Studies on the Genetic Basis for Thermotolerance in *Arabidopsis thaliana*

A thesis submitted to University of Glasgow for the degree of M.Sc

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Abstract

Vast tracts of land are available for arable food production but much of this is located in hot, arid regions. For crops to thrive in these conditions they will need to show improved drought tolerance and also improved thermotolerance as low water availability reduces transpiration resulting in increased leaf temperatures. Identification of traits and genes involved in drought tolerance has been one of the major areas of plant research over the last decade, but thermotolerance has received little attention. In this study two approaches were used to identify the genetic basis for improved thermotolerance in the model plant *Arabisopsis thaliana*.

In one set of experiments a gain-of-function heat stress screen (44 ^{o}C for 3 hours) was performed on a collection of Activation Tagged lines where individual plants were engineered to transcriptionally activate random sequences in the Arabidopsis genome. Preliminary experiments confirmed prior exposure to 37 ^{o}C for 1-3 hours acclimates Arabidopsis so that it survives better a subsequent heat stress event. A total of ~14,600 lines were screened and three mutants were isolated; secondary screens confirmed their improved thermotolerance phenotype, but in subsequent generations one of the lines developed a hypersensitive phenotype, another reverted to wild type, whilst the third retained its thermotolererant phenotype. This loss-of-phenotype through generations was attributed to gene silencing events which are not uncommon in dominant mutants. Further experiments on these three lines are now required to identify the loci of the disrupted gene(s) in each of these lines.

In the other set of experiments transgenic lines carrying a construct designed to constitutively express a MYB transcription factor were characterized. This MYB has been shown to confer salinity tolerance in Arabidopsis, and transcript profiling using cDNA microarrays had identified several sequences may be under the control of this MYB. Quantitative PCR (QRT-PCR) demonstrated that

compared with wild type MYB expression in the transgenic lines was over 500 times greater, and that transcript for a small heat shock protein AtHSP17.6, is 17 times more abundant. These transgenic lines were shown to have an improved thermotolerance. Treatment of wild type plants with 5×10^{-4} M ABA increased the expression of this MYB seven-fold, suggesting this transcription factor forms part of the ABA-dependent pathway for the activation of abiotic stress responses in Arabidopsis.

Declaration

I declare that this thesis has been written in accordance with University regulations and is less than 50,000 words in length. All work contained herein was performed by the author unless otherwise stated.

Agustina Dwi Retno Nurcahyanti

February 2009

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May Jesus bless them all.

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Abbreviations

ABA	Abscisic acid
abi	ABA insensitive
A.U.	Arbitrary unit
AOS	Activated oxygen species
AP2	Anthocyanin pigment 2
bZIP	Basic-region leucine zipper protein
CaM	Calmodulin
CBF	Calcium binding factor
CDPK	Calcium dependent protein kinase
DNA	Deoxyribonucleic acid
DRE	Dehydration-responsive element
EREBP	Ethylene-responsive element binding protein
ERF	Ethylene responsive factor
GPCR	G-protein coupled receptor
HSF	Heat shock transcription factor
HSP	Heat shock protein
InsP	Inositol phosphates
LEA	Late embryogenesis-abundant
MAPK	Mitogen activated protein kinase
MS	Mirashise and Skoog
OTS	Overly tolerant to salt

PKS	Protein kinase
PS	Photosystems
QRT-PCR	Quantitative reverse transcript polymerase chain reaction
RLK	Receptor-like kinase
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SOS	Salt-overly-sensitive
SQR-TPCR	Semi-quantitative reverse transcript polymerase chain reaction
TE	Tris EDTA
TF	Transcription factor
w/v	weight per volume (expressed as percentage)
v/v	volume per volume (expressed as percentage)

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CHAPTER 1

INTRODUCTION

1.1. Salinity and Heat Stress: A Worldwide Problem in Agriculture

1.1.1. Global Abiotic Stress

The greatest challenge for humanity in the next few decades will be how to increase and sustain arable production without degrading land. Land degradation is proceeding rapidly. Therefore many countries will not be able to achieve sustainable agriculture in the future. The Global Assessment of Land Degradation (GLASOD) estimated that a total of 1964 million Ha have degraded, 910 million Ha to at least a moderate degree (with significantly reduced productivity), and 305 million Ha strongly degraded (no longer suitable for agriculture). Based on these data, water erosion was the most common problem, affecting almost 1,100 million Ha.

Beside water erosion, the greatest cause of decreasing production in many agriculture lands, particularly in arid and semi-arid areas, is increasing soil salinization. Salinization occurs in irrigated areas, usually when inadequate drainage causes salts to concentrate in the upper soil layers where plants root. It is a problem mainly in the arid and semi-arid zones, where 10 to 50 percent of the irrigated area may be affected. Salinization can cause yield decreases of 10 to 25 percent for many crops, and may prevent cropping altogether when it is severe. It is estimated that 3 percent of the world's agricultural land is affected. In East Asia, however, the proportion is 6 percent and in South Asia is 8 percent. For the arid and semi-arid tropics as a whole, 12 percent of agricultural land may be affected (FAO, 2002).

Salinity also occurs through natural processes from the accumulation of salts over long periods of time in the soil or groundwater. It is caused by two natural processes: (1) the weathering of parent materials containing soluble salts and (2) the deposition of oceanic salt carried in wind and rain. Salinization caused by

natural or human-induced processes also result in the accumulation of dissolved salts in the soil water and subsequently inhibits plant growth (Oldeman *et al*., 1991).

Beside the salinity problem, agronomist worldwide are also greatly concerned with the threat of rising temperatures due to global warming which will impact on achieving maximum output from crop plants. High surface temperatures are a common problem faced by agriculture especially during periods of drought or in many arid and semi-arid regions in the world. Normally, plants grow in environments with sufficient water supply to maintain leaf temperatures at or below air temperatures through transpiration. However, in arid areas or when the plants are exposed to drought conditions, plants experience stomata closure and reduced transpiration. As a consequence of reduced transpiration, leaf temperatures increase above the temperature of the surrounding air and the elevated temperatures may limit dry matter accumulation because of increased respiration, reduced photosynthesis, and cellular damage (Burke, 2001). High temperatures are frequently experienced in seedlings, which leads to reduction in the yield (Zhu, 2002; Chakraborty & Tongden, 2005). In a study on kentucky bluegrass, a combination of heat and drought stress significantly reduced root dry weigh (Jiang and Huang, 2000). Kernel fresh and dry matter accumulation were severely disrupted by the long-term heat stress (8 days at 35 ^{0}C) and did not recover when transferred back to 25 ^{0}C, resulting in the abortion of 97% of the kernels (Cheikh and Jones, 1994).

1.1.2. Crop Improvement through Biotechnology

To solve the salinity and other stress-associated problem, an understanding at the mechanisms by which plants perceive environmental signals and transmit them into activate adaptive responses is of fundamental importance. In addition, most salinity problems arise in the arid and semi-arid zones where plants are also faced with high leaf temperatures. Biotechnology and genetic modification of plants to

tolerate multi- environmental stresses would be a valuable development. Knowledge about stress responses in plant is also vital for the continued development of rational breeding programs as well as transgenic strategies for developing improved crops.

Two common biotechnological approaches to understand and improve plant stress tolerance are marker assisted selection (MAS) and genetic transformation. These approaches have contributed greatly to better understanding of the genetic and biochemical bases of plant-stress tolerance and led to the development of plants with enhanced tolerance to abiotic stress. MAS has emerged as an effective approach to improve plant stress tolerance (Foolad, 2005). The use of this approach requires the identification of genetic markers that are correlated with genes or QTLs affecting whole plant stress tolerance. Other common biotechnological approaches, such as advanced genetic transformation techniques, have provided a significantly a better understanding of the genetic and biochemical bases of plant stress-tolerance. Significant progress has been made in the identification of genes, proteins or compound that have a remarkable effect on plant stress tolerance at the cellular or organismal level (Apse and Blumwald, 2002; Bohnert *et al*., 2006). Manipulation of the expression or production of the identified genes, proteins, or compounds through transgenic approaches have resulted in the development of plants with enhanced stress tolerance in different plant species (Zhang *et al*., 2001). Progress has been made using analysis of expressed sequences tags, analysis of global gene expressions, targeted or random mutagenesis, and gain-of-function or mutant complementation (Cushman and Bohnert, 2000; Xiong *et al*., 2001).

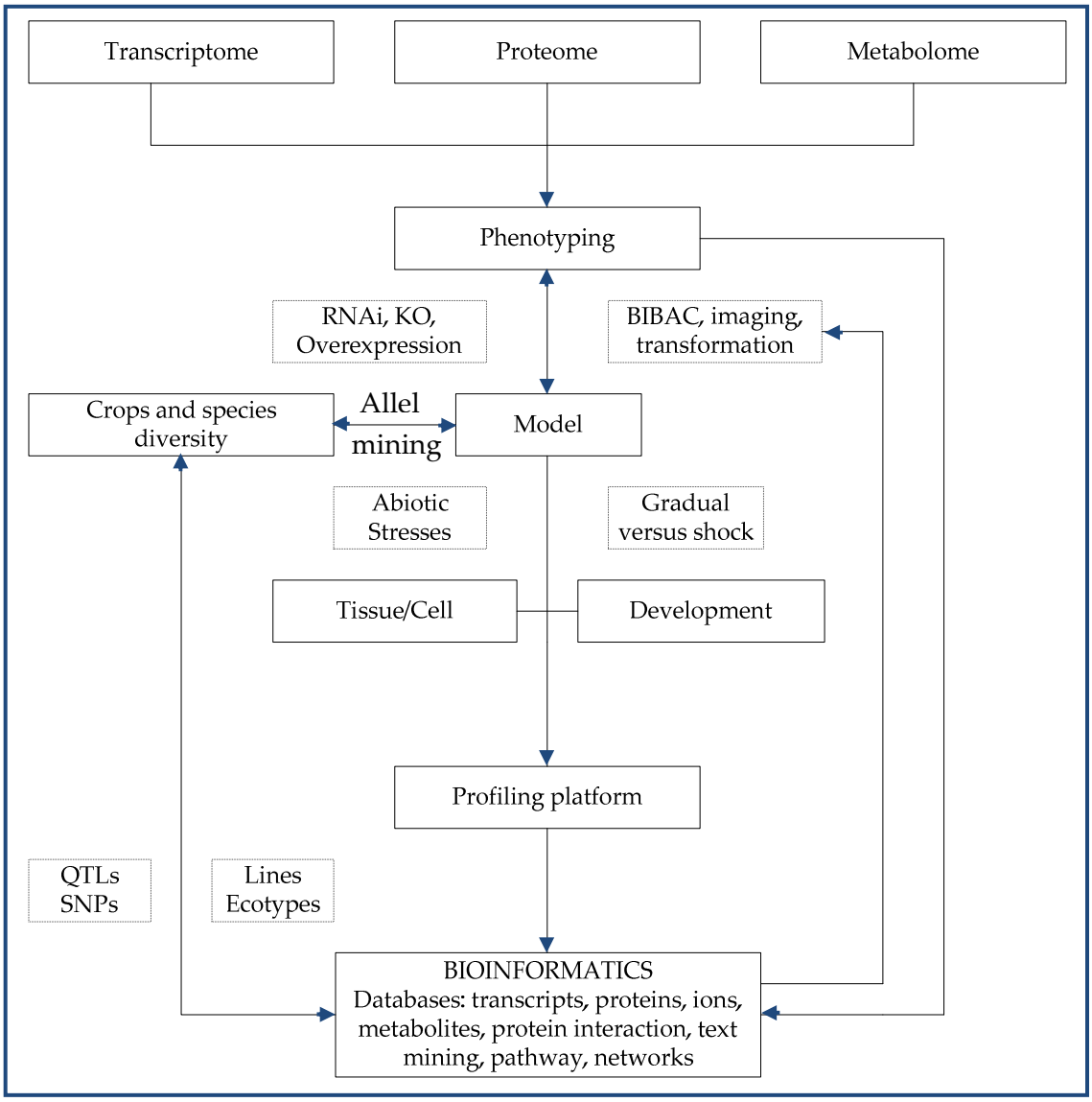


Figure 1.1. Flow Chart of Stress Systems Biology

The chart connects the systems approach to the analysis of plant stress response pathways with gene mining and the transfer of knowledge from models to crops. Figure adapted from Bohnert *et al*. (2006).

The development of advanced biotechnology techniques may improve stress tolerance in crops. This will result in increased productivity, leading to higher incomes for producers, lower prices for consumers, reduced environmental impact (particularly insecticides), new crop varieties for sustainable cultivation in marginal areas, improved food security, and higher nutritional value (protein quality, levels of vitamins and micronutrients, etc.; Burke, 2001; FAO, 2002; Wang *et al*., 2003).

1.2. Salt Tolerance Mechanism

There are three aspects of salt tolerance in plants: homeostasis, detoxification, and growth control (Figure 1.2.).

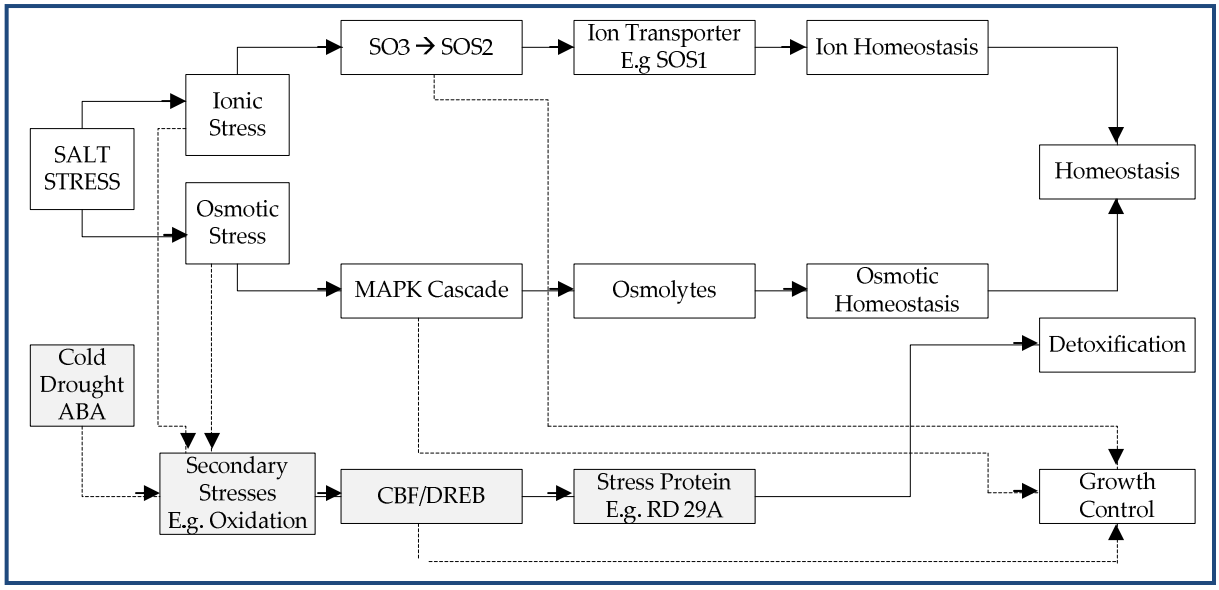


Figure 1.2. A Simplified View of Abiotic Stress Tolerance Network in Plants

The SOS pathway mediates ionic homeostasis and Na^+ tolerance. Two primary stresses, ionic and osmotic, cause damage and often result in secondary stress such as oxidation. CBFs/DREB transcription factor mediate some of the stress protein gene expression in response to secondary stresses caused by high salt concentration, cold, drought, or abscisic acid (ABA). The ionic homeostasis, osmotic homeostasis, and detoxification pathway are proposed to contribute actively cell division and expansion regulation to control plant growth.
Figure adapted from Seaman (_).

Homeostasis is broken down into ionic and osmotic homeostasis (Munns and Tester, 2008).

1. Osmotic homeostasis

The major effect of osmotic stress in plants is a reduction in cell turgor pressure. This impairs cell expansion in root tips and young leaves, and induces stomatal closure. Stomatal closure prevents the acquisition of CO_2 for photosynthesis, the movement of nutrients ions from the soil to the shoot via the transpiration stream, and transcriptional cooling. Further, osmotic stress in plants is usually more apparent when plants enter the reproductive phase of growth (late spring, early summer) and thus compromises yield (Abebe *et al*., 2003).

2. Ionic homeostasis

a. Na^+ Exclusion

Under salt stress, there is ionic imbalance with excess sodium and chloride ions having a deleterious effect on many cellular systems. By removing Na^+ from the transpiration stream, Na^+ will not accumulate to toxic concentrations in leaves. However, a failure to exclude Na^+ from the shoot arises after days or weeks, depending on the species, premature death of older leaves will occur (Munns and Tester, 2008).

b. Tissue Tolerance

Tolerance at the tissue level involves partition of Na^+ and Cl^- at the cellular and intracellular level to avoid toxic concentration within the cytoplasm, especially in mesophyll cells. Toxicity occurs with time after the concentration of Na^+ increase to high concentration in older leaves.

		Osmotic Stress	Ionic Stress	
Process Involved	**Candidate Gene**	**Osmotic Tolerance**	**Na^+ Exclusion**	**Tissue Tolerance**
Sensing and signalling in roots	*SOS3, SnRKs*	Modification of long-distance signalling	Control of net ion transport to shoot	Control of vacuolar loading
Photosynthesis	*ERA1, PP2C, AAPK, PKS3*	Decrease stomatal closure	Avoidance of ion toxicity in chloroplast	Delay ion toxicity in chloroplast
Accumulation of Na^+ in shoots	*HKT, SOS1*	Increased osmotic adjustment	Reduced long distance transport of Na^+	Reduce energy spent on Na^+ exclusion
Accumulation of Na^+ in vacuoles	*NHX, AVP*	Increased osmotic adjustment	Increased sequestration of Na^+ intro root vacuoles	Increased sequestration of Na^+ into leaf vacuoles
Accumulation of Na^+ in solutes	*P5CS, OTS, MT1D, M6PR, S6PDH, IMT1*	Increased osmotic adjustment	Alteration of transport processes to reduce Na^+ accumulation	Accumulation of high concentration of compatible solutes in cytoplasm

Table 1.1.Transport Processes that Contribute to Salinity Tolerance in Plants

Source : Munns and Tester (2008).

1.3. Plant Response to Heat Stress

Abiotic stress, such as heat stress, negatively influences survival, biomass production and accumulation, and the grain yield of most plants. Plants from different habitats are affected by different levels of heat stress. Importantly, the level of susceptibility differs from species to species and often affected by the developmental stage of the plant (Grover *et al*., 2001). Exposed to high temperatures, an overall reduction in plant performance occurs. These reductions can be manifest at three different levels of organization; morpho-anatomical and phenotypic, physiological, and molecular.

1.3.1. Morpho-anatomical and Phenotypic Responses

1.3.1.1. Morphological symptoms

High temperatures can cause pre- and post-harvest damage. Morphological damage includes scorching of leaves and twigs, leaf senescence and abscission, desiccation, failure of seeds to imbibe, loss of turgor, delayed germination and loss of vigour of seeds, fruit discoloration and damage, and ultimately reduced yield (Geisler and Vearasilp, 1998; Guilioni *et al*., 1997; Ismail and Hall, 1999; Vollenweider and Gunthardt-Georg, 2005). Plant responses to high temperatures vary with plant species. For example, high temperatures caused significant reduction in shoot dry mass, relative growth rate, and net assimilation in maize, pearl millet, and sugarcane (Ashraf and Hafeez, 2004; Wahid, 2007). Reductions also occurred in starch, protein and oil content of maize kernels (Wilhelm *et al*., 1999) and grain quality in other cereals (Maestri *et al,*. 2002).

1.3.1.2. Anatomical changes

High temperatures considerably affect the anatomy at both the cellular and sub-cellular level. At the cellular level, high temperatures lead to closure of stomata and reduced water loss, increased densities of stomata and trichomes, and a greater density of xylem vessels in both root and shoot (Añon *et al*., 2004). At the

sub-cellular level, major modifications occur in chloroplast structure leading to significant changes in photosynthesis (Karim *et al*., 1997; Zhang *et al*., 2005).

1.3.1.3. Phenotypic symptoms

A change in air temperature is perceived by plants and induce responses that result in phenotypic changes. The phenotype of plant depends on the severity of heat stress and differs between genotype and between species (Howarth, 2005). Thus, for crop production under high temperatures, it is important to know the developmental stages and plant processes that are most sensitive to heat stress (high day or high night temperature; Wahid *et al*., 2007).

1.3.2. Physiological Responses

1.3.2.1. Water balance

Under field conditions, high temperature stress is frequently associated with reduced water availability. High temperatures appear to cause water loss in plants more during the daytime than at night. During the daytime, elevated transpiration due to high temperatures induces water deficiency, causing a decrease in leaf water potential and perturbations of many physiological processes (Tsukaguchi *et al*., 2003; Wahid *et al*., 2007).

1.3.2.2. Accumulation of compatible solutes

Under stress conditions, different plant species accumulates a variety of compatible solutes. The accumulation of solutes may provide an increased stress tolerance of plants. Compatible solutes include sugars, proline, quartenary ammonium compounds (e.g. glycinebetaine), and tertiary sulphonium compounds such as choline O-sulphate (Sairam and Tyagi, 2004).

1.3.2.3. Photosynthesis

Photosynthetic processes are a good indicator of plant responses to heat stress because they often correlate well with other physiological processes, such as transpiration and respiration which cause alterations in plants growth and development. Heat stress usually causes a decrease in photosynthetic rates before respiratory rates. As a result, storage carbohydrate supplies decline and a reduction in sweetness of fruit and vegetable follows (Taiz and Zeiger, 2002). The effects of heat stress are dependent on the developmental stage, inhibition of photosynthesis and respiration rates leading to changes in organs size and shape (Stone, 2001).

1.3.2.4. Cell membrane thermostability

The integrity and function of biological membranes are sensitive to high temperatures because heat stress changes membrane function such as alterations in permeability either by denaturation of proteins or alteration in the fatty acids composition (Savchenko *et al*., 2002). Cell membrane thermostability (CMT) is an indirect measure of heat-stress tolerance in various plants and is often reflected by increased cell solute leakage (Wahid *et al*., 2007).

1.3.2.5. Hormonal changes

Plant hormone plays an important role in the response of plants to hostile environmental conditions either by causing adaptation to or tolerance of specific stresses. Under stress condition, the biosynthesis and/or degradation of plant growth regulators can be altered or the abundance or activity of their receptor proteins resulting in changes in the hormone signalling networks (Wahid *et al*., 2007).

Abscisic acid (ABA), ethylene (C_2H_4), and salicylic acid (SA) are plant hormones that have been implicated in heat stress tolerance mechanisms in plant either

during or post-heat exposure (Maestri *et al*., 2002; Arshad and Frankenberger, 2002).

The effects of gibberellins and cytokinins on high temperature tolerance are believed to be opposite to those of ABA. For example in barley, application of gibberellic acid reduced heat tolerance, whereas ABA usually accumulates in heat stressed plants (Vettakkorumakankav *et al*., 1999).

1.3.2.6. Secondary metabolites

High temperature conditions induce production of secondary products, such as phenolic compound including flavonoids, anthocyanin, lignin and phenylpropanoids (Chalker-Scott, 2002; Wahid and Ghazanfar, 2006). Phenylpropanoids are synthesized by the principal enzyme, phenylalanine ammonia-lyase (PAL), through the phenylpropanoid pathway. Under heat stress conditions, PAL activity increases and induces the biosynthesis of phenolic compound. The accumulation of phenolic compound is believed to suppress oxidation which results from heat stress in high light conditions (Rivero *et al*., 2001).

1.3.3. Molecular Responses

1.3.3.1. Oxidative stress and antioxidants

Oxidative stress can be induced by heat stress resulting in further cellular damage. This stress results from the generation of activated oxygen species (AOS) that causes the autocatalytic peroxidation of unsaturated membrane lipids and pigments leading to a modification in membrane permeability (Liu and Huang, 2000; Xu *et al*., 2006).

However, plants possess a series of both enzymatic and non-enzymatic detoxification system against AOS and these provide protection (Sairam and Tyagi, 2004). Further research is required to study the signalling pathways involved in activating antioxidant mechanisms in heat stressed plant cells.

1.3.3.2. Stress Proteins

1.3.3.2.1. Heat shock proteins

When plant or seedlings are heated to a critical level, essential proteins in plants become denatured resulting in the formation of insoluble aggregates which hamper cell recovery after heat shock. Some endogenous protective systems have evolved in all organisms that provide thermal tolerance. One of these protection systems involves an acquired heat resistance mechanism associated with the synthesis and accumulation of specific protein called 'heat shock protein's (HSPs). Induction of HSPs is a conserved mechanism in prokaryotes and eukaryotes for thermotolerance. However, each major HSP family has a unique mode of action which includes chaperone activity, and also interaction of HSPs in other stress-response mechanisms (Wang *et al*., 2004) such as the production of compatible solutes (Diamant *et al*., 2001) and antioxidants (Panchuk *et al*., 2002). The expression of HSPs positively correlates with the acquisitions of thermo tolerance, and the over expression of HSPs often results in enhanced thermo tolerance (Zhu, 2002; Chakraborty & Tongden, 2005).

1.3.3.2.2. Other heat induced proteins

Besides HSPs, a number of proteins are also expressed under heat stress conditions such as ubiquitin (Sun and Callis, 1997), cytosolic Cu/Zn-SOD (Herouart and Inze, 1994), and Mn-POD (Brown *et al*., 1993). For example, in *Chenopodium murale* under heat stress, Cu/Zn- SOD isolated from the stromal fraction of chloroplasts was heat tolerant and was suggested to be responsible for chloroplastic stability under heat stress (Khanna-Chopra and Sabarinath, 2004).

1.4. Regulation of Thermotolerance Mechanisms

Thermotolerance is defined as the ability of an organism to cope with excessively high temperatures (Vierling, 1991). The acquisition of thermotolerance is an autonomous cellular phenomenon and normally results from prior exposure to

sublethal high temperatures that protect cells and tissues from a subsequent lethal heat stress. This phenomenon is known as heat acclimation (Burke, 2001; Chakraborty & Tongden, 2005; Loeschcke & Sørensen, 2005; Lim *et al.*, 2006, Wahid *et al*., 2007).

Plants possess several mechanisms to survival at high temperature. These include mechanisms that support the adaptation and protection of plants at different type of stress at different of developmental stages, and in specific tissues (Queitsch *et al*., 2000). Some significant tolerance mechanisms include ion transporters, osmoprotectants, free radical scavengers, late embryogenesis abundant proteins, and factors involved in signalling cascades and the control of transcription (Wang *et al*., 2004).

Sung *et al*. (2003) and Wahid *et al*. (2007) have reviewed how plants sense environmental stresses and translate these stimuli into signalling pathways that control transcription and post-translational events to activate tolerance mechanisms (Figure 1.3).

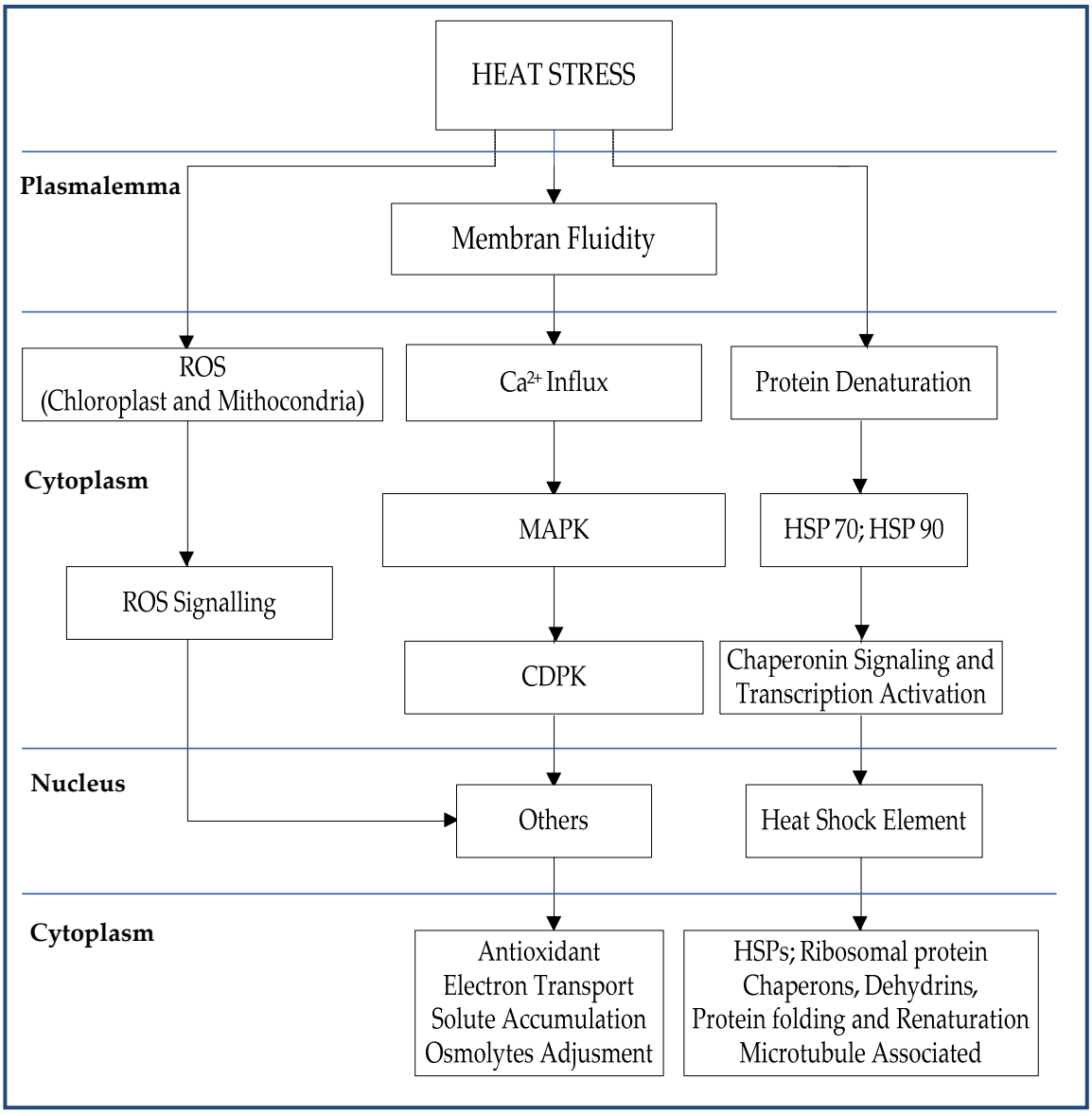


Figure 1.3. Heat Stress Sensing in Plants

Figure adapted from Sung *et al*. (2003); Wahid *et al*. (2007).

Heat stress is sensed at various locations in the cell the plasma membrane, the cytosol, and organelles. Heat stress causes an initial increase in fluidity of the plasmalemma that leads to Ca^{2+} influx and cytoskeletal reorganization, effecting in the upregulation of mitogen activated protein kinase (MAPK) and calcium dependent protein kinase (CDPK; Sung *et al*., 2003; Wahid *et al*., 2007). At the nuclear level, signalling of these cascade leads to transcription of sequences involved in the production of antioxidants and compatible osmolytes for cell water balance, and osmotic adjustment. Generation of ROS in the cytoplasm also acts as an important signal for the production of antioxidants (Bohnert *et al*., 2006). The antioxidant activity is a part of the heat stress adaptation response and correlates well with the acquisition of thermotolerance (Maestri *et al*., 2002). The induction of HSPs is one of the most closely studied mechanisms of thermotolerance and is comprised at several evolutionarily conserved protein families. The HSPs/chaperones can play a role in stress signal transduction and gene activation/expression (Nollen and Morimoto, 2002) as well as in regulating cellular redox state (Arrigo, 1998), production of compatible solutes (Diamant *et al*., 2001) and, antioxidant activity (Panchuk *et al*., 2002).

1.5. Abiotic Stress Signal Transduction in Plants

The cellular and molecular responses of plants exposed to abiotic stress has been studied intensively (Tomashow, 1999; Diamant *et al*., 2001; Kotak *et al*., 2007). Signal transduction, which is defined as the process by which plants perceive environmental signals and transmit them into activating adaptive response is of fundamental importance to biology. Knowledge about those stress signal transduction is also essential for continued development of rational breeding and transgenic strategies for improving stress tolerance in crops (Xiong and Jian, 2001; Xiong *et al*., 2002).

Many signal transduction networks have been identified in microbial and animal systems. In plants, the signal transduction pathways for environmentally induced

developmental changes, several phytohormones, and responses to the pathogen are being elucidated. However, the physiological and molecular details of how plants sense and transduce abiotic stress signals are still limited due to the complexity of environmental stresses. To overcome this limitation, molecular genetics approaches, such as reporter gene expression using the promoters of defence genes, have been explored and appear very promising (Xiong and Jian, 2001).

Generally, a signal transduction pathway begins with signal perception (primary signal), resulted by generation of second messengers (secondary signal). Second messengers can regulate intracellular Ca^{2+} levels within in turn that initiate a protein phosphorylation cascade that targets protein directly involved in cellular protection or transcription factors controlling specific sets of stress-responsive genes (Figure 1.4.; Xiong *et al*., 2002).

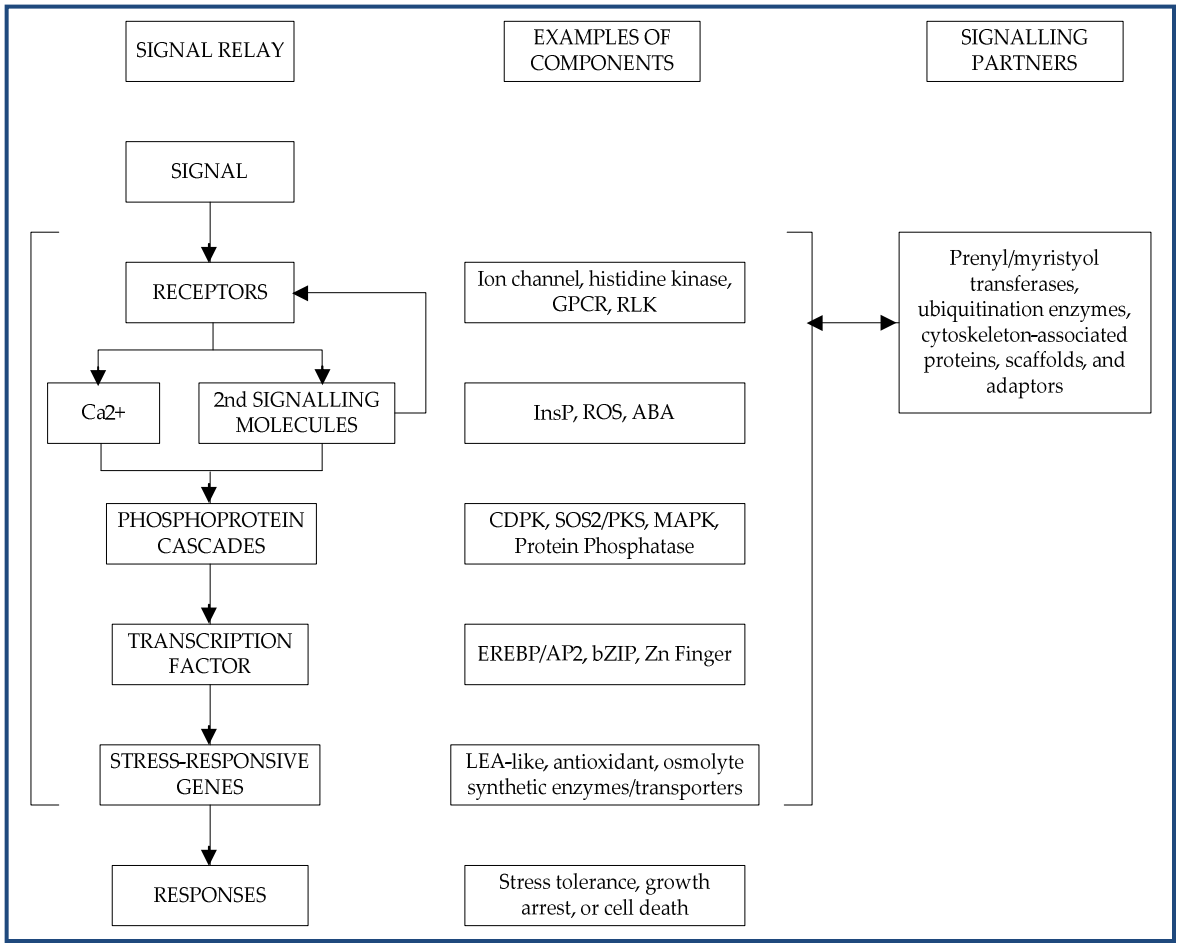


Figure 1.4. Generic Pathway for the Transduction of Abiotic Stress Signals in Plants

Secondary signalling molecules can cause receptor-mediated Ca^{2+} release (indicate with a feedback arrow). Signalling partners can be regulated by the main pathway. Signalling can also bypass Ca^{2+} or secondary signalling molecules in early signalling steps. Figure adapted from Xiong *et al*. (2002).

Signal transduction involves the appropriate spatial and temporal co-ordination of all signalling molecules. Therefore, there are certain molecules that participate in the modification, delivery, or assembly of signalling component but do not directly relay the signal. Secondary signals can initiate other cascades of signalling events which can differ from primary signalling in time and in space. Secondary signals may also differ in specificity from the primary stimuli, may be active in different stress response pathways, and may cause interaction between the signalling pathways for different stresses. One primary stress signal may, therefore, modulate multiple signalling pathways (Xiong *et al*., 2002).

1.5.1. ABA Dependent and ABA Independent Processes Regulate Stress-Responsive Genes

ABA is a ubiquitous plant hormone in vascular plants and has been detected in all major organs and tissues from the root cap to the apical bud. ABA is synthesized in almost all cells that contain chloroplasts or amyloplasts (Taiz and Zeiger, 2002). ABA plays a primary regulatory role in the initiation and maintenance of seed and bud dormancy, and in the plant response to stress. During exposure to stress such as heat shock, low temperature, and salt stress, ABA has been shown to regulate the expression of many downstream responsive genes (Rock, 2000). This is presumed to contribute to induced stress tolerance.

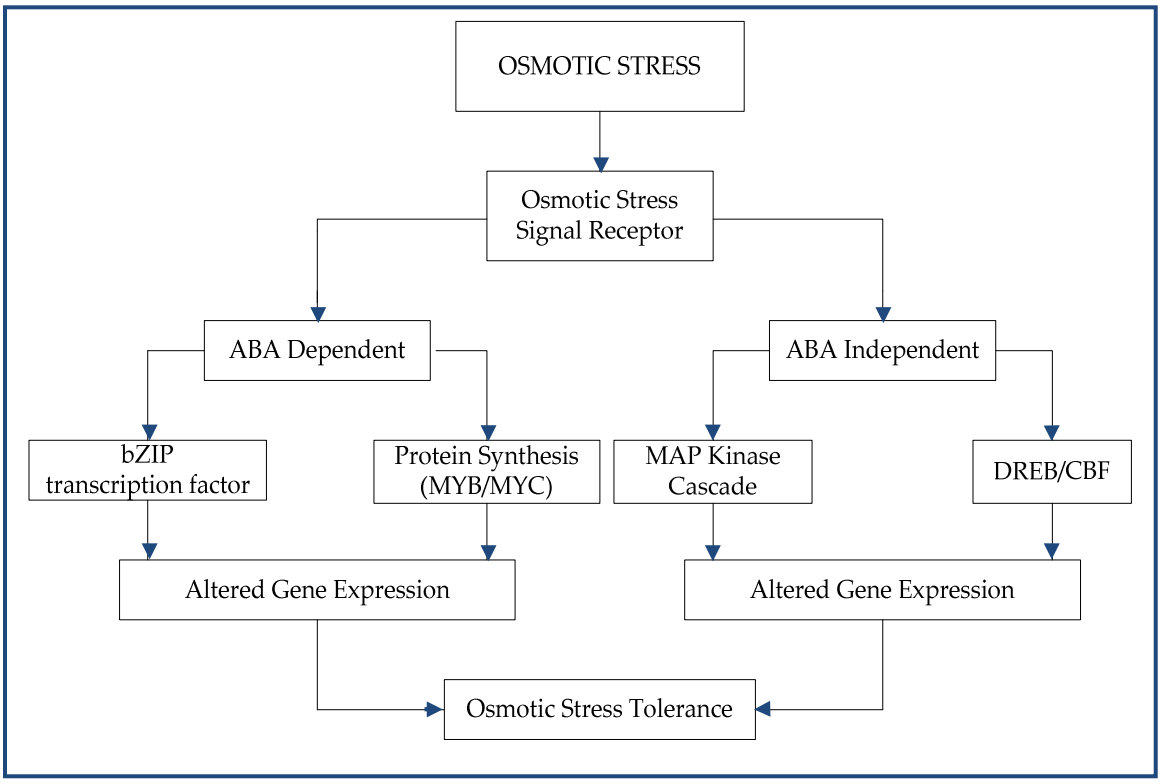


Figure 1.5. ABA Dependent and Independent Signal Transduction Pathways for Osmotic Stress in Plant Cells

Figure adapted from Taiz and Zeiger (2002).

The promoters of these ABA dependent genes contain a six nucleotide sequence element referred to as the ABA response element (ABRE) which bind transcription factors that are themselves regulated by ABA. On the other hand, the promoters of the activated genes can also be activated by osmotic stress in an ABA-independent manner and these contain an alternative nine-nucleotide regulatory sequence element, which called dehydration response element (DRE). Therefore, the genes activated by osmotic stress appear to be regulated through signal transduction pathways involving the actions of ABA (ABA-dependent genes), or by ABA-independent pathways.

Both ABA-dependent and –independent osmotic stress signalling modifies the expression of transcription factors leading to the activation of downstream stress responsive genes (Jian, 2002). The ABA-dependent pathway involves signalling through both MYC and MYB transcription factors, and the ABA-independent pathways involve signalling both MAP kinase cascade and DREB/CBF.

Although the involvement of ABA in environmental stress responses has long been recognized and the basic mechanisms have been resolved, the extent and the molecular basis of ABA involvement in stress-responsive gene expression and stress tolerance are not understood clearly. Studies on the relationship between ABA and different stress-signalling pathways have been inhibited by the complexity of signalling mutants. The study of mutations with differential responses to stress, ABA, or combinations of the stimuli has revealed a complex signal transduction network and suggest that there are extensive connections between cold, drought, salinity, and ABA signalling pathways (Ishitani *et al.,* 1997; Xiong *et al*., 1999).

1.5.2. Transcription Factors Involved in Stress Signal Transduction

One important factor in the activation of stress responses is transcriptional control. Numerous studies have shown that transcription factors are important in regulating plant genes that are responsive to environmental stress (Jalali et al.,

2006; Yang *et al.,* 2006). Gene expression is controlled through the interaction of regulatory proteins (transcription factors) and other proteins with binding domains that interact with specific motifs (elements) in the promoters of the genes they regulate.

The transcription factors that are known to play a role in modulating defence gene expression belong to the following families defined by their DNA-binding domains (Table 1.2.; Maleck *et al*., 2000; Eulgem, 2005; Guo *et al*., 2005).

TF Family	DNA-binding domain	*Cis*-element	Reference
WRKY	60 amino acid containing conserved sequences WRKYGQK and zinc finger motif	W-box sequences varies. Conserved core TGAC	Eulgem et al. (2000), Twick et al., (2004)
ERF	58 amino acid AP2 domain forming α-helix and β-sheet DNA binding by β-sheet	GCC-box. Conserved GCCGCC	Allen et al. (1998), Guterson and Reuber (2004)
bZIP	25 amino acid region rich in basic amino acid adjacent to a leucine-rich domain 2α-helix	TGA-box. Conserved sequence TGACGTCA (core ACGT)	Meshi and Iwabuchi (1995), Fan and Dong (2002)
		ABRE-box. Conserved sequence CACGTG	Kang et al. (2002)
MYB	52 amino acid helix-turn-helix domain	Sequence varies. Conserved core TAAC	Martin and Paz-Ares (1997), Jin and Martin (1999), Stracke et al. (2001)
DOF	Single zinc finger motif of C_2C_2 type	Sequence varies. Conserved core AAAG	Yanagasiwa (2002)
Whirly	Tetramic proteins. Conserved domain of β-sheet and α-helices bind ssDNA	Conserved sequences GTCAAA(AA)	Desveaux et al. (2004)
MYC	Basic helix-loop-helix domain	Conserved sequences CANNTG	Toledo-Ortz et al. (2003)
NAC	Twisted β-sheet surround by few helical elements	Conserved sequences AGGGATG	Duval et al. (2002), Ernst et al. (2004)

Table 1.2. Transcription Factor Families Involved in Stress-Responsive Gene Regulation in Plants
Source : Jalali *et al*. (2006)

Activation of abiotic stress-responsive genes in plants appears to occur through several classes of transcriptional factor and possibly, several transcriptional factors can cooperatively activate the same gene or set of response genes (desiccation or salinity, for example) some of these are themselves transcription factor that regulate the expression of other specific transcription factors. Studies on the promoters of several stress-induced genes have led to the identification of specific regulatory sequences for genes involved in different stresses (Yamaguchi-Shinozaki and Shinozaki 1994; Stockinger *et al*., 1997; Taiz and Zeiger, 2002).

1.6. MYB and HSP Transcription Factors

1.6.1. MYB

1.6.1.1. MYB Family

MYB transcription factors are a family of proteins that contain the conserved MYB DNA binding domain. 'Classical' MYB transcription factors are related to *c*-MYB that is involved in the control of the cell cycle in the animals, plants, and other higher eukaryotes (Stracke *et al.*, 2001). Regulation of the activity of the MYB protein occurs at two levels, by pre-translational and by subsequent post-translational modification. Together, these mechanisms direct the activity of MYB protein to the period around the G_1/S cell cycle transition (Weston, 1998). Pre-translational control is evident from the many differences in the organ specific and temporal pattern of RNA accumulation of different plant MYB transcripts in response to environmental stimuli such as light, salt stress, heat stress, or the application of plant hormones (eg. gibberelic acid and abscisic acid). Post-translational control operates by different mechanisms, such as cellular redox potential, protein phosphorylation, and protein-protein interactions (Martin and Paz-Ares, 1997).

1.6.1.2. Classification

In plants, MYB proteins can be categorized into three subfamilies depending on the number of adjacent repeats in the MYB domain (one, two, or three). Stracke *et al*. (2001) refer to MYB-like proteins with one repeat as 'MYB1R' factors, with two repeats 'R2R3-type MYB' factors, and with three repeat as 'MYB3R' factors.

The R2R3-type MYB sub-family is the largest MYB gene family in plants (Jin & Martin, 1999; and Stracke *et al*., 2001). In addition, three-repeat MYBs have recently been identified in plants together with a growing number of MYB proteins with a single MYB domain (Jin and Martin, 1999). The large size of this gene family was apparent from the work on *Arabidopsis thaliana* (Romero *et al*., 1998) and was also observed in *Zea mays* (Rabinowicz *et al*., 1999). More than 80 different *A.thaliana* genes have been characterized (Romero *et al*., 1998) and this number increased to 132 through the combined efforts of a European Community funded consortium (Stracke *et al*., 2001).

Stracke *et al*. (2001) compared the amino acid sequence of R2 and R3 repeats from all 125 Arabidopsis R2R3 MYB proteins to deduce a consensus sequence and to determine the frequency of the most prevalent amino acids at each position within a repeats. The 125 R2R3-type MYB genes found in Arabidopsis have been categorized into 25 groups on the basis of conserved amino acid sequence motifs present at the C-terminal end of the MYB domain (Stracke *et al*., 2001). These conserve domain might represent activation or suppression domains, and/or domains for the interaction with other proteins. The binding site preference and affinity of MYB proteins is likely to be strongly influenced by other protein factors that interact with them.

1.6.1.3. Function

MYBs can be activators, repressors, or both. All MYB-related proteins have a wide diversity of function. These are, for example, in primary biological roles such as binding telomeric sequences, their role as transcription factor, and the biochemical

function of some of these are related to the rhythmic changes in gene expression associated with the carcidian clock (Jin & Martin, 1999).

In the R2R3-MYB gene sub-family, the largest of the three in plants, function is large and diverse. No functional data are available for most of the 125 R2R3-type AtMYBs. However, some studies on knockout lines have been initiated (Meissner *et al*., 1999) and the number of AtMYBs for which functional information has become available has been grown significantly during the past year. Some functions of R2R3-type MYBs that have been observed are the regulation of phenylpropanoid metabolism in *A. thaliana*. For example, overexpression of AtMYB75 /PAP1 (Production of Anthocyanin Pigment1) and AtMYB90/PAP2 results in accumulation of anthocyanins (Borevitz *et al*., 2000). Another important function for R2R3-type MYB factor is the control of development and determination of cell fate and identity. For example, AtMYB0/GLABROUS 1 (GL 1) and AtMYB66/WEREWOLF (WER) are involved in epidermal cell patterning. Some evidence suggests that R2R3-type MYB factors often are involved in the combinatorial interaction of transcription factors for the generation of highly specific expression patterns. R2R3-type MYB factors also participate in plant responses to environmental factors and in mediating hormone action (Jin and Martin, 1999).

Overall, R2R3-type MYB proteins are involved predominantly in controlling 'plant specific' processes such as the control of secondary metabolism, or response to secondary metabolites unique to plants or cellular morphogenesis (Martin and Paz-Ares, 1997; Stracke *et al*., 2001). So, plants appear to have used R2R3-type MYB transcription factors selectively to control their specialized physiological functions.

