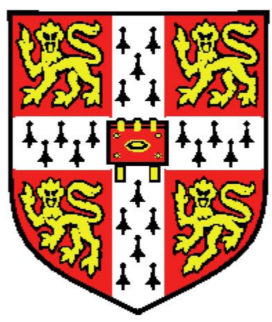
Investigating temperature signalling pathways in *Arabidopsis thaliana* using small molecules



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A thesis submitted for the degree of
Doctor of Philosophy in Plant Sciences

April 2018

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David Schoepfer

Upon exposure to heat or cold, *Arabidopsis thaliana* seedlings undergo rapid transcriptional reprogramming of several hundreds of genes that promote stress tolerance. Despite extensive characterisation of the transcriptional responses to these stimuli, however, relatively little is known about the mechanisms by which temperature signals are perceived and transduced in plant cells. High or low seasonal temperatures have large impacts on crop productivity and are expected to intensify given current global climatic projections. It is therefore of agricultural importance to better understand temperature signalling pathways in plants in order to find solutions to this problem. In this thesis, a chemical genomics screen for molecules activating or repressing heat-inducible genes in *A. thaliana* was performed in collaboration with Syngenta and the biological targets of these chemicals were predicted based on structural similarities to compounds with known modes of action. Many molecules that affect the function of chloroplasts or mitochondria either activate or repress heat-responsive genes, thus implicating these organelles in the regulation of plant temperature responses. In addition, the translation inhibitor cycloheximide was identified as a repressor of heat-inducible genes and an activator of early cold-inducible genes. Diverse translation inhibitors trigger a cytosolic influx of calcium ions and several inhibitors of translation elongation were found to strongly activate cold-inducible gene expression in a calcium-dependent manner. Furthermore, it was demonstrated that cold shock causes rapid translation repression in *A. thaliana* seedlings and that the elongation factor LOS1 is involved in cold- or cycloheximide-induced gene expression, thus implicating translational machinery in the regulation of temperature signalling in plants. Finally, one of the chemicals identified in the screen, S01A463859Y, was found to improve heat resilience in *A. thaliana* and may therefore be of potential use in enhancing crop productivity during thermal stress.

Acknowledgements

I would like to thank my PhD supervisor Philip Wigge for accepting me as a PhD student in his group, for his guidance over the last four years and for showing me how to conduct high-impact scientific research. Thank you to all past and present members of the Wigge lab and researchers from the Sainsbury Laboratory who have helped me along my journey as a PhD student and contributed to this work; Varodom (Yod) Charoensawan, Sandra Cortijo, Daphne Ezer and Hugo Tavares for useful discussions and their help with bioinformatic analyses, particularly the Bash script written by Yod for analysing RNA-sequencing data and the cold-shock *A. thaliana* RNA-seq dataset produced by Sandra that is referred to in *Chapter 4*; Poppy Marriott and Marie Pireyre for being great lab managers, helping to keep the lab running smoothly and organising social events in the group; and Mathew Box, Betty Chung, Jaehoon Jung and Fu Xiang Quah for scientific discussions and making the lab a fun place to work.

I am grateful to the Biotechnology and Biological Sciences Research Council (BBSRC) for the iCASE PhD studentship that supported me during my PhD. I extend my gratitude to Syngenta for their collaboration during my PhD, for their financial support and for providing a library of compounds that formed the basis for this thesis. Thank you to Mikael Courbot and Katrin Hermann, my industrial supervisors at Syngenta in Stein (Switzerland), for their support during my PhD. Thank you to David Brocklehurst, who supervised me during my iCASE PhD placement, as well as all scientists at Syngenta at Jealott's Hill (UK) with whom I interacted during my placement, for their time and for giving me a taste of industrial research. Thank you also to Carol Yarrow, statistician at Syngenta, for her analyses of my data and for her advice regarding replicates and randomisation.

I would like to extend my gratitude to Alex Webb (University of Cambridge) for allowing me to carry out experiments using his luminometer, and together with members of the 201 Lab, particularly María del Carmen Martí Ruiz, Matthew Stancombe and Elsa Matthus, for technical advice and useful discussions about calcium. Special thanks to Alex Webb as my PhD second supervisor, for his guidance throughout my PhD, for taking the time to read and provide constructive comments for my thesis, and for his friendly support in academia since 2010, when I worked as a summer student in his group. Thank you also to my Graduate Education Committee advisor Uta Paszkowski for her counsel during my PhD and for persuading Syngenta to disclose for this thesis the structures of the bioactive molecules identified in the chemical genomics screen. I am thankful to Alex Costa (University of Milan) for useful discussions about calcium signalling in plants and for confirming my aequorin-based cytosolic free calcium measurements with his Cameleon reporter lines.

I am grateful to the researchers who kindly provided biological materials for this thesis: Michael Thomashow (Michigan State University) and Kazuko Yamaguchi-Shinozaki (University of Tokyo) for the *Arabidopsis thaliana camta* mutants; Norihito Nakamichi (Nagoya University) for the *A. thaliana prr5-11 prr7-11 prr9-10* mutant; Jaehoon Jung (Sainsbury Laboratory, University of Cambridge) for the *A. thaliana ice1-2/+ ice2-2* mutant; Alex Webb for the *A. thaliana* Col-0 *pCaMV35S::APOAEQUORIN* line; Devin O'Connor (Sainsbury Laboratory, University of Cambridge) for the *Brachypodium distachyon* Bd21-3 seeds; and Kerry Bloom (University of North Carolina) for the *Saccharomyces cerevisiae* YEF473a strain. Thank you to the Nottingham Arabidopsis Stock Centre (NASC) and Arabidopsis Biological Resource Center (ABRC) for supplying

seeds of additional *A. thaliana* T-DNA insertion lines. I am also grateful to Ian Brierley (University of Cambridge) and to the Developmental Therapeutics Program of the National Cancer Institute (NCI; National Institute of Health, NIH) for kindly providing the translation inhibitors edeine A1 and phyllanthoside, cryptopleurine, narciclasine and nagilactone C, respectively.

I am thankful to the people who facilitated the research presented in this thesis, including the Laboratory, Media preparation and Stores technicians and the Horticultural and Facilities staff of the Sainsbury Laboratory, and to the people who made the laboratory a friendly environment: Toufik Saadaoui, Pritish Rudhun, Jay Pal and Reggie Nagel.

Thank you to my friends and colleagues in Cambridge with whom I share great memories from the last four years: Clement Quan, Matthias Benoit, Matthieu Bourdon, Diego Sanchez, Justine Toulotte, Thomas Rey, Marie-Amelie Farreny, Emilie Fernandez, Liliana Antunes, Christian Schwall, Stuart Fawke, Louis Kaplan, Chloé Orland, Thibault Laurent, Maxime Gaullier, Sam Elton, Ula Kania, Pawel Baster, Dorothee Stoeckle, Tom Jonesman, Bo Larsen, Bernhard Blob, Eva Hellman, Marina Linardic, Aleksandr Gavrin, Sofia Otero, Mai Chu, José Ramos, Yanniv Dorone, Melis Kucukoglu, George Artavanis…

Thank you to my parents and family for their endless support, particularly my mother Maria Isabel Lea Guillaume, without whom I would not be where I am today. Thank you to my grandfather Henri Alphonse Guillaume, for igniting my passion for biology. Finally, thank you to Noémie Vimont for her understanding and encouragement during the last four years and for always being there for me.

Declaration

This dissertation is the result of my own work and includes nothing that is the outcome of work done in collaboration except as declared in the Preface and specified in the text.

It is not substantially the same as any that I have submitted, or, is being concurrently submitted for a degree or diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text. I further state that no substantial part of my dissertation has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text.

It does not exceed the word limit prescribed by the Degree Committee for the Faculty of Biology.

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List of abbreviations

Abbreviation	Full form
$[Ca^{2+}]_{cyt}$	Free cytosolic calcium concentration
0.5×/1×MS medium	Half- or full-strength Murashige-Skoog medium
½MMG medium	Half-strength Murashige-Skoog-MES-glucose medium
17-DMAG	17-Dimethylaminoethylamino-17-demethoxygeldanamycin
4-HBA	4-Hydroxybenzoate acid
ABA	Abscisic acid
ABF	ABRE-binding factor
ABRC	Arabidopsis Biological Resource Center
ABRE	Abscisic acid-responsive element
ACA	Autoinhibited Ca^{2+}-ATPase
AFP	Anti-freeze protein
AHA labelling	L-azidohomoalanine labelling
ANN	Annexin
APX	Ascorbic acid peroxidase
BA	Benzyl alcohol
BAPTA	1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
bHLH	Basic helix-loop-helix
bp	Base pairs
BT	Basal thermotolerance
cADPR	Cyclic ADP ribose
CaM	Calmodulin
CAMTA	Calmodulin-binding transcription activator
CAT	Catalase
CAT tail	C-terminal alanine and threonine tail
CAX	Ca^{2+}/H^{+} exporter
CBF	CRT-binding factor
CBL	Calcineurin B-like protein
CCAMK	Calcium- and calmodulin-dependent protein kinase
CCCP	Carbonyl cyanide *m*-chlorophenylhydrazone
CCX	K^{+}-dependent Ca^{2+}/Na^{+} exporter
CDPK	Calcium-dependent protein kinase
CHX	Cycloheximide
CIPK	CBL-interacting protein kinase
CML	Calmodulin-like protein
CNGC	Cyclic nucleotide-gated channel
COR	Cold-responsive
CRT	C-repeat
CXIP	CAX-interacting protein
DAB	3,3'-Diaminobenzidine
DACC	Depolarisation-activated calcium channel
DARTS	Drug affinity responsive target stability
DBMIB	2,5-Dibromo-6-isopropyl-3-methyl-1,4-benzoquinone
DCIC	3,4-Dichloroisocoumarin
DCMU	3-(3,4-Dichlorophenyl)-1,1-dimethylurea
DGDG	Digalactosyl-diacylglycerol
DMSO	Dimethylsulfoxide
DRE	Dehydration-responsive element
DREB	DRE-binding factor
DTT	Dithiothreitol

EC_{50}	Half-maximal effective concentration
ECA	ER-type Ca^{2+}-ATPase
EMS	Ethyl methanesulfonate
ER	Endoplasmic reticulum
FV channel	Fast vacuolar channel
GABA	γ-Aminobuturic acid
$GABA_A$	GABA-regulated anion channel
GECO	Genetically-encoded calcium indicator for optical imaging
GLR	Glutamate receptor-like channel
GO	Gene ontology
GPCR	G-protein-coupled receptor
GPX	Glutathione peroxidase
H3K4me2/me3	Di/tri-methylation of histone H3 lysine 4
HACC	Hyperpolarisation-activated calcium channel
HAMK	Heat shock-activated MAP kinase
HDS	1-Hydroxy-2-methyl-2-[E]-butenyl 4-diphosphate synthase
HGA	Homogentisic acid
HHP	Heptahelical protein
HR-A/B/C	Heptad repeat-A/B/C
HSBP	HSF-binding protein
HSE	Heat shock element
HSF	Heat shock factor
HSP	Heat shock protein
HSR	Heat shock response
HST	Homogentisate solanesyl transferase
HTH	Helix-turn-helix
IC_{50}	Half-maximal inhibitory concentration
ICE	Inducer of *CBF* expression
$InsP_3R$	IP_3 receptor
IP_3	Inositol-1,4,5-trisphosphate
IPOD	Insoluble protein deposit
IRES	Internal ribosome entry site
JA	Jasmonic acid
JUNQ	Juxtanuclear quality control compartment
KFB	Kelch repeat F-box protein
LAT	Long-term acquired thermotolerance
LB medium	Luria-Bertani medium
LB primer	Left T-DNA border primer
LEA	Late embryogenesis abundant protein
LIC	Ligation-independent cloning
lncRNA	Long noncoding RNA
LP primer	Left genomic primer
MAP kinase	Mitogen-activated protein kinase
MCA	Mid1-complementing activity
MCU	Mitochondrial calcium uniporter
MEcPP	Methylerythritol cyclodiphosphate
MES	2-[N-morpholino]ethanesulfonic acid
MG132	N-benzyloxycarbonyl-L-leucyl-L-leucyl-L-leucinal
MS channel	Mechanosensitive channel
MSL	MscS-like
MVAP	Mevalonate-5-phosphate
NAC	Nascent polypeptide-associated complex
NAD(P)H	Nicotinamide adenine dinucleotide (phosphate)
NASC	Nottingham Arabidopsis Stock Centre
NBT	Nitro blue tetrazolium chloride
NCI	National Cancer Institute

NGD	No-Go Decay
NIH	National Institute of Health
nt	Nucleotide
P-body	Processing body
PAL	Phenylalanine ammonia-lyase
PBR	Peripheral-type benzodiazepine receptor
PCR	Polymerase chain reaction
PIF	Phytochrome-interacting factor
PlantTFDB	Plant Transcription Factor Database
ppm	Parts per million
PPT	4-Hydroxybenzoate polyprenyl transferase
PQ / PQH_2	Plastoquinone / plastoquinol
PRX	Peroxiredoxin
PSI / PSII	Photosystem I / photosystem II
PTM	Post-translational modification
PVDF	Polyvinylidene fluoride
qRT-PCR	Quantitative real-time polymerase chain reaction
QTL	Quantitative Trait Locus
RAC	Ribosome-associated complex
RNA-seq	RNA-sequencing
RNAi	RNA interference
ROS	Reactive oxygen species
RP primer	Right genomic primer
RQC	Ribosome quality control
RyR	Ryanodine receptor
S1P	Sphingosine 1-phosphate
SA	Salicylic acid
SAMK	Stress-activated MAP kinase
SAT	Short-term acquired thermotolerance
SAUR	Small auxin-up RNA
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SKOR	Stelar K^+ outward rectifying channel
SOCE	Store-operated calcium entry
SOD	Superoxide dismutase
Sph	Sphingosine
SPROX	Stability of proteins from rates of oxidation
SUnSET	Surface sensing of translation
SV channel	Slow vacuolar channel
TAIR	The Arabidopsis Information Resource
TBARS	Thiobarbituric acid reactive substances
TBST	Tris-Buffered Saline with Tween-20
TIC	Translocon of the inner chloroplast membrane
TMHT	Thermotolerance to moderately high temperatures
TPCK	N-α-tosyl-L-phenylalanine chloromethyl ketone
TPM	Transcripts per million
TPP	Thermal proteome profiling
TRP	Transient receptor potential
TTC	Triphenyl-tetrazolium chloride
uORF	Upstream open reading frame
UQ / UQH_2	Ubiquinone / ubiquinol
UTR	Untranslated region
YPD	Yeast extract-peptone-dextrose

Chapter 1

Introduction

1.1 Sensitivity of plants to ambient temperature

As sessile organisms, plants have the necessity to adapt to fluctuating environmental conditions. The ability to perceive and respond to changes in ambient temperature is one of the key determinants of their survival. Plants may experience large daily temperature fluctuations, for example up to 40˚C in certain regions (Rigg, 1964), and these temperature variations affect vital processes such as photosynthesis and respiration (Hew *et al.*, 1969; Crafts-Brandner & Salvucci, 2000). In addition, seasonal temperatures determine key decisions in the lifecycle of plants, for example reproduction or dormancy (Fu *et al.*, 2012; Heide & Prestrud, 2005).

A difference in ambient temperature of only a few degrees Celsius can determine how rapidly plants undergo the transition from vegetative growth to reproductive growth. In certain ecotypes of the facultative long-day plant *Arabidopsis thaliana*, for example, flowering under short-day photoperiods is delayed at 23˚C but rescued at 25˚C (Balasubramanian *et al.*, 2006). In contrast, an ambient temperature of 20˚C induces flowering in the short-day plant *Chrysanthemum morifolium*, while increases in temperature delay reproduction (Nakano *et al.*, 2013).

Similarly to reproduction, dormancy is tightly regulated according to ambient temperature, allowing growth suppression under sub-optimal environmental conditions. In cultivated lettuce seeds, for example, germination may occur at 29˚C but is inhibited when the temperature rises to 30˚C (Yoong *et al.*, 2016). In apple and pear trees, six-week exposure to an ambient temperature of 9˚C promotes growth cessation and dormancy, whereas trees at 12-15˚C resume growth (Heide & Prestrud, 2005).

This high sensitivity of plants to temperature is of agricultural importance, as global climatic changes are expected to have major impacts on crop productivity. Indeed, during the European heat wave of 2003, crop yields dropped by about 30% (Ciais *et al.*, 2005). Even without accounting for associated water shortages, modelling studies predict up to 16% decreases in crop production following an increase in seasonal temperatures of only 1˚C (Battisti & Naylor, 2009). Conversely to mild winter temperatures, which may prevent dormancy release in fruit trees (Pagter *et al.*, 2015),

extreme low temperatures may damage emergent reproductive or fruiting structures, a phenomenon which causes losses of billions of euros in the European fruit industry (Snyder & de Melo-Abreu, 2005; Canellas *et al.*, 2017). In order to find solutions to such problems, it is important to develop a comprehensive understanding of the mechanisms by which plants perceive temperature changes and transduce these signals into physiological responses.

1.2 Plant responses to elevated temperatures

1.2.1 Cellular responses to heat shock

In contrast to warm temperature-induced developmental responses such as hypocotyl elongation and flowering (Koini *et al.*, 2009), which are usually studied over the temperature range of 16˚C to 28˚C, exposure of *Arabidopsis* plants to temperatures above roughly 28˚C triggers a stress pathway known as the 'heat shock response' (HSR). This pathway has been extensively studied, predominantly in yeast and mammalian model systems, but it is highly conserved across eukaryotes (*Figure 1.1*).

At the cellular level, heat stress triggers the generation of reactive oxygen species (ROS), *via* disruption of the electron transport chain in chloroplasts and mitochondria (Verghese *et al.*, 2012; Sun & Guo, 2016), and these may cause oxidative damage to nucleic acids, proteins and membranes. High temperatures cause unfolding and aggregation of proteins, which may lead to the loss of essential protein functions and the build-up of toxic aggregates. These misfolded proteins accumulate in discrete areas of the cytosol, for example in the 'juxtanuclear quality control compartment' (JUNQ), in which proteasomes co-localise with soluble proteins that have been targeted for degradation by ubiquitination, and in the 'insoluble protein deposit' (IPOD), in which aggregated, insoluble proteins build up in response to prolonged or extreme heat stress (Kaganovich *et al.*, 2008).

To counteract these deleterious effects, cells undergo rapid transcriptional reprogramming. Approximately 4% of genes are rapidly induced or upregulated by heat in *A. thaliana*, most of which encode cytoprotective proteins (Yángüez *et al.*, 2013). These include genes encoding ROS-detoxifying enzymes, for example ascorbic acid peroxidase (APX), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and peroxiredoxin (PRX) (Mittler *et al.*, 2004), as well as many genes encoding heat shock proteins (HSPs). The latter function as molecular chaperones, neutralising the effects of elevated temperatures on protein stability. High-resolution expression

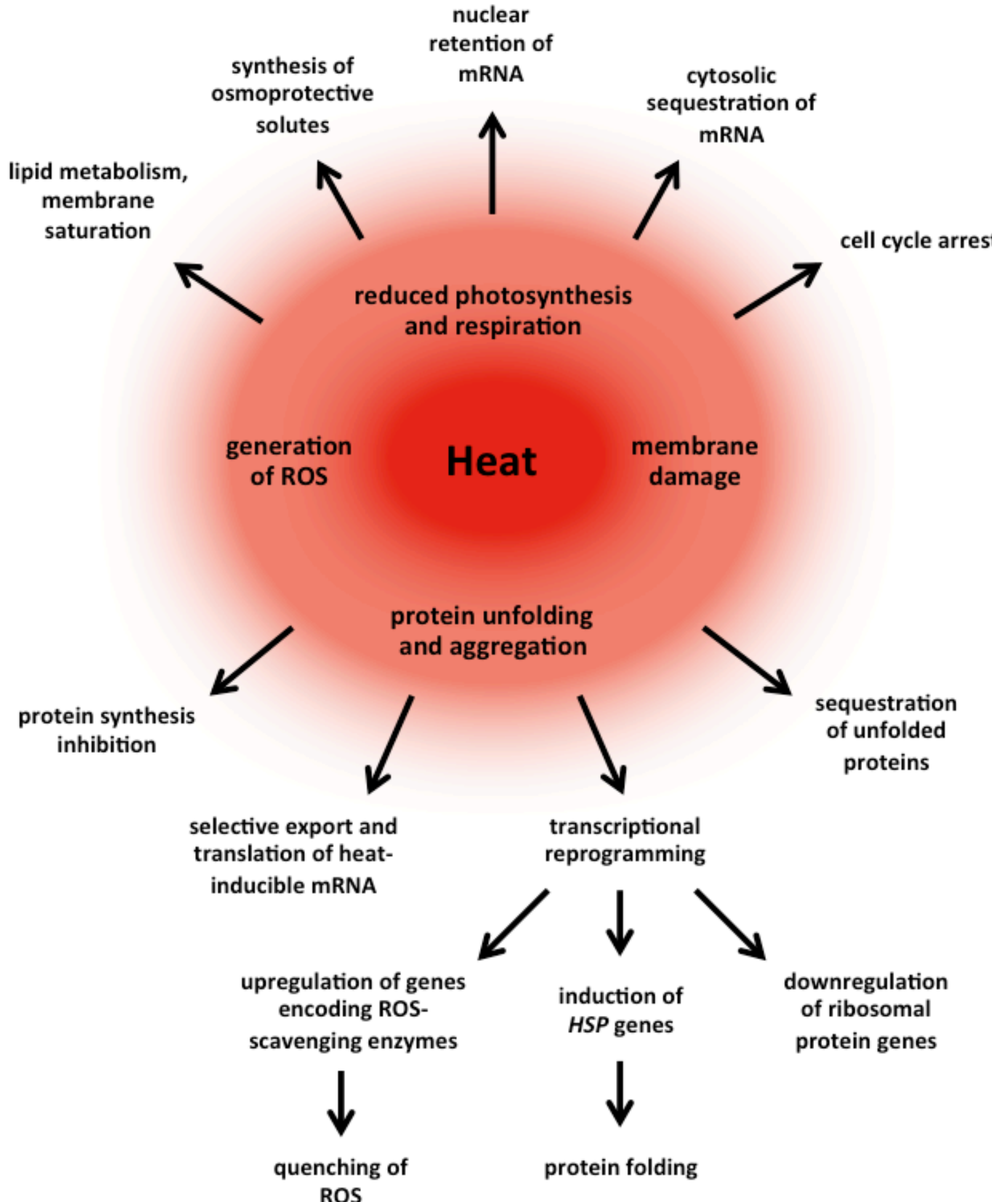


Figure 1.1: Summary of the heat shock response (HSR) in plants. Major deleterious effects of heat shock are shown in the red circle, and cellular responses triggered to neutralise these effects are listed above and below, in no particular order relative to the phrases in the circle. These responses include an activation of genes encoding protein chaperones, such as heat shock proteins (HSPs), or enzymes involved in reactive oxygen species (ROS) homeostasis, while genes encoding ribosomal proteins are repressed (Yángüez *et al.*, 2013); selective nuclear export and translation of heat-inducible mRNAs, whereas other mRNAs are sequestered to stress granules or processing bodies in the cytosol and their nuclear export and translation (Saavedra *et al.*, 1996; Yángüez *et al.*, 2013) are inhibited; sequestration of unfolded and aggregated proteins to discrete cytosolic compartments (Kaganovich *et al.*, 2008); inhibition of DNA replication and cellular division (Rowley *et al.*, 1993); and increased activity of enzymes involved in the synthesis of osmoprotective solutes (Kaplan *et al.*, 2004) and in membrane integrity and saturation (Falcone *et al.*, 2004; Mueller *et al.*, 2015).

analyses in *Saccharomyces cerevisiae* have shown that the expression of *HSP* genes is highly induced within minutes of heat shock and is rapidly attenuated, reaching steady-state levels above that of non-stressed cells (Gasch *et al.*, 2000). Transcription factors of the heat shock factor (HSF) family are key regulators of this response and bind to pentameric motifs known as heat shock elements (HSEs) in the promoters of many heat-inducible genes. Other classes of transcription factors are also involved to a lesser extent in regulation of heat shock responses in plants. These include certain stress-associated WRKY (Tryptophan-Arginine-Lysine-Tyrosine domain) proteins (Wu *et al.*, 2009; Li *et al.*, 2010, 2011a), many of which are induced by thermal stress (Qiu *et al.*, 2004; Wu *et al.*, 2008), and a small number of transcription factors from the AP2 (APETALA 2), NAC (NO APICAL MERISTEM/ARABIDOPSIS TRANSCRIPTION ACTIVATOR FACTOR/CUP-SHAPED COTYLEDON) or MBF1c (MULTIPROTEIN BRIDGING FACTOR 1c) families (Lim *et al.*, 2006; Kang *et al.*, 2011; Suzuki *et al.*, 2011; Shahnejat-Bushehri *et al.*, 2012; Barah *et al.*, 2016).

Up to 15% of the *A. thaliana* transcriptome is downregulated following transient exposure to heat (Kumar & Wigge, 2010; Yángüez *et al.*, 2013), including many constitutive genes such as those encoding ribosomal proteins. Little is known about the mechanisms of heat-induced repression of ribosomal protein genes. In the budding yeast *S. cerevisiae*, reduced binding of the transcriptional activator IFH1 (INTERACTS WITH FORK HEAD 1) (Wade *et al.*, 2004), combined with the rapid turnover of ribosomal protein mRNA (Li *et al.*, 1999), contribute to this response. The dual activator-repressor RAP1 (REPRESSOR/ACTIVATOR SITE-BINDING PROTEIN 1) also appears to regulate the shutdown of ribosomal protein genes *via* an unknown mechanism (Li *et al.*, 1999).

During thermal stress, newly synthesised mRNAs are retained in the nucleus (Saavedra *et al.*, 1996). In *S. cerevisiae*, this nuclear retention requires the MAP kinase SLT2/MPK1 (SUPPRESSOR OF THE LYTIC PHENOTYPE 2/MAP KINASE 1), which phosphorylates the mRNA-binding protein NAB2 (NUCLEAR POLYADENYLATED RNA-BINDING 2) (Carmody *et al.*, 2010). During normal growth, NAB2 is loaded co-transcriptionally onto mRNA and recruits the mRNA export receptor MEX67 (MESSENGER RNA EXPORT FACTOR 67kDA) (Iglesias *et al.*, 2010). The NAB2-MEX67 association decreases at elevated temperatures, thereby blocking mRNA export (Carmody *et al.*, 2010). Cytosolic mRNAs are sequestered into two distinct compartments known as stress granules and processing bodies (P-bodies). Stress granules form only upon stressful conditions and are enriched with components of translation initiation, whereas P-bodies are present in cells under normal conditions but

increase in abundance in response to heat stress and are enriched with enzymes involved in mRNA decay (Decker & Parker, 2012).

To counteract the potentially lethal effects of proteotoxicity, *de novo* protein synthesis is inhibited during severe heat shock (Yángüez *et al.*, 2013). In mammalian cells, the chaperone HSP27 contributes to translation inhibition by binding to the initiation factor eIF4G at elevated temperatures, causing it to be sequestered to stress granules (Cuesta *et al.*, 2000). The yeast heat shock factor HSF1 has been shown to indirectly inhibit the TOR (TARGET OF RAPAMYCIN) kinase complex (TORC1), which is a positive regulator of translation (Bandhakavi *et al.*, 2008). Similarly, the energy-sensing kinase SNF1 (SUCROSE NON-FERMENTING 1), which is activated during stressful conditions, is known to inhibit TORC1 activity by phosphorylating its regulator protein KOG1 (KONTROLLER OF GROWTH 1) (Hughes Hallett *et al.*, 2015). TORC1 is itself transiently sequestered to stress granules during heat shock (Takahara & Maeda, 2012). In addition, the chaperone HSP70, which folds nascent proteins emerging from the ribosomal exit tunnel, decreases its association with ribosomes during heat shock in mouse cells, thereby contributing to translational stalling (Shalgi *et al.*, 2014).

Although mRNA export and translation are inhibited during heat shock, the mRNAs of many heat-inducible genes are efficiently exported and translated. In *S. cerevisiae*, mRNA transcripts of HSP70 genes *SSA1* and *SSA4* (*STRESS SEVENTY A1/4*) have been shown to be efficiently exported *via* a heat-specific pathway, requiring nuclear pore protein NUP42/RIP1 (NUCLEAR PORE 42/REV-INTERACTING PROTEIN 1) (Saavedra *et al.*, 1997) and RNA-decapping enzymes EDC1 and EDC2 (ENHANCER OF mRNA DECAPPING 1/2) (Neef & Thiele, 2009). Zander *et al.* (2016) have shown that replacing the promoters of non-heat-inducible genes, such as *GPM1* (*GLYCERATE PHOSPHOMUTASE 1*) and *CYC1* (*CYTOCHROME C1*), with the *HSP12* promoter, or introducing a HSE motif into the native promoters, allows their mRNA transcripts to be efficiently exported during heat stress in budding yeast. The authors demonstrated that the mRNA export receptor MEX67 is loaded onto nascent mRNA by HSF1, thus circumventing the normal mRNA export pathway (Carmody *et al.*, 2010).

Sequences in the 5' and 3' UTRs of *HSP* mRNAs have also been shown to facilitate their export and translation. *GAL1* (*GALACTOSE METABOLISM 1*) mRNA, which is usually retained in the nucleus during heat stress, can be exported efficiently at elevated temperatures if fused to the sequence 1600 bp upstream or 500 bp downstream of the coding sequence of the HSP70 gene *SSA4* (Saavedra *et al.*, 1996). In human cells, *HSP70* mRNA has been shown to contain a 5' sequence complementary to 18S rRNA that allows for selective translation initiation during heat stress (Yueh & Schneider, 2000). The

mRNAs of the human HSP gene *BIP1* (*LUMINAL BINDING PROTEIN 1*) and co-chaperone gene *BAP-1* (*BRCA1-ASSOCIATED PROTEIN 1*) contain an internal ribosome entry site (IRES) that allows them to recruit ribosomes independently of the heat-repressed eIF4F initiation complex (Cuesta *et al.*, 2000; Coldwell *et al.*, 2001).

In addition to mRNA sequestration and translation inhibition, heat shock causes transient arrest of the cell cycle in yeast and mammalian cells, thereby preventing potentially lethal cell divisions under unfavourable conditions. Rowley *et al.* (1993) have shown that in *S. cerevisiae* this is a result of transcriptional repression of G_1/S cyclin genes *CLN1* and *CLN2* (*CYCLIN 1/2*) and can be overcome by expressing these cyclins under a constitutive promoter. Although heat stress effects on cell division have yet to be studied in plants, the expression of cyclin genes decreases in *A. thaliana* plants exposed to 37˚C (Cortijo *et al.*, 2017), suggesting a similar mechanism may be in play.

Heat shock may also disrupt essential cellular functions such as photosynthesis and respiration, *via* damage to membranes and electron carriers (Dekov *et al.*, 2000; Sinsawat *et al.*, 2004). Beside the synthesis of ROS-scavenging enzymes and protein chaperones, an alteration of metabolism is triggered during heat stress, whereby cells produce cytoprotective solutes to stabilise membranes and proteins and to balance cell osmotic pressure. In yeast cells, the disaccharide trehalose is rapidly synthesised during thermal stress (Hottiger *et al.*, 1994; Ribeiro *et al.*, 1997; Conlin & Nelson, 2007) and it displaces the 'water shell' around macromolecules (Madden *et al.*, 1985), thereby stabilising proteins and preventing their aggregation (Singer & Lindquist, 1998).

The amount of trehalose in *A. thaliana* plants has also been found to increase during heat stress (Kaplan *et al.*, 2004). Overexpression of the endogenous trehalose biosynthetic gene *AtTPS1* (*TREHALOSE 6-PHOSPHATE SYNTHASE 1*) (Avonce *et al.*, 2004) or ectopic expression of *S. cerevisiae TPS1* and *TPS2* under the stress-induced *RD29A* (*RESPONSIVE TO DESICCATION 29A*) promoter (Miranda *et al.*, 2007) in *A. thaliana* increases resistance to high temperatures. Exogenous trehalose treatment has also been shown to improve recovery from heat stress in wheat seedlings (Luo *et al.*, 2014). Metabolic analysis of *A. thaliana* seedlings exposed to 40˚C for up to four hours identified many other osmolytes that also increase during heat shock, including β-alanine, several glycerol derivatives and both raffinose and its precursor galactinol (Kaplan *et al.*, 2004). The latter two metabolites increase over 40-fold after four hours of heat stress, in contrast to the more modest two-fold increase of trehalose.

To counteract the effects of heat on the mechanical properties of membranes, lipid metabolism is altered in response to elevated temperatures. In *A. thaliana* seedlings, elevated temperatures cause a slow increase in saturated 16-carbon and

dienoic unsaturated 18-carbon fatty acids and a decrease in trienoic unsaturated fatty acids (Falcone *et al.*, 2004). While *de novo* synthesis of fatty acids occurs over several days after exposure to elevated temperatures, Mueller *et al.* (2015) have shown that membrane remodelling and the accumulation of triacylglycerol lipids occur within hours of heat exposure.

1.2.2 Heat shock proteins

Heat shock proteins (HSPs) are molecular chaperones whose functions include: (1) the folding of proteins into their native state, both nascent proteins as they leave the ribosome and denatured proteins during cellular stresses, (2) maintenance of proteins in specific conformations, for example keeping proteins in an unfolded state during membrane translocation, and (3) de-aggregating and unfolding proteins that have been denatured. There are seven main classes of HSPs, distinguished by their approximate size in kilodaltons: HSP70, HSP90, HSP100, HSP110, HSP60, HSP40 and sHSP (*Figure 1.2*).

These proteins are highly conserved across evolution, for example *A. thaliana* HSP70s share up to 76% homology and 59% identity with *Escherichia coli* homologue DnaK (DNA REPLICATION K). HSPs have diverse subcellular localisations, which include the cytosol, endoplasmic reticulum, mitochondria, chloroplasts and nucleus, and their expression patterns vary according to developmental stage and type of stress. Although HSPs were named because of the early observation that their genes are activated during heat treatments (Ritossa, 1962; Schedl *et al.*, 1978), *HSP* gene expression may be induced by numerous different stresses, including oxidative, cold, osmotic, salt or UV stresses, *Pseudomonas syringae* or elicitor infiltrations, and applications with hormone inhibitors TIBA or $AgNO_3$ or with the photosynthesis inhibitor PNO8 (Sung *et al.*, 2001; Swindell *et al.*, 2007; Kilian *et al.*, 2007). While many *HSP* genes are induced by multiple stresses, others have stress-specific patterns of expression, for example in *A. thaliana* shoots *HSP70-9* and *HSP70-11* are induced by UV light and osmotic or salt stress, respectively, but not heat shock, while *HSP70-8* is induced by heat shock but not UV, osmotic or salt stresses (Swindell *et al.*, 2007).

HSP70 proteins are characterised by an N-terminal ATPase domain and a C-terminal peptide-binding domain, the latter of which is stimulated by unfolded proteins and in turn activates the ATPase domain (Boston *et al.*, 1996; Verghese *et al.*, 2012). The *A. thaliana* genome contains eighteen *HSP70* genes, which exhibit distinct expression patterns in response to heat shock, for example some are induced up to twenty-fold

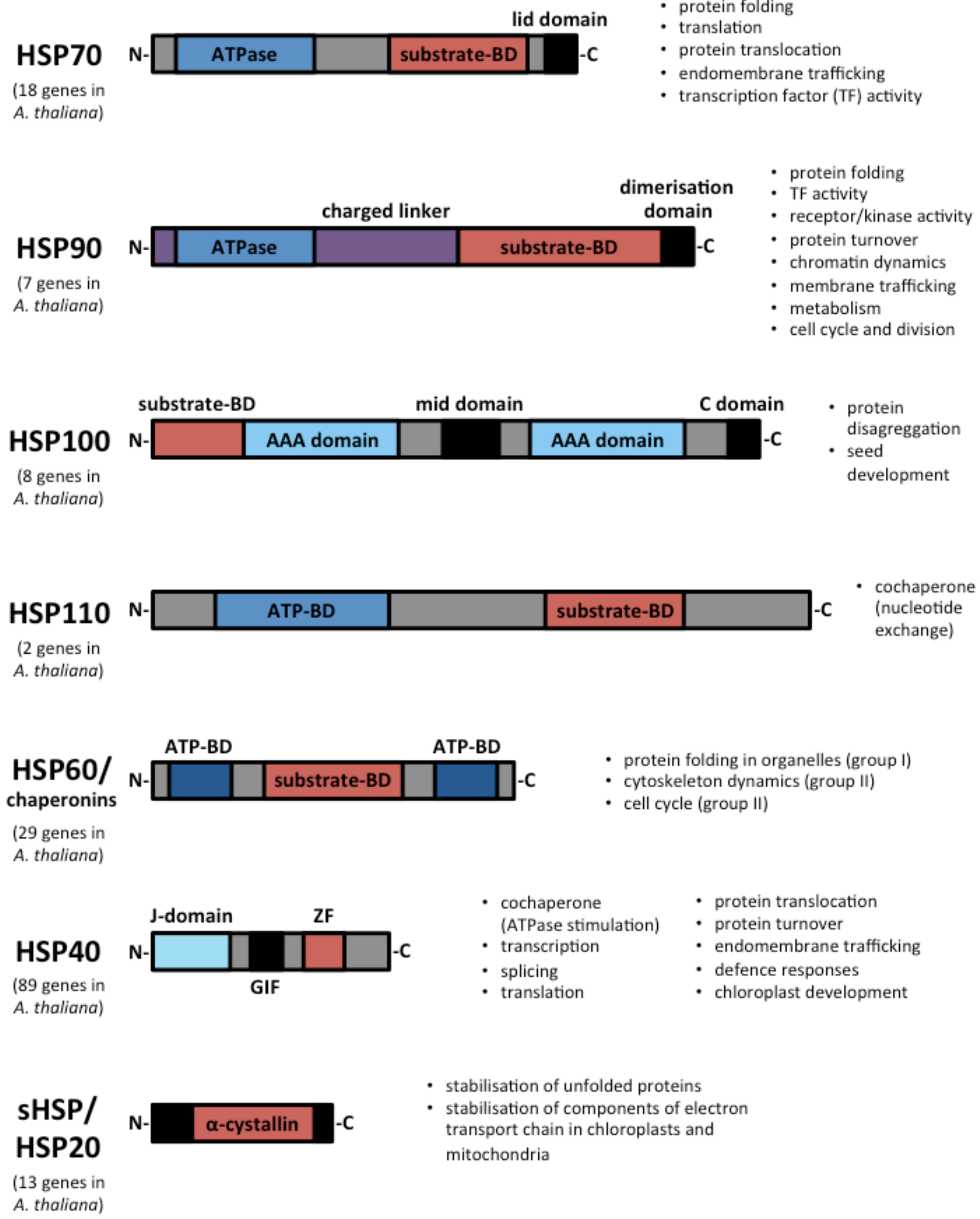


Figure 1.2: Domain structure and functions of heat shock protein (HSP) families.
Substrate-DB: substrate-binding domain, binds unfolded peptides. ATPase and AAA domain: HSP70/90- or HSP100-type ATPase domains, hydrolyse ATP for chaperone activity. HSP70 lid domain: involved in peptide folding. HSP100 mid- and C-domains: regulation of ATPase activity and hexamer assembly. HSP40 J-domain: stimulates the ATPase domain of HSP70/90. HSP40 GIF domain: regulates the interaction with HSP70/90. HSP40 ZF domain: zinc-finger domain, binds substrate. sHSP α-crystallin domain: binds unfolded peptides.

during heat treatment while others, such as the constitutive *HSC70* (*HEAT SHOCK COGNATE 70kDa*)-type *HSP70* genes, are upregulated only two-fold or are not heat-responsive (Sung *et al.*, 2001). These proteins are essential for viability, as shown in *S. cerevisiae*, where at least one of the HSP70 homologues SSA1, SSA2, SSA3 or SSA4 must be expressed for cell survival (Werner-Washburne *et al.*, 1987). Similarly, expressing antisense cDNA to the HSP70 gene *HSC70-1* results in lethality in *A. thaliana* (Sung & Guy, 2003). The dosage of HSP70 proteins appears to be carefully regulated, as *A. thaliana* plants transformed with an *HSC70-1* overexpression construct yield few viable transformants and those surviving have a dwarfed phenotype with abnormal root structure (Sung & Guy, 2003). *HSP70* overexpression has also been shown to have deleterious effects on growth and development in *Drosophila melanogaster* and mammalian cells (Krebs & Feder, 1997; Volloch & Sherman, 1999).

HSP70 proteins contribute to protein folding during normal growth and development as well as in response to proteotoxic stress. *HSP70* expression is diurnally regulated in *A. thaliana*, with a sharp upregulation at dawn, coinciding with the activation of photosynthesis (Dickinson *et al.*, 2018). HSP70 proteins play a key role in the translocation of proteins across membranes, for example into chloroplasts or the endoplasmic reticulum (Miernyk *et al.*, 1992; Chotewutmontri & Bruce, 2015). They regulate endomembrane trafficking by dissociating clathrin-coated vesicles, which are reassembled by the HSP40 protein auxilin (Braell *et al.*, 1984; Ungewickell *et al.*, 1995). In yeast, the HSP70 protein SSZ1 (STRESS SEVENTY Z1) forms a complex with the HSP40 protein ZUO1, known as the ribosome-associated complex (RAC), which associates with the exit tunnel of the ribosome. Together with HSP70 proteins SSB1 and SSB2 (STRESS SEVENTY B1/2), RAC contributes to the biogenesis of ribosomes and the folding of the nascent peptide chain (Albanèse *et al.*, 2010; Koplin *et al.*, 2010). HSP70 proteins are also regulators of gene expression, as they can either repress transcription factor activity, as shown for yeast HAP1 (HEME ACTIVATOR PROTEIN 1) (Hon *et al.*, 2001), or promote activity, as demonstrated for GAL4 (GALACTOSE METABOLISM 4) and MAL63 (MALTOSE FERMENTATION 63) in *S. cerevisiae* (Floer *et al.*, 2008; Ran *et al.*, 2008).

HSP90 proteins are characterised by an N-terminal ATP-binding domain and a highly charged linker region, which, unlike the C-terminus of HSP70s, can bind unfolded proteins in a nucleotide-independent manner (Boston *et al.*, 1996, Verghese *et al.*, 2012). HSP90s often act in complexes with HSP70s and they too are essential for viability (Borkovich *et al.*, 1989). While yeast and mammalian HSP90s have been partially characterised, very little is known about plant HSP90 proteins and their substrates. The

A. thaliana genome contains seven *HSP90* genes, four of which are heat-responsive (Krishna & Gloor, 2001; Swindell *et al.*, 2007).

Unlike HSP70s, which do not discriminate between misfolded proteins, HSP90 proteins interact with a large but seemingly specific set of proteins and aid in their maturation and assembly into complex macromolecular structures. HSP90s are thus key regulators of the activity of many proteins, for example components of signal transduction cascades. HSP90 proteins stabilise the ligand-binding domain of mammalian steroid receptors and promote their relocation to the nucleus in the presence of steroid hormones, while inhibiting their activity in the absence of hormones (Echeverria & Picard, 2010). In yeast, HSP90s have also been found to regulate the activity of kinases (Xu & Lindquist, 1993; Louvion *et al.*, 1998; Donzé & Picard, 1999), as well as that of transcription factors such as HAP1 (Zhang *et al.*, 1998), GAL4 (Floer *et al.*, 2008) and MAL63 (Ran *et al.*, 2008), whose target gene induction is severely compromised in HSP90-underexpressing cells.

HSP90s have been shown to stabilise the F-box proteins ZTL (ZEITLUPE) and TIR1 (TRANSPORT INHIBITOR RESPONSE 1) in *A. thaliana*, as treatments with the HSP90 inhibitor geldanamycin enhances the degradation of these proteins (Kim *et al.*, 2011a; Wang *et al.*, 2016c). HSP90s further contribute to protein turnover by aiding in the assembly and maintenance of the 26S proteasome, and drugs or mutations affecting HSP90 function in budding yeast cause disassembly of the proteasome (Imai *et al.*, 2003). By a combined use of chemical genetics, proteomics and interaction studies, Zhao *et al.* (2005) and McClellan *et al.* (2007) have identified many more HSP90 'client' proteins in yeast, implicating this chaperone in the cell cycle, reproduction, chromatin remodelling, protein trafficking and secretion.

Members of the HSP100 family contain either two ATP-binding domains (subclass I) or one such domain (subclass II), which, as for HSP70s, are stimulated by unfolded proteins (Boston *et al.*, 1996, Verghese *et al.*, 2012). These proteins form homohexamers and are termed "disaggregases" as they have a unique ability to pull apart aggregated proteins and, in association with HSP70 and HSP40 proteins, refold them into their soluble form (Parsell *et al.*, 1994; Glover & Lindquist, 1998). HSP100s are required for the release of mRNAs from stress granules and resumption of translation during heat shock recovery (Merret *et al.*, 2017). While they are essential for long-term survival at elevated temperatures or in the presence of other stresses, HSP100s are not required for growth at normal temperatures in both *S. cerevisiae* and *A. thaliana* (Sanchez & Lindquist, 1990; Sanchez *et al.*, 1992; Hong & Vierling, 2000; Quietsch *et al.*, 2000).

Although no homologues have been identified in metazoans, HSP100 proteins are highly conserved from bacteria to plants (Mokry *et al.*, 2017). The *A. thaliana* genome contains eight HSP100 proteins, three of which have heat-inducible expression (Agarwal *et al.*, 2001; Swindell *et al.*, 2007). Five of the *A. thaliana* HSP100 proteins are predicted to localise to organelles, two of which have been confirmed to localise to chloroplasts or mitochondria, respectively (Agarwal *et al.*, 2001; Lee *et al.*, 2006a). Among *A. thaliana* HSP100 genes, *HSP101* has the greatest change in expression during heat stress and its cytosolic protein is the most studied of the HSP100 family. The *HSP101* gene, as well as homologues from *Nicotiana tabacum* and *Triticum aestivum* (Wells *et al.*, 1998), *Glycine max* (Lee *et al.*, 1994) and *Oryza sativa* (Agarwal *et al.*, 2003), have been shown to rescue thermotolerance in HSP100-deficient yeast (Schirmer *et al.*, 1994). Overexpression of HSP100 genes in a variety of plants has been shown to enhance thermotolerance (Malik *et al.*, 1999; Queitsch *et al.*, 2000; Murakami *et al.*, 2004).

Though expressed at low levels in vegetative tissues in *A. thaliana* (Hong & Vierling, 2000), *HSP101* is expressed at higher levels in reproductive tissues (Schmid *et al.*, 2005). Supporting the role of HSP101 in reproduction, null *HSP101* mutants produce fewer siliques, both at normal growth temperatures and during heat stress (Tonsor *et al.*, 2008). Expression is particularly high in developing seeds, and Boston *et al.* (1996) have speculated that HSP100s may play a role in solubilising proteins during rehydration of dried seeds. The elevated expression of *HSP101* in *A. thaliana* seeds was shown to be responsible for the high level of thermotolerance observed during germination (Queitsch *et al.*, 2000).

HSP40 and HSP110 proteins are co-chaperones for HSP70s, HSP90s and HSP100s. HSP40s, also known as J-proteins because they contain a 70-residue domain homologous to the *E. coli* DnaJ co-chaperone (Pellecchia *et al.*, 1996), stimulate the ATPase activity of chaperones, while HSP110s facilitate their exchange of ADP for ATP. The HSP110 family has received relatively little attention compared to other classes of HSPs. The *A. thaliana* genome contains at least two *HSP110* genes (Storozhenko *et al.*, 1996), which remain to be characterised. *S. cerevisiae* cells express two HSP110 genes, the constitutively expressed *SSE1* and heat-inducible *SSE2* (*STRESS SEVENTY E1/2*) (Gasch *et al.*, 2000). Null *sse1* mutants have growth defects, while double *sse1 sse2* mutants are lethal (Trott *et al.*, 2005).

In contrast, plant HSP40 proteins have received more attention. The *A. thaliana* genome encodes 89 such proteins, which are predicted to localise to all subcellular organelles as well as the cytosol and membranes (Miernyk, 2001). Consistent with these

predictions, it has been shown that a subpopulation of the yeast HSP40 protein YDJ1 (YEAST DNAJ) is associated with the endoplasmic reticulum membrane *via* C-terminal farnseylation (Caplan *et al.*, 1992). Unlike HSP110s, which share no homology between bacteria and eukaryotes, HSP40s are highly conserved across evolution, as demonstrated by the fact that the *E. coli* DnaJ protein is capable of stimulating heterologously-expressed *Zea mays* HSP70 (Miernyk & Hayman, 1996). HSP40s have either target-specific or promiscuous binding, and experiments in budding yeast have implicated these proteins in a wide variety of cellular processes, including transcription (Johnson & Craig, 2000; Hon *et al.*, 2001), RNA splicing (Sahi & Craig, 2007), translation (Meyer *et al.*, 2007), membrane trafficking (Ungewickell *et al.*, 1995), peroxisomal protein import (Hettema *et al.*, 1998) and protein turnover (Youker *et al.*, 2004). In plants, HSP40 proteins have been associated with disease resistance (Liu & Whitham, 2013) and chloroplast development (Wang *et al.*, 2016b), in addition to thermotolerance (Yang *et al.*, 2009).

HSP60 proteins, also known as group I 'chaperonins', are exclusively found in eubacteria and organelles. These heat-inducible proteins form large homo-oligomer structures composed of two rings of seven subunits, which assist in folding newly synthesised and translocated proteins (Verghese *et al.*, 2012). Null mutants are lethal as they result in mitochondrial malfunction (Cheng *et al.*, 1989). In plants, HSP60 proteins are key regulators of photosynthesis, as they are required for the assembly of Rubisco holoenzyme (Aigner *et al.*, 2017) and the heat-stabilisation of Rubisco activase (Salvucci, 2008), which is particularly sensitive to elevated temperatures (Crafts-Brandner & Salvucci, 2000, 2002). In eukaryotes, the cytosolic TCP1 (TAILLESS COMPLEX POLYPEPTIDE 1) ring complex (TriC; group II chaperonin) forms a related structure composed of two rings of eight different proteins, which are constitutively expressed and essential for survival (Vinh & Drubin, 1994). Conditional yeast mutants have shown that the TriC chaperonin plays vital roles in the assembly of actin and tubulin (Vinh & Drubin, 1994) and regulation of the cell cycle (Camasses *et al.*, 2003).

The final class of heat shock proteins is the sHSP or HSP20 family, which encompasses proteins typically 16 to 30 kDa in size that share a signature 100-residue domain in their C-terminus, though they have little overall sequence homology among eukaryotes. Higher plants contain large sHSP gene families, unlike most other eukaryotic lineages, which contain one or few sHSP genes (Vierling, 1991). The *A. thaliana* genome encodes thirteen sHSPs, grouped into six classes according to conservation and cellular localisation (Scharf *et al.*, 2001). Similarly to HSP90s, sHSP proteins bind unfolded peptides in a nucleotide-independent manner, and in parallel to HSP100s, they are

expressed in vegetative tissues only during proteotoxic stress as well as during normal reproductive development (DeRocher *et al.*, 1991; Sun *et al.*, 2002). Unlike other chaperones, sHSP proteins lack an ATPase domain and act as 'holdases', stabilising unfolded proteins, preventing their aggregation and facilitating their interaction with HSP70 and HSP100 chaperones (Verghese *et al.*, 2012). Lee & Vierling (2000) demonstrated that *Pisum sativum* HSP18.1-CI could re-solubilise heat-denatured luciferase *in vitro* in the presence of HSP70 and HSP40 proteins.

Together, the three sHSP proteins HSP12, HSP26 and HSP42 in yeast can accumulate to over 3% of total proteins during proteotoxic stress (Welker *et al.*, 2010; Haslbeck *et al.*, 2004). Yeast HSP42 forms homo-oligomeric macrostructures of up to 26 subunits that are stabilised at elevated temperatures (Haslbeck *et al.*, 2004). HSP26, in contrast, forms 24-subunit homo-oligomers in the absence of stress, which dissociate into dimers upon heat shock (Haslbeck *et al.*, 1999). Similarly, sHSP proteins in *A. thaliana* form homo-oligomers of 9- to 24-subunits, which may undergo heat-responsive structural alterations (Scharf *et al.*, 2001). The dodacameric sHSP complexes of the cysotol, for example, either dissociate into dimers (class I) or retain their oligomeric state (class II) upon heat shock (Basha *et al.*, 2010). In contrast, oligomerisation of the chloroplast HSP25.3-P protein appears to increase during oxidative stress, owing to the sulfoxidation of methionine residues (Gustavsson *et al.*, 1999).

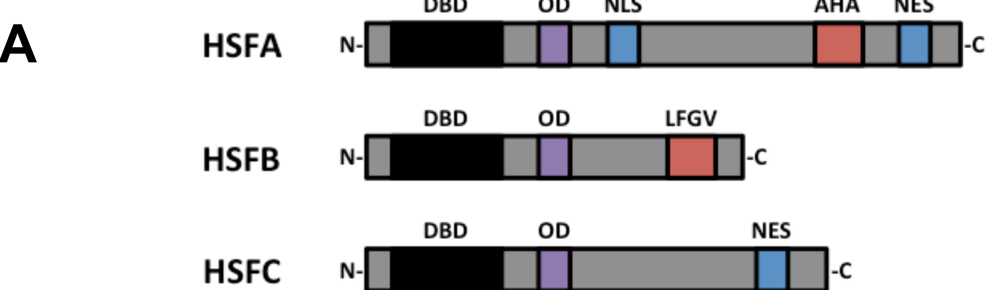
Plant sHSP proteins are critical for photosynthesis under heat stress. Elevated temperatures drastically decrease photosynthetic rates in plants, as a result of disruption of photosystem II (PSII) and disordering of thylakoid membranes (Dekov *et al.*, 2000; Crafts-Brandner & Salvucci, 2000; Sinsawat *et al.*, 2004). Chloroplast sHSP proteins counteract these deleterious effects by associating with the oxygen-evolving complex and stabilising PSII (Downs *et al.*, 1999; Preczewski *et al.*, 2000; Heckathorn *et al.*, 2002). They have been shown to stabilise PSII in response to other stresses, for example heavy metal toxicity (Heckathorn *et al.*, 2004), and sHSP protection of components of the mitochondrial electron transport chain has also been demonstrated (Neumann *et al.*, 1993; Lund *et al.*, 1998; Heckathorn *et al.*, 1999; Rhoads *et al.*, 2005). Although only a few plant sHSP proteins have been fully characterised, the overexpression of many of these proteins enhances resistance to heat stress, while reducing their expression can compromise thermotolerance (Yeh *et al.*, 1997; Malik *et al.*, 1999; Soto *et al.*, 1999; Wang *et al.*, 2017a).

1.2.3 Heat shock factors

Heat shock factors (HSFs) are transcriptional regulators of the helix-turn-helix (HTH) family and bind as oligomers to heat shock elements (HSEs) containing inverted repeats of the sequence nGAAn. Three different types of HSEs have been described: 'perfect' HSEs (nGAAnnTTCnnGAA), 'gap' HSEs (nGAAnnTTCn[5nt]nTTC) and 'step' HSEs (nGAAn[5nt]nGAAn[5nt]nGAAn) (Sakurai & Takemori, 2007). All HSF proteins contain a hydrophobic leucine-zipper oligomerisation domain, containing two regions known as heptad repeats A and B (HR-A/B), as well as the HTH domain. Plant genomes typically encode 20 to 55 HSF proteins, in contrast to animal or fungal genomes, which encode between one and four HSFs. The genomes of *S. cerevisiae*, *Caenorhabditis elegans* and *Drosophila melanogaster* harbour a single HSF protein and the *Homo sapiens* genome encodes four HSFs, without accounting for HSF-like proteins, whereas the *A. thaliana* and *O. sativa* genomes encode 21 and 25 HSFs, respectively. Unlike the *S. cerevisiae* HSF (HSF1), which is characterised by two activation domains on N- and C-terminus of the protein, metazoan and plant HSFs contain a single C-terminal activation domain. While yeast and mammalian HSF proteins contain a 'winged' HTH motif, which stabilises interactions between monomers as well as binding DNA, plants lack the wing motif in their DNA-binding domain (Littlefield & Nelson, 1999; Cicero *et al.*, 2001).

Plant HSFs are classified into three groups based on their conserved structural features (*Figure 1.3A*). HSFs of class A (HSFA; 15 members in *A. thaliana*) contain the longest oligomerisation domain, with a 21-amino acid insertion between HR-A and HR-B regions, followed by HSFs of class C (HSFC; a single member in *A. thaliana*), which contain a seven-amino acid insertion in the same region, and class B HSFs (HSFB; five members in *A. thaliana*), with no insertion between HR-A and HR-B regions (Nover *et al.*, 2001). HSFAs contain one or more AHA domains, enriched with aromatic, hydrophobic and acidic amino acids, which play a key role in transcriptional activation (Döring *et al.*, 2000). HSFBs and HSFCs, however, lack AHA domains and HSFBs contain a C-terminal LFGV motif, which confers repressor activity (Ikeda & Ohme-Takagi, 2009).

The A-, B- and C-class HSFs are further divided into subgroups based on their phylogenetic clustering among plant species, with nine HSFA, four HSFB and one HSFC subgroups in *A. thaliana* (Nover *et al.*, 2001). These different HSF proteins have evolved divergent functions and patterns of expression. Six of the *A. thaliana* HSF genes (*HSFA2, HSFA7a, HSFA7b, HSFB1, HSFB2a* and *HSFB2b*) have heat-inducible expression, while the expression of the other fifteen HSF genes is not affected by heat but is influenced by other stresses such as salt, osmotic, drought or cold treatment (Swindell *et al.*, 2007).



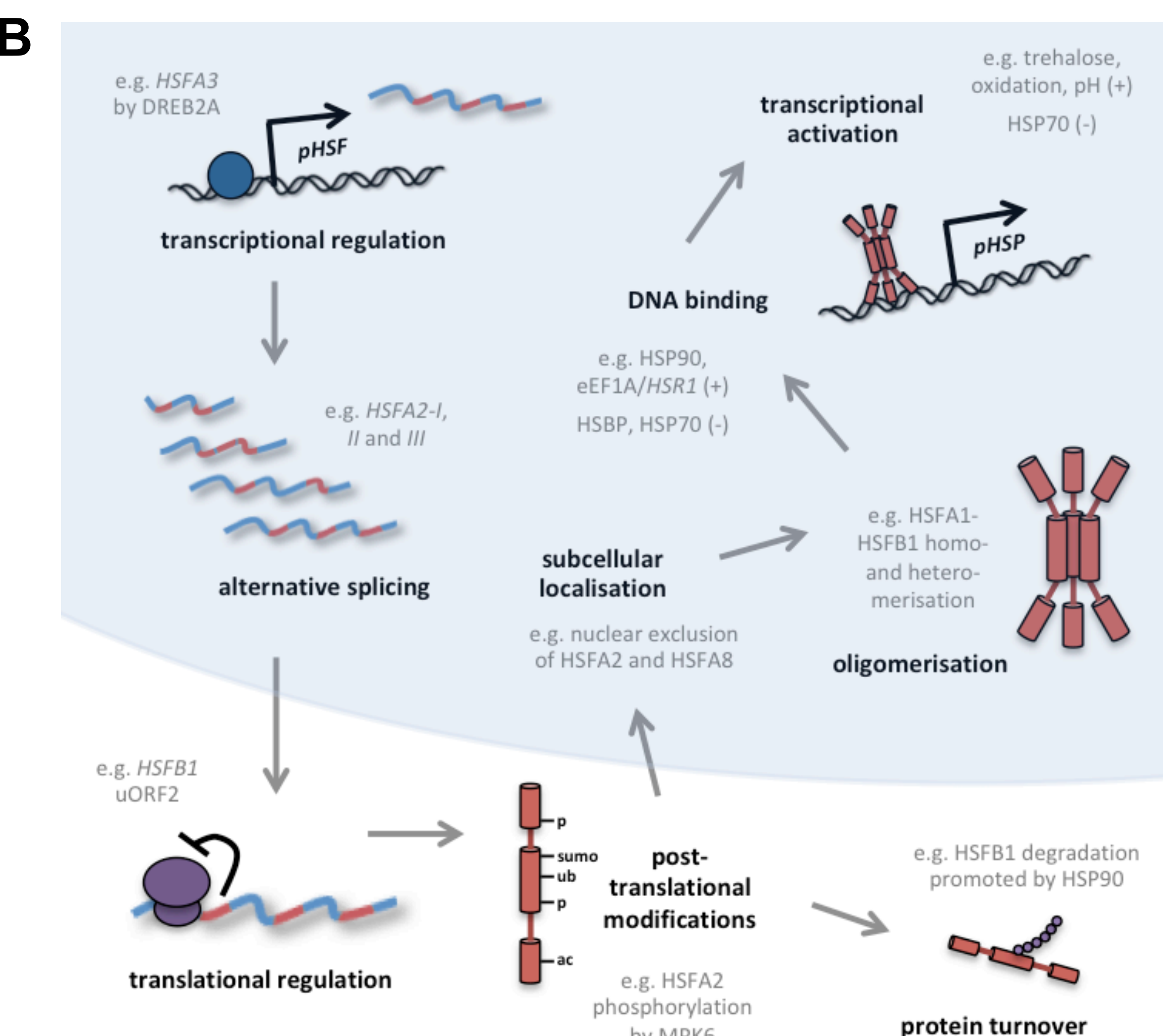


Figure 1.3: Plant heat shock factors (HSFs).
(**A**) Main domain features of the three *A. thaliana* HSF families. N-/C-: N- and C-termini, DBD: DNA-binding domain, OD: oligomerisation domain, NLS: nuclear localisation signal, NES: nuclear export signal, AHA: activation domain, LFGV: repressor domain (absent in HSFB5). Based on Guo *et al.* (2016).
(**B**) HSF activity is regulated at multiple steps that are dependent on ambient temperature. In plants (unless stated otherwise *A. thaliana*), these include: transcriptional activation of *HSFA3* by the drought-associated transcription factor DREB2A (Sakuma *et al.*, 2006); differential splicing of *HSFA2* into *HSFA2-I*, *HSFA2-II* and *HSFA2-III* variants (Sugio *et al.*, 2009; Liu *et al.*, 2013b); translational repression by upstream open reading frame uORF2 in *HSFB1* mRNA (Zhu *et al.*, 2012); phosphorylation of HSFA2 by the mitogen-activated kinase MPK6 (Evrard *et al.*, 2013); targeted degradation of HSFB1 by the chaperone HSP90 (Hahn *et al.*, 2011); nuclear exclusion of HSFA2 and HSFA8 (Kotak *et al.*, 2004); homo- and hetero-oligomerisation of HSFs, such as HSFA1 and HSFB1 in tomato (Bharti *et al.*, 2004); differential DNA binding, which is promoted by both HSP90 (Hahn *et al.*, 2011) and, in human cells, the elongation factor eEF1A (in complex with the long noncoding RNA *HSR1*) (Shamovsky *et al.*, 2006) but repressed by the transcriptional repressor HSBP (Hsu *et al.*, 2010) and the chaperone HSP70 (Hahn *et al.*, 2011); and levels of transcriptional activation, which are enhanced in the presence of oxidising agents, high or low pH (Liu *et al.*, 2013a) or, in budding yeast, the sugar trehalose (Conlin & Nelson, 2007) but are repressed by HSP70 (Hahn *et al.*, 2011).

Similarly, some HSF genes are expressed only during certain developmental stages, for example *HSFA9* is induced during seed maturation and *HSFA4c* and *HSFA5* are expressed predominantly in pollen tubes (Kotak *et al.*, 2007).

HSFA1a was found to be a master regulator of the heat shock response in *Solanum lycopersicum*, in which the HSF family encompasses 24 proteins, as post-transcriptional silencing of *HSFA1a* but not that of *HSFA2* or *HSFB1* resulted in compromised thermotolerance (Mishra *et al.*, 2002). Members of the HSFA1 group, particularly HSFA1a and HSFA1d, were subsequently shown to be master regulators in *A. thaliana*, as thermotolerance is severely compromised in T-DNA insertion mutants lacking both *HSFA1a* and *HSFA1d* and is completely abolished in triple mutants additionally lacking *HSFA1b* (Liu *et al.*, 2011). Heat-induction of *HSFA2* expression is mediated by HSFA1a, HSFA1b and HSFA1d, and the HSFA2 protein plays a key role in transcriptional amplification of the HSR initiated by these A-class HSFs (Liu *et al.*, 2011). Other A-class HSFs have roles in a variety developmental processes and abiotic stress responses. HSFA3 regulates drought-responsive genes as well as heat-responsive genes, and its own expression is induced by the drought-associated transcription factor DREB2A (DRE-BINDING PROTEIN 2A) during these stresses (Sakuma *et al.*, 2006). Members of the HSFA4 family are regulators of ROS homeostasis, and mutants in *A. thaliana* and *O. sativa* are characterised by low expression of the ROS-detoxifying enzyme *APX1* (*ASCORBATE PEROXIDASE 1*) and leaf lesions resulting from uncontrolled bursts of ROS (Yamanouchi *et al.*, 2002; Davletova *et al.*, 2005). Currently, the sole function of HSFA5 appears to be the regulation HSFA4 proteins, with HSFA5 forming repressive hetero-oligomers with HSFA4a and HSFA4c *in vivo* (Baniwal *et al.*, 2007). HSFA9 has been found to regulate embryogenesis in *A. thaliana* and *Helianthus annuus*. It interacts with transcription factors of the DREB2 family to control *HSP* expression in developing seeds (Almoguera *et al.*, 2002; Díaz-Martín *et al.*, 2005), and its own expression is in turn regulated by the seed-specific transcription factor ABI3 (ABA INSENSITIVE 3) (Kotak *et al.*, 2007).

HSFB1 and HSFB2b have been shown to transcriptionally repress the promoters of several *sHSP* genes and heat-inducible *HSF* genes, including *HSFA2* and *HSFA7a* as well as their own promoters (Ikeda *et al.*, 2011). In accordance with this finding, overexpression of a HSFB2-type transcription factor from *Vitis pseudoreticulata* in tobacco plants was found to decrease survival when exposed to severe heat stress (Peng *et al.*, 2013). When acclimated with a less intensive heat pre-treatment, though, these plants had higher survival rates relative to wild-type (Peng *et al.*, 2013). Similarly, *A. thaliana hsfb1 hsfb2b* double mutant seedlings showed reduced viability under such

conditions (Ikeda *et al.*, 2011), indicating that HSFB proteins exert both positive and negative effects on heat shock responses. In tomato, though not in *Arabidopsis*, HSFB1 was shown to promote gene activation by forming a complex with HSFA1a and the histone-modifying enzyme HAC1 (HISTONE ACETYLTRANSFERASE OF THE CBP FAMILY 1), the latter of which enhances promoter activity by acetylating histones (Bharti *et al.*, 2004).

Little is known about C-type HSFs, which are scarce in dicots but more numerous in monocots (Guo *et al.*, 2016). *HSF1C* expression is rapidly induced by cold shock in *A. thaliana*, and Park *et al.* (2015) have shown that overexpression of *HSFC1* affects the expression around 120 genes involved in cold acclimation and enhances freezing tolerance. The *rcf2-1* (*regulator of CBF gene expression 2-1*) mutant, which has lower expression of a small number of *HSF* genes including *HSFC1*, is hypersensitive to both heat and freezing stresses, suggesting that HSFC1 may play a role in both responses (Guan *et al.*, 2014). As it lacks an activation domain, HSFC1 most likely regulates expression in cooperation with other transcription factors, but further experiments are required to ascertain the function of C-type HSFs.

1.2.4 Regulation of HSF activity

Multiple mechanisms control the activity of HSF proteins, including regulation at the levels of transcription, alternative splicing, translation, post-translational modifications, subcellular localisation and interactions with proteins, RNA or small molecules (*Figure 1.3B*). As discussed above, the expression of *HSF* genes is controlled by many different transcription factors according to environmental or developmental cues. The mRNAs of some *HSF* genes, including *HSFA2*, *HSFA4c*, *HSFA7b*, *HSFB1* and *HSFB2b* in *A. thaliana*, have been found to undergo temperature-dependent alternative splicing (He *et al.*, 2007; Sugio *et al.*, 2009; Amano *et al.*, 2012; Liu *et al.*, 2013b; Chang *et al.*, 2014). The *HSFA2* gene, for example, encodes three splice variants: the functional full-length *HSFA2* (*HSFA2-I*); the *HSFA2-II* splice variant expressing a mini-exon that is absent in *HSFA2-I* and that contains a premature stop codon, thereby targeting HSFA2-II mRNA for degradation by nonsense-mediated mRNA decay (Sugio *et al.*, 2009); and the *HSFA2-III* splice variant induced by severe heat shock, in which the first intron is retained, yielding a truncated protein containing the DNA-binding domain and a novel hydrophobic region that acts to amplify *HSFA2* transcription (Liu *et al.*, 2013b). Heat-dependent alternative splicing has also been shown for transcriptional regulators of HSF genes, for example rice DREB2B (DRE-BINDING PROTEIN 2B) (Matsukura *et al.*, 2010).

Translational regulation of *HSF* mRNA by upstream open reading frames (uORFs) has recently been demonstrated in *A. thaliana*. Inhibitory uORFs may prevent ribosomes from initiating translation at the start codon of the main open reading frame. Zhu *et al.* (2012) have demonstrated that the second uORF in the 5' UTR of *HSFB1* represses translation under normal growth conditions but not during heat shock, allowing the HSFB1 protein to accumulate at elevated temperatures. uORFs have been identified in a third of *A. thaliana* HSF genes, including *HSFA1d*, *HSFA1e*, *HSFA2*, *HSFA4a*, *HSFB2b* and *HSFC1* in addition to *HSFB1* (Zhu *et al.*, 2012), but their functions remain to be characterised.

HSF proteins are subject to a number of post-translational modifications (PTMs) that may alter their activity, and such modifications have been extensively studied in budding yeast and mammalian cells using the master regulator HSF1 as a model system. One of the earliest indications that HSF1 is biochemically modified *in vivo* was the characteristic retardation in SDS-PAGE migration observed for heat-treated HSF1 relative to non-treated protein, which was shown to be caused by phosphorylation (Sorger *et al.*, 1987, 1989). Basal levels of phosphorylation in the absence of heat stress contribute to repression of transcriptional activity (Chu *et al.*, 1996, 1998; Kline & Morimoto, 1997; Wang *et al.*, 2006). In contrast, during heat shock, HSF1 undergoes hyperphosphorylation and these modifications enhance its capacity for transcriptional activation (Calderwood *et al.*, 2010). In budding yeast, 73 phosphorylation sites have been identified on HSF1 (Zheng *et al.*, 2016), and different stress treatments were found to induce distinct phosphorylation patterns (Liu & Thiele, 1996).

Less is known about post-translational modifications of plant HSFs. Liu *et al.* (2008) have demonstrated that *A. thaliana* HSFA1a can be phosphorylated *in vitro* by the calmodulin-regulated kinase CBK3 (CALMODULIN-BINDING KINASE 3), which promotes its DNA-binding ability. In contrast, *in vitro* phosphorylation by the cyclin-dependent kinase CDKA1 (CYCLIN-DEPENDENT KINASE A1) was found to repress the DNA association of HSFA1a (Reindl *et al.*, 1997). Tomato HSFA3, though not HSFA1a, is phosphorylated *in vitro* by extracts from heat-treated tomato cells (Link *et al.*, 2002). *Arabidopsis* HSFB2a was reported to be phosphorylated *in vitro* by two calcium-dependent protein kinases involved in defence responses (Kanchiswamy *et al.*, 2010). More recently, Evrard *et al.* (2013) showed that HSFA2 is phosphorylated *in vivo* at threonine 249 by the MAP kinase MPK6 (MAP KINASE 6) and that this modification regulates its nuclear localisation.

Phosphorylation of human HSF1 at S216 can stimulate its polyubiquitination and subsequent degradation (Lee *et al.*, 2008a). Human HSF2 is ubiquitinated at four

lysine residues *in vivo*, though the functions of these modifications are unknown (Kim *et al.*, 2011b; Wagner *et al.*, 2011). Phosphorylation of human HSF1 at S303 can also trigger sumoylation at lysine 298, which represses transcriptional activity (Hietakangas *et al.*, 2003, 2006). While sumoylation of transcription factors is often associated with transcriptional repression, as is the case for human HSF4 (Hietakangas *et al.*, 2006), this modification has been shown to enhance the DNA-binding ability of human HSF2 (Goodson *et al.*, 2001). In *A. thaliana*, this modification has also been shown stabilise transcription factors that are prone to degradation by polyubiquitination (Miura *et al.*, 2007, 2009). Two additional modifications identified on human HSF1 are the acetylation of residues M1 and K80, which appear to affect subcellular localisation (Forte *et al.*, 2011) and to negatively regulate DNA binding (Westerheide *et al.*, 2009), respectively.

In addition to chemical modifications that affect DNA-binding and transactivation, HSF proteins are regulated by their cellular distribution and by their ability to oligomerise. By transient expression in tobacco protoplasts, Kotak *et al.* (2004) showed that most *A. thaliana* HSFs, while concentrated in the nucleus, are also present in the cytosol, with the exception of HSFA8, which is absent from the nucleus under normal conditions. Both HSFA2 and HSFA8 are actively exported from the nucleus, as shown by their nuclear accumulation after treatment with the nuclear export inhibitor leptomycin B (Kotak *et al.*, 2004). During heat shock, HSFA1a, HSFA1b, HSFA1d and HSFA2 localisation is exclusively nuclear (Yoshida *et al.,* 2011; Evrard *et al.*, 2013), and it has been demonstrated that the heat-induced nuclear retention of HSFA2 is dependent on phosphorylation of T249 by MPK6 (Evrard *et al.*, 2013). Unlike other plant HSFs, HSFA2 can also localise to cytosolic stress granules following prolonged heat shock (Scharf *et al.*, 1998).

Based on the triplet nature of HSE motifs and the migration size of HSF proteins separated by polyacrylamide gel electrophoresis (Liu *et al.*, 2013a), the latter are generally believed to bind DNA as trimers. This has been confirmed for human HSF1 using X-ray crystallography (Neudegger *et al.*, 2016). Human HSF2, however, was found to bind as a dimer even when presented with a perfect HSE (Jaeger *et al.*, 2016). Similarly, tomato HSFB1 appears to bind preferentially as a dimer (Bharti *et al.*, 2004), while HSFA1 and HSFA2 proteins form trimers *in vivo* (Chan-Schaminet *et al.*, 2009). HSF monomers may also bind to nGAAn repeats but at lower affinity than trimers (Kim *et al.*, 1994). In addition, dimers or trimers may bind cooperatively to form higher-order complexes (Chan-Schaminet *et al.*, 2009; Jaeger *et al.*, 2016). Plant HSFA1 proteins can form homo-oligomers or hetero-oligomers with other HSFA1 (Yoshida *et al.*, 2011),

HSFA2 (Heerklotz *et al.*, 2001) or HSFB1 proteins (Bharti *et al.*, 2004), suggesting that different HSF combinations may fine-tune the heat shock response.

Hentze *et al.* (2016) have recently shown that in human HSF1 a hydrophobic region adjacent to the activation domain, named HR-C, interacts with the HR-A/B regions in the oligomerisation domain to maintain the protein as an inactive monomer, whereas heat causes dissociation HR-C from HR-A/B and favours inter-molecular HR-A/B interactions, thus promoting oligomerisation. Yeast and plant HSFs lack the conserved HR-C domain found in metazoans, suggesting divergent mechanisms of regulating oligomerisation.

