the screening and identification of potentially heat-tolerant plant materials a heat tolerance assay protocol was standardized for the quick estimation of relative heat tolerance and heat acclimation ability (HAA) in a large plant collection of *Solanum spp.* For this purpose, a heat tolerance assay was developed by an indirect assessment of leaf cell membrane structural integrity that results in ion leakage following exposure to heat stress (thermostability). In this procedure excised leaf tissue was immersed in distilled water maintained at 50°C in an oscillating water bath for 20 to 70 minutes. The conductance of the water inside the test tube was then recorded twice, once initially after being pulled from the water bath after the specified time (R_1) and again after autoclaving the sample to get the total conductance (R_2). The percent leakage (R_1/R_2)*100 was used as a sensitivity to heat stress. These leakage values were used to compare genotypes for an assessment of relative heat tolerance. These measurements were made on plants grown under control (20/15°C, day/night) conditions and following exposure to warm growing temperatures (35/25°C, day/night). The reduction in ion leakage following exposure to plants to warm growing temperatures was used as an estimation of heat acclimation ability (HAA). Multiple plant genotypes including commercial cultivars, wild potato species, and exotic heat tolerant selections obtained from the potato gene bank were used. With this protocol we can assess the differences in heat sensitivity as well as HAA among cultivated germplasm and wild species. These results highlight three mechanisms at work that could be potentially genetically distinct (1) heat-tolerance in the unadapted state and (2) heat tolerance in the

adapted state and (3) the ability to acclimate after long-term heat exposure. To our knowledge, no other reports exist comparing unadapted heat sensitivity/ tolerance, adapted heat sensitivity/tolerance, and HAA in long-term heat-adapted potato. Future studies should be directed to determine the possible relationship between the heat sensitivity measured by this *in vitro* assay and plant performance (growth and tuber yield) under heat stress conditions.

Dedication

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Chapter 1 General Introduction and Research Objectives

The Potato Plant

The potato is the most widely consumed vegetable in the United States and in the world (Consortium, 2011; Spillman, 2003). The most common species of potato that is utilized is *Solanum tuberosum* L., which is an autotetraploid species with a basic chromosome number of 12 (Consortium, 2011). It is a part of the agronomically important genus Solanaceae that also includes tomato, pepper, eggplant, petunia, and tobacco (Consortium, 2011). The potato is normally clonally propagated, has a highly heterozygous genome due to polyploidy and cannot be inbred without suffering from inbreeding depression. The potato genome is 844 megabases in length, of which 86% has been sequenced and assembled. It is predicted that the genome encodes for 39,031 protein-coding genes. The transcriptome of potato has also been investigated and reveals tissue-specific expression of genes in addition to restricted expression of genes under certain conditions (Massa, et al., 2011), which are important to investigate further to understand which genes contribute to adaptation to certain biotic and abiotic stresses. Linkage disequilibrium in potato has been estimated to be around 5 – 10 cM (Bjorn, et al., 2010; Simko, et al., 2006). The genus of *Solanum* is very large with over 2000 species identified across the world. However, most species of *Solanum* are non-tuber bearing species and only approximately 200 species bear tubers. Among the tuber-bearing species only about 8 are cultivated for food with the most popular cultivated species being *Solanum tuberosum* L. (Burton, 1989). Previous estimates have put production of potato at 250 million tons/ year and it is estimated that potato is grown on more than 17.6 million hectares in more than 125 countries (Rowe,

1993). A more current estimate puts production around 330 million tons as of 2009 (Consortium, 2011). The American continent is the exclusive home to tuber-bearing Solanum species where they provide an extremely important role to breeders as important sources of genetic variation for resistance to several insect pests, pathogens and environmental extremes (Hawkes, 1992). Potato is considered to have a narrow genetic base outside of its origins in South America due originally to limited germplasm introductions in Europe that later came to the United States. This limited genetic base, polyploidy and other factors are barriers to the improvement of potato genetics using classical breeding approaches (Consortium, 2011).

Growth Stages

The potato plant goes through five distinct growth phases in its lifetime. The first stage includes planting to emergence, where upon planting a seed tuber, the sprouts and roots develop before the emergence of the shoot from the soil and solely rely on the energy reserve in the seed tuber piece (Rowe & Secor, 1993). The second stage includes the vegetative growth of stems, leaves and underground stolons which are produced from the collection of nutrients and water from the soil and this marks the beginning of the independency of the potato plant from the energy stored in its “seed” piece and reliance on photosynthesis for energy production. Tuber initiation begins the third phase of potato plant growth and the stolon tips begin to swell, which often coincides with flowering. The fourth growth phase begins tuber initiation and bulking and the final stage follows the senescence of the potato vines, decreased photosynthesis and tuber growth and tuber skin set (Rowe & Secor, 1993).

Propagation

Potato is an interesting crop in that it is clonally propagated, and is perfectly suited for this type of reproduction, as the tubers can be used to naturally propagate and are commonly referred to as “seed tubers” (Burton, 1989). Sexual propagation in potato is rarely done for the agronomic production of potatoes but plays an important part in breeding and genetics, though a fair amount of research effort has been directed around the practical propagation of potato from true seed (Cutter, 1992). The fruits of potato look like small green tomatoes and the true botanical seed found within these fruits are generally yellow to yellow-brown, around 1mm in length, can be covered in hairs and can vary in shape from flat, oval or kidney-shaped (Dean, 1994). Sexual propagation is not used for agronomic production or propagation due to the highly heterozygous nature of potato, which creates large genetic variability in potato progeny and results in variability in tuber quality (tuber shape and size) and typically lower yields (Dean, 1994). Additionally, potato experiences rapid inbreeding depression and therefore homozygous lines are not possible for use as parents to derive heterozygous, uniform progeny for crop production (Massa, et al., 2011). However, sexual seed production is practiced in some parts of the world, such as in Peru, where the importance of uniformity and quality is not an issue (Dean, 1994).

Potato plants are most commonly propagated by *in vitro* tissue culture techniques on artificial media that allow the growth of single cells or individual plant organs into new plants that allow for the rapid production of potato plantlets (Espinoza, et al., 1986). The plant material introduced to tissue culture conditions must be adequately surface sterilized through a series of ethanol (~70%) and bleach (10%) treatments with an adequate surfactant

to help penetrate otherwise water-inaccessible spaces on the plant surface (Joy, et al., 2011). This must be done adequately otherwise the growth rate of fungi or bacteria will easily overcome the growth rate of the plantlet and it will die. Tissue culture production can also be used for pathogen elimination in potato as well, which is very important given the asexually-produced nature of the potato that can accumulate viruses and pass them on in propagation materials. Viruses are much more difficult to eradicate and this is one of the biggest and most significant problems affecting potato in the field (Espinoza, et al., 1986). Fortunately, tissue culture offers a method to eliminate viruses from potato plant propagules in vitro through thermotherapy in conjunction with anti-viral media additives and the eventual excision and culturing of meristem tips. Thermotherapy is given over a four-week period with constant lighting and thermocycling of 36/30°C for 16/8 hrs, respectively, with the goal of reducing virus replication and increasing meristem growth rate. This method takes several weeks to regenerate an entire plant, after which the material can then be ramped up for propagation and disease testing can begin. This method offers a way to control virus proliferation in an asexually produced crop and provide pathogen-free “clean seed” material for the entire potato industry (Joy, et al., 2011).

Several tissue culture methods are used for the in vitro production of potato including single node, shaken liquid cultures or cell and callus culture. The latter is not used as commonly as the prior methods since clonal stability can be impacted through the reproduction of a potato plant from a single cell and therefore the former methods are used which focus on propagation from organs and whole tissues that allow the release of an axillary bud. Potato plants can then be produced every 3-4 weeks (shaken liquid cultures 2-3

weeks) from single node cuttings and at that time they may be sub-cultured further, planted in artificial potting mix as mothers for conventional clonal propagation or planted in seed beds or seed fields for the production of certified disease-tested “seed” (Espinoza, et al., 1986).

Production

The potato plant is adapted to cooler environments such as those found in the Andes Mountains of South America where the potato is thought to have originated (Hawkes, 1978). Therefore, the areas most used for potato production (*Solanum tuberosum* L.) are similar in climate to the ancestral home of potato. To prevent pest and pathogen contamination the certified disease-tested potato “seed” obtained from tissue culture seed programs are usually produced in areas far from commercial potato production areas. Typical areas in the western United States used for growing disease-free seed include mountain valleys and plateaus that have lower populations of pest insects and do not support other insects such as the green peach aphid during non-summer months. These areas are typically poorly accessible to commercial markets but offer a quarantined area for production of clean seed for the rest of the industry.

The eastern seaboard region (ESR) has been used in potato production for a long portion of history but has been a minor contributor compared to overall US production with recent production at 6.5% of the US total (Resop, et al., 2013). While there are some areas with large production of potato, particularly in the northern portion of the ESR, the production of potato has been in decline in this region despite yield gains within the last half century that are mainly the cause of decreasing harvested potato area (National, 2012). Since

1900 the agricultural land in this region has been shrinking due to a population shift to urban areas, industrialization and reforestation (Bell, 1989; Foster, 1992). The erosion of the topsoil of prime farmland has also been of concern, where removal of field stones and continuous cropping have led to high amounts of erosion and depleted soil in the region (Lal, 1998). The result of these impacts has been a reduction in potato production in this area leading to reliance on the importation of potato to meet consumer demands as the yearly consumption of potatoes in this region is calculated at 4.17 million Mg while the production is at only 1.34 million Mg. Looking into the constraints on the production of potato in this region would provide important insight into how to increase potato production in this region to meet local demand without importation (Resop, et al., 2013). Potato imports to this region are primarily from Canada and are produced in New Brunswick, Prince Edward Island and Quebec (Cheng, 2005).

Potatoes are a high value crop that are typically in high production on a large amount of acreage in the US including the pacific northwest (PNW). This region includes Idaho, Washington, and Oregon. As of 1999 the total in-use land was approximately 256,000 hectares that produced a total of $11.7x10^6$ Mg. Compared to the overall potato production and acreage in the U.S. the PNW potato industry accounts for 45 and 54% of total U.S acreage and total production, respectively. This area contains the most highly productive land for potatoes in the entire U.S. called the Columbia Basin that has the capacity to produce 78 Mg/ha compared to the U.S. average of 38 Mg/ha. The soil conditions in this production area provide course, sandy soils low in organic matter that are subject to wind and water erosion. Due to these soil components this soil is prone to leaching of water and water-soluble

chemicals used during agricultural production that eventually makes its way below the root zone and can be exacerbated by poor irrigation practices. Good irrigation practices in addition to good nutrient management, especially with Nitrogen, and scheduling are important for sustaining large production on these soils, for helping maintain good water quality and for preventing water stress, which can be detrimental to tuber quality and yields in this region (Alva, et al., 2002).

The U.S. Midwest is another important production zone for potato that includes Minnesota, North Dakota, Michigan, and Wisconsin. The total acreage is considered small, with 94,000 hectares but an economic value of approximately $857 million/year (NASS, 2013). Potatoes in this region are grown primarily on sandy, well-drained soils that have a low water-holding capacity. Irrigation and nitrogen management are therefore especially important considerations. Nitrogen is recommended here in high amounts (270 kg N/ha) and therefore university guidelines suggest that nitrogen be applied as slow release or applied as split applications (fertigation) throughout the season to avoid losses due to leaching (Rosen & Bierman, 2008). Approximately 66% (NASS, 2013) of the total acreage in this region is irrigated due to the low water holding capacity of the sandy soil and the sensitivity that potatoes show to drought stress (Shock, et al., 2007; Bohman, et al., 2019).

Climate Change and Potato Production

In the last 10 to 20 years the potato has been introduced to areas with higher average daily temperatures such as tropic and sub-tropic areas (Tai, et al., 1994). The biophysical limitations of the potato plant in these new areas have contributed to reduced yields or outright failure of the crop. High temperature or heat stress has been identified as one of the

most significant uncontrollable factors that affect potato plant growth, development, and tuber yield (Smith, 1968). Although, in commercial production areas conducive for potato plant growth potatoes experience an array of conditions not in accordance with what is considered optimum for potato plant performance and growth such as temperature, light, water, and nutrition (Burton, 1989). For a time, high temperatures were not considered to be a serious limiting factor in potato production since most production occurred in areas considered to be within its threshold for temperature adaptation (Mendoza & Estrada, 1979).

Additionally, with the potential of climate change shifting temperatures in current production areas the importance of the effects of high temperatures on the growth and productivity of potato has become more important than ever before and the need to identify genetic resources and develop plant materials that are able to remain productive under temperatures higher than what is considered to be the optimum temperature for potato plant growth is paramount to protecting our food security in the face of a changing climate. Within the next 40 years the global average temperature is estimated to increase significantly around 2.1 – 3.2 °C (Hijmans, 2003). From 1900 to 2003, the average global warming trend was 0.6 °C and this trend is expected to continue and is estimated to increase to 1.4 and 5.8 °C over the period from 1990 to 2100 (Houghton, et al., 2001). When weighting the area of potato production into their estimate for average global temperature increase, especially in addition to the consideration of the adaptation of cultivars and planting times at higher latitudes, Hijman's (2003) estimates this temperature increase to be between 1 and 1.4 °C. He further estimates that the global potato production could decrease in the range of 18-34% without adaptation of cultivars and planting times and could decrease by 9 to 18% with

adaptation of these factors. It is further suggested that the predicted change in climate may not have a large impact on potato production at higher latitudes when considering the potential of cultivar adaptation (later maturing cultivars), the time of planting and changes in locations of potato production that are feasible in these areas. However, changing locations or planting times to adapt to a changing environment in lower latitudes, such as the tropics and subtropics, may not be feasible and the importance of cultivar selection and adaptation becomes the most feasible method of combating these potential future climatic changes. Areas that are likely to see changes in temperature, especially increased temperature, that significantly effect tuber yields will also be important to identify in addition to the effectiveness of heat tolerant germplasm in mitigating the effects of higher than normal temperatures in these areas where cultivar selection is one of the most important considerations in potato production in these regions (Hijmans, 2003).

Potatoes and Calcium

Potato is the most popular vegetable in the United States but suffers from several tuber-related defects that reduce marketability, storage life and overall yield (Spillman, 2003). Some of these defects have been negatively correlated with TC concentration (Spillman, 2003). For example, calcium has been associated with reduced severity of internal brown spot (Ozgen, et al., 2006; Tzeng, et al., 1986), reduced hollow heart (Arteca, et al., 1980; Kleinhenz, et al., 1995; Kleinhenz, et al., 1999; Levit, 1942; Rex & Mazza, 1989) reductions of soft rot in storage caused by the *Pectobacterium* carotovorum pathogen (McGuire & Kelman, 1984), improved yield and tuber grade (Simmons & Kelling, 1987), reduced internal heat necrosis (Yencho, et al., 2008), reduced blackspot bruise (Karlsson, et

al., 2006) and increased abiotic stress tolerance to both heat (Kleinhenz & Palta, 2002) and freezing stress (Vega, et al., 1996).

Calcium is very important to plant growth and development, membrane and cell wall function and structure, regulation of cell metabolism and stress signaling response (Palta, 2010). The highest proportion of calcium is found in the cell-wall where it is known to help maintain structural integrity of the plasmalemma by stabilizing pectins through calcium cross-links (White & Broadley, 2003; Murayama, et al., 2017). Calcium also supports the structure of the cell membrane through phospholipid linkages (Palta, 2010).

Tuber Calcium Accumulation

The ability to increase the calcium concentration in the potato tuber is important for tuber quality and this factor can be increased by both the modification of the growing environment through cultural practices and by genetic gain (Bamberg, et al., 1993). An improved understanding of how potato tubers accumulate calcium changed the way we think about calcium nutrition and fertilization and how we can specifically increase the naturally low calcium concentration in the potato tuber through the modification of the environment that we provide them. This improved understanding came about through the discovery of both a main root system, servicing the main stem and leaves, in addition to a tuber and stolon root system, servicing the tubers. Calcium preferentially follows the transpiration-driven mass flow of water in the xylem and this flow is determined by the gradient of water potential that favors transport from the main root system to the above-ground stems and leaves. However, the water potential surrounding the tubers is much lower than the potential surrounding above-ground stems and leaves due to a more humid environment around the

tubers that restricts transpiration and hence water and soluble/ exchangeable calcium transport from the soil around the tuber roots to the tubers (Palta, 2010). This results in tubers, from cultivated potato, accumulating around $1/5^{th}$ of the calcium found in the above-ground stems (Spillman, 2003). The result of this discovery motivated the implementation of targeted applications of water-soluble forms of calcium during the tuber bulking period to increase TC concentrations (Kratzke & Palta, 1986; Simmons & Kelling, 1987). Calcium is now used regularly as part of a normal potato fertilization program by routinely adding it in a water-soluble form, such as $CaNO_{3,}$ through the irrigation water during the tuber bulking period at a rate of approximately 112 – 224 kg/ha (100 – 200 lbs/acre) (Spillman, 2003).

ATL x SUP Population and the Study of Tuber Calcium Accumulation

The genetic variation for TC accumulation in the cultivated potato, *Solanum tuberosum*, was reported to be low compared to wild species (Chung, et al., 2016), but advanced breeding clones of potato have been observed to display significant sources of genotypic variation, and large broad-sense heritability's, for this trait under naturally available conditions of calcium (Brown, et al., 2012). Efforts are being made to develop materials that demonstrate a greater ability to accumulate TC to increase tuber quality and marketable yield using the large variation for TC found in wild potato species (Chung, et al., 2016). However, introgression of germplasm from other potato species into the cultivated varieties can be difficult due to crossing barriers, variations in ploidy levels, variations in endosperm balance number and the inherent small tuber size of wild species (Bamberg, et al., 1993; Jansky, 2006). A simpler approach could utilize the existing variation in cultivated tetraploid potato by developing populations using parents that exhibit contrasting

characteristics for the trait of interest and allowing the F1 population progeny to segregate for the trait (Vega, et al., 2006; Zorrilla, et al., 2014). F1 populations of highly heterozygous polyploid species generated from parents with contrasting traits, such as the population in the current study, show sufficient variation in these traits as demonstrated in other studies of potato (Heldak, et al., 2007; Meyer, et al., 1998) and other polyploid species such as alfalfa and sugarcane (Julier, et al., 2003; Lyrene, 1977). Using cultivated varieties as parents has the advantage of being more likely to obtain progeny with desired commercial qualities such as tuber uniformity (Zorrilla, et al., 2014).

'Atlantic' and 'Superior' Parental Characteristics

The current study utilizes the cultivated chipping varieties 'Atlantic' and 'Superior' as parents, which contrast each other in several important factors. 'Atlantic' is known to have low TC (Karlsson, et al., 2006), high yields (Webb, et al., 1978), high amount of internal defects such as internal tuber necrosis (Henninger, et al., 1979; Webb, et al., 1978), moderate scab susceptibility (Haynes, et al., 2010), high specific gravity (Webb, et al., 1978), good chip and fry quality (Webb, et al., 1978) and uniform tuber size (Webb, et al., 1978), while 'Superior' is known to have high TC (Karlsson, et al., 2006), lower yields (Zorrilla, et al., 2014), lower amounts of internal quality issues including hollow heart, internal necrosis and vascular discoloration (Rieman, 1962), moderate scab resistance (Haynes, et al., 2010; Rieman, 1962), poor fry quality (Zorrilla, et al., 2014) and low specific gravity (Karlsson, et al., 2006). The current study evaluated this population in the field and observed TC concentrations under two calcium treatments: (1) naturally occurring soil calcium (endogenous calcium supply) was used as the control and (2) applied soluble calcium in the

field during the tuber-bulking period (exogenous calcium supply) was used for the supplied calcium treatment.

Potato and High Temperature Stress

Potato (*Solanum tuberosum* L.) is adapted to cooler climates (Hawkes, 1978), such as those found in the Andes Mountains of South America where the relatives of cultivated potatoes are thought to have originated (Hawkes, 1992). High temperature stress is one of the most significant uncontrollable factors that affects potato plant growth, development, and tuber yield (Smith, 1968). High temperature stress affects many factors in potato, such as photosynthesis (Dwelle, et al., 1981; Reynolds, et al., 1990), transpiration (Ku, et al., 1977), respiration (Thornton, et al., 1996), stomatal conductance (Dwelle, et al., 1981), chlorophyll content (Reynolds, et al., 1990; Steffen, et al., 1995), assimilate partitioning (Ewing, 1981; Van Dam, et al., 1996), tuber initiation and development (Borah & Milthorpe, 1962; Ewing, 1981; Reynolds & Ewing, 1989a), as well as morphological and anatomical alterations (Ewing, 1981; Khedher & Ewing, 1985; Reynolds & Ewing, 1989a; Kleinhenz & Palta, 2002). Plants, such as potato, that originate in environments with large fluctuations in temperature, including regular exposure to long-term high temperatures, tend to demonstrate considerable variation in their adaptation of the photosynthetic system to high temperature (Berry & Björkman, 1980). Most studies in potato report on the short-term or acute effects of heat stress on photosynthesis. Reynolds et al. (1990) studied the photosynthetic rates of heat tolerant and heat susceptible potato species of *Solanum chacoense*, *Solanum demissum*, *Solanum stoloniferum*, and *Solanum bulbocastaunum* at 25/20°C and 40/30°C temperature (day/night) up to 9 days and observed lower photosynthesis rates, at higher temperatures, in

heat-unadapted leaf disks from heat susceptible genotypes (S. chacoense). However, the heat tolerant genotype showed an increased rate of CO_2 fixation under heat stress as compared to the control (Reynolds, et al., 1990). Another previous report, evaluating the short-term effects of heat stress on potato by Lafta and Lorenzen (1995), found increased photosynthetic rates after 8 days under high temperatures for two potato cultivars (Norchip & Up-to-Date) using 31/29°C temperatures (day/night). While many studies in potato focus on the short-term effects of high temperature stress; however, studies are lacking for the effects of long-term high temperature on potato including information on how the potato plant alters its morphology, anatomy, and physiology to adapt to these high temperatures. A study evaluating the long-term effects of five potato cultivars (Alpha, Desiree, Katahdin, Norchip, and LT1) grown under 22/12°C, 27/12°C, or 32/12°C (day/night) conditions, with an 8-hour photoperiod in a phytotron, found that the photosynthesis rate did not decrease significantly in heat-unadapted leaves after these plants had been grown for up to 4-6 weeks at higher than optimum temperatures (Wolf, et al., 1990). The same study also evaluated the three plants Desiree, Up-to-date, and Cl-884 in a greenhouse using 31/20°C ($\pm$ 1°C) or 41/20°C ($\pm$ 1°C) (day/night) growing conditions and found that the photosynthesis rate decreased significantly in the variety Up-to-date after long-term high temperature exposure compared to the control (Wolf, et al., 1990). Generally, over the course of 24 days the rate of photosynthesis declined overall in both temperature treatments and in all three varieties tested (Wolf, et al., 1990).

In the last several decades, large-scale potato production has been introduced to tropic and sub-tropic regions with high day and night temperatures (Mendoza & Estrada, 1979; Tai, et al., 1994) providing new challenges to crop production in a new climate that often

experiences high temperature extremes. Several studies suggest an optimum temperature for photosynthesis in potato (Ku, et al., 1977; Wheeler, et al., 1986). Ku et al. (1977) reported the optimum temperature for photosynthesis in cultivated potato plants of *Solanum tuberosum* L. W729R to be between 16 and 25°C. Similarly, Wheeler et al. (1986) concluded the optimal temperature for photosynthesis in potato was 20°C based on the yields of tuber fresh weight and foliage dry weight.

Breeding Potatoes for Adaptation to New Locations and High Temperatures

The collective efforts of breeders, agronomist and plant scientists have adapted potatoes to long day conditions, although the cooler temperature requirements have remained unaltered, when compared to the native species in South America (Hawkes, 1978). We have previously discussed the recent introduction of potato to the tropics and subtropics (Tai, et al., 1994). These regions are unlike the conditions faced by potatoes in their native climate, where they are periodically exposed to higher day and night temperatures than what is considered optimal for potato growth. Additionally, they can also see large variations in humidity from high humidity in the tropics to dry conditions in the subtropics. The general result of these climatic conditions, in many of the potato cultivars that have been bred for more temperate climates, is a reduction in yield and tuber quality (Ivans & Milthorpe, 1963; Ewing, 1981; Levy, 1983; 1984). The environmental constraints imposed on potato plant growth and development in warmer climates can be overcome through breeding for adaptation to these conditions. An important consideration in breeding for climatic adaptation is the genotype-environment (GXE) interaction experienced by several different genotypes in a number of different climates, locations or even years that may concern a number of

different plant factors/ traits. GxE interactions are frequently found in traits that show multigenic control, such as heat tolerance, and it is important to consider the stability of genotypes and the assessed heat tolerance over the years and locations where they are grown so that materials that consistently show heat tolerant qualities can be identified (Reynolds & Ewing, 1989b). Reynold and Ewing (1989b) evaluated 319 accessions from 59 *Solanum* species over a two-year period and additionally evaluated 51 accessions from 7 species over 3 years. They assessed heat tolerance by the ability of plants to produce shoot growth under long photoperiods and high temperature in addition to looking at how the same plants produced tubers under high temperatures and shorter photoperiods. After a particular accession or species showed good shoot growth under high temperatures the same plants were then evaluated for tuber production under the same conditions and although tuberization was found to be variable there were several species and accessions that were consistent in tuber production under these high temperature conditions (Reynolds & Ewing, 1989b).

It is suggested that efforts in increasing heat tolerance in potato be directed at the ability of the potato plant to initiate and produce tubers as opposed to gas exchange measurements such as net photosynthesis or dark respiration that are seen as less sensitive to high temperatures (Haverkort, et al., 2008; Muthoni & Kabira, 2015). Additional traits that could be important in a breeding program for heat tolerance are the ability of the potato plant to initiate tubers under high night temperatures, formation of a lower shoot/root ratio at higher temperatures, early maturity (Hijmans, 2003), high water-use efficiency, increased length of roots, and disease resistance (David & Richard, 2007).

There are several institutions and breeding programs that have studied the effects of high temperature in potato and that have been engaged in trying to create heat tolerant potato varieties including the James Hutton Institute, the Scottish Crop Research Institute, the Russian Potato Breeding Program, the Central Potato Research Institute in India, and the International Potato Center (CIP) among others (Minhas, et al., 2006; Hancock, et al., 2013; Raymundo, et al., 2014). These programs are an important part in creating genetic resources, such as parental material and heat tolerant clones (i.e. heat tolerant variety *Kufri Sirya in* India), and such programs can help identify genetic markers that are associated with heat tolerance that would aid in the rapid improvement of heat tolerance. CIP has been instrumental in potato breeding effort across the world, and they have been engaged in breeding for heat tolerance to benefit developing countries in lowland tropic areas since the 1970's. A major objective in 1975 was to develop populations that would be adapted to the lowland tropics and that displayed superior performance and stability in similar locations. Earliness and yield potential were used to select 34 tetraploid clones from these populations that were later evaluated in Peru. From the populations used in these evaluations the *neo-tuberosum* material with a wider genetic base demonstrated a greater response to the prevailing conditions than the S. *tuberosum* material, but the best performing materials were found to have had S. *tuberosum* as one parent providing both earliness and relative heat tolerance (CIP, 1975). The widening of the genetic base was also required to provide for disease resistance by using such crosses as *neo-tuberosum* or *Phureja-Stenotomum* crossed with *S. tuberosum* using 2n gametes. In the 1980's CIP continued to develop germplasm resources and cultural practices that showed improved performance and tolerance under the

conditions of the lowland tropics (Midmore, 1992; Midmore & Rhoades, 1987; Midmore & Prange, 1991). Progeny testing was performed to find superior progeny after recurrent selection; the result of which increased the allele frequency of those genes that provided adaptation to higher temperature environments such as providing for early plant maturity and resistance/ tolerance to both abiotic and biotic stresses. It is this manner of breeding that is the most practical and efficient for improvement of tetraploid potato and the complex tetrasomic inheritance, high amounts of heterozygosity, and asexual nature of its reproduction. Heat tolerant plant materials showed several generally similar traits including earliness of maturity, the ability to initiate tubers under high temperatures, and the conversion of electromagnetic energy into dry matter (Sleper & Poehlman, 2006). Several different sources of germplasm were used by CIP for the breeding of heat tolerant germplasm such as the native *S. andigena*, the long-day-adapted *S. andigena* also known as *neo-tuberosum*, *S. tuberosum* cultivars and elite materials from the United States and Europe, *S. stenotomum* and *S. phureja* diploid Peruvian and United States material in addition to a number of other species including S. *acaule*, S. *demissum*, S. *raphanifolium*, S. *sparsipilum* and S. *stoloniferum* (CIP, 2011).

It is well-known that high temperatures have a significant impact on potato plant growth, development, biomass production and photosynthate distribution, but currently little is known about the genotypic diversity of high temperature tolerance and avoidance that exists among all the many sources of potato germplasm. It is likely that adaptation to high temperatures involves a complex array of genes and relationships in potato, and therefore, an adapted genotype would be expected to express a complex collection of genes to create a

high temperature adapted genotype (Marshall, 1982). It is important to consider the multigenic nature of heat tolerance means that transgenic approaches would likely be limited in improving heat tolerance, but several other new methodologies in breeding for multigenic traits give us a method by which heat tolerance may be improved such as by association genetics in addition to marker assisted selection (Levy & Veilleux, 2007; Hancock, et al., 2013). Identifying heat tolerance traits and mechanisms in potato starts with understanding the genes involved in the control of heat tolerance (Gangadhar, et al., 2014). As potato production is expanded into new climates considered to be characterized by higher than optimum temperatures for potato growth, the need to produce genotypes and assess germplasm that can tolerate these conditions has received increased attention. Genetic studies have shown that variation for heat tolerance and other abiotic stress tolerance exists among potato germplasm both within species and cultivars (Levy & Veilleux, 2007). Heat tolerance has also been associated with plant maturity, where earlier varieties were found to show more heat tolerance characteristics than later-maturing genotypes, although differences exist between genotypes within a maturity class as well (Levy, et al., 1991). Even though little is currently known about the diversity of heat tolerance in the *Solanum*, efforts continue to focus on breeding and developing germplasm for these higher temperature environments (Marshall, 1982).

Solanum tuberosum L. ssp. *Tuberosum*, a tetraploid, is widely adapted to the prevailing environment in temperate climates (cooler day/night, longer photoperiod) but despite this fact some of the germplasm that exists within this subspecies is still able to initiate tuber production (Ewing, et al., 1987) and provide decent yields under heat stress

(Levy, 1986). Heat tolerance has also been observed in diploid tuber-bearing potato accessions such as *S. bulbocastanum*, *S. chacoense*, *S. demissum*, S. berthaultii and *S. stoloniferum* that could be used for breeding for tuber production under high temperatures (Reynolds & Ewing, 1989b; Muthoni & Kabira, 2015). Introgression of these diploid species into the tetraploid species is possible through sexual polyploidization using 2n gamete production, somatic hybridization, or chromosome doubling. Furthermore, the use of *S. tuberosum* dihaploids is also possible for breeding at the diploid level (Muthoni & Kabira, 2015).

To assess diversity, in a wide array of germplasm and progenies, and breed for adaptation to high temperature environments there needs to be a simple, relatively fast, and efficient method of assessing heat tolerance to produce new potato genotypes with excellent heat tolerance (Levy, et al., 1991).

Assessing Heat Tolerance in Potato

Assessing high temperature tolerance in potato has been performed in several ways by assessing various morphological and physiological characteristics after high-temperature exposure either in the field, greenhouse/ controlled-environments, or *in vitro*. Tai et al. (1994) assessed heat tolerance in potato by looking at tuber dry weights after high temperature exposure and then using this to calculate a heat susceptibility index for specific genotypes. Levy et. al (1991) used a similar method to assess heat tolerance by looking at the ability of the potato plant to form tubers under high temperatures. Reynolds and Ewing (Reynolds & Ewing, 1989b) evaluated over 300 accessions of 59 tuber-bearing *Solanum* species for the ability to produce shoots under high temperatures, including a stability

analysis of heat tolerance for 51 accessions of 7 species over a three-year period. Veilleux et. al (1997) assessed heat tolerance in the progeny of Atlantic and 2n gamete producing diploids by looking at tuber numbers, tuber weights and the percent of plants producing tubers. Heat tolerance has also been assessed in several accessions of potato by evaluating tuber initiation on stem cuttings of plants that were grown under high temperature (Reynolds & Ewing, 1989b). Other methods assess heat tolerance by measuring growth rates (dry weight), chlorophyll fluorescence, and using gas exchange/ carbon assimilation measurements such as net photosynthesis and maintenance dark respiration (Midmore & Prange, 1991). Nowak and Colbourne (1989) used *in vitro* tuberization in tissue culture and assessment of the accumulation of the protein patatin and other 22 kDa proteins to assess the heat tolerance of four common commercial varieties of *S. tuberosum*. The ability of the nodal cutting to tuberize in vitro after exposure to high temperature (28-30 °C) was used as the basis for heat tolerance where a greater amount of tuber initiation was taken as an indicator of heat tolerance (Nowak & Colborne, 1989). Additionally, the ability to produce and accumulate proteins after heat exposure was similarly used to assess tolerance to high temperature stress where a reduction in these proteins were used as an indicator of susceptibility to high temperatures (Nowak & Colborne, 1989). Additionally, heat tolerance has also been assessed using measurements of cell membrane thermostability through the measurement of ion leakage from cells of excised leaves after several timed exposures to high temperature (Nagarajan & Bansal, 1986).

As referenced above, these methods assess heat tolerance of potato in many ways based on growth rates (dry matter accumulation), tuber initiation, shoot production ability,

tuber numbers, tuber yield, the percent of plants producing tubers, evaluating tuber initiation on stem cuttings, chlorophyll fluorescence, gas exchange/ carbon assimilation measurements (net photosynthesis and dark respiration, respectively), ability of a nodal cutting to tuberize *in vitro*, protein accumulation, stomatal conductance (Demirel, et al., 2017), leaf area index (Demirel, et al., 2017), leaf/ canopy temperature (Demirel, et al., 2017), SPAD (Demirel, et al., 2017), transpiration rate (Demirel, et al., 2017), and measurements of cell membrane thermostability (Nagarajan & Bansal, 1986). The current study utilizes the latter method of cell-membrane thermostability to assess the heat tolerance of potato, both before and after a long-term (3-4 weeks) heat stress, and to determine a heat adaptation response. Determining a reliable method to assess heat tolerance in potato is important for the identification of useful genetic resources for use in breeding heat-tolerant potatoes for the future in the face of a changing climate.

High Temperature & Photosynthetic Rate

Reduced potato plant growth under high temperature conditions may result from a reduction in both photosynthetic capacity and carbon fixation (Ewing, 1981). The ability of the photosynthetic capacity of a plant to adapt to heat stress is very important since this process is very sensitive to high temperatures (Björkman, 1975). Burton (1981) reports that for every 5°C increase in leaf temperature above optimum there is a reduction in the rate of photosynthesis by 25% in potato. Burton (1975) and Bushnell (1925) both claim that potato growth is completely inhibited under field conditions when the temperature exceeds 29°C. They conclude this lack of growth results from the consumption of carbohydrates in respiration equivalent to that produced by photosynthesis (zero net photosynthesis). It has

been estimated, under field conditions, that 15 to 40% of assimilated carbon in potato is consumed in dark respiration and this consumption is estimated to double for each 10°C increase in temperature between 10 and 35°C (Winkler, 1971). Dwell et al. (1981) found the maximum rate of photosynthesis in Russet Burbank potato to be between 24 and 30°C. Heat stress may also cause imbalances in metabolic processes through effects on the relationships between hormones, membranes and enzymes that ultimately reduce the total photosynthate available to plants for growth (Ewing, 1981).

Reynolds et al. (1990) studied the photosynthetic rates of heat tolerant and heat susceptible potato species of *Solanum chacoense*, *Solanum demissum*, *Solanum stoloniferum*, and *Solanum bulbocastaunum* at 25/20°C and 40/30°C temperature (day/night) up to 9 days and observed lower photosynthesis rates, at higher temperatures, in heat-unadapted leaf disks from heat susceptible genotypes (S. chacoense). However, the heat tolerant genotype showed an increased rate of CO_2 fixation under heat stress as compared to the control (Reynolds, et al., 1990). A previous report by Lafta and Lorenzen (1995) found increased photosynthetic rates after 8 days under high temperatures for two potato cultivars (Norchip & Up-to-Date) using 31/29°C temperatures (day/night). Most studies show a reduction in the photosynthesis rate of plant leaves grown at a lower temperature that are exposed to higher temperatures (heat-unadapted leaves), while only a few studies report the photosynthetic capacity of heat-adapted plant leaves as compared to heat-unadapted plant leaves. A long-term study (weeks) using potato plants grown at 12°C for 4 weeks were found to have 3 times the photosynthetic rate than plants grown for 4 weeks at 28°C (Steffen, et al., 1995). Another long-term study evaluating three potato cultivars grown at 31/20°C or 41/20°C (day/night) in a greenhouse

found that the photosynthesis rate decreased significantly in the potato variety Up-to-date in the higher temperature treatment compared to the control (Wolf, et al., 1990a). Generally, over the course of 24 days the rate of photosynthesis declined overall in both temperature treatments and in all three varieties tested (Wolf, et al., 1990a). The two previous studies provide limited examples of changes in potato leaf photosynthesis rate of plants grown at high temperatures for extended periods of time (heat-adapted leaves) compared to leaves grown solely under cooler conditions and considered to be near the optimum temperature for potato plant growth.

High Temperature & Transpiration and Respiration

High temperature stress also influences transpiration and respiration. Ku et al. (1977) found that transpiration rates in potato leaves, in a controlled-environment chamber, decreased sharply when exposed to short-term temperatures from 25°C to 35°C. This decrease in transpiration under temperatures greater than 25°C was found to be associated directly with changes in diffusive processes, such as stomatal and mesophyll resistance, on the potato leaf surface. They also found that high temperature indirectly effects transpiration through effects on stomatal opening, where high temperatures have the tendency to cause closure of stomata; which they conclude may be the result of adaptation to a cooler environment (Ku, et al., 1977). In contrast, Lafta and Lorenzen (1995) found that transpiration rates of young potato plants, in a controlled environment, were increased after 3 and 8 days of exposure to the high temperature treatment of 31/29°C, day/night, compared to the control, 19/17°C day/night. However, these previous studies used potato plants that were not adapted to high temperatures. To our knowledge there is very limited information

comparing transpiration rates in potato between heat-adapted leaves, that are grown and developed solely under high temperatures, and those leaves that were developed solely under optimal temperatures. However, a long-term study in three potato varieties by Wolf et al. (1990a) found that transpiration significantly increased under heat stress (41/20°C, day/night) for 5-6 weeks as compared to the control (31/20°C, day/night). Studies in other plant species have also shown effects of extended high temperature stress on transpiration. Hofstra and Hesketh (1969) studied transpiration and stomatal aperture over 4 – 6 weeks in several different plant species (*Zea mays* L., *Helianthus annus* L., *Glycine max* L., *Pisum sativum* L., *Triticum aestivum* L., *Phaseolus vulgaris* L., and others) under five temperature regimes (15/10, 21/16, 27/22, 33/28, and 36/31°C, day/night) and found that in most of the species both stomatal aperture and transpiration rate increased with increasing air temperature (up to 36°C) under well-watered conditions.

Steffen et al. (1995) found that respiration rates in potato increased by 56 and 46%, when measured at 2 & 4 weeks, respectively, after the temperature increased from 12 to 28°C. Similarly, Thornton et al. (1996), using heat tolerant (*S. tuberosum* L. cvs. DTO-28 and Desiree) and heat susceptible (*S. tuberosum* L. cv Russet Burbank), studied dark respiration rates in plants grown under two temperature regimes (25/12°C, 35/25°C, day/night). They found that dark respiration rates of immature and mature leaves increased under 25°C night temperatures as compared to 12°C night temperatures.

Stomatal Conductance and Leaf Temperature Under Heat Stress

It is well-known that the rate of stomatal conductance influences leaf temperature. An increase in the rate of stomatal conductance is important in cooling plant leaves under heat

stress and can allow a continuation of photosynthetic rates like those observed under optimum temperatures. However, limited studies in potato investigate the relationship between high temperatures and stomatal conductance. A study by Dwelle et al. (1981), in Russet Burbank potato plants grown in a greenhouse for 7-8 weeks with a temperature range of 15-30 ℃, found that stomatal conductance was at its maximum value at around 24 ℃ and remained at that rate under higher temperatures up to 34℃. Wolf et al. (1990b) studied the effects of acute heat stress (32/12℃ for 1, 2 and 24 hours) on 5-week old 22/12℃ grown potato plants and found a negative linear relationship between stomatal resistance and air temperature. Ku et al. (1977) found a positive relationship between stomatal resistance and leaf temperature, above 25℃, in excised potato leaves grown under 20/15℃ (heat-unadapted), day/night, conditions subjected to increasing temperatures. The previous studies observed leaves that were not adapted to heat stress and to our knowledge no studies investigate effects of high temperature on stomatal conductance and leaf temperature in potato plant leaves that have developed solely under, and therefore adapted to, high temperature stress. However, the effects of high temperature on stomatal conductance and leaf temperature have been studied in other plant species. In plants of pima cotton (*Gossypium barbadense* L.) the relationship between leaf temperature and stomatal conductance has been investigated both under field conditions and in controlled environment conditions under air temperatures of 30°C (Lu, et al., 1994). It was shown that selection for higher yield potential and heat resistance resulted in inadvertent selection for higher stomatal conductance. Results showed positive correlations between stomatal conductance and lint yield, where under heat stress the lowest yielding line, Pima 32, had the lowest stomatal

conductance and the highest yielding line, P70, had the greatest stomatal conductance (Lu, et al., 1994). Their data shows a negative linear relationship between the difference in leaf and air temperature versus stomatal conductance, demonstrating the leaf cooling effect afforded by higher stomatal conductance under high temperatures in cotton (Lu, et al., 1994). The advanced heat resistant lines also showed a smaller leaf area both under field and controlled conditions (Lu, et al., 1994). Lu et al. (1994) demonstrated inadvertent selection for greater stomatal conductance and evaporative cooling, resulting in lower leaf temperatures, after selection for greater yield and heat tolerance. This study demonstrated the importance of considering physiological traits as selection criteria in breeding for heat tolerance in other plant species that may also have important implications in breeding potato plants with enhanced heat tolerance.

Effect of high temperature stress on Photosystem II

The photosynthetic activity of chloroplasts is very sensitive to the effects of high temperature, in particular the most sensitive component of this apparatus is photosystem II (PSII) (Havaux, 1993; Havaux & Tardy, 1996). Plants can adapt to high temperature stress by increasing the optimum temperature of photosynthesis after exposure to high temperatures (Berry & Björkman, 1980; Yordanov, et al., 1986; Havaux & Gruszecki, 1993). For instance, Havaux (1993) showed that PSII of 25°C-grown potato leaves quickly (~ 2 hours) increased their threshold temperature (T_c, temperature at which chlorophyll fluorescence begins to rise), above which PSII denatures, after exposure to temperatures (35°C for 2 hours) below their T_c (38°C); and also showed an increase in the temperature of peak chlorophyll fluorescence (T_p). In this study control leaf discs (cv. Haig) were kept at 25°C and heat

treated leaf discs were kept at 35°C for 2 hours and showed that T_c and T_p changed from 38 and 45°C in the control to 43 and 52°C in heat-treated leaf discs, respectively. This acquired thermo-tolerance was also shown to be reversible, but at a much slower rate than it was acquired (Havaux, 1993). The production of O_2 by photosynthesis was also measured under high (2000 $\mu mol \bullet m^{-2} \bullet s^{-1}$) and low (130 $\mu mol \bullet m^{-2} \bullet s^{-1}$) irradiance for both treatments and showed very little reduction in oxygen evolution in the heat pre-treated leaf disks compared to the control (Havaux, 1993). Estimates for the control and pre-treatment leaves showed nearly zero and slight reduction, respectively, in quantum yield after exposure to 40°C (Havaux, 1993). Furthermore, Sahel (+8 °C) had a greater and more rapid capacity to heat-adapt upon exposure to 35°C than Haig (+4 °C). Havaux and Gruszecki (1993) found a similar photosynthetic adaptation to increase in both T_c and T_p. In this study, control plants grown at 25°C showed T_c and T_p values of 34 and 38°C, respectively, while plants grown at 35°C for 5 days showed values of 37.5 and 41°C for T_c and T_p, respectively. A study in grapes (*Vitis vinifera* L.) under a short-term heat stress (38°C for 10 h) found that high temperature severely damaged mesophyll cells, increased the permeability of the plasma membrane, made mesophyll cells rounder, swelled the stroma lamellae, induced clumping in vacuoles, disrupted cristae and caused emptying of the mitochondria, which resulted in reduced photosynthetic and respiratory activities through the formation of antenna-depleted photosystem-II (Zhang, et al., 2005). A study in maize showed large modifications in chloroplasts in response to heat stress through alterations in thylakoid organization resulting in changes in photosynthetic capacity (Karim, et al., 1997). These sources give an idea to the effect of high temperature stress on photosystem II.

Genes Involved in Stomatal Patterning

MAPK's (Mitogen-activated protein kinase) are involved in abiotic stress response including temperature, salt, wind, touch, wound responses, O_3 exposure, water stress or UV radiation exposure (Colcombet & Hirt, 2008). MAPK cascades also have functions indirectly related to stress including the production of developmental signals important in stomatal development and patterning possibly through its association with auxin signaling (Dai, et al., 2006; Bergmann, et al., 2004; Gray & Hetherington, 2004; Le, et al., 2014; Mizoguchi, et al., 1994; Mockaitis & Howell, 2000). The recessive mutation of the gene TMM *(tmm*) was found in Arabidopsis that alters the ability of cells to orient properly to create the normal patterning of stomata (Nadeau & Sack, 2002). Another gene, SDD1, plays a similar role in stomatal patterning and distribution as TMM, but TMM seems to be more important for patterning and division orientation, while SDD1 seems to be most important for regulating stomatal density. Basic-helix-loop-helix (bHLH) transcription factors play an important part in the regulation of the differentiation of stomata (Liu, et al., 2009). The transcription factor SPCH (SPEECHLESS) has been found to be involved in the regulation of the asymmetric cell divisions and *spch* mutants have been shown to be unable to produce stomata (MacAlister, et al., 2007). Additionally, the two bHLH transcription factors, MUTE and FAMA, are involved in the differentiation of cells into stomata (MacAlister, et al., 2007; Ohashi-Ito & Bergmann, 2006; Pillitteri, et al., 2007). Control of SPCH, MUTE and FAMA have been predicted to occur through the physical interaction of two bHLH transcription factors ICE1/SCRM1 and SCRM2, which are required for the differentiation of cells into functional stomata (Kanaoka, et al., 2008). The stomatal one-cell spacing rule, positional

information, and prevention of stomatal differentiation may be related to *EPF1* (Hara, et al., 2007) and *EPF2* (Hara, et al., 2009; Hunt & Gray, 2009), respectively. *PIF4* (*PHYTOCHROME-INTERACTING FACTOR 4*) has been indicated in stomatal regulation through light (red) and temperature interactions (Casson, et al., 2009; Koini, et al., 2009). PIN-FORMED (PIN) proteins have been implicated in the development and patterning of stomata (Le, et al., 2014).

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Research Objectives

Chapter 2 - Adaptation of photosynthesis in potato leaves to long-term heat stress: Physiological explanations

The present study was conducted to investigate the physiological adaptations (photosynthesis, stomatal conductance, and leaf temperature) of the potato plant leaf after several weeks of exposure to long-term high temperatures in commercially cultivated potato varieties (*Solanum tuberosum L.*). We provide the first observations of these adaptations in potato. An understanding of how long-term high temperature effects potato and the ways by which the potato plant can adapt, especially concerning physiological adaptations, is extremely important in breeding varieties that are able to produce acceptable tuber yields and tuber quality in areas with average temperatures greater than the established optimum temperature for potato growth of 20°C.