1.6.1.4. AtMYB64

AtMYB64 contains two repeats and so is included in the R2R3 subfamily, the largest subfamily in Arabidopsis that contains 125 genes (Stracke *et al*., 2001). AtMYB64 demonstrates a high sequence homology (57%) to the consensus

sequence identified by Stracke *et al*. (2001). AtMYB64 contains three tryptophan residues in the R3 repeat, which although common in animal and yeast R3 repeats, are not present in most plants R2R3 MYB proteins.

1.6.2. HSP

1.6.2.1. HSP Family

Heat shock proteins are synthesized by all eukaryotes, including plants and were identified as proteins that are strongly induced by heat stress. HSPs or highly homologous proteins are also expressed in some cells either constitutively synchronized with developmental programmes (Waters *et al*., 1995). Some HSPs are also found in normal, unstressed cells and some essential cellular proteins are homologous to HSPs but do not increase in response to thermal stress (Vierling, 1991).

During heat exposure, HSPs play an important role helping cells withstand heat stress by acting as molecular chaperons (Taiz and Zeiger, 2002). Due to heat stress, many cell proteins become unfolded or misfolded and this leads to a loss of proper enzyme structure and activity. HSPs, as molecular chaperones, aid unfolded or misfolded proteins (those produced by elevated temperature) in re-folding into an active form (Alberts *et al.,* 2008).

Some studies have revealed a correlation of HSP expression with cellular tolerance of high temperatures, and this has led the hypothesis that HSPs increase thermotolerance (Water *et al*., 1996; Queitsch *et al*., 2000; Taiz and Zeiger, 2002).

1.6.2.2. Classification and Function

Classes	Example Members	Intracelullar Localization	Major Functions
Hsp 100	Hsp 100		Disaggregation, unfolding
Subfamily :			
Class I	ClpB, ClpA/C, ClpD	Cytosol, mitochondria	
Class II	ClpM, ClpN,	Chloroplast	
	ClpX, ClpY	Chloroplast	
Hsp 90	Hsp 90		Facilitating maturation of signalling molecules, genetic buffering
	At-Hsp90-1	Cytosol	
	At-Hsp90-5	Chloroplast	
	At-Hsp90-6	Mithocondria	
	At-Hsp90-7	Endoplasmic Reticulum	
Hsp 70			Preventing aggregation, assisting refolding, protein import and translocation, signal transduction, and transcriptional activation
Subfamily :			
DnaK	Hsp/Hsc70	Cytosol	
	Hsp 70	Chloroplast, mitochondria	
Hsp110/SSE	Bip	Endoplasmic Reticulum	
	Hsp91	Cytosol	
Hsp 60			Folding and assisting refolding
Subfamily :			
Group I	Cpn60	Chloroplast, mitochondria	
Group II	CCT	Cytosol	
sHsp			Preventing aggregation, stabilizing non-native protein
Subfamily :			
I	Hsp 17.6	Cytosol	
II	Hsp 17.9	Cytosol	
III	Hsp 21	Chloroplast	
	Hsp 26.2		
IV	Hsp 22	Endoplasmic Reticulum	
V	Hsp 23	Mitochondria	
VI	Hsp 22.3	Membrane	

Table 1.3. The Major Plant Heat Shock Protein Family
Source : Wang *et al*. (2004)

Low-molecular-weight (15 to 30 kDa) or small HSP (smHSPs) are more abundant in higher plants than in other organism and they show little homology with low molecular-weight HSPs in animals or microorganism. In addition, five or six classes of smHSPs have been identified in plants whereas other eukaryotes possess only one class (Buchanan *et al*., 2000 in Taiz and Zeiger, 2001; Sun *et al*., 2001).

The current model of smHSPs function has been derived from studies of plant and cyanobacterial smHSP (Kim *et al*., 1998; Montfort *et al*., 2001; Kotak *et al*., 2007). Most smHSP form large oligomer (of 8 or more monomers) in the native state. The only available X-ray structure of a eukaryotic oligomeric smHSP is that of the dodecameric cytosolic from HSP16.9 in wheat (Figure 1.6; Monffort *et al*., 2001). In the current model of smHSP function, smHSP oligomer dissociates into dimers and bind substrate through their non-conserved amino-terminal domain. Under heat stress, cyanobacterial smHSPs associated with diverse proteins, which might protect the substrates (Basha *et al*., 2004). In addition to their chaperone function, smHSP have also been proposed to modulate membrane fluidity and composition (Balogi *et al*., 2005).

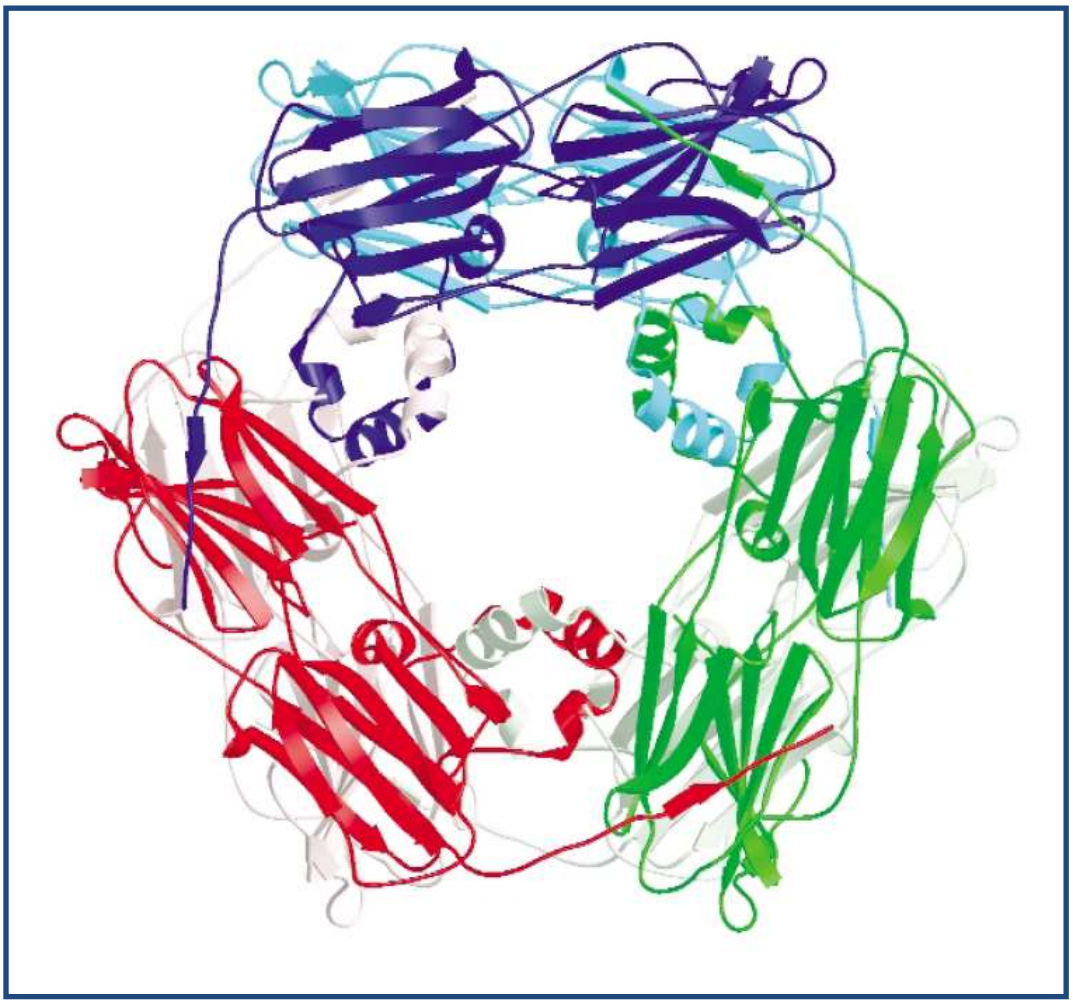


Figure 1.6. Structure of smHSP16.9 Dodecamer in Wheat

The HSP16.9 dodecamer is arranged as two disks whose overall assembly is ~95 Å wide and 55 Å high with a ~25 Å-wide central hole. The construction comprises 12 conserved α-crystallin domains (β-sheet) with 12 C-terminal extension, and six ordered and six disordered N-terminal arms. The basic building block is a nonsymmetric dimer that allows flanking sequences to form variable higher-order interaction that weave the assembly together around the 32 symmetry axes. The view is looking down the crystallographic three-fold axis, which is perpendicular to three crystallographic two-fold axes. The non-crystallographic dimer interface is located between any two molecules of the same colour. Dimmers in the top disk are displayed in red, green, and blue; dimmers in the buttom disk in pink, sage, and turquoise. The dimer-dimer interface that forms an eclipsed tetramer is located between red-pink, blue-turquoise and green-sage dimmers. Pairs of ordered N-terminal arms form an interface, resulting in three helical domains located in the centre of the double disk between red-sage, green-turquoise and blue-pink monomers, thus binding dimmers from the upper and lower disks form 'staggered' tetramers. This intermolecular interface results in an N-terminal arm making an intramolecular interface with its own α-crystallin domain (Figure 1.7(a)). The C-terminal extensions form equivalent interfaces around crystallographic two-fold and three-fold axes. The interfaces located around two-fold axes are between disks (between red-pink, blue-turquoise and green-sage monomers), whereas those located around the three–fold are within disks (between red-green, green-blue, and blue-red monomers in the top disk and between pink-turquoise, turquoise-sage, and sage-pink monomers in the bottom disk). Interfaces 3 and 2 are organized about the same crystallographic two-fold as seen in Figure 1.7(c) and Figure 1.6. Figure from Montfort *et al*. (2001).

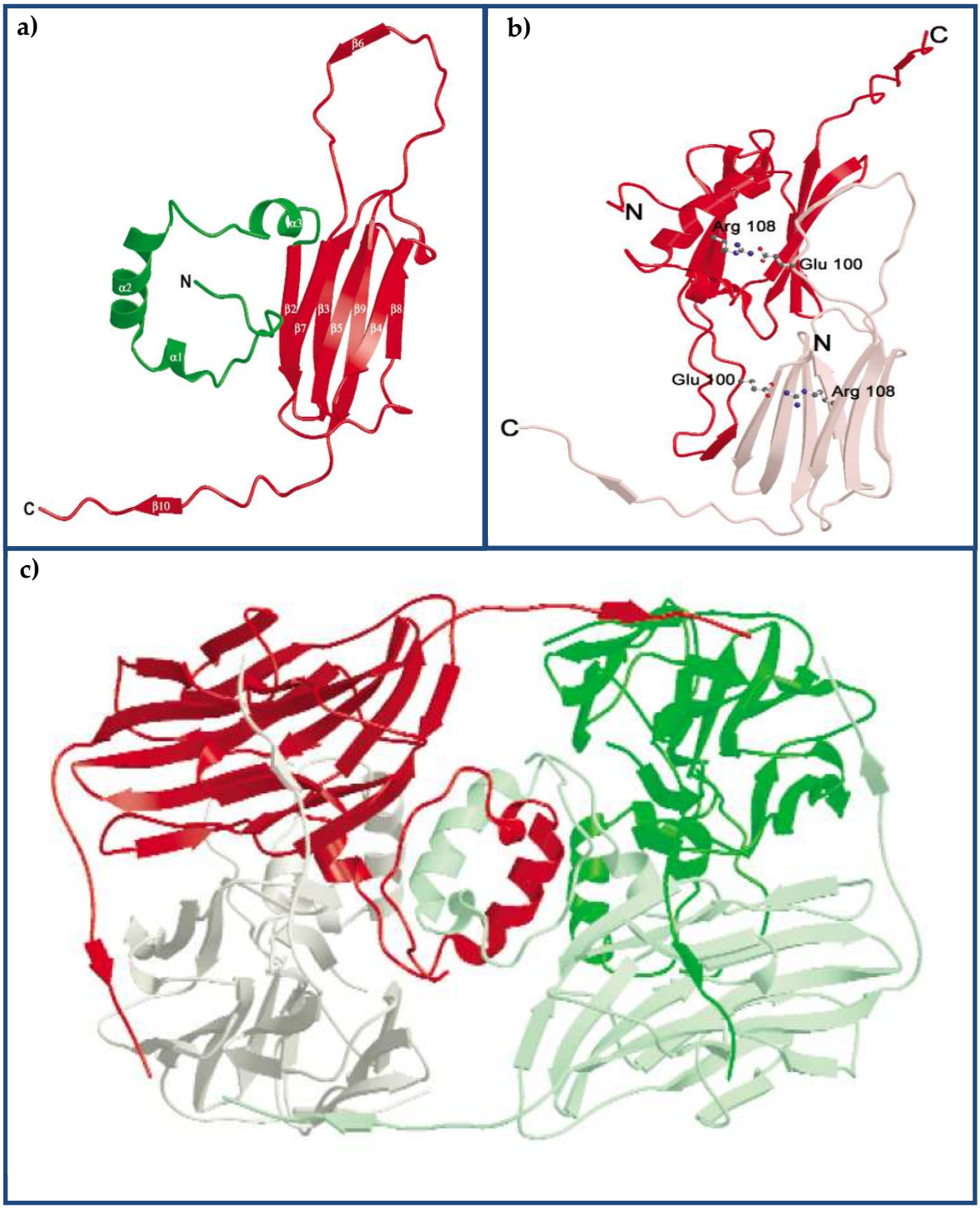


Figure 1.7. Construction and Fold of the smHSP16.9 Wheat Subunits

a) Ribbon diagram of the monomer with the ordered N-terminal arm show in green, and the α-crystallin domain and C-terminal extension in red. b) The noncrystallographic dimer with the complete monomer in red and the monomer with the disordered N-terminal arm in pink. N- and C-termini are labelled, and Glu 100 and Arg 108 are displayed in ball- and –stick representation using standard atom colours. c) Side view of one of the three tetramers of the dodecamer. Blue and turquoise dimmers are omitted for clarity. N-terminal arms of the red and sage dimmers form a loose knot organized about a crystallographic two-fold axis. Pictures from Montfort *et al*. (2001).

In many organisms members of high-molecular-weight HSPs such as HSP70, HSP90, and HSP100 families are most strongly induced by heat, primarily within 0.5–3 hours (Swindell *et al.*, 2007). Some studies also revealed that HSP90 and HSP70 are also responsive to other stresses and endogenous signals (Winter and Sinibaldi, 1991; DeRocher and Vierling, 1995; Yabe *et al.*, 1994; Wang *et al.*, 2004). These high molecular weight HSPs are also abundant components of most unstressed cells and their induction represents increased synthesis of one or more HSP isoforms (Wang *et al.*, 2004).

1.6.2.3. Chaperone Network for Other Environmental Stress Responses

During stress, many enzymes and structural proteins undergo deleterious conformational changes and removal of non-functional and potentially harmful polypeptides (denatured or/and aggregated) is particularly important for cell survival under stress. Thus the different classes of HSPs/chaperons cooperate in cellular protection and play complementary and sometimes overlapping roles in the protection of protein from stress (Wang *et al.*, 2004). Other studies suggest that in plants, various HSPs are also induced by low temperature, dehydration, high salinity, oxidative stress, high irradiation, wounding, exposure to heavy metals, and ABA treatment (Vierling, 1991; Taiz and Zeiger, 2001; Swindell *et al.*, 2007). These studies have identified a role for HSPs in crosstalk or overlap between cellular responses to different environmental stresses. However, no comparative analysis has been carried out to determine which particular stress treatments are the weakest and strongest inducers of HSP expression.

1.7. Functional Analysis by Gene Mutation

1.7.1. Loss-of-Function Mutation

Most genetic mutations are loss-of-function mutations. They alter the gene sequence resulting in the production of transcripts that are truncated or contain codon (amino acid) changes, producing proteins with no or altered function. This

results the inhibition of biochemical activity and/or a decrease in normal protein production (Hartwell *et al.*, 2008). There are, however, significant limitations with classical loss-of-function screens to study stress response. Mutants are usually hypersensitive (the mutant dies while wild type survives the screen) and gene redundancy, where several separate loci encode the same or similar proteins, limits the usefulness of this approach (Tani *et al.*, 2004).

1.7.2. Gain-of-Function Mutation

Gain-of-function mutants can be isolated as hypertolerant individuals (the mutant survives whilst wild type dies). These mutants arise by gene activation or by knockout of a suppressor transcription factor. The alleles from this mutation can be differentiated from recessive null alleles by the fact they are dominant mutations. Another advantage of this approach is that activation of single dominant allele can reveal the function of a redundant gene. Further, if gene activation is achieved with transcriptional enhancers, rather than strong constitutive promoters (Hartwell *et al.*, 2008), problems associated with ectopic gene expression are minimized (Dr. Peter Dominy, University of Glasgow, per. comm.)

1.8. *Arabidopsis thaliana* as a Plant Model

1.8.1. *Arabidopsis thaliana*

Arabidopsis thaliana is a small plant from the mustard family (Brassicaceae) that has become the model plant of choice for research in plant biology. Using this simple angiosperm, significant advances in understanding plant growth and development have been made by focusing on the molecular genetics. The 180-megabase genome of Arabidopsis is organized into five chromosomes and contains an estimated 25,000 genes. The complete genomic sequence of Arabidopsis has been completed and deposited in GenBank by consortium of laboratories in Europe, Japan, and the United States (Meinke *et al.*, 1998). Using

this data, the value of Arabidopsis as a model system has been realized not only for plant biology but also for analysis of complex organisms in general.

Figure 1.8. ***Arabidopsis thaliana***
Source : Meinke *et al*. (1998)

1.8.2. Arabidopsis Activation Tagged Lines

Many powerful research tools have been developed in Arabidopsis. One of these is collections of ~100,000 individuals that carry a randomly inserted activation tag. These collections provide a population for undertaking gain-of-function genetic screens in plants. Plant are randomly transformed with a T-DNA vector that contains four copies of an enhancer element from the promoter of the cauliflower mosaic virus (CaMV 35S). The tetrameric CaMV 35S enhancers can mediate transcriptional activation of nearby genes. The pSKI015 vector was developed to allow large scale application of enhancer 'activation tagging' in Arabidopsis. This activation tagging confers resistance to the antibiotic kanamycin or to the herbicide glufosinate and has been used to generate a collection of > 100,000 randomly transformed plants (Kardailsky *et al*., 1999; Weigel *et al*., 2000).

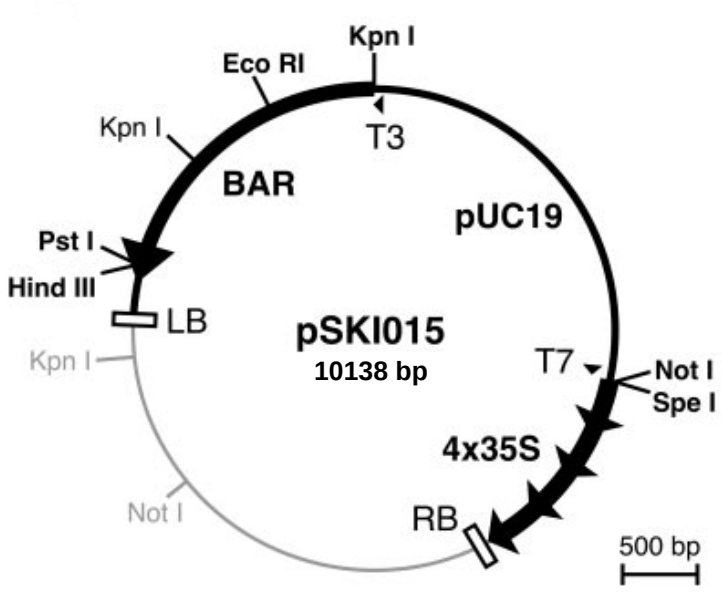


Figure 1.9. The pSKI015 Vector Used to Transform Arabidopsis Activation Tagged Lines
Figure from Weigel *et al*. (2000)

The pSKI015 vector (GenBank accession number AF187951) consists of:

1. BAR gene, this confers resistance to the herbicide glufosinate (Basta) for selection of transgenic plants grown in soil
2. pUC19 sequences with a bacterial origin of replication and an ampicilin resistance gene for plasmid rescue in bacteria
3. 4 copies of 35S Couliflower Mosaic Virus (CaMV) promoter enhancers.

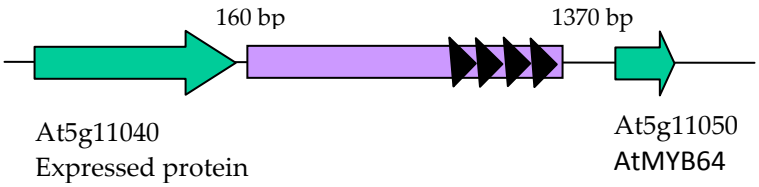
1.8.3. The Advantages of Arabidopsis Activation Tagged

There are three major advantages of Activation Tagged technology over conventional (knockout) genetic screens. First, unlike conventional (loss-of-function) screening, with gene activation gain-of-function screens can be undertaken; surviving plant can be easily identified for further experimentation. Second, it is difficult to identify a single mutant if there are several loci encoding the same (redundant gene); however, only one allele of one loci of a redundant gene needs to be activated to observe gene activation. Finally, the use of the 35S enhancer (as opposed to full, constitutive promoters) reduces the problems that are normally associated with ectopic expression.

1.9. Isolation and Characterization of Arabidopsis Activation Tagged Salt Tolerant Mutants

Screening of Arabidopsis Activation Tagged lines has been used to identify sequences that allow the plant to survive better under high salinity conditions. Weigel's Arabidopsis Activation Tagged collection contains ~30,000 independent mutants and these were screened for salt tolerance. Seven putative salt tolerant mutants were isolated from pools N23153 N23858 (NASC, European Arabidopsis Stock Centre). In one mutant (JP1) the Activation Tagged inserted in the promoter region of a putative SUMO protease (Price, 2005). Subsequence analysis confirmed a role for this protease in a SUMO-dependent salinity tolerance mechanism (Conti *et al*., 2008). Another of the mutants (JP5) demonstrated a strong salt tolerance phenotype when compared with wild type and subsequent analysis showed a MYB transcription was activated (Price, 2005).

A.



B.

Figure 1.10. Identification of Activation Tagged Insertion Site within JP5

(A) The Tagged inserted with left border adjacent to At5g11040, the right border and enhancer elements adjacent to At5g11050. At5g11040 is an expressed protein of unknown function and At5g 11050 is a putative MYB transcription factor (MYB64). The figure represents ~20 Kb of chromosome 5 in JP5; green arrows represent genes showing their direction of transcription. The purple box represents the T-DNA insertion (~10 Kb) and the 4 35-S transcriptional enhancers are shown as black arrows. (B) AtMYB64 gene structure; white box indicates 5′ or 3′ UTR: black box indicates exons.
Figure adapted from Price (2005).

The CaMV 35S promoter enhancers can cause transcriptional activation of nearby genes. At5g11050 is a MYB transcription factor (AtMYB64) which is a family of proteins that include the conserved MYB DNA binding domain. In contrast to animals, plant contain a MYB-protein subfamily that is characterised by the R2R3-type MYB domain. R2R3-type MYB genes control many aspects of plant secondary metabolism, as well as the identity and fate of plant cells. There are 132 members of the MYB family in Arabidopsis, 125 of these are R2R3-type (Stracke *et al*., 2001) and AtMYB64 includes in R2R3-type (See Section 1.6.1.4.).

Further experiments with Arabidopsis lines expressing the AtMYB64 gene have been shown to be salt tolerance. In addition, these are also more thermotolerant than wild type lines. DNA microarray experiments with the original JP5 mutant have shown smHSPs to be upregulated. It is intresting, therefore, to speculate that AtMYB64 regulates the synthesis of these smHSPs, and these in turn confer tolerance of high salinity and high temperatures.

1.10. The Aims and Objectives of this Study

The purpose of this study was to investigate the mechanism of thermotolerance in plants. One part of the study involved further characterization of a MYB transcription factor previously shown to confer salt and thermotolerance on *Arabidopsis thaliana,* probably through the action of small Heat Shock Protein (smHSPs). Part of these investigations involved determining whether this MYB transcription factor operates through an ABA-dependent or ABA-independent signalling pathway.

Transcript profiling was undertaken using QRT-PCR on wild type and transgenic lines expressing the MYB gene under the control of the constitutive 35-S Cauliflower Mosaic Virus promoter.

In addition, a gain-of-function genetic screen was developed to identify Arabidopsis Activation Tagged lines with improved thermotolerance. Experiments were initially performed on heat acclimated and non-acclimated

plants to establish the importance of acclimation in the development of thermotolerance. The screen was performed on non-acclimated plants (~14,800 lines) and several thermotolerant mutants were isolated.

CHAPTER 2
MATERIALS AND METHODS

2.1. Materials

2.1.1. Plant Material

2.1.1.1. *Arabidopsis thaliana* Activation Tagged

Weigel's Arabidopsis Tagged Lines were purchased from the Nottingham Arabidopsis Stock Centre (http://nasc.nott.ac.uk/). The background line of these collections is Columbia (Col-7). These lines are available in 3 sets. Set 1 (N21995) contains 86 pools of 100 lines; set 2 (N21991) contains 82 pools of 96 lines and set 3 (N23153) contains 62 pools of 100 lines. Combined these 3 sets provide 22,672 lines divided into 230 pools. This study used set 1 (N21995) and set 3 (N23153).

2.1.1.2. JP5 and p35S:AtMYB64 Transgenic Lines

JP5 and p35S:AtMYB64 transgenic lines were obtained from previous studies. The JP5 mutant was isolated from a salt tolerance screen of Weigel's Arabidopsis Tagged Lines by screening pool N23858 Weigel set N23153.

p35S:AtMYB64 lines were produced by insertion of T-DNA containing the MYB64 cDNA fused to the CaMV 35S promotor. The p35S:AtMYB64 over expression used in this experiment was line 141 transgenic lines were obtained using the GATEway vector pEARLEYgate100 and line 127 transgenic lines were obtained using the GATEway vector pB7Wg2.

2.1.2. Chemicals

All chemicals were purchased from Sigma-Aldrich Chemical Co. Ltd., Dorset UK, and Fisher Scientific Ltd., Loughborough UK. Antibiotic was supplied by Sigma-Aldrich Chemical Co. Ltd., Dorset UK and were dissolved in appropriate solvent by filter sterilised before use. DNA-*free*™ DNase Treatment for RNA extraction was purchased from Applied Biosystems UK. Enzymes were purchased from

Promega Ltd., Southampton UK. PCR primers were designed using Primers 3 software (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3.cgi; Rozen and Skaletsky, 2000) and purchased from MWG Biotech AG, Ebersberg Germany. The primers were supplied desalted and were resuspended to the appropriate concentration in sterile water before use. Brilliant SYBR Green Master Mix for Quantitative PCR was supplied by Stratagene (Agilent Technologies Company Ltd., Stockport Cheshire UK).

2.1.3. Kits

QRT-PCR tubes and caps were purchased from Stratagene Agilent Technologies Company Ltd., Stockport Cheshire UK (Cat No. #410022 and #410024). A set of sterile filter tips and 50 µl sterile *Corbett Robotic* tips (Cat No.#R1028-5819) were supplied by STARLAB Blakelands Milton Keynes, UK.

2.2. Methods

2.2.1. Surface Sterilization of Seeds

One Covclor 1000 chlorine tablet (Conventry chemicals Ltd UN 2465) was dissolved in 35ml dH_2O. Five ml of this solution was added in 45ml ethanol, mixed gently by inversion and left at room temperature for 5 min. White percipitate was removed by centrifugation at 2,000 g for 5 min. Under sterile conditions, seeds were transferred to 1500µl eppendorf tubes. The seeds were soaked in 1ml bleach for 7 min, mixing occasionally by gentle inversion. The bleach was removed and the seed washed twice with 70% ethanol. Once all traces of ethanol were removed, the seeds were washed 4 times in sterile dH_2O. The seed were then left in the final wash of sterile water for 48 hours at 4^0 C for stratification before sowing.

2.2.2. Germination of Seeds and Heat Treatment for Wild Type and Activation Tagged Lines of *Arabidopsis thaliana*

2.2.2.1. Germination of Seeds

After stratification, surface sterilised seeds (Section 2.2.1) were germinated on agar plates containing 1/10 MS media (Mirashise and Skoog, Sigma M5519, See Appendix 1), 0.75% (w/v) sucrose, and 0.8% (w/v) Micro Agar (Duchefu Biochemie, M1002.0500) adjusted to pH 5.8 with 1M KOH. Approximately 1ml of sterile water was dispensed into an eppendorf tube containing ~150 sterilised stratified seed, and these were sown on a 90mm petri disk containing growth media by placing onto the matrix and adding directly on top approximately 5ml of Top Agar (0.2% (w/v) Micro Agar); the seeds in Top Agar were dispersed across the plate by hand agitation. Seeds were germinated under continuous white light (PPFD 150µmols m^{-2} s^{-1}) at 22 ^{0}C for 24 hours photoperiod for 7 days before heat treatment (Figure 2.1.).

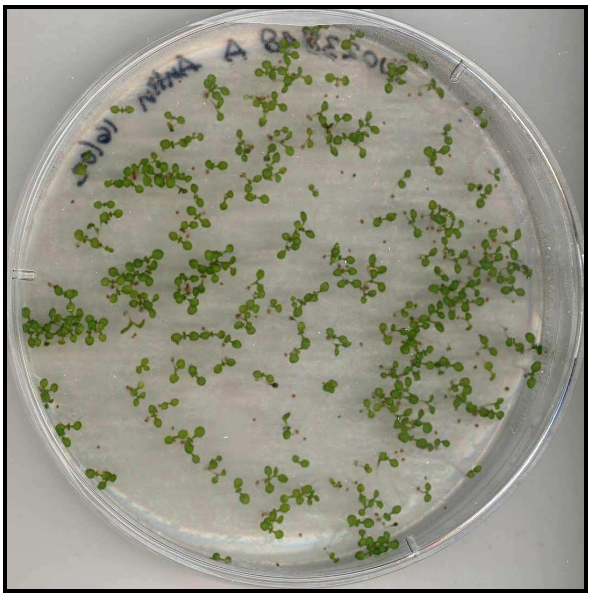


Figure 2.1. Germination condition of seven-day-old seedlings for Col-0 WT and Activation Tagged lines before heat treatment

2.2.2.2. Screening for Critical 'Killing' Temperature for Wild Type *Arabidopsis thaliana*

Incubators were pre-set to 35.0 ± 0.2 ^{0}C a day before to stabilize. The plates containing Col-0 WT germinated on 1/10 MS Media (Section 2.2.2.1) were exposed by heat for 1 hour. After acclimation treatment at 35.0 ± 0.2 ^{0}C, plates were returned to the controlled growth room for 3 days to allow plants to recovery after heat exposure. After three days, plates were exposed to heat stress at various temperatures to assess the optimum temperature for a thermotolerance screen. Non-acclimation plants were included in this experiment. Seven different temperatures were chosen; 35.0 ± 0.2, 40.0 ± 0.1, 44.0 ± 0.2, 48.0 ± 0.2, 52.0 ± 0.1, 56.0 ± 0.2, and 60.0 ± 0.3 ^{0}C. Plates were exposed for 3 hours at each temperature. This heat treatment period was chosen as a mimic of physiological response of the plant during the hot period in a day, from 12 to 3pm. After 3 hours heat exposure, plates were returned to the growth room for 7 days. Plates were examined every day and surviving seedlings counted and compared (by temperature and by pre-treatment).

2.2.2.3. Screening of Thermotolerance Mutant from Arabidopsis Activation Tagged Line

Incubator was pre-set to 44.0 ± 0.2 ^{0}C a day before to stabilize. The plates containing Weigel's Arabidopsis Activation Tagged lines (Section 2.1.1.1.) germinated on 1/10 MS Media (Section 2.2.2.1) were exposed to heat for 3 hours (Figure 2.1.). After heat shock treatment at 44.0 ± 0.2 ^{0}C, plates were returned to the controlled growth room to allow plants to recovery after heat exposure.

After one week recovery, plates were observed for surviving seedlings. The surviving seedlings were transferred to the soil, grown to maturity and the seeds harvested (M1). The M2 generation seeds were screened again under the same condition to confirm thermotolerance (Figure 2.2.). The percentage of seed

germination and seedlings survival were noted. The false positive lines were discarded.

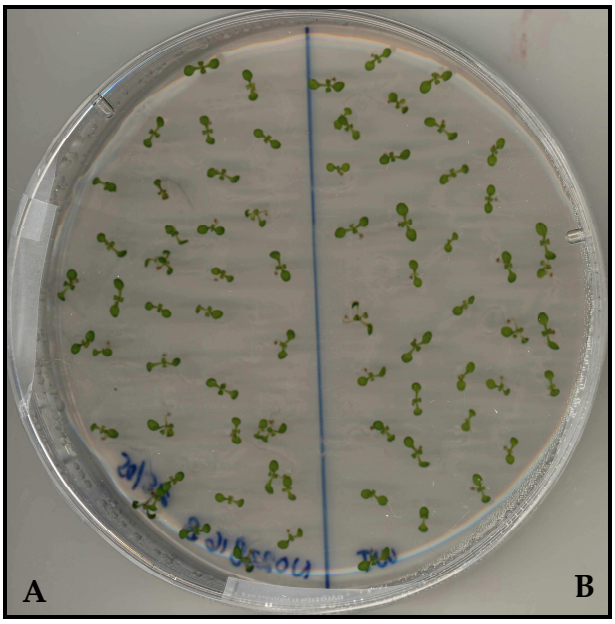


Figure 2. 2. Germination condition of seven-day-old seedlings of M2 putative thermotolerant mutant isolated from Arabidopsis Activation Tagged (A) compared with Col-0 WT (B) before heat exposure treatment

2.2.3. Germination of Seeds and Heat Treatment for p35S:AtMYB64 Transgenic Lines

2.2.3.1. The Plate-Based Phenotypic Thermotolerance Analysis of p35S:AtMYB64 Transgenic Lines

2.2.3.1.1. Germination of Seeds

Ten sterilised stratified seeds of p35S:AtMYB64 transgenic lines and Col-0 WT as control (section 2.1.1.2.; 2.2.1.) were sown on 120mm square plate containing 1/10 MS Media (Section 2.2.2.1.) at a density of ~1 cm spacing by placement on 2µl of top agar (0.2% (w/v) Micro Agar). Seeds were germinated under white light (PPFD 150µmols m^{-2} s^{-1}) at 22^0 C for 24 hours photoperiod for 7 days before exposed to heat acclimation and non-acclimation (Figure 2.3).

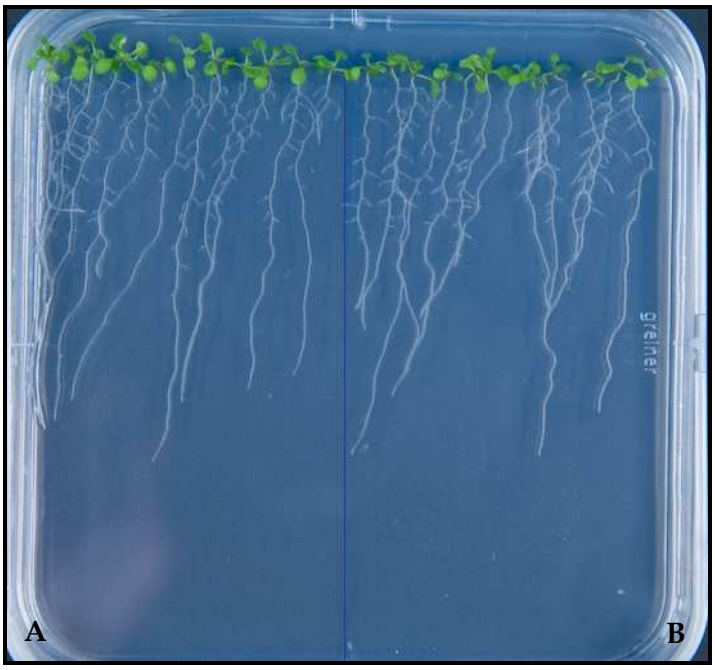


Figure 2. 3. Germination condition of seven-day-old seedlings of p35S:AtMYB64 transgenic lines (B) compared with Col-0 WT (A) before exposed to heat acclimation

2.2.3.1.2. Heat Exposure Treatment

There are two heat exposure treatments: acclimated and non-acclimated stress. For the acclimated treatment, the seven-day-old seedlings were incubated at 37.0 ± 0.2 ^{0}C for 1 hour and then returned to the growth room for a week recovery before expose to higher temperature. For the second treatment, non-acclimated, the seedling did not allowed acclimation but directly exposed to high temperature. Therefore both of acclimated and non-acclimated seedlings were incubated at 44.0 ± 0.2 ^{0}C for 3 hours. After 3 hours heat exposure, plates were returned to the growth room for 7 days. Plates were examined every day and surviving seedlings were counted.

2.2.3.2. Analysis of p35S:AtMYB64 Over Expression Lines by Heat Acclimation Treatment

2.2.3.2.1. Germination of Seeds

Approximately 25 sterilised stratified seeds (Section 2.2.1.) were sown on 120mm square plate containing 1/10 MS Media (Section 2.2.2.1.) at a density of ~1 cm spacing by placement on 2µl of top agar (0.2% (w/v) Micro Agar). Seeds were germinated under white light (PPFD 150µmols m^{-2} s^{-1}) at 22^0 C for 9 hours photoperiod for 14 days before heat acclimation treatment (Figure 2.4).

Figure 2. 4. Germination condition of fourteen-day-old seedlings of p35S:AtMYB64 transgenic lines before exposed to heat acclimation

2.2.3.2.2. Heat Acclimation Treatment

The stress treatments at 37.0 ± 0.2 ^{0}C for 3 hours were applied to the plant after two weeks germination. Plants were harvested after 0 hour, 3 hours, 6 hours, and 12 hours after heat acclimation treatment. Plant without heat acclimation was used as a control.

2.2.4. Genotyping Mutant and Transgenic Lines with Selectable *Bar* Marker Gene

Approximately 150 surface sterilized seeds (Section 2.2.1.) of mutant and transgenic lines carrying the p35S:AtMYB64 construct were placed on 1/10-strength MS Media with or without the herbicide Basta (20 μgml^{-1}); Col-0 WT plates were included as controls. Plates were placed in the growth room with continuous white light (PPFD 150$\mu mols\ m^{-2}\ s^{-1}$) at 22^0 C for 24 hours photoperiod. After 14 days germination, Basta resistance was assessed. Sensitive seedlings were defined as those with cotyledons but no true leaf emergence, whereas resistant lines were defined as a seedling with both cotyledons and true leaves.

2.2.5. Isolation of Plant Genomic DNA

Total extraction of genomic DNA was performed using Extraction Buffer containing 200mM TRIS, 250mM NaCl, 25mM EDTA, and 0.5% SDS at pH 7.5. Approximately 100 mg fresh weight of plant material was ground to a fine powder in liquid nitrogen using pestle and mortar. The powder was transferred to a sterile eppendorf and 350µl Extraction Buffer was added. The mixture was centrifuged at 13,000 g for 5 min at 4 ^{0}C. The supernatant was transferred into a fresh-eppendorf tube, mix with 450µl of isopropanol and allow to stand at room temperature for 2 minutes before centrifugation at 13,000 g for 5 minutes at 4 ^{0}C. After centrifugation, the supernatant was removed and DNA pellet was washed in three changes of ice cold 95% ethanol. The DNA pellet was then air dried, resuspended in 1 × TE buffer pH 8.5 and store at 4 ^{0}C.

2.2.6. Isolation of Total RNA

Total RNA isolation was performed using TRI reagent (Sigma-Aldrich Chemical Co. Ltd., Dorset, UK). Approximately 100mg fresh weight plant material was ground to a fine powder in liquid nitrogen using a pestle and mortar. The powder was transferred to a sterile eppendorf and 1ml TRI reagent added. The mixture

was vortexed briefly and incubated at 4 ^{0}C for 5 min. Then 200µl chloroform was added and shaken vigorously for 15 seconds before incubating at 10 ^{0}C for 10 min. The mixture was then centrifuged at 12,000 g for 15 min at 4 ^{0}C. The top colourless phase containing the RNA was transferred to a fresh eppendorf and mixed with 0.5 ml of isopropyl alcohol. The sample was allowed to stand at 4 ^{0}C for 10 min. The solution was then centrifuged at 10,000 g for 10 min at 4 ^{0}C and the supernatant discarded. The pellet was washed once with 1ml ice cold 75% ethanol. After all traces of ethanol had been removed, the pellet was resuspended in 20µl DEPC treated water and then incubated at 37 ^{0}C for 20 min. RNA were treated with DNase Inactivation Reagent (Ambion, Cat No.#AM1907 Lot No.#0806013) following the manufacturer's instructions.

2.2.7. Quantification and Qualification RNA using Spectrophotometer

The quantity and quality of RNA were measured using spectrophotometer by measuring absorbance at 260 and 280 nm (Sambrook and Rusell, 2001). The quantity of RNA was revealed by absorbance at 260nm of 1 which is equivalent to a RNA concentration of 38µg/ml and a DNA concentration of 50µg/ml. The quality (purity) of RNA is determined by dividing the absorbance at 260nm by absorbance at 280nm. An $A_{260/280}$ ratio of between 1.8 and 2.0 indicates that the nucleic acid is free from protein contamination.

2.2.8. Denaturing Agarose Gel Electrophoresis of RNA

In addition, the quality of RNA was determined by agarose gel electrophoresis and the bands checked for degradation. One µg aliquots RNA from fresh leaves were separated on a 1.5% (w/v) agarose gel containing 10% formaldehyde and 1 × MOPS buffer, pH 7.0 (20mM MOPS, 5mM sodium acetate, 1mM EDTA; Sambrook and Rusell, 2001). The RNA was mixed with 1% (v/v) formaldehyde, 30% (v/v) formamide, 1 × MOPS pH 8.0, and 0.1 volume of ethidium bromide. The mixtures were heated at 65 ^{0}C for 10 min; snap cooled on ice and loaded on the gel with 2µl

loading dye (Promega UK Ltd, Southampton, UK) for each sample. The gel was run for 2 hours at 100V in 1 × MOPS pH 7.0.

2.2.9. Isolation of PCR Product from Agarose Gel

The DNA fragment from the gene of interest was separated from residual agarose gel. The fragment was excised from the gel using a clean, sharp, razor blade and transferred to a sterile eppendorf tube. The DNA fragment was purified using a QIAquick Gel Extraction Kit (Qiagen, West Sussex, UK) following the manufacturer's instructions.

2.2.10. TOPO Cloning of PCR Products

PCR product was cloned into TOPO vector and then transformed into One Shot TOP10 Chemically Competent *E. coli* cells following the manufacturer's instruction of the TOPO TA Cloning Kit for Sequencing (Invitrogen Ltd., Paisley, UK). After successful transformation, emerging colonies were confirmed by colony PCR.

2.2.11. Colony PCR

Emerging colonies were inoculated into sterile LB media containing 50µg/ml kanamycin. A sterile pipette tip was dabbed onto a bacterial colony then soaked into 9µl dH_2O (Sambrook and Rusell, 2001). This was then mixed with 1 × ReddyMix (Abgene, Epsom, UK) which contained 1.25 units Thermoprime Plus DNA polymerase; 75mM Tris-HCl pH 8.8; 20mM $(NH_4)_2SO_4$; 1.5mM $MgCl_2$; 0.01% (v/v) Tween 20; 0.2mM each of dATP, dCTP, dGTP, and dTTP; and a precipitant and red dye for electrophoresis. Primer of 25pmol for each (forward and reverse) was added into the reaction solution. The PCR programme had an initial denaturing step at 96 ^{0}C for 10 min. This was then followed by 25 cycles of a denaturing step at 96 ^{0}C for 15 sec, an annealing step for 30 sec (temperature based on primer Tm) and an extension step at 72 ^{0}C for 1 min. This was then followed by final extension step at 72 ^{0}C for 5 min.

2.2.12. Plasmid DNA Isolation

A single colony was used to inoculate 5ml of LB broth supplemented with 50μg/ml kanamycin. The culture was grown overnight at 37 ^{0}C constant shaking at 200 rpm. The plasmid DNA was isolated from the overnight culture using the QIAprep Spin Miniprep Kit (Qiagen, West Sussex, UK) according to the manufacturer's instructions.

2.2.13. Semi-Quantitative Reverse Transcript Polymerase Chain Reaction (SQRT-PCR)

Semi-quantitative reverse transcript polymerase chain reaction (SQRT-PCR) was performed to investigate transcript levels of genes of interest in JP5 and p35S:AtMYB64 lines. To synthesize cDNA used for SQRT-PCR amplification, the RNA sample (2.5 μg) from extracted-plant tissue using TRI reagent (Section 2.2.6.) were mixed with 0.25μM oligodT at 70 ^{0}C for 10 min and cooled at 4 ^{0}C. Reverse transcription was carried out in a reaction mixture (25μL) containing AMV reverse transcription buffer (Promega UK Ltd, Southampton, UK), 1 mM dNTPs (Promega UK Ltd, Southampton, UK), 1 U μL $^{-1}$ RNase inhibitor (Promega UK Ltd, Southampton, UK) and 0.4 UμL $^{-1}$ AMV reverse transcriptase (Promega UK Ltd, Southampton, UK). The reaction was performed at 48^{0} C for 45 min and then the enzyme was denatured at 95 ^{0}C for 5 min. SQRT-PCR reactions were performed using 1μL of each cDNA sample in a reaction mixture (20μL) containing 1 x ReddyMix (Abgene, Epson, UK) and 0.5μM of each primer. The primer sequences are showed in Table 2.1. PCR reactions were conducted using 28 cycles: 94 ^{0}C for 5 min, 94 ^{0}C for 30 sec, 55 ^{0}C for 30 sec, 72 ^{0}C for 1 min, and a 5 min final extension step at 72 ^{0}C. After amplification, the PCR products were separated on 1% agarose gel and stained with Syber Safe (Invitrogen, Oregon, USA). Actin was used as a constitutive control.

AGI	Name	F/R	Sequence
At5g11050	AtMYB64	Forward	CTTGGATGATCCTTATGACGAAG
		Reverse	TTTCTGTCTTCCAACAACAATGA
At3g18780	Actin-2	Forward	CTTACAATTTCCCGCTCTGC
		Reverse	GTTGGGATGAACCAGAAGGA
At5g12030	smHSP17.6A	Forward	GGAAACCTTCCTAAATCCAT
		Reverse	ACACCATATCCCTCACGCAT
At5g12020	smHSP17.6	Forward	CCTTCCAAACTCCAAATCCA
		Reverse	TAGTTTGCTTATCGATTACATT
At3g12580	HSP70	Forward	GGGAAAGTTCGAGCTCAGTG
		Reverse	AGATGGGAATCAACTGGCTG
At1g74310	HSP101	Forward	GTGCTTCAGGGGACACAAAT
		Reverse	TGGTGCTACAACGCTTGAAG
At3g43810	Calmodulin 7	Forward	ATCACCACAAAGGAGCTTGG
		Reverse	TTCGTCAAAGTCATGATGGC

Table 2. 1. The Primers used for Semi-Quantitative Reverse Transcription PCR

Primer were design to coding sequence for each gene using online Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi, Rozen and Skaletsky 2000).

2.2.14. Quantitative Reverse Transcript Polymerase Chain Reaction (QRT-PCR)

QRT-PCR was performed to investigate transcript levels of heat tolerant genes in JP5 and p35S:AtMYB64 lines. The advantage of real time PCR is a precise quantification of mRNA level of genes of interest when expression levels are compared under different condition of treatment.

2.2.14.1. Determination of Reaction Efficiency

All primer sets were tested to determine the reaction efficiency before used in QRT-PCR analysis. Reaction efficiency was determined using plasmid contain gene of interest to generate standard curve. Six 10-fold dilutions were made from 100pg plasmid of gene of interest (GOI) stock solution in order to generate a series reaction solution for standard curve. Each dilution was mixed with 12.5µl SYBR Green and 0.5µl of each primer and run to QRT-PCR using programs that have been mentioned in Section 2.2.14.2.

For calculation of reaction efficiency, the log of the RNA concentration was plotted on the X-axis and Ct (number of cycle) values on the Y-axis. A line of best fit was generated and reaction efficiencies (RE) were determined using the equation: RE=[10^(-1/m)]/2*100, where m is the slope of the line. Each data point was tested in duplicate (instrumental replication). Two control tubes were also run exactly the same with the sample to generate standard curve except that a plasmid was not placed into the reaction solution (no template control tube).

All of primers used in QRT-PCR and efficiency reaction for each primer are shown on Table 2.2.