While a large number of proteins have been shown to regulate the post-translational modifications of HSFs in mammals (Xu *et al.*, 2012), few proteins are known to interact with HSFs in plants. Along with the aforementioned HSF kinases, plant HSFs are regulated by a class of negative regulators known as HSF-binding proteins (HSBPs), which trigger the dissociation of HSFs from their target DNA (Fu *et al.*, 2006; Hsu *et al.*, 2010; Rana *et al.*, 2012). Human HSF1 is additionally regulated by a ribonucleoprotein complex containing the elongation factor eEF1A and the long noncoding RNA (lncRNA) *HSR1* (*HEAT SHOCK RESPONSE 1*). Silencing of either eEF1A or *HSR1 in vivo* impairs HSF1 activation in human cells (Shamovsky *et al.*, 2006; Vera *et al.*, 2014). eEF1A was reported to re-localise to the nucleus during heat stress, where it facilitates the DNA-binding ability of HSF1, interacts with RNA polymerase II, stabilises the 3' UTR of *HSP70* mRNA and facilitates its nuclear export and translation (Vera *et al.*, 2014). Abundance of eEF1A increases upon heat shock and is correlated to heat resilience in different varieties of potato, suggesting that this elongation factor may also play a role in regulating the HSR in plants (Momčilović *et al.*, 2016).

Another group of key HSF regulators consists of the products of HSF target genes. Several yeast chaperone mutants were found to exhibit constitutive activation of HSF1, for example the double *ssa1 ssa2* mutant deficient in the two constitutive *HSP70* genes (Halladay & Craig, 1995), the double *hsc82 cpr7* (*heat shock cognate 82 / cyclosporin-sensitive proline rotamase 7*) mutant lacking the constitutive HSP90 gene and its cochaperone (Duina *et al.*, 1998) or single *sse1* or *sti1* (*stress inducible 1*) mutants compromised in the expression of a HSP110 or HSP70/90 cochaperone, respectively (Liu *et al.*, 1999). Subsequently, HSF1 was found to physically interact with HSP70 proteins SSB1 and SSB2, the double mutant of which has enhanced HSF1 activation during stress (Bonner *et al.*, 2000). HSP70 proteins SSA1 and SSA2 have also been identified as HSF1 interactors by mass spectrometry (Zheng *et al.*, 2016). Based on these genetic and physical interactions, it was suggested that chaperones, in particular

HSP70s, repress HSF1 activity in the absence of stress, whereas the accumulation of unfolded proteins during proteotoxic stress favours their dissociation from HSF1. Zheng *et al.* (2016) have recently confirmed this hypothesis by demonstrating that HSF1 can be activated *in vivo* by titrating away HSP70 *via* inducible overexpression of either HSF1 or a non-functional HSF1 'decoy' lacking oligomerisation and activation domains.

From *in vitro* assays, it was found that human HSF1 can physically associate with a number of chaperones, including not only HSP70 but also HSP90 and HSP40 (Zou *et al.*, 1998). The importance of HSP90 in the regulation of HSF1 activity has been established using pharmacological inhibitors such geldanamycin or herbimycin A, which specifically inhibit HSP90 activity and are potent activators of HSF1 (Murakami *et al.*, 1991). Zou *et al.* (1998) demonstrated that antibody-depletion of HSP90, though not of HSP70, from human cell lysates can induce the DNA-binding ability of HSF1, suggesting that HSP90 is the key regulator of HSF1 in mammalian cells. In addition, Wang *et al.* (2005) have shown that phosphorylation of human HSF1 at S121 can promote HSP90 association.

HSP90 inhibitors also induce heat-inducible genes in *A. thaliana*, and physical interactions between HSP90 and HSFA1d, HSFA7a and HSF1B have been demonstrated *in vivo* (Yamada *et al.*, 2007). A small increase in the expression of heat-inducible genes is observed in *A. thaliana* plants constitutively overexpressing either *HSFA1a* (Qian *et al.*, 2014) or a non-functional *HSP90.2* gene (Yamada *et al.*, 2007). In addition, prolonged HSFA1a activity is observed in *A. thaliana* plants heat-inducibly expressing an antisense *HSP70* gene (Lee & Schöffl, 1996). These findings support the model whereby chaperones regulate HSF activity in plants. Kim & Schöffl (2002) have shown that HSFA1a can interact with both heat-inducible (HSP70-4) and constitutive (HSP70-1) *A. thaliana* HSP70 proteins in yeast two-hybrid assays. Hahn *et al.* (2011) established that tomato HSFA1a and HSFA2, but not HSFB1, can interact *in vivo* with both HSP70 and HSP90. Furthermore, the authors demonstrated that HSP70 represses the DNA-binding and activation of HSF proteins, while HSP90 promotes DNA-binding and additionally regulates the mRNA and protein degradation of different HSFs.

In addition to regulation by protein-protein interactions, HSF activity appears to be influenced by small molecules or ions. Early experiments identified the importance of calcium ions, which are well-known secondary messengers in all organisms, for the DNA-binding ability of human HSF1 and transcription of *HSP70* (Mosser *et al.*, 1990; Price & Calderwood, 1991), whereas excess cytosolic calcium was found to have a negative impact on HSF1 activation (Soncin *et al.*, 2000). Indeed, a cytosolic increase in calcium ions is one of the earliest responses of plant cells to elevated temperatures

(Gong *et al.*, 1998; Zheng *et al.*, 2012; Gao *et al.*, 2012), and inhibitors of calcium signalling have been shown to block the accumulation of heat-induced proteins (Suri & Dhindsa, 2008; Saidi *et al.*, 2009) and to compromise thermotolerance in plants (Gong *et al.*, 1998; Larkindale & Knight, 2002).

Conlin & Nelson (2007) have demonstrated that the cellular concentration of trehalose positively influences the activation of HSF1 in budding yeast. Trehalose stimulates tertiary folding of the C-terminus of HSF1, as do elevated temperatures and low pH (Bulman & Nelson, 2005; Pattaramanon *et al.*, 2007). *In vitro* and *in vivo* analyses of *A. thaliana* HSFA1a have shown that heat, oxidation and high or low pH trigger its trimerisation and DNA-binding, while incubation with reducing agents dithiothreitol (DTT) or reduced nicotinamide adenine dinucleotide phosphate (NADPH) counteract effects (Liu *et al.*, 2013a). Further supporting the role of ROS in activating plant HSF proteins, Jung *et al.* (2013b) showed that mutating two cysteine residues in HSFA1d compromised the induction of the ROS-scavenger gene *APX2* (*ASCORBATE PEROXIDASE 2*) during high light stress.

1.2.5 Thermotolerance

Thermotolerance is the ability to withstand severe, typically lethal, heat stress. Transient exposure to elevated sub-lethal temperatures (heat acclimation) leads to enhanced heat resilience, known as acquired thermotolerance. Basal thermotolerance (BT), on the other hand, refers to the levels of tolerance in the absence of acclimation (Yeh *et al.*, 2012). Acquired thermotolerance may be separated into short-term (SAT) and long-term (LAT) responses, depending on the period of recovery between heat treatments (typically less and greater than 24 hours, respectively). In contrast to basal and acquired thermotolerance, which are typically studied using relatively short treatments at elevated temperatures (38-45°C for 30 minutes up to 16 hours, in *A. thaliana*), a fourth type of thermotolerance, known as thermotolerance to moderately high temperatures (TMHT), is assessed using long incubations at lower temperatures (30-35°C for several days to weeks in *A. thaliana*) (Yeh *et al.*, 2012).

The most frequently used phenotype for scoring thermotolerance in *A. thaliana* seedlings is viability, which is usually assessed visually but has also been quantified using the colorimetric triphenyl-tetrazolium chloride (TTC) reduction assay (Zhou *et al.*, 2012). Plant health following heat shock can be assessed by measuring fresh weight (Routaboul *et al.*, 2012), hypocotyl elongation or root growth (Hong & Vierling, 2000), or by quantifying the abundance of metabolites such as sucrose (Van der Westhuizen,

2017) or of chlorophyll, either visually based on leaf colour (Chen *et al.*, 2006a) or biochemically (Burke *et al.*, 2000). The extent of heat-damage to membranes can be measured using electrolyte leakage assays (Clarke *et al.*, 2009) and thiobarbituric acid reactive substances (TBARS) assays (Larkindale *et al.*, 2005). Levels of ROS and the ROS-scavenging ability of cells can also be estimated using 3,3'-diaminobenzidine (DAB) or nitro blue tetrazolium chloride (NBT) staining (Lv *et al.*, 2011) and glutathione reductase assays (Van der Westhuizen, 2017).

As photosynthesis is highly sensitive to heat, thermotolerance can be evaluated by photosynthetic efficiency in plants subjected to heat stress. Chlorophyll fluorescence techniques give an estimate of the ratio of energy used for photosynthesis relative to that dissipated as heat or fluorescence (Routaboul *et al.*, 2012). Although chlorophyll fluorescence is the most common analysis used to study photosynthetic thermotolerance, measurements of oxygen evolution from leaves have also been used to determine photosynthetic activity after heat shock (Murakami *et al.*, 2000). Most studies have assessed thermotolerance in vegetative tissues, particularly in *A. thaliana* seedlings, but a growing number of studies are studying thermotolerance in seeds, by measuring germination rates after heat shock, or in reproductive tissues, by quantifying seed yield or pollen viability (Yeh *et al.*, 2012).

Basal and acquired thermotolerance are positively correlated to the expression level of heat shock proteins. Thermotolerance is enhanced in *A. thaliana* plants overexpressing *HSP70* (Sung & Guy, 2003) or in seedlings pre-treated with HSP90 inhibitors that induce *HSP* gene expression (Yamada *et al.*, 2007). In a genetic screen for heat-sensitive *A. thaliana* mutants, Hong & Vierling (2000) identified the *hot1-1* mutant, which contains a missense mutation in the *HSP101* gene and is severely compromised in hypocotyl growth following severe heat stress. Overexpression of *HSP101* was subsequently shown to enhance thermotolerance in *A. thaliana* (Queitsch *et al.*, 2000). Numerous studies have investigated the effects of *sHSP* expression on thermotolerance, and overexpression of native *sHSP* genes (Malik *et al.*, 1999; Murakami *et al.*, 2004; Wang *et al.*, 2015b) or heterologous expression of plant or fungal homologues (Katiyar-Agarwal *et al.*, 2003; Ramakrishna *et al.*, 2003; Sanmiya *et al.*, 2004; Ahn & Zimmerman, 2006; Mu *et al.*, 2011; Sun *et al.*, 2012; Zou *et al.*, 2012) has been reported to increase thermotolerance in a large number of plant species. Plant *sHSP* genes expressed in bacteria or yeast cells have also been shown to increase survival during heat stress (Yeh *et al.*, 1997; Soto *et al.*, 1999; Murakami *et al.*, 2004; Xue *et al.*, 2010; Wang *et al.*, 2017a). Comparative studies have shown positive correlations between *sHSP* expression levels and heat resilience among different crop cultivars (Ristic *et al.*, 1991, 1996; Jorgensen *et*

al., 1992; Park *et al.*, 1996). Furthermore, overexpression of *HSFA1a* (Lee *et al.*, 1995), *HSFA1b* (Prändl *et al.*, 1998), *HSFA2* (Ogawa *et al.*, 2007) or *HSFA3* (Yoshida *et al.*, 2008) enhances basal thermotolerance in *A. thaliana*.

Other studies have demonstrated the complexity of thermotolerance responses, as acclimated wild-type *A. thaliana* plants still have substantially higher thermotolerance than non-acclimated plants overexpressing *HSP101* (Queitsch *et al.*, 2000), and in many cases the overexpression of *sHSP* genes has either no effect on thermotolerance (Sun *et al.*, 2001) or in fact compromises thermotolerance (Sun *et al.*, 2016). Null mutations in *HSF* genes can have complex consequences on heat resilience, for example HSFA2 is required for long- but not short-term acquired thermotolerance (Charng *et al.*, 2007), and the HSFB1 and HSFB2b proteins play a role in promoting acquired thermotolerance but negatively regulate basal thermotolerance (Ikeda *et al.*, 2011). In addition, a large number of genes that do not belong to *HSP* or *HSF* families appear to play a role in thermotolerance.

Multiple genetic screens have been carried out to isolate mutants compromised in thermotolerance (Wu *et al.*, 2000, 2010; Hong *et al.*, 2003; Lee *et al.*, 2006b, 2008b; Kwon *et al.*, 2007; Wang *et al.*, 2013b). Because of difficulties associated with screening for reduced survival, many thermotolerance-associated genes have been identified indirectly, for example based on their expression patterns (Larkindale & Vierling, 2008; Charng *et al.*, 2006, 2007) or from biochemical data (Liu *et al.*, 2008; Hsu *et al.*, 2010). These genes encode proteins with roles in diverse cellular processes.

Not surprisingly, in addition to *HSP* and *HSF* genes, many loci involved in thermotolerance have been identified as encoding cell damage response genes (Larkindale *et al.*, 2005), proteins interacting with HSFs or HSPs (Hsu *et al.*, 2010; Zhang *et al.*, 2010b) or regulators of protein folding or turnover (Yan *et al.*, 2003; Chen *et al.*, 2006b; Larkindale & Vierling, 2008; Meiri & Breiman, 2009; Wang *et al.*, 2009; Meiri *et al.*, 2010; Basak *et al.*, 2014; Sedaghatmehr *et al.*, 2016). The accumulation of certain metabolites, in particular proline, is associated with reduced thermotolerance, as observed in plants overexpressing the proline-biosynthesis gene *P5CS1* (*DELTA1-PYRROLINE-5-CARBOXYLATE SYNTHASE 1*) (Lv *et al.*, 2011) or mutants of the proline-catabolising gene *PDH1* (*PROLINE DEHYDROGENASE 1*) (Larkindale & Vierling, 2008).

Other than HSFs, transcription factors playing a role in thermotolerance include WRKY25, WRKY26 and WRKY33 (Li *et al.*, 2011a), MBF1c (Suzuki *et al.*, 2008), DREB2A (Sakuma *et al.*, 2006), DREB2C (DRE-BINDING PROTEIN 2C) (Lim *et al.*, 2007), bZIP28 (BASIC LEUCINE ZIPPER 28) (Gao *et al.*, 2008) and NFXL1 (NFX-LIKE 1) (Larkindale & Vierling, 2008), as demonstrated by the heat-sensitivity of their null or overexpression

mutants. The SUMO E3 ligase SIZ1 (SAP AND MIZ 1) has also been found to contribute to basal thermotolerance (Yoo *et al.*, 2006), presumably by affecting transcription of heat-responsive genes, since sumoylation of transcriptional regulators and histone proteins affects gene expression (Verger *et al.*, 2003). Wu *et al.* (2010) have shown that the nuclear export receptor XPO1A (EXPORTIN 1A) is required for both basal thermotolerance and long-term growth at elevated temperatures and have suggested that it controls the distribution of transcriptional regulators between the nucleus and cytoplasm.

Two components of the cell wall have been found to promote thermotolerance. The pectin methylesterase PME34 (PECTIN METHYLESTERASE 34) is necessary for remodelling of the cell wall in guard cells to allow stomatal movement in response to elevated temperatures (Huang *et al.*, 2017). The *hot2* mutant, which produces a defective endochitinase-like protein CTL1 (CHITINASE-LIKE 1), has reduced recovery after heat stress and has been proposed to lack an essential interaction in the plasma membrane-cell wall interface that is required for membrane integrity during heat stress (Kwon *et al.*, 2007).

Membrane properties during heat shock are additionally regulated by lipid metabolising enzymes, lipid carriers and membrane tethering factors. Fatty acid desaturases FAD3, FAD7 and FAD8 (FATTY ACID DESATURASES 3/7/8), double or triple mutants of which are characterised by increased basal thermotolerance yet reduced growth at sustained high temperatures, regulate the abundance of trienoic fatty acids in thylakoid membranes and are necessary for photosynthesis at elevated temperatures (Murakami *et al.*, 2000; Routaboul *et al.*, 2012). The digalactosyl-diacylglycerol (DGDG) synthase DGD1 (DIGALACTOSYL DIACYLGLYCEROL DEFICIENT 1) regulates DGDG levels in thylakoid membranes and is also required for sustained photosynthesis under conditions of constant heat (Chen *et al.*, 2006a). Acquired thermotolerance relies in part on the choline kinase CEK2 (CHOLINE/ETHANOLAMINE KINASE 1) (Larkindale & Vierling, 2008), an enzyme whose activity has been shown to increase during heat stress in plants (Johnston *et al.*, 2007) and which is involved in the synthesis of phosphatidylcholine, the major phospholipid of eukaryotes and a precursor of thylakoid membrane lipids (Mishra & Grover, 2014). Chi *et al.* (2009) have demonstrated that the lipid-binding protein TIL1 (TEMPERATURE-INDUCED LIPOCALIN 1) is important for both basal and thermotolerance and have suggested that it sequesters oxidised lipids during heat stress. The membrane-tethering factor VPS53 (VACUOLAR PROTEIN SORTING-ASSOCIATED 53), which is part of the retrograde pathway to the Golgi, is required for membrane trafficking during heat stress and its

mutant *hit1* (*heat intolerant 1*) has excessive membrane leakage during growth at elevated temperatures (Wang *et al.*, 2011a).

As well as playing structural roles in cellular membranes, lipids such as phosphatidylcholine and DGDG are known to act as secondary messengers (Chen *et al.*, 2006a; Mishra & Grover, 2014) and may play roles signal transduction pathways leading to heat acclimation. Zheng *et al.* (2012) have shown that the phospholipase C mutant *plc9* has lower temperature-dependent accumulation of inositol-1,4,5-trisphosphate (IP_3), which in turn affects calcium signalling and *sHSP* gene induction, thereby decreasing tolerance to high temperatures. Thermotolerance defects have been identified in calcium signalling mutants, for example in null lines of *CAM3* (*CALMODULIN 3*), one of the genes encoding the calcium-binding protein calmodulin (CaM) (Zhang *et al.*, 2009), and of *CBK3*, which encodes a CaM-binding kinase that phosphorylates HSFA1a *in vitro* (Liu *et al.*, 2008). The phosphatase PP7 (SERINE/THREONINE PHOSPHATASE 7) (Liu *et al.*, 2007a) and nitric oxide signalling-associated proteins NOA1 (NITRIC OXIDE-ASSOCIATED 1) (Xuan *et al.*, 2010) and GSNOR1 (GSNO REDUCTASE 1) (Lee *et al.*, 2008b) also regulate thermotolerance, probably *via* the same IP_3- and calcium-dependent pathway. PP7 interacts with both CaM and HSFA1a (Liu *et al.*, 2007a), while NOA1 and GSNOR1 stimulate the production of nitric oxide, the levels of which increase during heat stress (Xuan *et al.*, 2010). DNA-association of HSFs is reduced in the *noa1* mutant, while CAM3 overexpression can partially rescue this phenotype (Xuan *et al.*, 2010).

ROS play a complex role in thermotolerance, as they are essential secondary messengers for activating the HSR yet can cause oxidative damage when overproduced. This is demonstrated by the fact that genes encoding the ROS-detoxifying enzymes CSD1, CSD2 (COPPER/ZINC SUPEROXIDE DISMUTASE 1/2) and CCS (COPPER CHAPERONE FOR SOD1) are post-transcriptionally silenced by heat-induced microRNA *miR398* at elevated temperatures and their null mutants have enhanced heat resilience (Guan *et al.*, 2013b). Similarly, the *tylapx* (*thylakoid ascorbate peroxidase*) mutant lacking a chloroplast-localised isoform of the ROS-scavenging enzyme ascorbate peroxidase has increased thermotolerance (Miller *et al.*, 2007). On the other hand, increased heat sensitivity is observed in the *apx1* and *apx2* mutants, which are deficient in cytosolic ascorbate peroxidases (Suzuki *et al.*, 2012). In the *shot1* (*suppressor of hot1-4*) mutant, which lacks a mitochondrial transcription termination factor, enhanced heat resilience is associated with lower levels of heat-induced ROS (Kim *et al.*, 2012b).

Nearly all phytohormones are implicated in high-temperature responses. In *A. thaliana* seedlings, for example, basal thermotolerance is promoted by abscisic acid

(ABA), salicylic acid (SA) and jasmonic acid (JA) whereas it is negatively regulated by ethylene, as indicated by the phenotypes of their null signalling mutants (Clarke *et al.*, 2004, 2009; Larkindale *et al.*, 2005). Exogenous applications of *A. thaliana* seedlings with ABA, SA or JA have been shown to improve survival to lethal heat shock (Larkindale & Knight, 2002; Clarke *et al.*, 2009). Similarly, treatments with gibberellins, brassinosteroids, auxins or cytokinins in a variety of plants have been demonstrated to enhance certain aspects of growth at elevated temperatures, for example metabolism and reproduction (Ogweno *et al.*, 2008; Sakata *et al.*, 2010; Asthir & Bhatia, 2014; Sobol *et al.*, 2014).

Mutants with altered responses to long-term acquired thermotolerance have provided an insight into mechanisms of heat stress-associated memory in plants. HSP101 and the sHSP HSA32 (HEAT STRESS ASSOCIATED 32) stabilise each other at the protein level and delay their degradation (Charng *et al.*, 2006; Wu *et al.*, 2013). The chromatin-binding protein FGT1 (FORGETTER 1) binds to the promoters of heat-responsive genes and recruits chromatin remodellers of the SWI/SNF (SWITCH/SUCROSE NON-FERMENTING) and ISWI (IMITATION SWI) families to maintain low nucleosome occupancy for sustained gene expression (Brzezinka *et al.*, 2016). In addition, HSFA2 binding to many *HSP* genes promotes di- and tri-methylation of histone H3 lysine 4 (H3K4me2/me3), which is associated with prolonged gene activation (Lämke *et al.*, 2016).

1.3 Plant responses to low temperatures

1.3.1 Cold acclimation

Cold temperatures alter the physical properties of membranes, nucleic acids and proteins and reduce enzyme kinetics. As a consequence of lower enzyme activity, the efficiency of photosynthesis is diminished and an imbalance occurs between light harvesting and energy dissipation *via* metabolic activity (Öquist & Huner, 2003). Under these conditions, photosystem II (PSII) becomes prone to over-excitation, and as a result ROS are generated and cellular oxidative damage may occur. At sub-zero temperatures, formation of ice crystals can disrupt cellular membranes, leading to cellular electrolyte leakage and disruption of key functions such as those of chloroplasts and mitochondria. Freezing temperatures also impose an osmotic stress to cells, because, in addition to an overall decrease in availability of liquid water in the environment, cellular water is lost by osmosis as a result of ice formation in the intercellular space (Thomashow, 1999).

Unlike plants originating from tropical regions, *A. thaliana* and other plants from temperate climates are capable of surviving sub-zero temperatures upon prior exposure to non-freezing temperatures. In winter rye, for example, temperatures of around -5°C are usually lethal, but crops can withstand freezing down to -30°C following a priming period at low non-freezing temperatures (Thomashow, 1999). During this process, known as cold acclimation (*Figure 1.4*), plant cells induce a diverse array of cold-responsive (*COR*) genes, many of which are also expressed during drought or osmotic stress and ABA treatment (Thomashow *et al.*, 1997). Zhao *et al.* (2016) have identified over 2500 genes that are induced by different durations of cold treatment in *A. thaliana*. Although the expression patterns of *COR* genes have been extensively studied and a number of these genes are common markers of cold stress, for example *COR15a*, *COR47*, *RD29a* (*RESPONSIVE TO DESSICATION 29a*, also known as *COR78*) and *KIN1* (*COLD INDUCED 1*), the precise functions of most COR proteins remain to be characterised.

A considerable number of *COR* genes encode LEA (LATE EMBRYOGENESIS ABUNDANT) proteins, which act as 'dehydrins', binding both water molecules and divalent cations and stabilising cellular structures during osmotic stress (Hundertmark & Hincha, 2008). COR15a, for example, localises to the chloroplast stroma, where it regulates the physical properties of the thylakoid membrane (Steponkus *et al.*, 1998) and confers cryoprotection to enzymes, thus enhancing photosynthesis at low temperatures (Nakayama *et al.*, 2007). Some *COR* genes encode extracellular chitinases and thaumatin-like proteins that act as antifreeze proteins (AFPs) by binding to ice in

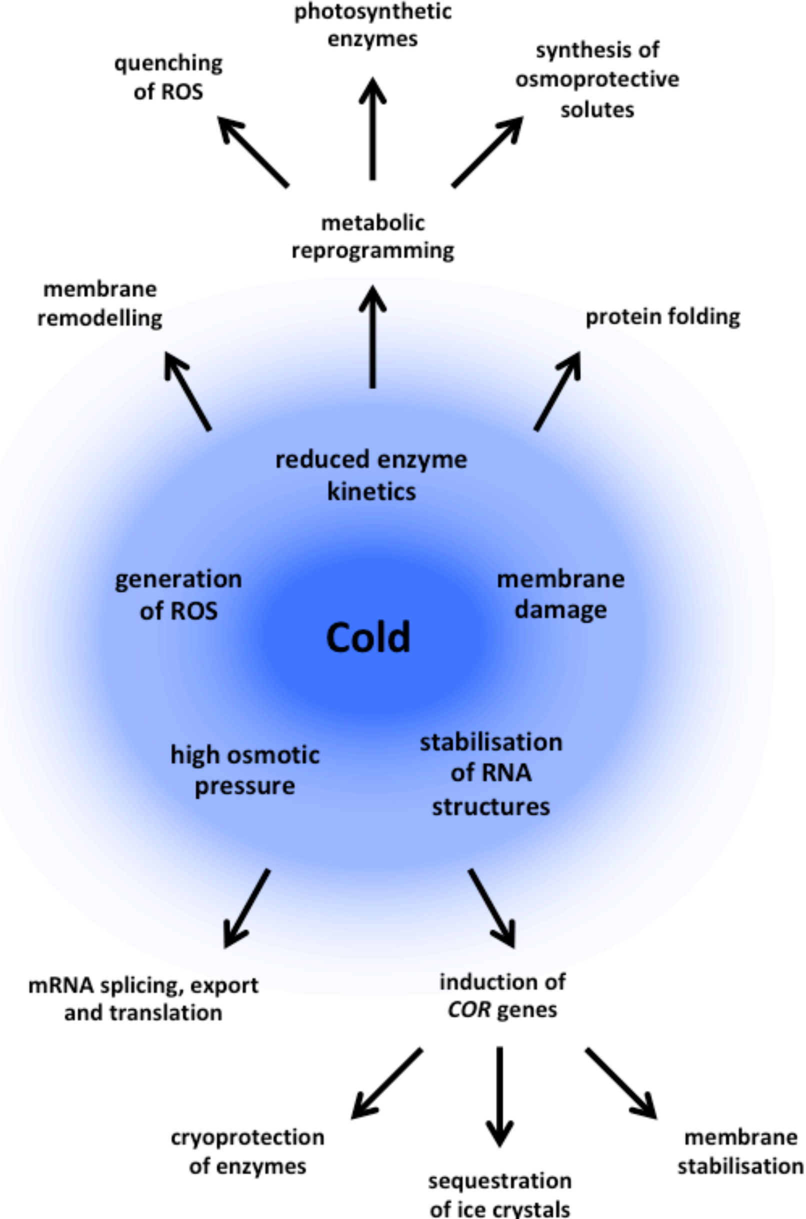


Figure 1.4: Summary of cellular responses to cold stress in plants, which lead to cold acclimation and, in chilling-tolerant species, freezing tolerance. Major deleterious effects of cold shock are shown in the blue circle, and cellular responses triggered to counteract these effects are listed above and below, in no particular order relative to the phrases in the circle. These responses include the activation of cold-responsive (*COR*) genes, encoding proteins that protect membranes and enzymes (Steponkus *et al.*, 1998; Nakayama *et al.*, 2007) and bind ice crystals to prevent their accumulation in plant tissues (Yeh *et al.*, 2000); increased expression of genes involved in mRNA splicing, export and translation (Usadel *et al.*, 2008); and increased activity of enzymes involved in stabilising labile proteins (Guy *et al.*, 1998), membrane integrity or desaturation (Schapire *et al.*, 2008), photosynthesis, reactive oxygen species (ROS) homeostasis or osmoprotective solute synthesis (Usadel *et al.*, 2008).

the apoplast (Yeh *et al.*, 2000).

The expression of metabolic enzymes is altered during cold exposure to increase the efficiency of temperature-sensitive processes such as photosynthesis and to promote the biosynthesis of osmoprotective solutes (Usadel *et al.*, 2008). These include polyamines and organic acids, as well as carbohydrates such as fructose, maltose, glucose and raffinose and the amino acids proline, glutamine and glycine (Gilmour *et al.*, 2000; Cook *et al.*, 2004; Usadel *et al.*, 2008; Maruyama *et al.*, 2009). Proteomics of *A. thaliana* plants identified an accumulation of starch-degrading enzymes and reduced abundance of a maltose-catabolising enzyme during cold treatment (Li *et al.*, 2011b). Mutants incapable of breaking down starch are hypersensitive to freezing (Yano *et al.*, 2005), while mutants accumulating high levels of raffinose and proline have enhanced tolerance to sub-freezing temperatures (Gilmour *et al.*, 2000; Takagi *et al.*, 2003; Nakamichi *et al.*, 2009; Jiang *et al.*, 2015).

As well as protecting cellular structures from dehydration, metabolites produced during cold exposure may protect from oxidative stress, for example tocopherol, phenylpropanoids and flavonoids are known to have ROS-scavenging properties and genes regulating their biosynthesis are upregulated during cold exposure (Usadel *et al.*, 2008). Tomato and *Arabidopsis* plants overexpressing ROS-scavenging enzymes, such as glutaredoxin or peroxidases, have enhanced growth at low temperatures (Kim *et al.*, 2012a; Hu *et al.*, 2015). Some LEA proteins also appear to have ROS-scavenging properties, as demonstrated by their ability to counteract lipid peroxidation *in vitro* (Hara *et al.*, 2003).

Wang *et al.* (2017b) have shown that polysome fractions are enriched in ribonucleoprotein pull-downs from cold-treated *A. thaliana* seedlings relative to those from untreated plants, suggesting that low temperatures slow down translation elongation or induce ribosome stalling, possibly by stabilising RNA secondary structures or reducing enzyme kinetics. Concomitantly, genes involved in protein synthesis are upregulated during cold stress, as are those involved in other processes that are affected by RNA secondary structures, such as RNA splicing and export (Usadel *et al.*, 2008; Guan *et al.*, 2013a). A number of chloroplast RNA-binding proteins are essential for plastid protein synthesis at low temperatures and their mutants have reduced viability when grown at 4°C (Wang *et al.*, 2016a). Low temperatures also affect the stability of plant proteins, as indicated by their increased interactions with HSP70 chaperones during cold stress (Guy *et al.*, 1998) and by the accumulation of various HSP100 disaggregases in cold-treated *A. thaliana* seedlings (Li *et al.*, 2011b).

The functions of many cold-inducible genes have been studied using reverse genetics in *A. thaliana*, and genes required for cold acclimation have additionally been identified in forward genetic screens of mutants with altered freezing tolerance. Such studies have identified multiple genes involved in regulating membrane composition, such as the lipid-binding proteins SYT1 (SYNAPTOTAGMIN 1) (Schapire *et al.*, 2008), LTP3 (LIPID TRANSPORTER 3) (Guo *et al.*, 2013) and ACBP1 (ACYL-CoA BINDING PROTEIN 1) (Du *et al.*, 2010), the lipid-metabolising enzymes PLDδ (Li *et al.*, 2004) and PLDα1 (Rajashekar *et al.*, 2006) (PHOSPHOLIPASE D δ/α1) and the regulator of vesicular trafficking APY2 (APYRASE 2) (Deng *et al.*, 2015). Enzymes affecting cell wall composition have also been shown to influence freezing tolerance, for example the mutants of ESK1 (ESKIMO 1) (Xin *et al.*, 2007; Yuan *et al.*, 2013) and BCB (BLUE COPPER BINDING PROTEIN) (Ji *et al.*, 2015), deficient in hemicellulose acetylation and lignin formation, respectively, are more tolerant to freezing than wild-type plants. In contrast, the *sfr3* (*sensitive to freezing 3*) mutant lacking a key enzyme in cuticle wax deposition is hypersensitive to freezing (Amid *et al.*, 2012).

The role of phytohormones in cold acclimation has been investigated from freezing tolerance phenotypes of biosynthetic or signalling mutants or of wild-type plants exogenously treated with hormones. ABA, which accumulates during drought or osmotic stress, induces many *COR* genes (Baker *et al.*, 1994; Xiong *et al.*, 2001), and plants overexpressing the ABA signalling component ABI3 have increased tolerance to freezing (Tamminen *et al.*, 2001) while those overexpressing the negative regulator of ABA signalling ABI1 (ABA INSENSITIVE 1) (Ding *et al.*, 2015) or those lacking the ABA biosynthetic enzyme ABA3 (ABA DEFICIENT 3) (Xiong *et al.*, 2001) are hypersensitive to freezing. Additional studies have implicated jasmonates (Hu *et al.*, 2013) and brassinosteroids (Eremina *et al.*, 2016) in the positive regulation of freezing tolerance, while ethylene (Shi *et al.*, 2012; Catalá *et al.*, 2015) and salicylic acid (Miura & Ohta, 2010; Yang *et al.*, 2010c) also appear to regulate this response, though the effects of exogenous applications with these phytohormones vary depending on the concentration of treatments and the developmental stage of treated plants.

1.3.2 The CBF regulon

The pathways regulating transcriptional changes upon cold exposure are less understood than those occurring during heat shock and are not conserved across eukaryotes. Promoter motif analyses identified the pentameric sequence CCGAC, known as the C-repeat (CRT) or dehydration-responsive element (DRE), that is required for

cold- and drought-inducibility of *COR* genes (Baker *et al.*, 1994; Yamaguchi-Shinozaki & Shinozaki, 1994). The transcription factors binding to CRT/DRE motifs were identified using yeast one-hybrid screens (Stockinger *et al.*, 1997; Liu *et al.*, 1998) and named CBFs (CRT-BINDING FACTORs). These proteins belong to the AP2/ERF (ETHYLENE RESPONSIVE FACTOR) family of transcriptional regulators, and while the *Arabidopsis* genome encodes six *CBF*-like genes only three of them are implicated in the cold induction of *COR* genes (Akhtar *et al.*, 2012). These three genes, *CBF1*, *CBF2* and *CBF3*, also known as *DREB1b*, *DREB1c* and *DREB1a* (*DRE-BINDING FACTORS 1a, b, c*), respectively, are clustered in tandem on chromosome four (Shinwari *et al.*, 1998; Gilmour *et al.*, 1998) and are rapidly induced by cold though not by drought or osmotic stress (Gilmour *et al.*, 1998; Medina *et al.*, 1999; Xiong *et al.*, 2001). Some studies suggest that ABA treatment induces *CBF* expression (Knight *et al.*, 2004; Lee & Seo, 2015b), while others contradict this finding (Medina *et al.*, 1999; Xiong *et al.*, 2001). This discrepancy has been attributed to mechanical stimulation when transferring seedlings, which also induces *CBF* expression (Gilmour *et al.*, 1998; Zarka *et al.*, 2003). Interestingly, treatment with the translation inhibitor cycloheximide has additionally been found to induce *CBF* expression (Zarka *et al.*, 2003).

CBF genes have been identified in a large number of plant species belonging to both chilling-tolerant plants, capable of cold acclimation, and chilling-sensitive plants, such as rice (Ito *et al.*, 2006), tomato (Jaglo *et al.*, 2001) and maize (Liu *et al.*, 2015). Overexpression of *A. thaliana CBF* genes in chilling-sensitive plants has been shown to enhance chilling tolerance and promote growth at low temperatures (Hsieh *et al.*, 2002; Yang *et al.*, 2010d). Overexpression of endogenous *CBF* genes (Jaglo-Ottosen *et al.*, 1998; Gilmour *et al.*, 2000; Alonso-Blanco *et al.*, 2005) or of orthologues from other species, including those from chilling-sensitive plants (Zhang *et al.*, 2004; Ito *et al.*, 2006), enhances survival during freezing stress in *A. thaliana*. The *CBF* gene cluster has been identified as a major contributor to freezing tolerance by Quantitative Trait Loci (QTL) mapping among *A. thaliana* ecotypes (Alonso-Blanco *et al.*, 2005). *CBF*-overexpressing *A. thaliana* plants are characterised by constitutive expression of *COR* genes, accumulation of osmoprotective metabolites and morphology resembling cold-grown plants, including a dwarfed phenotype, increased leaf thickness, reduced petiole length and delayed flowering (Jaglo-Ottosen *et al.*, 1998; Gilmour *et al.*, 2000; Park *et al.*, 2015).

The importance of CBFs in freezing tolerance in *A. thaliana* has been further demonstrated by mutational analysis of the *CBF* gene cluster. Freezing-associated electrolyte leakage is significantly increased in double but not single *cbf* null mutants, indicating a degree of redundancy between the three CBFs (Zhao *et al.*, 2016). The *CBF2*

gene, however, appears to be particularly important, as it is induced to highest levels during cold treatment (Novillo *et al.*, 2004) and the *cbf2* mutant has the least cold acclimation relative to other single mutants (Zhao *et al.*, 2016). Triple *cbf123* null mutants induce *COR* genes to only 4% to 20% of levels in wild-type plants after 24 hours of cold treatment (Zhao *et al.*, 2016). These mutants are severely compromised in tolerance to freezing temperatures, with 100% mortality when frozen at -7°C after cold acclimation, compared to only 40% mortality in wild-type plants (Zhao *et al.*, 2016). Even in the absence of cold acclimation, *cbf123* mutants have significantly greater electrolyte leakage relative to wild-type plants when frozen at -4°C, confirming the crucial roles of CBFs in freezing tolerance.

The expression of nearly 60% of cold-induced genes is decreased at least two-fold in the *cbf123* mutant relative to wild-type after 24 hours of cold treatment, half of which have greater than ten-fold reductions in expression in the mutant (Zhao *et al.*, 2016). From 414 genes differentially regulated both in cold-treated plants and in *cbf123* mutants, 212 contain CRT/DRE motifs in their promoters, suggesting that they are direct targets of CBFs (Zhao *et al.*, 2016). Bioinformatic analyses of genes differentially expressed between wild-type and *cbf123* plants have implicated these transcription factors in the regulation of diverse processes, including carbohydrate and lipid metabolism, cell wall organisation, chloroplast function and signalling pathways involving kinases, secondary messengers and hormones (Zhao *et al.*, 2016). In addition, 39 transcription factor genes are downregulated in the *cbf123* mutant, suggesting that the CBFs are master regulators of the cold-inducible transcriptional network. Phenotypic characterisation of *CBF*-overexpressing plants or *cbf* null mutants confirms their involvement in these processes, for example levels of *CBF* expression correlate with metabolite profiles (Gilmour *et al.*, 2000), photosynthetic efficiency (Yang *et al.*, 2010d), levels of auxin (Li *et al.*, 2017c) and gibberellins (Achard *et al.*, 2008), circadian clock function (Chow *et al.*, 2014), seed dormancy (Kendall *et al.*, 2011) and rates of growth, reproduction and senescence (Seo *et al.*, 2009; Sharabi-Schwager *et al.*, 2010a,b).

1.3.3 Regulators of *CBF* expression

A large number of *Arabidopsis* mutants have been isolated that have altered basal or cold-induced expression of *CBF* genes (*Figure 1.5*). Zhu and colleagues, for example, have identified such mutants by forward genetics from ethyl methanesulfonate (EMS)-mutagenised plants expressing *RD29a*- or *CBF3*-promoter driven reporters

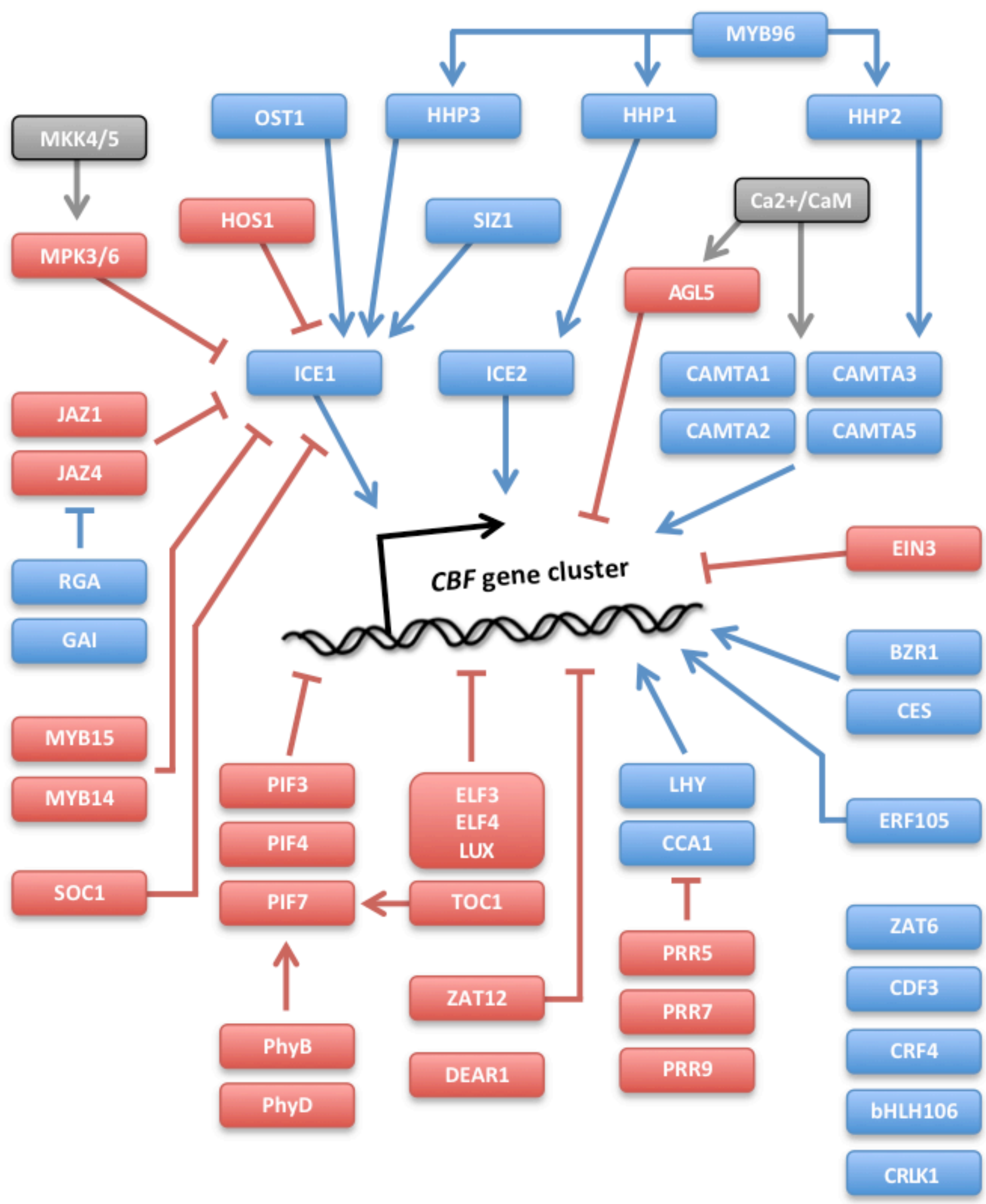


Figure 1.5: Proteins associated with the regulation of *CBF* expression in *A. thaliana*.
Positive regulators are shown in blue, negative regulators in red and regulators with both positive and negative roles are shown in grey. Positive interactions are represented with an arrow and repressive interactions with a blunt arrow. Regulators that might have indirect effects on *CBF* expression lack an arrow.

(Ishitani *et al.*, 1997; Chinnusamy *et al.*, 2003). From these screens, the MYC (MYELOCYTOMATOSIS)-type bHLH transcription factor ICE1 (INDUCER OF *CBF* EXPRESSION 1) was found to be a key regulator of *CBF* expression, with diminished *CBF* induction and increased freezing sensitivity in the dominant negative *ice1-1* mutant (Chinnusamy *et al.*, 2003). *ICE1* is constitutively expressed and has been reported to bind to MYC sequences in *CBF* promoters, though it activates *CBF* expression exclusively during cold exposure, *via* an unknown mechanism (Chinnusamy *et al.*, 2003). ICE1 also plays a role in stomatal development, as demonstrated by the over-production of stomata in the *ice1-1* mutant (Kanaoka *et al.*, 2008), as well as in reproduction (Lee *et al.*, 2015), endosperm breakdown (Denay *et al.*, 2014) and ABA signalling (Liang & Yang, 2015). The *A. thaliana* genome encodes a second ICE1-like protein, ICE2, which shares a role in stomatal development and cold signalling (Kanoaka *et al.*, 2008; Kurbidaeva *et al.*, 2014; Kim *et al.*, 2015). The severe stomatal phenotype in the double *ice1-2 ice2-2* T-DNA insertion mutant results in seedling lethality, and cold-induction of *CBF* genes is lower in the heterozygous *ice1-2 ice2-2/+* mutant than in single *ice1-2* or *ice2-2* null mutants, indicating a level of redundancy between ICE1 and ICE2 (Kim *et al.*, 2015). Nevertheless, the *ice2-2* mutant has a milder phenotype than *ice1-1* or *ice1-2* mutants, suggesting that ICE1 is the more important of the two *CBF* regulators (Kanoaka *et al.*, 2008; Kim *et al.*, 2015).

Various transcriptional regulators modulate ICE1 activity, for example MYB15 (MYELOBLASTOSIS-DOMAIN PROTEIN 15) has been shown to interact in vitro with both recombinant ICE1 and *CBF* promoter sequences containing *MYB* motifs (Agarwal *et al.*, 2006). *MYB15* expression negatively correlates with both cold-inducibility of *CBF* genes and freezing tolerance (Agarwal *et al.*, 2006). Null mutants of floral regulators FVE (FLOWERING LOCUS VE), GI (GIGANTEA) and SOC1 (SUPPRESSOR OF OVEREXPRESSION OF CO 1) have higher basal expression of *CBFs*, and SOC1 has been shown to bind directly to *CBF* promoters and inhibit the DNA-binding ability of ICE1 (Seo *et al.*, 2009; Lee *et al.*, 2015). The jasmonate signalling repressors JAZ1 and JAZ4 (JASMONATE ZIM DOMAIN PROTEIN 1/4) also interact with ICE1 and repress its activity (Hu *et al.*, 2013), whereas DELLA (Aspartic acid-Glutamic acid-Leucine-Leucine-Alanine-domain) proteins involved in gibberellin signalling sequester JAZ proteins and thereby promote ICE1 activity (Zhou *et al.*, 2017). Plants overexpressing *JAZ1* or *JAZ4* (Hu *et al.*, 2013) or deficient in DELLA proteins GAI (GIBBERELLIN ACID INSENSITIVE) and RGA (REPRESSOR OF GAI1-3) (Achard *et al.*, 2008) are hypersensitive to freezing.

ICE1 stability is regulated by post-translational modifications, for example polyubiquitination by the E3 ligase HOS1 (HIGH EXPRESSION OF OSMOTICALLY-

RESPONSIVE GENES 1) targets ICE1 for degradation (Dong *et al.*, 2006a), whereas sumoylation of ICE1 on lysine 393 by the SUMO E3 ligase SIZ1 prevents its degradation (Miura *et al.*, 2007). Consequently, cold-induction of *CBF* genes is compromised in the *siz1* mutant (Miura *et al.*, 2007) but enhanced in the *hos1* mutant (Lee *et al.*, 2001). The OST1 (OPEN STOMATA 1) kinase phosphorylates ICE1 on serine 278, thereby stabilising the protein by reducing its association with HOS1 (Ding *et al.*, 2015). Phosphorylation of ICE1 by MAP kinases MPK3 (MAP KINASE 3) and MPK6, in contrast, promote ICE1 degradation, and both *mpk3* or *mpk6* mutants (Li *et al.*, 2017b) and *OST1*-overexpressing plants (Ding *et al.*, 2015) have increased freezing tolerance. Intriguingly, MPK6 also regulates *CBF* expression by phosphorylation of MYB15, which acts to reduce its occupancy at *CBF* promoters (Kim *et al.*, 2017b). While the MKK4/5 (MPK KINASE 4/5)-MPK3/6 kinase cascade is associated with attenuation of the cold response, the MEKK1 (MAPK/ERK KINASE KINASE 1)-MKK2 (MPK KINASE 2)-MPK4 (MAP KINASE 4) pathway promotes *CBF* expression and freezing tolerance, *via* an unknown mechanism (Teige *et al.*, 2004; Zhao *et al.*, 2017).

Basal *CBF* expression oscillates throughout the day, with a peak in transcript abundance eight hours after dawn in both long- and short-day photoperiods (Lee & Thomashow, 2012). Cold-inducibility of *CBF* genes is also regulated in a circadian manner, with highest inducibility at dawn and lowest inducibility after dusk (Fowler *et al.*, 2005). Several clock regulators have been shown to directly modulate *CBF* expression. Dawn-expressed components CCA1 (CIRCADIAN CLOCK ASSOCIATED 1) and LHY (LATE ELONGATED HYPOCOTYL), for example, positively regulate the expression of *CBF* genes by binding to their promoters and are required for the circadian oscillations of both basal and cold-induced expression (Dong *et al.*, 2011). Null mutants of PRR5, PRR7 and PRR9 (PSEUDORESPONSE REGULATORS 5/7/9) have increased basal expression of *CBF* genes and enhanced freezing tolerance, probably owing to an increased expression of *CCA1*/*LHY*, which are repressed by the PRR proteins (Nakamichi *et al.*, 2009; Keily *et al.*, 2013). Dusk-expressed components TOC1 (TIMING OF CAB EXPRESSION 1) and ELF3 (EARLY FLOWERING 3) also bind to *CBF* promoters and repress their expression (Keily *et al.*, 2013). As a consequence, the double *cca1 lhy* mutant is hypersensitive to freezing (Dong *et al.*, 2011) whereas the *toc1*, *elf3* and *prr5,7,9* mutants are more resistant to freezing (Keily *et al.*, 2013). Interestingly, LUX (LUX ARRYTHMO), which functions with ELF3 in the repressive Evening Complex and is itself a target of CBF1, appears to promote freezing tolerance despite repressing the expression of *CBF* genes (Keily *et al.*, 2013; Chow *et al.*, 2014).

Sequence analysis of *CBF* promoters identified many conserved motifs, including the G-box (CACGTG) as well as the CAM box (ACGCGT). The G-box is bound by bHLH transcription factors PIF3, PIF4 and PIF7 (PHYTOCHROME-INTERACTING FACTOR 3/4/7) (Kidokoro *et al.*, 2009; Lee & Thomashow, 2012; Jiang *et al.*, 2017), which repress the diurnal peak in *CBF* expression, particularly under long-day conditions, and as a consequence freezing tolerance is greater in short-day photoperiods than in long-day photoperiods (Lee & Thomashow, 2012). Knock-out mutations in these PIFs or in phytochromes PhyB and PhyD (PHYTOCHROME B/D), with which they interact, result in increased basal *CBF* expression and freezing tolerance (Franklin & Whitelam, 2007; Lee & Thomashow, 2012; Jiang *et al.*, 2017), as does exposure to far red-enriched light, which inactivates phytochromes (Franklin & Whitelam, 2007).

The CAM-box, in contrast, has been found to interact *in vitro* with positive regulators of the CAMTA (CALMODULIN-BINDING TRANSCRIPTION ACTIVATOR) family (Doherty *et al.*, 2009). Kim *et al.* (2013) have demonstrated that CAMTA1, CAMTA2 and CAMTA3 all contribute to the activation of *CBF* genes during cold exposure and that they also repress salicylic acid-biosynthetic genes at ambient growth temperatures. Kidokoro *et al.* (2017) have recently shown that CAMTA3 and CAMTA5 play a major role in *CBF* induction during rapid cooling. CAMTAs interact with the calcium sensor protein calmodulin *in vitro* in the presence of calcium (Bouché *et al.*, 2002), the cytosolic concentration of which increases during cold treatment (Plieth *et al.*, 1999). In contrast, the calmodulin-binding transcription factor AGL15 (AGAMOUS-LIKE 15) contributes to repression of basal *CBF* expression (Hill *et al.*, 2008).

Lee & Seo (2015b) have identified three membrane-associated proteins, known as heptahelical proteins (HHPs), which interact with ICE and CAMTA transcription factors in a target-specific manner in *A. thaliana*. HHP1, HHP2 and HHP3 (HEPTAHELICAL PROTEIN 1/2/3) were found to interact *in vivo* with ICE1, CAMTA3 and ICE2, respectively, and to promote the expression of specific *CBF* genes (Lee & Seo, 2015b). Interestingly, while full-length HHP1 localises to the plasma membrane, the N-terminus localises to the nucleus, where it co-localises with ICE1 (Chen *et al.*, 2010). The exact functions of these transmembrane proteins is unknown, though the single *hhp1*, *hhp2* and *hhp3* mutants exhibit diminished freezing tolerance (Lee & Seo, 2015b).

A considerable number of transcription factors appear to directly or indirectly affect *CBF* expression, for example components of hormone signalling pathways. The brassinosteroid-associated regulators BZR1 (BRASSINAZOLE-RESISTANT 1) (Li *et al.*, 2017a) and CES (CESTA) (Eremina *et al.*, 2016) associate with *CBF* promoters *in vivo* and promote their induction, and consequently freezing tolerance is enhanced in plants

after exogenous application of brassinosteroids (Eremina *et al.*, 2016) or in plants overexpressing BZR1 or deficient in BIN2 (BRASSINOSTEROID INSENSITIVE 2), BIL1 and BIL2 (BIN2-LIKE 1/2), that phosphorylate BZR1 and thereby promote its degradation (Li *et al.*, 2017a). The cytokinin response factor CRF4 (CYTOKININ RESPONSE FACTOR 4) appears to positively regulate *CBF* genes as the *crf4* mutant has lower basal expression of *CBF* genes, though it may also regulate *COR* genes directly, as expression of *COR15a* but not that of *CBF* genes is increased in *CRF4*-overexpressing plants (Zwack *et al.*, 2016). In contrast, the ethylene signalling regulator EIN3 (ETHYLENE INSENSITIVE 3), which accumulates during cold exposure, binds and represses *CBF* promoters, and the *ein3* mutant, as well as other ethylene-insensitive mutants *ein2*, *ein4* (*ethylene insensitive 2/4*) and *etr1* (*ethylene response 1*), have enhanced tolerance to freezing (Shi *et al.*, 2012).

Expression of the transcription factors ERF105 (ETHYLENE RESPONSIVE FACTOR 105) and ZAT12 (ZINC FINGER OF *ARABIDOPSIS THALIANA* 12) is induced within one hour of cold shock (Vogel *et al.*, 2005; Bolt *et al.*, 2017), and though the former appears to promote *CBF* expression (Bolt *et al.*, 2017), the latter contributes to attenuation of the *CBF* response (Bolt *et al.*, 2017). Elevated basal expression of *CBF* genes and enhanced freezing tolerance have been reported in plants overexpressing abiotic stress-associated transcription factors CDF3 (CYCLING DOF FACTOR 3) (Corrales *et al.*, 2017), ZAT6 (ZINC FINGER OF *ARABIDOPSIS THALIANA* 6) (Shi *et al.*, 2014b) or bHLH106 (BASIC HELIX LOOP HELIX 106) (Ahmad *et al.*, 2015). In addition, overexpression of the AP2/ERF-type transcription factor DEAR1 (DREB AND EAR MOTIF PROTEIN 1) (Tsutsui *et al.*, 2009) or the MYB15 homologue MYB14 (Chen *et al.*, 2013), or null alleles of MYB96 (MYELOBLASTOSIS-DOMAIN PROTEIN 96), which induces *HHP* genes (Lee & Seo, 2015b), have been found to repress cold-induction of *CBF* genes.

Genetic screens for mutants with altered *CBF* expression have implicated a number of proteins associated with epigenetics, regulation of RNA polymerase activity, mRNA processing, mRNA export or translation in cold responses. The histone deacetylase subunit FVE acts to repress *CBF* and *COR* genes, and its null mutant has earlier cold-induction of these genes (Kim *et al.*, 2004). The transcriptional regulator RDM4 (RNA-DIRECTED DNA METHYLATION 4) and phosphatase RCF2/FRY2 (REGULATOR OF CBF EXPRESSION 2/FIERY 2) both interact with RNA polymerase II and have opposing roles in cold responses, with reduced cold-induction of *CBF* genes in the *rdm4* mutant (Chan *et al.*, 2016) though enhanced induction in the *rcf2* mutant (Guan *et al.*, 2014). The RNA helicase RCF1 (REGULATOR OF CBF EXPRESSION 1) and

RNA chaperones CSP2 and CSP4 (COLD SHOCK PROTEIN 2/4) are involved in pre-mRNA splicing, and mutations in these genes were found to increase *CBF* inducibility (Guan *et al.*, 2013a; Sasaki *et al.*, 2013), presumably by disrupting expression of cold signalling repressors, for example the *PRR5* gene, which is misspliced in the *rcf1* mutant (Guan *et al.*, 2013a). Mutations in the *SOAR1* (*SUPPRESSOR OF ABAR OVEREXPRESSOR 1*) gene, which is associated with RNA processing in organelles, also affects levels of *CBF* expression during cold responses (Jiang *et al.*, 2015).

Lower cold-induction of *CBFs* resulting from defects in mRNA export have been reported in mutants of the RNA helicase LOS4 (LOW EXPRESSION OF OSMOTICALLY-RESPONSIVE GENES 4) (Gong *et al.*, 2002) and the nucleoporin NUP160 (NUCLEAR PORE 160) (Dong *et al.*, 2006b). Interestingly, an allele of *LOS4* was identified which results in mRNA export defects at normal growth temperatures but enhanced mRNA export during cold exposure, and *CBF* induction was found to be increased in this mutant (Gong *et al.*, 2005). In addition to its involvement in the degradation of ICE1, the E3 ligase HOS1 was found to interact with nucleoporin NUP43 (NUCLEAR PORE 43) and RNA-export factor RAE1 (RIBONUCLEIC ACID EXPORT 1), and nuclear accumulation of polyadenylated mRNA is observed in the *hos1* mutant (Tamura *et al.*, 2010). HOS1 is additionally implicated in a variety of other processes including epigenetic regulation (Jung *et al.*, 2013a), post-transcriptional gene silencing (Wang *et al.*, 2015a), hormone signalling (Lee & Seo, 2015a), flowering (Jung *et al.*, 2012) and circadian clock function (MacGregor *et al.*, 2013). Lastly, the translation elongation-associated eEF2-like protein LOS1 (LOW EXPRESSION OF OSMOTICALLY-RESPONSIVE GENES 1) was found to be necessary for both protein synthesis and regulation of *CBF* genes at low temperatures, though the basis for the *los1* mutant phenotype is not yet clear (Guo *et al.*, 2002).

1.3.4 CBF-independent pathways

Although the CBF transcription factors are major contributors to cold acclimation, experimental evidence suggests that there are also transcriptional pathways acting independently of CBFs. Transcriptomic analysis has revealed that the expression of a considerable number of *COR* genes is either unaffected or only slightly reduced in the *cbf123* mutant (Zhao *et al.*, 2016). It is possible that CBF-like transcription factors, which also bind CRT/DRE motifs, may compensate for the CBF proteins. A fourth *CBF* gene, *CBF4/DREB1d*, which is induced by ABA but not by cold, and the CBF-like gene *DDF1/DREB1f* (*DWARF AND DELAYED FLOWERING 1/DRE-BINDING FACTOR 1f*) both improve freezing tolerance when overexpressed in *A. thaliana*

(Haake *et al.*, 2002). In addition to the ABA-independent CBF-mediated pathway, cold regulation of *COR* genes is also regulated by ABA-dependent pathways. The *ABA3*/*LOS5* (LOW EXPRESSION OF OSMOTICALLY-RESPONSIVE GENES 5) gene encodes an ABA-biosynthetic gene that contributes to the cold-induction of *COR* genes, and, while expression of the latter is severely reduced in the *los5* mutant, *CBF* gene expression is unaltered in these plants (Xiong *et al.*, 2001). The ABA-dependent and -independent pathways of *COR* gene induction are interlinked, for example the MYB96 transcription factor, which also influences *CBF* expression *via* its regulation of *HHP* expression, is necessary for expression of certain *COR* genes during cold and ABA treatment (Lee & Seo, 2015b).

Some transcription factors that regulate *CBF* expression, for example JAZ1 and JAZ4 (Hu *et al.*, 2013), CES (Eremina *et al.*, 2016) and BZR1 (Li *et al.*, 2017a), have been shown to additionally regulate cold-inducible genes that are not part of the CBF regulon. Kim *et al.* (2013) demonstrated that CAMTA1, CAMTA2 and CAMTA3 influence the expression of 15% of cold-responsive genes, most of which are not regulated by CBFs. The MKK2-MPK4/6 kinase pathway was found to regulate both *CBF* genes and CBF-independent genes during cold exposure (Teige *et al.*, 2004).

Park *et al.* (2015) have demonstrated that twenty-seven transcription factor genes are induced with the same dynamics as the *CBF* genes, within one hour of cold treatment. While many of these 'first wave' transcription factor genes are downregulated in the *cbf123* mutant, the expression of *HSFC1* (*HEAT SHOCK FACTOR C1*), *ZAT12* and *CZF1* (*CCCH-ZINC FINGER 1*) is unaltered in the absence of CBFs (Zhao *et al.*, 2016) and, likewise, overexpression of these genes does not affect basal *CBF* expression (Park *et al.*, 2015), indicating that they form separate transcriptional pathways. Plants overexpressing 'first wave' transcription factors have dwarfed phenotypes, resembling cold-grown plants, and some have altered basal expression of *COR* genes (Park *et al.*, 2015). While all of these lines have fewer upregulated *COR* genes than *CBF2*-overexpressing plants, the 'first wave' transcription factors HSFC1, ZAT12, ZF (ZINC FINGER), ZAT10 (ZINC FINGER OF *ARABIDOPSIS THALIANA* 10) and CZF1 together positively regulate over 130 *COR* genes, a quarter of which are also positively regulated by CBF2, and also negatively regulate an equal number of *COR* genes (Park *et al.*, 2015). Sequence analysis of the promoters of these transcription factors has identified conserved motifs, including potential CAMTA binding sites as well as several uncharacterised motifs (Vogel *et al.*, 2005). An atypical transcriptional regulator, LOS2 (LOW EXPRESSION OF OSMOTICALLY-RESPONSIVE GENES 2), which also acts as a glycolytic enzyme, has been found to repress one of these 'first wave' transcription

factor genes, *ZAT10* (Lee *et al.*, 2002). *ZAT10* is positively regulated by the MKK2-MPK4/6 pathway, as is *RAV1* (*RELATED TO ABI3/VP1*), another 'first wave' transcription factor gene (Teige *et al.*, 2004). Further investigation, though, is required to understand the pathways regulating the induction of such transcription factor genes during cold exposure.

A number of additional transcriptional regulators have been found to play a role in cold-induction of *COR* genes and freezing tolerance without affecting *CBF* expression, for example GI (Cao *et al.*, 2005), MYBC1 (MYELOBLASTOSIS-DOMAIN PROTEIN C1) (Zhai *et al.*, 2010), HAP5A (HEME-ASSOCIATED PROTEIN 5A) (Shi *et al.*, 2014a), HY5 (ELONGATED HYPOCOTYL 5) (Catalá *et al.*, 2011; An *et al.*, 2017), HOS9 (HIGH EXPRESSION OF OSMOTICALLY-RESPONSIVE GENES 9) (Zhu *et al.*, 2004) and RAP2.1 (RELATED TO AP2 1) (Dong & Liu, 2010), but these cold signalling pathways are still poorly understood. Furthermore, transcriptomic analyses have revealed several other motifs, in addition to CRT/DRE sequences, which are enriched in the promoters of cold-responsive genes (Lindlöf *et al.*, 2009), and interestingly, diverse plant species have different patterns of enrichment of cold-associated promoter motifs (Maruyama *et al.*, 2012), for example some have higher reliance on WRKY transcription factors (Chen *et al.*, 2014). Finally, certain *Arabidopsis* mutants, such as those of cell wall-associated genes *ESK1* (Xin *et al.*, 2007) and *BCB* (Ji *et al.*, 2015), lipid-metabolism gene *ACBP1* (Du *et al.*, 2010) and histone acetylation gene *ADA2* (*TRANSCRIPTIONAL ADAPTOR 2*) (Vlachonasios *et al.*, 2003), have no discernible changes to *CBF* or *COR* marker genes but exhibit an enhanced tolerance to freezing, indicating that many aspects of freezing tolerance *via* CBF-independent pathways remain to be investigated.

1.4 Biological thermosensors

Several different classes of biological temperature sensors have been discovered in the last two decades, which are based on temperature-associated structural changes in nucleic acids, lipids or proteins (*Figure 1.6*). One of the simplest thermosensory systems is the 'RNA thermometer', in which secondary structures in RNA conceal a ribosomal binding site at low temperatures but melt at elevated temperatures, thus allowing temperature-specific translation to occur. Regulatory structures of this type have been described in many bacteria (Johansson *et al.*, 2002; Waldminghaus *et al.*, 2005, 2007; Kortmann *et al.*, 2010) and are proposed to exist in eukaryotes. In budding yeast, for example, over 1800 RNAs are predicted to form secondary structures centred around ribosomal binding sites that may melt specifically at high temperatures (Wan *et al.*, 2012).

In a variety of thermophilic and pathogenic bacteria, ambient temperature has been shown to regulate the secondary and tertiary structures of DNA. For example, heat shock induces positive supercoiling of DNA while cold shock triggers its negative supercoiling, which alters the efficiency of transcription and contributes to the expression of stress-inducible genes (Kataoka *et al.*, 1996; López-García & Forterre, 1997). Promoters that form inactive loops at low temperatures but open in response to heat have also been described in these organisms (Falconi *et al.*, 1998; Prosseda *et al.*, 2004).

These DNA alterations rely at least in part on temperature-specific changes to protein function, for example DNA topoisomerases and gyrases, which appear to have distinct temperature-dependent enzymatic activity (López-García & Forterre, 1997). Certain bacterial proteins exhibit temperature-sensitive DNA binding, for example the genome-structuring nucleoid-associated H-NS (HISTONE-LIKE NUCLEOID-STRUCTURING) proteins, which lose their DNA-binding ability during heat stress and thereby allow target genes to be expressed (Ono *et al.*, 2005). This is reminiscent of chromatin responses to heat stress in eukaryotes, where nucleosome remodelling occurs during thermal stimulation (Shivaswamy *et al.*, 2008; Kumar & Wigge, 2010), though the dissociation of H-NS proteins appears to be a direct response to heat resulting from structural changes in these proteins (Ono *et al.*, 2005), unlike temperature-associated changes in nucleosome occupancy, which is an active process involving chromatin remodellers (Erkina *et al.*, 2008; Shivaswamy & Iyer, 2008). Temperature-sensitive conformational changes in DNA-binding domains have also been shown in several transcription factors in bacteria, for example the

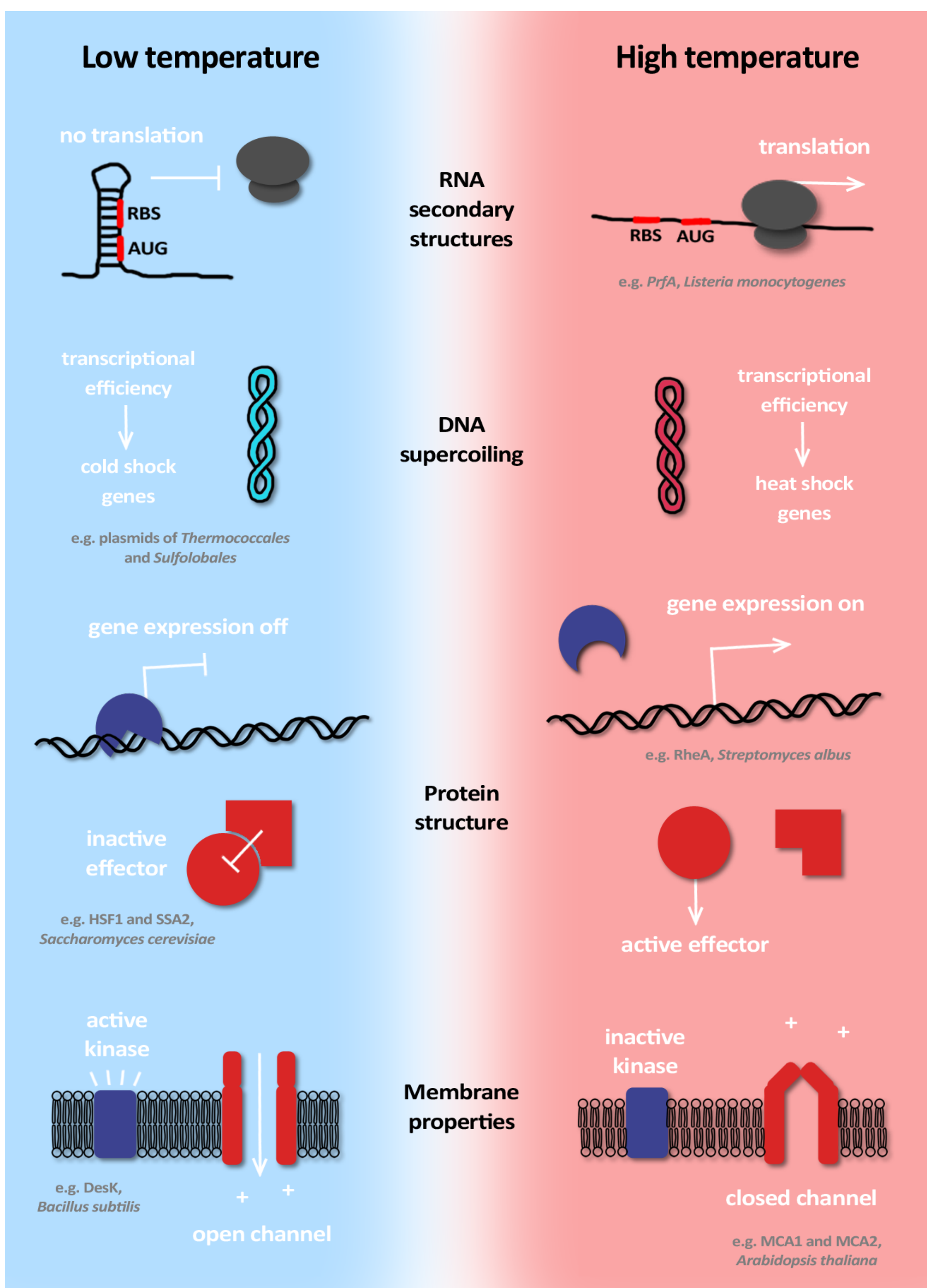


Figure 1.6: Overview and examples of biological thermosensors from various organisms. In RNA-based thermosensors, such as the *PrfA* (*POSITIVE REGULATORY FACTOR A*) RNA of *Listeria monocytogenes*, mRNA secondary structures typically conceal a ribosomal binding site (RBS) upstream of the start codon (AUG) at low temperature but melt at high temperature, thereby allowing ribosome binding and translation (Johansson *et al.*, 2002). Changes in ambient temperature can cause either positive or negative supercoiling of DNA, which in turn affects the efficiency of transcription at target genes, which may include heat- or cold-responsive genes (López-García & Forterre, 1997). Temperature changes can trigger conformational changes in proteins, for example leading to an differential DNA association, as is the case for the transcription factor RheA (REPRESSOR OF HSP EIGHTEEN A) in *Streptomyces albus* (Servant *et al.*, 2000), or altered protein-protein interactions, as observed between the transcription factor HSF1 (HEAT SHOCK FACTOR 1) and its repressor SSA2 (STRESS SEVENTY A2) in *S. cerevisiae* (Zheng *et al.*, 2016). Lastly, membrane-based thermosensors are differentially activated by changes in membrane structure. The DesK (DESATURASE K) kinase of *Bacillus subtilis* is activated as membrane thickness increases at low temperatures (Cybulski *et al.*, 2010). Similarly, the mechanosensitive calcium-permeable channels MCA1 and MCA2 (MID1-COMPLEMENTING ACTIVITY 1/2) in *A. thaliana* are activated during cold shock, presumably by an increase in membrane rigidity (Mori *et al.*, 2018).

Streptomyces albus RheA (REPRESSOR OF HSP EIGHTEEN A) and *Pyrococcus furiosus* Phr (PYROCOCCUS HEAT SHOCK REGULATOR) proteins that repress heat-inducible genes at normal growth temperatures but dissociate from DNA at higher temperatures to allow the expression of these genes (Servant *et al.*, 2000; Liu *et al.*, 2007b). The TlpA (TIR-LIKE PROTEIN A) repressor from *Salmonella enterica* forms homodimers that repress virulence genes at low temperatures, though reversible unfolding of the dimerisation domain at high temperatures prevents dimer formation and thus triggers gene expression (Hurme *et al.*, 1997).

Similarly, in eukaryotes, changes in ambient temperature may induce alterations in protein structure, a notable example being human HSF1. Elevated temperatures disrupt domain interactions that normally maintain HSF1 in a monomeric state, thereby triggering oligomerisation and DNA-binding (Hentze *et al.*, 2016). The trimerisation and DNA-binding ability of *A. thaliana* HSFA1a is also regulated by temperature *in vitro*, raising the possibility that it could directly sense changes in ambient temperature (Liu *et al.*, 2013a). A number of proteins involved in thermosensory responses in *A. thaliana*, such as HSFA1a, ICE2 and ELF3, have regions containing tandem repeats of amino acids such as glutamine or asparagine that could potentially destabilise these proteins at high temperatures and confer thermosensor function. In support of this hypothesis, the length of poly-glutamine (polyQ) tracts in ELF3 influence the thermosensitivity of hypocotyl elongation and flowering in *A. thaliana* (Press *et al.*, 2016), however the mechanism behind this observation is still unclear. Recent data have implicated the photoreceptors phytochrome B (Jung *et al.*, 2016; Legris *et al.*, 2016) and phototropin (Fujii *et al.*, 2017) in sensing ambient temperature changes, with changes in their activity arising from the temperature-dependent lifetime of their photoactivated chromophores.

In addition, some HSPs undergo temperature-dependent conformational changes that correlate with their chaperone activity (Haslbeck *et al.*, 1999, 2004; Gustavsson *et al.*, 1999; Scharf *et al.*, 2001; Basha *et al.*, 2010), and may thus regulate HSF activity in a temperature-dependent manner. Another mechanism of heat perception, for which an abundance of supporting evidence has accumulated in the last two decades, is the temperature-dependent unfolding or misfolding of cellular proteins, which exposes normally inaccessible hydrophobic peptide domains, thereby activating the chaperone activity of HSPs, titrating them away from HSF proteins and allowing the latter to activate transcription of stress-responsive genes (Zheng *et al.*, 2016).

As well as influencing DNA association and protein-protein interactions, ambient temperature also affects the physical properties of cellular membranes, which in turn

influences the activity of ion channels or membrane-associated enzymes. In mammalian cells, for example, six members of the transient receptor potential (TRP) family of ion channels are associated with perception of heat or cold in sensory neurons and are activated over different ranges of temperatures thus allowing fine-tuned responses to temperature stimuli (Belvisi *et al.*, 2011). Temperature-responsive TRP channels (Rosenzweig *et al.*, 2005), as well as bidirectional cyclic nucleotide-gated ion channels that open in response to heating and close upon cooling (Ramot *et al.*, 2008), have been described in invertebrates. Recently, the MCA1 and MCA2 (MID1-COMPLEMENTING ACTIVITY 1/2) stretch-activated calcium channels in *A. thaliana* have been found to regulate cold-induced calcium flux into the cytoplasm, suggesting that they are involved in the perception of low temperatures (Mori *et al.*, 2018).

The histidine kinase DesK (DESATURASE K) from *Bacillus subtilis* is active at low temperatures, when its sensor domain is embedded in the membrane, but is inactivated at elevated temperatures, when the membrane becomes thinner and the sensor domain is exposed to water molecules beyond the lipid bilayer (Cybulski *et al.*, 2010). Membrane-associated kinases that are affected by temperature-dependent changes in membrane fluidity have been described in other bacteria, for example *Synechocystis* sp. PCC6803 (Mikami *et al.*, 2002).