Chapter 3 - Potential Genes Involved in Heat Stress Adaptation in Potato: Analysis of Differential Gene Expression between Control and Heat-adapted Plant Leaves of the Cultivated *Solanum tuberosum* L. cv. 'Atlantic' and wild *Solanum microdontum* Bitter

This study was conducted to understand what genes and gene pathways in potato are involved in adaptation to a sustained (at least 3.5 weeks) high temperature stress. An understanding of the genes that are involved in heat stress tolerance will help give us an understanding on the genetics of heat stress tolerance in potato that can be used for breeding heat tolerant plants in the future. Enabling potato plants to tolerate higher temperatures can help facilitate the growth and production of potato crops in warmer areas where the production of potato is currently not possible due to higher average temperatures not conducive to potato growth.

Chapter 4 - Standardization of an *in vitro* assay to estimate relative heat sensitivity/tolerance and heat acclimation ability (HAA) of potato germplasm using excised whole leaves

A heat tolerance assay protocol was developed to evaluate the heat acclimation ability of an array of genotypes using heat-adapted and unadapted leaves from the plant canopy grown, respectively, under high (35/25 °C, day/night) and optimal (20/15 °C, day/night) temperatures for at least 3 weeks. Heat tolerance was evaluated for each leaf type by measuring percent ion leakage after exposure to 50°C in a heat bath for 30, 40, and 50 minutes. Heat acclimation ability (HAA) was estimated as the average of the heat-adapted percent ion leakage divided by the unadapted percent ion leakage. The HAA can be used to evaluate the genetic variation in a collection of *Solanum* species for the response to high temperature that reflects possible heat tolerance potential and the likelihood of survivability in the event of a sudden high temperature event. The screening and identification of plant materials for heat-tolerance would be an important tool for potato breeding that would serve to increase the possible range of agricultural production of potato and expand food production to areas with higher than optimum average temperatures for this crop. The purpose of this study was to: (1) create a simple and efficient method of screening for unadapted and adapted heat sensitivity that provides sufficient variation to determine statistically significant differences between genotypes (2) to allow for selection of genotypes based on their HAA by comparing leakage assays of unadapted and adapted leaves after growth and adaptation of the same plants to two temperature conditions including an optimal temperature (20/15°C day/night for at least 8 weeks) and a high temperature stress environment (35/25°C day/night for at least 3.5 weeks) and (3) determine if there is evidence

for genetically distinct mechanisms between unadapted heat sensitivity/tolerance and adapted heat sensitivity/tolerance in potato.

Chapter 2 Adaptation of photosynthesis in potato leaves to long-term heat stress: Physiological explanations

Abstract

Potatoes are known to grow well in cool climates, while higher temperatures generally reduce foliage growth, photosynthetic rates, and partitioning to tubers. Most studies to date have documented the adverse impact of heat stress on potato plant growth and development. However, very little has been reported on long-term adaptation of potato plants to heat stress. Our recent studies have shown that under long-term heat stress (4-6 weeks) the newly developed leaves have reduced size and dramatically higher stomatal density. The present study was conducted to investigate strategies employed by potato plant leaves to adapt to long-term heat stress. These adapted leaves are able to maintain chlorophyll and survive under heat stress as opposed to the leaves produced under non-stress conditions on the same plant. This experiment used shoot culture plantlets of *Solanum tuberosum* L. cvs. Atlantic (ATL) and Dark Red Norland (DRN) that were grown in 12.7 L pots, using an artificial soil mix (Metro Mix) and subjected to a 14-hour photoperiod with a 290 $\mu Mol \cdot m^{-2} \cdot s^{-1}$ average PAR light intensity, 60% relative humidity, and ambient CO_2 levels. Plants were irrigated every day more than saturation with ¼ strength Hoagland's solution. After growing in cool (20ºC/ 15ºC, day/night) temperatures for six weeks the plants were transferred to a controlled environment room for heat stress treatment (35ºC/ 25ºC, day/night). Measurements (photosynthesis, stomatal consuctance, leaf temperature) were taken within the first two days of heat stress and weekly thereafter for 2 weeks in the same leaves (unadapted). After 4 weeks the measurements were made on the newly developed (adapted) leaves. Photosynthetic rates were 30-40% lower in the unadapted leaves as compared to control

(prior to heat stress) after two weeks of heat stress. Photosynthetic rates were significantly higher in adapted leaves as compared to unadapted leaves under heat stress in both cultivars. In DRN the adapted leaves had similar photosynthetic rates under heat stress as the control leaves under non-stress conditions. As compared to control (non-stress), the stomatal conductance (SC) increased initially (1 day of heat stress) under heat stress but declined again to control level in DRN and to a significantly lower as compared to the control in ATL. The heat adapted leaves in both varieties showed higher SC as compared to unadapted leaves under heat stress. Leaf temperatures were lower in the adapted leaves as compared to unadapted leaves under heat stress suggesting that higher SC in the adapted leaves lowered the leaf temperatures and mitigated the heat stress. Scatter plots demonstrated that as SC increased the photosynthetic rates increased to almost four-fold. Furthermore, photosynthetic rates decreased dramatically as leaf temperatures increased from 32-35°C under heat stress. These results suggest that leaflets produced under heat stress (adapted) are able to maintain lower leaf temperatures by increasing the transpiration (higher SC) and lowered leaf temperatures allowed these adapted leaves to maintain higher photosynthetic rates. These adaptations appear to help potato plants tolerate the effects of long-term heat stress.

Abbreviations/ Key Words *Solanum tuberosum*; evaporative cooling; heat adaptation; ATL = Atlantic; DRN = Dark Red Norland; SC = Stomatal Conductance

Introduction

Potato (*Solanum tuberosum* L.) is adapted to cooler climates (Hawkes, 1978), such as those found in the Andean Mountains of South America where the relatives of cultivated potatoes are thought to have originated (Hawkes, 1992). High temperature stress is one of the most significant uncontrollable factors that affects potato plant growth plant growth,

development, and tuber yield (Smith, 1968). High temperature stress affects many factors in potato, such as inhibition of photosynthesis (Dwelle, et al., 1981; Reynolds, et al., 1990), decreased transpiration (Ku, et al., 1977), stomatal conductance (Dwelle, et al., 1981), chlorophyll content (Reynolds, et al., 1990; Steffen, et al., 1995), assimilate partitioning (Ewing, 1981; Van Dam, et al., 1996), tuber initiation and development (Borah & Milthorpe, 1962; Ewing, 1981; Reynolds & Ewing, 1989a), but an increase in respiration (Thornton, et al., 1996). In addition, heat stress influences morphological and anatomical changes that are very similar to that induced by longer day lengths where there is a shift from root growth and tuberization to foliage growth, increased above-ground axillary bud growth (Ewing, 1981), taller plants, and smaller leaves (Khedher & Ewing, 1985; Kleinhenz & Palta, 2002). Several studies suggest an optimum temperature for photosynthesis in potato (Ku, et al., 1977; Wheeler, et al., 1986). Ku et al. (1977) reported the optimum temperature for photosynthesis in cultivated potato plants of *Solanum tuberosum* L. W729R to be between 16 and 25°C. Similarly, Wheeler et al. (1986) concluded the optimal temperature for photosynthesis in potato was 20°C based on the yields of tuber fresh weight and foliage dry weight. Most studies in potato focus on the acute short-term (hours or days) effects of high temperature stress. However, studies are lacking for the long-term effects of high temperature on potato. Furthermore, there is little information on how the potato plant physiology may adapt to high temperatures. In the last several decades, large-scale potato production has been introduced to tropic and sub-tropic regions with high day and night temperatures (Tai, et al., 1994) providing new challenges to crop production in a new climate that often experiences high temperature extremes.

Reduced potato plant growth under high temperature conditions may result from a reduction in both photosynthetic capacity and carbon fixation (Ewing, 1981). The ability of the photosynthetic capacity of a plant to adapt to heat stress is very important since this process is very sensitive to high temperatures (Björkman, 1975). Burton (1981) reports that for every 5°C increase in leaf temperature above optimum there is a reduction in the rate of photosynthesis by 25% in potato. Burton (1975) and Bushnell (1925) both claim that potato growth is completely inhibited under field conditions when the temperature exceeds 29°C. They conclude this lack of growth results from the consumption of carbohydrates in respiration equivalent to that produced by photosynthesis (zero net photosynthesis). It has been estimated, under field conditions, that 15 to 40% of assimilated carbon in potato is consumed in dark respiration and this consumption is estimated to double for each 10°C increase in temperature between 10 and 35°C (Winkler, 1971).

Most studies in potato show a reduction in the photosynthesis rate of plant leaves exposed to a short-term (days) high temperature (Reynolds, et al., 1990; Wolf, et al., 1990a) but the information on long-term (weeks) effect of heat stress on photosynthesis is lacking.

High temperature stress also influences transpiration and respiration. Most of the studies in potato focus on the short-term effects of heat stress on respiratory factors. For example, Ku et al. (1977) found that transpiration rates in potato leaves, in a controlled-environment chamber, decreased sharply when exposed to short-term (days) temperatures from 25°C to 35°C. This decrease in transpiration under temperatures greater than 25°C was found to be associated directly with changes in diffusive processes, such as stomatal and mesophyll resistance, on the potato leaf surface. They also found that high temperature

indirectly effects transpiration through effects on stomatal opening, where high temperatures have the tendency to cause closure of stomata, which they conclude may be the result of adaptation of potatoes to a cooler environment (Ku, et al., 1977). In contrast, Lafta and Lorenzen (1995) found that transpiration rates of young potato plants, in a controlled environment, were increased after 3 and 8 days of exposure to the high temperature treatment of 31/29°C, day/night, compared to the control, 19/17°C day/night. However, these previous studies focused on the short-term effects of heat stress on transpiration in potato plant leaves that were not adapted to high temperatures after a long-term exposure of several weeks or more. To our knowledge there is very limited information comparing transpiration rates in potato between heat-adapted leaves, that are grown and developed solely under high temperatures, and those leaves that were developed solely under optimal temperatures. However, a long-term study in three potato varieties by Wolf et al. (1990a) found that transpiration significantly increased under heat stress (41/20°C, day/night) for 5-6 weeks as compared to the control (31/20°C, day/night). Studies in other plant species have also shown effects of extended high temperature stress on transpiration. Hofstra and Hesketh (1969) studied transpiration and stomatal aperture over 4 – 6 weeks in several different plant species (*Zea mays* L., *Helianthus annus* L., *Glycine max* L., *Pisum sativum* L., *Triticum aestivum* L., *Phaseolus vulgaris* L., and others) under five temperature regimes (15/10, 21/16, 27/22, 33/28, and 36/31°C, day/night) and found that in most of the species both stomatal aperture and transpiration rate increased with increasing air temperature (up to 36°C) under well-watered conditions.

Thornton et al. (1996), using heat tolerant (*S. tuberosum* L. cvs. DTO-28 and Desiree) and heat susceptible (*S. tuberosum* L. cv Russet Burbank), studied dark respiration rates in plants grown under two temperature regimes (25/12°C, 35/25°C, day/night). They found that dark respiration rates of immature and mature leaves increased under 25°C night temperatures as compared to 12°C night temperatures.

There is only limited information on the relationship between leaf temperatures and stomatal conductance in potato. However, the effects of high temperature on stomatal conductance and leaf temperature have been studied in other plant species. For example, in pima cotton (*Gossypium barbadense* L.) the relationship between leaf temperature and stomatal conductance has been investigated both under field conditions and in controlled environment conditions under air temperatures of 30°C (Lu, et al., 1994). In this study it was found that selection for higher yield potential under heat stress resulted in inadvertent selection for higher stomatal conductance. Results showed positive correlations between stomatal conductance and lint yield for various genotypes, suggesting the beneficial effect of leaf cooling afforded by higher stomatal conductance under high temperatures (Lu, et al., 1994).

Clearly, a lot is known on the short-term effects of heat stress on potatoes, but there is a lack of information on the impact of prolonged heat stress. Very little is known on the physiology of leaves that have developed completely under heat stress, and therefore adapted to high temperature stress. A previous study showed that long-term adaptation to high temperature results in modified leaf morphology including reduced leaf size, reduced cell division and reduced cell enlargement (Kleinhenz & Palta, 2002). We also found these

morphological adaptations of potato leaves developed under prolonged heat stress. The present study was conducted to investigate the physiological adaptations of the potato plant leaf after exposure to long-term high temperatures in two commercially cultivated potato varieties Atlantic and Dark Red Norland. Here we provide the first evidence for the adaptation of photosynthesis, stomatal conductance, and leaf temperature. An understanding of how long-term high temperature effects potato and the ways by which the potato plant can adapt, especially concerning physiological adaptations, is extremely important in breeding varieties that are able to produce acceptable tuber yields and tuber quality under heat stress.

Materials and Methods

Experimental Conditions

Shoot culture plantlets of cultivated potato *Solanum tuberosum L. cv.* 'Atlantic' (ATL) and 'Dark Red Norland' (DRN) were grown in 12.7 L pots containing an artificial soil mix (Sungro Metro Mix®) in a controlled environment room at the UW-Madison Biotron. Six plants of each cultivar were used for analysis. Plants were kept under a 14-hour photoperiod, light intensity averaged around 300 $\mu Mol \cdot m^{-2} \cdot s^{-1}$ within the zone of measurement in the leaf canopy and the relative humidity was maintained at 60%. Plants were irrigated daily more than saturation with a ¼ strength Hoagland's solution. The room was provided with constant fresh air circulation to supply adequate CO_2 at ambient levels. Six weeks after growing in cool (20ºC/ 15ºC, day/night) temperatures (control) plants were subjected to heat stress treatment for at least 3 weeks (35ºC/ 25ºC, day/night).

Leaf Sampling

For control leaves, fully-expanded terminal leaflets from the youngest leaves (3-4 nodes below the youngest growth) were sampled. For this purpose, plants were grown at 20/15°C day/night temperatures for 6 weeks. For heat stress adapted leaves, fully expanded terminal leaflets from youngest leaves (3-4 nodes below the youngest growth) were sampled. For this purpose, the control plants were subjected to a long-term heat stress (35/25 °C, day/night). During this period, the leaves produced under control conditions stayed healthy and showed no signs of damage for at least 2 weeks. The heat-adapted leaves, that emerged after at least 3 weeks of heat stress, remained healthy under the stress conditions for the entire duration of our study.

Physiological Measurements

Starting at 6 weeks after planting, measurements of photosynthesis (μMol $CO_2 \cdot m^{-2} \cdot s^{-1}$), stomatal conductance (moles $H_2O \cdot m^{-2} \cdot s^{-1}$), and leaf temperature (°C) were made using a LI-6400XT Portable Photosynthesis System (LI-COR, Inc., Lincoln, Nebraska, USA). The reference CO_2 concentration, flow rate and relative humidity of the leaf chamber were set to 400 μMol $CO_2 \cdot m^{-2} \cdot s^{-1}$, 500 μMol $\cdot s^{-1}$ and 57%, respectively. PAR in the leaf chamber averaged 450 μMol $\cdot m^{-2} \cdot s^{-1}$. All measurements were taken after readings had stabilized under chamber conditions. Measurements were taken for the control plants 1-2 days before initiation of heat stress. These leaves were tagged and followed for these measurements 1, 7, and 14 days after the start of heat stress conditions (DAHS). These measurements were then repeated on the newly developed (heat-adapted) leaves on the same plant, after 21 and 28 DAHS. Four measurements/leaf were taken on 1-3 of the youngest, fully-expanded terminal

leaflets from 4 – 6 plants that were unshaded and located about 3-4 nodes below the youngest growth (Table A1).

Statistical Analysis

Data were analyzed, and plots were made, using RStudio (Version 1.1.447, RStudio, Inc). ANOVA was used to test for significant differences between two treatment means. A Least Significant Difference (LSD) test was used in R with the functions anova() and LSD.test() for the comparison of more than two treatment means. For all figures, means followed by a different letter were significantly different ($p \leq 0.05$). Error bars in the figures are standard errors of the mean.

Results

Plant morphology was significantly altered for the leaves produced under long-term (4 weeks) heat stress. The newly developed, fully-expanded and heat-adapted, leaflets were darker and approximately 1/5 to 1/4 the size of leaflets on the same plant that had developed for 6 weeks under control conditions prior to heat stress exposure. These plants, subjected to heat stress, continued to produce new and healthy leaves (adapted leaves), while the leaves developed under control conditions (prior to heat stress treatment) on the same plant (unadapted leaves) began to show signs of stress as leaf curling and chlorosis.

Photosynthesis decreased significantly under two weeks of heat stress in the leaves developed prior to exposure of heat stress (unadapated leaves). This was consistent for both the varieties although the decline was more pronounced for ATL (Fig. 1). As compared to unadapted leaves, the photosynthetic rates were significantly greater in the heat adapted leaves (Fig. 2). These differences were more pronounced in DRN as compared to ATL.

Interestingly, in DRN adapted leaves had photosynthetic rates under heat stress condition similar to the control (undapted) leaves under non-stress condition.

Stomatal conductance (SC) significantly increased after one day of heat stress and decreased subsequently to similar rate as control (Fig. 3). Rates of SC in heat-adapted leaves of both varities, under heat stress, were significantly greater than the unadapted leaves (Fig. 4). The SC of the heat-adapted leaves of ATL were no different than the SC rates of the leaves in the control (Fig. 4). However, the rates of SC of the heat-adapted leaves of DRN were significantly greater, and nearly three times the rates in the control (Fig. 4).

Leaf temperature, in both ATL and DRN unadapted leaves under initial heat stress, significantly increased after 1 and 2 weeks of heat stress. There was no change in leaf temperature between weeks 1 and 2 unadapted leaflets for both ATL and DRN (Table 1). The fully heat-adapted leaves of both ATL and DRN had a significantly lower temperature (33.7 and 32.8, respectively) than the unadapted leaflets in week 2 (34.0 and 34.1, respectively, Table 2).

Relationship between SC and photosynthesis (Ps) as well as leaf temperature and Ps

The correlation between the three factors Ps, SC, and leaf temperature were evaluated for both ATL and DRN after 1, 7, and 14 days after heat stress in unadapted leaflets. Additional information, after 21 days of heat stress in heat-adapted leaflets and after 28 days of heat stress in unadapted leaflets from ATL, was utilized to demonstrate the trend of these three factors more clearly as they relate to leaf adaptation (Figs. 5, 7, and 9). Similar leaflets (fully expanded 3rd - 4th node) were selected for all measurements and outliers may be due to the accidental deviation from the consistent selection of leaflets. Ps is related to SC by a

positive 2nd degree polynomial relationship, for both ATL and DRN (Figs. 5 and 6). The relationship between leaf temperature and Ps fitted to a 2nd degree negative polynomial for both varieties' data (Figs. 7 and 8). In both varieties Ps rates dropped dramatically as leaf temperature increased from 32-34°C. The relationship was linear and negative, for ATL and DRN (r=-0.82 and -0.64, respectively), between SC and leaf temperature that is shown fitted to the data (Fig. 9 and Fig. 10, respectively).

Discussion

Adaptation of Photosynthesis to Heat Stress

In general, as expected photosynthetic rates declined significantly under heat stress. However, this was true for leaves developed under non-stress (unadapted) conditions (Fig. 1). These results agree with previous reports. For example, a study evaluating three potato cultivars grown at 31/20°C or 41/20°C (day/night) in a greenhouse found that photosynthesis rate decreased significantly in control-grown leaves after 1, 2, and 24-hour measurement intervals under heat stress (Wolf, et al., 1990a). In this study, leaves developed under control condition were measured for Ps under heat stress. However, Ps of heat adapted leaves (truly developed under heat stress) has not been studied in combination with either control or unadapted leaves under heat stress. Ours is the first study comparing heat adapted and unadapted leaves under heat stress (Figs. 2 & 4). These observations clearly demonstrate a long-term adaptation to heat stress and increase in heat tolerance of heat-adapted leaves.

Leaves of potato are known to decrease Ps and conductance with leaf age (Vos & Oyarzun, 1987; Wolf, 1993). Vos et. al. (1987) showed that from 25-75 days after the appearance of a leaf the photosynthesis and stomatal conductance declined to 1/3 and 1/5 of

the initial values, respectively. Wolf (1993) found that the older leaves (leaf # 13 & 21) of plants grown at cool temperatures (24/16°C, day/night) had a significantly lower rate of photosynthesis than the newest, fully expanded leaves (#'s 5 & 6) on the same plant. Measurements of Ps and stomatal conductance in the current study occurred on newly formed, fully expanded control-grown leaves after a maximum of 14 days after heat stress (Figs. 1 & 3), and it was assumed that due to the young age of these measured leaflets that the decrease in both Ps and stomatal conductance under heat stress was not due to leaf age in this case but because of the effect of heat stress on the subsequent performance of both photosynthesis and stomatal conductance.

Our results on SC and leaf temperatures shed light on mechanisms that may be involved in the maintenance of higher photosynthetic rates in adapted leaves as compared to unadapted leaves under heat stress. We found higher SC and lower leaf temperatures in the adapted leaves as compared to unadapted leaves (Figs. 2 and 4, Table 2). Greater stomatal conductance has been associated with reduced leaf temperatures via evaporative cooling (Lu, et al., 1994). For example, Radin et. al. (1994) found a negative relationship between SC and leaf temperature. We found similar results for the negative relationship between leaf temperature and stomatal conductance (Figs. 9 and 10). Furthermore, under heat stress, higher leaf temperatures have been correlated with reduced photosynthesis. For example, Ku et. al (1977) showed a negative linear relationship between Ps and leaf temperatures above 30°C in potato plants subjected to heat stress. Studies have shown thermal damage to the photosystem at elevated temperatures in potato and other crops (Isoda & Isoda, 2005; Ku, et al., 1977; Lu, et al., 1994). Thus, reduced leaf temperatures would allow plants to maintain

higher Ps through reduced thermal damage. We found a dramatic drop in Ps when leaf temperature increased from 32-34°C (Figs. 7 and 8). Lastly, our study showed an increase in Ps with increased SC (Figs, 5 & 6). This demonstrated the effect of evaporative cooling from increased SC that allowed for higher Ps. Taken together, our results suggest that adapted leaves can maintain higher photosynthesis under heat stress by lowering leaf temperature through higher stomatal conductance.

The identification of traits for improvement of plant productivity and function under heat stress is becoming increasingly important. It has been proposed that an ideotype for an adapted crop under heat stress should be able to maintain photosynthesis and carbon fixation by maintaining stomatal density and the opening of stomata, which also helps in cooling the leaf surface (George, et al., 2017). Additionally, an ideotype should protect the photosynthetic apparatus by producing heat shock proteins, antioxidants, and osmoprotectants that can maintain leaf function for as long as possible (George, et al., 2017). Additionally, they state that understanding and manipulating the ABA response pathway may be a way to optimize the opening of stomata, saturation of membrane lipids can help in maintaining membrane structural integrity, and that production of epicuticular waxes can help protect against desiccation (George, et al., 2017). Our previous anatomical studies looking at the adaptation ability of unadapted and heat-adapted leaves showed that stomatal density increases in heat-adapted leaves (Schabow, 2017). Our current, unpublished RNAseq data between the same leaf types highlights the importance of down-regulation of ABA, increased heat shock proteins, antioxidants, and lipid saturation. This study highlights the importance of the effect of increased transpiration (SC) and evaporative cooling in heat-

adapted leaves that helps maintain the function of photosynthesis. All these considerations together offer traits that would be important in an ideal genotype adapted to heat stress.

Conclusions

Our results demonstrate that leaves of both the cultivars can adapt to heat stress by producing new leaves under long-term heat stress. We found a greater rate of both photosynthesis and stomatal conductance under heat stress in heat-adapted leaves as compared to the unadapted control leaves on the same plant. Furthermore, we found a significant decrease in leaf temperature in heat-adapted leaves compared to heat-unadapted control leaves of the same plant. Results suggest that the adapted leaves can reduce leaf temperature and maintain higher photosynthesis by increased evaporative cooling. Understanding the mechanisms of heat adaptation in the potato physiological response is an important step in understanding the ways the potato plant is able to adapt, grow and survive outside of the native climatic conditions from which it arose, and offers valuable knowledge in developing plants with better performance under high temperature stress. Our results provide some insight that may one day allow breeders to develop varieties with enhanced characterietics that allow the growth of potatoes in areas with greater average temperature.

Figures

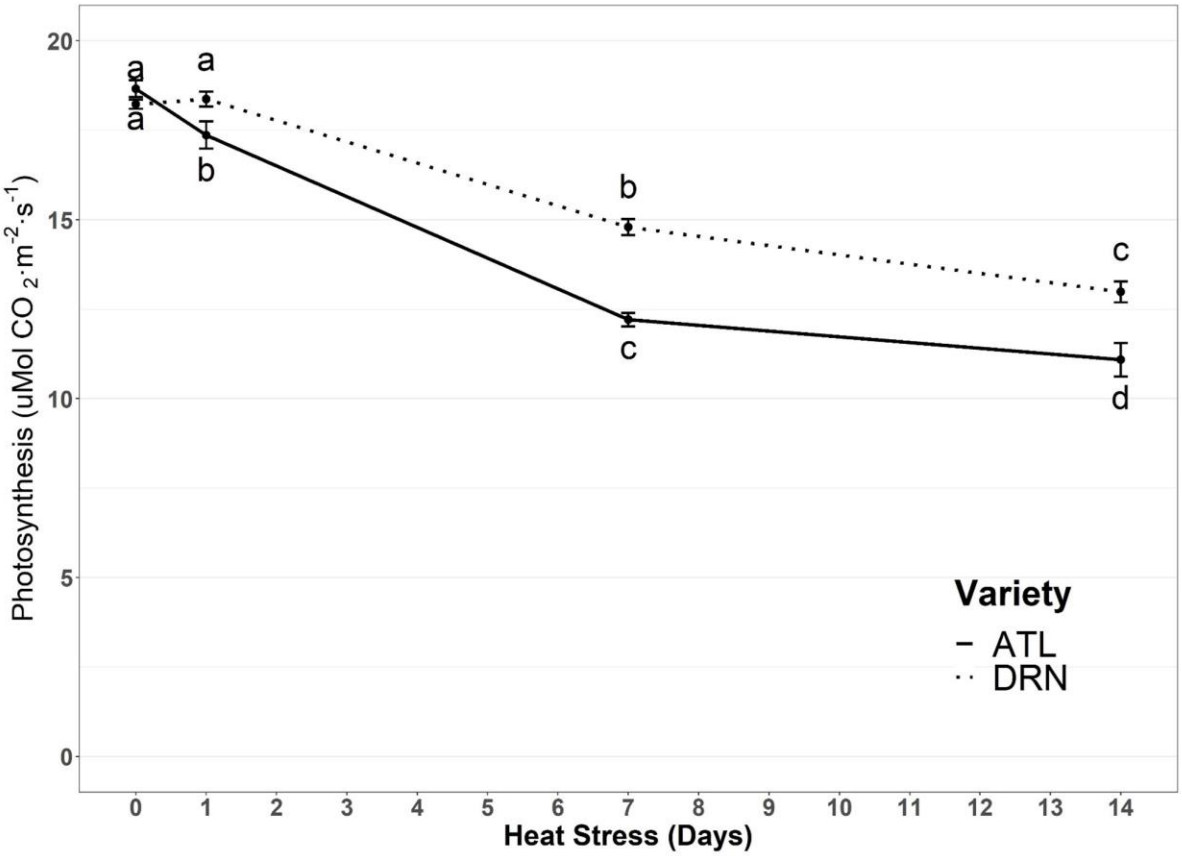

Figure 1: Influence of heat stress on photosynthesis rates in two varieties (ATL, Atlantic; DRN, Dark Red Norland) of potato leaves (unadapted). Measurements were made either prior to heat stress (Control day 0) or 1, 7, and 14 days of heat stress. Daytime air temperature in the control plants was maintained at 20°C and at 35°C during the heat stress. Values are means ± standard errors (n = 36, n=56, ATL and DRN, respectively). Significant differences determined within a variety by Duncan's Multiple Range test ($p < 0.05$). Means with similar letters are not significantly different.

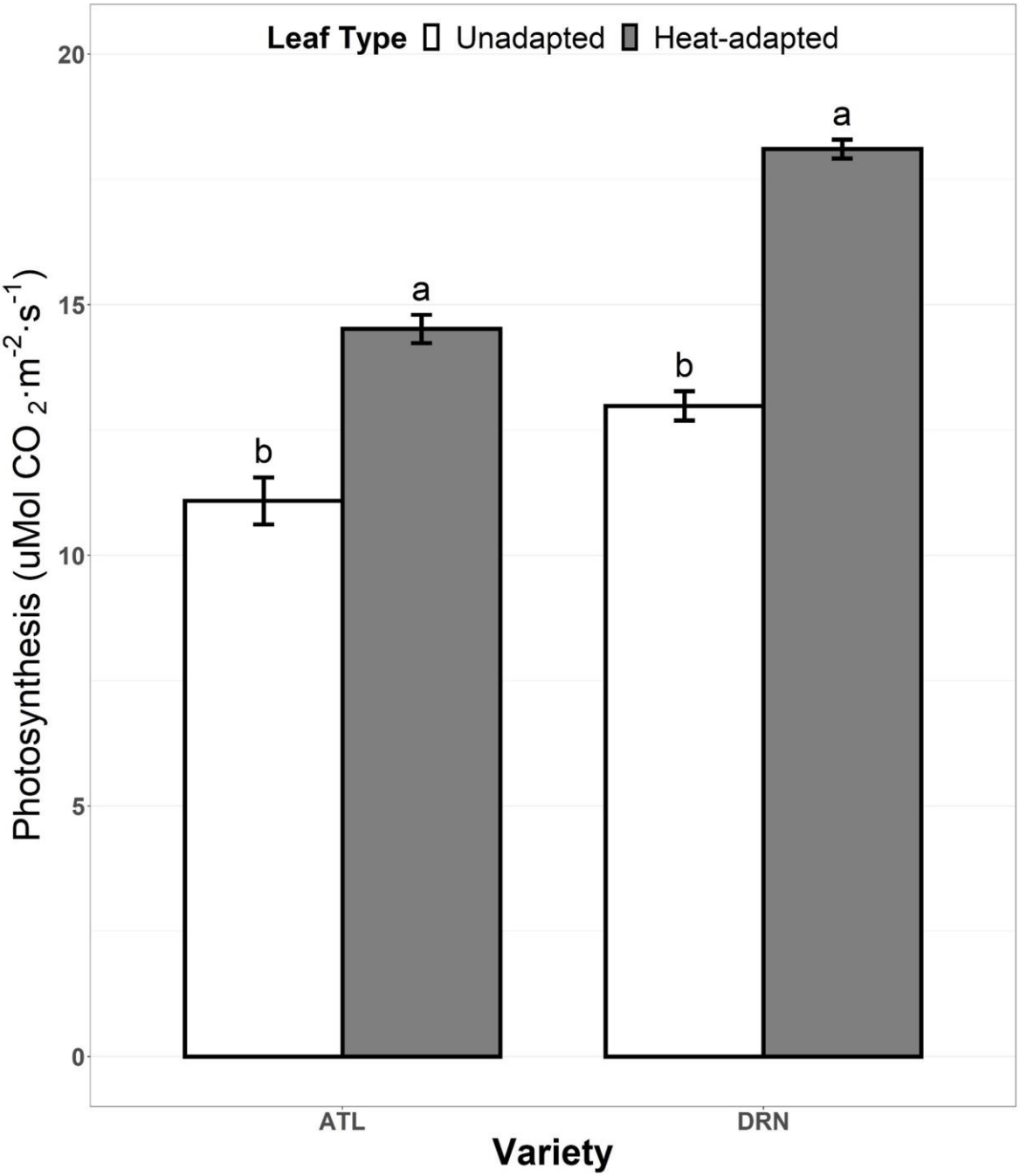

Figure 2: Comparison of photosynthetic rates of heat-adapted and unadapted leaves of two potato varieties; ATL, Atlantic; DRN, Dark Red Norland. Adapted leaves were the newly developed leaves after 21 days of heat stress for DRN and 28 days of heat stress for ATL. The unadapted leaves matured under control conditions prior to heat stress and photosynthetic rates were measured after 14 days of heat stress. All measurements were made at mid-day and the daytime temperature was maintained at 35°C. Significant differences determined among the two leaf types within a cultivar by Duncan's Multiple Range test ($p < 0.05$). Means with similar letters are not significantly different. Values are means $\pm$ standard errors ($n = 36$ and 148 for ATL unadapted and heat-adapted leaf measurements, respectively; $n = 56$ and 40 for DRN unadapted and heat-adapted leaf measurements, respectively).

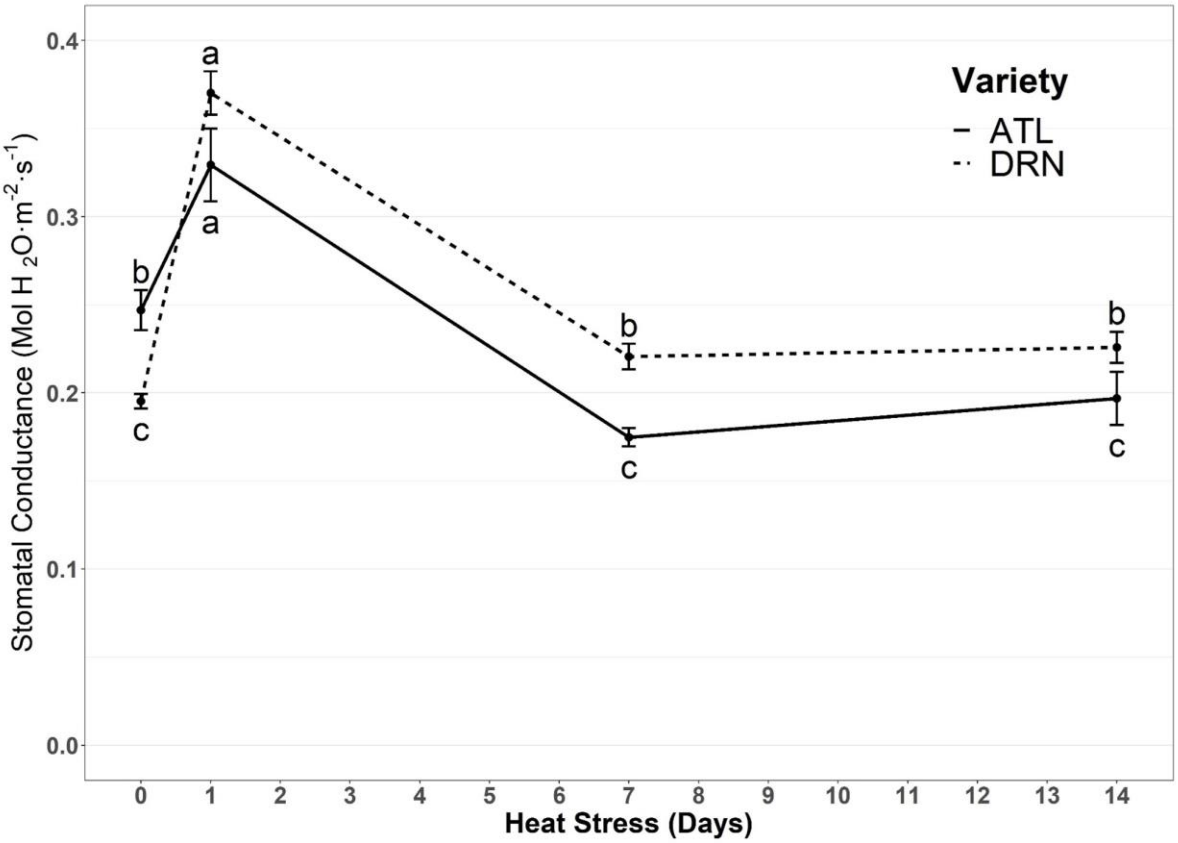

Figure 3: Influence of heat stress on rates of stomatal conductance in two varieties (ATL, Atlantic; DRN, Dark Red Norland) of potato leaves (unadapted). Measurements were made either prior to heat stress (Control day 0) or 1, 7, and 14 days of heat stress. Daytime air temperature in the control plants was maintained at 20°C and at 35°C during the heat stress. Values are means $\pm$ standard errors (n = 36, n=56, ATL and DRN, respectively). Significant differences determined within a variety by Duncan's Multiple Range test ($p < 0.05$). Means with similar letters are not significantly different.

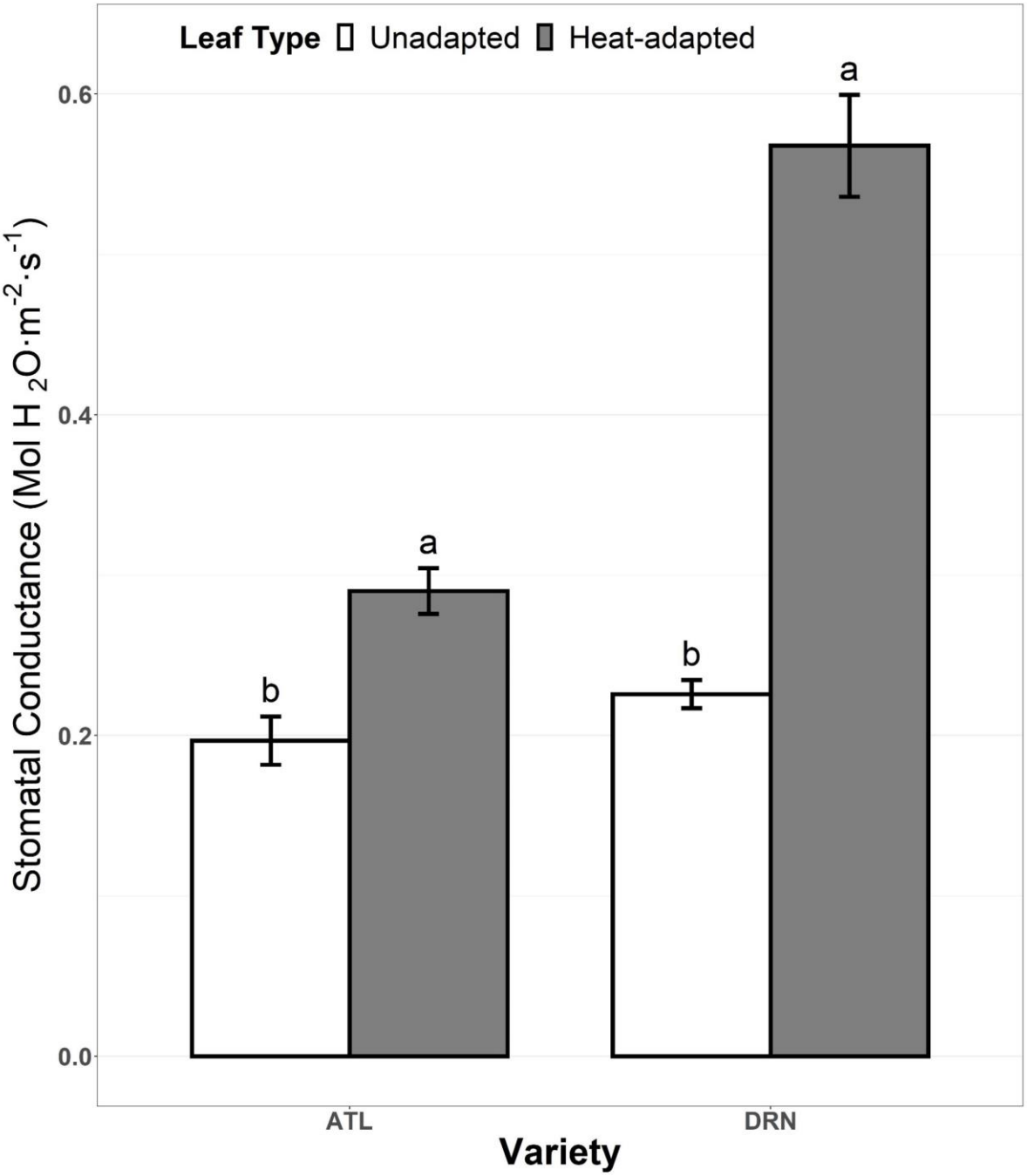

Figure 4: Comparison of rates of stomatal conductance for heat-adapted and unadapted leaves of two potato varieties; ATL, Atlantic; DRN, Dark Red Norland. Adapted leaves were the newly developed leaves after 21 days of heat stress for DRN and 28 days of heat stress for ATL. The unadapted leaves matured under control conditions prior to heat stress and photosynthetic rates were measured after 14 days of heat stress. All measurements were made at mid-day and the daytime temperature was maintained at 35°C. Significant differences determined among the two leaf types within a cultivar by Duncan's Multiple Range test ($p < 0.05$). Means with similar letters are not significantly different. Values are means $\pm$ standard errors ($n = 36$ and 148 for ATL unadapted and heat-adapted leaf measurements, respectively; $n = 56$ and 40 for DRN unadapted and heat-adapted leaf measurements, respectively).

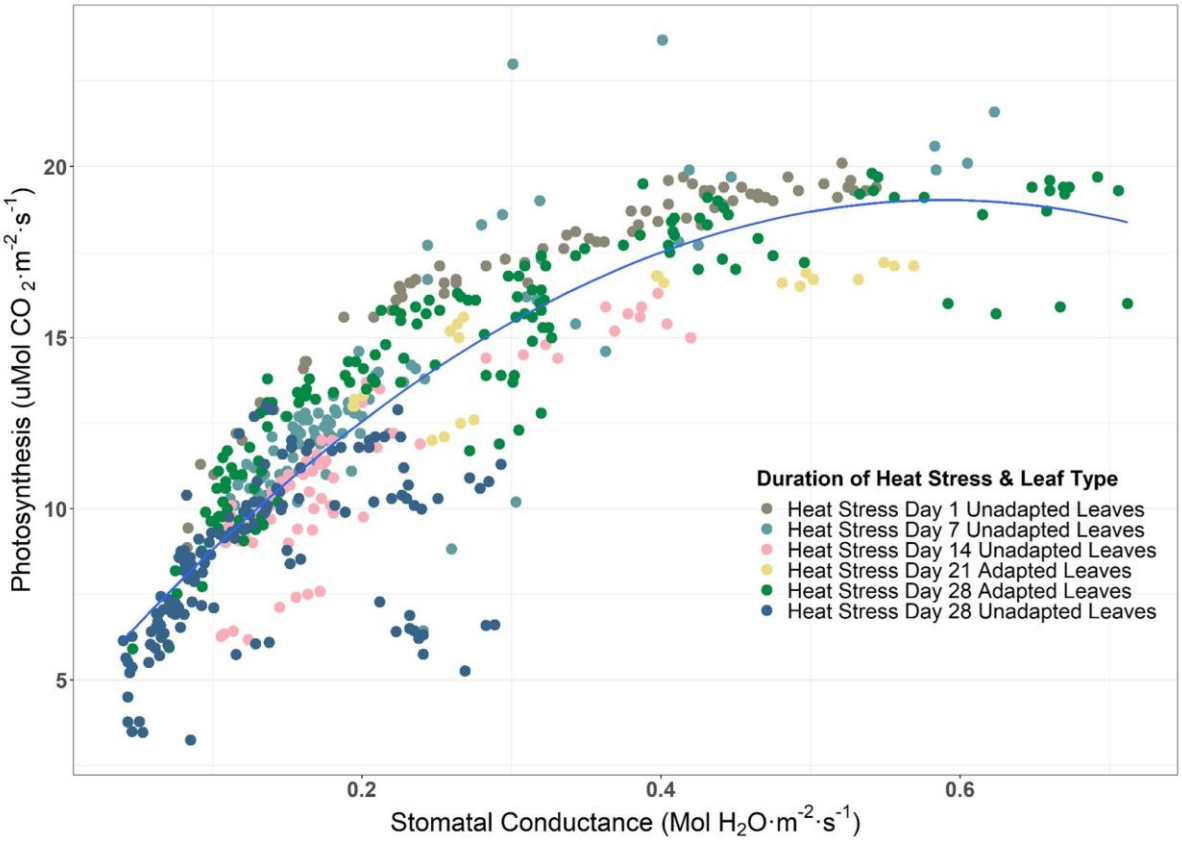

Figure 5: Correlation plot of all data between stomatal conductance and photosynthesis for the cultivar ATL in unadapted and heat-adapted leaflets. All measurements were made at mid-day and the air temperature was maintained at 35°C. Measurements were made after 1, 7, 14, 21, and 28 days of heat stress treatment in unadapted or heat-adapted leaflets as indicated.

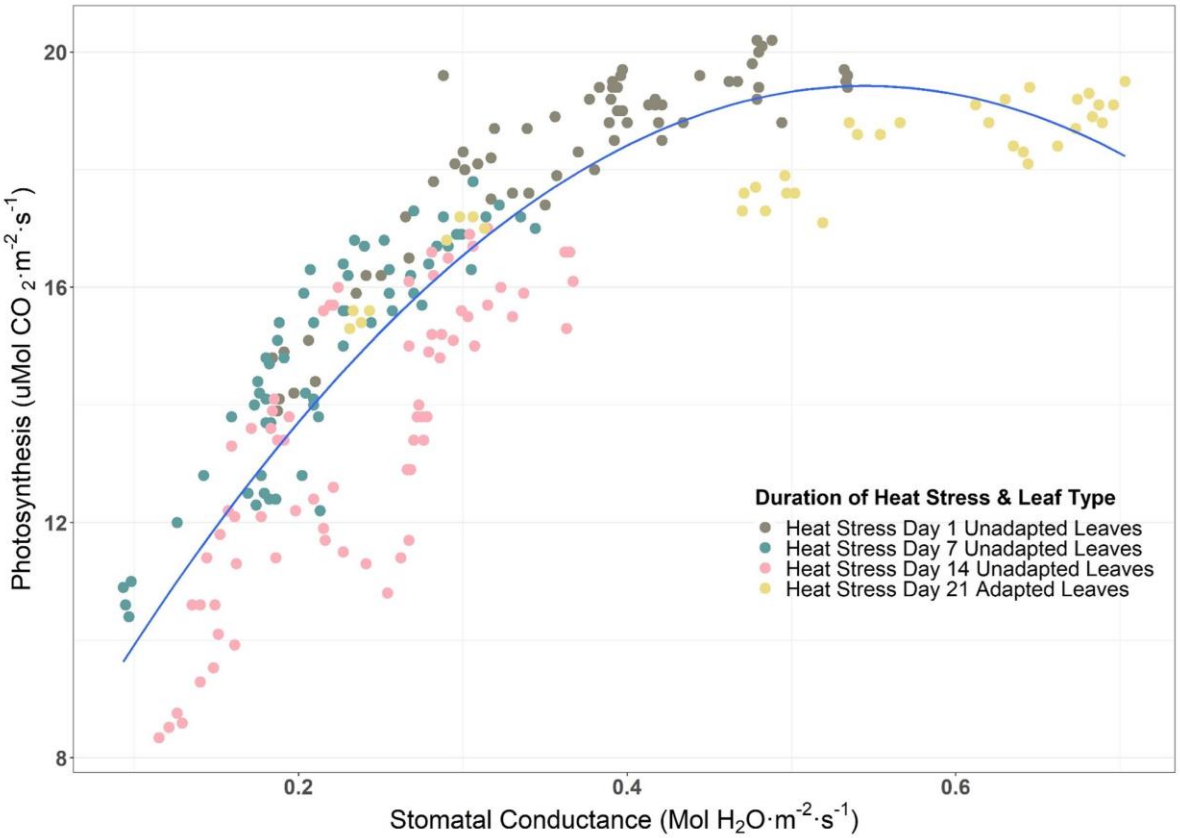

Figure 6: Correlation plot of all data between stomatal conductance and photosynthesis for the cultivar DRN in unadapted and heat-adapted leaflets. All measurements were made at mid-day and the air temperature was maintained at 35°C. Measurements were made after 1, 7, 14, and 21 days of heat stress treatment in unadapted or heat-adapted leaflets as indicated.