AGI	Name	F/R	Sequence	Efficiency (%)
At5g11050	AtMYB64	Forward Reverse	CTTGGATGATCCTTATGACGAAG TTTCTGTCTTCCAACAACAATGA	96.2
At3g18780	Actin-2	Forward Reverse	ctaagctctcaagatcaaaggctta aaccgctttcgttttgcgttttagt	89.1
At3g62250	Ubiquitin-5	Forward Reverse	ATCGCCATTACTGTGGTAAG CCAGAACGAAAGTTCA	94.6
At3g26650	GAPDH	Forward Reverse	AGAGAGGGTAACTTGATTTGG GCTGTTGATGTCTTTAGTG	96.1
At5g12020	smHSP17.6A	Forward Reverse	GGAAACCTTCCTAAATCCAT acaccatatccctcacgcat	90.9
At5g12030	smHSP17.6	Forward Reverse	CCTTCCAAACTCCAAATCCA tagttgcttatcgattacatt	96.1
At3g12580	HSP70	Forward Reverse	GGGAAAGTTCGAGCTCAGTG AGATGGGAATCAACTGGCTG	104.5
At1g74310	HSP101	Forward Reverse	GTGCTTCAGGGGACACAAAT TGGTGCTACAACGCTTGAAG	91.1
At3g43810	Calmodulin 7	Forward Reverse	ATCACCACAAAGGAGCTTGG TTCGTCAAAGTCATGATGGC	95.5

Table 2. 2. Primer Sequences of Six Heat Acclimation Responsive Genes Used for Quantitative Reverse Transcription PCR

Primers were design to coding sequence for each gene using online Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

2.2.14.2. Reaction Setup of QRT-PCR

QRT-PCR reactions were set up using an automated liquid handling system (CAS-3200; *Corbett Robotics*, Sydney, Australia). One microgram of total RNA was converted into cDNA and each cDNA was diluted 1:3. PCR reactions were performed using 5 μL of each cDNA sample in a reaction mixture (20μL) containing 1 x SYBR Green (Stratagene-The Agilent Technologies Division, UK) and 0.5 μM of each primer (forward and reverse). All of experiments were repeated 3 times for cDNA prepared from three batches of plants (biological replicates) and duplication of each sample (experimental replicates). QRT-PCR reaction was conducted at four segments; segment 1 was conducted at 95 ^{0}C for 10 min, 1 cycle; segment 2: 95 ^{0}C for 30 sec, 55 ^{0}C for 1 min, 72 ^{0}C for 1 min, 40 cycles; segment 3 was at 95 ^{0}C for 1 min, 1 cycle; and segment 4 as an dissociation step was conducted from 55 to 85 ^{0}C for 30 sec.

2.2.14.3. The Uses of *Corbett Robotic* in QRT-PCR Reaction Setup

In order to guarantee the accuracy of amplification reaction, QRT-PCR is critical controlled by pipetting steps. Preparation sample using the CAS-3200 *Corbett Robotic* (Figure 2.5.) eliminates the pipetting errors so less replicates need to be run and the need to re-run assay is reduced.

MASTER MIXES

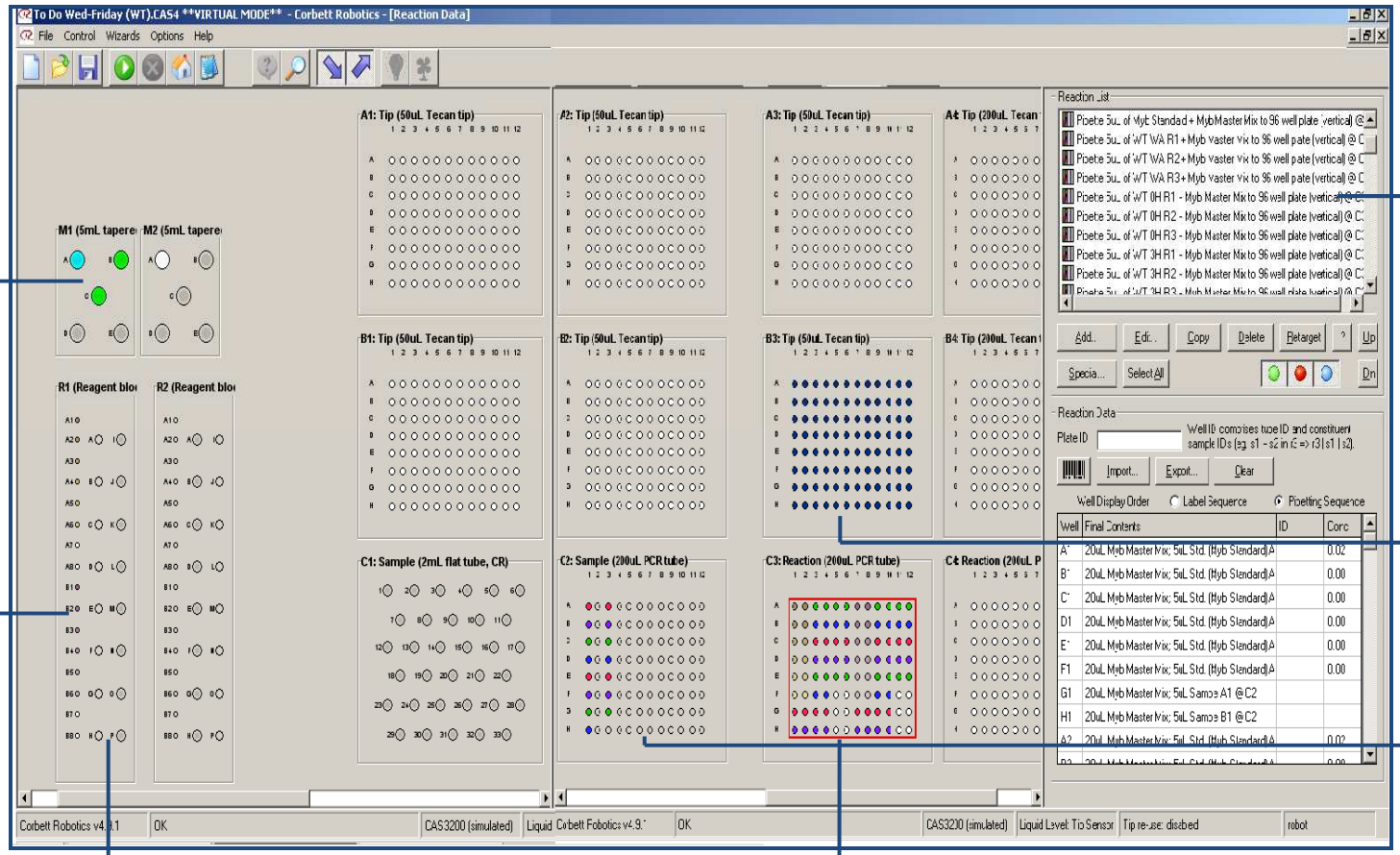
Multiple master mix was prepared within a run or pre-made mixes can be used. The pre-made master mix contain SYBR Green, primers, and water with equivalent amount to number of

DILUTION SERIES

A DNA standard dilution series was setup from a single known concentration. These dilution are then run in replicate to generate a standard curve on a QRT-PCR

REAGENTS

Primers, Probes, Taq and dNTPs can all be loaded into the reagent block. The bilk reagent can then be pipette to form a master mix.



SAMPLE MANAGEMENT SYSTEM

Setup sample format and the management of robotic to mix reaction to generate on a QRT-PCR.

GRAPHITE TIPS

The table can hold between 1 and 5 × 96 trays of tips. This tips useful when pipetting from large reagent vessels and ensures no excess fluid is left on the outer surface of the tip.

DNA SAMPLES

Up to 96 samples can be loaded on the table in a standard 96 well tray.

REACTION PLATE

All QRT-PCR sample types are catered for on the deck by setup the sample format option in the software.

Figure 2. 5. CAS-3200 *Corbett Robotic* Software Used in Sample Preparation for QRT-PCR Analysis

2.2.14.4. Analysis and Interpretation of QRT-PCR Data

Data were analysed and interpreted using Standard Curve Method (Livak, 1997). The quantities of the standard were generated using plasmid DNA of Gene of Interest (GOI) with known concentration. The expressions of amplicons detected by QRT-PCR were plotted to the standard curve for each GOI and Housekeeping Gene (HKG), Actin-2, to produce the quantity value of both GOI and HKG in picogram unit. The quantity value of GOI was then normalized to HKG quantity value. The results from this analyses method were revealed as the abundance level of GOI in Arbitrary Unit (A.U.).

2.2.15. Genotyping Arabidopsis Salt Tolerance Mutants using PCR

To identify homozygous and heterozygous plants for each mutant, primers were designed for the plant gDNA flanking the site of insertion of the pSKI015 vector (generally product size of ~400bp). If the plant contained a wild type version gene (heterozygous) then a PCR product was obtained. If the plant was homozygous for the pSKI015 insertion (did not contain a wild type version of the gene) then no PCR product would be obtained using these primers as the pSKI015 vector would increase the distance between the primers by ~80kb. To distinguish between heterozygous and wild type plants, one plant primer (P1 or P2) was used with primers that hybridized only to the pSKI015 vector. A product would be obtained with heterozygous plants but not with wild type plants.

2.2.16. Statistical Analyses

2.2.16.1. Statistic Analysis for Critical 'Killing' Temperature Screen for Wild Type *Arabidopsis thaliana*

Data were analysed by ANOVA using General Linear Model Analysis and was performed on Arcsin transformed data to observe the significance between acclimated and non-acclimated treatments.

2.2.16.2. Statistic Analysis for The Plate-Based Phenotypic Thermotolerance Analysis of p35S:AtMYB64 Transgenic Lines

Data were analysed by ANOVA using General Linear Model Analysis and was performed on Log transformed data to observe the thermotolerance characteristic of p35S:AtMYB64 T141 and T127 compared with Col-0 WT lines.

2.2.16.3. Statistic Analysis for Analysis of the Expression of Stress Responsive Genes in p35S:AtMYB64 Transgenic Lines

Data were analysed by ANOVA using General Linear Model Analysis and was performed on Log transformed data to observe the expression level of AtMYB64, HSPs, and Calmodulin 7 post heat acclimation in p35S:AtMYB64 T127 compared with Col-0 WT lines.

2.2.16.4. Statistic Analysis for Analysis of ABA Signalling Pathway of AtMYB64 Transcription Factor

Data were analysed by ANOVA using General Linear Model Analysis and without transformed data to observe the expression of AtMYB64 exposed to the application of ABA endogenous in time-dependent manner.

CHAPTER 3

GENETIC SCREEN OF ARABIDOPSIS ACTIVATION TAGGED LINES FOR THERMOTOLERANCE

3.1. Introduction

All plants sense and adapt to adverse environmental conditions, however crop plants exhibit less genetic diversity for abiotic stress tolerance than their wild relatives, indicating a genetic basis exists for improving stress tolerance. Genetic resources have been developed for model plants that greatly enhance studies for the identification of abiotic stress-response mechanisms. In this study, a large-scale gain-of-function screen using the Weigel Arabidopsis Activation Tagged lines (Weigel *et al*., 2000) was undertaken to identify sequences that enable plants to survive heat stress.

3.2. Weigel Arabidopsis Activation Tagged Lines

Only set N21995 and Set N23153 of the Weigel Arabidopsis Activation Tagged lines (Section 2.1.1.1.), were screened (~14,800 plants).

In previous studies this collection was screened for salinity tolerant mutants and considerable success was achieved (Dr. Peter Dominy, Pers. Comm). Ten mutants were isolated that showed a strong salt tolerance phenotype; a further 36 had a weak phenotype (Price, 2005). Several of these mutant lines have now been characterized at the genetic, cellular, and physiological level and the role of the tagged genes in stress responses confirmed. One of these lines showed activation of a SUMO protease enzyme (Conti *et al*., 2008). Another, showed elevated levels of a MYB transcription factor. A third sequences is involved in NO signalling. Clearly, gene activation technology can be used successfully to identify sequences involved in plant responses to abiotic stress.

3.3. Screen of Wild Type *Arabidopsis thaliana* for Thermotolerance

To identify the critical 'killing' temperature of heat exposure for screening the Weigel Arabidopsis Activation Tagged lines, a 'killing temperature' curve was constructed using *Arabidopsis thaliana* Col-0 WT using five different temperatures. Two separate experiments were conducted. In one, seedlings were first heat acclimated at 35.0 ± 0.2 ^{0}C for 1 hour to induce heat stress protection mechanism before exposure to higher 'killing' temperatures. In the second experiment, seedlings were exposed to high 'killing' temperatures directly without acclimation to a mild-heat stress. Approximately 150 seeds were germinated on agar plate containing 1/10 strength MS Media (Section 2.2.2.1.) and after one week 90-98% seeds germinated. One week after germination, plants were heat acclimated (35.0 ± 0.2 ^{0}C for 1 hour) and allowed a 3 day recovery period before heat exposure to higher temperatures. Both sets of plants, acclimated and non-acclimated, were exposed to higher temperature (44 ± 0.2 ^{0}C) for 3 hours. The number of surviving seedlings, their size and vigour, were observed every day during the 7 days post treatment.

Several important criteria were considered in the design of the screen. The first was the heat balance of the shoot. In the wild it is the shoot, more specifically the leaves, of plants that experience the highest temperatures. Leaf temperature is determined by the rate of heating (the level of irradiance and the air temperature if $T_{air} > T_{leaf}$), and the rate of cooling (by the transpiration stream and air temperature if $T_{leaf} > T_{air}$). Leaf temperature, therefore, is rarely the same as air temperature and can vary from leaf-to-leaf and plant-to-plant. This variability confounds genetic screens to identify sequences involved in thermotolerance. For this reason heat-stress was applied in darkness for 3 hours to seedlings grown on moist sealed plates with a RH of 100%. This approach removed the effects of heating (through irradiance) and cooling (through transpiration) so that T_{leaf} was dependent only on T_{air}. The screen was performed in thermostatted incubators that regulated T_{air} to <

0.2 ^{0}C of the set temperature, and T_{leaf}, therefore, should also have been controlled to this tolerance.

Figure 3.1 presents the percentage of surviving seedlings from 1 to 7 days after 3 hours exposure to high temperatures. For up to day 3, more than forty percent of seedlings survived exposure to 48.0 ± 0.2 ^{0}C. After 3 days a dramatic decline in the survival of seedlings exposed to temperatures above 40 ^{0}C was observed; by day 7, survival was less than 25%. It is also apparent that heat acclimated plants better survive a subsequent heat stress. An analysis of variance test showed that for seedlings exposed to 44.0 ± 0.2 ^{0}C a significant ($p<0.0001$) improvement in the survival of acclimated seedlings was seen from day 4 onwards (See Table 1 in Appendix 2). These experiments provide clear evidence that Arabidopsis undergoes an acclimation process that allows better survival of heat stress. These data are consistent with other reports of heat acclimation on *Arabidopsis thaliana* (Burke, 2001), *Cicer arietinum* L (Chakraborty & Tongden, 2005), and *Arabidopsis thaliana* suspension-culture cells (Lim *et al.,* 2006).

These experiments suggest the appropriate temperature for screening the Activation Tagged lines for thermotolerant mutants are between 44-48 ^{0}C for non-acclimated seedling and between 48-52 ^{0}C for acclimated seedlings. Screening of non-acclimated Activation Tagged lines for thermotolerance was already underway using an incubation temperature of 44 ^{0}C and selection after 7 days. It was decided to continue screening at this temperature even though 7% of Col-0 WT plants appear to survive after 7 days (Figure 3.1.) and a high number of false positive lines are expected to be rescued.

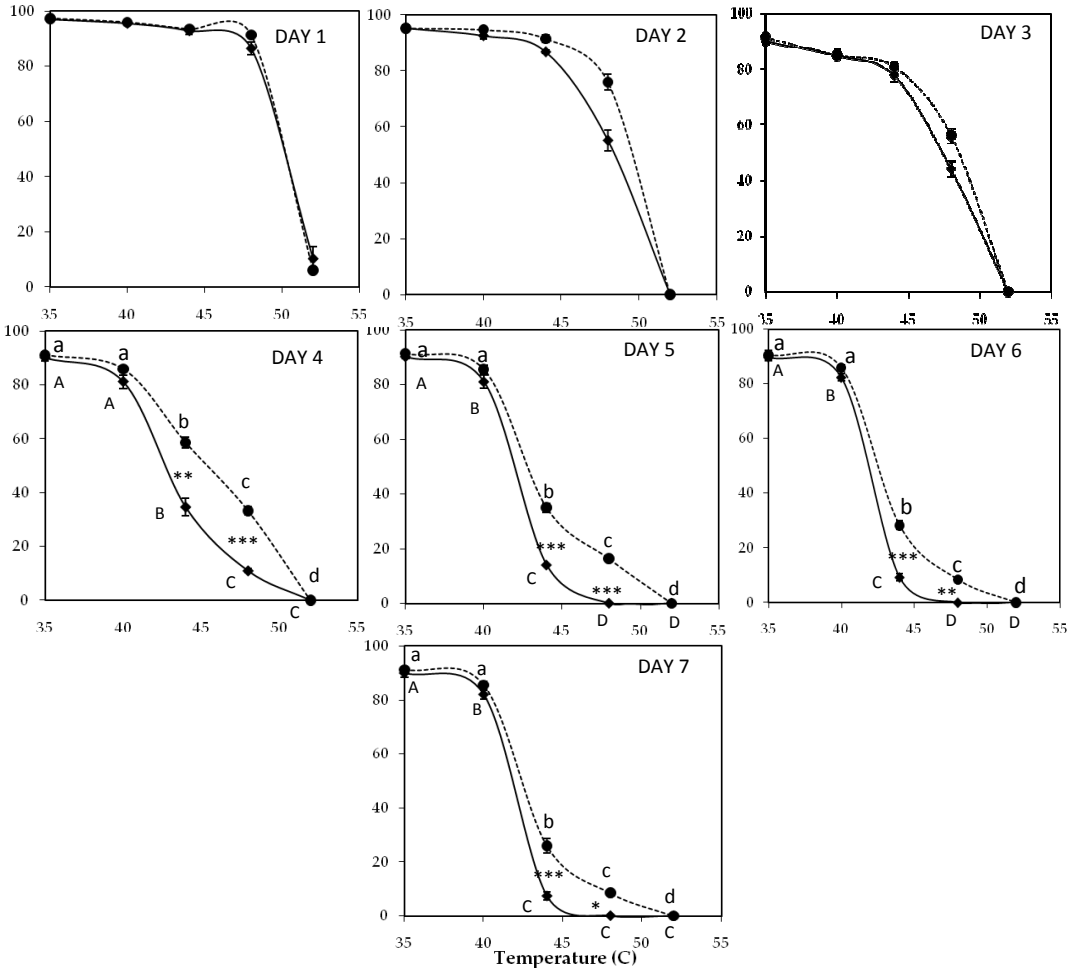


Figure 3. 1. Percentage Survival of Acclimated and Non Acclimated Arabidopsis Col-0 WT Seedlings Exposed to High Temperatures.

Seeds were sterilized and germinated on plates for 7 days in a controlled environment growth room (Section 2.2.2.1.). Plates containing seven-day-old seedlings were placed in the dark in a thermostatically controlled incubator (35, 40, 44, 48, and 52 ^{0}C) for 1 hour and returned to the growth room to recover. Percentage survival with (dashed line) and without (solid line) acclimation was observed every day after heat exposure. The data shown are averages and standard errors from 2 replicate plates. Analysis of variance tests were performed on Arcsin transformed data. Symbols with different Roman characters indicate significant differences ($p<0.05$) between temperatures for acclimated (lower case) and non-acclimated (upper case) seedlings. Significant acclimation-temperature interactions are indicated by asterices (* $p<0.05$; ** $p< 0.01$; *** $p<0.001$; See Table 1 in Appendix 2).

3.4. Screen of Non-Acclimated Arabidopsis Activation Tagged Lines for Thermotolerance

Primary screens were conducted on M1 progeny lines (Weigel's Arabidopsis Activation Tagged population). The Activation Tagged seeds were germinated on agar plates containing 1/10 strength of MS media (Section 2.2.2.1.). Each pool of the Activation Tagged lines was spread onto four plates, therefore, approximately 150 seeds were germinated per plate. After one week, approximately 90% of the Activation Tagged seeds germinated and the first true leaves had emerged.

Heat exposure at 44.0 ± 0.2 ^{0}C for 3 hours was applied to seven-day-old seedlings in the dark, and these were then incubated at growth room temperatures in the light to allow recovery after heat exposure (Section 2.2.2.3). Putative heat-tolerant mutants were identified as plants that survived after 5 days after heat exposure compared with the wild type (Figure 3.2). The surviving plants were removed to plates containing fresh-1/10MS media and allowed to recover for 2 weeks. After two weeks, plants were rescued and transplanted to sterile soil, grown to maturity, allowed to self and seed collected (M2).

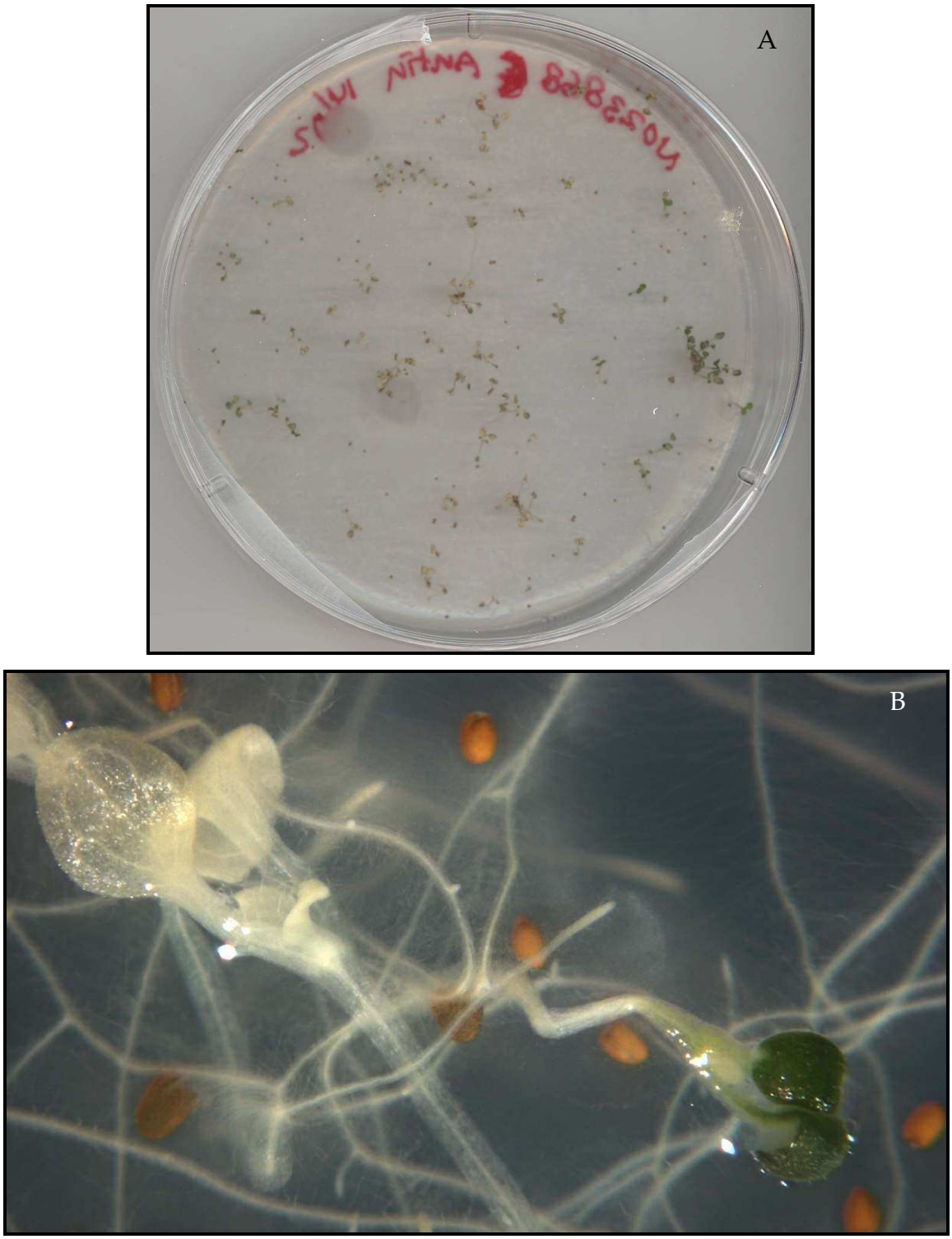


Figure 3. 2. Primary Screen of Arabidopsis Activation Tagged Lines for Heat Tolerant Mutants

(A) Five-day-post-heat stress exposure showed about 5% plant survived. (B) Putative heat-tolerant Activation Tagged mutants were identified as plants with some green colouration whereas dying seedlings showed a translucent colouring to the whole plant.

M2 seeds from surviving individual M1 plants were subjected to two secondary screens. One to confirm authentic thermotolerance phenotype, and the other to characterize the genetic basis of the mutation using *Bar* selectable marker (BASTA).

The secondary thermotolerance screen was conducted to confirm the thermotolerance phenotype. Half-plates were set up containing Col-0 WT and putative mutant (M2) seeds and germinated for 7 days before exposure to 44.0 ± 0.2 ^{0}C for 3 hours (Section 2.2.2.3).

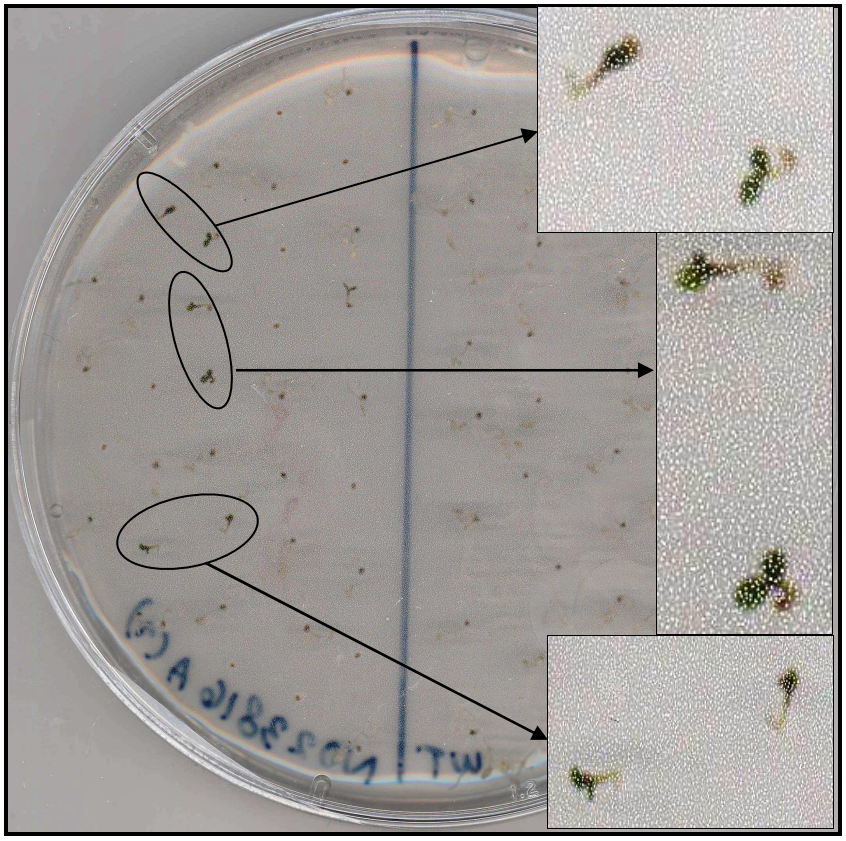


Figure 3. 3. Secondary Screen of Putative Thermotolerant Arabidopsis Activation Tagged Mutants

The activation lines isolated from the Primary Screen (left side of plate) were compared with Col-0 WT (right side of plate). Five-day-post-heat stress exposure showed 10% of the putative heat tolerant plants survived and Col-0 WT growth was totally prevented after heat stress exposure at 44.0 ± 0.2 ^{0}C for 3 hours.

Figure 3.3 shows the survival of non-acclimated Col-0 WT and putative mutant (M2) seedlings after exposure to a 3 hours 44.0 ± 0.2 ^{0}C heat shock. Table 3.1 summarizes the results from the primary and secondary thermotolerance screens of the original 18 putative mutants identified from the primary screen. Only 3 lines were confirmed as thermotolerant.

Weigel Set	Name of Pool	Primary Screen	Secondary Screen
N21995	4 Pools :	7 Individuals :	0 Individual
	N21321	1	0
	N21346	3	0
	N21374	1	0
	N21391	2	0
N23153	9 Pools :	11 Individuals :	13 Individuals
	N23814	1	1
	N23816	2	8
	N23822	1	4
	N23824	1	0
	N23826	1	0
	N23840	2	0
	N23843	1	0
	N23847	1	0
	N23873	1	0

Table 3. 1. Primary and Secondary Screen of Activation Tagged Lines for Thermotolerance.

A total of 18 individual putative thermotolerant mutants were selected by primary screening. Seeds was harvested from these plants and screened under similar condition (secondary screen) to confirm heat tolerance and 13 putative heat tolerant mutants were rescued, grown to maturity self and seed collected (M2).

In the BASTA screen, putative mutants (M2) seeds were spread on 1/10 MS plates containing the herbicide 20μg/ml glufosinate (BASTA); the activation tag T-DNA carries the dominant *Bar* (BASTA) resistance gene as a selectable marker (Section 2.2.4.). Segregation analysis of this M2 population can provide evidence for the number of independent T-DNA insertions and identify homozygous and heterozygous lines. The most thermotolerant line from each of the donor pools was selected for BASTA segregation analysis (Table 3.2.). After 14 days germination, sensitive seedlings were defined as those with cotyledons but no true leaf emergence, whereas resistant lines were defined as a seedling with both cotyledons and true leaves.

Chi Square Test Ratio	16:0 = B^R:B^S	15:1 = B^R:B^S	3:1 = B^R:B^S	1:3 = B^R:B^S	1:15 = B^R:B^S	0:16 = B^R:B^S
Lines	N23816A	N23814A	-	N23822A	-	-
p	0.975	0.895		0.651		

Table 3. 2. Chi-Square Test of BASTA Segregation Analysis of the Most Tolerant Lines Identified from The Secondary Screen.

The calculation of the Chi-Square test was performed using the Chi-test function for the observed and expect ratio. The highest probability value (P) revealed the most likely ratio between resistant and sensitive alleles from the observed plants.

Using the Chi-Square test, the most probable ratio of segregation in the 3 mutants isolated by the secondary screen were 16:0 = B^R:B^S, 15:1 = B^R:B^S, and 1:3 = B^R:B^S . A ratio of 3:1 (B^R:B^S) would indicate a heterozygous line with a single insertion. Ratios higher than this suggest multiple insertions most probably at a different loci. A ratio of 16:0 (B^R:B^S) could indicate a homozygous line with a single insertion at one or more loci. Ratio of less than 3:1 (B^R:B^S) suggest the fitness of the line has been compromised by the T-DNA insertion. Line N23822A appears to have reduced fitness as fewer individuals survive than expected from a heterozygous

line. Line N23814A appears to contain multiple independent heterozygous insertions. Line N23816A could be homozygous with a single insertion but further experiments (backcrossing) will be required to confirm that it does not contain additional insertions.

3.5. Characterization of Thermotolerance in Lines N23814A, N23816A, and N23822A

Screen of non-acclimated Arabidopsis Activation Tagged lines for thermotolerance (Section 3.4) has found three putative mutants: N23814A, N23816A, and N23822A. To characterize the thermotolerance phenotype of those lines in more detail, M3 generation seedlings of each mutant were exposed to a range of temperatures from 40 to 45 ^{0}C.

Temperature (^{0} C)	Putative Thermotolerant Mutant Activation Tagged % Survival									Wild Type		
	N23814 A			N23816 A			N23822 A			R I	R II	R III
	R I	R II	R III	R I	R II	R III	R I	R II	R III			
40	95.0	97.14	95.12	97.36	95.12	97.56	97.36	97.78	93.73	97.65	97.59	96.86
41	94.28	94.28	97.5	94.44	97.36	92.5	97.61	97.43	97.72	95.21	96.83	95.12
42	61.76	48.57	56.75	4.76	18.42	28.57	75.00	64.28	80.00	79.76	36.12	79.60
43	7.50	12.82	20.51	2.56	0	2.63	0	2.63	2.5	1.68	0	2.63
44	0	0	0	0	0	0	0	0	0	0	0	0
45	0	0	0	0	0	0	0	0	0	0	0	0

Table 3. 3. Percentage Survival of Thermotolerant Mutants N23814A, N23816A, and N23822A.
Abbrv. R: Replication Plate

Seeds of M3 mutants isolated from the secondary screen were germinated on one-half of the plates for 7 days in a controlled growth room; Col-0 WT was germinated on the other half as a control. Plates containing seven-day-old seedlings were placed in thermostatically controlled incubators for 3 hours in the dark and then returned to growth room to recover. Percentage survival was estimated after five days recovery (See Section 2.2.2.3 for full details).

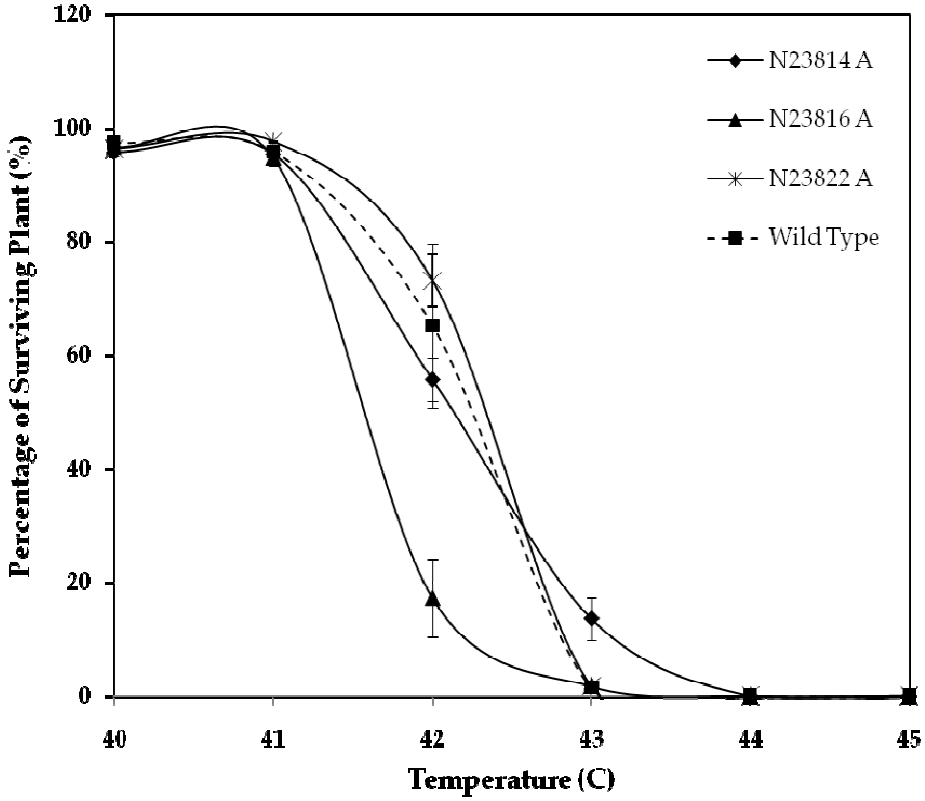


Figure 3. 4. Percentage Survival of Col-0 WT and M3 Putative Thermotolerant Mutants.

These data were obtained from seedling after five-day-post heat exposure. Sterilised stratified seeds of each mutant were sown on one half of 90mm petri dishes and Col-0 WT as control on another half (Section 2.2.2.3.). Each data point is the average of 3 replicates; error bar indicates standard error from three biological replications.

Based on Table 3.3 and Figure 3.4, there was a similar pattern of survival for the M3 mutants and Col-0 WT plants with increasing temperature. The M3 progeny of the N23814A line appears to be significantly more thermotolerant than Col-0 WT at 43.0 ± 0.2 ^{0}C ($p<0.05$) although no difference was observed below this temperature. In contrast, line M2 N23816A was significantly more thermosensitive ($p<0.001$; See Table 2 in Appendix 2) compared with Col-0 WT, and N23822A was equally sensitive. Based on the results from this experiment, it appears that 43.0 ± 0.2 ^{0}C is a more appropriate 'killing temperature' than the 44.0 ± 0.2 ^{0}C used. From these results it is recommended that in future all thermotolerance screens on non-

acclimated Arabidopsis seedlings should be performed at 43.0 ± 0.2 ^{0}C in the dark for 3 hours.

3.6. Discussion

Thermotolerance is defined as the ability of plants to cope with the high temperature by recruiting complex multigenetic processes that may differ with the developmental stage of growth. To understand the genetic basis for thermotolerance, resources in the model plant *Arabidopsis thaliana* such as Activation Tagged lines, can be used to identify the sequences involved in plant responses to high temperatures. The experiments reported in this Chapter have used these Activation Tagged collections to screen for thermotolerant mutants.

The results presented in this Chapter show that Arabidopsis undergoes thermal acclimation by exposure to high, non-lethal temperatures (37 ^{0}C for 1 hour). Non-acclimated Col-0 WT plants do not survive temperatures over 48 ^{0}C, whereas after thermal acclimation approximately 10% survive. These findings are consistent with many studies (Burke, 2001; Lim *et al.,* 2006). Given the time constraints of this project, it was only possible to screen one complete set of the Weigel Arabidopsis Activation Tagged collection although part of a second set was also screened (14,800 lines in total). These screens were performed on non-acclimated plants only. It is recommended that acclimated plants should also be screened.

Three mutants with improved thermotolerance in the M2 generation were identified from this screen and preliminary analysis of these plants confirmed they were more thermotolerant than Col-0 WT. Two of the progeny (M3) from these lines showed altered phenotypes from their parents; one had reverted to wild type, the other was hypersensitive. The secondary screen to confirm the thermotolerance phenotype was robust; mutant lines were screened on the same plates as Col-0 WT, and the same stock of wild type seeds was used throughout. It is likely, therefore, that the observed change in the M3 phenotype was genuine and not attributable to poorly controlled experimental conditions. Loss of

phenotype of dominant mutants isolated from Activation Tagged population is not unusual; a more detail explanation is provided in Chapter 5.

It is recommended that all secondary and confirmatory thermal screens are performed on 'half-plates' with Col-0 WT included as an internal control. This approach is robust and provides confidence in the observed phenotypes. Also, to ensure leaf temperature is controlled precisely, thermostatted incubators with temperature tolerances of ± 0.2 ^{0}C should be used, and plates sealed with *Nescofilm* (not *Parafilm*) or similar heat resistant products.

Despite the partial loss of phenotypes in the M3 generations it is recommended that the three identified mutants are characterized further. First, homozygous lines containing a T-DNA insertion at a single locus should be isolated; this can be achieved by segregation analysis of Basta resistance or from genomic Southern Blot analysis using probes that hybridize to the T-DNA. Second, the site of T-DNA insertion should be identified using TAIL-PCR or similar methods. Following these studies appropriate knockout lines and transgenic lines can be acquired to confirm the tagged genomic sequences are genuinely involved in activating thermotolerance mechanisms.

CHAPTER 4

ANALYSIS OF THERMOTOLERANCE IN p35S:AtMYB64 TRANSGENIC LINES

4.1. Introduction

The Arabidopsis transcription factor, AtMYB64, has been shown from previous investigations to activate salt tolerance mechanisms. In these experiments a gain-of-function salinity tolerance screen was performed on a collection of Arabidopsis Activation Tagged lines. The tolerant line JP5 was isolated and subsequently shown to be homozygous for a single T-DNA insertion in the promoter region of a gene AtMYB64 (At5g11050) encoding a MYB transcription factor. Line JP5 was analyzed using DNA microarrays (University of Arizona, http://www.ag.arizona.edu/microarray/) to profile the transcriptome. The list of differentially abundant sequences was analysed by Rank Product analysis (Breitling *et al*., 2004; Price, 2005) and several heat shock proteins (HSPs) were shown to be 'upregulated', and a calcium binding protein, Calmodulin 7 (CaM 7), was 'downregulated'.

From these findings of elevated levels of HSPs, further studies were conducted on the thermotolerance of the JP5 mutant and on transgenic Arabidopsis lines expressing the AtMYB64 cDNA under the control of the CaMV 35S promoter (p35S:AtMYB64). Results showed that compared with Col-0 WT, the JP5 line was as heat sensitive but the p35S:AtMYB64 lines were thermotolerant. These results suggest the hyper accumulation of AtMYB64 that occurs in the p35S:AtMYB64 lines leads to thermotolerance, but the lower levels that accumulate in the JP5 line, although effective at recruiting salinity tolerance mechanisms, are not sufficient to activate thermotolerance. Preliminary experiments also suggested that AtMYB64 is activated by ABA although this observation requires further confirmation.

The major objectives of the experiments reported in this Chapter are to investigate extensively the role of AtMYB64 in controlling downstream stress responsive

genes. This was achieved by exposing Col-0 WT and p35S:AtMYB64 transgenic lines to heat stress and monitoring the expression of HSPs and CaM7 over time. In addition, experiments were undertaken to determine whether this MYB transcription factor operates through an ABA-dependent or ABA-independent signalling pathway. This was investigated using QRT-PCR to analyse the expression level of AtMYB64 on Col-0 WT plants treated with exogenous ABA.

4.2. Quantitative Reverse Transcription PCR (QRT-PCR)

4.2.1. Definition of QRT-PCR

QRT-PCR is the technique of quantifying mRNA abundance by collecting data during the PCR process, thus combining amplification and detection into a single step (Wong and Medrano, 2005). The abundance in a sample is determined from the amplification cycle where the target sequence is first detected. The detection is based on the incorporation of fluorescent probes into the PCR product so that fluorescence intensity increases with each cycle of amplification.

The advantage of using QRT-PCR over other methods is that quantitative data can be generated over a large dynamic range of target sequences concentrations. This type of PCR is also more sensitive than the other RNA quantification methods.

4.2.2. General Method of QRT-PCR

One of the first points to consider in the experimental design is which quantitative PCR chemistry to use in the QRT-PCR experiment. Three general chemistries for quantitative detection are DNA-binding agents, hydrolysis probes, and hybridization probes.

1. DNA-binding agents

DNA-binding agents are chromophores that increase their fluorescence yield when bound to double stranded DNA. The more double-stranded DNA that is present, the more binding sites there are for the dye, so fluorescence increases

proportionately with DNA concentration. As the target sequence is amplified by the PCR, the increasing concentration of double stranded DNA in the solution can be directly measured by the increase in fluorescence signal (Figure 4.1.). Double-stranded DNA-binding agents include SYBR Green, ROX, CY5, and HEX. One limitation of this assay is the inherent non-specificity of this method because the dye can bind to any double stranded DNA. A non-specific signal cannot always be eliminated, but its presence can be easily and reliably detected by performing melting curve analysis on the PCR product from every run (Figure 4.2.).

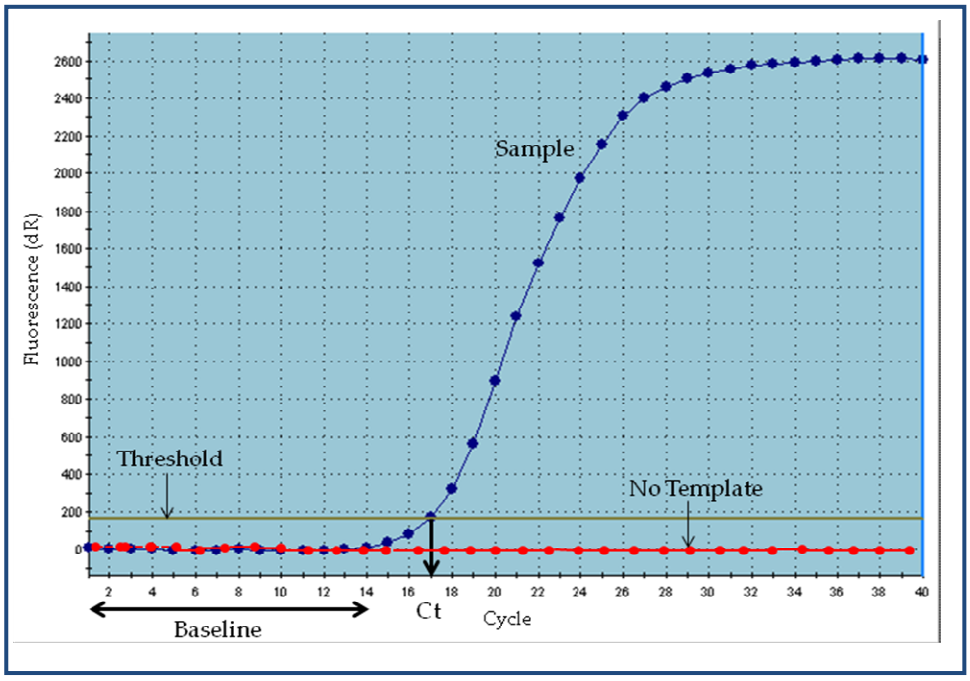


Figure 4. 1. Principle of QRT-PCR Detection

mRNA is isolated from a sample and cDNA synthesized by reverse transcription. This cDNA is then used as template in the QRT-PCR reaction. Fluorescence detection, and therefore QRT-PCR target concentration, is measured at the threshold cycle (Ct). The Ct is inversely proportional to the initial target sequence copy number. Only when the DNA concentration has reached the fluorescence detection threshold can it be reliably inferred from the fluorescence intensity. A higher initial copy number will correlate to a lower threshold cycle (Ct).
Figure adapted from Stratagene, Methods and Application Guide (2004).

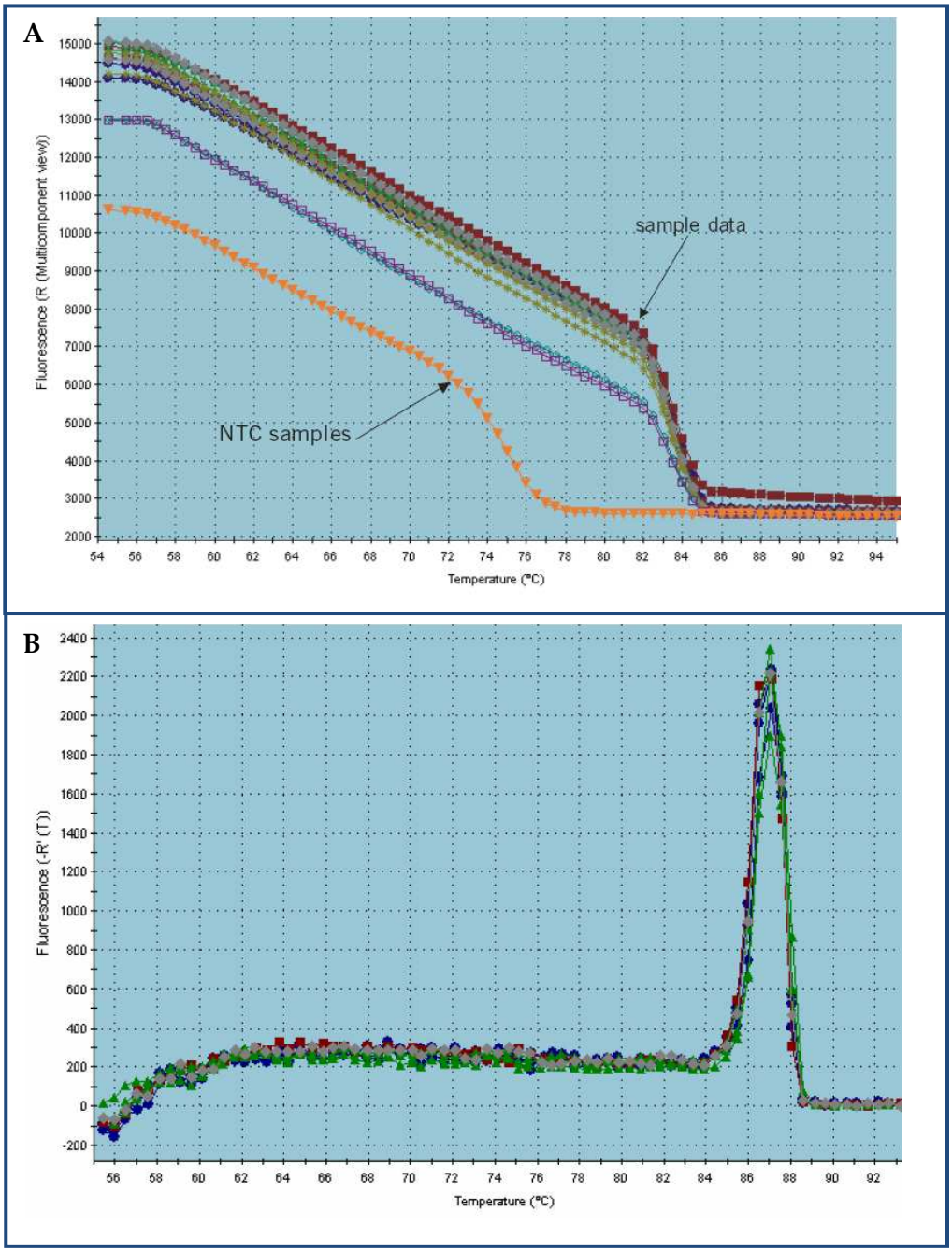


Figure 4. 2. Melt Curve Analysis of QRT-PCR Products

(A) Fluorescence signal of product plotted as a function of increasing temperature. After completion of the QRT-PCR the sample is heated until all duplex sequence has melted. The upper traces show rapid melt of product between 82 ^{0}C and 84 ^{0}C. The Non Template Control (NTC) sample shows a different melt temperature of around 72 ^{0}C. The presence of different melt temperatures indicates non-specific amplification. (B) The first derivative of raw fluorescence plotted against an increase in temperature. The single melt peak at 86.5 ^{0}C indicates a single PCR product is being amplified in these samples.
Figure adapted from Stratagene, Methods and Application Guide (2004).

2. Linear probes

Linear Probes or Hydrolysis or TaqMan probes are used widely as detection chemistries for QRT-PCR applications. During amplification with PCR primers, this chemistry includes a third oligonucleotide as the probe that anneals to one strand of the target sequence just slightly downstream of one of the primers. As the polymerase extends the primer, it will encounter the 5′ end of the probe. *Taq* DNA polymerase has 5′-3′ nuclease activity, so when *Taq* DNA polymerase encounters the probe it degrades the 5′ end, releasing free reporter dye in solution. The separation of reporter dye and quencher result in increasing of fluorescence from the reporter dye (Figure 4.3.).

