# Role of RAE1 in *Arabidopsis thaliana* heat tolerance

by

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## Abstract

Plants need to adapt to environmental conditions such as heat stress. Studies in *Arabidopsis thaliana* have implicated Damaged DNA Binding protein 1 (DDB1) in plant heat tolerance. DDB1 interacts with DDB1-binding WD40 (DWD) proteins, and six heat-induced DWD genes were identified as candidate genes. Analysis of mutant alleles of these candidates revealed that an *RNA EXPORT 1* (*rae1*) mutant exhibited heat sensitivity. We examined the effect of this allele on the *RAE1* transcript and found that *RAE1* was still expressed in control conditions, however heat stress resulted in failure of intron 1 splicing in the mutant. In stronger *rae1* alleles we were unable to identify homozygotes due to female gametophytic lethality. Nonetheless, heterozygotes of one of these stronger alleles also exhibit increased heat sensitivity. In addition, RAE1 overexpression resulted in heat sensitivity. Thus, both loss and gain of function alleles of *RAE1* result in decreased heat tolerance in Arabidopsis.

## Acknowledgments

After my effort to get to where I am now, a student has a master's degree from the University of Manitoba, I express with all my heart my gratitude and thanks to my supervisor Dr. Dana Schroeder for accepting me, and her support and her guidance through my years of study. I faced many difficulties and problems but ultimately got to the right answer with her patience. I appreciate her role in inspiring and pushing me to work, think, and research better. Many thanks to my committee members Dr. Jake Stout and Dr. Rob Duncan for their help and advice to get my work and research on the right track. Also, I would like to thank Charlene Ibbetson, Amy Clark and everyone who helped me here, including my previous and current lab members.

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## List of Abbreviations

AS Alternative splicing

ATCSA *Arabidopsis thaliana* COCKAYNE SYNDROME TYPE A

Col-0 Colombia-0

CUL Cullin

DDB1 Damaged DNA Binding protein 1

DWD DDB1-binding WD40 protein

DWH DWD REGULATED by HEAT

HSFs Heat shock transcription factors

HSP Heat shock proteins

IR Intron retention

NPC Nuclear Pore Complex

NUP Nucleoporin

PEX7 PEROXIN7

RAE1 RNA EXPORT FACTOR 1

SAR3 SUPPRESSOR OF AUXIN RESISTANCE 3

Ub Ubiquitin

ULCS1 Ubiquitin Ligase Complex Subunit 1

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# 1. LITERATURE REVIEW

## 1.1 Introduction

Abiotic stresses include various environmental effects such as high and low temperature, drought, salinity, chemical toxicity and oxidative stress, which are dangerous factors that affect plant germination, growth and development (Wang et al., 2003). These stresses force plants to adapt to these changes. Under stress, plants utilize a variety of strategies, including their ability to degrade proteins via the ubiquitination pathway, which is one of the most complex regulation mechanisms in eukaryotes (Jackson & Xiong, 2009). In this thesis, I will focus on high temperature response in plants. I demonstrate that a component of the Nuclear Pore Complex (NPC), RNA export 1(RAE1), is involved in Arabidopsis heat tolerance. In addition, I show that RAE1 is also required for development of the female gametophyte.

## 1.2 Plant response to high temperature

Year after year, global temperatures rise due to greenhouse gases, including carbon dioxide (CO2), methane (CH4) and nitrous oxide (N2O), which are disseminated by human activity and absorb thermal infrared radiation (Ma, 1998). These gases remain locked in the atmosphere changing the climate and heating up the earth's surface (Center for Sustainable Systems, 2017). Many studies have demonstrated the severe influence of high temperature on plant growth, pollination, photosynthesis, and production (Hatfield & Prueger, 2015).

There are many cells that die after being exposed to high temperature due to toxic lipid accumulation, drought, or lack of nutrients (Distéfano et al., 2017; Walbot, 2011). Heat affects

protein stability as well as cytoskeleton and RNA structure (Walbot, 2011). In addition, high heat reduces absorption of nutrients and the levels of proteins in the roots, directly affecting plant mass (Giri et al., 2017). Despite high temperatures reaching up to 50°C, plants can adapt and perform all light and chemical processes in these difficult conditions (Walbot, 2011). Plants regulate thermal stress responses through genetic and epigenetic mechanisms using physiological and molecular signaling pathways, including responsive genes, kinases proteins, heat shock proteins and catalases (Qu et al., 2013). For example, during heat stress, chromatin can stabilize the interaction of the DNA and proteins for transcription and translation; many proteins act to stabilize ribosomes to allow weak interactions to continue their function accurately and efficiently. These stabilization factors work together to regulate the cellular procedures (Walbot, 2011). In plants, to respond to an abiotic stress, genes and biochemical mechanisms work to regulate the gene expression associated with a particular stress (Wang et al., 2003).

### 1.2.1 Plant transcriptional response to heat

For their response to high temperature, plants depend mainly on the regulation of signaling pathways that in turn activate gene transcription. Many transcription factors exist in the plant genome, some of them act differently to resist various abiotic stresses (Wang et al., 2003). Through the use of transcriptomics and proteomics, genes and proteins involved in thermal stress response were identified (Xu et al., 2011). Heat responsive gene expression is often regulated by heat shock transcription factors (HSFs). HSFs under normal heat are latent, but activate under heat stress condition (Wu, 1995). Plants have at least 21 HSFs; each one has a role in regulation of genes. In heat stress, they work together to generate stress response (Asthir, 2015). In rice, Spl7, which is a type of HSF, participates in the control of cell death caused by high temperature.

HSFs stimulate transcription of functional genes like heat shock proteins (HSPs) (Qu et al., 2013). HSPs function to activate and control the heat stress response, repairing heat stress damage in signal transduction, transcriptional control, transfer of ions, regulating cellular oxidation, and repairing damaged proteins and membranes (Asthir, 2015).

### 1.2.2 Effect of heat on alternative splicing

Alternative splicing (AS) is another mechanism by which plants respond to heat stress (Keller et al., 2017). AS occurs in pre-mRNA via the action of the spliceosome, which removes introns from splicing sites to create the mature transcript. The spliceosome contains five small nuclear ribonucleoproteins (snRNPs) and many other proteins which when localized in introns to either enhance or inhibit splicing (Chen & Moore, 2015). AS allows the creation of many different mature mRNA from the same gene (Mao et al., 2014) possibly encoding proteins which function differently (Keller et al., 2017). Thus, AS is employed as a mechanism to increase the functions and the organizational capacities of the genome (Staiger, 2015). Intron retention (IR) is the most common event from AS, which occurs in *Arabidopsis thaliana* at a rate of 40 % (Liu et al., 2014). Splicing sites are determined by spliceosome components, RNA-binding proteins, serine/arginine-rich proteins (SR), and heterogeneous nuclear ribonucleoproteins (hnRNPs), which link with cis-regulatory elements that exist in exons and introns. Abiotic stresses alter AS (Laloum et al., 2018); in grape, heat stress leads to AS of more than 1000 genes (Jiang et al., 2017). In addition, heat stress induced AS activates HSFs, for example, by resulting in an HSFA2 isoform which activates its own transcription (Liu et al., 2013). These HSFs controls HSPs in plants to activate gene transcription that are responsible for responses under altered conditions (Keller et al., 2017).

### 1.2.3 Effect of heat on protein degradation

Proteins are often exposed to cellular damage under heat stress (McClellan et al., 2005), causing defects in the folding of proteins, resulting in toxic and insoluble aggregates in the cells. Cells need to either fix the damage or degrade and replace the damaged macromolecules (Flick & Kaiser, 2012). It is essential for plants to dispose of these aggregates in order to maintain protein quality control (Gil et al., 2017). This mechanism acts via refolding and degradation of proteins via the ubiquitin-proteasome pathway. This operation is initiated by recognition by molecular chaperones like HSP70 and HSP90 of the hydrophobic parts of misfolded proteins. The ubiquitin proteasome pathway then eliminates misfolded proteins in insoluble protein aggregates (McClellan et al., 2005). HSP90 is considered one of the main molecular chaperones, controlling the cell cycle as well as signaling pathways under stress conditions, response to environmental stresses, and refolding proteins upon exposure to high heat (Dickey et al., 2007). Notably, the protein concentration of HSP90 increases following heat (Meiri & Breiman, 2009).

HSP90 acts as a molecular chaperone to stabilize the ZEITLUPE (ZTL) protein in Arabidopsis (Kim et al., 2011). ZTL interacts with a ubiquitin ligase to activate proteasomal degradation of TIMING OF CAB EXPRESSION 1 (TOC1) and PSEUDORESPONSE REGULATOR 5 (PRR5) that are components of the circadian clock (Más et al., 2003). ZTL overexpression assists polyubiquitination elevation following high heat, which works to recognize misfolded proteins and its aggregates, then eliminate them through the ubiquitin proteasome pathway (McClellan et al., 2005). Accordingly, ZTL overexpression is important for the plant to remove insoluble protein aggregates. In addition, the interactions of ZTL with HSP90 protein regulate polyubiquitination after exposure to high heat (Kim et al., 2011). Thus

protein quality control, including ZTL and HSP90, is important for heat stress tolerance (Gil et al., 2017).

## 1.3 The ubiquitin proteasome pathway

Ubiquitination targets specific proteins for degradation using covalent attachment of ubiquitin. The pathway functions through three enzymes: the E1 ubiquitin activating enzyme, the E2 ubiquitin-conjugating enzyme and the E3 ubiquitin ligase (Pickart & Eddins, 2004). The E1 enzyme activates the C-terminal of Ub via ATP-dependent thioester formation. The E2 enzyme then transfers ubiquitin to the E3 enzyme, which is responsible for determining the protein substrate to bind with ubiquitin (Jackson & Xiong, 2009). This process is reiterated to attach multi-ubiquitin to the substrate, an important step for degradation via the 26S proteasome, whereupon the protein is reduced to peptides and the ubiquitin recycled (Figure 1.1) (Moon et al., 2004). There are two main types of E3 ubiquitin ligases, homologous to E6-AP C-terminus (HECT) and the RING domain. The RING domain can form complexes with cullins (Petroski & Deshaies, 2005). Each complex utilizes a particular adaptor to form multi-subunit cullin-RING E3 ligase complex (CRLs) (Ahn et al., 2011). Four cullin-like proteins exist in *Arabidopsis thaliana,* cullin 1, cullin 3a, cullin 3b, and cullin 4. The RING finger protein binds to the E2 enzyme and the C-terminal of the cullin. The N-terminal of the cullin interacts with an adapter protein(s) which, in turn, interacts with the substrate to be targeted for protein degradation (Bosu & Kipreos, 2008). Multiple members of substrate receptors families can interact with one cullin, resulting in many distinct CRL complexes (Jackson & Xiong, 2009).

Arabidopsis cullin family (CUL1-4) RING E3 ligase complexes contain the RING finger protein REGULATOR OF CULLINS1 (ROC1)/RING-BOX1 (RBX1). S-phase kinase-

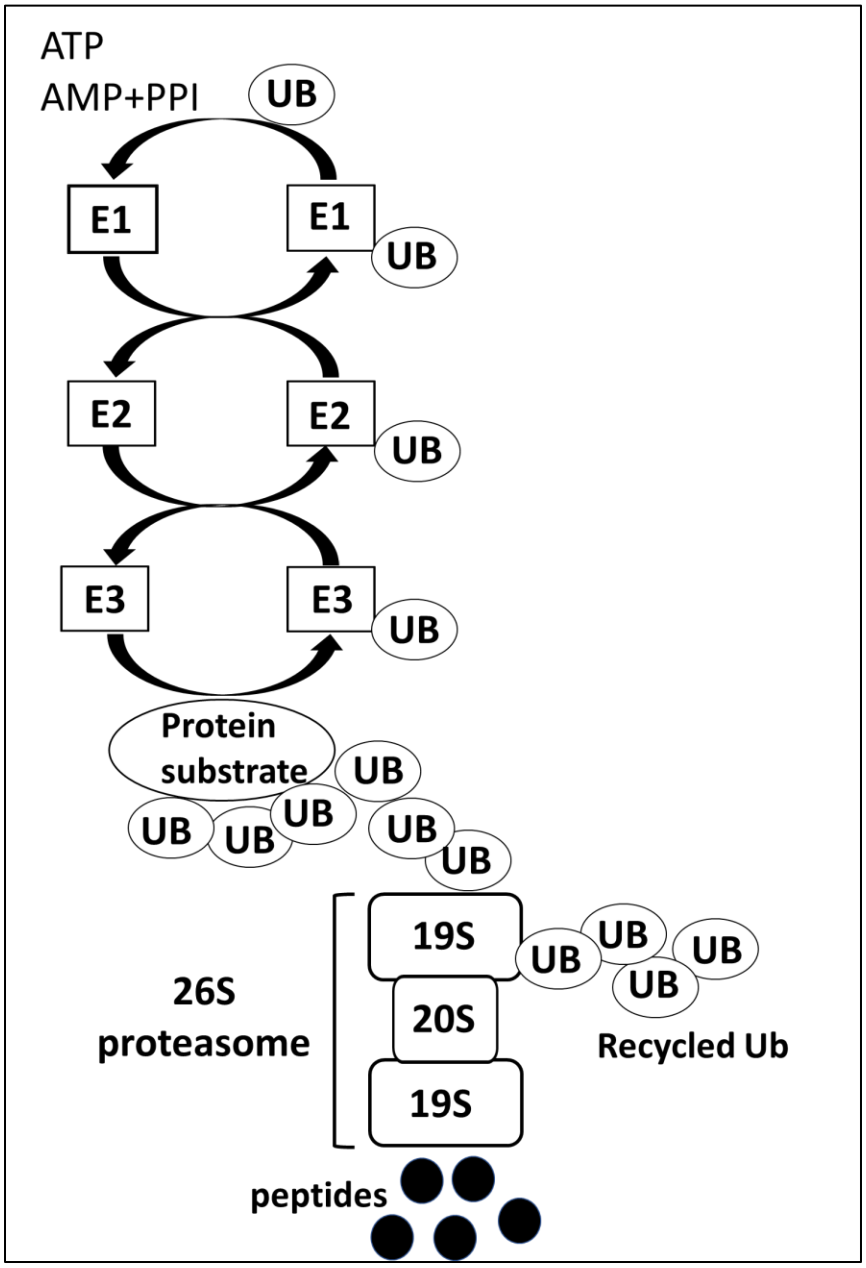


Figure 1.1 The ubiquitin proteasome pathway. Ubiquitin (UB) is activated by the E1 ubiquitin activating enzyme, then transferred to the E2 ubiquitin conjugating enzyme, then to the E3 ubiquitin ligase, which adds ubiquitin to the protein substrate. The ubiquitin tag targets the protein to the 26S proteasome (composed of 20S and 19S subunits), which degrades the protein, while ubiquitin is recycled. See text for details and references.

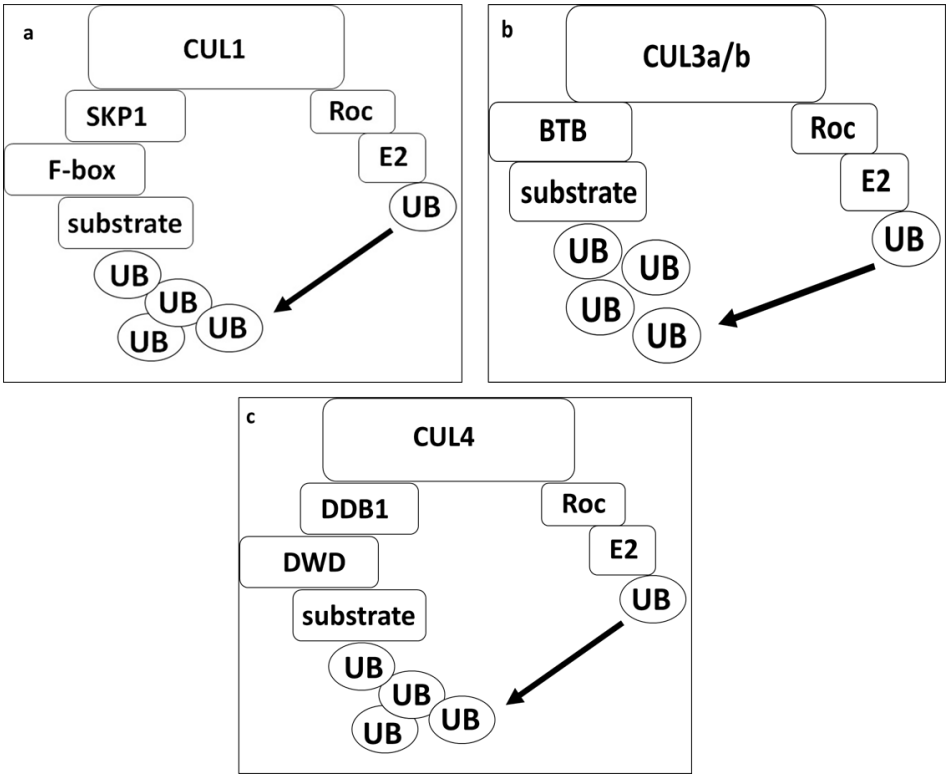


Figure 1.2 Cullin-based E3 ubiquitin ligases. a) Cullin 1 interacts with SKP1, which acts as an adaptor for F-box proteins. b) BTB domain proteins interact with Cullin 3a/b to form a receptor that binds directly the substrate. c) Cullin 4 interacts with DDB1, which acts an adaptor for DWD proteins. The C-terminus of the cullins links with Roc proteins.

associated protein 1 (SKP1) and Bric a brac, Tramtrack and Broad complex (BTB) domain proteins bind with CUL1 and CUL3, respectively. SCF complexes contain Cullin1, SKP1 and an F-box protein (Figure 1.2a) (Moon et al., 2004). SKP1 acts as a link between CUL1 and the F-box protein (Zheng et al., 2002) and CUL1 acts as the scaffold. The Arabidopsis genome encodes approximately 700 F-box proteins, which form different SCF complexes to recognize multiple substrate proteins, which collectively regulate many various biological processes (Hellmann & Estelle, 2002). The N-terminus and C-terminus of F-box proteins bind with SKP1 and substrate, respectively (Hua & Vierstra, 2011). CUL3-E3 ubiquitin ligase complexes contain

BTB domain proteins (Figure 1.2b) that function as substrate specific adaptors and in transcriptional regulation (Stogios et al., 2005).

### 1.3.1 Arabidopsis CUL4 E3 ligases

CUL4-based E3 ubiquitin ligases are a large family that consist of three core components: CULLIN4 (CUL4), a RING finger protein ROC1/ RBX1, and UV-DAMAGED DNA BINDING PROTEIN1 (DDB1) (Zhang et al., 2008) (Figure 1.2c). The N-terminal domain of CUL4 binds the β-propeller B domain of the adapter protein DDB1 to assemble a substrate receptor complex (Lee & Kim, 2011). The RING finger protein ROC1/RBX1 links the C-terminal portion of CUL4 and the E2 enzyme to form a catalytic core, so CUL4 forms the basic structure to determine the position of the substrate relative to the E2 enzyme and facilitates the transport of ubiquitin (Zhang et al., 2008; Lee & Kim, 2011).

CUL4 interacts with DNA damage-binding protein 1 (DDB1), which acts as an adaptor protein (Li et al., 2006) to assemble the E3 ligase complex. This complex regulates cell proliferation, survival, DNA repair, and genomic integrity (Lee & Zhou, 2007). Arabidopsis DDB1 has two homologs: DDB1A and DDB1B. Despite that *ddb1b* null mutants are lethal and *ddb1a* mutants do not show obvious developmental phenotypes, studies have shown that DDB1 has a role in plant development and contributes to the response to abiotic stresses (Zhang et al., 2008; Lee & Kim, 2011). CUL4-DDB1 interacts with proteins containing WD40 repeats, called DDB1-CUL4 ASSOCIATED FACTOR (DCAF) proteins, or DDB1 binding WD40 (DWD) proteins or CUL4- and DDB1-associated WD40-repeat proteins. Eighty five WD40 proteins have been identified in Arabidopsis with one or two copies of a conserved 16–amino acid

DDB1-interacting motif, therefore possibly functioning as substrate receptors in different CUL4 E3 ubiquitin ligases (Zhang et al., 2008).