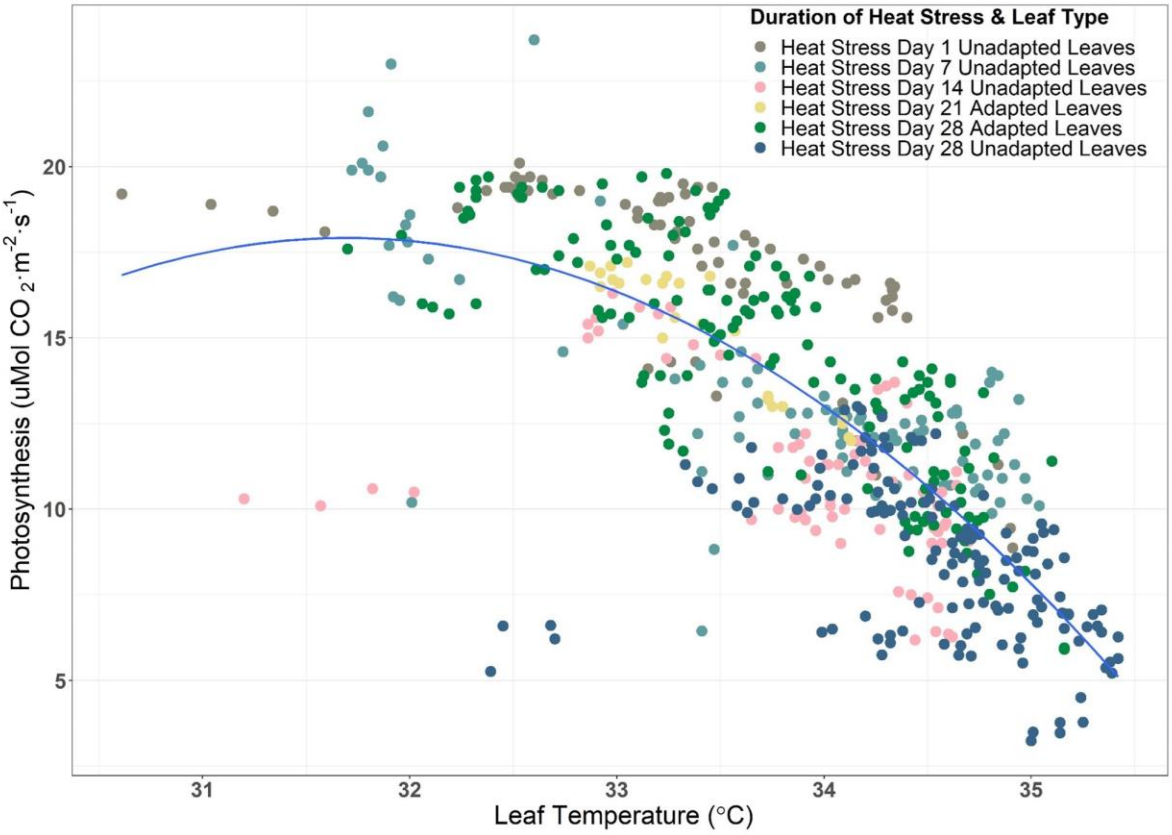

Figure 7: Correlation plot of all data between leaf temperature and photosynthesis for the cultivar ATL in unadapted and heat-adapted leaflets. All measurements were made at mid-day and the air temperature was maintained at 35°C. Measurements were made after 1, 7, 14, 21, and 28 days of heat stress treatment in unadapted or heat-adapted leaflets as indicated.

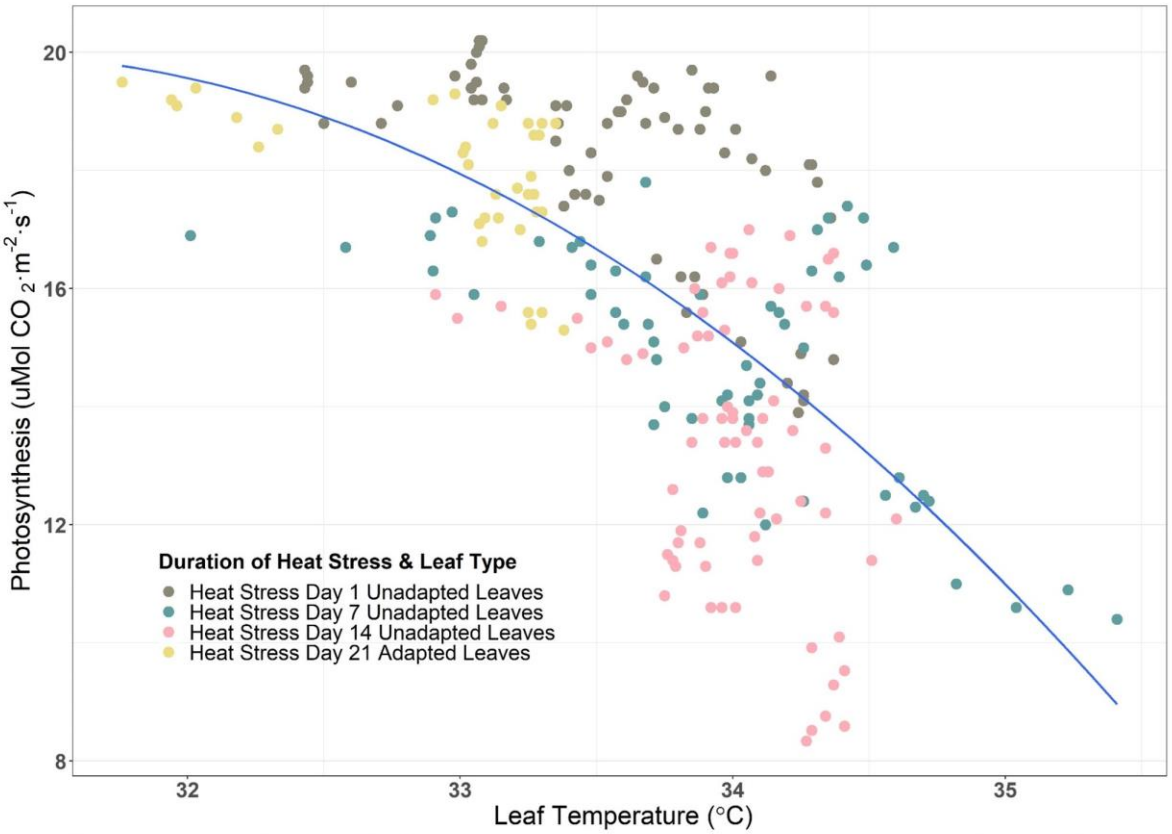

Figure 8: Correlation plot of all data between leaf temperature and photosynthesis for the cultivar DRN in unadapted and heat-adapted leaflets. All measurements were made at mid-day and the air temperature was maintained at 35°C. Measurements were made after 1, 7, 14, and 21 days of heat stress treatment in unadapted or heat-adapted leaflets as indicated.

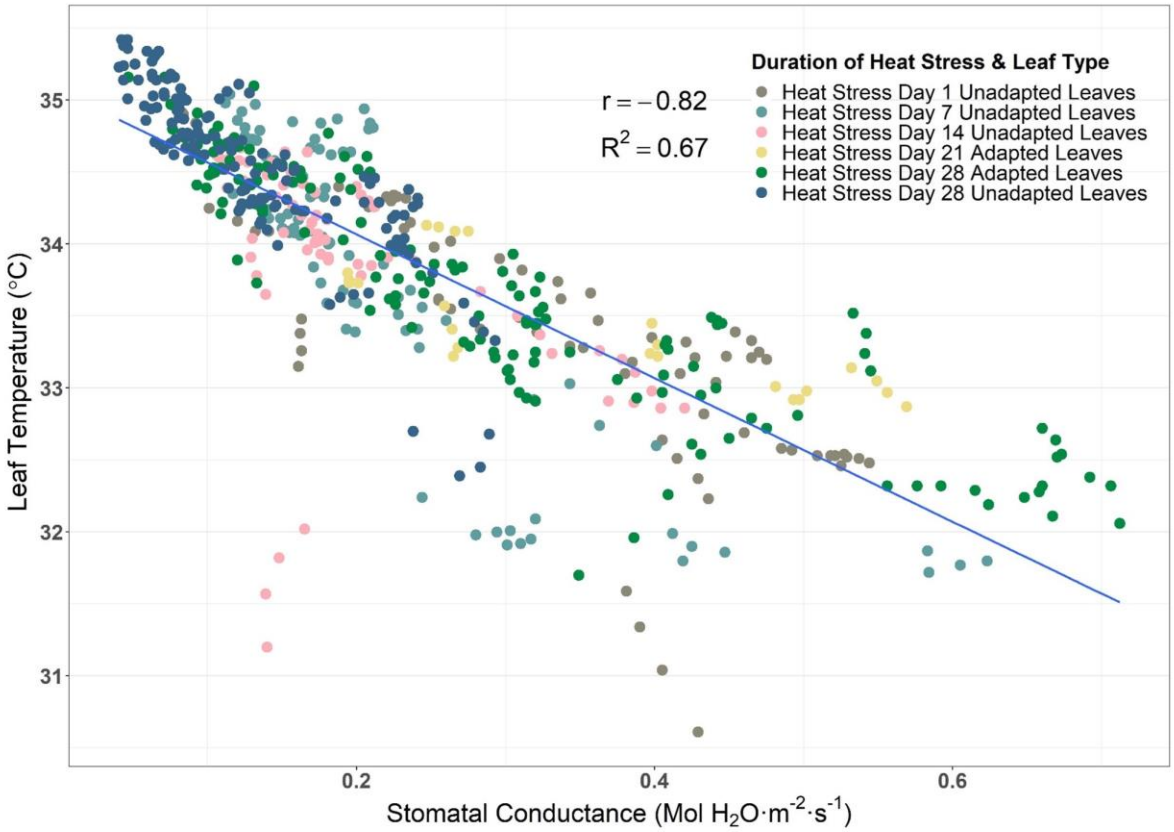

Figure 9: Correlation plot of all data between stomatal conductance and leaf temperature for the cultivar ATL in unadapted and heat-adapted leaflets. All measurements were made at mid-day and the air temperature was maintained at 35°C. Measurements were made after 1, 7, 14, 21, and 28 days of heat stress treatment in unadapted or heat-adapted leaflets as indicated.

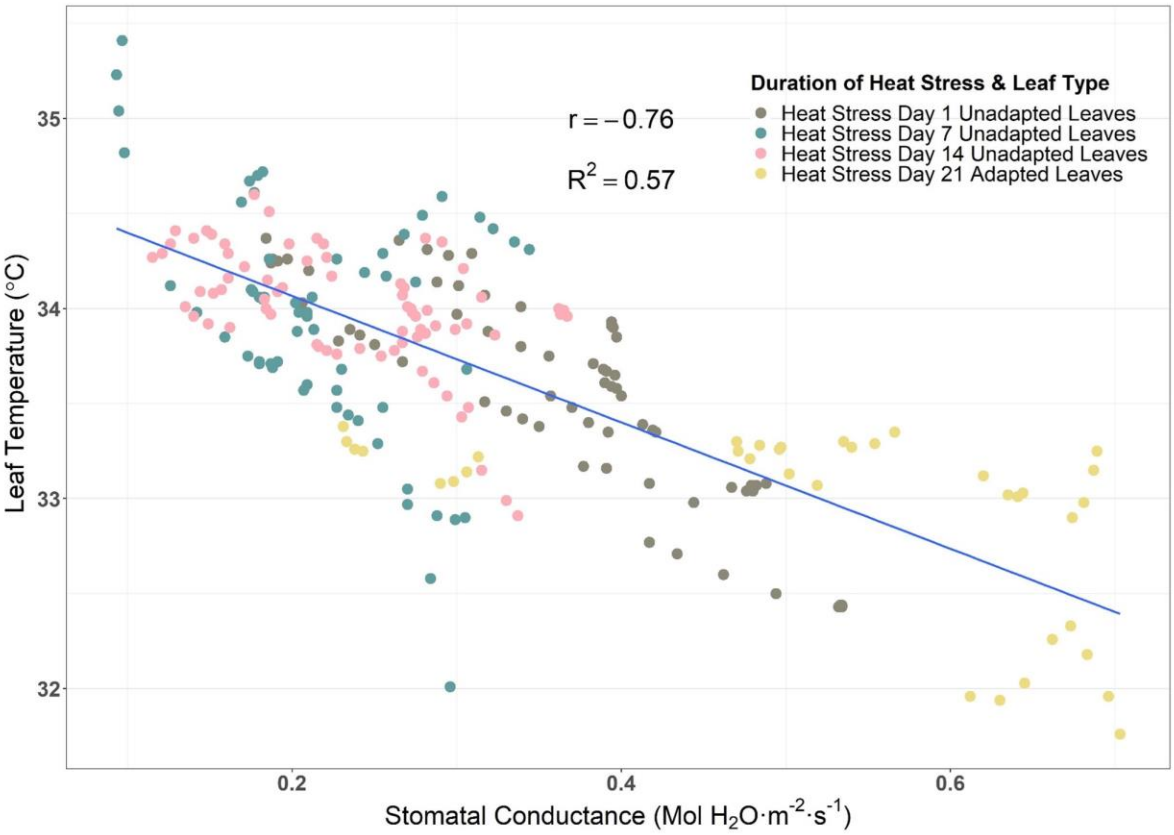

Figure 10: Correlation plot of all data between stomatal conductance and leaf temperature for the cultivar DRN in unadapted and heat-adapted leaflets. All measurements were made at mid-day and the air temperature was maintained at 35°C. Measurements were made after 1, 7, 14, and 21 days of heat stress treatment in unadapted or heat-adapted leaflets as indicated.

Tables

Table 1: Leaf temperature in unadapted leaves after 1, 7, and 14 days under heat stress for ATL and DRN. The leaf temperature increase was higher at 7 and 14 days of heat stress as compared to day 1. Significant difference determined by Duncan's Multiple Range test within a variety ($p < 0.05$). Values are means ($n = 36$, $n=56$, ATL and DRN, respectively).

Genotype	Duration of Heat Stress	Leaf Temp	
ATL	Day 1 Heat Stress	33.3	b
	Day 7 Heat Stress	34.1	a
	Day 14 Heat Stress	34.0	a
DRN	Day 1 Heat Stress	33.6	b
	Day 7 Heat Stress	34.4	a
	Day 14 Heat Stress	34.1	a

Table 2: Leaf temperature in unadapted leaves after 14 days of heat stress, and adapted leaves after 21 and 28 days of heat stress, for ATL and DRN, respectively. Heat-adapted leaves of both ATL and DRN have a lower leaf temperature than unadapted leaves. All measurements were made in the mid-day and the air temperature during the day was maintained at 35°C. Significant difference determined by Duncan's Multiple Range test within a variety ($p < 0.05$). Values are means ($n = 36$ and 148 for ATL unadapted and heat-adapted leaf measurements, respectively; $n = 56$ and 40 for DRN unadapted and heat-adapted leaf measurements, respectively).

Genotype	Leaf Type	Temperature Treatment	Leaf Temp	
ATL	Unadapted	Day 14 Heat Stress	34.0	a
	Adapted	Day 28 Heat Stress	33.7	b
DRN	Unadapted	Day 14 Heat Stress	34.1	a
	Adapted	Day 21 Heat Stress	32.8	b

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Chapter 3 Potential Genes Involved in Heat Stress Adaptation in Potato: Analysis of Differential Gene Expression between Control and Heat-adapted Plant Leaves of the Cultivated *Solanum tuberosum* L. cv. 'Atlantic' and wild *Solanum microdontum* Bitter

Abstract

High temperature stress, or heat stress, has been identified as one of the most significant uncontrollable abiotic factors that affect potato growth, development, and tuber yield. With the increase in temperatures attributable to climate change and the expansion of potato production to regions with higher average daily temperatures, it is crucial to elucidate the physiological and molecular mechanism of potato heat stress and explore resistance and adaptation genes for breeding heat tolerant cultivars.. While short-term heat stress experiments have produced considerable insights into the effects of heat stress on potato, less is known about the impacts of long-term heat stress and the mechanisms involved in heat stress adaptation. Therefore, the purpose of our study was to evaluate the differential expression of genes, in the leaves of potato plants grown at optimal (20/15 °C, day/night) and supra-optimal conditions (35/25°C, day/night, for 3 weeks), that may be involved in heat adaptation to prolonged high temperatures in two contrast heat tolerance potato genotypes. The two genotypes, *Solanum tuberosum* L. 'Atlantic' (ATL) and *Solanum microdontum* Bitter (MCD), used in this study show contrasting tolerance to high temperature, where ATL was sensitive to heat stress and MCD was found to perform well. Our current study evaluated the differences in gene expression between optimal and supra-optimal conditions in ATL and MCD and found several differentially expressed genes that were expressed under heat stress, in both ATL and MCD. We found significantly regulated genes involved in anthocyanin production, lipid and lignin modification, ABA biosynthesis, photosynthesis, heat shock

proteins, cell growth, expansion, and patterning. These data offer insight into potential genes involved in heat tolerance in potato that may be useful in breeding for heat-tolerant potato varieties.

Abbreviations/ Keywords

ATL, *Solanum tuberosum* L. 'Atlantic'; MCD, *Solanum microdontum* Bitter; DAP, Days After Planting; QS, ¼ Strength Hoagland's Solution; DAHS, Days After Heat Stress

Introduction

The genus *Solanum* represents approximately 1,500 species, is genotypically diverse, and widely adaptive to environmental conditions (Spooner, et al., 2014). In commercial production areas potatoes experience a diverse array of conditions, many of which are not considered optimum for potato plant performance and growth, such as temperature, light, water, and nutrition (Burton, 1989). The effects of heat stress on potato plant growth and productivity have become an important issue as production has expanded to areas with higher average daily temperatures, such as tropic and subtropic areas (Tai, et al., 1994), and temperatures have increased due to climate shifts in current areas of production to a range outside of that required for optimal potato plant production (Raymundo, et al., 2014). High temperature stress affects many factors in potato along with production, such as photosynthesis (Dwelle, et al., 1981; Reynolds, et al., 1990), transpiration (Ku, et al., 1977), respiration (Thornton, et al., 1996), stomatal conductance (Dwelle, et al., 1981), chlorophyll content (Reynolds, et al., 1990; Steffen, et al., 1995), assimilate partitioning (Ewing, 1981; Van Dam, et al., 1996), tuber initiation and development (Borah & Milthorpe, 1962; Ewing, 1981; Reynolds & Ewing, 1989a), as well as morphological and anatomical alterations

(Ewing, 1981; Khedher & Ewing, 1985; Reynolds & Ewing, 1989a; Kleinhenz & Palta, 2002). The direct and indirect effects of high temperature include the denaturation of proteins, changes in the fluidity of membrane lipids, chloroplast and mitochondrial enzyme inactivation, disrupted protein synthesis and degradation, and a loss of membrane structural integrity (Howarth, et al., 2005).

It is well-know that acclimation to a short-term high temperature result in an improved ability to tolerate a future high temperature event. Four major types of thermotolerance, or classes of acclimation ability, have been identified in the model plant *Arabidopsis thaliana* and can be used to describe heat adaptation ability in *Solanum tuberosum* L. These include basal thermotolerance (BT), short- and long-term acquired thermotolerance (SAT and LAT, respectively), and thermotolerance to moderately high temperatures (TMHT) which occurs over a prolonged period (Yeh, et al., 2012). BT refers to the inherent or non-acclimated ability of a plant grown at optimum temperature (e.g. 22°C) to tolerate and survive a sudden heat stress. SAT and LAT, respectively, refers to the short (hours) or long-term (days) period between an acclimating temperature (e.g. 37-38°C) and exposure to a sub-lethal high temperature (e.g. 44-45°C). TMHT refers to a prolonged period (days-weeks) of adaptation or acclimation to a sudden sub-lethal high temperature (e.g 35/25 °C, day/night) after a plant is grown at optimum temperature (e.g. 20/15 °C, day/night), without a prior acclimation/ exposure. Plants, such as potato, that originate in environments with large fluctuations in temperature, including regular exposure to long-term high temperatures, tend to demonstrate considerable variation in their adaptation of the photosynthetic system to high temperature (Berry & Björkman, 1980). With the potential of

shifting temperatures and the expansion of current production areas, the importance of the effects of high temperatures on the growth and productivity of potato and the genes responsible for tolerance and survival under these conditions is of significant importance (Tang, et al., 2020).

Identifying heat tolerance traits and mechanisms in potato starts with understanding the genes involved in the control of heat tolerance (Gangadhar, et al., 2014). Acclimation is the result of the sensing of changes in the environment (stress) which follow a signal transduction pathway that then relays the stress signal to secondary signaling molecules inducing a phosphorylation cascade and subsequently activates transcription factors that regulate the primary response to stress through appropriate activation of genes necessary for protection from the observed stress (Xiong, et al., 2002). For example, these changes in transcriptional regulation result in the adaptation and tolerance to high temperature (Xiong, et al., 2002) and in the case of a prolonged stress result in morphological and anatomical adaptation. A study by Liu et. al. (2021) evaluated the regulation of differentially expressed transcripts from potato leaf samples exposed to 3 days of heat stress with significant upregulation of genes in the metabolism overview analysis, related to secondary metabolism (isoprenoids and phenylpropanoids), cell wall degradation (mannan-xylose-arabinose-fucose), cell wall modification, and a minor CHO metabolism gene (raffinose) (Liu, et al., 2021). It is likely that adaptation to high temperatures involves a complex array of genes and relationships in potato, and therefore, an adapted genotype would be expected to express a complex collection of genes to create a high temperature adapted genotype (Marshall, 1982).

Many studies on potatoes focus on the acute heat stress effects of high-temperature stress. However, there is a knowledge gap on the impact of long-term high-temperature stress on potatoes, including information on morphological modification, physiological adaptations, and differences in gene responses between heat-sensitive and heat-tolerant genotypes.. Hence in the current experiment, plants were subjected to BT and TMHT conditions to understand acquirement of thermotolerance during these conditions by characterizing the morpho-physiological adaptation and molecular mechanisms involved in such adaptation in two different genotypes. MCD is a wild diploid species used mostly in breeding for disease resistance (Bisognin, et al., 2005; Tan, et al., 2008), high calcium accumulating tubers (Bamberg, et al., 1993; Bamberg, et al., 1998), tuber greening variation (Bamberg, et al., 2015), and reduced cold-induced sugars under long storage conditions (Bhardwaj, et al., 2011) and shown to be heat tolerant in our previous study (Unpublished). ATL is known to be a heat sensitive potato variety and often performs poorly in ion leakage analyses (Ahn, et al., 2004) and suffers from several heat-related defects in the field generally described as internal necrosis, but more specifically are referred to as internal heat necrosis or internal brown spot, that have a negative impact on the potato industry by reducing tuber quality, and overall useable yield (Yencho, et al., 2008).

Purpose

The objective of our study was to evaluate the differential gene expression (DGE) in the leaves of potato plants grown at optimal (20/15°C, day/night, at least 4 weeks) and supra-optimal conditions (35/25°C, day/night, for 3 weeks) and identify functional groups and pathways that may be involved in long-term heat adaptation in a heat sensitive tetraploid of

Solanum tuberosum (ATL) and a potentially heat tolerant wild diploid of *Solanum microdontum* (MCD). We aim to provide useful insight into the study of general adaptation mechanisms in *Solanum* that may be involved in thermotolerance through the evaluation of differential expression of the potato transcriptome between two contrasting species using RNAseq.

Materials and Methods

Experimental Conditions

Tissue culture plants of *Solanum tuberosum* L. cv. 'Atlantic' (ATL) and *Solanum microdontum* Bitter (MCD, PI 218225) were planted at the University of Wisconsin-Madison Biotron greenhouse facility in 100 pots (Nursery Supplies, Inc.) that were 11.5 cm in diameter. Initially, 12 plants each of ATL and MCD were planted and covered with clear plastic cups for 5 days to allow the tissue culture plants to acclimate to greenhouse conditions. These cups were vented on the 6th day and removed 7 days after planting (DAP). The ATL and MCD tissue culture material was maintained and sourced from our labs tissue culture collection. ATL and MCD were planted using a soilless media (Sungro Horticulture Professional Growing Mix) that was saturated initially with tap water. The fourth day after planting, the plants were fertilized with ¼ strength Hoagland's (QS) solution (1ml/L each of standard stocks A, B and C). Thereafter, the plants were watered every other day for about 15 days with tap water on the days between fertilizations. Plants were transplanted into #1200 pots 16 DAP and transferred from the greenhouse to two controlled environment rooms with equal numbers of replicates of each genotype per room with 14-hour photoperiod and temperatures set to 20/15°C (Day/Night). Daily fertilization with QS began 19 DAP using an

automatic drip irrigation system for 4 minutes beginning at 6AM. Plants were given a nutrient solution consisting of a total of 2.5 mM Ca consisting of a normal QS (1.25mM Ca from $CaNO_3$) plus 1.25 mM Ca from $CaCl_2 \cdot 2H_2O$. Heat stress was started 62 DAP in two controlled environment rooms with the temperature set to 35/25°C (Day/Night).

Ion leakage analysis

Leaflets were taken from plants under control conditions and several weeks after initiation of heat stress. Leaflets samples were placed into glass sample tubes in a 50°C water bath. A total of 3 whole leaflets were used for each of the 5 time treatments. The sample tubes were pulled out of the bath and the samples were shaken (G10 Gyratory® shaker, New Brunswick Scientific, Edison, NJ, USA) for one hour and then the conductivity of the sample was recorded after using a calibrated (cell constant = $0.57 \cdot cm^{-1}$) conductivity meter (Metler Toledo, SevenEasy™, Schwerzenbach, Switzerland) and conductivity probe (Metler Toledo, InLab® 731-ISM, Order No. 30014092). The initial ion leakage measurements were recorded as they were pulled off the shaker after 1 hour. All samples were then autoclaved for 10-15 minutes at 121.6°C with a pressure of 1.6 kg per cm using a liquid cycle. The final readings were recorded after the samples were cooled to room temperature with the help of a cool water bath, and the percent ion leakage was then calculated and summarized in a plot.

Illumina Sequencing, Quality Control, and RNAseq Data Analysis

Sequencing

A total of 12 samples were collected, six samples were derived from ATL and six were derived from MCD for the transcriptome profiling sequencing. Of the 6 samples collected for each genotype, 3 were sampled from the terminal leaflets of plants grown at

20/15 °C (Day/Night) for 56 days, while the other three samples were taken from the terminal leaflets of the same plants grown at 35/25 °C (Day/Night) for 33 days (95 DAP) (Fig. A1). Fresh leaflet samples (0.1 g) were taken and immediately placed in liquid nitrogen and stored at -80 °C freezer until processed for RNA extraction and sequencing.

Plant leaf samples were processed for RNA extraction and sequencing at UW-Madison Biotechnology Center (Madison, WI). The samples were sequenced using a NovaSeq6000 (Illumina, San Diego, California, USA). The differential gene expression (DGE) analysis was based on treatment and the primary contrast was the heat treatment and the control was the primary reference for ATL and MCD. The library type used was the Illumina TruSeq Stranded mRNA with polyA enrichment and samples were prepared following the Illumina TruSeq mRNA protocol (https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/samplepreps_truseq/truseqstrandedtotalrna/truseq-stranded-total-rna-sample-prep-guide-15031048-e.pdf).

Preliminary data inspection and mapping alignment

Issues with mislabeling or batch effects causing inconsistencies in expression values across these replicates were detected using multi-dimensional scaling (MDS) of read counts and a clustered image map (CIM/ heat map) of dissimilarities in read counts of samples within each condition (control and heat stress treatments). Gene expression normalization was carried out prior to gene expression analysis using the method of trimmed mean of M-values (TMM), which consists of estimating the fraction of reads per gene/transcript

compared to the total number of reads for each biological sample with respect to all 12 samples.

Read Mapping/ Alignment

The paired-end reads were aligned to the 23 using the STAR (Spliced Transcripts Alignment to a Reference) alignment software (Dobin, et al., 2013). In addition to the previously reported sequence-only metrics, transcriptome mapping statistics were computed for the obtained fastq files and we have evaluated the QC summaries that include sample, chromosome, and aggregate gene-level comparisons (Table A1). The sample-level mapping results were checked visually to confirm the reads aligned to the expected regions using the Integrative Genomics Viewer (IGV) application (Robinson, et al., 2011).

Enrichment Analysis

Gene Set Enrichment Analysis (GSEA)

Gene enrichment analysis is regularly used for the interpretation of genome-wide profiles of RNA expression (Subramanian, et al., 2005). Here it was performed using the web-based software ShinyGO 0.76 (Ge, et al., 2020). Ensembl gene ID's (e.g. PGSC0003DMG400000001) were submitted separately for significantly up- and down-regulated genes (FDR<0.05) from the RNAseq expression data of both ATL and MCD. All available database information (GO biological process, GO cellular component, GO molecular function, UniProt (annotated keywords), STRING (local network cluster), Pfam (protein domains), SMART (protein domains), and InterPro (protein domains and features)) was used for the eclectic construction of the enrichment plots. The fold enrichment is defined

as the percentage of genes in the test set for a particular pathway, divided by its corresponding percentage in the reference/background gene set. The background gene-set for the enrichment used the gene ID's of all expressed genes for ATL (16,575), MCD (15,838), and a joint analysis (17,427) detected in the experiment as suggested by Timmons et. al. (2015). Redundant pathways were removed, so those sharing 95% of genes and 50% of the words in their names were represented by the pathway that was most significant.

MapMan Analysis

Transcript analysis was performed using MapMan (Version 3.6.0RC1) to display the significantly up and down-regulated fold change data in the context of biological functions and pathways that may provide insight into transcripts involved in heat tolerance (Usadel, et al., 2009). The transcript expression data was inserted onto pathways using the "X4.2 solanum tuberosum" mapping file available from the MapMan website.

Validation of the RNA-Seq results using Q RT-PCR

Twelve validation gene transcripts were randomly selected (Table A2) from the list of significantly up and down-regulated genes that were shared between ATL and MCD in our study to verify their regulation from our results obtained from RNA sequencing (Table A3). Of the 12 validation genes selected, 6 were significantly upregulated and 6 were significantly downregulated. Potato genome sequencing consortium (PGSC) transcript ID's (e.g. PGSC0003DMG400000001) were used to identify sequences in the Spud DB Genome Browser using the potato (Solanum tuberosum group Phureja DM1-3) PGSC v4.03 pseudomolecules. Primers were designed for the 12 genes using PrimerQuest tool of IDT

technologies. The primer forward and reverse primers for the 12 genes were listed in the table A2.

Total plant RNA was extracted using the TRIzol method (Rio, et al., 2010) and was obtained from the same samples as those used for RNAseq. The purity and concentration of RNA was then quantified on a nandrop (Nanodrop One, ThermoFisher Scientific, USA). The cDNA was synthesized following the iScript™ protocol for cDNA synthesis (iScript™ Advanced cDNA Applied Biosystem kit for RT-qPCR, # 4368814, Applied Biosystem, USA).We determined the expression levels of the target genes with quantitative real-time PCR (qRT-PCR) using the Applied Biosystems QuantStudio™ 3 real-time PCR system (A28567, Applied Biosystems™, USA). The qRT-PCR was performed using the applied biosystems PowerUp™ SYBR™ Green Master Mix (A25741, Applied Biosystems™, USA). The expression of each gene was normalized and calibrated against the control and using a geo metric mean of the reference genes *EF1α* and *Peroxidase superfamily protein*. The relative stable expression level under different conditions was calculated using $2^{-\Delta\Delta Ct}$ method (Livik & Schmittgen, 2001).

Results

Physiological Adaptations

The current physiology and RNAseq study contributes additional knowledge on the many genes and regulatory pathways involved in the multigenic control of heat tolerance and adaptation in two distinct genotypes of potato. The adaptation of two contrasting potato cultivars, ATL and MCD, was evaluated by growing them at 20/15 °C (Day/Night) for 56

days and then exposing them to heat stress for 33 days. As shown in Figure A1, the newly adapted leaves of the two cultivars were slightly darker and smaller than those of the control plants. Additionally, we discovered that the potato plant adjusts its morphology, anatomy, and physiology in response to these sustained high temperatures (Data not shown). We also observed prolific foliage growth, flowering, retainment of flower and leaf health under long-term high temperatures *Solanum microdontum*. We found significant differences ($P<0.05$ to $P<0.01$) in ion leakage between ATL and MCD at all temperatures in he adapted leaves after exposure to 33 days of heat stress. The results shows higher tolerance in MCD to heat stress using membrane thermostability analysis compared to ATL (Fig. 1).

Sequencing data

The results of both MDS and CIM analyses show that our experiment was well-controlled with respect to sample acquisition and sample processing and that the greatest variation was between the condition/genotype combination (e.g. control ATL vs heat stress ATL) than between samples within these combinations. The inspection of both MDS (Fig. A2) and CIM (Fig. A3) plots confirm consistent expression values across the 3 biological replicates for each genotype within each condition and suggested that our specified contrasts (control vs heat-adapted) yield significant differences. In this experiment, TMM procedure produced uniform distributions centered on a common median between the 3 control and 3 heat-adapted samples for each genotype (Fig. A4).

DEG Analysis

We identified a total of 23,461 and 21,552 expressed transcripts and of those 4,400 and 2,819 were significantly ($FDR<0.05$) expressed for ATL and MCD, respectively (Fig. 2).

Of the total 4,400 significantly expressed transcripts in ATL, 1,974 were up-regulated and 2,426 were down-regulated, while for MCD, of the total 2,819 significantly expressed transcripts, 1,436 were up-regulated and 1,383 were down-regulated (Fig. 2B). Of the total 1,974 and 1,436 significantly up-regulated transcripts 1,498 and 960 were uniquely up-regulated for ATL and MCD, respectively, and 476 were shared. Of the total 2,426 and 1,383 significantly down-regulated transcripts 1,750 and 707 were uniquely down-regulated for ATL and MCD, respectively, and 676 were shared (Fig. 2B).

We identified several significant and highly expressed genes that may contribute to tolerance and adaptation to prolonged high temperature in both ATL and MCD (Fig. 3). Highly up-regulated transcripts in ATL represented genes related to photosynthetic processes including ribulose bisphosphate carboxylase/oxygenase activase (RCA1), phytochrome-interacting factors (PIF), leucoanthocyanidin dioxygenase (LDOX) essential for proanthocyanidin synthesis, ascorbate peroxidase (APX), a transcription factor (FAMA) involved in promoting guard cell differentiation, and several heat shock proteins (HSP's) including the chaperone protein dnaJ (HSP40) and several small HSP's (sHSP_17.6kD). Several highly down-regulated transcripts in ATL included the flowering locus T protein (FT) a mediator of the onset of flowering, the oxidation repair enzyme glutaredoxin (GTDX), heavy chain myosin ubiquitous actin-based motor proteins (MyosinHC) involved in cytokinesis, sphingolipid delta-8 desaturase (SLD1), inositol-3-phosphate synthase (INO1) involved in phospholipid production and signal transduction, and a component LHCb1/2/3 of LHC-II complex (Lhcb) involved in the light-harvesting of chlorophyll a/b. The highest up-regulated transcripts in MCD represented genes related to ascorbate peroxidase (APX),

ribulose bisphosphate carboxylase/oxygenase activase (RCA1), leucoanthocyanidin dioxygenase (LDOX), cytochrome (P-450) involved in catalyzing monooxygenation reactions in primary and secondary metabolism, DAWARF1 involved in steroid synthesis, and heat shock proteins (sHSP, sHSP_17.6kD_II). The highly down-regulated transcripts in MCD were phenylalanine ammonia-lyase (PAL) a primary enzyme in phenol accumulation and synthesis that is considered a primary inducible response to abiotic stress, elicitor-inducible LRR receptor (EILP), the NAD(P)H-quinone oxidoreductase subunit 4L (ndhE) involved in the photosynthetic chain, and peroxidase (PX).

Several highly enriched pathways of up-regulated genes that were shared between ATL and MCD were identified using GSEA that included heat shock protein synthesis (Fig. 4). Among the genes enriched in the heat shock pathways were HSP22.7 and HSP17.6. Other enriched pathways included carbohydrate metabolism (glycosyl hydrolase family 36), stress protection protein synthesis, oxidation, iron metabolism, and steroid biosynthesis. The highly enriched pathways of down-regulated genes that were shared between ATL and MCD included ribosome protein activity, ribosome biosynthesis, nucleic acid binding, and carbon metabolism (Fig. A7). The highly enriched pathways of up-regulated genes that were unique to ATL included ATP enzymatic activity, the patterning of anatomical development (homeobox), and leucine-rich repeat activity that provide a structural framework in protein-protein interactions, DNA-binding, and signaling (Figure 5). The highly enriched pathways of down-regulated genes that were unique to ATL and most significant were involved in photosynthesis with other less significantly enriched pathways including ribosomal activity and biosynthesis and ATP enzymatic activity (Figure A7). The highly enriched pathways of

up-regulated genes that were unique to MCD included RNA editing (DYW and PPR repeat, Figure A9), while the highly enriched pathways of down-regulated genes that were unique to MCD included lipid metabolism (desaturase, isoprenoid synthase), terpene synthesis, carbohydrate metabolism, leucine-rich repeat activity, signalling, and heme that is involved in redox reactions and secondary metabolism (Figure 6).

The Mapman regulation overview for ATL and MCD show a large percentage of down-regulated transcripts among all significantly regulated transcripts related to ABA, BA, ethylene, cytokinin, glutaredoxin, and dismutase/catalase. Jasmonate and IAA were similarly down-regulated in ATL, but jasmonate is more up-regulated in MCD (Figure 7). The Mapman metabolism overview for ATL shows many down-regulated transcripts related to various aspects of photosynthesis, tetrapyrrole biosynthesis, and oxidative phosphorylation. Lipid metabolism shows up- and down-regulation of transcripts related to phytosterols and fatty acids, respectively. Transcripts related to PO_3^{3-}, NH_3, SO_4^{2-}, purines, pyrimidines, and deoxy-nucleotides were mostly down-regulated, while NO_3^- and Fe^{3+} were up-regulated (Figure 8A). MCD shows many up-regulated transcripts related to the calvin cycle, phyto-sterols, NO_3^-, and pyrimidines and several down-regulated transcripts related to PO_3^{3-}, NH_3, SO_4^{2-}, and fatty acid metabolism (Figure 8B).

Discussion/Conclusion

Heat stress is becoming a more important concern in commercial potato production (Raymundo, et al., 2014) and plants can typically experience higher than optimum temperatures for several days to weeks. While the transcriptional effects of heat stress can occur within minutes of exposure (Mittal, et al., 2009), the effect on morphology and

anatomy requires a sustained stress to acquire a true adaptation to those conditions and these physical adaptations in turn allow for an adaptation in physiological response (i.e. increased stomatal density resulting in increased photosynthesis/transpiration and cooling). We found distinct morphological (Figure A1), physiological, and anatomical adaptations that allowed potato plants to fully adapt to these prevailing long-term (~4 weeks), moderate high temperatures (35/25°C, day/night) in both cultivars (data not shown). In heat-sensitive plants, degradation of proteins, chlorophyll concentration, and membrane stability, as well as an increase in electrolyte leakage, leads in a reduction in maximum photochemical efficiency of photosystem II (PSII) leading to reduced yield (Bhattarai et al., 2021). Our ion leakage results indicate significantly lower leakage rates in MCD demonstrating its higher cell membrane thermal stability and consequently heat tolerance compared to ATL (Figure 1).

To obtain a deeper understanding of the mechanisms involved in long-term, moderate heat stress adaptation and tolerance we investigated the genetic aspect of heat tolerance by RNAseq analysis to try and uncover genes or functional pathways related to these adaptations. Most studies investigating the effects of heat stress on potato and most other plants species make observations after several hours (Ahn, et al., 2004; Liu, et al., 2021; Rensink, et al., 2005) or days (Liu, et al., 2021; Tang, et al., 2020) of heat stress without acclimation. These studies in potato can be considered to investigate BT. However, this is the first study to investigate the transcriptional regulation and identifying the gene that results in the adaptation and tolerance to a prolonged heat stress (4.5 weeks) that results in morphological, anatomical, and physiological adaptation in two contrast genotypes.

Gene enrichment was used to determine which sets of pathways were significantly changed between conditions (control and heat stress) for a predetermined gene set that included up- and down-regulated sets of genes shared or unique between ATL and MCD (Figs. 4-6, Appendix Figs. 7-9). The GO analysis in our study found pathways related to abiotic stress tolerance, heat shock proteins and plant pathogen interaction including the up-regulated pathway involved in plant pathogen defense response (fusaric acid resistance protein) in both ATL and MCD (Figure 4). HSP's are an important class of proteins involved in many aspects of thermotolerance, such as their role in acting as molecular chaperones, preventing the denaturation and aggregation of target proteins, and facilitating the folding of proteins (Sarkar, et al., 2009; Perez, et al., 2009). The overwhelming majority of the most highly expressed genes in ATL and MCD found in this study relate to HSP's (Figs. 3 and 4). More specifically HSP17.6 class I and II were the most abundantly expressed among the HSP's. HSP17.6 from *Methanolobus psychrophilus* has been found to play a role in oxidative inactivation in *Escherichia coli* (Ma, et al., 2021) and may play a similar role is protecting against oxidative stress, such as that caused by photooxidation, in potato under high temperature. A study in potato screening for heat tolerance used the heat sensitive cultivar Desiree and heat tolerant Festival and found a similar accumulation of class 1 17.6 kD HSP's (HSP17.6_I) in addition to HSP101 and eEF1A (elongation factor-1 alpha) in shoots and microtubers. The presence of HSP17.6_I in micro tubers of Desiree did not help to alleviate the effects of heat stress on micro-tuberization in contrast to a potential positive effect of eEF1A on this trait in Festival (Pantelic, et al., 2018). EF1A in ATL and MCD was significantly down-regulated in our study and ranked high in the most DOWN-regulated

transcripts in MCD (#32). A study by Oljača et. al. (2018) looked at the expression of HSP17.6, HSP101 and EF1A in cultivars of potato in the field in Serbia and found a positive correlation for the accumulation of all three genes and tuber yield, in addition to a negative correlation for the same genes with primary shoot height and above-ground biomass. Our study found a similar up-regulation of HSP 17.6 and HSP101 in ATL (#9 and #20, respectively) and MCD for HSP 17.6, HSP101, and EF1A (#9 and #48, and #32, respectively, Figure 3). A similar experiment in *Solanum tuberosum* cv. 'Russet Burbank' utilized 20/18°C (day/night, sampled 45 DAP) and 35/28°C (day/night, sampled 3 DAHS or 45 DAP) conditions for control and heat stress treatments, respectively (Tang, et al., 2020). They found that after 3 days of heat stress that the most highly expressed heat stress transcription factors (StHsf's) and heat shock proteins (StHsp's) were HsfA2, HsfA3, HSP26-CP, and HSP70 which showed a -12.7, -3.5, 414.7, and 10.1-fold change, respectively, compared to the control. Of these four genes, only HSP70 was significantly down- and up-regulated in ATL and MCD, respectively, in our study (Figure 3). Lastly, of the three total heat shock factors (HSF's) that were expressed in our study (HSF8, HSF24, and HSF30) only HSF30 was significantly expressed and showed up- and down-regulation in MCD and ATL, respectively. These findings demonstrate that HSP's are an important component of long-term heat adaptation in potato and that HSF's may play a limited role after long-term adaptation has occurred.

In addition to unique up-regulated pathways in ATL we also found pathways related to plant pathogen defense response (virus X resistance protein, and NB-ARC domain) (Figure 5). Tang et. al. (2020) found a similar enrichment of genes in "plant pathogen

interaction" pathways after KEGG pathway analysis that were important after 3 days of heat stress. This may demonstrate the importance of genes related to pathogen attack in the long-term thermotolerance of potato.

Several abiotic factors including high light, drought, and heat stress are known to increase photosynthetic reactive oxygen species (ROS) generation (Ahuja, et al., 2010; Mittler, et al., 2012; Scarpeci, et al., 2008). Interestingly, it may be the case that heat stress initiates oxidative stress signaling (Desikan, et al., 2004) through a burst of production of H_2O_2 that may be sensed directly by heat shock transcription factors (Hsf's) (Miller & Mittler, 2006; Scarpeci, et al., 2008) that results in HSP expression. This may relate directly to the findings for the upregulation of HSP's in our study as HSP70 and HSP17.6 were highly up-regulated (Figure 3) and were found by Scarpeci et. al. (2008) to have a plausible role in oxidative stress mitigation. Oxidative stress is a common occurrence in plants under heat stress as heat affects many factors such as the stability of proteins, cell membranes, RNA species, the structure of the cytoskeleton, and the alteration of enzymatic reaction efficiency, which can disrupt the metabolic balance (McClung & Davis, 2010; Ruelland & Zachowski, 2010). The metabolic pathways in plants are sensitive to environmental change and imbalances in metabolic flux by high temperature can result in the accumulation of ROS, which are toxic to the cell through their oxidation of cellular components and subsequent effect on membrane integrity of organelles. A high-degree of metabolic coordination is required to maintain the flow of energy in the mitochondria and chloroplasts under adverse conditions to prevent the production of ROS (Suzuki, et al., 2012). Tang et. al. (2020) found "metabolic pathways", "secondary metabolite biosynthesis", "plant hormone signal

transduction", and "carbohydrate metabolism" were the pathways that had the greatest number of DEG's in their enrichment analysis and may be indicative of the enrichment of oxidative stress pathways that were highlighted by their GO analysis. Our GSEA analysis also showed up-regulation of pathways in both ATL and MCD related to genes involved in carbohydrate metabolism (glycosyl hydrolase), and oxidation (Figure 4). Unique down-regulated pathways in MCD were related to carbohydrate metabolism (glycosyl hydrolase), and heme that may be involved in redox reactions and secondary metabolism (Figure 6). Furthermore, most of the pathways that were uniquely down-regulated in MCD were related to terpene synthesis, while one pathway related to up-regulation of steroid/ terpenoid biosynthesis in both ATL and MCD (Figs. 6 and 4, respectively). Terpene may possess antioxidant activity (Gonzalez-Burgos & Gomez-Serranillos, 2012) and rice has been shown to produce volatile terpenes in response to oxidative stress caused by abiotic factors (Lee, et al., 2015). In addition, the production of terpenes in plants have also been shown to be produced as the result of ROS induced by abiotic stress including heat stress (Esnault, et al., 2010; Jenkins, 2009). Therefore, it is no surprise that plants have developed defenses against the production of ROS in response to high temperature, such as antioxidant enzyme systems that include peroxidase, catalase, and superoxide dismutase in addition to non-enzymatic antioxidant compounds such as carotenoids, ascorbate, and glutathione (Barta, et al., 2004; Berli, et al., 2010; Calogirou, et al., 1999; Jansen, et al., 1998). These results may suggest that terpene could play an important role as an antioxidant in potato and contribute to heat tolerance by mitigating oxidative stress resulting from long-term heat stress in fully adapted leaves of ATL and MCD.

ATL and MCD were also found to up-regulate pathways related to steroid/ sterol biosynthesis (Figure 4). Sterols, and specifically brassinosteroids, play a role in increasing the tolerance of plants to biotic and abiotic stress, including heat stress, in rice (Divi & Krishna, 2009; Krishna, 2003) and are also important in the regulation of cell elongation, photomorphogenesis, xylem differentiation, and seed germination. This finding may demonstrate that sterols may also be important in tolerance to heat stress in potato.

Lipids play an important role in the heat tolerance of plants and the saturation of lipids is known to increase under heat stress (Horvath, et al., 1998; Larkindale & Huang, 2004) making cell membranes less fluid. Several unique down-regulated pathways in MCD related to lipid metabolism were found including desaturase and isoprenoid synthesis (Figure 6). Additionally, 22 gene transcripts involving lipid desaturase were identified that were significantly regulated in both ATL and MCD and 17 of those were significantly down-regulated, including sphingolipid delta-8 desaturase (SLD1), W-3 desaturase (W3), delta-12 (D12) and sterol desaturase (SD) (Figure 3). The up-regulated desaturases common to both ATL and MCD were delta(7)-sterol-C5(6)-desaturase (D7, Figure 3). The down-regulation of desaturase transcripts in our study suggests an increase in the saturation of membrane lipids, such as sphignolipids which are known to play a vital role in cell plasma- and endomembrane structural integrity (Luttgeharm, et al., 2016), may be an important component of long-term heat stress in potato.