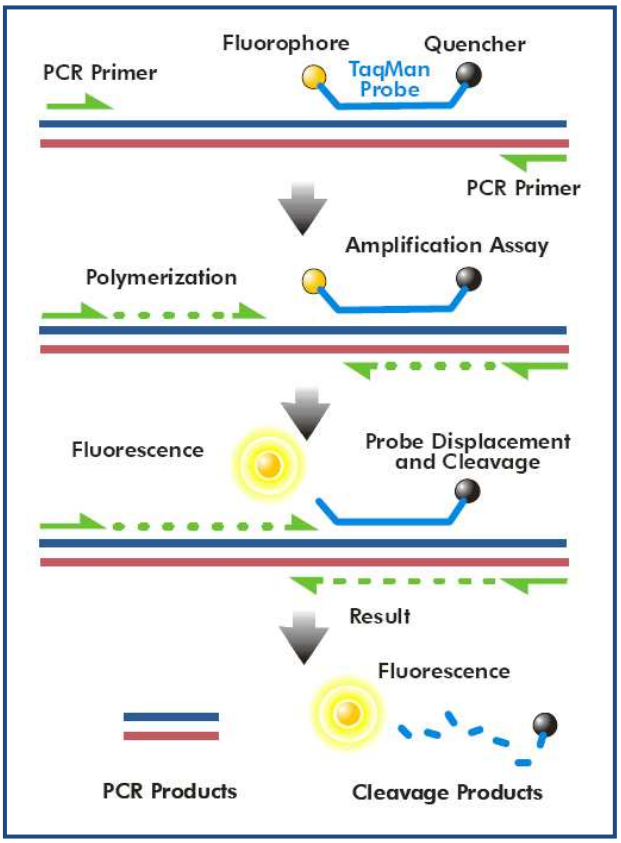


Figure 4. 3. TaqMan Probe Chemistry Mechanism

These probes rely on the 5′-3′ nuclease activity of *Taq* DNA polymerase to cleave a dual-labeled probe during hybridization to the complementary target sequence. Figure adapted from Stratagene, Methods and Application Guide (2004).

3. Hybridization probes (Light Cycler).

Light cycler is a combination between thermal cycler and fluorimeter method which allows PCR product detection and identification with a variety of fluorescence chemistries. This system provides rapid analyses because of very fast temperature transition rates and online analysis of the data, with a reduced risk of contamination (Steven *et al*., 2001). The signal from hybridization probes depends directly on hybridization, not on exonuclease activity and probe hydrolysis. Each probe is covalently labeled with only one dye, so they are inherently simpler to synthesize than double-labeled probes. One probe is labeled on the 3'-end. The other probe is labeled on the 5'-end and its 3'-end blocked to prevent extension. When both probes are hybridized in tandem, fluorescence emission occurs. Maximum fluorescence occurs with a one base separation between probes (Stratagene, Methods and Application Guide, 2004).

4.2.3. Experimental Design

There are two basic quantification methods and each is suitable for different applications: Absolute Quantification and Relative Quantification.

1. Absolute Quantification

The most direct and precise approach for analyzing quantitative data is to use a standard curve that is prepared from a dilution series of target gene template (eg. plasmid containing a clone of the gene of interest (GOI), genomic DNA, cDNA, synthetic oligos, in vitro transcripts, or total RNA) of known concentration.

Following amplification of the standard dilution series, the standard curve is generated by plotting the log of the known initial template copy number against the Ct generated for each dilution. The accuracy of aliquoting the standard series and stability of the amplification reaction over the range of template concentrations should generate a straight line, the standard curve. Comparing the Ct values of the unknown samples to this standard curve allows the direct quantification of initial copy number.

2. Relative Quantification

The majority of scientific questions regarding gene expression can be accurately and reproducibly answered by measuring the relative concentration of the gene of interest (GOI) in a sample compared to a 'calibrator', or control sequence. Using this method, differences in Ct value between a GOI sequence and calibrator sequence are expressed as fold-change (i.e. up or down regulated) relative to the calibrator.

4.3. Genotyping p35S:AtMYB64 Lines using Selectable *Bar* Marker Genes

Several transgenic Arabidopsis lines carrying a p35S:AtMYB64 construct had been generated by others before this investigation began. These lines had not been genotyped, however, to identify homozygous lines. The acquisition of homozygous transgenic lines will simplify the analysis of future experiments and is considered desirable. Two transgenic lines expressing p35S:AtMYB64, T141 and T127, were genotyped to identify homozygous individuals using segregation analysis of the selectable *Bar* marker gene that provides resistance to the herbicide Basta. All vectors used in this study carried the *Bar* gene.

Approximately 150 seeds of each line were germinated on plates containing 1/10 MS media and ± 20 μg/ml herbicide (Basta). Col-0 WT plates were included as controls. Plates were placed in the growth room and Basta resistance assessed after 2 weeks (See Section 2.2.4.). Germination efficiency was scored for each line on the Basta plates, and the number of seedlings producing true first leaves scored as resistant (B^R) on the medium containing Basta after 14 days. Seedlings that germinated but failed to develop first true leaves were scored as Basta sensitive (B^S). From these data, Chi-Square tests were applied to establish the genetic basis of the inheritance of Basta resistance.

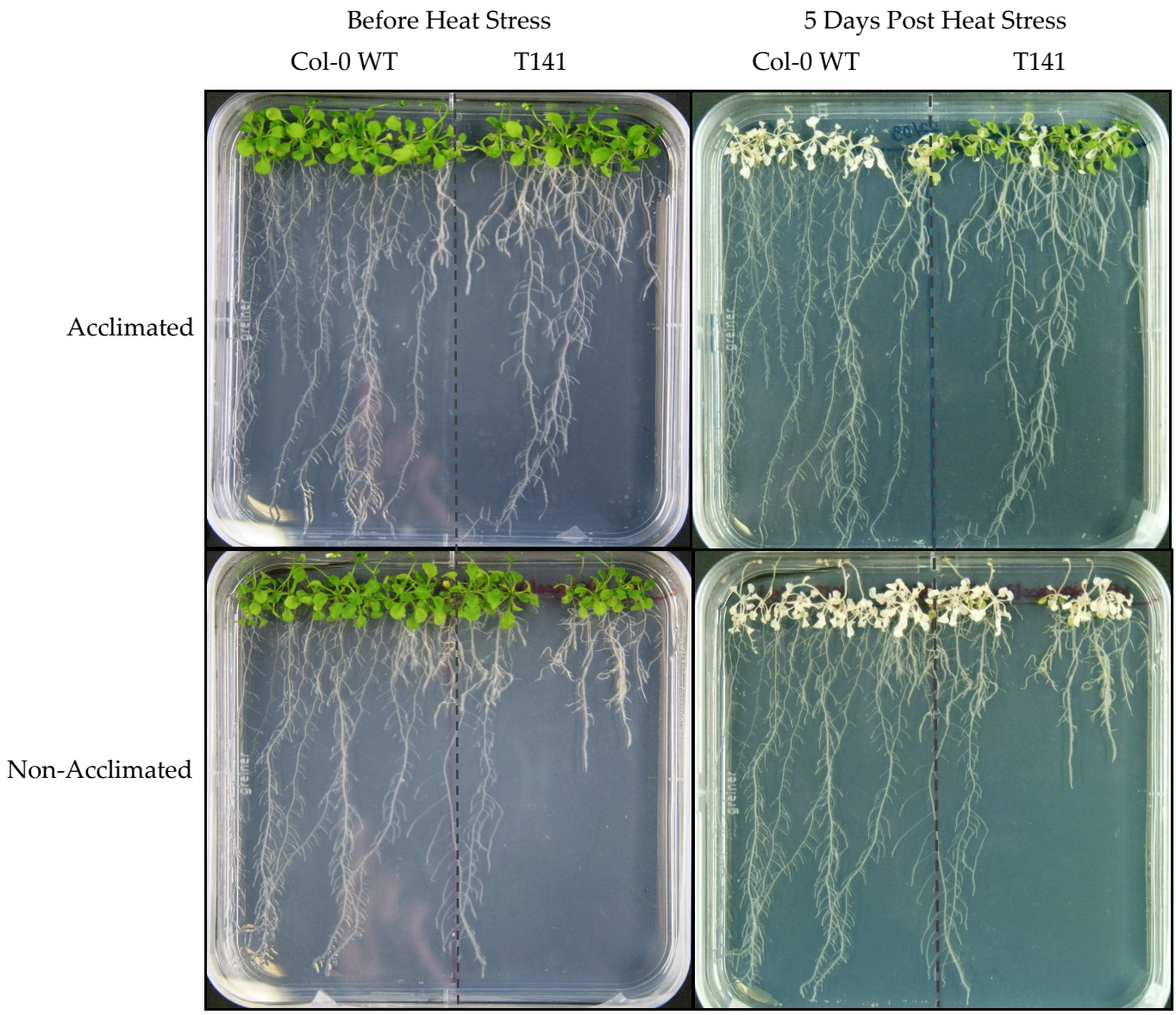
The *Bar* (Basta resistance) gene carried on the pEARLEYgate100 and pB7Wg2 GATEWAY vectors used is a dominant allele and therefore progeny of the B^R T141 and T127 lines would be expected to segregate at a ratio of 3:1 (B^R:B^S) if the parent

was heterozygous at one insertion site, rising to all B^R if the line was homozygous at one or more insertion sites. Ratios between 3:1 and 4:0 would indicate heterozygous insertions at more than one site (i.e. several independent insertional events).

Ten T141 lines and 18 T127 lines were assessed for Basta resistance and the data are presented in Appendix 3. Segregation analysis and Chi-square tests showed for the M3 generation of T141, 1 line showed a 16:0 (B^R:B^R) segregation, and 8 lines a (15:1) segregation. For T127, 1 line showed a 16:0 and 4 a 15:1 segregation. These data suggest that in both T141 and T127, homozygous lines may be present (16:0), although the high proportion of lines with 15:1 segregation suggests multiple insertions are probably present. Further work will be required to isolate homozygous individuals with insertions at a single locus.

4.4. The Plate-Based Phenotypic Thermotolerance Analysis of p35S:AtMYB64 Transgenic Lines

Thermotolerance of p35S:AtMYB64 transgenic lines was assessed using two heat treatments to analyze the thermotolerant mechanism. The first experiment assessed heat acclimation by transferring seven day-old plants grown in 22 ^{0}C to 37.0 ± 0.2 ^{0}C for 1 hour to acclimate before returning to the growth room (22 ^{0}C) for a week to recover before exposure to heat stress. In the second experiment, 14-day-old seedlings were transferred from 22 ^{0}C and exposed to heat stress without a period of heat acclimation. The germination level of p35S:AtMYB64 T141 transgenic lines were lower than those of the T127 lines. Note that T141 lines had a germination rate of about 80%; however, root length was generally shorter than both Col-0 WT and T127 lines. In contrast, the T127 lines had a germination rate of near 100% and there were no obvious differences in leaf area and root length compared with Col-0 WT.


Before Heat Stress
5 Days Post Heat Stress
Col-0 WT
T141
Col-0 WT
T141
Acclimated
Non-Acclimated

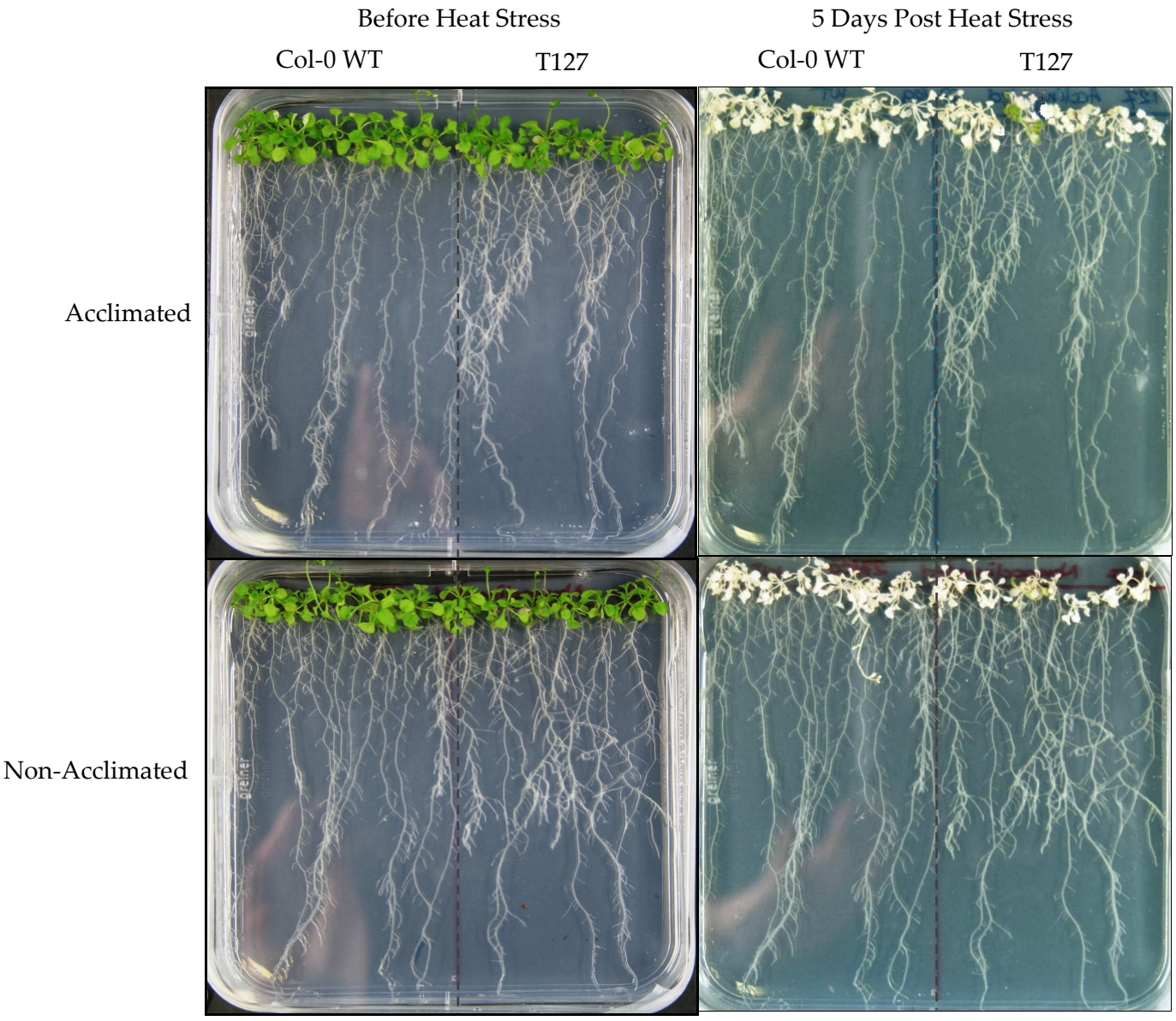


Figure 4. 4. The Effect of Heat Stress on Heat Acclimated and Non-Acclimated p35S:AtMYB64 Transgenic and Col-0 WTArabidopsis Lines.

Ten Col-0 WT (left half) and 10 transgenic lines (right half) sterilized seeds were placed on plates and germinated for 7 days at 22 ^{0}C (Section 2.2.3.1.1.). Heat acclimation was achieved by transferring seven-day-old seedlings to 37.0 ± 0.2 ^{0}C for 1 hour before returning to the growth room (22 ^{0}C) for a week to recover. Both acclimated and non-acclimated 14 day-old seedlings were transferred from 22 ^{0}C to 44 ± 0.2 ^{0}C for 3 hours (heat stress) and returned to the growth room at 22 ^{0}C for 5 days. The images presented are typical examples selected from 3 replicate plates. Thermotolerance was estimated as the number of surviving (green) seedlings.

Transgenic Line/ % Germination ± SE	Heat Treatment	% Surviving Plant After Heat Stress			Average Surviving Plant (% ± SE)
		R 1	R 2	R 3	
141 Transgenic Line	Acclimated	30	20	90	46.67 ± 21.86
83.33 ± 3.33	Non-Acclimated	10	10	30	16.67 ± 6.67
127 Transgenic Line	Acclimated	10	20	10	13.33 ± 3.33
100 ± 0.00	Non-Acclimated	10	0	0	3.33 ± 3.33
Col-0 WT	Acclimated	0	0	0	0.00 ± 0.00
100 ± 0.00	Non-Acclimated	0	0	0	0.00 ± 0.00

Table 4. 1. Percentage Surviving Seedlings of p35S:AtMYB64 Transgenic and Col-0 WT Lines 5-Day-Post Heat Stress.

Abbrv: R : Replication
These data were calculated from the experiment described in Figure 4.4 (3 replicates per treatment).

Figure 4.4 shows the phenotype of acclimated and non-acclimated lines of Col-0 WT, T141, and T127 after exposure to heat stress. Major differences were observed in the leaves of seedlings before and after heat exposure. After heat treatment, leaves appeared crinkled and were translucent-white compared with non-stressed controls.

The results shown in Figure 4.4 and Table 4.1, suggest that acclimated transgenic plants showed more tolerance to heat stress than non-acclimated plants. It appears that preliminary treatment of plants with a moderately elevated or non-lethal temperature can transform plants to be more resistant to subsequent potentially lethal temperatures. Line T141 demonstrated a greater thermotolerance than line T127 but both were more thermotolerant than Col-0 WT. A large variance in survival was observed between the replicates, particularly for acclimated T141 plants (Table 4.1). However, it is clear that acclimation improves survival, that T141 is more thermotolerant than T127, and both transgenic lines are more thermotolerant than Col-0 WT.

4.5. Analysis of the Expression of Stress Responsive Genes in Heat Acclimated p35S:AtMYB64 Transgenic and Col-0 WT Arabidopsis Lines.

4.5.1. Introduction

To assess the expression of stress responsive genes in heat acclimated p35S:AtMYB64 transgenic lines, 25 seeds were germinated on 1/10 MS media as mentioned in Section 2.2.3.2.1. After two weeks growth, plants were heat acclimated at 37.0 ± 0.2 ^{0}C for 3 hours and then returned to the growth room. During this procedure, samples were taken at various time points (0, 3, 6, and 12 hours) from the start of acclimation and RNA extracted for the synthesis of cDNA. cDNA was generated by reverse transcription PCR and the abundance of the cDNA for the following heat shock proteins was assessed (smHSP 17.6, smHSP 17.6A, HSP70, and HSP101). In addition, the abundance of Calmodulin 7 (CaM7) was also measured. Previous transcript profiling experiments using DNA microarrays had indicated these genes were differentially abundant in the JP5 mutant compared with Col-0 WT. This suggested that AtMYB64 controlled the expression of these heat shock proteins and CaM 7 and that these are involved in stress responses. The cDNAs prepared from heat acclimated plants were used to confirm a role for these stress response genes in thermotolerance.

4.5.2. Semi Quantitative Reverse Transcription Polymerase Chain Reaction (SQRT-PCR) Analysis

The expression of heat-stress responsive genes was analyzed using SQRT-PCR. Actin-2 was chosen as an internal control for the expression of genes in the samples. An ideal internal control should be uniformly expressed in all experimental treatments, however, as shown in Figure 4.5., the variation of Actin-2 expression between experimental treatments was very variable even though comparable levels of RNA were used to generate the cDNA. Careful adjustment in the concentration of template cDNA to achieve the same expression of Actin 2 between samples proved to be useless as erratic patterns of target gene

expressions were routinely generated. This was due in part to the large dynamic range of target sequence abundance in the samples.

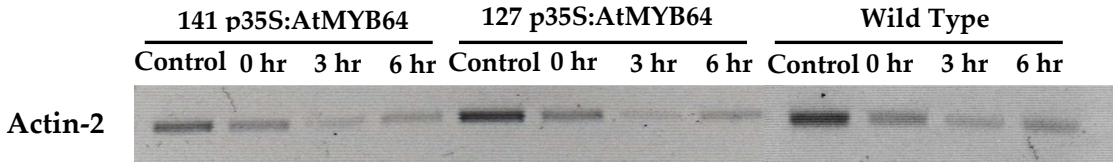


Figure 4. 5. SQRT-PCR Assessment of Actin-2 Abundance in p35S:AtMYB64 Transgenic and Col-0 WT Arabidopsis Lines After Heat Acclimation

RNA samples were obtained from 14-day-old seedlings heat-acclimated plants (37 ^{0}C for 3 hours); non-acclimated plants were used as controls; 0 hour (0H), 3 hour (3H), and 6 hour (6H) after heat acclimation. cDNA was then synthesized from those RNA and the abundance of Actin-2 in each cDNA sample was assessed by SQRT-PCR (See Section 2.2.13.).

Actin-2 abundance changed with heat acclimation showing a decrease 3 hours after acclimation, and levels were very variable between replicates.

Strenuous attempts were made to standardize Actin-2 expression in heat acclimated samples, but expression was too variable to be of use. Therefore, due to the limitations of SQRT-PCR to investigate heat-stress responsive gene expression, Quantitative RT-PCR (QRT-PCR) was used to overcome this problem.

4.5.3. Quantitative Reverse Transcription Polymerase Chain Reaction (QRT-PCR) Analysis.

4.5.3.1. Selection of Internal Standard Sequences to Normalize Gene Expression.

Genuine differences in the abundance of a specific transcript is masked by differences arising from variations in the amount of starting material of each sample. This is especially important for samples that have been obtained from different individuals and can result in misinterpretation of the expression level of the gene(s) of interest. The common method for minimizing the error and

correcting for sample-to-sample variations in total RNA abundance is to amplify a cellular RNA (cDNA) that operates as an internal reference against which the concentration of other cDNAs can be normalized (Karge *et al*., 1998). Ideal internal standards should be expressed at a constant level among different tissues of an organism, at all stages of development, and should be unaffected by experimental treatment. The three sequences that are most commonly used to normalize are actin, GAPDH, and ribosomal RNAs (Bustin, 2000). It is very important to decide on the appropriate standard as this constitutes an important aspect of experimental design.

Actin

Actin mRNA is expressed at moderately abundant levels in most cell types and encodes a ubiquitous cytoskeleton protein. It was one of the first RNAs to be used as an internal standard, and it is still recommended as a quantitative reference for QRT-PCR assays (Kreuzer *et al.*, 1999). However, there is some evidence to suggest that its transcription can vary widely in response to experimental conditions (Schmittgen *et al*., 2000; Thellin *et al*., 1999) in Arabidopsis (Sun *et al*., 2001, Volkov *et al*., 2003), human breast epithelial cells (Spanakis, 1993), blastomeres (Krussel *et al.*, 1998), porcine tissues (Foss *et al.*, 1998), and canine myocardium (Carlyle *et al.*, 1996). The varying expression levels of β-Actin have also been studied by Ruan and Lai (2007) who assessed mRNA levels using Northern Blots, QRT-PCR, Competitive RT-PCR, TaqMan PCR, and cDNA microarrays. Based on these studies, it is clear that Actin transcript levels depend on many factors, such as stage of development, and exposure to environmental stress.

GAPDH

The RNA encoding GAPDH (Glyceraldehyde 3-Phosphate dehydrogenase) is a ubiquitously expressed, moderately abundant message. GAPDH converts G3-P into 1,3 Bisphosphoglycerate and forms a central step in many metabolic processes

including glycolysis and the Calvin cycle. It is frequently used as an internal standard for QRT-PCR analysis because, in some experimental systems, its expression is reported to be constant even after experimental manipulation (Edwards & Denhardt, 1985; Winer *et al.*, 1999). However, there is significant evidence to suggest its use as an internal standard is inappropriate (Oliveira *et al.*, 1999; Thellin *et al.*, 1999). GAPDH concentrations vary significantly between different individuals (Bustin *et al.*, 1999), with developmental stage (Puissant *et al.*, 1994, Calvo *et al.*, 1997), and during the cell cycle (Mansur *et al.*, 1993). Besides that, numerous transcription regulatory domains have been identified in the yeast GAPDH promoter, again suggesting that this gene is subject to complex transcriptional regulation (Yagi *et al.*, 1994).

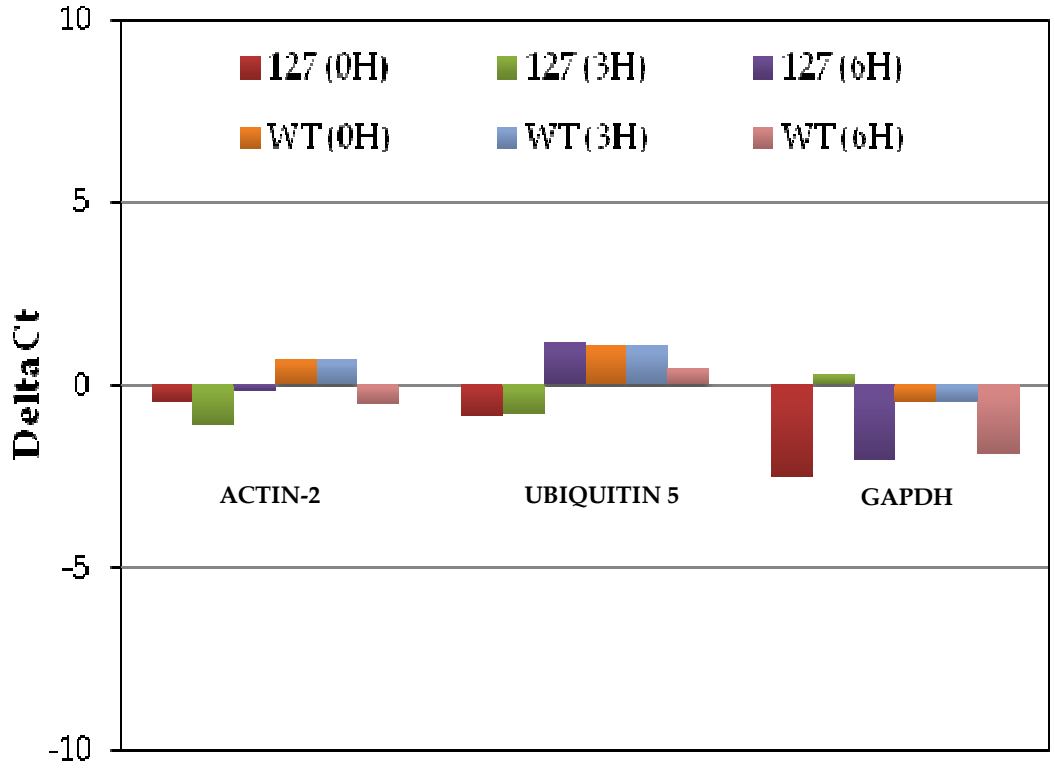


Figure 4. 6. Variations in 'Internal Standard' Sequence Concentration in Heat Acclimated p35S:AtMYB64 Transgenic and Col-0 WT Arabidopsis Lines

RNA samples were prepared from 14-day-old seedlings and cDNA synthesized as described in Section 2.2.13. QRT-PCR was performed as described in Section 2.2.14 with 2 instrumental replications and data shown in the diagram are the differences between average Ct_s of control plants and average Ct_s of treated plants. The control plants (non-acclimated) served as a baseline for the assay and is indicated as zero on the graph. Samples with values above zero indicate lower levels of target gene expression, whereas those below zero indicate a higher level of expression of the specific genes compared with controls. Genes that consistently showed little variation from the control (zero line) were chosen for use as reliable internal standards.

Figure 4.6., presents the variation in expression of three internal standard gene sequences (Actin-2, Ubiquitin 5, and GAPDH) between samples isolated from heat acclimated seedlings. To assess the stability of these sequences in various experimental treatments, the value of ΔCt (delta Ct) was calculated. ΔCt values are obtained by subtracting the Ct value for the sequence of interest in the treated sample from the Ct of a control; positive ΔCt indicate a decrease in sequence abundance in the treated sample, negative ΔCt an increase in treated sample. Based on Figure 4.6, it is clear that the Actin-2 expression varies in all samples but by not more than 1 ΔCt (2-fold change). GAPDH has the largest variation (3 ΔCt, 8-fold change) compared with the two other sequences. Actin-2 seems to be the best sequence for an internal standard and was chosen for use in the following experiments.

Figure 4. 7. Variation of Actin-2 Abundance in Heat Acclimated p35S:AtMYB64 Transgenic and Col-0 WT Arabidopsis Lines

Four different conditions were used; without heat acclimation (WA), immediately (0H), 3 hours (3H), 6 hours (6H), and 12 hours (12H) after acclimation, respectively are shown. The control samples (without acclimation (WA) served as a baseline for the assay and is shown as zero on the graph. Samples showing values above zero indicate lower levels of target gene expression. The values are the average of two experimental replicates from one biological replicate.

The results presented in Figure 4.7 indicates the expression level of Actin-2 among all experimental treatments is suppressed by up to 4 fold (2 ΔCt) after heat acclimation and persists for up to 6 hours post-acclimation, although there are signs of recovery at 12 hours post-acclimation. A similar pattern arises in both the p35S:AtMYB64 transgenic and the Col-0 WT lines.

4.5.3.2. QRT-PCR Analysis of AtMYB64 in Heat Acclimated p35S:AtMYB64 T127 Transgenic and Col-0 WT Arabidopsis Lines

Plants used in this experiment were from the p35S:AtMYB64 T127 transgenic line (Section 2.1.1.2.); Col-0 WT was used as a control. The plants were heat acclimated by exposure to 37 ^{0}C for 3 hours, followed by incubation at growth room temperatures (Section 2.2.3.2.2).

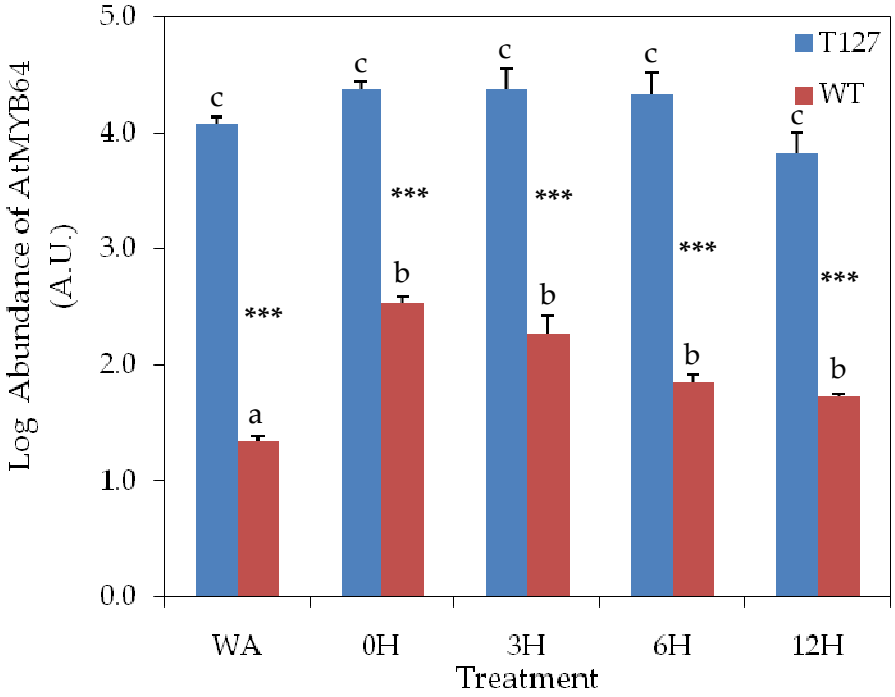


Figure 4. 8. QRT-PCR of AtMYB64 Abundance in Heat Acclimated p35S:AtMYB64 T127 Transgenic and Col-0 WT Arabidopsis Lines.

RNA samples were obtained from heat-acclimated plants (37 ^{0}C for 3 hours); non-acclimated plants (WA) were used as controls; 0 hour (0H), 3 hours (3H), 6 hours (6H), and 12 hours (12H) after heat acclimation. Three independent RNA preparations were made for each treatment to provide Biological Replication. cDNA was prepared from each of the Biological Replicates to generate 30 cDNA samples (2 lines × 5 treatments × 3 replicates). The abundance of AtMYB64 in each cDNA sample was assessed by QRT-PCR (See Section 2.2.14.) and log abundance level in Arbitrary Units (A.U.; ± SE) are presented. Different lower case Roman letter codes indicated significant differences between treatments (within line) at 95% confidence level. Asterisks indicate significant differences between lines at each treatment level (***, $p<0.001$)

Figure 4.8 indicates the changes in AtMYB64 transcript abundance before and after heat acclimation in Col-0 WT and T127 (p35S:AtMYB64) lines. This data set was transformed into a normal distribution by taking Log base 10 of the values and then analyzed by an Analysis of Variance test (GLIM Model, Minitab Ver. 15). The output from this analysis is presented in Table 4, Appendix 2. The apparent levels of AtMYB64 transcript in non-acclimated T127 lines was over five hundred times greater than those in Col-0 WT ($p<0.001$) confirming that the strong 35S promoter has resulted in a large increase in AtMYB64 expression (Col-0 WT WA versus T127 WA). Upon heat acclimation AtMYB64 expression increased about 17 times in Col-0 WT ($p< 0.05$; Col-0 WT WA versus Col-0 WT 0H), but declined with time and after 12 hours was only approximately double those of non-acclimated plants (0H versus 3H-12H). Heat acclimation also produced an increase and subsequent decline in the levels of AtMYB64 in T127 lines although this was only a doubling; large variances were incurred with T127 heat acclimated samples and this resulted in a failure to detect significant changes between treatments (Figure 4.8.). Regardless of these large variances in the T127 heat acclimated samples, AtMYB64 transcript levels were between 70 and 540 times greater ($p<0.001$) in T127 samples compared with Col-0 WT samples at comparable treatment levels (Figure 4.8.). These data confirm that AtMYB64 transcript levels are dramatically elevated in the p35S:AtMYB64 lines (~500 times, $p<0.001$; Col-0 WT versus T127), and that the AtMYB64 promoter is responsive to heat acclimation.

4.5.3.3. QRT-PCR Analysis of Heat Shock Proteins (HSPs) in Heat Acclimated p35S:AtMYB64 T127 Transgenic and Col-0 WT Arabidopsis Lines

Figure 4.9, presents the apparent abundance of smHSP17.6, smHSP17.6A, HSP70, and HSP101 in Col-0 WT and p35S:AtMYB64 T127 transgenic lines before and after heat acclimation. The data set for smHSP17.6 and smHSP17.6A was transformed (Log base 10) to provide a normal distribution and an Analysis of Variance test performed on these data; the output is presented in Table 5 and 6 of Appendix 2.

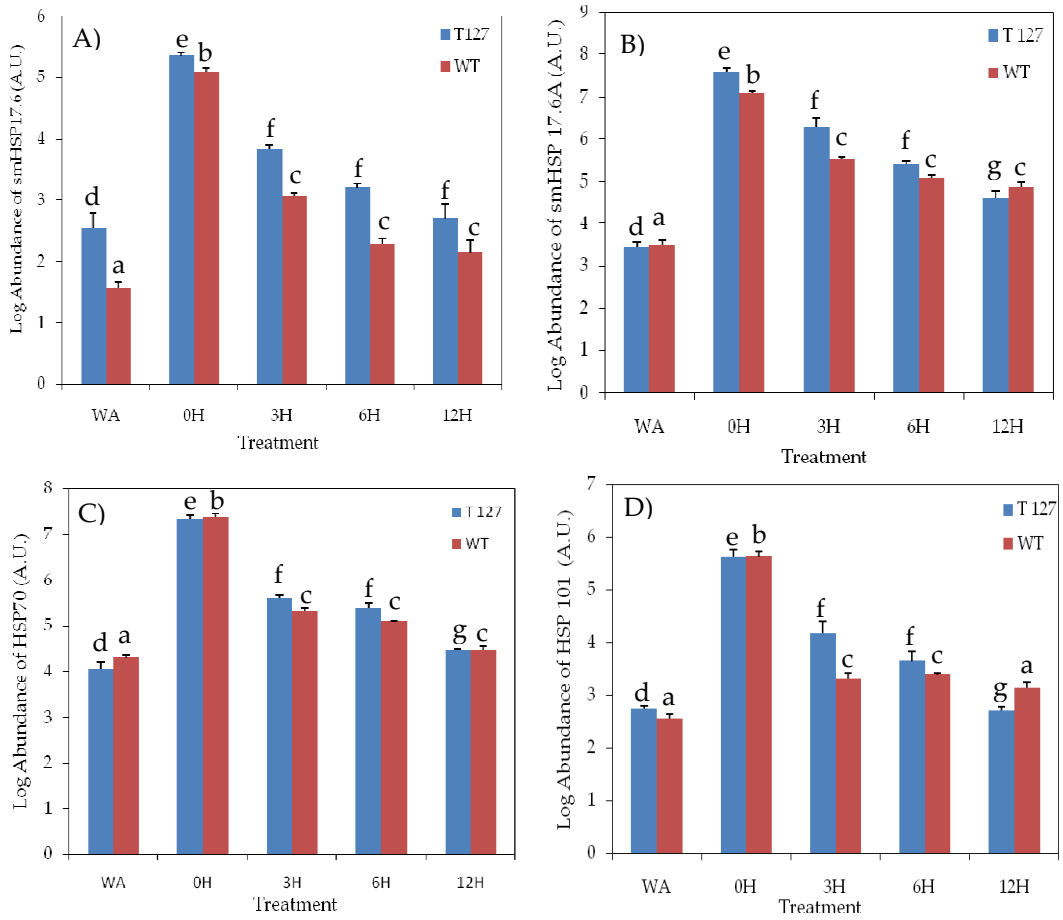


Figure 4. 9. QRT-PCR of 4 Heat Shock Proteins (HSPs) Abundance in Heat Acclimated p35S:AtMYB64 T127 Transgenic and Col-0 WT Lines.

(A) smHSP 17.6; (B) smHSP 17.6; (C) HSP 70; and (D) HSP 101. Line T127 p35S:AtMYB64 transgenic line and Col-0 WT under non-heat acclimation conditions (WA), and with time after heat acclimation (0H, 3H, 6H, and 12H). The expression levels shown are the apparent transcript abundance levels of the sequences in Arbitrary Unit (A.U.). Values are the average and standard error of three biological replications per treatment. Different letter codes signify significant differences between treatments (within lines; $p<0.05$).

The levels of smHSP17.6 in non-acclimated Col-0 WT lines are low but approximately ten times higher in T127 lines (Figure 4.9. A, Col-0 WT WA versus T127 WA). It should be emphasised that this difference was not significant, however, despite the large difference in the transcript abundance. This appears to have been due to inherent variability between the abundance of template in the non-acclimated T127 samples, although it is not clear why this should be. Further biological replicates were prepared from non-acclimated Col-0 WT and T127 lines (3 each) and QRT-PCR performed on these but similar results were obtained; the transgenic line T127 contained more smHSP17.6 sequence but with a high inherent variance, no significant difference was detected between the transgenic and Col-0 WT line (data not presented).

Heat acclimation of Col-0 WT plants produced a dramatic increase in smHSP17.6 transcript abundance (>1000 times, $p<0.05$, Table 5 in Appendix 2), but this declined rapidly after removal from heat-acclimation conditions (Figure 4.9A). A similar pattern was observed for smHSP17.6 abundance in the T127 lines (Figure 4.9A). Comparison of the abundance of smHSP17.6 in Col-0 WT and T127 plants exposed to the same treatment showed no significant differences were present at any time (Col-0 WT 0H versus T127; Figure 4.9A). This might seem surprising given the standard errors presented in Figure 4.9 but it should be remembered these are Log base 10 of the values and these error bars, therefore, are distorted somewhat.

The pattern of change in apparent abundance of smHSP17.6A transcript in Col-0 WT and T127 lines before and after heat acclimation were similar (Figure 4.9B). Initially levels were low, but in both lines this increased over 1000 times ($p<0.05$) immediately after heat acclimation (WA versus 0H), but declined rapidly thereafter (0H versus 3H-12H). Comparison between smHSP17.6A levels in Col-0 WT and T127 were not significantly different at any stage suggesting the presence of elevated levels of AtMYB64 in the T127 line (over 500 times) does not affect the abundance of smHSP17.6A (Figure 4.9B and Table 6, Appendix 2). A similar

pattern of change in HSP abundance with heat acclimation was observed for HSP70 and HSP101 (a 100 to 1000 fold change; $p<0.0001$) followed by a rapid decline, but no significant differences were observed at any time between the Col-0 WT and T127 lines. It appears that HSP70 and HSP101 expression are also not under the control of AtMYB64 (Figure 4.6 C and D, Table 7 and 8 in Appendix 2).

4.5.3.4. QRT-PCR Analysis of Calmodulin 7 (CaM7) in Heat Acclimated p35S:AtMYB64 T127 Transgenic and Col-0 WT Arabidopsis Lines.

Analysis of Calmodulin 7 cDNA (RNA) levels using quantitative reverse transcription PCR revealed it was marginally more abundant in the T127 transgenic lines and Col-0 WT after heat acclimation. The data set was transformed (Log 10) to provide a normal distribution and this analysed by Analysis of Variance (GLIM, Minitab Ver. 15; See Table 9 in Appendix 2).

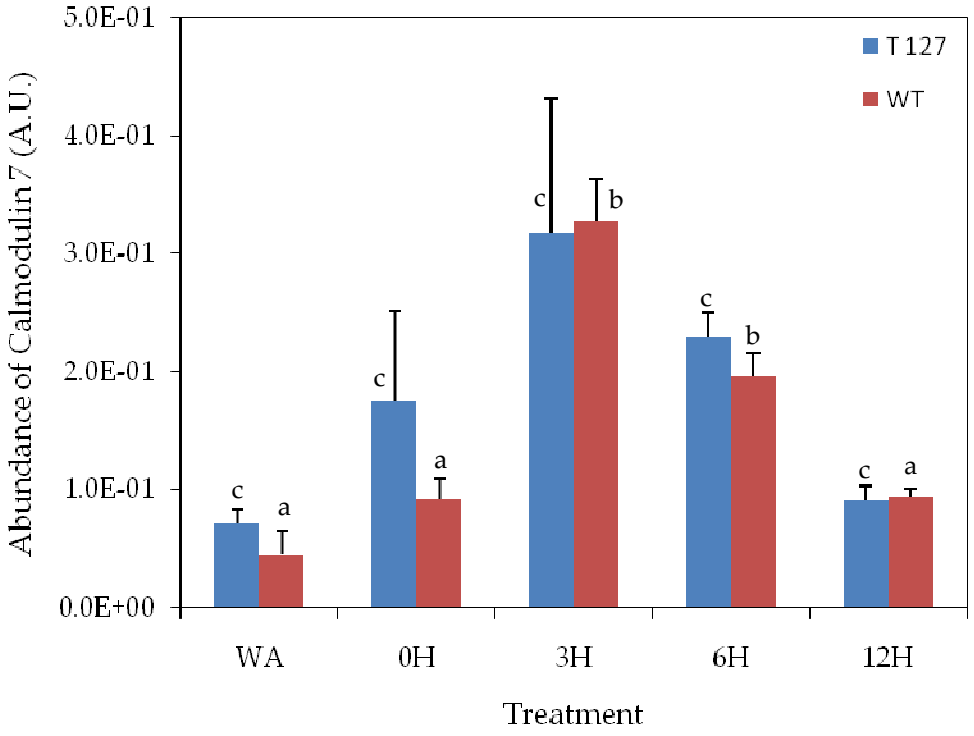


Figure 4. 10. QRT-PCR of Calmodulin 7 (CaM7) Abundance in Heat Acclimated p35S:AtMYB64 T127 and Col-0 WT Arabidopsis Lines.

RNA samples were obtained from heat-acclimated plants (37 ^{0}C for 3 hours); non-acclimated plants (WA) were used as controls; 0 hour (0H), 3 hours (3H), 6 hours (6H), and 12 hours (12H) after heat acclimation. Three independent RNA preparations were made for each treatment to provide Biological Replication. cDNA was prepared from each of the Biological Replicates to generate 30 cDNA samples (2 lines × 5 treatments × 3 replicates). The abundance of AtMYB64 in each cDNA sample was assessed by QRT-PCR (See Section 2.2.14.) and log abundance level in Arbitrary Units (A.U.; ± SE) are presented. Different lower case Roman letter codes indicated significant differences between treatments (within line) at the $p<0.05$ level.

In both lines the peak in Calmodulin 7 abundance occurred 3 hours after ABA treatment but this was only a 4 – 7 fold increase and was significant only in the Col-0 WT line ($p<0.05$; See Table 9 in Appendix 2). After 3 hours, the levels of Calmodulin 7 declined in both lines. The pattern of apparent expressions pattern CaM7 transcript abundance is similar in the p35S:AtMYB64 T127 transgenic and Col-0 WT lines and there was no clear difference in the abundance between the two lines at any time.

4.6. Characterization of The AtMYB64 Transcription Factor Signalling Pathway

4.6.1. Introduction

The important role of the plant hormone abscisic acid (ABA) during many phases of a plant's life cycle, such as in plant responses to various environmental stresses, has been studied in detail (Abe *et al*., 2003; Knight *et al*., 2004). Genetic and molecular studies have suggested that there is a complex interaction between osmotic stress, temperature stress, and plant ABA levels (Xiong *et al*., 1999; Koiwa *et al*., 2006). Adaptive responses to abiotic stress require the regulation of gene expression and this operates either through an ABA-dependent or an ABA-independent signalling pathway. The promoters of genes activated in the ABA-dependent pathway often contain the ABA Response Element (ABRE) which binds transcription factors involved in gene activation. Sequences that are regulated in the ABA-independent pathway appear to be controlled either post-translationally by MAP kinase cascades, or transcriptionally by the presence of a Drought Response Element Box (DREB) (also known as the C-Repeat Binding Factor (CBF)), cis-element in their promotors. The aim of the experiments described in this section was to determine if the AtMYB64 transcription factor forms part of the ABA-dependent or ABA-independent signalling pathway. The approach used was to monitor the levels of AtMYB64 expression in Col-0 WT Arabidopsis treated with 50 μM ABA.

4.6.2. Semi Quantitative Reverse Transcription Polymerase Chain Reaction (SQRT-PCR) Analysis

The effect of exogenous ABA application on AtMYB64 expression in wild type seedlings was analyzed using SQRT-PCR (Figure 4.11.). The application of a single factor, ABA, in the absence of any other stress factor should determine if AtMYB64 is under the control of ABA.

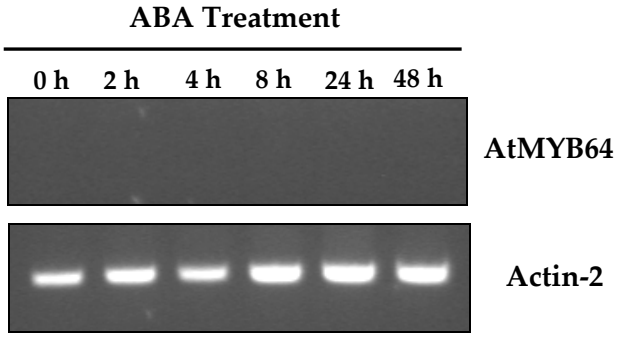


Figure 4. 11. SQRT-PCR Analysis of The Expression of AtMYB64 in Col-0 WT Treated with ABA

RNA samples were obtained from 14-day-old whole seedlings treated by 50μM ABA. RNA extraction was isolated on 0 hour, 2 hours, 4 hours, 8 hours, 24 hours, and 48 hours after ABA application. The abundance of AtMYB64 was assessed by SQRT-PCR (See Section 2.2.13.) Amplification reaction was performed for 35 cycles. Actin-2 was used as a control.

Figure 4.11 presents the results from a SQRT-PCR experiment designed to detect AtMYB64 expression in Arabidopsis seedlings. Actin-2 transcript was used as an internal control and was clearly visible in all samples; In contrast, AtMYB64 transcript was not detectable. There are two possibilities to account for this result. Firstly, AtMYB64 may be expressed at levels below the detection limits of the protocols used here. Secondly, this MYB transcription factor may be absent at this stage of development (14-day-old seedlings). AtMYB64 might be abundant in the mature leaves or at specific stages of the life cycle. Increasing the number of

amplification cycles to 40 produced bands in all of the samples (data not presented) but with this level of amplification artefacts can be incurred. For this reason it was decided to use QRT-PCR as it is both more sensitive and more reliable for transcripts that are not abundant.

4.6.3. Quantitative Reverse Transcription Polymerase Chain Reaction (QRT-PCR) Analysis

4.6.3.1. Selection of Endogenous Control to Normalize Gene Expression

As has been mentioned in Section 4.5.3.1 any variation in the total amount of RNA in the starting material, especially related to samples which have been obtained from different individuals, will result in an error of mRNA transcript quantification and a misinterpretation of the expression level of the gene of interest. Therefore, to minimize this error and correct for sample-to-sample variations in total RNA abundance, normalization on an internal standard RNA is used.

The ideal internal standard should be expressed at a constant level among different tissues of an organism, at all stages of development, and should be unaffected by experimental treatments such as the application of ABA. Hence, the determination of an appropriate standard is very important for drawing accurate conclusions. In this experiment, three sequences that are often used as standards (Actin-2, Ubiquitin 5, and GAPDH) were amplified to evaluate the appropriate internal control. Figure 4.12 shows the results of QRT-PCR analyses for the expression of Actin-2, Ubiquitin-5, and GAPDH in 14-day-old whole seedlings of Col-0 WT exposed to ABA.