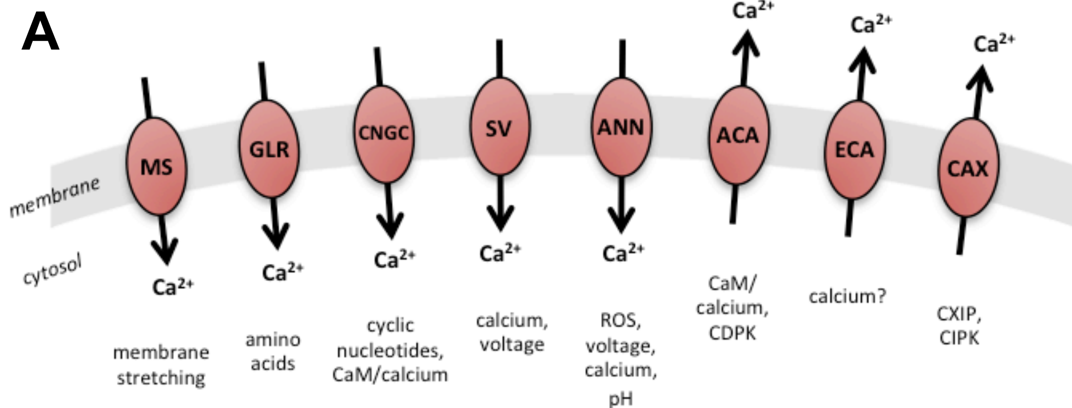
Two MAP kinases that are activated either by cold or heat, named SAMK (STRESS-ACTIVATED MAP KINASE) and HAMK (HEAT SHOCK-ACTIVATED MAP KINASE) respectively, have been identified immunologically in alfalfa and tobacco cell extracts (Jonak *et al.*, 1996; Sangwan *et al.*, 2002; Suri & Dhindsa, 2008). Dimethylsulfoxide (DMSO) and benzyl alcohol (BA), which cause membrane rigidification and fluidisation respectively, were shown to differentially affect these kinases, whereby BA could stimulate and DMSO repress the heat-activation of HAMK and vice versa for the cold-activation of SAMK. This observation led the authors to suggest that plants also perceive temperature stimuli *via* changes in the fluidity of membranes. By using the same chemicals in *A. thaliana*, Furuya *et al.* (2014) have proposed that membrane rigidification can activate the cold-responsive MEKK1-MKK2-MPK4 kinase cascade and have shown that DMSO treatment induces the expression of *COR15a*, albeit weakly, and enhances freezing tolerance. However, the mechanism by which membrane fluidity could regulate temperature perception in plants is unclear, and such a model is highly debated, because DMSO and BA affect not only the physical properties of membranes but also those of other cellular macromolecules.

1.5 Calcium signalling in plants

1.5.1 Calcium influx and efflux

Calcium ions are toxic to cells at high concentrations, as they may react with phosphate ions to form insoluble precipitates and can inhibit mitochondrial function and promote cell death (Clapham, 2007; Vygodina *et al.*, 2013). For this reason, both eukaryotic and bacterial cells maintain low cytosolic free calcium concentrations (50 nM to 100 nM) by chelating and actively extruding these ions from the cytosol (Dominguez, 2004; Stael *et al.*, 2012; Choi *et al.*, 2014a; Riveras *et al.*, 2015). In plant cells, free calcium concentrations in the apoplast, vacuole and endoplasmic reticulum are estimated to be three orders of magnitude higher than in the cytosol (Stael *et al.*, 2012). Owing to this large electrochemical potential gradient, calcium ions are ideal secondary messengers and are involved in signal transduction in response to abiotic and biotic stresses as well as developmental cues such as hormone signalling and gravitropic or phototropic responses (Poovaiah *et al.*, 1993). A large number of stimuli have been found to trigger increases in cytosolic free calcium in plants, including treatments with low or high temperatures (Knight *et al.*, 1996; Gao *et al.*, 2012), drought and salinity (Knight *et al.*, 1997), oxidative stress (Evans *et al.*, 2005; Jiang *et al.*, 2013), heavy metals (Yeh *et al.*, 2007), light (Shacklock *et al.*, 1992), gravitational and mechanical stimulation (Knight *et al.*, 1991; Plieth & Trewavas, 2002), hormones (Bai *et al.*, 2009; Shih *et al.*, 2015), amino acids (Qi *et al.*, 2006), extracellular ATP (Choi *et al.*, 2014a), nitrates (Riveras *et al.*, 2015), defence elicitors (Knight *et al.*, 1991; Lecourieux *et al.*, 2002; Thor & Peiter, 2014) and rhizobial symbionts (Ehrhardt *et al.*, 1996; Navazio *et al.*, 2007). More cytosolic responses to stimuli have been described for calcium ions than any other secondary messenger.

Because of the toxicity of these ions, calcium signalling is mediated by the interplay between calcium efflux and influx pathways (*Figure 1.7*). Calcium efflux is mediated by two groups of carriers, namely the low-affinity Ca^{2+} exchangers and the high-affinity Ca^{2+}-ATPases. The *A. thaliana* genome encodes six Ca^{2+}/H^{+} exchangers (CAX1-6; <u>CA</u>TION E<u>X</u>CHANGER 1-6) and five K^{+}-dependent Ca^{2+}/Na^{+} exchangers (CCX1-5; <u>C</u>ATION/<u>C</u>A^{2+} E<u>X</u>CHANGER 1-5) (Shigaki *et al.*, 2006). Two categories of calcium-specific ATPases are found in plants, type IIA, which includes the ECA family (<u>E</u>R-type <u>C</u>a^{2+}-<u>A</u>TPase, four genes in *A. thaliana*), and type IIB, which comprises the ACA family (<u>a</u>utoinhibited <u>C</u>a^{2+}-<u>A</u>TPase, eleven genes in *A. thaliana*) (Kamrul Huda *et al.*, 2013). Whereas CAX proteins are reported to localise to the tonoplast and plasma membrane



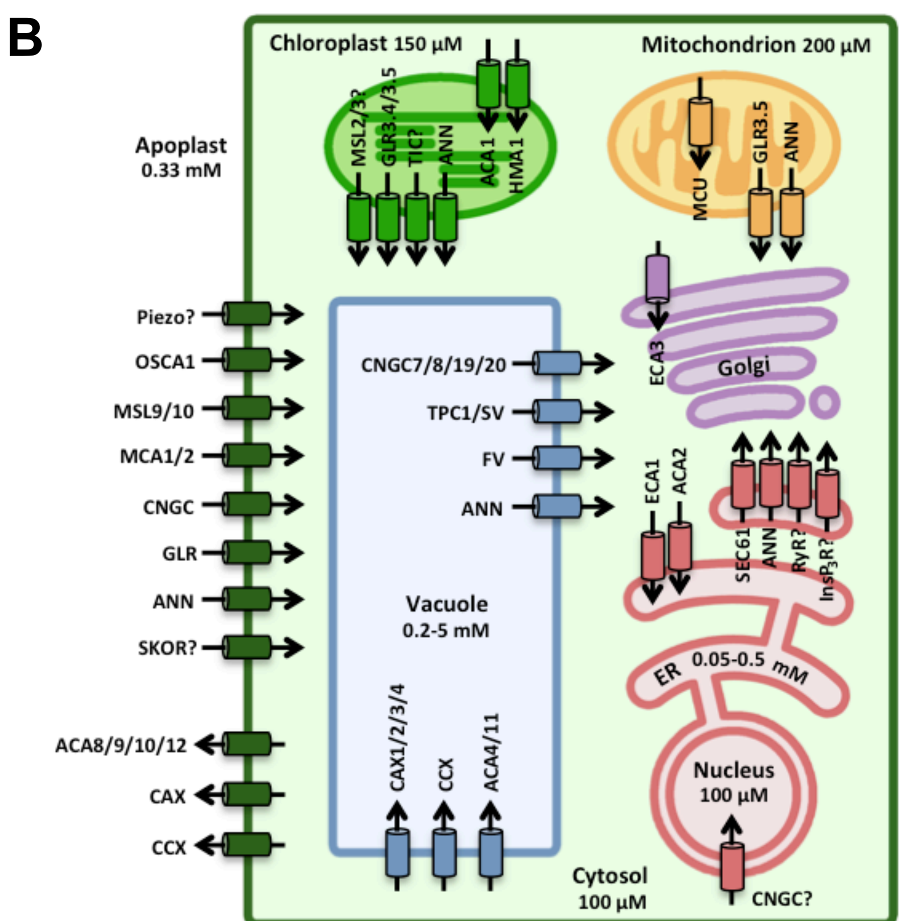


Figure 1.7: Plant calcium-permeable channels.
(**A**) Classes of calcium influx and efflux channels that have been characterised in plants. MS: mechanosensitive channels (MCA, MSL, OSCA, Piezo), GLR: glutamate receptor-like channels, CNGC: cyclic nucleotide-gated channels, SV: slow-vacuolar channels (TPC1), ANN: annexins, ACA: autoinhibited Ca^{2+}-ATPases, ECA: ER-type Ca^{2+}-ATPases, CAX: Ca^{2+}/H^{+} exporters (also CCX: K^{+}-dependent Ca^{2+}/Na^{+} exporters). Components that regulate the activity of these channels are indicated. CXIP: CAX-interacting protein, CIPK: CBL-interacting protein kinase.
(**B**) Cellular localisation of calcium channels in *A. thaliana*. Channels with speculative identity, localisation or calcium permeability are indicated by question marks. While the identities of nuclear calcium channels are unknown in *A. thaliana*, CNGC15a, CNGC15b and CNGC15c are known to localise to nuclear membranes in *Medicago truncatula* (Charpentier *et al.*, 2016). Concentrations of free calcium are indicated (based on Stael *et al.*, 2012). FV: fast vacuolar channels, RyR: putative ryanodine receptor, $InsP_3R$: putative IP_3 receptor, SEC61: endoplasmic reticulum translocon.

(Kasai & Muto, 1990; Cheng *et al.*, 2002) and ECA proteins to the endoplasmic reticulum and Golgi membranes (Liang *et al.*, 1997; Mills *et al.*, 2008), ACA proteins have diverse subcellular localisations, including the plasma membrane, tonoplast, endoplasmic reticulum and plastid envelope (Huang *et al.*, 1993; Hong *et al.*, 1999; Bonza *et al.*, 2000; Geisler *et al.*, 2000; Schiøtt *et al.*, 2004). ACA proteins are characterised by an N-terminal autoinhibitory domain that interacts with calcium-bound calmodulin, the latter of which relieves repression and activates the ATPase (Bose *et al.*, 2011). CAX proteins are also regulated by autoinhibition, though *via* a calmodulin-independent mechanism (Pittman *et al.*, 2002). A type IB ATPase, HMA1 (HEAVY METAL ATPASE 1), which is associated with transport of heavy metals across the plastid envelope, has also been implicated in calcium mobilisation (Moreno *et al.*, 2008).

Cytosolic calcium influx is mediated by calcium-permeable channels, which have been reported in the plasma membrane, tonoplast, endoplasmic reticulum, Golgi apparatus, nuclear membrane and mitochondrial and plastid membranes (Sanders *et al.*, 2002). Whereas selective calcium channels exist in animals, plant calcium channels are also permeable to other monovalent or divalent cations (Hetherington & Brownlee, 2004). These channels have been studied using two different approaches in plants, firstly electrophysiological characterisation using patch-clamp recordings and more recently genetic and molecular approaches based largely on homology to channels in animals, fungi and bacteria.

Electrophysiological experiments have identified voltage-dependent calcium channels in the plasma membranes of plants, including depolarisation-activated calcium channels (DACCs) and hyperpolarisation-activated calcium channels (HACCs) (Swarbreck *et al.*, 2013). A number of biotic and abiotic stresses are known to trigger membrane depolarisation (Ehrhardt *et al.*, 1992; Lhuissier *et al.*, 2001; Okazaki *et al.*, 2002), and DACCs have been proposed to regulate such stress responses (White, 2000). DACC activity has been studied in *Arabidopsis* (Thion *et al.*, 1998), maize (Marshall *et al.*, 1994) and carrot cells (Thuleau *et al.*, 1994). In contrast to DACCs, which are difficult to characterise because of their rapid kinetics and low currents, HACCs have received relatively more attention (Hetherington & Brownlee, 2004). HACC activity has been reported in a variety of cell types and is implicated in growth and development (Véry & Davies, 2000) as well as stress responses (Hamilton *et al.*, 2000). In addition to membrane hyperpolarisation, HACCs may be stimulated by ROS, cytosolic calcium, cyclic nucleotides and phosphorylation (Swarbreck *et al.*, 2013).

Among the most studied families of calcium-permeable channels in plants are the cyclic nucleotide-gated channels (CNGCs) and glutamate receptor-like channels

(GLRs), both of which are homologous to their animal counterparts. Twenty *CNGC* genes have been identified in *A. thaliana*, some of which have tissue-specific expression, such as the pollen-specific genes *CNGC7*, *CNGC8* and *CNGC16* (Tunc-Ozdemir *et al.*, 2013a,b), or stress-inducible expression, for example the salt-inducible genes *CNGC19* and *CNGC20* (Kugler *et al.*, 2009). CNGCs contain two distinct cyclic nucleotide binding domains, one of which overlaps with a calmodulin-binding domain, allowing calcium-bound calmodulin to negatively regulate CNGC activity (Dietrich *et al.*, 2010). The *A. thaliana* genome encodes twenty GLRs, which are differentially activated by the six amino acids glycine, alanine, serine, cysteine, aspartic acid and glutamic acid as well as glutathione (Qi *et al.*, 2006). Although most CNGCs and GLRs have been predicted or confirmed to localise to the plasma membrane, GLR3.4 has also been detected at the plastid envelope (Teardo *et al.*, 2011), GLR3.5 is reported to localise to both chloroplasts and mitochondria (Teardo *et al.*, 2015) and CNGC7, CNGC8, CNGC19 and CNGC20 reside exclusively at the tonoplast (Chang *et al.* 2007; Yuen & Christopher, 2013).

Another relatively well-studied calcium-permeable channel in plants is the slow-vacuolar (SV) channel, the sole member in *A. thaliana* of which is encoded by the *TPC1* (*TWO-PORE CHANNEL 1*) gene. Unlike the rice OsTPC1 channel that localises to the plasma membrane (Hashimoto *et al.*, 2005), the *Arabidopsis* TPC1 protein is confined to the tonoplast and is characterised by large currents with slow activation kinetics (Peiter *et al.*, 2005). It is activated both by positive voltages on the cytosolic side of the membrane and by cytosolic calcium, which binds two EF hands on a cytosolic loop of the protein (Ward *et al.*, 2016). In the loss-of-function *tpc1-2* mutant, the speed at which calcium waves are propagated in roots during salt stress is reduced (Choi *et al.*, 2014b). This mutant is reported to have defects in germination and stomatal responses (Peiter *et al.*, 2005) and attenuated induction of salt-responsive genes during salt stress (Choi *et al.*, 2014b). The gain-of-function allele *TPC1*^{D454N}, in contrast, results in reduced growth and overproduction of jasmonates (Bonaventure *et al.*, 2007). Fast-vacuolar (FV) calcium-permeable channels characterised by rapid kinetics with low current have also been identified in plants using electrophysiological techniques however their molecular identities are unknown (Allen & Sanders, 1996).

Annexins are ubiquitous membrane-binding proteins that can reversibly form calcium-permeable pores. Eight annexins are present in *A. thaliana*, which may localise to the cytosol and even the extracellular space, as well as the plasma membrane, tonoplast, endoplasmic reticulum, mitochondria and chloroplasts (Davies, 2014). The membrane association of animal annexins is known to be regulated by calcium, pH, ROS, voltage and membrane curvature (Laohavisit & Davies, 2011). Annexin (*ANN*) gene

expression is induced by a number of abiotic stresses in *A. thaliana* (Cantero *et al.*, 2006; Konopka-Postupolska *et al.*, 2009), and the loss-of-function *ann1* mutant has altered cytosolic calcium responses to salt stress (Laohavisit *et al.*, 2013). Annexins are also implicated in the growth of the hypocotyl, primary roots and root hairs (Clark *et al.*, 2005; Laohavisit *et al.*, 2012), and their expression is highest in actively growing cells (Laohavisit & Davies, 2011). Another ROS-activated channel (Garcia-Mata *et al.*, 2010) that has been proposed to be calcium-permeable is the SKOR (STELAR K+ OUTWARD RECTIFIER) potassium-channel. Gaymard *et al.* (1998) have shown that current through the SKOR channel increases proportionally with the extracellular concentration of calcium and is repressed by the calcium chelator BAPTA.

The last group of calcium-permeable channels is characterised by activation through membrane stretching, though there is no homology between the four families of mechanosensitive channels in *Arabidopsis*. Ten MSL (MscS-LIKE) channels have been identified in *A. thaliana*, based on their homology to the bacterial MscS (MECHANOSENSITIVE CHANNEL OF SMALL CONDUCTANCE S) channel. While calcium permeability has been demonstrated for MSL9 and MSL10 (Haswell *et al.*, 2008), experimental evidence suggests that they may be preferentially stimulated by anions (Maksaev & Haswell, 2012). These two proteins localise predominantly at the plasma membrane (Haswell *et al.*, 2008), unlike MSL2 and MSL3, which are exclusively found at the plastid envelope (Veley *et al.*, 2012). Two MCA-type channels were identified in *A. thaliana* from their ability to rescue a yeast mutant deficient in the mechanosensitive calcium channel MID1 (MATING PHEROMONE-INDUCED DEATH 1) (Nakagawa *et al.*, 2007). Both MCA1 and MCA2 are plasma membrane-localised and appear to function non-redundantly as their single null mutants have distinct calcium homeostasis and growth phenotypes (Yamanaka *et al.*, 2010). Rice cells with RNAi-silenced *OsMCA1* have lower increases in cytosolic calcium in response to hypoosmotic stress (Kurusu *et al.*, 2012). Recently, another mechanosensitive calcium channel, OSCA1 (REDUCED HYPEROSMOLARITY-INDUCED CA^{2+}_{i} INCREASE 1), has been identified in a forward genetic screen for *A. thaliana* mutants with altered cytosolic calcium responses to hyperosmotic stress (Yuan *et al.*, 2014). A gene potentially encoding a homologue of the Piezo ('Pressure' in Greek) mechanosensitive channels present in animals has been identified in *A. thaliana* (*AT2G48060*) but is yet to be characterised (Hedrich, 2012).

In addition to the tonoplast-specific TPC1 channel, other organelle-specific channels are being uncovered. Inositol-1,4,5-trisphosphate (IP_3) and cyclic ADP ribose (cADPR) have been shown to trigger calcium efflux from plant endoplasmic reticulum preparations (Muir & Sanders, 1997; Navazio *et al.*, 2001), suggesting that IP_3- and

cADPR-dependent calcium channels are present in plants. Such channels have been characterised in animals but no orthologous channels have been identified in plants (Stael *et al.*, 2012). In animals, the protein translocon of the endoplasmic reticulum is calcium-permeable (Van Coppenolle *et al.*, 2004), and the *A. thaliana* translocon of the inner chloroplast membrane (TIC) has been shown to conduct potassium ions (Kikuchi *et al.*, 2013; Swarbreck *et al.*, 2013), suggesting that these complexes could potentially act as calcium channels in plants. A recent study has characterised a calcium uniporter (MCU) in the inner mitochondrial membrane in *A. thaliana* (Teardo *et al.*, 2016). Empirical evidence indicates distinct calcium dynamics between the cytosol and nucleus (Baum *et al.*, 1999), suggesting the presence of nuclear calcium channels rather than passive calcium diffusion *via* nuclear pores. Patch-clamp experiments have identified calcium-permeable channels in the plant nuclear envelope (Grygorczyk & Grygorczyk, 1998), and three CNGCs have been found to localise to nuclear membranes in the legume *Medicago truncatula* (Charpentier *et al.*, 2016), however the identities of nuclear calcium channels in *A. thaliana* are currently unknown.

1.5.2 Calcium signal transduction

Although diverse stimuli trigger increases in cytosolic calcium, each appears to induce a 'calcium signature' with distinct characteristics, for example they may differ in amplitude, frequency, duration or spatial and temporal properties. In addition, calcium fluxes have also been detected in subcellular compartments, such as the nucleus (Krebs *et al.*, 2012), mitochondria (Loro *et al.*, 2012), chloroplasts (Loro *et al.*, 2016), endoplasmic reticulum (Bonza *et al.*, 2013) and peroxisomes (Costa *et al.*, 2010). Calcium signals are decoded by two groups of proteins, known as 'sensor responders', which contain effector domains of enzymatic function as well as calcium-binding domains, and 'sensor relays', which also bind calcium but lack enzymatic activity and relay the calcium signal to other proteins (Sanders *et al.*, 2002) (*Figure 1.8*).

Calcium-dependent protein kinases (CDPKs) form the major sensor responders in *A. thaliana*, in which thirty-four CDPK (*CPK*) genes have been identified. These proteins contain an N-terminal kinase domain that is negatively regulated by an adjacent autoinhibitory domain and one to four calcium-binding EF hand motifs in their C-terminus, which contribute to the derepression of the kinase domain (DeFalco *et al.*, 2010). Certain CDPKs can additionally be activated by phospholipids or *via* interactions with auxiliary proteins (Harper *et al.*, 1993; Camoni *et al.*, 1998). These kinases are targeted to diverse subcellular compartments and also to cellular membranes *via* lipid

modifications (Simeunovic *et al.*, 2016). CDPK targets include transcription factors, metabolic enzymes, transporters and cytoskeletal proteins (Sanders *et al.*, 2002), and CDPKs have been shown to regulate plant development and hormone signalling (Choi *et al.*, 2005; Myers *et al.*, 2009), as well as of biotic and abiotic stress responses (Saijo *et al.*, 2000; Romeis *et al.*, 2001). Calcium- and calmodulin-dependent protein kinases (CCamKs), which contain an additional calmodulin-binding domain as well as calcium-binding motifs and a kinase domain, are absent in *A. thaliana* but found in certain plant taxa such as the legumes (DeFalco *et al.*, 2010).

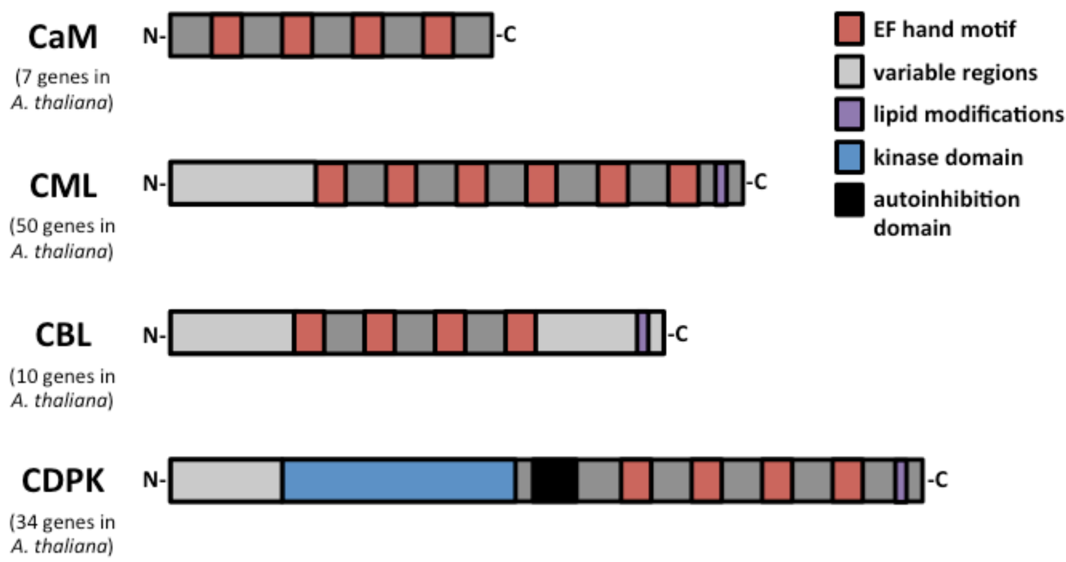


Figure 1.8: Structural features of major calcium sensors in *A. thaliana*.
CaM: calmodulin, CML: calmodulin-like protein, CBL: calcineurin B-like protein, CDPK: calcium dependent protein kinase. CMLs contain 1-6 EF hands and CDPKs contain 1-4 EF hands. CMLs, CBLs and CDPKs may associate with membranes *via* lipid modifications. Based on DeFalco *et al.* (2010).

Calcineurin B-like proteins (CBLs), calmodulins (CaMs) and calmodulin-like proteins (CMLs) are the key sensor relays in *A. thaliana*. CBLs are calcium-binding proteins that function as regulators of a group of kinases known as CBL-interacting protein kinases (CIPKs). The *A. thaliana* genome encodes ten CBLs and twenty-six CIPKs, and these proteins can interact in different combinations to fine-tune calcium signalling. For example, CIPK1 is targeted to the plasma membrane when interacting with CBL1 but to the tonoplast when interacting with CBL2 (Batistič *et al.*, 2008). As well as associating with membranes *via* lipid modification motifs, CBLs have been reported to localise to the

cytoplasm and nucleus (Batistič *et al.*, 2010). CBLs promote CIPK activity by binding to an autoinhibition domain in the C-terminus of CIPKs and possibly compete with PP2C protein phosphatases for their association with these kinases (DeFalco *et al.*, 2010). CBL-CIPK signalling has been studied in abiotic stress responses, for example CBL4/SOS3 and CIPK24/SOS2 (SALT OVERLY SENSITIVE 2/3) are key regulators of salt tolerance in *A. thaliana* (Halfter *et al.*, 2000).

CaMs are small proteins containing four EF hands that undergo conformational changes upon calcium binding, thereby exposing hydrophobic domains that can promote or repress interactions with target proteins (Clapham, 2007). CMLs contain between one and six EF hands and have varying degrees of homology to CaMs. Seven CaMs and fifty CMLs are found in *A. thaliana*, most of which are uncharacterised. While CaMs are found ubiquitously across eukaryotes, CMLs, in addition to CDPKs and CBLs, are specific to plants and certain unicellular eukaryotes (DeFalco *et al.*, 2010). A vast number of CaM/CML-binding proteins have been described, including transcription factors, enzymes, structural proteins and transporters (Bouché *et al.*, 2005). Various calcium channels or transporters contain CaM/CML-binding motifs, including CNGCs, ACAs and the endoplasmic reticulum translocon, and pharmacological studies have shown that CaM/CMLs regulate calcium mobilisation (Kaplan *et al.*, 2006). CaM-binding transcription factors include the CG-1 family, comprising parsley CG-1 (CGCG-BINDING PROTEIN 1), *Arabidopsis* CAMTAs and EICBP (ETHYLENE-INDUCED CALMODULIN BINDING PROTEIN), rice CBT (CALMODULIN-BINDING TRANSCRIPTION ACTIVATOR) and tobacco ER1 (ETHYLENE-RESPONSIVE 1), which contain calcium-dependent and -independent calmodulin binding sites (Kim *et al.*, 2009). The *A. thaliana* transcriptional regulators TGA3 (TGACG-BINDING PROTEIN 3) (Szymanski *et al.*, 1996), MYB2 (MYELOBLASTOSIS-DOMAIN PROTEIN 2) (Yoo *et al.*, 2005), CBNAC (CALMODULIN-BINDING NAC PROTEIN) (Kim *et al.*, 2007), WRKY7, WRKY43 and WRKY53 (Park *et al.*, 2005; Popescu *et al.*, 2007), SCL4 (SCARECROW-LIKE PROTEIN 4) (Popescu *et al.*, 2007), CBP60g (CALMODULIN-BINDING PROTEIN 60-LIKE g) (Zhang *et al.*, 2010a) and GTL1 (Yoo *et al.*, 2010) also interact with CaMs, and the latter were found to promote the DNA association of both TGA3 and MYB2. By binding to phosphatases, such as PP7, or kinases, such as CBK3, CaMs indirectly regulate transcription factor activity, as shown for HSFA1a, which is modified by both PP7 and CBK3 (Liu *et al.*, 2007a, 2008). Kushwaha *et al.* (2008) have demonstrated that calmodulin CAM7 regulates gene expression by directly association with promoters of light-responsive genes *CAB1* (CHLOROPHYLL A/B-BINDING PROTEIN 1) and *RBCS1A* (RIBULOSE BISPHOSPHATE CARBOXYLASE SMALL CHAIN 1A).

Using electrostimulation of *A. thaliana* seedlings to create different cytosolic calcium signatures, Whalley *et al.* (2011) found four calcium-responsive promoter elements, including the CAM box (ACGCGT) recognised by CAMTAs, as well as the CRT/DRE motif (CCGAC), abscisic acid response element (ABRE; ACGTG) and Site II motif (GGCCC). In addition to CaM-mediated transcriptional regulation, some transcription factors may directly bind calcium, for example the single EF hand-containing *A. thaliana* transcription factor NIG1 (NaCl-INDUCIBLE GENE 1), which is associated with abiotic stress responses (Kim & Kim, 2006).

1.5.3 Calcium signalling in temperature responses

Although both heat and cold treatments induce an influx of calcium ions in plant cells, the cytosolic calcium signatures differ between these two stimuli. Knight *et al.* (1991) found that an instantaneous drop in temperature from 20˚C to 5˚C triggers a large spike in cytosolic calcium that rapidly subsides in *A. thaliana*, and subsequent experiments showed that slower rates of cooling alter the calcium signature into a more gradual increase (Plieth *et al.*, 1999). In contrast, no change in cytosolic calcium was detected by Knight and colleagues upon a transient increase from 20˚C to 50˚C. When plant cells are exposed to sustained elevated temperatures, however, cytosolic calcium increases gradually over several minutes (Gong *et al.*, 1998; Saidi *et al.*, 2009; Zheng *et al.*, 2012; Gao *et al.*, 2012; Finka *et al.*, 2012; Liao *et al.*, 2017).

Calcium channels in the plasma membrane, and CNGCs in particular, have been implicated in the cytosolic calcium response to heat. An inward current has been detected across the plasma membrane in response to heat shock in *A. thaliana* and *Physcomitrella patens* protoplasts, which can be inhibited by treatment with channel blockers lanthanum and gadolinium (Saidi *et al.*, 2009; Gao *et al.*, 2012). This current appears to be induced by cAMP, cytosolic levels of which rapidly increase during heat shock, and occurs in a CNGC6-dependent manner (Gao *et al.*, 2012). Cellular abundance of CNGC6 correlates positively with the heat-induction of *sHSP* and *HSP70* genes and with the acquisition of thermotolerance (Gao *et al.*, 2012). Interestingly, Finka *et al.* (2012) have found that the loss-of-function mutants of *A. thaliana CNGC2* and *CNGC4* and *P. patens CNGCb* are more thermotolerant than wild-type plants, with induction of the heat shock response at lower temperatures. The authors demonstrated that current through one of three heat-activated calcium channels is abolished in the *P. patens CNGCb* mutant, and as a consequence the other two channels are more likely to be activated during heat shock. CNGC16 has additionally been found to contribute to

thermotolerance in pollen tubes and is required for full heat-induction of *HSFA2* and *HSFB1* genes (Tunc-Ozdemir *et al.*, 2013b).

A recent study has demonstrated that annexins are involved in calcium signalling during heat stress. A large increase in calcium influx is measured in the *myb30-2* mutant, which has greater expression of *ANN1-4* genes, in response to both high temperature and hydrogen peroxide treatments, whereas this phenotype is diminished in *myb30-2 ann4* double mutants or *myb30-2 ann1 ann4* triple mutants (Liao *et al.*, 2017). The heat-induced calcium signal mediated by CNGCs and annexins appears to be transduced by calmodulin CAM3, the abundance of which positively correlates with the DNA-binding ability of HSFs, the heat-induction of *HSP* genes and thermotolerance (Zhang *et al.*, 2009). These effects are likely to be mediated by regulating the activities of the aforementioned HSFA1a-interactors CBK3 (Liu *et al.*, 2008) and PP7 (Liu *et al.*, 2007a).

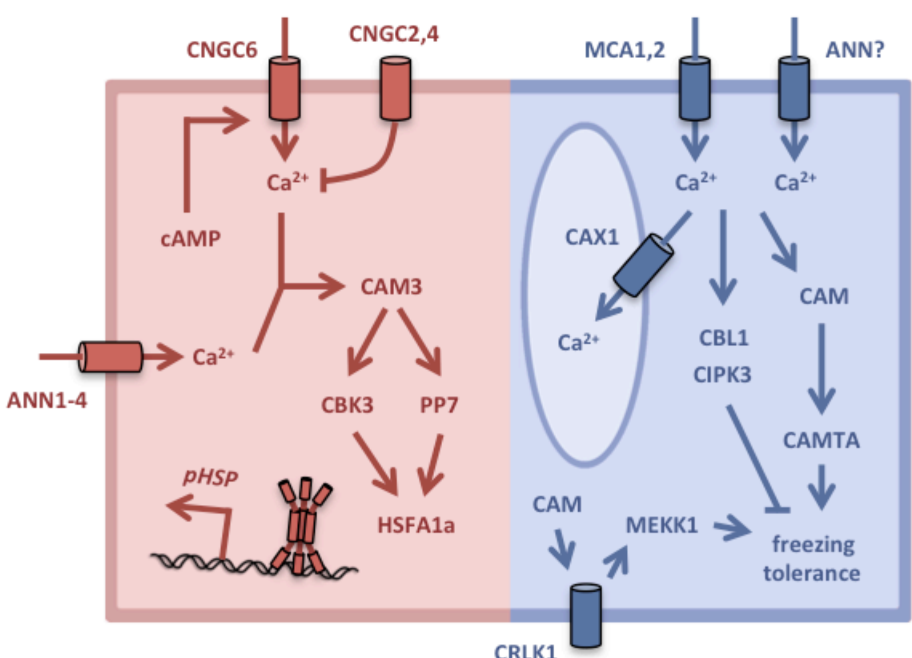


Figure 1.9: Calcium signalling components known to be involved in temperature responses in *A. thaliana*.
The rectangle represents a plant cell. Calcium influx and signalling associated with elevated temperatures are indicated in red and those associated with low temperatures are shown in blue. Heat-induced calcium influx is mediated by cyclic nucleotide-gated calcium channel CNGC6 (triggered by cAMP) and annexins ANN1-4. Null-mutants of CNGC2 and CNGC4 have enhanced heat-induced calcium influx. Calmodulin CAM3 interacts with kinase CBK3 and phosphatase PP7, which both positively regulate HSFA1a.
Cold-induced calcium influx is mediated in part by mechanosensitive channels MCA1 and MCA2. Annexins re-localise to membranes upon cold treatment. Calmodulin-regulated CAMTA transcription factors promote and calcineurin B-like protein CBL1 represses freezing tolerance. Calmodulin activates membrane kinase CRLK1, which promotes MEKK1-mediated MAP kinase signalling to promote freezing tolerance. Cytosolic calcium is extruded into the vacuole by the calcium exporter CAX1.

Several calcium influx or efflux genes are upregulated by low temperatures, including *CAX1* (Catalá *et al.*, 2003), *ACA8* (Schiøtt & Palmgren, 2005), *GLR3.4* (Meyerhoff *et al.*, 2005) and *ANN1*, *ANN3*, *ANN4*, *ANN5*, *ANN7* and *ANN8* in *A. thaliana* (Cantero *et al.*, 2006) and *TPC1* in wheat (Wang *et al.*, 2005), while *ACA10* expression is repressed at low temperatures (Schiøtt & Palmgren, 2005), suggesting that these proteins may play a role in cold-associated calcium signalling. Annexins have been shown to associate with membranes in cold-treated wheat shoots (Breton *et al.*, 2000), and the rice *OsAnn3* null mutant is hypersensitive to chilling stress (Shen *et al.*, 2017).

Cold-induced cytosolic calcium appears to originate from both the apoplast and vacuole, as indicated from treatments with pharmacological inhibitors and by cytosolic calcium measurements in the tonoplast microdomain (Knight *et al.*, 1996). Calcium dynamics during cold shock are unaltered in loss-of-function mutants of the calcium channels TPC1 or CNGC2 or of the V-ATPase DET3 (DE-ETIOLATED 3) (Carpaneto *et al.*, 2007; Ranf *et al.*, 2008). The null *cax1* mutant, however, has enhanced *CBF* and *COR* gene induction during cold shock and enhanced freezing tolerance, as a result of higher levels of cytosolic calcium (Catalá *et al.*, 2003).

Despite cold shock being one of the first stimuli found to induce a calcium signature in *A. thaliana* (Knight *et al.*, 1991) and multiple studies characterising the electrophysiology of cold-activated calcium channels in plants (Ding & Pickard, 1993; Carpaneto *et al.*, 2007), the molecular identities of such channels are only beginning to be resolved. Reduced cold-induced calcium influx has recently been reported in a rice mutant deficient in a regulator of G-protein signalling (*OsCOLD1*; *CHILLING TOLERANCE DIVERGENCE 1*), though the exact role of this regulator in cold responses is unclear (Ma *et al.*, 2015). Mori *et al.* (2018) have recently demonstrated that MCA1 and MCA2 contribute to cold-induced calcium influx, with a diminished peak in cytosolic calcium in single and double mutants upon instantaneous cooling to 3°C. Interestingly, slight increases in the induction of *CBF* genes were reported in the *mca1 mca2* double mutant during cold exposure, while that of some CBF-independent cold-inducible genes was reduced.

Cold-associated calcium signalling appears to converge on CAMTA transcription factors (Doherty *et al.*, 2009), as well as CBL1 (Cheong *et al.*, 2003; Albrecht *et al.*, 2003) and CIPK3 (Kim *et al.*, 2003), mutants of which have altered expression of some cold-inducible genes and, in the case of the *CBL1*-overexpressing plants (Cheong *et al.*, 2003) and double *camta1 camta3* loss-of-function mutants (Doherty *et al.*, 2009), reduced freezing tolerance. Cold-induced calcium influx appears to regulate the membrane-bound kinase CRLK1 (CALMODULIN-REGULATED RECEPTOR-LIKE KINASE 1) *via* its

interaction with calmodulin, and CRLK1 is proposed to activate the MEKK1 kinase, leading to MAP kinase signalling that ultimately contributes to freezing tolerance (Yang *et al.*, 2010a,b). The calcium signalling pathways involved in heat and cold responses are summarised in *Figure 1.9*.

1.6 Objectives

This thesis investigates temperature-signalling pathways in plants using a 'chemical genetics' approach, in which small molecules are used to perturb protein function, in contrast to classical genetics, in which the genes encoding the proteins are altered by mutagenesis. Chemical genetics provides the advantage of being able to overcome two major obstacles of classical genetics, gene lethality and redundancy, owing to rapid, reversible and dose-dependent chemical effects that can be triggered in a regulated spatiotemporal manner. Genome-wide chemical genetics studies, known as 'chemical genomics', have contributed to key discoveries in cell wall metabolism (Peng *et al.*, 2001; Desprez *et al.*, 2002; Yoneda *et al.*, 2010), defence responses (Zheng *et al.*, 2006; Serrano *et al.*, 2007), hormone signalling (Walsh *et al.*, 2006; Gleason *et al.*, 2011; Prigge *et al.*, 2016) and membrane trafficking processes (Zouhar *et al.*, 2004; Norambuena *et al.*, 2008).

Small molecules are immensely diverse and can interact with proteins in a specific manner. They are thus useful tools to probe biological systems. The fact that all known biological thermosensors, as well as components of signalling pathways, are dependent on specific structural alterations for their activity suggests that chemical genomics may be used to study temperature-signalling pathways in plants. In collaboration with the agrochemical company Syngenta, a screen for molecules that induce or repress the heat shock response was conducted in *A. thaliana* seedlings, with the aim of better understanding heat perception in plants and to identify chemical agents that could potentially be used to protect crops from heat stress. To identify such compounds affecting warm temperature responses, a heat-responsive bioluminescent reporter previously developed by Kumar & Wigge (2010) was used, and chemical effects on heat-inducible genes were subsequently validated by quantitative PCR and RNA sequencing (RNA-seq). Compounds of interest were additionally tested in the model monocotyledon *Brachypodium distachyon* and in the unicellular model eukaryote *Saccharomyces cerevisiae*, to assess where their effects observed in *A. thaliana* are conserved across more distant phylogenetic lineages. Potential biological targets of

these chemicals were predicted using cheminformatic searches for structurally similar molecules with known modes of action, in conjunction with molecular and physiological assays.

In addition to studying warm temperature responses in plants using a diverse library of small molecules, the mechanism of cold perception in *A. thaliana* was examined using protein synthesis inhibitors. As mentioned previously, some of these chemicals, such as cycloheximide, are known to induce *CBF* genes in *A. thaliana* (Zarka *et al.*, 2003). A second major aim of this thesis was to elucidate the mechanism by which such inhibitors trigger the expression of early cold-responsive genes in plants and to investigate the involvement of translation in cold signalling. Bioinformatic analyses were used to compare the transcriptomic changes brought about by cycloheximide treatment with those triggered by temperature or abiotic stresses. Analyses of conserved motifs in the promoters of cycloheximide-inducible genes identified candidate regulators of this response, which were validated by *CBF* expression studies in several mutants of signalling- and translation-associated genes. The conditions necessary for *CBF* gene induction were dissected using a variety of inhibitors that affect different steps of translation or that bind to different sites in the ribosome, combined with an *in vivo* translation assay developed by Schmidt *et al.* (2009) and optimised for *A. thaliana* seedlings in this thesis.

Finally, the role of calcium signalling in the induction of *CBF* genes was investigated, with the aim of gaining an understanding of the regulation of calcium mobilisation during cold temperature signalling. *CBF* expression studies were performed in *A. thaliana* seedlings treated with a calcium chelator or calcium channel blocker in combination with cold shock or cycloheximide treatments. In addition, the bioluminescent calcium reporter aequorin was used to measure potential changes in cytosolic free calcium occurring during treatments with a variety of inhibitors with different effects on *CBF* gene expression.

Chapter 2

Materials and methods

2.1 Plant growth conditions

Arabidopsis thaliana seeds were sterilised in 70% v/v ethanol containing 0.05% v/v Tween-20 for five minutes, followed by a brief wash in 96% v/v ethanol and air-drying on sterile filter paper. Sterilised seeds were stratified at 4˚C in the dark for 72 hours, in liquid medium or on agar plates, and seedlings were maintained at 170 μmol/m^2/s light and 65% relative humidity for all experiments (Conviron).

All luciferase experiments were carried out using *A. thaliana* Col-0 *pHSP70::LUC* (Kumar & Wigge, 2010). Sterilised seeds were stratified in full-strength Murashige-Skoog medium (1×MS; 0.44% w/v Murashige-Skoog mix including vitamins; M0222, Duchefa) containing 0.2% w/v agar (P1001, Duchefa), adjusted to pH 5.7 and supplemented with 0.1% w/v glucose after autoclaving, with approximately 10 mg of seeds per millilitre of medium. Seed-medium mix (20 μl) and 80 μl liquid 1×MS medium were dispensed into white 96-well microplates (655075, Greiner Bio-One) using wide-end tips (11972295, Fisher Scientific). Microplates were closed with clear lids (L3911, Sigma) and sealed with Micropore tape (1530-0, 3M). Seedlings were grown at 22˚C with 16-hour long-day photoperiod.

For all expression analysis experiments, *A. thaliana* seedlings were grown in half-strength Murashige-Skoog liquid medium (½×MS; 0.22% w/v Murashige-Skoog mix including vitamins; M0222, Duchefa) containing 0.05% w/v MES (2-[N-morpholino]ethanesulfonic acid; 69892, Sigma), adjusted to pH 5.7 and supplemented with 0.1% w/v glucose after autoclaving (½MMG medium). The seedlings were cultured in 12- or 6-well plates (657-160 and 665-180, Greiner Bio-One), with 1 ml or 2 ml of liquid medium per well, respectively. The plates were sealed with Micropore tape and maintained either at 21˚C in constant light or at 22˚C with a 16-hour long-day photoperiod. Seedlings were cultured in the same manner for analyses of HSP70 protein levels, using a Col-0 line expressing a FLAG-tagged HSP70B/AT1G16030 protein (*pHSP70B::HSP70B::FLAG×3*; Philip A. Wigge, unpublished).

For cytosolic free calcium measurements, *A. thaliana* Col-0 *pCaMV35S::APOAEQUORIN* (Xu *et al.*, 2007) were grown for eight to twelve days on

½×MS medium (pH 5.7) with 0.8% w/v agar (P1001, Duchefa) or in 6-well plates with 2 ml ½MMG medium per well, sealed with micropore tape, at 20°C with a 12-hour photoperiod.

For transformations and seed harvesting, *A. thaliana* plants were grown on Levington F2 soil in large (base $25cm^2$, height 7cm) or medium (base 12.25 cm^2, height 6 cm) pots, at 22°C with a 16-hour long-day photoperiod. Transgenic *A. thaliana* T1 and T2 seeds were stratified and grown for selection on ½×MS medium (pH 5.7) with 0.8% w/v agar (P1001, Duchefa) supplemented with 50 to 100 µg/ml kanamycin.

After removing the lemma, *Brachypodium distachyon* Bd21-3 seeds (kindly provided by Dr Devin O'Connor, University of Cambridge) were sterilised by shaking for five to ten minutes in 70% v/v ethanol containing 0.05% v/v Tween-20, followed by a brief wash in 96% v/v ethanol and air-drying on sterile filter paper. Sterilised seeds were stratified and germinated in sterile plastic boxes (base 42.25 cm^2, height 9 cm) containing 10 ml ½MMG medium. Seedlings were grown at 20°C with a 16-hour long-day photoperiod, at 170 $\mu mol/m^2/s$ light and 65% relative humidity, and were individually transferred to 14 ml Falcon tubes containing 1 ml ½MMG medium after emergence of the first true leaf.

2.2 Temperature and chemical treatments

Chemicals from the Syngenta library were obtained in 96-well plates at 100 ppm concentration in 100 µl DMSO. Publically available inhibitors were prepared as indicated in *Table 2.1*. Edeine A1 was provided by Ian Brierley (University of Cambridge) and phyllanthoside, cryptopleurine, narciclasine and nagilactone C were obtained from the Developmental Therapeutics Program of the National Cancer Institute (NCI), National Institute of Health (NIH).

Chemical treatments were performed on seedlings grown for seven to ten days in liquid medium, with a final DMSO concentration of no more than 0.1% v/v. For expression analyses, the liquid medium was removed and replaced with an equivalent volume of fresh ½MMG medium containing chemicals dissolved to the indicated concentrations and equilibrated to the growth temperature of the seedlings. Where stock solutions were prepared in water, DMSO was added to maintain a constant concentration in all treatments. Pre-treatments with calcium-signalling inhibitors were performed as above, by replacing the ½MMG liquid medium (800 µl per well, in 12-well plates), and subsequent treatments were carried out by adding concentrated chemicals dissolved in a small amounts fresh medium (200 µl) without removing the calcium-

signalling inhibitors. Other than the MES present in the ½MMG medium, no additional buffers were added to the medium during chemical treatments.

Table 2.1: Inhibitors used in this study

Chemical	Supplier	Catalogue number	Stock concentration (mM)	Stock solvent
17-DMAG	Invivogen	ant-dgl-5	30	DMSO
Anisomycin	Sigma	A9789	30	DMSO
BAPTA	Abcam	ab120449	-	water
Blasticidin S	Sigma	15205	30	DMSO
Chloramphenicol	Sigma	C7795	30	DMSO
Cryptopleurine	NIH/NCI	NSC 19912	30	DMSO
Cycloheximide	Sigma	46401	30	DMSO
DBMIB	Sigma	271993	30	DMSO
Deoxynivalenol	Sigma	D0156	30	DMSO
Edeine A1	Ian Brierley (Cambridge)	-	2	water
G418	Sigma	A1720	30	water
Gentamicin	Melford	G0124	30	water
Homoharringtonine	Sigma	SML1091	30	DMSO
Hygromycin B	Sigma	H9773	30	DMSO
Kanamycin	Fisher	BP906-5	30	water
Lactimidomycin	Merck Millipore	506291	4	DMSO
Lanthanum	Sigma	262072	-	water
Lycorine	Sigma	L5139	30	DMSO
MG132	Sigma	C2211	10	DMSO
Nagilactone C	NIH/NCI	NSC 211500	30	DMSO
Narciclasine	NIH/NCI	NSC 266535	30	DMSO
Nourseothricin	Cambridge Bioscience	2882-100	30	water
Paromomycin	Sigma	P5057	30	water
Phyllanthoside	NIH/NCI	NSC 328426	30	DMSO
Puromycin	Sigma	P8833	30	DMSO
Spectinomycin	Melford	S0188	30	DMSO
Streptomycin	Sigma	S6501	30	water
Tetracycline	Sigma	87128	30	DMSO
Verrucarin A	Sigma	V4877	30	DMSO

Temperature treatments were carried out by transferring the plates to Microclima Snijder cabinets (BRS) set to the indicated temperatures, with 170 $\mu mol/m^2/s$ light and 65% relative humidity. Seedlings were harvested in collection microtubes (19560, Qiagen) or 2 ml microcentrifuge tubes, by flash freezing on dry ice or in liquid nitrogen. Temperature measurements of liquid medium were made using a

thermocouple logger (Tinytag View-2 logger with a thermistor probe; TV-4020 and PB-5001-1M5; Gemini).

For the cold-shock RNA-seq experiment performed by Sandra Cortijo and Philip A. Wigge, *A. thaliana* Col-0 seedlings were grown for seven days at 17˚C with a 16-hour long-day photoperiod, on rafts (consisting of nylon mesh [pore size 1 mm] lined by pieces of plastic straws for support) placed on ½×MS medium (pH 5.7) with 0.8% w/v agar (P1001, Duchefa). At ZT1, rafts were transferred to liquid ½×MS medium at 17˚C or pre-cooled liquid medium on ice at 4˚C.

For the SUnSET (Surface Sensing of Translation) assay (Schmidt *et al.*, 2009), seedlings were incubated in 100 or 150 μM puromycin (in ½MMG) for 30 or 15 minutes, respectively, and then immediately harvested in liquid nitrogen.

Saccharomyces cerevisiae YEF473a cells (Bi & Pringle, 1996; kindly provided by Kerry Bloom, University of North Carolina) were cultured in YPD medium (1% w/v yeast extract, 2% w/v peptone, 2% w/v glucose) at 28˚C and 230 rpm shaking (Ecotron, Infors HT), from OD_{600} 0.2 to OD_{600} 0.8 (Ultrospec 10, Amersham Biosciences). Yeast cells was transferred to 2 ml tubes, with 1 ml culture per tube, centrifuged at maximum speed for 12 seconds to harvest the cells, resuspended in 1 ml fresh YPD medium containing chemicals and returned to 28˚C. Heat treatment was performed by incubating the tubes at 37˚C in a ThermoMixer (Eppendorf) at 1000 rpm. Cells were harvested by centrifugation as above, decanting the liquid and immediately flash freezing the tubes in liquid nitrogen.

2.3 Luciferase screening

Fifty microlitres 400 μM sodium-D-luciferin (L3708, Melford) and 50 μl 20 ppm chemicals from Syngenta, both prepared in deionised water, were simultaneously added to microplates containing ten-day-old *A. thaliana* seedlings in 1×MS medium. Microplates were returned to 22˚C and for one hour, after which luminescence was measured for 1 second per well, using a microplate reader (TriStar LB941, Berthold) coupled with data-capture software (Microwin 2000; Berthold). Microplates were then placed at 30˚C for one hour, and luminescence was quantified as before. Average photon counts were taken for each temperature, for sixteen biological replicates per chemical for the repressor screen and for two biological replicates per chemical for the activator screen. Luminescence images were captured using a photon-counting camera (NightOwl LB983-NC100, Berthold) coupled with image-capture software (IndiGO v.2.0.3.0, Berthold).

2.4 Chemical lethality assays

A. thaliana Col-0 seedlings were grown in 12-well plates with 1 ml ½MMG medium per well, at 17˚C or 27˚C with an eight-hour short-day photoperiod. Two days after germination, the medium was replaced with 1 ml fresh ½MMG medium containing 10, 1 or 0.1 ppm chemicals (0.1%, 0.01% or 0.001% v/v DMSO), and the plates were returned to 22˚C or 27˚C. After seven days, the plates were scanned at 800 dpi (Perfection V700 Photo, Epson). Hypocotyl measurements were made using ImageJ (NIH).

2.5 Thermotolerance assays

A. thaliana Col-0 seeds stratified in ½MMG were plated in clear sterile F-bottom 96-well plates (655180; Greiner Bio-One) with one seed and 100 µl ½MMG medium per well. Plates were sealed with Micropore tape and maintained at 22˚C with a 16-hour long-day photoperiod. Seedlings were treated with chemicals at 22˚C by replacing the growth medium with 100 µl fresh ½MMG medium containing chemicals from the Syngenta library at 10 or 1 ppm (0.1% or 0.01% v/v DMSO). Six-day-old seedlings were chemically treated for 24 hours and seven-day-old seedlings for three hours. For the latter, chemicals were then removed, the wells replenished with 100 µl fresh ½MMG medium and the seedlings incubated for one hour at 22˚C. The plates were sealed with micropore tape and placed in a water bath (SUB28, Grant) at 42˚C for 30 minutes. The heat treatments were performed at ZT8 and the plates were then returned to 22˚C.

After seven days of recovery, the plates were imaged using a camera (EOS Rebel T5i, Canon; ISO100, 1/30, F13) fixed 81 cm above the plates, illuminated by two sidelights. The green content of the plates was measured as follows. Using the "Threshold Colour" plug-in in ImageJ (NIH), the RGB filter was set to exclude 170-255 Red, include 0-255 Green and exclude 0-90 Blue. The image type was converted to 8-bit, the threshold adjusted to 0-200, and using the 'Analyze particles' function the masked content (total green area) was calculated. Thermotolerance was calculated as the percentage green area of treated plates relative to control plates.

For chlorophyll fluorescence, Fm and Fo values were measured in dark-adapted seedlings (maintained in the dark for at least 20 minutes prior to measurements) 24 hours after heat treatments, using an 800-millisecond pulse of actinic light at 2000 µmol/m^2/s in a chlorophyll fluorescence imager (CFImager, Technologica).

2.6 RNA extraction and transcriptomic analysis

For expression analyses by quantitative PCR, total RNA was isolated from *A. thaliana* and *B. distachyon* seedlings using phenol-chloroform extraction, as described by Box *et al.* (2011). RNA extractions from *S. cerevisiae* cells were performed as described by Del Aguila *et al.* (2005). Genomic DNA was eliminated by DNase I treatment using the TURBO DNA-free kit (AM1907, Invitrogen) and purified RNA was reverse-transcribed using the Transcriptor First-Strand cDNA Synthesis Kit (04379012001, Roche), according to manufacturer instructions.

***Table 2.2**:* Primers used for qRT-PCR

Primer number	Gene	Sequence (5'-3')
5295 5296	*HSP70/AT3G12580*	CTGACAGCGAGCGTCTCAT GGATCACTGTATCTTCTTCCGATT
10964 10965	*CBF2/AT4G25470*	TGGATGAAGAGGCGATGTTGGG GGCGACGGTAAAAGCATCCCT
5343 5344	*PP2A/AT1G13320*	GCGGTTGTGGAGAACATGATACG GAACCAAACACAATTCGTTGCTG
5297 5298	*UBC21/AT5G25760*	TCCTCTTAACTGCGACTCAGG GCGAGGCGTGTATACATTTG
11757 11758	*ICE1/AT3G26744*	GACTGGGATTGAGGTTTCTGGG GAACACTCTCAGCCGCTTTACCG
13619 13620	*AT1G07930/eEF1A*	CGAATCCTCAAAACTCTATCCGC CCTCCGATCAAGAACCCAGTT
13640 13641	*AT1G13950/eEF5*	CTGTAGGCTTCCATTACTCATCGG CGCACGAGTAAAGACTTCGCTCATC
9843 9844	*HSP70/BRADI2G23250*	TGAACCCCACCAACACCG GCTTGTCGCCAGGTCCAG
9962 9963	*SamDC/BRADI5G14640*	GCTTCTCTGAGGAGGTTGATGTC AAGCATTGCCACCAGATTTCA
9966 9967	*UBC18/BRADI4G00660*	GCATCCGCACATTTACAGCA ATAGCGGTCATTGTCTTGCG
5483 5484	*SSA4/YER103W*	TTGAGGGTGAAAGGACAAGG ATTTGTGGTACGCCTCTTGG
5473 5474	*ACT1/YFL039C*	TCACCAACTGGGACGATATG TCTGGGGCAACTCTCAATTC
9078 9079	*CDC19/YAL038W*	GACTTACAACCCAAGACCAACCAGA CGGTTTCACCAGACAACATAACACAG

Quantitative real-time PCR (qRT-PCR) was carried out using LightCycler 480 DNA SYBR Green I Master mix (04707516001, Roche), with 0.3 µM primers and 2 µl cDNA diluted 1:10 after synthesis. Fluorescence was quantified for 45 PCR cycles with 15-second 94˚C denaturation, 30-second 60˚C annealing and 30-second 72˚C extension (LightCycler 480, Roche). Primers used for qRT-PCR are given in *Table 2.2*. Crossing point (Cp) values were calculated using the 'Second Derivative Maximum Method' in the

LightCycler software, and relative gene expression was subsequently calculated using the following formula:

$$Relative\ gene\ expression = \frac{2^{-Cp_A}}{\sqrt{(2^{-Cp_B}) \times (2^{-Cp_C})}}$$

where A is the gene of interest and B and C are control genes used for normalisation.

For RNA-sequencing (RNA-seq), total RNA was extracted and DNase-treated using MagMAX-96 Total RNA Isolation Kit (AM1830, Thermo Fisher Scientific). RNA was quantified using the Qubit fluorometer (Thermo Fisher Scientific) and RNA profiles were analysed using the TapeStation 2200 (Agilent) with RNA ScreenTapes (5067-5576, Agilent). Paired-end libraries for sequencing were prepared from 1 μg RNA using the NEBNext Ultra Directional RNA Library Prep Kit for Illumina (E7420L, NEB), with the NEBNext Poly(A) mRNA Magnetic Isolation Module (E7490, NEB), according to manufacturer instructions. DNA libraries were quantified using the Qubit fluorometer, and library profiles were analysed using the TapeStation 2200 with High Sensitivity D1000 ScreenTapes (5067-5584, Agilent). Libraries were diluted to 1 nM, and two 1 nM pools of 24 libraries were sequenced on the NextSeq-500 (Illumina; paired-end 75bp reads), according to Illumina guidelines.

2.7 Bioinformatic methods

A. thaliana genomic sequences were obtained from The *Arabidopsis* Information Resource (TAIR; arabidopsis.org). Sequences were analysed using BLAST (www.ncbi.nhlm.nih.gov/BLAST/) and aligned using ClustalW-MView (bar.utoronto.ca/ ntools/cgi-bin/ntools_multiplealign_w_mview.cgi). Primers were designed using Biosoft NetPrimer (www.premierbiosoft.com/netprimer/index.html) and Sigma DNA Calculator (www.sigma-genosys.com/calc/DNACalc.asp). Restriction digests were designed using NEBcutter V2.0 (http://tools.neb.com/NEBcutter2/). Chemical structures were drawn using MolView (http://molview.org/) or ChEMBL (ChEMBL_23; https://www.ebi.ac.uk/ chembl/). Cheminformatic searches were carried out with SIMCOMP (http://www.genome.jp/tools/simcomp/) and ChEMBL.

Sequencing data were mapped using a Bash script written by Varodom Charoensawan (Mahidol University, Thailand). The quality of raw reads was assessed using FastQC (www.bioinformatics.babraham.ac.uk/projects/fastqc/), and adaptor contamination and low-quality trailing sequences were removed using Trimmomatic (Bolger *et al.*, 2014). Trimmed reads were mapped to the TAIR10 transcriptome using Tophat (Trapnell *et al.*, 2009) and optical duplicates were removed using Picard tools

(http://github.com/broadinstitute/picard). Transcripts per million (TPM) values were calculated (Wagner *et al.*, 2012).

Z-scores were calculated from TPM values after filtering out genes that were lowly expressed (with an average TPM less than 1 across samples), not expressed in the majority of samples (with a TPM of 0 for more than three quarters of samples) or that showed little difference in expression across treatments (with a coefficient of variation less than 0.3). Z-score transcriptomes were clustered using heatmap.2 function in the gplots package of R.

Gene ontology enrichment was performed using GOrilla with two gene lists (http://cbl-gorilla.cs.technion.ac.il/). Analyses of promoter motif enrichment were carried out using HOMER (http://homer.ucsd.edu/homer/) with promoter sequences retrieved from TAIR (TAIR10 Loci Upstream Seq -1000bp; https://www.arabidopsis.org/tools/bulk/sequences/). *Arabidopsis* public microarray datasets were obtained from AtGenExpress (http://jsp.weigelworld.org/AtGenExpress/resources/).

2.8 Western blotting

Frozen seedlings were pulverised in 2 ml tubes containing a single tungsten carbide bead (69997, Qiagen) using the Tissue Lyser II (Qiagen). Proteins were extracted using the urea/SDS method outlined by Clontech (2009), with 200 µl cracking buffer per ten to twenty pulverised seedlings. The tubes were vigorously shaken for ten seconds to resuspend the powder, boiled at 96˚C for three minutes, placed on ice for one to two minutes to cool and then centrifuged at maximum speed for five minutes.

Protein extracts were separated by SDS-polyacrylamide gel electrophoresis at 120 V (456-1094, Bio-Rad) and transferred onto PVDF membranes (IPFL00010, Millipore) at 350 mA for one hour at 4˚C. Washes were carried out in TBST (20 mM Tris-HCl, 150 mM sodium chloride, 0.1% v/v Tween-20) and blocking and incubations were carried out with 5% w/v BSA (A7906, Sigma) or skimmed milk (232100, BD) in TBST. Proteins of interest were probed with 1:2500 horseradish peroxidase (HRP)-conjugated anti-FLAG (A8592, Sigma), 1:3000 anti-actin (A0480, Sigma) or 1:1000 anti-puromycin (MABE343, Sigma). Non-conjugated primary antibodies were detected with 1:10,000 fluorophore-conjugated secondary antibodies (AS12-2426, AS12-2302, AS12-2454 and AS12-2327, Agrisera) or 1:10,000 HRP-conjugated secondary antibodies (A9044 and A0545, Sigma) using Pierce ECL Western Blotting Substrate (32106, Thermo Scientific). Chemiluminescence and 600 nm, 700 nm or 800 nm emission detection were carried

out over a period of five to ten minutes and 30 seconds, respectively, using a dual-mode camera (Odyssey Fc, Li-Cor) coupled with an imaging suite (Image Studio v.2.1.10, Li-Cor). The intensity of protein bands was measured using ImageJ (NIH).

2.9 Cloning and generation of transgenic lines

pCYHr1 and *pCYHr2* vectors, containing the genes *RPL36aAΔP56Q* and *RPL27aCΔH39R*, respectively, were constructed using Ligation-Independent Cloning (LIC), as described by Li & Evans (1997). The binary vector *PW1211* (Philip A. Wigge, unpublished) was digested with Eco53kI and HindIII. The entire genes of *RPL36aA* and *RPL27aC* were amplified from Col-0 genomic DNA with primers 12878+12879 (1.9 kb) and 12896+12897 (1.6 kb), respectively. *RPL36aA* was re-amplified with primers 12880+12881 (1.1 kb), 12882+12883 (0.4 kb) and 12884+12885 (0.5 kb), and *RPL27aC* with primers 12898+12899 (0.4 kb), 12900+12901 (0.1 kb), 12902+12903 (0.3 kb) and 12904+12905 (0.9 kb). Purified amplicons were mixed at 150 nM and joined by overlap PCR in two steps, firstly without primers for 15 cycles in a 30 µl volume (98˚C 10 seconds, 50˚C 20 seconds, 72˚C 60 seconds) and secondly with primers at 0.5 µM, specific for the full-length insert, for 35 cycles in a 50 µl volume (98˚C 10 seconds, 50˚C 20 seconds, 72˚C 60 seconds). Primers used for LIC are given in *Table 2.3*.

***Table 2.3**:* Primers used for cloning

Primer number	Sequence (5’-3’)	Purpose
12878	AAGATGAGGAACAAAGAAAGAGAGTG	Amplification of inserts for *pCYHr1*
12879	TTCTCAAAGCTTTGACATGTAAAGA	
12880	GACCATGATTACGAATTCGAGCTCAAGATGAGGAACAAAGAAAGAGAGTG	
12881	CTGACCACCATAACCAGACTGCTTTCGATCGTATCGACGCTTTCCTTGAGCAGC	
12882	CAGTCTGGTTATGGTGGTCAGACTAAGCAAGTCTTCCACAAAAAGGTAACATTG	
12883	CTTACCTTTCTTATCTCCTCCGATCTCGAAATGCTTGCAC	
12884	TCGGAGGAGATAAGAAAGGTAAGGGAACATCTCTGTTTTAAGTTG	
12885	ACGACGGCCAGTGCCAAGCTTTTCTCAAAGCTTTGACATGTAAAGA	
12896	GTCACGTAAGGAAGAATCGTG	Amplification of inserts for *pCYHr2*
12897	GATTTTGGTTTTCTTTTGTGGG	
12898	GACCATGATTACGAATTCGAGCTCGTCACGTAAGGAAGAATCGTG	
12899	ACTAACATGTCCCCTCTTCTTCCTGTTCTTCTTGAATCTG	
12900	GAAGAAGAGGGGACATGTTAGTGCCGGACATGGGCGTATC	
12901	CTGTGGTGACGCATACCTCCAGCGTTACCA	
12902	GAGGTATGCGTCACCACAGGATCCTCTTCG	
12903	CCTCTTTGATCTTCTTTTCAGCAGTCTTCGAAATAAGCTTC	
12904	GCTGAAAAGAAGATCAAAGAGGCTGGTGGTGCTGTTGTGC	
12905	ACGACGGCCAGTGCCAAGCTTGATTTTGGTTTTCTTTTGTGGG	
6483	AGGCTTTACACTTTATGCTTCC	Genotyping and sequencing of *pCYHr1* and *pCYHr2*
12468	GCCTCTTCGCTATTACGCCA	
12872	GGAACGACGACGGCACAAA	
12873	TCGCAGAAAATAAGATGAAACCAAC	
12893	GTAAGGAAGAATCGTGTCAACCAAA	
12894	ATCTATGTGACTATGTTGACTCCGTTC	
12895	AACGATTGGGCAGAAGAACTTG	

The linearised vector and insert were isolated by gel electrophoresis, purified using the QIAquick Gel Extraction Kit (28706, Qiagen), concentrated by ethanol precipitation and then mixed in an equimolar ratio (100-200ng vector backbone) in a 10µl volume. The DNA was digested for 15-30 seconds with exoIII (NEB) at 14˚C, to create 5' sticky ends, and the digestion was halted by adding 50µl TE buffer and placing the tube on ice. The DNA was isolated by phenol-chloroform extraction, concentrated by ethanol precipitation and resuspended in 10µl deionised water. DNA was annealed at 60˚C for 3 minutes and then transformed into chemically-competent *Escherichia coli* DH5α cells using 42˚C heat shock for 30 seconds.

Bacteria were grown on LB (Luria-Bertani) medium (1% w/v sodium chloride, 1% w/v tryptone, 0.5% w/v yeast extract) supplemented with 1.5% w/v Bacto agar (214010, BD) in 10 cm round Petri plates. Positive clones were selected on spectinomycin and genotyped with primers 12872+12873 and 12893+12894, respectively. Overnight bacterial cultures were grown in 5 ml LB media in 50 ml Falcon tubes, with shaking at 225 rpm (Innova 44; Eppendorf). Plasmids were purified from overnight cultures using the QIAprep Spin Miniprep Kit (27106, Qiagen) and verified by Sanger sequencing with primers 6483 and 12468 and either 12872 and 12873 (*pCYHr1*) or 12894 and 12895 (*pCYHr2*). Plasmids were transformed into electrocompetent *Agrobacterium tumefaciens* GV3101 cells by electroporation. *A. thaliana* plants were transformed using the floral dip method (Clough & Bent, 1998).

2.10 Genotyping of T-DNA insertion mutants

Leaf discs 3×3 mm in size were harvested from three-week-old plants in collection microtubes (19560, Qiagen) and lysed with tungsten carbide beads (69997, Qiagen) in 300 µl extraction buffer (200 mM Tric-HCl pH 7.5, 25 mM EDTA pH 8, 250 mM NaCl, 0.5% w/v SDS), with two 60-second cycles at 26 sec^{-1} using the Tissue Lyser II (Qiagen) and a ten-minute incubation at -20˚C between cycles. Microtubes were centrifuged at maximum speed (5810R, Eppendorf) for 15 minutes at 4˚C. 200 µl supernatant was recovered and mixed with 200 µl isopropanol and centrifuged at maximum speed for 30 minutes at 4˚C. The tubes were inverted to decant the supernatant and briefly dried on paper towel. 300 µl cold 70% v/v ethanol was added to the DNA pellet and the tubes were centrifuged at maximum speed for 20 minutes at 4˚C. The supernatant was discarded as before and the tubes air-dried. The DNA pellet was resuspended in 50 µl deionised water.

Genotyping by PCR was performed in a 20 µl reaction volume with 3 µl DNA and three primers at 0.25 µM, specific to the T-DNA insertion (left T-DNA border primer, LB) and to the genomic regions on either side of the insertion (left genomic primer, LP; right genomic primer, RP). Thirty-five cycles of PCR with one minute at 95˚C, 1:30 minutes at 55˚C, two minutes at 72˚C were used, and amplicons were separated by gel electrophoresis on 1.3% w/v agarose. Genotyping primers are given in *Table 2.4*.

The *camta1*, *camta2*, *camta3*, *camta23* and *camta123* mutants comprise the following T-DNA insertions: SALK_008187, SALK_007027 and SALK_001152 (Kim *et al.*, 2013). The *camta5*, *camta35*, *camta12346*, *camta12456* and *camta123456* mutants include the following T-DNA insertions: SALK_108806, SALK_139868, SALK_001152, SALK_087870, SALK_134491 and SALK_078900 (Kidokoro *et al.*, 2017). The *prr579* mutant (*prr5-11 prr7-11 prr9-10*) consists of T-DNA insertions SALK_064538, SALK_030430 and SALK_007551 (Nakamichi *et al.*, 2005), and the *ice1-2/+ ice2-2* mutant consists of T-DNA insertions SALK_003155 and GABI_175D04 (Jaehoon Jung, University of Cambridge, unpublished). All other mutants were obtained from the Nottingham *Arabidopsis* Stock Centre (NASC) or *Arabidopsis* Biological Resource Center (ABRC).

Table 2.4*:* Primers used for genotyping T-DNA insertion mutants

Primer number	Primer type	Sequence (5’-3’)	Locus	Mutant
9723	LB	ATTTTGCCGATTTCGGAAC	SALK T-DNA	-
1629	LB	TAGCATCTGAATTTCATAACCAATCTCGATACAC	SAIL T-DNA	-
10824	LB	CCCATTTGGACGTGAATGTAGACAC	GABI T-DNA	-
11189 11190	LP RP	ATTCTTTGCTCTGCCTCTTCC TTTGTAGGGCCTTTGTTGTTG	*ICE1/* *AT3G26744*	*SALK_003155* *(ice1-2)*
6244 6245	LP RP	GCCAACTCTGTTTCAGAAACG ACAATGGCATTTTGCTCAAAG	*ICE2/* *AT1G12860*	*GABI_175D04* *(ice2-2)*
11775 11776	LP RP	CTACCCCCAAGTACTCCAAGG TTCGAATGTGATACACGCAAC	*eEF1A/* *AT1G07920*	*SAIL_261_F03*
12545 12546	LP RP	CATTCTTCAAGAATTTGGGCTC CACATCAACATTGTGGTCATTG	*eEF1A/* *AT1G07930*	*GABI_513G04*
11779 11780	LP RP	TCTGGATTTGAGGGTGACAAC AACTTCAGAGGCTGATCGTTG	*eEF1A/* *AT1G07940*	*SALK_049659*
11465 11466	LP RP	GGTGGGTACTCGGAGAAAGTC GATTACTGGTACCTCCCAGGC	*eEF1A/* *AT5G60390*	*SALK_063369*
12922 12924	sequencing	CATTTGCCATCTCCCCACACT CAGACGGTTATGCTTGTTAGGAGAC	*LOS1/* *AT1G56070*	*los1-1*
11771 11772	LP RP	TTGCTTCTCAGCACCTCTCTC ATCACCCTCTTGATCGTTTCC	*eEF2/* *AT2G45030*	*SAIL_1240_C06*
11455 11456	LP RP	TTTTTGGTCGTCCATTTTCTG GGATCGTAAACACAACATCCG	*eEF2/* *AT3G12915*	*SALK_001421*
11767 11768	LP RP	AGACGTGTTGCATATTCAGCC ACTGTTTGTGAGTCATGCGTG	*eEF2/* *AT5G25230*	*SAIL_327_E09*
11463 11464	LP RP	AACATTCTTTCTCATCGCCG CGTCGGGTCAGTCTTATGAAG	*eEF2/* *AT5G39900*	*SALK_108014*
12541 12542	LP RP	AGTACGGTTGACATGAGGAACC GCTGGACTCTAAAATTAGGCCC	*eEF5/* *AT1G13950*	*SALK_019584*
11761 11762	LP RP	AGGCCCACTCCATAGCATAAC AAAGGTGGTCACATCGTCATC	*eEF5/* *AT1G69410*	*SALK_059807*

2.11 Measurements of cytosolic free calcium

A single *A. thaliana* Col-0 *pCaMV35S::APOAEQUORIN* seedling grown on ½×MS agar or three seedlings grown in ½MMG medium (section 2.1), both for eight to twelve days, were transferred with a toothpick to 500 µl freshly-prepared 2 µM coelenterazine solution (303-500, Nanolight Technology; 3.2 nmol dissolved in 8 µl methanol and diluted in 1.6 ml deionised water) in luminometer cuvettes (diameter 12 mm, height 51 mm; Sarstedt). The cuvettes were incubated in the dark at 20˚C overnight to reconstitute aequorin.

One hour after subjective dawn until ZT8, bioluminescence was measured using a photon-counting luminometer (9899A photomultiplier tube) cooled to -20°C with a FACT50 housing (Electron Tubes), as follows. The coelenterazine was removed from the cuvette and replaced with 500 µl chemicals dissolved in deionised water at 20˚C. The cuvette was immediately placed in the luminometer turret and photon counts were measured every second. To estimate total aequorin in the samples, 1 ml of discharge solution (1 M $CaCl_2$, 10% v/v ethanol, final concentrations) was injected into the cuvette using a 1 ml syringe attached to a 75 mm needle inserted into a light-tight port in the luminometer turret. Measurements were continued until photon counts/sec had reached <10% of the peak after injection of the discharge solution. Cytosolic free calcium levels were calculated according to Fricker *et al.* (1999) using the following formula:

$[Ca^{2+}]_{cyt}$ (nM) = 10 ^ - (0.332588 (- log k) + 5.5593) * 10^9

where k = photon count / total photon count over course of experiment.

For heat shock experiments, luminescence was measured over 15 seconds before and immediately after a five-minute incubation of the cuvettes in a water bath (NE1-2.5, Nickel-Electro) at 42˚C, following one-hour treatments with 500 µl chemical solutions at 20˚C in the dark. Aequorin was discharged and average calcium levels over the 15-second measuring periods were quantified, as above.

Chapter 3

Chemical genomics of the heat shock response

3.1 Introduction

Prolonged heat stress is deleterious to plants, as it interferes with key processes required for vegetative and reproductive growth (Hew *et al.*, 1969; Crafts-Brandner & Salvucci, 2000) and ultimately results in reduced seed yield and viability (Young *et al.*, 2004; Boote *et al.*, 2005). As the mean surface temperature of the earth is rising constantly, with predicted increases of up to 5.8°C in the next century (Cubasch *et al.*, 2001), current agricultural losses to high seasonal temperatures are likely to be exacerbated in the future. It is thus of importance to understand plant responses to warm temperatures, in order to develop solutions to sustain efficient food production.

Chemical genetics has been key in elucidating the mechanisms by which heat shock proteins (HSPs) regulate the activity of heat shock factor (HSF) transcription factors in mammalian cells. HSP90 inhibitors, such as geldanamycin and herbimycin A, were identified as activators of the heat shock response (HSR) (Murakami *et al.*, 1991). These molecules specifically bind to the N-terminal ATPase domain of HSP90, thereby repressing chaperone activity and reducing their inhibitory interaction with HSF proteins (Murakami *et al.*, 1991). Regulation of the HSR is of importance in human health, as inadequate HSF activation can lead to neurodegenerative diseases while HSF over-activation is associated with cancer development (West *et al.*, 2012). For this reason, a large number of HSR-modulating compounds have been isolated by forward chemical genomics screens in mammalian cells, and many of these chemicals, including geldanamycin, have potent anti-tumour activity (Westerheide & Morimoto, 2005). In plants, geldanamycin has been shown to enhance survival during severe heat shock (Yamada *et al.*, 2007).