The plant hormone ABA usually plays a key role is response to drought by regulating stomatal aperture to prevent water loss (Lim, et al., 2015). Like terpene, ABA is an isoprenoid but is a phytohormone that functions in the regulation of physiological processes

such as stomatal aperture and acts as a signaling mediator that regulates the adaptive response to stress (Sah, et al., 2016). For example, abiotic stresses (e.g. drought, salinity, low temperature) that cause osmotic stress tend to significantly increase the production of ABA that subsequently leads to the closure of stomata (Cutler, et al., 2010). Our results in the MapMan "Regulation Overview" showed that transcripts related to ABA were largely down-regulated in both ATL and MCD (Figure 7) demonstrating the importance of lowering ABA expression levels in long-term heat stress in potato. This gene regulation of ABA fits the physiological data in the first chapter where we found higher stomatal conductance in heat-adapted leaves compared to control. Transcripts related to other plant hormones were down-regulated in both ATL and MCD that were related to IAA, cytokinin, jasmonate, BA, and ethylene (Figure 7). Several transcripts related to redox compounds were also found to be significantly down-regulated that included heme, glutaredoxin, and dismutase/catalase (Figure 7).

The MapMan "Metabolism Overview" showed many down-regulated transcripts related to photosynthetic processes including light reactions, the Calvin cycle, photorespiration, tetrapyrrole biosynthesis, and oxidative phosphorylation (Figure 8). For MCD, the same categories were ambiguous for a trend in regulation, but the Calvin cycle showed a contrasting trend towards upregulation of transcripts involved in the process.

In conclusion, the RNAseq analysis and subsequent down-stream analyses highlighted the key involvement of heat shock proteins, oxidative stress response, lipid metabolism, hormone signaling, and photosynthesis in the long-term heat tolerance of both ATL and MCD. The data presented here may also contribute to understanding the effect of seasonal

variations and high temperature effects and genes involved in adaptation in potato and other related plant systems to breed tolerant genotypes.

Figures

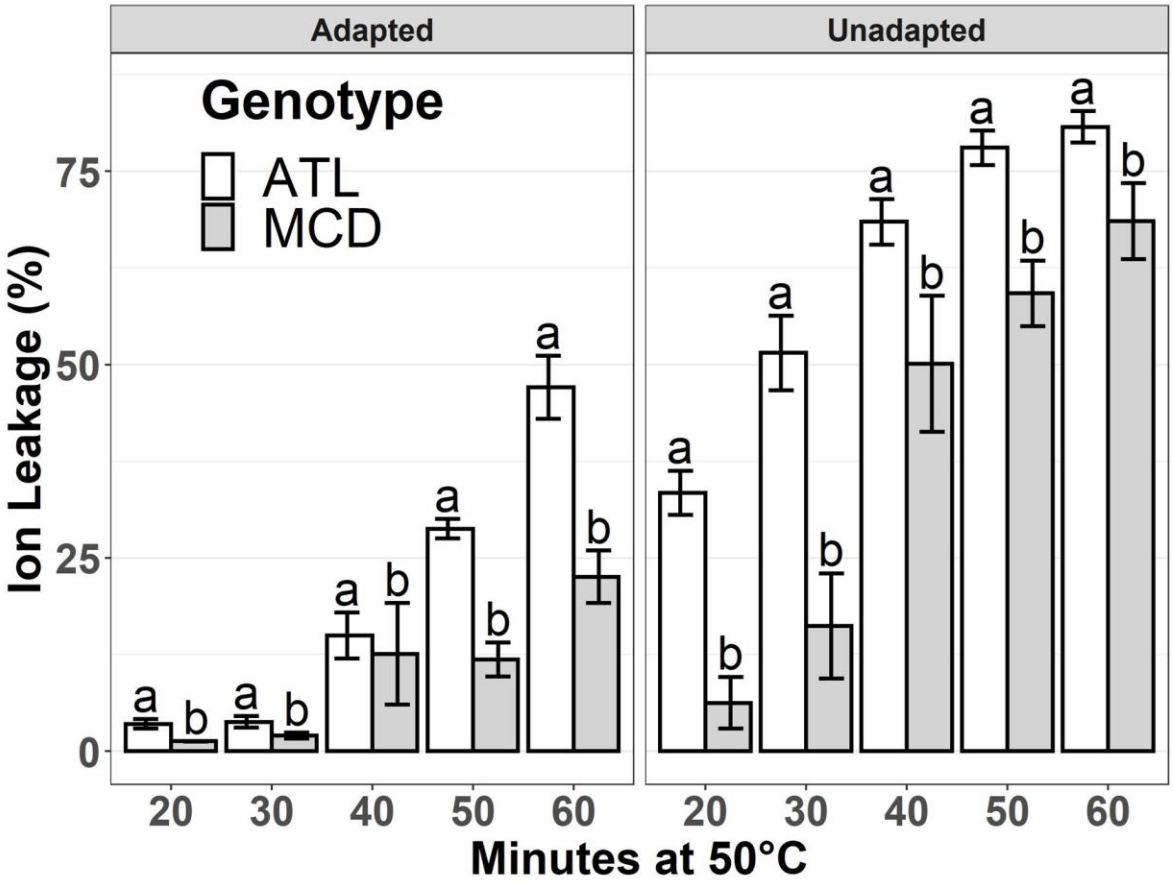

Figure 1: Ion leakage Profiles for *Solanum tuberosum* L. cv. Atlantic (ATL) and *Solanum microdontum* Bitter (MCD) from heat-adapted and unadapted leaves from the same plant. Unadapted leaves were produced under 20/15°C (day/night) temperatures and adapted leaves were produced under 35/25°C (day/night) temperatures. Lower ion leakage in MCD as compared to ATL is indicative of higher leaf cell membrane thermotolerance. Values are means (± SE), n=3. Significant differences determined between genotypes within a time treatment by t-test and ($p<0.05$). Means with similar letters are not significantly different.

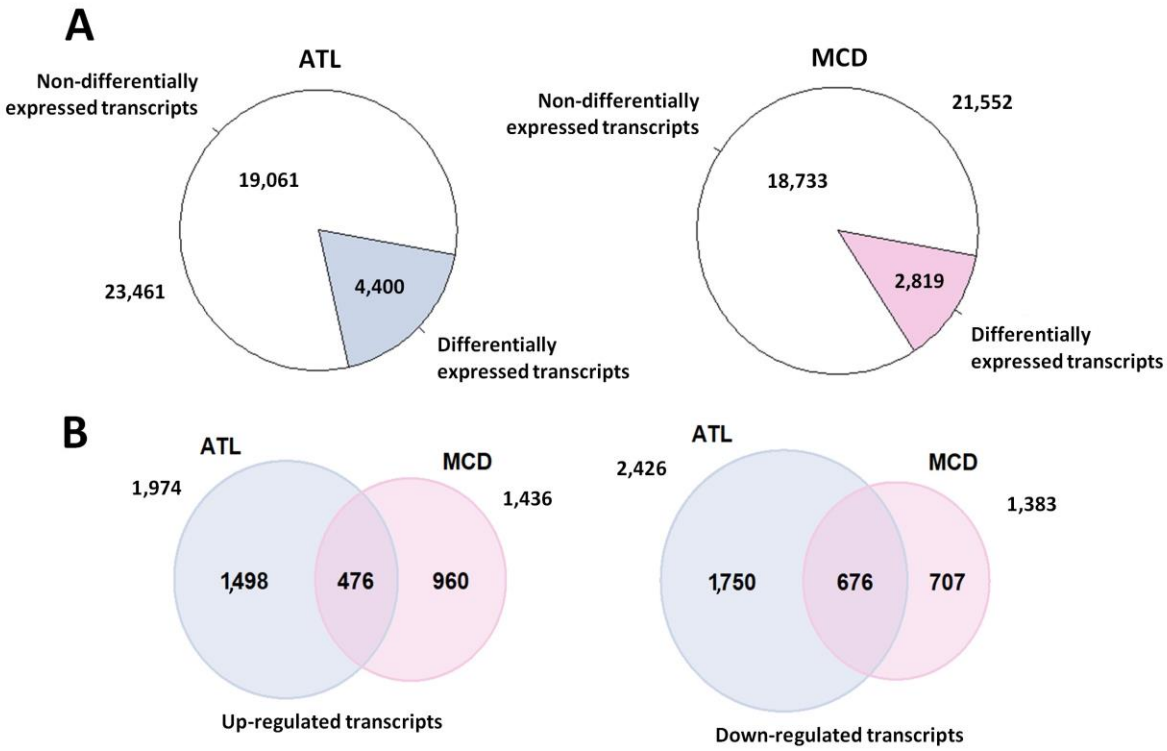

Figure 2: **A)** The number of non-differentially expressed and differentially expressed (up- and down-regulated) transcripts (FDR<0.05) between ATL and MCD potato leaves grown under control (20/15°C, day/night) and long-term high temperature (35/25°C, day/night for 4.5 weeks). The total number of ATL or MCD expressed transcripts is given on the outer circumference. A total of 23,461 and 21,552 expressed transcripts were identified in ATL and MCD, respectively, after trimming and alignment of reads to the *Solanum tuberosum* assembly (SolTub_3.0). A total of 19,061 and 18,733 of those transripts were not significantly expressed (FDR>0.05), while 4,400 and 2,819 of those transcripts were significantly expressed (up- or down-regulated, FDR<0.05) in ATL and MCD, respectively. **B)** The number of significantly up- and down-regulated transcripts (FDR<0.05) that are shared and unique to both ATL and MCD after growth under control (20/15°C, day/night) and long-term high temperature (35/25°C, day/night for 4.5 weeks). The total number of ATL or MCD up- or down-regulated transcripts is given on the outer circumference. Of the total 1,974 and 1,436 significantly up-regulated transcripts 1,498 and 960 were uniquely up-regulated for ATL and MCD, respectively, and 476 were shared. Of the total 2,426 and 1,383 significantly down-regulated transcripts 1,750 and 707 were uniquely down-regulated for ATL and MCD, respectively, and 676 were shared.

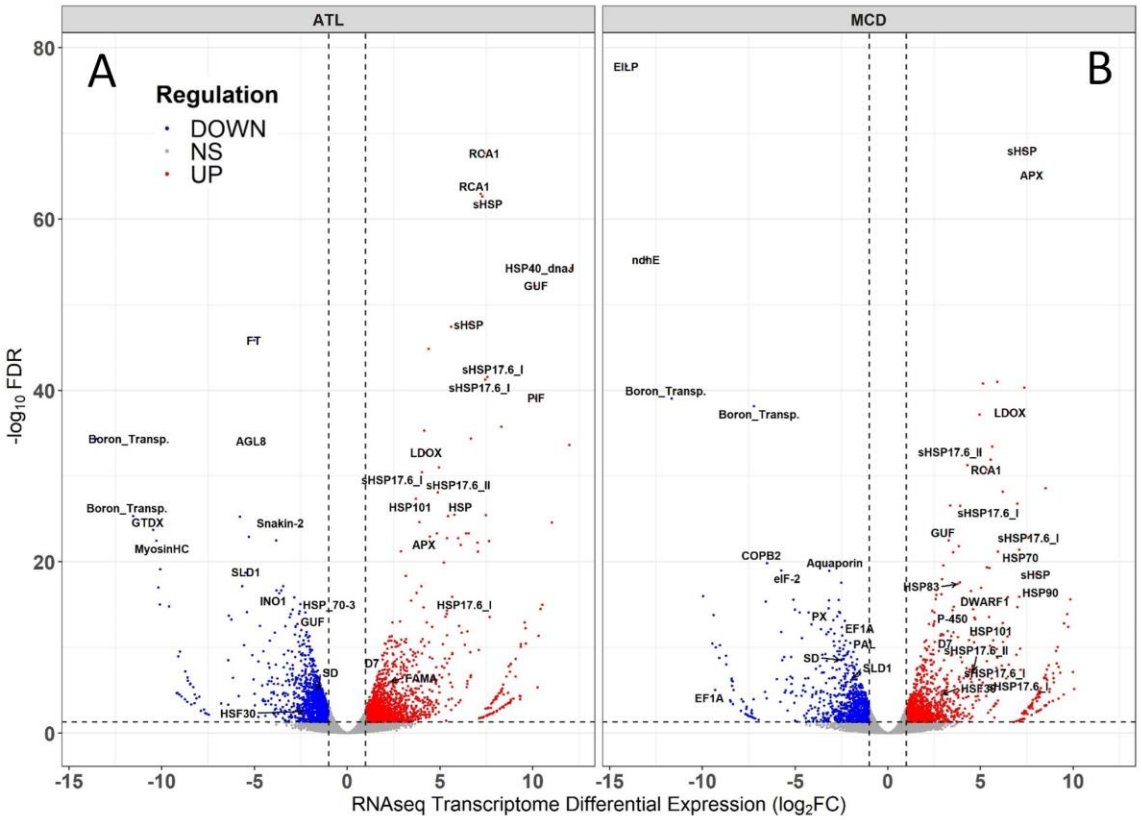

Figure 3: The most significantly expressed up- and down-regulated transcripts in ATL and MCD. **A)** Highest up-regulated transcripts in ATL representing genes related to photosynthetic processes including ribulose bisphosphate carboxylase/oxygenase activase (RCA1), phytochrome-interacting factors (PIF), leucoanthocyanidin dioxygenase (LDOX) essential for proanthocyanidin synthesis, ascorbate peroxidase (APX), delta(7)-sterol-C5(6)-desaturase (D7) a transcription factor (FAMA) involved in promoting guard cell differentiation, and several heat shock proteins (17.6kD HSP's, HSP101) inluding the chaperone protein dnaJ (HSP40). Several highly down-regulated transcripts include the flowering locus T protein (FT) a mediator of the onset of flowering, the oxidation repair enzyme glutaredoxin (GTDX), sterol desaturase (SD), sphingolipid delta-8 desaturase (SLD1), heavy chain myosin ubiquitous actin-based motor proteins (MyosinHC) involved in cytokinesis, sphingolipid delta-8 desaturase (SLD1), inositol-3-phosphate synthase (INO1) involved in phospholipid production and signal transduction, and a component LHCb1/2/3 of LHC-II complex (Lhcb) involved in the light-harvesting of chlorophyll a/b. **B)** Highest up-regulated transcripts in MCD representing genes related to ascorbate peroxidase (APX), delta(7)-sterol-C5(6)-desaturase (D7), ribulose bisphosphate carboxylase/oxygenase activase (RCA1), leucoanthocyanidin dioxygenase (LDOX), cytochrome (P-450) involved in catalyzing monooxygenation reactions in primary and secondary metabolism, DAWARF1 involved in steroid synthesis, and heat shock proteins (sHSP, sHSP_17.6kD_II). Highly down-regulated transcripts wereelongation factor 1 alpha (EF1A), sterol desaturase (SD), sphingolipid delta-8 desaturase (SLD1)phenylalanine ammonia-lyase (PAL) a primary enzyme in phenol accumulation and synthesis considered a primary inducible response to abiotic stress, HSF30, Elicitor-inducible LRR receptor (EILP), the NAD(P)H-quinone oxidoreductase subunit 4L (ndhE) involved in the photosynthetic chain, and peroxidase (PX). GUF = Gene of Unknown Function.

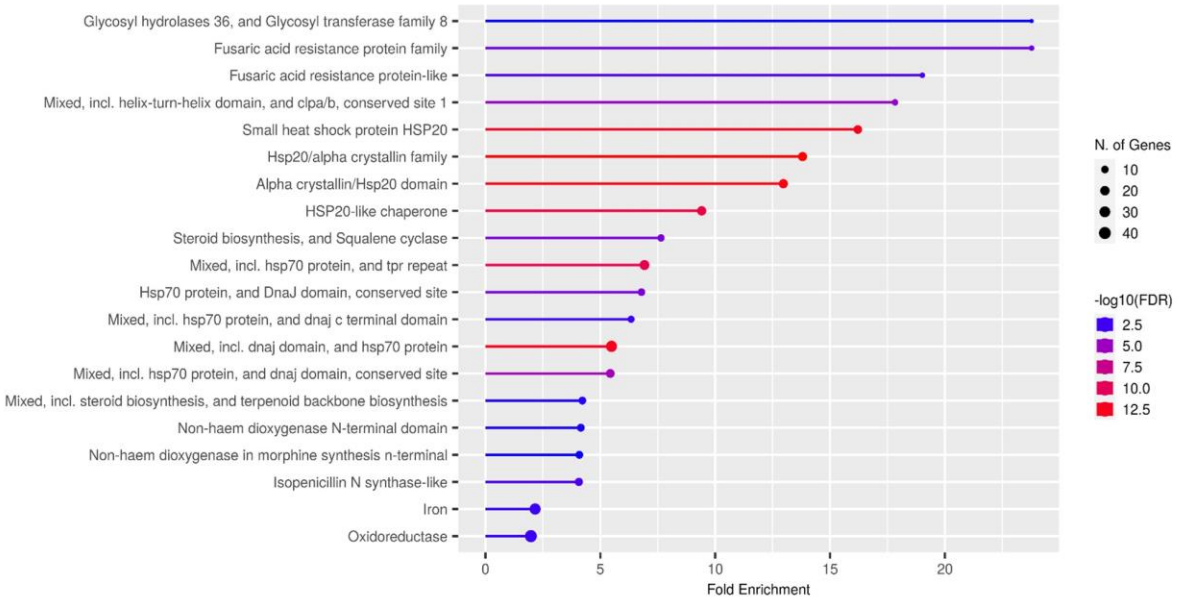

Figure 4: Enrichment analysis derived from ensembl gene ID's for significantly (FDR<0.05) up-regulated genes that are shared between ATL and MCD from the RNAseq expression data. The up-regulated gene pathways that are highly enriched are involved in carbohydrate metabolism, heat shock protein synthesis, pathogen defense response (fusaric acid resistance protein), stress protection protein synthesis, oxidation, iron metabolism, and steroid biosynthesis. For this analysis the FDR tells us the likelihood of enrichment by chance and is calculated from the p-value obtained from the hypergeometric test and is displayed here as the -log10(FDR), which displays greater significance with a higher value. The fold enrichment is defined as the percentage of genes in the test set for a particular pathway, divided by its corresponding percentage in the reference/background gene set. The background gene-set for the enrichment uses the unique gene ID's of all expressed genes for ATL and MCD (17,427) detected in the experiment. Redundant pathways have been removed, so those sharing 95% of genes and 50% of the words in their names are represented by the pathway that is most significant.

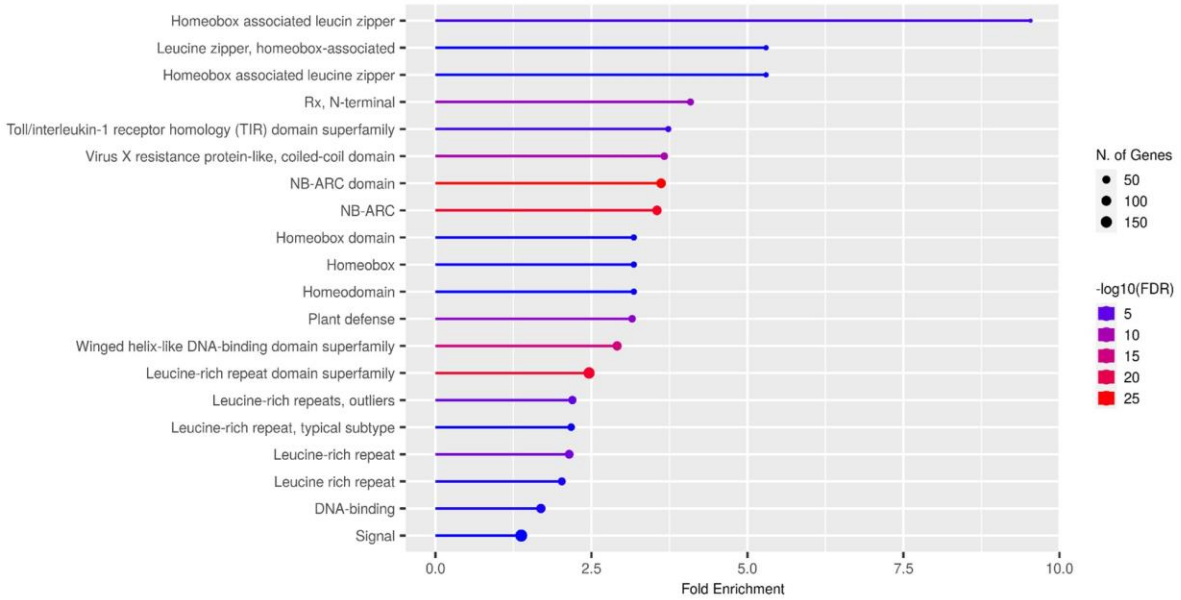

Figure 5: Enrichment analysis derived from ensembl gene ID's for significantly (FDR<0.05) up-regulated genes unique to ATL from the RNAseq expression data. The up-regulated gene pathways that are unique to ATL that are enriched are mainly involved in ATP enzymatic activity, plant pathogen defense response (virus X resistance, NB-ARC domain) the patterning of anatomical development (homeobox), and leucine-rich repeat activity that provide a structural framework in protein-protein interactions, DNA-binding, and signaling. For this analysis the FDR tells us the likelihood of enrichment by chance and is calculated from the p-value obtained from the hypergeometric test and is displayed here as the -log10(FDR), which displays greater significance with a higher value. The fold enrichment is defined here as the percentage of genes in the test set for a particular pathway, divided by its corresponding percentage in the reference/background gene set. The background gene-set for the enrichment uses the gene ID's of all expressed genes for ATL (16,575) detected in the experiment. Redundant pathways have been removed, so those sharing 95% of genes and 50% of the words in their names are represented by the pathway that is most significant.

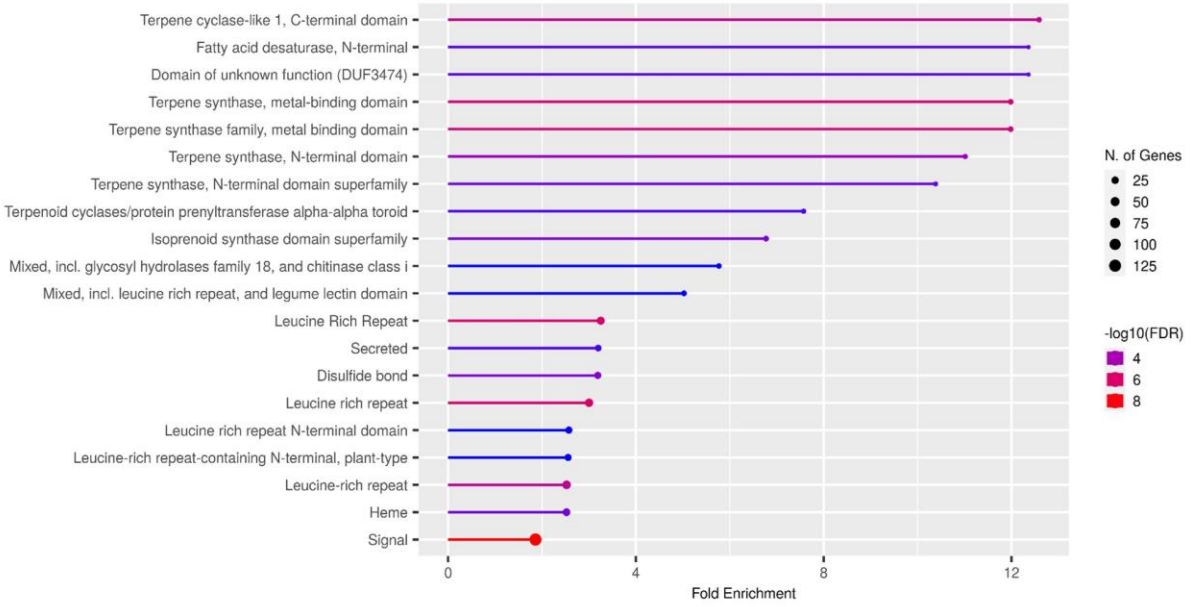

Figure 6: Enrichment analysis derived from ensembl gene ID's for significantly (FDR<0.05) down-regulated genes unique to MCD from the RNAseq expression data. The down-regulated gene pathways that are unique to MCD that are enriched are mainly involved in lipid metabolism (desaturase, isoprenoid synthase), terpene synthesis, carbohydrate metabolism, leucine-rich repeat activity, signalling, and heme involved in redox reactions and secondary metabolism. For this analysis the FDR tells us the likelihood of enrichment by chance and is calculated from the p-value obtained from the hypergeometric test and is displayed here as the -log10(FDR), which displays greater significance with a higher value. The fold enrichment is defined here as the percentage of genes in the test set for a particular pathway, divided by its corresponding percentage in the reference/background gene set. The background gene-set for the enrichment uses the gene ID's of all expressed genes for MCD (15,838) detected in the experiment. Redundant pathways have been removed, so those sharing 95% of genes and 50% of the words in their names are represented by the pathway that is most significant.

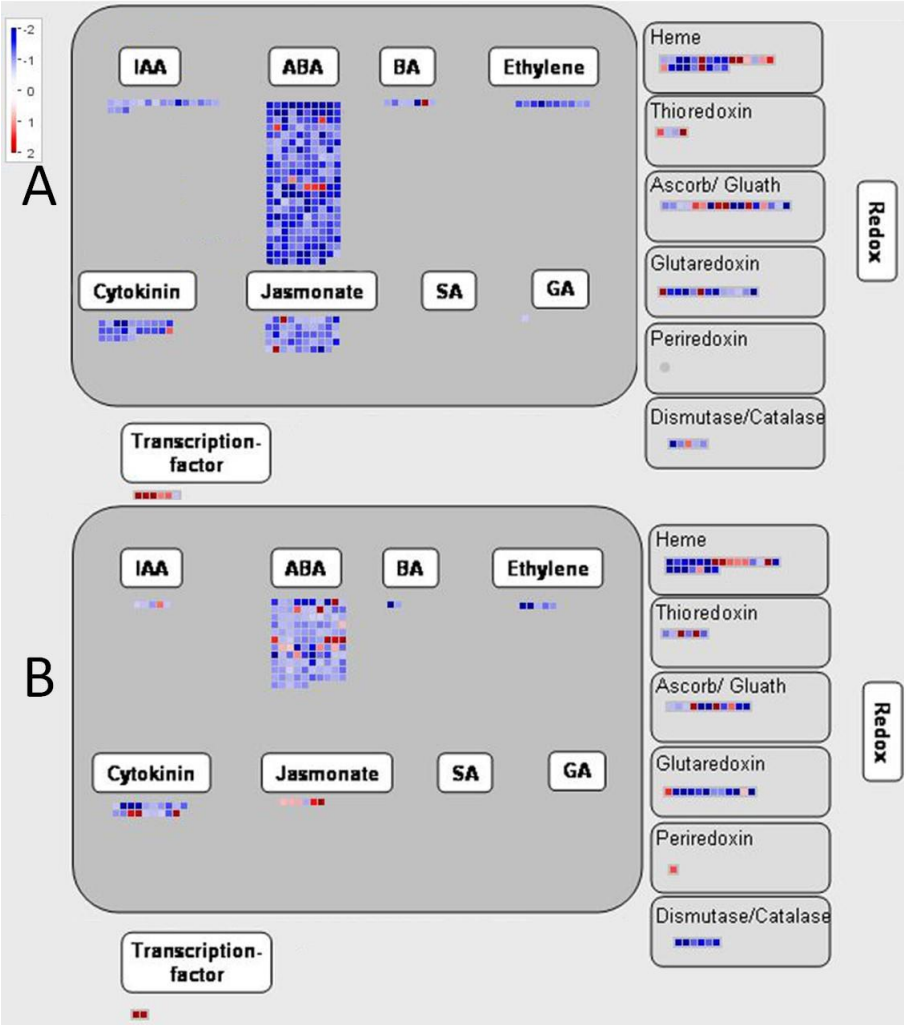

Figure 7: Mapman regulation overview for ATL (A) and MCD (B) showing a large percentage of down-regulated transcripts among all significantly regulated transcripts related to ABA, BA, ethylene, cytokinin, glutaredoxin, and dismutase. Jasmonate and IAA are similarly down-regulated in ATL, but jasmonate is more up-regulated in MCD.

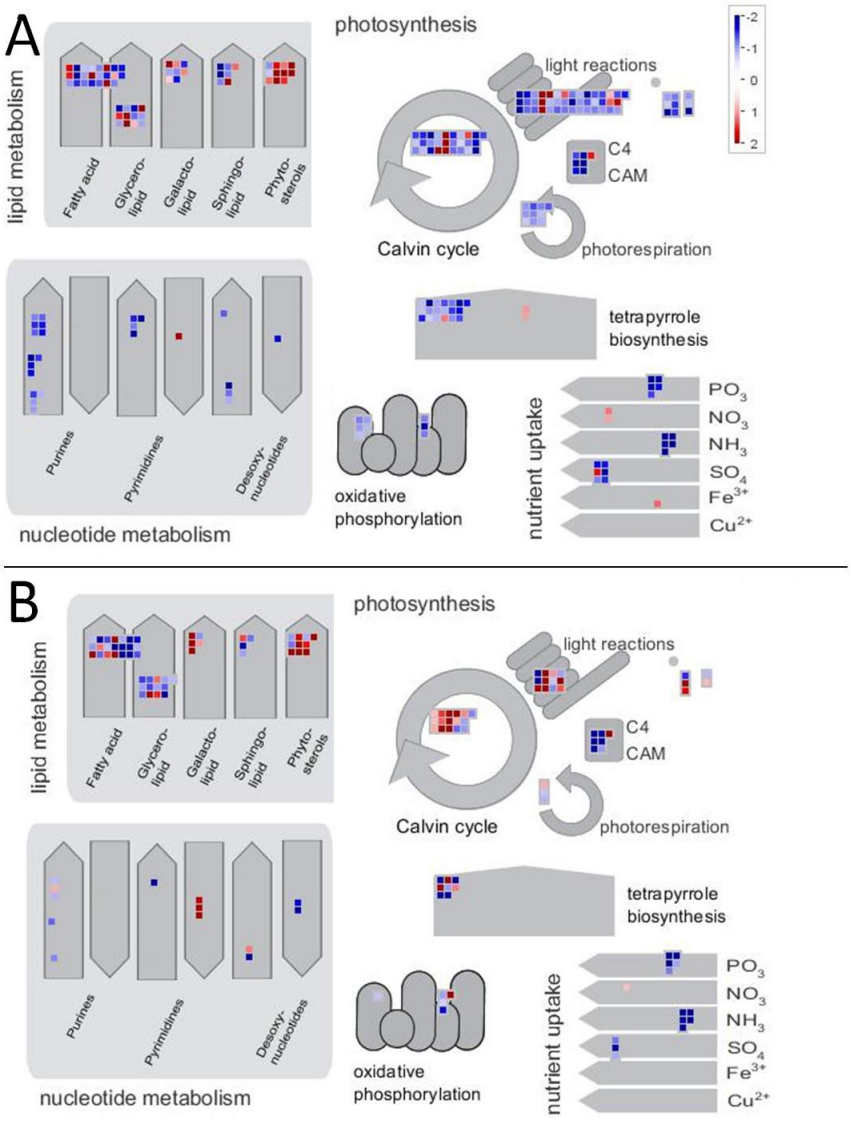

Figure 8: Mapman metabolism overview for ATL and MCD. A) ATL shows many down-regulated transcripts related to various aspects of photosynthesis, tetrapyrrole biosynthesis, and oxidative phosphorylation. Lipid metabolism shows up- and down-regulation of transcripts related to phytosterols and fatty acids, respectively. Transcripts related to PO_3^{3-}, NH_3, SO_4^{2-}, purines, pyrimidines, and deoxy-nucleotides are mostly down-regulated, while NO_3^- and Fe^{3+} are up-regulated. B) MCD shows many up-regulated transcripts related to the calvin cycle, phyto-sterols, NO_3^-, and pyrimidines and several down-regulated transcripts related to PO_3^{3-}, NH_3, SO_4^{2-}, and fatty acid metabolism.

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Chapter 4 Standardization of an *in vitro* assay to estimate relative heat sensitivity/tolerance and heat acclimation ability (HAA) of potato germplasm using excised whole leaves

Abstract

Breeding for enhanced temperature or abiotic stress tolerance in agricultural production has become an important consideration in recent decades with interest in the expansion in the range of production, concerns over climate change, and an ever-increasing world population. The screening, identification, and breeding of heat-tolerant potato genotypes in this regard would serve to increase the possible range of agricultural production of potato and expand food production to areas with higher than optimum average temperatures for this crop. To facilitate the screening and identification of potentially heat-tolerant plant materials a heat tolerance assay protocol was standardized for the quick estimation of relative heat tolerance and heat acclimation ability (HAA) in a large plant collection of *Solanum spp.* For this purpose, a heat tolerance assay was developed by an indirect assessment of leaf cell membrane structural integrity that results in ion leakage following exposure to heat stress (thermostability). In this procedure excised leaf tissue was immersed in distilled water maintained at 50°C in an oscillating water bath for 20 to 70 minutes. The conductance of the water inside the test tube was then recorded twice, once initially after being pulled from the water bath after the specified time (R_1) and again after autoclaving the sample to get the total conductance (R_2). The percent leakage (R_1/R_2)*100 was used as a sensitivity to heat stress. These leakage values were used to compare genotypes for an assessment of relative heat tolerance. These measurements were made on plants grown under control (20/15°C, day/night) conditions and following exposure to warm growing temperatures (35/25°C, day/night). The reduction in ion leakage following exposure to plants

to warm growing temperatures was used as an estimation of heat acclimation ability (HAA). Multiple plant genotypes including commercial cultivars, wild potato species, and exotic heat tolerant selections obtained from the potato gene bank were used. With this protocol we can assess the differences in heat sensitivity as well as HAA among cultivated germplasm and wild species. These results highlight three mechanisms at work that could be potentially genetically distinct (1) heat-tolerance in the unadapted state and (2) heat tolerance in the adapted state and (3) the ability to acclimate after long-term heat exposure. To our knowledge, no other reports exist comparing unadapted heat sensitivity/ tolerance, adapted heat sensitivity/tolerance, and HAA in long-term heat-adapted potato. Future studies should be directed to determine the possible relationship between the heat sensitivity measured by this *in vitro* assay and plant performance (growth and tuber yield) under heat stress conditions.

Abbreviations/ Key Words

ATL , *Solanum tuberosum* L. cv. 'Atlantic'; BUR , *Solanum tuberosum* L. cv. 'Russet Burbank'; SNW, *Solanum tuberosum* L. cv. 'Snowden'; MCD, *Solanum microdontum* Bitter; KTZ, *Solanum kurtzianum* Bitter & Wittm.; COM, *Solanum commersonii* Poir.; TUB, *Solanum tuberosum* L.; HAA, Heat Acclimation Ability; DAP, days after planting; DAHS, days after heat stress; membrane ion leakage; thermostability, heat stress

Introduction

The potato (*Solanum tuberosum* L.) has its origins in the Andean regions of South America (Hawkes, 1992) and is therefore adapted to a cooler climate (Hawkes, 1978). Upon its commercial introduction to Europe and other northern temperate regions, the potato has been adapted to provide its greatest yield under the long photoperiods and relatively cooler temperatures of these environmental regions (Levy & Veilleux, 2007). It is generally considered that a daytime temperature of around 20°C is the optimum for potato production

(Ku, et al., 1977; Wheeler, et al., 1986). In recent decades potato cultivation has been extended to areas that regularly experience higher-than-optimum temperatures, including tropical (Tai, et al., 1994; Van Der Zaag & Horton, 1983), sub-tropical (Gaur & Pandey, 2000), and semi-arid regions (Susnoschi & Shimshi, 1985). This is, in part, driven by increase in world population (Bita & Gerats, 2013).

High temperature stress is one of the most uncontrollable abiotic factors (Smith, 1968) that can have a major effect on potato plant growth and development by its negative effects on photosynthesis (Reynolds, et al., 1990), transpiration and respiration (Ku, et al., 1977; Thornton, et al., 1996), stomatal conductance (Dwelle, et al., 1981), chlorophyll content (Steffen, et al., 1995), assimilate partitioning/ source-sink relationships, tuber initiation and bulking (Reynolds & Ewing, 1989a; Van Dam, et al., 1996), and tuber quality (Levy & Veilleux, 2007). In addition,heat stress in potato has been shown to result in morphological and anatomical alterations (Kleinhenz & Palta, 2002), alteration of cellular structure and membrane function (Weis & Berry, 1988), altered protein synthesis including the production of heat shock proteins (Ahn, et al., 2004; Bray, et al., 2000), and the production of hormones and antioxidants (Aien, et al., 2011; Maestri, et al., 2002). Tolerance to high temperature stress involves many complex processes and the current understanding of the mechanisms of resistance to this stress are poor (Jha, et al., 2014; Trapero-Mozos, et al., 2018) and has resulted in little significant improvement in heat tolerance of food crops despite breeding efforts in the last several decades (Parent & Tardieu, 2012). Elucidating the mechanisms of adaptive morpho-physiological traits involved in heat tolerance and providing a response to this stress in the form of new plant varieties through selection and breeding will

involve precise phenotyping efforts to assess the genetic diversity of tolerance traits in potato germplasm (Jha, et al., 2014). Under field conditions heat stress is also frequently accompanied by water stress thus making it difficult to study the impact to elevated temperature per se. When carrying out an assay of thermotolerance it is important to consider 1) the heat stress regime utilized (duration and degree) 2) the stage of plant development when encountering heat stress and 3) the phenotype that is assayed (Yeh, et al., 2012), in addition to the genotype and inter- and intra-specific variations among germplasm (Barnabas, et al., 2008; Bita & Gerats, 2013).

Several types of thermotolerance have been identified in the model plant *Arabidopsis thaliana* L. and include basal thermotolerance (unadapted control plant) and short- and long-term acquired thermotolerance (adapted plants after growth at elevated temperatures) (Yeh, et al., 2012). Unadapted tolerance refers to the inherent ability of a plant to survive heat stress without a prior exposure to sub-lethal high temperatures and adapted tolerance refers to the ability of the plant to survive heat stress with prior exposure to a sub-lethal high temperature. This prior exposure to a sub-lethal high temperature is known to increase thermotolerance, which varies among germplasm (Yeh, et al., 2012).

Several methods have been employed to evaluate the heat tolerance of several plant species that includes measurements of gas exchange during photosynthesis (Thornton, et al., 1996), chlorophyll fluorescence measurements (Smillie & Hetherington, 1983), measurements of chlorophyll concentration (Ristic, et al., 2007), metabolomics (Raza, 2020), infrared thermography (Amani, et al., 1996; Prashar & Jones, 2014) and cell membrane thermostability (Tawfik, et al., 1996). Measurements of thermostability are carried out *in*

vitro by measuring the conductivity of the leachate of the solution surrounding stressed plant material in test tubes or water bath and is an indirect measure of the damage to plant leaf cell membranes (Ibrahim & Quick, 2001; Sullivan, 1972). For *Solanum tuberosum* the greatest differences in ion leakage (membrane thermostability) response to high temperature have been reported near 52.5°C (Nagarajan & Bansal, 1986).

Previous studies have used cut leaf discs (Agarie, et al., 1995; Blum & Ebercon, 1981; Martineau, et al., 1979; Rahman, et al., 2004; Shanahan, et al., 1990; Sullivan, 1972) to evaluate thermotolerance from leaves grown initially at optimal conditions that are subjected to a simulated heat stress without acclimation and after acclimation for a period of hours or days. Heat tolerance can be estimated in either acclimated or unacclimated conditions where leaf discs are immersed in water and maintained at a fixed elevated temperature (50°C) for various lengths of time (20, 30, 40, 50 minutes, etc.) to determine the time needed to cause 50% ion leakage (heat killing time) or exposed to various temperatures (30°C, 40°C, 50°C, etc.) at a fixed amount of time (30 minutes) to determine the temperature needed to cause 50% ion leakage (heat killing temperature) (Chen, et al., 1982). Between these two different methods of estimating heat tolerance, significant differences between heat susceptible and heat tolerant genotypes of potato (Red Pontiac and DTO-33, respectively) were found to be more discernable using heat killing time. Furthermore, by using the method of heat killing time, Chen et. al. (1982) found that a prior exposure of potato plants to 35/35°C (day/night) for 24 hours prior to assay, at an elevated temperature of 50°C, resulted in a significantly ($p<0.05$) longer heat killing time than prior exposure to 20/15°C (day/night). Interestingly, no further improvement in heat tolerance was found with prior exposure to 35/35°C (day/night)

for more than 24-48 hours. Furthermore, they found no significant difference ($p<0.05$) in heat killing time between heat susceptible and tolerant potato after prior exposure to temperatures below 30°C. However, heat killing time increased significantly more in the heat tolerant genotype than the susceptible after prior exposure to temperatures above 30°C. In the current study, a modified assay similar to the method of heat killing time used by Chen et. al. (1982) was designed to measure relative heat tolerance of whole leaves of potato germplasm before and after adaptation to prolonged (3.5 weeks) sub-lethal heat stress. A heat stress index was devised to differentiate the germplasm for response to heat stress. We provide evidence that sensitivity of unadapted leaf tissue may be genetically distinct from the sensitivity of heat-adapted leaf tissue.

Materials and Methods

General plant growth and treatment conditions

Plants were raised either from seed tubers or tissue culture plantlets and included genotypes representing various *Solanum* species such as *tuberosum*, *kurtzianum*, *commmersonii*, and *microdontum*. Plants were potted in a 5.1L pot (classic #400, Nursery Supplies, Inc., 19.05cm diameter) using a soilless media (Pro-mix BX Growing Mix Sungro or Sungro Horticulture Professional Growing Mix). Anywhere from 36 to 73 days after planting (DAP) all plants were transplanted into classic #2000 pots (Nursery Supplies, Inc.) and supported with a cylindrical 122 cm (4 ft) galvanized metal cage and, if applicable, were transferred to a controlled environment room at the same time. Plants were grown initially at either a greenhouse location or in a controlled environment room. Initially, plants were fertilized with either Peters 20-10-20 (Everris NA Inc, Dublin, OH), a modified ¼ strength Hoagland's Solution (Standard Hoagland's stock A is replaced with 1ml/L 0.25M $CaCl_2$ and

1ml/L 2.5M NH_4NO_3 plus 1ml/L each of standard stocks B and C), or a ¼ strength standard Hoagland's Solution (1ml/L each of standard stocks A, B and C). Fertilizer was given initially by hand or by a drip irrigation system twice a week to every other day and were later fertilized daily and at least seven days prior to the first leaf assay. Fertilizer was always applied in excess so that the soil was saturated and flushed of excess fertilizer salts. Light intensity averaged from ~ 250 - 400 $\mu mol \cdot m^{-2} \cdot s^{-1}$ as measured from the top of the 122 cm cage. Rooms were maintained at a 14-hour photoperiod with a relative humidity of 50% $\pm$ 30%. Temperatures were set at 20/15°C (day/night) for the control and 35/25°C (day/night) for the heat treatment, with all other factors held constant. Temperature was recorded at 19°C $\pm$ 1° for the control and 32.5°C $\pm$ 3°C for the heat treatment. RH was measured at 47% $\pm$ 3% in the control and 36% $\pm$ 13% in the heat stress. Heat stress conditions were initiated from 71 to 118 DAP. Control leaves were assayed from 56 to 95 DAP. Heat-adapted leaves were assayed from 97 to 151 DAP or 26 to 34 days after heat stress (DAHS). Specific details for each experiment can be found in the appendix for chapter 4 (Chapter 4 Appendix).

Plant material selection

A group of 7 genotypes from 3 accessions (6, 35, and 36) of *Solanum kurtzianum* Bitter & Wittm. (KTZ) were obtained as seedlings from the Sturgeon Bay potato gene bank and utilized in this experiment as this species is known to have useful traits for heat tolerance (Hawkes & Hjerting, 1969; Machida-Hirano, 2015). KTZ accession number 6 (PI 230584) was found to perform the best in drought trials (Bamberg, 2022). Several genotypes (OKA and FER) of the wild potato species *Solanum commersonii* Poir. (COM) were utilized in this experiment as it is known to have high tolerance to both biotic and abiotic stresses (Zuluaga,

2015). Three commercially cultivated varieties of *Solanum tuberosum* L. (TUB) from the United States such as Atlantic (ATL) and Russet Burbank (BUR), which are two reportedly heat sensitive cultivars (Tibbitts, et al., 1992) and Snowden (SNW) were included in this experiment. ATL is also a poor performer in ion leakage analyses (Ahn, et al., 2004) and is susceptible to several heat-related defects in the field such as internal necrosis or internal brown spot (Yencho, et al., 2008). Several exotic genotypes of *Solanum tuberosum* were also included in this experiment such as Zarewo (PI 587054) from Ukraine, Olalla (PI 665406) from Spain, DTO-2 (PI 491544) from Peru, Papa Cacho (PI 611078) from Chile, and Bora Valley (PI 634776) from South Korea to assay a random sampling of these materials for this protocol. Several genotypes (EBS525, PI 218225; EBS626, PI 218226) of *Solanum microdontum* Bitter (MCD) were used in the current study. MCD is a wild diploid species used mostly in breeding for disease resistance (Bisognin, et al., 2005; Tan, et al., 2008), selection for high calcium accumulating tubers (Bamberg, et al., 1993; Bamberg, et al., 1998), for tuber greening variation (Bamberg, et al., 2015), and breeding for reduced cold-induced sugars under long storage conditions (Bhardwaj, et al., 2011). Our previous unpublished work has observed *Solanum microdontum* (MCD15) with prolific foliage growth and flowering, retainment of flower and leaf health, and lower ion leakage in membrane thermostability analysis compared to ATL and may be a potential source for abiotic stress resistance.

Heat Bath Setup

72 large, consecutively-numbered test tubes (See Appendix Figure A1) with a 75 ml volume were filled with exactly 33 ml of double-distilled H_2O using a step dispenser and

placed directly next to one another in an oscillating heat bath (Thermo Scientific Precision SWB 15, model number TSSWB15). Initial evaluations were conducted at 45°C using a 50, 55, or 80-rpm oscillation in the water bath but later evaluations were conducted and fixed at 50°C and a 40-rpm bath oscillation. The temperature inside the sample tubes has been measured to be consistently around 48.1 °C when the bath is set to 50 °C. Another small test tube with a 35 ml volume was used as an "insulating cap" for the large test tube and was filled entirely with distilled water, capped, taped with colored tape for color coding, and placed into the 75 ml tube. The cap prevented the small tube from falling into the large tube and the volume of double-deionized H_2O in the large test tube was adjusted to 33 ml so that the small test tube just touched the water. Due to the size of the water bath and the amount of samples that can be read between time treatments a maximum of 72 samples could be used to evaluate heat stress tolerance within a single run (See Appendix Figure A1), which can accommodate two genotypes or one genotype with two treatment conditions (e.g. Calcium Treatment) per run using 6 time treatments (allowing for 6 leaf reps·genotype^{-1} · time treatment^{-1}). Alternatively, 4 genotypes or two genotypes with two treatment conditions can be assessed per run using only 3 time treatments with 6 leaf reps · genotype^{-1} · time treatment^{-1}. As the bath and tubes are being heated the leaflet samples can be collected and prepared as follows in the next section.

Leaflet Sampling

Leaflets were taken from several experiments that were used to develop a rapid assay method for the determination of (1) heat sensitivity and (2) HAA. When taking samples, similar leaflets were taken according to leaflet size, health, and location on the plant canopy.