Studies have been shown that DDB1–CULLIN4 E3 ubiquitin ligase complexes participate in many abiotic responses (Biedermann & Hellmann, 2011). Constitutive photomorphogenic (COP) and De-etiolated (DET) proteins interact with DDB1 to form the COP10-DET1-DDB1 (CDD) complex (Schroeder et al., 2002; Yanagawa et al., 2004). *cop* and *det* mutants exhibit a similar phenotype to plants grown in light even when grown in the darkness (Chory et al., 1989). The CDD complex enhances ubiquitin chain activity and activates CUL4-DDB1-COP1-SUPPRESSOR OF PHTOCHROME A-105 (SPA) E3-ubiquitin ligases (COP1-SPA) (Fernando & Schroeder, 2016; Lau & Deng, 2012). Photomorphogenesis has been inhibited, thus, allowing hypocotyl elongation under complete darkness due to the COP1-SPA complex targeting ELONGATED HYPOCOTYL 5 (HY5) for proteasomal degradation, which is considered a negative regulator of hypocotyl elongation growth (Koornneef et al., 1980; Oyama et al.,1997). Thus, the CDD and COP1-SPA complexes are regulators of plant light response (Figure 1.3).

CUL4-DDB1 complexes are also involved in response to damaging UV rays. Nucleotide excision repair (NER) is a key pathway for DNA repair that removes the UV-induced photolesions. NER includes two sub-pathways: global genomic repair (GGR) and transcription coupled DNA repair (TCR). CUL4-DDB1 complexes with two DWD proteins, DDB2 and CSA, that are involved in GGR and TCR, respectively, thus maintaining genome integrity (Figure 1.3) (Groisman et al., 2003; Sarker et al., 2005). In Arabidopsis, *ddb2* mutants exhibit increased UV sensitivity (Koga et al., 2006). Under UV irradiation, DDB2 binds CUL4-DDB1A to regulate GGR-type DNA repair (Molinier et al., 2008). Arabidopsis CSA1A and B connect with the CUL4-DDB1A complex to regulate TCR, thus UV stress response (Zhang et al., 2010).

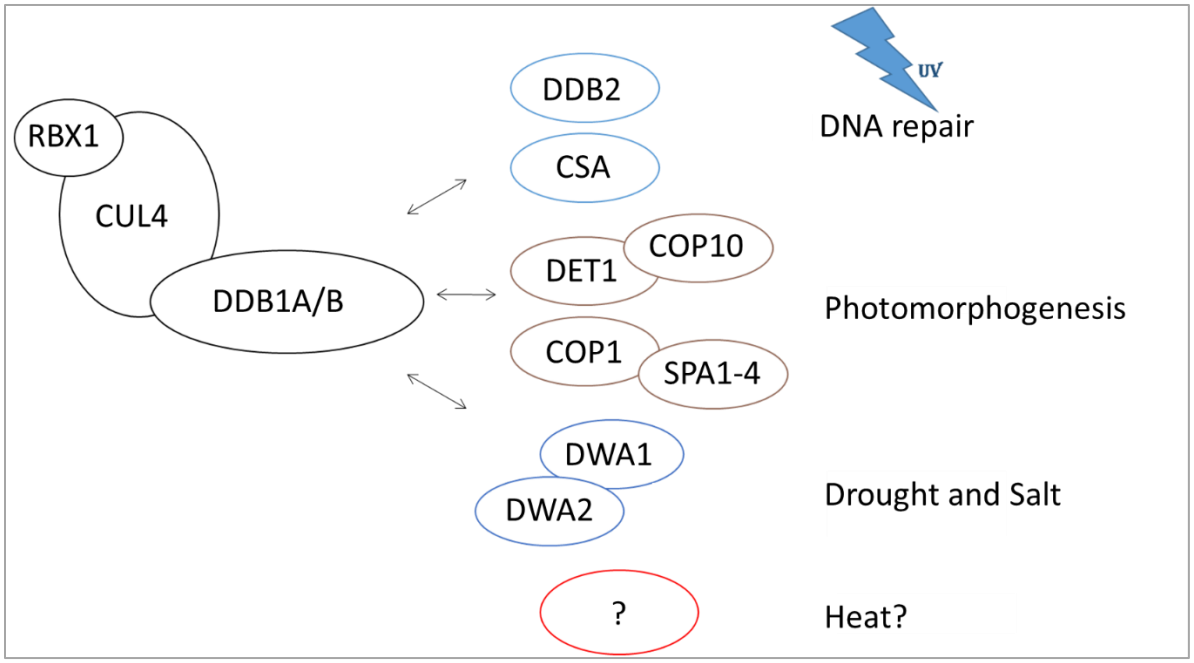

Figure 1.3 CUL4-DDB1 proteins complexes are involved in abiotic stresses responses. See text for details and references.

CUL4-DDB1 complexes are also involved in drought and salt stress response. Abscisic acid (ABA) is a key hormone in drought and salt stress response. DWD hypersensitive to ABA1/2 (DWA1/2) proteins regulate levels of ABA signaling component Abscisic Acid Insensitive 5 (ABI5). DWA1/2 are components of CUL4 E3 ligase complexes that target ABI5 for degradation (Lyzenga & Stone, 2012). Therefore, DWA1/2 targets ABI5 for proteasomal degradation and function as negative regulators of ABA signaling (Lee et al., 2010) (Figure 1.3).

Previous studies have been performed on Arabidopsis *ddb1a* and weak *ddb1b* mutants under many abiotic stresses using *ddb1a* and *ddb1b* single mutants as well as *ddb1a ddb1b-2/+* and *ddb1b-2 ddb1a/+* heterozygotes (*ddb1a ddb1b* double mutants are embryonic lethal). One of these studies was under heat stress, to examine if *ddb1a* and *ddb1b* mutants are heat sensitive. The results showed a reduction in the hypocotyl length of *ddb1b-2*, *ddb1b-2 ddb1a/+*, *ddb1a*, and *ddb1a ddb1b-2/+* heterozygotes compared with *Col-0* wildtype (Figure 1.4), which indicates that *DDB1A* and *DDB1B* have roles in Arabidopsis heat tolerance (Ganpudi & Schroeder, 2013).

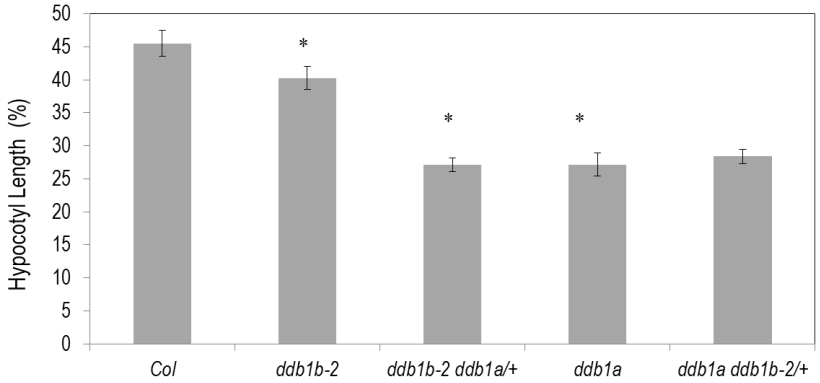


Figure 1.4 *ddb1a* and *ddb1b* mutant seedlings are heat sensitive. Hypocotyl length is relative to unheated control. Error bars indicate SE and $*P \leq 0.05$ of single mutants relative to *Col*, or *ddb1a ddb1b-2/+* and *ddb1b-2 ddb1a/+* relative to *ddb1a* and *ddb1b-2*, respectively (Ganpudi & Schroeder, 2013).

### 1.3.2 Heat-induced DWD genes

To determine the regulation of genes under abiotic conditions, the level of gene expression has been compared under a variety of environmental conditions (Kilian et al., 2007). Eighty-five *Arabidopsis thaliana* proteins containing the conserved 16-amino acid DWD motif have been identified (Lee et al., 2008). The effect of heat on the gene expression level of these 85 genes was examined using data from Kilian et al. (2007) accessed via AtGenExpress. Analysis of the 85 genes revealed that six candidate genes, *DWD REGULATED by HEAT 1* (*DWH1/At2g47410*), *DWH2 (At5g66240*), *DWH3 (At5g54520*), Arabidopsis *PEROXIN 7* (*PEX7/At1g29260*), *Arabidopsis thaliana* COCKAYNE SYNDROME TYPE A protein (*AT-CSA*/*At1g27840),* and RNA EXPORT FACTOR 1 (*RAE1*/*At1g80670),* were upregulated when exposed to heat treatment. While *DWH1* and *DWH3* are not yet characterized, previous studies have examined the role of the other four proteins in plants.

#### 1.3.2.1 DWH2/ULCS1

DWD regulated by heat 2 (DWH2), also known as *Arabidopsis thaliana* Ubiquitin Ligase Complex Subunit 1 (ULCS1), localizes to the nucleus and cytoplasm. DWH2 is a subunit of a CUL4-RING E3 ubiquitin ligase complex and interacts with DDB1a in plants. ULCS1/DWH2 contains 4 WD40 repeats. The gene expression appears in two phases of reproductive development: during pollen maturity and in the developing embryonic and endosperm tissues (Beris et al., 2016). *ULCS1* silenced plants display a sterile phenotype and produce tiny siliques. Decreased productivity is not from the female or male gamete; the defect is from the inability of the anther to release pollen grains due to the cellulose deposition and lignin in the secondary wall of endothecium tissues (Beris et al., 2016; Zhong & Ye, 2009).

#### 1.3.2.2 PEX7

There are many *Peroxisome Biogenesis Peroxin (PEX*) genes; these genes encode proteins that import the responsible proteins for stimulating metabolic reactions in the peroxisome (Woodward & Bartel, 2005). In Arabidopsis at least 22 *PEX* genes have been reported (Nito et al., 2007), and in mammals and yeast there are more than 30 *PEX* genes. PEX proteins function to import matrix proteins. PEX7 is a soluble receptor that recognizes proteins containing Peroxisomal Targeting Signal type 2 (PTS2), which is a signal to import a matrix protein to peroxisomes (Rehling et al., 1996). Peroxisomes are organelles that host important metabolic reactions (Beevers, 1979). Mutation of human *PEX7* causes peroxisome biogenesis disorder Refsum disease (van den Brink et al., 2003). *Saccharomyces cerevisiae* PEX7 interacts with other proteins such as PEX18p and PEX21p to import PTS2 proteins (Purdue et al., 1998). In Arabidopsis, PEX7 interacts with PEX5 to import PTS2 proteins as well (Sparkes & Baker,

2002). The Arabidopsis *pex7-1* mutant is resistant to the auxin Indole-3-butyric acid (IBA), which is processed in peroxisomes into a auxin indole-3-acetic acid (IAA) (Zolman et al., 2000).

1.3.2.3 CSA

Cockayne Syndrome A (CSA) protein acts as a substrate receptor for the CUL4-DDB1 E3 ubiquitin ligase during transcription-coupled repair (TCR) under UV stress (O'Connell & Harper, 2007). CSA is recruited to DNA damage by aggregates of RNA polymerases, initiating DNA repair (Groisman et al., 2003). *Arabidopsis thaliana* CSA is expressed in almost all tissues, has four WD40 repeats and also functions in UV damage repair (Biedermann & Hellmann, 2010; Zhang et al., 2010).

1.3.2.4 RAE1

RNA EXPORT FACTOR 1 (RAE1), called Gle2p in *Saccharomyces cerevisiae*, has been linked with mRNA export under heat shock and ethanol-stressed conditions. Under heat shock, *gle2* mutants exhibit decreased mRNA export, resulting in accumulation of bulk poly (A)+ mRNA in the nucleus (Izawa et al., 2004). In control conditions, Gle2p-GFP showed a fluorescent signal around the nuclear rim. Interestingly, following heat shock Gle2p-GFP separated from the nuclear envelope and transferred to the cytoplasm, exhibiting a speckled pattern (Izawa et al., 2004). In metazoans, *S. cerevisiae*, and *S. pombe*, RAE1 is an mRNA export factor associated with the nuclear pore complex, and its mutants accumulate nuclear poly (A) RNA (Pritchard et al., 1999). In mice, Rae1 is associated with Nucleoporin 98 (NUP98) and regulates the interaction between vesicular stomatitis virus (VSV) matrix M proteins. These interactions function to block mRNA export (Faria et al., 2006), but high gene expression of

*Rae1* can rework the interactions (Faria et al., 2005). Thus regulation in nuclear transport assists antiviral mechanisms (Faria et al., 2006). Human Rae1-Nup98 interaction has been analyzed in detail, and the Rae1-NUP98 complex shown to directly interact with single stranded RNA (Ren et al., 2010). In *Drosophila melanogaster,* RAE1 is localized to the nuclear envelope, and is required in the G1 stage of mitosis (Sitterlin, 2004). Moreover, it participates in differentiation of male germline after meiosis. RAE1 is required in the nuclear envelope of primary spermatocytes to regulate meiotic chromatin condensation (Volpi et al., 2013).

*RAE1* has been studied in plants to identify its functions (Lee et al., 2009). One of the *RAE1* homologs is *Nicotiana benthamiana Rae1 (NbRae1),* which was found to play a variety of roles in plants such as mRNA export and spindle assembly. *NbRae1* encodes a protein with four WD40 repeats, which is linked with the nuclear envelope during interphase. *NbRae1* silencing resulted in accumulated poly (A) RNA in the nuclei of leaf cells, and defects in mRNA export. NbRAE1 has been shown to attach to microtubules and regulate bipolar spindle formation (Lee et al., 2009). In addition, *Arabidopsis thaliana* RAE1 has also been implicated in mRNA export and spindle assembly. By using a green fluorescent protein (GFP) tag, RAE1-GFP was shown to localize to the nuclear pore complex (Tamura et al., 2010).

## 1.4 Plant Nuclear Pore Complex

The nuclear pore complex (NPC) is the largest multiprotein complex within the nuclear envelope, which separates the nucleus and cytoplasm in the cell (Kiseleva et al., 2013). It facilitates the transfer of proteins and RNA and directly affects gene expression (Parry, 2014). mRNA transport receptor interacts with nucleoporins Phe-Gly (FG) repeats thus allowing mRNA permeability through NPC wall (Köhler & Hurt, 2007). A large part of the NPC mass consists of

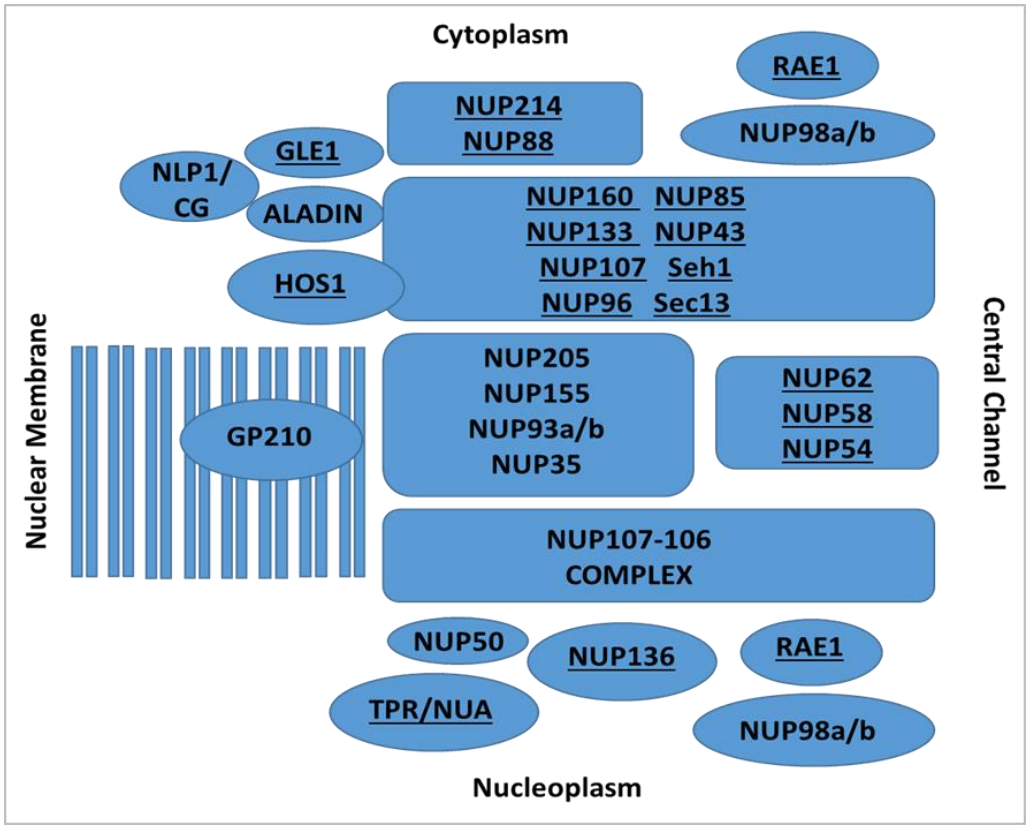


Figure 1.5 The Arabidopsis Nuclear Pore Complex (NPC). Sub-complexes are indicated. Underlined proteins have been described. Acronyms are as follows: Nucleoporin (NUP), GLFG lethal 1 (GLE1), RNA export factor 1 (RAE1), HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES1 (HOS1), NUCLEAR PORE ANCHOR (TPR/NUA), Glycoprotein 210 (GP210), Nodule inception protein-like protein 1 (NLP1/CG) (Parry, 2014). See text for details and additional references.

nucleoporins (NUPs) (Figure 1.5), which in turn contain a FG repeat domain that binds to transport receptors and mediates active transport through the NPC (Tamura et al., 2010) and have a role in structure and function processes (Rollenhagen et al., 2004). Some nucleoporins are members of the WD repeat family that form a β-propeller structure. In higher plants, 8 proteins have been described for the ~ 30 different nucleoporins (Meier & Brkljacic, 2009). Both *RAE1* and the gene next to it on chromosome 1, *NUP96 (SAR3)*, encode components of the nuclear pore complex.

### 1.4.1 SAR3

SUPPRESSOR OF AUXIN RESISTANCE 3 (SAR3 / NUP96) is part of the nuclear pore complex, SAR3 is involved in mRNA export and participates in auxin signaling (Parry et al., 2006). In Arabidopsis, *sar3* mutants showed higher mRNA accumulation in the nucleus due to export disruption, as well as impaired disease-resistance (Zhang & Li, 2005). Arabidopsis *sar3* mutants display early flowering, reduced flower bud size, and reduced stem thickness phenotypes. *SAR3* was identified in a screen for suppressors of Arabidopsis *auxin-resistant 1* (*axr1*) phenotypes, such as the formation of the lateral root. *sar3* also represses the auxin-resistance granted by *RUP-conjugating enzyme* (*rce1*) (Parry et al., 2006). In yeast and vertebrates, the SAR3 homolog, NUP96, is involved in mRNA export (Faria et al., 2006). In addition, study of the immune system in mice determined that NUP96 participates in immune response mediated by interferons via regulation of mRNA export (Faria et al., 2006). Furthermore, the inhibition of mRNA export is stopped by vesicular stomatitis virus (VSV) matrix M protein, which is restored by treating cells with interferon or by NUP98/NUP96 (Enninga et al., 2002).

## 1.5 Female gamete lethality

Gametophyte evolution and transition from diploid and haploid cell is considered an essential stage of plant proliferation. Female gametophyte development occurs in two stages. The first stage is mega-sporogenesis. Meiotic division occurs in megaspore mother cells (MMC) resulting in four haploid nuclei. In Arabidopsis, megasporogenesis takes a monosporic pattern where two meiotic divisions produce four one-nucleate megaspores, then three of them degenerate (Haig, 1990; Willemse & Went, 1984; Yadegari & Drews, 2004). The second stage is

mega-gametogensis. The single megaspore undergoes three rounds of mitosis without cytokinesis resulting in eight nuclei. Subsequently, phragmoplasts and cell plates shape between the sister cells. During cellularization, one nuclei emigrates from each pole to the gamete center then merge together, forming the central cell. Thus seven cells are produced during mega-gametogensis including three antipodal cells, one central cell, two synergid cells, and one egg cell (Drews et al., 1998; Haig, 1990; Willemse & Went, 1984; Yadegari & Drews, 2004).