In addition to HSP90 inhibitors, molecules triggering protein misfolding or aggregation are known to induce the HSR. These include the non-protein amino acids azetidine-2-carboxylate and canavanine, which may be incorporated into proteins in the place of proline and arginine residues, respectively, and result in disruptions to protein structure and function (Li & Laszlo, 1985). Likewise, the translation inhibitor puromycin is incorporated by ribosomes into nascent peptide chains, whereby it triggers premature translation termination and results in the accumulation of truncated proteins

(Lee & Dewey, 1987). Proteasome inhibitors, such as lactacystin and N-benzyloxycarbonyl-L-leucyl-L-leucyl-L-leucinal (MG132), and some proteinase inhibitors, including 3,4-dichloroisocoumarin (DCIC) and N-α-tosyl-L-phenylalanine chloromethyl ketone (TPCK), induce the HSR by preventing the degradation of unfolded proteins (Rossi *et al.*, 1998; Holmberg *et al.*, 2000).

The HSR may also be triggered by oxidants, organic electrophiles and heavy metals, which react with biological molecules, in particular with thiol-containing peptides, and can cause protein cross-linking or misfolding (West *et al.*, 2012). Potent electrophilic inducers of the HSR include certain animal fatty acids, such as arachidonic acid (Jurivich *et al.*, 1994) and cyclopentenone prostaglandins (Ohno *et al.*, 1988), and the naturally-occurring plant metabolites celastrol, gedunin and withaferin A (Santagata *et al.*, 2012). Metal ions can also trigger HSF activation *via* the production of reactive oxygen species (ROS), either directly, in the case of iron or copper, or indirectly by disrupting cellular enzymes (Shahid *et al.*, 2014).

In contrast, reducing agents and ROS scavengers repress the induction of the HSR (Liu *et al.*, 2013a; Volkov *et al.*, 2006). In plants, the plastid electron transport chain inhibitors 2,5-Dibromo-6-isopropyl-3-methyl-1,4-benzoquinone (DBMIB) and 3-(3,4-Dichlorophenyl)-1,1-dimethylurea (DCMU) trigger an increase and decrease in chloroplast hydrogen peroxide levels, respectively, which correlate with induction or repression of the HSR (Mühlenbock *et al.*, 2008; Dickinson *et al.*, 2018). A small number of molecules have been reported to specifically inhibit HSF1 in animal cells, though by unknown mechanisms, including triptolide, quercetin and KNK437 (Nagai *et al.*, 1995; Yokota *et al.*, 2000; Westerheide *et al.*, 2006). A more recent study, however, demonstrated that these chemicals act as more general repressors of gene expression (Santagata *et al.*, 2013).

As well as chemicals affecting protein homeostasis or chaperone activity, a range of small molecules are known to specifically stimulate warm or cold signalling in mammalian cells, by interacting with thermosensory TRP (TRANSIENT RECEPTOR POTENTIAL) channels. For example, capsaicin and menthol, the active ingredients of chilli peppers and mint, activate the heat-receptor TRPV1 and cold-receptor TRPM8, respectively (Takaishi *et al.*, 2016). While capsaicin induces the HSR by acting as an agonist of TRPV1, menthol diminishes the HSR by antagonising TRPV1 (Bromberg *et al.*, 2013; Takaishi *et al.*, 2016). Chemical genetics has been key in characterising these temperature sensors in animals. In plants, although ion channels associated with heat or cold responses have been described (Finka *et al.*, 2012; Gao *et al.*, 2012; Mori *et al.*,

2018), such molecules that specifically activate or repress temperature-associated channel activity have not yet been identified.

Wigge and colleagues have used forward classical genetics to screen for mutants with altered temperature signalling, by measuring the activity of a heat-responsive reporter comprising the promoter of *HSP70/AT3G12580* and the coding sequence of firefly luciferase (*pHSP70::LUC*). Mutants of ARP6 (ACTIN-RELATED PROTEIN 6), a component of the SWR1 chromatin remodelling complex (Kumar & Wigge, 2010), and of SS4 (STARCH SYNTHASE 4), a starch synthase (Dickinson *et al.*, 2018), were isolated in this screen, based on their constitutively high levels of *HSP70* expression. However, no biological temperature sensor has been identified from classical genetics screens in plants, and little is known about the temperature signals that converge on plant HSFA1 transcription factors. Chemical genomics can overcome the possible problems of gene lethality and redundancy, which could account for the limitations of classical genetics in the field of temperature signalling. To address this issue, molecules affecting heat responses in *Arabidopsis thaliana* were screened using the *pHSP70::LUC* reporter. From an agronomical perspective, the motive for this screen was to identify a compound capable of enhancing plant responses to heat stress. The objectives of this project are as follows:

1. To identify small molecules that activate or repress the HSR.
2. To investigate the biological targets of these chemicals.
3. To characterise chemical effects on thermotolerance.

3.2 Results

3.2.1 Identification of small molecules affecting *HSP70* expression

A. thaliana Col-0 *pHSP70::LUC* seedlings emit a level of photons that is proportional to the ambient growth temperature and that is increased upon a shift from 12°C to 27°C (Kumar & Wigge, 2010). As demonstrated in *Figure 3.1*, luminescence rapidly increases 10- or 100-fold respectively during short heat treatments at 30°C or 37°C in these seedlings. This reporter therefore allows warm temperature responses to be quantified *in vivo* with high sensitivity.

In order to identify potential chemical modifiers of the HSR, *pHSP70::LUC* seedlings were screened with 3,073 small molecules from Syngenta (Jealott's Hill, United Kingdom). Compounds included in this library were selected by Syngenta based on their predicted cell permeability and on the diversity of chemical structures

represented (Mikael Courbot, Syngenta, personal communication). Each compound was supplied by Syngenta in DMSO at a concentration of 10,000 ppm, and this unit of concentration is used hereafter for treatments with these chemicals. Seedlings were grown in liquid culture and treated with chemicals at 10 ppm, which on average is equivalent to a concentration of approximately 35 μM for these compounds. Following one hour of chemical treatment, the seedlings were subjected to mild heat shock for an additional hour and luminescence was measured before and after the heat treatment (*Figure 3.2A*), with the aim of identifying both activators and repressors of the HSR. All treatments and measurements were carried out between ZT8 and ZT12, to minimise diurnal effects on the *pHSP70::LUC* reporter, as endogenous *HSP70/AT3G12580* expression varies little during this period though it peaks in the morning and evening (Mockler *et al.*, 2007).

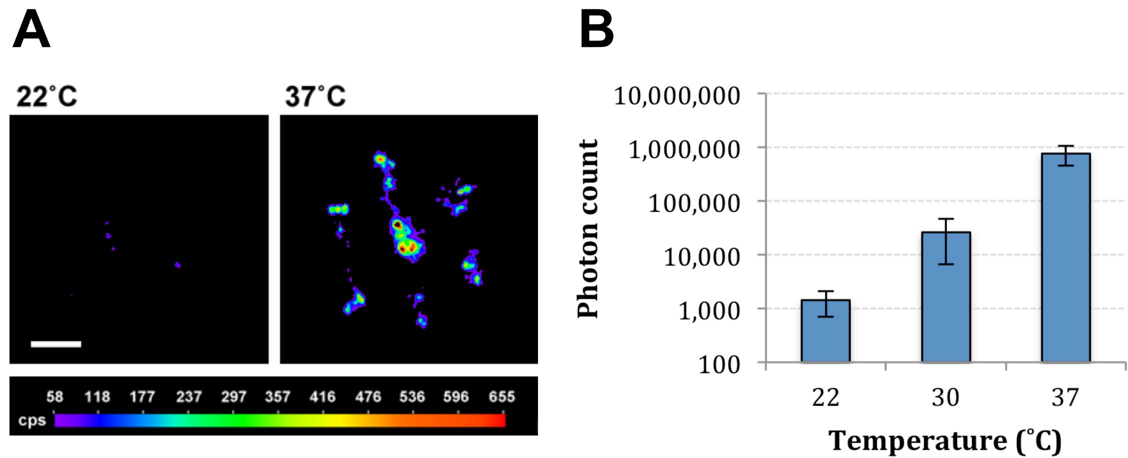


Figure 3.1: The *pHSP70::LUC* reporter.
(**A**) Luminescence images of seven-day-old *A. thaliana* Col-0 *pHSP70::LUC* seedlings grown at 22˚C and heat-treated for one hour at 37˚C. Images were captured for ten seconds after three minutes incubation with 1 μM luciferin. cps: counts per second. The scale-bar represents 10 mm.
(**B**) Photon emissions from ten-day-old *pHSP70::LUC* seedlings grown in liquid culture in 96-well plates at 22˚C and heat-treated for 30 minutes at 30˚C or 15 minutes at 37˚C. Photon counts were measured for one second, after one-minute incubation with 1 μM luciferin. Error bars represent standard deviation for 20 biological replicates, with approximately 10 seedlings per replicate. The y-axis is scaled logarithmically.

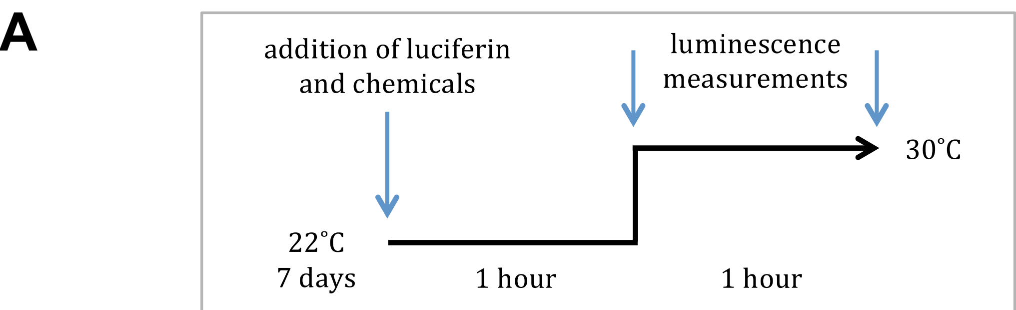
Eighty-seven candidate modifiers of the HSR were identified from the primary screen, based on a two-fold cut-off relative to the mock controls. As shown in *Figure 3.2B* and *Figure 3.2C*, chemicals affecting either heat-inducible luminescence or absolute luminescence were detected. The chemicals are denoted by an eleven-digit Individual

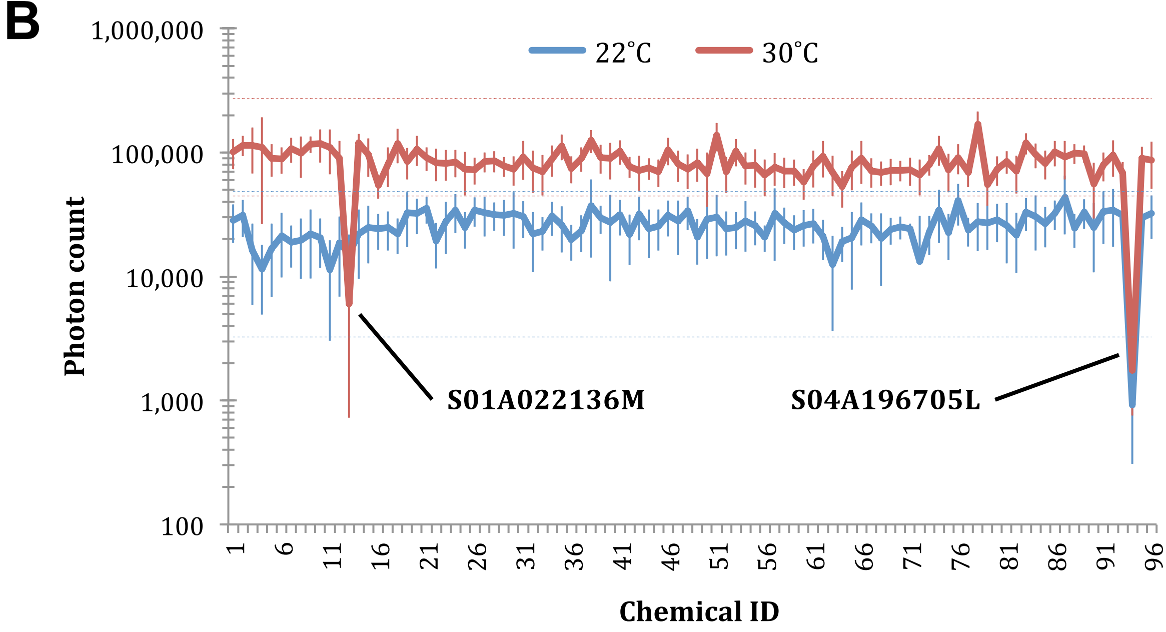
Serial Number (ISN) that refers to the Syngenta production batch for a particular compound.

Luminescence was found to vary depending on the position of wells in the plates, with increased variation from plants growing at the periphery of the plates. To eliminate this 'edge effect', the eighty-seven candidates were analysed in a secondary luminescence screen in which the positions of the chemical treatments were randomised. The results of the secondary screen are given in *Figure 3.3*. Only two chemicals, S03A111138F and S01A463859Y, were found to increase *pHSP70::LUC* luminescence greater than two-fold relative to mock controls in the absence of heat, whereas six molecules, including these two activators, increased heat-induced luminescence over two-fold. In contrast, thirty-nine chemicals caused at least two-fold repression of luminescence during heat shock, thirty of which also reduced photon counts in the absence of heat.

As only two activators of *pHSP70::LUC* were identified after one hour of chemical treatment, a smaller-scale 'activator' screen was carried out at a lower growth temperature with a longer chemical treatment in the absence of heat shock (*Figure 3.4A*). *pHSP70::LUC* seedlings were treated with chemicals for three hours, in order to allow sufficient time for chemically-triggered *de novo* synthesis of the luciferase enzyme. As shown in *Figure 3.4B*, this longer incubation period allowed for the identification of *pHSP70::LUC* activators. Forty-seven such molecules were identified, three of which were also identified in the first 'repressor' screen. These three chemicals, S01A463859Y, S01E974935C (=S03A498457F) and S04A100314B, induced modest increases in *pHSP70::LUC* luminescence of 1.3- to 2.1-fold relative to mock controls after one hour of treatment, whereas they triggered up to 24-fold higher luminescence after three hours.

Fifty-one candidates from the secondary screen were selected for further analyses, based on their effects on *pHSP70::LUC* luminescence differing to mock controls by at least a factor of two in either the presence or absence of heat shock. Together with the forty-seven *pHSP70::LUC* activators, the effects of these chemicals on endogenous *HSP70* expression were assessed by quantitative PCR (*Supplementary Figures 1 and 2, Appendix*). Wild-type seedlings were treated with chemicals for 30 minutes and then either maintained at the same growth temperature or subjected to mild heat shock for an additional 30 minutes. Fourteen compounds were confirmed to induce *HSP70* expression at least two-fold under these conditions and eighteen were confirmed to repress the 25-fold increase in *HSP70* expression observed during heat treatment, twelve of which also reduced expression in the absence of heat shock. No chemicals were found to increase the heat-induction of the *HSP70* gene. To reduce the number of





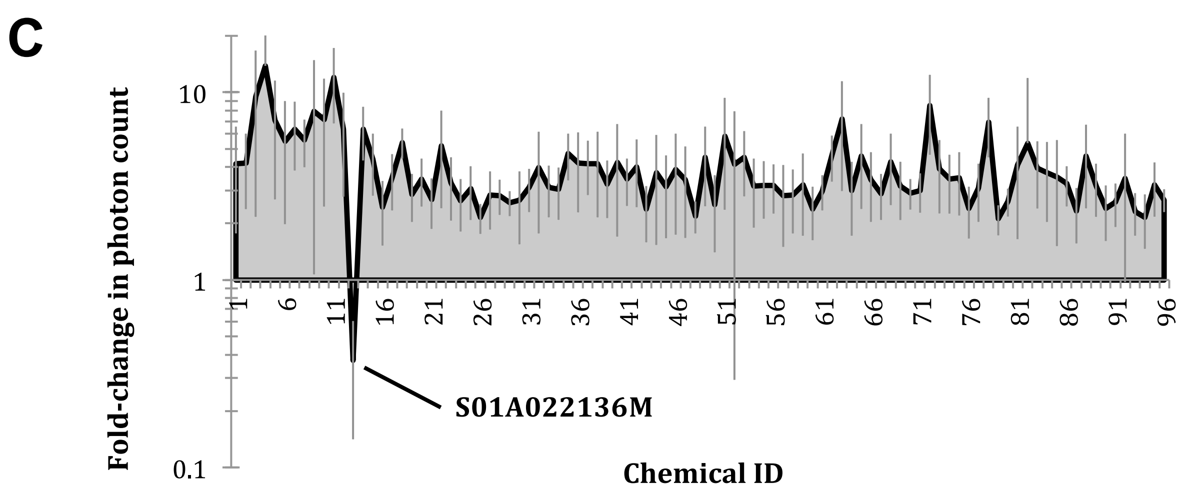


Figure 3.2: Results of the primary *pHSP70::LUC* chemical screen.
(**A**) Screen experimental layout.
(**B**,**C**) Luminescence measurements before and after heat treatment (**B**) and fold-change in luminescence after heat treatment (**C**), for *A. thaliana* Col-0 *pHSP70::LUC* seedlings treated with one plate of chemicals (10 ppm, 0.1% v/v DMSO). Dotted lines represent average maximum and minimum luminescence for mock controls (0.1% v/v DMSO). Chemicals affecting absolute luminescence (e.g. S04A196705L) or heat-induced luminescence (e.g. S01A022136M) were identified. Error bars represent standard deviation for sixteen biological replicates, with approximately 10 seedlings per replicate. Counting time for luminescence measurements: one second. The y-axes are scaled logarithmically.

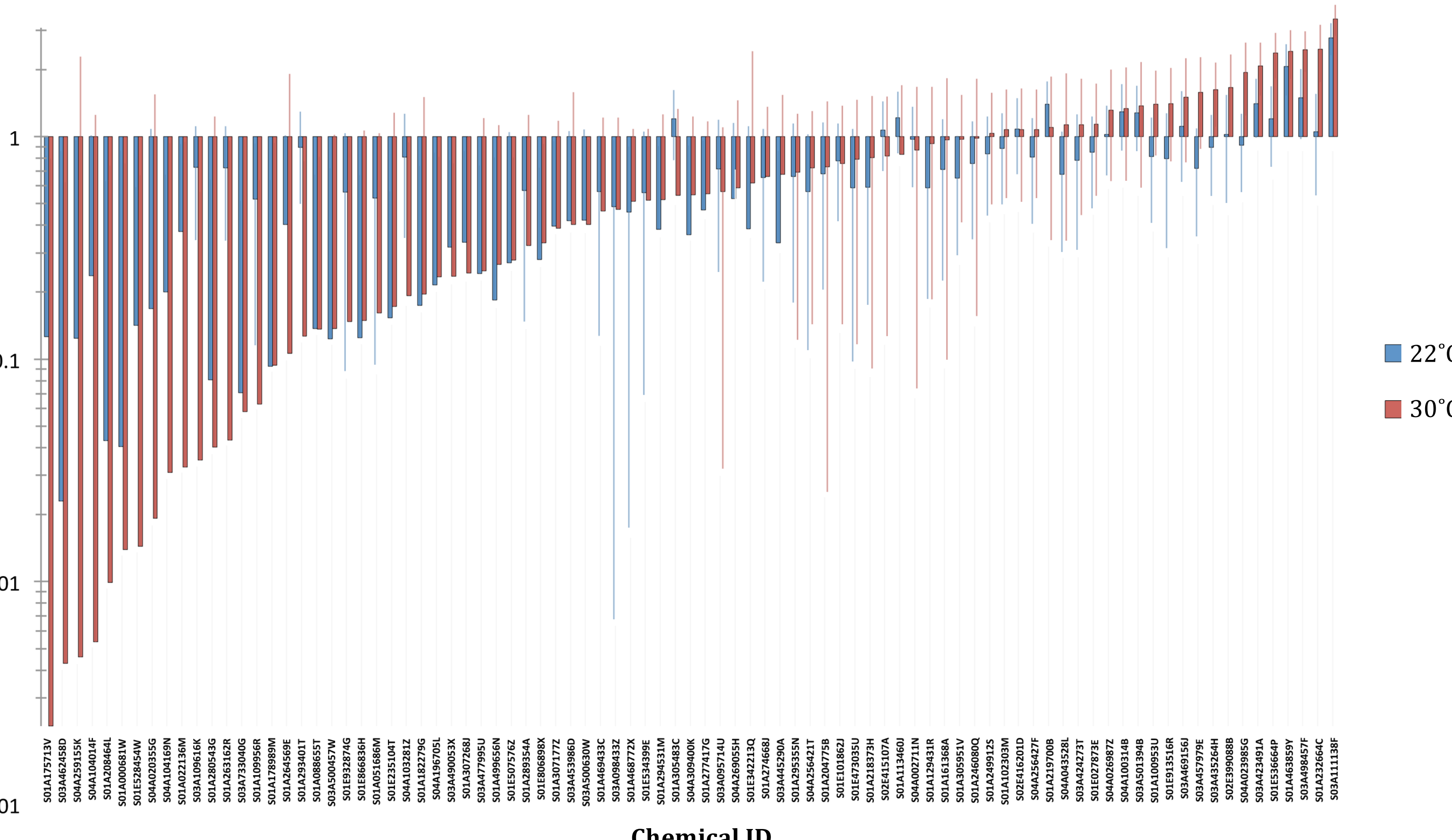
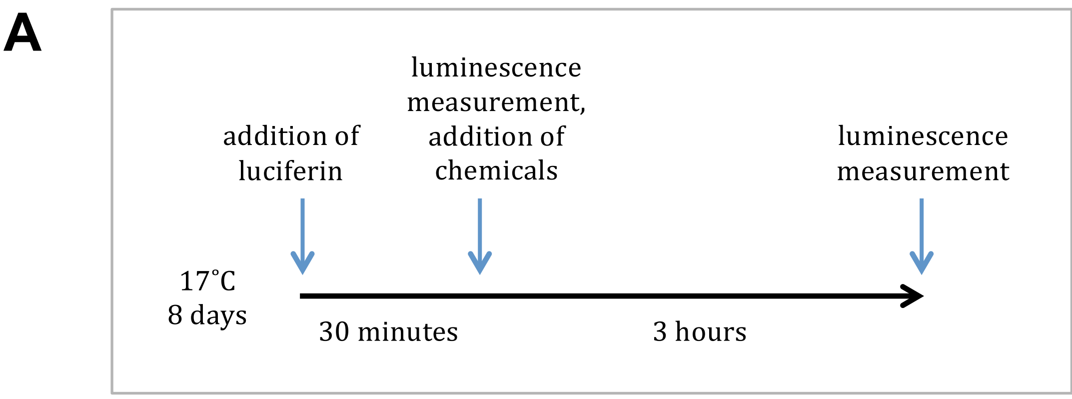


Figure 3.3: Randomised secondary luminescence screen of the candidates identified from the primary screen.
Photon counts before and after 1.5 hours of heat treatment, normalised to mock controls (0.1% v/v DMSO), following 1.5-hour chemical treatments (10 ppm, 0.1% v/v DMSO) in seven-day-old *A. thaliana* Col-0 *pHSP70::LUC* seedlings. Error bars represent standard deviation for twelve biological replicates, with approximately 10 seedlings per replicate. Counting time for luminescence measurements: one second. The y-axis is scaled logarithmically.



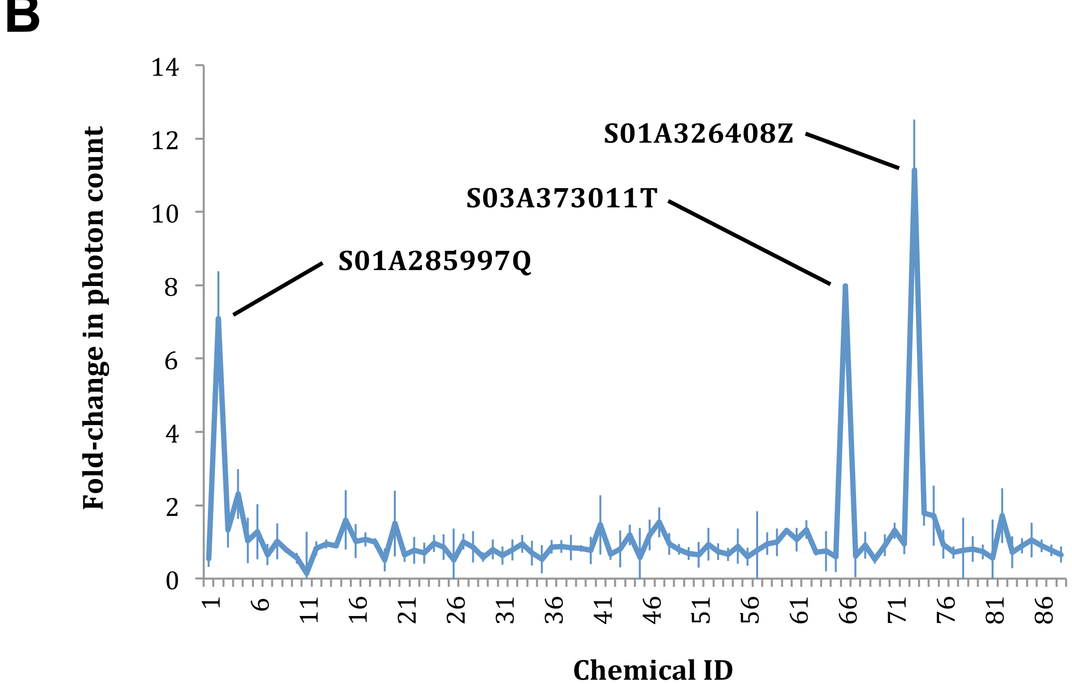


Figure 3.4: Results of the *pHSP70::LUC* activator screen.
(**A**) Screen experimental layout.
(**B**) Fold-change in luminescence of eight-day-old *A. thaliana* Col-0 *pHSP70::LUC* seedlings after three hours of chemical treatment (10 ppm, 0.1% v/v DMSO) at 17˚C, relative to the t=0 time-point and normalised to changes during mock treatments (0.1% v/v DMSO), for one plate of chemicals. Error bars represent standard deviation for two biological replicates, with approximately 10 seedlings per replicate. Counting time for luminescence measurements: one second.

molecules to a manageable number for further analyses, a four-fold cut-off in expression differences relative to mock controls was selected. The effects on *HSP70* expression of the twenty candidates selected, including five *HSP70*-activators and fifteen *HSP70*-repressors, are displayed in *Figure 3.5*.

Interestingly, S03A111138F was found to have a positive effect on the activity of the *pHSP70::LUC* reporter, with 2.8- and 3.4-fold higher luminescence than for the mock controls at 22°C and 30°C, respectively. However, no differences in endogenous *HSP70* expression were detected following chemical treatment, with expression levels of 0.7 and 1.0 at 22°C and 30°C, respectively, relative to mock controls. S03A111138F may therefore enhance the activity of the luciferase enzyme rather activating the *HSP70* promoter.

Chemical effects on the HSR were confirmed by analysing HSP70 protein levels in *A. thaliana* seedlings expressing under its native promoter an epitope-tagged HSP70 homologue, HSP70B/AT1G16030, which is 85% identical to HSP70/AT3G12580 at the protein level. As demonstrated in *Figure 3.6*, the HSP70B protein accumulates in a heat-dependent manner, with approximately 10- and 70-fold increases in abundance after three hours at 30°C or 30 minutes at 37°C, respectively. All five *HSP70*-activators induce the accumulation of HSP70B in the absence of heat, after three hours of chemical treatment (*Figure 3.6*), though to levels observed during moderate (37°C) rather than mild (30°C) heat shock. Similar levels of HSP70B accumulate during treatments with known HSR activators, including DBMIB and the geldanamycin analogue 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG) (*Figure 3.6*).

The structures of the *HSP70*-activators and *HSP70*-repressors are presented in *Figure 3.7* and *Figure 3.8*, respectively. Two activators, S01A052378F and S01A052379R, are highly similar in structure, with the only difference being the substitution of two fluorines in S01A052378F for chlorines in S01A052379R. There do not appear to be any conserved chemical structures among *HSP70*-repressors, although seven of these molecules contain trifluoromethyl moieties, which are lacking in *HSP70*-activators. This chemical group is commonly added to agrochemicals to increase lipophilicity, thus enhancing cell permeability, and to delay their catabolism *in vivo* (John Delaney, Syngenta, personal communication). Based on their structures, S01A052378F, S04A100314B and S04A259155K could potentially act as weak electrophiles, and S01A305483C could potentially be aromatised, thus making it a reductant (Katrin Hermann, Syngenta, personal communication).

Two of the *HSP70*-repressors are chemicals known from the literature. S03A109616K has the same structure as cycloheximide, a eukaryotic protein synthesis

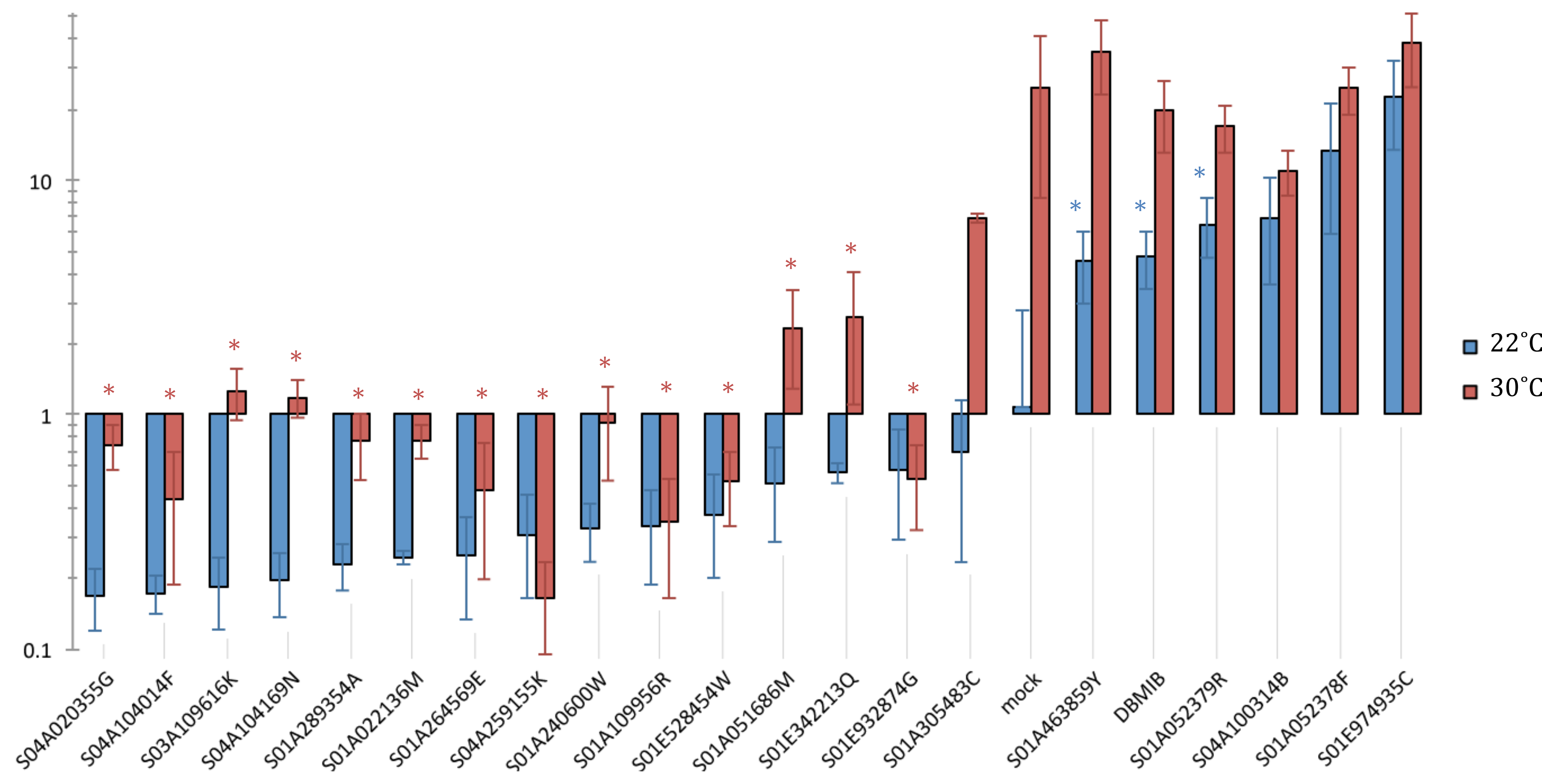
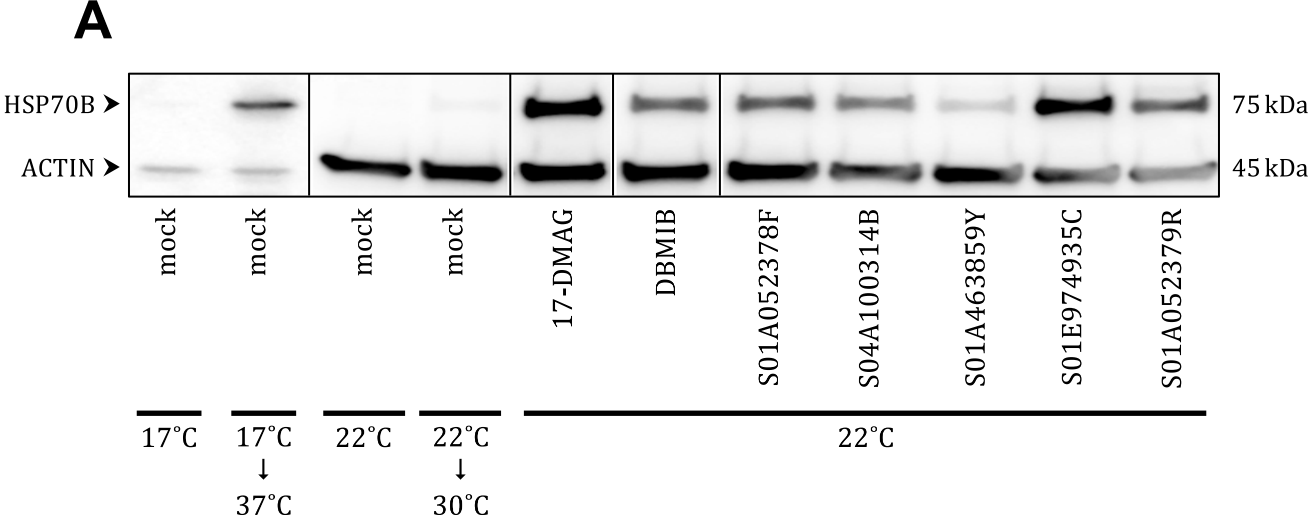


Figure 3.5: Chemical effects on *HSP70* expression.
Expression of *HSP70* in *A. thaliana* Col-0 seedlings treated with chemicals identified from the primary screens (10 ppm, 0.1% v/v DMSO) for one hour at 22˚C with or without a 30-minute heat treatment at 30˚C. Expression was normalised to *PP2A* and *UBC21* levels. Only chemicals with at least four-fold differences relative to the mock controls (0.1% v/v DMSO) at either 22˚C or 30˚C are shown. S01A240600W and S01E974935C are the same chemicals as S01A263162R and S03A498457F, respectively, in *Figure 3.3*. DBMIB is included as a positive control for *HSP70* activation. Error bars represent standard deviation for three biological replicates, with 10-15 seedlings per replicate. Asterisks indicate significant differences relative to mock controls, in blue for 22˚C treatments and in red for 30˚C treatments (two-tailed *t*-test, p-value <0.05).



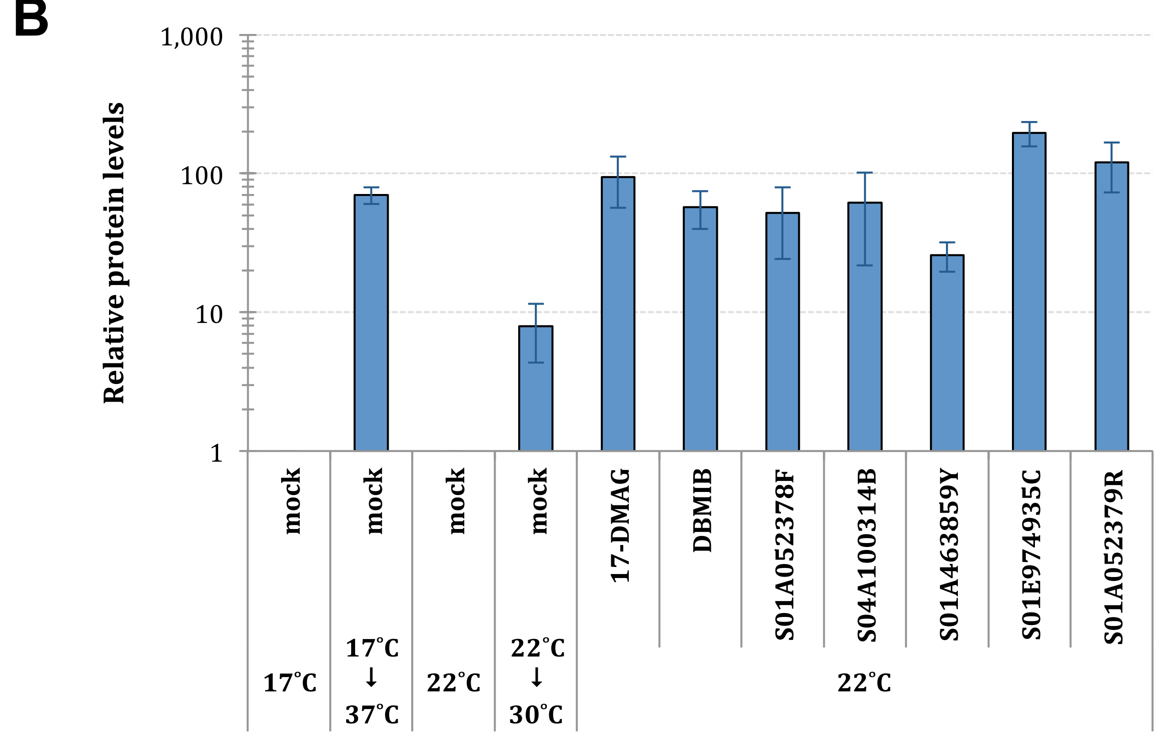


Figure 3.6: Accumulation of HSP70B protein during treatment with *HSP70*-activators.
(**A**) Seven-day-old *A. thaliana* Col-0 seedlings expressing FLAG-tagged HSP70B/AT1G16030 were grown at 17°C in short-days or 22°C in long-days, as indicated, and treated with 10 ppm *HSP70*-activators, including 17-DMAG and DBMIB, or with mock treatments (0.1% v/v DMSO). Mock-treated seedlings were either maintained at the respective growth temperatures or transferred to 37°C for 30 minutes or 30°C for three hours. Western blots were performed independently for seedlings grown at 17°C or at 22°C. Approximate sizes of HSP70B::FLAG×3 and ACTIN bands are given in kilodaltons.
(**B**) Quantification of HSP70B protein levels, normalised to actin and to mock treatments in the absence of heat shock, for the chemical treatments in (A). Error bars represent standard deviation for two biological replicates, with 10-15 seedlings per replicate. The y-axis is scaled logarithmically.

inhibitor that blocks the translocation step of translation and was discovered from the soil bacterium *Streptomyces griseus* (Schneider-Poetsch *et al.*, 2010). S01A289354A has the same structure as Tralopyril, a biocide that is used in ship antifouling paint and that acts as a protonophore, carrying protons across cellular membranes and thereby disrupting the proton gradient necessary for ATP synthesis in chloroplasts and mitochondria (European Chemicals Agency, 2014). As they inhibit the coupling between electron transport and ATP synthase reactions in these organelles, such compounds are known as 'uncouplers'.

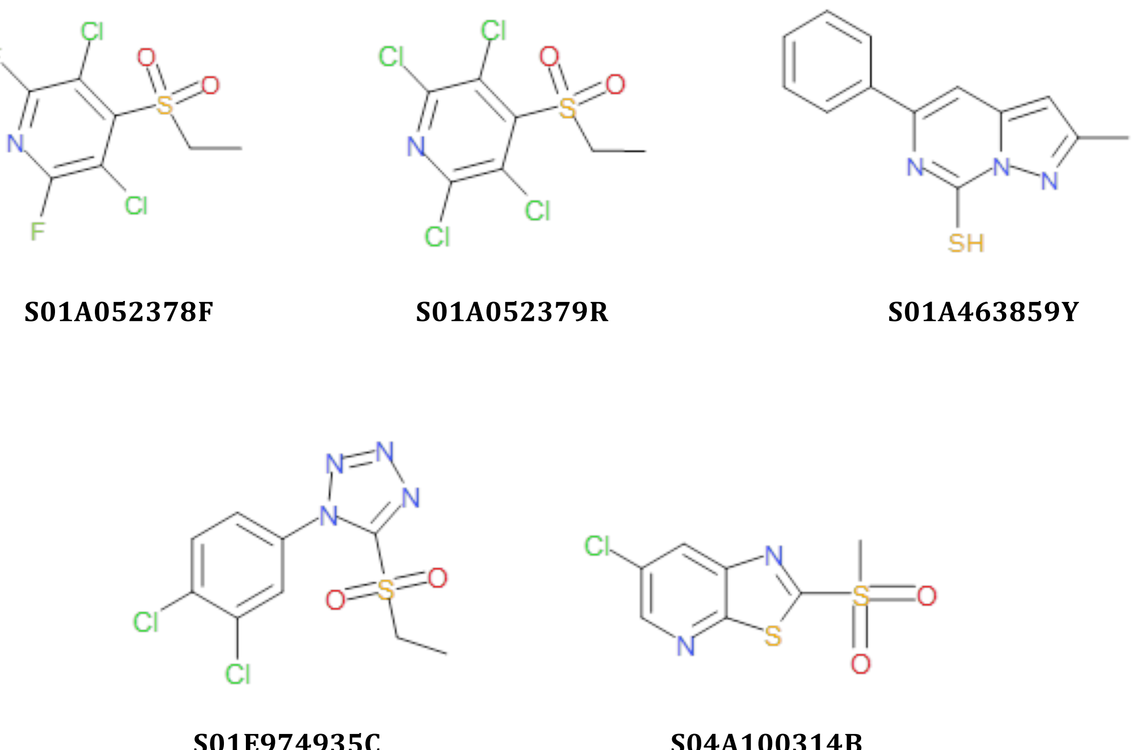


Figure 3.7: Chemical structures of *HSP70*-activators.

3.2.2 Characterisation of chemical effects

The dynamics of *HSP70* induction or repression by the HSR modifiers were studied by quantitative PCR. Wild-type *A. thaliana* seedlings were treated with *HSP70*-activators and harvested every 15 minutes up to one hour, and in the case of *HSP70*-repressors a rapid heat shock was applied following these periods of incubation. Insufficient chemical was available to include S01A305483C in these analyses, and because Syngenta was not able to re-synthesise the compound for this project it was omitted from subsequent experiments. As shown in *Figure 3.9*, *HSP70* induction by

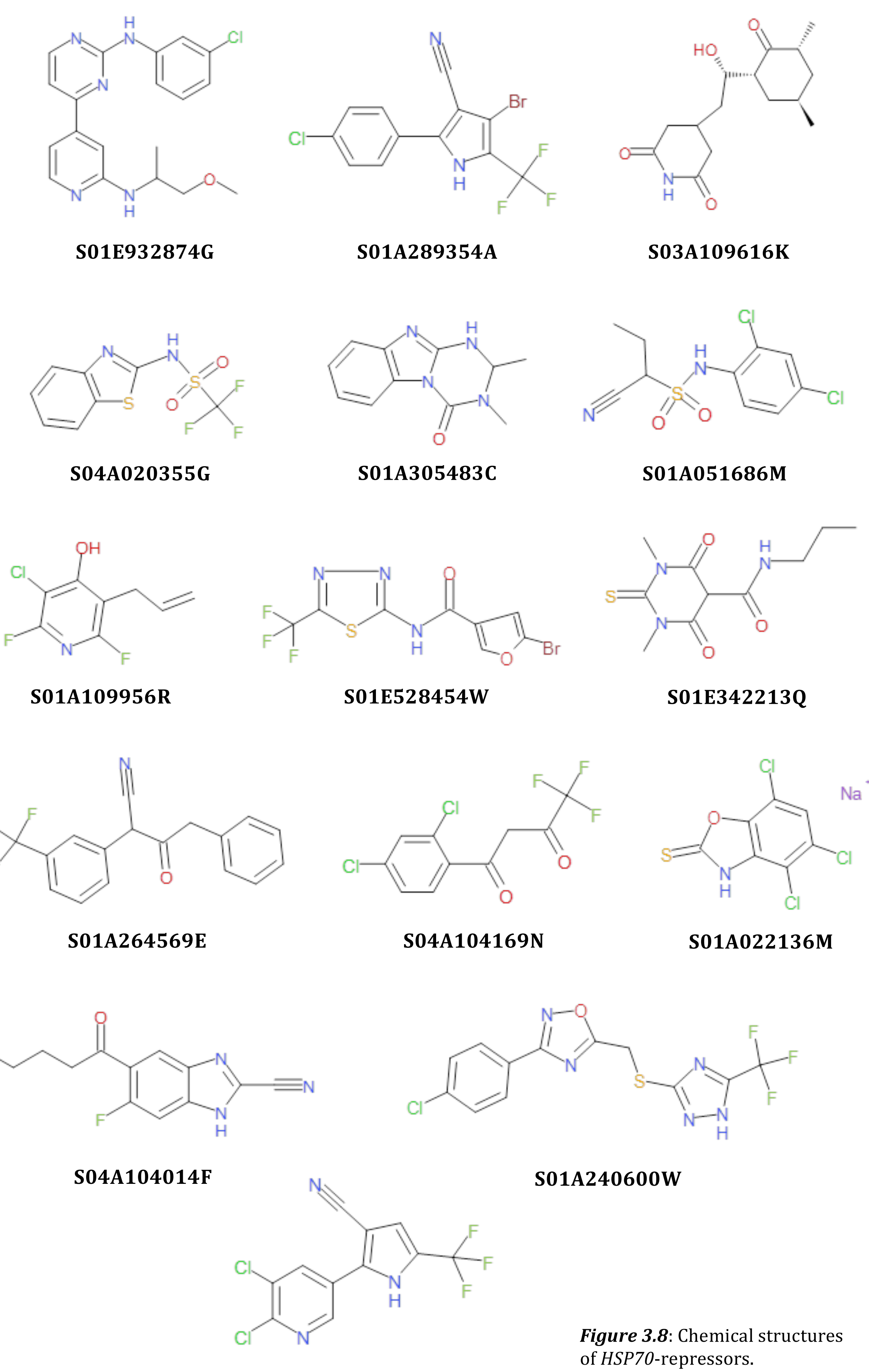


Figure 3.8: Chemical structures of *HSP70*-repressors.

activators is detectable at 30 minutes of chemical treatment, after which expression increases exponentially, except for S01A463859Y, which triggers a sharp rise in *HSP70* expression after 45 minutes of treatment. The dynamics of *HSP70* activation by these chemicals are similar to that by DBMIB (*Figure 3.9*). In contrast, *HSP70*-repressors act extremely rapidly, with heat-induced *HSP70* levels reduced on average by a factor of four relative to the mock controls even after 15 minutes of treatment (*Figure 3.10*). A notable exception is S03A109616K/cycloheximide, for which a large reduction in the heat-inducibility of *HSP70* is observed only after 60 minutes of chemical treatment.

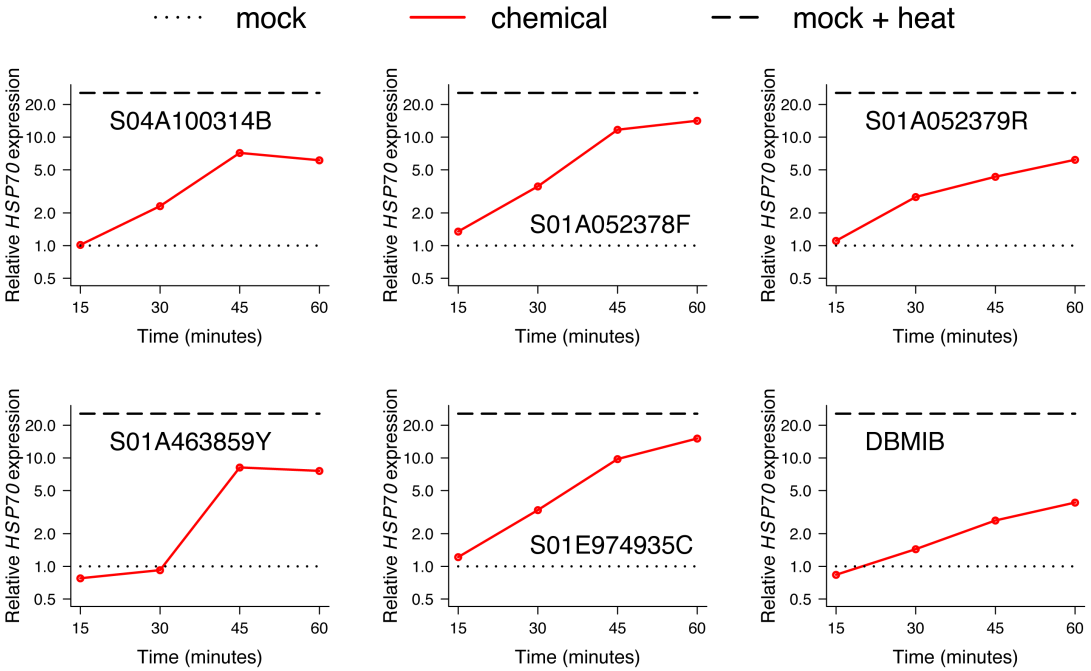


Figure 3.9: Dynamics of *HSP70* induction by *HSP70*-activators.
Seven-day-old *A. thaliana* Col-0 seedlings grown in liquid culture were incubated with chemicals at 10 ppm at 22˚C and harvested after 15, 30, 45 or 60 minutes. *HSP70* expression was normalised to *PP2A* and *UBC21* levels and to expression in the mock controls (0.1% v/v DMSO). Heat shock levels of *HSP70* represent expression in mock-treated seedlings (0.1% v/v DMSO, 60 minutes) subjected to heat shock at 37˚C for ten minutes. Expression values are averages of two biological replicates, with 10-15 seedlings per replicate. The y-axes are scaled logarithmically.

In order to determine the potency of these chemicals, dose-response curves for *HSP70* induction or repression were generated using quantitative PCR. Wild-type *A. thaliana* seedlings were treated for one hour with a ten-fold dilution series of chemicals, and, as above, a brief heat shock was applied following treatments with *HSP70*-repressors. The results are presented in *Figure 3.11* and *Figure 3.12* for *HSP70*-activators and *HSP70*-repressors, respectively. By applying a logarithmic regression line to the

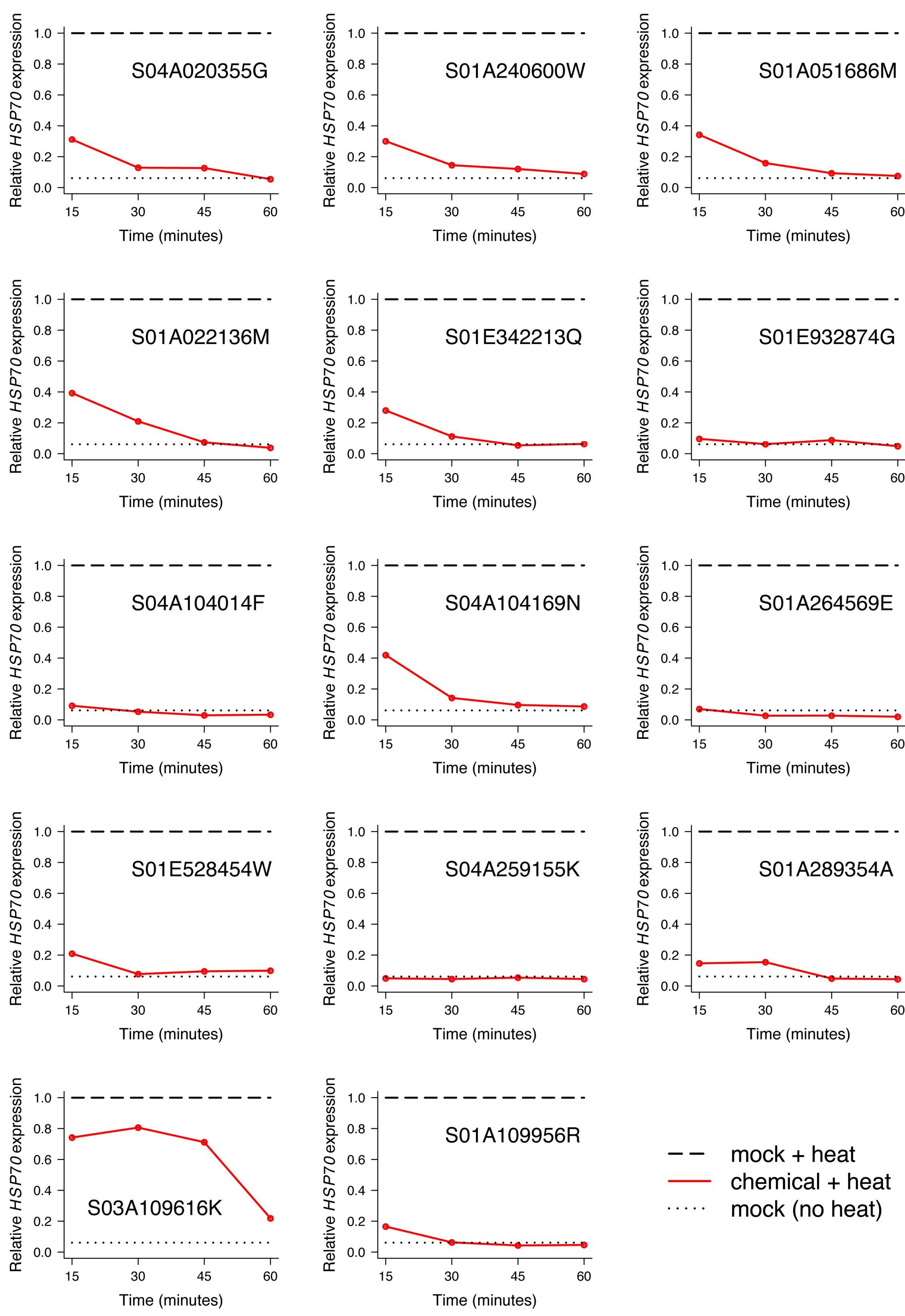


Figure 3.10: Dynamics of *HSP70* repression by *HSP70*-repressors.
Seven-day-old *A. thaliana* Col-0 seedlings grown in liquid culture were incubated with chemicals at 10 ppm at 22˚C for 15, 30, 45 or 60 minutes followed by heat shock at 37˚C for ten minutes. *HSP70* expression was normalised to *PP2A* and *UBC21* levels and to expression in the heat-treated mock controls (0.1% v/v DMSO). *HSP70* levels in the absence of heat shock represent expression in mock-treated seedlings (0.1% v/v DMSO, 60 minutes) at 22˚C. Expression values are averages of two biological replicates, with 10-15 seedlings per replicate.

data points, the half-maximal effective concentration (EC_{50}) of *HSP70*-activators and the half-maximal inhibitory concentration (IC_{50}) of *HSP70*-repressors were estimated (*Table 3.1* and *Table 3.2*, respectively). For both classes of chemicals, effective concentrations vary twenty-fold from most potent to least potent. The IC_{50} value estimated for S03A109616K/cycloheximide in *A. thaliana* is similar to that obtained from translation assays in mammalian cells (Schneider-Poetsch *et al.*, 2010).

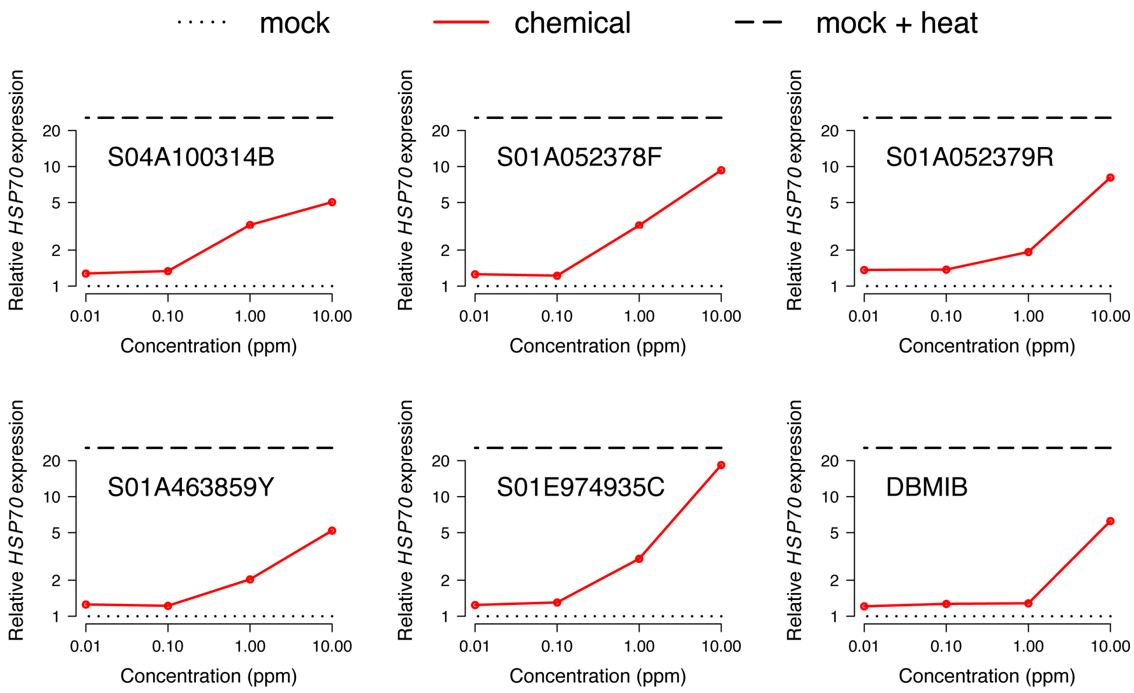


Figure 3.11: Dose-response curves of *HSP70* induction by *HSP70*-activators.
Seven-day-old *A. thaliana* Col-0 seedlings grown in liquid culture were incubated with chemicals at 10, 1, 0.1 or 0.01 ppm at 22°C and harvested after one hour. *HSP70* expression was normalised to *PP2A* and *UBC21* levels and to expression in the mock controls (0.1% v/v DMSO). Heat shock levels of *HSP70* represent expression in mock-treated seedlings (0.1% v/v DMSO, 60 minutes) subjected to heat shock at 37°C for ten minutes. Expression values are averages of two biological replicates, with 10-15 seedlings per replicate. The x- and y-axes are scaled logarithmically.

Table 3.1: Estimated potency of *HSP70*-activators

HSP70-activator	EC_{50} (μM)
S04A100314B	1.41
S01A052378F	7.24
S01A052379R	8.09
S01A463859Y	8.29
S01E974935C	11.39
DBMIB	21.74

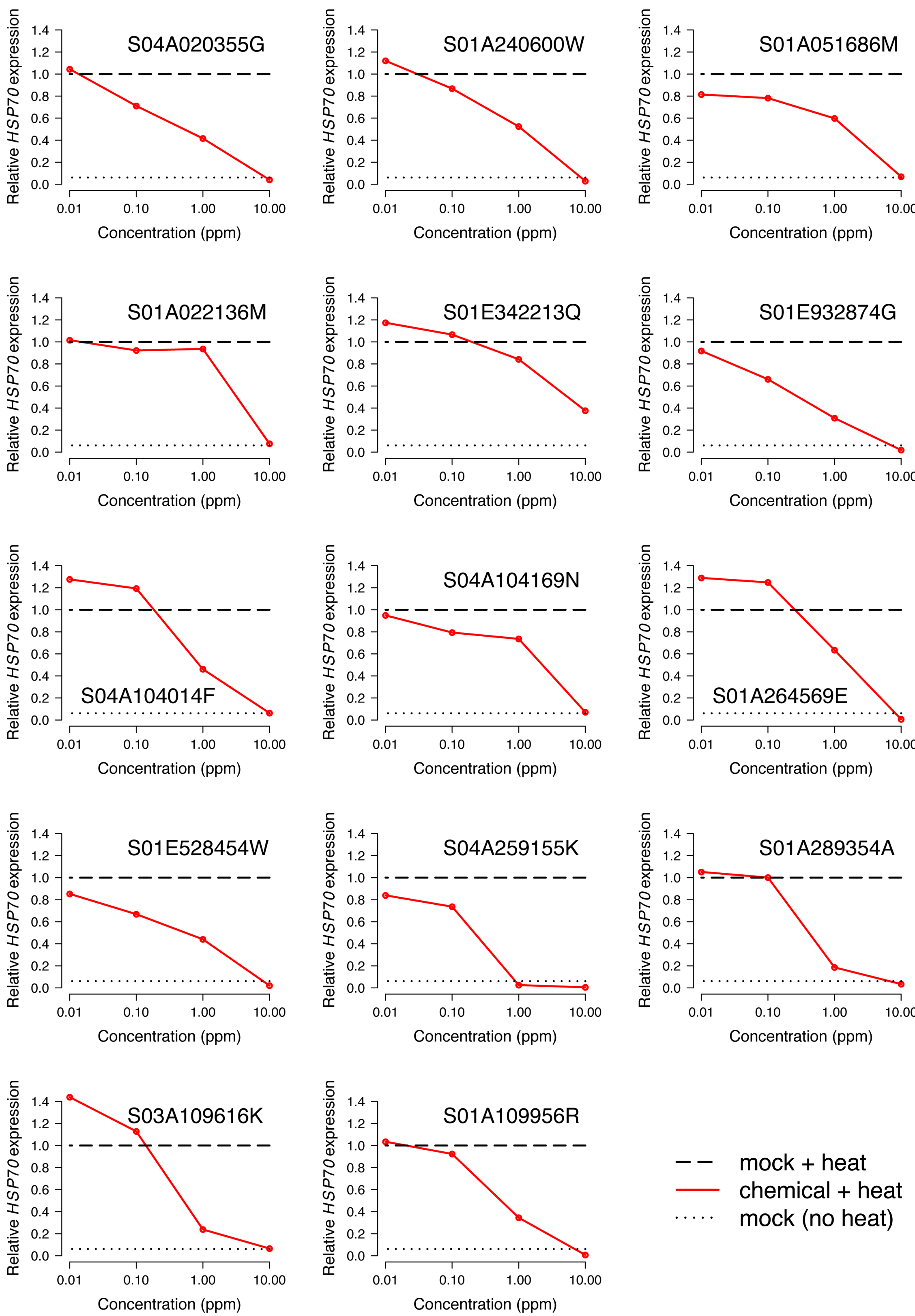


Figure 3.12: Dose-response curves of *HSP70* repression by *HSP70*-repressors.
Seven-day-old *A. thaliana* Col-0 seedlings grown in liquid culture were incubated with chemicals at 10, 1, 0.1 or 0.01 ppm at 22˚C for 60 minutes followed by heat shock at 37˚C for ten minutes. *HSP70* expression was normalised to *PP2A* and *UBC21* levels and to expression in the heat-treated mock controls (0.1% v/v DMSO). *HSP70* levels in the absence of heat shock represent expression in mock-treated seedlings (0.1% v/v DMSO, 60 minutes) at 22˚C. Expression values are averages of two biological replicates, with 10-15 seedlings per replicate. The x-axes are scaled logarithmically.

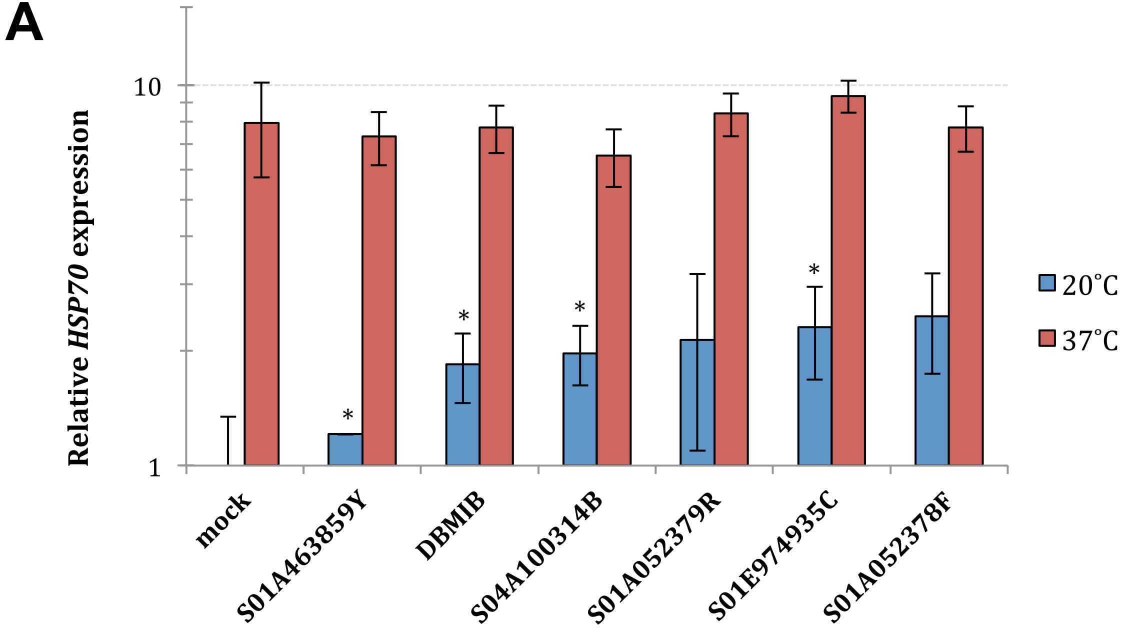
Table 3.2: Estimated potency of *HSP70*-repressors

HSP70-repressor	IC_{50} (μM)
S01A289354A	0.86
S01E932874G	0.98
S04A259155K	0.98
S03A109616K	1.07
S04A020355G	1.39
S01A240600W	1.41
S01A109956R	1.46
S04A104014F	2.31
S01A264569E	4.06
S01E528454W	4.33
S04A104169N	8.85
S01A051686M	9.30
S01A022136M	10.02
S01E342213Q	21.97

Because most of the major staple food crops around the world are monocotyledonous species, it was of interest to characterise chemical effects on *HSP70* expression in the model monocotyledonous plant *Brachypodium distachyon*. As *B. distachyon* seedlings are much larger than *A. thaliana* seedlings and contain a thick cuticle on their leaves, chemical treatments were carried out for twice as long as for *A. thaliana*. Hydroponically-grown *B. distachyon* seedlings were treated by submerging leaves in liquid for up to two hours, with or without a moderate 30-minute heat shock before harvesting tissues. As shown in *Figure 3.13A*, an increase in *HSP70* expression of 1.8- to 2.5-fold relative to the mock controls is observed after two-hour treatments with *HSP70*-activators in the absence of heat, except for S01A463859Y, which brings about a slight but significant increase, 1.2-fold higher than the average *HSP70* level in mock controls (two-tailed *t*-test, p-value 1.72×10^{-4}). As in *A. thaliana*, these chemicals do not affect heat-induced levels of *HSP70*. All *HSP70*-repressors, except for S01A240600W, caused a decrease in average *HSP70* induction during heat shock (*Figure 3.13B*). The effects of both *HSP70*-activators and *HSP70*-repressors were overall less pronounced than in *A. thaliana*, presumably as a result of lower rates of penetration.

Chemicals were also tested on the model unicellular eukaryote *Saccharomyces cerevisiae*, in order to see if effects are conserved across more distant phylogenetic taxa. Because individual cells are expected to take up molecules more rapidly than tissues, yeast cells were treated with chemicals for half of the duration of treatments in *A. thaliana*. For *HSP70*-activators, cells were treated for 30 minutes in the absence of heat,

A



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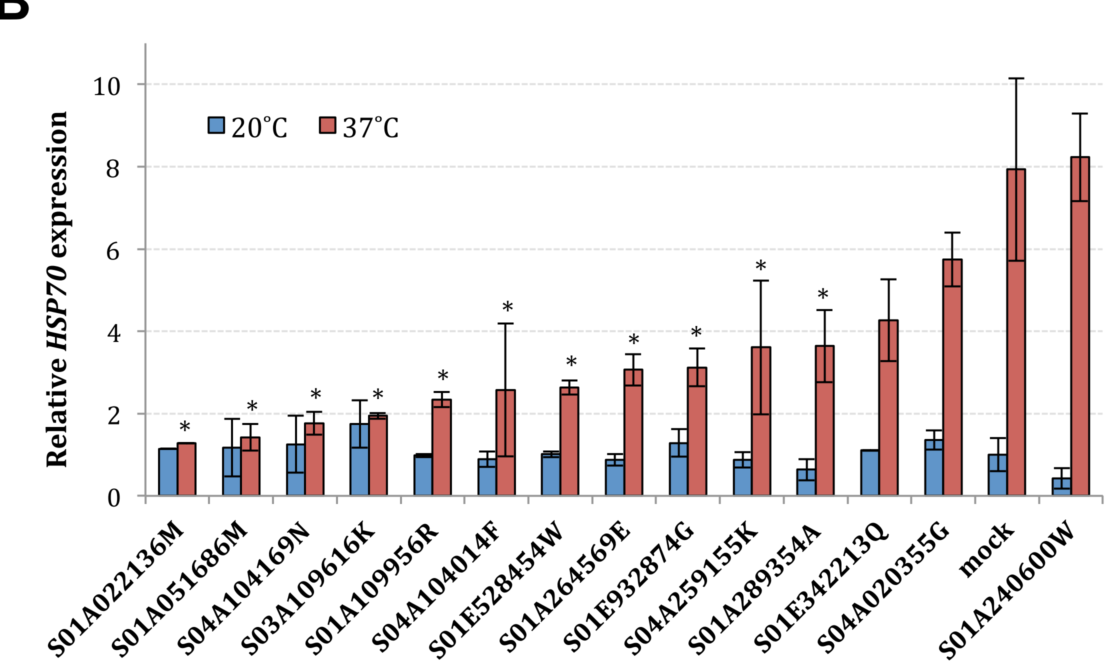


Figure 3.13: Chemical effects on *HSP70* expression in *B. distachyon*.
(**A**,**B**) Bd21-3 seedlings were grown hydroponically at 20˚C for ten days and treated with chemicals at 10 ppm (0.1% v/v DMSO for the mock controls) for 1.5 hours at 20˚C with or without heat shock treatment at 37˚C for 30 minutes, for *HSP70*-activators (**A**) and *HSP70*-repressors (**B**). *HSP70* expression in whole seedlings was normalised to *SamDC* and *UBC18* levels. Error bars represent standard deviation for at least two biological replicates (two whole plants). The y-axis is scaled logarithmically in (A). Asterisks indicate significant differences relative to mock controls (two-tailed *t*-test, p-value <0.05).

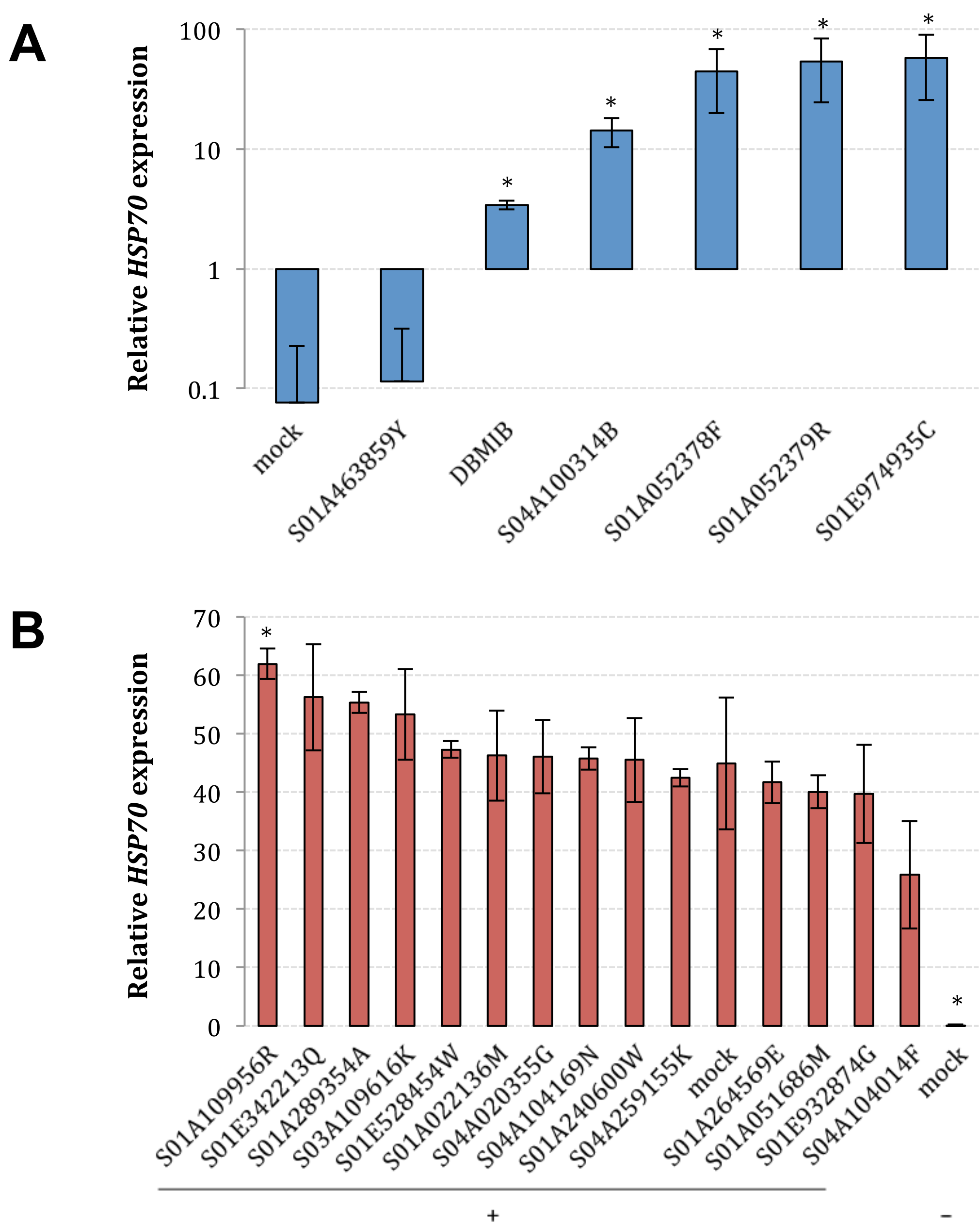


***Figure 3.14**:* Chemical effects on *HSP70* expression in *S. cerevisiae*.
(**A**,**B**) YEF473a cells were grown in liquid culture at 28˚C till mid-log phase and then treated with 10 ppm *HSP70*-activators for 30 minutes at 28˚C (**A**) or with 10 ppm *HSP70*-repressors for 15 minutes at 28˚C followed by heat shock at 37˚C for 15 minutes (**B**). The presence or absence of heat shock is denoted by '+' and '-', respectively. Mock controls are treatments with 0.1% v/v DMSO. *HSP70* (*SSA4*) expression was normalised to *ACT1* and *CDC19* levels. Error bars represent standard deviation for two biological replicates. The y-axis is scaled logarithmically in (A). Asterisks indicate significant differences relative to 28˚C (A) or 37˚C (B) mock controls (two-tailed *t*-test, p-value <0.05).

whereas in the case of *HSP70*-repressors 30-minute treatments included a 15-minute heat shock prior to harvesting. While four of the *HSP70*-activators induced *HSP70* expression over 100-fold, no increase in *HSP70* was detected after treatment with S01A463859Y (*Figure 3.14A*). DBMIB was noticeably less potent than other *HSP70*-activators though it still induced *HSP70* over 40-fold relative to the mock control. In contrast to *HSP70*-activators, only one of the *HSP70*-repressors (S04A104014F) was found to reduce average *HSP70* induction by more than 1.5-fold during heat shock after 15 minutes of chemical treatment, though not significantly (two-tailed *t*-test, p-value 0.322) (*Figure 3.14B*). This is somewhat surprising, because as shown in *Figure 3.10*, most of these chemicals decreased heat-induction of *HSP70* after 15-minute treatments in *A. thaliana* seedlings. With the exception of S03A109616K/cycloheximide, which is known to be active in *S. cerevisiae* but whose inhibitory effects on *HSP70* repression in *A. thaliana* occur more slowly than other *HSP70*-repressors (*Figure 3.10*), the results suggest that these chemicals may be specific to plant cells or may simply be less potent in yeast cells and may require longer treatments for similar expression changes to be observed.