All leaflets taken for any single assay were located on new growth (approximately the 3rd or 4th node), were fully-expanded, and exposed to the light and were from plants given similar nutrition when possible. These leaflets were then rinsed in bulk several times with distilled/ purified water and transported in a cooler that was kept at the temperature of the samples growing conditions. Planning and preparation of leaflets samples well in advance was required for efficient execution of this method. For all evaluations in this method we utilized the evaluation of samples from clonal plant replicates when possible. The number of leaflet samples per replicate was determined according to the number of replicates available per genotype so that the total number of samples per genotype per time was equal to 6, but that utilized an equal number of samples from each genotype replicate (See Appendix Table A1). For example, if only 1 plant replicate for a genotype is available (See Appendix Table A1 inset) then 6 leaflet samples per rep per time may be used and a total of 36 leaflets per replicate are needed from this single plant to make a reliable evaluation of heat tolerance when using 6 time treatments, while if 2 plant replicates per genotype are available then 3 leaflet samples per rep per time treatment may be used and a total of 18 leaflet samples per replicate are needed for a total of 36 leaflet samples per genotype when using 6 time treatments. The total number of time treatments evaluated in all experiments was 6 (20, 30, 40, 50, 60, and 70 minutes) and so 36 total samples per genotype were needed (6 time treatments x 6 leaflet samples $\cdot$ genotype^{-1} $\cdot$ time^{-1} = 36 leaflet samples $\cdot$ genotype^{-1}) that includes a particular number of clonal replicates (See Appendix Table A1). These examples allowed for evaluation of two genotypes at one time as the maximum number of samples that fit in the bath is equal to 72. However, it was found that only a subset of time treatments was

needed to determine significant differences between genotypes within a species or accession in each experiment and allowed for a similar mean separation of average leakage values just as well as all six time treatments together. The three best time treatments, which displayed the most variation between genotypes (See Appendix Table A2), were found to be the 30, 40, and 50-minute times and so these were chosen in the tables below for comparison of estimated heat sensitivities. This finding allowed an increase in assessment efficiency by 100% allowing for 4 genotypes to be assessed at one time while using 3 time treatments compared to the 2 genotypes that were possible using 6 time treatments (See Appendix Table A1).

Plots of Leakage Profiles

All plots were made using RStudio (Version 1.4.1103) and all plots display the standard error (SE) of the mean.

Loading Samples into the Heat Bath

The leaflets were then gently placed into the large test tubes using a large forceps. The size of the plants' compound leaves and leaflets varied depending on the genotype and the growing conditions experienced by the plants (e.g. control or heat stress temperatures) such that heat stress-exposed plants (e.g. 35/25°C, Day/Night) produced compound leaves and leaflets that were much smaller in size than those compound leaves and leaflets of plants grown under optimum temperature (e.g. 20/15°C, Day/Night). A single terminal leaflet or compound leaf was used as a single sample depending on the size. For example, some evaluations used one large leaflet (6-10 cm, e.g. *S. tuberosum* or control-adapted leaflets) per sample tube or if the leaflets were significantly smaller (3-6 cm, e.g. *S. commersonii* or heat-

adapted leaflets) between samples being compared then the first three to five terminal leaflets of the compound leaf were taken as one sample. Larger leaflets were placed into the tube so that the bottom surface of the leaflets faced outward, while the distal end of either leaflets or compound leaves pointed up. The goal was to fill the 33 ml volume of water in the large test tube with the leaf sample to be tested, while keeping the sample submerged. Next, the times in which the sample tubes went into the bath was recorded in a spreadsheet that helped track samples. The samples were placed into the bath 3 tubes at a time, starting with either the highest time treatment (when using 6 time treatments/ 2 genotypes) or starting with the lowest time treatment (when using 3 time treatments/ 4 genotypes). For example, if we were doing 6 samples per genotype per time treatment and 6 time treatments (20-70-minute) then 3 samples would be placed into the bath at one time starting with the higher time treatment (e.g. 70-minute). Otherwise, if we were doing 6 samples per genotype per time treatment and 3 time treatments (20-40-minute) then 3 samples would be placed into the bath at one time starting with the lower time treatment (e.g. 20-minute). The former method with 6 time treatments, starting with the highest time treatment first, made the most efficient use of time, but was not possible for evaluations using 3 time treatments due to overlapping times. The use of 6 total time treatments and 6 samples per genotype per time treatment would result in the ability to assess 2 different plant genotypes at one time (6 time treatments x 6 samples $\cdot$ genotype^{-1} $\cdot$ time treatment^{-1} x 2 genotypes = 72 total samples), while evaluating 3 total time treatments and 6 samples $\cdot$ genotype^{-1} $\cdot$ time treatment^{-1} would allow the assessment of 4 different plant genotypes at one time (3 time treatments x 6 samples $\cdot$ genotype^{-1} $\cdot$ time treatment^{-1} x 4 genotypes = 72 total samples). It was found that 6 samples (reps) per time

treatment was necessary to obtain acceptable standard errors to provide significant differences in leakage values between time treatments and allowed for the assessment of variation in heat tolerance based on leakage values between commercial cultivars (*S. tuberosum*), accessions (*S. kurtzianum*), and diverse germplasm (*S. commersonii*, *S. microdontum*).

Ion Leakage Evaluation

The sample tube groups were pulled out of the bath, the insulating tubes were removed, and the samples were shaken (G10 Gyratory® shaker, New Brunswick Scientific, Edison, NJ, USA) for one hour and then the conductivity of the sample was recorded after using a calibrated (cell constant = $0.57 \cdot cm^{-1}$) conductivity meter (Metler Toledo, SevenEasy™, Schwerzenbach, Switzerland) and conductivity probe (Metler Toledo, InLab® 731-ISM, Order No. 30014092). Leaflets typically showed consecutively increasing damage with time in the form of darkened, water-soaked areas that were clearly visible on the leaflet surface. The initial ion leakage measurements were recorded as they were pulled off the shaker after 1 hour. The samples were then autoclaved for 10-15 minutes at 121.6°C with a pressure of 1.6 kg per cm using a liquid cycle. The final readings were recorded after the samples were cooled to room temperature with the help of a cool water bath, and the percent ion leakage was then calculated and summarized in a plot using RStudio and the unadapted and adapted ion leakage profiles were calculated using all 6 time treatments.

Calculation of Heat Sensitivity of Unadapted and Adapted Leaf Tissues

The unadapted or adapted sensitivity value estimate of a genotype is calculated simply as the average of the sample ion leakage data from the 6 time treatments (20-70-minutes) or subsets of those times' (e.g. 30-40-50-minute) as follows:

Unadapted Sensitivity (% leakage) = mean(unadapted sample ion leakage value (%))

Adapted Sensitivity (% leakage) = mean(adapted sample ion leakage value (%))

Calculating Heat Acclimation Ability (HAA)

It was necessary to create a value for the response of a genotype after heat adaptation to determine a genotypes HAA. To do this the unadapted leakage profile (leakage at 20, 30, 40, 50, 60, and 70 minutes) was determined after a plant's growth under optimum conditions of 20/15 °C (day/night) for no less than 8 weeks. Similarly, the adapted leakage profile was determined after a plant's growth under moderately high temperature conditions of 35/25 °C (day/night) for no less than 3.5 weeks. Only those leaves that were fully-adapted, both physiologically and morphologically to the growth conditions, were selected to determine a genotypes leakage profile. The unadapted and adapted values were then used to calculate the HAA of a genotype that served as an index of heat adaptation or acclimation potential. A method for determining this index value from the unadapted and adapted leakage data to produce an indicator of heat acclimation capacity/adaptability (HAA) follows.

While the samples in the unadapted and adapted assays are not the same sample (but originate from the same plant), they are paired by location in the water bath for calculation of the difference used to calculate the HAA. An estimate for HAA can then be calculated

simply as the average of the difference between each of the paired leaf sample's (unadapted and adapted) percent ion leakage data from the 6 time treatments (20-70-minutes) or subsets of those time's (e.g., 30-40-50-minute) as follows:

$$\text{HAA (\% Leakage)} = \text{mean}(\text{Unadapted Leakage} - \text{Adapted Leakage})$$

This value estimate of mean differences in leakage between leaf adaptation types serves as an index of the acclimation potential of a genotype. By this definition, a HAA of zero would represent no heat adaptation potential of a genotype, while a larger HAA would indicate a larger adaptation potential of a genotype to heat stress and potentially greater heat tolerance. This method was utilized in the evaluation of several potentially heat tolerant materials to determine their HAA estimates (Tables 1-5) and offers a fast quantitative method for ordering genotypes by HAA and for determining significant differences in HAA between genotypes. The heat assay profiles for these genotypes are plotted in Figures 1-5 and the calculated HAA estimates are displayed in Tables 1-5 by experiment and species. Ordering the leakage profile plots by HAA value estimate gives a plot that aligns quite well with the visual/ qualitative analysis (plots not shown) of the magnitude of the response in reduced leakage after morphological and physiological heat acclimation/adaptation.

Heat Tolerance Ability Protocol

The final protocol for assessing ion leakage of leaves of a genotype to determine unadapted heat sensitivity, adapted heat sensitivity, and HAA follows:

1. Plant material to be tested was initially grown in a controlled-environment room or a temperature-controlled greenhouse at 20/15 °C (day/night) for 4 – 6 weeks when

plants were around 122 cm tall. At this time, whole plant leaflets/leaves were sampled and subjected to steps 2-10 that follow. These initially tested leaflets/leaves from the control (grown at 20/15 °C, day/night) are referred to as “unadapted”, meaning unadapted to heat stress. These same plants are then subjected to 35/25 °C (day/night) for a minimum of 3.5 weeks when new, “heat-adapted” leaves/leaflets have developed. These new heat-adapted leaves are subjected to the same test as their control-grown counterparts in steps 2-10 that follow. The control (unadapted) and heat-treated (adapted) assay data that are obtained are then used to provide an estimate of unadapted sensitivity (% leakage) and adapted sensitivity (% leakage), respectively, and both are used to calculate the HAA (% leakage) of a particular genotype.

2. The heat bath was turned on and filled with double-deionized water at least one hour prior to leaf collection. The water bath was set to 50°C and was set to shake at 40 rpm. 72 large 75 ml test tubes were filled with 33 ml of double-deionized water and a smaller 35 ml test tube (also filled with deionized water) was inserted into this larger tube to help keep the sample at 50°C, keep the leaf sample underwater inside the large test tube, and to keep the sample below the surface of the water bath.
3. Whole compound leaves or leaflets from replicated clones of the desired genotype were then selected from a uniform height in the plant canopy that were healthy, exposed to light and air circulation, similar in size, and preferably fully-expanded (3^{rd} or 4^{th} node). Note that sample leaves should be taken from plants that have received the same source of water/nutrition and that have been grown in the same location.

4. Leaves were cut, rinsed in distilled or purified water, placed into a labelled Ziplock bag, and transported in a cooler to the lab for assessment.
5. Leaves were then laid out by plant replicate into weigh boats.
6. Using a 25 cm forceps, the leaf/leaflet was submerged (one leaf/leaflet · $tube^{-1}$) in the 33 ml of double-deionized water in the large test tube, with the basal end of the leaf inserted first, and with the bottom of the leaf/leaflet facing outward. After three sample leaves/leaflets were prepared in this way, they were capped with the insulating tube and placed into the bath at the same time. Six samples were prepared per time treatment and 6 time treatments (20,30,40,50,60, and 70-minute) were utilized per genotype. The time the tubes were placed into the bath was recorded in a spreadsheet and the heat-treatment end time was calculated. The tubes were placed into the bath 3 at a time with a minute between each set of three tubes. For 72 tubes this would take exactly 24 minutes to get all the leaf samples into the bath, whether starting with the 20 or 70-minute time.
7. The tubes were then pulled out (3 at a time), after they had been in the bath for the required amount of time. Next, the insulating tube is removed/rinsed, and the large sample tube is placed onto a rotary shaker for 1 hour at 220 rpm to cool the samples to near room temperature and to make sure the leakage from the damaged plant leaf cells was stabilized. Sample tubes were held at an angle of 30° for the 1-hour duration to help with cooling.

8. Once they were removed from the shaker the samples were vortexed for several seconds, read with a conductivity meter until a stable value was determined, and the values were recorded.
9. When all the initial readings were completed, the samples were autoclaved for 10-15 minutes at 121.6°C with a pressure of 1.6 $kg \cdot cm^{-1}$ using a liquid cycle to prevent boil-over/sample contamination. The sample tubes were then cooled to near room temperature in a room temperature water bath for several minutes. The final readings were recorded, and the percent ion leakages were calculated.
10. Value estimates of unadapted heat sensitivity, adapted heat sensitivity, and HAA were then calculated for each genotype after obtaining leakage values of both the control (unadapted) and heat-adapted (adapted) leaves. Plots were created of the leakage profiles of control and heat-adapted leaves by genotype for a given species along with a table of the unadapted heat sensitivity, adapted heat sensitivity, and HAA value estimates.

Results

Experiment #1

A group of 7 genotypes from 3 accessions (6, 35, and 36) of *Solanum kurtzianum*, a species known to be arid-adapted (Hawkes & Hjerting, 1969) and potentially heat-tolerant, were assessed in experiment #1. This experiment calculated the unadapted sensitivity, adapted sensitivity, and HAA value estimates between seedlings of these different accessions and between seedlings within an accession (Table 1) and the data is plotted in Figure 1. The unadapted sensitivity was the least in seedling 35-3 and the most sensitive was 35-2. Regarding the adapted sensitivity, 6-1 was the most sensitive and the least sensitive was 6-3

and 36-3. Four selection classes can be described in experiment #1 based on unadapted sensitivity, adapted sensitivity, and HAA (Table 1). For example, 35-3 is categorized as class 4 and is the least sensitive to heat in the unadapted condition, shows poor tolerance compared to other genotypes in this group, and does not acclimate well after long-term heat exposure. 6-1 is categorized as class 6 as it doesn't meet the selection criteria for any of the three measured factors. 6-3 is categorized as class 8 as it only met the selection criteria for acceptable adapted heat tolerance. 6-2 and 35-3 are categorized as class 3 by meeting the selection criteria for good performance in both adapted heat tolerance and HAA.The best heat acclimator was 35-2 and the worst was 35-3 and 6-1 (Table 1). The best genotypes were 6-2 and 35-2 as they had the best response in at least two (class 3) of the categories across the unadapted sensitivity, adapted sensitivity, and HAA (see Table 6 for class designations).

Experiment #2

A group of 4 genotypes representing exotic *Solanum tuberosum* were assessed in experiment #2. This experiment similarly calculated the unadapted sensitivity, adapted sensitivity, and HAA value estimates between clones of these different species (Table 2) and the data is plotted in Figure 2. With respect to the unadapted sensitivity within *Solanum tuberosum*, Zarewo is the best with the least sensitivity and Olalla performed the worst. However, the adapted sensitivity showed that Zarewo was the poorest and most sensitive, while Olalla and Papa Cacho were the best with the least sensitivity to heat after a prolonged acclimation period. Looking at the acclimation capacity, the best acclimators were Olalla and Papa Cacho, while the worst acclimator was Zarewo (Table 2). There are three selection classes that can be described in experiment # 2 based on unadapted sensitivity, adapted

sensitivity, and HAA (Table 2). For example, Papa Cacho is categorized as class 2 as it performs well in at least two categories (unadapted and adapted heat tolerance). Olalla is categorized as class three as it does not meet the criteria for unadapted heat tolerance but performs well for adapted heat tolerance and HAA. Zarewo and DTO-2 are categorized as class 4 as they both meet the criteria for unadapted heat tolerance but not for adapted or HAA. The best genotypes were Papa Cacho and Olalla as they had the best response in at least two (class 2 and 3) of the categories across the unadapted sensitivity, adapted sensitivity, and HAA (see Table 6 for class designations).

Experiment #3

A group of 12 genotypes representing two species (*Solanum microdontum* and *Solanum commersonii*) were assessed in experiment #3. This experiment similarly calculated the unadapted sensitivity, adapted sensitivity, and HAA value estimates between these different species (Table 3) and the data is plotted in Figure 3. With respect to the unadapted sensitivity within *Solanum microdontum*, EBS525-3 is the best with the least sensitivity while the genotypes EBS626-1, EBS626-2, and EBS626-3 performed the worst. Similarly, the adapted sensitivity showed that EBS525-3 was the least sensitive, while EBS525-2, EBS626-1, EBS626-2, and EBS626-3 were the poorest with the most sensitivity to heat after a prolonged acclimation period. Looking at the acclimation capacity, the best acclimator was EBS525-3, while the worst acclimator was EBS626-2. However, there were no significant differences between these *Solanum microdontum* genotypes for HAA. With respect to the unadapted sensitivity within *Solanum commersonii*, FER-2 is the best with the least sensitivity while the genotypes OKA5059-1, OKA5040-2, and FER-1 performed the worst.

Similarly, adapted sensitivity showed that FER-2 and OKA5059-2 were the best and the least sensitive, while OKA5059-3 and OKA5059-1 were the poorest with the most sensitivity to heat after a prolonged acclimation period. Looking at the acclimation capacity, the best acclimators were OKA5059-2 and FER-1, while the worst acclimator was OKA5059-3. There are five selection classes that can be described in experiment #3 based on unadapted sensitivity, adapted sensitivity, and HAA (Table 3). EBS525-3 is categorized as class 1 as it meets all selection criteria for each of the three measured factors within the *Solanum microdontum* group. FER-2 is categorized as class 2 as it meets the selection criteria in the *Solanum commersonii* group for unadapted sensitivity and adapted sensitivity, but not for HAA. OKA5059-2 is categorized as class 3 as it does not meet the selection criteria for unadapted heat tolerance, but it meets the criteria for adapted heat tolerance and HAA. FER-1 is categorized as class 5 as it does not meet the selection criteria for unadapted or adapted heat tolerance, but it meets criteria for HAA. The last class in experiment #3, class 6, is represented by many genotypes in both *Solanum microdontum* and *Solanum commersonii* and for example is shown by OKA5059-3, which does not meet the criteria for any of the three measured factors. The best genotype for *Solanum microdontum* was EBS525-3 as it had the best response in all three (class 1) of the categories across the unadapted sensitivity, adapted sensitivity, and HAA (see Table 6 for class designations). The best genotypes *Solanum commersonii* were FER-2 andOKA5059-2 as they had the best response in at least two (class 2 and 3) of the categories across the unadapted sensitivity, adapted sensitivity, and HAA (see Table 6 for class designations).

Experiment #4

A group of 10 genotypes representing three species (*Solanum tuberosum, Solanum microdontum*, and *Solanum commersonii*) were assessed in experiment #4. This experiment similarly calculated the unadapted sensitivity, adapted sensitivity, and HAA value estimates between clones of these different species (Table 4) and the data is plotted in Figure 4.

With respect to the unadapted sensitivity within *Solanum tuberosum*, Papa Cacho is the best with the least sensitivity while the genotypes Bora Valley, Olalla, and DTO-2 performed the worst. Similarly, the adapted sensitivity showed that Papa Cacho and Olalla were the best and least sensitive, while DTO-2 and Bora Valley were the poorest with the most sensitivity to heat after a prolonged acclimation period. Looking at the acclimation capacity, the best acclimators were Olalla and DTO-2, while the worst acclimators were Papa Cacho and Bora Valley (Table 4). There are four distinct classes of selection for heat tolerant genotypes that can be described in experiment #4 based on unadapted sensitivity, adapted sensitivity, and HAA by classifying genotype as either the best or worst performing in each of these 3 observed factors (Table 4). The 2nd class, as demonstrated by Papa Cacho, is the least sensitive to heat in both the unadapted condition and adapted conditions but does not acclimate to a large extent. The 3rd class, as demonstrated by Olalla, is the most sensitive to heat in the unadapted condition but is the least sensitive in the adapted condition and is a good acclimator. The 6th class is demonstrated by Bora Valley and DTO-2, which classify as poor for unadapted and adapted sensitivity and HAA.The best genotypes were Papa Cacho and Olalla as they had the best response in at least two (class 2 and 3) of the categories across the unadapted sensitivity, adapted sensitivity, and HAA (see Table 6 for class designations).

Experiment #5

A group of 3 popular cultivated varieties of *Solanum tuberosum* were assessed in experiment #5. This experiment calculated the unadapted sensitivity, adapted sensitivity, and HAA value estimates between cultivars (Table 5) and the data is plotted in Figure 5. The unadapted sensitivity was the least in BUR and the most sensitive was ATL (Table 5). Regarding the adapted sensitivity, BUR was the least sensitive and the most sensitive was SNW. The best heat acclimator was ATL and the worst were BUR and SNW. Three classes of selection for heat tolerant genotypes can be described (i.e. 2, 5, and 6) in experiment #5 based on unadapted sensitivity, adapted sensitivity, and HAA by classifying genotype as either the best or worst performing in each of these 3 observed factors (Table 5). BUR can be described as class 2, which has the best score for unadapted and adapted heat sensitivity but a lower acclimation ability than the highest performer. ATL can be described as class 5, which has the worst score for unadapted and adapted heat sensitivity but a higher acclimation ability than the lowest performer. SNW can be described as class 6 as it does not meet the selection criteria for any of the three measured factors compared to the best performers in each of the three respective categories. The best genotype was BUR as it had the best response in at least two (class 3) of the categories across the unadapted sensitivity, adapted sensitivity, and HAA (see Table 6 for class designations).

Experiment #2 and #4 Reproducibility

With respect to the reproducibility of reponses between experiments for *Solanum microdontum*, the unadapted sensitivity for MCD24 and MCD35 in experiment # 2 and #4 showed that MCD24 is the best for all three categories (Class 1) of unadapted sensitivity, adapted sensitivity, and HAA in both experiments, while MCD35 performed below MCD24

in these three areas (Class 6) in both experiments (Table A4). With respect to the reproducibility of responses between experiments #2 and #4 for exotic *Solanum tuberosum*, the results for Papa Cacho are consistently lower than Olalla for all three categories and both ranked as class 2 and 3, respectively, in each experiment (Tables 2 and 4).

Discussion and Conclusions

In these experiments we can group genotypes into 8 possible classes based on unadapted sensitivity, adapted sensitivity, and HAA by classifying them as either the "best" or "worst" performing in each of these 3 observed factors and the 8 possible classes are described in Table 6. For the unadapted and adapted heat tolerance the "best" selections were based on the lower quarter of the range of ion leakage values within a species of an experiment, while the "best" selections for HAA were based on the upper quarter of the range of ion leakage values within a species of an experiment. The best classification is class 1 as it denotes a genotype that performs well in all three measured categories. Class 2 and 3 are the 2nd best classification as they denote genotypes that perform well in at least two categories. Classes 4, 5, 7, and 8 are the 3rd best classification as they denote genotypes that perform well in at least one category. Class 6 denotes genotypes that perform the worst in all 3 categories.

These results highlight mechanisms at work that could be potentially genetically distinct (1) heat-tolerance in the unadapted state and (2) heat tolerance in the adapted state that gives the ability to acclimate after long-term heat exposure. To our knowledge, no other reports exist comparing unadapted heat sensitivity/ tolerance, adapted heat sensitivity/tolerance, and HAA in long-term heat-adapted potato. However, a study by Chen

et. al. (1982) implicitly shows a difference in HAA between a heat sensitive (Red Pontiac) and a heat-tolerant (DTO-33) potato variety after a short-term period of acclimation (24 hr.) and explicitly shows a difference in heat sensitivity/ tolerance, measured as heat killing time at 50°C, after a 24 hr. acclimation period. However, this same report does not describe the genetic mechanisms between heat tolerance in the non-acclimated versus the acclimated state and was unable to show a difference in heat sensitivity/ tolerance for these two varieties under a non-acclimated condition. Additionally, while there are not a lot of data describing HAA in potato there are a lot of data available for cold acclimation ability and non-acclimated freezing tolerance. A study by Stone et. al. (1993) showed two distinct mechanisms of genetic control between non-acclimated freezing tolerance and the ability to cold acclimate using an F1 and backcross populations between two species of wild diploid potato, *Solanum commersonii* and *Solanum cardiophyllum*, that represented the extremes of these two factors where *Solanum cardiophyllum* is unable to cold acclimate but *Solanum commersonii* is able to cold acclimate from -4.5°C to -9.6°C. Similar to the current study, this study utilized the ion leakage method to determine the freezing tolerance of these populations under both non-acclimated and acclimated conditions in order to determine the acclimation ability. There was no correlation between these non-acclimated and acclimated freezing tolerance measurements suggesting separate genetic control. In addition, relatively few genes were suggested to control these traits due to the parental phenotypes of both characteristics being retrieved in the test populations. Finally, the means of the population distributions for both the non-acclimated and acclimated freezing tolerance laid closest to the sensitive parent *Solanum cardiophyllum* suggesting that the genes controlling these traits may be recessive,

and the utilization of a generational means analysis on the test populations lent evidence for an additive-dominance control of the underlying genes influencing these traits (Stone, et al., 1993).

Future work studying the control of non-acclimated heat tolerance, acclimated heat tolerance, and the resulting HAA could utilize the methodology in the current study in determining unadapted (non-acclimated) and adapted (acclimated) heat tolerance in collections of S*olanum* species and follow the work of Stone et. al (1993) in deriving F_1 and backcross populations between extreme genotypes to determine the underlying genetic control of these traits to aid in breeding for future heat tolerant potato varieties. Additionally, it is important to note that future studies need to establish that the results of this assay correspond to the performance of a genotype in terms of plant growth and tuber yield under heat stress.

The procedure in the current study was able to (1) provide sufficient variation in average unadapted and adapted heat sensitivity/tolerance value estimates to determine significant differences in all 5 experiments between seedlings of different accessions and between seedlings within an accession of *Solanum kurtzianum* (experiment #1), between genotypes within a species of exotic *Solanum tuberosum* and between genotypes within a species of wild *Solanum commersonii* and *Solanum microdontum* (experiments #2-4), and between cultivated varieties of *Solanum tuberosum* (experiment #5). (2) The procedure used in the current study was also able to determine significant differences between genotypes based on their HAA value estimates that was generated after comparing the average difference between 30, 40, and 50-minute leakage assay data of unadapted and adapted

leaves at 50°C after growth and adaptation of the same plants to two temperature conditions. (3) The current study was able to provide evidence for two potentially genetically distinct mechanisms for unadapted heat tolerance (non-acclimated) and adapted heat tolerance (acclimated). These findings may be of use in selecting for heat tolerant potato varieties by identifying genotypes that demonstrate high heat tolerance in both the unadapted and adapted conditions and good HAA. This methodology could also be used to select genotypes for the creation of F1 and backcross test populations to characterize the genetic control of the studied traits more clearly.

Figures

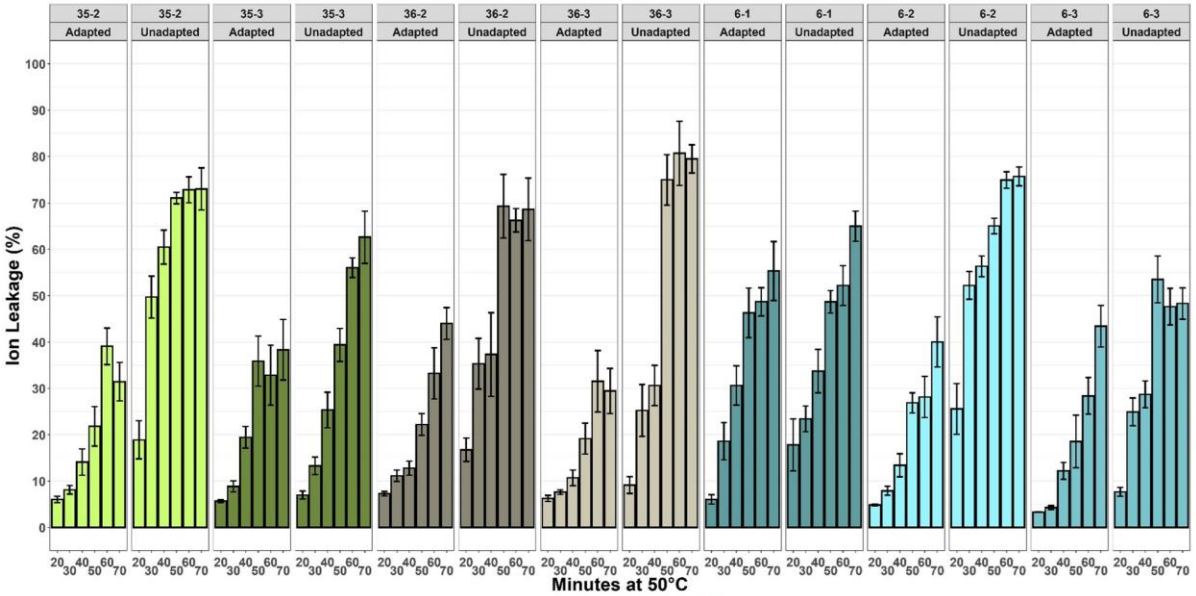

Figure 1: Ion leakage from leaf tissue of accessions (6, 35, and 36) of *Solanum kurtzianum* Bitter and Wittm. from experiment #1 following exposure to 50°C for various time periods. The control (unadapted) measurements were made on fully-expanded, newly developed leaves after 11.5 weeks of control temperatures (20/15°C, day/night). The heat-treated (adapted) measurements were made on fully-expanded, newly developed leaves from the same plants after 3.5 weeks of heat stress (35/25°C, day/night). Values are means (± SE), n = 6.

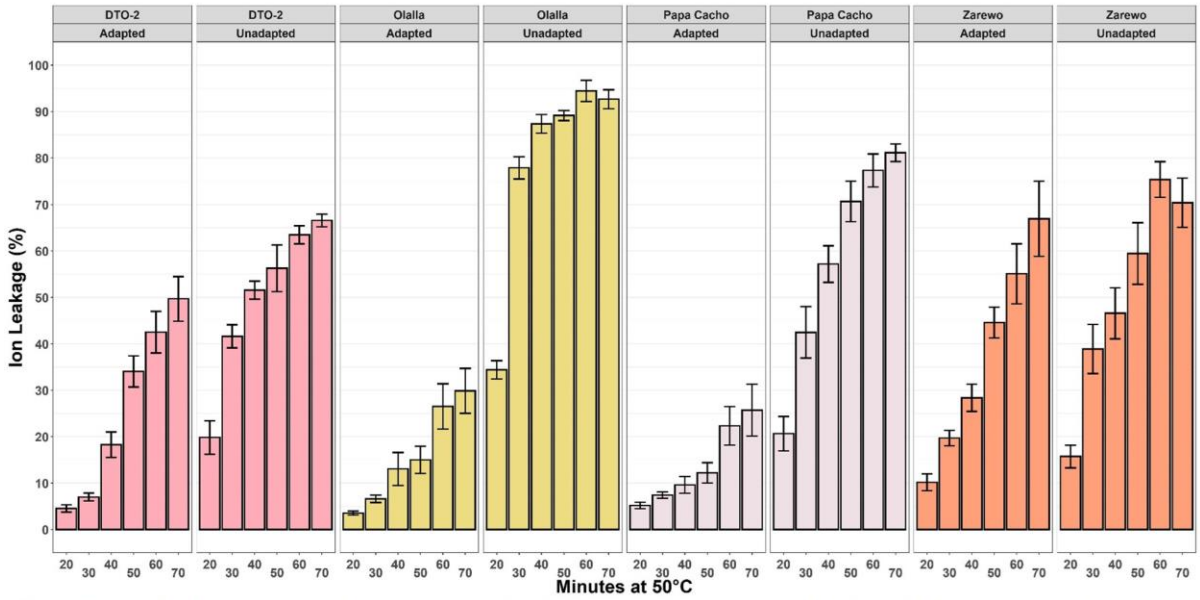

Figure 2: Ion leakage from leaf tissue of exotic *Solanum tuberosum* L. (DTO-2, Olalla, Papa Cacho, and Zarewo) from experiment #2 following exposure to 50°C for various time periods. The control (unadapted) measurements were made on fully-expanded, newly developed leaves after 8 weeks of control temperatures (20/15°C, day/night). The heat-treated (adapted) measurements were made on fully-expanded, newly developed leaves from the same plants after 3.5 weeks of heat stress (35/25°C, day/night). Values are means (+ SE), n = 6.

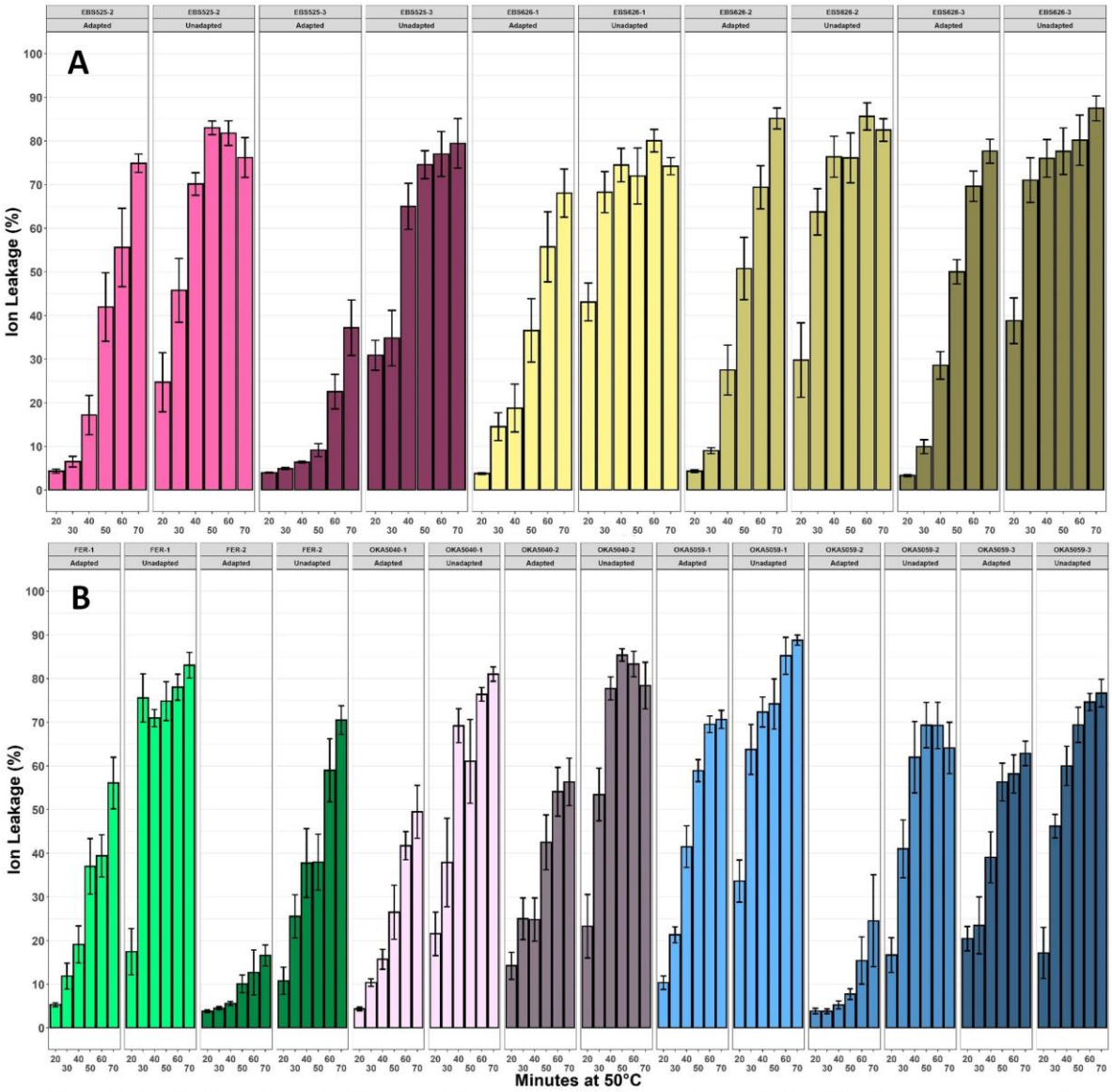

Figure 3: Ion leakage from leaf tissue of *Solanum microdontum* Bitter **(A)** and *Solanum commersonii* Poir. **(B)** from experiment #3 following exposure to 50°C for various time periods. The control (unadapted) measurements were made on fully-expanded, newly developed leaves after 11.5 weeks of control temperatures (20/15°C, day/night). The heat-treated (adapted) measurements were made on fully-expanded, newly developed leaves from the same plants after 5 weeks of heat stress (35/25°C, day/night). Values are means (± SE), n = 6.

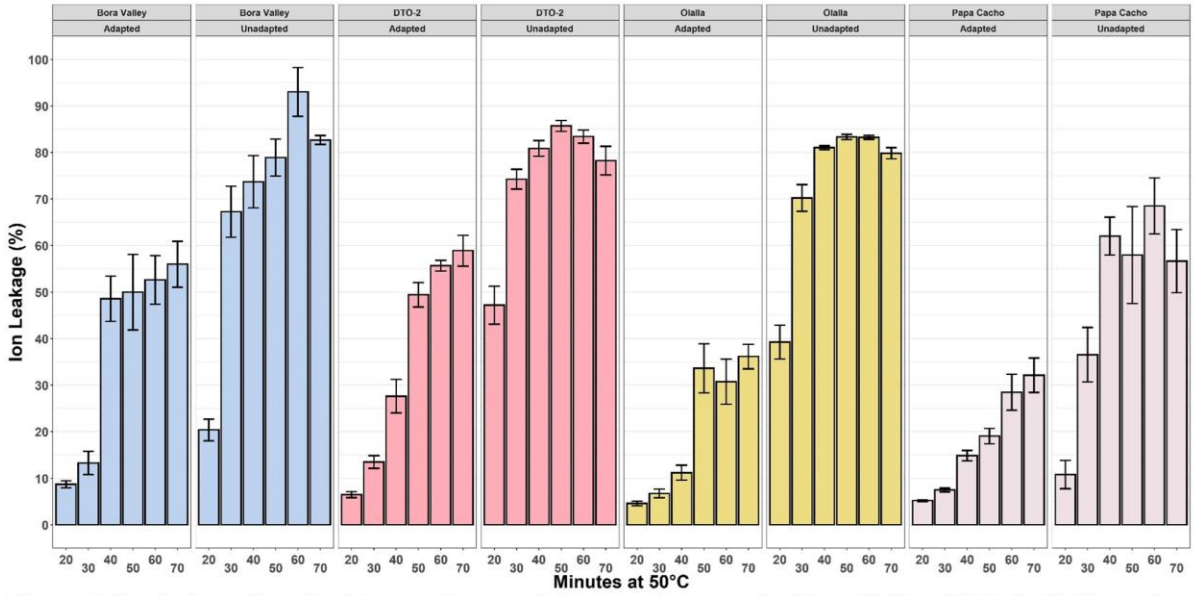

Figure 4: Ion leakage from leaf tissue of exotic *Solanum tuberosum L.* (Bora Valley, DTO-2, Olalla, and Papa Cacho) from experiment #4 following exposure to 50°C for various time periods. The control (unadapted) measurements were made on fully-expanded, newly developed leaves after 13.5 weeks of control temperatures (20/15°C, day/night). The heat-treated (adapted) measurements were made on fully-expanded, newly developed leaves from the same plants after 4.5 weeks of heat stress (35/25°C, day/night). Values are means (+ SE), n = 6.

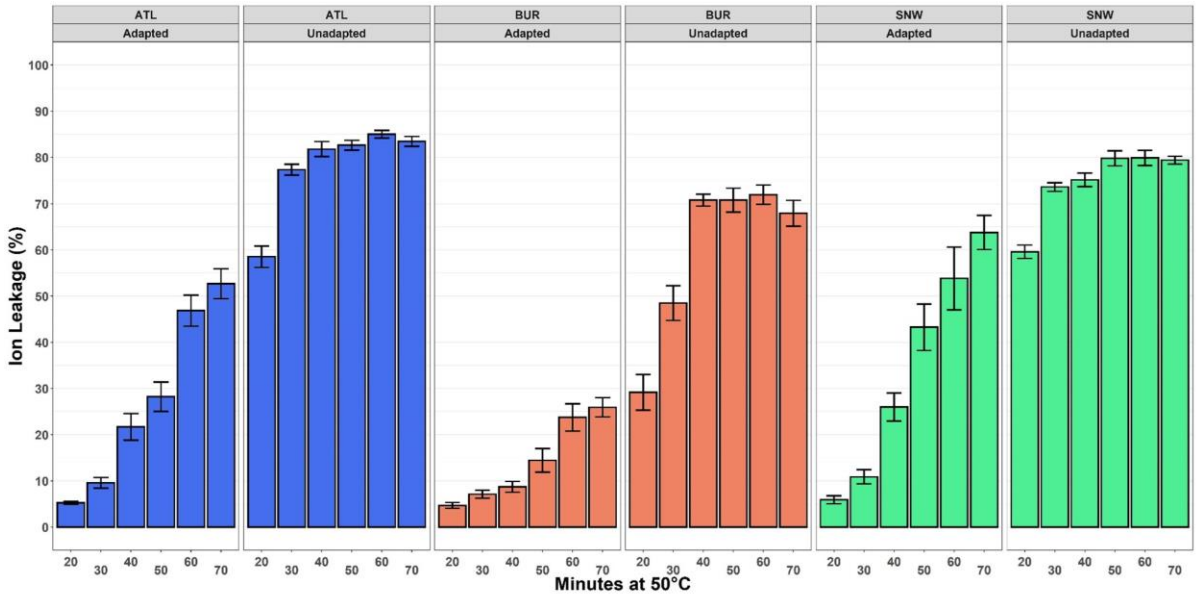

Figure 5: Ion leakage from leaf tissue of clones of three cultivars (Atlantic, ATL; Russet Burbank, BUR; Snowden, SNW) of *Solanum tuberosum L.* from experiment #5 following exposure to 50°C for various time periods. The control (unadapted) measurements were made on fully-expanded, newly developed leaves after 12.5 weeks of control temperatures (20/15°C, day/night). The heat-treated (adapted) measurements were made on fully-expanded, newly developed leaves from the same plants after 4.5 weeks of heat stress (35/25°C, day/night). Values are means (+ SE), n = 12.

Tables

Table 1: Summary of calculated heat sensitivity of various accessions of unadapted and adapted leaves of Solanum kurtzianum. For these calculations data given in Figure 1 was used to calculate heat sensitivity, which is defined here as the average of ion leakage for 30-40-50-minute time treatments. [a]

Genotype	Species	Unadapted Sensitivity (% leakage)	Adapted Sensitivity (% leakage)	HAA (% leakage)[b]	Class[UAH]
35-3	*S. kurtzianum*	26 d	21 b	5 c	4
6-1	*S. kurtzianum*	35 cd	32 a	3 c	6
6-3	*S. kurtzianum*	36 cd	12 c	24 b	8
36-3	*S. kurtzianum*	44 c	12 c	31 b	8
36-2	*S. kurtzianum*	47 bc	15 bc	32 b	8
6-2	*S. kurtzianum*	58 ab	16 bc	42 a	3
35-2	*S. kurtzianum*	60 a	15 bc	46 a	3

[a] Significant differences determined by Duncan's Multiple Range Test within a column. Means with similar letters are not significantly different. [b] Heat Acclimation Ability, HAA = mean(Unadapted Leakage - Adapted Leakage), is calculated from the average of the 30, 40, and 50-minute time leakage data. At each time-point 6 separate measurements were made. Values are means, n = 18. [UAH] Class designation as described in Table 6.

Table 2: Summary of calculated heat sensitivity of unadapted and adapted leaves of various clones of exotic selections of *Solanum tuberosum* . For these calculations data given in Figure 2 was used to calculate heat sensitivity, which is defined here as the average of ion leakage for 30-40-50-minute time treatments. [a]

Genotype	Species	Unadapted Sensitivity (% leakage)	Adapted Sensitivity (% leakage)	HAA (% leakage)[b]	Class[UAH]
Zarewo	*S. tuberosum*	48 b	31 a	17 d	4
DTO-2	*S. tuberosum*	50 b	20 b	30 c	4
Papa Cacho	*S. tuberosum*	57 b	10 c	47 b	2
Olalla	*S. tuberosum*	85 a	12 c	73 a	3

[a] Significant differences determined by Duncan's Multiple Range Test within a column. Means with similar letters are not significantly different. [b] Heat Acclimation Ability, HAA = mean(Unadapted Leakage - Adapted Leakage), is calculated from the average of the 30, 40, and 50-minute time leakage data. At each time-point 6 separate measurements were made. Values are means, n = 18. [UAH] Class designation as described in Table 6.

Table 3: Summary of calculated heat sensitivity of unadapted and adapted leaves of various clones of *Solanum microdontum* and *Solanum commersonii* . For these calculations data given in Figure 3 was used to calculate heat sensitivity, which is defined here as the average of ion leakage for 30-40-50-minute time treatments. [a]

Genotype	Species	Unadapted Sensitivity (% leakage)	Adapted Sensitivity (% leakage)	HAA (% leakage)[b]	Class[UAH]
EBS525-3	*S. microdontum*	58 b	7 b	51 a	1
EBS525-2	*S. microdontum*	66 ab	22 a	44 a	6
EBS626-1	*S. microdontum*	72 a	23 a	48 a	6
EBS626-2	*S. microdontum*	72 a	29 a	43 a	6
EBS626-3	*S. microdontum*	75 a	29 a	45 a	6
FER-2	*S. commersonii*	34 c	7 d	27 cd	2
OKA5040-1	*S. commersonii*	56 b	18 c	39 abc	6
OKA5059-2	*S. commersonii*	57 b	6 d	52 a	3
OKA5059-3	*S. commersonii*	59 b	40 a	19 d	6
OKA5059-1	*S. commersonii*	70 a	41 a	30 bcd	6
OKA5040-2	*S. commersonii*	72 a	31 ab	41 ab	6
FER-1	*S. commersonii*	74 a	23 bc	51 a	5

[a] Significant differences determined by Duncan's Multiple Range Test within a species and column. Means with similar letters are not significantly different. [b] Heat Acclimation Ability, HAA = mean(Unadapted Leakage - Adapted Leakage), is calculated from the average of the 30, 40, and 50-minute time leakage data. At each time-point 6 separate measurements were made. Values are means, n = 18. [UAH] Class designation as described in Table 6.

Table 4: Summary of calculated heat sensitivity of unadapted and adapted leaves of various clones of exotic selections of *Solanum tuberosum* . For these calculations data given in Figure 4 was used to calculate heat sensitivity, which is defined here as the average of ion leakage for 30-40-50-minute time treatments.[a]

Genotype	Species	Unadapted Sensitivity (% leakage)	Adapted Sensitivity (% leakage)	HAA (% leakage)[b]	Class[UAH]
Papa Cacho	*S. tuberosum*	52 b	14 b	38 b	2
Bora Valley	*S. tuberosum*	73 a	37 a	36 b	6
Olalla	*S. tuberosum*	78 a	17 b	61 a	3
DTO-2	*S. tuberosum*	80 a	30 a	50 a	6

[a] Significant differences determined by Duncan's Multiple Range Test within a column. Means with similar letters are not significantly different. [b] Heat Acclimation Ability, HAA = mean(Unadapted Leakage - Adapted Leakage), is calculated from the average of the 30, 40, and 50-minute time leakage data. At each time-point 6 separate measurements were made. Values are means, n = 18. [UAH] Class designation as described in Table 6.

Table 5: Summary of calculated heat sensitivity of various cultivars of unadapted and adapted leaves of *Solanum tuberosum* . For these calculations data given in Figure 5 was used to calculate heat sensitivity, which is defined here as the average of ion leakage for 30-40-50-minute time treatments. [a]

Genotype	Species	Unadapted Sensitivity (% leakage)	Adapted Sensitivity (% leakage)	HAA (% leakage)[b]	Class[UAH]
BUR	*S. tuberosum*	63.3 c	10.1 c	53.3 b	2
SNW	*S. tuberosum*	76.2 b	26.7 a	49.5 b	6
ATL	*S. tuberosum*	80.6 a	19.8 b	60.8 a	5

[a] Significant differences determined by Duncan's Multiple Range Test within a column. Means with similar letters are not significantly different. [b] Heat Acclimation Ability, HAA = mean(Unadapted Leakage - Adapted Leakage), is calculated from the average of the 30, 40, and 50-minute time leakage data. At each time-point 12 separate measurements were made. Values are means, n = 36. [UAH] Class designation as described in Table 6.

Table 6: The 8 possible classes of selection for heat tolerant genotypes in this study based on unadapted sensitivity, adapted sensitivity, and heat acclimation ability (HAA).[a]

Unadapted Sensitivity	Adapted Sensitivity	HAA	Class
BEST	BEST	BEST	1
BEST	BEST	WORST	2
WORST	BEST	BEST	3
BEST	WORST	WORST	4
WORST	WORST	BEST	5
WORST	WORST	WORST	6
BEST	WORST	BEST	7
WORST	BEST	WORST	8

[a] For the unadapted and adapted sensitivity "BEST" refers to those genotypes with sensitivity value estimates less than the lower quarter of the range of observed values, while for HAA "BEST" refers to those genotypes with an acclimation ability greater than the upper quarter of the range of observed values.