Therefore, it is important to study female gametophyte mutations to determine the function and development of the embryo sac (Christensen et al., 2002). Research has identified female gametophyte specific mutations necessary for gamete development and divisions in all stages of mega-gametogenesis, initiating with female gametophyte polarity and ending with seed development (Drews et al., 1998). These mutants include genes which encode proteins involved in proteolysis, including regulators of ubiquitin mediated proteolytic pathway, such as EDA1, EDA2, EDA4, and EDA18 (Pagnussat et al., 2005).

The objectives of my thesis research are as follows: 1) to characterize T-DNA insertion mutants of the six candidate heat-induced DWDs with respect to both heat sensitivity and growth phenotypes; 2) to determine the molecular basis of the phenotypes exhibited by the *rae1* mutant; 3) to examine additional alleles of *RAE1* and the neighbouring *SAR3* gene; and 4) to determine the effect of RAE1 overexpression on growth and heat sensitivity. Together, these analyses will determine the role of RAE1 in Arabidopsis heat tolerance.

# 2. Role of RAE1 in *Arabidopsis thaliana* heat tolerance

Azizah Alharthy, Charlene Ibbetson, Amy Clark and Dana Schroeder

In preparation for Plant Science

This study was initiated by Charlene Ibbetson, who performed the experiments in Figures 2.2, 2.5, 2.7, 2.10, 2.13, and 2.14. Amy Clark did the analysis of YFP-RAE1 lines presented in Figure 2.26 and 2.30. All other analyses performed by Azizah Alharthy.

## 2.1. Introduction

In plants, environmental factors can influence the ability to survive and reproduce, thus, plants have mechanisms to adapt or respond to these factors such as heat stress. Signaling pathways regulate heat stress responses via transcription, kinases, and heat shock proteins (Qu et al., 2013) that repair damaged macromolecules thus facilitate cellular operation under stress.

Protein degradation is another mechanism of adaptation to abiotic stresses (Flick & Kaiser, 2012). Through the function of three enzymes: E1, the ubiquitin activating enzyme; E2, the ubiquitin conjugating enzyme; and E3, the ubiquitin ligase (Jackson & Xiong, 2009), ubiquitin is attached to specific substrate proteins, targeting them for degradation via the 26S proteasome (Moon et al., 2004). There are several types of E3 ubiquitin ligases. One of them is composed of a cullin family member along with a small RING protein to form a cullin-RING E3 ligase (CRLs). *Arabidopsis thaliana* has four cullins: CUL1, CUL3A, CUL3B, and CUL4 (Jackson and Xiong, 2009). CUL4 interacts with the DNA damage-binding protein 1 (DDB1) adaptor which in turn interacts with substrate-specific WD40 proteins (Lee and Zhou, 2007). The two homologs of DDB1 in Arabidopsis are DDB1a and DDB1b. Studies have shown that CUL4-DDB1 E3 ubiquitin ligase complexes are involved in multiple abiotic responses, such as to light, UV and drought (Lee & Kim, 2011). Arabidopsis DDB1 has also been implicated in heat stress response (Ganpudi & Schroeder, 2013). In this study we identify heat-induced DWD-motif (DDB1 binding WD40) containing WD40 proteins and examine their role Arabidopsis heat tolerance.

## 2.2 Materials and methods

### 2.2.1 Plant material and growth conditions

*Arabidopsis thaliana* mutants including *rae1* (SALK_149413), *rae1-236* (SALK_030236), *rae1-GK* (GABI_037E10), *dwh1-2* (SALK_141782), *dwh1-3* (SALK_020927), *dwh2-2* (SALK_051819), *dwh3-1* (SALK_132649), *sar3-4* (SALK_135920), *sar3-3* (SALK_109957), *pex7* (SALK_005356), and *at-csa* (SALK_030558) T-DNA insertion allele mutants were ordered through the Arabidopsis Biological Resource Centre (ABRC) (Alonso et al., 2003; Kleinboelting et al., 2012). *Arabidopsis thaliana* Colombia-0 (*Col-0*) was used as the wildtype control. Crosses were done using standard protocols (Weigel & Glazebrook, 2002).

After sterilization, seeds were plated on Linsmaier and Skoog (LS) media (Caisson) with 0.6% sucrose and 0.86% Phytoblend (Caisson). After 2 days at 4°C, plates were transferred to an incubator at 20°C with 50% R.H. for long day growth (16 hr light, 8 hr dark). 14-day old seedlings were transplanted to soil (Sunshine mix #1) in long day conditions (16 hr light, 8 hr dark) at 20°C with 50% R.H.

### 2.2.2 DNA extraction and genotyping analysis

DNA was isolated from seven-day old seedlings using standard protocols (Weigel & Glazebrook, 2002) for PCR analysis. Top Taq Master Mix (Qiagen) was used for all genotyping reactions. Primer sequences used for genotyping are presented in Table 2.1. T-DNA insertion alleles were genotyped using reverse primers and LBb1.3 (ATTTTGCCGATTTCGGAAC) for the SALK lines, and GK-8409 (ATATTGACCATCATACTCATTGC) for the *rae1-GK* line. All

T-DNA genotyping primers were designed using the iSect Primer tool on the T-DNA Express website (http://signal.salk.edu/tdnaprimers.2.html).

### 2.2.3 Adult growth analysis

Adult growth parameters were measured using standard protocols (Ganpudi & Schroeder, 2013). Flowering time was scored as the day of bud emergence from the rosette, and subsequently as the total number of rosette and cauline leaves. Rosette diameter was scored after four weeks of growth in long day conditions. Apical dominance (cm), silique length (cm), and height (cm) were measured after maximum height was achieved, at approximately six weeks.

### 2.2.4 Adult heat experiments

One week after transplanting, 21-day old plants were exposed to 37°C in a growth chamber for consecutive days (0, 1, 2, or 3), then returned to 20°C for a recovery period. One week after the initiation of treatment, the fraction leaf damage (damaged or yellow leaves / total leaves) and rosette diameter were determined. Growth data parameters were collected as above and compared with *Col-0*.

### 2.2.5 Seedling heat experiments

Approximately 20-25 seeds were sterilized (10 min shaking in 70% ethanol, 0.5% Triton X-100 followed by 10 min in 95% ethanol) and plated on 35 ml of LS media. After 2 days at 4°C plates were transferred to light at 20°C for 4-6 hours, then plates were wrapped in foil and returned to 20°C. After three days of dark growth plates were incubated at 45°C for 0, 45, or 90

minutes then returned to 20°C for recovery in the dark. Plates were scanned four days after treatment and hypocotyls measured using NIH Image J software version 1.48.

### 2.2.6 Generation of RAE1 overexpression lines

The *RAE1* cDNA in Gateway entry vector pENTR223 (ABRC G82498) was cloned into pEarleygate pEG104 and pEG203 overexpression vectors (Earley et al., 2006) using LR recombinase (Invitrogen), followed by DNA transfer into Agrobacterium (Weigel & Glazebrook, 2002). Agrobacterium solution was sprayed onto *Col-0* flowers for transformation. Transformed T1 seeds were grown for approximately 10 days then sprayed with Basta (Bayer CropScience Canada) to identify transformed lines. Transgenic lines were confirmed via genotyping using the primers in Table 2.2 and homozygous T3 lines used for all experiments.

### 2.2.7 RNA extraction and semi-quantitative RT-PCR analysis

Total RNA was extracted using the RNeasy plant mini kit (Qiagen, Hilden, Germany) from approximately 50 seven-day-old seedlings per line based on Kim et al. (2012) protocol. RNA concentrations were measured on a Nano-drop spectrophotometer (Thermo Scientific, Canada). cDNA was prepared using the Maxima First Strand cDNA synthesis kit (Fermentas, Waltham, MA, USA). For semi-quantitative RT-PCR, *Actin* was used as the loading control. *RAE1* and *SAR3* were amplified for 28 cycles (30 sec 94°C, 30 sec 60°C, 1 min 72°C), while *Actin* was amplified for 24 cycles (30 sec 94°C, 1 min 58°C, 1.5 min 72°C). Semi-quantitative RT-PCR primer sequences are shown in Table 2.3. To analyze the effect of heat on RNA level, 50 seeds were plated on 35 ml LS media. After two days of stratification at 4°C, plates were incubated at 20°C in long day conditions for seven days, then plates wrapped in foil and heat

treated at 45°C for 0 or 3 hours, then returned to 20°C. After one hour of recovery, the total RNA was isolated using the procedure above.

### 2.2.8 Quantitative Real-Time PCR

Analysis was performed using SsoFast EvaGreen Supermix (Bio-rad, Hercules, CA, USA). Quantitative PCR was performed in triplicate for each sample and *EF1α* (At5g60390) was used as the loading control (Hossain et al., 2012; Jain et al., 2006). Data was analyzed using the comparative $C_t$ ($2^{-\Delta\Delta Ct}$) method, where $\Delta C_T$ is calculated as the difference between the *Ct* of the gene of interest and the *Ct* of the reference gene (e.g. *EF1α*) (Schmittgen & Livak, 2008). The primer sequences used for qPCR are presented in Table 2.4.

### 2.2.9 Protein extraction and Western blot

Total protein was isolated from 5-7 days old seedlings in 50 µl of 1X protein loading dye (50 mM Tris pH 6.8, 100 mM DTT, 2% SDS, 10% glycerol, 0.01% bromophenol blue) for each sample. Samples were heated at 95°C for 5 min before separation on 7% polyacrylamide gels. Proteins were then transferred to nitrocellulose membrane (Bio-rad). The total protein was visualized using 1X of IDGel™ Ponceau S Staining Solution and scanned. Membranes were then rinsed and blocked in 1X TBST milk (10 mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween, 5% milk) and incubated in primary antibody (1/200 α-GFP (Santa Cruz) in TBST milk) overnight. The next day the membrane was washed, incubated in secondary antibody (1/5000 HRP α-rabbit (Bio-rad)) for 45 minutes and detected using SuperSignal West Dura Extended Duration Substrate (Pierce). Chemiluminescent signal was detected with CL-XPosure film (Thermo Scientific) exposed for around 15 min.

### 2.2.10 Microscopy

Samples were prepared using pre- and post-fertilization carpels from *rae1-236* heterozygotes. The wall tissue was removed to reveal the ovules. Samples were soaked in Hoyer's solution (Liu & Meinke, 1998) for ~15 minutes. Images were obtained using differential interference contrast on an AXIO Imager ZI microscope equipped with Axio vision 4.8 software.

YFP-RAE1 localization was examined in the hypocotyls of four-day old dark grown seedlings treated for 0, 10, 40, 75, 100, or 170 minutes at 37°C. Following heat treatment, images were taken at 40X magnification using YFP Filter Set YFP-2427B-000 (Semrock Inc.) on an AXIO Imager ZI microscope with Axio vision 4.8 software.

### 2.2.11 Statistical analysis

All the experiments were repeated at least twice and representative results are shown. Student's t-test in Excel software was used to compare the data and P values of 0.05 or less considered statistically significant.

Table 2.1 Primers used for genotyping T-DNA insertion mutants

| Gene | Allele | T-DNA | Primers | Sequence |
|---|---|---|---|---|
| *RAE1* | *rae1* | SALK | RAE1-F | ATCCTTCTTTTCTTTCGCGAG |
| | | 149413 | RAE1-R | TTGTTAGGGTTACTGTTGGCG |
| | *rae1-236* | SALK | rae1-236-F | TCACAAGAAAGTAAGCCCGTC |
| | | 030236 | rae1-236-R | AAAGAAAGCGACAAGCTAGGG |
| | *rae1-GK* | GABI | rae1-GK-F | AACATTGGGCCATAATAAGGC |
| | | 037E10 | rae1-GK-R | TATTTCCCAACACCTCACCTG |
| *SAR3* | *sar3-4* | SALK | Sar3-1F | CGAAATGTAAGACCTTCGCAG |
| | | 135920 | Sar3-1R | TGAACTGTCGGGAGGTAGATG |
| | *sar3-3* | SALK | Sar3-2F | GATGCACGAAGCTCTGGTAAG |
| | | 109957 | Sar3-2R | AACTTGGGCTGTGTTGTCATC |
| *PEX7* | *pex7* | SALK | Pex7-F | TCGACATCAGTGTACTGCTGC |
| | | 005356 | Pex7-R | AGGAGGAGGAAGAGCTGTGTC |
| *DWH1* | *dwh1-2* | SALK | DWH1-2F | CAACGAATTAGCAGCCAAGAG |
| | | 141782 | DWH1-2R | ATACCCACTTTGCGACAGTTG |
| | *dwh1-3* | SALK | DWH1-3F | GCAAGCAAGGATAACAGCAAG |
| | | 020927 | DWH1-3R | GCATCCATAGCTCCCTCTTTC |
| *DWH2* | *dwh2-2* | SALK | DWH2-2F | AGTTTGGGGTTTGGAACTCAC |
| | | 051819 | DWH2-2R | TCTCCGCCTCATCATGATATC |
| *DWH3* | *dwh3* | SALK | DWH3-F | GGGTGGGAAGCAGTTTATCTC |
| | | 132649 | DWH3-R | GAGCGCAGTGGATTTGTAGTC |
| *At-CSA* | *at-csa* | SALK | At-CSA-F | TGTGGATTTTAAAATGCCTGG |
| | | 030558 | At-CSA-R | CCAGCAGATGCTGCCTATAAC |

Table 2.2 Primers used to screen transgenic lines

| **Gene** | **Primers** | **Sequence** |
|---|---|---|
| *Myc-RAE1&* | RAE1-C-SAC | CCCCGAGCTCTCATTTTCTGCCGGTTGCTC |
| *YFP-RAE1* | KPN-N-RAE1 | GGGGGGTACCATGGCAACTTTTGGTGCGCC |

Table 2.3 Primers used for semi-quantitative RT-PCR

| **Gene** | **Primers** | **Sequence** |
|---|---|---|
| *SAR3* | Sar3-F | ATTGGCTGTTTGGGGTGACA |
| | Sar3-R | TGACACAGCGTCTTGCAGAT |
| | RAE1-F | ATCCTTCTTTTCTTTCGCGAG |
| | SAR3-c118F | GAAAGGTCGCGGTAGAGTCA |
| *RAE1* | RAE1-R | TTGTTAGGGTTACTGTTGGCG |
| | RAE1-c3F | CGTCAATGTGACGCAGAGAG |
| | RAE1-GK-R | TATTTCCCAACACCTCACCTG |
| | KPN-N-RAE1 | GGGGGGTACCATGGCAACTTTTGGTGCGCC |
| *Actin* | Act2-F | CTGGAACAAGACTTCTGGGC |
| | Act2-R | GGTGATGAAGCACAATCCAAG |

Table 2.4 Primers used for quantitative RT-PCR

| **Gene** | **Primers** | **Sequence** |
|---|---|---|
| *RAE1* | RAE1-in324-R | TTCAATTCAAGAGACAGAATCACAT |
| | RAE1-c267-R | CGAGGTCTGATTAACTAGAGAGAAG |
| | RAE1-c3-F | CGTCAATGTGACGCAGAGAG |
| *SAR3* | Sar3-F | ATTGGCTGTTTGGGGTGACA |
| | Sar3-R | TGACACAGCGTCTTGCAGAT |
| *Myc-RAE1&* | RAE1-c850F | TCGAGGTGCAATCAGCCAAT |
| *YFP-RAE1* | RAE1-C-SAC | CCCCGAGCTCTCATTTTCTGCCGGTTGCTC |
| *EEF* | EEF-F | CTGGAGGTTTTGAGGCTGGTAT |
| | EEF-R | CCAAGGGTGAAAGCAAGAAGA |

## 2.3 Results

### 2.3.1 Analysis of mutants of heat-induced DWD genes

Eighty five DWD-motif containing WD40 proteins have been identified in Arabidopsis that possibly function as substrate receptors in different CUL4 E3 ubiquitin ligases (Lee et al., 2008). The effect of abiotic stress on the expression of these 85 genes was examined using data from Kilian et al. (2007) accessed via AtGenExpress. This analysis revealed that six candidate genes, *DWD REGULATED by HEAT 1* (*DWH1), DWH2, DWH3*, Arabidopsis *PEROXIN7 (PEX7), RNA EXPORT FACTOR1 (RAE1),* and *Arabidopsis thaliana COCKAYNE SYNDROME TYPE A* protein *(AT-CSA)*, are at least two-fold up-regulated by heat in either root or shoot (Figure 2.1). T-DNA insertion alleles for each candidate gene were genotyped to identify homozygous lines. Homozygotes were found for *DWH1, DWH2, RAE1, At-CSA* and *PEX7* but not for *DWH3*, indicating that *dwh3* mutants exhibit gametic or zygotic lethality.

With respect to adult growth in control conditions, *rae1* displayed significantly earlier flowering, as well as a significant decrease in rosette diameter, height, and silique length and increased stem number (decreased apical dominance) compared with *Col-0* wildtype (Figure 2.2). *dwh1* alleles exhibited decreased height and stem number, while *dwh2* exhibited early flowering and decreased height (Figure 2.2). The heat sensitive control *uvh6* also exhibited decreased rosette diameter, height, and silique length as previously described (Kim et al. 2012). *csa* adults did not differ significantly from wildtype *Col-0* (Figure 2.3), while *pex7* mutants exhibited delayed flowering and increased rosette diameter (Figure 2.4).

Heat tolerance experiments were conducted on *rae1* and *csa* mutants, along with the *Col-0* wild-type and the *uvh6* heat sensitive control (Jenkins et al., 1997). Following heat exposure, neither *rae1* nor *csa* showed increased leaf damage like *uvh6* (Figure 2.5a). However, *rae1*

mutants displayed significantly shorter height and higher stem number compared with *Col-0* following heat treatment (Figure 2.5c, d), suggesting that *rae1* mutants have a heat sensitive phenotype. In *csa* and *pex7* mutants, no consistent significant heat tolerance phenotype was observed in adults (Figure 2.5, 2.6).

In seedling heat assays, *rae1* seedlings, along with *uvh6* and *ddb1a*, showed a significant decrease in hypocotyl length compared with *Col-0* following heat treatment, while *dwh1* and *dwh2* did not exhibit seedling heat phenotypes (Figure 2.7). *csa* seedlings showed slightly increased relative hypocotyl length compared to wildtype following 45 min of heat treatment but not at 90 min (Figure 2.8). In contrast, *pex7* mutants showed slight heat sensitivity at 90 min (Figure 2.9). Additional assays with *rae1* displayed consistently decreased hypocotyl length (Figure 2.10), suggesting that *RAE1* has a role in heat response.

Interestingly, over generations, while *rae1* maintained its early flowering phenotype (Figure 2.11a, b), it exhibited decreased heat sensitivity, as indicated by decreased height and increased stem number following heat treatment (Figure 2.11e, g).

### 2.3.2 Analysis of effect of *rae1* T-DNA insertion on *RAE1* and *SAR3* levels

The *rae1* mutant exhibited flowering time and heat sensitivity phenotypes. The *rae1* T-DNA insertion is located in the *RAE1* promoter (Figure 2.12), thus we examined its effect on *RAE1* transcript level. Semi-quantitative RT-PCR analysis of RNA extracted from *rae1* seedlings grown in control conditions showed that *RAE1* is still expressed is this allele (Figure 2.13a), thus the T-DNA insertion allele had not resulted in complete loss of *RAE1* function. Therefore, the expression of the neighboring gene to *RAE1, SAR3* (Suppressor of Auxin Resistance 3), was examined, since the T-DNA insertion is also in its promoter (Figure 2.12). Semi-quantitative RT-

PCR of RNA extracted from the *rae1* allele showed *SAR3* is also still expressed (Figure 2.13b).