The effects of these compounds on the growth of *A. thaliana* seedlings was determined using growth assays in ten-fold dilution series of chemicals. These assays were carried out at 17˚C and 27˚C, to observe potential temperature-specific effects on growth or lethality. As displayed in *Figure 3.15A*, the two *HSP70*-activators S04A100314B and S01A052379R caused complete lethality at 10 ppm and S01E974935C caused approximately 30% lethality at 10 ppm, though no seedling death was observed at lower concentrations. Interestingly, no chemical lethality was evident in seedlings treated with S01A052378F, an analogue of the S01A052379R, indicating that the fluorine substitutions decrease the toxicity of this compound. However, chlorosis was visible in seedlings treated with either 10 ppm S01A052378F or 1 ppm S01A052379R, suggesting that these chemicals affect chlorophyll levels. No differences in lethality were observed for these chemicals between 17˚C and 27˚C. In comparison, seedling viability is not compromised by treatments with the known HSR-activators DBMIB, puromycin or 17-DMAG at either 17˚C or 27˚C (*Figure 3.15B*). Reduced size of cotyledons is evident from seedlings grown in 10 ppm puromycin at both 17˚C and 27˚C (*Figure 3.15D*). Interestingly, seedlings treated with 10 ppm S01E974935C have shorter hypocotyls than the mock-treated controls (two-tailed *t*-test, p-values 1.68×10^{-3} at 17˚C, 8.16×10^{-7} at 27˚C) and do not undergo warm temperature-associated hypocotyl elongation at 27˚C (two-tailed *t*-test between S01E974935C-treated seedlings at 17˚C and 27˚C, p-value 0.256) (*Figure 3.15C,D*).

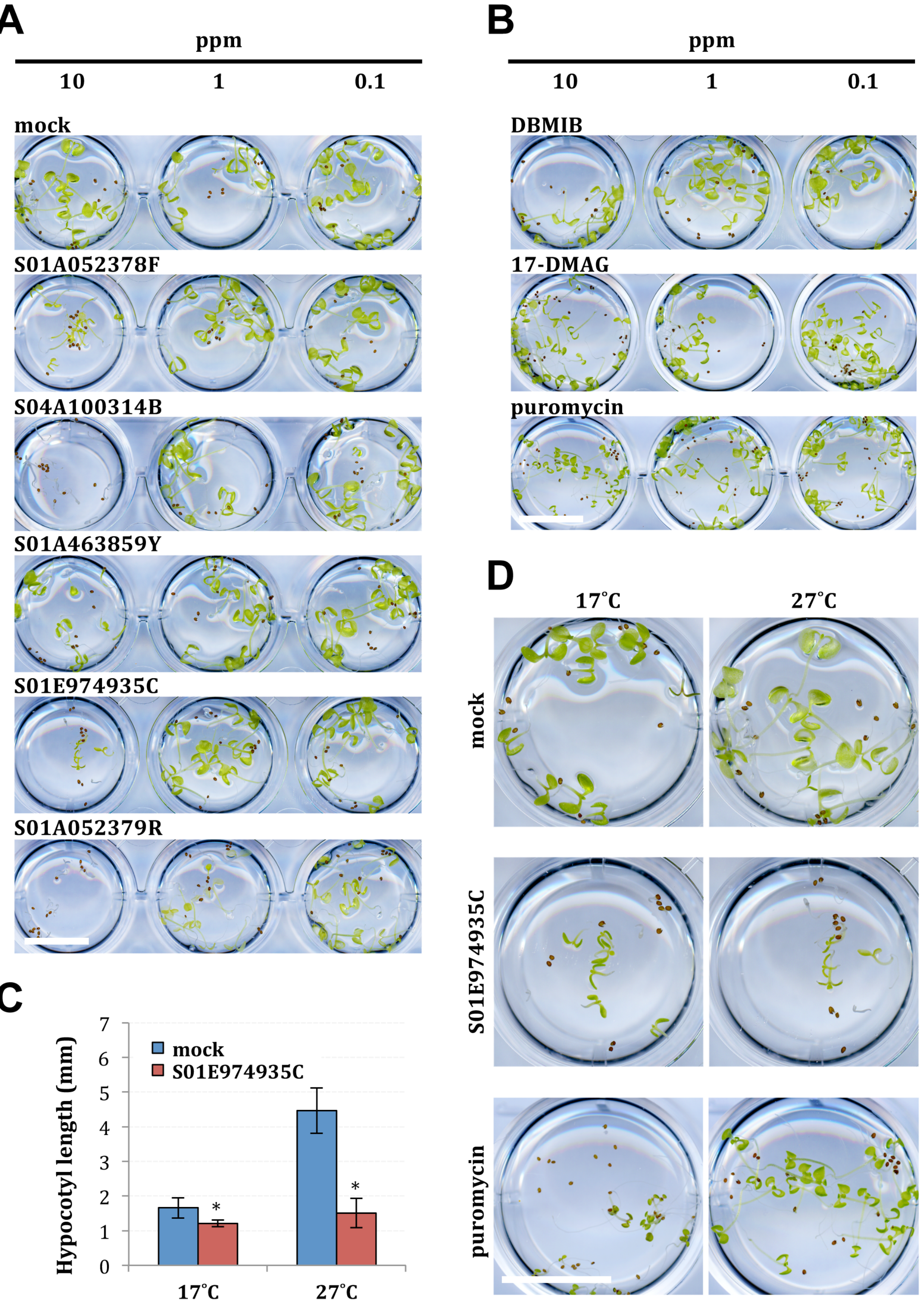


Figure 3.15: Phenotypes of plants grown in the presence of *HSP70*-activators.
(**A**,**B**) *A. thaliana* Col-0 seeds were germinated in liquid culture at 27˚C for two days and then grown for seven days in media containing chemicals at 10, 1 or 0.1 ppm (0.1% v/v, 0.01% v/v or 0.001% v/v DMSO for the mock controls), for *HSP70*-activators identified from the *pHSP70::LUC* screen (**A**) and from the literature (**B**). Chemicals were applied just before the beginning of the night. All scale bars represent 15 mm, and photos are from single biological replicates.
(**C**) Hypocotyl measurements of seedlings treated with 10 ppm S01E974935C or 0.1% v/v DMSO (mock). Error bars represent standard deviation for nine seedlings. Asterisks indicate significant differences relative to mock controls (two-tailed *t*-test, p-value <0.05).
(**D**) Seedling morphology affected by 10 ppm S01E974935C or puromycin.

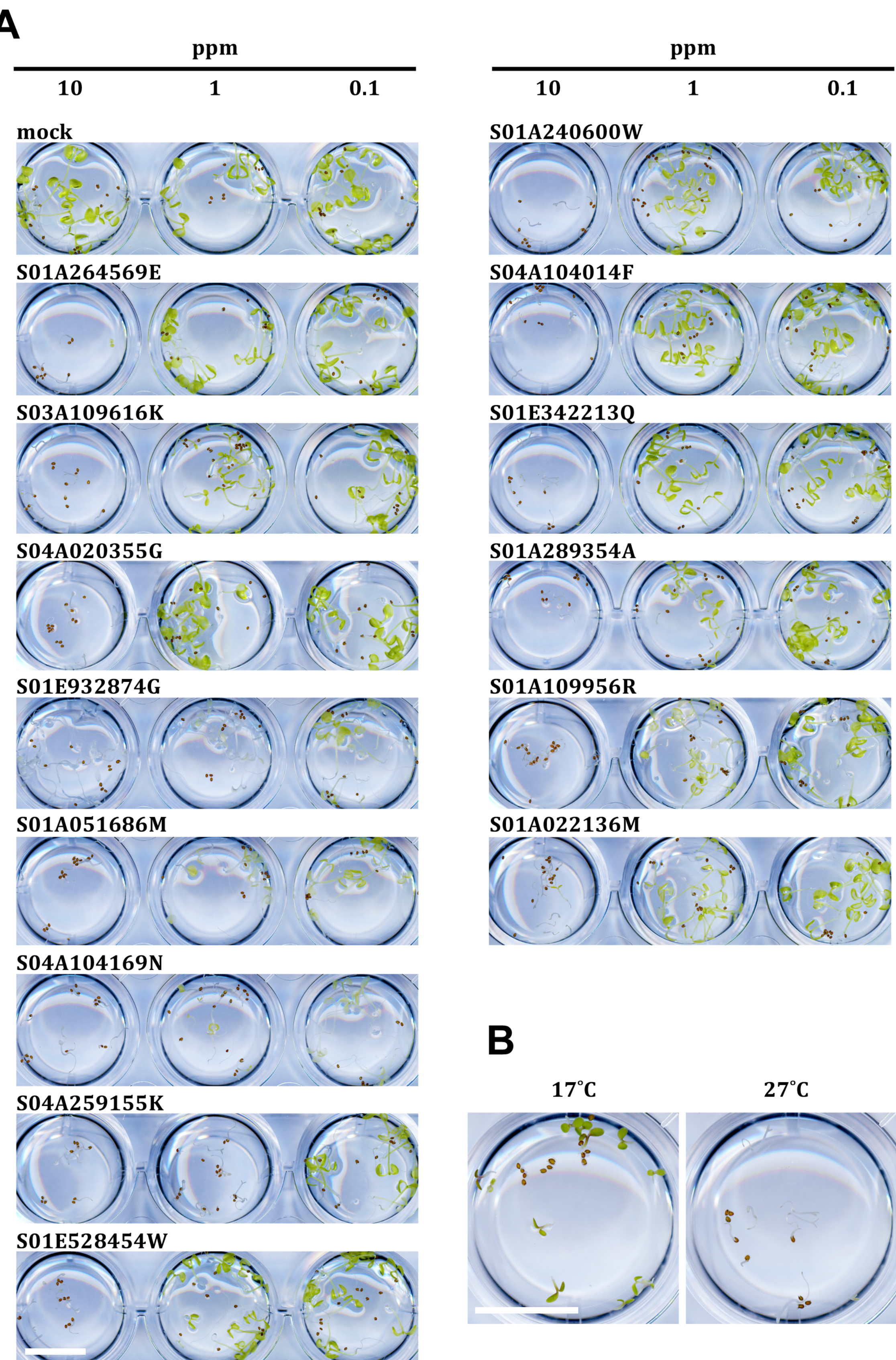


Figure 3.16: Phenotypes of plants grown in the presence of *HSP70*-repressors.
(**A**) *A. thaliana* Col-0 seeds were germinated in liquid culture at 27˚C for two days and then grown for seven days in media containing chemicals at 10, 1 or 0.1 ppm (0.1% v/v, 0.01% v/v or 0.001% v/v DMSO for the mock controls). Chemicals were applied just before the beginning of the night. All scale bars represent 15 mm, and photos are from single biological replicates.
(**B**) Treatment with 10 ppm S01E342213Q is lethal only at warm temperatures (photos of mock-treated samples are given in *Figure 3.15D*).

In contrast, all *HSP70*-repressors caused seedling lethality at 10 ppm both at 27˚C and 17˚C, with the exception of S01E342213Q, for which the lethality observed at 27˚C was completely rescued at 17˚C, though the seedlings were considerably smaller compared to the mock-treated controls (*Figure 3.16A*,*B*). Most of these chemicals also caused either chlorosis or lethality at 1 ppm, and treatments with S01E932874G, S01A051686M and S04A104169N resulted in chlorosis even at 0.1 ppm. This indicates that the *HSP70*-repressors affect vital biological processes in plant cells and are highly potent, up to ten-fold more so than predicted from their IC_{50} values for *HSP70* repression.

3.2.3 Chemical effects on cytosolic calcium

As a cytosolic influx of calcium ions is one of the earliest cellular responses to heat shock, the possibility that *HSP70*-activators or *HSP70*-repressors might affect this response was investigated. *A. thaliana* Col-0 seedlings expressing the cytosol-localised bioluminescent calcium reporter aequorin (*pCaMV35S::APOAEQUORIN*, reconstituted with coelenterazine; Xu *et al.*, 2007) were treated with chemicals for one hour and luminescence was measured before and after a five-minute heat shock at 42˚C. Examples of the raw traces obtained in these experiments are given in *Supplementary Figure 3*, *Appendix*. As shown in *Figure 3.17*, a three-fold increase in cytosolic free calcium concentration is detected in mock-treated seedlings exposed to this heat shock treatment (two-tailed *t*-test; p-value 3.69×10^{-4}). This increase in calcium concentration is approximately twice as high as that observed in previous studies in which a more moderate heat shock temperature of 37˚C was used (Gao *et al.*, 2012; Zheng *et al.*, 2012). A similar increase in calcium concentration of 100 to 150 nM was observed after most treatments with *HSP70*-activators or *HSP70*-repressors. However, it was found that cytosolic calcium levels prior to heat shock following the one-hour chemical treatments were higher than in the mock-treated seedlings for most of the compounds tested, particularly for *HSP70*-repressors.

One chemical induced a strikingly different calcium response to heat shock: seedlings treated with S03A109616K/cycloheximide for one hour had higher cytosolic calcium levels relative to mock-treated seedlings but did not have the same response to heat shock, with an increase in calcium concentration of only 20 nM, equivalent to a fold-change of approximately 1.1. In contrast, treatments with all other *HSP70*-repressors that resulted in elevated cytosolic calcium levels did not considerably compromise the heat-induced influx in calcium. Apart from S01A052379R, which caused

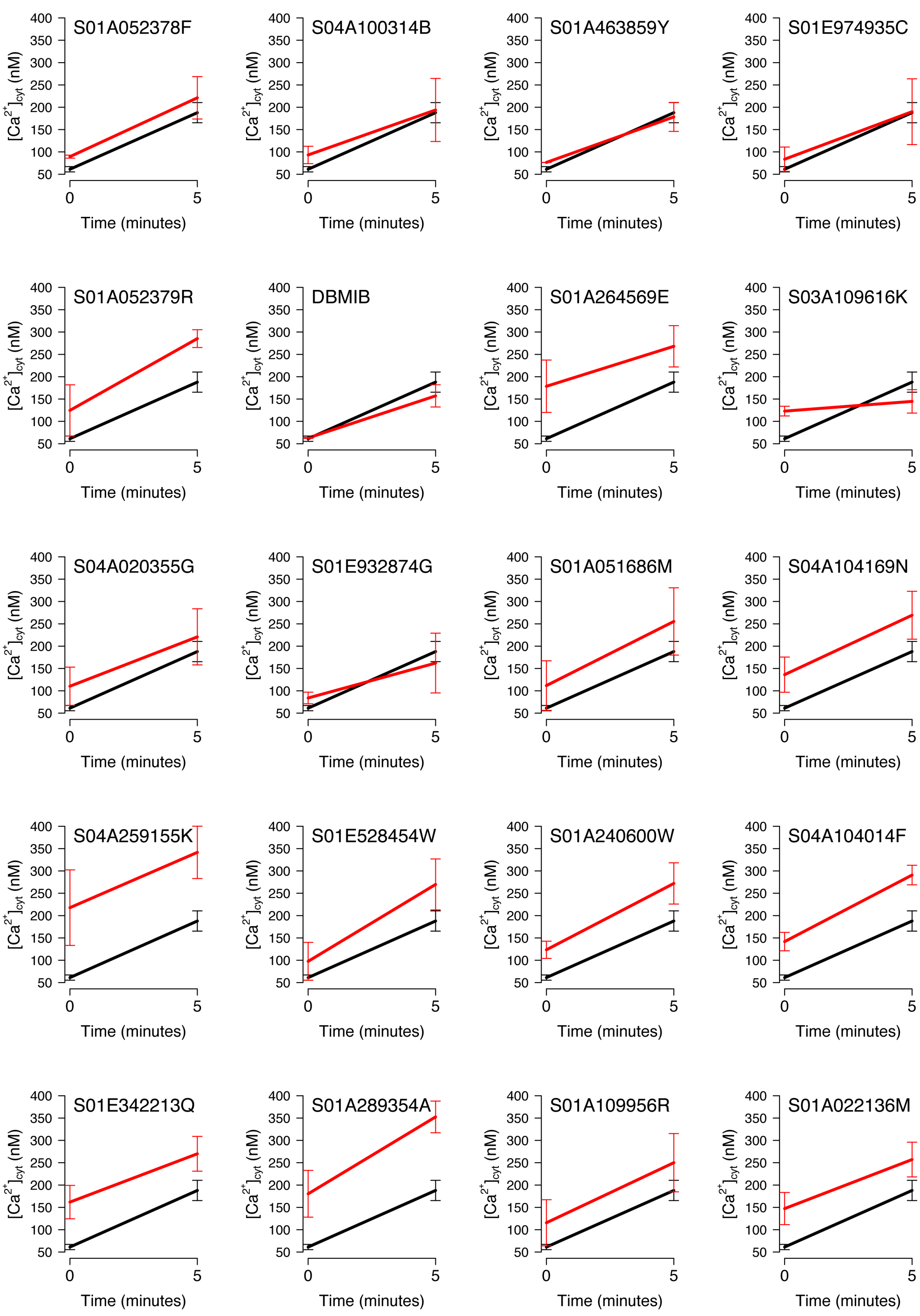


Figure 3.17: Effects of chemicals on heat-induced cytosolic calcium influx
A. thaliana Col-0 *p35SCaMV::APOAEQUORIN* seedlings were grown on ½×MS agar for 12 days at 20˚C and, following aequorin reconstitution, treated for one hour at 20˚C with chemicals at 10 ppm in deionised water (0.1% v/v DMSO for mock controls). Cytosolic calcium levels were quantified luminometrically before and after heat shock treatment at 42˚C for five minutes. Black line: mock treatment (all graphs have the same mock samples), red line: chemical treatment. Error bars represent standard deviation for at least three biological replicates, each comprising a cuvette with a single seedling.

an increase in cytosolic calcium after one hour of treatment, other *HSP70*-activators, including DBMIB, did not affect resting or heat-induced calcium levels. None of the chemicals were found to cause a significantly higher or lower bioluminescence emission upon calcium discharge relative to the mock controls (two-tailed *t*-tests; p-values >0.05).

Because many chemicals were found to induce an increase in cytosolic calcium levels after one hour of treatment, immediate responses to chemicals were recorded in high-resolution 'blind trial' experiments in which luminescence was quantified every second during one-hour treatments. The calcium signatures of reconstituted *pCaMV35S::APOAEQUORIN* seedlings treated with *HSP70*-activators or *HSP70*-repressors are given in *Figure 3.18* and *Figure 3.19*, respectively. The cytosolic calcium responses to these chemicals differ in their amplitude and temporal characteristics, suggesting that these chemicals may affect distinct components of calcium homeostasis pathways or that they perturb these pathways by different mechanisms.

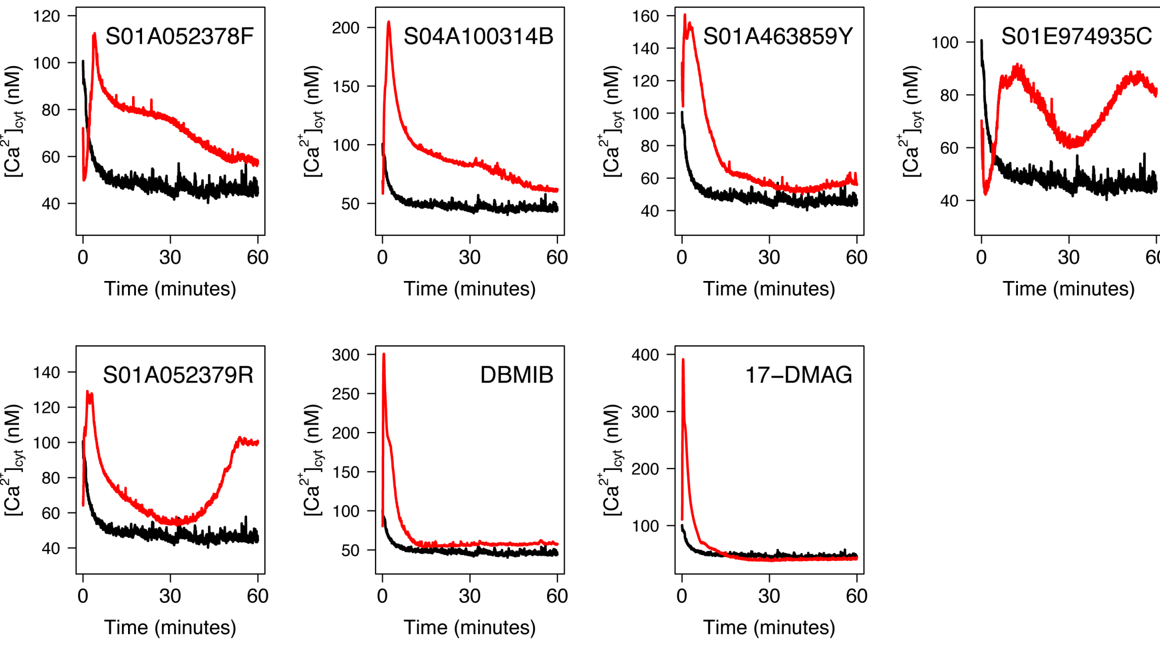


Figure 3.18: Cytosolic calcium signatures induced by *HSP70*-activators.
A. thaliana Col-0 *p35SCaMV::APOAEQUORIN* seedlings were grown in liquid culture for eight days at 20°C and, after reconstitution of aequorin, cytosolic calcium levels were quantified luminometrically during one-hour treatments with chemicals at 10 ppm in deionised water (0.1% v/v DMSO for mock controls). 17-DMAG treatments were carried out at 2.5 ppm. Cuvettes were placed in the luminometer immediately after the addition of chemicals. Black line: mock treatment (all graphs have the same mock samples), red line: chemical treatment. Traces represent single measurements with three seedlings per cuvette. Y-axes are scaled according to the amplitude of the calcium responses.

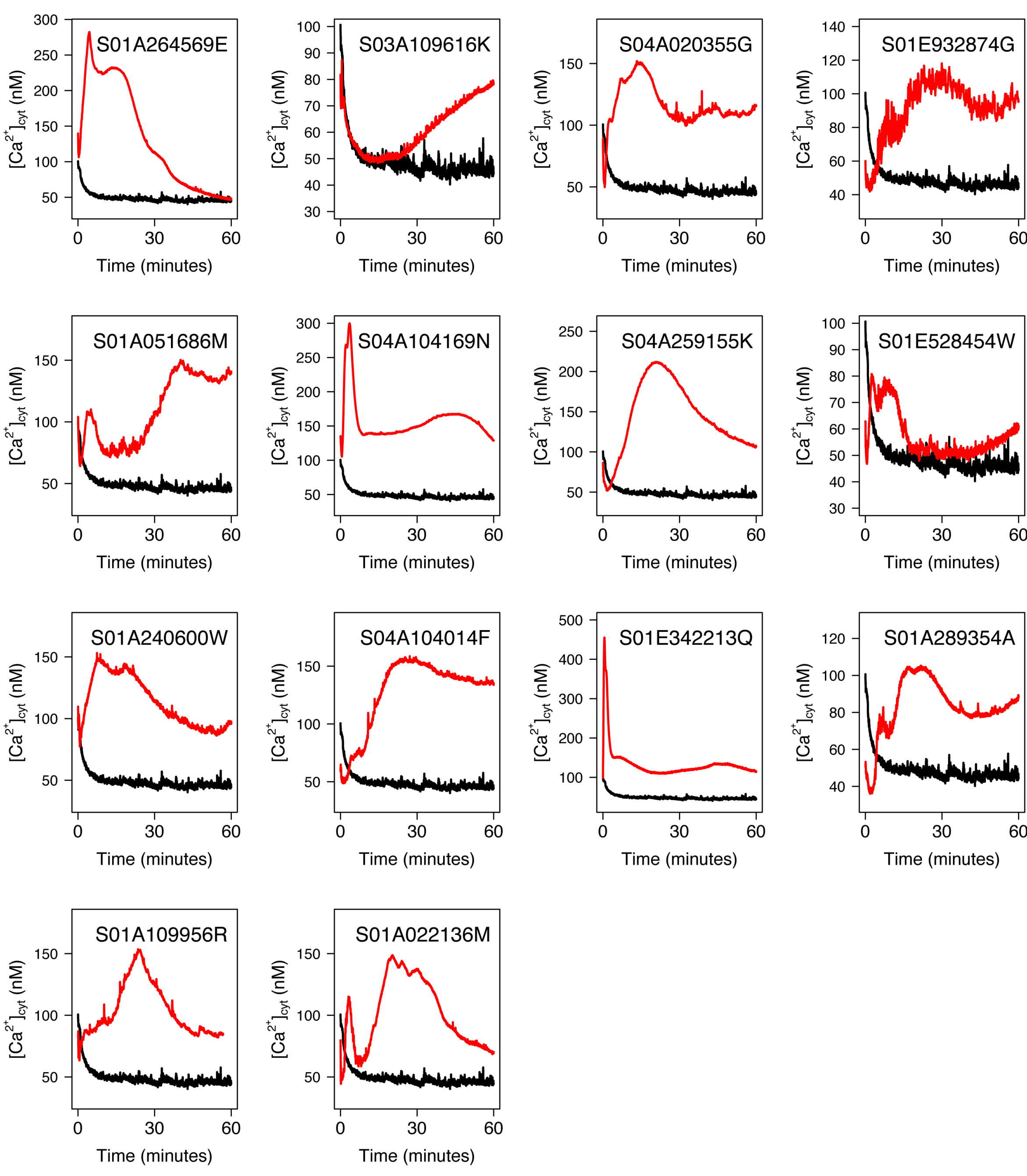


Figure 3.19: Cytosolic calcium signatures induced by *HSP70*-repressors.
A. thaliana Col-0 *p35SCaMV::APOAEQUORIN* seedlings were grown in liquid culture for eight days at 20°C and, after reconstitution of aequorin, cytosolic calcium levels were quantified luminometrically during one-hour treatments with chemicals at 10 ppm in deionised water (0.1% v/v DMSO for mock controls). Cuvettes were placed in the luminometer immediately after the addition of chemicals. Black line: mock treatment (all graphs have the same mock samples), red line: chemical treatment. Traces represent single measurements with three seedlings per cuvette. Y-axes are scaled according to the amplitude of the calcium responses.

Four of the five *HSP70*-activators (S01A052378F, S04A100314B, S01A463859Y and S01A052379R) induce a sharp increase in cytosolic calcium within two to three minutes of application, which is distinct from the transient calcium response observed during mechanical stimulation of seedlings upon application of chemicals, the tail-end of which is visible in the first five minutes of measurements in the mock controls. Interestingly, an immediate increase in cytosolic calcium is also observed after treatment with DBMIB or 17-DMAG, though with a 1.5- to 4-fold higher amplitude than for the other *HSP70*-activators (*Figure 3.18*). Similar calcium responses to geldanamycin have been reported in human cells and are necessary for induction of the HSR by this inhibitor (Shu *et al.*, 2005; Chang *et al.*, 2006).

A rapid cytosolic calcium increase is also observed during treatments with *HSP70*-repressors S01A264569E, S04A104169N and S01E342213Q, though the calcium signatures are distinct from those induced by *HSP70*-activators. S01A289354A/Tralopyril causes a rise in cytosolic calcium that peaks after about 20 minutes of treatment and remains high throughout the course of the experiment. Given its protonophore activity, this chemical is likely to cause membrane depolarisation and thereby could trigger mobilisation of calcium into the cytosol. The calcium signatures of many of the other *HSP70*-repressors, including S04A020355G, S01A051686M, S01A240600W, S04A104014F, S01A109956R and S01A022136M, appear superficially similar to that induced by S01A289354A/Tralopyril, suggesting that these molecules could potentially also be uncouplers.

3.2.4 Chemical effects on thermotolerance

Given the deleterious effects of heat shock on the growth and reproduction of plants, a chemical protectant that could enhance crop performance during high seasonal temperatures would be invaluable for agriculture. A thermotolerance assay was developed in *A. thaliana* seedlings to test whether any of the *HSP70*-activators are capable of improving survival rates during exposure to severe heat shock, as has been demonstrated previously for the HSP90-inhibitors geldanamycin and radicicol (Yamada *et al.*, 2007). To facilitate the uptake of chemicals, seedlings were grown in liquid culture, similarly to the assays performed by Yamada and colleagues, except that individual plants were cultured in separate wells in 96-well plates. Well separation was used to eliminate potential interactions between seedlings, as a large number of extracellular enzymes such as peroxidases and chitinases are predicted to be secreted in

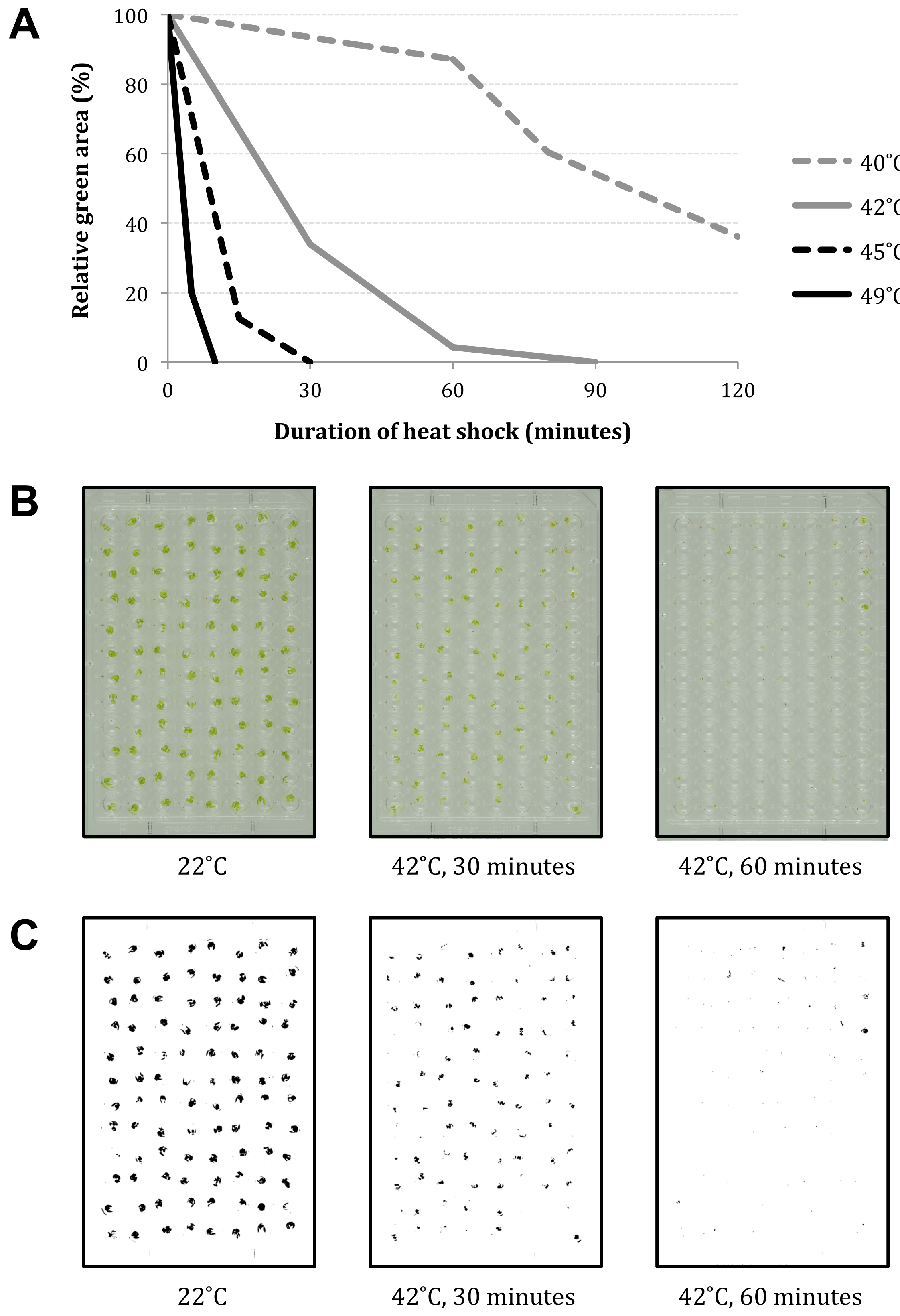


Figure 3.20: Development of a thermotolerance assay for liquid-grown *A. thaliana* seedlings.
(**A**) Seven-day-old Col-0 seedlings grown hydroponically in 96-well plates at 22˚C were incubated at 40˚C, 42˚C, 45˚C or 49˚C for up to 2 hours and then returned to 22˚C for seven days before imaging. Green area is given for single plates relative to a plate maintained at 22˚C, all containing 96 seedlings.
(**B**,**C**) Images of plates of seedlings subjected to heat shock at 42˚C before (**B**) and after (**C**) filtering for green area.

A. thaliana (Lum *et al.*, 2014), some of which have been associated with thermotolerance (Kwon *et al.*, 2007).

Most assays for basal thermotolerance in *A. thaliana* plants have been developed for seedlings grown on agar, with temperature treatments of 45˚C for 20 to 60 minutes (Yeh *et al.*, 2012). Seedlings grown in liquid culture are highly sensitive to heat shock, as a 20-minute incubation at 45˚C results in nearly 100% mortality (*Figure 3.20A*). This is likely due to the fact that liquid media conducts heat more efficiently than agar, as well as because liquid-grown seedlings are not as resilient as plants grown on agar. Seedlings were exposed to various heat shock regimes to identify the optimal temperature and duration of treatment that result in 25% to 50% of seedling survival and that could thus be used to measure increases in viability brought about by chemical treatments. As chlorophyll content and viability are positively correlated, seedling survival in response to severe heat shock can be quantified colorimetrically, with dead seedlings having a bleached appearance (*Figure 3.20B*). In order to measure the proportion of seedlings surviving severe heat shock, the 96-well plates were imaged and the number of pixels remaining after applying a green filter to the images was recorded (*Figure 3.20C*). Using this method, a 30-minute heat shock at 42˚C was found to result in a third of seedlings surviving (34% green area relative to plates for which no heat treatment was applied; *Figure 3.20A*).

Seven-day-old *A. thaliana* seedlings were exposed to the above heat shock treatment following either three hours or 24 hours of incubation with *HSP70*-activators at concentrations of 10 or 1 ppm. Only concentrations that did not result in seedling death, as determined in *Figure 3.15*, were used. In addition to the five *HSP70*-activators identified from the *pHSP70::LUC* screen, DBMIB, puromycin and 17-DMAG were included in these assays. *Figure 3.21A* and *Figure 3.21B* show the green area of plates seven days after recovery from the lethal heat shock for chemical treatments of three hours or 24 hours, respectively, relative to plates of seedlings treated with 0.1% v/v or 0.01% v/v DMSO as mock treatments prior to heat shock. A slight but not statistically significant increase in thermotolerance was observed for 17-DMAG treatments, with on average 1.3- and 1.2-fold higher green content of seedlings relative to mock controls after three-hour and 24-hour treatments, respectively (two-tailed *t*-test; p-values 0.133 and 0.113 for respective treatments). Similar results were obtained after three hours of incubation with 1 ppm S01A463859Y (p-value 0.188), though this effect was observed neither at 10 ppm nor after 24 hours of treatment.

Apart from S01A463859Y, all other *HSP70*-activators identified from the *pHSP70::LUC* screen either had no effect on thermotolerance or, particularly after three

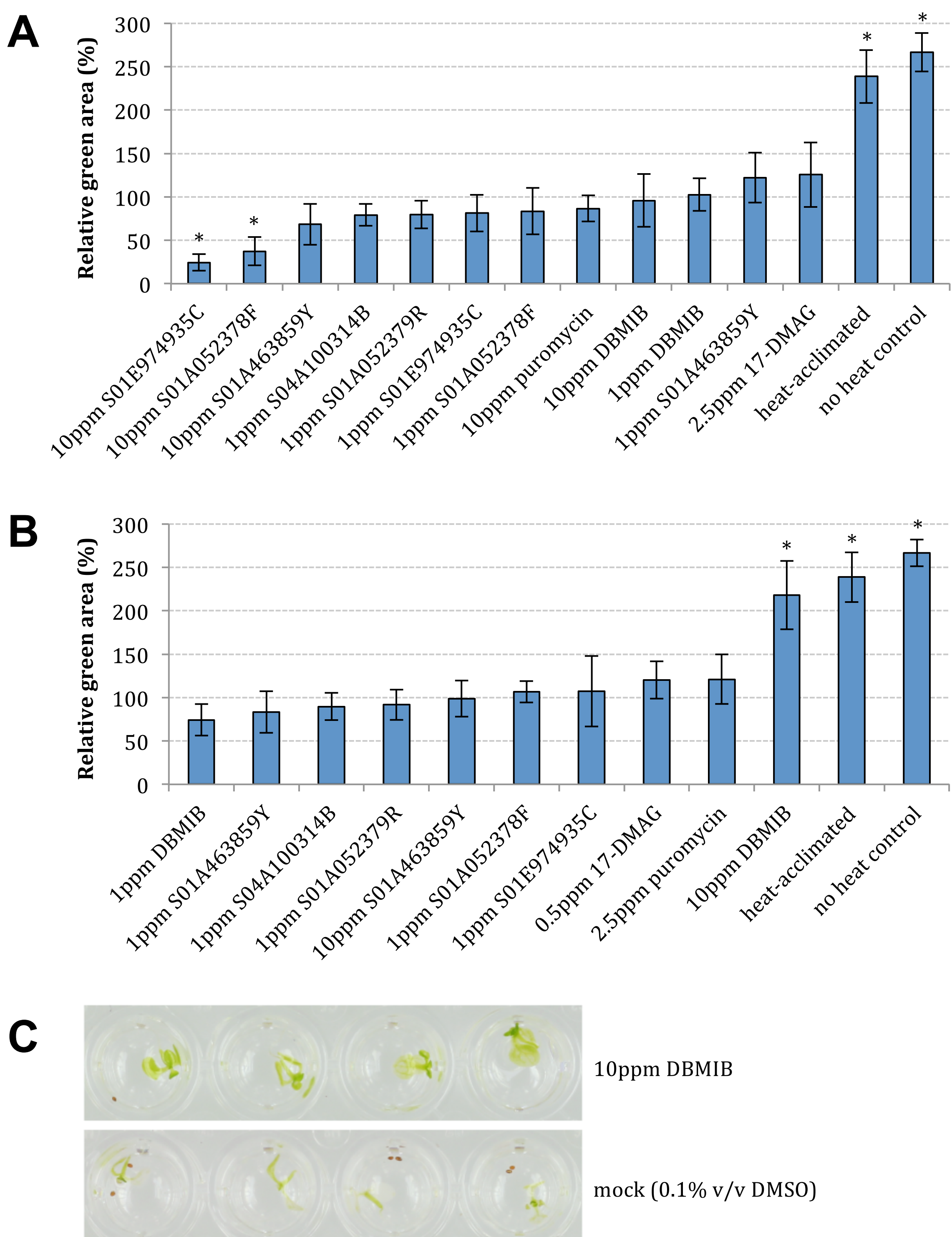


Figure 3.21: Chemical effects on thermotolerance in *A. thaliana* seedlings, measured by green area.
(**A**,**B**) Green area of chemically-treated seedlings after heat shock, relative to mock controls (0.1% v/v or 0.01% v/v DMSO). (**A**) *A. thaliana* Col-0 seedlings were grown hydroponically in 96-well plates for seven days and treated with chemicals at 10 or 1 ppm for three hours, followed by one-hour recovery in fresh medium, before heat shock at 42˚C for 30 minutes. (**B**) Six-day-old seedlings were chemically treated for 24 hours before heat shock. Plates were imaged seven days after heat shock. Heat-acclimated seedlings were pre-treated for one hour at 36˚C followed by three hours of recovery prior to 42˚C heat shock. Error bars represent standard deviation for three biological replicates (three plates of 96 seedlings). Asterisks indicate significant differences relative to mock controls (p-value <0.05, two-tailed *t*-test).
(**C**) Images of seedlings treated with 10ppm DBMIB or 0.1% v/v DMSO (mock control) for 24 hours before heat shock.

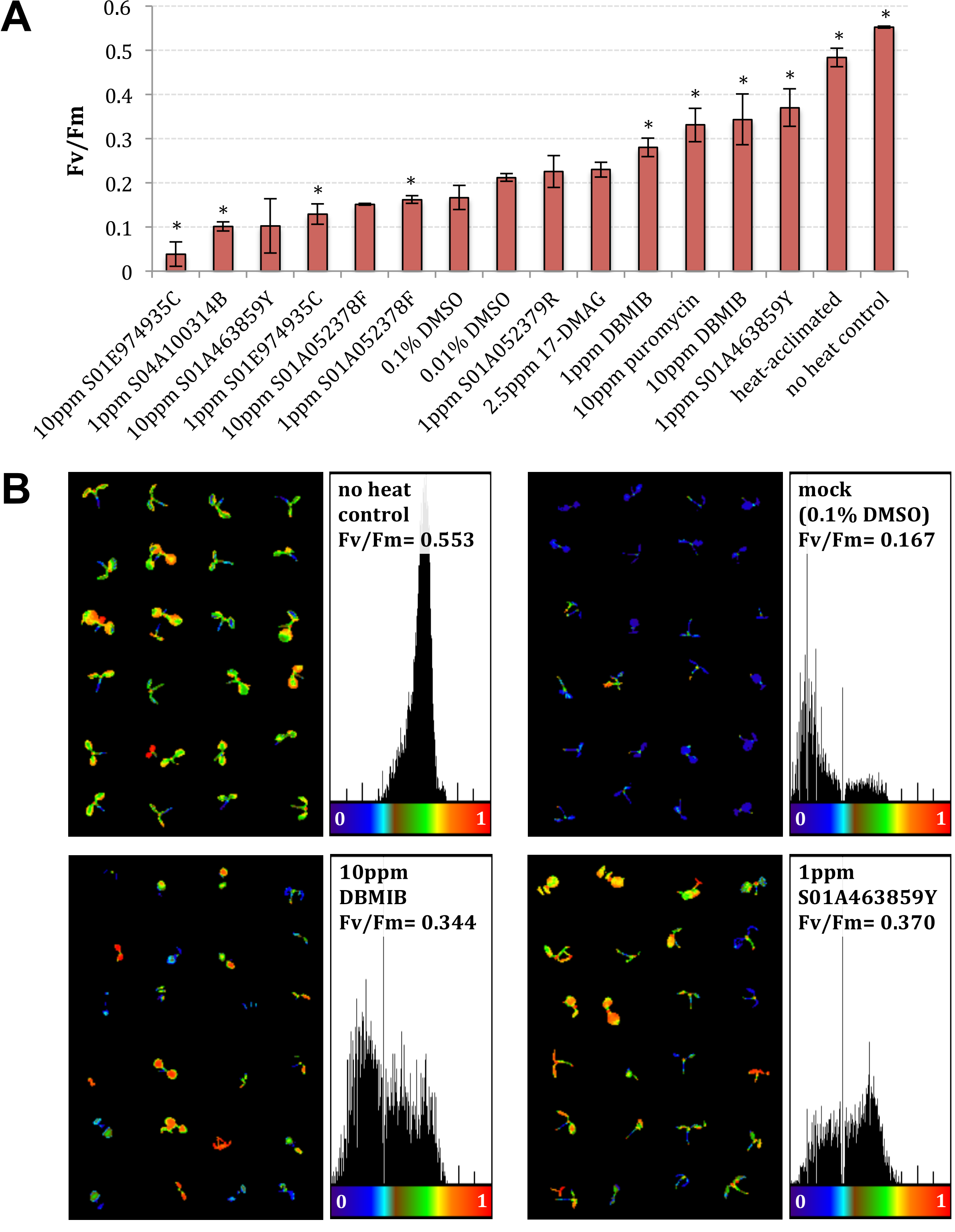


Figure 3.22: Chemical effects on thermotolerance in *A. thaliana* seedlings, measured by photosynthetic capacity.

(**A**) Fv/Fm values of *A. thaliana* Col-0 seedlings treated with chemicals for three hours, followed by one-hour recovery in fresh medium and heat shock at 42˚C for 30 minutes. Measurements were taken 24 hours after heat shock. Heat-acclimated seedlings were pre-treated for one hour at 36˚C followed by three hours of recovery prior to 42˚C heat shock. Error bars represent standard deviation for two biological replicates (two plates of 96 seedlings). Asterisks indicate significant differences relative to mock controls (two-tailed *t*-test, p-value <0.05).

(**B**) Histograms of Fv/Fm values and false-colour fluorescence images for seedlings treated with 10 ppm DBMIB, 1 ppm S01A463859Y or 0.1% v/v DMSO (mock) prior to heat shock or control plates in the absence of heat shock.

hours of treatment, reduced the proportion of seedlings surviving lethal heat shock. It is unclear why some chemical treatments, such as 10 ppm S01A463859Y, 1 ppm S01A052378F or 1 ppm S01E974935C, resulted in a decrease in survival following three hours but not 24 hours of incubation. Given the toxicity of S01A052378F and S01E974935C at high concentrations, it is possible that short treatments with these chemicals prior to heat shock results in increased cellular stress and thus higher susceptibility to heat, whereas longer treatments allow sufficient time for detoxification or compartmentalisation of these chemicals.

The strongest chemical enhancement of thermotolerance was observed after 24 hours of treatment with 10 ppm DBMIB, which resulted in a two-fold increase in green area relative to mock-treated controls (p-value 0.018). As shown in *Figure 3.21C*, the bleaching of cotyledons that is evident in mock-treated seedlings is strongly reduced in DBMIB-treated seedlings and the latter are able to resume growth, as the emergence of true leaves is visible in these seedlings. The level of thermotolerance conferred by 10 ppm DBMIB treatment is similar to that obtained during heat acclimation by pre-treatment at 36˚C for one hour (*Figure 3.21B*). In contrast, 24-hour treatments with 1 ppm DBMIB have the opposite effect on thermotolerance.

Other than seedling viability, a second phenotype that is commonly used as a measure of thermotolerance is photosynthetic capacity, which is drastically reduced following severe heat shock. Photons absorbed by PSII may either drive photochemistry by entering the electron transport chain or they may be dissipated as heat or as fluorescence. A higher level of fluorescence is emitted from PSII during excitation in healthy photosynthetic tissues compared to those that have been damaged by abiotic stress (Butler, 1978; Baker, 2008). The maximal efficiency of PSII can therefore be quantified by measuring the ratio between maximal fluorescence in actinic light, termed 'Fm', and minimum fluorescence in non-actinic light, termed 'Fo', and is calculated according to the formula: Fv/Fm, where Fv=Fm-Fo. As demonstrated in *Figure 3.22*, the efficiency of photosynthesis from PSII is decreased over three-fold in seedlings exposed to heat shock at 42˚C for 30 minutes.

Photosynthetic efficiency was measured following 24 hours of recovery in seedlings pre-treated for three hours with chemicals prior to heat shock. In accordance with the thermotolerance assay based on green area of seedlings, no enhancement of photosynthesis was observed after treatments with S01A052378F, S04A100314B, S01E974935C or S01A052379R at either 10 or 1 ppm (*Figure 3.22A*). However, both 10 ppm DBMIB and 1 ppm S01A463859Y increased photosynthetic recovery after heat shock, as indicated by the two-fold higher average Fv/Fm value in these seedlings

(*Figure 3.22A,B*). Three hours of pre-treatment with 10 ppm puromycin also doubled photosynthetic capacity relative to the mock controls, while pre-treatment with 2.5 ppm 17-DMAG did not rescue photosynthesis (*Figure 3.22A*).

3.2.5 Transcriptomic analysis of chemical treatments

RNA sequencing was used to characterise the transcriptomes of plants treated with compounds identified from the *pHSP70::LUC* screen. As for quantitative PCR analyses of *HSP70* expression, *A. thaliana* Col-0 seedlings were treated with chemicals for 30 minutes and then exposed to mild heat (30˚C) or maintained at ambient growth temperature (22˚C) for 30 minutes, after which whole seedlings were harvested. DBMIB was included among *HSP70*-activators as a positive control.

The expression of *HSP* and *HSF* genes was examined to validate the effects on *HSP70* expression observed by quantitative PCR. *Table 3.3* illustrates gene expression changes in heat-inducible and heat-insensitive or heat-repressed members of four *HSP* families, *HSP70*, *HSP90*, *HSP100* and *sHSP*, and the *HSF* family, as well as in three constitutively-expressed house-keeping genes. All five *HSP70*-activators induce the expression of these heat-responsive genes with similar patterns as observed in the mock-treated 30˚C sample, the genes most highly induced by these chemicals being the ones that are the most heat-responsive and *vice versa* for lowly induced genes. Comparable expression patterns are observed in DBMIB-treated plants. In contrast, little change in heat-insensitive genes is observed after chemical treatment.

The expression of heat-inducible *HSP* and *HSF* genes during heat shock is lower in the presence of *HSP70*-repressors, though little change in the expression of house-keeping genes and constitutively-expressed *HSP* or *HSF* genes is observed under these conditions. S01E932874G, however, appears to cause the down-regulation of a number of constitutively-expressed *HSP* and *HSF* genes as well as heat-inducible genes (*Table 3.3*).

The transcriptomes of chemically-treated seedlings were clustered according to similarities in genome-wide patterns of expression. As shown in *Figure 3.23*, some chemicals cause large-scale changes in gene expression. This is particularly evident for treatments with S03A109616K/cycloheximide and S01E932874G. Seven clusters of transcriptomes are defined in *Figure 3.23*, comprising transcriptomes either clustering closely with the mock-treated 22˚C sample (22˚C cluster, indicated by a blue bar) or the mock-treated 30˚C sample (30˚C clusters, indicated by red bars) or clustering apart from

Table 3.3: Transcriptional effects of chemicals on *HSP* and *HSF* genes.

Expression changes of heat-inducible (≥1.5-fold upregulated) and non-heat-inducible (<1.5-fold upregulated) genes of *HSF* and *HSP* families, and three house-keeping genes, in *A. thaliana* Col-0 seedlings treated with chemicals and/or heat shock. Seven-day-old liquid-grown seedlings were treated at ZT8 with chemicals at 10 ppm (0.1% v/v DMSO for mock controls) for 30 minutes at 22°C and either subjected to heat shock at 30°C or maintained at 22°C for 30 minutes. Log2 TPM ratios relative to 22°C mock controls are given for heat shock (mock 30°C), 22°C treatments with *HSP70*-activators or 30°C treatments with *HSP70*-repressors. Cells are coloured according to expression changes, red indicating up-regulated genes and blue indicating down-regulated genes. Grey cells indicate gene transcripts that were not detected after chemical treatments. *HSP* and *HSF* genes whose transcripts were not detected in more than half of the samples were omitted from the table.

Gene family		Gene name	Gene identifier	mock 30°C	DBMIB 22°C	S01E974935C 22°C	S01A463859Y 22°C	S01A052378F 22°C	S04A100314B 22°C	S01A052379R 22°C	S04A020355G 30°C	S03A109616K 30°C	S01E932874G 30°C	S01A264569E 30°C	S01A022136M 30°C	S01A051686M 30°C	S01A109956R 30°C	S01A240600W 30°C	S01A289354A 30°C	S01E342213Q 30°C	S01E528454W 30°C	S04A104014F 30°C	S04A104169N 30°C	S04A259155K 30°C
heat-inducible	HSP70	*HSP70-15*	*AT1G79920*	2.5	0.8	1.9	1.3	1.6	1.7	1.2	0.4	0.6	0.0	-0.3	0.0	0.6	0.1	0.0	0.1	0.0	-0.2	-0.2	1.3	-0.3
		HSP70-5	*AT1G16030*	7.0	5.8	8.1	4.7	6.9	6.9	6.7	0.3	0.6	0.5	0.7	0.4	4.4	2.7	3.2	3.1	-0.8	0.3	-0.9	4.9	-0.1
		HSP70-8	*AT2G32120*	6.4	3.9	6.1	2.9	5.5	5.3	5.1	0.6	0.4	-1.6	-0.4	1.0	3.5	1.0	2.7	3.1	-0.8	-0.2	0.1	4.6	-1.1
		HSP70-6	*AT4G24280*	1.0	0.5	0.8	0.5	0.5	1.0	0.5	0.5	0.2	-0.1	-0.2	-0.1	0.4	0.1	-0.2	-0.1	0.1	0.3	0.0	0.9	-0.1
		HSP70-1	*AT5G02500*	0.7	-0.3	0.5	0.6	0.4	0.0	0.1	0.2	-0.4	-0.9	0.3	-0.5	0.0	-0.2	-0.2	0.2	-0.1	-0.1	0.3	0.7	0.3
		HSP70-10	*AT5G09590*	3.8	1.7	3.3	1.9	3.1	3.0	2.8	0.4	0.9	-0.1	0.2	0.0	1.4	0.2	0.7	1.3	0.1	-0.1	0.2	2.3	-0.2
		HSP70-7	*AT5G49910*	1.1	-0.1	0.7	0.5	0.6	0.6	0.4	0.1	-0.7	-0.9	-1.0	-1.2	0.0	-0.3	-0.5	-0.8	-0.1	-0.1	-0.7	0.4	-1.0
		HSP70-3	*AT3G09440*	2.0	0.5	2.2	1.5	1.8	0.9	0.9	-1.2	0.5	-2.1	-2.0	-1.1	-0.4	-1.4	-1.1	-0.5	-1.4	-1.5	-1.3	0.4	-1.7
		HSP70-2	*AT5G02490*	1.7	1.6	2.1	1.9	2.7	1.0	1.5	0.1	5.1	1.6	0.2	2.1	0.2	-0.8	-0.3	0.9	-0.2	-0.4	0.5	2.0	-0.7
		HSP70-4	*AT3G12580*	5.4	3.9	5.1	3.7	4.7	4.0	4.2	0.6	3.2	0.5	-0.8	0.9	3.0	1.5	2.2	2.4	-1.2	-0.2	-0.6	3.6	-1.4
	sHSP	*HSP17.6-CII*	*AT5G12020*	8.3	5.6	8.0	4.0	7.0	6.5	6.3	0.3	1.3	1.5	0.1	1.6	5.0	3.0	4.1	4.1	-2.0	-0.1	0.0	5.6	-1.3
		HSP23.6-M	*AT4G25200*	10.1	6.1	8.4	3.7	7.8	7.0	7.7	1.2	3.1	0.3	0.7	4.0	7.3	3.8	6.5	7.1	-0.1	1.4	2.4	8.4	-
		HSP22.0-ER	*AT4G10250*	11.1	7.9	10.8	5.3	9.8	9.2	9.4	3.6	1.9	2.6	-	5.4	8.5	6.0	7.2	7.4	1.6	3.7	4.0	8.7	-
		HSP23.5-M	*AT5G51440*	5.8	3.3	5.0	2.7	4.7	4.0	3.9	-0.3	2.7	-1.0	-1.7	0.6	3.0	1.2	1.5	2.0	-1.0	0.4	-0.7	3.3	-1.9
		HSP17.6B-CI	*AT2G29500*	6.9	4.4	7.0	3.8	6.2	5.6	5.6	0.4	1.7	1.7	-1.9	0.6	4.0	1.8	3.2	3.1	-0.1	-0.2	-0.5	4.4	-
		HSP17.7-CII	*AT5G12030*	7.8	5.4	7.5	4.2	6.9	5.8	6.4	0.6	2.8	1.9	-0.2	2.1	5.3	2.8	4.5	4.8	-2.1	0.2	-0.1	5.3	-3.0
		HSP17.6A-CI	*AT1G59860*	8.5	5.6	7.2	4.1	6.6	6.3	5.9	2.4	3.3	0.7	1.8	2.0	5.7	3.7	5.1	5.3	-0.3	1.3	0.5	5.8	-0.9
		HSP18.1-CI	*AT5G59720*	8.0	5.6	8.8	3.5	7.3	7.3	6.9	0.7	1.7	2.4	0.7	2.6	5.0	3.3	3.7	3.7	-0.1	0.5	-0.5	5.4	-
		HSP17.6C-CI	*AT1G53540*	11.0	8.4	10.8	7.2	10.1	9.5	9.5	3.0	3.7	3.8	3.0	4.6	7.8	5.8	7.1	7.3	0.0	1.4	1.9	8.6	1.0
		HSP17.4-CI	*AT3G46230*	9.2	6.8	8.8	6.1	8.0	7.5	7.3	2.3	3.7	3.2	-0.7	2.6	6.0	3.6	5.2	5.4	-1.1	0.3	1.8	6.8	-0.1
		HSP15.7-CI(r)	*AT5G37670*	8.2	5.2	7.5	3.5	6.3	6.4	6.3	1.8	0.9	-1.0	1.6	2.8	5.4	3.6	4.7	4.6	0.4	1.2	-0.8	5.4	-0.1
		HSP17.4-CIII	*AT1G54050*	7.8	4.7	6.6	3.7	6.2	5.7	5.8	1.8	1.7	0.5	0.9	1.6	5.3	3.6	4.7	4.7	-1.4	1.2	1.1	5.3	-1.4
		HSP18.5-CI(r)	*AT2G19310*	3.2	0.8	2.5	0.4	2.0	1.5	1.4	-1.2	-0.1	-1.8	-3.0	-1.1	0.4	-0.6	-0.3	-0.9	-0.7	-0.8	-1.8	0.0	-2.3
	HSF	*HSFA2*	*AT2G26150*	6.4	2.7	5.1	3.0	4.6	4.7	4.0	0.4	3.1	-2.4	-1.1	0.4	3.6	0.4	2.7	3.3	-4.4	-2.0	-1.7	4.4	-3.0
		HSFA7a	*AT3G51910*	5.4	2.2	3.3	2.3	3.7	3.0	3.4	0.4	2.1	-0.3	0.7	1.7	2.3	1.3	1.7	1.9	0.5	1.0	0.7	2.3	1.4
		HSFB1	*AT4G36990*	3.8	2.6	3.6	2.4	3.0	2.6	2.7	-0.2	1.4	-1.0	1.0	0.4	1.4	0.7	1.1	0.9	0.1	0.4	1.0	1.3	1.1
		HSFB2a	*AT5G62020*	5.6	4.6	5.4	3.5	4.3	4.3	4.1	1.5	5.3	-1.1	0.9	2.0	3.6	1.9	2.3	2.4	0.2	1.7	0.4	3.0	0.7
		HSFB2b	*AT4G11660*	3.9	2.4	3.3	2.3	3.0	2.9	2.4	0.6	3.4	-1.3	-0.8	0.1	1.8	0.5	1.0	0.8	-0.8	-0.1	-0.3	2.0	-0.2
	HSP90	*HSP90-6*	*AT3G07770*	2.1	0.8	0.8	0.9	0.9	1.2	0.7	0.3	0.3	-0.2	0.1	0.0	0.5	0.0	0.0	0.3	0.1	0.3	0.3	1.2	-0.3
		HSP90-5	*AT2G04030*	1.3	0.3	0.5	0.4	0.3	0.7	0.3	0.3	-0.1	-0.2	-0.4	-0.3	0.4	0.1	0.0	0.0	0.1	0.3	-0.1	0.8	-0.5
		HSP90-1	*AT5G52640*	5.6	3.9	5.6	3.6	4.9	4.6	4.3	0.6	2.4	0.1	-0.6	0.4	3.1	1.4	2.2	2.2	-0.9	-0.6	-0.5	3.6	-1.2
		HSP90-2	*AT5G56030*	2.6	0.4	2.0	1.4	1.6	1.2	1.0	-0.4	-0.1	-2.0	-0.7	-1.3	0.5	-0.7	-0.2	0.2	-1.0	-0.9	-0.5	1.3	-0.9
	HSP100	*HSP100-1*	*AT1G74310*	6.9	4.8	6.6	4.3	6.0	5.8	5.6	1.3	2.1	-0.7	-0.8	1.2	4.5	2.2	3.5	3.7	-1.2	-0.1	0.1	5.1	-1.4
		HSP100-4	*AT2G25140*	5.6	2.5	4.4	2.5	4.1	4.4	3.6	0.5	1.7	0.1	0.8	1.3	3.1	1.1	1.5	2.1	0.3	0.4	0.8	3.6	0.6
		HSP100-3	*AT5G15450*	3.8	0.9	3.1	1.0	2.4	2.7	2.1	0.1	0.0	-0.1	-0.7	0.1	1.5	0.3	0.6	0.4	-0.2	-0.2	-0.6	1.6	-0.8
not heat-inducible	HSP70	*HSP70-17*	*AT4G16660*	0.1	-0.4	-0.2	0.2	-0.2	-0.2	-0.2	0.0	1.0	1.3	-0.1	0.2	-0.3	-0.1	-0.6	-0.3	0.0	-0.1	0.1	0.2	-0.1
		HSP70-9	*AT4G37910*	-0.2	0.0	0.2	0.5	0.1	0.4	0.3	0.4	0.4	-0.2	-0.1	0.3	0.4	0.1	-0.2	-0.2	0.2	0.2	0.1	0.6	0.0
		HSP70-11	*AT5G13820*	-0.6	0.9	1.0	0.7	1.2	0.7	0.7	-1.0	2.7	0.3	1.7	2.6	0.0	-0.1	0.4	0.7	0.4	0.0	0.9	0.6	0.3
	sHSP	*HSP21.7-CI(r)*	*AT5G54660*	-0.1	-0.5	-0.6	-0.5	-0.4	-0.5	-0.4	-0.8	-0.5	-2.6	-0.3	-0.1	-0.4	-0.8	-1.2	-0.4	-0.8	-0.4	-0.9	-0.8	-0.5
		HSP15.4-CI(r)	*AT4G21870*	-0.5	-1.2	-1.2	-1.4	-1.0	-1.0	-1.1	-0.6	-1.4	-3.0	-0.9	-1.3	-0.4	-0.4	-0.5	-0.7	-0.3	0.0	-0.7	-1.0	-0.7
	HSF	*HSFA1a*	*AT4G17750*	-0.7	0.3	-0.2	0.6	-0.7	-0.2	-0.2	0.3	1.5	-2.4	1.1	0.6	-0.4	0.2	-0.2	-0.1	-0.2	0.0	-0.9	-0.4	0.4
		HSFA1b	*AT5G16820*	-0.6	0.1	0.4	0.4	-0.1	0.7	0.1	1.1	1.4	-0.7	0.7	1.3	0.7	0.9	-0.1	0.5	0.6	0.8	0.8	0.4	1.1
		HSFA1d	*AT1G32330*	-0.5	0.0	0.5	0.4	0.7	1.9	0.4	0.2	0.8	-3.4	0.4	0.3	-0.2	0.1	-0.5	-0.5	0.0	-0.1	-0.1	0.2	0.3
		HSFA1e	*AT3G02990*	-0.4	-0.5	0.4	0.1	-0.1	0.6	-0.2	-0.5	0.4	-1.7	-0.9	-0.2	-0.6	-0.5	-0.7	0.0	-0.3	-0.1	-0.1	-0.4	0.4
		HSFA3	*AT5G03720*	0.6	1.4	1.9	1.2	1.6	1.8	1.2	0.5	3.8	0.1	0.2	2.5	0.9	0.8	0.6	-0.1	0.4	0.5	0.1	1.0	-0.1
		HSFA4a	*AT4G18880*	0.3	2.2	3.5	1.4	2.9	3.0	2.4	0.0	2.9	-2.4	0.8	0.7	2.2	0.8	0.3	0.1	-0.6	0.7	0.1	0.1	0.6
		HSFA4c	*AT5G45710*	-0.5	0.8	0.2	0.0	0.5	0.2	0.2	-0.9	1.3	-1.6	0.3	0.5	-0.1	-0.1	-0.5	-1.3	-0.4	-0.3	-0.9	-0.7	-0.8
		HSFA5	*AT4G13980*	-0.1	0.4	0.0	0.3	0.2	0.3	-0.1	0.0	0.1	-5.1	0.3	0.1	0.1	-0.2	-0.4	-0.1	-0.4	-0.1	-0.1	0.0	0.0
		HSFA6b	*AT3G22830*	-1.6	2.9	1.1	0.6	1.3	1.4	1.3	0.3	4.4	-2.4	1.0	-0.1	0.9	1.3	0.7	1.9	0.5	1.6	1.5	0.6	0.2
		HSFA8	*AT1G67970*	0.1	1.4	1.1	0.9	0.8	1.6	1.1	0.5	3.8	-2.2	0.6	1.1	1.4	0.3	0.4	0.1	-0.1	0.7	-0.3	0.6	0.1
		HSFB4	*AT1G46264*	-0.8	-2.1	-1.8	-0.5	-1.3	-1.4	-1.4	-0.6	-1.5	-	-1.4	-3.5	-1.4	-1.1	-1.1	-0.4	-0.9	-1.7	-0.4	-0.3	-0.9
		HSFC1	*AT3G24520*	-1.3	-1.4	0.3	-0.5	-0.9	0.5	0.5	-0.4	0.5	-3.0	0.6	0.0	-0.1	0.0	-0.4	-0.2	1.0	-0.8	0.6	-0.6	0.5
	HSP90	*HSP90-4*	*AT5G56000*	0.3	-0.6	-0.3	0.3	-0.1	-0.6	-0.4	-0.5	-1.0	-2.6	0.0	-1.5	-0.7	-0.7	-1.0	-0.4	-0.5	-0.7	0.0	0.2	-0.1
		HSP90-7	*AT4G24190*	0.0	-0.8	-0.4	-0.1	-0.5	-0.3	-0.5	0.0	-0.4	0.2	-0.4	-0.5	-0.2	-0.3	-0.5	-0.4	-0.2	-0.1	-0.1	0.1	-0.5
	HSP100	*HSP100-5*	*AT5G50920*	0.2	0.3	0.3	0.4	0.2	0.5	0.2	0.4	0.3	1.1	-0.6	0.4	0.2	0.3	-0.2	-0.6	0.2	0.2	-0.5	0.3	-0.3
		HSP100-8	*AT5G57710*	-0.3	-0.6	-0.1	0.5	0.0	-0.6	-0.3	-1.2	0.1	-4.4	-1.3	-0.8	-1.6	-1.6	-1.8	-1.5	-1.5	-1.8	-1.2	-1.0	-0.7
		HSP100-7	*AT5G51070*	0.3	1.0	1.1	1.1	0.9	0.7	0.7	0.4	0.9	2.1	-1.3	1.3	0.9	0.7	0.3	-0.6	0.4	0.6	-0.8	0.0	-0.4
House-keeping genes		*ACT2*	*AT3G18780*	0.0	0.2	0.2	0.3	0.2	0.3	0.3	0.5	-0.1	0.5	0.6	0.7	0.4	0.6	0.4	0.5	0.3	0.4	0.5	0.4	0.6
		GAPA2	*AT1G12900*	-0.1	-0.1	-0.3	-0.2	-0.3	0.0	-0.4	0.1	-1.1	-0.5	-1.1	-0.9	-0.2	-0.3	-0.5	-0.8	-0.4	-0.2	-0.8	-0.5	-0.6
		RCA	*AT2G39730*	0.2	0.2	0.2	0.2	0.1	0.4	0.1	0.6	-0.2	-0.3	-0.6	-0.2	0.1	0.2	0.1	-0.3	0.0	0.1	-0.4	0.0	-0.2

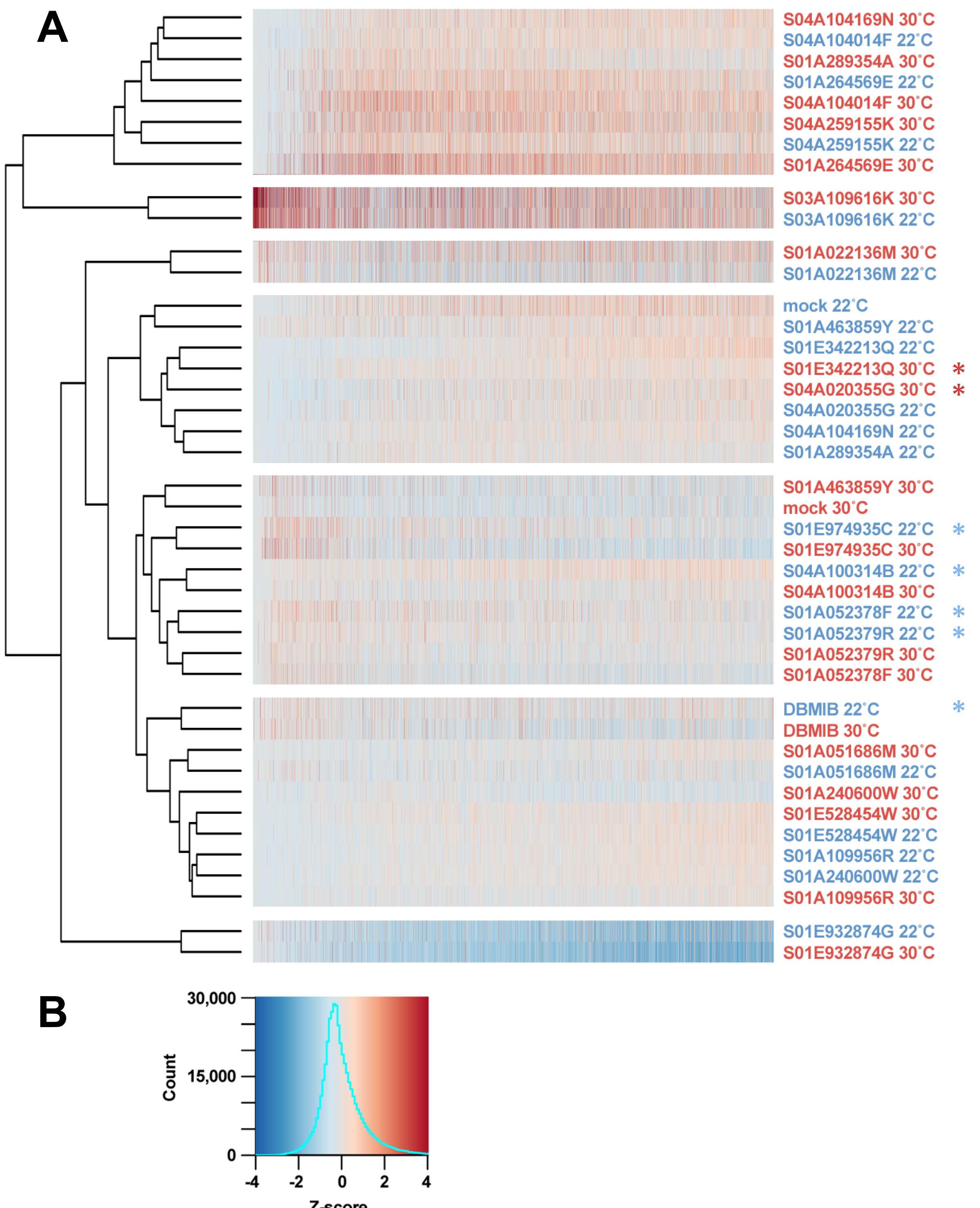


Figure 3.23: Clustering analysis of chemical treatments.
(**A**) Hierarchical clustering of transcriptomes of seven-day-old *A. thaliana* Col-0 seedlings treated at ZT8 with chemicals at 10ppm (0.1% v/v DMSO for mock controls) for 30 minutes at 22˚C and either subjected to heat shock at 30˚C or maintained at 22˚C for 30 minutes. Each row represents a treatment and each column a gene. Z-scores of TPM values are represented in red for up-regulated genes or blue for down-regulated genes, for a total of 10,993 genes. Clustering was carried out using complete agglomeration and Euclidean distance. Blue asterisks indicate treatments with *HSP70*-activators at 22˚C that cluster with the mock 30˚C sample, and red asterisks indicate treatments with *HSP70*-repressors at 30˚C that cluster with the mock 22˚C sample.
(**B**) Histogram plot of the distribution of Z-scores across all samples (mean = 0).

these samples, as do the transcriptomes of both S03A109616K/cycloheximide and S01E932874G treatments.

Treatments with *HSP70*-activators S01A052378F, S04A100314B, S01E974935C and S01A052379R at 22˚C cluster tightly with the mock treatment at 30˚C, indicating that these seedlings have warm temperature transcriptomes even though they were not exposed to heat. The S01A463859Y treatment at 22˚C clusters more closely with the mock treatment at 22˚C than that at 30˚C, suggesting only partial induction of the HSR by this chemical. Weaker induction of *HSP* genes by S01A463859Y relative to other *HSP70*-activators is also visible from *Table 3.3* and *Figure 3.6*. Treatments at 22˚C with DBMIB or the *HSP70*-repressors S01A051686M, S01E528454W, S01A109956R or S01A240600W also cluster with the mock treatment at 30˚C, albeit more distantly than the above *HSP70*-activators (*Figure 3.23*). Only two of the *HSP70*-repressor treatments at 30˚C cluster more closely to the mock treatment at 22˚C than that at 30˚C (S01E342213Q, S04A020355G), consistent with their inhibitory effects on the HSR. This suggests that these chemically-treated plants have cool temperature transcriptomes even though they were exposed to elevated temperatures. Other *HSP70*-repressors appear to have strong effects on gene expression that are independent of temperature, resulting in transcriptomes that cluster apart from mock treatments at 22˚C or 30˚C.

To obtain potential information on the pathways targeted by the different compounds, the enrichment of gene ontology (GO) terms was analysed for genes up-regulated by chemical treatments. *Table 3.4* lists the most highly enriched GO terms, including the function and localisation of gene products as well as the biological processes associated with their expression. GO terms associated with genes induced by heat (30˚C) or DBMIB are included in the table, as a comparison for *HSP70*-activators.

All *HSP70*-activators, as well as heat and DBMIB treatments, induce genes associated with responses to ROS, such as hydrogen peroxide, or to high light intensity, which is known to trigger an elevation of plastid levels of ROS (Jung *et al.*, 2013b). GO enrichment of genes induced by DBMIB, S01A052378F, S01E974935C and S01A052379R include ‘response to oxygen-containing compound’, while those induced by all *HSP70*-activators except S01A463859Y include ‘response to nitrogen compound’. These genes are implicated in biotic and abiotic stress responses and include a number of genes encoding pathogenesis-related proteins, peroxidases or transcription factors associated with oxidative stress responses. Only for S01A463859Y and S01E974935C treatments is the GO term ‘response to heat’ highly enriched. Interestingly, 15% of the genes up-regulated by S01A463859Y encode proteins associated with transmembrane receptor or kinase function, including many receptor kinases.

Table 3.4: Gene ontology enrichment of *A. thaliana* genes upregulated at least four-fold by chemical treatments at 22°C or mock treatment (0.1% v/v DMSO) at 30°C, relative to the 22°C mock-treated sample. A total of 16,487 genes expressed in all treatments were included in the analyses. Top eight GO terms and p-values are listed in descending order of significance.