Chapter 5 General Discussion and Conclusions

Chapter 2 Discussion

In general, as expected photosynthetic rates declined significantly under heat stress. However, this was true for leaves developed under non-stress (unadapted) conditions (Fig. 1). These results agree with previous reports. For example, a study evaluating three potato cultivars grown at 31/20°C or 41/20°C (day/night) in a greenhouse found that photosynthesis rate decreased significantly (Wolf, et al., 1990a). In this study, leaves developed under control condition were measured for Ps under heat stress. However, Ps of heat adapted leaves (truly developed under heat stress) has not been studied in combination to either control or unadapted leaves under heat stress. Ours is the first study comparing heat adapted and unadapted leaves under heat stress (Fig. 2). These observations clearly demonstrate a long-term adaptation to heat stress and increase in heat tolerance of heat-adapted leaves.

Our results on SC and leaf temperatures shed light on mechanisms that may be involved in the maintenance of higher photosynthetic rates in adapted leaves as compared to unadapted leaves under heat stress. We found higher SC and lower leaf temperatures in the adapted leaves as compared to unadapted leaves (Figs. 2 and 4, Table 2). Greater stomatal conductance has been associated with reduced leaf temperatures via evaporative cooling (Lu, et al., 1994). For example, Radin et. al. (1994) found a negative relationship between SC and leaf temperature. We found similar results for the negative relationship between leaf temperature and stomatal conductance (Figs. 9 and 10). Furthermore, under heat stress, higher leaf temperatures have been correlated with reduced photosynthesis. For example, Ku et. al (1977) showed a negative linear relationship between Ps and leaf temperatures above

30°C in potato plants subjected to heat stress. Studies have shown thermal damage to the photosystem at elevated temperatures in potato and other crops (Isoda & Isoda, 2005; Ku, et al., 1977; Lu, et al., 1994). Thus, reduced leaf temperatures would allow plants to maintain higher Ps through reduced thermal damage. We found a dramatic drop in Ps when leaf temperature increased from 32-34°C (Figs. 7 and 8). Taken together, our results suggest that adapted leaves can maintain higher photosynthesis under heat stress by lowering leaf temperature through higher stomatal conductance.

Chapter 2 Conclusions

- We found a greater rate of both photosynthesis and stomatal conductance under heat stress in heat-adapted leaves as compared to the unadapted control leaves on the same plant.
- We found a significant decrease in leaf temperature in heat-adapted leaves compared to heat-unadapted control leaves of the same plant.
- Overall, both cultivars can adapt to heat stress by producing new leaves (adapted) under long-term (weeks) heat stress that can reduce leaf temperature by increased evaporative cooling (greater transpiration/stomatal conductance) thereby maintaining higher rates of photosynthesis compared to control (unadapted) leaves.

Chapter 3 Discussion

To obtain a deeper understanding of the mechanisms involved in long-term, moderate heat stress adaptation and tolerance we investigated the genetic aspect of heat tolerance by RNAseq analysis to try and uncover genes or functional pathways related to these adaptations. Most studies investigating the effects of heat stress on potato and most other

plants species make observations after several hours (Ahn, et al., 2004; Liu, et al., 2021; Rensink, et al., 2005) or days (Liu, et al., 2021; Tang, et al., 2020) of heat stress without acclimation. These studies in potato can be considered to investigate BT. However, this is the first study to investigate the transcriptional regulation and identifying the gene that results in the adaptation and tolerance to a prolonged heat stress (4.5 weeks) that results in morphological, anatomical, and physiological adaptation in two contrast genotypes.

Chapter 3 Conclusions

- Our RNAseq analysis and subsequent down-stream analyses highlighted the key involvement of heat shock proteins, oxidative stress response, lipid metabolism, hormone signaling, and photosynthesis in the long-term heat tolerance of both ATL and MCD. The data presented here may also contribute to understanding the effect of seasonal variations and high temperature effects and genes involved in adaptation in potato and other related plant systems to breed tolerant genotypes.

Chapter 4 Discussion

These results highlight mechanisms at work that could be potentially genetically distinct (1) heat-tolerance in the unadapted state and (2) heat tolerance in the adapted state that gives the ability to acclimate after long-term heat exposure. To our knowledge, no other reports exist comparing unadapted heat sensitivity/ tolerance, adapted heat sensitivity/tolerance, and HAA in long-term heat-adapted potato. However, a study by Chen et. al. (1982) implicitly shows a difference in HAA between a heat sensitive (Red Pontiac) and a heat-tolerant (DTO-33) potato variety after a short-term period of acclimation (24 hr.) and explicitly shows a difference in heat sensitivity/ tolerance, measured as heat killing time

at 50°C, after a 24 hr. acclimation period. However, this same report does not describe the genetic mechanisms between heat tolerance in the non-acclimated versus the acclimated state and was unable to show a difference in heat sensitivity/ tolerance for these two varieties under a non-acclimated condition.

Compared to many other procedures describing similar methodology as the current work (Chen, et al., 1982; Nagarajan & Bansal, 1986; Rahman, et al., 2004), our study provides a simpler and more efficient method that can provide significant differences between diverse germplasm under both non-acclimated and heat-adapted conditions.

Chapter 4 Conclusions

- We provided sufficient variation in average unadapted and adapted heat sensitivity/tolerance value estimates to determine significant differences in all 5 experiments between seedlings of different accessions and between seedlings within an accession of *Solanum kurtzianum* (experiment #1), between genotypes within a species of exotic *Solanum tuberosum* and between genotypes within a species of wild *Solanum commersonii* and *Solanum microdontum* (experiments #2-4), and between cultivated varieties of *Solanum tuberosum* (experiment #5).
- The procedure used in the current study was also able to determine significant differences between genotypes based on their HAA value estimates that was generated after comparing the average difference between 30, 40, and 50-minute leakage assay data of unadapted and adapted leaves at 50°C after growth and adaptation of the same plants to two temperature conditions.

- The current study was able to provide evidence for two potentially genetically distinct mechanisms for unadapted heat tolerance (non-acclimated) and adapted heat tolerance (acclimated).

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Appendix

Appendix Chapter 1 - Genetic analysis of tuber calcium concentration and pitted scab traits in a segregating bi-parental ('Atlantic' x 'Superior') F1 tetraploid population of potato (*S. tuberosum*)

Abstract

In the United States growers routinely apply soluble calcium during the tuber bulking period in the production of potatoes, which is known to have a positive effect on tuber quality. Atlantic is a popular potato cultivar desired by the chip industry for high yields and high specific gravity. However, this cultivar suffers from poor internal quality, high susceptibility to pitted scab, and is known to have low tuber calcium concentration (TC). In contrast, the cultivar Superior is known to have high TC, good internal tuber quality, and resistance to pitted scab, but has lower yields and specific gravity. Recent studies have provided evidence that in the absence of seasonal applied calcium TC has genetic control. However, little is known on the genetic control of TC uptake for the seasonally added calcium. The purpose of this study was to: (1) determine the effect of soluble in-season calcium fertilization during tuber bulking on the TC of the parents and F1 population (2) determine if genetic variation exists (H^2) in this population for TC traits using the control and calcium-treated data (3) determine the extent of genotype x environment (year) interactions on the measured TC traits in the F1 population (4) determine whether separate genetic control between TC treatments exist (5) determine the effect of in-season calcium application and (6) estimate the broad-sense heritability for the tuber quality traits hollow heart, pitted scab, and internal brown spot. A segregating bi-parental F1 population of potato derived from the commercially important cultivars *Solanum tuberosum* L. cvs. Atlantic (ATL) and Superior (SUP), was evaluated for TC concentration under control and applied calcium

conditions, over the 3 years 2011, 2013, and 2014 at the UW-Madison Hancock Agricultural Research Station using standard agronomic practices for this area. A total of 47 to 77 F1 genotypes were evaluated in the field each year with 3 replicates per F1 genotype, two treatment groups and 8 hills per plot in a completely randomized design (CRD). Parents were evaluated alongside the progeny and were analyzed similarly. Calcium treatments consisted of 3 separate applications of a soluble calcium product during the tuber bulking period given in two-week increments. Different calcium sources were used in different years. $CaCl_2$ was used in 2011(224 $kg \cdot ha^{-1}$ Ca), while $CaSO_4$ and $Ca(NO_3)_2$ were used in combination in 2013 (306 $kg \cdot ha^{-1}$ Ca), and only $CaNO_3$ (224 $kg \cdot ha^{-1}$ Ca) was used in 2014. All control plants did not receive any seasonal calcium but received the same amount of N as that received by the calcium treated plots using NH_4NO_3. The application of soluble in-season applied calcium, during the bulking period, significantly increased the TC concentration in the tuber and significantly reduced the occurrence of hollow heart, pitted scab, and internal brown spot. The mixed model analysis of variance for the control TC and calcium-treated TC showed high genetic variation (458 and 923.1, respectively) and heritability (0.75 and 0.84, respectively) in the F1 population. The phenotypic and genotypic correlations for control TC vs. calcium treated TC were 0.825 and 0.846, respectively. We conclude that the application of soluble in-season applied calcium, during the bulking period, can significantly increase the TC concentration in the tuber and significantly reduce the occurrence of pitted scab, hollow heart, and internal brown spot. The genetic variation, presented as heritability estimates, for both the control and calcium-treated plots in this study were moderately-high (H^2=0.75) to high (H^2=0.84), respectively, over the three years of this study. We found moderately-low,

moderately-high, and high genetic variation for hollow heart (0.79), pitted scab (0.70), and internal brown spot (0.39), respectively, suggesting that improvement of these traits within this population is likely to respond to selection. We further conclude, as supported by our mixed model analysis, that control TC and calcium-treated TC share similar genetic components and are not under independent control. While our data shows high genetic variation for these two traits the response to added calcium (ΔTC, the difference in TC between the calcium-treated and control plots) did not show high genetic variation, which may be due to confounding effects from the variation in the field soil Ca. A repeat of the current study under a more controlled environment would be a logical next step to determine the true genetic variation in ΔTC in this population and help to understand the genetic control of calcium in this population so that it can be practically applied in a farm setting. Improving the capacity of tubers to increase their TC is important as farmers now routinely use soluble calcium fertilizers and being able to understand the genetic control of calcium and being able to identify genetic materials that can efficiently utilize added calcium would be of great benefit to the industry by increasing useable yield, by increasing tuber health and quality, and lowering fertilizer input costs.

Abbreviations/ Key Words ATL, Atlantic; BUR, Russet Burbank, DRN, Dark Red Norland; SNW, Snowden; SUP, Superior; CI, confidence interval; CRBD, completely randomized block design; CRD, completely randomized design; HSD, honest significant difference; TC, tuber calcium; H^2, broad-sense heritability, genetic correlation, phenotypic correlation

Introduction

Potato is the most popular vegetable in the United States but suffers from several tuber-related defects that reduce marketability, storage life and yield (Spillman, 2003). Some of these defects have been negatively correlated with tuber calcium concentration (TC) (Spillman, 2003). For example, TC has been associated with reduced severity of internal brown spot (Ozgen, et al., 2006; Tzeng, et al., 1986), reduced hollow heart (Arteca, et al., 1980; Kleinhenz, et al., 1995; Kleinhenz, et al., 1999; Levit, 1942; Rex & Mazza, 1989) reductions of soft rot in storage caused by the *Pectobacterium carotovorum* pathogen (McGuire & Kelman, 1984), improved yield and tuber grade (Simmons & Kelling, 1987), reduced internal heat necrosis (Yencho, et al., 2008), reduced blackspot bruise (Karlsson, et al., 2006) and increased abiotic stress tolerance to both heat (Kleinhenz & Palta, 2002) and freezing stress (Vega, et al., 1996).

Calcium is very important to plant growth and development, membrane and cell wall function and structure, regulation of cell metabolism and stress signaling response (Murayama, et al., 2017; Palta, 2010; White & Broadley, 2003; Wilkins, et al., 2016). The highest proportion of calcium is found in the cell-wall where it is known to help maintain structural integrity of the plasmalemma by stabilizing pectins through calcium cross-links (White & Broadley, 2003; Murayama, et al., 2017). Calcium also supports the structure of the cell membrane through cross-linking phospholipid polar head groups (Palta, 2010).

The ability to increase the calcium concentration in the potato tuber is important for tuber quality (Chung, et al., 2016). It has been found that the incidence of internal quality issues in the tuber decreased from 20 to 5% when the average TC is increased from 100ppm to 250 ppm (dry weight) (Palta, 2010). An improved understanding of how potato tubers

accumulate calcium has changed the way we think about calcium nutrition of potato and how we can practically increase TC. This improved understanding of water and calcium uptake came about through the discovery of both a main root system, servicing the main stem and leaves, in addition to a tuber and stolon root system, servicing the tubers (Kratzke & Palta, 1985; Kratzke & Palta, 1986). Calcium preferentially follows the transpiration-driven mass flow of water in the xylem and this flow is determined by the gradient of water potential that favors transport from the main root system to the above-ground stems and leaves. However, the water potential surrounding the tubers is much lower than the potential surrounding above-ground stems and leaves due to a more humid environment around the tubers that restricts transpiration and hence water and calcium transport from the soil around the tuber roots to the tubers (Kratzke & Palta, 1985; Palta, 2010). The result of this discovery motivated the implementation of targeted applications of water-soluble forms of calcium during the tuber bulking period to increase TC concentrations (Kratzke & Palta, 1986; Simmons & Kelling, 1987). Calcium is now used regularly as part of a normal potato fertilization program by routinely adding it in a water-soluble form, such as $CaNO_{3,}$ through the irrigation water during the tuber bulking period at a rate of approximately 112 – 224 kg/ha (100 – 200 lbs/acre) (Palta, 2010).

Studies have provided evidence for the genetic variation for TC among cultivated (Ozgen, et al., 2006) and wild (Bamberg, et al., 1993; Bamberg, et al., 1998; Vega, et al., 1996) germplasm. A study using a representative sampling from the US potato germplasm collection and was able to identify both high and low calcium accumulating germplasm among species, populations, and fine-screening of genotypes. At the accession level, the best

accumulators of calcium were found in *S. gourlayi* and *S. microdontum* populations, which on average accumulated more than 6 times the TC (8 times at the genotype level) than the lowest TC accumulating populations among *S. kurtzianum*. They concluded that selection was able to act on TC in either direction after evaluation of intercrosses made between the progeny of these populations (Bamberg, et al., 1998). However, the genetic variation for TC accumulation in the cultivated potato, *Solanum tuberosum*, was reported to be low compared to wild species (Chung, et al., 2016), but advanced breeding clones of potato have been observed to display significant sources of genotypic variation, and large broad-sense heritability's, for this trait under naturally available conditions of calcium (Brown, et al., 2012). Efforts are being made to develop materials that demonstrate a greater ability to accumulate TC to increase tuber quality and marketable yield using the large variation for TC found in wild potato species (Chung, et al., 2016). However, introgression of genes from other potato species into the cultivated varieties can be difficult due to crossing barriers, variations in ploidy levels, variations in endosperm balance number, and the inherent small tuber size of wild species (Bamberg, et al., 1993; Jansky, 2006). A simpler approach could utilize the existing variation in cultivated tetraploid potato by developing populations using parents that exhibit contrasting characteristics for the trait of interest and allowing the F1 population progeny to segregate for the trait (Vega, et al., 2006; Zorrilla, et al., 2014). F1 populations of highly heterozygous polyploid species generated from parents with contrasting traits, such as the population in the current study, have shown sufficient variation in traits as demonstrated in other studies of potato (Heldak, et al., 2007; Meyer, et al., 1998) and other polyploid species such as alfalfa and sugarcane (Julier, et al., 2003; Lyrene, 1977).

Using cultivated varieties as parents has the advantage of being more likely to obtain progeny with desired commercial qualities such as tuber uniformity (Zorrilla, et al., 2014). However, it is not known whether selection for calcium traits in a tetraploid F1 population derived from divergent parents is effective. Additionally, *Solanum tuberosum* subsp. *tuberosum* is considered to have a narrow genetic base outside of its origins in South America due to limited germplasm introductions in Europe that later came to the United States (Consortium, 2011). This limited genetic base may limit the efficiency of the improvement of TC in potato.

An additional consideration in the improvement of TC is the effect of physiology, such as maturity, on this factor. It has been found that later-maturing potato varieties tend to have higher specific gravity (SG) (Vakis, 1978) and SG is known to be lower in genotypes with higher average TC (Zorrilla, et al., 2014). For example, the parent SUP is known to have higher average TC but low SG (Zorrilla, et al., 2014) and is known to be a medium-early maturing (Chang, et al., 2018), while the parent ATL is known to have lower average TC but a higher SG (Zorrilla, et al., 2014) and is known to be medium-late maturing (Webb, et al., 1978). The physiology of parents is an important consideration in the improvement of TC and other important characteristics such as SG.

Due to the highly variable native availability of calcium in the field environment, experiments that evaluate the TC in the field must be designed in a way that the environmental effects of the field environment on TC can be separated from those genetic effects. The current study utilized restricted maximum likelihood (REML) mixed models in the analysis of TC in this population. Mixed models have been used in previous experiments (de Souza, et al., 1998; Zorrilla, 2013) to determine effects due to the environment and those

due to genetic components of various factors, through the evaluation of variance components obtained using best linear unbiased prediction (BLUP) to estimate fixed effects, or REML to estimate random variance components due to genotype, environment, or random error; which can be used to calculate the broad-sense heritability and the genetic and phenotypic correlations between traits. The current study could estimate only broad-sense heritability, in contrast to narrow-sense heritability, in this population, as dominance effects must be estimated to calculate an estimate of narrow-sense heritability, and the calculation of dominance effects is not possible in an F1 population such as that used in the current study. While the use of broad-sense heritability is limited, it can be used to assess the presence of genetic variation for a particular trait. This information can be used to determine whether a particular trait will likely respond to selection during breeding.

It is well-known that internal tuber quality is associated with calcium. However, there is no good way right now to select for tuber quality in potato breeding programs because in the first years of evaluation and selection there are not enough tubers to cut and evaluate for this purpose. Growers must save seed to produce more tubers to make a good evaluation of internal quality and many genotypes are discarded early before this can take place. In the seventh or eighth year of a breeding program there are enough potatoes to evaluate internal quality but by this time 95% of the germplasm from year one has been discarded. Therefore, by the time we have the possibility to select for internal quality we have lost a vast majority of the variation that we had from year one. It is easier to select for calcium in the early years instead of tuber quality 7-8 years down the line as we have a better chance of acquiring higher quality tubers by selecting those genotypes that demonstrate a higher TC as higher TC

is known to be advantageous in internal quality. We want a clone that will have high TC concentration without fertilizing and one that will have a high response in TC after fertilization. Therefore, selection for higher tuber calcium in this population should be able to occur under the conditions of endogenous or exogenously applied calcium. However, due to the significant variation in available calcium across the field macro and micro-environment it would be more effective to select for this trait under better-controlled conditions, but controlled environment studies come with the added challenges of scale and cost.

The current study utilizes the cultivated chipping varieties ‘Atlantic’ and ‘Superior’ as parents, who contrast each other in several important factors. ‘Atlantic’ is known to have low TC (Karlsson, et al., 2006), high yields (Webb, et al., 1978), high amount of internal defects such as internal tuber necrosis (Henninger, et al., 1979; Webb, et al., 1978), moderate scab susceptibility (Haynes, et al., 2010), high specific gravity (Webb, et al., 1978), good chip and fry quality (Webb, et al., 1978) and uniform tuber size (Webb, et al., 1978), while ‘Superior’ is known to have high TC (Karlsson, et al., 2006), lower yields (Zorrilla, et al., 2014), lower amounts of internal quality issues including hollow heart, internal necrosis and vascular discoloration (Rieman, 1962), moderate scab resistance (Haynes, et al., 2010; Rieman, 1962), poor fry quality (Zorrilla, et al., 2014) and low specific gravity (Karlsson, et al., 2006). In addition to increasing the TC, it would also be of great interest to the potato industry to develop breeding materials with the other positive characteristics of both cultivars. The current study evaluated this population in the field and observed TC concentrations under two calcium treatments: (1) naturally occurring soil calcium

(endogenous calcium supply) was used as the control and (2) applied soluble calcium in the field during the tuber-bulking period (exogenous calcium supply).

Purpose

The purpose of this study was to: (1) determine the effect of soluble in-season calcium fertilization during tuber bulking on the TC of the parents and F1 population (2) determine if genetic variation exists (H^2) in this F1 population for TC under control and calcium-treated conditions (3) determine the extent of genotype x environment (year) interactions on the measured TC under control and calcium-treated conditions (4) determine whether there is likely separate genetic control between TC uptake between control and calcium-treated plots and (5) determine the effect of in-season calcium application and estimate the genetic variation (H^2) for the tuber quality traits hollow heart, pitted scab, and internal brown spot.

Materials and Methods

Experimental Setup

This study was performed over a period of 5 years, from 2011 – 2015, at the UW-Madison Hancock Agricultural Research Station (HARS). TC was evaluated only in the 3 years 2011, 2013, and 2014, while non-calcium traits were evaluated for up to 5 years of the study. The experiment in each year of this study followed a completely randomized design concerning the evaluation of the F1 population progeny and the parental materials. Experiments were planted in each of the five years in late April or early May and harvested in early to mid-September. Not including the parents, a total of 53 genotypes were evaluated in 2011, 67 in 2012, 77 in 2013, 47 in 2014 and 71 in 2015 with a total of 133 unique genotypes evaluated overall in the 5 years. The same genotypes were not used in all years of

this study due to availability or quality of seed potatoes at the time of planting. A total of 12 genotypes were available that were represented in all three years. Seed in this experiment was saved year-after-year from 2011 and so yield effects may not be consistent due to possible virus contamination of some genotypes. All experiments were planted in crop rotated fields (low disease pressure) in a Plainfield loamy sand soil and used standard agronomic practices for potato including nutrition, pesticide/herbicide application and overhead irrigation. For calcium treatment applications, a total of roughly 90.7 $kg \cdot ha^{-1}$ Ca was given every year in three separate applications, 2 weeks apart, during the bulking period containing one or a mix of $Ca(NO_3)_2$, $CaSO_4$ or $CaCl_2$ depending on the year. In 2011, three equal applications of $CaCl_2$ were given for a total of 224 $kg \cdot ha^{-1}$ Ca. In 2012 three separate applications of $CaCl_2$ were given at 84 $kg \cdot ha^{-1}$ Ca and two other applications of 81 $kg \cdot ha^{-1}$ Ca for a total of 414 $kg \cdot ha^{-1}$ Ca. In 2013 142 $kg \cdot ha^{-1}$ Ca as $CaSO_4$ was applied in addition to two other 82 $kg \cdot ha^{-1}$ Ca applications of $Ca(NO_3)_2$ for a total of 306 $kg \cdot ha^{-1}$ Ca. In 2014 and 2015 three equal applications of $Ca(NO_3)_2$ were given for a total of 224 $kg \cdot ha^{-1}$ Ca. All control plants were fertilized with solid NH_4NO_3 and received the same amount of nitrogen given to the calcium treated plants when $Ca(NO_3)_2$ was used as the source of calcium. Clones of individual genotypes were planted in a 6.1m long row with plants spaced 30.5 cm apart within a row and with rows 91 cm apart. To facilitate calcium fertilizer treatments a total of 16 clones per genotype were planted per row with half of these rows separated by 4 plants of the cultivar Dark Red Norland. This allowed for calcium treatment and control plots to be right next to one another and allowed for the obvious separation between treatments during machine harvest. A total of 16 of these 6.1 m long rows were planted within a planting block

with the outer rows planted as a guard row using cultivar material (14 rows of experimental material). Planting blocks were separated by 2.44m long alleys. A total of 16 of these planting blocks were planted per field with the two outer blocks planted as guards leaving 14 experimental blocks per planted field.

Trait Evaluation

Tuber Calcium (TC) Sample Collection and Quantification

TC data was collected only in the years 2011, 2013, and 2014. TC data was not collected in 2012 and was partially collected in 2015 due to an issue with calcium contamination during processing and problems with sample preparation, respectively. TC concentrations were measured similarly to the method of Kratzke and Palta (1986) with some minor differences. A sample of 10 A-grade tubers were taken per plot during harvest and these samples were pooled together for calcium analysis at UW-Horticulture. Anywhere from a 1mm to 5mm thick longitudinal section of potato was taken from the center of a tuber, that was rinsed with double deionized H_2O, followed by the removal of the cortical tissue by hand with a razor blade and with the aid of a light box. The remaining medullary tissue was then scrapped with the razor blade to remove any excess liquid or was rinsed with double deionized H_2O depending on the year of evaluation. The medullary tissue was then cut into roughly 5mm chunks and, depending on the year, either dried in a drying oven and ground to a fine powder using a grinding mill or dried in a lyophilizer (freeze-dryer) and powdered by hand. Calcium was then extracted according to the method of Kratzke and Palta (1986) and quantified using an atomic absorption spectrometer (Varian SpectrAA 55B). TC concentration was reported as micrograms of calcium per gram of dried tuber weight ($\mu g \cdot g^{-1}$ or ppm).

Non-calcium Traits

Many other traits, other than TC, were evaluated throughout the 4 years of this study including hollow heart, pitted scab, and internal brown spot. Only A-grade tubers were used for the visual evaluation of counts of internal defects that included hollow heart, pitted scab, and internal brown spot. These tubers were visually evaluated at the research station within a few days of harvest after cutting no more than 100 tubers longitudinally per plot. Data for internal tuber quality is presented as a percent of the total number of tubers cut per plot.

Statistical Analysis

Statistical analyses in this study were carried out using RStudio (Version 1.1.447 - © 2009-2018 Rstudio, Inc.). The quality of the data was evaluated first by plotting and removing obvious outliers followed by evaluation of the conformity of the data to statistical assumptions (normality and homogeneity of residual variances). All factors were assessed for normality using the qqnorm() and skewness() functions (e1071 package), while residual homogeneity was assessed on the factor model using the plot() function in Rstudio. Any factor data with an absolute value of skewness greater than 0.9 was chosen to be transformed with either a $\log_{10}$ or squared-root transformation before performing statistical analysis. Choosing whether to use a $\log_{10}$ or squared-root transformation was determined by comparing the normality of the transformed data (qqplot or skewness function) from both methods. All internal quality data (hollow heart and internal brown spot) and data on pitted scab were transformed using a log10 transformation. Data was transformed before performing statistical analysis and the statistical significance is reported from the transformed data, while the presentation of data means, standard errors, mean comparisons, and confidence intervals use the original data without transformation. Linear models, lm(),

and anova() were used for statistical analysis in determining main effects in Rstudio for normally distributed quantitative data. The ANOVA main effects for both offspring and parents were determined using the following:

(1) Full model: $\gamma_{ijkln} = \mu + G_i + Y_j + GxY_{ij} + T_k + R(Y)_{l(j)} + \varepsilon_{ijkln}$

(2) Within-year full model: $\gamma_{ikln} = \mu + G_i + T_k + R_l + \varepsilon_{ikln}$

(3) Reduced model : $\gamma_{jklmn} = \mu + G_i + Y_j + GxY_{ij} + R(Y))_{l(m(j))} + \varepsilon_{jklmn}$

(4) Within-year reduced model: $\gamma_{klmn} = \mu + G_i + R_{l(m)} + \varepsilon_{klmn}$

Where, γ_{ijkln} is the *n*th observation of the *i*th *genotype* in the *j*th *year*, *k*th *treatment*, and *l*th *replication* nested in the *j*th *year*, μ is the grand mean, G is the *genotype*, Y is the *year*, GxY is the *genotype x year* interaction, T is the calcium *treatment* and R(Y) is the *replication* nested in *year*, R is the *replication*, and ε is the *residual* error. Model (1) was used to evaluate traits between years and calcium treatments. Model (2) was used to evaluate traits within a year and between calcium treatments. Model (3) was used to evaluate differences in traits between years within the control or the calcium treatment and model (4) was used to evaluate trait differences between genotypes within a given year and calcium treatment. Both full models include the calcium *treatment* term (T) , while both reduced models drop the calcium *treatment* term (T) in the models above. The full and reduced models include *year* and *genotype x year*, while the within-year models drop all *year* terms.

Mean Comparisons

Mean differences and their confidence intervals (CI's) were determined using Tukey's HSD (Honest Significant Difference) and were determined between the calcium and control treatments within the F1 population, Atlantic or Superior groups within a single year and across all years (grand mean). These tests were determined using the Rstudio functions

aov() and TukeyHSD() and the mean differences and CI's were presented using the original data and the ANOVA model is the same as above. The adjusted p-values for the contrasts were calculated from the normalized/transformed data where appropriate. Means are significantly different if the adjusted p-value is below 0.05 or if the confidence interval does not contain zero.

Heritability Estimates

The broad-sense heritability (H^2) was calculated using nested mixed models in Rstudio using the lme4 package and lmer() for normal or normalized data depending on the trait analyzed. The models used to evaluate the F1 population were:

(5) Nested Full Model: $\gamma_{ijklm} = \mu + G_i + GxY_{ij} + T_k(R_l(Y_j)) + \varepsilon_{ijklm}$

(6) Nested Reduced Model: $\gamma_{iklm} = \mu + G_i + T_k(R_l) + \varepsilon_{iklm}$

(7) Nested Replicate(Year) Full Model: $\gamma_{ijlm} = \mu + G_i + GxY_{ij} + R(Y)_{l(j)} + \varepsilon_{ijlm}$

(8) Crossed Reduced Model: $\gamma_{ilm} = \mu + G_i + R_l + \varepsilon_{ilm}$

Where, γ_{ijklm} is the *k*th observation of the *i*th *genotype* in the *j*th *year* and *k*th calcium *treatment* nested in the *l*th *replication* of the *j*th *year*, μ is the overall mean response of the F1 population, G is the *genotype*, GxY is the *genotype x year* interaction, R is the *replicate*, T(R(Y)) is the *calcium treatment* (T) nested within *replicate* (R) nested within *year* (Y). T(R) is the *calcium treatment* (T) nested within *replicate* (R), R(Y) is the *replicate* nested within *year* and ε is the error term. All terms were treated as a random effect. The reduced models drop the *year* and *genotype x year* terms. The full models (5) and (7) were used to estimate variance components across years, while the reduced models (6) and (8) were used to estimate variance components within years. Models (5) and (6) retained the *treatment* term (T), while models (7) and (8) drop the *treatment* term (T). The broad-sense heritability is

calculated on a genotype-mean basis, from the variance components of the random effects mixed model, as presented by Holland et al. (2003).

$$H^2 = \sigma^2_G / (\sigma^2_G + (\sigma^2_{GxY}/y) + (\sigma^2_\varepsilon/ry))$$

Where H^2 is the genotype-mean broad-sense heritability, σ^2_G is the variation due to *genotype*, σ^2_{GxY} is the variation due to the *genotype x year* interaction, σ^2_ε is the *residual* variation, r is number of *replications* and y is number of *years/ environments*. For the purposes of discussion heritability values are classified into five groups, low ($0.0<H^2\leq0.2$), low moderate ($0.20<H^2\leq0.4$), moderate ($0.4<H^2\leq0.6$), high moderate ($0.6<H^2\leq0.8$) or high ($0.8<H^2\leq1.0$).

Phenotypic and Genotypic Correlations

Genetic correlations were calculated using the random effects mixed model variance components to assess the relationship of genetic control (Falconer, 1989) between control TC and calcium-treated TC data of the 12 genotypes that had TC data available in all three of the years 2011, 2013, and 2014. The phenotypic correlations are calculated and are included similarly.

Degree of Dominance

A simple calculation for the estimate of the degree of dominance (Falconer, 1989) was calculated for the F1 population using the parental and F1 population mean values for both the calcium and control TC data for the years 2011, 2013 and 2014. This was calculated as the departure of the F1 population from the mid-parent value (*h*) which is then divided by the departure of the lesser-value value parent from the mid-parent (*d*). A value of 0 for *h/d* is

attributable to variance due to completely additive effects, while values closer to 1 indicates variance due to completely dominant effects.

Results and Discussion

Summary of Effects of Calcium Application on TC

Application of in-season calcium had a significant effect on TC in the parents and the F1 population. The combined 2011, 2013 and 2014 distribution of TC from the F1 population is shifted positively after soluble calcium fertilization (Fig. 1). This increase in TC is significant in the F1 population, Superior and Atlantic within all years and across all years, except for Superior 2014 (Table 1). This data demonstrates the ability of ATL, the F1 population, and SUP to significantly increase their TC concentration (182.2, 235.8, 299.3, respectively) in response to added soluble calcium during the growing season as compared to the control (151.2, 187.5, and 233.8 respectively). The grand means of the TC for the F1 population, for both the control and calcium-treated plots, were in between the average TC values of the parents (Table 1, Fig. 1).

ANOVA Main Effects and Broad-sense Heritability of Calcium Traits

ANOVA

All the effects of the independent variables, over all 3 years, were significant in the F1 population for the combined treatment data, including the genotype x year interaction (Table 2). Additionally, the effect of the calcium treatment for each of the three years and overall was significant (Table 2, data for both control and ca-treated combined). The genotype x year (GXE) interaction for the combined data was also significant, suggesting that genotypes, in general, significantly change their TC from year-to-year (Table 2).

Similarly, the effects of the independent variables were significant for the control TC data over the 3 years combined (Table 2). Generally, the TC under control conditions (no calcium fertilizer) can be expected to change significantly for genotype in this population depending on the year (GXE). Zorrilla et al. (2014) showed a similar response in this population under similar conditions as the control of the current study. However, the independent variables for the TC data for the calcium-treated plots, in the current study, were all significant except for the genotype x year, which generally suggests that TC variance for a particular genotype will be stable from year to year when fertilizing with soluble calcium during the tuber bulking period in this population (Table 2).

Comparing the genotype x year interaction between the control and calcium treatment data, we show that the TC variance for genotypes will not increase consistently year to year when there is no soluble calcium applied and plants of this population are reliant on endogenous calcium. This could be due to the uniform spread of calcium fertilizer in the calcium treated plots versus the non-uniform availability of soluble calcium naturally present in the field in the control.

Broad-Sense Heritability (H^2) Estimates of TC Traits

To estimate the broad-sense heritability of a given factor, the genetic and environmental effects must be partitioned. It is important to note that the existence of a large heritability does not necessarily mean the response to selection for the given trait will be large, as we see in the breeder's equation ($R_s=h^2S$), where R_s is the response to selection, h^2 is the narrow-sense heritability, and S is the selection differential, the response to section is the product of the narrow-sense heritability and the selection differential. If the selection

differential is small, even though the heritability is high, the response to selection will be low (Nyquist, 1991).

The H^2 estimates for the years 2011, 2013, and 2014 of our combined treatment data were 0.78, 0.71, and 0.87, respectively (Table 2). The heritability for the analysis over all three years together, which includes GXE, was 0.84 (Table 2). This demonstrates a moderately high amount of genotypic variation within this population for TC, regardless of calcium treatment, and provides evidence for the segregation of TC in this population without considering Ca treatment.

The H^2 estimates for the years 2011, 2013, and 2014 of the control data only were 0.87, 0.71, and 0.90, respectively (Table 2), while the H^2 estimates for the years 2011, 2013, and 2014 of the calcium-treated data were 73, 0.73, and 0.87, respectively (Table 2). Accounting for the genotype x year variation, over the three years 2011, 2013 and 2014, the H^2 estimate for the TC in the control data alone was 0.75 overall, while the H^2 estimates within the calcium TC data was 0.83 over the same years (Table 2) and provides evidence for the genetic variation of TC under both control and calcium-treated conditions in this population. A similar study, utilizing a population derived from the same parents, found a moderate H^2 estimate for TC of 0.54 under the conditions of naturally existing control calcium over a period of two years (Zorrilla, et al., 2014). Another study by Brown et al. (2012) found a wide range of H^2 estimates within regional trials of advanced potato breeding clones not given supplemental calcium. They found low to moderate values of 0, 0.37 and 0.65 for the western regional red/ specialty (13 clones), western regional (13 clones) and tri-state trials (10 clones), respectively. The current study found a moderately high H^2 (0.75)

under the control conditions in our F1 population over a period of three years (Table 2). Ours is the first study, to our knowledge, that demonstrates a high H^2 (0.83) in TC concentration under calcium-treated conditions (Table 2). Our data lends evidence for high genotypic variation and segregation of TC concentration after the application of in-season calcium like that found for the control data (Table 2) and suggests that selection can occur under both control and calcium-treated conditions.

Depending on the amount of additivity of the genetic components of heritability for these calcium tuber traits, the transmission of these characteristics to future generations would be as low or lower than these H^2 estimates. The component of additive genetic variation cannot be directly calculated for a trait in a population without data from a mating scheme that removes the effects of dominance on that trait (i.e. backcross or inbred lines). Therefore, this estimate could not be directly calculated in our F1 population. A simple estimate of the degree of dominance was used to estimate a value of dominance for TC before and after soluble calcium was applied in the field, using the mid-parent means analysis (Falconer, 1989). These values were estimated to be 0.54 and 0.56, respectively for control and calcium treatments. The additivity of this trait could be expected to be around 0.46 and 0.44, respectively, with the assumption of the absence of epistatic effects. This would mean the narrow-sense heritability of this trait would be about half that of the broad-sense heritability for control and calcium applied treatments (Table 1) and therefore a smaller portion of the genetic variability in this population for these traits could be expected to be transmitted to the next generation resulting in more time to obtain higher calcium accumulating progeny.

Genotypic, Phenotypic and Simple Correlations

Control vs Ca-treated

The simple correlations for the F1 population, for the years 2011, 2013, and 2014, between control TC versus the calcium-treated TC were positive (r=0.81, r=0.68, and r=0.81, respectively) and significant ($p<0.01$, Fig. 2). The correlation for these two traits over all three years (r=0.91) also showed a significant relationship ($p<0.001$, Table 3, Figure 3). In agreement with this, the mixed model analysis showed high genotypic and phenotypic correlations between the control versus the calcium-treated (1.06 and 0.95, respectively, Table 3). These results suggest that the TC in the control and the calcium-treated traits are under similar genetic control.

Ours is the first study evaluating phenotypic and genotypic relationship among TC's of control and in-season applied calcium in a segregating population in tetraploid potato. Like the current study, a previous study showed a similar, positive trend, in the correlation between control and calcium-treated TC among 5 potato cultivars (Karlsson, et al., 2006). In this study, plants were grown for 3 seasons (1999-2001) under field conditions and looked at differences in tuber bruising between calcium treatments. Using the data from Karlsson et al. (2006) we made a scatter plot between control versus calcium-treated TC (Figure 4) and found a strong positive relationship between the control and calcium-treated TC (r=0.98, $p<.001$, Figure 4). This lends evidence to the similar genetic control of TC under both control and calcium-treated conditions.

Physiology and TC

Additionally, it is important to consider the effect of physiology, such as maturity, on the TC accumulation ability. It has been found that later-maturing potato varieties tend to have

higher specific gravity (SG) (Vakis, 1978) and SG is known to be lower in genotypes with higher average TC (Zorrilla, et al., 2014). For example, the parent SUP is known to have higher average TC but low SG (Zorrilla, et al., 2014) and is known to be a medium-early maturing (Chang, et al., 2018), while the parent ATL is known to have lower average TC but a higher SG (Zorrilla, et al., 2014) and is known to be medium-late maturing (Webb, et al., 1978). These trends in TC, SG, and maturity were confirmed between the parents ATL and SUP in the present study (data not shown) demonstrate the importance and complexity of considering the physiology of parents in the improvement of TC and other important characteristics such as SG.

Importance of Breeding for TC

Our data shows that a genotype in our population with a given high level of TC, without addition of soluble calcium, can generally be expected to also have a high level of TC after soluble calcium is given during the growing season (Table 4). However, there are some genotypes that have low control TC but have a higher added-calcium TC, such as B-252 and B-301 (Table 4) and shows that some genotypes are not consistent in TC increase between control or calcium-treated conditions or from year to year. This may reflect soil Ca variability, which may have a confounding effect on selection and future experiments that may address this issue is discussed in the following section. We have found that the selection for increased TC in the potato tuber should be possible in similar populations under control and calcium-treated conditions. Additionally, selection for increased tuber calcium should also be possible under both control and calcium-treated conditions in the field. This is important as farmers now routinely use soluble calcium fertilizers and being able to identify

genetic materials that can better utilize this added calcium would be of great benefit to the industry by increasing tuber health and quality and lowering fertilizer input costs.

Future Studies

Under control conditions in the field environment the distribution and availability of calcium in the soil is highly variable and may contribute to the variation in control and calcium-treated TC concentrations that we see in our experiment. Even the micro-environment around individual tubers can contribute to significant variations in TC within the tubers of a plot. The current study observed a low genetic variation for the change in Ca (ΔTC) between the calcium-treated and control plots, which may reflect the variation in soil Ca present under field conditions. An experiment that can more precisely control the supplied calcium in the tuber environment would be able to observe any genetic effects on TC more effectively than in a field study where these environmental soil calcium variances exist and are difficult to account for experimentally. Furthermore, the careful evaluation of crosses between diverse cultivars and species or the utilization of F2 or BC populations could lend more insight into the genetics of TC and provide important parental materials for the improvement of TC. A repeat of the current study under a more controlled environment would be a logical next step to determine the true genetic variation in ΔTC in this population and help to understand the genetic control of calcium in this population so that it can be practically applied in a farm setting.

Effects of In-Season Soluble Calcium Application on Non-calcium Traits

In addition to the measurement of tuber calcium concentration values, we also measured several other non-calcium-related factors, in the parents and F1 population, that were evaluated up to a period of four-years.

Hollow Heart

Hollow heart was significantly decreased in the F1 population with calcium application in 2012 and over the 4 years that this trait was evaluated (Table 4). In general, there were more genotypes with lower incidence of hollow heart under calcium-treatment as compared to the control. Neither of the parents showed a significant reduction in hollow heart with calcium fertilization (Table 4). However, hollow heart was reduced by calcium fertilization in ATL in all 4 years and overall, but this effect was not significant (Table 4). In contrast, SUP showed hollow heart was increased by calcium fertilization in all years and overall but was only significantly increased by calcium fertilization in 2011 and over the 4 years that this trait was evaluated (Table 4). Previous studies have shown a similar effect on the reduction of hollow heart from supplemental calcium fertilization (Arteca, et al., 1980; Kleinhenz, et al., 1995; Kleinhenz, et al., 1999; Levit, 1942; Rex & Mazza, 1989). Zorrilla (2013) showed a similar relationship between these two factors in the same ATL x SUP population. However, this experiment was performed only under control conditions, but TC was negatively correlated with hollow heart in this study (Zorrilla, 2013).

Pitted Scab

Pitted scab was significantly decreased in the F1 population with calcium fertilization in 2011 and 2012, and over the four years 2011 - 2014 (Table 4). In general, there were more genotypes with lower incidence of pitted scab under calcium-treatment as compared to the control. Atlantic showed a decrease in pitted scab in the year 2012 and over the four years 2011-2014 (Table 4). Superior is resistant to pitted scab and had a low occurrence of this defect in all 4 years it was evaluated. A study by Lambert and Manzer (1991), on a Caribou gravel-silt loam with a 4.2 starting pH and a CEC of 5.5 meq/100g, showed no positive effect

of applied calcium (960 kg·ha^{-1} $CaCO_3$ or a Ca equivalent 4.5 MT·ha^{-1} $CaSO_4$) on scab over the two years of the study and showed no correlation with soil calcium concentration, healthy periderm, or medullary tuber tissue. In fact, the concentration of calcium in the periderm of the healthy and scabby tubers from the control, gypsum, and lime treatments all showed higher levels of calcium in the scabby tubers as compared to the control. Calcium treatment did, however, increase the tuber calcium concentration of both the periderm and the medullary tissues. Calcium was significantly increased in the periderm for the gypsum, but not the lime treatment. The medulla tissue showed the same trend, with gypsum significantly increasing the calcium content compared to the control or lime treatments. However, in support of our results for this trait, Zorrilla (2013) utilized the same population as the current study and found a negative correlation between tuber calcium concentration, under conditions of no applied calcium, and pitted scab.

Non-pathogenic Necrosis - Internal Brown Spot (IBS)

IBS and BC, measured in the current study, can be generally categorized as non-pathogenic internal necrosis of the tuber, and may be referred to by other names such as internal heat necrosis (IHN),internal browning, heat necrosis, internal rust spot, internal physiological necrosis or brown fleck among others (Tzeng, et al., 1986). IHN is a similar term in the eastern production regions of the United States for a tuber disorder which is similar or possibly the same as IBS in the mid-west (Yencho, et al., 2008).

The current study shows a significant, positive effect of calcium on IBS for the F1 population in years 2011, 2012, and over all 4 years evaluated for this trait (Table 4). In general, there were more genotypes with lower incidence of IBS under calcium-treatment as compared to the control. Only the parent ATL had a significant positive effect of calcium on

IBS in 2012 (Table 4), which was a very hot summer. Hot conditions during the growing season are known to worsen internal necrosis (Tzeng, et al., 1986) and so providing calcium during these conditions may be especially beneficial in reducing IBS. Several other studies cite the effect of calcium on non-pathogenic necroses of potato (Collier, et al., 1978; Karlsson, et al., 2006; Tzeng, et al., 1986). Karlsson et al. (2006) and Tzeng et al. (1986) demonstrated the reduction of IBS by increasing the concentration of TC. Collier et al. (1978) showed that rust spot was minimized in plants supplied within the range of 9-27 mM $CaCl_2$.

The parent ATL in our study is highly susceptible to non-pathogenic tuber necrosis disorders (Henninger, et al., 1979) and although these disorders can be difficult to distinguish from one another they are generally characterized by a red-brown darkening in internal tuber medullary parenchyma tissue or near or within the vascular ring. They can often be difficult to evaluate due to their similarity in appearance, but some of these disorders are categorized by where they are found in the tuber. For example, IHN is characterized as a non-pathogenic necrosis primarily found near the apical portion of the tuber (Yencho, et al., 2008). However, in our study, this necrosis was scored as IBS, which the current study characterized as being more than 1 necrotic lesion away from the tuber center and closer to the cortex. Additionally, these disorders occur under many similar environmental conditions, such as heat and drought, including the presence of genotype x environment interactions that contribute to variations in expression (Yencho, et al., 2008).

Heritability's of TC and Hollow Heart, IBS, and Pscab

Additional information is provided for the F1 population progeny on all non-calcium trait main effects from the ANOVA including genotype, year, calcium treatment, genotype x

year and rep in addition to the mixed model variance components and broad-sense heritability (Table 2).

The broad-sense heritability for the non-pathogenic necroses IBS (4 years) were estimated to be 0.402 (Table 5). Other studies have looked at the genetics of IHN and have estimated high broad-sense heritability's (0.83 – 0.92) for this defect in tubers greater than 64 mm in diameter (Henninger, et al., 2000). This is greater than the heritability values of IBS found in the current study (0.402). Sterrett et al. (2003) concluded that a population of *S. phureja* x *S. stenotomum* has the potential to be introgress improved IHN resistance alleles into cultivated *S. tuberosum* (4x).

The current study assessed pitted scab as incidence (% of tubers having pitted scab) and estimated heritability at 0.717. A previous study found heritability values for common scab based on the arcsine proportion of scabby tubers from 0.30-0.80 (Haynes, et al., 2010). A study by Bradshaw (2008) estimated heritability for common scab to be 0.660.