Even though no complete loss of function of *RAE1* or *SAR3* was observed in *rae1*, the plants display a heat sensitive phenotype, thus we hypothesized that the molecular defect may be heat specific. Therefore, semi-quantitative RT-PCR was conducted on RNA extracted from control and heat-treated *rae1* and *Col-0* seedlings. While *RAE1* is still expressed in the *rae1* allele in control conditions, following heat treatment a reduction in the intensity of the *RAE1* band and an increase in the intensity of a higher molecular weight product was observed (Figure 2.14a). Subsequent recovery and sequencing of this higher molecular weight product revealed that it is an un-spliced product that retains *RAE1* intron 1. In contrast, the level and band size of the *SAR3* product, which spans *SAR3* intron 5, was not altered in heat-treated *rae1* (Figure 2.14b). Splicing of other introns in *RAE1* and *SAR3* was examined to determine the specificity of the splicing defect. It was found that *RAE1* intron 2 and 3 and *SAR3* intron 1 and 5 showed normally spliced products in both control and heat conditions (Figure 2.15). Therefore, the splicing defect appears specific to *RAE1* intron 1, which is located within the *RAE1* 5' UTR. Thus, the *rae1* heat sensitive phenotype seems to be associated with changes in the splicing of *RAE1* intron 1 under heat stress. Given the effect of seed age on heat sensitivity in *rae1*, we examined the effect of seed age on intron 1 splicing. Both older (2012) and newer (2015) *rae1* seeds still exhibit the heat-induced intron 1 splicing defect, although the residual fraction of correctly spliced product may be higher in the newer seed (Figure 2.16).

In order to analyze the effect of the *rae1* allele on *RAE1* and *SAR3* transcript levels more quantitatively, we performed quantitative RT-PCR on control and heat-treated samples. Using an intron 1 specific primer (Figure 2.17), we found that after heat treatment *rae1* showed a significant increase in the un-spliced product, consistent with the semi-quantitative PCR data

(Figure 2.18a). When an exon-junction spanning primer, specific to the spliced product, was used, we found that in control conditions the spliced *RAE1* transcript was up-regulated (~5x) in *rae1* (Figure 2.18b). This pattern was not altered by heat treatment, in contrast to the semi-quantitative RT-PCR data, which indicated a decrease in the spliced product in *rae1* following heat treatment. *SAR3* transcript levels also increased 1.7-fold in *rae1* in control conditions, while *SAR3* transcript levels dropped 2-3-fold in both wildtype and *rae1* following heat treatment (Figure 2.18c). Thus, *rae1* exhibits increased levels of both *RAE1* and *SAR3* in control conditions and decreased levels of *SAR3* and increased levels of un-spliced *RAE1* following heat treatment.

### 2.3.3 Analysis of additional *RAE1* and *SAR3* alleles

Since our original *rae1* allele altered the levels of both *RAE1* and *SAR3* transcripts, we examined additional alleles of both genes to determine their contribution to the *rae1* flowering time and heat sensitivity phenotypes. For *SAR3*, we examined two T-DNA alleles, SALK 135920, *sar3-4*, in the second exon, and SALK_109957, *sar3-3*, previously described by Parry et al. (2006), in the fifth exon (Figure 2.12). In control conditions, *sar3-3* homozygous lines exhibit early flowering, as well as decreased rosette diameter, silique length, and apical dominance as previously described (Parry et al., 2006) (Figure 2.19). Surprisingly, *sar3-4* did not display any growth phenotypes. In adult heat tolerance experiments, *sar3-3* showed a significant increase in leaf damage following heat treatment. However, heat did not affect *sar3-3* or *sar3-4* relative rosette diameter (Figure 2.20). In seedlings, *sar3-3* exhibited slightly increased tolerance at 45 min (Figure 2.21). Thus, while *sar3-3* resulted in increased cell death in response to heat, it did not appear to result in decreased growth.

Additional *RAE1* alleles GABI_037E10 (*rae1-GK*) and SALK_030236 (*rae1-236*) (Figure 2.12) were obtained and segregating populations genotyped. Viable homozygotes were not identified for either allele, suggesting that these alleles result in gametic or embryonic lethality. Siliques were examined for both lines and the fraction abnormal seeds determined. *RAE1/rae1-236* heterozygotes exhibited an increase in missing seeds compared with *RAE1* wildtype (Table 2.5, Figure 2.22). In contrast, *rae1-GK* siliques showed abnormal rather than missing seeds (Figure 2.23), and this phenotype was also present in "wildtype" siblings lacking the *rae1-GK* allele (Table 2.5, Figure 2.23). Thus, another mutation affecting seed development appears to be segregating in the *rae1-GK* background. Therefore, we focused on characterizing the *rae1-236* lethal phenotype.

*rae1-236* heterozygotes were self-crossed and crossed with wildtype *Col-0.* The progeny were genotyped to determine if *RAE1* is required in male or female gametes or embryos. No heterozygous progeny were produced by crossing *rae1-236* heterozygous mothers with wildtype fathers (Table 2.5). In contrast, both self-crosses of heterozygotes and crosses of wildtype mothers with *rae1-236* heterozygous fathers resulted in ~1:1 ratios of heterozygous to wildtype progeny. These data suggests that *RAE1* is required in the female gamete. To further study this defect, pre-fertilization carpels of *rae1-236* heterozygotes were examined. In contrast to presumed wildtype ovules within the same silique, *rae1-236* ovules lacked well-defined egg cells, central cells, and synergid cells, and recently fertilized *rae1-236* ovules lacked embryos and suspensors (Figure 2.24). Thus, while it is not clear at which stage this defect has occurred, *RAE1* is required for female mega-gametogenesis.

Since *rae1-236* homozygotes were not available, we examined development and heat tolerance in segregating *rae1-236* heterozygotes. In control conditions, *rae1-236* heterozygotes

exhibited early flowering, increased height, and decreased silique length (Figure 2.25). *rae1-236* heterozygotes did not show any differences in apical dominance in either control or heat conditions (data is not shown). Following heat treatment, *rae1-236* heterozygotes showed increased leaf damage, but did not show other adult heat stress phenotypes. Thus, like the *rae1* promoter allele, *rae1-236* heterozygotes show early flowering and decreased silique length in control conditions, but exhibit increased leaf damage rather than decreased growth and apical dominance following heat treatment.

### 2.3.4 Investigation of the effect of YFP-RAE1 overexpression

The role of *RAE1* in growth and heat tolerance was also examined using overexpression lines. *RAE1* was cloned into the pEarleygate pEG104 vector (Earley et al. 2006), resulting in CaMV 35S-driven YFP-RAE1 expression. Anti-YFP western blots of homozygous lines confirmed YFP-RAE1 expression (Figure 2.26a). Quantitative RT-PCR analysis of *RAE1* transcript level showed results consistent with the western blot (Figure 2.26b), with lines YFP-RAE1 2-5 and 4-1 up-regulated ~100 fold, and YFP-RAE1 3-2 overexpressed 35 fold.

Analysis of growth and heat sensitivity in YFP-RAE1 overexpression lines was performed. In control conditions, overexpression of YFP-RAE1 resulted in early flowering with respect to number of leaves, as well as decreased rosette diameter, height, and silique length (Figure 2.27). Following heat treatment, YFP-RAE1 line 4-1 showed a significant increase in leaf damage, however YFP-RAE1 overexpression did not have a significant relative effect on other adult growth parameters (Figure 2.27, 2.28). YFP-RAE1 overexpression did not result in a hypocotyl length phenotype in heat-treated seedlings (Figure 2.29). Thus YFP-RAE1 overexpression results in decreased flowering time, rosette diameter, height, and silique length

like the *rae1* promoter allele. However, the increased heat-induced leaf damage phenotype more closely resembles *rae1-236* than the *rae1* promoter allele.

RAE1 has been shown to be nuclear localized in several systems such as *Saccharomyces cerevisiae* (Izawa et al., 2004), *Drosophila melanogaster* (Sitterlin, 2004), and *Arabidopsis thaliana* (Tamura et al., 2010). We examined YFP-RAE1 cellular localization in our transgenic lines, where it was detected in nuclei (Figure 2.30). We also examined YFP-RAE1 localization following heat treatment and observed increased localization to cytoplasmic speckles. A similar pattern of RAE1 localization in response to heat treatment has been observed in yeast (Izawa et al., 2004).

### 2.3.5 Effect of Myc-RAE1 overexpression

To control for the effect of the large YFP tag, we also overexpressed RAE1 with a smaller N-terminal Myc tag using the pEarleyGate pEG203 vector (Earley et al. 2006). Analysis of Myc-RAE1 overexpressing lines via quantitative RT-PCR revealed 30-80 fold levels of overexpression (Figure 2.31). Adult growth and heat treatment assays were performed with these lines. In control conditions, Myc-RAE1 overexpressing lines showed early flowering as well as decreased rosette diameter, silique length, and apical dominance (Figure 2.32, 2.33). Following heat treatment, Myc-RAE1 overexpression lines exhibited significantly increased leaf damage, as well as a relative decrease in rosette diameter and height.

In heat-treated seedlings, Myc-RAE1 overexpression resulted in a significant increase in hypocotyl length after 45 min (line #2) but a decrease in hypocotyl length after 90 min (line #1,3) (Figure 2.34). To further investigate the basis of this seemingly contradictory pattern, we repeated the experiment and examined both absolute and relative hypocotyl lengths (Figure

2.35). In the absolute data, it is clear that while the Myc-RAE1 overexpressing lines are shorter in control conditions, they are similar length after 45 min, resulting in an apparent relative increase. However after 90 min the overexpression lines are shorter with respect to both absolute and relative values. Thus, it appears that the apparent tolerance at 45 min is a consequence of the shorter hypocotyl length of the overexpression lines in control conditions.

Overall, like the *rae1-236*, *rae1* promoter allele and YFP-RAE1 overexpression lines, Myc-RAE1 overexpression resulted in decreased flowering time and silique length in control conditions. Following heat treatment, Myc-RAE1 overexpression resulted in decreased relative height and hypocotyl length, like *rae1*, but also increased leaf damage, like the *rae1*-236 and YFP-RAE1 overexpression lines. Thus, RAE1 loss of function and overexpression appear to result in similar phenotypes.

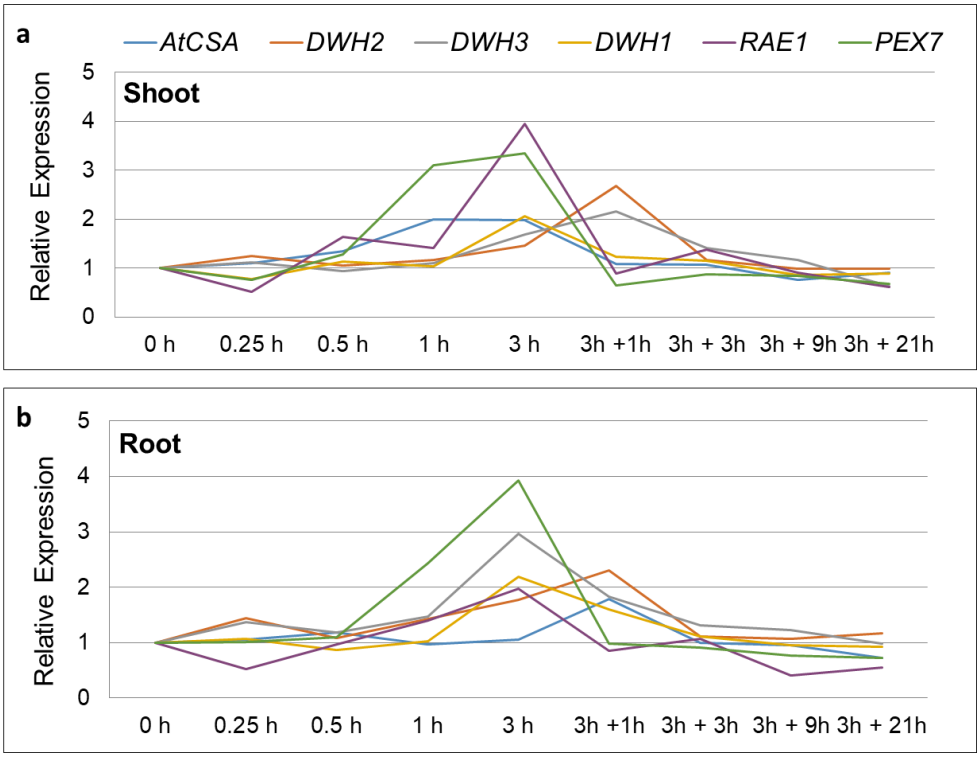


Figure 2.1 Expression of heat-induced DWD genes. *DWH1*, *DWH2*, *DWH3*, *PEX7*, *AT-CSA*, and *RAE1* transcript level in a) shoot and b) root during 3 hrs of 38ºC treatment followed by recovery at 25ºC. Data from Kilian et al. (2007) accessed via AtGenExpress.

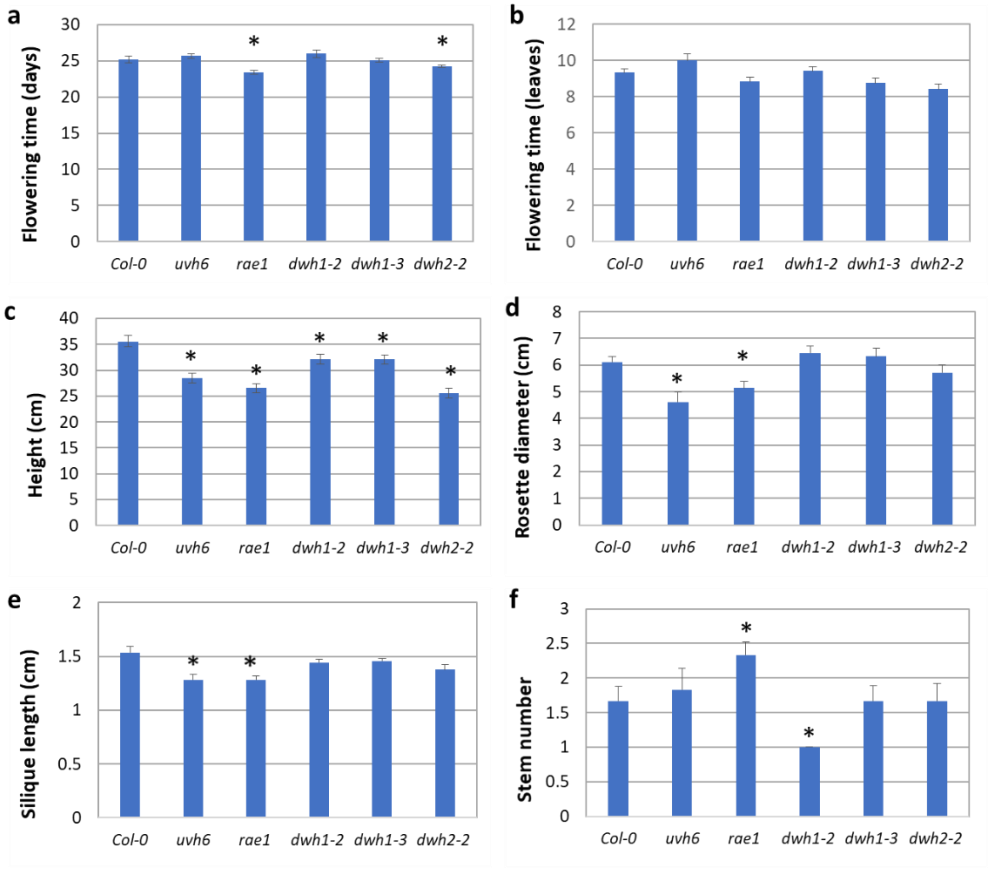


Figure 2.2 Adult growth analysis of *rae1*, *dwh1* and *dwh2*. a) Flowering time in days, b) flowering time in leaves, c) height, d) rosette diameter, e) silique length, f) stem number. Error bars indicate SE (n=6). * = P <0.05 relative to *Col-0*.

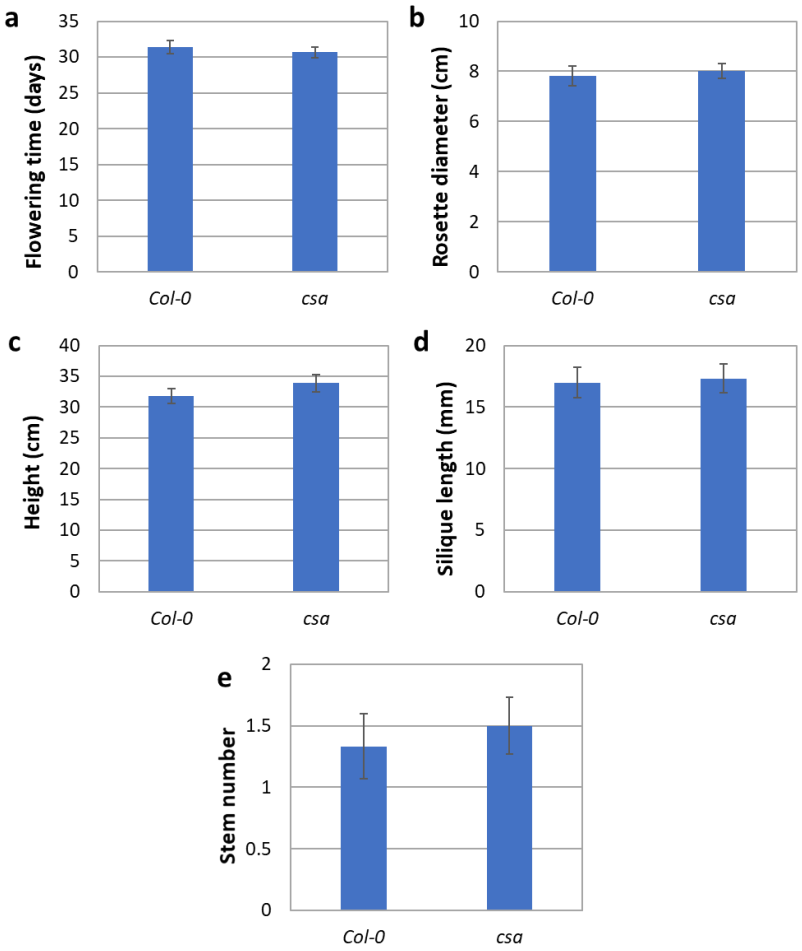


Figure 2.3 Adult growth analysis of *csa*. a) Flowering time in days, b) rosette diameter, c) height, d) silique length, e) stem number. Error bars indicate SE (n=6). * = P <0.05 relative to *Col-0*.

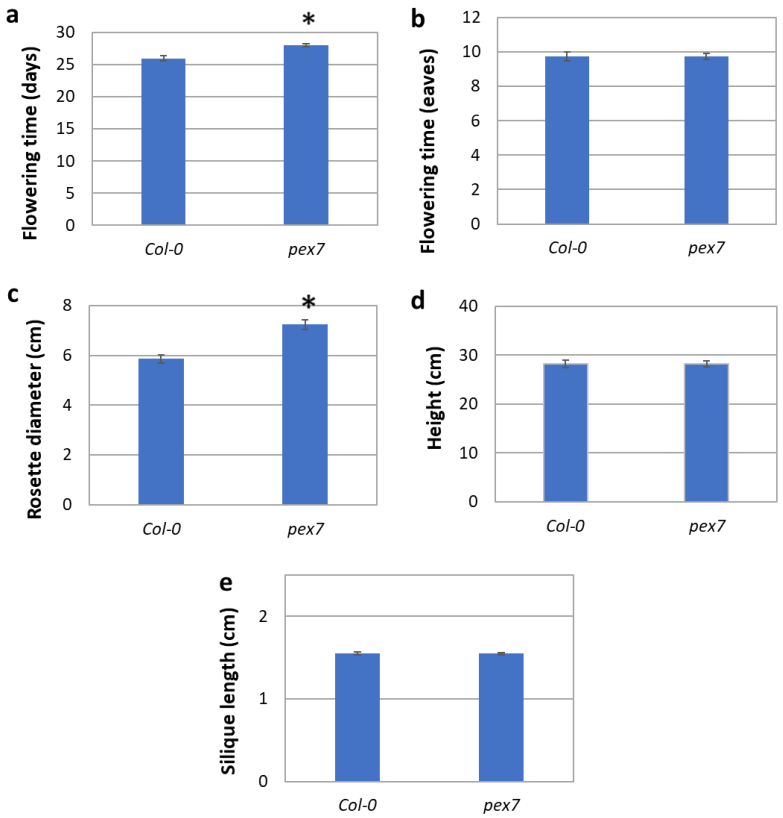


Figure 2.4 Adult growth analysis of *pex7*. a) Flowering time in days, b) flowering time in leaves, c) rosette diameter, d) height, e) silique length. Error bars indicate SE (n=6). * = P <0.05 relative to *Col-0*.