Treatment		Biological Process	Molecular Function	Cellular Component
HSP70-activators	30°C	response to heat (2.51×10^{-12}); response to high light intensity (2.51×10^{-8}); response to hydrogen peroxide (9.03×10^{-8}); response to temperature stimulus (1.35×10^{-7}); response to toxic substance (3.62×10^{-6}); response to light intensity (7.36×10^{-6}); response to reactive oxygen species (3.85×10^{-5}); protein folding (5.61×10^{-5})	no enrichment	no enrichment
	DBMIB	response to stress (6.67×10^{-9}); response to stimulus (1.05×10^{-8}); response to oxygen-containing compound (1.06×10^{-7}); cellular respiration (5.61×10^{-7}); anaerobic respiration (5.61×10^{-7}); response to abiotic stimulus (5.91×10^{-7}); response to abscisic acid (6×10^{-7}); response to alcohol (6×10^{-7})	lipase activity (3.04×10^{-5})	no enrichment
	S01A052378F	response to drug (1.79×10^{-10}); response to chitin (7.55×10^{-10}); response to stimulus (3.06×10^{-9}); response to organonitrogen compound (4.71×10^{-9}); response to nitrogen compound (1.48×10^{-8}); response to stress (2.5×10^{-7}); response to chemical (3.34×10^{-7}); response to oxygen-containing compound (1.18×10^{-6})	no enrichment	no enrichment
	S04A100314B	response to drug (8.61×10^{-8}); response to chitin (2.21×10^{-6}); response to organonitrogen compound (3.97×10^{-6}); response to stress (4.07×10^{-6}); response to nitrogen compound (8.7×10^{-6}); cellular respiration (1.08×10^{-5}); anaerobic respiration (1.08×10^{-5}); response to high light intensity (1.91×10^{-5})	no enrichment	no enrichment
	S01A463859Y	cellular respiration (1.08×10^{-8}); anaerobic respiration (1.08×10^{-8}); energy derivation by oxidation of organic compounds (1.47×10^{-7}); response to stimulus (7.64×10^{-7}); response to heat (1.89×10^{-6}); generation of precursor metabolites and energy (5.04×10^{-6}); response to drug (1.19×10^{-5}); response to hydrogen peroxide (1.52×10^{-5})	protein serine/threonine kinase activity (1.71×10^{-4}); transmembrane receptor protein serine/threonine kinase activity (2.15×10^{-4}); transmembrane receptor protein kinase activity (3.33×10^{-4}); phosphotransferase activity, alcohol group as acceptor (3.4×10^{-4}); transmembrane receptor activity (5.54×10^{-4}); transmembrane signalling receptor activity (5.54×10^{-4}); signalling receptor activity (5.54×10^{-4}); lipid transporter activity (6.35×10^{-4})	no enrichment
	S01E974935C	response to drug (1.66×10^{-12}); response to chitin (2.8×10^{-10}); response to stimulus (4.59×10^{-10}); response to nitrogen compound (9.07×10^{-10}); response to organonitrogen compound (3.15×10^{-9}); response to stress (5.58×10^{-8}); response to oxygen-containing compound (7.53×10^{-8}); response to heat (1.66×10^{-7})	molecular function (1.92×10^{-4})	no enrichment
	S01A052379R	response to drug (3.54×10^{-10}); response to nitrogen compound (4.5×10^{-8}); response to chitin (9.08×10^{-8}); response to stimulus (1.99×10^{-7}); response to organonitrogen compound (2.26×10^{-7}); response to stress (1.37×10^{-6}); response to oxygen-containing compound (3.26×10^{-6}); response to oxidative stress (4.06×10^{-6})	calmodulin binding (8.66×10^{-4})	no enrichment

Table 3.4 Continued

HSP70-repressors	S01A264569E	no enrichment	no enrichment	no enrichment
	S03A109616K/ cycloheximide	defence response (2.19×10^{-21}); response to stimulus (1.88×10^{-19}); response to chitin (2.12×10^{-15}); response to stress (6.15×10^{-15}); response to organonitrogen compound (1.76×10^{-12}); response to nitrogen compound (1.48×10^{-11}); response to drug (3.67×10^{-11}); response to organic substance (3.08×10^{-10})	ADP binding (1.88×10^{-10}); protein kinase activity (4.55×10^{-8}); adenyl ribonucleotide binding (8.73×10^{-8}); adenyl nucleotide binding (8.73×10^{-8}); phosphotransferase activity, alcohol group as acceptor (1.58×10^{-7}); purine ribonucleotide binding (1.5×10^{-6}); purine nucleotide binding (1.5×10^{-6}); carbohydrate derivative binding (2.07×10^{-6})	intrinsic component of membrane (1.77×10^{-5}); integral component of membrane (1.23×10^{-4}); plasma membrane (3.42×10^{-4}); membrane part (8.6×10^{-4})
	S04A020355G	regulation of phenylpropanoid metabolic process (2.19×10^{-4}); regulation of secondary metabolic process (7.92×10^{-4})	lipase activity (8.83×10^{-4})	no enrichment
	S01E932874G	generation of precursor metabolites and energy (3.88×10^{-4}); response to gibberellin (5.89×10^{-4}); cellular response to oxygen levels (6.61×10^{-4}); cellular response to hypoxia (6.61×10^{-4}); cellular response to decreased oxygen levels (6.61×10^{-4})	no enrichment	plasma membrane region (1.12×10^{-4})
	S01A051686M	response to oxygen-containing compound (2.49×10^{-7}); response to chitin (4.42×10^{-7}); response to organic substance (4.56×10^{-7}); response to organonitrogen compound (6.56×10^{-7}); response to drug (1.04×10^{-6}); response to nitrogen compound (1.47×10^{-6}); cellular respiration (4.17×10^{-6}); anaerobic respiration (4.17×10^{-6})	O-methyltransferase activity (2.11×10^{-4}); S-adenosylmethionine-dependent methyltransferase activity (5.15×10^{-4})	no enrichment
	S04A104169N	no enrichment	no enrichment	no enrichment
	S04A259155K	cell wall organization or biogenesis (6.68×10^{-6}); carbohydrate metabolic process (1.05×10^{-5}); cell wall organization (2.61×10^{-5}); external encapsulating structure organization (5.69×10^{-5})	structural constituent of cell wall (6.28×10^{-5})	no enrichment
	S01E528454W	cellular respiration (2.44×10^{-7}); anaerobic respiration (2.44×10^{-7}); energy derivation by oxidation of organic compounds (1.68×10^{-6}); regulation of phenylpropanoid metabolic process (9.02×10^{-4})	no enrichment	no enrichment
	S01A240600W	cellular respiration (2.08×10^{-5}); anaerobic respiration (2.08×10^{-5}); energy derivation by oxidation of organic compounds (8.01×10^{-5}); regulation of phenylpropanoid metabolic process (7.86×10^{-4})	no enrichment	no enrichment
	S04A104014F	cellular homeostasis (1.71×10^{-4}); regulation of phenylpropanoid metabolic process (3.54×10^{-4}); cell redox homeostasis (6.08×10^{-4})	no enrichment	no enrichment
	S01E342213Q	response to oxygen-containing compound (1.58×10^{-4}); response to organic substance (3.4×10^{-4}); response to chemical (4.02×10^{-4}); response to acid chemical (4.46×10^{-4}); response to abscisic acid (9.48×10^{-4}); response to alcohol (9.48×10^{-4})	no enrichment	no enrichment
	S01A289354A/ Tralopyril	regulation of phenylpropanoid metabolic process (4.17×10^{-4})	no enrichment	no enrichment
	S01A109956R	regulation of phenylpropanoid metabolic process (3.69×10^{-4}); hormone-mediated signalling pathway (6.82×10^{-4}); cellular respiration (6.85×10^{-4}); anaerobic respiration (6.85×10^{-4})	no enrichment	no enrichment
	S01A022136M	response to stress (6.28×10^{-7}); response to stimulus (6.3×10^{-7}); response to external stimulus (4.57×10^{-6}); response to biotic stimulus (2.1×10^{-5}); response to other organism (3.93×10^{-5}); response to external biotic stimulus (6.03×10^{-5}); multi-organism process (6.46×10^{-5}); drug catabolic process (1.65×10^{-4})	sequence-specific DNA binding (5.78×10^{-4}); L-gulonolactone oxidase activity (6.72×10^{-4}); D-arabinono-1,4-lactone oxidase activity (6.72×10^{-4})	extracellular region (6.68×10^{-4}); plasma membrane region (6.72×10^{-4}); intrinsic component of membrane (8.34×10^{-4})

Similarly, treatments with the three *HSP70*-repressors S03A109616K/ cycloheximide, S01E932874G and S01A022136M trigger the up-regulation of genes whose proteins localise to the plasma membrane, including regulators of endocytosis and, in the case of S03A109616K, a large number of receptor kinases, transporters and cell wall-associated enzymes. Another potentially interesting enrichment of GO terms is seen for genes upregulated by S04A259155K, 8% of which encode structural proteins of the cell wall or enzymes associated with cell wall metabolism. The GO term 'regulation of phenylpropanoid metabolic process' is enriched for genes upregulated by six *HSP70*-repressors, including S01A289354A/Tralopyril and four other chemicals that were hypothesised to be uncouplers based on their cytosolic calcium signatures. These genes include members of the Kelch repeat F-box (KFB) family, which negatively regulate the activity of phenylalanine ammonia-lyase (PAL) during carbon limitation (Zhang *et al.*, 2013).

3.2.6 Cheminformatic analyses of chemical structures

While the chemical genetics method has clear advantages over classical genetics with regards to overcoming gene redundancy and lethality, its main limitation is the difficulty associated with the identification of biological targets of active molecules. Typical approaches to target identification in the literature include proteomic analysis using a tagged or labelled compound or forward genetics to identify mutants with altered responses to the compound of interest.

These two options were not feasible for this project, given technical and time constraints with Syngenta, and therefore biological targets were predicted using cheminformatics, by searching public databases of chemicals for structural similarities to compounds identified from the *pHSP70::LUC* screen. Two databases were used to search for chemical analogues: the KEGG COMPOUND database, which contains diverse xenobiotics as well as naturally-occurring metabolites, and the ChEMBL database, which is enriched with small molecules from drug tests in medical field and includes data from assays of biological activity or protein binding. For each chemical, the two most similar structures identified from each database are given in *Table 3.5* and *Table 3.6*. Most of the molecules identified from the KEGG COMPOUND database are herbicides or pesticides and overall show lower structural similarity to the chemicals from Syngenta than those identified from the ChEMBL database, though the similarity scores are not directly comparable between database searches.

Table 3.5*:* Top compounds with structural similarity to *HSP70*-activators and *HSP70*-repressors from the KEGG COMPOUND database. Structures, similarity scores and modes of action from the literature are given.

	ISN number and chemical structure	KEGG COMPOUND database					
		Most similar chemical			Second most similar chemical		
		Score	Name and target	Structure	Score	Name and target	Structure
HSP70-activators	S01A052378F	0.62	Haloxydine: inhibitor of homogentisate solanesyl transferase (Sadre *et al.*, 2010)		0.47	Picloram: activator of AFB4 and AFB5 (Prigge *et al.*, 2016)	
	S04A100314B	0.60	2-Benzothiazole-sulfonamide: inhibitor of carbonic anhydrase (Miller *et al.*, 1950)		0.57	2-Methylthio-benzothiazole: unknown	
	S01A463859Y	0.58	Pyrimethanil: inhibitor of cystathionine-β-lyase (Leroux, 1996)		0.57	Mepanipyrim: unknown, inhibits retrograde Golgi-endoplasmic reticulum trafficking (Nakamura *et al.*, 2003)	
	S01E974935C	0.66	1-Phenyl-5-mercaptotetrazole: unknown, antifungal (Nesměrák *et al.*, 2000)		0.62	F5231: protoporphyrinogen oxidase (Theodoridis *et al.*, 2012)	
	S01A052379R	0.62	Haloxydine: inhibitor of homogentisate solanesyl transferase (Sadre *et al.*, 2010)		0.47	Picloram: activator of AFB4 and AFB5 auxin receptors (Prigge *et al.*, 2016)	

Table 3.5 Continued

HSP70-repressors	S01A264569E	0.64	Fluridone: inhibitor of phytoene desaturase (Chamovitz *et al.*, 1993)		0.61	2,4-Diphenyl-1-butene: unknown	
	S04A020355G	0.68	Riluzole: inhibitor of tetrodotoxin-sensitive sodium channels (Song *et al.*, 1997)		0.68	2-Benzothiazole-sulfonamide: inhibitor of carbonic anhydrase (Miller *et al.*, 1950)	
	S01E932874G	0.54	Mepanipyrim: unknown, inhibits retrograde Golgi-endoplasmic reticulum trafficking (Nakamura *et al.*, 2003)		0.50	Carbinoxamine: antagonist of histamine H_1 receptor (Corey & Helal, 1996)	
	S01A051686M	0.53	Fenson: insecticide, suspected antagonist of GABA-gated chloride channels (PPDB, 2016)		0.52	Albendazole-2-aminosulfone: unknown, metabolite of antihelminthic albendazole (Shaikh *et al.*, 2006)	
	S04A104169N	0.71	Tridiphane: inhibitor of glutathione S-transferase (Lamoureux & Rusness, 1986)		0.58	4-(2-Aminophenyl)-2,4-dioxobutanoate: unknown	
	S04A259155K	0.53	Nicotyrine: inhibitor of cytochrome P450 2A6 and 2A13 (Kramlinger *et al.*, 2012)		0.51	4,5,6,7-Tetrachloro-2-trifluoromethyl-benzimidazole: uncoupler (Romaschenko *et al.*, 2015)	
	S01E528454W	0.48	Thiazafluron: inhibitor photosynthetic electron transport chain (Jurado *et al.*, 2011)		0.46	Flufenacet: very long chain fatty acid elongase (Wakabayashi & Böger, 2002)	

Table 3.5 Continued

	Compound						
HSP70-repressors	S01A240600W	0.45	Fenchlorazole-ethyl: unknown, safener for herbicide Fenoxaprop-ethyl (Yaacoby *et al.*, 1991)		0.42	Pyrimethamine: competitive inhibitor of dihydrofolate reductase (Peterson *et al.*, 1988)	
	S04A104014F	no similar compounds					
	S01E342213Q	0.53	Thiopental: agonist of $GABA_A$ receptor anion channel, antagonist of voltage-gated calcium channels (Zhan *et al.*, 1998; Garcia *et al.*, 2010)		0.51	Thiamylal: agonist of $GABA_A$ receptor anion channel, antagonist of voltage-gated calcium channels (Zhan *et al.*, 1998; Garcia *et al.*, 2010)	
	S01A109956R	no similar compounds					
	S01A022136M	0.69	2,4,5-Trichlorophenol: uncoupler of mitochondrial respiration (Stockdale & Selwyn, 1971)		0.66	3,4,6-Trichloro-catechol: unknown	

***Table 3.6**: Top compounds with structural similarity to *HSP70*-activators and *HSP70*-repressors from the ChEMBL database. Structures, similarity scores and most potent interactions are given.*

	ISN number and chemical structure	ChEMBL database					
		Most similar chemical			Second most similar chemical		
		Score	Name and target	Structure	Score	Name and target	Structure
HSP70-activators	S01A052378F	0.76	CHEMBL1974011: unknown, antitumour activity		0.75	CHEMBL1468738: inhibitor of human histone 3 lysine 9 N-methyltransferase [potency 2.8 µM]	
	S04A100314B	0.85	CHEMBL158743: unknown, antitumour activity		0.85	CHEMBL161036: unknown, antitumour activity	
	S01A463859Y	0.79	CHEMBL99210: interacts with rat $GABA_A$ receptor anion channel [K_i >10 µM] (Colotta *et al.*, 1996)		0.76	CHEMBL156288: unknown, antischistosomal activity	
	S01E974935C	0.93	CHEMBL1486934: inhibitor of human sphingosine 1-phosphate receptor [IC_{50} 951 nM], inhibitor of human chromobox protein homolog 1 [potency 447 nM]		0.92	CHEMBL1402153: inhibitor of human mTORC1 kinase [potency 37 nM], inhibitor of microtubule-associated protein tau [potency 1.3 µM]	
	S01A052379R	0.77	CHEMBL1974011: unknown, antitumour activity		0.74	CHEMBL1468738: inhibitor of human histone 3 lysine 9 N-methyltransferase [potency 2.8 µM]	

Table 3.6 Continued

HSP70-repressors	S01A264569E	no similar compounds					
	S04A020355G	0.83	CHEMBL3134334: inhibitor of human lysine-specific histone demethylase 1 [IC_{50} 44 μM]		0.78	CHEMBL17868: unknown, inhibitor of leukotriene-induced muscle contraction	
	S01E932874G	0.84	CGP060476 / CHEMBL584384: inhibitor of maize casein kinase II α [IC_{50} 9.6 μM] (Sarno *et al.*, 2003)		0.83	CGP-60474/ GNF-Pf-88/ CHEMBL300389: inhibitor of human cyclin-dependent kinases 1 and 2 [IC_{50} <5.2 nM], human glycogen synthase kinase 3 [IC_{50} 10 nM], human SMG1 kinase [IC_{50} 0.18 μM], human TOR kinase [IC_{50} 1.5 μM] (Kuo *et al.*, 2005; Gopalsamy *et al.*, 2012)	
	S01A051686M	no similar compounds					
	S04A104169N	0.77	CHEMBL421036: disruptor of mitochondrial membrane potential (potency 15.6 μM)		0.75	CHEMBL1205211: unknown, insecticide activity	

Table 3.6 Continued

HSP70-repressors	S04A259155K	0.83	CHEMBL2253356: unknown, insecticide activity		0.72	CHEMBL2253349: unknown, insecticide activity	
	S01E528454W	0.92	CHEMBL1520808: inhibitor of human parathyroid hormone receptor [potency 0.77 μM]		0.92	CHEMBL3718206: agonist of T1R2/T1R3 receptor [EC_{50} 1.55 μM]	
	S01A240600W	0.85	CHEMBL1592907: inhibitor of *Escherichia coli* AmpC beta-lactamase [potency 0.89 μM]		0.84	CHEMBL1554964: interacts with human cellular tumour antigen p53 [potency 6.3 μM]	
	S04A104014F	positive uncoupler activity (Syngenta uncoupling assays)					
	S01E342213Q	0.86	CHEMBL1712671: unknown, antitrypanosomal activity		0.82	CHEMBL2136061: inhibitor of human PLK1 kinase [potency 6.7 μM]	
	S01A109956R	positive uncoupler activity (Syngenta uncoupling assays)					
	S01A022136M	no similar compounds					

Based on the chlorosis observed in *Figure 3.15* and on their structural similarity to the herbicide haloxydine (*Table 3.5*), it is likely that S01A052378F and S01A052379R interfere with chloroplast function. Haloxydine inhibits the enzyme homogentisate solanesyl transferase involved in the biosynthesis of the electron carrier plastoquinone (Sadre *et al.*, 2010). S01A052378F and S01A052379R also show some structural similarity to the herbicide picloram (*Table 3.5*), which acts as an auxin mimic and induces hypocotyl growth while reducing leaf development in *A. thaliana* (Delarue *et al.*, 1998). As depicted in *Figure 3.15*, however, seedlings grown in the presence of S01A052378F or S01A052379R do not have morphologies associated with overactive auxin signalling, with no effects on hypocotyl or leaf size.

S04A100314B shows high similarity to benzothiazole-sulfonamide (*Table 3.5*), an inhibitor of carbonic anhydrases, which catalyse the interconversion of carbon dioxide and bicarbonate ions. The *A. thaliana* genome encodes cytoplasmic, mitochondrial and chloroplast isoforms of carbonic anhydrases, and loss of function of many of these proteins results in reduced viability (Ferreira *et al.*, 2008; Wang *et al.*, 2012a; Córdoba *et al.*, 2016; Fromm *et al.*, 2016), consistent with the lethal effects of S04A100314B treatment at high concentration (*Figure 3.15*). S04A100314B is also structurally similar to the active benzothiazole moieties of YM-1 and MKT-077, molecules that have been shown to interact with HSP70 and stimulate its chaperone activity *in vitro* (Wadhwa *et al.*, 2000; Wang *et al.*, 2013a).

The compound with the highest structural similarity to S01A463859Y is CHEMBL99210 (*Table 3.6*), which binds with low affinity (>10 μM) to the mammalian benzodiazepine receptor (Colotta *et al.*, 1996). The latter forms a complex with γ-aminobuturic acid (GABA)-regulated anion channels ($GABA_A$) and, when activated, increases their activity by promoting higher affinity for GABA, resulting in membrane hyperpolarisation (Asadi-Pooya & Sperling, 2016). Numerous molecules with structural similarity to S01A463859Y identified from the ChEMBL database are reported to bind and activate G-proteins. CHEMBL1328326, CHEMBL1332549, CHEMBL1341309, CHEMBL1429335 and CHEMBL1586091, for example, interact with the human G-protein α subunit with affinities down to 1.6 μM. All of these structures have ChEMBL similarity scores above 0.7, indicating that S01A463859Y could potentially exhibit similar activities to some of these analogues in plant cells.

Additionally, the four compounds CHEMBL82532, CHEMBL84353, CHEMBL337860 and CHEMBL3703528 are reported to act as inhibitors of phosphodiesterases with affinities up to 24 nM (Senga *et al.*, 1982). Such activity is less likely for S01A463859Y, given the stimulation of the heat-responsive calcium channel

CNGC6 by such inhibitors (Gao *et al.*, 2012) and the fact that S01A463859Y did not cause any alterations to heat-induced calcium influx (*Figure 3.17*). In contrast, S01A463859Y does not appear to affect protein synthesis, as would be predicted based on the results from the KEGG COMPOUND database, because accumulation of HSP70B protein was observed after chemical treatment (*Figure 3.6*) and *pHSP70::LUC* seedlings treated with S01A463859Y did not have diminished levels of luciferase activity, unlike those treated with S03A109616K/cycloheximide.

S01E974935C is structurally highly similar to CHEMBL1486934 and CHEMBL1402153 (*Table 3.6*), both of which are reported to interact with multiple proteins in human cells. The most relevant target is the sphingosine 1-phosphate receptor, the human orthologue of which is inhibited by CHEMBL1486934 with high affinity (<1 μM). Sphingosine 1-phosphate is known to act as a signalling molecule in plants, playing roles in calcium signalling, growth and cell death (Spiegel & Milstien, 2002), in accordance with the effects of S01E974935C on seedling growth and viability and the calcium oscillations observed during treatments with this chemical. CHEMBL1402153 is reported to potently inhibit the human TORC1 kinase, which positively regulates translation. However, S01E974935C does not appear to affect translation in plants as it induces the accumulation of HSP70B protein, while the TORC1 kinase inhibitor rapamycin is known to repress HSF1 activation in human cells (Chou *et al.*, 2012). Similarly, although S01E974935C shares some structural features with the herbicide F5231, which inhibits the chlorophyll biosynthesis enzyme protoporphyrinogen oxidase, chlorosis is not visible in seedlings treated with S01E974935C and thus the latter is unlikely to function analogously to F5231. Interestingly, the third most similar chemical to S01E974935C identified from the ChEMBL database, CHEMBL1367316, was found to be a potentiator of mouse HSF1.

Given their predicted electrophilicity, the enrichment of gene ontology terms associated with oxidative stress and their similarities to compounds known to disrupt chloroplast function, it was hypothesised that some of the *HSP70*-activators might activate *HSP70* expression in a manner dependent on redox stress. This possibility was tested by treating *A. thaliana* Col-0 seedlings simultaneously with these chemicals and with low concentrations of reductant, which quenches ROS *in vivo* and results in reduced oxidative stress-mediated induction of the HSR (Volkov *et al.*, 2006; Liu *et al.*, 2013a). As shown in *Figure 3.24*, the reducing agent dithiothreitol (DTT) abolishes the increase in *HSP70* expression triggered by DBMIB, consistent with the positive effect of this chemical on hydrogen peroxide levels in chloroplasts. Conversely, no effect on *HSP70* induction by 17-DMAG is observed in the presence of DTT, as expected based on

the derepression of HSF activity arising from inhibition of HSP90. DTT largely abolishes *HSP70* induction by S01A052379R and S04A100314B and causes a 2.6-fold decrease in induction by S01A052378F (*Figure 3.24*). These findings suggest that oxidative stress does play a role in the activity of these molecules. In contrast, DTT does not affect the responses to S01A463859Y and S01E974935C (*Figure 3.24*).

Based on their chemical structures, the majority of *HSP70*-repressors are predicted to be uncouplers (Fergus Earley, Syngenta, personal communication). Three chemical characteristics favour uncoupling activity, namely a dissociable proton, a strong electron-withdrawing group and a bulky hydrophobic moiety (Terada, 1990). The *HSP70*-repressors all contain hydrophobic carbon-heterocyclic groups, most contain electron-withdrawing trifluoromethyl or nitrile groups and all contain potentially dissociable protons from hydroxyl or secondary amine groups, some of the former of which are formed from carbonyl groups in aqueous solution. The cheminformatic analyses provide further support that S04A104169N and S04A259155K may have uncoupling activity, as they show structural similarity to the known uncouplers CHEMBL421036 (*Table 3.6*) and 4,5,6,7-tetrachloro-2-trifluoromethyl-benzimidazole (*Table 3.5*). In addition, three *HSP70*-repressors were confirmed to have uncoupler activity in uncoupling assays performed by Syngenta, including S01A289354A/Tralopyril as well as S04A104014F and S01A109956R (David Brocklehurst, Syngenta, personal communication).

Based on differences in their chemical structures, these molecules are likely to have different uncoupling strengths and may have additional biological targets. S04A104169N, for example, shares some structural features with the herbicide synergist tridiphane (*Table 3.5*), which inhibits glutathione S-transferases required for glutathione-mediated detoxification of ROS and xenobiotics (Hatzios, 1991). Accordingly, S04A104169N causes bleaching of seedlings even at a concentration of 0.1 ppm, unlike S01A289354A/Tralopyril, which was estimated to have a 10-fold lower IC_{50} value than S04A104169N based on dose-dependent inhibition of heat-induced *HSP70* expression. Though a likely uncoupler, S01E528454W has high structural similarity to CHEMBL1520808 and CHEMBL3718206 (*Table 3.6*), both of which interact with G-protein coupled receptors with high affinity. However, the biological relevance of potential *HSP70*-repressor interactions predicted from similar molecules in the ChEMBL database is questionable as uncoupler activity is likely to mask any other potential cellular effects, including expression levels of *HSP70* and chemical lethality.

S01E342213Q is of interest because it causes seedling lethality at 27˚C but not 17˚C. It is possible that it could be a weak uncoupler whose effects are exacerbated by

elevated growth temperatures. Based on results from the KEGG COMPOUND database, the two most similar molecules are thiopental and thiamylal (*Table 3.5*), both of which bind to and promote activation of the mammalian $GABA_A$ anion channel (Franco, 2008). Therefore, the altered levels of cytosolic calcium triggered by S01E342213Q (*Figure 3.19*) could potentially be a result of direct interactions with ion channels, independently of the potential weak uncoupling activity of this compound.

Finally, one compound that is unlikely to be an uncoupler is S01E932874G, as it lacks a strong electron-withdrawing group. Results from the ChEMBL database suggest that it is a kinase inhibitor (*Table 3.6*). This is consistent with the apparent genome-wide repression of transcription in *Figure 3.23*, as RNA polymerase II activity is tightly regulated by kinases (Oelgeschläger, 2002).

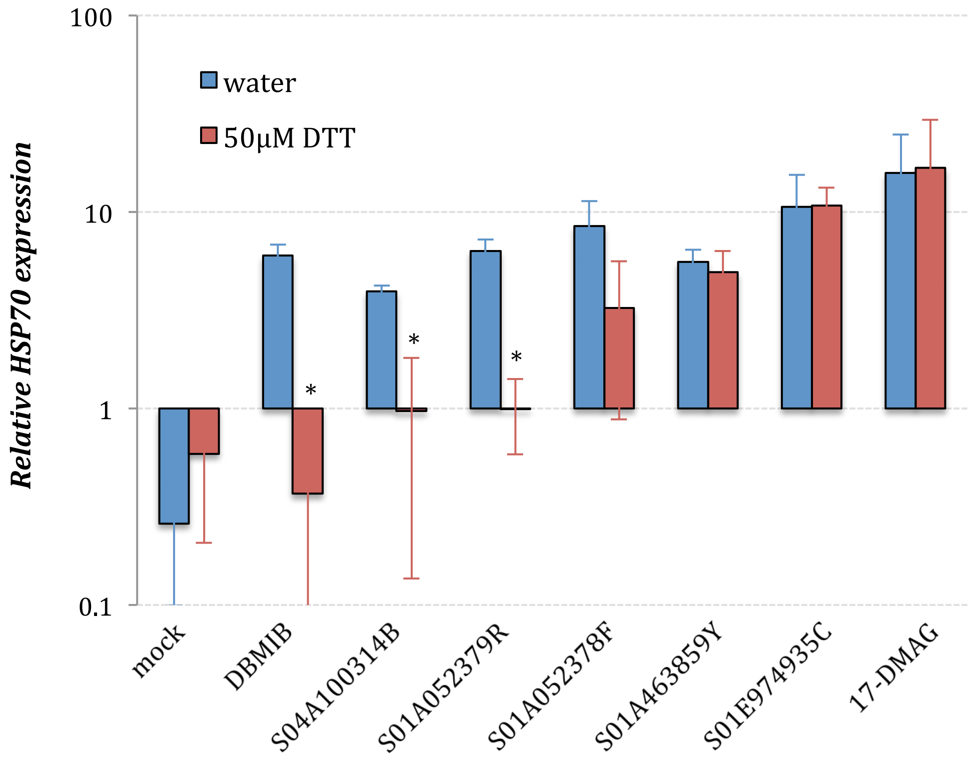


Figure 3.24: Effect of DTT on *HSP70* induction by *HSP70*-activators.
Seven-day-old liquid-grown *A. thaliana* Col-0 seedlings were co-treated with chemicals at 10 ppm (0.1% v/v DMSO for mock controls) and either water or 50 µM DTT for one hour at 22°C. Expression was normalised to *PP2A* and *UBC21*. Error bars represent standard deviation for three biological replicates, with 10-15 seedlings per replicate. Asterisks indicate significant differences between water and DTT treatments (two-tailed *t*-test, p-value <0.05).

3.3 Discussion

3.3.1 Identification of molecules affecting the expression of *HSP70*

Transcriptional responses of plants to elevated temperatures are complex and appear to involve multiple signalling pathways (Misra *et al.*, 2007; Suri & Dhindsa, 2008; Xuan *et al.*, 2010; Hahn *et al.*, 2011; Gao *et al.*, 2012; Zheng *et al.*, 2012; Sun & Guo, 2016). A large number of proteins that regulate the activity of the conserved heat shock factor (HSF) transcriptional regulators have been identified in mammalian cells, whereas relatively few HSF-interacting proteins have been described in plant cells. To identify potential novel regulators of warm temperature signalling pathways in plants, a chemical genomics screen was carried out in collaboration with Syngenta.

Two primary screens were performed to isolate molecules activating or repressing the transcriptional response to heat shock, using the heat-inducible bioluminescent reporter *pHSP70::LUC* to measure the heat signalling status of *A. thaliana* seedlings (Kumar & Wigge, 2010). This reporter was previously used in classical genetics screens to identify mutants with misregulation of warm temperature marker genes (Kumar & Wigge, 2010; Dickinson *et al.*, 2018), however it was questionable whether this reporter could be used with the Syngenta chemical library to study warm temperature signalling pathways. *HSP70* expression is induced by numerous stresses other than heat shock, though the dynamics of expression vary. These include oxidative, cold, osmotic, salt or UV treatments, *Pseudomonas syringae* or elicitor infiltrations, and applications with hormone inhibitors TIBA or $AgNO_3$ or with the photosynthesis inhibitor PNO8 (Sung *et al.*, 2001; Swindell *et al.*, 2007; Kilian *et al.*, 2007).

Unlike classical genetics, in which harmful mutations are automatically selected against, chemical treatments may bring about cytotoxic effects that could either induce general stress pathways or block transcriptional responses, leading to a high discovery rate of false positives for activators or repressors of *HSP70* expression, respectively. The proportion of chemicals found to affect the *pHSP70::LUC* reporter was surprisingly small, given the potential for chemical toxicity and the relatively high concentration (10 ppm) at which the primary screens were carried out. From over 3000 chemicals, approximately 1.5% were found to activate the *pHSP70::LUC* reporter in the absence of heat shock and an equivalent proportion repressed its inducibility during heat shock.

Expression analyses revealed that only a third of candidates from the screen induced or diminished *HSP70* expression within one hour of treatment. This suggests

that the activator screen predominantly identified chemicals with indirect effects on HSF regulation, for example by triggering proteotoxic or oxidative stress. Supporting this idea is the observation that toxic compounds, such as those containing heavy metals, induce the HSR with slower kinetics than do *HSP90* inhibitors (West *et al.*, 2012). Selecting only those chemicals with rapid effects is likely to enrich for activators with direct rather than indirect effects on *HSP70* expression. The majority of molecules identified from the repressor screen appear to act at the protein level, perhaps blocking protein synthesis or enzyme activity. As a large number of chemicals were found to reduce absolute luminescence rather than heat-induced luminescence, it is likely many of these chemicals inhibit vital biological processes, resulting in loss of luciferase expression.

Chemical lethality assays showed that all of the *HSP70*-repressors and three of the five *HSP70*-activators decreased seedling viability at high concentrations. On the one hand, such lethality may be expected from chemical modifiers of the HSR, since the expression of *HEAT SHOCK PROTEIN* (*HSP*) genes is highly regulated and both knockdown mutations or overexpression of *HSP70* or *HSF* genes can compromise viability, growth and development (Sung & Guy, 2003; Ogawa *et al.*, 2007; Liu *et al.*, 2011). In contrast, the known *HSP70*-activators DBMIB, puromycin and 17-DMAG were not found to cause seedling lethality, even at high concentrations. These compounds trigger the upregulation of plastid ROS, induce the misfolding of nascent peptides or inhibit the ATPase activity of HSP90, respectively. However, they do not directly target HSP70 or HSF proteins. Based on the possibility that temperature signalling components may be essential for cell survival, chemicals that reduced seedling viability were not excluded from further analyses.

3.3.2 Investigating the potential biological targets of candidate molecules

Some of the pitfalls of the chemical genomics approach were highlighted in this project. A clear limitation in the screen was the screening phenotype, namely the lack of sensitivity of the *pHSP70::LUC* reporter in differentiating between molecules specifically affecting warm temperature signalling and those affecting general stress responses. Another fundamental obstacle associated with chemical genomics is identifying the biological targets of compounds.

Because classical genetics or proteomic approaches to target identification were not feasible for this project, a combination of experimental and cheminformatic analyses were used to build hypotheses as to how the chemicals induce or repress warm

temperature signalling pathways. The potency and dynamics of chemical effects in *A. thaliana* were assessed by expression analyses of *HSP70*. Transcriptional responses in this dicotyledonous plant were compared with those in the monocotyledon *B. distachyon* and the unicellular fungus *S. cerevisiae*, to test for conserved effects across phylogenetic taxa. Impacts of chemical treatments on plant morphology and viability were investigated by growth assays with *A. thaliana* seedlings in media supplemented with compounds at various concentrations. Effects on seedling viability were further studied using thermotolerance assays, to determine whether *HSP70*-activating chemicals could enhance heat resilience. Cytosolic calcium was monitored in *A. thaliana* plants during treatments to investigate potential secondary messenger responses to the compounds. Transcriptome sequencing was carried out to characterise genome-wide effects of treatments, and finally, chemical structures were compared to known compounds from public databases to predict potential modes of action.

Transcriptomics can be an invaluable tool to characterise mutants identified by classical genetics, but it may not necessarily provide any information regarding the biological targets of compounds identified by chemical genomics, because of pleiotropic effects of chemical treatments. The gene ontology terms enriched for genes induced by S03A109616K/cycloheximide and S01A289354A/Tralopyril, for example, do not evidently suggest mechanisms of protein synthesis inhibition and mitochondrial uncoupling, respectively. Accordingly, the RNA sequencing dataset provided limited information on the cellular interactions of candidates from the screen, except for S01E932874G, which appears to cause genome-wide down-regulation of gene expression, suggesting that it is an inhibitor of RNA polymerase. Nevertheless, hierarchical clustering of transcriptomes provided evidence that most *HSP70*-repressors are not specific inhibitors of the HSR, as the transcriptomes of seedlings treated with these chemicals tended to cluster separately from those of mock-treated plants at either 22˚C or 30˚C. In addition, gene ontology analyses for loci upregulated by chemical treatments provided evidence that many of these molecules may be uncouplers, as they triggered the expression of genes that regulate carbon flux into phenylpropanoid metabolism according to sugar availability (Zhang *et al.*, 2013), consistent with defects in respiration as a result of chloroplast or mitochondrial dysfunction.

Cheminformatic searches of structurally similar compounds provided the strongest indications of potential modes of action. The main limitation of this approach, however, is that overall similarity between two compounds can be a poor predictor of their shared interactions. For example, the translation inhibitors cycloheximide and phyllanthoside bind to the same site in eukaryotic ribosomes but are structurally very

different (Garreau de Loubresse *et al.*, 2014). In contrast, slight modifications of groups in cycloheximide can completely alter its activity, as demonstrated by the inactive analogues anhydrocycloheximide, lacking the hydroxyl group of cycloheximide, and cycloheximideoxime, containing a ketoxime group instead of a ketone group in the cyclohexanone moiety (Wheatley & Oaks, 1978). Testing analogues of candidates from the *pHSP70::LUC* screen for *HSP70* induction or repression would provide useful information on chemical moieties that are important for activity and would allow the list of similar compounds to be narrowed down.

While the ChEMBL database provides useful empirical data of protein interactions, the targets bound with the highest affinity in mammalian cells do not necessarily represent proteins that would be bound by the compounds in plant cells, because of evolutionary differences between these organisms. Given the bias for proteins of medical interest to human health, the data likely represent a small subset of potential interactions that could occur in plants, particularly at the high concentration used for chemical treatments, which can promote lower-affinity, off-target interactions.

Nevertheless, the cheminformatic analyses were useful in providing potential protein targets, the most likely of which could be selected based on information from the above experiments. From these analyses, three of the five *HSP70*-activating chemicals (S04A100314B, S01A052378F, S01A052379R) are predicted to affect cellular or plastid redox homeostasis, the latter two of which might trigger retrograde signalling from chloroplasts. The other two *HSP70*-activators (S01A463859Y, S01E974935C) may also interact with components of signalling pathways, though the mechanisms by which they differentially regulate the HSR and thermotolerance are currently unclear.

Among *HSP70*-repressing chemicals, one was found to be the translation inhibitor cycloheximide (S03A109616K) and another is predicted to be a kinase inhibitor that causes transcriptional repression by inhibiting RNA polymerase kinases (S01E932874G). S01A305483C was not included in the above analyses because of insufficient supply of the compound but is predicted to be a reductant based on its structure (Katrin Hermann, Syngenta, personal communication), which would explain its mild repression of *HSP70* expression during heat shock, similar to that observed with the reducing agent dithiothreitol (DTT) (Volkov *et al.*, 2006). All other *HSP70*-repressors are predicted to be membrane uncouplers, of which S04A104014F, S01A109956R and S01A289354A have confirmed protonophore activity. Potential cellular interactions for the above chemicals are discussed below and summarised in *Figure 3.25*.

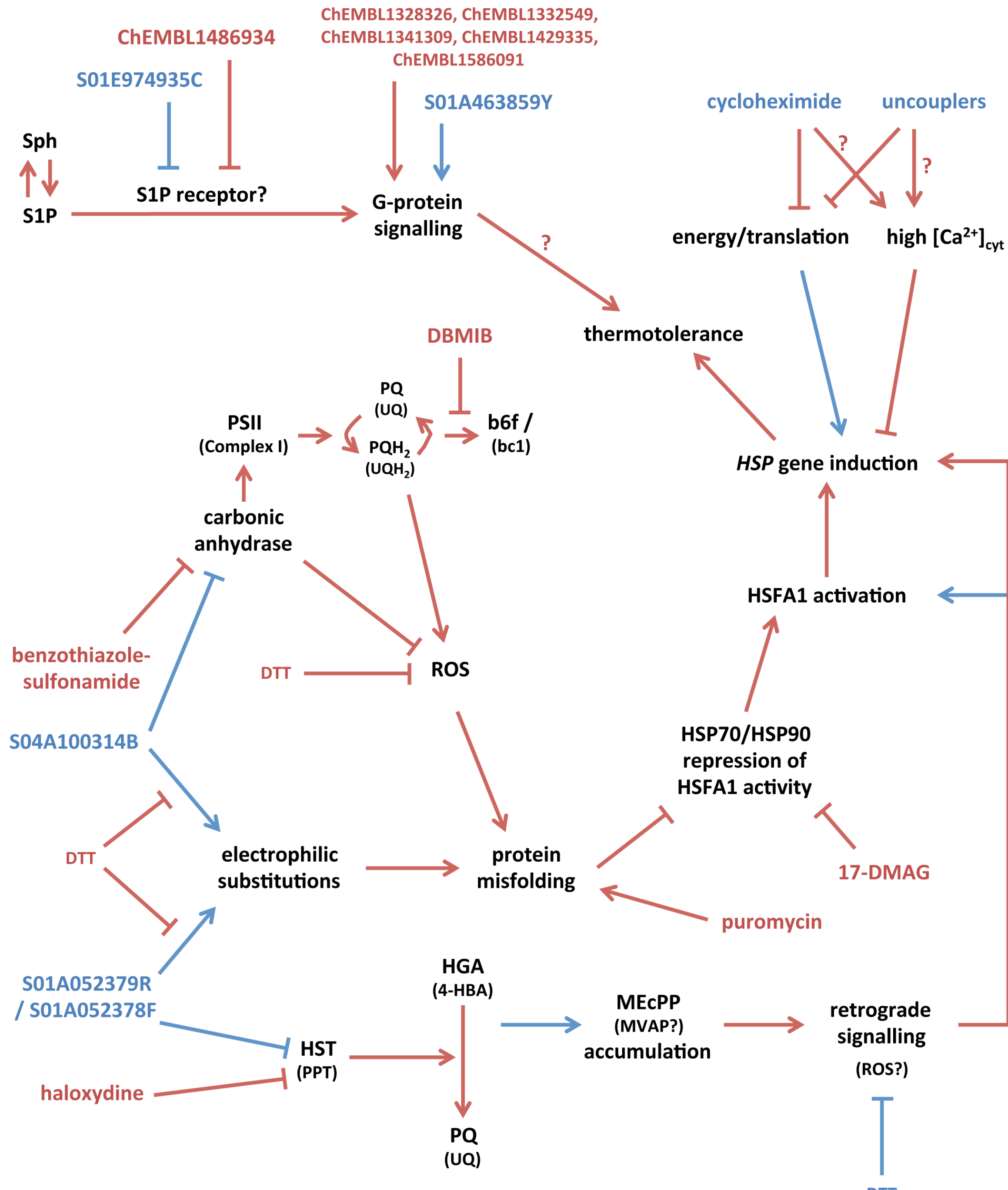


Figure 3.25: Potential modes of action of chemicals identified from the *pHSP70::LUC* screen. Compounds from the Syngenta chemical library are indicated in blue, whereas compounds from the literature or ChEMBL database are indicated in red. Pointed arrows represent activation and blunt arrows represent repression. Blue arrows indicate hypothetical interactions, whereas red arrows indicate interactions that have been demonstrated either in the literature or in this study. Mitochondrial equivalents to chloroplast components or metabolites are indicated in parentheses. Question marks represent hypothetical components or unknown mechanisms.
$[Ca^{2+}]_{cyt}$: cytosolic calcium concentration; 4-HBA: 4-hydroxybenzoate acid; DTT: dithiothreitol; HGA: homogentisic acid; HST: homogentisate solanesyltransferase; MEcPP: methylerythritol cyclodiphosphate; MVAP: mevalonate-5-phosphate; PPT: 4-hydroxybenzoate polyprenyl transferase; $PQ(H_2)$: (reduced) plastoquinone; ROS: reactive oxygen species; S1P: sphingosine 1-phosphate; Sph: sphingosine; $UQ(H_2)$: (reduced) ubiquinone.

3.3.3 Regulation of redox homeostasis by carbonic anhydrases

The simplest explanation for the induction of *HSP70* expression by S04A100314B is that it could be a weak electrophile. Given its electrophilicity, the chlorine atom in this compound is likely to attract electrons from the sulfonyl group, resulting in a localised positive charge. Such molecules can react with nucleophiles, such as the thiol group of cysteine residues or the amine group of lysine or histidine residues (West *et al.*, 2012). These oxidative interactions can cause protein misfolding or cross-linking, which may activate HSF transcription factors by sequestering HSP70 and HSP90 proteins. Electrophiles can also activate the HSR in a direct manner by reacting with chaperones (Wang *et al.*, 2012b), which are among the most abundant of cellular proteins (Sreedhar *et al.*, 2004).

The reducing agent DTT has previously been demonstrated to repress the activation of the HSR by heat (Liu *et al.*, 2013a) and by thiol-reactive molecules, such as iodoacetamide (Liu *et al.*, 1996) and celastrol (Trott *et al.*, 2008). Likewise, the induction of *HSP70* by S04A100314B was largely abolished in the presence of DTT. Organic electrophiles have been shown to enhance thermotolerance, for example yeast cells treated with celastrol for one hour prior to severe heat shock at 47˚C for 20 minutes are able to resume growth, whereas a complete loss of viability is observed in untreated cells under these conditions (Trott *et al.*, 2008). In contrast, S04A100314B reduced thermotolerance in *A. thaliana*, even at low concentrations (1 ppm) that are not lethal to seedlings. This suggests that this chemical might have other cellular interactions.

S04A100314B is structurally similar to the carbonic anhydrase inhibitor benzothiazole-sulfonamide. Such activity could account for the redox-dependent activation of *HSP70* expression by S04A100314B and its negative impact on heat resilience. Carbonic anhydrases play vital roles in photosynthesis (Suorsa & Aro, 2007; Ferreira *et al.*, 2008) and amino acid biosynthesis (DiMario *et al.*, 2016) and they are components of the electron transport chain in mitochondria and chloroplasts (Ferreira *et al.*, 2008; Soto *et al.*, 2015). Repressed carbonic anhydrase activity in mitochondria is associated with elevated levels of ROS (Soto *et al.*, 2015; Córdoba *et al.*, 2016; Zhang *et al.*, 2018), while overexpression of mitochondrial carbonic anhydrase has been shown to reduce ROS levels in *A. thaliana* (Villarreal *et al.*, 2009). This has been attributed to lower activity of Complex I, which requires mitochondrial carbonic anhydrases for its assembly in plants. As a result of lower NADH dehydrogenase activity, the ratio of NADH to NAD^+ is increased and this enhances the production of superoxide radicals (Soto *et al.*, 2015). In addition, loss of carbonic anhydrase activity results in reduced intracellular

availability of carbon dioxide, thereby increasing photorespiration, in which the by-product glycolate is oxidised to glyoxylate and hydrogen peroxide (Soto *et al.*, 2015).

Similarly, chloroplast carbonic anhydrases have been implicated in regulation of ROS levels in response to high light stress (Rudenko *et al.*, 2017, 2018), and null mutations in either mitochondrial or chloroplast enzymes result in reduced viability (Yi *et al.*, 2005; Ferreira *et al.*, 2008; Wang *et al.*, 2012a; Córdoba *et al.*, 2016; Fromm *et al.*, 2016). Tianpei *et al.* (2015) have shown that heat stress upregulates the expression of a carbonic anhydrase gene in rice and that heterologous expression of this gene in *Escherichia coli* enhances thermotolerance, suggesting that these enzymes play important roles in responses to heat shock. These findings are in accordance with the effects of S04A100314B on seedling lethality at high concentrations and suggest that this chemical induces the heat shock response and reduces thermotolerance in *A. thaliana via* over-production of ROS. In addition, the role of carbonic anhydrases in regulation of ROS levels appears to be conserved in yeast, as the carbonic anhydrase mutant *nce103* (*non-classical export 103*) is hypersensitive to hydrogen peroxide (Götz *et al.*, 1999), consistent with the effects of S04A100314B on *HSP70* expression in *S. cerevisiae*.

3.3.4 Regulation of stress genes by intermediates of chloroplast metabolites

S01A052378F and S01A052379R are structural analogues, the latter containing two chlorine substitutions of fluorine atoms in S01A052378F, and both were identified as activators of *HSP70* expression. S01A052378F and to a lesser extent S01A052379R could potentially act as weak electrophiles *in vivo*, as their electrophilic fluorine or chlorine atoms may result in a positive charge at the opposing end of the molecules. Indeed, co-treatment with DTT reduced the induction of *HSP70* expression by these chemicals. However, these compounds decreased thermotolerance, similarly to S04A100314B, and they also caused chlorosis in *Arabidopsis* seedlings, suggesting that they have alternative or additional activities in plants and likely interfere with chloroplast function.

S01A052378F is structurally similar to haloxydine, an inhibitor of homogentisate solanesyl transferase (HST), the latter of which is required for plastoquinone biosynthesis in chloroplasts. Plastoquinones (plastoquinols, in their reduced form) are essential electron carriers necessary for channelling electrons from PSII to the cytochrome b6f complex in the chloroplast electron transport chain and for oxidation of phytoene in the carotenoid biosynthesis pathway (Norris *et al.*, 1995; Sadre

et al., 2010). A diminished plastoquinone pool is likely to trigger the production of ROS, as a result of over-excitation of PSII caused by disruptions to the electron transport chain and to carotenoid-dependent energy dissipation.

In addition, it is likely that inhibition of HST leads to the build-up of intermediates of the plastoquinone biosynthesis pathway, such as methylerythritol cyclodiphosphate (MEcPP). This metabolite is thought to be involved in retrograde signalling from chloroplasts to the nucleus. Accumulation of MEcPP in the *A. thaliana ceh1* (*constitutively expressing HPL*) mutant, defective in an upstream enzyme in the same pathway (1-hydroxy-2-methyl-2-[E]-butenyl 4-diphosphate synthase, HDS), is associated with upregulation of stress-associated nuclear genes (Xiao *et al.*, 2012). Although the mechanism of gene regulation is unknown, MEcPP has been shown to trigger nucleoid decondensation in bacteria (Grieshaber *et al.*, 2004), suggesting that it might affect chromatin dynamics in plants. Similarly, the retrograde signalling molecules heme and Mg-protoporphyrin IX were found to trigger the upregulation of nuclear stress genes in the alga *Chlamydomonas reinhardtii*, half of which were also induced by heat shock (Voss *et al.*, 2011). It is therefore possible that the inhibition of HST by haloxydine or S01A052378F may cause an accumulation of plastoquinone biosynthesis intermediates that trigger the upregulation of *HSP70* expression. This may account for the observation that S01A052378F induces *HSP70* expression over ten-fold even in the presence of DTT.

Retrograde signalling may also be mediated directly *via* ROS, for example *via* stromules that link chloroplasts to the nucleus. These structures form upon chloroplast stress (Brunkard *et al.*, 2015) and have recently been found to carry hydrogen peroxide (Caplan *et al.*, 2015). Given the repressive effects of DTT on *HSP70* induction by S01A052378F and S01A052379R, and the chlorosis observed in seedlings treated with these chemicals, it is possible that chloroplast stress triggered by the inhibition of HST may trigger ROS-dependent retrograde signalling.

Sadre *et al.* (2010) proposed that haloxydine inhibits HST by structurally mimicking homogentisate, which would imply that it might also inhibit 4-hydroxybenzoate polyprenyl transferase (PPT), a mitochondrial enzyme required for the biosynthesis of ubiquinone from the homogentisate analogue 4-hydroxybenzoate acid (4-HBA). S01A052378F and S01A052379R could therefore potentially trigger stress-responsive genes *via* retrograde signalling from mitochondria. This would provide one possible explanation for their potent activation of *HSP70* in yeast cells. Alternatively, the latter may simply be a result of their propensity to react with thiol or amine groups in peptides. It is intriguing that although S01A052378F and S01A052379R

are equally potent activators of *HSP70* expression, the latter is far more cytotoxic than S01A052378F in *A. thaliana*, as observed in chemical lethality assays. This highlights the requirement for further analyses to better understand the mechanisms by which these compounds act in plants.

3.3.5 G-protein-associated signalling in plant abiotic stress responses

S01A463859Y and S01E974935C were found to induce *HSP70* expression independently of ROS and were predicted to interact with receptor complexes or signalling regulators, based on similar compounds from the ChEMBL database. The most similar chemical identified for S01A463859Y (CHEMBL99210) binds to a mammalian anion channel complex known as the $GABA_A$/benzodiazepine receptor, which is activated by γ-aminobutyric acid (GABA). Though no homologues to mammalian $GABA_A$ receptors are found in plant cells, the latter do contain both GABA-regulated ion channels (Ramesh *et al.*, 2015) and GABA-independent benzodiazepine receptor-like proteins (Lindemann *et al.*, 2004). Despite the fact that GABA accumulates at extreme temperatures in plants (Kinnersley & Turano, 2000) and promotes thermotolerance (Li *et al.*, 2016), as does S01A463859Y, CHEMBL99210 is reported to bind to the benzodiazepine receptor moiety rather than the $GABA_A$ channel moiety of the complex, and therefore it is unlikely that S01A463859Y influences GABA signalling in plants.

The *A. thaliana* protein TspO (TRYPTOPHAN-RICH SENSORY PROTEIN O) is homologous to the mammalian peripheral-type benzodiazepine receptor (PBR) (Lindemann *et al.*, 2004) and could therefore be potentially bound by S01A463859Y. The *TSPO* gene is upregulated by abiotic stress (Guillaumot *et al.*, 2009; Hachez *et al.*, 2014) and its protein localises to chloroplasts, mitochondria, the endoplasmic reticulum and the Golgi network (Guillaumot *et al.*, 2009; Balsemão-Pires *et al.*, 2011) and functions to sequester excess heme, preventing its interaction with heme-peroxidases and NADPH oxidases that could generate superoxide radicals (Balsemão-Pires *et al.*, 2011; Vanhee *et al.*, 2011). Such an interaction, however, would not explain the ROS-independent activation of *HSP70* expression observed for S01A463859Y, and furthermore CHEMBL99210 is reported to bind only weakly to the mammalian benzodiazepine receptor, raising the question of whether this potential interaction would be biologically relevant in plant cells.

A number of other chemicals with high similarity to S01A463859Y from the ChEMBL database were reported to potently bind and activate G-protein α subunits. Interestingly, the most similar compound to S01E974935C (CHEMBL1486934) binds

and represses a mammalian G-protein-coupled receptor (GPCR), the sphingosine 1-phosphate receptor. Little is known about the involvement of G-proteins in heat shock responses in plants. One study has shown that genes encoding G-protein α and β subunits are induced by heat and hydrogen peroxide in pea plants and that their overexpression in tobacco enhances thermotolerance (Misra *et al.*, 2007). In contrast, Chakraborty *et al.* (2015) have reported that *A. thaliana* mutants of the G-protein subunit α GPA1 (G-PROTEIN ALPHA SUBUNIT 1) and the GPCR GCR1 (G-PROTEIN-COUPLED RECEPTOR 1) have lower levels of oxidative damage during heat shock.

While no homologues of mammalian sphingosine 1-phosphate receptors have been identified in plant genomes (Spiegel & Milstien, 2002), the signalling molecule sphingosine 1-phosphate (S1P) is involved in abiotic stress responses and appears to act in a G-protein-dependent manner. S1P accumulates during drought stress and triggers stomatal closure *via* calcium mobilisation and GPA1 activity (Ng *et al.*, 2001; Coursol *et al.*, 2003). In budding yeast, the mutants of the S1P phosphatases *LBP1/YSR2* and *LBP2/YSR3* (LONG-CHAIN BASE PHOSPHATASE 1/2 / YEAST SPHINGOSINE RESISTANCE 2/3), accumulate phosphorylated long-chain sphingoid bases and have elevated thermotolerance, whereas their overexpression causes a reduction in thermotolerance (Mandala *et al.*, 1998; Mao *et al.*, 1999). Furthermore, Dickson *et al.* (1997) have shown that exogenous sphingolipid treatments can activate heat-inducible promoters, though the mechanism behind this transcriptional response is unknown.

In addition to regulating calcium mobilisation and thermotolerance, phosphorylated sphingolipids are known to promote growth and suppress cell death (Spiegel & Milstien, 2002; Liang *et al.*, 2003). Considering its structural similarity to an inhibitor of the mammalian S1P receptor and its effect on cytosolic calcium levels, seedling size and viability, hypocotyl elongation, thermotolerance and *HSP70* expression in both plants and yeast, it is tempting to speculate that S01E974935C may interfere with S1P signalling. However, it is unclear why inhibition of S1P signalling by S01E974935C would reduce thermotolerance yet induce the HSR. In addition, it is unknown whether seedling lethality and compromised thermotolerance brought about by S01E974935C treatments are signalled responses, or whether these effects are simply a result chemical toxicity. Furthermore, the absence of a S1P receptor homologue in plants raises the question as to how this signalling molecule is perceived in *A. thaliana*.

Although S01A463859Y had a positive effect on thermotolerance, consistent with the effects of over-active G-protein α activity reported by Misra *et al.* (2007) in tobacco, the potential involvement of this compound in G-protein signalling in *A.*

thaliana is based solely on its structural similarity to agonists of human G-protein α. While S01A463859Y induced *HSP70* expression in plants, no effect was observed in yeast cells, even though they also contain Gα proteins. While there are clearly many unanswered questions regarding the activities of S01E974935C and S01A463859Y, the cheminformatic analyses provide a basis for further investigation into the modes of action of these two compounds, both of which have interesting effects as potential regulators of the heat shock response.

3.3.6 Repression of the heat shock response by translation inhibition and protonophore activity

The inhibitory effect of S03A109616K/cycloheximide on the expression of heat-inducible genes is intriguing, as RNA-sequencing showed that many genes are upregulated by this chemical. Short pre-treatments with cycloheximide (45 minutes or less) do not severely affect *HSP70* induction during 37˚C heat shock whereas a 60-minute pre-treatment largely abolishes this response. This is in accordance with previous studies showing that induction of heat-inducible gene expression is unaffected by short cycloheximide treatments up to 30 minutes in other organisms (Ashburner, 1970; Kropat *et al.*, 1995), even though *de novo* protein synthesis is rapidly inhibited, within several minutes of treatment (Zimarino & Wu, 1987). Schneider-Poetsch *et al.* (2010) have found that cycloheximide represses transcription in mammalian cells incubated for two hours at concentrations greater than 10 μM. This appears to be a secondary effect arising from inhibition of translation, rather than direct interaction between cycloheximide and transcriptional machinery, as a single point mutation in ribosomal protein RPL28 (RIBOSOMAL PROTEIN LARGE SUBUNIT 28) allows yeast cells to thrive at high concentrations of cycloheximide (Huang *et al.*, 2013). It is unlikely, however, that this is the result of rapid protein turnover of HSF proteins, because even six-hour cycloheximide treatments were not found to diminish protein levels of HSF1 in mammalian cells (Santagata *et al.*, 2013), and components of transcriptional machinery in yeast and mammals have half-lives of at least two hours (Belle *et al.*, 2006; Schwanhäusser *et al.*, 2011).

The inhibition of *HSP70* expression by cycloheximide coincides with increasing cytosolic calcium levels and a diminished heat-induced influx of calcium ions. One possibility is that protein synthesis inhibition causes the internalisation of heat-responsive calcium channels that are required for the HSR, as has been shown for the PIN2 (PIN-FORMED 2) auxin transporter following cycloheximide treatment (Jásik *et al.*,

2013). Gao *et al.* (2012) have demonstrated that a knock-out mutation in *CNGC6* in *A. thaliana* decreases the expression of *HSP* genes, while overexpression of *CNGC6* increases their expression. Decreased plasma membrane-association of such calcium channels could trigger the activation of other channels, resulting in the increased cytosolic calcium levels observed during cycloheximide treatment. A null-mutation of the heat-activated calcium channel CNGCb in *Physcomitrella patens*, for example, increases the likelihood of two other channels opening (Finka *et al.*, 2012). Sustained high levels of cytosolic calcium have been shown to repress HSF1 activity in human cells (Soncin *et al.*, 2000).

An alternative possibility is that cycloheximide-mediated repression of heat-inducible genes is a signalled response, whereby active translation is required for full induction of these genes. This hypothesis has been proposed in human cells to account for the repressive effects of various translation inhibitors on the heat shock response. Santagata *et al.* (2013) suggested that the extent of ribosome activity reflects the available energy in cells and regulates HSF-mediated transcriptional responses accordingly. Such regulation would not be surprising, given that the heat shock response is an energy-intensive process, requiring a large pool of ATP for the chaperone-dependent refolding of damaged proteins.

Another indication that HSF activity may be regulated by the energy status of cells is demonstrated by the identification of uncouplers as potent repressors of the heat shock response. These chemicals remain uncharged under acidic conditions, such as those of the apoplast, thylakoid lumen or mitochondrial intermembranal space, and this favours their diffusion across cellular membranes. However, upon entering cellular compartments with higher pH, such as the cytosol, chloroplast stroma or mitochondrial matrix, these weak acids gain negative charge *via* proton dissociation. As a result, uncouplers diffuse across membranes, shuttling protons down their concentration gradient and preventing synthesis of ATP in chloroplasts and mitochondria. Although uncouplers repressed the heat-induction of *HSP70* expression, many of these chemicals upregulated stress-associated genes in the absence of heat, including genes associated with low energy responses, such as the Kelch repeat F-box (KFB) genes (Zhang *et al.*, 2013).

In contrast to the effects observed in plants, these uncouplers did not repress *HSP70* induction during heat shock in yeast cells after 15-minute chemical treatments, even though pre-treatments of similar durations in *A. thaliana* largely abolished *HSP70* expression. This could be explained by the larger proton gradient between the chloroplast lumen and stroma than between the mitochondrial intermembrane space

and matrix, with up to 30,000-fold and 10-fold differences in proton concentrations, respectively (Jagendorf & Uribe, 1966; Porcelli *et al.*, 2005). Pyatrikas *et al.* (2014), however, have demonstrated that a two-hour treatment with the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) activates *HSP101* expression in yeast cells yet decreases its induction during heat shock. These data implicate chloroplasts and mitochondria in the regulation of the heat shock response.

Contrary to cycloheximide, the repressive effects of uncouplers on *HSP70* expression are observed extremely rapidly in *A. thaliana*, within 15 minutes of chemical treatment. This may be a result of altered levels of cytosolic pH and calcium, the latter of which was detected during chemical treatment using the calcium reporter aequorin. The effects of uncouplers on cytosolic calcium have previously been described in mammalian cells and result from membrane depolarisation followed by activation of voltage-gated calcium channels (Buckler & Vaughan-Jones, 1998; Vaur *et al.*, 2000). While a transient increase in calcium is detected in the cytosol during heat treatment and contributes to heat stress signalling (Gong *et al.*, 1998; Suri & Dhindsa, 2008; Saidi *et al.*, 2009; Gao *et al.*, 2012), sustained high levels of cytosolic calcium triggered by cycloheximide or uncouplers may repress HSFA1 activity (Soncin *et al.*, 2000).

Increases in cytosolic calcium concentrations triggered by uncouplers may simply be a consequence of their protonophore activity, which disrupts cellular ion homeostasis and causes membrane depolarisation. Alternatively, this may be a signalled event from organelles, which acts to repress the heat shock response to conserve cellular energy. In support of this hypothesis, inhibitors of mitochondrial ATP synthase or of the mitochondrial electron transport chain also trigger membrane depolarisation and calcium mobilisation in mammalian cells (Wyatt & Buckler, 2004).

3.3.7 Thermotolerance analyses

A thermotolerance assay based on green content of *A. thaliana* seedlings in 96-well plates was developed for testing whether *HSP70*-activating chemicals could enhance survival of lethal heat shock. While these compounds induce many thermotolerance marker genes, including the *sHSP* and *HSP100* gene families, three of the five chemicals were found to reduce seedling viability and a fourth caused chlorosis at high concentrations. It was therefore not surprising that these molecules did not promote thermotolerance but in many cases diminished seedling green area relative to mock controls. Very slight increases in thermotolerance were detected for S01A463859Y (1 ppm, three hours), the only candidate from the *pHSP70::LUC* screen

that did not have any signs of cytotoxicity, as well as for 17-DMAG (0.5 ppm, 24 hours; and 2.5 ppm, three hours) and puromycin (2.5 ppm, 24 hours), while a large increase in thermotolerance was observed for DBMIB (10 ppm, 24 hours).

Using a second assay based on photosynthetic efficiency, larger positive effects on thermotolerance were measured for S01A463859Y, as well as for DBMIB and puromycin. Discrepancies in chemical effects between the two assays could be explained by the different durations of recovery of the seedlings. Strong effects on photosynthesis detected 24 hours after heat treatment may have subsided after seven days of recovery, because unlike young seedlings two-week-old plants grown in liquid medium start to exhibit chlorosis, with thin, folded leaves, and eventually die. The assay could be improved by decreasing the periods of growth before and after chemical and heat treatments or improving the growth conditions of liquid-grown seedlings, for example by supplementing the growth medium with sucrose or simply shaking the plates to provide better gas exchange.

The fact that pre-treatments with geldanamycin have previously been shown to have large positive effects on viability during lethal heat shock in *A. thaliana* seedlings (Yamada *et al.*, 2007), whereas only small enhancements to green content were measured in thermotolerance assays with 17-DMAG, may be attributed to differences in the duration and concentration of chemical treatments. In their thermotolerance assays, Yamada and colleagues used six-hour treatments with 50 µM geldanamycin, rather than three-hour treatments at 5 µM (2.5 ppm).

While 24-hour treatments with DBMIB at 10 ppm were found to protect seedlings from lethal heat shock, treatments at 1 ppm had the opposite effect. This might be explained by the fact that at concentrations higher than 10 µM DBMIB acts as an electron carrier and increases hydrogen peroxide levels in chloroplasts, whereas at lower concentrations it blocks the flow of electrons from PSII to the b6f complex in the chloroplast electron transport chain, thereby inhibiting photosynthesis (Gould & Izawa, 1973; Karpinski *et al.*, 1997; Pfannschmidt *et al.*, 2001; Mühlenbock *et al.*, 2008). While DBMIB, S01A052378F, S01A052379R and S04A100314B all appear to trigger ROS production in seedlings, the latter three chemicals are likely to do so by inhibiting essential organellar enzymes, while DBMIB does not cause any reductions in viability. It is currently unclear why three-hour pre-treatments with S01A463859Y improve thermotolerance at 1 ppm but not at 10 ppm, given that neither concentration yields toxic effects in seedlings. Nevertheless, given their enhancing effects on the HSR, DBMIB, 17-DMAG and S01A463859Y are all promising candidates for chemical protection of crops from heat stress.

Chapter 4

Induction of *CBF* expression by translation inhibitors

4.1 Introduction

Plant cells may be exposed to large temperature extremes, unlike most mammalian cells, and are thus likely to have evolved sophisticated mechanisms of perceiving cold stimuli. Although a large number of proteins have been identified as regulators of basal or cold-induced expression of the CBF (C-REPEAT BINDING FACTOR) transcription factors, the means by which low temperatures affect the activity of these proteins is unknown. The key *CBF*-activators ICE1 (INDUCER OF *CBF* EXPRESSION 1) and CAMTAs (CALMODULIN-BINDING TRANSCRIPTION ACTIVATOR), for example, are constitutively expressed and nuclear-localised but only activate *CBF* expression upon cold exposure (Chinnusamy *et al.*, 2003; Kidokoro *et al.*, 2017).

A number of plant-derived molecules have been found to specifically activate cold-responsive TRP (TRANSIENT RECEPTOR POTENTIAL) ion channels in mammalian cells. These include menthol, eucaliptol, linalool and geraniol for TRPM8, which is activated at temperatures below 25˚C, and menthol, allicin and cannabinol for TRPA1, which is activated below 17˚C (Belvisi *et al.*, 2011). Though such interactions with low-temperature sensors have not yet been described in plants, chemical genetics has provided an interesting insight into the regulation of cold-responsive genes. Cycloheximide, which blocks the elongation step of translation, has been shown to induce *CBF* expression in *Arabidopsis thaliana* (Zarka *et al.*, 2003). Similarly, cycloheximide was reported to induce the expression of the three cold-responsive genes *MLIP15* (*MAIZE LOW-TEMPERATURE INDUCED PROTEIN 15*), *CDPK1* (*CALCIUM DEPENDENT PROTEIN KINASE 1*) and *ADH1* (*ALCOHOL DEHYDROGENASE 1*) in maize (Berberich & Kusano, 1997). The mechanism by which cycloheximide induces the expression of these cold genes is currently unknown.

This project investigates how cycloheximide regulates *CBF* expression in *A. thaliana*. The objectives are as follows:

1. To characterise the transcriptional response to cycloheximide.
2. To identify regulators of cycloheximide-induced *CBF* expression.
3. To characterise the effects of different translation inhibitors on *CBF* expression.
4. To assess the involvement of translation in cold signalling in plants.

4.2 Results

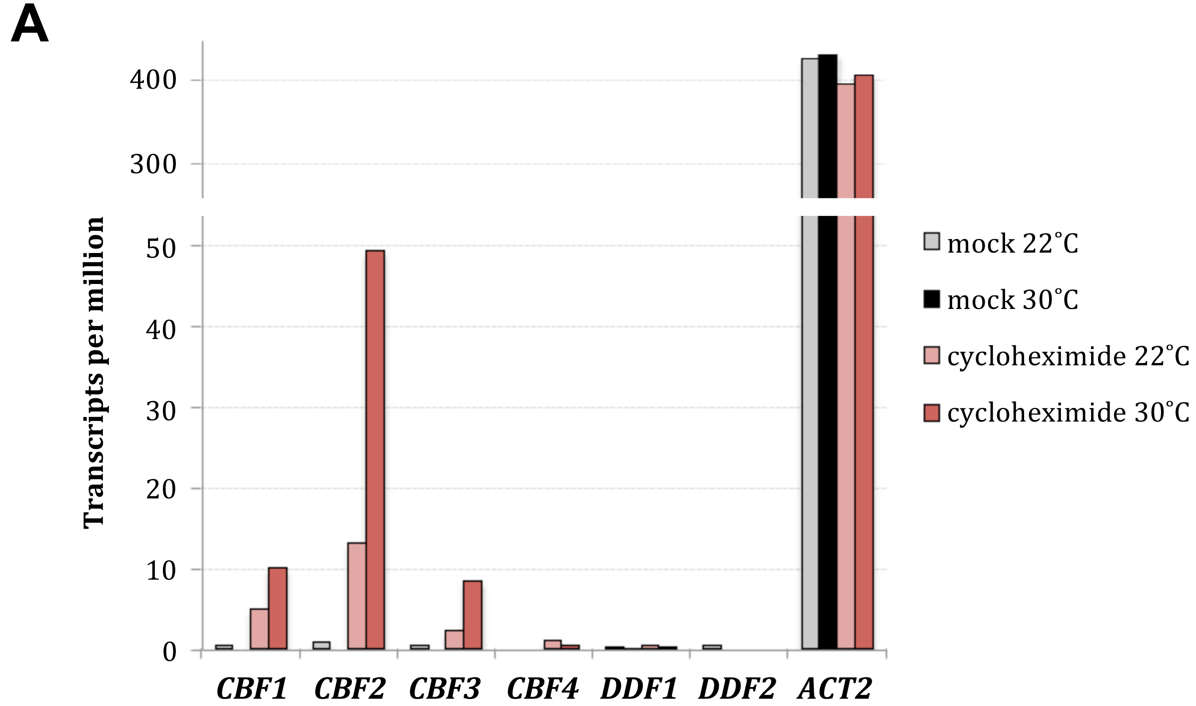
4.2.1 Early cold-responsive genes are induced by cycloheximide

Cycloheximide was identified in the *pHSP70::LUC* screen for chemical modifiers of the heat shock response (*Chapter 3*). Though this molecule represses the induction of *heat shock protein* genes during heat treatment, transcriptomic analyses using RNA-seq identified a large number of genes that are induced by cycloheximide. From 33,557 loci annotated in the *A. thaliana* genome, 11% (3,756) were found to have at least two-fold higher expression in seedlings treated with cycloheximide for one hour relative to mock-treated seedlings, and 5% (1,628) were found to have at least four-fold higher expression between these two treatments.

A number of cold-inducible genes were identified among these upregulated genes, including *CBF1, CBF2* and *CBF3*, which were induced six-fold to 17-fold after one hour of treatment at the ambient growth temperature (22°C) (*Figure 4.1A*). This transcriptional response was further enhanced at high temperature (30°C), with expression 20- to 62-fold higher than in mock-treated seedlings at 22°C. In contrast, the expression of *CBF4* and the *CBF*-like genes *DDF1* and *DDF2* (*DWARF AND DELAYED FLOWERING 1/2*), which are induced by drought or salinity but not low temperatures (Akhtar *et al.*, 2012), remained low at both 22°C and 30°C, while levels of actin (*ACTIN 2*), which are known to be stable, remained high (*Figure 4.1A*).

Changes in gene expression induced by cycloheximide were compared to those triggered by cold shock for these seven genes (Sandra Cortijo & Philip A. Wigge, unpublished). As shown in *Figure 4.1B*, *CBF1*, *CBF2* and *CBF3* are rapidly induced at low temperatures, whereas *CBF4*, *DDF1* and *DDF2* show little or no induction. The patterns of expression for the *CBF* genes are similar between cold and cycloheximide, with highest induction of *CBF2*.

As illustrated in *Figure 4.2*, the induction of cold-responsive *CBF* genes by cycloheximide is extremely rapid, with approximately two-fold increases in expression after 15 minutes of chemical treatment and five- to nine-fold increases after 30 minutes. A time-course experiment was performed in order to characterise the dynamics in expression of *CBF* and *COR* genes in response to cycloheximide or cold treatments (*Figure 4.3*). *CBF2* was chosen based on its high inducibility from *Figure 4.1* and *COR15a* because it is the most studied of *COR* genes. *CBF2* expression was found to peak after two hours of cycloheximide or cold treatment. In contrast, cold but not cycloheximide induced the expression of *COR15a*, with levels increasing after four hours and peaking at



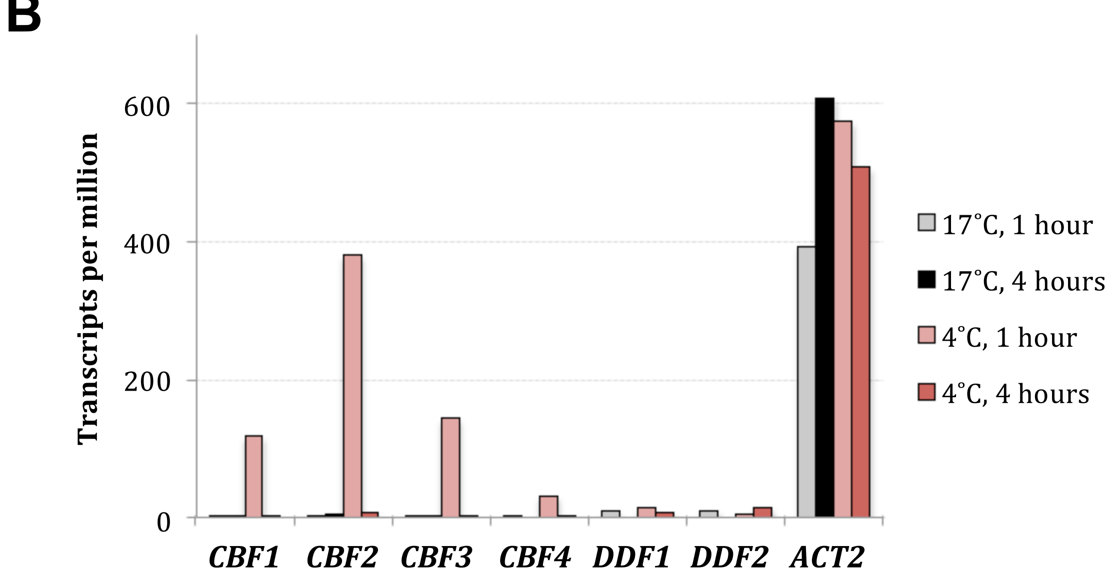


Figure 4.1: Cold-responsive *CBF* genes are induced by cycloheximide.
Expression of *CBF*-like genes and actin (*ACT2*) during cycloheximide (**A**) or cold (**B**) treatment, determined by RNA-seq (single experiment without replicates). (**A**) *A. thaliana* Col-0 seedlings were grown in liquid culture for seven days at 22˚C with a long-day photoperiod and treated with 35 μM cycloheximide or 0.1% v/v DMSO (mock control) for one hour at ZT8, with or without a 30-minute incubation at 30˚C prior to harvesting. (**B**) Seedlings were grown on solid media at 17˚C with a long-day photoperiod and at ZT1 were transferred to liquid media at 17˚C or at 4˚C (pre-cooled media) for one or four hours (data generated by Sandra Cortijo and Philip A. Wigge).

12 hours of treatment, in accordance with the dependency of *COR* gene induction on *de novo* protein synthesis.