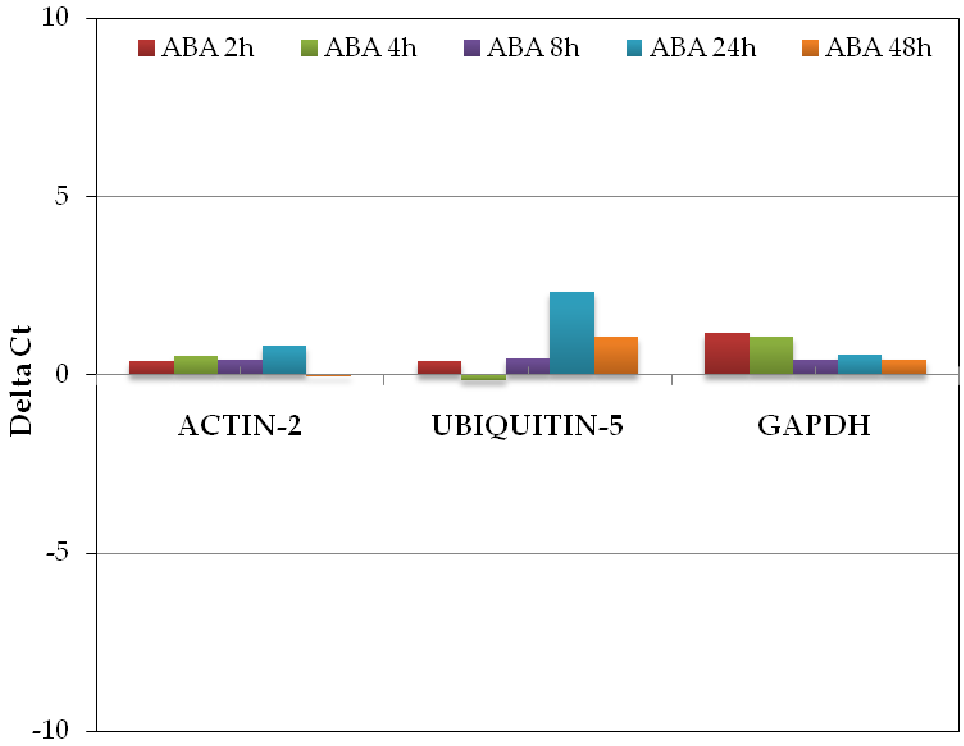


Figure 4. 12. Variations in Potential QRT-PCR Normalization Sequences in ABA-treated Arabidopsis Seedlings.

Plants were treated with 50μM ABA and RNA was isolated after 0 h, 2 h, 4 h, 8 h, 24 h, and 48 h. Data shown are the apparent abundance levels calculated as ΔCts. ΔCt can be obtained by subtracting the Ct value of gene of interest in the treated samples from the Ct value of the gene of interest in untreated samples (as a control). Samples with values above zero indicate lower levels of target gene abundance in the treated sample, whereas those below zero indicate a higher level of abundance of the sequence compared with control.

Referring to data shown in Figure 4.12., the expression level of RNA for Actin-2 was consistently within 1 Ct of controls indicating no more than a two-fold change in abundance. In contrast, Ubiquitin-5 and GAPDH showed 2-3 Ct differences which is equivalent to a 4-8 fold change in abundance and this was considered too excessive to be reliable for standardization. For this reason, Actin-2 was chosen as the standardization sequence for assessing the effects of ABA application on AtMYB64 mRNA levels.

4.6.3.2. QRT-PCR Analysis of AtMYB64 in Col-0 WT Arabidopsis Seedlings Treated with ABA

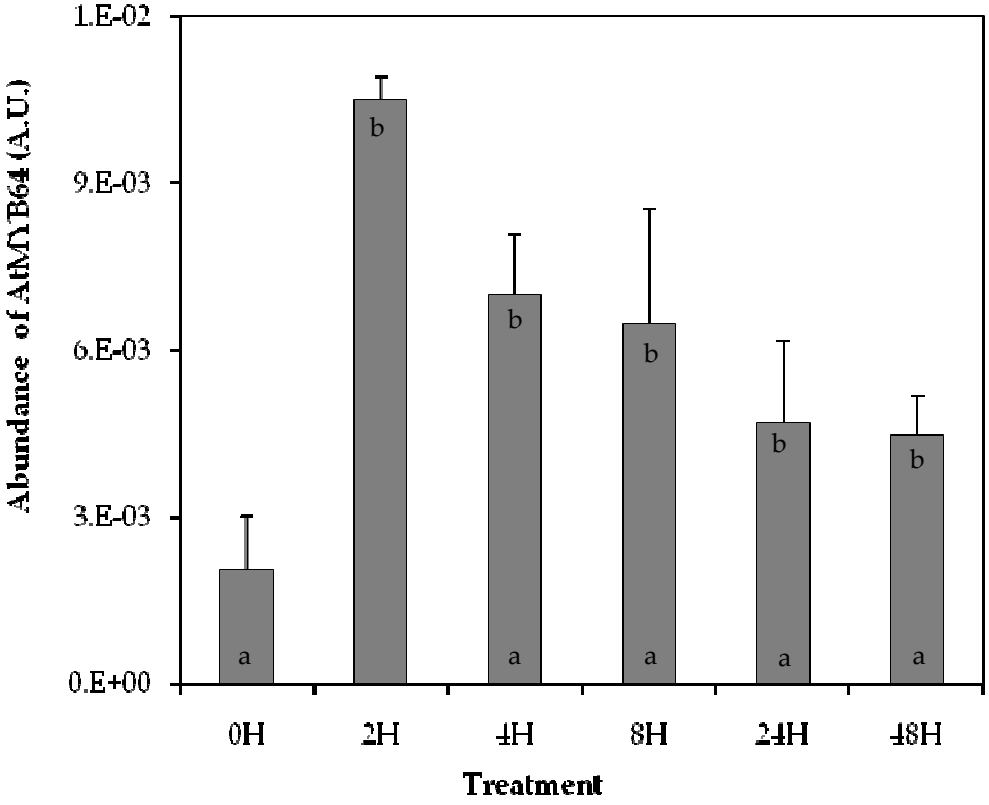


Figure 4. 13. QRT-PCR of AtMYB64 Abundance in Col-0 WT Arabidopsis Seedlings Treated with ABA.

RNA preparations obtained from 14-day-old wild type seedlings treated with 50μM ABA for 0 h, 2 h, 4 h, 8 h, 24 h, and 48 h. The expression levels shown are the average (±SE) of 3 biological replicates in Arbitrary Unit (A.U.) after normalization using Actin-2 as a standard. Different Roman letter codes signify significant differences at the $p<0.01$ level (See Table 10 in Appendix 2).

Figure 4.13 presents the results of QRT-PCR analyses of the apparent abundance of AtMYB64 RNA in 14-day-old Col-0 WT seedlings treated with 50 μM ABA (0, 2, 4, 8, 24 and 48 hours post-treatment). Statistical analysis using ANOVA General Linear Model; Main Factors, ABA Application (+/-) and Time (0, 2, 4, 8, 24, and 48 hours; replication level =3; 2×5×3=30 samples) showed that 2 hours after the application of 50 μM ABA to leaf tissue AtMYB64 cDNA (mRNA) levels had increased 500% ($p<0.01$). These levels subsequently declined and 48 hours post ABA treatment, AtMYB64 cDNA (mRNA) levels had fallen to approximately 40% of those at 2 hours.

4.7. Discussion

Other experiments conducted in the host laboratory prior to the start of this project had identified a transcription factor, AtMYB64 (At5g11050) that confers salinity tolerance to an Arabidopsis mutant (JP5). Transcript profiling using DNA Microarrays on JP5 indicated several heat shock protein and Calmodulin 7 may be under the control of AtMYB64. Preliminary experiments had also indicated that transgenic lines overexpressing AtMYB64 were thermotolerant. In this Chapter result are presented from experiments to characterize thermotolerance signalling components upstream and downstream of AtMYB64.

Early attempts to quantify transcript abundance of the HSPs, AtMYB64, and Calmodulin 7 in heat shocked Arabidopsis seedlings proved fruitless. It immediately became clear that even short periods of exposure to temperature over 40 ^{0}C caused a major decrease in the amount of total RNA isolated. It appears that temperatures above 40 ^{0}C caused a major decrease in general transcription and/or RNA stability, or an inhibition in the yield of RNA isolation. For this reason the effects of heat acclimation on Col-0 WT and transgenic lines were assessed at 37 ^{0}C only.

The notion that AtMYB64 confers thermotolerance was tested in two transgenic lines containing a p35S:AtMYB64 construct. T141 and T127, were compared with

Col-0 WT and this was confirmed. Line T141 was subsequently shown to be more thermotolerant than line T127, but both were more thermotolerant than Col-0 WT. These transgenic lines were then used to profile HSPs and CaM7 expression.

Rigorous attempts were made to determine the best sequence to use as internal standard for quantitative PCR; Actin-2 was found to vary by no more than two-fold between any of the samples and was subsequently used in all experiments.

QRT-PCR on RNA isolated from non-acclimated seedlings of Line T127 showed AtMYB64 abundance was increased over 500 times of those of Col-0 wild type. Heat acclimation increased AtMYB64 abundance in Col-0 wild type by 17 times confirming this transcription factor is activated by thermal acclimation. The abundance of two small heat shock protein transcripts, smHSP17.6 and smHSP17.6A, appear to be elevated in the T127 lines but this was significant only in the former. The abundance of CaM7 transcript and that of HSP70 and HSP101 do not appear to be regulated by AtMYB64 abundance. QRT-PCR analysis of AtMYB64 transcript abundance in seedlings treated with 5×10^{-4} M ABA indicated this transcription factor is activated in an ABA-dependent manner.

The work presented in this Chapter should be continued. Clearly the transgenic lines T141 shows an impressive improvement in thermotolerance and it is important that the molecular mechanisms under pinning this should be investigated. One important area for further study will be to determine if this improvement in thermotolerance is also manifest in mature plants. Whilst it is comparatively easy to control the leaf temperature of seedlings growing on agarose plates, it will be considerably more difficult to control leaf temperature of plants grown in soil. Nonetheless it is important to confirm the AtMYB64-dependent improvement in thermotolerance is not confined to seedlings. Attempts by others to establish the tissue-specific expressions of AtMYB64 has had limited success, probably due to the very low abundance of AtMYB64 transcript in any tissues (Price, 2005). This is supported by the Arabidopsis transcriptome profile

databases that report AtMYB64 transcript expression is non-specific and extremely low.

The data presented here indicate AtMYB64 transcription is regulated by ABA and by thermal acclimation. This suggest the AtMYB64 promoter contains and ABA response element (ABRE) and possibly also a drought response element (DRE). These possibilities are discussed further in Chapter 5.

It appears that AtMYB64 activates smHSP17.6 and confers thermotolerance. What is not clear is how this smHSP confers thermotolerance; further studies on smHSP knockout and transgenic lines may provide some insight into its role. In addition studies at the biochemical level may identify proteins that interact with smHSP17.6 during the acclimation process. One possible approach would be also to perform these experiments in the *abi* and *aba* mutant background; further discussion on this point is provided in Chapter 5.

CHAPTER 5

GENERAL DISCUSSION

5.1. Introduction

Soil salinity and high temperature are the most important environmental constraints which limit plant growth and agricultural productivity. Salinity is a common feature on arid and semiarid land, and in some irrigated farmland. Due to water limitations, leaf temperature rises above the temperature of the surrounding air as a consequence of reduced transpiration. High leaf temperatures cause severe cellular injury and even cell death. Direct injuries that arise from high temperatures include protein denaturation and aggregation of membrane lipids, inactivation of enzymes in chloroplast and mitochondria, inhibition of protein synthesis, protein degradation and loss of membrane integrity (Howarth, 2005). Those environmental factors that impose water-deficit stress, such as salinity and temperature extremes, impact severely environmental quality and decrease of crops productivity.

To overcome these limitations and improve the efficiency of crop production in the face of a growing world population, more stress tolerant crops must be developed. An understanding of salt tolerance and heat tolerance mechanisms in model plants will contribute to identifying genes in crops for improving important tolerance traits. Gain-of-function genetic screen in Arabidopsis is one way of identifying important sequences for manipulation in crop plants.

5.2. Genetic Screen of Arabidpsis Activation Tagged Lines for Thermotolerance

Robust genetic screens of field-grown plants that provide full genome coverage are difficult to implement due to the sheer magnitude of the undertaking. This task is considerably easier with the model plant due to its small physical size, small genome, and wealth of experimental resources. Collections of Arabidopsis

Activation Tagged lines are available where T-DNAs have been randomly inserted into the genome. The T-DNA contains multimerized transcriptional enhancers from the Cauliflower Mosaic Virus (CaMV) 35S promoter that can activate the transcription of nearby genes allowing gain-of-function screen for abiotic stress tolerance. Around 100,000 Activation Tagged lines are available and provide 95% genome coverage. Currently, there are two collections of Arabidopsis Activation Tagged lines that could be used in screens for stress tolerance. These are the Weigel Arabidopsis (23,000 lines, 3 sets; Section 2.1.1.1) and Scheible & Somerville (~63,000 lines) collections. In the experiments reported in this thesis set 1 (N21995; 8,600 lines) and set 3 (N23153; 6,200 lines) of the Weigel collection were screened. The remaining Weigel set (~8,200 lines) and the full Scheible & Somerville should also be screened, but this was not possible in this study due to time constraints. Initially screening was performed on both acclimated and non-acclimated plants exposed to a range of high temperatures to determine the ideal conditions for a heat tolerance screen.

Thermal tolerance in plants has been studied for more than 5 decades (Barnet *et* al., 1980; Lin *et al*., 1984; Binelli and Mascarenhas, 1990; Ortiz and Cardemil, 2001; Sung *et al*., 2003; Hong *et* al., 2003; Adamo *et al*., 2008). Each study has developed different protocols for heat treatment depending on the species of plant, stage of development, and questions posed. In all these studies, however, the experimental design focussed on controlling air temperature (T_{air}), not leaf temperature (T_{leaf}), and this presents considerable disadvantages for reliably assessing thermal damage as many factors (water extraction from the soil, water transport to the shoot, stomatal function, leaf absorbance, as well as cellular heat tolerance mechanisms) contribute to survival. The experiments reported in this thesis were designed to control T_{leaf} to within 0.2 ^{0}C of the set T_{air}. This approach removes the confounding effects of between-plant differences in water supply, stomatal function and leaf absorbance, to reveal differences that arise at the cellular level.

5.2.1. Genetic Screen of Wild Type *Arabidopsis thaliana* for Thermotolerance

The thermotolerance of Arabidopsis plants was assessed on acclimated and non-acclimated plants. For the acclimated plants experiment, seedlings were exposed to a high, nonlethal temperature of 35 ± 0.2 ^{0}C initially to induce thermotolerance before exposure to lethal temperatures, whereas in the non-acclimated experiment, seedlings were exposed directly to high temperatures. The effect of the temperature acclimation on thermotolerance in plants has been investigated by several groups (Burke, 2001; Lim *et al.,* 2006). The results from the experiments presented here clearly demonstrate Arabidopsis does acclimate to high temperatures (Figure 3.1.). Based on Figure 3.1 there clearly was a significant difference between the survival of acclimated and non-acclimated plants exposed to high temperatures. Those differences were noticeable after 4 days from heat stress, but were more noticeable after 7 days.

The critical conditions where acclimated plants can be distinguished from non-acclimated plants was a 3 hour exposure period within the temperature range of 44 ^{0}C to 48 ^{0}C. Under these conditions the survival rate of acclimated plants is at least double that of non-acclimated plants. Studies on the acquisition of thermotolerance in *Arabidopsis thaliana* using either plate-based or soil-based experiments, differ in the range of temperatures used. Larkindale *et al.* (2005) studied the heat stress phenotypes of an Arabidopsis mutant using 45 ^{0}C for 1 hour as the appropriate screening temperature. Other experiments used 50 ^{0}C for 15 minutes and 42 ^{0}C for 2 hours as a lethal temperature on non-acclimated plants (Binelli and Mascarenhas, 1990). Hong and Vierling (2000) also assayed a mutant defective in temperature stress response by exposure at plants to 45 ^{0}C for 2 hours. The experiments reported here suggest that a 44 to 50 ^{0}C temperature range is appropriate for screening for heat acclimation plants (Figure 3.1.). In contrast, a range of 40 – 45 ^{0}C is appropriate for screening non-acclimated plants (Figure 3.4).

Even though the exposure temperature determined here for stress screening is consistent with other studies, the appropriate exposure period for either

acclimation or non-acclimation was still unclear. This experiment used a single 1 hour period for acclimation; a 3 hour period was also used for heat stress, whereas most of studies have used shorter exposure. The rationale for using 3 hour periods was to mimic the conditions plants would experience during the hot period of a day, from 12.00 to 15.00. In the experiments conducted by Binelli and co-workers over 50% cellular electrolyte leakage occurred in Arabidopsis leaves during a 15 mins exposure period to 50 ^{0}C, indicating a killing time of less than 15 minutes (Binelli and Mascarenhas, 1990). It is clear that exposure time is a critical factor to consider when designing an appropriate methods for thermotolerance screens.

5.2.2. Genetic Screen of Arabidopsis Activation Tagged Lines for Thermotolerance

As has been shown in Figure 3.2, plants that were considered to be tolerant of heat stress exposure grew with a green leaf colour whereas 'sensitive' plants suffered and developed a translucent colouring to the whole of plant. The surviving plants isolated from the primary screen were rescued and transferred into soil, grown to maturity, and allowed to flower and set seed (M2). The seed isolated from the primary screen (M2) was re-screened under similar conditions of heat treatment and analysed for segregation of the heat tolerant phenotype. Based on Mendel's First Law of Inheritance, the M2 generation from a homozygous mutant parent (M0) carrying a dominant mutation would show a segregation ratio of all 'mutant phenotype'. If the M0 parent was heterozygous for a dominant allele, the M2 generation phenotype would segregate as 3:1 (mutant: wild type; Figure 5.1.). If the parent (M0) carried a recessive mutation, the phenotype would be observed in this generation only if it was homozygous, in which case all subsequent generations would segregate as all 'mutant phenotype'.

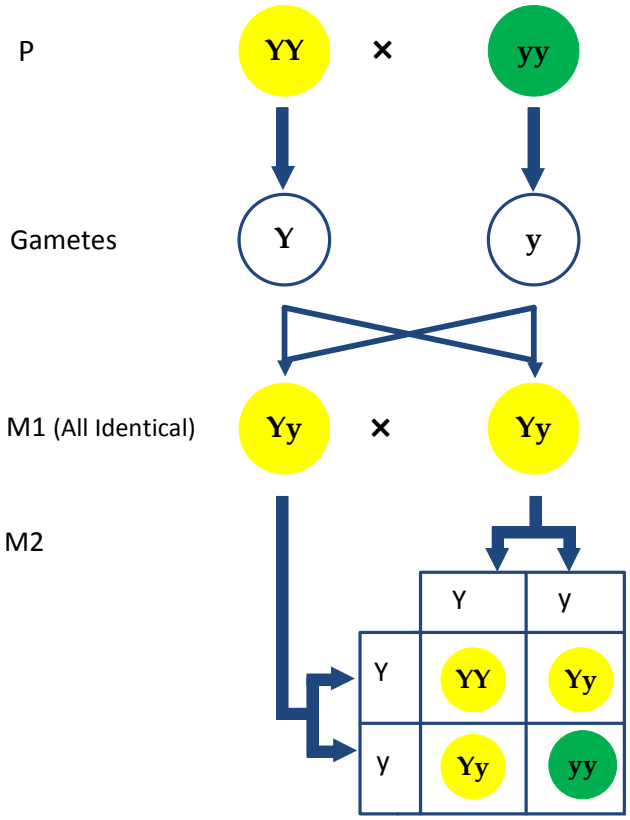


Figure 5. 1. The Punnet Square: Visual Summary of a Cross between a Wild Type and Homozygous Dominant Mutant

This Punnet square illustrates the combination that can arise when a heterozygous M1 hybrid carrying a dominant mutation undergoes gamet formation and self-fertilization. The M2 generation should have a 3:1 ratio of heat tolerant (Dominant, yellow) to heat sensitive (Recessive, green).

In the experiments reported here, however, a ratio of heat tolerant to heat sensitive M2 plants segregated with a ratio nearer 1:15 (tolerant : sensitive; Figure 3.3; data not presented). This ratio was clearly not consistent with Mendel's Law for inheritance of single genes. This low ratio can be attributed to the narrow temperature range over which wild type and mutant plants survive. To avoid isolating a large number of false positives from the screen, a high temperature (43 – 44 ^{0}C) was chosen, and this inevitably resulted in a high proportion of fatalities of thermotolerant plants.

5.2.3. Partial Loss of Thermotolerance Phenotype in M3 Generation

The secondary screen clearly identified three pools with authentic thermotolerant M2 generation mutants (Table 3.1. and Figure 3.3.). These secondary screens were performed as 'half plate' experiments with Col-0 WT seedlings included as an internal control. However for one line, the M3 seedlings generated from the surviving M2 plants were hypersensitive to heat stress, whilst another reverted to the Col-0 WT phenotype (Figure 3.4.). In contrast, line N23814A had retained its improved thermotolerance. This observation of loss-of-phenotype in subsequent generations is not unusual in dominant mutants.

Screens of Activation Tagged populations have identified and confirmed several M2 generation mutants that are tolerant of abiotic stress factors, only for the phenotype to disappear in the M3 or subsequent generations (Dr. Peter Dominy, University of Glasgow; Dr. Mark Tester, Waite Institute; Pers. Comm.). The suggestion is that these dominant mutations give rise to gene silencing through small interfering RNAs. This notion is consistent with the data presented in Figure 3.4. The reversion of line N23822A to the Col-0 WT phenotype can be explained by the partial silencing of the mutant allele, whilst the hypersensitivity observed in M3 seedlings of line N23816A can be explained by the complete silencing of both Col-0 WT and mutant alleles. Further experiments are required to validate this hypothesis. The other possibility is that the experimental design is not sufficiently well established to generate consistent results, but this is difficult to accept. In these experiments (Figure 3.3. and 3.4.) survival of mutant and Col-0 WT seedlings were assessed on the same plate (half-plate experiments) so the underlying cause of the observed thermotolerance appears to have a biological basis. Further, throughout these studies, the same stock of Col-0 WT seed was used so comparative differences cannot be attributed to different WT lines.

Experimental evidence that dominant mutants generated from Activation Tagged populations are unstable has been established. A salt-tolerant Arabidopsis mutant was isolated from the Weigel Activation Tagged collection and secondary screens

on the M2 generation confirmed its phenotype. Experiments on the M3 generation showed the line had reverted to Col-0 WT. The disrupted gene in this tagged line was shown to be a putative SUMO protease (subsequently called OTS1). Double knockouts lines of OTS1 and OTS2 (the closest homologue of OTS1) were hypersensitive to high salinity, and transgenic lines over expressing OTS1 were more tolerant than WT lines (Conti *et al*., 2008). Studies on some, but not all, mutants isolated from this salt screen showed a similar loss-of-phenotype through generations. The dominant mutant JP5 described in Section 1.9 was also salt-tolerant and the phenotype persisted up to the M5 generation but no further; transgenic lines over expressing the activated gene (AtMYB64) are also salt-tolerant. It appears that individuals from the M2 generation of N23816A, and N23822A were probably thermotolerant but have undergone loss-of-phenotype in the M3 generation. For this reason, it seems sensible to identify the site of insertion of the activation tag in all three lines using TAIL-PCR as subsequent experiments using knockout or transgenic lines may confirm an involvement of the tagged loci in thermotolerance.

Control of Leaf Temperature

What is important in these experiments is the control of leaf temperature, not air temperature. Leaf temperature is dependent on the irradiance (leaf gain), transpiration (heat loss), and heat exchange with the surrounding air. In the experiments described here, heat stress was applied in the dark to seedlings in sealed plates (i.e. 100% RH). Under these conditions, leaf temperature should be the same as air temperature and therefore controlled to within ± 0.2 ^{0}C of the set temperature.

However, close investigation of the *Parafilm* seal around the plates suggested temperature of ~44 ^{0}C may have resulted in small pores appearing. This partial melting could have resulted in RH declining below 100% in some plates, or in some areas on a plate, resulting in transpirational cooling in these regions, and

therefore differential leaf temperatures within and between plates in the same incubator. It is recommended to use *Nescofilm* in future screens as this appears less affected by high temperatures, and in addition to seal the plates with plastic tape.

Development Stage of Seedling

The stage of development of the plant could be associated with the capacity of the plant to acquire heat tolerance. Previous work has shown that a thermotolerance phenotype can vary at different stages of growth (Hong and Vierling, 2000; Hong *et al*., 2003; Clarke *et al*., 2004). Larkindale *et al.* (2005) compared the elongation of 2.5-day-old hypocotyls and 4-day-old root growth after heat exposure and concluded a time-dependent difference in response to heat stress. Further studies have shown the leaves from 25-day-old plants appeared more thermotolerant than those of 7-day-old plants (Binelli and Mascarenhas, 1990). In this experiment 7-day-old acclimated and non-acclimated plants were subjected to a thermotolerance screen to evaluate heat tolerance of photosynthetically active, autotrophic seedlings (Larkindale *et al*., 2005). Young tissue may respond very differently to mature leaves and experiments should clearly be undertaken on plants at different stages of development.

5.3. Analysis of Thermotolerance in p35S:AtMYB64 Transgenic Lines

5.3.1. Actin-2 Chosen as Endogenous Control

Many studies on the activation of defence and stress responses in plants have focussed on transcriptional control (Kirch *et al*., 1997; Volkov *et al*., 2003; Mohamed *et al*., 2005). Transcriptome studies have provided a better understanding of plant stress responses and suggest that there are core components as well as specific responses to a wide range of stimuli (Sung *et al*., 2003). In order to determine the role of stress responsive genes, the analysis of gene expression requires sensitive, precise, and reproducible measurement of specific mRNA sequences. PCR-based methods, such as QRT-PCR, are the most sensitive methods for the detection of

low abundance mRNA (Bustin, 2000) and can be used for many different applications, including plant studies (Gachon *et al*., 2004). To achieve accurate conclusions, QRT-PCR is typically referenced to an internal control gene sequences whose transcript abundance should not be influenced by the experimental conditions (Schmittgen and Zakrajsek, 2000). However, several studies have shown this is not the case for sequences that are routinely used for this purpose (Thellin *et al*., 1999; Volkov *et al*., 2003; Nicot *et al*., 2005; Ruan and Lai, 2007).

Heat acclimation induces very high levels of several heat shock protein transcripts which presumably are involved in plant protection against heat stress, whereas the mRNA levels of other sequences, including 'housekeeping genes' decrease. There are three different possible mechanisms to explain the decrease of mRNA of housekeeping sequences (internal standard; eg. Actin-2) under heat shock conditions, these are: (i) direct effects of high temperature on transcriptional repression/activation, (ii) direct effects of high temperature on total mRNA stability, and (iii) temperature-dependent effects on specific transcripts (Volkov *et al*., 2003). Other studies have revealed that in soybean seedlings, the complexity and abundance of mRNA is significantly reduced after heat stress (Schoffl and Key, 1982) and that some 'constitutively expressed' sequences were down regulated during heat stress, while under the same conditions the abundance of heat shock transcripts increased.

Actin-2, a member of a family of 10 actin genes in *Arabidopsis thaliana* is a popular standard for the analysis of RNA abundance which is strongly expressed in vegetative tissues (An *et al*., 1996; Kandasamy *et al*., 2002). Volkov *et al*. (2003) revealed that the mRNA level of ribosomal proteins and actin genes are differentially modulated in different vegetative tissues. Upon heat stress, there was not only an increase in HSP transcript but also a down-regulation of mRNA for other sequences, including Actin-2.

Many studies have focussed on the reliability of sequences often used as internal controls under different growth conditions and stages of development (Schmittgen and Zakrajsek, 2000; Selvey *et al*., 2001; Sturzenbaum and Kille, 2001; Volkov *et al*., 2003; Nicot *et al*., 2005; Ruan and Lai, 2007). The conclusion from these studies is that no sequence is an ideal internal standard.

Erroneous results can arise from differences in the quantity or quality of the starting RNA. RNA abundance is usually estimated from UV absorption measurements, but this approach does not distinguish between full-length and degraded mRNA, and mRNA and t-RNA/r-RNA. Further, all microarray and PCR-based mRNA quantification methods rely on the synthesis of cDNA, and the assumption is that the efficiency of the reverse transcription step is the same for all sequences in all samples; there is, however, little evidence to support this assumption. Errors can also be introduced during the PCR amplification of the cDNA, but with QRT-PCR, these can usually be minimized. Taken together, PCR-based methods for assessing transcript abundance are sensitive to artefacts at several points in the process. Those associated with cDNA amplification can be minimized. Those resulting from the quality and/or quantity of mRNA can be controlled by normalization of the gene of interest on an 'internal standard'. Those associated with the efficiency of the reverse transcriptase step are unknown and often ignored (Schmittgen and Zakrajsek, 2000; Sturzenbaum and Kille, 2001).

The results from the experiments presented here suggest that Actin-2, although not ideal, is the best sequence to use as an internal control when studying changes in transcription resulting from heat acclimation. It remains to be seen if Actin-2 is also an acceptable internal control to use for studying changes in transcription that result from heat stress.

5.3.2. The Abundance of AtMYB64 in Heat Acclimated Plants

One of the largest families of transcription factors in Arabidopsis is the MYB family. The *MYB* gene was first identified in the oncogenic component of avian

myoblastoma virus, *v*-MYB, which has a cellular protooncogenic counterpart in animals designated *c*-MYB (LÜscher and Eisenman, 1990). After identification, members of the MYB gene family were found in all major eukaryotic groups (Rosinski and Atchley, 1998). In animals and yeast, the number of identified *MYB* genes is small (Thompson and Ramsay, 1995; Rosinski and Atchley, 1998), in contrast, a large number of *MYB* genes has been identified in plants (Martin and Paz-Ares, 1997). MYB factors denote a family of proteins that include the conserved MYB DNA binding domain and consist of one to three imperfect helix-turn-helix repeats and then can be classified further into three subfamilies depending on the number of repeats found in the MYB domain. MYB-like proteins with one repeat are referred to as 'MYB1R' factors, with two as 'R2R3' type factors, and with three repeats 'MYB3R' factors (Rosinski and Atchley, 1998; Meissner *et al*., 1999; Stracke *et al*., 2001). The largest group contain two imperfect MYB-like repeats in their DNA binding domains and are called the R2R3 class (Romero *et al*., 1998). To date, there are 125 R2R3-type *MYB* genes that have been characterized in Arabidopsis (Stracke *et al*., 2001).

The function of MYBs has been recognized to be multifunctional and diverse with only a few intensely studied, the rest remain much less clear. Those roles associated with their involvement in 'plant-specific processes' are linked to particular environmental conditions or developmental stages. Some plant R2R3 MYBs are understood to regulate secondary metabolism, especially in the phenylpropanoid pathway (Meissner *et al*., 1999; Jin and Martin, 1999), and in tryptophan biosynthesis (Bender and Fink, 1998). Other roles are involved in processes of cellular morphogenesis (Noda *et al*., 1994; Waites *et al*., 1998; Jin and Martin, 1999), signal transduction in plant growth (Gubler *et al*., 1995; Iturriaga *et al*., 1996), abiotic stress (Urao *et al*., 1993; Magaraggia, 1997; Hoeren *et al*., 1998), and pathogen defense (Yang and Klessig, 1996).

Studies have revealed R2R3 *MYB* genes have a unique expression pattern. *MYB*2 is transiently induced by dehydration, salt, and abscisic acid, but not by heat or

cold stress (Urao *et al*., 1993). Stracke *et al*., (2001) also revealed that AtMYB2 has been found to regulate the AtADH1 (Alcohol Dehydrogenase 1) gene promoter and it is also involved in responses to low oxygen. Studies on AtMYB30 suggests that its expression is strongly correlated with cell death during the hypersensitive response (HR) upon pathogen attack or elicitor treatment (Vailleau *et al*., 2002).

There is some evidence that MYBs may have a different function in different tissues: For example, AtMYB7, AtMYB44, and AtMYB73 are expressed in all plant organs, whereas AtMYB46 was only detected in siliques and AtMYB21 only in flower buds (Kranz *et al*., 1998). Feng *et al*. (2004) studied the role of Arabidopsis AtMYB68 in the control of development, and in responses to high temperatures. Analyses of AtMYB68 expression indicated MYB68 is expressed specifically in root pericycle cells. AtMYB68 was elevated in roots during high temperature exposure, even though in vegetative growth, that expression was reduced compared with wild type.

AtMYB64 is a member of the R2R3 type subfamily. This transcription factor contains three tryptophan residues in the R3 repeat, which even though common in animal and yeast R3 repeats, are not present in most plant R2R3 MYB proteins. Tryptophan residues are central to the formation of a hydrophobic core of amino acid that plays an important role in sequence-specific DNA binding (Kanei-Ishii *et al*., 1990). AtMYB64 shows a high sequence homology (57%) at the amino acid level to the consensus sequence generated by Stracke *et al*. (2001) who compared the amino acid sequences of R2 and R3 repeats from all 125 Arabidopsis R2R3 MYB subfamily.

Previous studies undertaken at Glasgow University indicated AtMYB64 is involved with salt tolerance in Arabidopsis (Price, 2005). The expression of AtMYB64 was enhanced in the original mutant JP5 and over expression of AtMYB64 in a wild type background improved salt tolerance. It is clear that AtMYB64 activates specific mechanisms for salt tolerance in plants. However, which plant organs and stages of development are most affected remains unclear.

One objective is to now identify the signalling components both upstream and downstream of AtMYB64. Identification of signalling components downstream of AtMYB64 was attempted using DNA Microarrays of transcripts isolated from JP5 and Col-0 WT grown in high salinity. Results from these experiments suggest several heat shock proteins and Calmodulin 7 have different levels of expression in JP5 compared with Col-0 WT after long-term salt exposure. It was tempting to speculate that AtMYB64 was also involved in activating mechanisms for heat tolerance in plants through induction of heat shock proteins. It is important, therefore, to establish the downstream targets of AtMYB64 to confirm its role in conferring tolerance to salinity and other abiotic stresses.

It is now widely believed that plant responses to various stresses are regulated by a complex network of signalling molecules and transcriptional regulators (Larkindale and Knight, 2002; Xiong *et al*., 2002; Jalali *et al*., 2006). Transcription factors bind to specific *cis*-elements that are present in the promoters of the stress responsive genes. Several *cis*-elements probably lie within the same promoter indicating that different transcription factor may lead to induction of the same stress responsive genes (Jalali *et al*., 2006).

The idea that a single transcription factor has several targets has some experimental support. For example, the overexpresssion of single transcription factors that could bind to CBF1/DREB1 promotor element can increase the tolerance of transgenic plants to low temperature, salt, and drought stress (Kasuga *et al*., 1999; Shinozaki and Yamaguchi-Shinozaki, 2000; Tomashow, 2001). The ability of AtMYB64 transcription factor to activate the HSF class of stress-responsive genes was demonstrated by overexpression of AtMYB64 which could activate the target genes whereas non-overexpression is less activated. Advance of multi-abiotic stress experiments, such as low temperature, osmotic stress, and drought stress could be conducted to characterize the signal transduction network of AtMYB64 transcription factor. Subsequently, the regulation of the AtMYB64 in plants is essential for development of stress tolerance.

In addition, the result presented here suggests that AtMYB64 can activate both salt tolerance and thermotolerance mechanisms. There are some interesting correlations between MYB genes based on the comparison of the sequence. AtMYB74 and AtMYB102, which both belong to subgroup 11, are up-regulated by drought stress, and subgroup 10 contains only genes with a low expression levels; AtMYB21 and AtMYB57 from subgroup 19 are specifically expressed in flower buds (Kranz *et al*., 1998); AtMYB75 and AtMYB90 from subgroup 6 are specifically detected in anthocyanin biosynthesis (Borevitz *et al*., 2000). At the protein level, AtMYB119 has the most similarity to AtMYB64, and both belong to subgroup 25 (Stracke *et al*., 2001; See Appendix 4). The high degree of similarity between AtMYB119 and AtMYB64 may mean they have similar roles in *planta* thus AtMYB119 may also be involved in salt tolerance and/or heat tolerance.

5.3.3. The Abundance of HSPs Family in Heat Acclimated Plants.

HSPs are thought to be involved in minimizing the damaging effects of denatured proteins that arise from stress. They achieve this by degrading or refolding damaged protein (HSP100, HSP70, HSP40), or by acting as molecular chaperones that interact with target proteins to prevent denaturation (small HSPs, e.g. smHSP17.6 and smHSP17.6A). The abundance of these proteins is controlled largely at the level of transcription and levels are usually very low in vegetative tissues in the absence of heat stress (Vierling, 1991; Sun *et al*., 2001; Volkov *et al*., 2003). The accumulation of heat shock proteins (HSPs) under the control of heat stress transcription factors (HSFs) is assumed to play a central role in the heat stress response (HSR) and in acquired thermotolerance in plants and other organisms. Exposure of plants to moderately high temperatures (heat acclimation, 37 ^{0}C) results in an increased thermal tolerance, which protects the plant from a second exposure to lethal temperature (Lindquist, 1980; Howarth and Ougham, 1993; Burke, 2001; Sharkey *et al*., 2001) and this correlates with the accumulation of heat shock proteins (Lim *et al*., 2006).

Members of the HSP family are targeted to the nuclear–cytosolic compartment, chloroplast, mithocondria, endoplasmic reticulum (ER), and peroxisomes (Waters *et al*., 1996; Kotak *et al*., 2007). For example, smHSP17.6 is located in cytosol, whereas HSP70 and HSP101 are located in cytosol, chloroplast, and mitochondria (Wang *et al*., 2004). The most complex group of HSPs in plants is the small HSP (smHSPs). Transcripts of all major smHSPs accumulate dramatically during heat stress even though specific small HSPs are also expressed during development and upon exposure to some other stresses (Sun *et al*., 2001; Kotak *et al*., 2007).

Some studies have been conducted to understand the mechanism of heat shock protein induction under stress conditions. Sun *et al*. (2001) studied the abundance of AtHSP17.6A transcripts and concluded it was rapidly increased upon heat stress, with the highest level appearing after 1.5 - 2 hours treatment at 37 ^{0}C (temperature optimum for expression of smHSP in Arabidopsis; RA Volkov, F Schoffl, unpublished data). Generally, all other heat shock genes were activated only while heat stress was directly applied (0.5-3 hours). Members of the HSP70, HSP90, and HSP100 families were also strongly induced by heat, primarily over the early portion (0.5-3 hours) of the time course (Swindell *et al*., 2007). In this experiment, HSP70 has the highest expression when compared with the 3 others HSP under growth normal condition, as has been presented on Figure 4.9. Some studies revealed that HSP90 and HSP70 are not only responsive to other stresses and endogenous signals (Winter and Sinibaldi, 1991; Yabe *et al*., 1994; DeRocher and Vierling, 1995; Wang *et al*., 2004), but are also abundant in most unstressed tissues and their induction represents increased synthesis of one or more HSP isoforms (Wang *et al*., 2004). Results from this experiment were consistent with these literatures.

Based on the result shown in Figure 4.9 (A) and (B), the expression level of smHSP17.6 (At5g12020) in the transgenic lines was always greater (2-3 times) than in those Col-0 WT but only significantly so before heat acclimation. smHSP17.6A (At5g12030) transcript levels in the transgenic line were generally higher than

those in Col-0 WT, but not significantly so. This suggests the expression of smHSPs is controlled partly by AtMYB64. In contrast, the expression of HSP70 and HSP101 do not appear to be under the control of AtMYB64 because, as shown in Figure 4.9 (C) and (D) HSP70 and HSP101 expression in the transgenic lines was always similar to those in Col-WT.

In addition to the observation that the AtMYB64 controls the expression of small heat shock proteins (smHSPs), other heat shock transcription factors (HSFs) are also reported to control the expression of HSP genes especially under heat stress conditions. Kotak *et al.* (2007) studied the heat shock transcription factors in tomato, *HsfA1a* and *HsfA1b,* and found they are important during the initial phase of heat stress, and *HsfA2* during prolonged heat stress and recovery. Referring to those studies, further experiments are required to clearly understand the interaction between of heat shock transcription factors and AtMYB64 in Arabidopsis.

In Arabidopsis and other plant species, various HSPs have been induced by other stresses in addition to heat stress, such as low temperature (Sabehat *et al*., 1998), osmotic stress (Sun *et al*., 2001), salt (Liu *et al*., 2006), oxidative stress (Lee *et al*., 2000; Volkov *et al*., 2006), desiccation (Liu *et al*., 2006), exposure to intense light (Hihara *et al*., 2001; Rossel *et al*., 2002), wounding (Cheong *et al*., 2002), and heavy metal exposure (Gyorgyey *et al*., 1991). Sun *et al*. (2001) observed that both AtHSP17.6A and AtHSP17.6 (AtHSP17.6II) were induced by 200mM NaCl and 20% PEG. It seems clear that the expression of these smHSP is under the control of both AtMYB64 and HSFs, and that these transcription factors are regulated by both salinity and high temperatures. However, it remains unclear which stress-response pathways overlap most extensively with this important part of the Arabidopsis heat shock regulatory network. If the primary stress conditions interacting with HSP response pathway can be identified, it would be of substantial interest to understand how HSF and HSP contribute to tolerance under such stress conditions. Clearly, further studies are required to establish the

physiological role of HSFs and HSPs in promoting tolerance of different stress imposed upon the plants.

5.3.4. The Abundance of Calmodulin 7 in Heat Acclimated Plants.

Many second messengers have been identified in the signalling pathways of plants cell (e.g. Ca^{2+}, lipids, pH, and cyclic GMP; Sanders *et al*., 1999). However, changes in cytoplasmic free Ca^{2+} levels have been implicated in most responses to stimuli. Calmodulin (CaM) is a ubiquitous and multifunctional Ca^{2+} sensor that binds Ca^{2+} and interacts with a wide variety of cellular proteins to modulate their function and regulate diverse cellular processes (Li *et al*., 2004). Calcium has a vital role in mediating plant responses to external stimuli of both abiotic stresses (e.g. light, cold, heat, movement, hypoxia, and drought), and biotic stresses (e.g. phytohormone, pathogen, and interaction with symbionts). Thus, Ca^{2+} triggers a myriad of cellular processes that influence growth, development, and physiology, and which allows plants to adapt to the changing environment (Snedden and Fromm, 1998).

Snedden and Fromm (1998) reported the induced expression of at least some of these CaM–related genes is mediated by a rise in cytosolic Ca^{2+} in response to the external stimulus such as physical (e.g. touch, dark, light, heat) and chemical (e.g. auxin and NaCl) stimuli. Many papers address the possible role of Ca^{2+}-dependent signalling in the heat shock response, in both animal (Calderwood *et al*., 1988) and plant cells (Biyaseheva *et al*., 1993; Gong *et al*., 1998; Liu *et al*., 2003; Li *et al*., 2004). The involvement of calcium and calcium-activated CaM in heat shock signal transduction in wheat (*Triticum aestivum*) has been investigated (Liu *et al*., 2003). It was found that the increase in intracellular free calcium ion concentration started within 1 min after a 37 °C heat stress. The levels of CaM mRNA and protein then increased during heat shock at 37 °C because of the presence of calcium ions concentration. This suggested that CaM accumulation is dependent on cytosolic Ca^{2+} levels that are elevated by heat stress. Heat stress also regulates the level of

CaM protein in maize seedlings (Gong *et al*., 1997). In addition, studies on maize under heat stress conditions revealed that DNA-binding activity of heat shock transcription factors was increased by directly adding $CaCl_2$ to whole cell extracts under non-heat stress conditions (Li *et al*, 2004). The summary of Ca^{2+} -bound-CaM-mediated signal transduction in plants is presented in Figure 5.2.

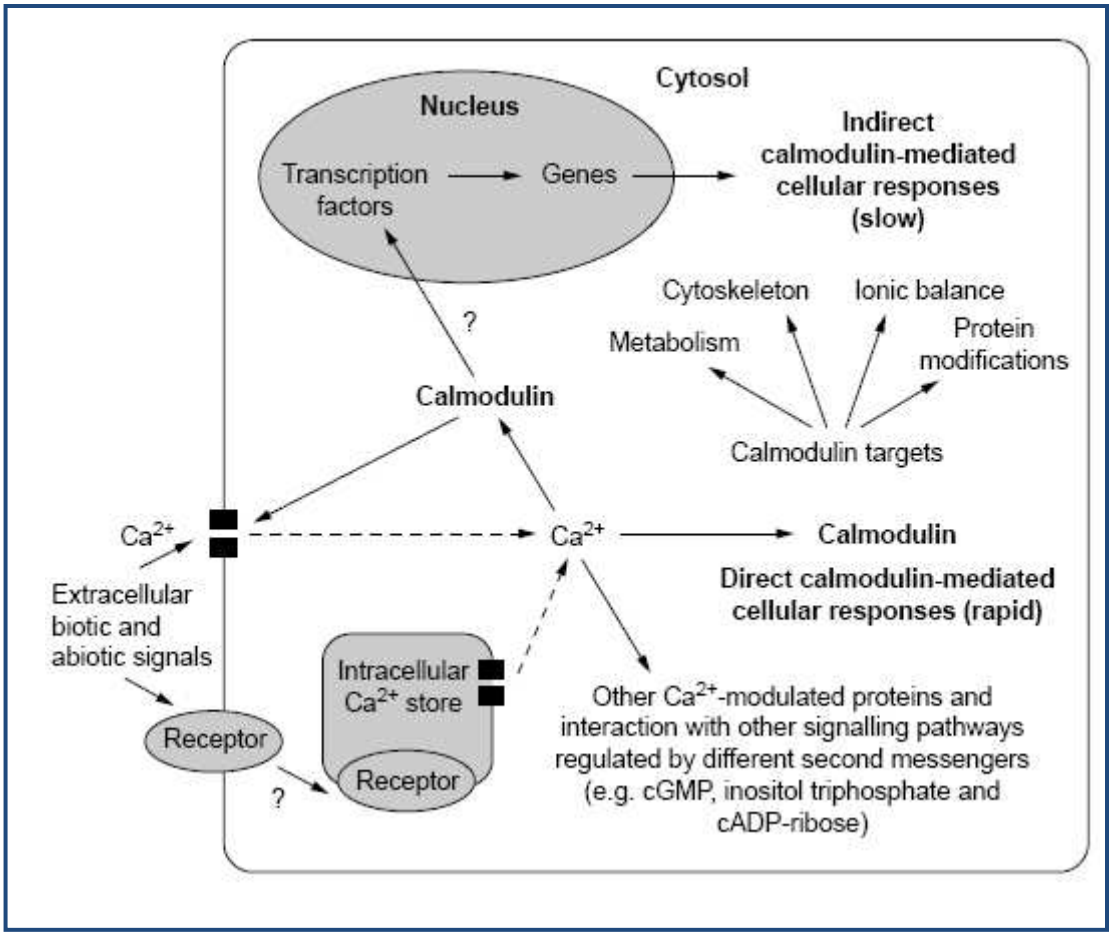


Figure 5. 2. Ca^{2+}-CaM-mediated Signal Transduction in Plants

The presence of biotic and abiotic stresses are perceived by receptors, leading to transient changes in Ca^{2+} concentrations in the cytosol and/or organelles (e.g. nucleus). Increases in free Ca^{2+} concentrations initiating from either extracellular or intracellular stores are capable of binding to Ca^{2+}-modulated proteins including CaM and CaM-related proteins. These proteins undergo structural modifications that enable them to interact with numerous cellular targets and control a multitude of cellular functions, such as metabolism, ion balance, the cytoskeleton and protein modifications. In addition, Ca^{2+} and CaM might also regulate the expression of genes by complex signalling cascades (rapid response) or by direct binding to transcription factors (slower response). Rapid changes in cellular functions result from direct interactions of CaM and CaM-related proteins with their targets (within seconds to minutes). Slower responses require gene transcription, RNA processing and protein synthesis (variable times from minutes to days). These CaM-mediated processes, together with cellular changes triggered by other signaling pathways, constitute the response of the plant to the external signals. Broken arrows represent Ca^{2+} fluxes from extracellular or intracellular stores, and question marks mean unknown signal transduction intermediates.
Figure from Snedden and Fromm (1998).

Some studies have focused on the role of Ca^{2+}-CaM and found it is directly involved in the heat shock signal transduction pathway (Li *et al*., 2002; Liu *et al*., 2003; Li *et al*., 2004). The heat shock signals are perceived by an unidentified receptor. Receptor activation is closely followed by an increase in cytosolic free [Ca^{2+}] through the opening of Ca^{2+} channels in the plasma membrane or the membranes of intracellular stores (e.g. ER). Elevated cytoplasmic calcium i.e. $[Ca^{2+}]_i$ directly activates CaM and promotes the expression and accumulation of CaM. Activated CaM promotes the activity of heat shock transcription factors (HSFs) which initiate the transcription and translation of HSP genes. However, the mechanism by which the activity of the HSFs is regulated by CaM is still unclear. Li *et al*. (2004) suggest that Ca^{2+}-CaM signalling mechanism involves the regulation of HSP gene expression probably trough by regulating HSF phosphorylation by CaM-dependent kinases or phosphatases. Another possibility is through the HSP70 pathway; a CaM-binding site within HSP70 suggests a direct interaction (Stevenson and Calderwood, 1990; Sun *et al*., 2000). Thus CaM can directly interact with HSP70 that caused HSP70-HSF complex to release and activate HSF.