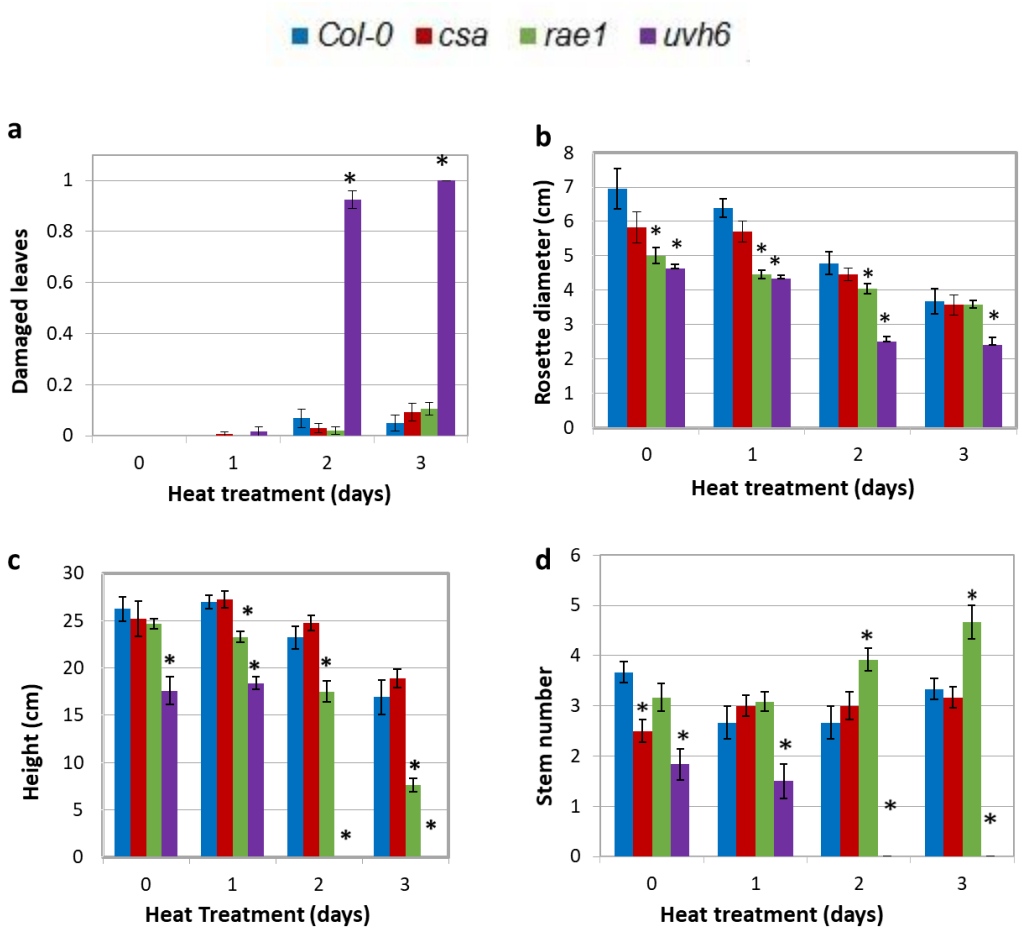


Figure 2.5 *csa* and *rae1* adult heat tolerance analysis. a) Fraction damaged leaves, b) rosette diameter, c) height, d) stem number following 0-3 days at 37ºC. Error bars indicate SE (n=6) and * = P <0.05 relative to *Col-0*. *uvh6* is the heat sensitive control.

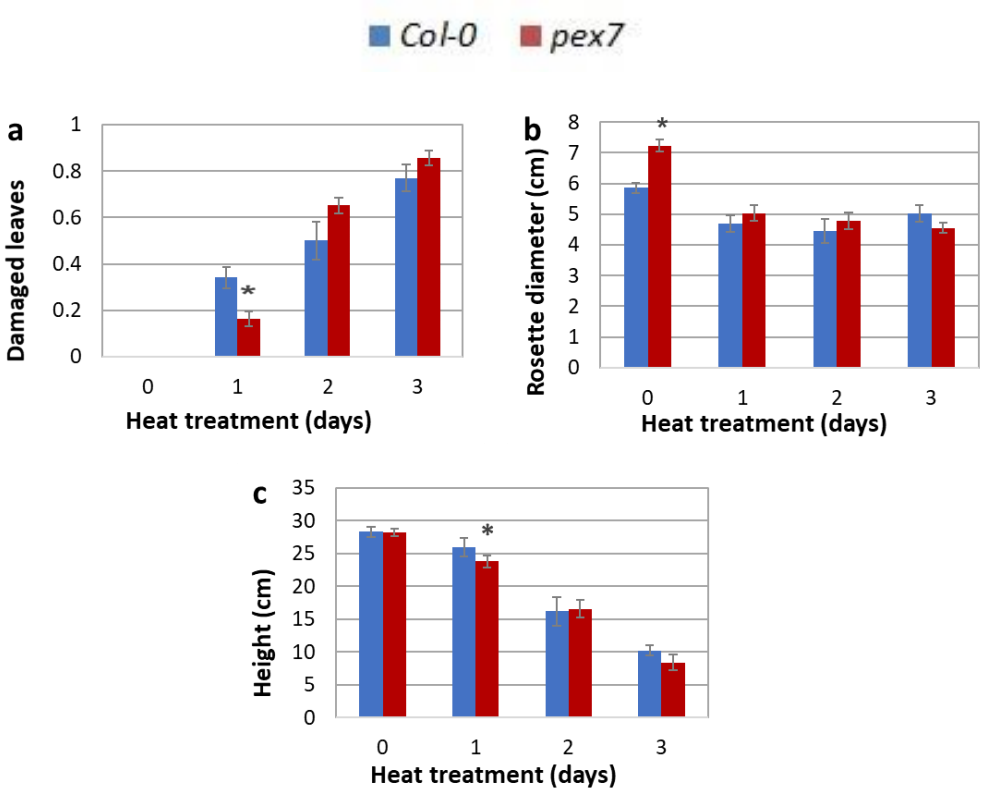


Figure 2.6 *pex7* adult heat tolerance analysis. a) Fraction damaged leaves, b) rosette diameter, c) height following 0-3 days at 37ºC. Error bars indicate SE (n=6) and *= P <0.05 relative to *Col-0*.

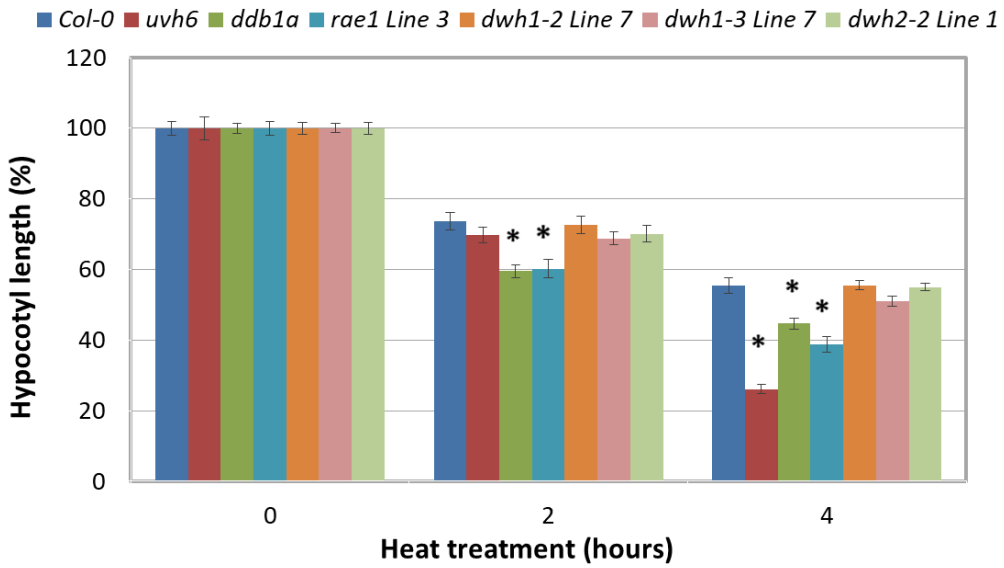


Figure 2.7 Seedling heat tolerance analysis of *rae1*, *dwh1*, and *dwh2*. Hypocotyl length of heat (45ºC) treated seedlings relative to untreated controls of the same genotype. Error bars indicate SE (n=15) and * = P <0.05 relative to *Col-0*. *uvh6* is the heat sensitive control.

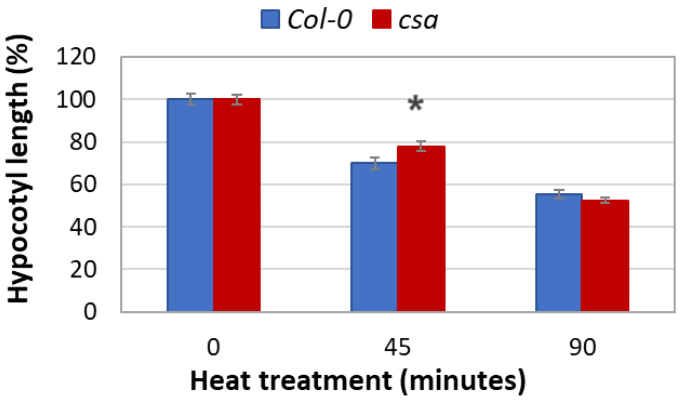


Figure 2.8 *csa* seedling heat tolerance. Hypocotyl length of heat (45ºC) treated seedlings relative to untreated controls of the same genotype. Error bars indicate SE (n=18) and * = P <0.05 relative to *Col-0*.

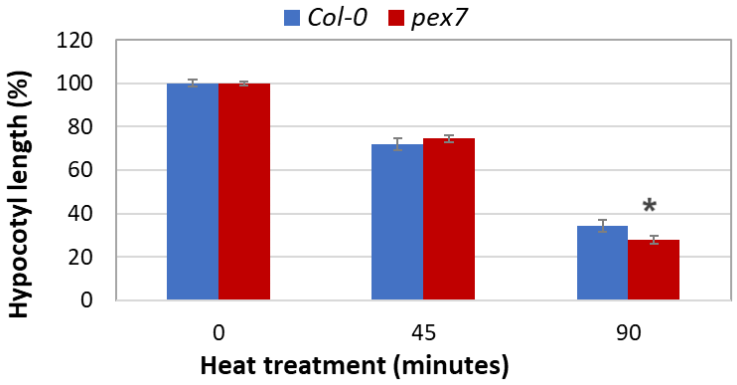


Figure 2.9 *pex7* seedling heat tolerance. Hypocotyl length of heat (45°C) treated seedlings relative to untreated controls of the same genotype. Error bars indicate SE (n=16) and * = P <0.05 relative to *Col-0*.

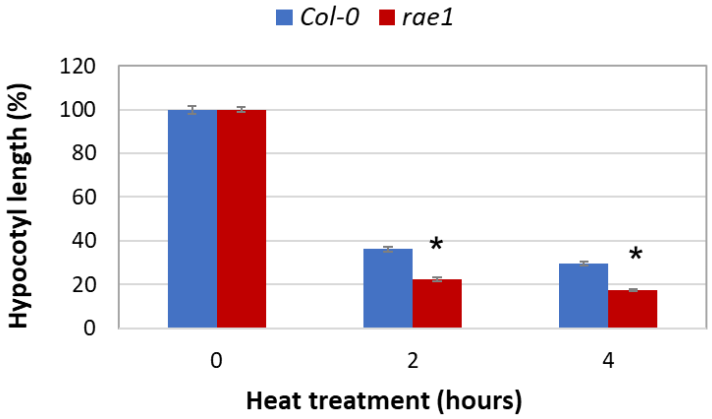


Figure 2.10 *rae1* seedling heat tolerance. Hypocotyl length of heat (45°C) treated seedlings relative to untreated controls of the same genotype. Error bars indicate SE (n=15) and * = P <0.05 relative to *Col-0*.

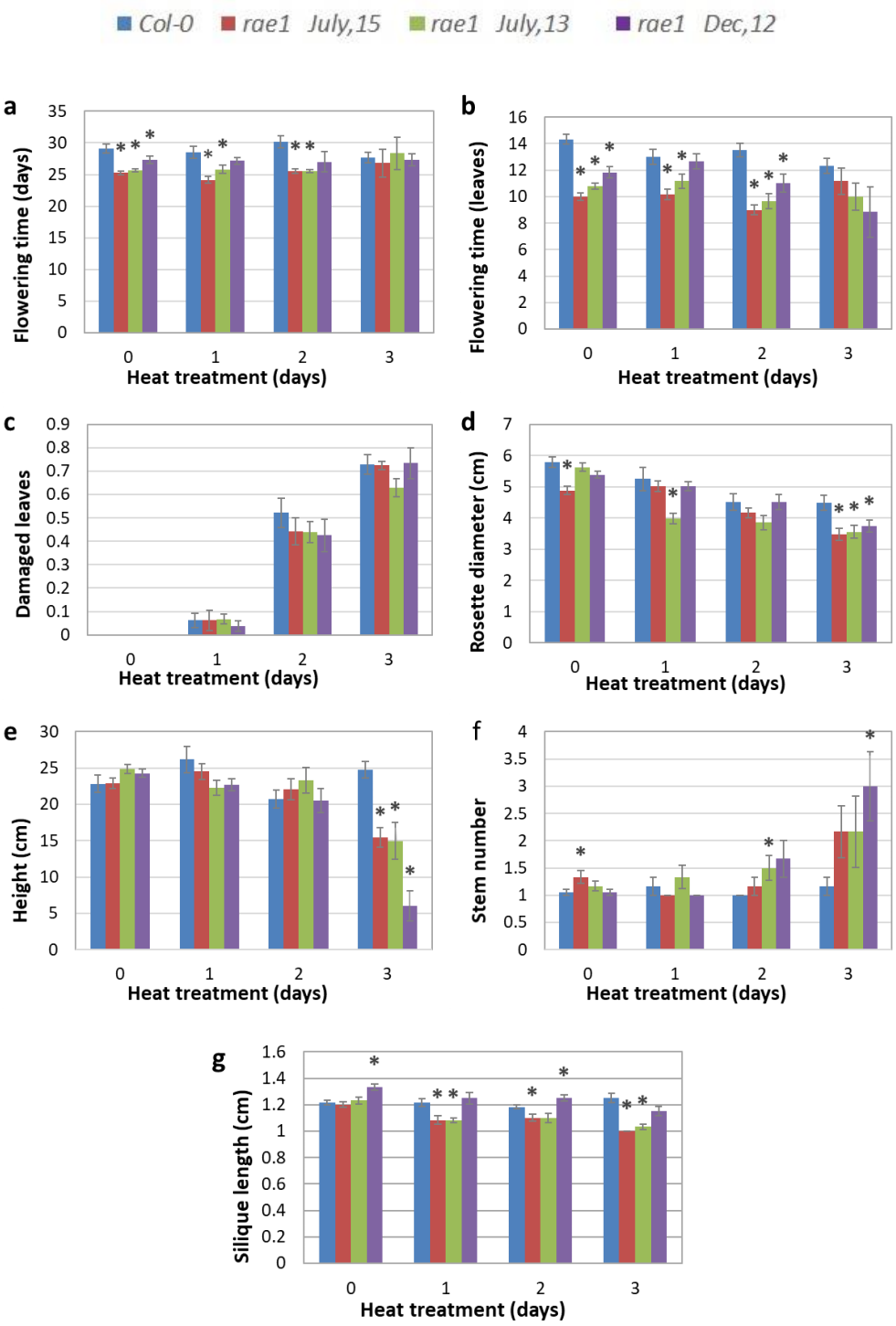


Figure 2.11 Adult growth and heat tolerance analysis of different batches of *rae1* seed. a) Flowering time in days, b) flowering time in leaves, c) fraction damaged leaves, d) rosette diameter, e) height, f) stem number, and g) silique length following 0-3 days at 37ºC. Error bars indicate SE (n=6) and * = P <0.05 relative to *Col-0*.

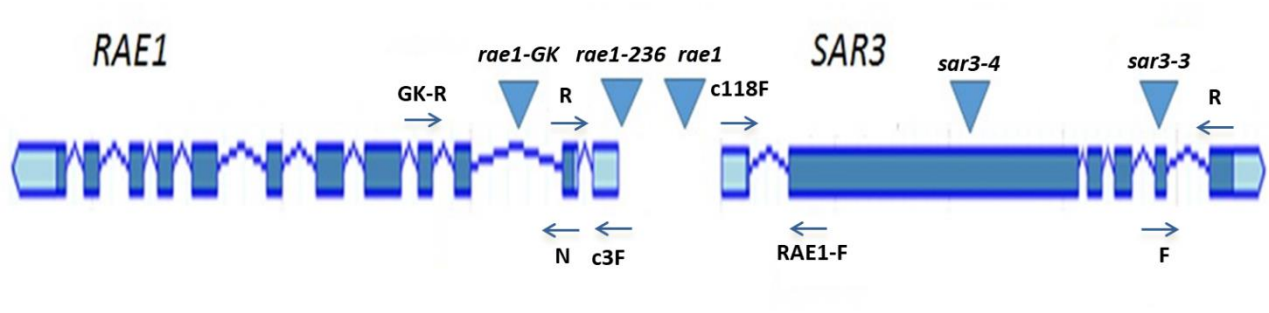


Figure 2.12 Map of *RAE1* and *SAR3* genes. *RAE1* is transcribed to the left and *SAR3* to the right. T-DNA allele insertion sites are indicated with triangles and primers used in semi-quantitative RT-PCR with arrows.

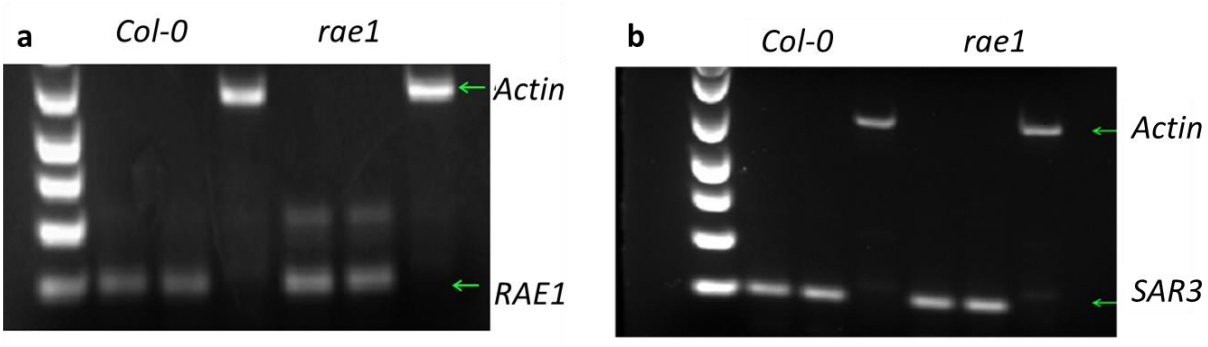


Figure 2.13 Semi-quantitative RT-PCR of *Col-0* and *rae1* RNA in control conditions. a) *RAE1* (RAE1-c3F/R primers), b) *SAR3* (SAR3-F/R primers) with *Actin* loading control.

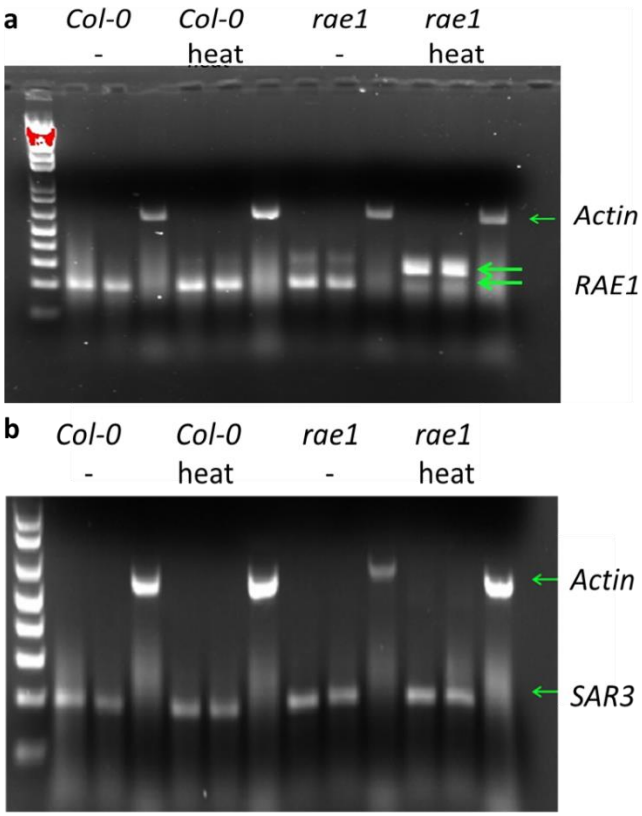


Figure 2.14 Semi-quantitative RT-PCR of *Col-0* and *rae1* in control and heat-treated conditions. a) *RAE1* (RAE1-c3F/R primers), b) *SAR3* (SAR3-F/R primers) with *Actin* loading control.