Lee *et al.* (2005) have characterised early and late cold-induced genes in *A. thaliana*, which are upregulated within three or six hours (220 genes) and after 24 hours (435 genes) of exposure to 0˚C, respectively. Using RNA-seq analyses, the expression patterns of these two groups of genes in response to cycloheximide were investigated (*Figure 4.4A*). Late cold-induced genes are on average slightly upregulated (1.59-fold relative to the mock control) and early cold-induced genes, though varying in expression patterns, are highly upregulated (7.12-fold, on average), with 71% of which are upregulated greater than two-fold relative to the mock control. Park *et al.* (2015) identified 30 transcription factor genes, including *CBF1*, *CBF2* and *CBF3*, that are induced within one hour of cold shock. These 'first wave' transcription factors were found to be highly upregulated by cycloheximide, with an average expression 10.77-fold higher than that in mock controls and 85% of which were induced greater than two-fold by cycloheximide (*Figure 4.4B*). In contrast, the average expression of 1760 transcription factors from the Plant Transcription Factor Database (PlantTFDB) was found to be only 1.37-fold higher than in mock controls. These findings suggest that cycloheximide preferentially induces the expression of early cold-inducible genes.

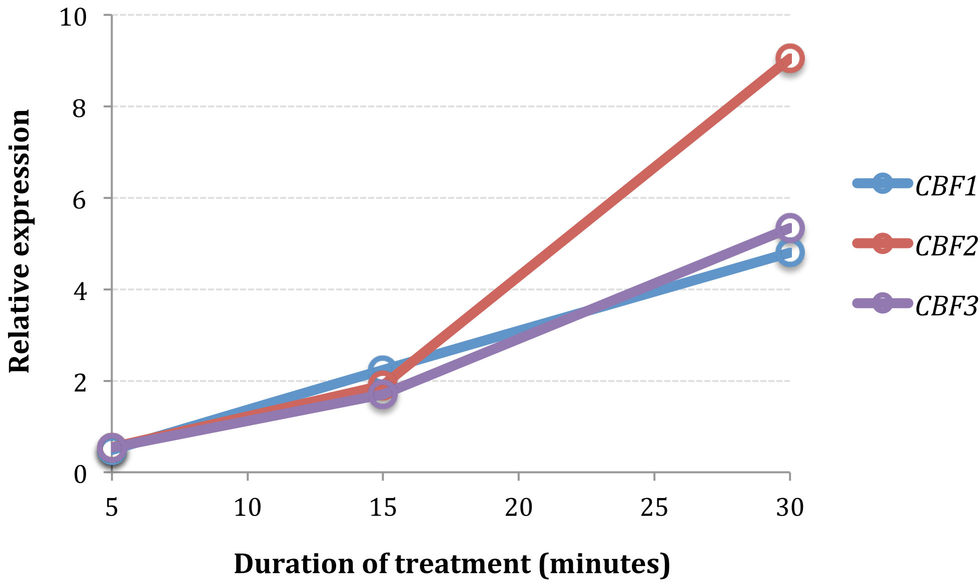
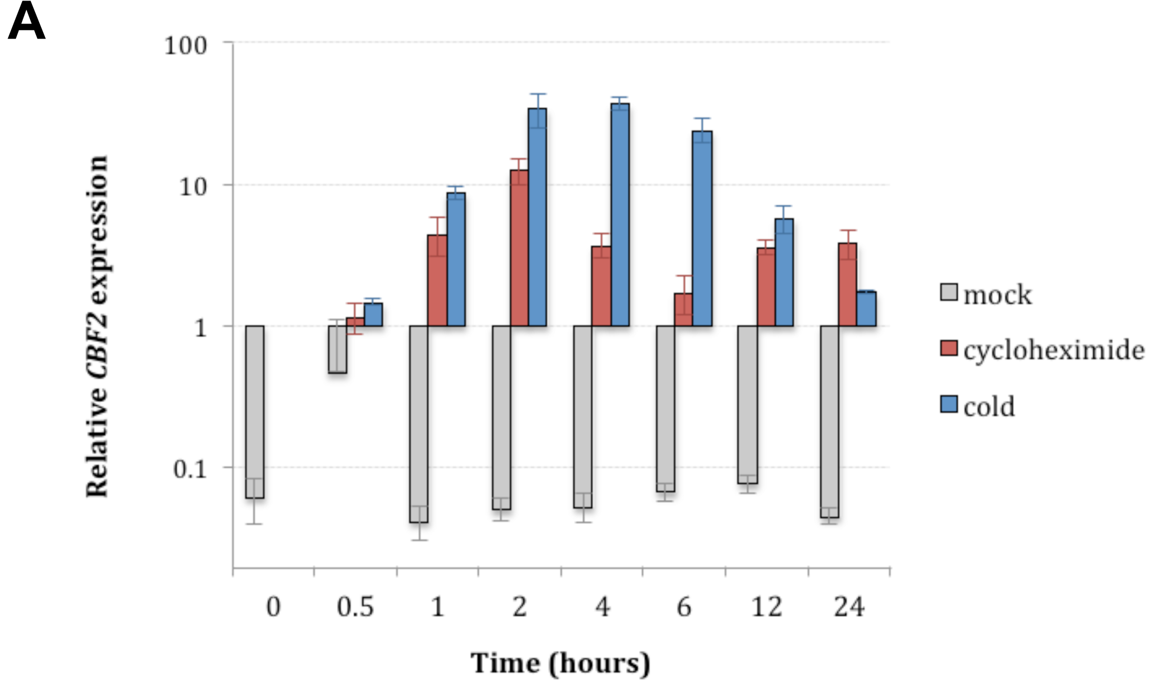


Figure 4.2: *CBF1*, *CBF2* and *CBF3* are rapidly induced by cycloheximide.
A. thaliana Col-0 seedlings were grown in liquid culture for seven days at 22˚C with a long-day photoperiod and treated with 30 μM cycloheximide or 0.1% v/v DMSO (mock control) for 5, 15 or 30 minutes at ZT8. Gene expression was determined by RNA-seq (single experiment without replicates). Relative expression of *CBF* genes is given in seedlings treated with cycloheximide relative to that in mock-treated seedlings.

A



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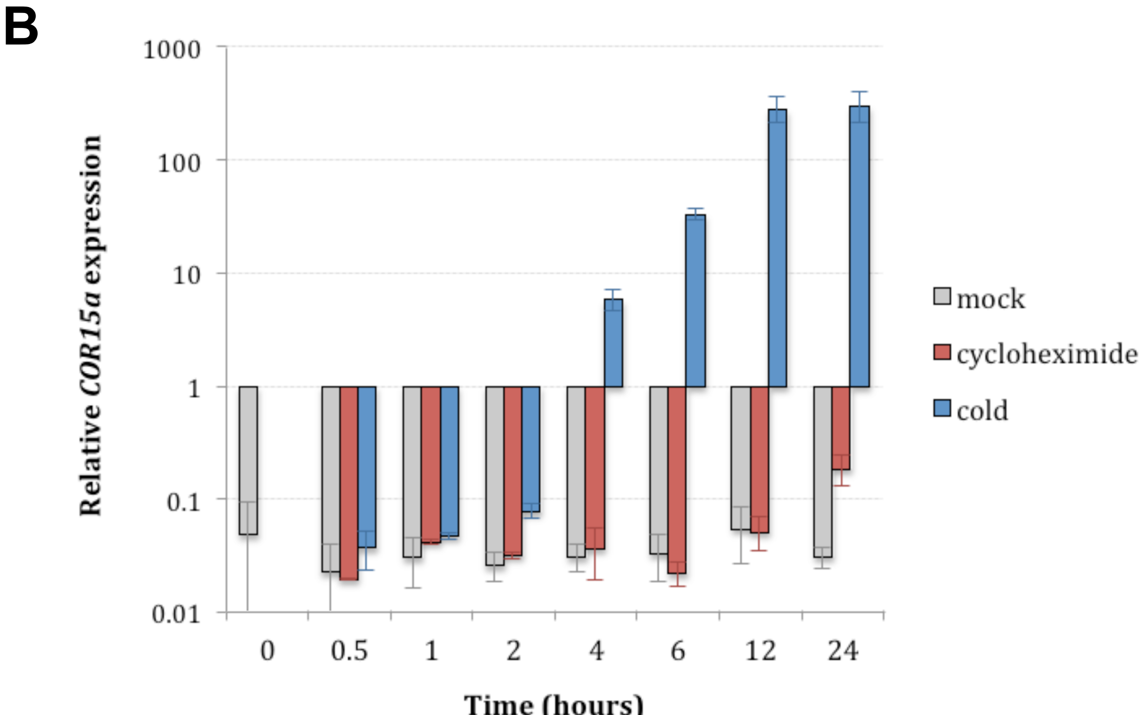
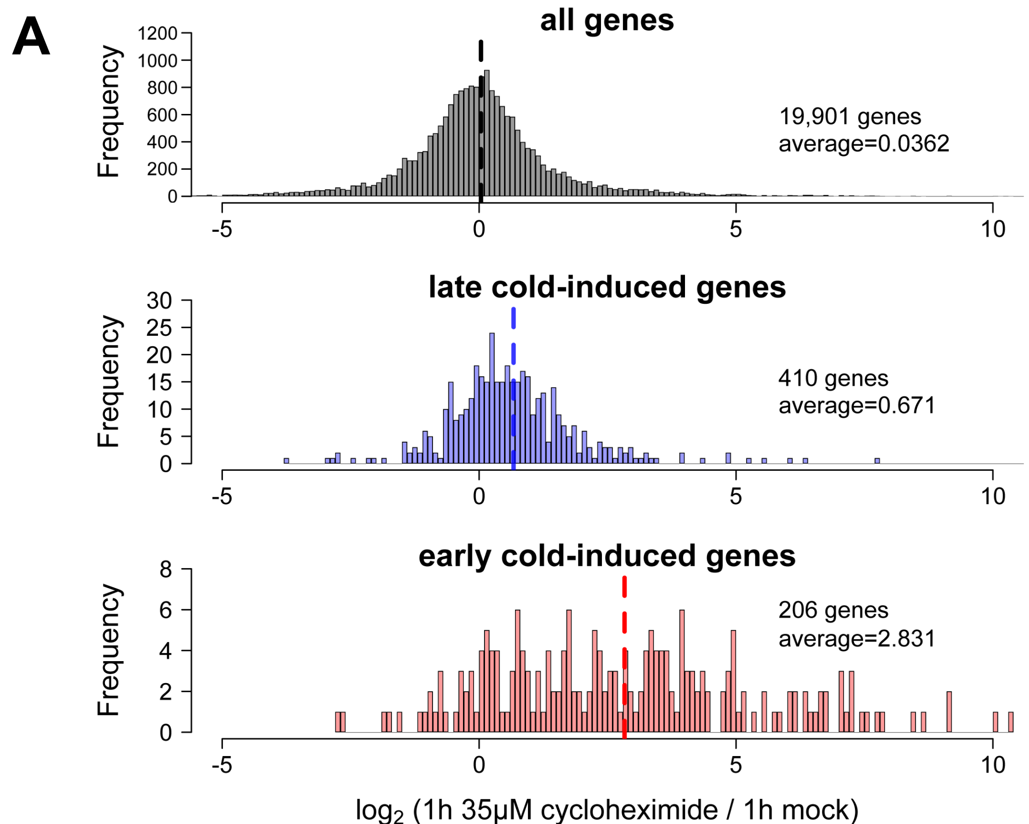


Figure 4.3: Cycloheximide upregulates *CBF* but not *COR* genes.
A. thaliana Col-0 seedlings were grown in liquid culture for seven days at 21˚C in constant light and treated with 30 μM cycloheximide at 21˚C, 0.1% v/v DMSO at 21˚C (mock control) or 0.1% v/v DMSO at 4˚C (cold treatment) for the incubation periods indicated. Expression of *CBF2* and *COR15a* was determined by quantitative PCR and normalised to that of *PP2A* and *UBC21*. Error bars represent standard deviation for three biological replicates, with 10-15 seedlings per replicate.



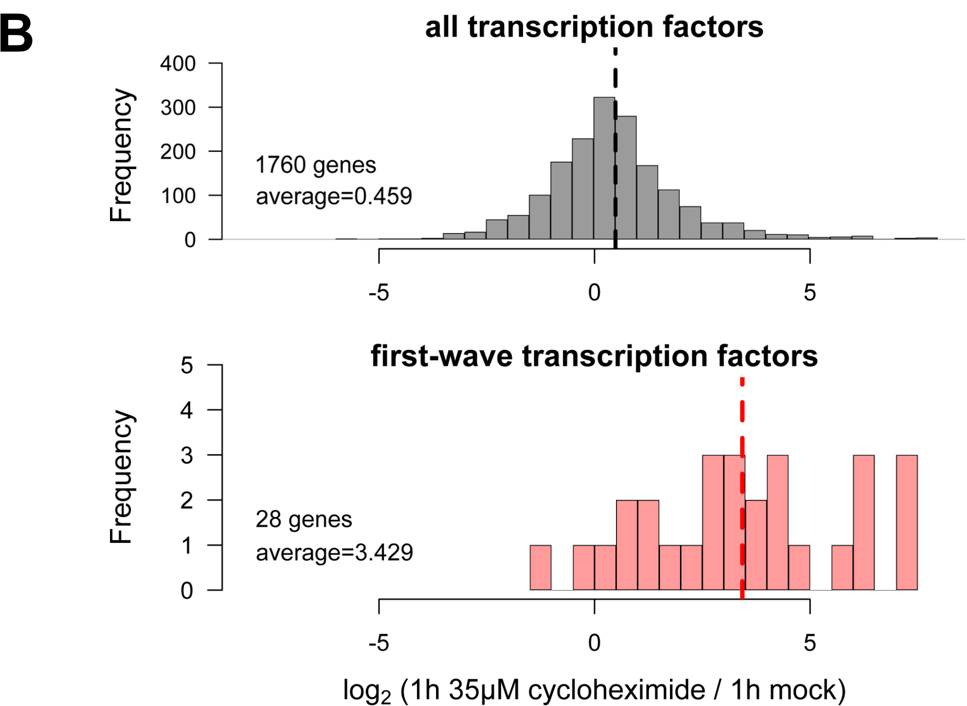


Figure 4.4: Cycloheximide preferentially upregulates early cold-inducible genes. Histograms of gene expression during cycloheximide treatments for different classes of cold-regulated genes. Dotted lines represent the mean. Cycloheximide treatments (22˚C, RNA-seq) are described in *Figure 4.1*. Only genes whose transcripts were detected in both mock and cycloheximide treatments are included.

To determine the overlap between transcriptomic changes induced by cycloheximide and those triggered by abiotic stresses, the RNA-seq dataset was compared to the AtGenExpress datasets (Kilian *et al.*, 2007). *Figure 4.5* shows the gene expression changes during one-hour treatment with cycloheximide or three-hour treatments with cold, osmotic, wounding, heat, oxidative or genotoxic stresses. The strongest correlation with cycloheximide-induced gene expression is seen for cold shock, with a Pearson correlation coefficient of 0.352. Interestingly, stronger correlations were observed for transcriptomic changes induced by osmotic stress and wounding, which are known to induce the expression of some cold-responsive genes (Thomashow *et al.*, 1997; Cheong *et al.*, 2002), than for those induced by heat, oxidative or genotoxic stresses. While there appears to be a positive correlation between genes induced by cycloheximide and cold, osmotic stress or wounding, the reverse is not true for genes that are repressed by these treatments.

A positive correlation is observed between transcriptomic changes induced by one hour of cycloheximide treatment and those induced by three or six hours of cold treatment (4˚C) (*Figure 4.6*), time-points at which *CBF* expression is at its highest. Interestingly, a negative correlation is observed between the transcriptomes of seedlings treated with cycloheximide and those exposed to severe heat shock (38˚C) for 30 minutes (*Figure 4.6*), the time-point at which the expression of many heat-inducible genes, such as *HSFA2* (*HEAT SHOCK FACTOR A2*), is at a maximum. As well as repressing the expression of heat-upregulated genes, including those encoding heat shock proteins, cycloheximide induces many genes that are repressed during heat shock, most of which encode metabolic enzymes, transporters and regulators of hormone signalling.

4.2.2 *CBF* responses to cycloheximide are mediated by known cold regulators

The induction of *CBF* genes is known to be regulated by the circadian clock, with highest cold-inducibility at the beginning of the day and lowest cold-inducibility at the beginning of the night (Fowler *et al.*, 2005). This regulation can be abolished by disrupting the clock components CCA1 (CIRCADIAN CLOCK ASSOCIATED 1) or LHY (LATE ELONGATED HYPOCOTYL), which bind to promoters of cold-inducible *CBF* genes (Fowler *et al.*, 2005; Dong *et al.*, 2011). Circadian regulation of cycloheximide-mediated *CBF* induction would imply a common mechanism of transcriptional regulation with cold signalling pathways.

A. thaliana seedlings were grown in long-day or short-day photoperiods and subjected to cold or cycloheximide treatments at regular intervals for 24 hours. As

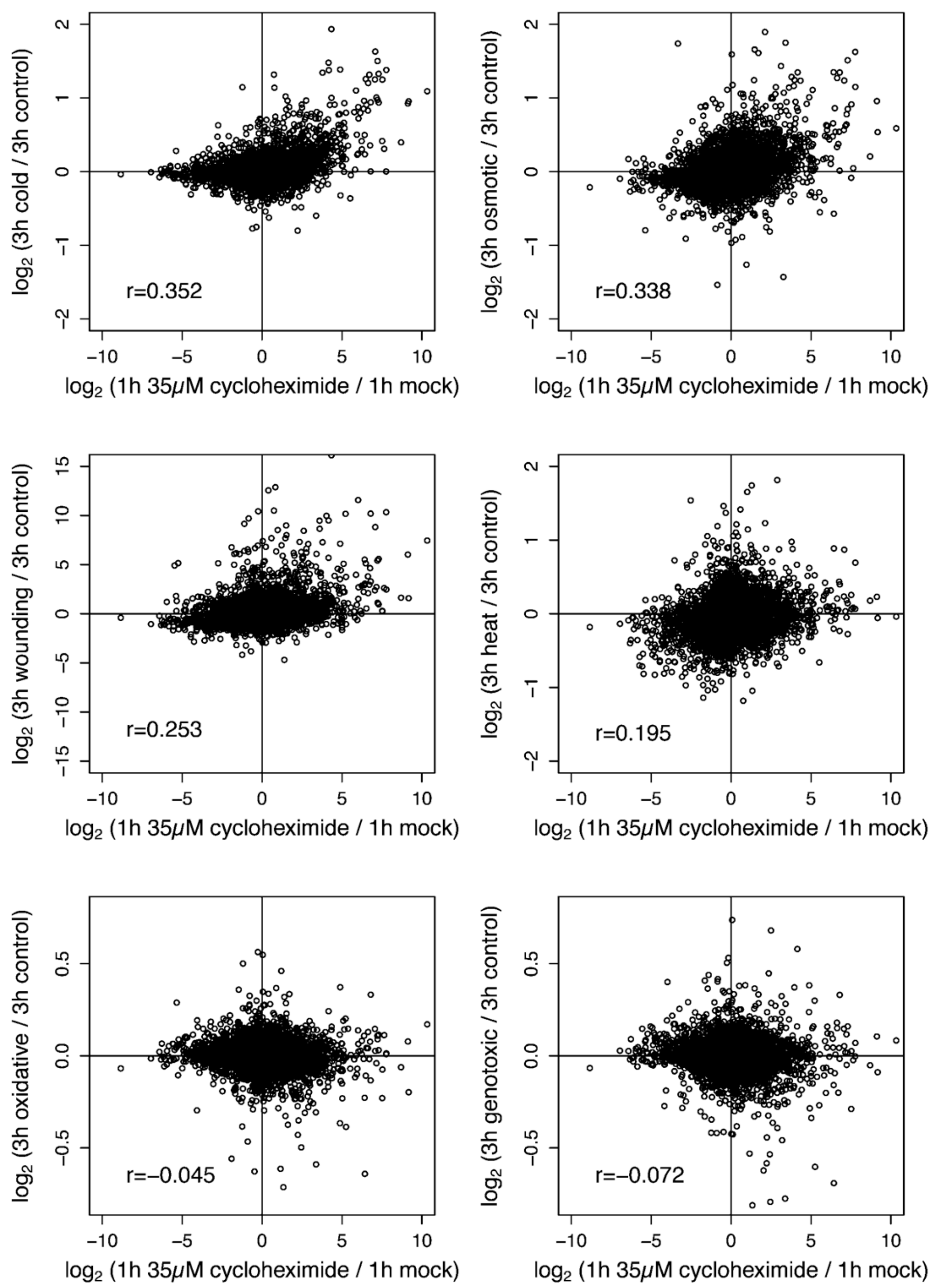
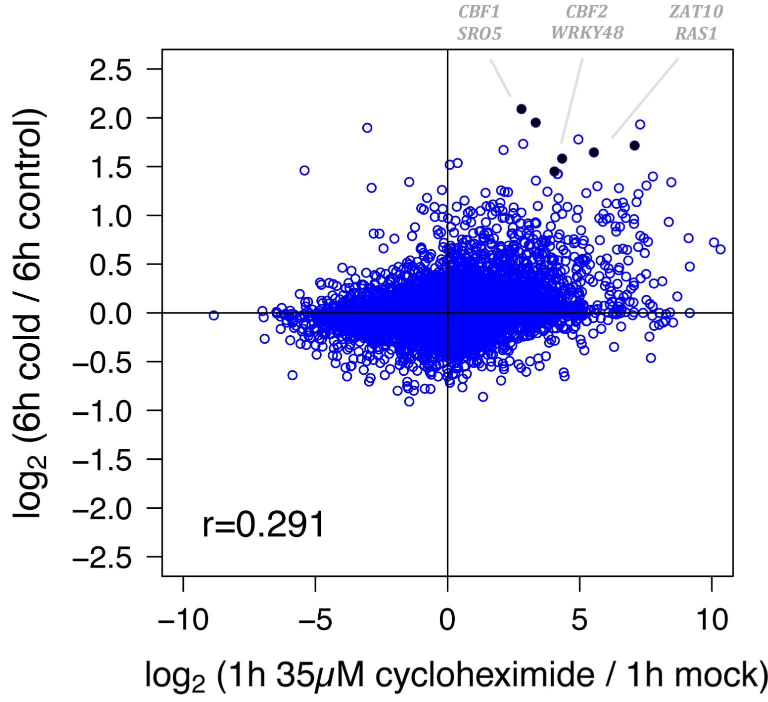


Figure 4.5: Correlations in gene expression between cycloheximide and stress treatments. Cycloheximide treatments (22°C, RNA-seq) are described in *Figure 4.1*. Three-hour stress treatments from the AtGenExpress expression dataset (Kilian *et al.*, 2007): cold (4°C), osmotic (300mM mannitol), wounding (leaf punctures), heat (38°C), oxidative (10μM methyl viologen) and genotoxic (1.5μg/ml bleomycin, 22μg/ml mitomycin). Each plot contains 10,379 genes. The Pearson correlation coefficient (r) is given for each plot.



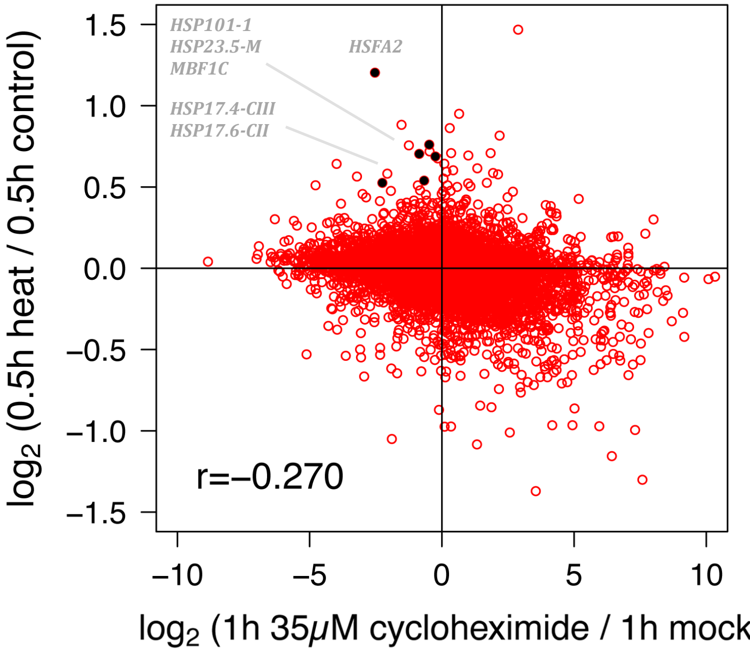


Figure 4.6: Correlations in gene expression between cycloheximide and temperature treatments. Cycloheximide treatments (22˚C, RNA-seq) are described in *Figure 4.1*. Three-hour stress treatments from the AtGenExpress expression dataset (Kilian *et al.*, 2007): cold (six hours, 4˚C) and heat (30 minutes, 38˚C). Six cold- or heat-responsive marker genes are indicated in black. Each plot contains 16,491 genes. The Pearson correlation coefficient (r) is given for each plot.

shown in *Figure 4.7*, highest cold-induction of *CBF2* was observed at dawn and lowest cold-induction was detected after dusk in both long and short days, in accordance with data from Fowler *et al.* (2005). A two-fold difference in cold-induction was measured between these time-points. Fowler and colleagues reported a larger difference of approximately ten-fold after one or four hours of cold exposure between these time points. This discrepancy in *CBF* induction is likely due to differences in cold treatments, as the rate of cooling has recently been shown to affect the amplitude of *CBF* induction during cold shock (Kidokoro *et al.*, 2017).

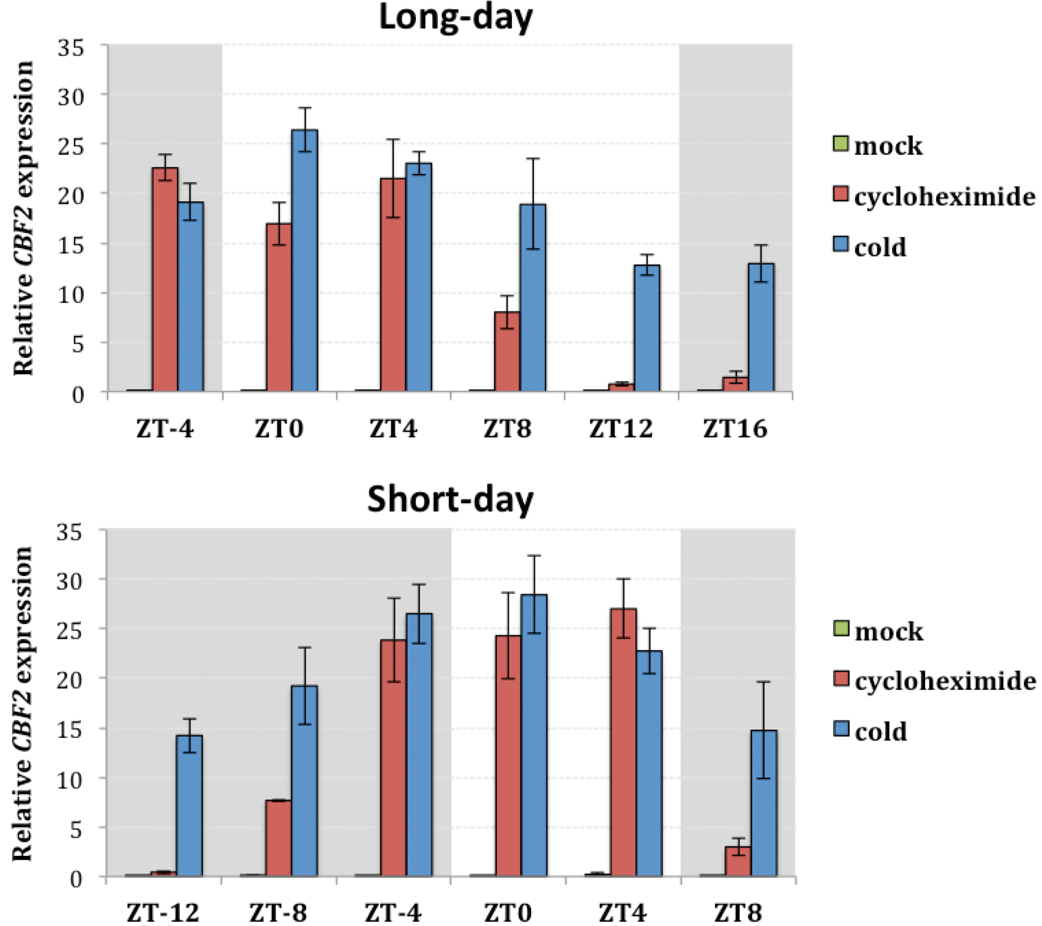


Figure 4.7: *CBF2* induction by cold or cycloheximide treatments varies across the 24-hour cycle. *A. thaliana* Col-0 seedlings were grown in liquid culture for nine days at 22˚C with a long-day (16-hour) or short-day (8-hour) photoperiod and harvested after two-hour treatments with 0.1% v/v DMSO ('mock') at 22˚C, 30 µM cycloheximide at 22˚C or 0.1% v/v DMSO at 4˚C ('cold'), at the indicated intervals. *CBF2* expression was normalised to *PP2A* and *UBC21*. Grey boxes represent treatments performed in the dark. Error bars represent standard deviation for three biological replicates, with 10-15 seedlings per replicate.

Similarly to cold shock, cycloheximide treatment was found to induce *CBF2* to highest levels after dawn, with lowest effects after dusk (*Figure 4.7*). Differences in *CBF2* expression of 25- to 90-fold were observed between these time-points during cycloheximide treatment. Both cold shock and cycloheximide treatments were performed under light/dark cycles, rather than after a shift to continuous light, as were the experiments conducted by Fowler *et al.* (2005). Nevertheless, the data presented in *Figure 4.7* suggest that the circadian clock regulates *CBF2* induction by cycloheximide as well as that by cold shock, because the level of induction is not dependent on light (*i.e.*, it is not diurnal).

Using the RNA-seq dataset of seedlings treated with cycloheximide or mock control (DMSO) for one hour at ambient growth temperature (22˚C), genes were clustered into groups based on their transcriptional changes upon chemical treatment (*k*-means clustering, using Pearson correlation coefficients). Among 11,078 expressed genes, a third (3,723) were found to form a cycloheximide-upregulated cluster. The promoters of these genes were analysed for conserved DNA motifs that could contribute to cycloheximide-inducibility. The top three motifs that were significantly enriched in the cycloheximide-upregulated gene cluster relative to total expressed genes are given in *Table 4.1*. These include the W-box, CAM box and G-box, which are bound by WRKY (Tryptophan-Arginine-Lysine-Tyrosine domain) (Eulgem *et al.*, 2000), CAMTA (Han *et al.*, 2006) and group B bHLH (Jones *et al.*, 2004) transcription factors, respectively, the latter of which include MYC-type regulators such as ICE1 and PIFs (PHYTOCHROME-INTERACTING FACTORs). Members of these three groups of transcription factors are known to be involved in the regulation of cold-responsive genes and freezing tolerance (Chinnusamy *et al.*, 2003; Doherty *et al.*, 2009; Kidokoro *et al.*, 2009; Lindlöf *et al.*, 2009; Lee & Thomashow, 2012; Kim *et al.*, 2013; Le *et al.*, 2015; Li *et al.*, 2017a).

Table 4.1: Top three promoter motifs enriched in genes upregulated by cycloheximide treatment (targets; 3,723 genes) relative to total expressed genes (background; 11,078 genes).

Motif	Description	Transcription factor	p-value	Percentage of targets	Percentage of background
	W-box	WRKY	1×10^{-23}	34.71	23.96
	CAM-box	CAMTA	1×10^{-20}	11.88	5.76
	G-box	Group B bHLH	1×10^{-3}	25.44	21.78

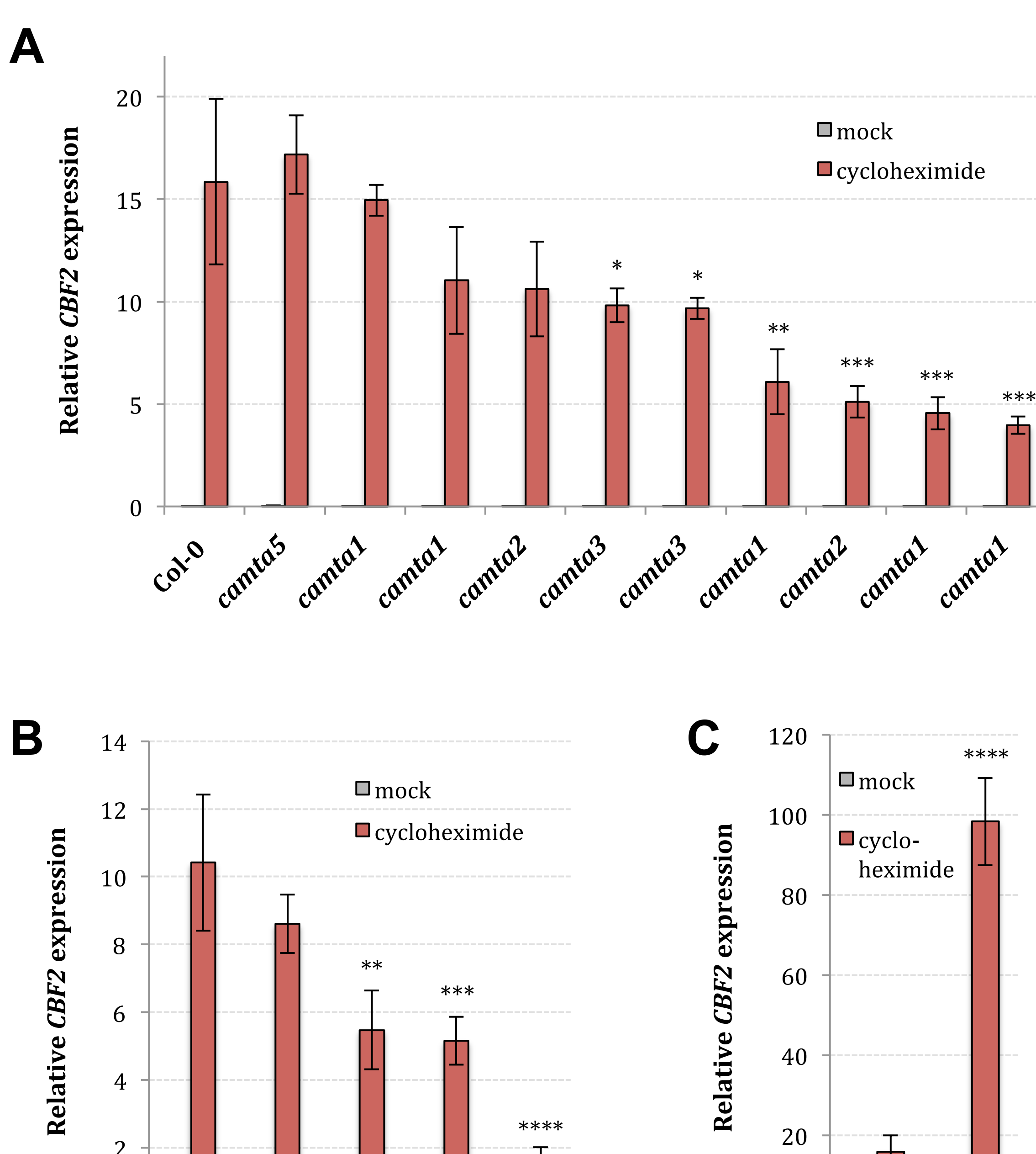


Figure 4.8: Known cold regulators contribute to *CBF2* induction by cycloheximide.
A. thaliana Col-0 or T-DNA insertion mutants of *CAMTA* (**A**), *ICE* (**B**) or *PRR* (**C**) genes were grown in liquid culture for seven days at 21˚C in constant light and treated with 30 µM cycloheximide or 0.1% v/v DMSO (mock control) for 2 hours. *CBF2* expression was normalised to that of *PP2A* and *UBC21*. Error bars represent standard deviation for three biological replicates, with 10-15 seedlings per replicate. Asterisks indicate significant differences relative to Col-0 (two-tailed *t*-test, p-values <0.05 *, <0.01 **, <0.005 ***, <0.001 ****).

To confirm the involvement of such regulators in transcriptional responses to cycloheximide, mutants of ICE and CAMTA genes were tested for altered induction of *CBF2*. CAMTA1, CAMTA2, CAMTA3 and CAMTA5 have been shown to contribute to cold-induction of *CBF* genes in *A. thaliana* (Kim *et al.*, 2013; Kidokoro *et al.*, 2017). Single and multiple *camta* mutants were treated with cycloheximide for two hours, and as shown in *Figure 4.8A* CAMTA proteins are required for full induction of *CBF2* by this inhibitor. CAMTA2 and CAMTA3 play particularly important roles in this regulation, as a three-fold lower induction is detected in the double *camta2 camta3* (*camta23*) mutant relative to wild-type plants, while a four-fold lower induction is observed in the triple *camta1 camta2 camta3* (*camta123*) mutant.

Two alleles of *ICE1* have been reported to contribute to lower *CBF* induction during cold shock: the *ice1-1* allele, which encodes an ICE1 protein with a missense mutation (R236H) of unknown effect (Chinnusamy *et al.*, 2003), and the *ice1-2* allele, which contains a T-DNA insertion in the third exon (Kim *et al.*, 2015). Both *ice1* mutants were found to have reduced *CBF2* expression during cycloheximide treatment, with 1.9- and 6.5-fold decreases in expression relative to wild-type in the *ice1-2* and *ice1-1* mutants, respectively (*Figure 4.8B*). Though the *ice1-2* mutant was reported to be a null allele (Kanaoka *et al.*, 2008), the T-DNA insertion is downstream of the C-terminal DNA-binding domain and the region upstream of the insertion, which encodes 85% of the protein, is expressed at normal levels in this mutant (data not shown). This suggests that *ice1-2* is a weak allele, which explains why a smaller reduction in *CBF2* induction is observed in these plants relative to the dominant negative *ice1-1* mutant. ICE2, in contrast, was not found to play a key role in cycloheximide-mediated expression of *CBF2*, as induction was not significantly compromised in the *ice2-2* T-DNA insertion mutant relative to wild-type, nor in the double *ice1-2 ice2-2* mutant relative to the single *ice1-2* mutant. While the homozygous *ice1-2 ice2-2* mutant cannot be propagated owing to seedling lethality arising from a failure to produce stomata (Kim *et al.*, 2015), homozygous mutants were isolated from *ice1-2/+ ice2-2* progeny based on their considerably smaller size and were genotyped to verify zygosity following RNA extractions.

Lastly, *CBF2* induction was investigated in plants lacking the circadian clock regulators PRR5, PRR7 and PRR9 (PSEUDORESPONSE REGULATOR 5/7/9), which accumulate in the evening and repress the expression of *CCA1* and *LHY*, thereby contributing to low basal levels of *CBF* expression at night (Nakamichi *et al.*, 2009). As shown in *Figure 4.8C*, *CBF2* expression after cycloheximide treatment was 6.2-fold higher in the triple *prr5-11 prr7-11 prr9-10* (*prr579*) mutant than in wild-type plants.

These findings indicate that transcriptional responses to cycloheximide converge on cold signalling pathways in *A. thaliana* and are mediated at least in part by known *CBF* regulators.

4.2.3 *CBF* induction by cycloheximide is mediated *via* ribosomes

Cycloheximide and anisomycin, the latter of which also blocks the elongation step of eukaryotic translation, have both been shown to induce the expression of certain genes in mammalian cells (Radulovic & Tronson, 2008). It has been speculated that such transcriptional changes may be brought about by off-target interactions with signalling enzymes such as kinases (Mahadevan & Edwards, 1991; Edwards & Mahadevan, 1992; Hazzalin *et al.*, 1998). To test whether cycloheximide-mediated *CBF* expression is a result of specific binding to eukaryotic ribosomes or secondary interactions with other cellular components, *A. thaliana* plants producing cycloheximide-resistant ribosomes were generated.

Point mutations in two ribosomal proteins, RPL28 and RPL42 (RIBOSOMAL PROTEIN LARGE SUBUNIT 28/42), confer cycloheximide resistance in the budding yeast *Saccharomyces cerevisiae*. These include the H39R mutation or substitutions of Q38 for E, K, R, L or H in RPL28 (Huang *et al.*, 2013) and the P56Q mutation in RPL42, which is encoded by two genes in *S. cerevisiae* (Kawai *et al.*, 1992). Similarly, the *A. thaliana* encodes two RPL42 orthologues (RPL36aA and RPL36aB), both containing the conserved proline at residue 56 (*Figure 4.9*). Three RPL28 orthologues are present in *A. thaliana* (RPL27aA, RPL27aA and RPL27aA), the first of which lacks the region that interacts with cycloheximide in yeast RPL28 and the other two proteins of which contain the conserved histidine at residue 39 but lack the adjacent glutamine (*Figure 4.9*).

Point-mutated isoforms of RPL36aA and RPL27aC were expressed under their native promoter and terminator regions in *A. thaliana* Col-0 plants (*RPL36aAΔP56Q* and *RPL27aCΔH39R*, hereafter referred to as *CYHr1*/*CYCLOHEXIMIDE RESISTANCE 1* and *CYHr2*/*CYCLOHEXIMIDE RESISTANCE 2*). As shown in *Figure 4.10A*, the *CYHr1* gene allowed *A. thaliana* seedlings to grow in the presence of cycloheximide even at a concentration as high as 30 μM. No growth was observed in the wild-type or plants expressing the *CYHr2* gene, suggesting differences in interactions of cycloheximide and RPL28-like ribosomal proteins between yeast and *Arabidopsis*.

A 90% reduction in cycloheximide-induced *CBF2* expression was observed in T2 plants expressing the *CYHr1* gene, which confirms that this transcriptional response is a

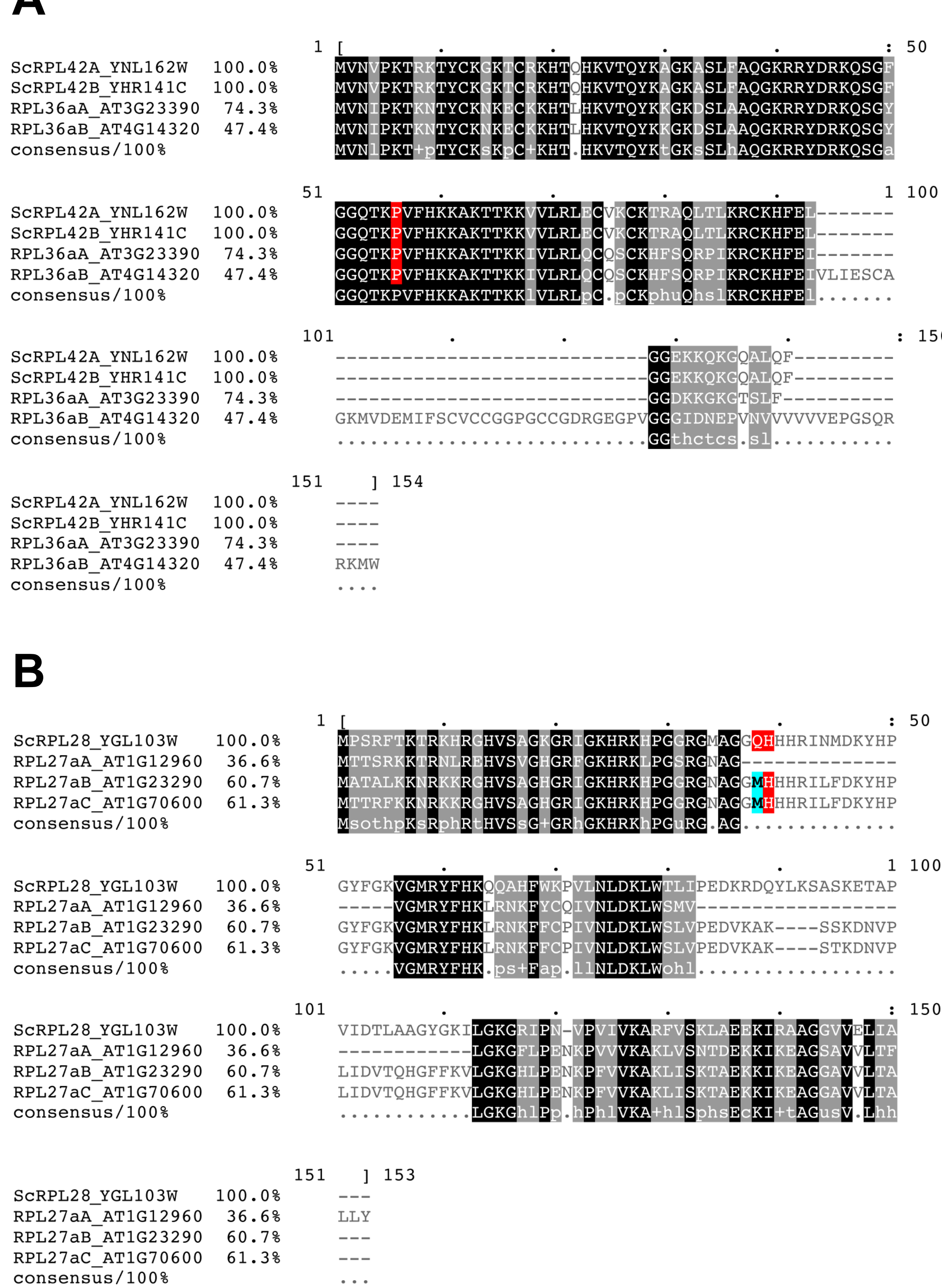


Figure 4.9: Alignments of ribosomal proteins known to interact with cycloheximide in budding yeast.
(**A**) RPL42 homologues from *S. cerevisiae* (ScRPL42A, ScRPL42B) and *A. thaliana* (RPL36aA, RPL36aB).
(**B**) RPL28 homologues from *S. cerevisiae* (ScRPL28) and *A. thaliana* (RPL27aA, RPL27aB, RPL27aC).
Identical or biochemically similar amino acid residues are highlighted in black or grey, respectively. Residues that contribute to cycloheximide resistance in yeast are indicated in red. Conserved or non-conserved residues in equivalent regions of *A. thaliana* proteins are indicated in red or blue, respectively.

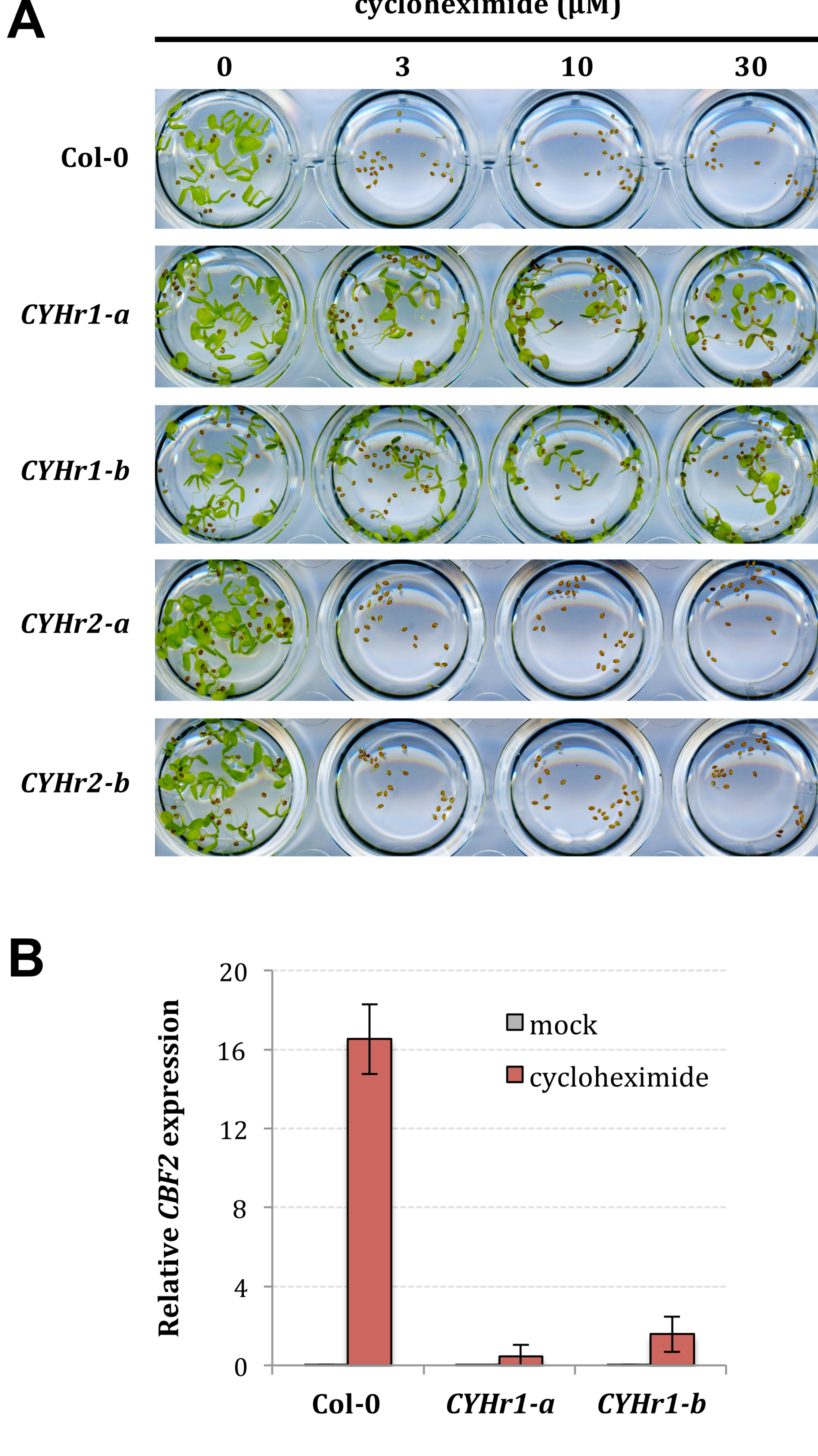


Figure 4.10: *CBF2* induction by cycloheximide is mediated *via* ribosomes.
(**A**) *A. thaliana* Col-0 seedlings expressing *RPL36aAΔP56Q* (*CYHr1*) or *RPL27aCΔH39R* (*CYHr2*) genes were grown in liquid culture supplemented with cycloheximide at the given concentrations (0.1% v/v DMSO) for seven days at 21˚C in constant light. Results for two independent T2 lines, named '*a*' and '*b*', are given.
(**B**) The *RPL36aAΔP56Q* (*CYHr1*) mutation abolishes *CBF2* induction by cycloheximide. Seedlings were grown in liquid culture for seven days at 21˚C in constant light and treated with 30 μM cycloheximide or 0.1% v/v DMSO (mock control) for two hours (T2 plants). Expression was normalised to *PP2A* and *UBC21* and given relative to that in wild-type seedlings. Error bars represent standard deviation for three biological replicates, with 10-15 seedlings per replicate.

result of inhibition of ribosome function rather than direct activation of signalling components (*Figure 4.10B*).

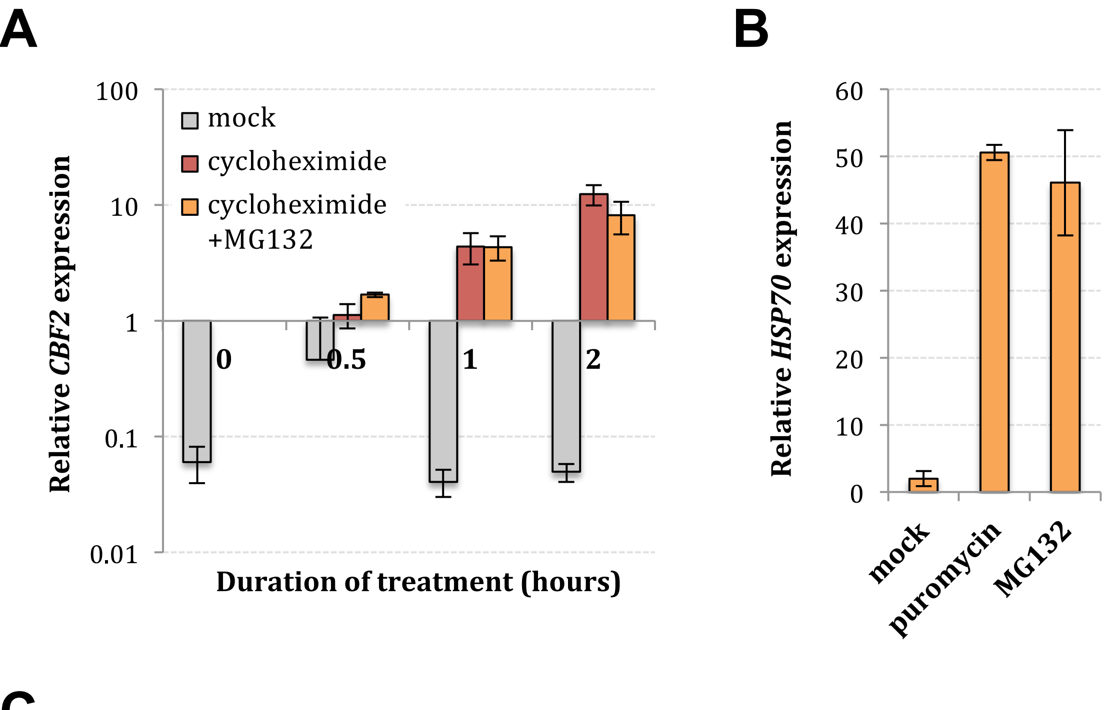
4.2.4 *CBF* induction is not a result of protein turnover

As transcriptional changes brought about by cycloheximide treatment are a consequence of translation inhibition, it was hypothesised that the latter could cause a decrease in abundance of proteins required for transcriptional repression or mRNA decay, owing to rapid protein turnover. This theory of labile repressors has been proposed from previous studies in mammalian cells to explain the upregulation of certain genes by cycloheximide (Wall *et al.*, 1986; Ma *et al.*, 2000; Bi *et al.*, 2006). To explore this possibility, *A. thaliana* seedlings were treated either with cycloheximide alone or co-treated with cycloheximide and the proteasome inhibitor MG132, which stabilises proteins targeted for ubiquitin-mediated protein degradation. Both of these treatments induced *CBF2* expression over 150-fold relative to mock treatments (*Figure 4.11A*). Expression was not significantly different between cycloheximide treatments with or without MG132 after one or two hours of treatment (two-tailed *t*-test, p-values 0.95 and 0.11, respectively).

Protein synthesis was additionally blocked using an inhibitor with a distinct mode of action to cycloheximide. Seedlings were treated with puromycin, an inhibitor that mimics the 3' end of aminoacyl-tRNAs, allowing it to be incorporated into nascent peptides, upon which it terminates translation. Prolonged incubations in puromycin at concentrations as high as 150 μM were not found to induce any changes in the expression of *CBF2* (*Figure 4.11C*). As both puromycin and MG132 are known to trigger the heat shock response as a result of a build-up of misfolded or aggregated proteins (Lee & Dewey, 1987; Holmberg *et al.*, 2000), the induction of *HSP70* (*HEAT SHOCK PROTEIN 70*) expression by these inhibitors was verified to confirm their uptake and activity in seedlings (*Figure 4.11B*). The above findings suggest that translation repression *per se* is insufficient to trigger *CBF* expression and that labile repressors do not contribute to this transcriptional response.

4.2.5 *CBF* gene induction is specific to some translation inhibitors

In order to investigate the mechanism of cycloheximide-induced *CBF* expression, a variety of translation inhibitors were tested for their ability to induce similar transcriptional responses. Inhibitors were selected based on their different binding sites



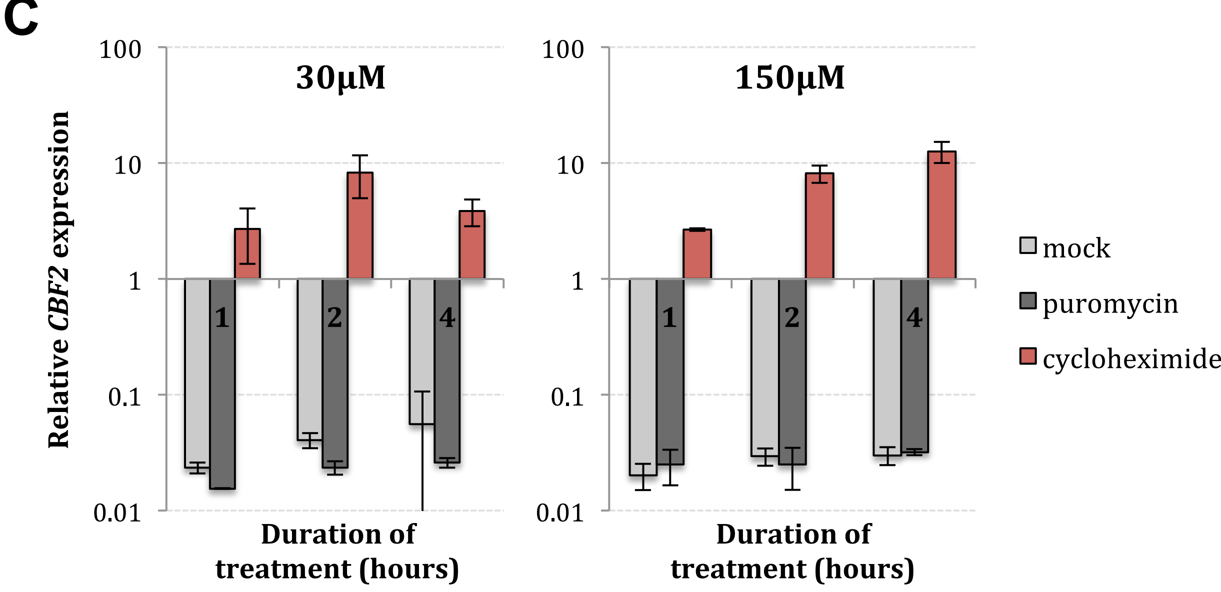


Figure 4.11: *CBF2* induction by cycloheximide is not a result of protein turnover.
A. thaliana Col-0 seedlings were grown in liquid culture for seven days at 21°C in constant light and treated with inhibitors for the durations of time indicated. Expression of *CBF2* (**A**, **C**) or *HSP70* (**B**) was normalised to *PP2A* and *UBC21*. Error bars represent standard deviation for three biological replicates, with 10-15 seedlings per replicate. The y-axis is logarithmically scaled in (A) and (C).
(**A**) Treatments with 30 µM cycloheximide, 30 µM cycloheximide + 100 µM MG132 or 1% v/v DMSO (mock control).
(**B**) Two-hour treatments with 30 µM puromycin, 10 µM MG132 or 0.1% v/v DMSO (mock control).
(**C**) Treatments with cycloheximide or puromycin at 30 µM or 150 µM, or with DMSO (mock control; 0.02% v/v or 0.1% v/v, respectively).

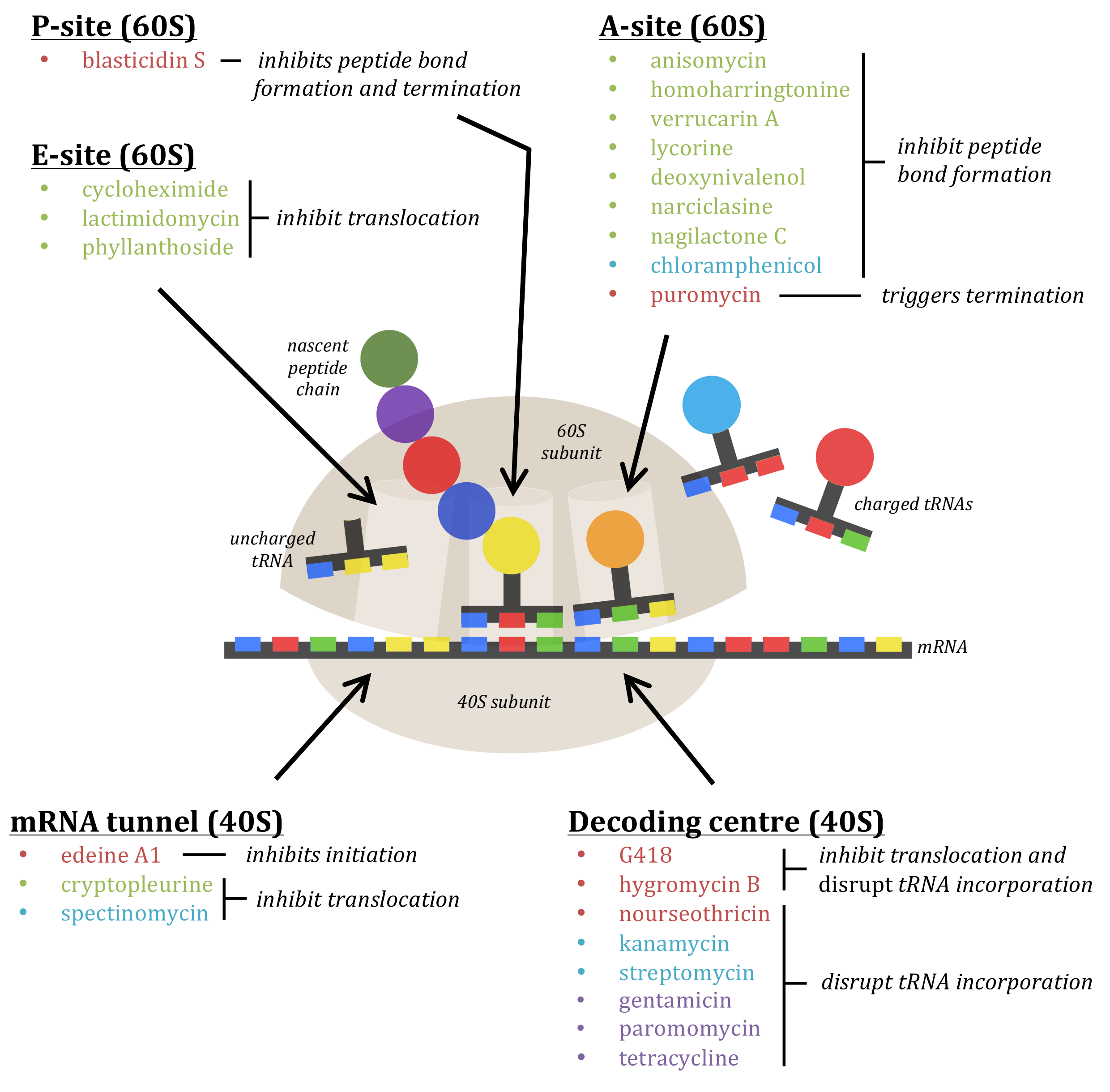


Figure 4.12: Summary of the binding sites and modes of action of translation inhibitors used. Inhibitors that specifically target 80S or 70S ribosomes are indicated in green and blue, respectively. Those targeting both types of ribosomes are indicated in red, except for those that display weak inhibition *in vivo*, which are indicated in purple. A-site: aminoacyl site; P-site: peptidyl site; E-site: exit site. Binding sites and mechanisms are based on Wilson (2009) and Garreau de Loubresse *et al.* (2014).

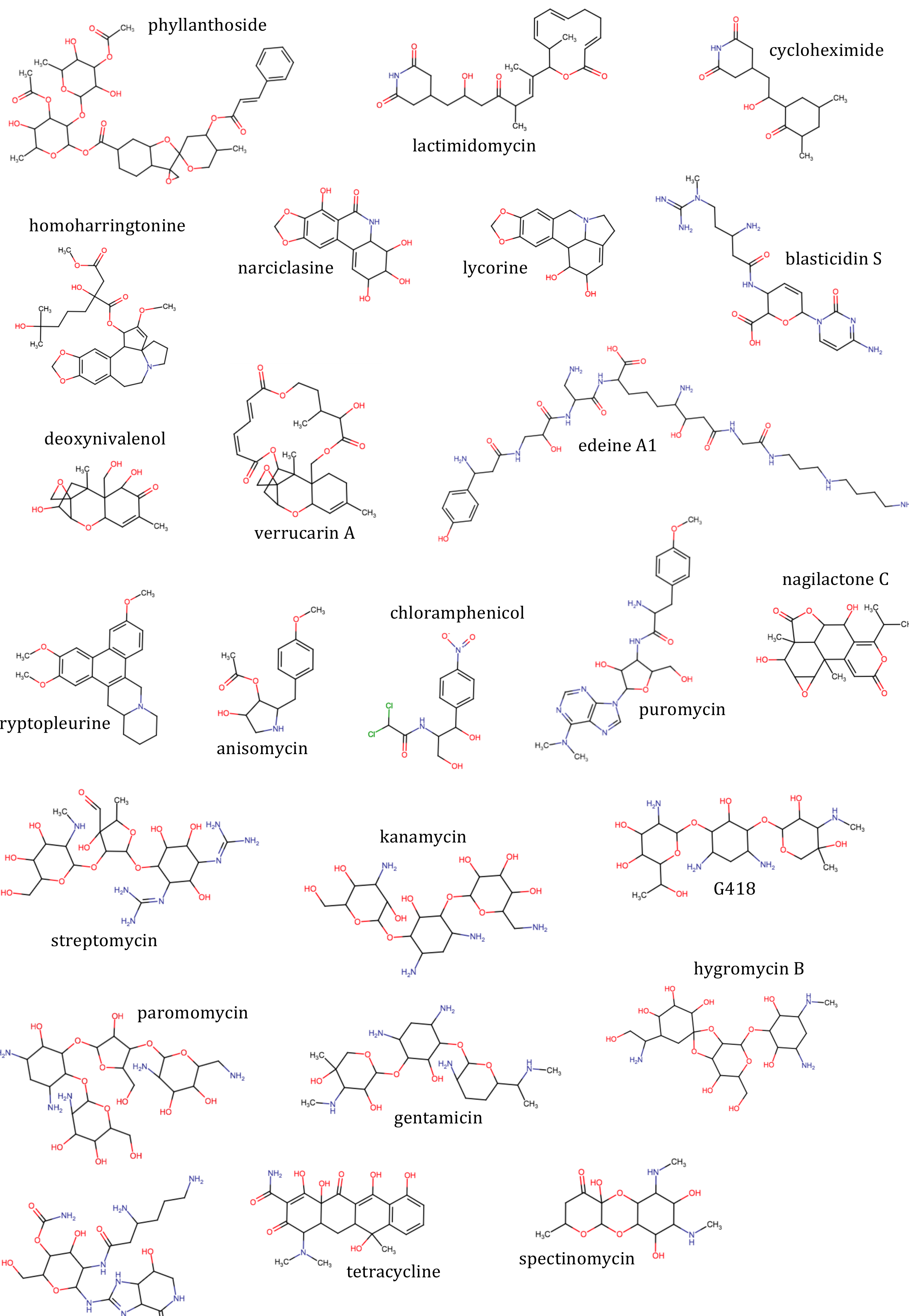
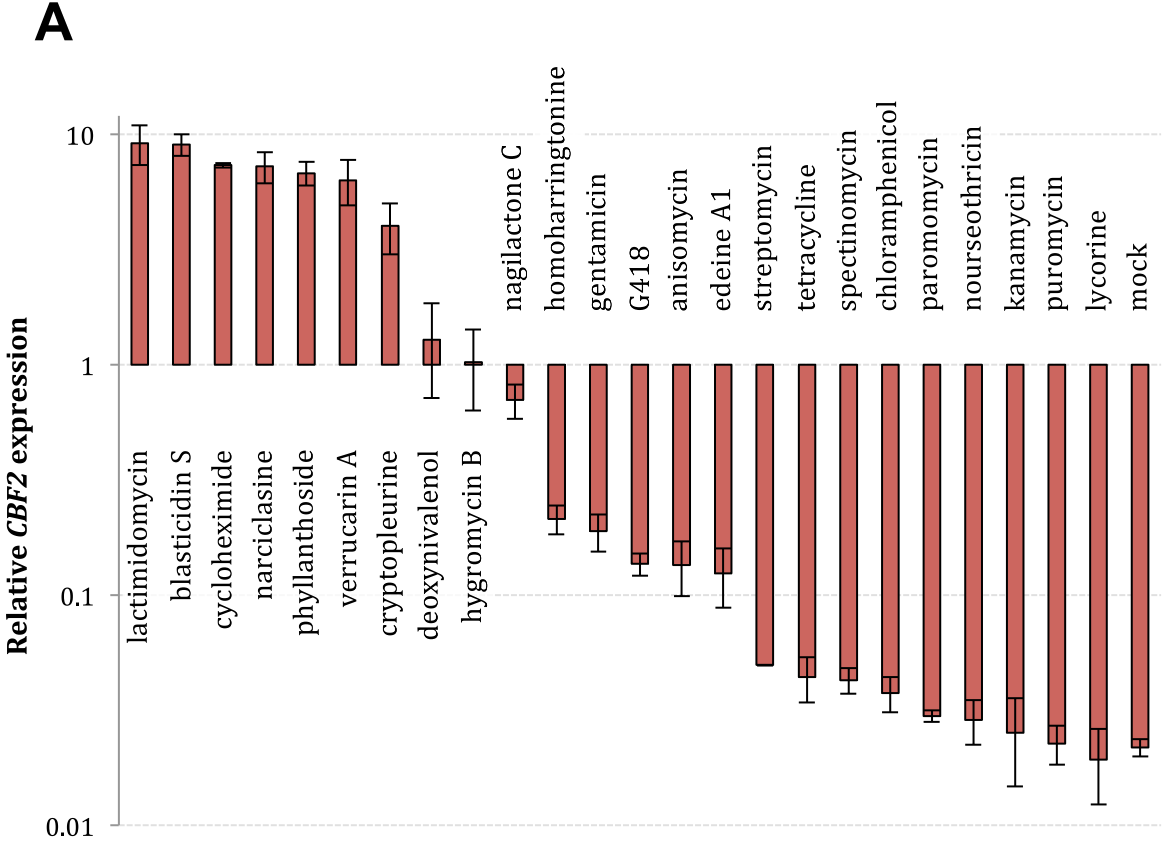


Figure 4.13: Structures of translation inhibitors used.



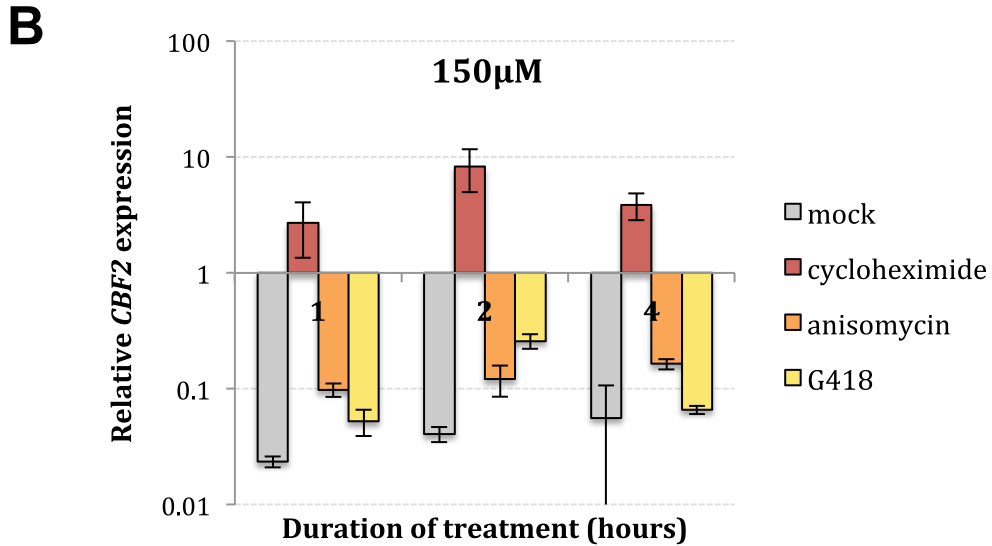


Figure 4.14: *CBF2* induction by different translation inhibitors.
A. thaliana Col-0 seedlings were grown in liquid culture for seven days at 21˚C in constant light and treated with inhibitors at 30 µM (4 µM in the case of lactimidomycin) (**A**) or 150 µM (**B**) for two hours. For mock controls, seedlings were treated with 0.1% v/v DMSO. *CBF2* expression was normalised to *PP2A* and *UBC21*. Error bars represent standard deviation for three biological replicates, with 10-15 seedlings per replicate.

on the ribosome and their distinct mechanisms of repressing protein synthesis, which are summarised in *Figure 4.12*. Inhibitors of 70S ribosomes were also included to test whether chloroplast or mitochondrial ribosomes play a role in this response. The chemical structures of the inhibitors used are given in *Figure 4.13*.

As shown in *Figure 4.14A*, inhibitors of the 70S ribosome had no effect on *CBF2* expression. Similarly, edeine A1, which inhibits translation initiation in both 70S and 80S ribosomes, was not found to induce high levels of *CBF2* expression, further supporting the finding that *CBF* upregulation is not an intrinsic outcome of translation arrest. In contrast, several inhibitors of translation elongation were found to induce high levels of *CBF2* expression, including verrucarin A, narciclasine and blasticidin S, which block the peptidyl transferase reaction of elongation, and phyllanthoside, lactimidomycin and cryptopleurine, which block the translocation step of elongation (Garreau de Loubresse *et al.*, 2014). However, not all inhibitors of translation elongation trigger large increases in *CBF2* expression. For example, deoxynivalenol and hygromycin B induce *CBF2* expression to five- and seven-fold lower levels than cycloheximide, respectively, while anisomycin and G418 induce minor differences in the levels in *CBF2* expression, over 50-fold lower than levels triggered by cycloheximide (*Figure 4.14A*).

Neither high doses (150 μM) nor prolonged incubation times (four hours) were found to induce *CBF2* expression during treatments with the A-site inhibitor anisomycin or the decoding centre inhibitor G418 (*4.12B*). Therefore, there appears to be no correlation between *CBF2* induction and either inhibitor binding sites or mode of inhibition, as treatments with certain A-site inhibitors that block peptide bond formation brought about a strong transcriptional response whereas others did not.

To confirm the uptake and activity of these inhibitors in seedlings, *in vivo* rates of translation were measured using the SUnSET (surface sensing of translation) assay, in which puromycin is incorporated by ribosomes into *de novo* synthesised proteins, thereby labelling them for subsequent detection and quantification using a puromycin-specific antibody (Schmidt *et al.*, 2009). As demonstrated in *Figure 4.15A* and *Figure 4.15B*, the inhibition of translation by cycloheximide is detected within 15 minutes of treating seedlings with puromycin. This method is therefore sufficiently sensitive to measure differences in translation *in vivo*.