Based on the experimental results presented here (Figure 4.10), the basal level of CaM 7 cDNA (mRNA) before heat acclimation in the p35S:AtMYB64 and Col-0 WT lines are similar. After the acclimation period, levels of CaM 7 levels arise to a peak four times their basal levels 3 hours after the heat acclimation period. The delay in CaM 7 rise suggests an indirect effect of heat on the abundance of this transcript, possibly involving the synthesis of new protein. After 3 hours, the levels of CaM 7 mRNA declined, and by 12 hours they have returned to near basal levels. The results presented here indicate that at no time was a significant different in the abundance of CaM 7 mRNA between Col-0 WT and the p35S:AtMYB64 transgenic lines. This suggests the expression of CaM 7 is not under the control of the AtMYB64 transcription factor.

5.3.5. The Role of ABA in Activation of AtMYB64 Transcription Factor

Osmotic and temperature stress increase the cellular levels of the phytohormone ABA (Chandler and Robertson, 1994) and the expression of many osmotic stress-responsive genes can be induced by high endogenous levels of ABA (Xiong *et al*., 1999). It has been established that the expression of some stress genes is mediated by ABA, but that of others are independent of ABA. Acclimation to extreme temperatures, both high and low appears to involve ABA signalling but is thought to be more critical for acquired thermotolerance than for basal thermotolerance (Larkindale *et al*., 2005). Most of the drought induced genes studied to date are also induced by ABA. It appears that dehydration triggers the production of ABA, which in turn induces the transcription of various genes. *cis*- and *trans*-acting factors involved in ABA-induced gene expression have been studied (Chandler and Robertson, 1994; Ingram and Bartels, 1996; Shinozaki and Yamaguchi-Shinozaki, 1996; Bray, 1997). The experiments reported in this thesis have shown that both heat acclimation and ABA application induce the activation of AtMYB64 which in turn induces stress-responsive genes. The highest levels of AtMYB64 transcript induced by both ABA application and heat acclimation were achieved within 2-3 hours. The highest expression of heat stress-responsive genes, HSPs, was also achieved within 3 hours. This suggest the activation of heat shock proteins in heat acclimated plants is controlled in part by AtMYB64 through an ABA-dependent signalling pathway.

Based on the result presented here (Figure 4.8 and 4.13) the transcription factor AtMYB64 appears to be induced by the application of exogenous ABA as well as by high temperatures. Genes that are activated by ABA normally possess the ABA Response Element (ABRE) in their promoter region which contain a consensus six nucleotide sequence element (CGTGGC; Nakashima *et al.* 2009) which bind transcription factors that mediate the expression of 3′ open reading frame (ORF). The suggestion is, therefore, that the *AtMYB64* promoter should contain an ABRE in its promoter.

At this stage it is not possible to say whether AtMYB64 is also to some extent activated through the ABA-independent pathway. As endogenous ABA levels were not measured in these experiments no direct correlation between endogenous ABA and AtMYB64 transcript levels can be established. Stress response genes that are transcriptionally activated through the ABA-independent pathway usually contain a Drought Response Element Box (DREB; consensus TACCGACAT, Nakashima *et al.* 2009) in their promoter.

Analysis of the putative promoter region of AtMYB64 (4,000 bp 5′ sequence) revealed no perfect match for either a consensus ABRE or DREB element although close matches were observed (aGTGGC and ctaCGACAT, respectively). Further experiments need to be conducted to confirm if either of these putative *cis*-elemets are involved in the regulation of *AtMYB64* transcription.

One useful approach might be to study AtMYB64 expression in the *aba* (ABA-deficient) or *abi* (ABA-insensitive) Arabidopsis mutant background to characterize further the involvement of ABA. If expression is controlled solely through the ABA-dependent pathway, then in the *aba1, aba2,* and *aba3* mutant background (which are deficient in ABA synthesis), AtMYB64 transcript abundance should not change with heat acclimation (Rock, 2000). The ABA signalling mutants, *abi1* and *abi2*, have been shown to be hyper-sensitive to heat stress (Larkindale and Knight, 2002; Larkindale and Huang, 2004); studying AtMYB64 transcript abundance in these lines might also provide a deeper insight into the role of ABA in the activation of AtMYB64 and genes under its direct control.

Another approach for assessing the role of AtMYB64 in thermotolerance would be to study the phenotype of AtMYB64-null lines. Several possible knockout lines are listed in the SALK T-DNA collection, but studies have shown that none of these has a clearly defined phenotype and it is concluded that AtMYB64 is a redundant gene (Dr Peter Dominy, per. comm.). There are also potential knockout lines for the closest homologue of AtMYB64, AtMYB119; these have been acquired and crosses between homozygous At*myb64* and At*myb119* have been made, but the

double knockout lines were not available for this study. Once homozygous double-knockout lines are available, studies on thermotolerance (and salinity tolerance) should be undertaken.

5.3.6. Indication of Interaction between Temperature and Abscisic Acid (ABA) in Regulation of AtMYB64

Evidence has been provided to suggest osmotic stress and ABA act synergistically at normal growth temperatures to activate the transcription of stress response genes (Xiong *et al*., 1999). In this study the effects of normal and low temperatures on transcription of a stress response gene (*RD29A*) was monitored in transgenic lines carrying an in-frame construct of the RD29A promoter fused 5′ of the luciferase (*LUC*) gene (*pRD29A:LUC*). These experiments showed that higher levels of expression were obtained when plants were exposed to low temperatures and treated with ABA than when exposed to low temperatures alone. A similar synergism may occur with ABA application and acclimation to high temperatures. The experiments reported in this thesis have shown that in the Col-0 wild type line, application of 5 x 10^{-4}M ABA increased AtMYB64 expression by approximately 5 times, whereas thermal acclimation increased it approximately 17 times. Within the term of this project it was not possible to study the effects of a range of concentrations of ABA on AtMYB64 abundance or to study the interactions between ABA applications and thermal acclimation. These experiments should be undertaken, either using QRT-PCR on the Col-0 wild type line, or on transgenic lines carrying a construct of the AtMYB64 promoter fused 5′ and in-frame of a reporter gene such as LUC (i.e. pAtMYB64:LUC).

APPENDICES

Appendix 1 - Growth Media

Table 1. Component and Organics Compound of Murashige & Skoog Basal Medium (Sigma-Aldrich, 2008)

Component	Amount (mg/L)
Ammonium nitrate	1650.0
Boric acid	6.2
Calcium chloride anhydrous	332.2
Cobalt chloride * $6H_2O$	0.025
Cupric sulphate * 5 H_2O	0.025
Na_2-EDTA	37.26
Ferrous sulphate * $7H_2O$	27.8
Magnesium sulphate	180.7
Manganese sulphate * H_2O	16.9
Molybdic acid (sodium salt) * $2H_2O$	0.25
Potassium iodide	0.83
Potassium nitrate	1900.0
Potassium phosphatise monobasic	170.0
Zinc sulphate * 7 H_2O	8.6
Organics	
Glycine (free base)	2.0
Myo-Inositol	100.0
Nictotinic acid (free acid)	0.5
Pyrodixine * HCl	0.5
Thiamine * HCl	0.1
Grams of powder to prepare	4.4

Appendix 2 - Statistic Analysis

Table 1. Statistic Analysis for Genetic Screen Wild Type *Arabidopsis thaliana* for Thermotolerance

Example Analysis on DAY 4

General Linear Model: Arcsin versus Temperature, Acclimation (95% Confidence Level)

```
Factor       Type   Levels  Values
Temperature  fixed       5  35, 40, 44, 48, 52
Acclimation  fixed       2  0, 1

Analysis of Variance for Arcsin, using Adjusted SS for Tests

Source                   DF   Seq SS   Adj SS   Adj MS        F      P
Temperature               4  5.97399  5.97399  1.49350  1111.15  0.000
Acclimation               1  0.13414  0.13414  0.13414    99.80  0.000
Temperature*Acclimation   4  0.13633  0.13633  0.03408    25.36  0.000
Error                    10  0.01344  0.01344  0.00134
Total                    19  6.25790
S = 0.0366619   R-Sq = 99.79%   R-Sq(adj) = 99.59%

Tukey 95.0% Simultaneous Confidence Intervals
Response Variable Arcsin
All Pairwise Comparisons among Levels of Temperature
Temperature = 35  subtracted from:
Temperature   Lower  Center   Upper     +---------+---------+---------+------
40           -0.196  -0.111  -0.026                              (-*)
44           -0.776  -0.691  -0.606                   (-*-)
48           -1.161  -1.076  -0.990            (*-)
52           -1.509  -1.423  -1.338     (-*)
                                        +---------+---------+---------+------
                                     -1.50     -1.00     -0.50      0.00
Temperature = 40  subtracted from:

Temperature   Lower  Center   Upper     +---------+---------+---------+------
44           -0.666  -0.580  -0.495                    (*-)
48           -1.050  -0.965  -0.879              (-*)
52           -1.398  -1.312  -1.227       (-*)
                                        +---------+---------+---------+------
                                     -1.50     -1.00     -0.50      0.00
Temperature = 44  subtracted from:

Temperature    Lower   Center    Upper
48           -0.4696  -0.3844  -0.2992
52           -0.8174  -0.7322  -0.6469

Temperature     +---------+---------+---------+------
48                                (*-)
52                           (*-)
                +---------+---------+---------+------
              -1.50     -1.00     -0.50      0.00
Temperature = 48  subtracted from:

Temperature    Lower   Center    Upper
52           -0.4330  -0.3478  -0.2625

Temperature     +---------+---------+---------+------
52                                (-*-)
                +---------+---------+---------+------
              -1.50     -1.00     -0.50      0.00
```

```
Tukey 95.0% Simultaneous Confidence Intervals
Response Variable Arcsin
All Pairwise Comparisons among Levels of Temperature*Acclimation
Temperature = 35
Acclimation = 0  subtracted from:
Temperature  Acclimation   Lower  Center   Upper
35           1            -0.128   0.017   0.163
40           0            -0.285  -0.139   0.006
40           1            -0.210  -0.065   0.080
44           0            -1.016  -0.871  -0.726
44           1            -0.639  -0.494  -0.349
48           0            -1.387  -1.242  -1.097
48           1            -1.037  -0.892  -0.746
52           0            -1.560  -1.415  -1.269
52           1            -1.560  -1.415  -1.269
Temperature  Acclimation  ------+---------+---------+---------+
35           1                                    (-*--)
40           0                                (--*-)
40           1                                 (--*-)
44           0                       (-*--)
44           1                             (--*-)
48           0                  (-*--)
48           1                       (-*--)
52           0            (-*--)
52           1            (-*--)
                          ------+---------+---------+---------+
                             -1.20     -0.60      0.00      0.60
Temperature = 35
Acclimation = 1  subtracted from:
Temperature  Acclimation   Lower  Center   Upper
40           0            -0.302  -0.157  -0.012
40           1            -0.227  -0.082   0.063
44           0            -1.033  -0.888  -0.743
44           1            -0.657  -0.512  -0.366
48           0            -1.405  -1.259  -1.114
48           1            -1.054  -0.909  -0.764
52           0            -1.577  -1.432  -1.287
52           1            -1.577  -1.432  -1.287
Temperature  Acclimation  ------+---------+---------+---------+
40           0                                (-*--)
40           1                                 (--*-)
44           0                       (-*--)
44           1                             (-*--)
48           0                  (-*-)
48           1                       (--*-)
52           0            (-*--)
52           1            (-*--)
                          ------+---------+---------+---------+
                             -1.20     -0.60      0.00      0.60
Temperature = 40
Acclimation = 0  subtracted from:
Temperature  Acclimation   Lower  Center   Upper
40           1            -0.071   0.075   0.220
44           0            -0.876  -0.731  -0.586
44           1            -0.500  -0.355  -0.210
48           0            -1.248  -1.103  -0.957
48           1            -0.897  -0.752  -0.607
52           0            -1.420  -1.275  -1.130
52           1            -1.420  -1.275  -1.130
Temperature  Acclimation  ------+---------+---------+---------+
40           1                                  (-*--)
44           0                         (--*-)
44           1                               (-*--)
48           0                    (--*-)
48           1                         (-*--)
52           0              (--*-)
52           1              (--*-)
                          ------+---------+---------+---------+
                             -1.20     -0.60      0.00      0.60
```

```
Temperature = 40
Acclimation = 1  subtracted from:

Temperature  Acclimation   Lower  Center   Upper
44           0            -0.951  -0.806  -0.661
44           1            -0.575  -0.429  -0.284
48           0            -1.322  -1.177  -1.032
48           1            -0.972  -0.827  -0.682
52           0            -1.495  -1.350  -1.205
52           1            -1.495  -1.350  -1.205
Temperature  Acclimation  ------+---------+---------+---------+
44           0                      (--*-)
44           1                           (--*-)
48           0                 (-*--)
48           1                      (-*--)
52           0              (--*-)
52           1              (--*-)
                          ------+---------+---------+---------+
                             -1.20     -0.60      0.00      0.60
Temperature = 44
Acclimation = 0  subtracted from:

Temperature  Acclimation    Lower   Center    Upper
44           1             0.2314   0.3765   0.5217
48           0            -0.5165  -0.3714  -0.2262
48           1            -0.1661  -0.0209   0.1243
52           0            -0.6891  -0.5439  -0.3987
52           1            -0.6891  -0.5439  -0.3987
Temperature  Acclimation  ------+---------+---------+---------+
44           1                                        (-*--)
48           0                           (--*-)
48           1                                (--*-)
52           0                         (-*-)
52           1                         (-*-)
                          ------+---------+---------+---------+
                             -1.20     -0.60      0.00      0.60
Temperature = 44
Acclimation = 1  subtracted from:

Temperature  Acclimation   Lower   Center    Upper
48           0            -0.893  -0.7479  -0.6027
48           1            -0.543  -0.3974  -0.2523
52           0            -1.066  -0.9204  -0.7753
52           1            -1.066  -0.9204  -0.7753
Temperature  Acclimation  ------+---------+---------+---------+
48           0                      (--*-)
48           1                           (-*--)
52           0                   (--*-)
52           1                   (--*-)
                          ------+---------+---------+---------+
                             -1.20     -0.60      0.00      0.60
Temperature = 48
Acclimation = 0  subtracted from:

Temperature  Acclimation    Lower   Center     Upper
48           1             0.2053   0.3505   0.49564
52           0            -0.3177  -0.1725  -0.02737
52           1            -0.3177  -0.1725  -0.02737
Temperature  Acclimation  ------+---------+---------+---------+
48           1                                     (--*-)
52           0                               (-*--)
52           1                               (-*--)
                          ------+---------+---------+---------+
                             -1.20     -0.60      0.00      0.60
Temperature = 48
Acclimation = 1  subtracted from:
Temperature  Acclimation    Lower   Center    Upper
52           0            -0.6682  -0.5230  -0.3778
52           1            -0.6682  -0.5230  -0.3778
```

```
Temperature  Acclimation  ------+---------+---------+---------+
52           0                        (-*--)
52           1                        (-*--)
                          ------+---------+---------+---------+
                             -1.20     -0.60      0.00      0.60
Temperature = 52
Acclimation = 0  subtracted from:

Temperature  Acclimation    Lower    Center   Upper
52           1            -0.1452  0.000000  0.1452
Temperature  Acclimation  ------+---------+---------+---------+
52           1                              (-*-)
                          ------+---------+---------+---------+
                             -1.20     -0.60      0.00      0.60
```

General Linear Model: Arcsin versus Temperature, Acclimation (99% Confidence Level)

```
Factor       Type   Levels  Values
Temperature  fixed       5  35, 40, 44, 48, 52
Acclimation  fixed       2  0, 1

Analysis of Variance for Arcsin, using Adjusted SS for Tests
Source                  DF   Seq SS   Adj SS   Adj MS        F      P
Temperature              4  5.97399  5.97399  1.49350  1111.15  0.000
Acclimation              1  0.13414  0.13414  0.13414    99.80  0.000
Temperature*Acclimation  4  0.13633  0.13633  0.03408    25.36  0.000
Error                   10  0.01344  0.01344  0.00134
Total                   19  6.25790
S = 0.0366619   R-Sq = 99.79%   R-Sq(adj) = 99.59%

Tukey 99.0% Simultaneous Confidence Intervals
Response Variable Arcsin
All Pairwise Comparisons among Levels of Temperature
Temperature = 35  subtracted from:
Temperature   Lower  Center   Upper    -+---------+---------+---------+-----
40           -0.223  -0.111   0.002                              (-*-)
44           -0.804  -0.691  -0.579                   (-*-)
48           -1.188  -1.076  -0.963           (-*--)
52           -1.536  -1.423  -1.311    (--*-)
                                       -+---------+---------+---------+-----
                                    -1.50     -1.00     -0.50      0.00
Temperature = 40  subtracted from:

Temperature   Lower  Center   Upper    -+---------+---------+---------+-----
44           -0.693  -0.580  -0.468                 (-*--)
48           -1.077  -0.965  -0.852           (--*-)
52           -1.425  -1.312  -1.200     (--*-)
                                       -+---------+---------+---------+-----
                                    -1.50     -1.00     -0.50      0.00
Temperature = 44  subtracted from:

Temperature    Lower   Center    Upper    -+---------+---------+---------+-----
48           -0.4969  -0.3844  -0.2718                      (-*--)
52           -0.8447  -0.7322  -0.6196                (-*--)
                                         -+---------+---------+---------+-----
                                      -1.50     -1.00     -0.50      0.00

Temperature = 48  subtracted from:

Temperature    Lower   Center    Upper    -+---------+---------+---------+-----
52           -0.4603  -0.3478  -0.2352                       (-*-)
                                         -+---------+---------+---------+-----
                                      -1.50     -1.00     -0.50      0.00
```

```
Tukey 99.0% Simultaneous Confidence Intervals
Response Variable Arcsin
All Pairwise Comparisons among Levels of Temperature*Acclimation

Temperature = 35
Acclimation = 0  subtracted from:

Temperature  Acclimation   Lower  Center   Upper
35           1            -0.170   0.017   0.205
40           0            -0.327  -0.139   0.048
40           1            -0.252  -0.065   0.122
44           0            -1.058  -0.871  -0.684
44           1            -0.681  -0.494  -0.307
48           0            -1.429  -1.242  -1.055
48           1            -1.079  -0.892  -0.704
52           0            -1.602  -1.415  -1.227
52           1            -1.602  -1.415  -1.227
Temperature  Acclimation  -------+---------+---------+---------
35           1                                  (--*--)
40           0                               (--*--)
40           1                                (--*--)
44           0                      (--*---)
44           1                             (--*--)
48           0                   (--*--)
48           1                      (--*--)
52           0            (--*---)
52           1            (--*---)
                          -------+---------+---------+---------
                              -1.20     -0.60      0.00
Temperature = 35
Acclimation = 1  subtracted from:
Temperature  Acclimation   Lower  Center   Upper
40           0            -0.344  -0.157   0.030
40           1            -0.269  -0.082   0.105
44           0            -1.075  -0.888  -0.701
44           1            -0.699  -0.512  -0.324
48           0            -1.447  -1.259  -1.072
48           1            -1.096  -0.909  -0.722
52           0            -1.619  -1.432  -1.245
52           1            -1.619  -1.432  -1.245
Temperature  Acclimation  -------+---------+---------+---------
40           0                               (--*---)
40           1                                 (--*--)
44           0                      (--*--)
44           1                           (--*---)
48           0                   (--*--)
48           1                      (--*--)
52           0            (--*--)
52           1            (--*--)
                          -------+---------+---------+---------
                              -1.20     -0.60      0.00
Temperature = 40
Acclimation = 0  subtracted from:

Temperature  Acclimation   Lower  Center   Upper
40           1            -0.113   0.075   0.262
44           0            -0.918  -0.731  -0.544
44           1            -0.542  -0.355  -0.168
48           0            -1.290  -1.103  -0.915
48           1            -0.939  -0.752  -0.565
52           0            -1.462  -1.275  -1.088
52           1            -1.462  -1.275  -1.088

Temperature  Acclimation  -------+---------+---------+---------
40           1                                  (--*--)
44           0                         (--*--)
44           1                              (--*--)
48           0                   (--*--)
48           1                        (--*---)
```

```
52          0              (--*--)
52          1              (--*--)
                        -------+---------+---------+---------
                            -1.20     -0.60      0.00
Temperature = 40
Acclimation = 1  subtracted from:

Temperature  Acclimation   Lower  Center   Upper
44           0            -0.993  -0.806  -0.619
44           1            -0.617  -0.429  -0.242
48           0            -1.364  -1.177  -0.990
48           1            -1.014  -0.827  -0.640
52           0            -1.537  -1.350  -1.163
52           1            -1.537  -1.350  -1.163
Temperature  Acclimation  -------+---------+---------+---------
44           0                      (---*--)
44           1                             (--*--)
48           0               (--*--)
48           1                      (--*--)
52           0            (---*--)
52           1            (---*--)
                        -------+---------+---------+---------
                            -1.20     -0.60      0.00
Temperature = 44
Acclimation = 0  subtracted from:

Temperature  Acclimation    Lower   Center    Upper
44           1             0.1894   0.3765   0.5637
48           0            -0.5585  -0.3714  -0.1842
48           1            -0.2081  -0.0209   0.1663
52           0            -0.7311  -0.5439  -0.3567
52           1            -0.7311  -0.5439  -0.3567
Temperature  Acclimation  -------+---------+---------+---------
44           1                                       (--*--)
48           0                          (--*--)
48           1                                (--*--)
52           0                       (--*--)
52           1                       (--*--)
                        -------+---------+---------+---------
                            -1.20     -0.60      0.00
Temperature = 44
Acclimation = 1  subtracted from:

Temperature  Acclimation   Lower   Center    Upper
48           0            -0.935  -0.7479  -0.5607
48           1            -0.585  -0.3974  -0.2103
52           0            -1.108  -0.9204  -0.7333
52           1            -1.108  -0.9204  -0.7333
Temperature  Acclimation  -------+---------+---------+---------
48           0                      (---*--)
48           1                             (--*--)
52           0                    (--*--)
52           1                    (--*--)
                        -------+---------+---------+---------
                            -1.20     -0.60      0.00

Temperature = 48
Acclimation = 0  subtracted from:
Temperature  Acclimation    Lower   Center    Upper
48           1             0.1633   0.3505  0.53763
52           0            -0.3597  -0.1725  0.01463
52           1            -0.3597  -0.1725  0.01463

Temperature  Acclimation  -------+---------+---------+---------
48           1                                       (--*--)
52           0                                 (--*--)
52           1                                 (--*--)
                        -------+---------+---------+---------
                            -1.20     -0.60      0.00
```

```
Temperature = 48
Acclimation = 1  subtracted from:

Temperature  Acclimation    Lower   Center    Upper
52           0            -0.7102  -0.5230  -0.3358
52           1            -0.7102  -0.5230  -0.3358

Temperature  Acclimation  -------+---------+---------+---------
52           0                        (--*--)
52           1                        (--*--)
                         -------+---------+---------+---------
                             -1.20     -0.60      0.00
Temperature = 52
Acclimation = 0  subtracted from:

Temperature  Acclimation    Lower    Center   Upper
52           1            -0.1872  0.000000  0.1872

Temperature  Acclimation  -------+---------+---------+---------
52           1                              (--*--)
                         -------+---------+---------+---------
                             -1.20     -0.60      0.00
```

General Linear Model: Arcsin versus Temperature, Acclimation (99.9% Confidence Level)

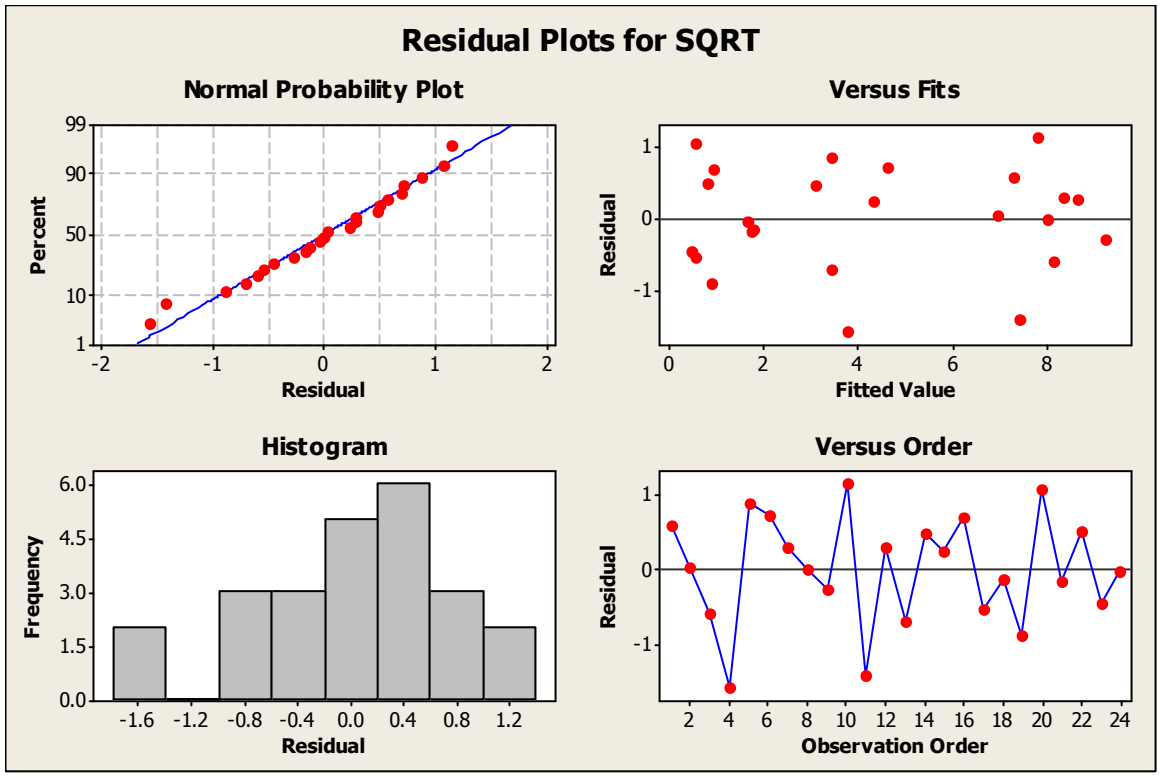
```
Factor       Type   Levels  Values
Temperature  fixed       5  35, 40, 44, 48, 52
Acclimation  fixed       2  0, 1

Analysis of Variance for Arcsin, using Adjusted SS for Tests

Source                  DF   Seq SS   Adj SS   Adj MS        F      P
Temperature              4  5.97399  5.97399  1.49350  1111.15  0.000
Acclimation              1  0.13414  0.13414  0.13414    99.80  0.000
Temperature*Acclimation  4  0.13633  0.13633  0.03408    25.36  0.000
Error                   10  0.01344  0.01344  0.00134
Total                   19  6.25790
S = 0.0366619   R-Sq = 99.79%   R-Sq(adj) = 99.59%

Tukey 99.9% Simultaneous Confidence Intervals
Response Variable Arcsin
All Pairwise Comparisons among Levels of Temperature
Temperature = 35  subtracted from:

Temperature   Lower  Center   Upper   --+---------+---------+---------+----
40           -0.266  -0.111   0.044                            (--*--)
44           -0.846  -0.691  -0.536                  (--*--)
48           -1.231  -1.076  -0.920          (--*---)
52           -1.578  -1.423  -1.268   (---*--)
                                      --+---------+---------+---------+----
                                    -1.50     -1.00     -0.50      0.00

Temperature = 40  subtracted from:

Temperature   Lower  Center   Upper   --+---------+---------+---------+----
44           -0.735  -0.580  -0.425                   (--*--)
48           -1.120  -0.965  -0.810            (--*--)
52           -1.468  -1.312  -1.157      (--*--)
                                      --+---------+---------+---------+----
                                    -1.50     -1.00     -0.50      0.00
Temperature = 44  subtracted from:

Temperature    Lower   Center    Upper   --+---------+---------+---------+----
48           -0.5395  -0.3844  -0.2293                        (--*--)
52           -0.8872  -0.7322  -0.5771                (--*--)
                                        --+---------+---------+---------+----
                                      -1.50     -1.00     -0.50      0.00
```

```
Temperature = 48  subtracted from:

Temperature    Lower   Center    Upper   --+---------+---------+---------+----
52          -0.5029  -0.3478  -0.1927                        (--*--)
                                        --+---------+---------+---------+----
                                      -1.50     -1.00     -0.50      0.00

Tukey 99.9% Simultaneous Confidence Intervals
Response Variable Arcsin
All Pairwise Comparisons among Levels of Temperature*Acclimation
Temperature = 35
Acclimation = 0  subtracted from:

Temperature  Acclimation   Lower  Center   Upper
35           1           -0.236   0.017   0.271
40           0           -0.393  -0.139   0.114
40           1           -0.319  -0.065   0.189
44           0           -1.125  -0.871  -0.617
44           1           -0.748  -0.494  -0.240
48           0           -1.496  -1.242  -0.988
48           1           -1.145  -0.892  -0.638
52           0           -1.668  -1.415  -1.161
52           1           -1.668  -1.415  -1.161
Temperature  Acclimation  ----+---------+---------+---------+--
35           1                            (--*---)
40           0                          (---*---)
40           1                           (---*---)
44           0                  (---*--)
44           1                      (---*---)
48           0              (--*---)
48           1                  (--*---)
52           0           (---*--)
52           1           (---*--)
                         ----+---------+---------+---------+--
                          -1.40     -0.70      0.00      0.70

Temperature = 35
Acclimation = 1  subtracted from:

Temperature  Acclimation   Lower  Center   Upper
40           0           -0.411  -0.157   0.097
40           1           -0.336  -0.082   0.172
44           0           -1.142  -0.888  -0.634
44           1           -0.765  -0.512  -0.258
48           0           -1.513  -1.259  -1.006
48           1           -1.163  -0.909  -0.655
52           0           -1.686  -1.432  -1.178
52           1           -1.686  -1.432  -1.178

Temperature  Acclimation  ----+---------+---------+---------+--
40           0                           (---*--)
40           1                            (---*--)
44           0                  (--*---)
44           1                      (---*--)
48           0             (---*---)
48           1                 (---*---)
52           0           (---*--)
52           1           (---*--)
                         ----+---------+---------+---------+--
                          -1.40     -0.70      0.00      0.70

Temperature = 40
Acclimation = 0  subtracted from:
Temperature  Acclimation   Lower  Center   Upper
40           1           -0.179   0.075   0.328
44           0           -0.985  -0.731  -0.477
44           1           -0.609  -0.355  -0.101
48           0           -1.356  -1.103  -0.849
48           1           -1.006  -0.752  -0.498
```

```
52           0            -1.529  -1.275  -1.021
52           1            -1.529  -1.275  -1.021
Temperature  Acclimation  ----+---------+---------+---------+--
40           1                                (---*---)
44           0                          (---*--)
44           1                               (---*---)
48           0                      (--*---)
48           1                          (--*---)
52           0                    (---*--)
52           1                    (---*--)
                          ----+---------+---------+---------+--
                           -1.40     -0.70      0.00      0.70
Temperature = 40
Acclimation = 1  subtracted from:
Temperature  Acclimation   Lower  Center   Upper
44           0            -1.060  -0.806  -0.552
44           1            -0.683  -0.429  -0.176
48           0            -1.431  -1.177  -0.923
48           1            -1.081  -0.827  -0.573
52           0            -1.604  -1.350  -1.096
52           1            -1.604  -1.350  -1.096
Temperature  Acclimation  ----+---------+---------+---------+--
44           0                    (--*---)
44           1                         (---*--)
48           0                 (--*---)
48           1                    (--*---)
52           0             (---*--)
52           1             (---*--)
                          ----+---------+---------+---------+--
                           -1.40     -0.70      0.00      0.70
Temperature = 44
Acclimation = 0  subtracted from:
Temperature  Acclimation    Lower   Center    Upper
44           1             0.1227   0.3765   0.6303
48           0            -0.6252  -0.3714  -0.1176
48           1            -0.2747  -0.0209   0.2329
52           0            -0.7977  -0.5439  -0.2901
52           1            -0.7977  -0.5439  -0.2901
Temperature  Acclimation  ----+---------+---------+---------+--
44           1                                   (--*---)
48           0                          (---*--)
48           1                               (---*--)
52           0                        (--*---)
52           1                        (--*---)
                          ----+---------+---------+---------+--
                           -1.40     -0.70      0.00      0.70
Temperature = 44
Acclimation = 1  subtracted from:

Temperature  Acclimation   Lower   Center    Upper
48           0            -1.002  -0.7479  -0.4941
48           1            -0.651  -0.3974  -0.1436
52           0            -1.174  -0.9204  -0.6666
52           1            -1.174  -0.9204  -0.6666
Temperature  Acclimation  ----+---------+---------+---------+--
48           0                    (--*---)
48           1                         (--*---)
52           0                 (---*--)
52           1                 (---*--)
                          ----+---------+---------+---------+--
                           -1.40     -0.70      0.00      0.70
Temperature = 48
Acclimation = 0  subtracted from:

Temperature  Acclimation    Lower   Center    Upper
48           1             0.0967   0.3505  0.60426
52           0            -0.4263  -0.1725  0.08125
52           1            -0.4263  -0.1725  0.08125
```

```
Temperature  Acclimation  ----+---------+---------+---------+--
48           1                                    (---*---)
52           0                               (---*--)
52           1                               (---*--)
                          ----+---------+---------+---------+--
                           -1.40     -0.70      0.00      0.70
Temperature = 48
Acclimation = 1  subtracted from:

Temperature  Acclimation    Lower   Center    Upper
52           0            -0.7768  -0.5230  -0.2692
52           1            -0.7768  -0.5230  -0.2692
Temperature  Acclimation  ----+---------+---------+---------+--
52           0                        (---*--)
52           1                        (---*--)
                          ----+---------+---------+---------+--
                           -1.40     -0.70      0.00      0.70
Temperature = 52
Acclimation = 0  subtracted from:

Temperature  Acclimation    Lower    Center   Upper
52           1            -0.2538  0.000000  0.2538
Temperature  Acclimation  ----+---------+---------+---------+--
52           1                             (---*---)
                          ----+---------+---------+---------+--
                           -1.40     -0.70      0.00      0.70
```

Table 2. Statistic Analysis for Characterization of Thermotolerance in Lines N23814A, N23816A, and N23822A



General Linear Model: SQRT versus Code Mutant, Code Temp, Code Repl

```
Factor       Type   Levels  Values
Code Mutant  fixed       4  0, 14, 16, 22
Code Temp    fixed       2  42, 43
Code Repl    fixed       3  1, 2, 3


Analysis of Variance for SQRT, using Adjusted SS for Tests

Source                 DF   Seq SS   Adj SS   Adj MS       F      P
Code Mutant             3   30.100   30.100   10.033   11.60  0.000
Code Temp               1  167.806  167.806  167.806  193.94  0.000
Code Repl               2    6.103    6.103    3.052    3.53  0.057
Code Mutant*Code Temp   3   23.413   23.413    7.804    9.02  0.001
Error                  14   12.113   12.113    0.865
Total                  23  239.535

S = 0.930175   R-Sq = 94.94%   R-Sq(adj) = 91.69%

Unusual Observations for SQRT
Obs     SQRT      Fit   SE Fit  Residual  St Resid
  4  2.18218  3.77107  0.60043  -1.58889     -2.24 R
 11  6.01048  7.43882  0.60043  -1.42834     -2.01 R

R denotes an observation with a large standardized residual.
```

```
Tukey 95.0% Simultaneous Confidence Intervals
Response Variable SQRT
All Pairwise Comparisons among Levels of Code Mutant*Code Temp
Code Mutant =  0
Code Temp = 42  subtracted from:
Code    Code
Mutant  Temp   Lower  Center   Upper  -------+---------+---------+---------
 0      43    -9.659  -6.980  -4.300   (---*----)
14      42    -3.180  -0.501   2.179             (---*----)
14      43    -7.018  -4.338  -1.659       (----*---)
16      42    -6.695  -4.015  -1.335        (---*----)
16      43    -9.559  -6.879  -4.199   (----*---)
22      42    -2.094   0.586   3.266                (---*---)
22      43    -9.566  -6.886  -4.206   (----*---)
                                      -------+---------+---------+---------
                                         -6.0       0.0       6.0
Code Mutant =  0
Code Temp = 43  subtracted from:
Code    Code
Mutant  Temp   Lower   Center   Upper  -------+---------+---------+---------
14      42     3.799  6.47915   9.159                        (----*---)
14      43    -0.039  2.64123   5.321                  (---*----)
16      42     0.285  2.96492   5.645                  (----*---)
16      43    -2.579  0.10068   2.780              (---*----)
22      42     4.886  7.56591  10.246                       (----*---)
22      43    -2.586  0.09397   2.774              (---*----)
                                     -------+---------+---------+---------
                                         -6.0       0.0       6.0
Code Mutant = 14
Code Temp = 42  subtracted from:
Code    Code
Mutant  Temp   Lower  Center   Upper  -------+---------+---------+---------
14      43    -6.518  -3.838  -1.158       (----*---)
16      42    -6.194  -3.514  -0.834        (---*----)
16      43    -9.058  -6.378  -3.699    (---*----)
22      42    -1.593   1.087   3.767                (----*---)
22      43    -9.065  -6.385  -3.705    (---*----)
                                      -------+---------+---------+---------
                                         -6.0       0.0       6.0
Code Mutant = 14
Code Temp = 43  subtracted from:
Code    Code
Mutant  Temp   Lower  Center   Upper  -------+---------+---------+---------
16      42    -2.356   0.324  3.0035              (----*---)
16      43    -5.220  -2.541  0.1393          (----*---)
22      42     2.245   4.925  7.6045                      (---*----)
22      43    -5.227  -2.547  0.1326          (----*---)
                                      -------+---------+---------+---------
                                         -6.0       0.0       6.0
Code Mutant = 16
Code Temp = 42  subtracted from:
Code    Code
Mutant  Temp   Lower  Center    Upper  -------+---------+---------+---------
16      43    -5.544  -2.864  -0.1844         (---*----)
22      42     1.921   4.601   7.2808                     (----*---)
22      43    -5.551  -2.871  -0.1911         (---*----)
                                     -------+---------+---------+---------
                                         -6.0       0.0       6.0

Code Mutant = 16
Code Temp = 43  subtracted from:
Code    Code
Mutant  Temp   Lower    Center   Upper  -------+---------+---------+---------
22      42     4.785   7.46524  10.145                           (---*----)
22      43    -2.687  -0.00671   2.673               (---*---)
                                      -------+---------+---------+---------
                                          -6.0       0.0       6.0
Code Mutant = 22
Code Temp = 42  subtracted from:
```

```
Code    Code
Mutant  Temp   Lower  Center   Upper  -------+---------+---------+---------
22      43    -10.15  -7.472  -4.792  (----*---)
                                      -------+---------+---------+---------
                                          -6.0       0.0       6.0
```

General Linear Model: SQRT versus Code Mutant, Code Temp, Code Repl

```
Factor       Type   Levels  Values
Code Mutant  fixed       4  0, 14, 16, 22
Code Temp    fixed       2  42, 43
Code Repl    fixed       3  1, 2, 3


Analysis of Variance for SQRT, using Adjusted SS for Tests
Source                 DF   Seq SS   Adj SS   Adj MS       F      P
Code Mutant             3   30.100   30.100   10.033   11.60  0.000
Code Temp               1  167.806  167.806  167.806  193.94  0.000
Code Repl               2    6.103    6.103    3.052    3.53  0.057
Code Mutant*Code Temp   3   23.413   23.413    7.804    9.02  0.001
Error                  14   12.113   12.113    0.865
Total                  23  239.535

S = 0.930175   R-Sq = 94.94%   R-Sq(adj) = 91.69%

Unusual Observations for SQRT
Obs     SQRT      Fit   SE Fit  Residual  St Resid
  4  2.18218  3.77107  0.60043  -1.58889     -2.24 R
 11  6.01048  7.43882  0.60043  -1.42834     -2.01 R

R denotes an observation with a large standardized residual.
Tukey 99.0% Simultaneous Confidence Intervals
Response Variable SQRT
All Pairwise Comparisons among Levels of Code Mutant*Code Temp
Code Mutant =  0
Code Temp = 42  subtracted from:
Code    Code
Mutant  Temp   Lower  Center   Upper  --------+---------+---------+--------
 0      43    -10.34  -6.980  -3.618   (----*-----)
14      42     -3.86  -0.501   2.861             (----*-----)
14      43     -7.70  -4.338  -0.977       (-----*----)
16      42     -7.38  -4.015  -0.653        (----*-----)
16      43    -10.24  -6.879  -3.517   (-----*----)
22      42     -2.78   0.586   3.948              (-----*-----)
22      43    -10.25  -6.886  -3.524   (-----*----)
                                       --------+---------+---------+--------
                                           -6.0       0.0       6.0

Code Mutant =  0
Code Temp = 43  subtracted from:
Code    Code
Mutant  Temp   Lower   Center   Upper  --------+---------+---------+--------
14      42     3.117  6.47915   9.841                       (-----*----)
14      43    -0.721  2.64123   6.003                 (----*-----)
16      42    -0.397  2.96492   6.327                 (-----*-----)
16      43    -3.261  0.10068   3.463              (----*-----)
22      42     4.204  7.56591  10.928                        (-----*----)
22      43    -3.268  0.09397   3.456              (----*-----)
                                       --------+---------+---------+--------
                                            -6.0       0.0       6.0

Code Mutant = 14
Code Temp = 42  subtracted from:
Code    Code
Mutant  Temp   Lower  Center   Upper  --------+---------+---------+--------
14      43    -7.200  -3.838  -0.476       (-----*----)
16      42    -6.876  -3.514  -0.152        (----*-----)
16      43    -9.740  -6.378  -3.017   (----*-----)
22      42    -2.275   1.087   4.449              (-----*----)
```

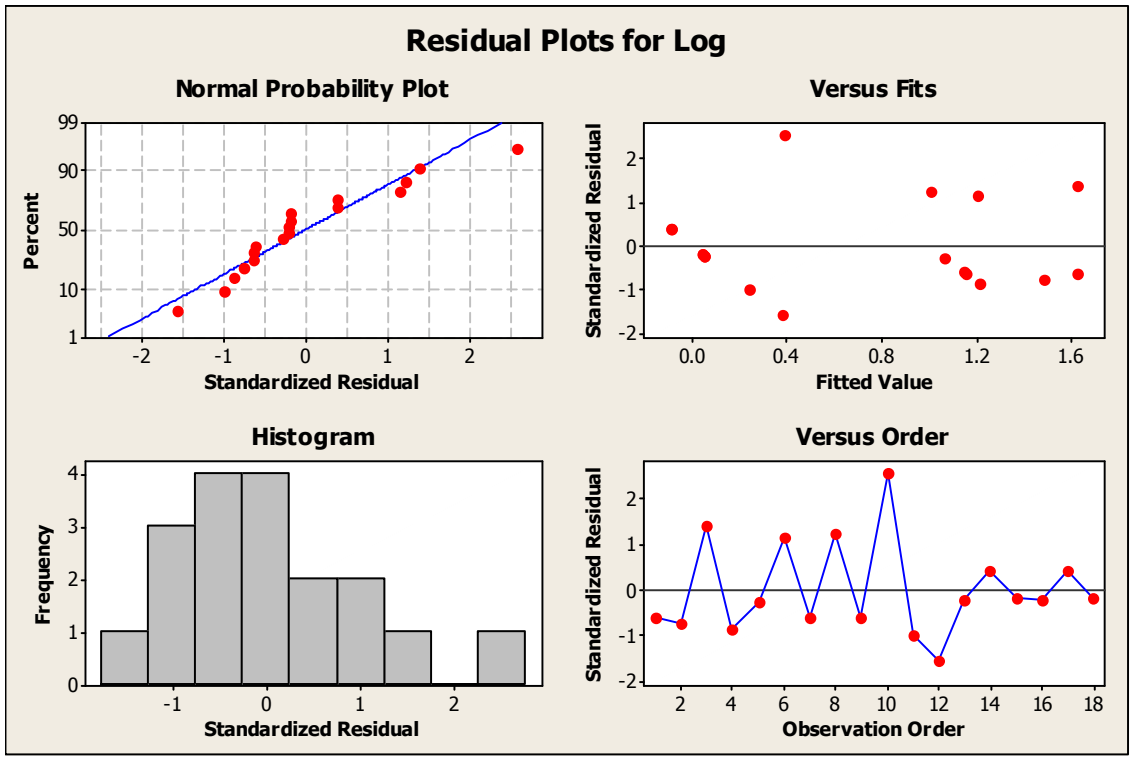
```
22      43    -9.747  -6.385  -3.023    (----*-----)
                                       --------+---------+---------+--------
                                            -6.0       0.0       6.0
Code Mutant = 14
Code Temp = 43  subtracted from:
Code    Code
Mutant  Temp   Lower  Center   Upper  --------+---------+---------+--------
16      42    -3.038   0.324  3.6855             (-----*----)
16      43    -5.902  -2.541  0.8213          (-----*----)
22      42     1.563   4.925  8.2865                       (----*-----)
22      43    -5.909  -2.547  0.8146          (-----*----)
                                       --------+---------+---------+--------
                                            -6.0       0.0       6.0
Code Mutant = 16
Code Temp = 42  subtracted from:
Code    Code
Mutant  Temp   Lower  Center   Upper  --------+---------+---------+--------
16      43    -6.226  -2.864  0.4976          (----*-----)
22      42     1.239   4.601  7.9628                       (-----*----)
22      43    -6.233  -2.871  0.4909          (----*-----)
                                       --------+---------+---------+--------
                                            -6.0       0.0       6.0
Code Mutant = 16
Code Temp = 43  subtracted from:
Code    Code
Mutant  Temp   Lower    Center   Upper  --------+---------+---------+--------
22      42     4.103   7.46524  10.827                           (----*-----)
22      43    -3.369  -0.00671   3.355             (-----*-----)
                                       --------+---------+---------+--------
                                          -6.0       0.0       6.0
Code Mutant = 22
Code Temp = 42  subtracted from:
Code    Code
Mutant  Temp   Lower  Center   Upper  --------+---------+---------+--------
22      43    -10.83  -7.472  -4.110  (-----*----)
                                       --------+---------+---------+--------
                                            -6.0       0.0       6.0
```

General Linear Model: SQRT 43 versus Code Mutant 43, Code R 43

```
Factor          Type   Levels  Values
Code Mutant 43  fixed       4  0, 14, 16, 22
Code R 43       fixed       3  1, 2, 3


Analysis of Variance for SQRT 43, using Adjusted SS for Tests

Source          DF   Seq SS   Adj SS  Adj MS     F      P
Code Mutant 43   3  14.9535  14.9535  4.9845  7.66  0.018
Code R 43        2   2.5963   2.5963  1.2981  1.99  0.217
Error            6   3.9067   3.9067  0.6511
Total           11  21.4564


S = 0.806913   R-Sq = 81.79%   R-Sq(adj) = 66.62%


Tukey 95.0% Simultaneous Confidence Intervals
Response Variable SQRT 43
All Pairwise Comparisons among Levels of Code Mutant 43
Code Mutant 43 =  0  subtracted from:

Code
Mutant
43       Lower   Center  Upper  ------+---------+---------+---------+
14       0.358  2.64123  4.924                  (-------*------)
16      -2.182  0.10068  2.383          (------*-------)
```

```
22      -2.189  0.09397  2.377           (------*-------)
                                  ------+---------+---------+---------+
                                     -3.0       0.0       3.0       6.0
Code Mutant 43 = 14  subtracted from:
Code
Mutant
43       Lower  Center    Upper  ------+---------+---------+---------+
16      -4.823  -2.541  -0.2578  (-------*------)
22      -4.830  -2.547  -0.2645  (-------*------)
                                  ------+---------+---------+---------+
                                     -3.0       0.0       3.0       6.0


Code Mutant 43 = 16  subtracted from:
Code
Mutant
43       Lower     Center  Upper  ------+---------+---------+---------+
22      -2.289  -0.006714  2.276          (-------*-------)
                                   ------+---------+---------+---------+
                                      -3.0       0.0       3.0       6.0
```

Table 3. Statistic Analysis for AtMYB64 Transgenic Line Plate-Based Phenotype Thermotolerance Analysis