Conclusion

We conclude, as supported by our mixed model analysis, that control TC and calcium-treated TC share similar genetic components and are not under independent control as given by our evidence for the simple correlation, the overall correlations, and after accounting for environmental variance that showed a high genetic correlation between these two factors. We observed genetic variation for TC in both control and calcium-treated conditions in this population and we have identified both high and low performing genotypes. While the control and calcium-treated TC showed high genetic variation, the response to added calcium (ΔTC, the difference in TC between the calcium-treated and control plots) did not show high genetic variation in this F1 population as expected, which may be due to confounding effects

from the variation in the field soil Ca and other confounding environmental factors that are often faced in the field. A repeat of the current study under a more controlled environment would be a logical next step to determine the true genetic variation in ΔTC in this population and help to understand the genetic control of calcium in this population so that it can be practically applied in a farm setting. Additionally, the utilization of divergent genotypes and species may aide in the identification of suitable parental materials with a wider genetic base than is currently found in the cultivated species and could contribute to the efficiency of selection for improved TC. Improving the capacity of tubers to increase their TC is important as farmers now routinely use soluble calcium fertilizers and being able to understand the genetic control of calcium and being able to identify genetic materials that can efficiently utilize added calcium would be of great benefit to the industry by increasing useable yield, by increasing tuber health and quality, and lowering fertilizer input costs.

Figures

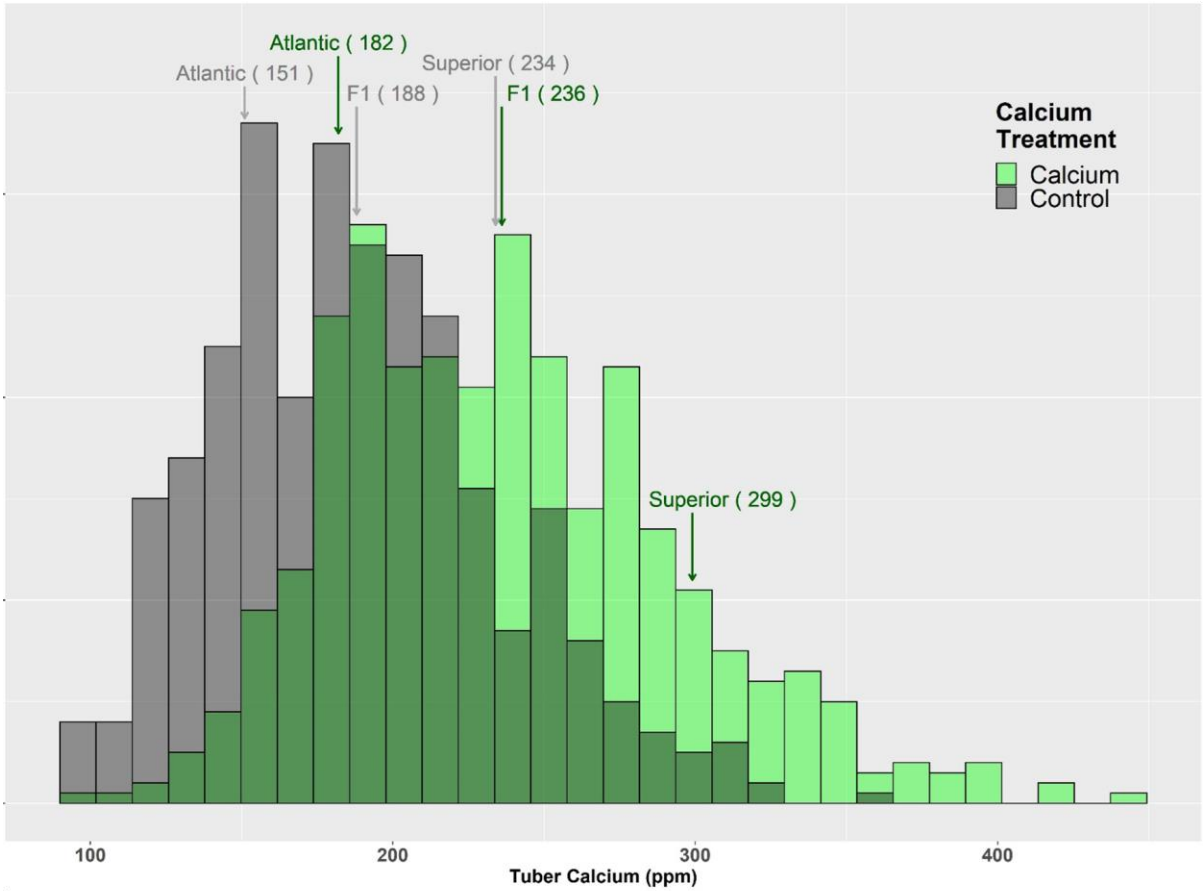

Figure 1. Distributions of tuber calcium concentrations (TC) in the F1 progeny with (green) and without (gray) added in-season calcium. Values are plotted as TC means from the data for 2011, 2013 and 2014 for the parents Atlantic and Superior and the F1 population.

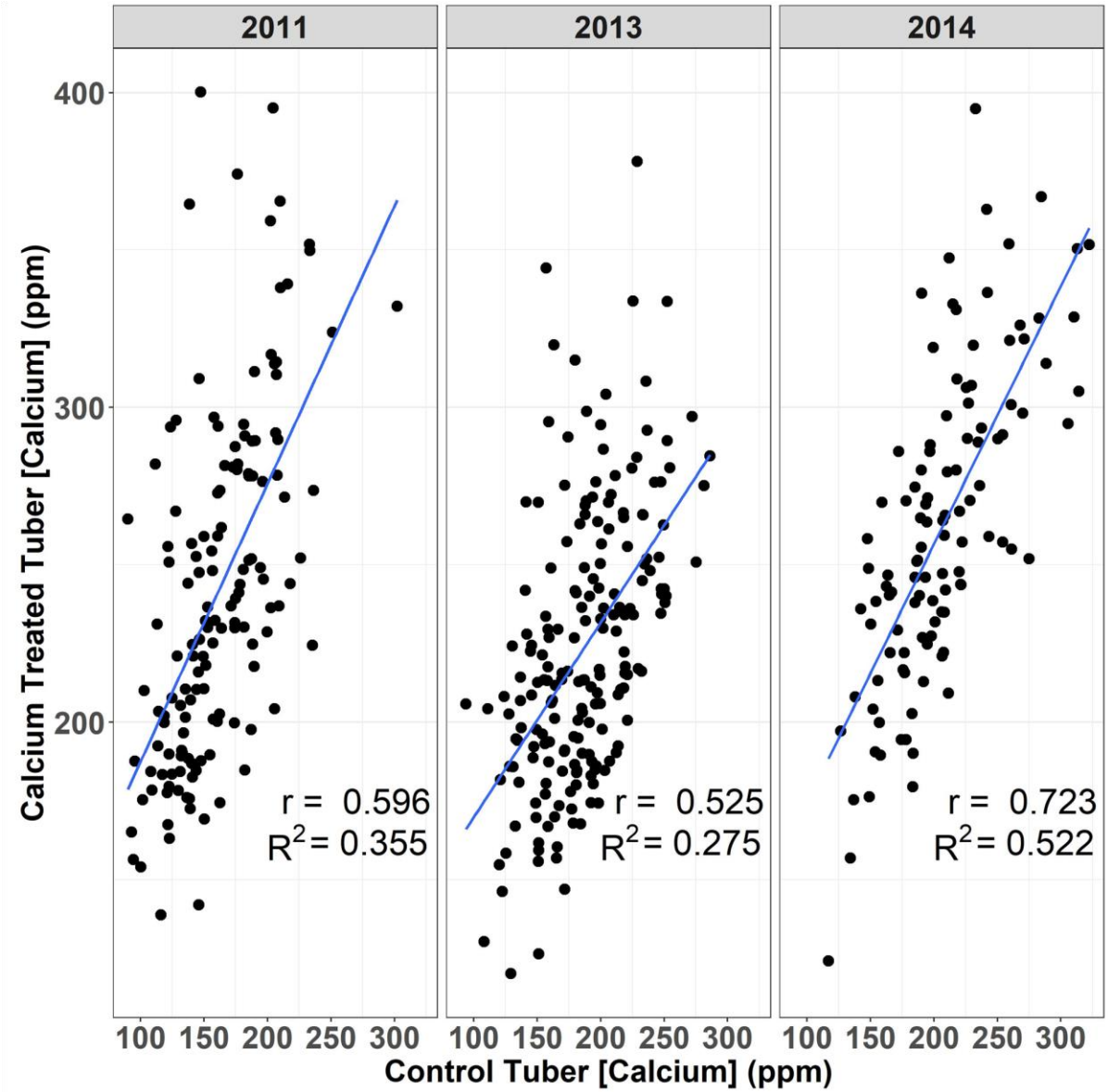

Figure 2. Correlations for the control versus the calcium-treated tuber calcium (TC). Data points are the replicate averages of all available genotypes within the years 2011, 2013 and 2014 for the F1 population.

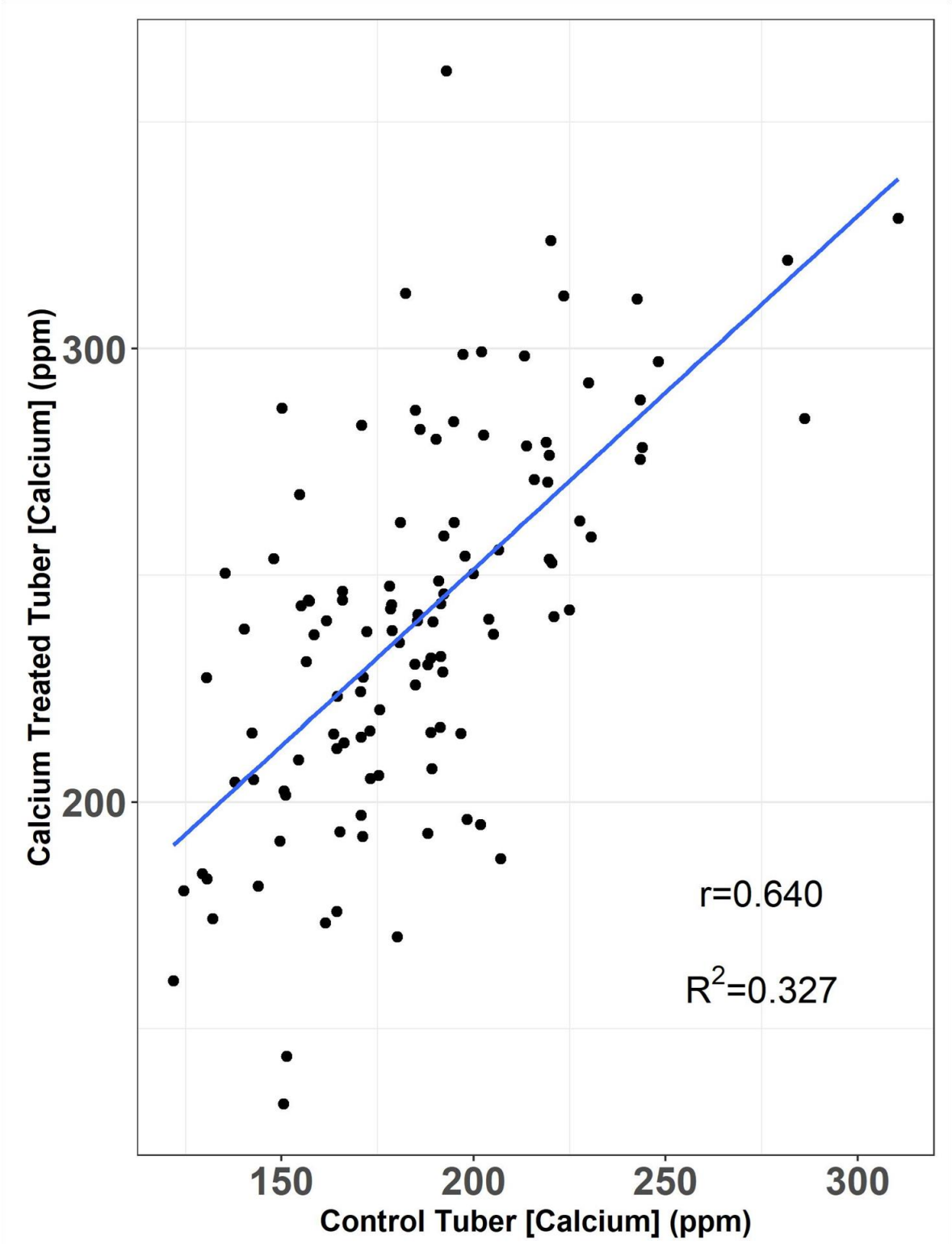

Figure 3. Correlations for the control versus the calcium-treated tuber calcium (TC). Data points are averaged over the 3 years years and reps for each genotype in the F1 population.

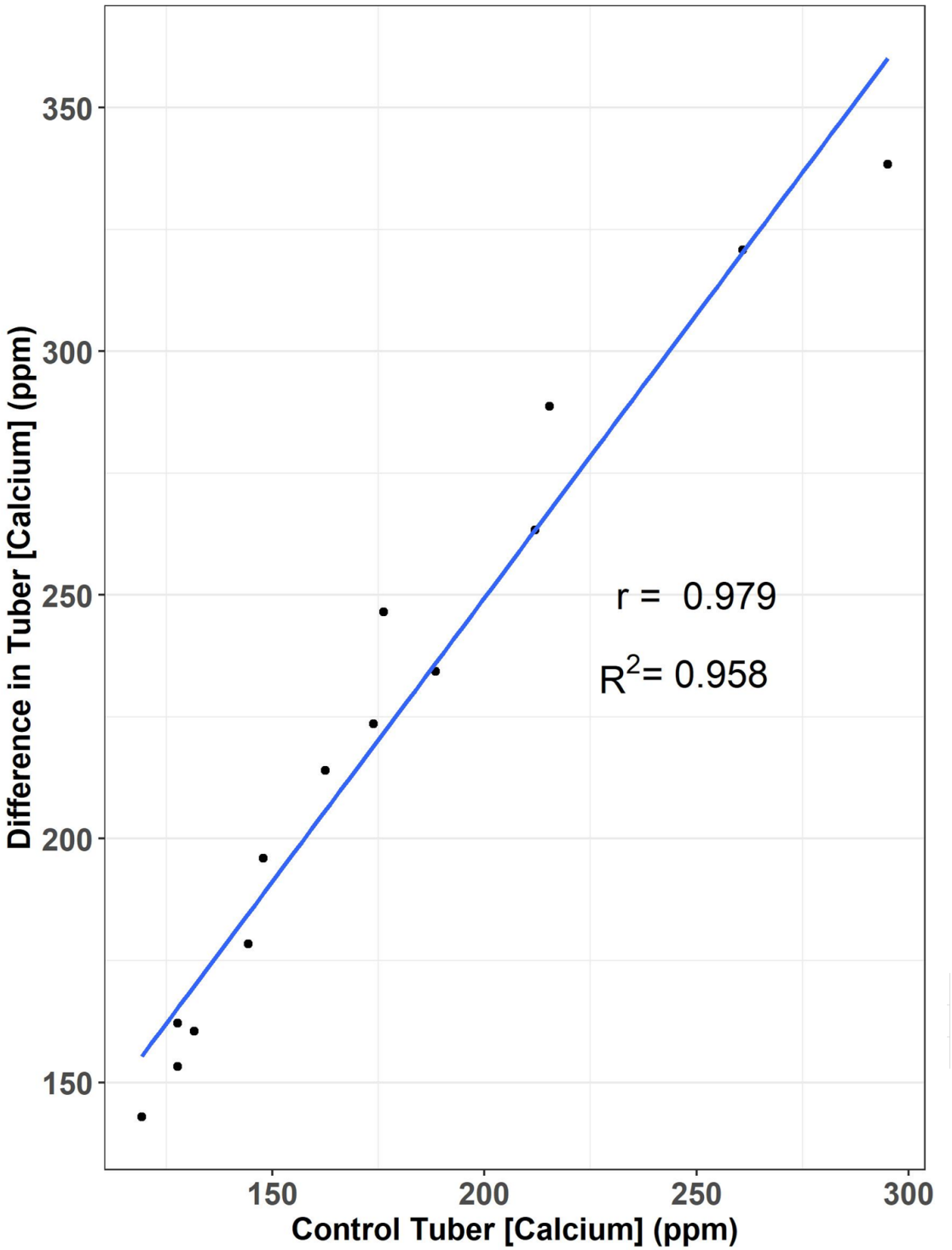

Figure 4. Karlsson et al. (1993) grew plants from 5 commercial cultivars averaged over the 3 years 1999, 2000, and 2001 of potato under field conditions with control and calcium-treated plots. The current study replotted this data and found a significant correlation ($p<0.001$) between the control TC and the calcium-treated TC.

Tables

Table 1: Means (SE), contrasts, and confidence intervals[a] for tuber calcium concentration of parents and 12 F1 genotypes observed in 2011, 2013, and 2014.

Year	Average Tuber Calcium Concentration ($mg \cdot kg^{-1}$ dry weight)					
	Control			Calcium		
	Atlantic	F1	Superior	Atlantic	F1	Superior
2011	144 (7.6)	150 (5.7)	220 (11)	178 (10.9)	223 (7.7)	343 (14.8)
2013	152 (4.2)	180 (6.3)	238 (8.2)	179 (5)	207 (6.5)	274 (11.5)
2014	159 (5.1)	199 (9)	264 (12.4)	206 (11.2)	256 (9.7)	280 (14.5)
Grand Mean	151 (3.2)	176 (4.5)	234 (6.4)	182 (4.5)	227 (4.9)	299 (9.8)

Year	Control-Calcium[b]											
	Atlantic				F1				Superior			
	Mean difference (95%CI)		Adjusted p-value		Mean difference (95%CI)		Adjusted p-value		Mean difference (95%CI)		Adjusted p-value	
2011	-33	(-53.5,-13.3)	3.1E-03	**	-73	(-84.1,-60.9)	3.6E-12	***	-123	(-158.5,-87.1)	2.0E-07	***
2013	-27	(-40.2,-13.6)	1.9E-04	***	-27	(-40.9,-13.5)	2.1E-04	***	-36	(-66,-6.5)	1.8E-02	*
2014	-48	(-70.3,-25.1)	1.6E-03	**	-56	(-71.8,-40.8)	2.7E-09	***	-15	(-50.8,20.1)	2.6E-01	NS
Grand Mean	-31	(-40.8,-21.1)	3.1E-08	***	-51	(-58.8,-42.4)	7.0E-15	***	-66	(-88.8,-42.3)	3.3E-07	***

Significance thresholds: p<0.1 (·), p<0.05 (*), p<0.01 (**), p<0.001 (***), NS=Not Significant

[a] Means, standard errors (SE), mean differences and confidence intervals (CI) presented from original data (not transformed).

[b] Tukey HSD tests - Differences between calcium treatment means for the F1 population or the parents, within or across years (grand mean).

Table 2: Broad-sense heritability estimates for tuber calcium concentration in 12 F1 genotypes observed in 2011, 2013, and 2014.

Year	Treatment Data	Main Effects					Variance Components				Broad-sense Heritability
		Genotype	Year	Calcium Treatment	Genotype x Year	Rep	σ^2_G	σ^2_Y	σ^2_{GxY}	σ^2_e	H^2
2011	Both[a]	***	-	***	-	***	718.3	-	-	606.5	0.78
2013		***	-	***	-	NS	670.5	-	-	820.4	0.71
2014		***	-	***	-	.	2021.1	-	-	909.6	0.87
all		***	***	***	***	***	1004.1	249.7	324.7	776.6	0.84
2011	Control	***	-	-	-	***	536.3	-	-	243.3	0.87
2013		*	-	-	-	NS	631.9	-	-	791.9	0.71
2014		***	-	-	-	*	1960.8	-	-	647.6	0.90
all		***	***	-	***	***	603.5	463.2	429.0	570.5	0.75
2011	Calcium Treated	**	-	-	-	**	892.8	-	-	968.9	0.73
2013		**	-	-	-	NS	719.1	-	-	797.2	0.73
2014		**	-	-	-	NS	2203.0	-	-	976.7	0.87
all		***	***	-	*	*	901.9	472.0	234.9	954.6	0.83

Significance thresholds: p<0.1 (·), p<0.05 (*), p<0.01 (**), p<0.001 (***), NS=Not Significant

[a] Analysis uses both the control and calcium treated data

Table 3: Pearson simple correlation tests between tuber calcium concentration (TC) in control and calcium-treated plots for 12 genotypes in the F1 population observed in the years 2011, 2013, and 2014. Correlations tests are given for the data averaged over genotypes. The genotypic and phenotypic correlations are provided from the mixed model analysis to assess the genetic relation between traits.

	Year	Simple Correlation Tests (95% CI)[a]			Genotypic Correlation	Phenotypic Correlation
Control vs. Ca-Treated	2011	0.81	(0.43,0.94)	***	-	-
	2013	0.68	(0.18,0.9)	**	-	-
	2014	0.81	(0.45,0.95)	***	-	-
	All years	0.91	(0.71,0.98)	***	1.06	0.95

Significance thresholds: p<0.1 (·), p<0.05 (*), p<0.01 (**), p<0.001 (***), NS=Not Significant

[a] The Pearson correlation coefficient (r).

Table 4: Grand means of tuber calcium concentration (TC) from 12 F1 genotypes observed over 2011, 2013, and 2014. Individual year means of TC from 12 F1 genotypes observed in 2011, 2013, and 2014. Genotypes are sorted according to ascending TC in the control. Increasing heat map density indicates higher values.

	3-year Grand Mean		2011		2013		2014	
Genotype	Control	Calcium-treated	Control	Calcium-treated	Control	Calcium-treated	Control	Calcium-treated
B-071	127	174	118	184	132	174	134	157
B-252	140	205	126	198	135	187	160	243
B-301	151	203	124	192	158	168	170	248
B-136	151	191	135	192	164	192	155	190
B-339	171	192	143	196	179	164	192	214
B-231	178	248	131	201	192	232	227	342
B-089	181	225	164	228	218	210	160	239
B-032	185	230	159	231	204	227	191	233
B-258	199	237	195	241	169	212	250	269
B-282	206	256	184	291	194	209	241	267
B-067	214	280	158	272	186	255	290	328
B-074	216	266	178	237	228	255	229	305

Table 5: Means (SE) , contrasts, and confidence intervals for hollow heart, pitted scab, and internal brown spot for parents and F1 population.

Traits	Year	Means (SE)					
		Control			Calcium		
		Atlantic	Superior	F1	Atlantic	Superior	F1
Hollow Heart (%)[a]	2011	20.44 (3.95)	0.35 (0.19)	5.82 (0.97)	13.14 (2.79)	2.35 (1.25)	4.46 (0.75)
	2012	0.37 (0.15)	0 (0)	2.09 (0.43)	0.34 (0.13)	0.1 (0.07)	1.11 (0.22)
	2013	5.98 (0.98)	0.57 (0.2)	9.56 (1.07)	5.46 (0.96)	1.32 (0.34)	8.95 (1.13)
	2014	11.74 (2.82)	0 (0)	8.38 (1.22)	8.53 (2.76)	2.89 (2.33)	8.92 (1.33)
	Grand Mean	6.18 (1.06)	0.25 (0.08)	6.43 (0.49)	4.63 (0.77)	1.14 (0.33)	5.73 (0.49)
Pitted Scab (%)[a]	2011	20.35 (4.74)	1.15 (0.58)	10.61 (1.33)	15.83 (4.98)	1.55 (0.62)	7.34 (0.89)
	2012	36.03 (4.07)	9 (1.93)	25.36 (1.8)	24.49 (2.87)	4.98 (0.82)	14.52 (1.14)
	2013	12.48 (3.16)	1.84 (0.69)	4.8 (0.64)	10.3 (2.94)	1.35 (0.46)	3.42 (0.42)
	2014	13.84 (4.96)	1.75 (1.09)	6.63 (0.93)	11.68 (2.94)	0.68 (0.68)	6.11 (0.8)
	Grand Mean	23.31 (2.52)	4.6 (0.92)	12.2 (0.72)	17.07 (1.91)	2.82 (0.44)	7.88 (0.45)
Internal Brown Spot (%)[a]	2011	3.74 (1.27)	8.6 (2.58)	10.02 (1.04)	3.72 (1.62)	16.41 (3.12)	6.47 (0.63)
	2012	11.62 (1.51)	0.3 (0.11)	8.16 (1.08)	6.1 (0.9)	0.65 (0.43)	5 (0.65)
	2013	3.16 (1.07)	3 (0.62)	3.05 (0.35)	4.73 (1.14)	2.94 (0.7)	3.59 (0.55)
	2014	11.15 (2.83)	1.13 (1.13)	3.66 (0.52)	3.48 (1.82)	0.8 (0.8)	3.81 (0.61)
	Grand Mean	7.43 (0.93)	2.91 (0.66)	6.13 (0.42)	5.06 (0.62)	4.6 (0.98)	4.66 (0.31)

		Contrast $_{Control-Calcium}$[b]								
		Atlantic			Superior			F1		
		Mean Difference (95%CI)	Adjusted p-value		Mean Difference (95%CI)	Adjusted p-value		Mean Difference (95%CI)	Adjusted p-value	
Hollow Heart (%)[a]	2011	7.3 (-3.8,18.4)	2.22E-01	NS	-2.01 (-4.56,0.55)	2.48E-02	*	1.36 (-0.15,2.88)	5.27E-02	.
	2012	0.03 (-0.36,0.43)	9.21E-01	NS	-0.1 (-0.24,0.04)	1.62E-01	NS	0.98 (0.28,1.68)	3.15E-02	*
	2013	0.52 (-2.43,3.46)	8.26E-01	NS	-0.75 (-1.66,0.15)	1.07E-01	NS	0.61 (-1.58,2.8)	9.07E-02	.
	2014	3.22 (-3,9.43)	2.95E-01	NS	-2.89 (-9.35,3.57)	2.11E-01	NS	-0.54 (-3.65,2.57)	5.35E-01	NS
	Grand Mean	1.55 (-0.24,3.34)	2.51E-01	NS	-0.89 (-1.54,-0.24)	1.27E-03	**	0.69 (-0.27,1.66)	2.52E-02	*
Pitted Scab (%)[a]	2011	4.52 (-8.33,17.36)	3.88E-01	NS	-0.41 (-2.38,1.57)	5.97E-01	NS	3.28 (0.51,6.04)	3.51E-02	*
	2012	11.55 (4.62,18.47)	1.31E-02	*	4.02 (0.09,7.95)	2.23E-01	NS	10.84 (7.85,13.83)	3.92E-08	***
	2013	2.18 (-1.08,5.43)	2.30E-01	NS	0.5 (-1.1,2.09)	7.00E-01	NS	1.38 (0.08,2.67)	1.46E-01	NS
	2014	2.16 (-16.71,21.03)	8.66E-01	NS	1.08 (-3.08,5.24)	5.64E-01	NS	0.52 (-1.75,2.8)	6.41E-01	NS
	Grand Mean	6.25 (2.62,9.87)	1.80E-02	*	1.78 (0.06,3.5)	2.96E-01	NS	4.32 (3.11,5.53)	1.81E-06	***
Internal Brown Spot (%)[a]	2011	0.02 (-3.69,3.73)	9.98E-01	NS	-7.81 (-13.99,-1.62)	4.88E-02	*	3.55 (1.72,5.38)	1.65E-02	*
	2012	5.52 (2.3,8.74)	3.72E-03	**	-0.36 (-1.21,0.5)	9.39E-01	NS	3.15 (1.61,4.7)	1.58E-02	*
	2013	-1.57 (-4.11,0.98)	1.42E-01	NS	0.07 (-2,2.13)	8.79E-01	NS	-0.54 (-1.69,0.62)	6.71E-01	NS
	2014	7.67 (-1.6,16.94)	2.02E-01	NS	0.33 (-3.95,4.61)	9.23E-01	NS	-0.15 (-1.78,1.48)	7.67E-01	NS
	Grand Mean	2.37 (0.44,4.29)	1.56E-01	NS	-1.68 (-3.22,-0.15)	2.62E-01	NS	1.47 (0.71,2.23)	6.44E-03	**

Significance thresholds: p<0.1 (·), p<0.05 (*), p<0.01 (**), p<0.001 (***), NS=Not Significant

Table 6: ANOVA main effects, mixed model variance components, and broad-sense heritability's for hollow heart, pitted scab, and internal brown spot for parents and F1 population.

Traits	Year	Main Effects					Variance Components				Broad-sense Heritability
		Genotype	Year	Calcium Treatment	Genotype x Year	Rep	σ^2_G	σ^2_Y	σ^2_{GxY}	σ^2_e	H^2
Hollow Heart (%)[a]	2011	***	-	.	-	***	0.14	-	-	0.12	0.78
	2012	***	-	*	-	.	0.08	-	-	0.05	0.82
	2013	***	-	.	-	NS	0.19	-	-	0.18	0.75
	2014	***	-	NS	-	*	0.09	-	-	0.26	0.51
	all	***	***	*	***	***	0.08	0.03	0.04	0.15	0.79
Pitted Scab (%)[a]	2011	***	-	*	-	***	0.10	-	-	0.21	0.59
	2012	***	-	***	-	***	0.15	-	-	0.17	0.74
	2013	***	-	NS	-	***	0.05	-	-	0.17	0.49
	2014	*	-	NS	-	*	0.02	-	-	0.26	0.21
	all	***	***	***	***	***	0.05	0.07	0.03	0.19	0.70
Internal Brown Spot (%)[a]	2011	***	-	*	-	NS	0.09	-	-	0.19	0.59
	2012	***	-	*	-	NS	0.18	-	-	0.13	0.80
	2013	***	-	NS	-	NS	0.05	-	-	0.16	0.50
	2014	NS	-	NS	-	NS	0.00	-	-	0.23	0.00
	all	***	***	**	***	NS	0.02	0.02	0.06	0.17	0.39

Significance thresholds: p<0.1 (·), p<0.05 (*), p<0.01 (**), p<0.001 (***), NS=Not Significant

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Appendix to Chapter 2

Cultivar	Factor Level	Leaf Adaptation/Time Period					
		Unadapted (DAHS)				Heat-adapted (DAHS)	
		Control	1	7	14	21	28
ATL	Plant Reps	4	4	4	4	NA	6
	Leaves/Rep	1-3	1-3	1-3	1-3	NA	6
	Measurement/Leaf	4	4	4	4	NA	4
	Total Measurements/ Time Period	36	36	36	36	NA	148
DRN	Plant Reps	6	6	6	6	5	NA
	Leaves/Rep	1-3	1-3	1-3	1-3	2	NA
	Measurement/Leaf	4	4	4	4	4	NA
	Total Measurements/ Time Period	56	56	56	56	40	NA

Table A1: Measurements of photosynthesis, conductance, and leaf temperature were taken using a LI-6400XT Portable Photosynthesis System. Leaf measurements were taken on the youngest, fully-expanded terminal leaflets (3-4th node). Unadapted leaf measurements were taken during the control, and 1, 7 and 14 days after heat stress (DAHS). Adapted leaf measurements were taken after 21 and 28 DAHS for DRN and ATL, respectively.

Appendix to Chapter 3

Appendix Figures

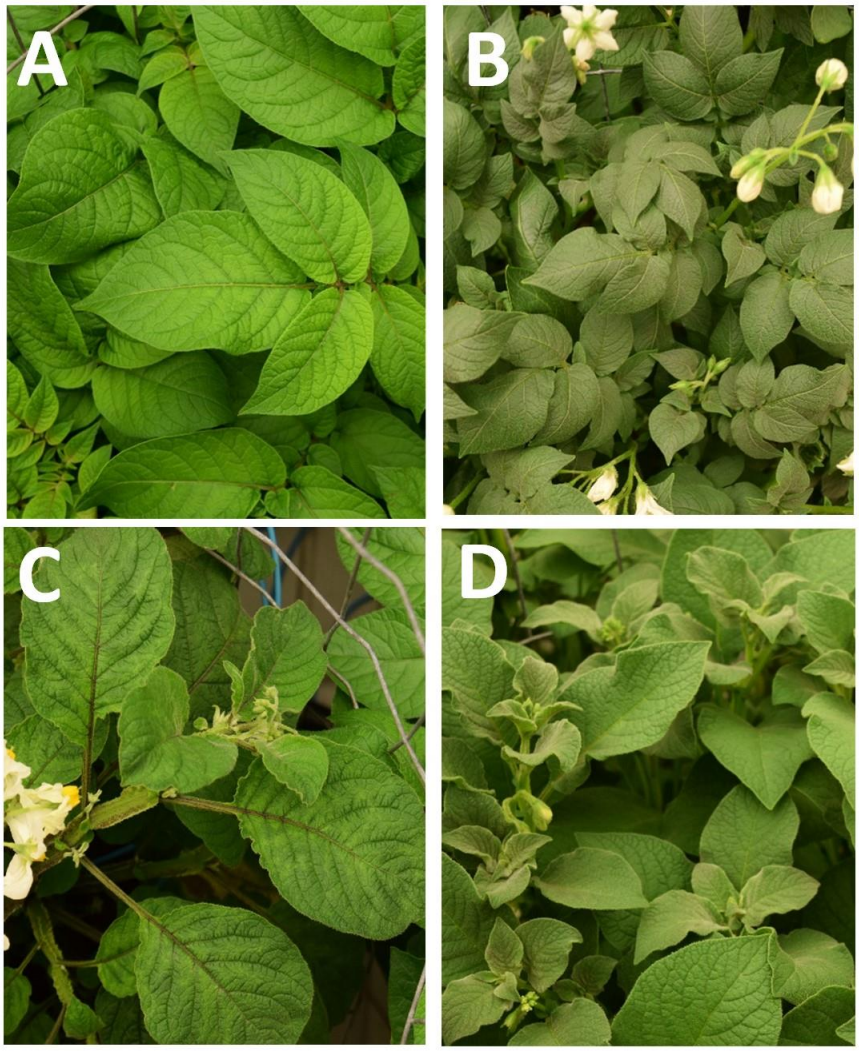

Figure A1: Leaves of *Solanum tuberosum* L. cv. 'Atlantic' (ATL) and *Solanum microdontum* (MCD 15) grown under control (20/15°C, day/night) and long-term high temperature (35/25°C, day/night for 4.5 weeks). A) ATL control B) ATL heat-adapted C) MCD control D) MCD heat-adapted.

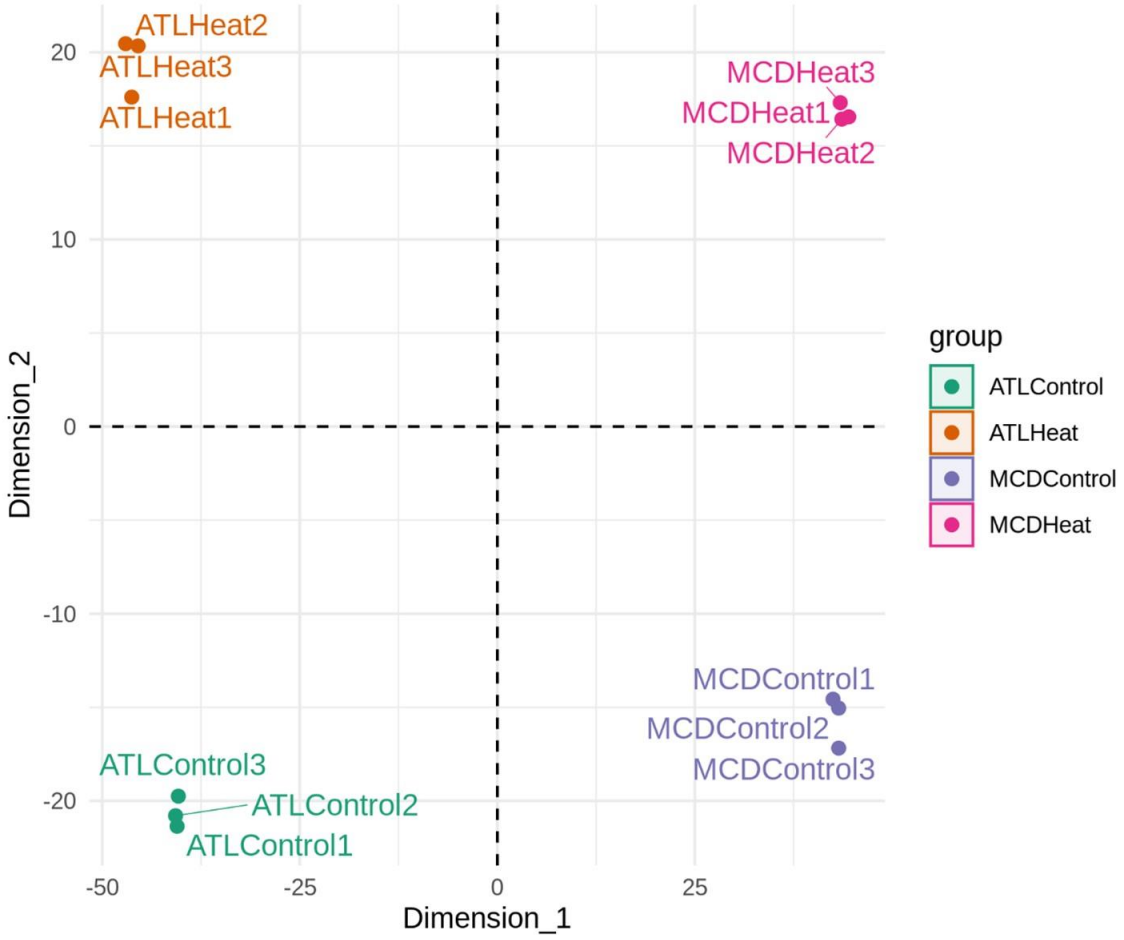

Figure A2: Multi-dimensional scaling (MDS) plot of normalized read counts of transcripts confirming the biological replicates of ATL and MCD in each condition (Control or Heat) share similar expression values.

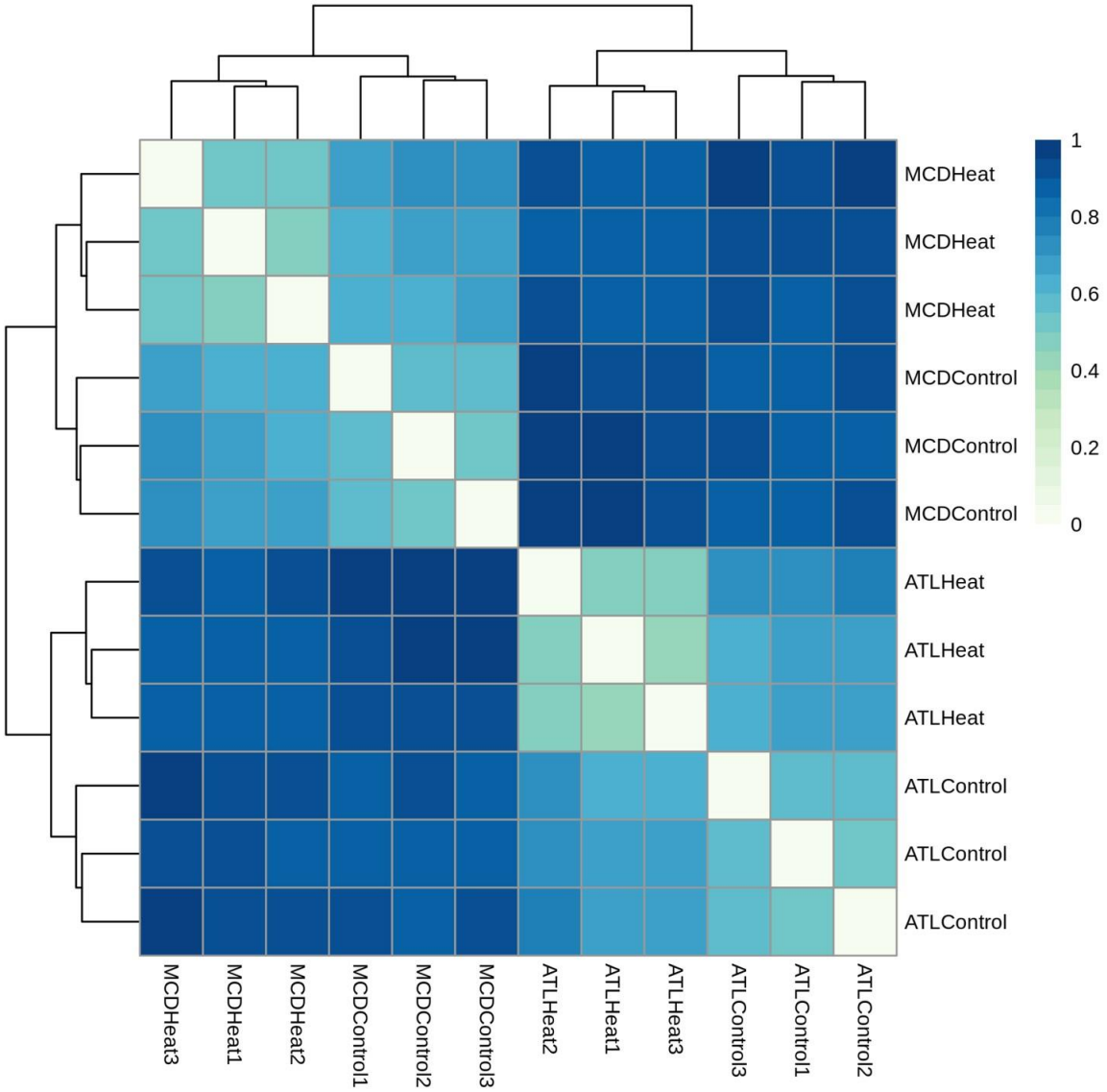

Figure A3: Cluster image map (heat map) of dissimilarities in read counts of transcripts confirming the biological replicates of ATL and MCD in each condition (Control or Heat) share similar expression values.

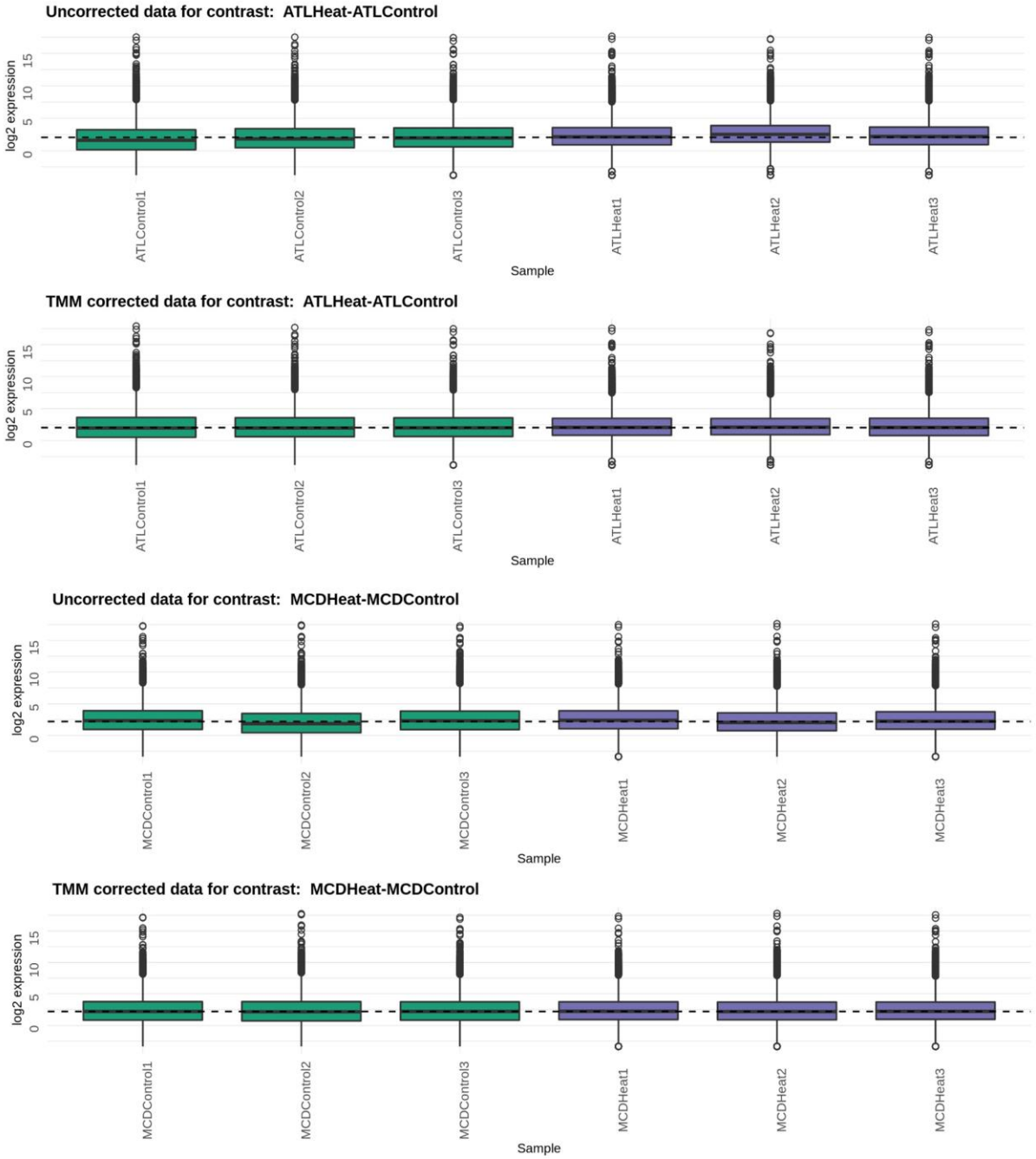

Figure A4: Raw (uncorrected) and normalized (corrected) library of per sample expression distributions before and after trimmed mean of M-values (TMM) normalization for ATL and MCD, respectively, showing a more uniform distribution centered on a common median after normaliztion. The effective library size (corrected/normalized) is the product of the TMM and the original library size (uncorrected).

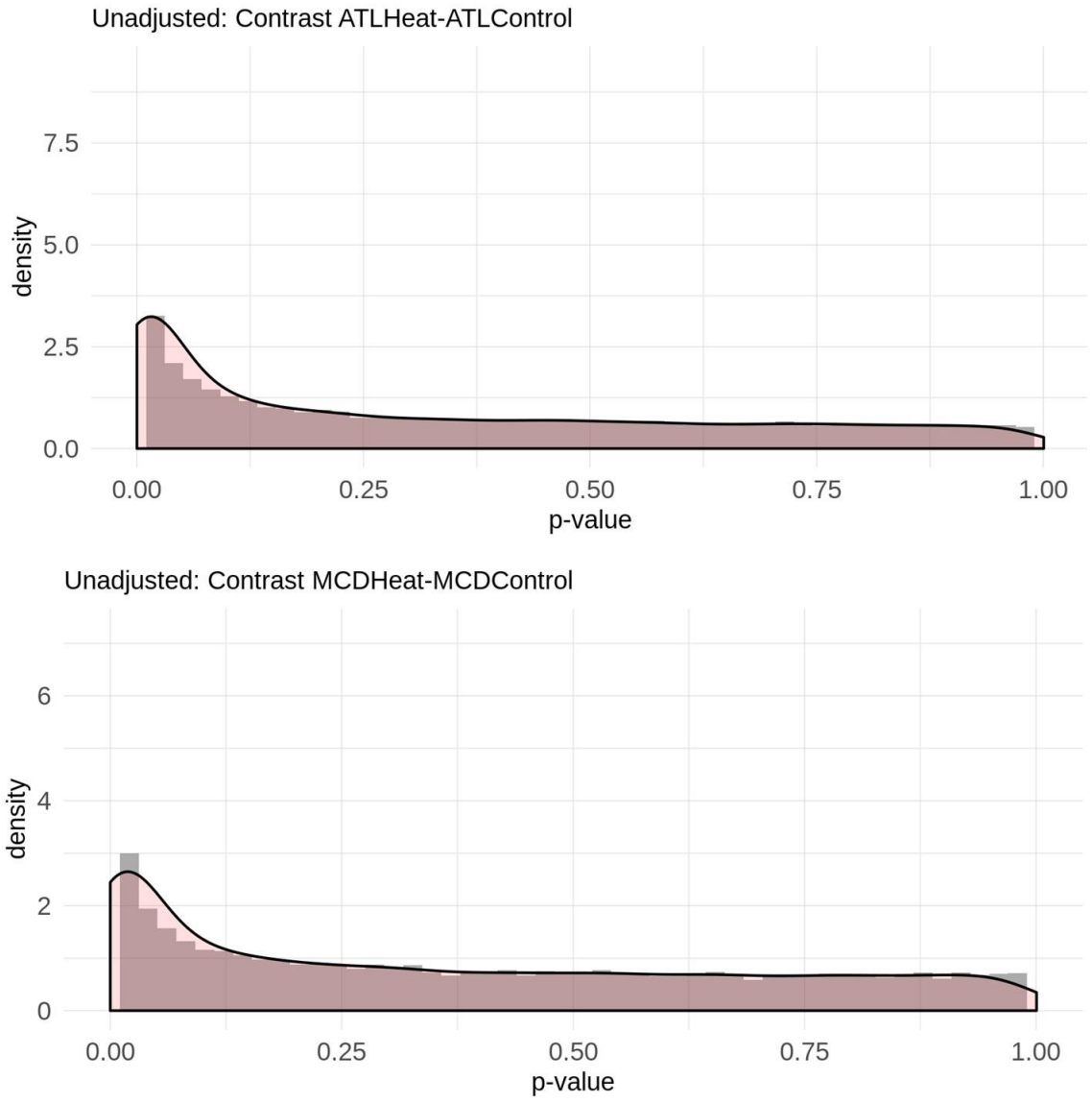

Figure A5: Uncorrected p-value histograms for ATL and MCD transcript expression indicating no issues with the mthods operating on p-values where the enrichment of smaller p-values comes from differentially expressed transcripts, while the transcripts that are not differentially expressed are spread evenly across the rest of the range.