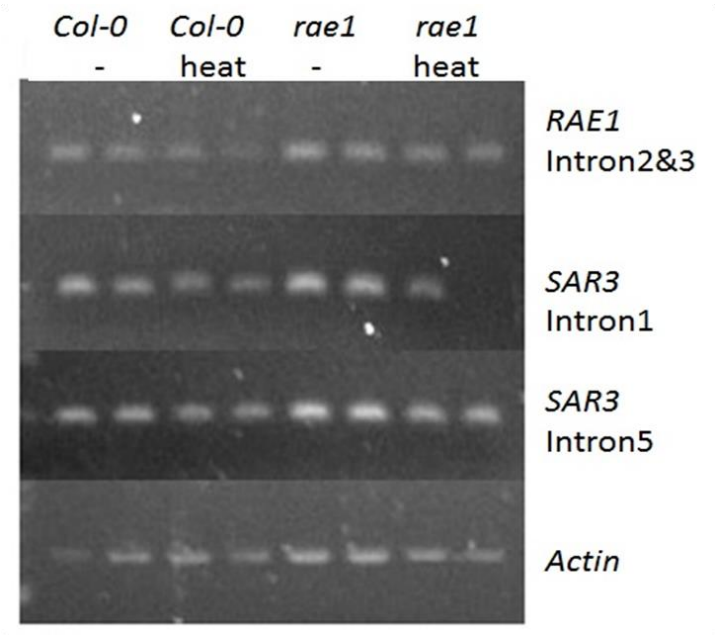


Figure 2.15 Semi-quantitative RT-PCR analysis of additional introns. Analysis of *Col-0* and *rae1* in control and heat-treated conditions with *RAE1* intron 2 & 3 primers (RAE1-GKR/N), *SAR3* intron 1 primers (SAR3-c118F/RAE1-F), *SAR3* intron 5 primers (SAR3-F/R), and *Actin* loading control.

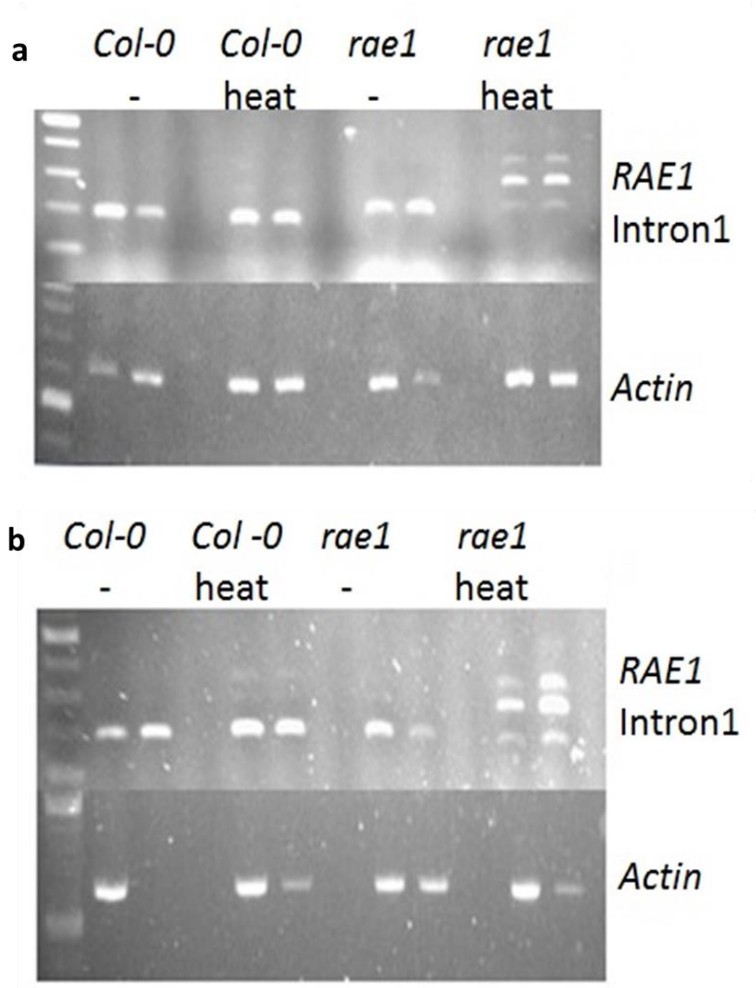


Figure 2.16 Semi-quantitative RT-PCR analysis of different *rae1* seed batches. Analysis of a) oldest *rae1* (2012) and b) newer *rae1* (2015) in control and heat-treated conditions with *RAE1* intron 1 primers (RAE1-R/c3F) and *Actin* loading control.

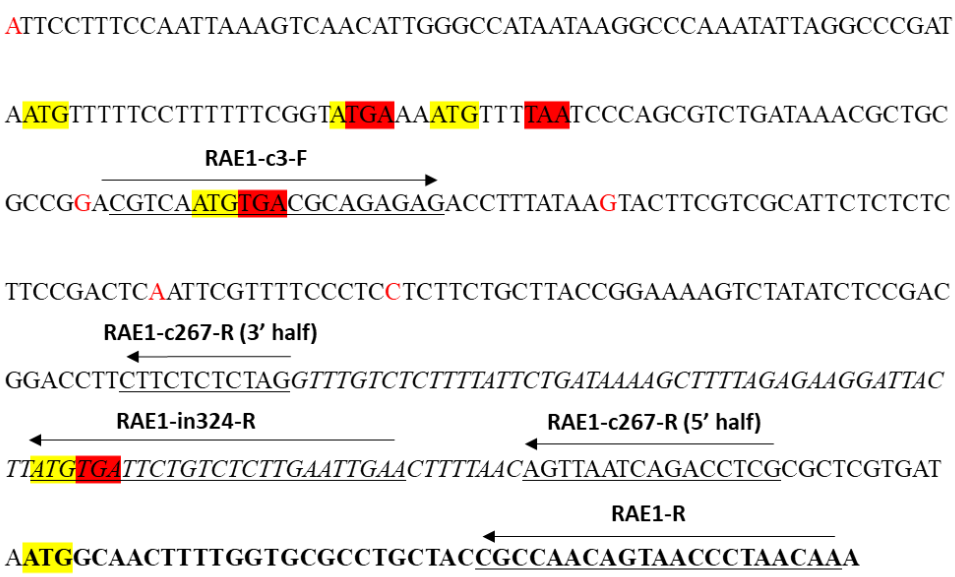


Figure 2.17 *RAE1* 5' untranslated region. Transcription start sites are indicated in red font, derived from Genbank mRNA sequences NM_106715, BP837699, BX840038, AY087683, and BX840152 respectively. Intron 1 is indicated in italics and coding sequence in bold. Start codons are highlighted in yellow and respective in frame stop codons in red. Primers used in semi-quantitative and quantitative RT-PCR analysis are indicated by arrows.

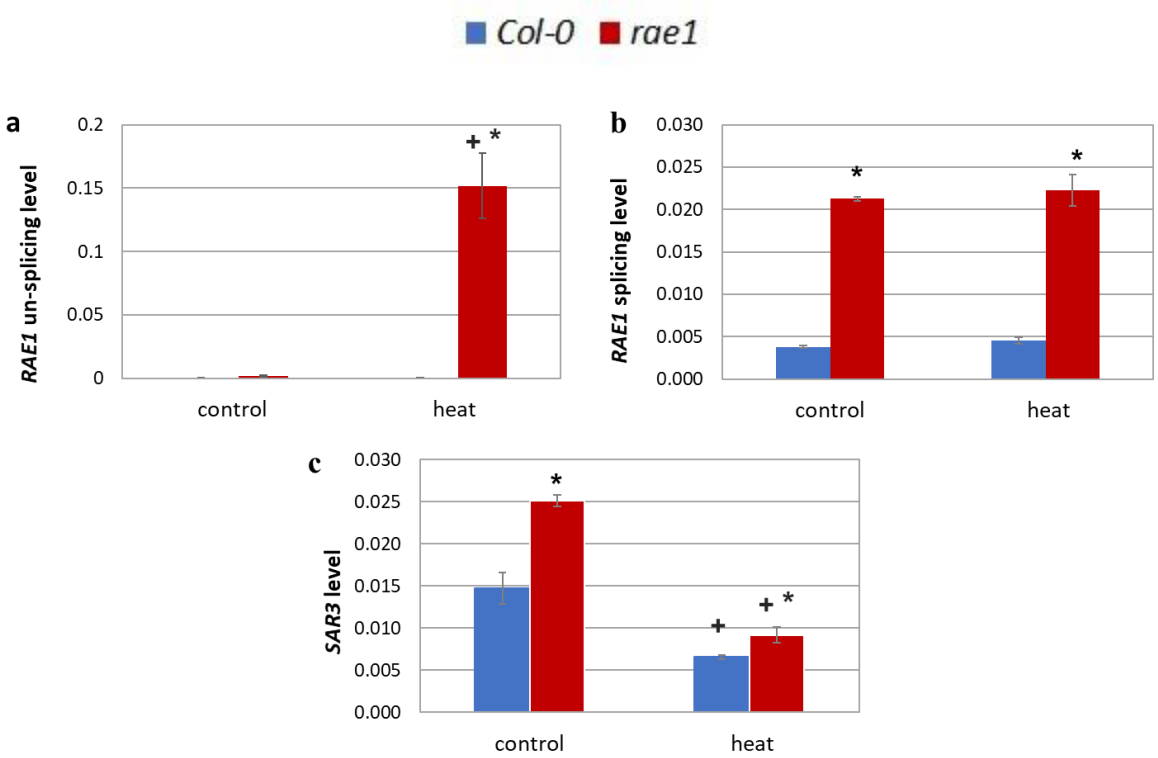


Figure 2.18 Quantitative RT-PCR analysis of *Col-0* and *rae1* in control and heat-treated conditions. a) unspliced *RAE1* intron 1 (RAE1-in324-R / c3-F primers), b) spliced *RAE1* intron 1 (RAE1-c267-R / c3-F primers), and c) *SAR3* intron 5 (SAR3-F/R primers). Values are normalized relative to reference gene *EF1α*. Error bars indicate SE of three technical replicates. * = P <0.05 relative to *Col-0* and + = significant effect of heat treatment.

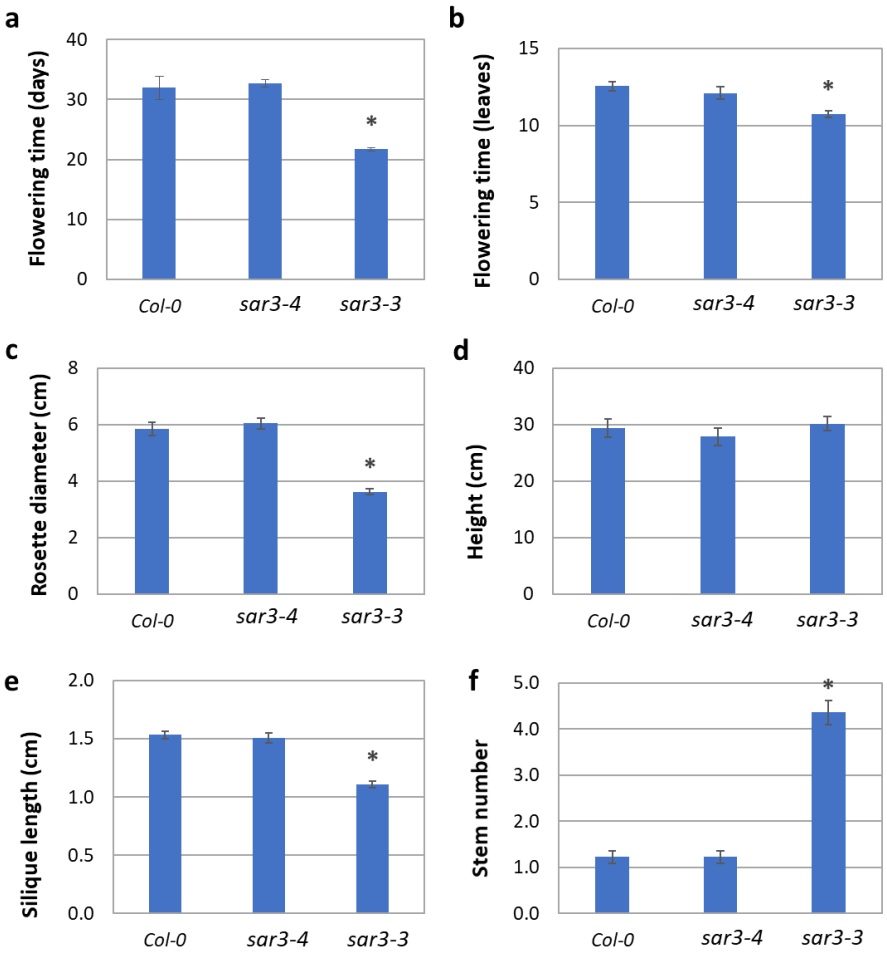


Figure 2.19 Adult growth analysis of *sar3* mutants in control conditions. a) Flowering time in days, b) flowering time in leaves, c) rosette diameter, d) height, e) silique length, and f) stem number. Error bars indicate SE (n=6) and * = P <0.05 relative to *Col-0*.

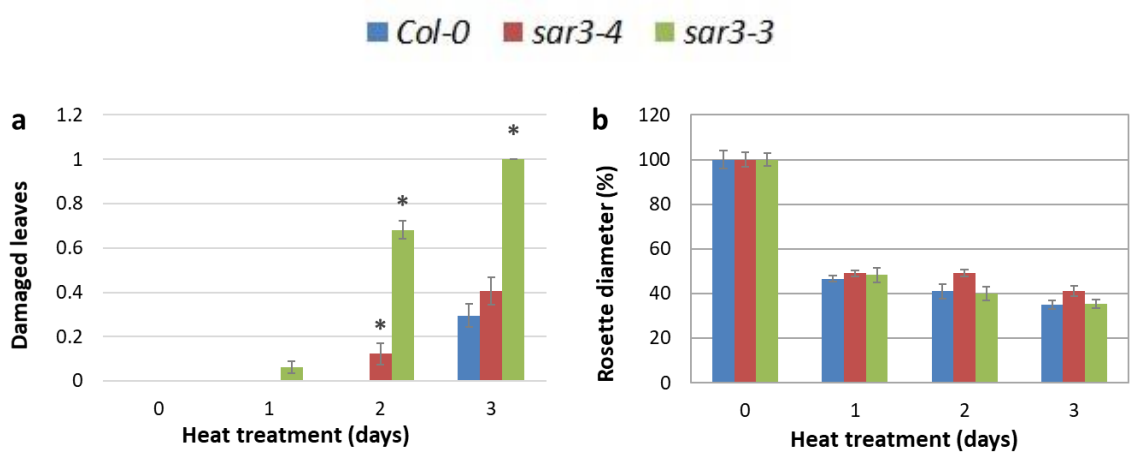


Figure 2.20 Adult heat tolerance of *sar3* mutants. a) Fraction damaged leaves and b) relative rosette diameter under control and heat conditions. Error bars indicate SE (n=6) and *= P <0.05 relative to *Col-0*.

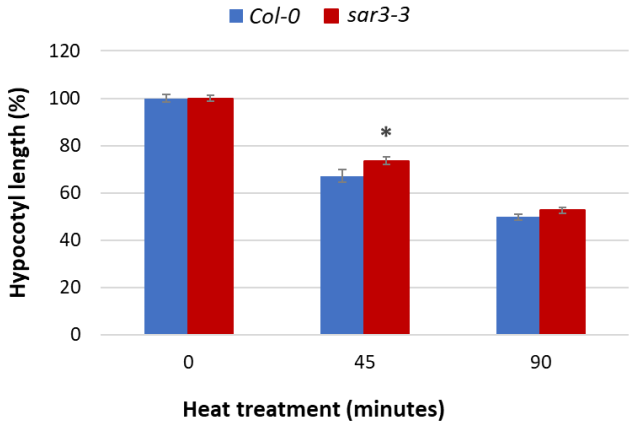


Figure 2.21 *sar3-3* seedling heat tolerance. Hypocotyl length of heat-treated seedlings relative to untreated controls of the same genotype. Error bars indicate SE (n=18) and * = P <0.05 relative to *Col-0*.

Table 2.5 Analysis of silique phenotypes and inheritance of strong *rae1* alleles.

| | *rae1-236* | | *rae1-GK* | |
|---|---|---|---|---|
| | wt | het | wt | het |
| Abnormal/total seeds | 7/38 | 17/31 | 11/34 | 13/37 |
| | 2/48 | 13/20 | 14/33 | 16/33 |
| | - | - | 13/36 | 15/37 |

| *rae1-236*/+ crosses | het X het | het (F) X wt (M) | wt (F) X het (M) |
|---|---|---|---|
| progeny genotype (het / total) | 11/24 | 0/19 | 22/39 |


Figure 2.22 Siliques derived from segregating *rae1-236*/+ population.


Figure 2.23 Siliques derived from segregating *rae1-GK/+* population.

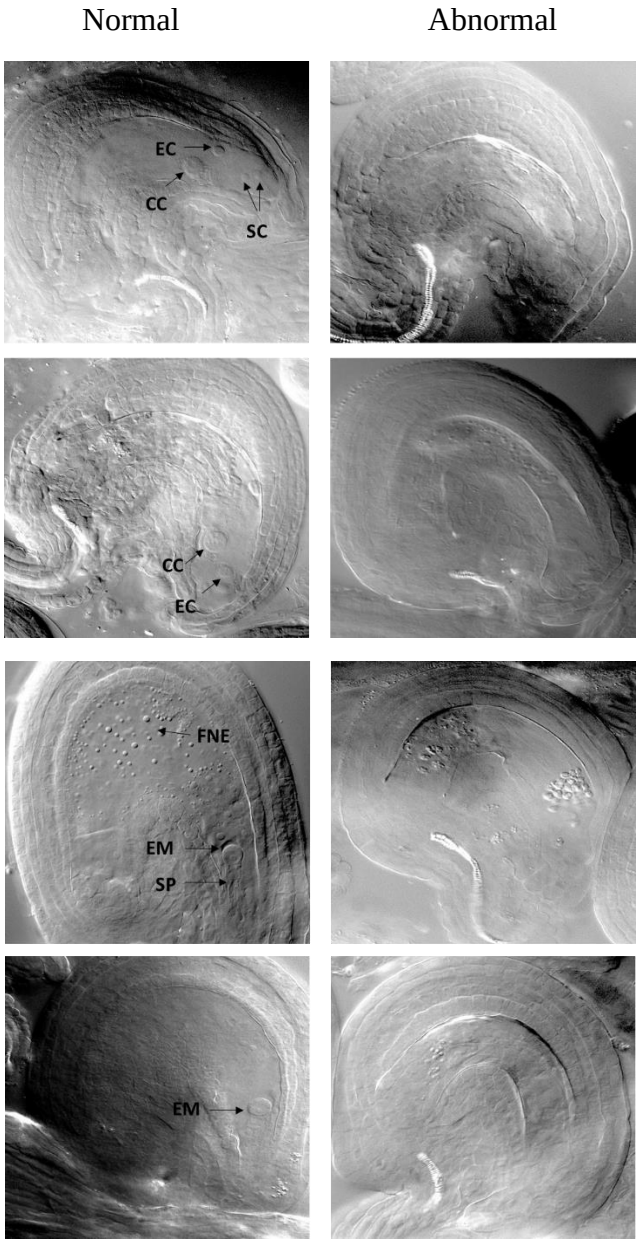


Figure 2.24 DIC images of female gamete phenotype of *rae1-236* heterozygotes. Each row shows normal (left) and abnormal (right) cleared ovules derived from the same *rae1-236/+* carpel/silique. Pre-fertilization carpels are shown at the top and early embryos at the bottom. (EC) egg cell, (SC) synergid cell, (CC) central cell, (EM) embryo, (SP) suspensor, and (FNE) free nuclear endosperm.