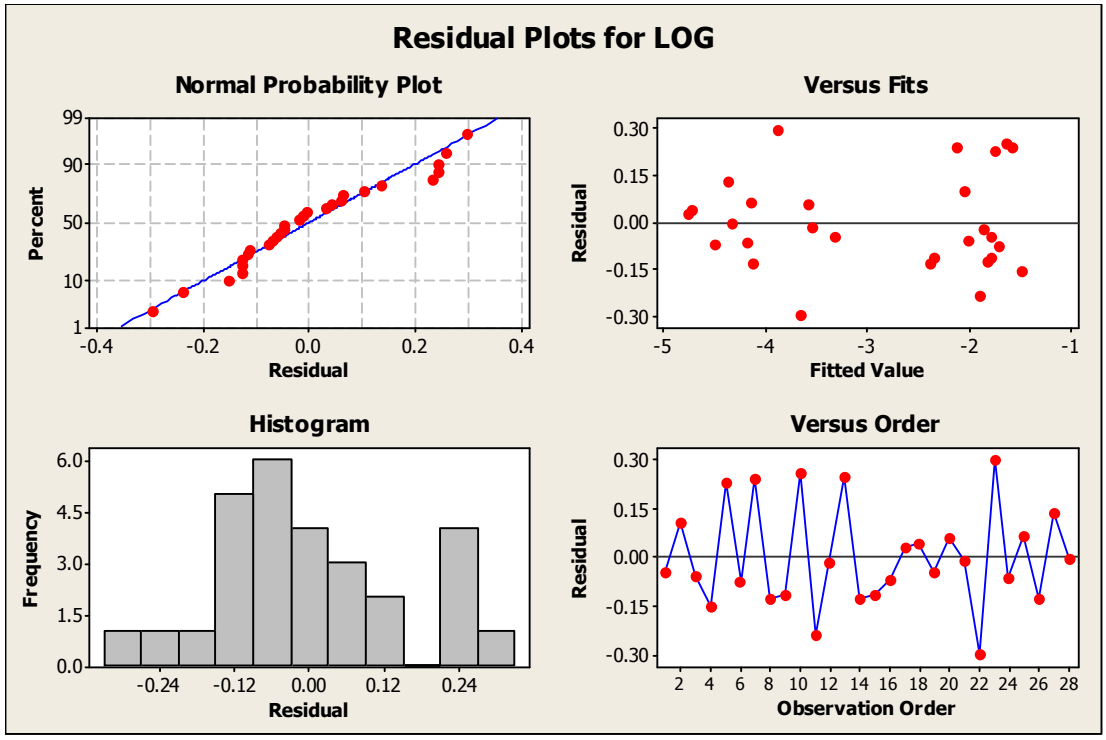


General Linear Model: Log versus Line, Acclim, Rep

```
Factor  Type   Levels  Values
Line    fixed       3  0, 127, 141
Acclim  fixed       2  -1, 1
Rep     fixed       3  1, 2, 3


Analysis of Variance for Log, using Adjusted SS for Tests

Source       DF  Seq SS  Adj SS  Adj MS      F      P
Line          2  5.6206  5.6206  2.8103  27.38  0.000
Acclim        1  0.7026  0.7026  0.7026   6.85  0.026
Rep           2  0.0809  0.0809  0.0404   0.39  0.684
Line*Acclim   2  0.4424  0.4424  0.2212   2.16  0.167
Error        10  1.0264  1.0264  0.1026
Total        17  7.8730


S = 0.320379   R-Sq = 86.96%   R-Sq(adj) = 77.84%

Unusual Observations for Log

Obs      Log      Fit   SE Fit  Residual  St Resid
 10  1.00000  0.38449  0.21359   0.61551      2.58 R

R denotes an observation with a large standardized residual.
```

```
Tukey 95.0% Simultaneous Confidence Intervals
Response Variable Log
All Pairwise Comparisons among Levels of Line*Acclim
Line =   0
Acclim = -1  subtracted from:
Line  Acclim    Lower    Center   Upper  ---------+---------+---------+-------
  0    1      -0.9082  -0.00000  0.9082  (--------*--------)
127   -1      -0.5749   0.33333  1.2415     (--------*--------)
127    1       0.1921   1.10034  2.0085              (--------*--------)
141   -1       0.2508   1.15904  2.0672               (--------*--------)
141    1       0.6693   1.57746  2.4857                   (--------*--------)
                                        ---------+---------+---------+-------
                                               0.0       1.0       2.0
Line =   0
Acclim =  1  subtracted from:
Line  Acclim    Lower  Center  Upper  ---------+---------+---------+-------
127   -1      -0.5749  0.3333  1.242     (--------*--------)
127    1       0.1921  1.1003  2.009              (--------*--------)
141   -1       0.2508  1.1590  2.067               (--------*--------)
141    1       0.6693  1.5775  2.486                   (--------*--------)
                                       ---------+---------+---------+-------
                                              0.0       1.0       2.0
Line = 127
Acclim = -1  subtracted from:
Line  Acclim    Lower  Center  Upper  ---------+---------+---------+-------
127    1      -0.1412  0.7670  1.675          (--------*--------)
141   -1      -0.0825  0.8257  1.734          (--------*--------)
141    1       0.3359  1.2441  2.152              (--------*---------)
                                       ---------+---------+---------+-------
                                              0.0       1.0       2.0
Line = 127
Acclim =  1  subtracted from:
Line  Acclim    Lower   Center   Upper  ---------+---------+---------+-------
141   -1      -0.8495  0.05870  0.9669   (--------*--------)
141    1      -0.4311  0.47712  1.3853       (--------*--------)
                                        ---------+---------+---------+-------
                                               0.0       1.0       2.0
Line = 141
Acclim = -1  subtracted from:
Line  Acclim    Lower  Center  Upper  ---------+---------+---------+-------
141    1      -0.4898  0.4184  1.327      (--------*--------)
                                       ---------+---------+---------+-------
                                              0.0       1.0       2.0
```

Table 4. Statistic Analysis for AtMYB64 Abundance



General Linear Model: LOG versus Treatment, Lines, Replication

```
Factor       Type   Levels  Values
Treatment    fixed       5  -1, 0, 3, 6, 12
Lines        fixed       2  0, 127
Replication  fixed       3  1, 2, 3

Analysis of Variance for LOG, using Adjusted SS for Tests

Source           DF   Seq SS   Adj SS   Adj MS       F      P
Treatment         4   3.3187   2.4496   0.6124   15.31  0.000
Lines             1  33.2685  32.5695  32.5695  814.35  0.000
Replication       2   0.3851   0.3402   0.1701    4.25  0.033
Treatment*Lines   4   0.7198   0.7198   0.1799    4.50  0.013
Error            16   0.6399   0.6399   0.0400
Total            27  38.3319

S = 0.199986   R-Sq = 98.33%   R-Sq(adj) = 97.18%

Unusual Observations for LOG

Obs       LOG       Fit   SE Fit  Residual  St Resid
 22  -3.95386  -3.65430  0.14888  -0.29957     -2.24 R
 23  -3.57748  -3.87705  0.14888   0.29957      2.24 R

R denotes an observation with a large standardized residual.


Tukey 95.0% Simultaneous Confidence Intervals
Response Variable LOG
All Pairwise Comparisons among Levels of Treatment*Lines

Treatment = -1
Lines =   0  subtracted from:
Treatment  Lines    Lower  Center   Upper
-1         127     2.1399  2.7346  3.3292
```

```
 0           0     0.6004  1.1950  1.7896
 0         127     2.4328  3.0274  3.6221
 3           0     0.1838  0.8562  1.5286
 3         127     2.3509  2.9455  3.5401
 6           0    -0.0883  0.5841  1.2565
 6         127     2.2919  2.8865  3.4812
12           0    -0.2026  0.3921  0.9867
12         127     1.7929  2.3875  2.9821
Treatment  Lines  ------+---------+---------+---------+
-1         127                          (--*--)
 0           0                    (--*--)
 0         127                          (--*--)
 3           0                  (--*---)
 3         127                          (--*--)
 6           0                 (--*--)
 6         127                       (--*--)
12           0               (--*--)
12         127                       (--*--)
                  ------+---------+---------+---------+
                     -2.0       0.0       2.0       4.0
Treatment = -1
Lines = 127  subtracted from:
Treatment  Lines   Lower  Center   Upper  ------+---------+---------+---------+
 0           0    -2.134  -1.540  -0.945       (--*--)
 0         127    -0.302   0.293   0.888                (--*--)
 3           0    -2.551  -1.878  -1.206     (---*--)
 3         127    -0.384   0.211   0.806                (--*--)
 6           0    -2.823  -2.150  -1.478    (--*---)
 6         127    -0.443   0.152   0.747                (--*--)
12           0    -2.937  -2.343  -1.748   (--*--)
12         127    -0.942  -0.347   0.248             (--*--)
                                          ------+---------+---------+---------+
                                            -2.0       0.0       2.0       4.0
Treatment =  0
Lines =   0  subtracted from:
Treatment  Lines   Lower   Center    Upper
 0         127     1.238   1.8324   2.4271
 3           0    -1.011  -0.3388   0.3336
 3         127     1.156   1.7505   2.3451
 6           0    -1.283  -0.6109   0.0615
 6         127     1.097   1.6915   2.2862
12           0    -1.398  -0.8029  -0.2083
12         127     0.598   1.1925   1.7872
Treatment  Lines  ------+---------+---------+---------+
 0         127                        (--*--)
 3           0               (--*---)
 3         127                        (--*--)
 6           0             (--*--)
 6         127                      (--*--)
12           0            (--*--)
12         127                      (--*--)
                  ------+---------+---------+---------+
                     -2.0       0.0       2.0       4.0

Treatment =  0
Lines = 127  subtracted from:
Treatment  Lines   Lower  Center   Upper  ------+---------+---------+---------+
 3           0    -2.844  -2.171  -1.499    (--*---)
 3         127    -0.677  -0.082   0.513              (--*--)
 6           0    -3.116  -2.443  -1.771  (---*--)
 6         127    -0.736  -0.141   0.454            (--*--)
12           0    -3.230  -2.635  -2.041  (--*--)
12         127    -1.235  -0.640  -0.045           (--*--)
                                          ------+---------+---------+---------+
                                             -2.0       0.0       2.0       4.0
Treatment =  3
Lines =   0  subtracted from:
Treatment  Lines   Lower   Center   Upper
 3         127     1.417   2.0893  2.7616
```

```
 6           0    -1.021  -0.2721  0.4773
 6         127     1.358   2.0303  2.7027
12           0    -1.137  -0.4642  0.2082
12         127     0.859   1.5313  2.2037

Treatment  Lines  ------+---------+---------+---------+
 3         127                        (--*---)
 6           0              (---*--)
 6         127                        (--*---)
12           0             (---*--)
12         127                    (---*--)
                  ------+---------+---------+---------+
                     -2.0       0.0       2.0       4.0
Treatment =  3
Lines = 127  subtracted from:
Treatment  Lines   Lower  Center   Upper  ------+---------+---------+---------+
 6           0    -3.034  -2.361  -1.689   (--*---)
 6         127    -0.654  -0.059   0.536               (--*--)
12           0    -3.148  -2.553  -1.959  (--*--)
12         127    -1.153  -0.558   0.037            (--*--)
                                           ------+---------+---------+---------+
                                              -2.0       0.0       2.0       4.0
Treatment =  6
Lines =   0  subtracted from:
Treatment  Lines    Lower   Center   Upper
 6         127     1.6300   2.3024  2.9748
12           0    -0.8645  -0.1921  0.4803
12         127     1.1310   1.8034  2.4758
Treatment  Lines  ------+---------+---------+---------+
 6         127                        (---*--)
12           0               (--*--)
12         127                     (--*--)
                  ------+---------+---------+---------+
                     -2.0       0.0       2.0       4.0
Treatment =  6
Lines = 127  subtracted from:
Treatment  Lines   Lower  Center   Upper  ------+---------+---------+---------+
12           0    -3.089  -2.494  -1.900   (--*--)
12         127    -1.094  -0.499   0.096            (--*-)
                                           ------+---------+---------+---------+
                                              -2.0       0.0       2.0       4.0
Treatment = 12
Lines =   0  subtracted from:
Treatment  Lines  Lower  Center  Upper  ------+---------+---------+---------+
12         127    1.401   1.995  2.590                       (--*--)
                                        ------+---------+---------+---------+
                                           -2.0       0.0       2.0       4.0
```

General Linear Model: LOG versus Treatment, Lines, Replication

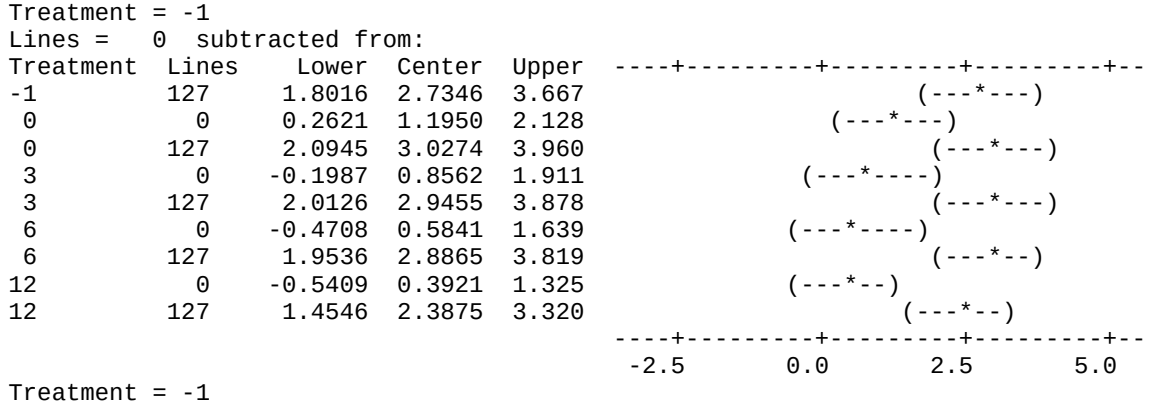
```
Factor       Type   Levels  Values
Treatment    fixed       5  -1, 0, 3, 6, 12
Lines        fixed       2  0, 127
Replication  fixed       3  1, 2, 3

Analysis of Variance for LOG, using Adjusted SS for Tests
Source           DF   Seq SS   Adj SS   Adj MS       F      P
Treatment         4   3.3187   2.4496   0.6124   15.31  0.000
Lines             1  33.2685  32.5695  32.5695  814.35  0.000
Replication       2   0.3851   0.3402   0.1701    4.25  0.033
Treatment*Lines   4   0.7198   0.7198   0.1799    4.50  0.013
Error            16   0.6399   0.6399   0.0400
Total            27  38.3319

S = 0.199986   R-Sq = 98.33%   R-Sq(adj) = 97.18%
Unusual Observations for LOG
Obs       LOG       Fit   SE Fit  Residual  St Resid
 22  -3.95386  -3.65430  0.14888  -0.29957     -2.24 R
 23  -3.57748  -3.87705  0.14888   0.29957      2.24 R
```

R denotes an observation with a large standardized residual.

Tukey 99.9% Simultaneous Confidence Intervals
Response Variable LOG
All Pairwise Comparisons among Levels of Treatment*Lines

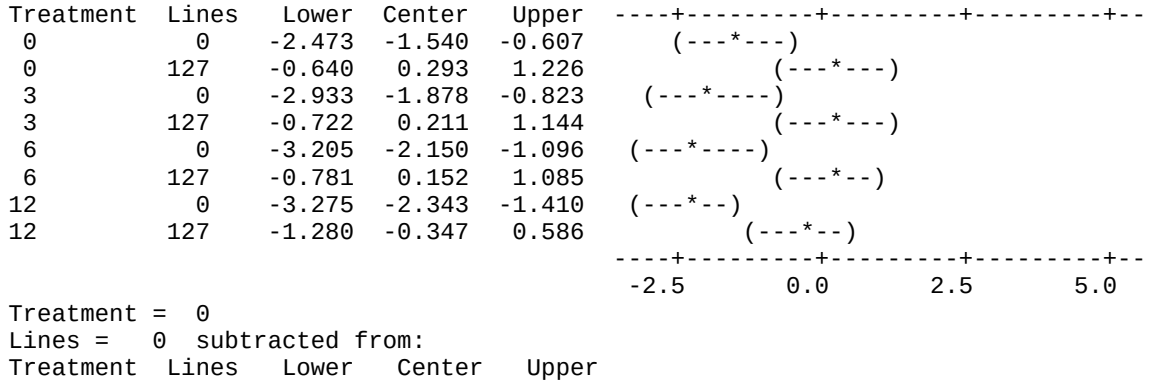
Treatment = -1
Lines = 0 subtracted from:

Treatment	Lines	Lower	Center	Upper
-1	127	1.8016	2.7346	3.667
0	0	0.2621	1.1950	2.128
0	127	2.0945	3.0274	3.960
3	0	-0.1987	0.8562	1.911
3	127	2.0126	2.9455	3.878
6	0	-0.4708	0.5841	1.639
6	127	1.9536	2.8865	3.819
12	0	-0.5409	0.3921	1.325
12	127	1.4546	2.3875	3.320



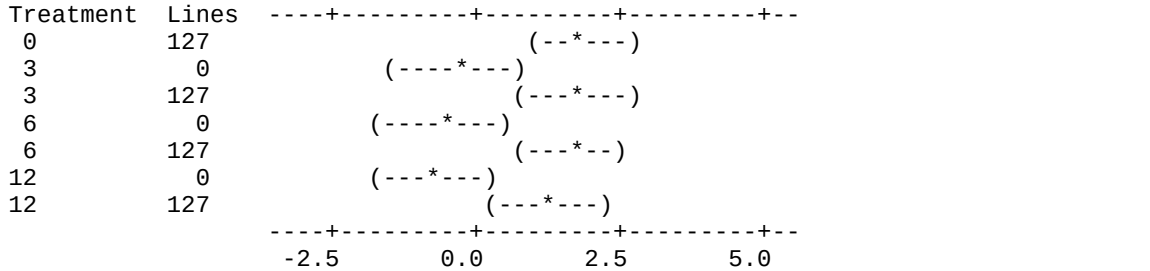
Treatment = -1
Lines = 127 subtracted from:

Treatment	Lines	Lower	Center	Upper
0	0	-2.473	-1.540	-0.607
0	127	-0.640	0.293	1.226
3	0	-2.933	-1.878	-0.823
3	127	-0.722	0.211	1.144
6	0	-3.205	-2.150	-1.096
6	127	-0.781	0.152	1.085
12	0	-3.275	-2.343	-1.410
12	127	-1.280	-0.347	0.586



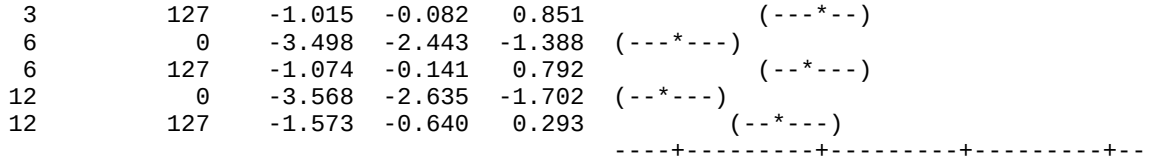
Treatment = 0
Lines = 0 subtracted from:

Treatment	Lines	Lower	Center	Upper
0	127	0.900	1.8324	2.7654
3	0	-1.394	-0.3388	0.7162
3	127	0.818	1.7505	2.6834
6	0	-1.666	-0.6109	0.4441
6	127	0.759	1.6915	2.6245
12	0	-1.736	-0.8029	0.1300
12	127	0.260	1.1925	2.1255



Treatment = 0
Lines = 127 subtracted from:

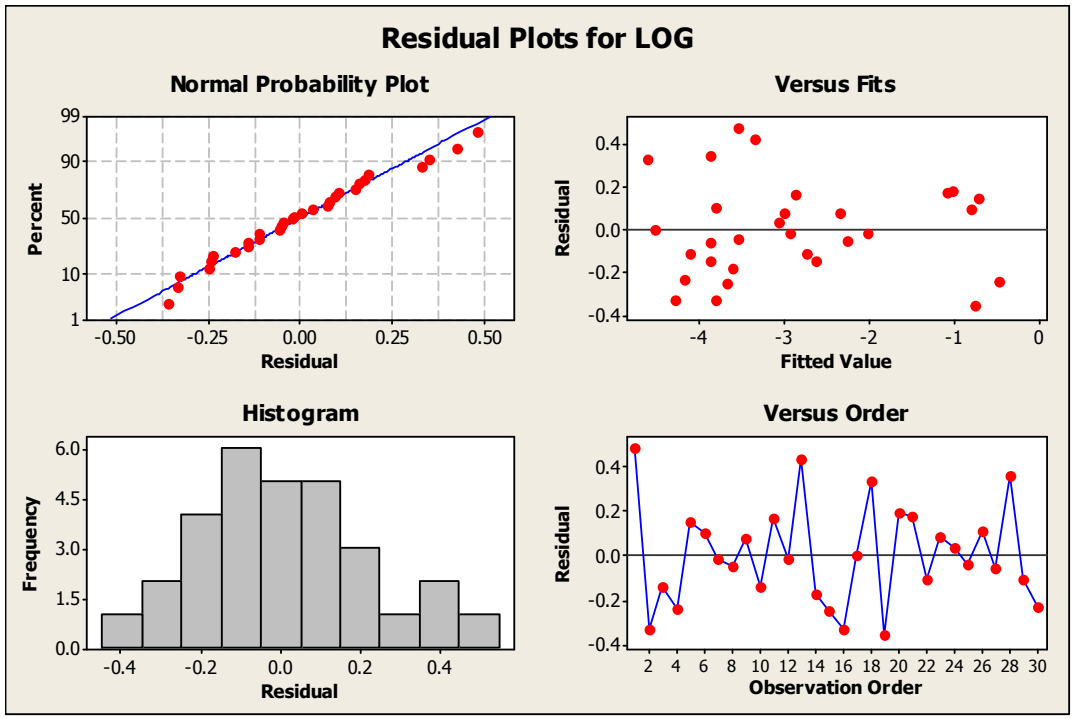
Treatment	Lines	Lower	Center	Upper
3	0	-3.226	-2.171	-1.116
3	127	-1.015	-0.082	0.851
6	0	-3.498	-2.443	-1.388
6	127	-1.074	-0.141	0.792
12	0	-3.568	-2.635	-1.702
12	127	-1.573	-0.640	0.293



```
Treatment =  3
Lines =   0  subtracted from:
Treatment  Lines   Lower   Center   Upper
 3         127     1.034   2.0893  3.1442
 6           0    -1.448  -0.2721  0.9036
 6         127     0.975   2.0303  3.0852
12           0    -1.519  -0.4642  0.5907
12         127     0.476   1.5313  2.5862

Treatment  Lines  ----+---------+---------+---------+--
 3         127                    (---*----)
 6           0            (----*----)
 6         127                    (---*---)
12           0            (---*---)
12         127                   (---*---)
                 ----+---------+---------+---------+--
                  -2.5       0.0       2.5       5.0
Treatment =  3
Lines = 127  subtracted from:
Treatment  Lines   Lower  Center   Upper  ----+---------+---------+---------+--
 6           0    -3.416  -2.361  -1.306  (----*---)
 6         127    -0.992  -0.059   0.874           (---*--)
12           0    -3.486  -2.553  -1.621  (---*---)
12         127    -1.491  -0.558   0.375          (---*--)
                                          ----+---------+---------+---------+--
                                           -2.5       0.0       2.5       5.0
Treatment =  6
Lines =   0  subtracted from:
Treatment  Lines   Lower   Center   Upper
 6         127     1.247   2.3024  3.3573
12           0    -1.247  -0.1921  0.8629
12         127     0.748   1.8034  2.8583
Treatment  Lines  ----+---------+---------+---------+--
 6         127                     (---*---)
12           0             (---*---)
12         127                    (---*---)
                 ----+---------+---------+---------+--
                  -2.5       0.0       2.5       5.0
Treatment =  6
Lines = 127  subtracted from:
Treatment  Lines   Lower  Center   Upper  ----+---------+---------+---------+--
12           0    -3.427  -2.494  -1.562  (---*---)
12         127    -1.432  -0.499   0.434          (---*---)
                                          ----+---------+---------+---------+--
                                           -2.5       0.0       2.5       5.0
Treatment = 12
Lines =   0  subtracted from:

Treatment  Lines  Lower  Center  Upper  ----+---------+---------+---------+--
12         127    1.063   1.995  2.928                   (---*---)
                                        ----+---------+---------+---------+--
                                         -2.5       0.0       2.5       5.0
```

Table 5. Statistic Analysis for HSP17.6 (At5g12020) Abundance

General Linear Model: Data versus Data T, Data R, Data L (95% Confidence Level)



General Linear Model: LOG versus Code L, Code T, Code R

```
Factor  Type   Levels  Values
Code L  fixed       2  0, 127
Code T  fixed       5  -1, 0, 3, 6, 12
Code R  fixed       3  1, 2, 3
Analysis of Variance for LOG, using Adjusted SS for Tests
Source         DF   Seq SS   Adj SS   Adj MS       F      P
Code L          1   3.0411   3.0411   3.0411   38.48  0.000
Code T          4  41.3661  41.3661  10.3415  130.86  0.000
Code R          2   0.5619   0.5619   0.2810    3.56  0.050
Code L*Code T   4   0.3591   0.3591   0.0898    1.14  0.371
Error          18   1.4225   1.4225   0.0790
Total          29  46.7507

S = 0.281115   R-Sq = 96.96%   R-Sq(adj) = 95.10%
Unusual Observations for LOG
Obs       LOG       Fit   SE Fit  Residual  St Resid
  1  -3.05731  -3.53641  0.17779   0.47909      2.20 R
R denotes an observation with a large standardized residual.

Tukey 95.0% Simultaneous Confidence Intervals
Response Variable LOG
All Pairwise Comparisons among Levels of Code L*Code T
Code L =   0
Code T = -1  subtracted from:
Code L  Code T    Lower  Center  Upper  ------+---------+---------+---------+
  0       0      2.7001  3.5230  4.346                            (--*--)
  0       3      0.7128  1.5357  2.359                   (--*--)
  0       6     -0.0896  0.7333  1.556                (--*--)
  0      12     -0.4057  0.4172  1.240             (---*--)
127      -1     -0.0883  0.7346  1.557                (--*--)
```

```
127      0       2.9861  3.8090  4.632                            (--*---)
127      3       1.4417  2.2646  3.087                       (--*--)
127      6       0.8343  1.6571  2.480                   (---*--)
127     12       0.1048  0.9277  1.751               (---*--)
                                          ------+---------+---------+---------+
                                             -2.5       0.0       2.5       5.0
Code L =   0
Code T =  0  subtracted from:
Code L  Code T   Lower  Center   Upper  ------+---------+---------+---------+
  0      3      -2.810  -1.987  -1.164      (--*--)
  0      6      -3.613  -2.790  -1.967    (--*--)
  0     12      -3.929  -3.106  -2.283  (---*--)
127     -1      -3.611  -2.788  -1.966    (--*--)
127      0      -0.537   0.286   1.109               (--*--)
127      3      -2.081  -1.258  -0.436         (--*--)
127      6      -2.689  -1.866  -1.043       (---*--)
127     12      -3.418  -2.595  -1.772    (---*--)
                                          ------+---------+---------+---------+
                                             -2.5       0.0       2.5       5.0
Code L =   0
Code T =  3  subtracted from:
Code L  Code T   Lower  Center    Upper  ------+---------+---------+---------+
  0      6      -1.625  -0.802   0.0205          (---*--)
  0     12      -1.941  -1.119  -0.2956         (---*--)
127     -1      -1.624  -0.801   0.0218           (--*--)
127      0       1.450   2.273   3.0962                     (--*--)
127      3      -0.094   0.729   1.5518                (--*--)
127      6      -0.701   0.121   0.9443             (--*---)
127     12      -1.431  -0.608   0.2149           (---*--)
                                           ------+---------+---------+---------+
                                              -2.5       0.0       2.5       5.0
Code L =   0
Code T =  6  subtracted from:
Code L  Code T   Lower   Center   Upper  ------+---------+---------+---------+
  0     12      -1.139  -0.3161  0.5067           (---*--)
127     -1      -0.822   0.0013  0.8241             (--*--)
127      0       2.253   3.0757  3.8986                          (--*---)
127      3       0.708   1.5313  2.3541                    (--*--)
127      6       0.101   0.9238  1.7467                (---*--)
127     12      -0.628   0.1944  1.0173              (---*--)
                                          ------+---------+---------+---------+
                                             -2.5       0.0       2.5       5.0
Code L =   0
Code T = 12  subtracted from:
Code L  Code T    Lower  Center  Upper  ------+---------+---------+---------+
127     -1      -0.5055  0.3174  1.140              (--*---)
127      0       2.5690  3.3919  4.215                         (---*--)
127      3       1.0245  1.8474  2.670                   (--*---)
127      6       0.4171  1.2400  2.063                 (--*--)
127     12      -0.3123  0.5106  1.333               (--*--)
                                          ------+---------+---------+---------+
                                             -2.5       0.0       2.5       5.0
Code L = 127
Code T = -1  subtracted from:
Code L  Code T    Lower  Center  Upper  ------+---------+---------+---------+
127      0       2.2516  3.0745  3.897                         (--*---)
127      3       0.7071  1.5300  2.353                    (--*--)
127      6       0.0997  0.9226  1.745                (---*--)
127     12      -0.6297  0.1932  1.016             (---*--)
                                          ------+---------+---------+---------+
                                             -2.5       0.0       2.5       5.0
Code L = 127
Code T =  0  subtracted from:
Code L  Code T   Lower  Center   Upper  ------+---------+---------+---------+
127      3      -2.367  -1.544  -0.722        (--*--)
127      6      -2.975  -2.152  -1.329     (--*---)
127     12      -3.704  -2.881  -2.058   (--*---)
                                          ------+---------+---------+---------+
                                             -2.5       0.0       2.5       5.0
```

```
Code L = 127
Code T =  3  subtracted from:

Code L  Code T   Lower  Center    Upper  ------+---------+---------+---------+
127      6      -1.430  -0.607   0.2155           (---*--)
127     12      -2.160  -1.337  -0.5140         (---*--)
                                          ------+---------+---------+---------+
                                             -2.5       0.0       2.5       5.0
Code L = 127
Code T =  6  subtracted from:

Code L  Code T   Lower   Center    Upper  ------+---------+---------+---------+
127     12      -1.552  -0.7294  0.09345           (--*--)
                                           ------+---------+---------+---------+
                                              -2.5       0.0       2.5       5.0
```

General Linear Model: LOG versus Code L, Code T, Code R

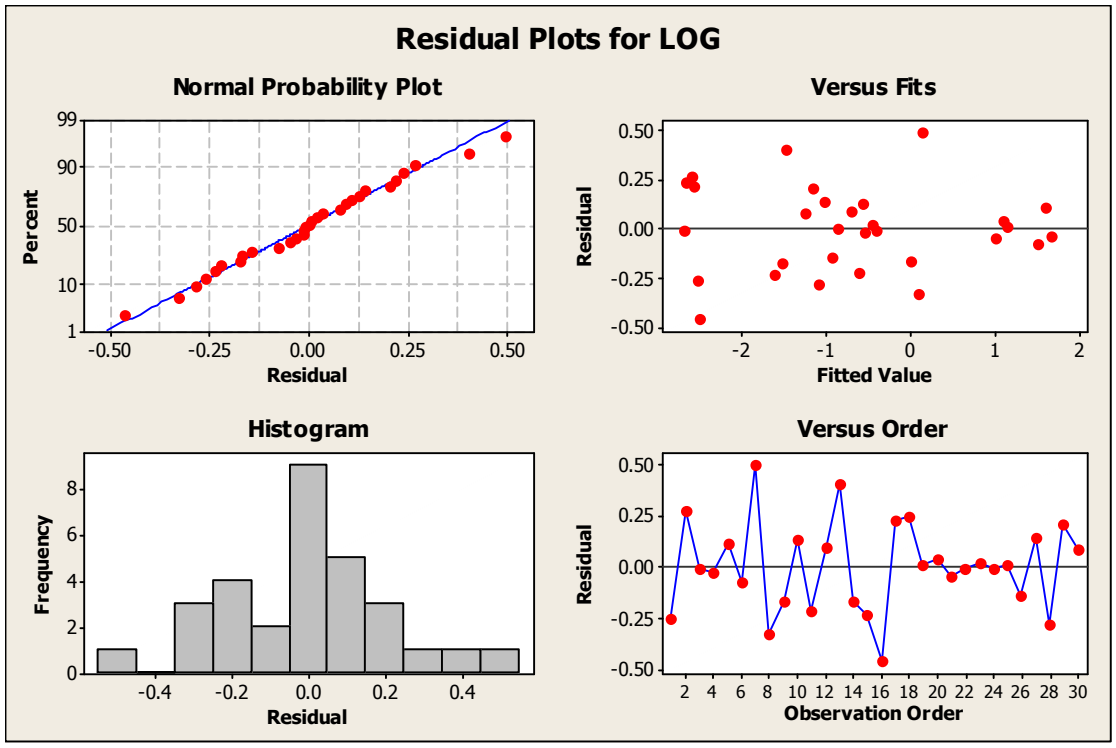
```
Factor  Type   Levels  Values
Code L  fixed       2  0, 127
Code T  fixed       5  -1, 0, 3, 6, 12
Code R  fixed       3  1, 2, 3

Analysis of Variance for LOG, using Adjusted SS for Tests
Source         DF   Seq SS   Adj SS   Adj MS       F      P
Code L          1   3.0411   3.0411   3.0411   38.48  0.000
Code T          4  41.3661  41.3661  10.3415  130.86  0.000
Code R          2   0.5619   0.5619   0.2810    3.56  0.050
Code L*Code T   4   0.3591   0.3591   0.0898    1.14  0.371
Error          18   1.4225   1.4225   0.0790
Total          29  46.7507

S = 0.281115   R-Sq = 96.96%   R-Sq(adj) = 95.10%
Unusual Observations for LOG
Obs       LOG       Fit   SE Fit  Residual  St Resid
  1  -3.05731  -3.53641  0.17779   0.47909      2.20 R
R denotes an observation with a large standardized residual.
```

Tukey 99.0% Simultaneous Confidence Intervals

```
Response Variable LOG
All Pairwise Comparisons among Levels of Code L*Code T
Code L =   0
Code T = -1  subtracted from:
Code L  Code T    Lower  Center  Upper  ------+---------+---------+---------+
  0      0       2.5167  3.5230  4.529                          (---*---)
  0      3       0.5294  1.5357  2.542                 (---*---)
  0      6      -0.2730  0.7333  1.740               (---*---)
  0     12      -0.5891  0.4172  1.423              (---*---)
127     -1      -0.2717  0.7346  1.741               (---*---)
127      0       2.8027  3.8090  4.815                          (---*---)
127      3       1.2583  2.2646  3.271                    (---*---)
127      6       0.6509  1.6571  2.663                  (---*---)
127     12      -0.0786  0.9277  1.934                (---*---)
                                          ------+---------+---------+---------+
                                             -2.5       0.0       2.5       5.0
Code L =   0
Code T =  0  subtracted from:
Code L  Code T   Lower  Center   Upper  ------+---------+---------+---------+
  0      3      -2.994  -1.987  -0.981      (---*---)
  0      6      -3.796  -2.790  -1.783   (---*---)
  0     12      -4.112  -3.106  -2.100  (---*---)
127     -1      -3.795  -2.788  -1.782   (---*---)
127      0      -0.720   0.286   1.292              (---*---)
127      3      -2.265  -1.258  -0.252        (---*---)
127      6      -2.872  -1.866  -0.860       (---*---)
127     12      -3.602  -2.595  -1.589    (---*---)
                                          ------+---------+---------+---------+
                                             -2.5       0.0       2.5       5.0
```

```
Code L =   0
Code T =  3  subtracted from:
Code L  Code T   Lower  Center    Upper  ------+---------+---------+---------+
  0       6     -1.809  -0.802   0.2039          (---*---)
  0      12     -2.125  -1.119  -0.1122         (---*---)
127      -1     -1.807  -0.801   0.2052          (---*---)
127       0      1.267   2.273   3.2796                    (---*---)
127       3     -0.277   0.729   1.7352               (---*---)
127       6     -0.885   0.121   1.1277             (---*----)
127      12     -1.614  -0.608   0.3983           (---*---)
                                        ------+---------+---------+---------+
                                           -2.5       0.0       2.5       5.0
Code L =   0
Code T =  6  subtracted from:
Code L  Code T   Lower   Center   Upper  ------+---------+---------+---------+
  0      12     -1.322  -0.3161  0.6901           (---*---)
127      -1     -1.005   0.0013  1.0075            (---*---)
127       0      2.069   3.0757  4.0820                      (---*---)
127       3      0.525   1.5313  2.5375                 (---*---)
127       6     -0.082   0.9238  1.9301                (---*---)
127      12     -0.812   0.1944  1.2007              (---*---)
                                        ------+---------+---------+---------+
                                           -2.5       0.0       2.5       5.0
Code L =   0
Code T = 12  subtracted from:
Code L  Code T    Lower  Center  Upper  ------+---------+---------+---------+
127      -1     -0.6889  0.3174  1.324            (---*---)
127       0      2.3856  3.3919  4.398                       (---*---)
127       3      0.8411  1.8474  2.854                  (---*---)
127       6      0.2337  1.2400  2.246                (---*---)
127      12     -0.4957  0.5106  1.517              (---*---)
                                       ------+---------+---------+---------+
                                          -2.5       0.0       2.5       5.0
Code L = 127
Code T = -1  subtracted from:
Code L  Code T    Lower  Center  Upper  ------+---------+---------+---------+
127       0      2.0682  3.0745  4.081                       (---*---)
127       3      0.5237  1.5300  2.536                  (---*---)
127       6     -0.0837  0.9226  1.929                (---*---)
127      12     -0.8131  0.1932  1.199            (---*---)
                                       ------+---------+---------+---------+
                                         -2.5       0.0       2.5       5.0
Code L = 127
Code T =  0  subtracted from:
Code L  Code T   Lower  Center   Upper  ------+---------+---------+---------+
127       3     -2.551  -1.544  -0.538        (---*---)
127       6     -3.158  -2.152  -1.146     (---*---)
127      12     -3.888  -2.881  -1.875  (---*---)
                                        ------+---------+---------+---------+
                                          -2.5       0.0       2.5       5.0
Code L = 127
Code T =  3  subtracted from:
Code L  Code T   Lower  Center    Upper  ------+---------+---------+---------+
127       6     -1.614  -0.607   0.3989           (---*---)
127      12     -2.343  -1.337  -0.3306         (---*---)
                                        ------+---------+---------+---------+
                                           -2.5       0.0       2.5       5.0
Code L = 127
Code T =  6  subtracted from:
Code L  Code T   Lower   Center   Upper  ------+---------+---------+---------+
127      12     -1.736  -0.7294  0.2769           (---*---)
                                        ------+---------+---------+---------+
                                           -2.5       0.0       2.5       5.0
```

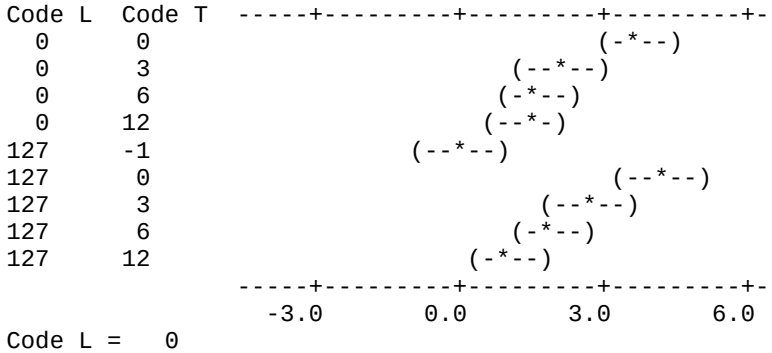
Table 6. Statistic Analysis for HSP17.6A (At5g12030) Abundance



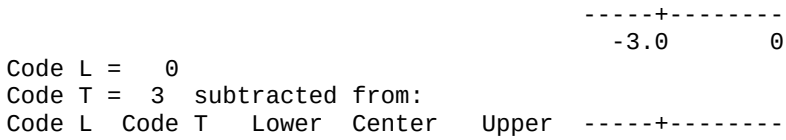
General Linear Model: LOG versus Code L, Code T, Code R

```
Factor  Type   Levels  Values
Code L  fixed       2  0, 127
Code T  fixed       5  -1, 0, 3, 6, 12
Code R  fixed       3  1, 2, 3

Analysis of Variance for LOG, using Adjusted SS for Tests
Source         DF   Seq SS   Adj SS   Adj MS       F      P
Code L          1   0.2815   0.2815   0.2815    3.73  0.069
Code T          4  50.6983  50.6983  12.6746  167.86  0.000
Code R          2   0.1041   0.1041   0.0521    0.69  0.515
Code L*Code T   4   0.9474   0.9474   0.2368    3.14  0.040
Error          18   1.3591   1.3591   0.0755
Total          29  53.3904
S = 0.274783   R-Sq = 97.45%   R-Sq(adj) = 95.90%

Unusual Observations for LOG
Obs       LOG       Fit   SE Fit  Residual  St Resid
  7   0.65139   0.15561  0.17379   0.49578      2.33 R
 16  -2.97692  -2.51658  0.17379  -0.46035     -2.16 R

R denotes an observation with a large standardized residual.

Tukey 95.0% Simultaneous Confidence Intervals
Response Variable LOG
All Pairwise Comparisons among Levels of Code L*Code T
Code L =   0
Code T = -1  subtracted from:
Code L  Code T    Lower    Center   Upper
  0       0       2.8638   3.66811  4.4724
  0       3       1.3077   2.11206  2.9164
  0       6       0.8415   1.64580  2.4501
```

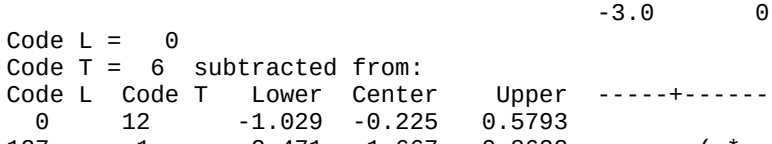
```
  0     12       0.6165   1.42080  2.2251
127     -1      -0.8251  -0.02073  0.7836
127      0       3.3776   4.18191  4.9862
127      3       1.8679   2.67219  3.4765
127      6       1.1438   1.94813  2.7525
127     12       0.2296   1.03392  1.8383
```



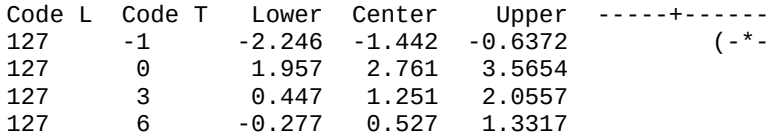

```
Code L =   0
Code T =  0  subtracted from:
Code L  Code T   Lower  Center   Upper
  0      3      -2.360  -1.556  -0.752
  0      6      -2.827  -2.022  -1.218
  0     12      -3.052  -2.247  -1.443
127     -1      -4.493  -3.689  -2.885
127      0      -0.291   0.514   1.318
127      3      -1.800  -0.996  -0.192
127      6      -2.524  -1.720  -0.916
127     12      -3.439  -2.634  -1.830
```




```
Code L =   0
Code T =  3  subtracted from:
Code L  Code T   Lower  Center   Upper
  0      6      -1.271  -0.466   0.338
  0     12      -1.496  -0.691   0.113
127     -1      -2.937  -2.133  -1.328
127      0       1.266   2.070   2.874
127      3      -0.244   0.560   1.364
127      6      -0.968  -0.164   0.640
127     12      -1.882  -1.078  -0.274
```




```
Code L =   0
Code T =  6  subtracted from:
Code L  Code T   Lower  Center    Upper
  0     12      -1.029  -0.225   0.5793
127     -1      -2.471  -1.667  -0.8622
127      0       1.732   2.536   3.3405
127      3       0.222   1.026   1.8307
127      6      -0.502   0.302   1.1067
127     12      -1.416  -0.612   0.1925
```



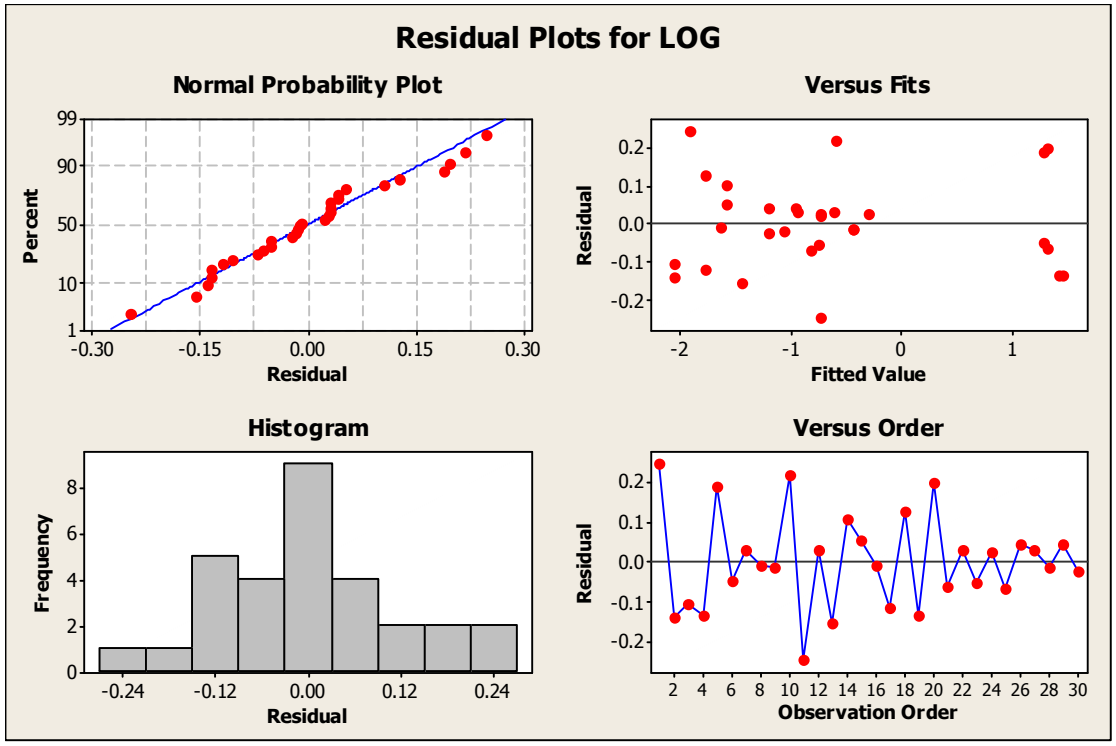

```
Code L =   0
Code T = 12  subtracted from:
Code L  Code T   Lower  Center    Upper
127     -1      -2.246  -1.442  -0.6372
127      0       1.957   2.761   3.5654
127      3       0.447   1.251   2.0557
127      6      -0.277   0.527   1.3317
127     12      -1.191  -0.387   0.4174
```



```
Code L = 127
Code T = -1  subtracted from:
Code L  Code T   Lower  Center  Upper
127      0      3.3983   4.203  5.007
```

```
127      3      1.8886   2.693  3.497                     (--*--)
127      6      1.1645   1.969  2.773                  (--*-)
127     12      0.2503   1.055  1.859               (--*-)
                                          -----+---------+---------+---------+-
                                             -3.0       0.0       3.0       6.0
Code L = 127
Code T =  0  subtracted from:
Code L  Code T   Lower  Center   Upper  -----+---------+---------+---------+-
127      3      -2.314  -1.510  -0.705         (--*--)
127      6      -3.038  -2.234  -1.429       (--*-)
127     12      -3.952  -3.148  -2.344    (--*-)
                                         -----+---------+---------+---------+-
                                            -3.0       0.0       3.0       6.0
Code L = 127
Code T =  3  subtracted from:
Code L  Code T   Lower  Center    Upper  -----+---------+---------+---------+-
127      6      -1.528  -0.724   0.0803            (--*-)
127     12      -2.443  -1.638  -0.8339         (--*-)
                                          -----+---------+---------+---------+-
                                             -3.0       0.0       3.0       6.0
Code L = 127
Code T =  6  subtracted from:
Code L  Code T   Lower   Center    Upper  -----+---------+---------+---------+-
127     12      -1.719  -0.9142  -0.1099           (--*--)
                                           -----+---------+---------+---------+-
                                              -3.0       0.0       3.0       6.0
```