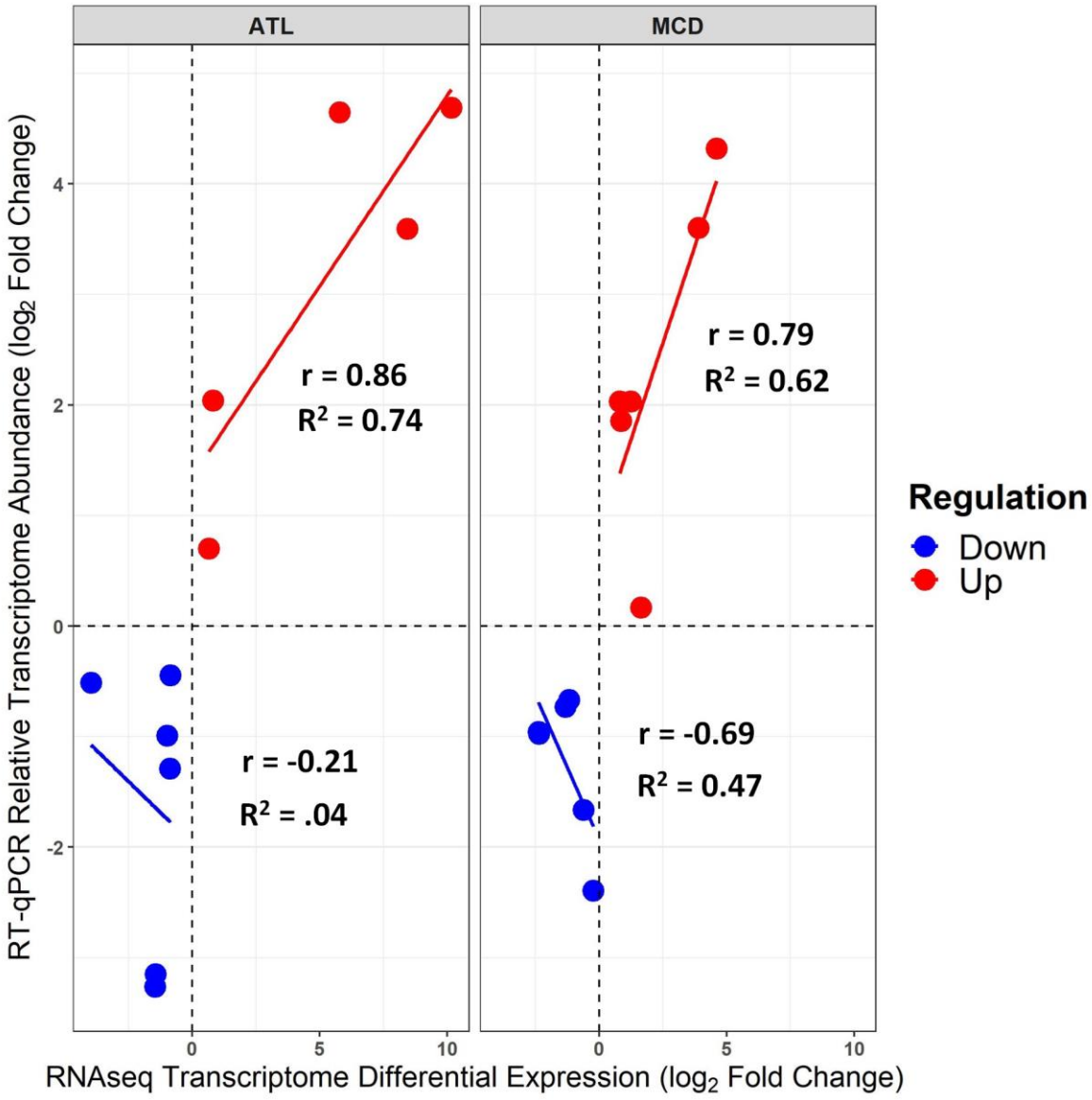

Figure A6: Correlation analysis of the results from the RNA-seq and the RT-qPCR analyses. Twelve significantly expressed transcripts were chosen from the RNA-seq analysis data and RT-qPCR was used to validate their change in expression between control (20/15°C, day/night) and long-term high temperature (35/25°C, day/night for 4.5 weeks). The values RNA-seq (log_2 Fold Change) and RT-qPCR (log_2 Fold Change) represent the change in expression of significantly up- and down-regulated transcripts under long-term high temperature compared to its expression under the control.

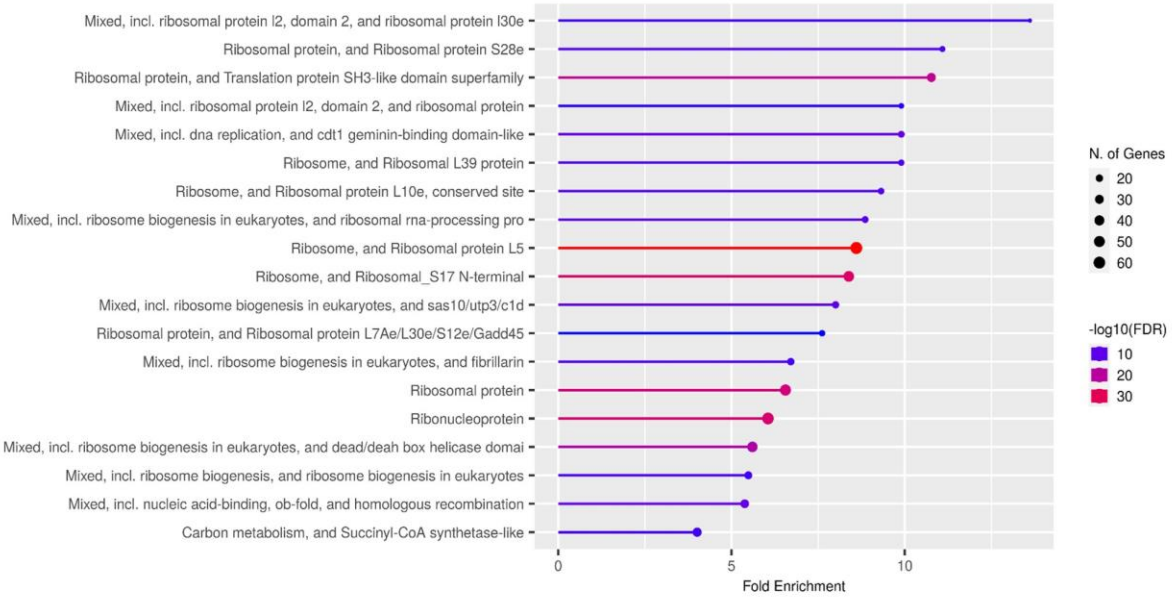

Figure A7: Enrichment analysis derived from ensembl gene ID's for significantly (FDR<0.05) down-regulated genes that are shared between ATL and MCD from the RNAseq expression data. The down-regulated gene pathways that are highly enriched are involved in ribosome protein activity, ribosome biosynthesis, nucleic acid binding, and carbon metabolism. For this analysis the FDR tells us the likelihood of enrichment by chance and is calculated from the p-value obtained from the hypergeometric test and is displayed here as the -log10(FDR), which displays greater significance with a higher value. The fold enrichment is defined here as the percentage of genes in the test set for a particular pathway, divided by its corresponding percentage in the reference/background gene set. The background gene-set for the enrichment uses the unique gene ID's of all expressed genes for ATL and MCD (17,427) detected in the experiment. Redundant pathways have been removed, so those sharing 95% of genes and 50% of the words in their names are represented by the pathway that is most significant.

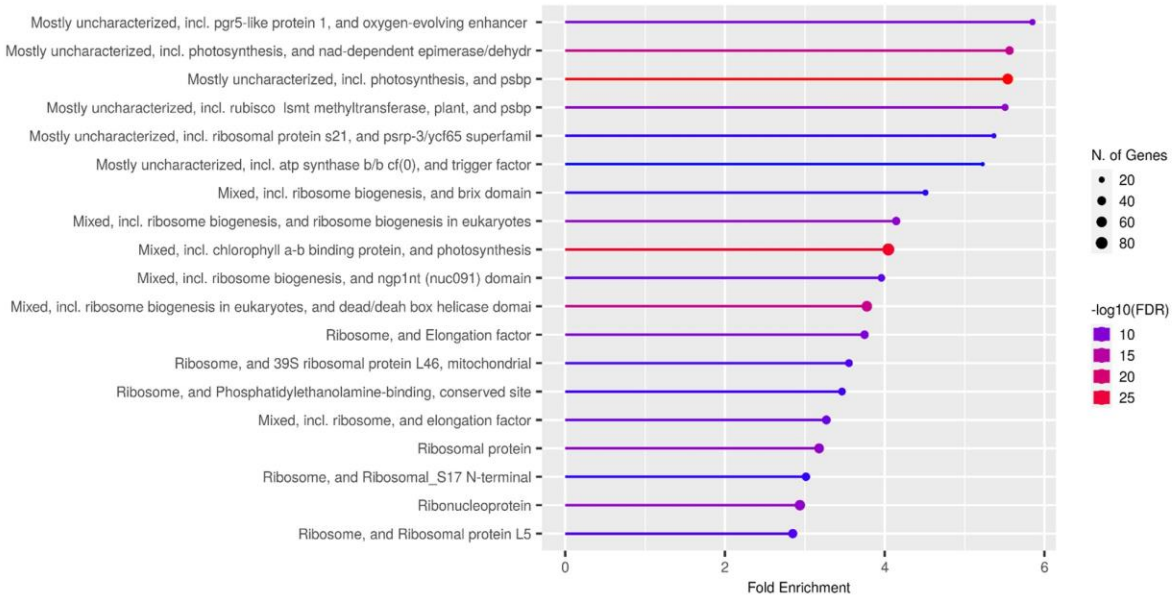

Figure A8: Enrichment analysis derived from ensembl gene ID's for significantly (FDR<0.05) down-regulated genes unique to ATL from the RNAseq expression data. The down-regulated gene pathways that are unique to ATL that are highly and significantly enriched are mainly involved in photosynthesis. Other enriched pathways include ribosomal activity and biosynthesis and ATP enzymatic activity. For this analysis the FDR tells us the likelihood of enrichment by chance and is calculated from the p-value obtained from the hypergeometric test and is displayed here as the -log10(FDR), which displays greater significance with a higher value. The fold enrichment is defined here as the percentage of genes in the test set for a particular pathway, divided by its corresponding percentage in the reference/background gene set. The background gene-set for the enrichment uses the gene ID's of all expressed genes for ATL (16,575) detected in the experiment. Redundant pathways have been removed, so those sharing 95% of genes and 50% of the words in their names are represented by the pathway that is most significant.

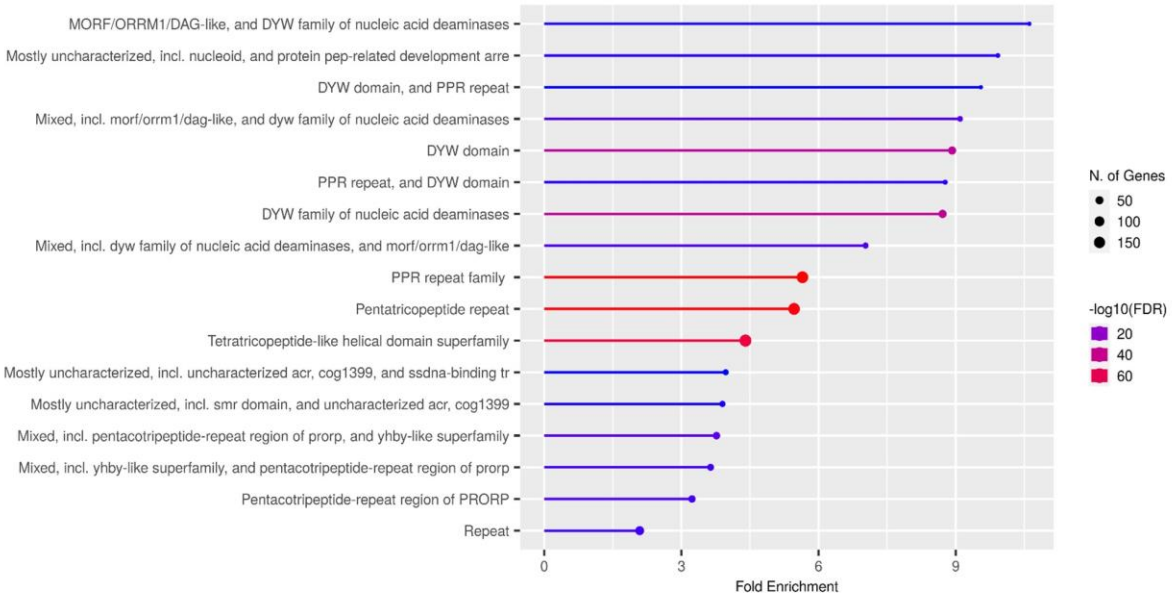

Figure A9: Enrichment analysis derived from ensembl gene ID's for significantly (FDR<0.05) up-regulated genes unique to MCD from the RNAseq expression data. The up-regulated gene pathways that are unique to MCD that are enriched are mainly involved in RNA editing (DYW and PPR repeat). For this analysis the FDR tells us the likelihood of enrichment by chance and is calculated from the p-value obtained from the hypergeometric test and is displayed here as the -log10(FDR), which displays greater significance with a higher value. The fold enrichment is defined here as the percentage of genes in the test set for a particular pathway, divided by its corresponding percentage in the reference/background gene set. The background gene-set for the enrichment uses the gene ID's of all expressed genes for MCD (15,838) detected in the experiment. Redundant pathways have been removed, so those sharing 95% of genes and 50% of the words in their names are represented by the pathway that is most significant.

Appendix Tables

Table A1: The sample-level mapping qaulity control (QC) statistics for each alignment file including the total number of fastq mapping reads, the total number of alignments, and the percentage of primary and secondary alignments (reads mapped to more than 1 sequence) showing how well the reads aligned (mapped) to each sample using the SolTub_3.0 genome assembly.

FileName	Factor	rawReads	Aligned	Primary	%	Secondary	%	Unaligned	%
ATLControl-1_S187_L002_R1_00Aligned.sortedByCoord.out.bam	ATLControl1	123916416	140437418	105549230	66.46484	34888188	21.96925	18367186	11.56590
ATLControl-2_S188_L002_R1_00Aligned.sortedByCoord.out.bam	ATLControl2	115682938	131003630	99575927	67.68778	31427703	21.36331	16107011	10.94891
ATLControl-3_S189_L002_R1_00Aligned.sortedByCoord.out.bam	ATLControl3	105350884	117791735	90836883	68.65680	26954852	20.37315	14514001	10.97005
ATLHeat-1_S190_L002_R1_00Aligned.sortedByCoord.out.bam	ATLHeat1	116341070	128329340	100832156	70.10107	27497184	19.11674	15508914	10.78219
ATLHeat-2_S191_L002_R1_00Aligned.sortedByCoord.out.bam	ATLHeat2	119115248	128251879	102784503	71.09049	25467376	17.61441	16330745	11.29510
ATLHeat-3_S192_L002_R1_00Aligned.sortedByCoord.out.bam	ATLHeat3	115680282	126048023	100129135	70.71308	25918888	18.30441	15551147	10.98251
MCDControl-1_S193_L002_R1_00Aligned.sortedByCoord.out.bam	MCDControl1	113168454	66403350	52174282	40.95392	14229068	11.16903	60994172	47.87705
MCDControl-2_S194_L002_R1_00Aligned.sortedByCoord.out.bam	MCDControl2	117027076	90709603	68808170	49.52775	21901433	15.76453	48218906	34.70771
MCDControl-3_S195_L002_R1_00Aligned.sortedByCoord.out.bam	MCDControl3	117796982	76546517	57602991	42.12577	18943526	13.85363	60193991	44.02060
MCDHeat-1_S196_L002_R1_00Aligned.sortedByCoord.out.bam	MCDHeat1	114163836	121946496	96111273	68.65137	25835223	18.45385	18052563	12.89477
MCDHeat-2_S197_L002_R1_00Aligned.sortedByCoord.out.bam	MCDHeat2	116762012	128123543	98763623	67.58987	29359920	20.09275	17998389	12.31738
MCDHeat-3_S198_L002_R1_00Aligned.sortedByCoord.out.bam	MCDHeat3	105909872	115088521	90220357	68.98739	24868164	19.01555	15689515	11.99706

Table A2: Validation set of 12 gene transcript primers used for RT-qPCR analysis to confirm regulation in our illumina expression anlaysis along with their log fold change values and sequences.

Name/ Annotation	ATL Log_2FC[a]	MCD Log_2FC[a]	PGSC Representative Gene Model	Set #	Forward Sequence 5'-3'	Reverse Sequence 3'-5'	Amplicon Length (bp)
Transcription factor FAMA	2.33	1.63	PGSC0003DMT400040761	1	TGAGGAAGTTGAGAGCCAAAG	CAGGCATGAGAGACCTCAATAC	101
				2	AAGTCTTGTTTGGCTGATGTTG	GAGTTGTCCTGGTCTTCTTCTT	87
EPIDERMAL PATTERNING	-0.85	-0.23	PGSC0003DMT400043275	1	CCGAGCCTACCGAGTAATAATG	CTACCGTGTTGTGGTCCATAG	85
				2	GGACCACAACACGGTAGAAT	GCTAGGGATTGTAGAAGTGACC	113
ER glycerol-phosphate acyltransferase	0.82	0.81	PGSC0003DMT400069797	1	AATGTCCTTCCTGCTGCTATC	CCCTCACAGCCATCATCTAAC	133
				2	CCCATCAATGACTGAGGAAGAG	AGAACTCATAAGGGTGCAGAAG	123
Heat shock protein	5.78	4.60	PGSC0003DMT400036856	1	CACAGCCTCATCAGGGTTAAT	GTTCTTGTTGGTGGCTCTACT	105
				2	CACTTCTCCACAGGTTCCATAC	GCTACAATTACCAGGGCAAGA	77
MAP protein kinase	0.65	0.87	PGSC0003DMT400026093	1	GCACGGATAAATCAAGTCCATAAC	AGTCATCTCAAGCACCCAAATA	98
				2	CCAAAGGAGGAAGTGGTAGTG	TAAGCCCACAGAGGAGTAGAA	138
Chlorophyll a-b binding protein 3C	-3.96	-2.38	PGSC0003DMT400034896	1	AGAACGGCAGACTTGCTATG	CCCAAGCGTTGTTGTTTACTG	123
				2	GTATGGTCCTGATCGTGTTAAGT	CAGCAGTATCCCATCCATAGTC	101
LRR receptor-like serine/threonine-protein	-0.88	-0.61	PGSC0003DMT400044535	1	CATGGTGCAGATGCCTCTTA	GCCTGTACAGTCTCCTCATTAC	109
				2	TGCCCTTGTCTACTGGTTTG	GAAGCACCTCTGGGTTCTAAAT	126
L-allo-threonine aldolase	-1.46	-1.17	PGSC0003DMT400029948	1	CGCTCAGACACAGTCACTAAA	CGTTGGGCTGTTGGATCATA	101
				2	CTGGAAGGAGATCACAGAAAGG	AGTTCCGCTGAGAAGTAAATAGG	119
Phenylalanine ammonia-lyase	-0.98	-2.36	PGSC0003DMT400080548	1	CCACCATGTAAAGCCTTGTTTC	TGGCCCTCAGATTGAAGTTATT	114
				2	GCCATTGTGGTGACGTTCTA	TTGGATGGGAGTGCTTATGTG	106
Phytochrome-interacting factor	10.16	3.90	PGSC0003DMT400048756	1	GGGATATAGGAGATCGGAGTAGAG	TCTGTTTATGCAGGAGGATGAG	97
				2	CCATCTCATCCTCCTGCATAAA	TGATGCGGTTATTCCCTCTG	98
				3	ATGGGACTTTAACCCACTCTAAC	ACAAAGGGCAGGGATAACTAAA	122
Mitogen-activated protein kinase kinase	-1.43	-1.31	PGSC0003DMT400057171	1	GCTTGTAGGAGAATCCCTCTTC	ATGAGCGTTGGACAGCTAAA	104
				2	ACCCAATCTTCTTCCTCAACC	CCGTTGATTCCGCTACAGATAG	97
Peroxidase 44	8.42	1.24	PGSC0003DMT400062402	1	GAGGGCTTGTGGAATTGTTAATG	AGCTGGAGGCCCAAATTATC	109
				2	CAGTGATGGAAGGGTCACTATT	CCATTTAGTTTCAGCCCAACTTC	119

[a]Log_2 of the fold change in gene expression under heat stress as compared to the control gene expression from the differential gene expression analysis using RNAseq. Tm (°C) for all primers was 62°C.

Table A3: Correlation analysis of the results from the RNA-seq and the RT-qPCR analyses. Twelve significantly expressed transcripts were chosen from the RNA-seq analysis data and RT-qPCR was used to validate their change in expression between control (20/15°C, day/night) and long-term high temperature (35/25°C, day/night for 4.5 weeks). The values RNA-seq (log_2 Fold Change) and RT-qPCR (log_2 Fold Change) represent the change in expression of significantly up- and down-regulated transcripts under long-term high temperature compared to its expression under the control.

Gene ID	Gene Name	Expression	Genotype	RNA-seq (log_2 fold change)	RT-qPCR (log_2 fold change)	Correlation	R^2
PGSC0003DMT400029948	threonine aldolase	Down	ATL	-1.46	-3.26	-0.21	0.04
PGSC0003DMT400034896	component LHCb1/2/3 of LHC-II complex			-3.96	-0.51		
PGSC0003DMT400043275	EPIDERMAL PATTERNING FACTOR 1			-0.85	-0.44		
PGSC0003DMT400044535	LRR receptor-like serine/threonine-protein kinase			-0.88	-1.29		
PGSC0003DMT400057171	Mitogen-activated protein kinase kinase kinase			-1.43	-3.15		
PGSC0003DMT400080548	Phenylalanine ammonia-lyase			-0.98	-0.99		
PGSC0003DMT400026093	MAP protein kinase	Up	ATL	0.65	0.70	0.86	0.74
PGSC0003DMT400036856	Heat shock protein			5.78	4.65		
PGSC0003DMT400040761	Transcription factor FAMA			2.33	-0.1[a]		
PGSC0003DMT400048756	Phytochrome-interacting factor			10.16	4.69		
PGSC0003DMT400062402	Peroxidase 44			8.42	3.59		
PGSC0003DMT400069797	SDIR1 signal transducer of abscisic acid perception			0.82	2.04		
PGSC0003DMT400029948	threonine aldolase	Down	MCD	-1.17	-0.67	-0.69	0.47
PGSC0003DMT400034896	component LHCb1/2/3 of LHC-II complex			-2.38	-0.96		
PGSC0003DMT400043275	EPIDERMAL PATTERNING FACTOR 1			-0.23	-2.39		
PGSC0003DMT400044535	LRR receptor-like serine/threonine-protein kinase			-0.61	-1.66		
PGSC0003DMT400057171	Mitogen-activated protein kinase kinase kinase			-1.31	-0.73		
PGSC0003DMT400080548	Phenylalanine ammonia-lyase			-2.36	-0.97		
PGSC0003DMT400026093	MAP protein kinase	Up	MCD	0.87	1.86	0.79	0.62
PGSC0003DMT400036856	Heat shock protein			4.60	4.32		
PGSC0003DMT400040761	Transcription factor FAMA			1.63	0.17		
PGSC0003DMT400048756	Phytochrome-interacting factor			3.90	3.60		
PGSC0003DMT400062402	Peroxidase 44			1.24	2.03		
PGSC0003DMT400069797	SDIR1 signal transducer of abscisic acid perception			0.81	2.03		

[a] Value not included in correlation calculation

Appendix to Chapter 4

Additional details for each experiment

Heat Tolerance Experiment # 1

Tuber-derived plantlets of *Solanum kurtzianum* genotypes were initially planted at the UW-Madison Walnut Street Greenhouse. Plants were potted using a soilless media (Sungro Horticulture Professional Growing Mix) in a 5.1L pot (classic #400, Nursery Supplies, Inc.) that were 19.05cm in diameter. Approximately 36 DAP all plants were transferred to a controlled environment room at the UW-Madison Biotron, transplanted into classic #2000 pots (Nursery Supplies, Inc.), and supported with a cylindrical 122 cm (4 ft) galvanized metal cage. Plants were initially fertilized with peter's 20-10-20 fertilizer twice weekly for 31 DAP. A ¼ strength (QS) standard Hoagland's Solution (1ml/L each of standard stocks A, B and C) was given by hand 38 DAP every other day and was given in excess to saturate and flush the soil to prevent excess accumulation of fertilizer salts. At 48 DAP plants were given this standard Hoagland's daily in the same manner. At 59 DAP a modified ¼ strength (QS) Hoagland's Solution (Standard Hoagland's stock A is replaced with 1ml/L 2.5M $CaCl_2$ and 1ml/L 2.5M NH_4NO_3 plus 1ml/L each of standard stocks B and C) was given every day in the same manner. Light intensity averaged ~400 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at the top of the 122 cm cage. Rooms were set to maintain a 14-hour photoperiod and relative humidity was not able to be controlled. Day and night temperatures were set at 20/15°C for the control and were used up to 85 DAP. At 86 DAP heat stress conditions were initiated with temperatures set at 35/25°C, day/night, with all other factors held constant. With these setpoints, temperatures and relative humidity were recorded in the growth room at ~18°C/44% RH for the control and ~35.8°C

/23% RH for heat stress conditions. The control leaf heat assay was performed 80 DAP. The heat-treated leaf heat assay was performed 26 days (112 DAP) after heat stress initiation.

Heat Tolerance Experiment #2

Tissue culture plants of *Solanum tuberosum* varieties Papa Cacho, DTO-2, Zarewo, and Olalla were initially planted at the UW-Madison Walnut Street Greenhouse. Additionally, tissue culture plants of *Solanum microdontum* genotypes MCD24 and MCD35 were also planted. Plants were potted using a soilless media (Promix Soilless Media) in a 5.1L pot (classic #400, Nursery Supplies, Inc.) that were 19.05cm in diameter. At 41 DAP plants were transplanted into classic #2000 pots (Nursery Supplies, Inc.) and supported with a cylindrical 122 cm (4 ft) galvanized metal cage. At 69 DAP all plants were transferred to a controlled environment room at the UW-Madison Biotron. Plants were initially fertilized with peter's 20-10-20 fertilizer twice weekly up until 68 DAP. At 70 DAP a modified ¼ strength (QS) Hoagland's Solution (Standard Hoagland's stock A is replaced with 1ml/L 0.25M $CaCl_2$ and 1ml/L 2.5M NH_4NO_3 plus 1ml/L each of standard stocks B and C) was given by hand every day in excess so that the soil was saturated and flushed of excess fertilizer salts. Light intensity averaged ~400 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at the top of the 122 cm cage. Rooms were set to maintain a 14-hour photoperiod and relative humidity was set to 50%. Day and night temperatures were set at 20/15°C for the control and plants and the control leaf heat assay was performed 56 DAP. Heat stress conditions were initiated 71 DAP with temperatures set at 35/25°C, day/night, with all other factors held constant. The heat-treated leaf heat assay was performed 26 days (97 DAP) after heat stress initiation. With these setpoints, temperature and relative humidity were recorded in the growth room at ~18°C/44%

RH for the control and ~35.8°C /23% RH for heat stress conditions during the day. For this experiment the control-adapted leaves were heat assayed when plants were given Peter's fertilizer, while the heat stress-adapted leaves were heat assayed when the plants were given the modified Hoagland's fertilizer.

Heat Tolerance Experiment #3

Tissue culture plants of *Solanum commersonii* genotypes OKA5059-1, OKA5059-2, OKA5059-3, OKA5040-1, OKA5040-2, FER-1, and FER-2 were initially planted at the UW-Madison Walnut Street Greenhouse. Additionally, tissue culture plants of *Solanum microdontum* genotypes EBS626-1, EBS626-2, EBS626-3, EBS525-2, AND EBS525-3 were also planted. Plants were potted using a soilless media (Promix Soilless Media) in a 5.1L pot (classic #400, Nursery Supplies, Inc.) that were 19.05cm in diameter. At 73 DAP plants were transferred to a controlled environment room at the UW-Madison Biotron, transplanted into classic #2000 pots (Nursery Supplies, Inc.), and supported with a cylindrical 122 cm (4 ft) galvanized metal cage. Plants were initially fertilized with peter's 20-10-20 fertilizer twice weekly up until 72 DAP. At 73 DAP a modified ¼ strength (QS) Hoagland's Solution (Standard Hoagland's stock A is replaced with 1ml/L 0.25M $CaCl_2$ and 1ml/L 2.5M NH_4NO_3 plus 1ml/L each of standard stocks B and C) was given by hand every day in excess so that the soil was saturated and flushed of excess fertilizer salts. Light intensity averaged ~400 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at the top of the 122 cm cage. Rooms were set to maintain a 14-hour photoperiod and relative humidity was set to 50%. Day and night temperatures were set at 20/15°C for the control conditions in the controlled environment room. Heat stress conditions were initiated 103 DAP with temperatures set at 35/25°C (day/night) with all other factors

held constant. With these setpoints, temperature and relative humidity were recorded in the growth room at ~18°C/44% RH for the control and ~34.6°C /17% RH for heat stress conditions during the day. For this experiment the control-adapted leaves and the heat stress-adapted leaves were heat assayed when the plants were given the modified Hoagland's fertilizer. The control leaf heat assay was performed 80 DAP. The heat-treated leaf heat assay was performed 34 days (137 DAP) after heat stress initiation.

Heat Tolerance Experiment #4

Tissue culture plants of *Solanum commersonii* genotypes OKA5059-2, OKA5040-2, and FER-1 were planted at the UW-Madison Biotron in a controlled environment room. Additionally, tissue culture plants of *Solanum microdontum* genotypes EBS626-2, MCD24, and MCD35 and tissue culture plants of *Solanum tuberosum* L. varieties Bora Valley, Papa Cacho, Zarewo, and DTO-2 were also planted. Plants were potted using a soilless media (Promix Soilless Media) in a 5.1L pot (classic #400, Nursery Supplies, Inc.) that were 19.05cm in diameter. At 34 DAP plants were transplanted into classic #2000 pots (Nursery Supplies, Inc.) and supported with a cylindrical 122 cm (4 ft) galvanized metal cage. Plants were initially fertilized with a modified ¼ strength (QS) Hoagland's Solution (Standard Hoagland's stock A is replaced with 1ml/L 0.25M $CaCl_2$ and 1ml/L 2.5M NH_4NO_3 plus 1ml/L each of standard stocks B and C) up to 45 DAP and was given by a drip irrigation system every day in excess so that the soil was saturated and flushed of excess fertilizer salts. A ¼ strength (QS) standard Hoagland's Solution (1ml/L each of standard stocks A, B and C) was given starting at 46 DAP and was allowed to saturate and flush the soil to prevent excess accumulation of fertilizer salts. Light intensity averaged ~400 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at the top of the

122 cm cage. Rooms were set to maintain a 14-hour photoperiod and relative humidity was set to 50%. Day and night temperatures were set at 20/15°C for the control conditions in the controlled environment room. Heat stress conditions were initiated 118 DAP with temperatures set at 35/25°C, day/night. With these setpoints, temperature and relative humidity were recorded in the growth rooms at ~18°C/44% RH for the controls and ~30-31°C /30-52% RH for heat stress conditions during the day. The control leaf heat assay was performed 95 DAP. The heat-treated leaf heat assay was performed 33 days (151 DAP) after heat stress initiation.

Heat Tolerance Experiment #5

Tissue culture plants of *Solanum tuberosum* cvs. 'Atlantic' (ATL), 'Russet Burbank' (BUR), and 'Snowden' (SNW) were planted at the UW-Madison Biotron in separate controlled environment rooms in a 5.1L pot (classic #400, Nursery Supplies, Inc.) that were 19.05cm in diameter. Upon planting all plants were covered with clear plastic cups for 3 days to allow the tissue culture plants to acclimate to the conditions of the controlled environment room. These cups were vented on the 2nd day and removed 3 days after planting (DAP). At 34 DAP all plants were transplanted into classic #2000 pots (Nursery Supplies, Inc.) and all plants were supported with a cylindrical 122 cm (4 ft) galvanized metal cage at this time. All plants were potted using a soilless media (Sungro Horticulture Professional Growing Mix). Plants were initially fertilized 6 DAP with a modified ¼ strength (QS) Hoagland's Solution (Standard Hoagland's stock A is replaced with 1ml/L 0.25M$CaCl_2$ and 1ml/L 2.5M NH_4NO_3 plus 1ml/L each of standard stocks B and C) and received this fertilizer everyday through an automatic drip irrigation system until the soil was completely saturated. A ¼ strength (QS)

standard Hoagland's Solution (1ml/L each of standard stocks A, B and C) was given 42 DAP and was allowed to saturate and flush the soil to prevent excess accumulation of fertilizer salts. Light intensity averaged ~400 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at the top of the 122 cm cage in all rooms. Rooms were set to maintain a 14-hour photoperiod and relative humidity (RH) was set to 50%. Day and night temperatures were set at 20/15°C and were used up to 110 DAP. At 111 DAP heat stress was initiated with temperatures set at 35/25°C, day/night, with all other factors held constant. With these setpoints, temperatures and RH for the control in each room were recorded around 18°C/44% RH and heat stress conditions were recorded in each room at ~32°C/40% for ATL, ~33.9°C/40% RH for BUR, ~33.1°C/44% RH for SNW, respectively, during the day. Plants were held at heat stress temperatures for no less than 28 days. The control leaf heat assay was performed 87 DAP. The heat-treated leaf heat assay was performed 33 days (144 DAP) after heat stress initiation.

Additional Experimental Details and Insights

Comparisons between the 5 experiments in this study were not assessed due to the finding from an initial comparison that was performed to determine whether the heat sensitivity assessment based on ion leakage can be used to compare the same genotypes grown under similar temperatures but different conditions (fertilizer, locations, and physiological age of the plant at the time of assessment, see Figure A2 and Table A3), which found that comparisons need to be made between materials under similar conditions (other than treatment conditions). The terms heat tolerance and heat sensitivity are used interchangeably in the following results, discussion, and conclusions.

A group of 7 genotypes from 3 accessions (6, 35, and 36) of *Solanum kurtzianum*, a species known to be arid-adapted (Hawkes & Hjerting, 1969) and potentially heat-tolerant,

were utilized in the first heat assay experiment named HTM1 (Heat Tolerant Materials Experiment #1). An initial comparison was performed to determine whether the heat sensitivity assessment based on ion leakage can be used to compare the same genotypes grown under similar temperature conditions but different fertilizer regimens and locations. Initially, the *S. kurtzianum* genotypes were grown in a greenhouse under control conditions (20/15°C, Day/Night) using peters 20-10-20 fertilizer and the unadapted sensitivity was assessed under these conditions for this species. The plants were then subsequently transferred to a controlled environment room where they received a ¼ strength modified Hoagland's solution containing 2.5 mM Ca under control conditions (20/15°C, Day/Night) for at least 1-2 weeks before another assessment of unadapted sensitivity was performed. The visual observation of plot results showed that all genotypes, except for 35-2, 36-3, and 6-1 showed a similar leakage profile between the leaves of the same plants grown under a similar temperature but different nutrient regimen and location (See Appendix Figure A2). The genotype x fertilizer/location interactions for the estimated unadapted sensitivity values caused an inconsistent ranking for genotype between locations (See Appendix Table A3). The results show a low correlation (0.26) between locations for unadapted sensitivity (See Appendix Table A3) and it is suggested to make comparisons between unadapted and adapted ion leakage data from the same plants under similar conditions (fertilizer, relative humidity, light intensity, physiological age of plant, etc.) other than the temperature treatment conditions required here. Comparisons between the 5 experiments in this study were not assessed due to this finding as several differences between experiments existed,

including growing environment conditions, locations, and physiological age of the plant at the time of assessment.

In this study we used leaves from the same plant as the control and so control leaves were from a different physiological age of the plant than the heat-adapted leaves that were assessed later after heat stress.

Appendix Figures

Figure A1: Water bath setup with the maximum 72 tubes and color-coded "insulating caps" that were used to help keep the whole-leaf sample up to temperature (50°C) and keep it completely submerged underwater inside the large test tube and under the bathwater surface.

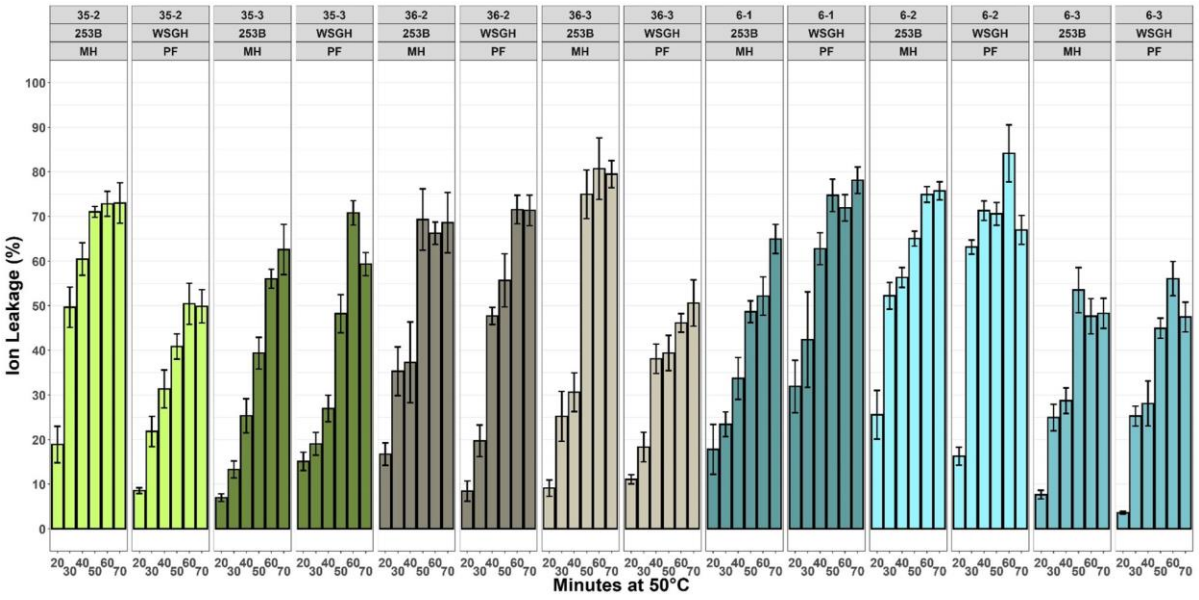

Figure A2: Ion leakage from leaf tissue of accessions of *Solanum kurtzianum Bitter and Wittm.* from experiment #1 following exposure to 50°C for various time periods. The control (unadapted) measurements in the greenhouse (WSGH) were made on fully-expanded, newly developed leaves after 4 weeks of control temperatures (20/15°C, day/night). The control (unadapted) measurements in the controlled-environment room (253B) were made on fully-expanded, newly developed leaves after 11.5 weeks of control temperatures (20/15°C, day/night). Values are means (+ SE), n = 6. MH = Modifed Hoagland's Solution Fertilizer; PF = Peters 20-10-20 Fertilizer.

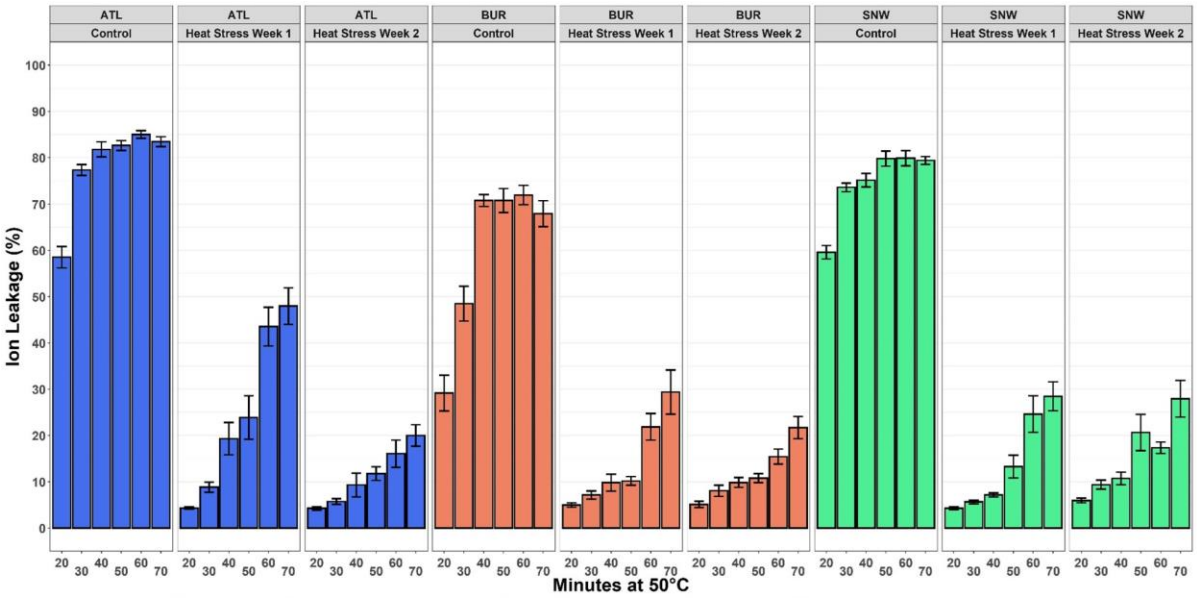

Figure A3: Ion leakage of unadapted leaves from the control (unadapted), heat stress week 1, and heat stress week 2 conditions from clones of three cultivars (Atlantic, ATL; Russet Burbank, BUR; Snowden, SNW) of *Solanum tuberosum* L. from experiment #5 following exposure to 50°C for various time periods. The control (unadapted) measurements were made on fully-expanded, newly developed leaves leaves after 12.5 weeks of control temperatures (20/15°C, day/night). The heat stress (unadapted) measurements were made on fully-expanded control-grown leaves (unadapted) from the same plants after 1 and 2 weeks of heat stress (35/25°C, day/night). Values are means (+ SE), n = 12.

Appendix Tables

Table A1: Quick reference table for determination of leaf sample number for assays with replicated genotypes.

# of Plant Reps Available · Genotype^{-1}		Samples · Rep^{-1} · Time^{-1}		Total # Samples · Genotype^{-1} · Time^{-1}		# of Time Treatments		Total # Samples · Genotype^{-1}	Samples · Rep^{-1}	# Genotypes Assessed · 72-Samples
1	x	6	=	6	x	6	=	36	36	2
2		3		6		6		36	18	2
3		2		6		6		36	12	2
1		6		6		3		18	18	4
2		3		6		3		18	9	4
3		2		6		3		18	6	4

Table A2: Determination of the three best time treatments with the most variation in ion leakage. Heat map density tends to red as values are increased.

Experiment	Minutes at 50°C	N	Average Percent Leakage	sd	se	ci
BE4	20	72	27	25.1	3.0	5.9
BE4	30	72	38	30.9	3.6	7.3
BE4	40	72	47	30.2	3.6	7.1
BE4	50	72	53	28.1	3.3	6.6
BE4	60	72	60	24.3	2.9	5.7
BE4	70	72	62	21.0	2.5	4.9
HTM1	20	84	10	8.7	0.9	1.9
HTM1	30	84	21	16.6	1.8	3.6
HTM1	40	84	28	17.6	1.9	3.8
HTM1	50	84	44	22.1	2.4	4.8
HTM1	60	84	49	20.4	2.2	4.4
HTM1	70	84	54	19.5	2.1	4.2
HTM2	20	84	15	12.4	1.4	2.7
HTM2	30	84	32	24.0	2.6	5.2
HTM2	40	84	42	25.9	2.8	5.6
HTM2	50	84	53	26.2	2.9	5.7
HTM2	60	84	61	23.8	2.6	5.2
HTM2	70	84	63	22.9	2.5	5.0
HTM3	20	156	16	14.9	1.2	2.4
HTM3	30	156	31	25.5	2.0	4.0
HTM3	40	156	43	27.9	2.2	4.4
HTM3	50	156	52	26.5	2.1	4.2
HTM3	60	156	61	24.2	1.9	3.8
HTM3	70	156	67	21.7	1.7	3.4
HTM4	20	120	20	19.8	1.8	3.6
HTM4	30	120	36	27.1	2.5	4.9
HTM4	40	126	50	27.6	2.5	4.9
HTM4	50	126	57	27.1	2.4	4.8
HTM4	60	120	64	24.1	2.2	4.4
HTM4	70	126	66	20.3	1.8	3.6
Overall	20	516	17	17.5	0.8	1.5
Overall	30	516	32	25.8	1.1	2.2
Overall	40	522	42	27.3	1.2	2.4
Overall	50	522	52	26.4	1.2	2.3
Overall	60	516	60	24.0	1.1	2.1
Overall	70	522	63	21.5	0.9	1.9

Table A3: Heat tolerance experiment #1 (HTM1) rank comparison between two locations with a similar temperature (20/15°C, day/night) but different fertilizer regime for the 30-40-50-minute time treatments. [a] The correlation between ion leakage from location 1 and 2 was low (0.26).

Genotype	Species	Location 1 Unadapted Sensitivity (% leakage)		Rank	Location 2 Unadapted Sensitivity (% leakage)		Rank
35-2	*S. kurtzianum*	31	b	1	60	a	7
35-3	*S. kurtzianum*	31	b	2	26	d	1
36-3	*S. kurtzianum*	32	b	3	44	c	4
6-3	*S. kurtzianum*	33	b	4	36	cd	3
36-2	*S. kurtzianum*	41	b	5	47	bc	5
6-1	*S. kurtzianum*	60	a	6	35	cd	2
6-2	*S. kurtzianum*	68	a	7	58	ab	6

[a] Significant differences determined by Duncan's Multiple Range Test. Means with similar letters are not significantly different.

Table A4: Summary of calculated heat sensitivity of unadapted and adapted leaves of various clones of *Solanum microdontum* . For these calculations data given in Figure 2 was used to calculate heat sensitivity, which is defined here as the average of ion leakage for 30-40-50-minute time treatments. [a]

Experiment	Genotype	Species	Unadapted Sensitivity	Adapted Sensitivity	HAA (%	Class[UAH]
#2	MCD24	*S. microdontum*	62 a	17 b	44 a	1
	MCD35	*S. microdontum*	66 a	31 a	35 a	6
#4	MCD24	*S. microdontum*	72 a	35 b	37 a	1
	MCD35	*S. microdontum*	75 a	51 a	24 b	6

[a] Significant differences determined by Duncan's Multiple Range Test within a column. Means with similar letters are not significantly different. [b] Heat Acclimation Ability, HAA = mean(Unadapted Leakage - Adapted Leakage), is calculated from the average of the 30, 40, and 50-minute time leakage data. At each time-point 6 separate measurements were made. Values are means, n = 18. [UAH] Class designation as described in Table 6.