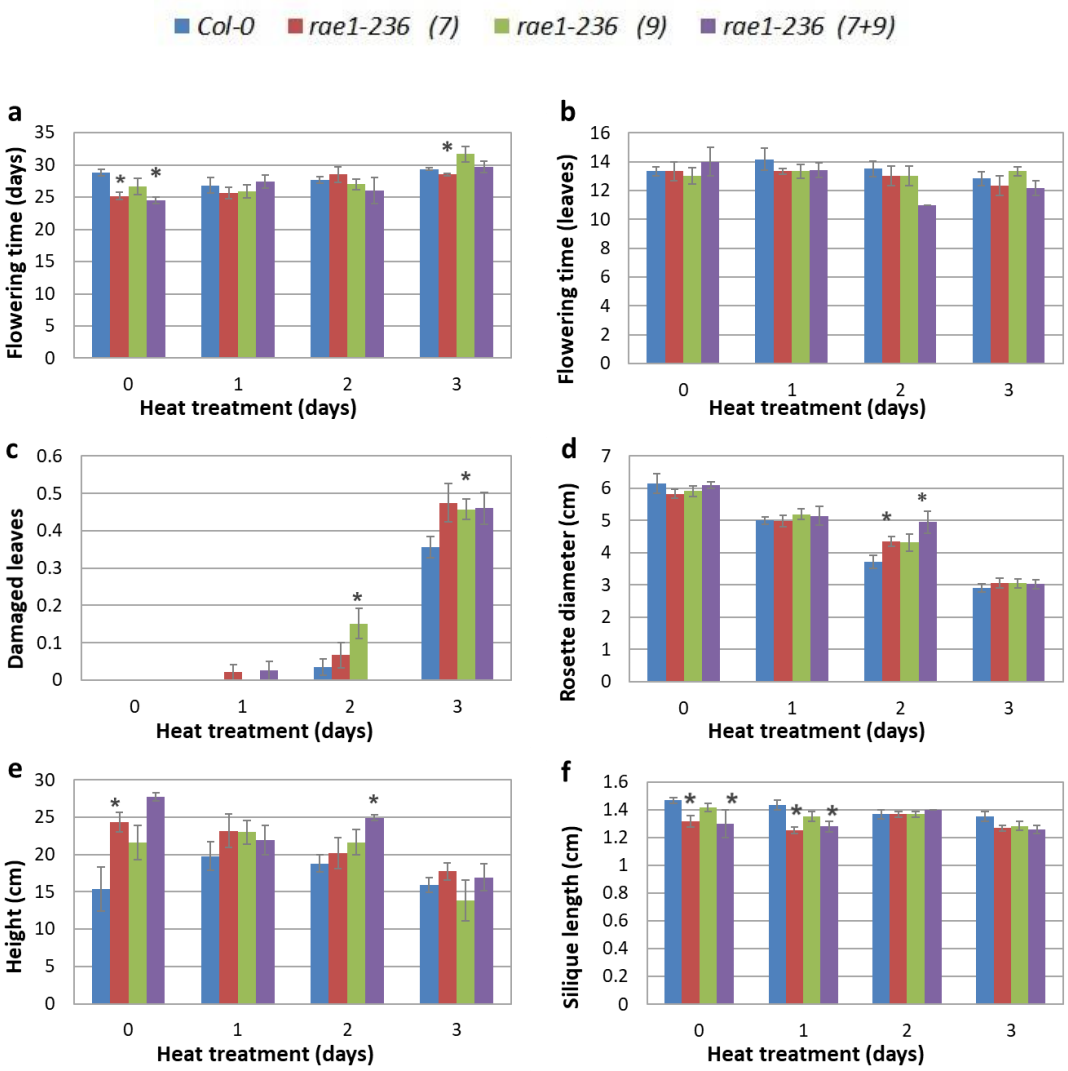


Figure 2.25 Adult growth and heat tolerance analysis of *rae1-236* heterozygotes. a) Flowering time in days, b) flowering time in leaves, c) fraction damaged leaves, d) rosette diameter, e) height, and f) silique length. #7 and #9 are segregating *rae1-236/+* populations while 7+9 includes confirmed heterozygotes from both lines. Error bars indicate SE (n=6) and * =P <0.05 relative to *Col-0.*

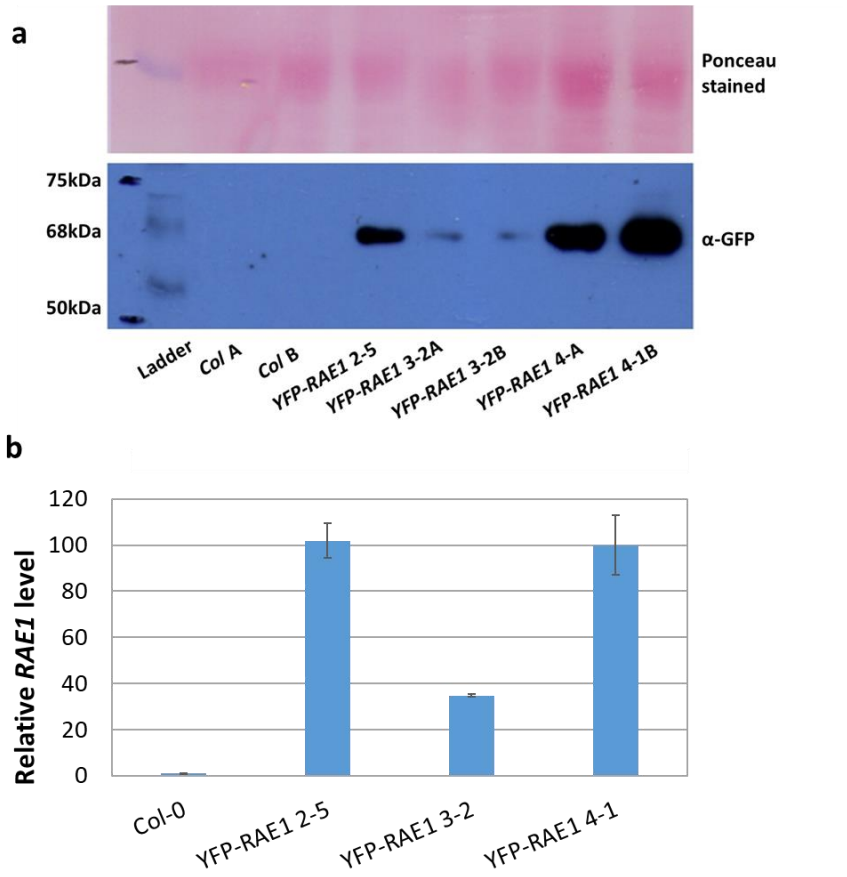


Figure 2.26 Expression analysis of YFP-RAE1 overexpression lines. a) Western blot of *Col-0* and YFP-RAE1 overexpression lines showing anti-GFP detection and Ponceau staining. b) Quantitative RT-PCR of YFP-RAE1 lines showing relative *RAE1* transcript level. Values were normalized with reference gene *EF1*□. Error bars indicate SE of three technical replicates.

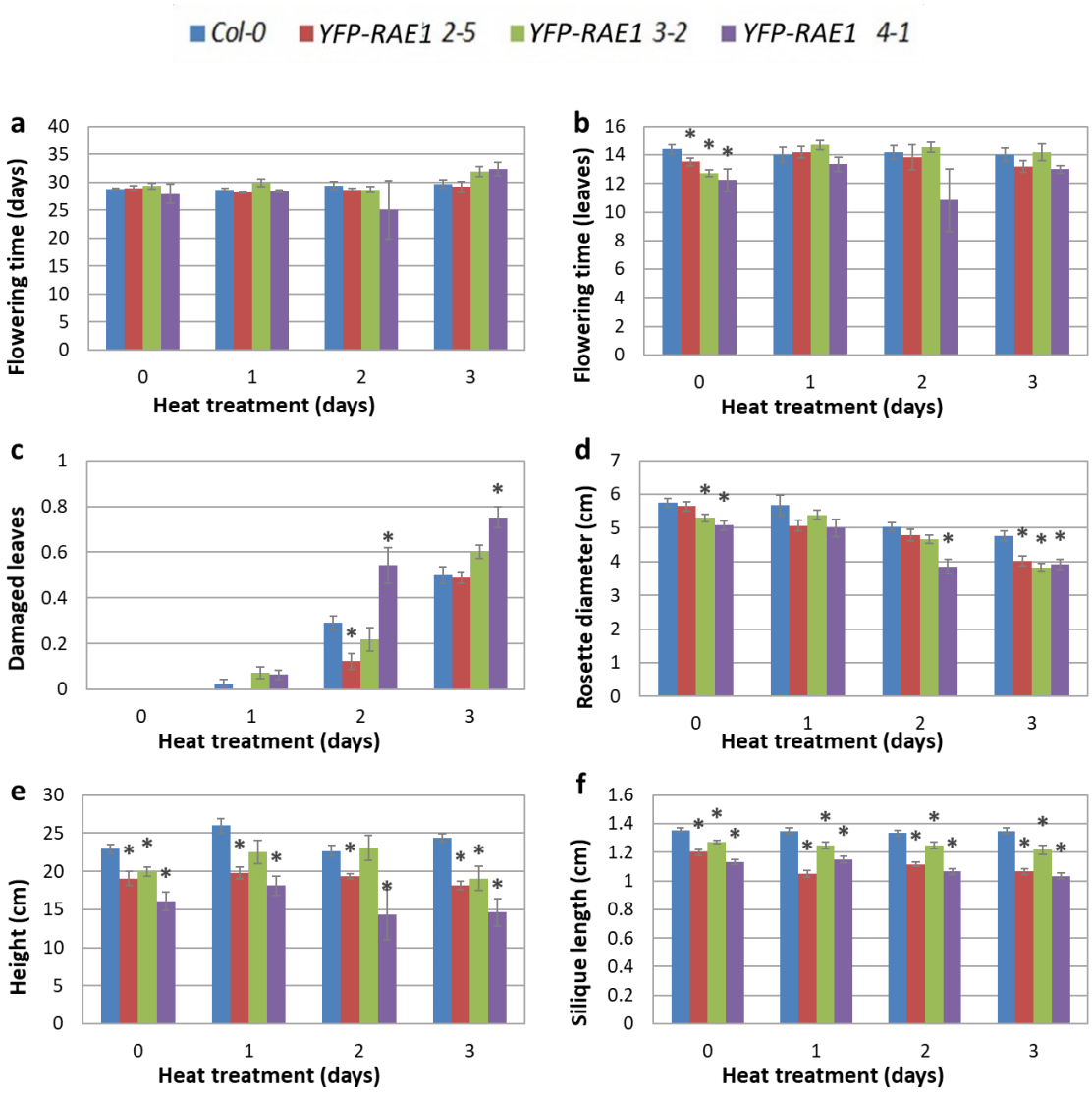


Figure 2.27 Adult growth and heat tolerance analysis of YFP-RAE1 overexpression lines. a) Flowering time in days, b) flowering time in leaves, c) fraction damaged leaves, d) rosette diameter, e) height, f) silique length. Error bars indicate SE (n=6) and * = P <0.05 relative to *Col-0*.


Figure 2.28 Representative adult *YFP-RAE1* overexpression lines after heat treatment.

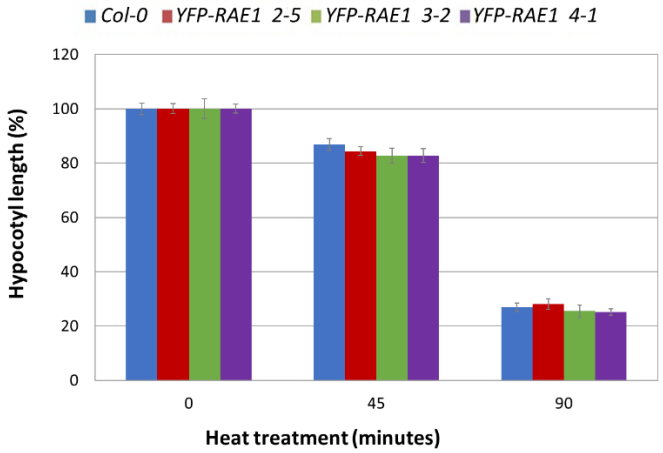


Figure 2.29 Seedling heat tolerance of *YFP-RAE1* overexpression lines. Hypocotyl length of heat-treated seedlings relative to untreated controls of the same genotype. Error bars indicate SE (n=15).

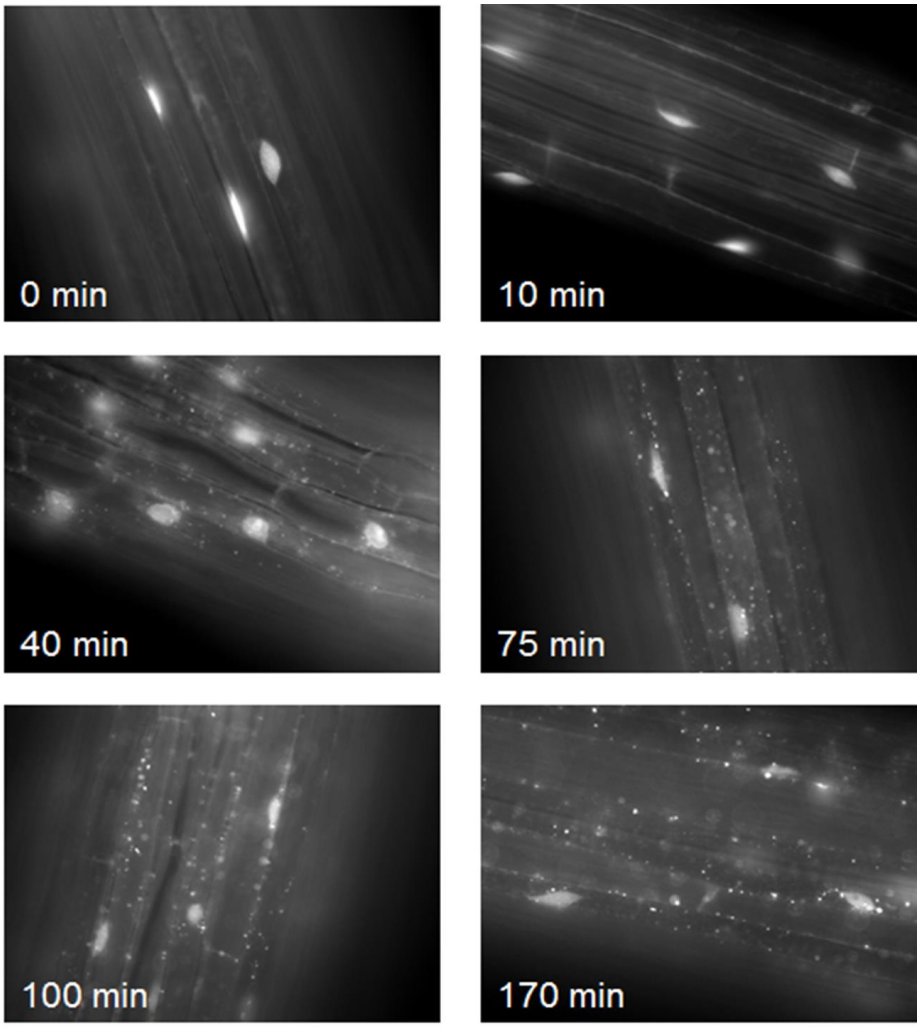


Figure 2.30 YFP-RAE1 localization following heat treatment. YFP-RAE1 in hypocotyl cells of 4-day old dark grown Arabidopsis seedlings viewed under 40X magnification following 37°C treatment for 0, 10, 40, 75, 100, and 170 minutes.

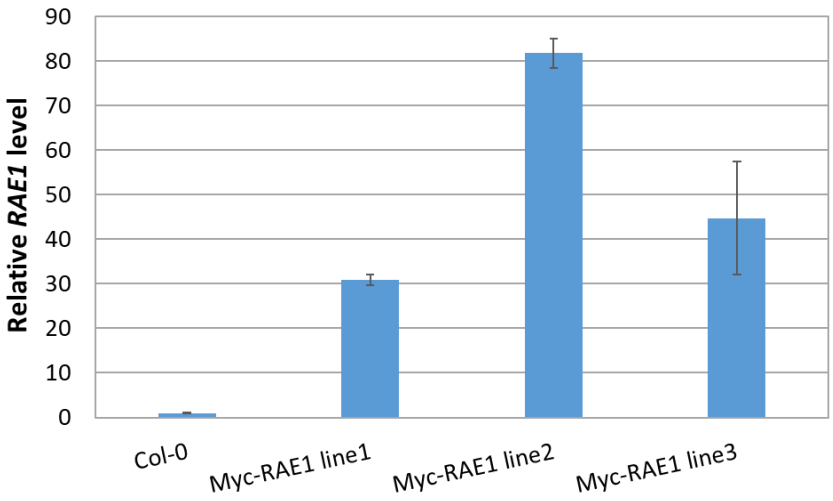


Figure 2.31 Quantitative RT-PCR of Myc-RAE1 overexpression lines showing relative *RAE1* transcript level. Values were normalized with reference gene *EF1α*. Error bars indicate SE of three technical replicates.

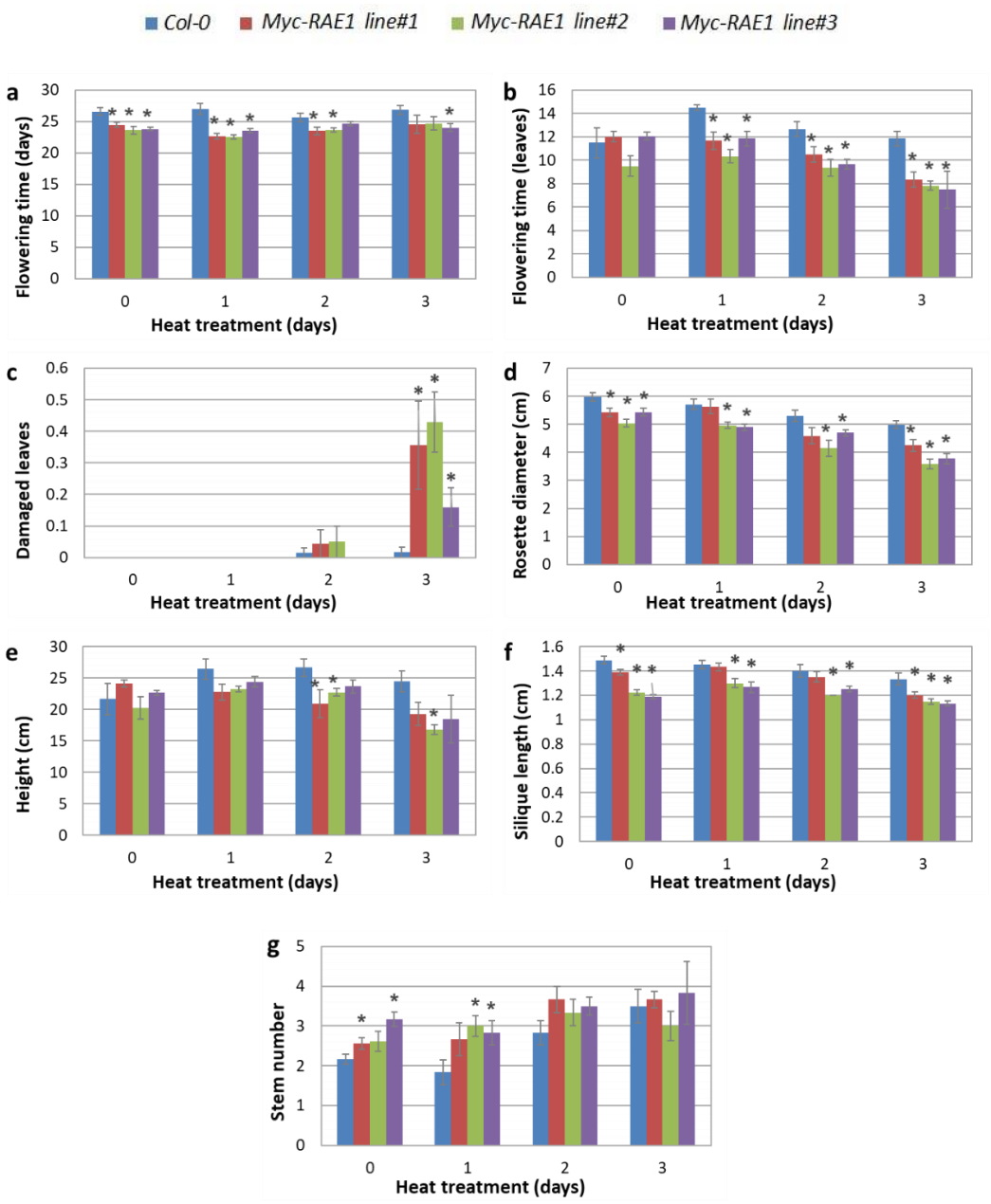


Figure 2.32 Adult growth and heat tolerance of Myc-RAE1 overexpression lines. a) Flowering time in days, b) flowering time in leaves, c) fraction damaged leaves, d) rosette diameter, e) height, f) silique length, g) stem number. Error bars indicate SE (n=6) and * = P <0.05 relative to *Col-0*.