Table 7. Statistic Analysis for HSP70 Abundance



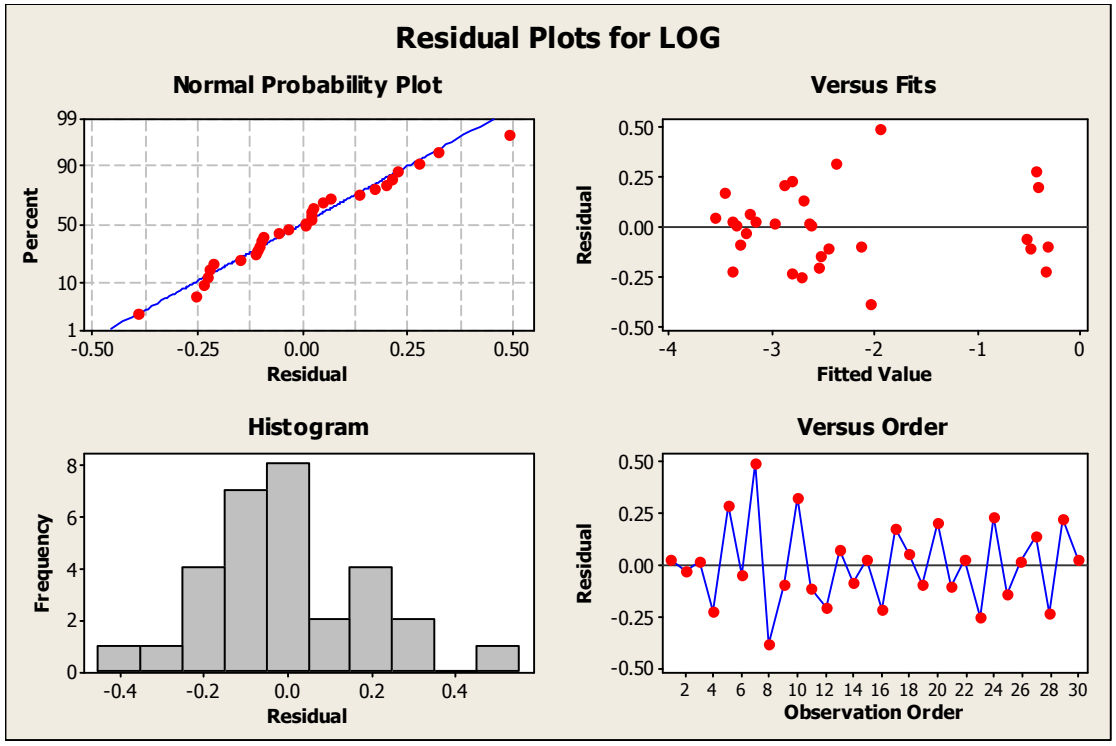
General Linear Model: LOG versus Code L, Code T, Code R

```
Factor  Type   Levels  Values
Code L  fixed       2  0, 127
Code T  fixed       5  -1, 0, 3, 6, 12
Code R  fixed       3  1, 2, 3

Analysis of Variance for LOG, using Adjusted SS for Tests
Source         DF   Seq SS   Adj SS  Adj MS       F      P
Code L          1   0.0093   0.0093  0.0093    0.42  0.526
Code T          4  35.8532  35.8532  8.9633  402.03  0.000
Code R          2   0.1251   0.1251  0.0625    2.81  0.087
Code L*Code T   4   0.5189   0.5189  0.1297    5.82  0.003
Error          18   0.4013   0.4013  0.0223
Total          29  36.9079

S = 0.149315   R-Sq = 98.91%   R-Sq(adj) = 98.25%

Unusual Observations for LOG

Obs       LOG       Fit   SE Fit  Residual  St Resid
  1  -1.66351  -1.90937  0.09444   0.24586      2.13 R
 11  -0.98215  -0.73457  0.09444  -0.24758     -2.14 R

R denotes an observation with a large standardized residual.

Tukey 95.0% Simultaneous Confidence Intervals
Response Variable LOG
All Pairwise Comparisons among Levels of Code L*Code T
Code L =   0
Code T = -1  subtracted from:

Code L  Code T    Lower   Center   Upper  -----+---------+---------+---------+-
  0       0      2.6521   3.0892  3.5263                          (*-)
  0       3      0.5921   1.0292  1.4663                (-*-)
  0       6      0.3847   0.8218  1.2589                (*-)
```

```
  0     12       0.1276   0.5646  1.0017                  (*-)
127     -1      -0.7162  -0.2791  0.1580              (-*-)
127      0       2.6155   3.0525  3.4896                          (-*-)
127      3       0.8908   1.3279  1.7650                    (*-)
127      6       0.5975   1.0345  1.4716                  (-*-)
127     12      -0.2445   0.1925  0.6296                (-*-)
                                          -----+---------+---------+---------+-
                                            -2.5       0.0       2.5       5.0
Code L =   0
Code T =  0  subtracted from:
Code L  Code T   Lower  Center   Upper  -----+---------+---------+---------+-
  0      3      -2.497  -2.060  -1.623       (-*-)
  0      6      -2.704  -2.267  -1.830      (-*-)
  0     12      -2.962  -2.525  -2.087     (-*-)
127     -1      -3.805  -3.368  -2.931  (-*)
127      0      -0.474  -0.037   0.400              (-*-)
127      3      -2.198  -1.761  -1.324        (-*-)
127      6      -2.492  -2.055  -1.618       (-*-)
127     12      -3.334  -2.897  -2.460    (*-)
                                         -----+---------+---------+---------+-
                                           -2.5       0.0       2.5       5.0
Code L =   0
Code T =  3  subtracted from:
Code L  Code T   Lower  Center    Upper  -----+---------+---------+---------+-
  0      6      -0.644  -0.207   0.2296             (-*-)
  0     12      -0.902  -0.465  -0.0275            (-*-)
127     -1      -1.745  -1.308  -0.8712         (-*-)
127      0       1.586   2.023   2.4604                     (-*-)
127      3      -0.138   0.299   0.7358                (-*-)
127      6      -0.432   0.005   0.4424              (-*-)
127     12      -1.274  -0.837  -0.3996           (-*)
                                          -----+---------+---------+---------+-
                                            -2.5       0.0       2.5       5.0
Code L =   0
Code T =  6  subtracted from:
Code L  Code T   Lower  Center    Upper  -----+---------+---------+---------+-
  0     12      -0.694  -0.257   0.1799             (-*-)
127     -1      -1.538  -1.101  -0.6638          (-*)
127      0       1.794   2.231   2.6678                      (-*-)
127      3       0.069   0.506   0.9432                (-*-)
127      6      -0.224   0.213   0.6498               (-*-)
127     12      -1.066  -0.629  -0.1922            (*-)
                                          -----+---------+---------+---------+-
                                            -2.5       0.0       2.5       5.0
Code L =   0
Code T = 12  subtracted from:
Code L  Code T   Lower   Center    Upper  -----+---------+---------+---------+-
127     -1      -1.281  -0.8437  -0.4067           (-*)
127      0       2.051   2.4879   2.9250                       (-*-)
127      3       0.326   0.7633   1.2003                  (-*-)
127      6       0.033   0.4699   0.9070                (-*-)
127     12      -0.809  -0.3721   0.0650              (-*)
                                          -----+---------+---------+---------+-
                                            -2.5       0.0       2.5       5.0

Code L = 127
Code T = -1  subtracted from:
Code L  Code T    Lower  Center   Upper  -----+---------+---------+---------+-
127      0      2.89456  3.3316  3.7687                            (*-)
127      3      1.16994  1.6070  2.0441                     (*-)
127      6      0.87655  1.3136  1.7507                    (*-)
127     12      0.03457  0.4716  0.9087                (-*-)
                                          -----+---------+---------+---------+-
                                            -2.5       0.0       2.5       5.0
Code L = 127
Code T =  0  subtracted from:
Code L  Code T   Lower  Center   Upper  -----+---------+---------+---------+-
127      3      -2.162  -1.725  -1.288       (-*-)
127      6      -2.455  -2.018  -1.581      (-*-)
```

```
127     12      -3.297  -2.860  -2.423    (-*)
                                        -----+---------+---------+---------+-
                                          -2.5       0.0       2.5       5.0

Code L = 127
Code T =  3  subtracted from:
Code L  Code T   Lower  Center    Upper  -----+---------+---------+---------+-
127      6      -0.730  -0.293   0.1437             (-*-)
127     12      -1.572  -1.135  -0.6983          (*-)
                                        -----+---------+---------+---------+-
                                          -2.5       0.0       2.5       5.0
Code L = 127
Code T =  6  subtracted from:
Code L  Code T   Lower   Center    Upper  -----+---------+---------+---------+-
127     12      -1.279  -0.8420  -0.4049           (-*)
                                         -----+---------+---------+---------+-
                                           -2.5       0.0       2.5       5.0
```

Table 8. Statistic Analysis for HSP101 Abundance



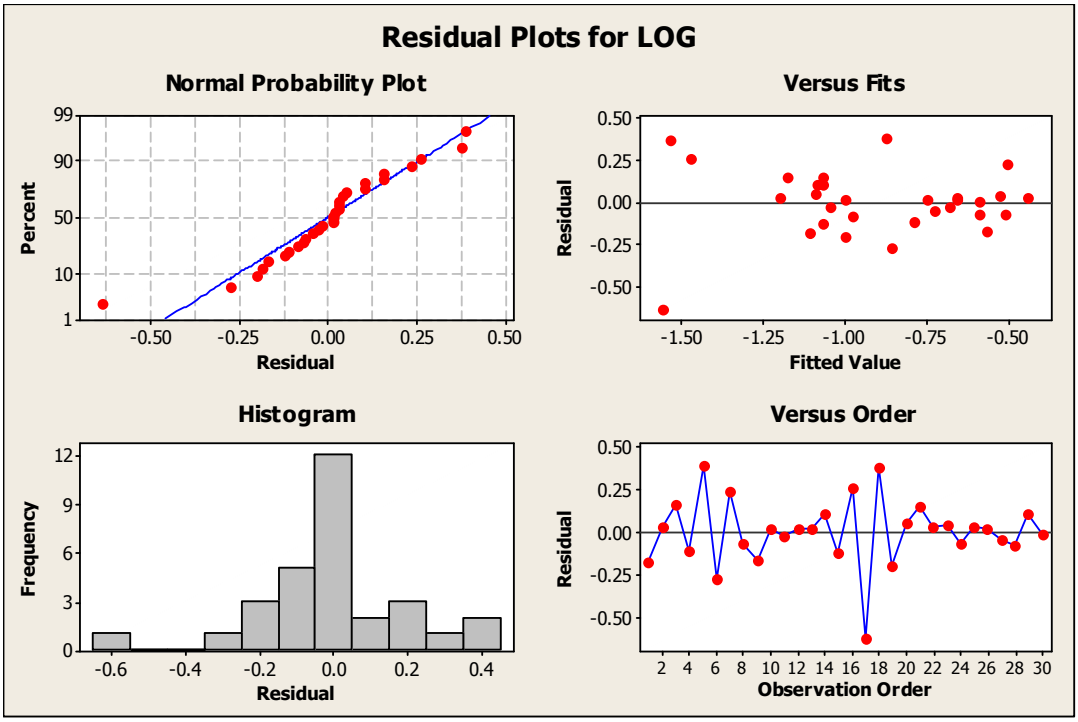
General Linear Model: LOG versus Code L, Code T, Code R

```
Factor  Type   Levels  Values
Code L  fixed       2  0, 127
Code T  fixed       5  -1, 0, 3, 6, 12
Code R  fixed       3  1, 2, 3

Analysis of Variance for LOG, using Adjusted SS for Tests
Source         DF   Seq SS   Adj SS  Adj MS       F      P
Code L          1   0.1088   0.1088  0.1088    1.75  0.202
Code T          4  32.2845  32.2845  8.0711  129.95  0.000
Code R          2   0.1452   0.1452  0.0726    1.17  0.333
Code L*Code T   4   0.9531   0.9531  0.2383    3.84  0.020
Error          18   1.1180   1.1180  0.0621
Total          29  34.6095

S = 0.249221   R-Sq = 96.77%   R-Sq(adj) = 94.80%

Unusual Observations for LOG

Obs       LOG       Fit   SE Fit  Residual  St Resid
  7  -1.46028  -1.95253  0.15762   0.49225      2.55 R
  8  -2.43099  -2.04016  0.15762  -0.39083     -2.02 R

R denotes an observation with a large standardized residual.

Tukey 95.0% Simultaneous Confidence Intervals
Response Variable LOG
All Pairwise Comparisons among Levels of Code L*Code T
Code L =   0
Code T = -1  subtracted from:
```

```
Code L  Code T    Lower  Center   Upper  -----+---------+---------+---------+-
  0      0       2.3397  3.0692  3.7987                       (--*--)
  0      3       0.0214  0.7509  1.4804               (--*--)
  0      6       0.1256  0.8551  1.5846                (-*--)
  0     12      -0.1494  0.5801  1.3097              (--*--)
127     -1      -0.5191  0.2104  0.9400             (--*--)
127      0       2.3159  3.0454  3.7749                       (--*--)
127      3       0.7010  1.4305  2.1600                  (--*--)
127      6       0.2819  1.0114  1.7409                (--*--)
127     12      -0.5698  0.1597  0.8892             (--*--)
                                      -----+---------+---------+---------+-
                                        -2.5       0.0       2.5       5.0
Code L =   0
Code T =  0  subtracted from:
Code L  Code T   Lower  Center   Upper  -----+---------+---------+---------+-
  0      3      -3.048  -2.318  -1.589    (--*--)
  0      6      -2.944  -2.214  -1.485    (--*--)
  0     12      -3.219  -2.489  -1.760   (--*--)
127     -1      -3.588  -2.859  -2.129  (--*-)
127      0      -0.753  -0.024   0.706            (--*--)
127      3      -2.368  -1.639  -0.909       (-*--)
127      6      -2.787  -2.058  -1.328     (--*--)
127     12      -3.639  -2.909  -2.180 (--*--)
                                      -----+---------+---------+---------+-
                                        -2.5       0.0       2.5       5.0
Code L =   0
Code T =  3  subtracted from:
Code L  Code T   Lower   Center   Upper  -----+---------+---------+---------+-
  0      6      -0.625   0.1041  0.8337            (--*--)
  0     12      -0.900  -0.1708  0.5587           (--*--)
127     -1      -1.270  -0.5405  0.1890          (--*--)
127      0       1.565   2.2944  3.0239                    (--*--)
127      3      -0.050   0.6796  1.4091               (--*--)
127      6      -0.469   0.2605  0.9900              (--*--)
127     12      -1.321  -0.5912  0.1383          (--*--)
                                      -----+---------+---------+---------+-
                                        -2.5       0.0       2.5       5.0
Code L =   0
Code T =  6  subtracted from:
Code L  Code T   Lower   Center    Upper  -----+---------+---------+---------+-
  0     12      -1.004  -0.2749  0.45458           (--*--)
127     -1      -1.374  -0.6446  0.08488          (-*--)
127      0       1.461   2.1903  2.91980                   (--*--)
127      3      -0.154   0.5754  1.30494              (--*--)
127      6      -0.573   0.1563  0.88586             (--*--)
127     12      -1.425  -0.6954  0.03415          (--*--)
                                      -----+---------+---------+---------+-
                                        -2.5       0.0       2.5       5.0
Code L =   0
Code T = 12  subtracted from:
Code L  Code T   Lower   Center   Upper  -----+---------+---------+---------+-
127     -1      -1.099  -0.3697  0.3598           (--*-)
127      0       1.736   2.4652  3.1947                    (--*--)
127      3       0.121   0.8504  1.5799               (--*--)
127      6      -0.298   0.4313  1.1608              (--*--)
127     12      -1.150  -0.4204  0.3091          (--*--)
                                      -----+---------+---------+---------+-
                                        -2.5       0.0       2.5       5.0
Code L = 127
Code T = -1  subtracted from:
Code L  Code T    Lower    Center   Upper
127      0       2.1054   2.83492  3.5644
127      3       0.4906   1.22007  1.9496
127      6       0.0715   0.80098  1.5305
127     12      -0.7802  -0.05072  0.6788

Code L  Code T  -----+---------+---------+---------+-
127      0                           (--*--)
127      3                    (--*--)
```

```
127      6                    (--*--)
127     12                (--*--)
                -----+---------+---------+---------+-
                  -2.5       0.0       2.5       5.0

Code L = 127
Code T =  0  subtracted from:
Code L  Code T   Lower  Center   Upper  -----+---------+---------+---------+-
127      3      -2.344  -1.615  -0.885        (--*-)
127      6      -2.763  -2.034  -1.304      (--*--)
127     12      -3.615  -2.886  -2.156   (-*--)
                                        -----+---------+---------+---------+-
                                          -2.5       0.0       2.5       5.0
Code L = 127
Code T =  3  subtracted from:
Code L  Code T   Lower  Center    Upper  -----+---------+---------+---------+-
127      6      -1.149  -0.419   0.3104           (--*--)
127     12      -2.000  -1.271  -0.5413         (--*--)
                                         -----+---------+---------+---------+-
                                           -2.5       0.0       2.5       5.0
Code L = 127
Code T =  6  subtracted from:
Code L  Code T   Lower   Center    Upper  -----+---------+---------+---------+-
127     12      -1.581  -0.8517  -0.1222           (--*--)
                                          -----+---------+---------+---------+-
                                            -2.5       0.0       2.5       5.0
```

Table 9. Statistic Analysis for Calmodulin 7 Abundance



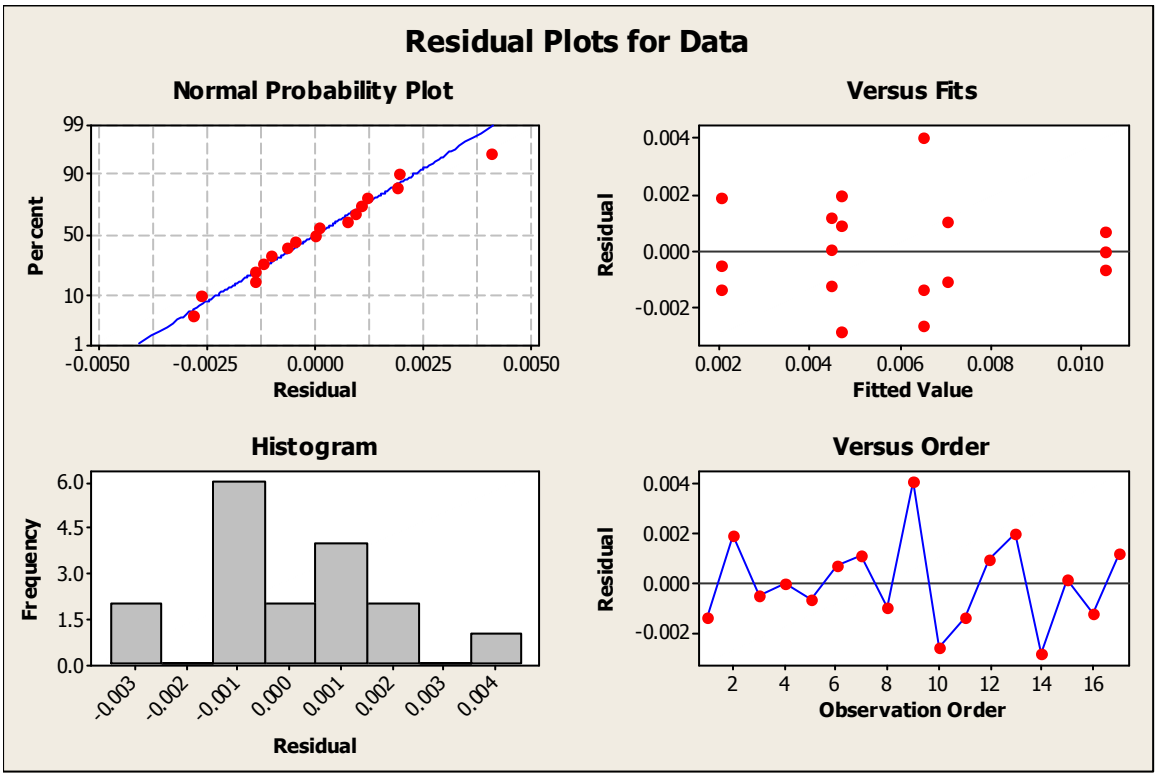
General Linear Model: LOG versus Code L, Code T, Code R

```
Factor  Type   Levels  Values
Code L  fixed       2  0, 127
Code T  fixed       5  -1, 0, 3, 6, 12
Code R  fixed       3  1, 2, 3


Analysis of Variance for LOG, using Adjusted SS for Tests

Source         DF   Seq SS   Adj SS   Adj MS     F      P
Code L          1  0.09387  0.09387  0.09387  1.52  0.234
Code T          4  2.46868  2.46868  0.61717  9.97  0.000
Code R          2  0.04309  0.04309  0.02154  0.35  0.711
Code L*Code T   4  0.18320  0.18320  0.04580  0.74  0.577
Error          18  1.11462  1.11462  0.06192
Total          29  3.90346
S = 0.248844   R-Sq = 71.45%   R-Sq(adj) = 54.00%

Unusual Observations for LOG
Obs       LOG       Fit   SE Fit  Residual  St Resid
  5  -0.48898  -0.87488  0.15738   0.38590      2.00 R
 17  -2.19360  -1.55973  0.15738  -0.63388     -3.29 R
R denotes an observation with a large standardized residual.

Tukey 95.0% Simultaneous Confidence Intervals
Response Variable LOG
All Pairwise Comparisons among Levels of Code L*Code T
Code L =   0
Code T = -1  subtracted from:
Code L  Code T    Lower  Center  Upper  ----+---------+---------+---------+--
  0       0     -0.2559  0.4725  1.201            (-------*------)
  0       3      0.3053  1.0337  1.762                  (------*-------)
  0       6      0.0841  0.8125  1.541                (------*------)
  0      12     -0.2335  0.4949  1.223             (------*------)
127      -1     -0.3670  0.3614  1.090           (-------*------)
```

```
127      0      -0.0436  0.6848  1.413               (------*------)
127      3       0.2439  0.9723  1.701                 (-------*------)
127      6       0.1532  0.8816  1.610                 (------*------)
127     12      -0.2556  0.4728  1.201           (-------*------)
                                          ----+---------+---------+---------+--
                                           -1.0       0.0       1.0       2.0

Code L =   0
Code T =  0  subtracted from:
Code L  Code T    Lower   Center   Upper  ----+---------+---------+---------+--
  0      3      -0.1673   0.5612  1.2896            (-------*------)
  0      6      -0.3884   0.3400  1.0684          (------*-------)
  0     12      -0.7060   0.0224  0.7508        (------*-------)
127     -1      -0.8395  -0.1111  0.6173       (------*------)
127      0      -0.5161   0.2123  0.9407         (------*------)
127      3      -0.2286   0.4998  1.2282           (------*------)
127      6      -0.3193   0.4091  1.1375          (------*------)
127     12      -0.7281   0.0003  0.7287        (------*------)
                                           ----+---------+---------+---------+--
                                            -1.0       0.0       1.0       2.0

Code L =   0
Code T =  3  subtracted from:
Code L  Code T   Lower   Center    Upper  ----+---------+---------+---------+--
  0      6      -0.950  -0.2212  0.50722      (------*------)
  0     12      -1.267  -0.5387  0.18967   (-------*------)
127     -1      -1.401  -0.6723  0.05612  (------*-------)
127      0      -1.077  -0.3488  0.37959    (-------*------)
127      3      -0.790  -0.0614  0.66703       (------*-------)
127      6      -0.880  -0.1521  0.57636      (------*-------)
127     12      -1.289  -0.5608  0.16757   (------*-------)
                                          ----+---------+---------+---------+--
                                         -1.0       0.0       1.0       2.0

Code L =   0
Code T =  6  subtracted from:
Code L  Code T   Lower   Center   Upper  ----+---------+---------+---------+--
  0     12      -1.046  -0.3175  0.4109     (------*------)
127     -1      -1.180  -0.4511  0.2773   (------*-------)
127      0      -0.856  -0.1276  0.6008      (-------*------)
127      3      -0.569   0.1598  0.8882         (-------*------)
127      6      -0.659   0.0691  0.7975        (-------*------)
127     12      -1.068  -0.3396  0.3888    (-------*------)
                                          ----+---------+---------+---------+--
                                           -1.0       0.0       1.0       2.0

Code L =   0
Code T = 12  subtracted from:
Code L  Code T    Lower   Center   Upper  ----+---------+---------+---------+--
127     -1      -0.8620  -0.1336  0.5949      (-------*------)
127      0      -0.5385   0.1899  0.9183          (------*------)
127      3      -0.2510   0.4774  1.2058            (-------*------)
127      6      -0.3417   0.3867  1.1151            (------*------)
127     12      -0.7505  -0.0221  0.7063        (-------*------)
                                           ----+---------+---------+---------+--
                                            -1.0       0.0       1.0       2.0

Code L = 127
Code T = -1  subtracted from:
Code L  Code T    Lower  Center   Upper  ----+---------+---------+---------+--
127      0      -0.4049  0.3235  1.0519           (------*-------)
127      3      -0.1175  0.6109  1.3393              (------*------)
127      6      -0.2082  0.5202  1.2486             (------*------)
127     12      -0.6169  0.1115  0.8399         (------*------)
                                          ----+---------+---------+---------+--
                                           -1.0       0.0       1.0       2.0

Code L = 127
Code T =  0  subtracted from:
Code L  Code T    Lower   Center   Upper  ----+---------+---------+---------+--
127      3      -0.4410   0.2874  1.0159           (------*------)
127      6      -0.5316   0.1968  0.9252          (------*------)
127     12      -0.9404  -0.2120  0.5164       (------*------)
                                           ----+---------+---------+---------+--
                                            -1.0       0.0       1.0       2.0
```

```
Code L = 127
Code T =  3  subtracted from:

Code L  Code T   Lower   Center   Upper  ----+---------+---------+---------+--
127      6      -0.819  -0.0907  0.6377        (------*------)
127     12      -1.228  -0.4995  0.2290    (------*------)
                                          ----+---------+---------+---------+--
                                           -1.0       0.0       1.0       2.0
Code L = 127
Code T =  6  subtracted from:

Code L  Code T   Lower   Center   Upper  ----+---------+---------+---------+--
127     12      -1.137  -0.4088  0.3196     (------*------)
                                          ----+---------+---------+---------+--
                                           -1.0       0.0       1.0       2.0
```

Table 10. Statistic Analysis for AtMYB64 Abundance in ABA-Treated Plants



General Linear Model: Data versus Code T

```
Factor  Type   Levels  Values
Code T  fixed       6  0, 2, 4, 8, 24, 48
Analysis of Variance for Data, using Adjusted SS for Tests
Source  DF     Seq SS     Adj SS     Adj MS     F      P
Code T   5  0.0001220  0.0001220  0.0000244  5.37  0.010
Error   11  0.0000500  0.0000500  0.0000045
Total   16  0.0001720
S = 0.00213137   R-Sq = 70.95%   R-Sq(adj) = 57.74%
Unusual Observations for Data
Obs      Data       Fit    SE Fit  Residual  St Resid
  9  0.010522  0.006479  0.001231  0.004043      2.32 R
R denotes an observation with a large standardized residual.
Tukey 95.0% Simultaneous Confidence Intervals
Response Variable Data
All Pairwise Comparisons among Levels of Code T
Code T =  0  subtracted from:
Code T      Lower    Center     Upper  -----+---------+---------+---------+-
 2       0.002530  0.008461  0.014392                   (-------*------)
 4      -0.001667  0.004964  0.011595              (-------*-------)
 8      -0.001500  0.004431  0.010362              (-------*------)
24      -0.003288  0.002643  0.008575            (------*-------)
48      -0.003504  0.002427  0.008358            (------*------)
                                  -----+---------+---------+---------+-
                                     -0.0080    0.0000    0.0080    0.0160
Code T =  2  subtracted from:
Code T      Lower     Center      Upper  -----+---------+---------+---------+-
 4      -0.01013  -0.003497   0.003134    (--------*-------)
 8      -0.00996  -0.004030   0.001901     (------*------)
24      -0.01175  -0.005817   0.000114  (-------*------)
48      -0.01196  -0.006034  -0.000102  (------*-------)
                                        -----+---------+---------+---------+-
                                          -0.0080    0.0000    0.0080    0.0160
```

```
Code T =  4  subtracted from:
Code T      Lower     Center     Upper  -----+---------+---------+---------+-
 8      -0.007164  -0.000533  0.006098       (-------*--------)
24      -0.008952  -0.002321  0.004311     (-------*-------)
48      -0.009168  -0.002537  0.004095     (-------*-------)
                                       -----+---------+---------+---------+-
                                         -0.0080    0.0000    0.0080    0.0160
Code T =  8  subtracted from:
Code T      Lower     Center     Upper  -----+---------+---------+---------+-
24      -0.007719  -0.001787  0.004144      (-------*------)
48      -0.007935  -0.002004  0.003927      (------*-------)
                                       -----+---------+---------+---------+-
                                         -0.0080    0.0000    0.0080    0.0160
Code T = 24  subtracted from:
Code T      Lower     Center     Upper  -----+---------+---------+---------+-
48      -0.006148  -0.000216  0.005715        (-------*------)
                                       -----+---------+---------+---------+-
                                         -0.0080    0.0000    0.0080    0.0160
```

General Linear Model: Data versus Code T

```
Factor  Type   Levels  Values
Code T  fixed       6  0, 2, 4, 8, 24, 48


Analysis of Variance for Data, using Adjusted SS for Tests

Source  DF     Seq SS     Adj SS     Adj MS     F      P
Code T   5  0.0001220  0.0001220  0.0000244  5.37  0.010
Error   11  0.0000500  0.0000500  0.0000045
Total   16  0.0001720


S = 0.00213137   R-Sq = 70.95%   R-Sq(adj) = 57.74%

Unusual Observations for Data

Obs      Data       Fit    SE Fit  Residual  St Resid
  9  0.010522  0.006479  0.001231  0.004043      2.32 R

R denotes an observation with a large standardized residual.
Tukey 99.0% Simultaneous Confidence Intervals
Response Variable Data
All Pairwise Comparisons among Levels of Code T
Code T =  0  subtracted from:
Code T      Lower    Center    Upper  ----+---------+---------+---------+--
 2       0.000770  0.008461  0.01615               (------*-------)
 4      -0.003635  0.004964  0.01356           (--------*--------)
 8      -0.003260  0.004431  0.01212            (------*-------)
24      -0.005047  0.002643  0.01033          (-------*------)
48      -0.005264  0.002427  0.01012          (------*-------)
                                       ----+---------+---------+---------+--
                                        -0.010     0.000     0.010     0.020
Code T =  2  subtracted from:
Code T      Lower     Center     Upper  ----+---------+---------+---------+--
 4      -0.01210  -0.003497  0.005102    (--------*-------)
 8      -0.01172  -0.004030  0.003661    (-------*-------)
24      -0.01351  -0.005817  0.001874  (-------*-------)
48      -0.01372  -0.006034  0.001657  (-------*-------)
                                       ----+---------+---------+---------+--
                                        -0.010     0.000     0.010     0.020
Code T =  4  subtracted from:
Code T      Lower     Center     Upper  ----+---------+---------+---------+--
 8      -0.00913  -0.000533  0.008066       (-------*--------)
24      -0.01092  -0.002321  0.006278     (--------*-------)
48      -0.01114  -0.002537  0.006062     (-------*--------)
                                       ----+---------+---------+---------+--
                                        -0.010     0.000     0.010     0.020
```

```
Code T =  8  subtracted from:
Code T      Lower     Center     Upper  ----+---------+---------+---------+--
24      -0.009478  -0.001787  0.005903       (------*-------)
48      -0.009695  -0.002004  0.005687      (-------*-------)
                                        ----+---------+---------+---------+--
                                         -0.010     0.000     0.010     0.020
Code T = 24  subtracted from:
Code T      Lower     Center     Upper  ----+---------+---------+---------+--
48      -0.007907  -0.000216  0.007475       (-------*------)
                                        ----+---------+---------+---------+--
                                         -0.010     0.000     0.010     0.020
```

General Linear Model: Data versus Code T

```
Factor  Type   Levels  Values
Code T  fixed       6  0, 2, 4, 8, 24, 48

Analysis of Variance for Data, using Adjusted SS for Tests
Source  DF     Seq SS     Adj SS     Adj MS     F      P
Code T   5  0.0001220  0.0001220  0.0000244  5.37  0.010
Error   11  0.0000500  0.0000500  0.0000045
Total   16  0.0001720
S = 0.00213137   R-Sq = 70.95%   R-Sq(adj) = 57.74%
Unusual Observations for Data
Obs      Data       Fit    SE Fit  Residual  St Resid
  9  0.010522  0.006479  0.001231  0.004043      2.32 R

R denotes an observation with a large standardized residual.
Tukey 99.9% Simultaneous Confidence Intervals
Response Variable Data
All Pairwise Comparisons among Levels of Code T
Code T =  0  subtracted from:
Code T      Lower    Center    Upper  ------+---------+---------+---------+
 2      -0.001913  0.008461  0.01883               (---------*----------)
 4      -0.006634  0.004964  0.01656           (-----------*-----------)
 8      -0.005943  0.004431  0.01480            (---------*----------)
24      -0.007730  0.002643  0.01302          (----------*---------)
48      -0.007946  0.002427  0.01280          (---------*----------)
                                       ------+---------+---------+---------+
                                          -0.010     0.000     0.010     0.020
Code T =  2  subtracted from:
Code T      Lower     Center     Upper  ------+---------+---------+---------+
 4      -0.01509  -0.003497  0.008101   (-----------*----------)
 8      -0.01440  -0.004030  0.006344    (---------*---------)
24      -0.01619  -0.005817  0.004556  (---------*----------)
48      -0.01641  -0.006034  0.004340  (---------*---------)
                                       ------+---------+---------+---------+
                                          -0.010     0.000     0.010     0.020
Code T =  4  subtracted from:
Code T      Lower     Center     Upper  ------+---------+---------+---------+
 8      -0.01213  -0.000533  0.011065     (----------*-----------)
24      -0.01392  -0.002321  0.009277   (-----------*----------)
48      -0.01413  -0.002537  0.009061   (-----------*----------)
                                       ------+---------+---------+---------+
                                          -0.010     0.000     0.010     0.020
Code T =  8  subtracted from:
Code T      Lower     Center     Upper  ------+---------+---------+---------+
24      -0.01216  -0.001787  0.008586     (---------*----------)
48      -0.01238  -0.002004  0.008370     (---------*---------)
                                       ------+---------+---------+---------+
                                          -0.010     0.000     0.010     0.020
Code T = 24  subtracted from:
Code T      Lower     Center    Upper  ------+---------+---------+---------+
48      -0.01059  -0.000216  0.01016      (----------*---------)
                                       ------+---------+---------+---------+
                                          -0.010     0.000     0.010     0.020
```

Appendix 3 - Analysis of Chi-Square Test for *Bar* Gene Selection

Table 1. *Bar* Gene Selection in 10 Seeds Stocks of T141 Transgenic Lines

Line	F	Screen	B^S	B^R	Total	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
						16	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1	0
					Sensitive	86.0	80.6	75.3	69.9	64.5	59.1	53.8	48.4	43.0	37.6	32.3	26.9	21.5	16.1	10.8	5.4	0.0
					Resistant	0.0	5.4	10.8	16.1	21.5	26.9	32.3	37.6	43.0	48.4	53.8	59.1	64.5	69.9	75.3	80.6	86.0
141 49 HS C	**T3**	**BASTA**	6	80	86	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.005	0.121	0.781	0.000
						80.0	75.0	70.0	65.0	60.0	55.0	50.0	45.0	40.0	35.0	30.0	25.0	20.0	15.0	10.0	5.0	0.0
						0.0	5.0	10.0	15.0	20.0	25.0	30.0	35.0	40.0	45.0	50.0	55.0	60.0	65.0	70.0	75.0	80.0
141 49 HS D	**T3**	**BASTA**	0	80	80	#DIV/0!	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.021	0.975
					0	95.0	89.1	83.1	77.2	71.3	65.3	59.4	53.4	47.5	41.6	35.6	29.7	23.8	17.8	11.9	5.9	0.0
					0	0.0	5.9	11.9	17.8	23.8	29.7	35.6	41.6	47.5	53.4	59.4	65.3	71.3	77.2	83.1	89.1	95.0
141 49 HS E	**T3**	**BASTA**	5	90	95	#DIV/0!	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.033	0.691	0.000
					0	48.0	45.0	42.0	39.0	36.0	33.0	30.0	27.0	24.0	21.0	18.0	15.0	12.0	9.0	6.0	3.0	0.0
					0	0.0	3.0	6.0	9.0	12.0	15.0	18.0	21.0	24.0	27.0	30.0	33.0	36.0	39.0	42.0	45.0	48.0
141 49 HS B^R B	**T3**	**BASTA**	3	45	48	#DIV/0!	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.003	0.027	0.190	1.000	0.000
					0	95.0	89.1	83.1	77.2	71.3	65.3	59.4	53.4	47.5	41.6	35.6	29.7	23.8	17.8	11.9	5.9	0.0
					0	0.0	5.9	11.9	17.8	23.8	29.7	35.6	41.6	47.5	53.4	59.4	65.3	71.3	77.2	83.1	89.1	95.0
141 49 HS B^R C	**T3**	**BASTA**	5	90	95	#DIV/0!	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.033	0.691	0.000
					0	97.0	90.9	84.9	78.8	72.8	66.7	60.6	54.6	48.5	42.4	36.4	30.3	24.3	18.2	12.1	6.1	0.0
					0	0.0	6.1	12.1	18.2	24.3	30.3	36.4	42.4	48.5	54.6	60.6	66.7	72.8	78.8	84.9	90.9	97.0
141 49 HS B^R D	**T3**	**BASTA**	7	90	97	#DIV/0!	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.004	0.116	0.694	0.000
					0	87.0	81.6	76.1	70.7	65.3	59.8	54.4	48.9	43.5	38.1	32.6	27.2	21.8	16.3	10.9	5.4	0.0
					0	0.0	5.4	10.9	16.3	21.8	27.2	32.6	38.1	43.5	48.9	54.4	59.8	65.3	70.7	76.1	81.6	87.0
141 49 HS B^R E	**T3**	**BASTA**	7	80	87	#DIV/0!	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.011	0.209	0.489	0.000

					0	78.0	73.1	68.3	63.4	58.5	53.6	48.8	43.9	39.0	34.1	29.3	24.4	19.5	14.6	9.8	4.9	0.0
					0	0.0	4.9	9.8	14.6	19.5	24.4	29.3	34.1	39.0	43.9	48.8	53.6	58.5	63.4	68.3	73.1	78.0
141 49 HS B^{R} F	**T3**	**BASTA**	8	70	78	#DIV/0!	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.003	0.055	0.549	0.144	0.000
					0	100.0	93.8	87.5	81.3	75.0	68.8	62.5	56.3	50.0	43.8	37.5	31.3	25.0	18.8	12.5	6.3	0.0
					0	0.0	6.3	12.5	18.8	25.0	31.3	37.5	43.8	50.0	56.3	62.5	68.8	75.0	81.3	87.5	93.8	100.0
141 49 HS B^{R} H	**T3**	**BASTA**	3	97	100	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.004	0.179	0.000
					0	84.0	78.8	73.5	68.3	63.0	57.8	52.5	47.3	42.0	36.8	31.5	26.3	21.0	15.8	10.5	5.3	0.0
					0	0.0	5.3	10.5	15.8	21.0	26.3	31.5	36.8	42.0	47.3	52.5	57.8	63.0	68.3	73.5	78.8	84.0
141 49 HS B^{R} I	**T3**	**BASTA**	4	80	84	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.032	0.573	0.000

Table 2. *Bar* Gene Selection in 18 Seeds Stocks of T127 Transgenic Lines

Line	F	Screen	B^{S}	B^{R}	Total	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
						16	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1	0
					0	100.0	93.8	87.5	81.3	75.0	68.8	62.5	56.3	50.0	43.8	37.5	31.3	25.0	18.8	12.5	6.3	0.0
					0	0.0	6.3	12.5	18.8	25.0	31.3	37.5	43.8	50.0	56.3	62.5	68.8	75.0	81.3	87.5	93.8	100.0
127 61 C	**T3**	**BASTA**	5	95	100	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.023	0.606	0.000
					0	64.0	60.0	56.0	52.0	48.0	44.0	40.0	36.0	32.0	28.0	24.0	20.0	16.0	12.0	8.0	4.0	0.0
					0	0.0	4.0	8.0	12.0	16.0	20.0	24.0	28.0	32.0	36.0	40.0	44.0	48.0	52.0	56.0	60.0	64.0
127 61 E	**T3**	**BASTA**	11	53	64	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.015	0.149	0.749	0.257	0.000	0.000
					0	80.0	75.0	70.0	65.0	60.0	55.0	50.0	45.0	40.0	35.0	30.0	25.0	20.0	15.0	10.0	5.0	0.0
					0	0.0	5.0	10.0	15.0	20.0	25.0	30.0	35.0	40.0	45.0	50.0	55.0	60.0	65.0	70.0	75.0	80.0
127 61 G	**T3**	**BASTA**	0	80	80	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.021	0.975
					0	56.0	52.5	49.0	45.5	42.0	38.5	35.0	31.5	28.0	24.5	21.0	17.5	14.0	10.5	7.0	3.5	0.0
					0	0.0	3.5	7.0	10.5	14.0	17.5	21.0	24.5	28.0	31.5	35.0	38.5	42.0	45.5	49.0	52.5	56.0
127 61 H 4	**T3**	**BASTA**	6	50	56	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.014	0.123	0.686	0.168	0.000

					0	63.0	59.1	55.1	51.2	47.3	43.3	39.4	35.4	31.5	27.6	23.6	19.7	15.8	11.8	7.9	3.9	0.0
					0	0.0	3.9	7.9	11.8	15.8	19.7	23.6	27.6	31.5	35.4	39.4	43.3	47.3	51.2	55.1	59.1	63.0
127 61 I	**T3**	**BASTA**	11	52	63	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.018	0.167	0.793	0.234	0.000	0.000
					0	98.0	91.9	85.8	79.6	73.5	67.4	61.3	55.1	49.0	42.9	36.8	30.6	24.5	18.4	12.3	6.1	0.0
					0	0.0	6.1	12.3	18.4	24.5	30.6	36.8	42.9	49.0	55.1	61.3	67.4	73.5	79.6	85.8	91.9	98.0
127 61 K	**T3**	**BASTA**	18	80	98	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.006	0.129	0.923	0.079	0.000	0.000
					0	69.0	64.7	60.4	56.1	51.8	47.4	43.1	38.8	34.5	30.2	25.9	21.6	17.3	12.9	8.6	4.3	0.0
					0	0.0	4.3	8.6	12.9	17.3	21.6	25.9	30.2	34.5	38.8	43.1	47.4	51.8	56.1	60.4	64.7	69.0
127 61 L	**T3**	**BASTA**	9	60	69	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.022	0.225	0.891	0.020	0.000
					0	87.0	81.6	76.1	70.7	65.3	59.8	54.4	48.9	43.5	38.1	32.6	27.2	21.8	16.3	10.9	5.4	0.0
					0	0.0	5.4	10.9	16.3	21.8	27.2	32.6	38.1	43.5	48.9	54.4	59.8	65.3	70.7	76.1	81.6	87.0
127 61 M	**T3**	**BASTA**	7	80	87	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.011	0.209	0.489	0.000	
					0	76.0	71.3	66.5	61.8	57.0	52.3	47.5	42.8	38.0	33.3	28.5	23.8	19.0	14.3	9.5	4.8	0.0
					0	0.0	4.8	9.5	14.3	19.0	23.8	28.5	33.3	38.0	42.8	47.5	52.3	57.0	61.8	66.5	71.3	76.0
127 61 AS	**T3**	**BASTA**	6	70	76	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.015	0.225	0.554	0.000
					0	55.0	51.6	48.1	44.7	41.3	37.8	34.4	30.9	27.5	24.1	20.6	17.2	13.8	10.3	6.9	3.4	0.0
					0	0.0	3.4	6.9	10.3	13.8	17.2	20.6	24.1	27.5	30.9	34.4	37.8	41.3	44.7	48.1	51.6	55.0
127 61 BR DA	**T3**	**BASTA**	19	36	55	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.022	0.169	0.651	0.598	0.102	0.003	0.000	0.000	0.000
					0	56.0	52.5	49.0	45.5	42.0	38.5	35.0	31.5	28.0	24.5	21.0	17.5	14.0	10.5	7.0	3.5	0.0
					0	0.0	3.5	7.0	10.5	14.0	17.5	21.0	24.5	28.0	31.5	35.0	38.5	42.0	45.5	49.0	52.5	56.0
127 61 BR DB	**T3**	**BASTA**	4	52	56	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.002	0.026	0.225	0.783	0.000	
					0	101.0	94.7	88.4	82.1	75.8	69.4	63.1	56.8	50.5	44.2	37.9	31.6	25.3	18.9	12.6	6.3	0.0
					0	0.0	6.3	12.6	18.9	25.3	31.6	37.9	44.2	50.5	56.8	63.1	69.4	75.8	82.1	88.4	94.7	101.0
127 61 BR DC	**T3**	**BASTA**	11	90	101	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.043	0.625	0.054	0.000	
					0	92.0	86.3	80.5	74.8	69.0	63.3	57.5	51.8	46.0	40.3	34.5	28.8	23.0	17.3	11.5	5.8	0.0
					0	0.0	5.8	11.5	17.3	23.0	28.8	34.5	40.3	46.0	51.8	57.5	63.3	69.0	74.8	80.5	86.3	92.0
127 61 BR DD	**T3**	**BASTA**	12	80	92	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.008	0.161	0.875	0.007	0.000	
					0	100.0	93.8	87.5	81.3	75.0	68.8	62.5	56.3	50.0	43.8	37.5	31.3	25.0	18.8	12.5	6.3	0.0

					0	0.0	6.3	12.5	18.8	25.0	31.3	37.5	43.8	50.0	56.3	62.5	68.8	75.0	81.3	87.5	93.8	100.0
127 61 BR DE	**T3**	**BASTA**	20	80	100	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.015	0.248	0.749	0.023	0.000	0.000
					0	68.0	63.8	59.5	55.3	51.0	46.8	42.5	38.3	34.0	29.8	25.5	21.3	17.0	12.8	8.5	4.3	0.0
					0	0.0	4.3	8.5	12.8	17.0	21.3	25.5	29.8	34.0	38.3	42.5	46.8	51.0	55.3	59.5	63.8	68.0
127 61 BR DF	**T3**	**BASTA**	8	60	68	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.012	0.140	0.855	0.060	0.000
					0	99.0	92.8	86.6	80.4	74.3	68.1	61.9	55.7	49.5	43.3	37.1	30.9	24.8	18.6	12.4	6.2	0.0
					0	0.0	6.2	12.4	18.6	24.8	30.9	37.1	43.3	49.5	55.7	61.9	68.1	74.3	80.4	86.6	92.8	99.0
127 61 BR DG	**T3**	**BASTA**	9	90	99	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.014	0.305	0.243	0.000
					0	75.0	70.3	65.6	60.9	56.3	51.6	46.9	42.2	37.5	32.8	28.1	23.4	18.8	14.1	9.4	4.7	0.0
					0	0.0	4.7	9.4	14.1	18.8	23.4	28.1	32.8	37.5	42.2	46.9	51.6	56.3	60.9	65.6	70.3	75.0
127 61 BR DH	**T3**	**BASTA**	15	60	75	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.002	0.036	0.317	0.782	0.050	0.000	0.000
					0	51.0	47.8	44.6	41.4	38.3	35.1	31.9	28.7	25.5	22.3	19.1	15.9	12.8	9.6	6.4	3.2	0.0
					0	0.0	3.2	6.4	9.6	12.8	15.9	19.1	22.3	25.5	28.7	31.9	35.1	38.3	41.4	44.6	47.8	51.0
127 61 BR DI	**T3**	**BASTA**	6	45	51	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.003	0.029	0.201	0.874	0.104	0.000

Appendix 4 - Relationship of R2R3 MYB Family in *Arabidopsis thaliana*

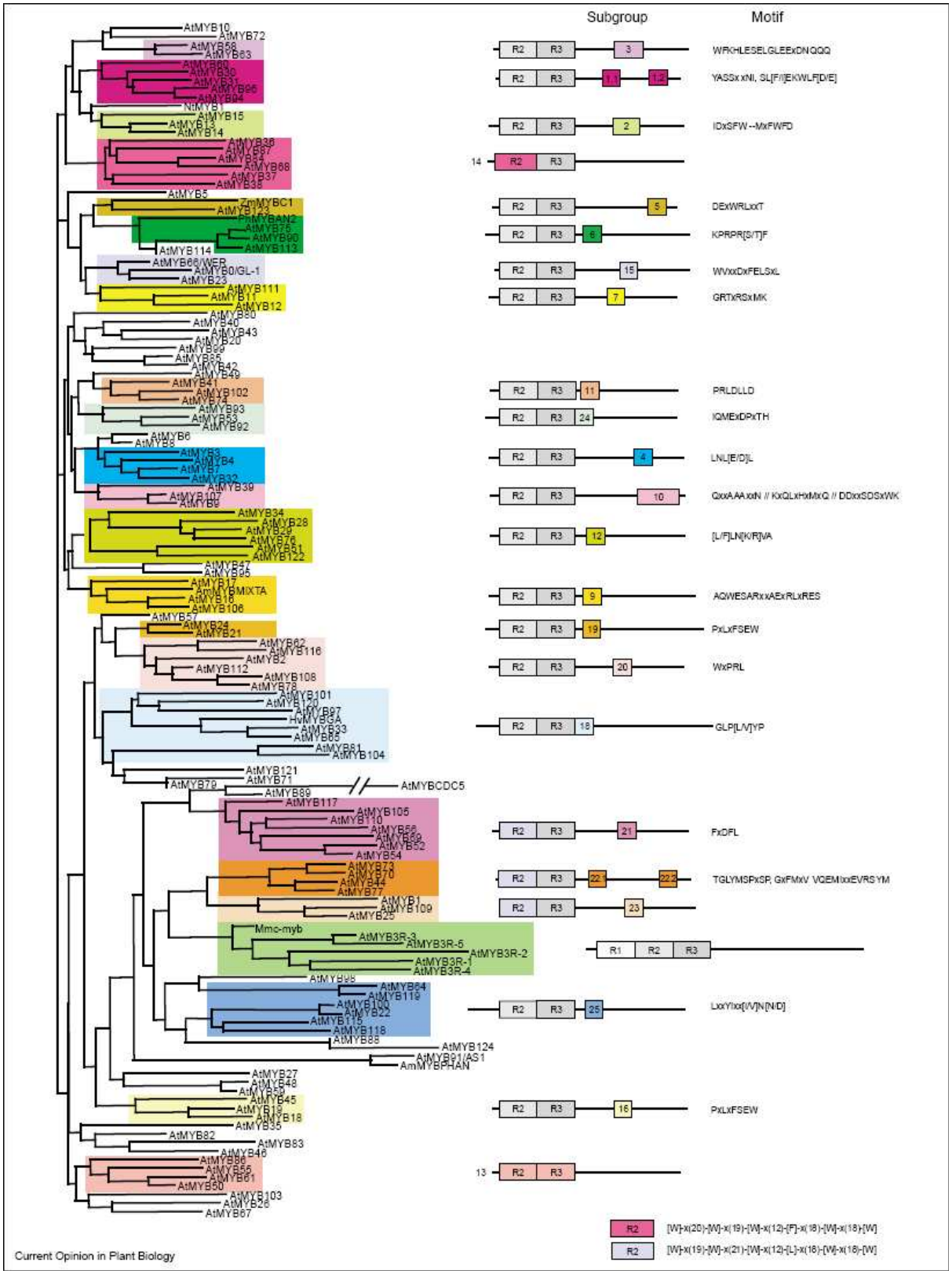


Figure from Stracke *et al.* (2001)

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