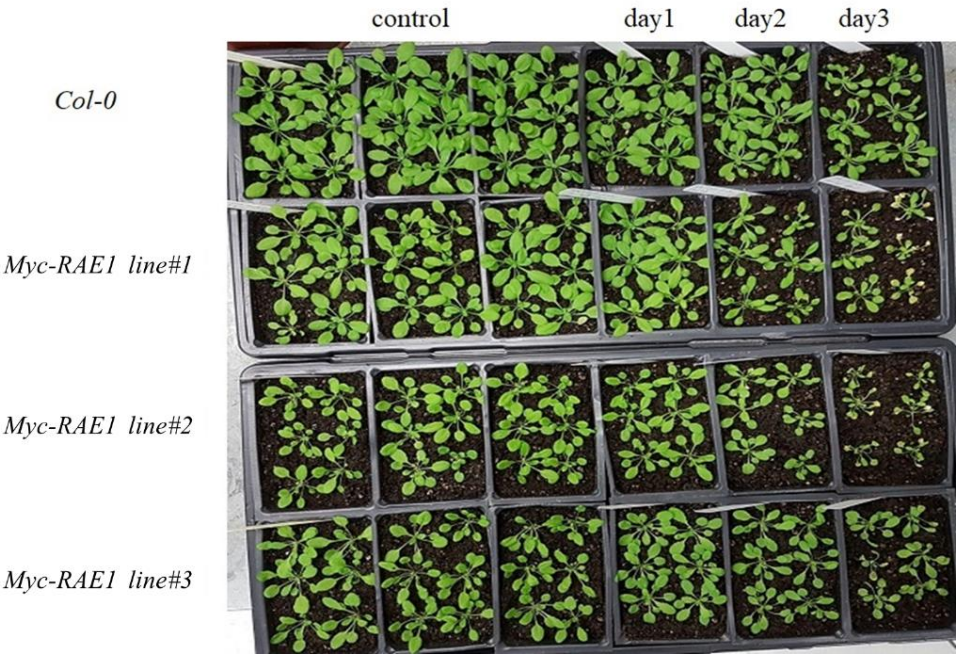


Figure 2.33 Representative adult *Myc-RAE1* overexpression lines after heat treatment.

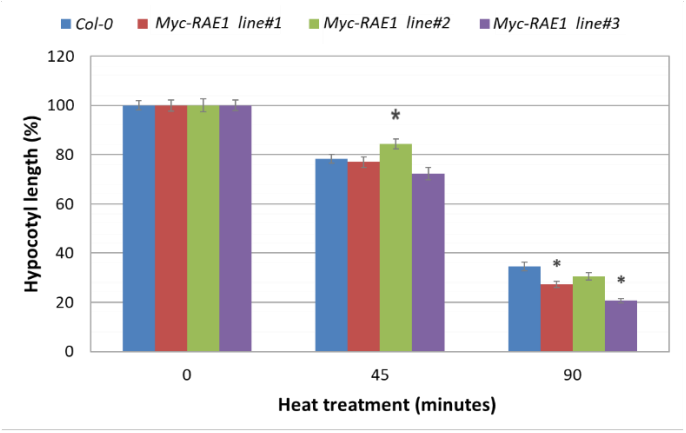


Figure 2.34 Seedling heat tolerance of Myc-RAE1 overexpression lines. Hypocotyl length of heat-treated seedlings relative to untreated controls of the same genotype. Error bars indicate SE (n=20) and * = P <0.05 relative to *Col-0*.

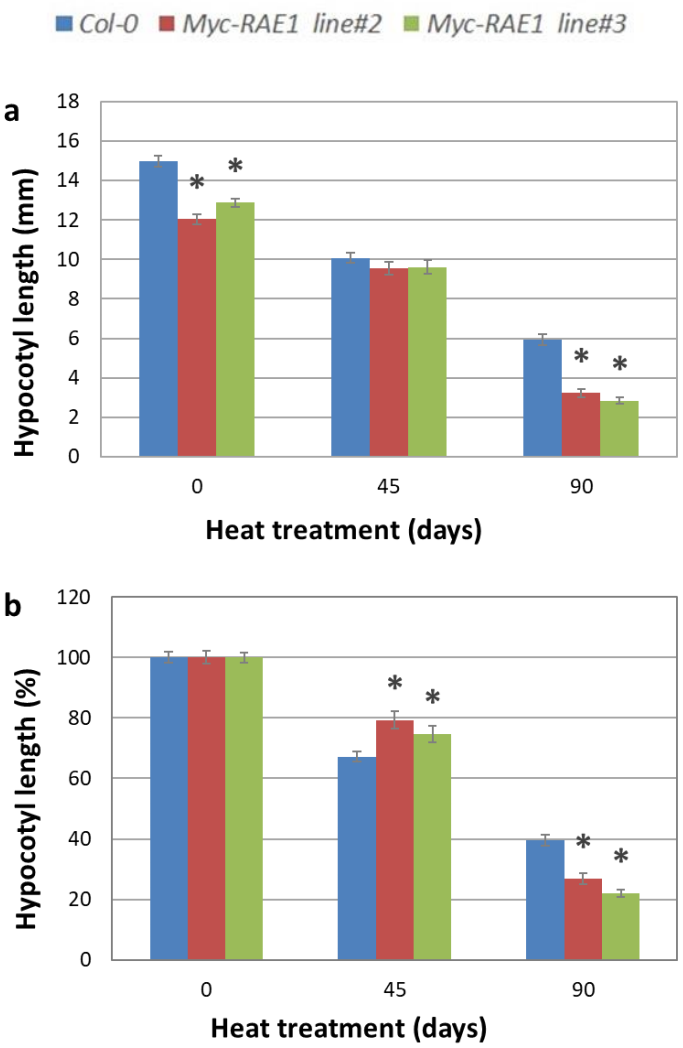


Figure 2.35 Absolute and relative hypocotyl length of heat-treated Myc-RAE1 overexpression lines. a) Absolute hypocotyl length and b) hypocotyl length of heat-treated seedlings relative to untreated controls of the same genotype. Error bars indicate SE (n=20) and * = P <0.05 relative to *Col-0*.

## 2.4 Discussion

### 2.4.1 Role of DWD genes in heat tolerance

We examined the role of heat-induced DWD genes in Arabidopsis growth and heat response, in order to identify heat specific components of DDB1-CUL4 complexes. *dwh1* mutants exhibited decreased height and apical dominance but did not show heat sensitivity. *dwh2* mutants were also not heat sensitive but showed early flowering and decreased height phenotypes. *DWH2*, also known as Arabidopsis Ubiquitin Ligase Complex Subunit 1 (*ULCS1*), silenced lines displayed abnormal phenotypes like increased growth, deformed leaves, and variable seed size (Beris et al., 2016). Given the contrasting nature of these phenotypes, perhaps our *DWH2* T-DNA promoter allele upregulates *DWH2* expression. We found *csa* mutants did not exhibit growth or heat sensitivity phenotypes while *pex7* mutants exhibited delayed flowering and increased rosette diameter, but did not display consistent heat phenotypes. Previously *PEX7* knockdown and overexpression resulted in wildtype Arabidopsis development (Hayashi et al., 2005; Singh et al., 2009).

In other studies, another heat-induced DWD, heat stress tolerant DWD1 (*HTD1*), was identified (Kim et al., 2014). *HTD1* exhibited an increase of transcript level following return to room temperature after exposure to high temperature (3 hr at 38 $^{o}$C plus 1 hr 25 $^{o}$C) (Kilian et al., 2007). HTD1 interacts with DDB1a as well as HSP90. Following heat, *htd1* mutants showed increased heat tolerance and increased levels of heat-induced gene expression (Kim et al., 2014). Thus, *htd1* mutants exhibit the opposite phenotype of *ddb1* mutants (Ganpudi & Schroeder, 2013), suggesting that HTD1 may be a negative regulator of DDB1-CUL4 activity.

In the current research, we found that both loss of function (e.g. *rae1-236* heterozygotes) and gain of function (e.g. *rae1* promoter allele, overexpressed YFP-RAE1 and Myc-RAE1) of

DWD protein RAE1 resulted in increased heat sensitivity, as well as flowering time and growth phenotypes. Other E3 ligase components, such as RBX1, also exhibit similar phenotypes in loss of function and overexpression lines (Gray et al., 2002). RAE1 interacts with DDB1a and DDB1b in yeast two hybrid assays (Lee et al., 2008; Li et al., 2018), and was recently found to interact with ABA receptor RCAR1 (Li et al., 2018); however, the role of these interactions in *RAE1* heat and growth phenotypes has yet to be determined.

### 2.4.2 Molecular analysis of *rae1* allele

In our research we discovered that levels of unspliced *RAE1* intron 1 increased in the *rae1* mutant in response to heat stress. Intron retention (IR) is one type of Alternative Splicing (AS), which is one of the main mechanisms of heat stress response (Guerra et al., 2015). In the development of tomato pollen, IR in transcripts, including those of heat shock proteins (HSP) and heat shock transcription factors (HSF) genes, increases under heat stress (Keller et al., 2017). In addition, AS of Arabidopsis Hsfa2 is regulated by heat (Liu et al., 2013). In rice, the *DEHYDRATION-RESPONSIVE ELEMENT BINDING PROTEIN2 (DREB2B)* transcript only encodes a functional protein when exposed to heat stress via exon 2 skipping, which allows the protein to respond to heat stress (Staiger & Brown, 2013). IR in the Arabidopsis *EARLY FLOWERING 3* (*ELF3β*) transcript occurred upon exposure to heat stress (Kwon et al., 2014). IR in grape leaves increased following heat treatment, in fact IR was the most common AS event observed (Jiang et al., 2017). Thus, IR is a common response to heat stress.

However, the functional importance of *RAE1* intron 1 retention is unclear. There are no functionally meaningful start codons within intron 1 (Figure 2.17), thus the RAE1 protein encoded should be unchanged. The levels of the unspliced product in *rae1* following heat

treatment exceed those of the spliced product (Figure 2.18), thus the *rae1* heat phenotype may be a gain of function effect. With respect to the spliced product, while semi-quantitative RT-PCR indicated decreased levels in *rae1* following heat treatment (e.g. Figure 2.14a), quantitative RT-PCR did not show this effect (Figure 2.18b). This difference may be due to the fact that slightly different primers were used for these analyses (Figure 2.17), or to technical differences between the techniques. *SAR3* was upregulated in *rae1* in control conditions, but downregulated following heat in both wildtype and *rae1* (Figure 2.18c). Thus, both *RAE1* and *SAR3* are upregulated in *rae1* in control conditions while the *RAE1* unspliced product is highly upregulated following heat treatment, suggesting that *rae1* may be acting as a gain of function allele and that the *rae1* T-DNA insertion alters regulation of *SAR3* and *RAE1* transcription and splicing. In addition, changes in the *RAE1* 5' UTR due to intron retention may alter RAE1 protein levels (Dvir et al., 2013).

### 2.4.3 *RAE1* phenotypes

We found that Arabidopsis *RAE1* gain of function and loss of function resulted in heat sensitivity, early flowering time, reduced growth, and female gamete lethality. In *Drosophila melanogaster,* studies have shown that Rae1 has an important role in cell proliferation. Rae1 depletion caused cells to accumulate in the G1 phase. Rae1 regulates the G1 and S phases that impact cyclin E expression required for DNA duplication (Sitterlin, 2004). In *S. cerevisiae*, Rae1/Gle2 interact with components of the anaphase promoting complex (APC), which is the main regulator for the correct timing of chromosome separation, as well as the cytoskeleton and kinetochore proteins. These proteins participate in cell cycle progression and regulation. Interaction between Gle2 and Cdc16, an APC-component, facilitates chromosome segregation

(Zander et al, 2017). In mammals and yeast, Rae1/Gle2 mutants accumulate poly(A) RNA in nucleus. Rae1 is also required for microtubule regulation (Blower et al., 2005). In human cells, Rae1/Gle2 participates with kinetochore checkpoint proteins to maintain mitotic division (Ren et al., 2010). *Rae1* loss of function lead to defect in chromosome separation in mouse (Babu et al., 2003) as well as tobacco (Lee et al., 2009). Tobacco *Rae1* silencing reduced plant growth due to defects in mitotic division of the meristem tissue (Lee et al., 2009).

### 2.4.4 *SAR3* phenotypes

We also examined mutants of *SAR3*, the gene adjacent to *RAE1,* that encodes another component of the nuclear pore complex, and again detected flowering time, growth, and heat sensitivity phenotypes. In our study, surprisingly no growth or heat sensitivity phenotypes were observed in *sar3-4.* Analysis of salt and ABA sensitivity of this allele did not detect a phenotype (Zhu et al., 2017). On the other hand, we found that *sar3-3* exhibited early flowering, as well as decreased rosette diameter, silique length, and apical dominance in control conditions. Following heat treatment, *sar3-3* showed increased leaf damage, but no growth phenotypes. Similar results have been observed in *uvh6 det1* double mutants following heat treatment, where double mutants exhibited enhanced death and reduced heat-induced growth inhibition (Kim et al., 2012). In other studies, *SAR3/MOS3/NUP96* mutants have been found to display developmental phenotypes and resist fungal and bacterial infections. The *sar3-3* mutant showed early flowering, decreased plant height and fertility, and short roots (Parry, 2013). *sar3* mutants also suppress auxin resistance and auxin is an important regulator of plant growth and development. Auxin transport deficient *aux1* seedlings displayed a reduction in root elongation following heat treatment, suggesting heat

sensitivity (Krishnamurthy & Rathinasabapathi, 2013). Thus, the *sar3* heat sensitivity phenotype may be due to interaction with auxin response.

### 2.4.5 Role of the plant NPC in abiotic stress response

Studies have shown that NPC components are involved in plant abiotic stress response (Yang et al., 2017). Arabidopsis NPC component high expression of osmotically responsive gene 1 (HOS1) regulates low temperature stress response through the ubiquitin proteasome pathway (Dong et al., 2006). *hos1* mutants stimulate C-repeat-binding factor (CBF) transcription factors and their downstream cold-responsive genes (COR) (Chimnnusamy et al., 2007). HOS1 encodes an E3 ligase component that accumulates in the nucleus during cold stress response. In addition, the transcription factor INDUCER OF CBF EXPRESSION 1 (ICE1) regulates CBF genes and cold stress response in Arabidopsis (Chinnusamy et al., 2003). This factor interacts with HOS1 to degrade it (Dong et al., 2006).

In addition to the function of NPC and NUPs in nucleocytoplasmic transport, they were also found to be involved in many other cellular functions. In plants, some nucleoporin mutants exhibit early flowering phenotypes (Tamura & Hara-Nishimura, 2014). One of these mutations, *tpr/nua,* reduced the expression of floral repressor genes and increased the expression level of floral activator genes, which suggests that TPR/ NUA is an inhibitor of flowering (Tamura & Hara-Nishimura, 2014).

Other NUPs are essential for plant viability. *Modifier of Snc 7* (*MOS7*) is the homolog of human and Drosophila *NUP88*. It is involved in mitosis in addition to its function in transport. Arabidopsis *MOS7/NUP88* complete loss of function alleles exhibit lethality. The mutant showed a deficiency in male and female gametophytes in either pre or post first mitotic division

and subsequent embryo abortion (Park et al., 2014). Furthermore, MOS7 localized to the spindle during mitosis, and has a role in microtubule dynamics (Tamura et al., 2010). In addition, mutations in other Arabidopsis NPC components such as LNO1, which also participates in mRNA export, also showed embryonic lethality (Braud et al., 2012).

### 2.4.6 Strong *RAE1* allele results in female gamete lethality

In *rae1-236* heterozygous mutants, we identified a defect in the female gamete, however it is not clear at which stage failure occurred. Some female gametophyte mutations in Arabidopsis exhibit defects in embryo sac development called embryo sac development arrest (EDA) where the failure is in mega-gametogenesis nuclear division (Pagnussat et al., 2005). However, the *gametophytic factor 2 (gfa2)* mutant affected the polar nuclear fusion phase, making the fusion fail (Christensen et al., 1998). Also, *gfa3* and *gfa7* mutants affected the polar nuclear fusion. *female gametophyte 4* (*fem4*) mutants impact cellular morphology, resulting in disorder in nuclear positions (Christensen et al., 1998). Furthermore, *nuclear fusion defective (nfd1-6)* mutants influence the fusion of polar nuclei because either of failure of the external membrane connection for the two nuclei or failure to initiate membrane fusion (Portereiko et al., 2006). Defects in *fam2, fem3, gfa4,* and *gfa5* mutants occurred during the mitosis stage resulting in FG1 arrest in embryo sac development (Christensen et al., 1997). FG2 and FG4 arrest results from *nomega* and *prl* (PROLIFERA) mutants due to their impact on the second and third divisions of nuclear mitosis (Liu & Qu, 2008; Springer et al., 2000).

Moreover, female gamete defects occur in meiosis as well (Liu & Qu, 2008). *Three-division mutant* (*ms5/tdm1*) initiation appeared normal after meiotic division II and soon it decomposes (Caryl et al., 2003). Some Arabidopsis mutations result in defects during

chromosome synapsis. Failure in chromosome synapsis creation in prophase 1 by *asynaplic 1* (*asy1*) mutant results in decreased male and female fertility (Ross et al., 1997). In *desynaptic 1* (*dsy1*) mutants, following the complement of diplotene homolog chromosome synapsis, it turns out that they are not properly connected together due to the disorder in chiasma formation in anaphase I (Caryl et al., 2003).

### 2.4.7 Future directions

Future research on this project may include the following experiments.

1) Generation of viable partial loss of function *RAE1* material via, for example, RNA interference, as well as tagless overexpression lines, to further examine the interplay between *RAE1* loss and gain of function and the effect of the RAE1 dose on heat tolerance. 2) Examination of the effect of *RAE1* level on mRNA export by using Oligo (dT) in situ hybridization. 3) Further investigation of the *rae1-236* female gametophyte lethality phenotype by examining earlier carpels using DAPI staining, so we can determine if the arrest is happening during meiotic or mitotic division. 4) Generation of *ddb1 rae1* double mutants and examine heat sensitivity in order to investigate the role of DDB1 RAE1 interaction in heat tolerance.

### 2.4.8 Conclusion

In this research, the role of six candidate heat-induced genes in growth and heat tolerance was examined. Only *rae1* exhibited growth and heat sensitivity phenotypes. The *rae1* promoter allele results in *RAE1* intron 1 retention after heat treatment. Strong *rae1* alleles result in female gamete lethality while heterozygotes exhibit growth and heat sensitivity phenotypes. RAE1

overexpression lines also displayed growth and heat sensitivity phenotypes. Thus, RAE1 plays an important role in Arabidopsis heat tolerance.

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