As shown in *Figure 4.15C*, a reduction in translation was observed for all eukaryotic inhibitors except the plant alkaloid lycorine, suggesting that the latter may not interact with 80S ribosomes in *Arabidopsis* as it does with yeast ribosomes *in vitro* (Garreau de Loubresse *et al.*, 2014). As a negative control, no decrease in translation was observed for kanamycin, which specifically inhibits 70S ribosomes. While cycloheximide

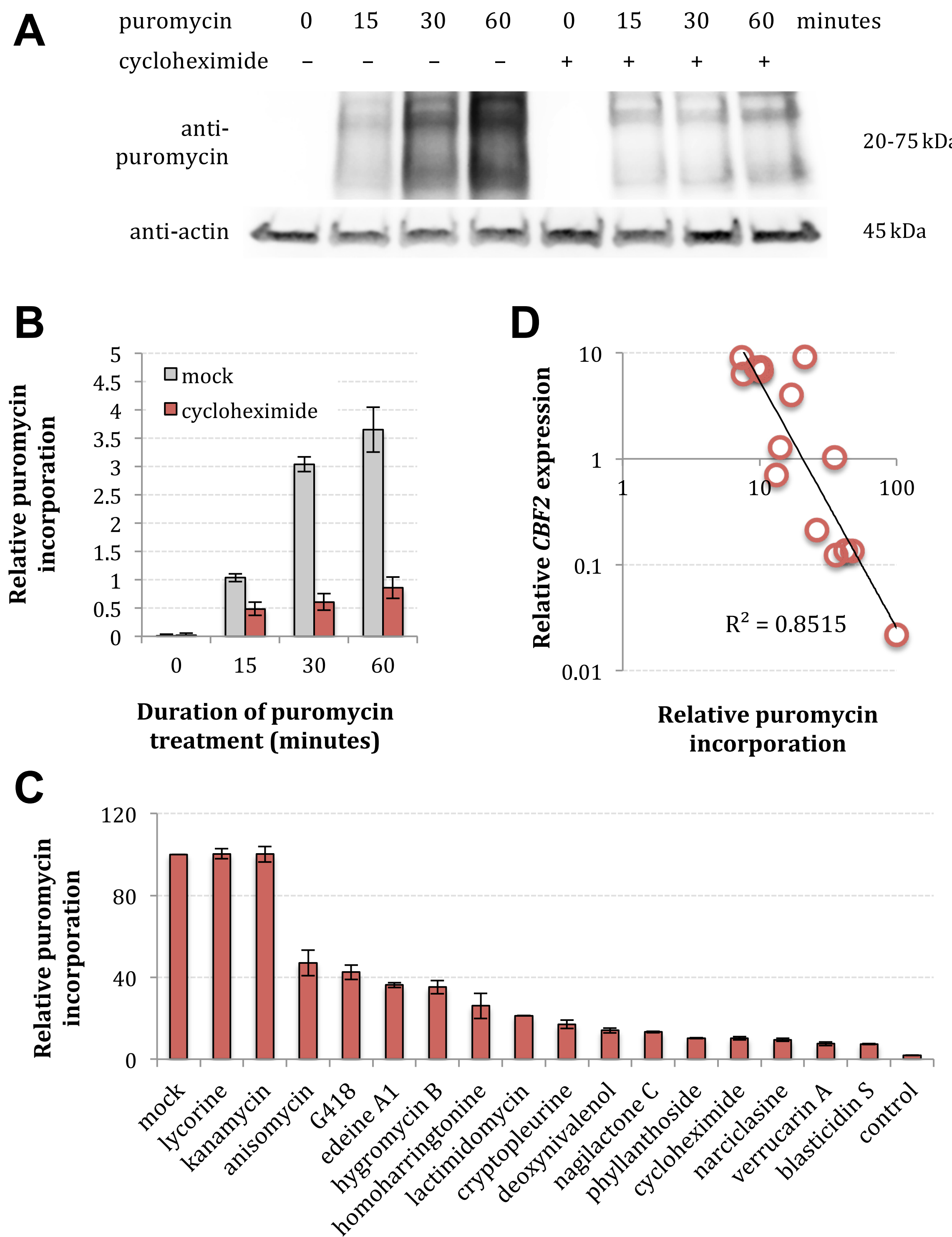


Figure 4.15: *CBF2* induction correlates with the extent of translation repression by inhibitors.
(**A**, **B**, **C**) Quantification of translation *in vivo* using the SUnSET assay. (**A**, **B**) *A. thaliana* Col-0 seedlings were grown in liquid culture for seven days at 21°C in constant light and treated for one hour with 30 µM cycloheximide or 0.1% v/v DMSO (mock control), followed by 0, 15, 30 or 60 minutes of incubation in 100 µM puromycin. (**C**) Seedlings were treated for one hour with inhibitors at 30 µM (4 µM in the case of lactimidomycin) or 0.1% v/v DMSO (mock control) and then for 30 minutes with 100 µM puromycin (control: no puromycin). The amount of puromycin-labelled proteins was normalised to actin levels (**B**, **C**) and is given as a percentage of levels in the mock control in (C). Error bars represent standard deviation for two biological replicates, with 10-15 seedlings per replicate.
(**D**) Correlation between extent of translation inhibition and induction of *CBF2* (from *Figure 4.12*) by translation inhibitors.

diminished protein synthesis to approximately 10% of the levels observed in the absence of inhibitors, anisomycin and G418 caused 50% reductions in translation. A strong correlation was observed between the induction of *CBF2* expression and the extent of translation repression triggered by inhibitors (*Figure 4.15D*).

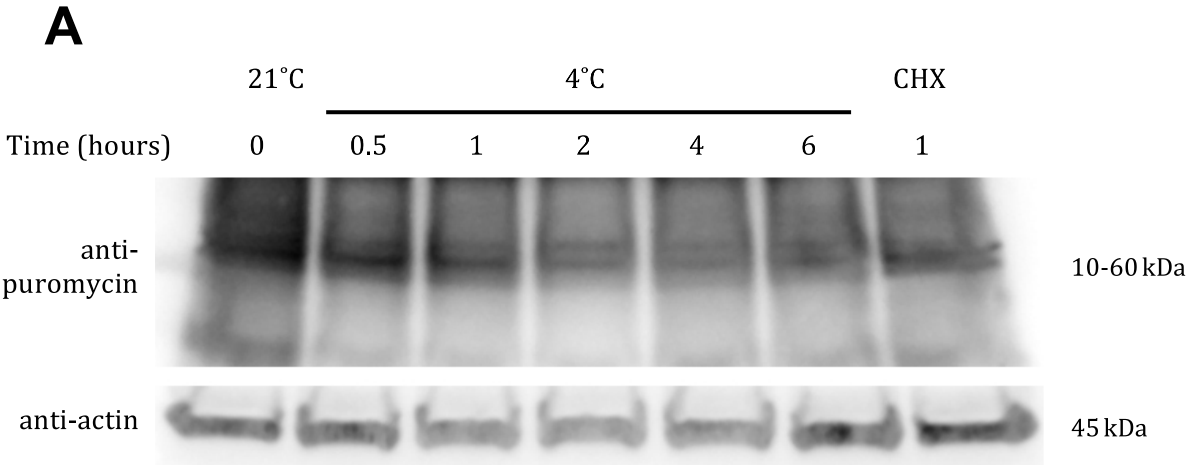
4.2.6 Low temperatures reduce the rate of translation

Low temperatures are known to stabilise RNA secondary structures, which restrict the association or movement of ribosomes on mRNA (Waldminghaus *et al.*, 2007; Wan *et al.*, 2012). Accordingly, a large number of genes involved in cellular processes that are affected by RNA secondary structures, such as mRNA splicing, mRNA export and protein synthesis are upregulated by cold stress in *A. thaliana* (Usadel *et al.*, 2008; Guan *et al.*, 2013a; Beine-Golovchuk *et al.*, 2018). Wang *et al.* (2017b) have shown that *Arabidopsis* seedlings exposed to low temperatures accumulate polysomal ribosomes relative to sub-polysomal fractions, consistent with ribosome stalling upon cold exposure. However, *in vivo* analyses of translation during cold shock are lacking in plants.

Given the correlation between *CBF* expression and translation arrest, it was predicted that cold shock would cause a rapid decrease in translation rate. To test this hypothesis, the SUnSET assay was used to measure *de novo* protein synthesis in *A. thaliana* seedlings exposed to cold treatments of different durations. In order to study rapid responses to cold shock (30 minutes), a short incubation in puromycin (15 minutes) was used following cold exposure. As a result, the dynamic range of translation measurements in this experiment was lower that for the translation inhibitors from *Figure 4.15C*, for which a 30-minute incubation in puromycin was used. Nevertheless, within 30 minutes of incubation at 4˚C, and for all subsequent time-points within the six-hour cold treatment, lower levels of translation were detected relative to the ambient growth temperature of 21˚C (*Figure 4.16A,B*). After two hours of cold-exposure, translation levels were similar to those after one hour of cycloheximide treatment. Lowest levels of translation after two hours coincide with the peak in induction of *CBF2* expression (*Figure 4.3A*).

4.2.7 LOS1 is necessary for full *CBF* induction

In a forward genetic screen for mutants with altered *CBF* expression, Guo *et al.* (2002) identified the elongation factor eEF2 mutant *los1-1* (*low expression of*



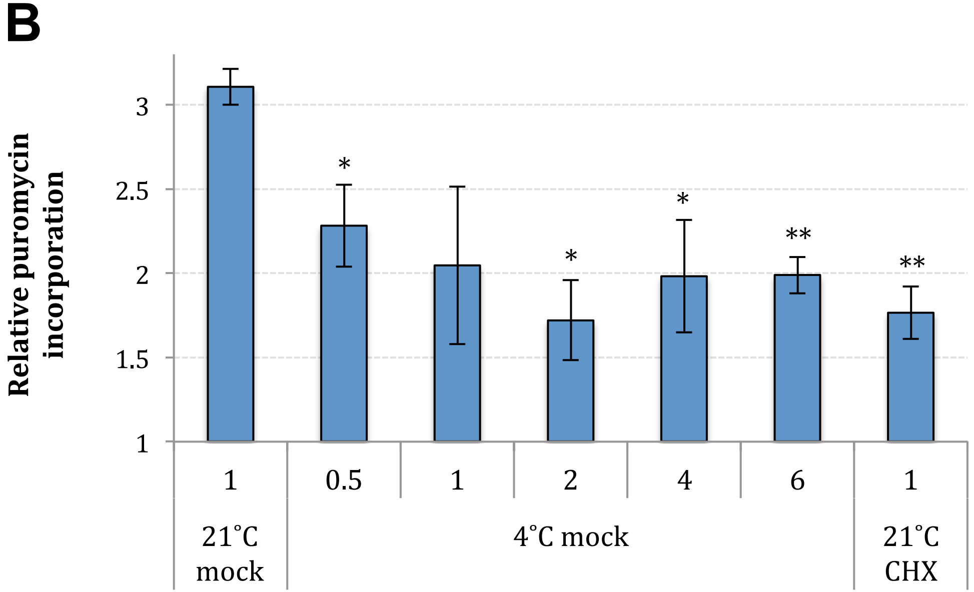


Figure 4.16: Cold shock induces a decrease in translation rate.
(**A**, **B**) Quantification of translation *in vivo* using the SUnSET assay. *A. thaliana* Col-0 seedlings were grown in liquid culture for seven days at 21˚C in constant light and treated with 30 µM cycloheximide (CHX) or 0.1% v/v DMSO (mock control) for one hour at 21˚C or with 0.1% v/v DMSO at 4˚C for 0.5, 1, 2, 4 or 6 hours. Seedlings were subsequently incubated in 150 µM puromycin at the respective temperatures for 15 minutes prior to harvesting. The amount of puromycin-labelled proteins was normalised to actin levels (**B**). Error bars represent standard deviation for two biological replicates, with 10-15 seedlings per replicate. Asterisks indicate significant differences relative to the 21˚C mock treatment (two-tailed *t*-test, p-values <0.05 *, <0.01 **).

Figure 4.17: LOS1 is important for *CBF2* induction by cycloheximide.
(**A**) Schema of mutations in translation elongation factor (eEF1A, eEF2, eEF5) genes, orientated 5’ to 3’. Dark blue boxes represent exons, light blue boxes represent UTRs and black lines represent introns. T-DNA insertions are indicated with arrowheads and the point mutation in *LOS1* (*AT1G56070*) is indicated with an arrow.
(**B**,**C**) *A. thaliana* wild-type and mutants of translation elongation factors (eEF1A, eEF2, eEF5) were grown in liquid culture for seven days at 21˚C in constant light and treated with 30 µM cycloheximide (**B**) or 0.1% v/v DMSO at 4˚C (cold shock) or 22˚C (mock control) (**C**) for two hours. *CBF2* expression was normalised to *PP2A* and *UBC21* and in (B) is given relative to wild-type plants (C24 for *los1-1* and Col-0 for T-DNA insertion mutants). Mock controls at 22˚C were not performed for the experiment in (A). Error bars represent standard deviation for three biological replicates, with 10-15 seedlings per replicate. Asterisks indicate significant differences relative to wild-type (two-tailed *t*-test, p-values <0.005 ***, <0.001 ****). Heterozygous lines are indicated (-/+).

osmotically-responsive genes 1-1), which was reported to be deficient in *de novo* protein synthesis at low temperatures though not at ambient growth temperatures. The authors showed that *CBF2* expression levels were highly upregulated while *COR* gene levels were downregulated after 24 hours of cold treatment, though short-term responses to cold shock were not studied. Given these phenotypes of the *los1-1* mutant and the apparent role of translation repression in cold responses in *A. thaliana*, the involvement of elongation factors in the regulation of *CBF2* expression was investigated.

T-DNA insertion mutants were isolated for three classes of elongation factors: eEF1A, involved in recruitment of aminoacyl-tRNAs to the A-site of ribosomes, eEF2, required for translocation, and eEF5, necessary for facilitating elongation of certain peptide sequences such as proline or glycine repeats (Browning & Bailey-Serres, 2015). Homozygous mutants were obtained for three of the four eEF1A genes (*AT1G07920*, *AT1G07940*, *AT5G60390*), four eEF2 genes, including one of the two canonical eEF2 genes (*AT3G12915*) and three non-canonical eEF2 genes (*AT2G45030*, *AT5G25230*, *AT5G39900*), and two of the three eEF5 genes (*AT1G13950*, *AT1G69410*). For the fourth eEF1A gene (*AT1G07930*), which was found to be essential for viability, a heterozygous mutant was isolated with on average 22% lower expression level of full-length *AT1G07930* than wild-type plants. T-DNA insertions were selected in exons (*Figure 4.17A*), except for the eEF5 gene *AT1G13950*, where an insertion was selected 100 bp upstream of the translation start site and was found to cause a 1.5-fold increase in *AT1G13950* expression. Transcripts from all of the elongation factor genes were detected in the RNA-seq datasets generated, confirming their expression during normal growth and development. As no T-DNA insertions were available for the canonical eEF2 *LOS1* gene, the *los1-1* mutant was used, though it contains a point mutation of unknown effect (C495Y) in the *A. thaliana* C24 ecotype (*Figure 4.17A*).

The induction of *CBF2* expression by cycloheximide was assessed in the elongation factor mutants. As shown in *Figure 4.17B*, the *los1-1* mutant has the strongest phenotype, with approximately a third of the levels of *CBF2* expression observed in wild-type plants after two hours of chemical treatment (two-tailed *t*-test, p-value 4.86×10^{-4}). Another eEF2 mutant, *AT5G39900*, was also found to have slightly lower *CBF2* expression (p-value 2.96×10^{-3}). Significant differences in cycloheximide-associated *CBF2* expression were found neither in the eEF1A or eEF5 mutants, nor in the other canonical eEF2 mutant (*AT3G12915*) (*Figure 4.17B*), suggesting either that these genes are redundant or that they do not contribute to this transcriptional response. LOS1, on the other hand, clearly plays an important role in regulating the expression of *CBF2*. The involvement of LOS1 in cold-induced gene expression was confirmed by subjecting the

los1-1 mutant to cold shock for two hours, and, as shown in *Figure 4.17C*, *CBF2* expression in this mutant was found to be considerably lower than in wild-type plants under these conditions (p-value 1.82×10^{-4}).

Translation levels were monitored in *los1-1* seedlings upon cold shock using the SUnSET assay, to see if these plants have weaker cold- or cycloheximide-induced protein synthesis inhibition, which could account for the reduced induction of *CBF2* expression detected in this mutant. Consistent with results from Guo *et al.* (2002), no differences in translation levels were detected between Col-0 and *los1-1* plants during normal growth at 21˚C (*Figure 4.18*). Similarly, no significant differences were observed between these plants during cold shock or cycloheximide treatment (*Figure 4.18*). This suggests that the effects of LOS1 on *CBF* expression are downstream of cold- or inhibitor-triggered repression of translation.

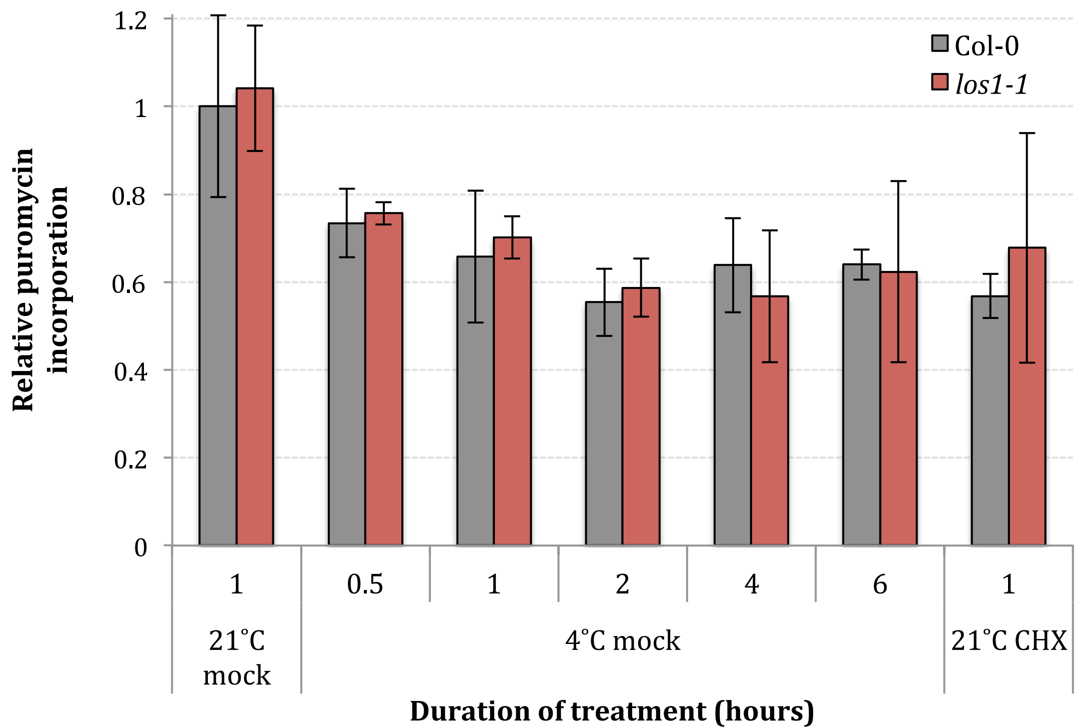


Figure 4.18: LOS1 is not required for cold-associated translation repression.
Quantification of translation *in vivo* using the SUnSET assay in Col-0 and *los1-1* seedlings. Treatments were performed as described in *Figure 4.16*. The amount of puromycin-labelled proteins was normalised to actin levels and is given relative to that in 21˚C mock-treated Col-0 seedlings. Error bars represent standard deviation for two biological replicates, with 10-15 seedlings per replicate.

4.3 Discussion

4.3.1 Gene superinduction by translation inhibitors

The upregulation of certain genes by translation inhibitors was demonstrated over three decades ago and numerous studies in mammalian cells using cycloheximide, anisomycin or puromycin have corroborated this finding (Wall *et al.*, 1986; Radulovic & Tronson, 2008). This phenomenon, known as 'gene superinduction', is characterised by the prolonged expression of genes that are usually rapidly and transiently induced in response to specific stimuli. It has further been demonstrated in bacteria (Jiang *et al.*, 1993), fungi (Tamaru *et al.*, 1994) and plants (Franco *et al.*, 1990; Shen *et al.*, 1993; Li *et al.*, 1994; Botella *et al.*, 1996; Berberich & Kusano, 1997; Zarka *et al.*, 2003). Despite these studies, however, the mechanisms of inhibitor-induced transcriptional responses are unclear.

Gene superinduction has been attributed to labile transcriptional repressors or mRNA decay machinery or to interactions with components of signalling pathways. In this study, it was shown that cycloheximide induces the cold-responsive gene *CBF2* in *A. thaliana via* an interaction with the ribosomal protein RPL36a, whereby a single point mutation in this protein abolishes *CBF2* induction. It was found that some translation inhibitors, such as the inhibitor of translation initiation edeine A1 or the aminoacyl-tRNA analogue puromycin, do not upregulate expression of *CBF2* even at high concentrations. Based on these observations, neither direct activation of signalling enzymes nor protein turnover appear to contribute to this superinduction.

Previous studies have demonstrated that mRNAs in yeast can be protected from degradation by steric hindrance of RNA decay machinery by stalled ribosomes, triggered either by cycloheximide or an accumulation of uncharged tRNAs (Peltz *et al.*, 1992; Beelman & Parker, 1994). *CBF2* mRNA has been shown to have a short half-life in *A. thaliana* seedlings upon a shift to ambient growth temperatures following a cold treatment (Zarka *et al.*, 2003). It is therefore possible that ribosome-associated mRNA stabilisation may contribute to the accumulation of *CBF2* mRNA during cycloheximide treatment. However, this *CBF2* upregulation by cycloheximide is predominantly a transcriptional response, as demonstrated by the similar effect of this inhibitor on stable *GUS* (*β-GLUCURONIDASE*) mRNA in *A. thaliana* plants expressing a *pCBF2::GUS* transgene (Zarka *et al.*, 2003). In addition, induction of *CBF2* by cycloheximide was found to be dependent on the transcriptional activators ICE1, CAMTA2 and CAMTA3, which are constitutively expressed and localised to the nucleus during normal growth and

development, though they do not activate *CBF* expression under these conditions, even when overexpressed (Chinnusamy *et al.*, 2003; Kidokoro *et al.*, 2017). Furthermore, cycloheximide was found to repress instead of stabilise the expression of heat shock protein genes, even though their mRNAs are known to be unstable, with half-lives as short as fifteen minutes (Petersen & Lindquist, 1988; Gasch *et al.*, 2000).

While *CBF2* upregulation by cycloheximide is dependent on the global state of translation, it is possible that gene superinduction in different organisms may involve distinct mechanisms. For example, some studies have reported that cycloheximide can upregulate certain mRNAs independently of translation in mammalian cells (Mahadevan & Edwards, 1991; Sensel *et al.*, 1994; Ohh & Takei, 1995; Joiakim *et al.*, 2004). In soybean, small auxin-up RNAs (SAURs) are highly induced by anisomycin (Franco *et al.*, 1990), the latter of which was found to have only a minor effect on *CBF2* expression in *A. thaliana*. These SAURs are also induced by puromycin (Franco *et al.*, 1990), which decreases the association of ribosomes with mRNA (Gao *et al.*, 2015), indicating that superinduction in this case is not a result of ribosome-associated increase in mRNA stability. Puromycin has also been reported to cause gene superinduction in mammalian cells (Joiakim *et al.*, 2004), though it does not bring about any changes in *CBF2* expression. The phenomenon of gene superinduction therefore appears to be a multifaceted response that involves some degree of specificity between species, genes or inhibitors.

4.3.2 Regulation of temperature-responsive genes by cycloheximide

While a large number of genes are upregulated by one hour of cycloheximide treatment, the strongest positive correlation in transcriptomes among abiotic stress treatments was observed from plants exposed to low temperatures for three to six hours, during which a peak in *CBF* gene expression is observed. The correlation is relatively weak (0.29 to 0.35), though this is not surprising as the abiotic stress transcriptome datasets were obtained from seedlings grown under different experimental conditions (age, photoperiod, temperature, growth substrate) and using different mRNA detection techniques (microarrays) (Kilian *et al.*, 2007). A large number of expressed genes were included in these analyses (10,000 to 16,000 loci), only a subset of which have large changes in expression during stress treatments. Cycloheximide affects the expression of a larger set of genes than cold stress, which may be expected, given the stabilising effect of ribosome stalling on certain mRNAs and the possibility that translation arrest may induce pleiotropic responses such as the

activation of stress-associated genes. In addition, transcriptional responses to low temperatures are likely to involve multiple signalling pathways (Miura *et al.*, 2007; Furuya *et al.*, 2014; Kidokoro *et al.*, 2017; Ding *et al.*, 2018), and while cycloheximide upregulates many cold-inducible genes it does not appear to affect cold-repressible genes.

Nevertheless, there appears to be some degree of specificity in the cold-inducibility of genes upregulated by cycloheximide, whereby genes that increase in expression within the first three to six hours of cold exposure (early cold-induced genes) have on average four-fold higher expression during cycloheximide treatment than genes upregulated after 24 hours of cold exposure (late cold-induced genes), such as the *COR* genes. The group of 'first wave' transcription factors that are induced within an hour of cold shock and regulate the expression of *COR* genes (Park *et al.*, 2015) were extremely highly upregulated by cycloheximide, over ten-fold on average.

Bioinformatic analyses of cycloheximide-inducible genes identified three enriched promoter motifs, which are bound by transcription factors of the WRKY, CAMTA and group B bHLH families that have previously been implicated in cold gene regulation and freezing tolerance (Chinnusamy *et al.*, 2003; Doherty *et al.*, 2009; Kidokoro *et al.*, 2009; Lindlöf *et al.*, 2009; Lee & Thomashow, 2012; Kim *et al.*, 2013; Le *et al.*, 2015; Li *et al.*, 2017a). The bHLH transcription factor ICE1 and the two calmodulin-binding transcriptional regulators CAMTA3 and CAMTA5 have been reported to bind and activate *CBF* promoter sequences *in vitro* (Chinnusamy *et al.*, 2003; Kidokoro *et al.*, 2017). The *A. thaliana* genome encodes over seventy transcriptional regulators of the WRKY family that are associated with abiotic stress responses (Wang *et al.*, 2011b), many of which are upregulated by cold treatment (Le *et al.*, 2015). Decreased freezing tolerance was reported in the *A. thaliana wrky6* mutant (Li *et al.*, 2017a), while enhanced tolerance has also been demonstrated in *Arabidopsis* plants heterologously expressing certain WRKY transcription factors from other species (Zhou *et al.*, 2008; Niu *et al.*, 2012). WRKY binding sites (W-boxes) are significantly enriched in the promoters of cold-inducible genes in *A. thaliana* (Lindlöf *et al.*, 2009), and the *CBF2* gene contains two consensus W-box motifs in its promoter. WRKY mutants with altered *CBF* expression have not yet been reported in *Arabidopsis*, but the bioinformatic analyses of cycloheximide-upregulated genes suggest that these transcription factors could potentially play a role in the regulation of early cold-induced genes.

In contrast to cold-responsive genes, a negative correlation was observed between transcriptomic changes brought about by cycloheximide and those induced by transient heat shock (-0.27). Many genes that are rapidly induced upon heat shock

(within 30 minutes at 38°C) were repressed by cycloheximide, even those with short half-lives that are rapidly attenuated during heat recovery (Petersen & Lindquist, 1988; Gasch *et al.*, 2000). Conversely, a large number of heat-repressed genes were upregulated by cycloheximide treatment. These findings are in accordance with a previous study in human cells, in which a high degree of transcriptome similarity was demonstrated between the transcriptomes induced by RNA silencing of the master heat shock transcriptional regulator HSF1 (HEAT SHOCK FACTOR 1) and those triggered by chemical inhibition of translation (Santagata *et al.*, 2013). The opposing effect of cycloheximide on genes responding to heat or cold treatments suggests that the global state of translation may control temperature-signalling pathways.

Low temperatures can stabilise RNA secondary structures and reduce enzyme kinetics, whereas warm temperatures diminish these RNA interactions and typically promote enzyme activity (Öquist & Huner, 2003; Waldminghaus *et al.*, 2007; Wan *et al.*, 2012). Ambient temperature would therefore be predicted to inherently affect the efficiency or rate of translation, with rapid cold shock triggering global ribosome stalling. Such a relationship has been demonstrated in *Escherichia coli*, in which the rate of translation elongation is directly proportional to the ambient growth temperature and also increases upon a shift to warmer temperatures (Farewell & Neidhardt, 1998). In these bacteria, translation repression *via* the 70S ribosome inhibitor chloramphenicol was reported to induce the expression of the cold-inducible gene *cspA* (*COLD SHOCK PROTEIN A*) (Jiang *et al.*, 1993). Furthermore, distinct ribosome-binding antibiotics were found to induce the accumulation of either heat shock proteins or cold shock proteins upon chemical treatment (VanBogelen & Neidhardt, 1990). A link between temperature and translation is also supported by genetic evidence. Mutants with defects in ribosomal subunit assembly in bacteria and budding yeast are hypersensitive to low temperatures (Guthrie *et al.*, 1969; Tai *et al.*, 1969; Ripmaster *et al.*, 1993; Li *et al.*, 2009), as are mutants of many genes encoding ribosomal proteins (Belyy *et al.*, 2016; Kim & Strich, 2016), translation initiation factors (Altman *et al.*, 1993; Goyer *et al.*, 1993), translation elongation factors (Kinzy *et al.*, 1995) or release factors (Merritt *et al.*, 2010). Overexpression of the elongation factor eEF3 in yeast rescues the cold sensitivity phenotype of ribosome assembly mutants (Kinzy *et al.*, 1994). Null mutations in certain genes associated with translation have also been reported to result in cold sensitivity in *A. thaliana*, for example those encoding the ribosome biogenesis factors REIL1 and REIL2 (REI1-LIKE 1/2) (Beine-Golovchuk *et al.*, 2018) or the regulator of translation initiation GCN1 (GENERAL CONTROL NON-DEREPRESSIBLE 1) (Wang *et al.*, 2017b). Furthermore, genes encoding proteins associated with ribosome biogenesis, translation

initiation and translation elongation are upregulated at low temperatures in *A. thaliana* (Usadel *et al.*, 2008; Beine-Golovchuk *et al.*, 2018), thus highlighting the importance of these components during cold stress.

4.3.3 Ribosome stalling as a potential signal for cold gene induction

Though cycloheximide upregulates early cold-induced genes, reducing *de novo* protein synthesis *per se* was insufficient to trigger *CBF2* expression. No induction of *CBF2* expression was observed during treatments with translation inhibitors that reduce the association of ribosomes with mRNA. This was the case for edeine A1, which binds to the 40S ribosome subunit and prevents translation initiation by interfering with start codon recognition and subunit joining (Garreau de Loubresse *et al.*, 2014), even though a two-thirds reduction in protein synthesis was observed during chemical treatment. Similarly, no *CBF2* upregulation was induced by puromycin, which is incorporated into the nascent peptide and releases it from the ribosome, thereby triggering ribosome dissociation and recycling (Wilson, 2009). Inhibitors that brought about the largest induction of *CBF2* expression were those that strongly block translation elongation, ultimately causing ribosomes to stall on mRNA.

Using puromycin incorporation as a measure of *de novo* protein synthesis, cold shock was shown to trigger rapid translation repression in *A. thaliana* seedlings. Based on a recent study by Wang *et al.* (2017b), in which cold-treated plants were shown to have an enrichment of polysomes relative to sub-polysomal fractions, this low temperature-induced translation repression may be a result of ribosome stalling during elongation. As both cold shock and inhibitors of translation elongation appear to halt elongating ribosomes, it is possible that an accumulation of stalled ribosomes may be a signal for the induction of cold responses in *A. thaliana*.

While the potential signalling mechanism for such stalled ribosomes is not yet clear, such pathways are known to exist in other organisms. In budding yeast, for example, dissociation of stalled ribosomes yields a peptidyl-tRNA-60S complex that is recognised by the ribosome quality control (RQC) complex. The RQC2 component of this complex binds the P-site tRNA and catalyses the formation of a C-terminal alanine and threonine (CAT) tail by recruiting charged tRNAs to the A-site (Shen *et al.*, 2015). The production of aberrant peptides containing CAT tails is a signal for translation stress and activates the HSF1 transcription factor, presumably be forming protein aggregates that trigger the heat shock response (Brandman & Hegde, 2016).

The extent to which protein synthesis is repressed by translation inhibitors was found to positively correlate with *CBF2* induction, consistent with the notion that inhibitors of translation elongation that have weaker effects in *A. thaliana*, such as anisomycin and G418 (approximately 55% reduction in protein synthesis, compared to 90% for cycloheximide), induce less ribosomal stalling. A mechanistic explanation for the difference in potency between these inhibitors is not yet clear. It is possible that anisomycin interacts with 80S ribosomes with lower affinity in *A. thaliana*, or that its incorporation into plant ribosomes is less efficient, relative to other A-site inhibitors. The latter is improbable, however, because although constraints on the ribosomal binding of large inhibitors such as lactimidomycin (Schneider-Poetsch *et al.*, 2010) or homoharringtonine (Ingolia *et al.*, 2011) have been demonstrated, anisomycin has one of the smallest structures among the inhibitors tested.

A second possibility is that these differences in potency are a result of structural alterations in ribosomes induced by the binding of inhibitors. Anisomycin has been shown to stabilise ribosomes in their rotated state, characterised by a movement of the 40S subunit that transfers the acceptor ends of the tRNAs from the A- and P-sites into the P- and E-sites, respectively (Lareau *et al.*, 2014). Matsuo *et al.* (2017) recently demonstrated that ribosomes stalled in their rotated state are targeted for dissociation and recycling by the RQC pathway. As a result the effects of anisomycin on translation inhibition are likely to be diluted by removal of stalled ribosomes, reducing the potential signal for *CBF* gene induction. Cycloheximide, in contrast, stalls ribosomes in their non-rotated state (Lareau *et al.*, 2014), which are not rescued by the RQC complex (Matsuo *et al.*, 2017).

A third possibility, which has previously been suggested to explain the differences in responses of *E. coli* cells treated with translation inhibitors that trigger the accumulation of either heat shock proteins or cold shock proteins (VanBogelen & Neidhardt, 1990), is the difference in occupancy of the A-site upon ribosomal stalling. Cycloheximide and lactimidomycin, which are potent inducers of *CBF2* expression, cause ribosomes to stall with an occupied A-site, as they prevent the movement of the P-site deacylated tRNA into the E-site and the A-site peptidyl-tRNA into the P-site (Schneider-Poetsch *et al.*, 2010). Anisomycin, in contrast, binds to post-translocation ribosomes (Lareau *et al.*, 2014) and prevents the incorporation of puromycin (Wilson, 2009), suggesting that it stalls ribosomes with a vacant A-site. Such ribosomes are detected and rescued *via* the No-Go mRNA decay (NGD) pathway. DOM34 (DUPLICATION OF MULTILOCUS REGION 34) and HBS1 (HSP70 SUBFAMILY B SUPPRESSOR 1), which are paralogues of the release factors eRF1 and eRF3 that terminate translation, bind the

vacant A-site and trigger ribosome dissociation (Shao *et al.*, 2016). This would explain the lower inhibition of translation by anisomycin and its inability to induce *CBF2* expression to the levels observed for cycloheximide or lactimidomycin. This model is in accordance with the effects of translation inhibitors in *E. coli*, in which an occupied A-site in stalled ribosomes correlates with the accumulation of cold-shock proteins (VanBogelen & Neidhardt, 1990). Nevertheless, verrucarin A and narciclasine bind to similar positions within the A-site as anisomycin but still induce *CBF2* expression to high levels, and it is not known whether these inhibitors cause ribosomes to stall in a rotated conformation or with a vacant A-site. Ribosome footprinting (*i.e.*, sequencing the RNA sequences that are occupied by ribosomes) and analyses of aminoacyl-tRNA binding would need to be carried out to address these issues.

4.3.4 The role of LOS1 in cold gene regulation

The rapid induction of *CBF2* expression by cold shock or cycloheximide was found to be dependent on the elongation factor eEF2 protein LOS1. The *los1-1* mutant, containing the missense mutation C495Y in LOS1 (Guo *et al.*, 2002), had only a third of the expression of *CBF2* observed in wild-type plants after two hours of treatment. In contrast, major differences in levels of *CBF* expression during cold shock are not apparent in the double *reil1 reil2* mutant compromised in ribosome biogenesis (Beine-Golovchuk *et al.*, 2018) and the *gcn1* mutant lacking a regulator of translation initiation (Wang *et al.*, 2017b). These mutants are chilling-sensitive (Wang *et al.*, 2017b; Beine-Golovchuk *et al.*, 2018), however they are not strongly compromised in cold acclimation and freezing tolerance, unlike the *los1-1* mutant (Guo *et al.*, 2002). This suggests that the effects of the *los1-1* mutation on cold gene regulation are not inherently linked to defects in translation.

LOS1 appears to be the dominant of two canonical eEF2 elongation factors in *A. thaliana* (Browning & Bailey-Serres, 2015), as its mRNA expression in seedlings is approximately 100-fold higher than that of the other eEF2 gene (*AT3G12915*), based on the RNA-seq dataset generated. The latter does not appear to be essential for *CBF2* induction, as a null mutant for this gene had wild-type levels of *CBF2* expression during cycloheximide treatment. Based on the dominant nature of the *los1-1* mutation and the high degree of similarity between LOS1 and AT3G12915 proteins (96% homology), including the conserved cysteine residue at position 495 that is mutated to a tyrosine residue in the *los1-1* mutant, it is likely that these proteins act redundantly. The mutant of another non-canonical eEF2 gene, *AT5G39900*, was found to have a small but

significant difference in *CBF2* induction by cycloheximide, though no significant differences were observed for mutants in eEF1A or eEF5 genes. Though the four eEF1A proteins are highly similar, they are not entirely redundant, as demonstrated by the embryo lethality of the homozygous null mutant of *AT1G07930*. While more detailed mutagenesis analyses are required to determine the possible involvement of eEF1A or eEF5 proteins in transcriptional responses to cold shock, it is evident from the *los1-1* and *AT5G39900* mutants that eEF2 proteins play an important role in this pathway.

No detectable differences in cold-induced translation repression were observed between the *los1-1* mutant and wild-type plants. It is possible that the SUnSET assay used to measure rates of protein synthesis *in vivo* was not sensitive enough to detect differences between these two genotypes. In addition, because of natural variability between seedlings and variations arising from the western blotting procedure, the experiment would need to be replicated several more times to determine if there are any subtle differences in translation between these plants. Based on the results presented, however, it is clear that cold-induced repression of translation also occurs in the *los1-1* mutant. This suggests that LOS1 is required for *CBF* induction downstream of cold shock-associated ribosome stalling.

Guo *et al.* (2002) previously reported that the *los1-1* mutant is incapable of *de novo* protein synthesis at low temperatures, while it does not have any translation defects at normal growth temperatures. These authors carried out measurements of protein synthesis over a period of 1.5 days after four days at 0˚C. In the current study, measurements using the SUnSET assay were carried out after a maximum of six hours of cold treatment. Longer incubations at low temperatures would be required to confirm these previous findings. Guo and colleagues also demonstrated that expression of the *CBF* genes after 24 hours of cold exposure is considerably higher in the *los1-1* mutant relative to wild-type plants, whereas that of *COR* genes is much lower. The authors speculated that the transient upregulation of *CBF* expression during cold shock is caused by negative feedback inhibition of *CBF* promoters by their target gene products and that this feedback is abolished in the *los1-1* mutant because of its defect in protein synthesis at low temperatures, though evidence to support this hypothesis is currently lacking (Guo *et al.*, 2002). Increased expression of *CBF2* after 24 hours of cold treatment may alternatively be a result of over-compensation in cold gene induction resulting from the activation of parallel cold signalling pathways. It is likely that perception of low temperatures occurs *via* multiple mechanisms, given that all the cold-signalling mutants that have been identified still induce the expression of *CBF* genes during cold treatment, albeit to lower levels.

Although it is not yet clear how LOS1 could be involved in cold signalling pathways, many translation elongation factors have been found to have additional roles outside general protein synthesis. For example, eEF1A in human cells was found to interact with both the transcriptional regulator HSF1 and RNA polymerase II to promote expression of *HSP70* (Vera *et al.*, 2014). This protein can also localise to the nucleus in the absence of heat shock (Mingot *et al.*, 2013) and binds both mRNAs (Liu *et al.*, 2002) and diverse types of proteins, including signalling kinases and receptors (McClatchy *et al.*, 2006; Amiri *et al.*, 2007; Bluem *et al.*, 2007), transcription factors (Mingot *et al.*, 2013) and cytoskeletal components (Liu *et al.*, 2002). The γ-subunit of eEF1B also regulates gene expression in yeast, for example by binding to the promoter of the redox regulator gene *MXR1* (*METHIONINE SULFOXIDE REDUCTASE 1*) (Hanbauer *et al.*, 2003). The fungal eEF3 protein binds certain mRNAs, possibly to regulate their localisation (Samra *et al.*, 2015), and is also required for protein targeting, for example in ferrying the calcium channel CCH1 (CALCIUM CHANNEL HOMOLOG 1) to the plasma membrane (Liu & Gelli, 2008). Elongation factors of the eEF5 family play roles in intracellular trafficking and RNA stability (Rosorius *et al.*, 1999; Schrader *et al.*, 2006), and in *A. thaliana* the eEF5 protein FBR12 (FUMONISIN B_1-RESISTANT 12) is required for cytokinin-mediated protoxylem development (Ren *et al.*, 2013).

Similar extra-translational functions have been demonstrated for certain ribosomal proteins in yeast and human cells (Bluem *et al.*, 2007; Schroder & Moore, 2005) and have also been proposed for ribosomal or ribosome-associated proteins in plants (Browning & Bailey-Serres, 2015). In addition to eEF1A, other translation-associated proteins may interact directly with transcription factors, as has been demonstrated for the yeast ribosome-associated protein RBG1 (RIBOSOME INTERACTING GTPASE 1), which physically binds to the oxidative stress-associated transcriptional regulator YAP1 (YEAST AP-1) (Wout *et al.*, 2009). Although the *los1-1* mutant does not have translation defects at normal growth temperatures, its flowering is delayed (Guo *et al.*, 2002), suggesting that LOS1 also has extra-translational roles in plant development.

During preparation of this thesis, a study was published demonstrating that *A. thaliana* plants with defects in the nascent polypeptide-associated complex (NAC), which shields nascent peptides from the cytosol, are hypersensitive to freezing and are characterised by lower *CBF* gene induction during cold exposure (Ding *et al.*, 2018). It is unclear how these proteins regulate *CBF* expression, however Ding and colleagues demonstrated that the NAC components BTF3 (BASIC TRANSCRIPTION FACTOR 3) and

BTF3L (BTF3-LIKE) can localise to the nucleus. It will be of interest to determine whether these proteins interact with LOS1 to jointly regulate cold gene expression.

Based on the results presented, it possible that LOS1 may sense global ribosome stalling induced by translation inhibitors or cold shock, perhaps as a result of reduced association with ribosomes, since eEF2 proteins bind to the rotated state of the ribosome, while cycloheximide stabilises ribosomes in their non-rotated state (Lareau *et al.*, 2014). Franke *et al.* (2001) have demonstrated that NAC subunits in yeast can re-localise from the cytosol to the nucleus when their binding to ribosomes is prevented, which suggests that nuclear re-localisation might also be possible for LOS1.

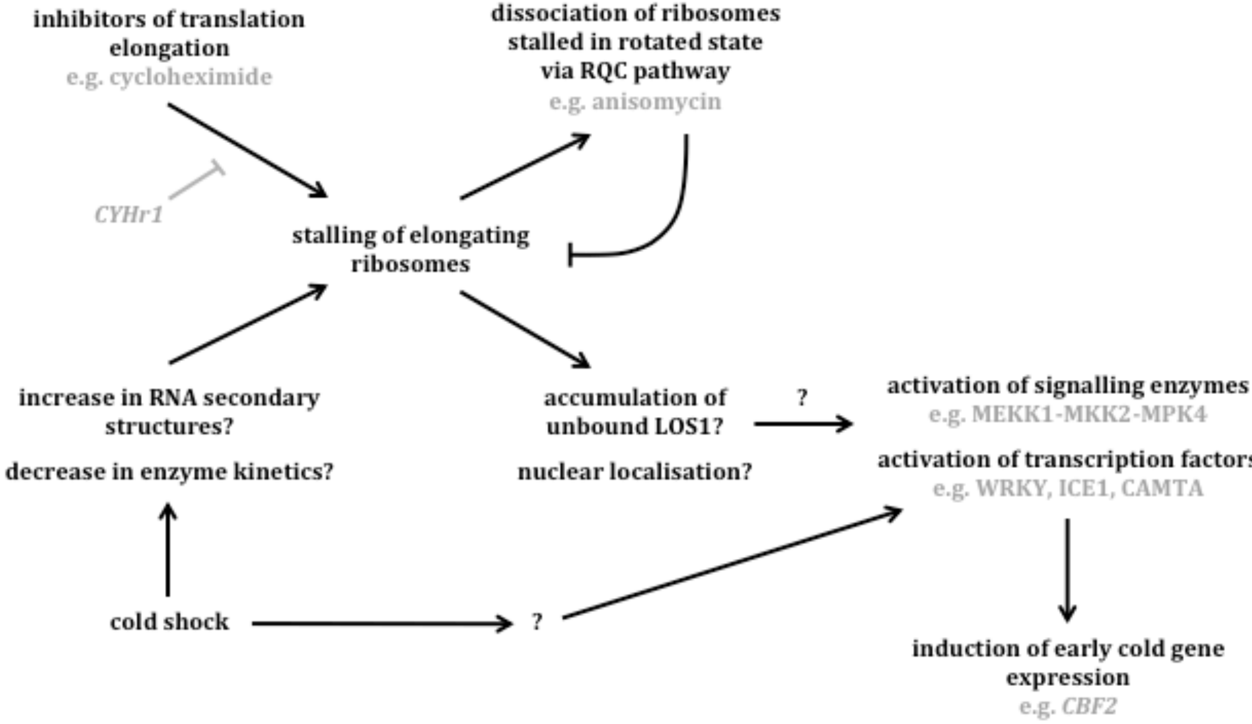


Figure 4.19: Proposed model of *CBF* gene induction by ribosome stalling triggered by translation inhibitors or cold shock. Question marks represent unknown mechanisms.

Given the interaction of eEF1A with transcription factors in human cells, LOS1 might be capable of interactions with components of cold temperature signalling pathways, such the known regulators ICE1 and CAMTAs or additional regulators of the WRKY family. As certain translation inhibitors have been shown to activate kinase signalling in human cells (Newton *et al.*, 1997; Hazzalin *et al.*, 1998; Joiakim *et al.*, 2004),

ribosome stalling triggered by cycloheximide or cold shock may also activate kinase pathways, such as that involving MPK4 (MAP KINASE 4), which is known to positively regulate *CBF* expression (Teige *et al.*, 2004; Zhao *et al.*, 2017). A possible model for translation-mediated regulation of low temperature signalling is given in *Figure 4.19*, which now needs to be experimentally tested.

Chapter 5

Calcium signalling in *CBF* gene induction

5.1 Introduction

Cold shock was among the first of many environmental stimuli reported to trigger an increase in cytosolic free calcium in plants (Knight *et al.*, 1991). Since this initial observation, several studies have implicated calcium in the regulation of cold-responsive genes. The induction of certain genes at low temperatures, for example those encoding putative antifreeze proteins KIN1 (*COLD-INDUCED 1*) and COR6.6/KIN2 (*COLD-RESPONSIVE 6.6/COLD-INDUCED 2*), the cell wall-modifying enzyme XTH22/TCH4 (*XYLOGLUCAN ENDOTRANSGLUCOSYLASE-HYDROLASE 22/TOUCH-INDUCED 4*) and the calmodulin-like proteins CML12/TCH3 (*CALMODULIN-LIKE 12/TOUCH-INDUCED 3*) and CML24/TCH2 (*CALMODULIN-LIKE 24/TOUCH-INDUCED 2*), can be repressed by pre-treating *Arabidopsis thaliana* seedlings with calcium chelators or channel blockers (Knight *et al.*, 1996; Polisensky & Braam, 1996; Tähtiharju *et al.*, 1997). Similar results have been reported for cold-inducible *CAS* (*COLD ACCLIMATION-SPECIFIC*) genes in alfalfa cells, in which treatments with inhibitors of calcium signalling have been shown to abolish cold acclimation, resulting in hypersensitivity to freezing (Monroy *et al.*, 1993).

When exposed to low temperatures, *A. thaliana* mutants lacking the mechanosensitive calcium-permeable channels MCA1 and MCA2 (MID1-COMPLEMENTING ACTIVITY 1/2) are characterised by lower expression of certain *COR* (*COLD-RESPONSIVE*) genes, such as *AT5G61820*, *AT3G51660* and *AT4G15490* (Mori *et al.*, 2018). Conversely, enhanced cold-induction of *CBF* (*CRT-BINDING FACTOR*) genes has been reported in mutants of the calcium efflux transporter CAX1 (CATION EXCHANGER 1), which are compromised in vacuolar calcium uptake (Catalá *et al.*, 2003). In addition, transcriptional regulators of the CAMTA (CALMODULIN-BINDING TRANSCRIPTION ACTIVATOR) family, which interact with calmodulin (Bouché *et al.*, 2002), are required for full induction of *CBF* genes during cold stress (Doherty *et al.*, 2009; Kim *et al.*, 2013).

As demonstrated in *Chapter 4*, cycloheximide and several other inhibitors of translation elongation trigger the induction of *CBF* genes. Multiple findings suggest that calcium signalling may be involved in the transcriptional response to these inhibitors.

Firstly, among abiotic stress datasets studied, the strongest positive correlation was observed between the transcriptomes of plants treated with cycloheximide and those subjected to either cold shock, osmotic stress or wounding, all of which trigger a rapid increase in cytosolic calcium (Knight *et al.*, 1991, 1996, 1997). The three motifs that were significantly enriched in the promoters of genes upregulated by cycloheximide (W-box, CAM-box and G-box) are known to be involved in calcium signalling. The W-box motif is bound by transcription factors of the WRKY (Tryptophan-Arginine-Lysine-Tyrosine domain) family and a large subgroup of these proteins has been found to interact with calmodulin (Park *et al.*, 2005; Popescu *et al.*, 2007). Whalley *et al.* (2011) demonstrated that cytosolic calcium elevations triggered by electrical impulses in *A. thaliana* upregulate the expression of genes containing CAM-box or G-box/ABRE (abscisic acid-responsive element) motifs, bound by transcription factors of the CAMTA and bHLH/ABF (ABRE-BINDING FACTOR) families, respectively. As demonstrated in *Chapter 4*, the induction of *CBF2* expression by cycloheximide was reduced in the double *camta2 camta3* mutant. Furthermore, Berberich & Kusano (1997) demonstrated that cycloheximide-mediated upregulation of the cold-responsive gene *MLIP15* was diminished in the presence of a calcium chelator.

The objectives of this project are as follows:

1. To determine to what extent the induction of *CBF* gene expression by cold shock or translation inhibitors is dependent on calcium signalling.
2. To characterise the effects of various translation inhibitors on cytosolic calcium levels.

5.2 Results

5.2.1 *CBF* gene induction is mediated *via* calcium signalling

Although the upregulation of *KIN* and *TCH* genes during low temperature was previously reported to involve calcium signalling (Knight *et al.*, 1996; Polisensky & Braam, 1996; Tähtiharju *et al.*, 1997), the requirement for calcium mobilisation in the induction of *CBF* genes has not been studied. To test this possibility, *A. thaliana* seedlings were pre-treated in medium supplemented with either calcium chloride or calcium signalling inhibitors and then subjected to cold shock or maintained at ambient growth temperature. Two calcium signalling inhibitors were used, namely lanthanum chloride, a competitive inhibitor of calcium-permeable channels, and sodium 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetate (BAPTA), a calcium chelator.

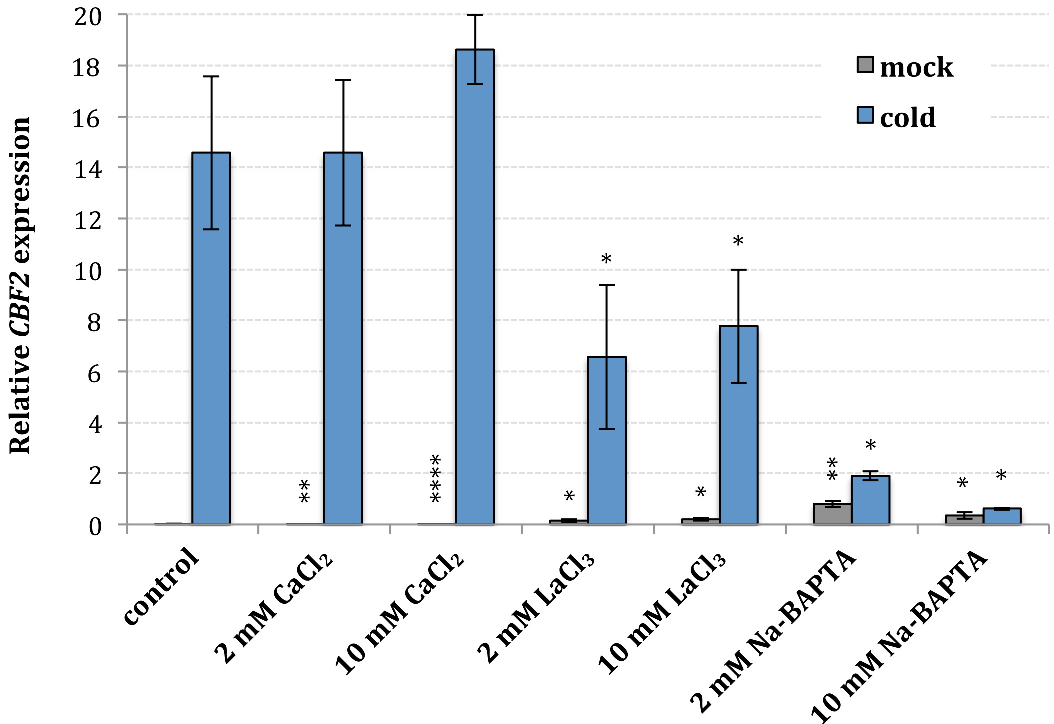


Figure 5.1: Inhibitors of calcium signalling block cold-induced *CBF2* expression.
A. thaliana Col-0 seedlings were grown in liquid culture for seven days at 21˚C in constant light, pre-treated for 30 minutes with fresh medium (control), calcium chloride ($CaCl_2$) or calcium signalling inhibitors and then either transferred to 4˚C (cold) or maintained at 21˚C (mock) for two hours. Treatments at 21˚C or 4˚C contained DMSO to a final concentration of 0.1% v/v. $LaCl_3$: lanthanum chloride, Na-BAPTA: sodium-BAPTA. *CBF2* expression was normalised to *PP2A* and *UBC21*. Error bars represent standard deviation for three biological replicates, with 10-15 seedlings per replicate. Asterisks indicate significant differences between chemical and control treatments (two-tailed *t*-test, p-value <0.05 *, <0.01 **, <0.005 ***, <0.001 ****).

As shown in *Figure 5.1*, increasing the extracellular pool of free calcium to 10 mM causes a slight though not significant increase in average *CBF2* expression during cold shock (two-tailed *t*-test, p-value 0.178). In contrast, expression levels were two-fold lower in the presence of lanthanum and eight-fold to 25-fold lower in the presence of 2 mM or 10 mM BAPTA, respectively. BAPTA caused a slight increase in basal *CBF2* expression in the absence of cold shock, similar to the level of expression after cold shock in seedlings pre-treated with 10 mM BAPTA.

Similar effects on *CBF2* expression were observed in *A. thaliana* seedlings upon treatment with cycloheximide (*Figure 5.2A*). Pre-treatment with calcium chloride increased cycloheximide-mediated *CBF2* induction (two-tailed *t*-test, p-value 0.006 at 2 mM, 0.067 at 10 mM), whereas lanthanum and BAPTA both diminished expression over six-fold. Interestingly, the negative effect of lanthanum on *CBF2* induction was more

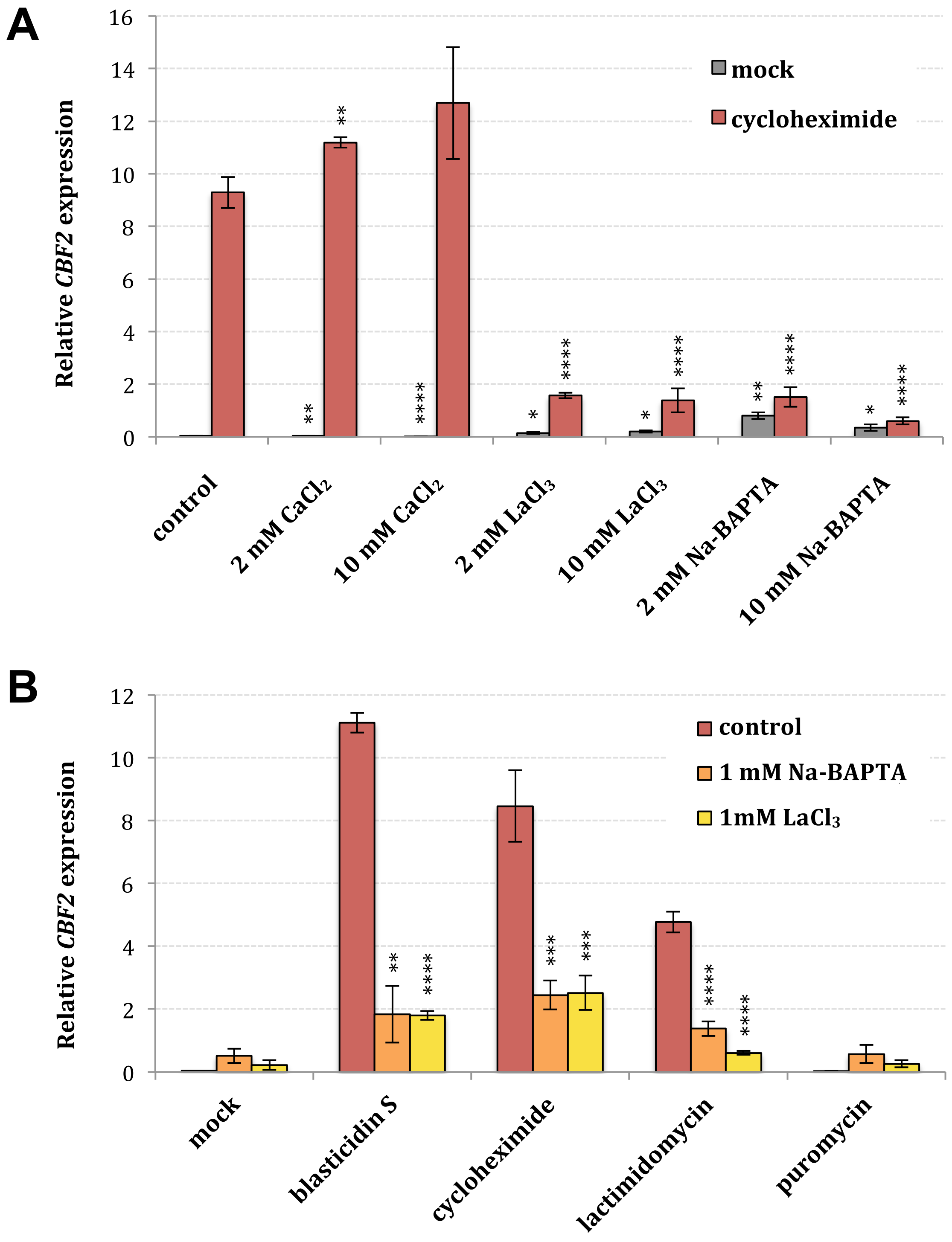


Figure 5.2: Inhibitors of calcium signalling block the induction of *CBF2* by translation inhibitors. *A. thaliana* Col-0 seedlings were grown in liquid culture for seven days at 21˚C in constant light and pre-treated for 30 minutes with fresh medium (control), calcium chloride or calcium signalling inhibitors and then treated for two hours either with 0.1% v/v DMSO (mock treatment) or 30 μM cycloheximide (**A**) or other translation inhibitors at 30 μM (2μM for lactimidomycin) (**B**). Translation inhibitors were added to the medium without removing the calcium chloride ($CaCl_2$), lanthanum chloride ($LaCl_3$) or sodium-BAPTA (Na-BAPTA). *CBF2* expression was normalised to *PP2A* and *UBC21*. Error bars represent standard deviation for three biological replicates, with 10-15 seedlings per replicate. Asterisks indicate significant differences between chemical and control treatments (two-tailed *t*-test, p-value <0.05 *, <0.01 **, <0.005 ***, <0.001 ****).

pronounced for cycloheximide treatment than for cold shock. Reductions in *CBF2* expression by lanthanum and BAPTA were also detected for other inhibitors of translation elongation, such as blasticidin S and lactimidomycin (*Figure 5.2B*). No significant differences in expression relative to mock treatments were observed for puromycin, which triggers translation termination but does not upregulate *CBF2*.

5.2.2 Cycloheximide triggers an increase in cytosolic free calcium

Given the inhibitory effect of calcium signalling inhibitors on cycloheximide-mediated *CBF2* expression, cytosolic free calcium levels in *A. thaliana* seedlings were measured in response to treatments with this inhibitor, using the calcium reporter aequorin. As shown in *Figure 5.3*, cycloheximide causes an increase in cytosolic calcium after 20 to 30 minutes of treatment, which levels off after 70 to 80 minutes at approximately 1.9-fold higher levels than those in mock-treated plants.

In the presence of lanthanum, the cycloheximide-induced increase in cytosolic calcium was completely abolished (*Figure 5.4A*), consistent with its effects on *CBF2* expression (*Figure 5.2B*). BAPTA, however, caused an increase in cytosolic calcium, both in the presence or absence of cycloheximide (*Figure 5.2B*), indicating that chelation of the apoplast pool of free calcium by this inhibitor may trigger the opening of depolarisation-activated calcium channels. The 2.6-fold increase in cytosolic calcium caused by BAPTA correlates with a minor increase in basal expression of *CBF2* (*Figure 5.2A,B*).

The effects of cold shock on cytosolic calcium levels have been most widely studied by discharging ice-cold water onto seedlings (Knight *et al.*, 1991, 1996; Ranf *et al.*, 2008; Krebs *et al.*, 2012). This immediate drop in temperature triggers an influx of calcium from the apoplast, at least partly by activating mechanosensitive calcium-permeable channels (Mori *et al.*, 2018). The calcium signature triggered by instantaneous cooling (*Figure 5.5A*) is transient and has high amplitude.

In contrast, decreasing the rate of cooling, for example to 3˚C per minute, induces a more gradual elevation in cytosolic calcium (Plieth *et al.*, 1999). Given the requirement for a specialised luminometer setup, experiments to monitor cytosolic calcium in real-time during different cooling regimes were not possible for this study, and attempts to replicate the results by Plieth and colleagues by incubating cuvettes at low temperature and subsequently monitoring luminescence were unsuccessful. Nevertheless, although the temporal characteristics differ between these two experiments, the shape of the increase in cytosolic calcium reported by Plieth *et al.*

(1999) during moderate cooling bears striking similarity to that triggered by cycloheximide.

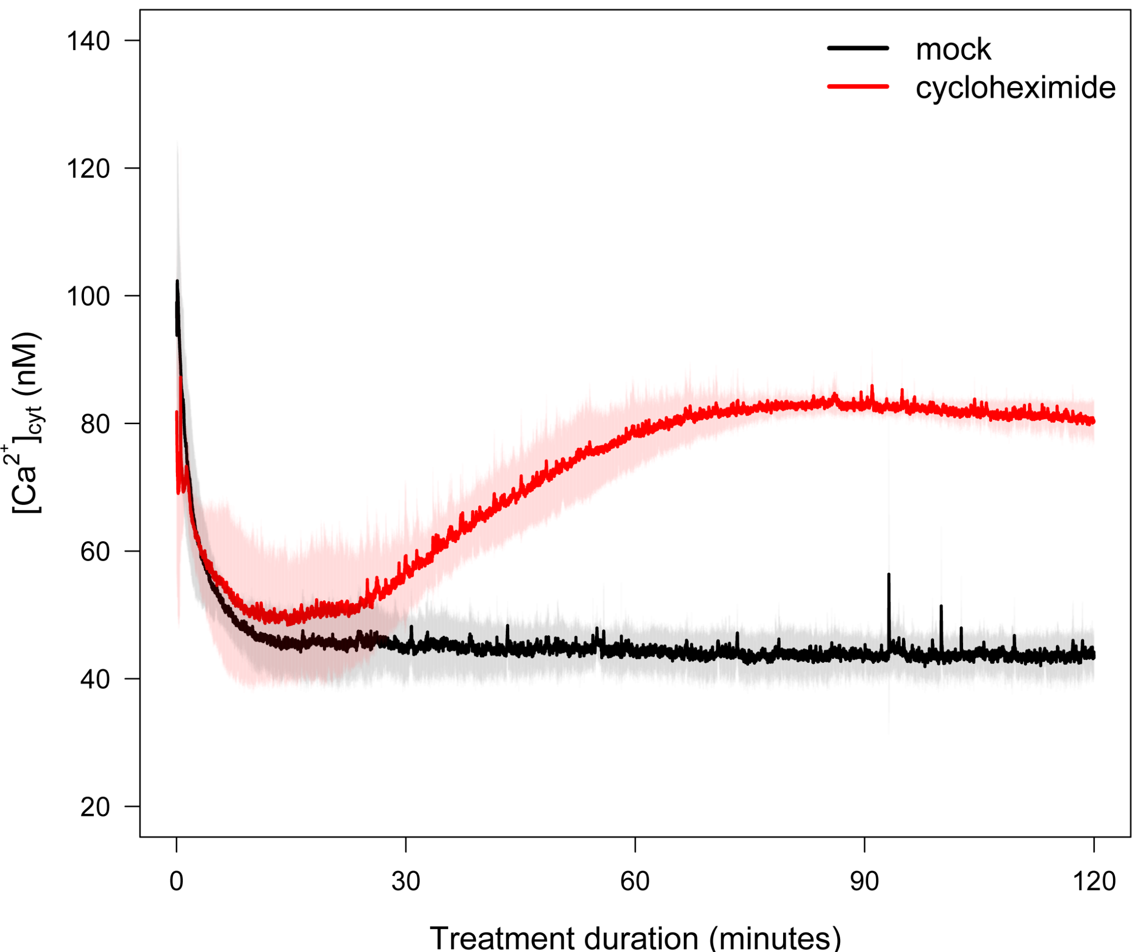


Figure 5.3: Cytosolic calcium signature induced by cycloheximide.
A. thaliana Col-0 *pCaMV35S::APOAEQUORIN* seedlings were grown in liquid culture for eight days at 20°C with a 12-hour photoperiod and cytosolic free calcium levels were quantified luminometrically during two-hour treatments with 30 μM cycloheximide or 0.1% DMSO (mock control) in deionised water. Cuvettes were placed in the luminometer immediately after the addition of chemicals. Shading indicates standard deviation for three biological replicates, each comprising a cuvette with three seedlings.

The cooling rates for cold shock treatments used in this thesis (*Figure 5.1* and *Chapter 4*) were measured using a thermocouple placed in liquid medium in tissue culture plates during a temperature shift from 24°C to 4°C. The cooling rate was found to be at most 3°C per minute, within three minutes of transfer to 4°C, with a gradual decrease in cooling until the temperature was stabilised at 4°C after 30 minutes (*Figure 5.5B*). These moderate rates of cooling suggest that the cold shock treatments used in

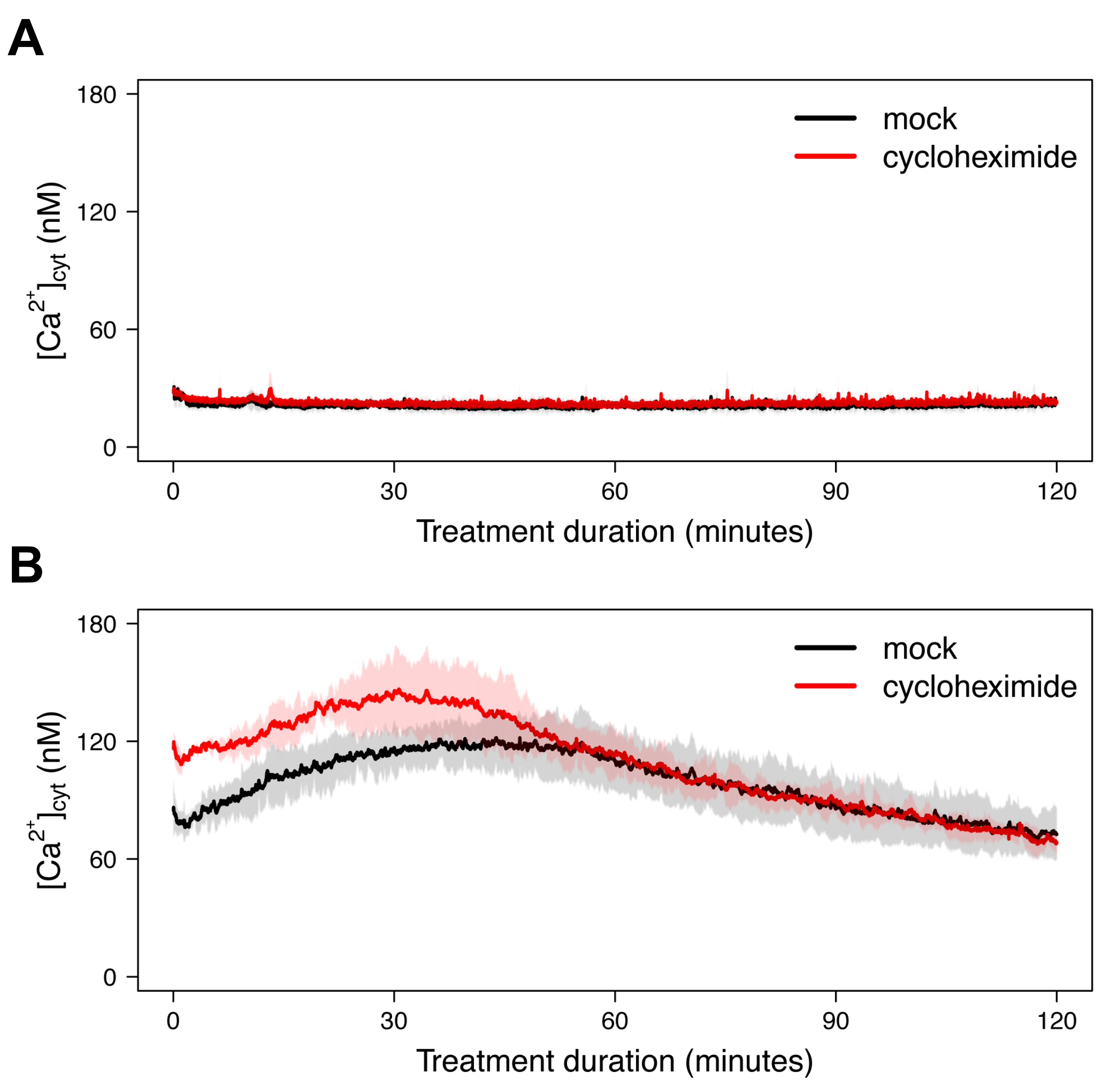
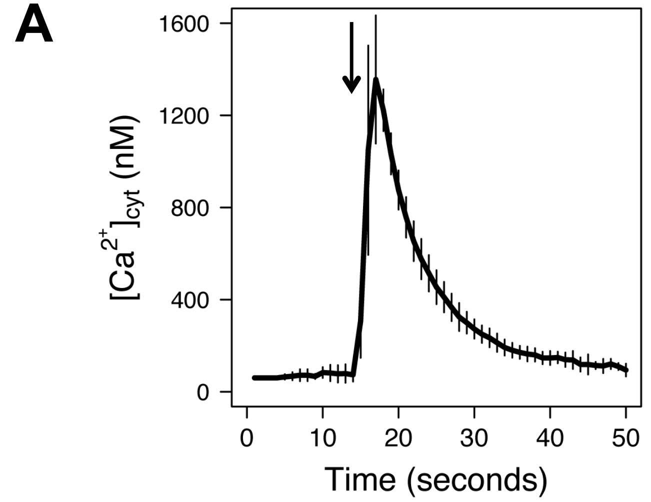


Figure 5.4: Effects of lanthanum and BAPTA on the cytosolic calcium dynamics during cycloheximide treatment.
A. thaliana Col-0 *pCaMV35S::APOAEQUORIN* seedlings were grown in liquid culture for eight days at 20˚C with a 12-hour photoperiod and pre-treated for 30 minutes in 1 mM lanthanum chloride (**A**) or 1 mM sodium BAPTA (**B**) in deionised water. The liquid was then replaced with fresh solutions containing lanthanum or BAPTA and either 30 µM cycloheximide or 0.1% v/v DMSO (mock control). Cuvettes were placed in the luminometer immediately after the addition of chemicals and cytosolic free calcium levels were quantified luminometrically for two hours. Shading indicates standard deviation for three biological replicates, each comprising a cuvette with three seedlings.



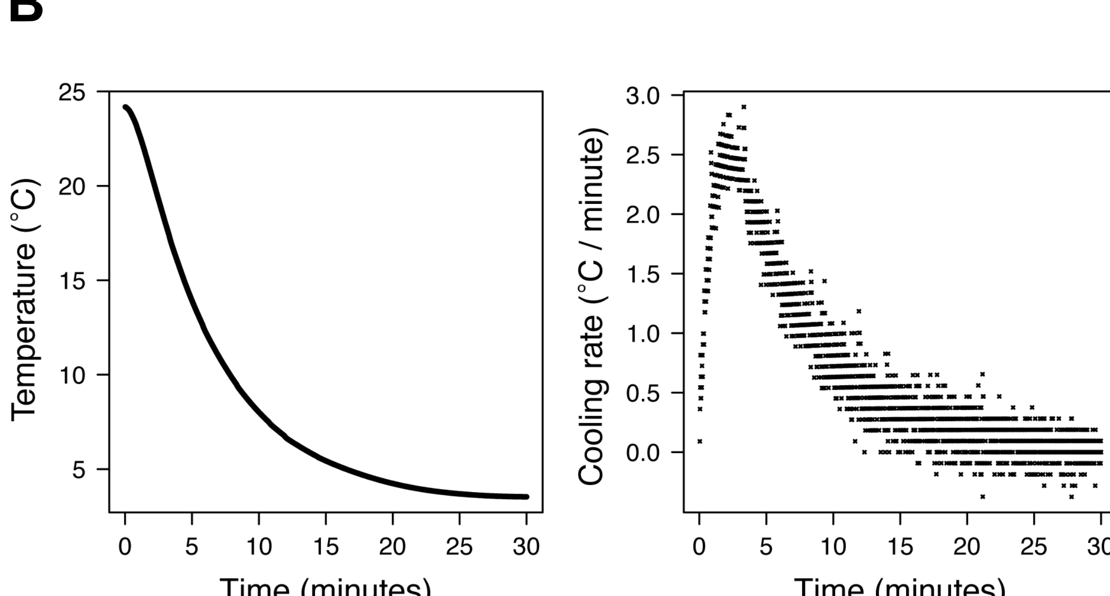


Figure 5.5: The rate of cooling affects cold-induced calcium signatures.
(**A**) *A. thaliana* Col-0 *pCaMV35S::APOAEQUORIN* seedlings were grown in liquid culture for eight days at 20˚C with a 12-hour photoperiod and cytosolic free calcium levels were quantified luminometrically during instantaneous cooling by injecting ice-cold water into the cuvettes, at the time-point indicated by the arrow. Error bars represent standard deviation for three biological replicates, each comprising cuvette with a three seedlings.
(**B**) Temperature and rate of temperature change of liquid medium during incubations at 4˚C, measured using a thermocouple.

this thesis invoke gradual cytosolic calcium elevations in *A. thaliana* seedlings similar to those reported by Plieth *et al.* (1999) rather than the rapid calcium transients detected during instantaneous cooling. Based on the similarities in transcriptional and calcium responses between moderate cooling and cycloheximide treatment, it was hypothesised that common calcium signalling mechanisms might be involved in regulating the induction of *CBF2* expression by these two different stimuli.

5.2.3 Translation inhibitors induce distinct calcium signatures

The effects of other protein synthesis inhibitors on cytosolic calcium levels were determined. Similarly to cycloheximide, inhibitors of translation elongation induced a gradual calcium increase after 20 to 30 minutes of treatment, which reached a maximum after 60 to 90 minutes (*Figure 5.6*). There was no evident difference in the shapes of the calcium signatures between inhibitors that induce high levels of *CBF2* expression, including blasticidin S, phyllanthoside and verrucarin A, and those that cause only minor differences in *CBF2* expression, such as homoharringtonine, anisomycin and deoxynivalenol (*Figure 4.14A*, *Chapter 4*).

Inhibitors targeting different steps of translation, however, were found to trigger distinct calcium signatures. As shown in *Figure 5.7*, edeine A1, which inhibits translation initiation, causes a steep increase in cytosolic calcium within five minutes of treatment, which peaks after 20 minutes and then gradually decreases. The aminoacyl-tRNA mimic puromycin, which triggers translation termination, brings about an instantaneous increase in cytosolic calcium that subsides to resting levels after approximately 30 minutes. Kanamycin, which inhibits 70S ribosomes, and hygromycin B, which targets both 80S and 70S ribosomes, also trigger calcium signatures with different characteristics (*Figure 5.7*).

Increases in cytosolic calcium were detected in response to all translation inhibitors. Despite a positive correlation between calcium levels and *CBF2* expression after two-hours treatment with inhibitors of translation elongation (Pearson correlation coefficient 0.908), elevations in cytosolic calcium levels are clearly not sufficient to trigger *CBF2* expression to levels observed during cycloheximide treatment (*Figure 5.8*).

5.2.4 LOS1 does not influence cycloheximide-induced calcium influx

The elongation factor eEF2 gene *LOS1* (*LOW EXPRESSION OF OSMOTICALLY-RESPONSIVE GENES 1*) was found to contribute to the induction of *CBF2* expression by

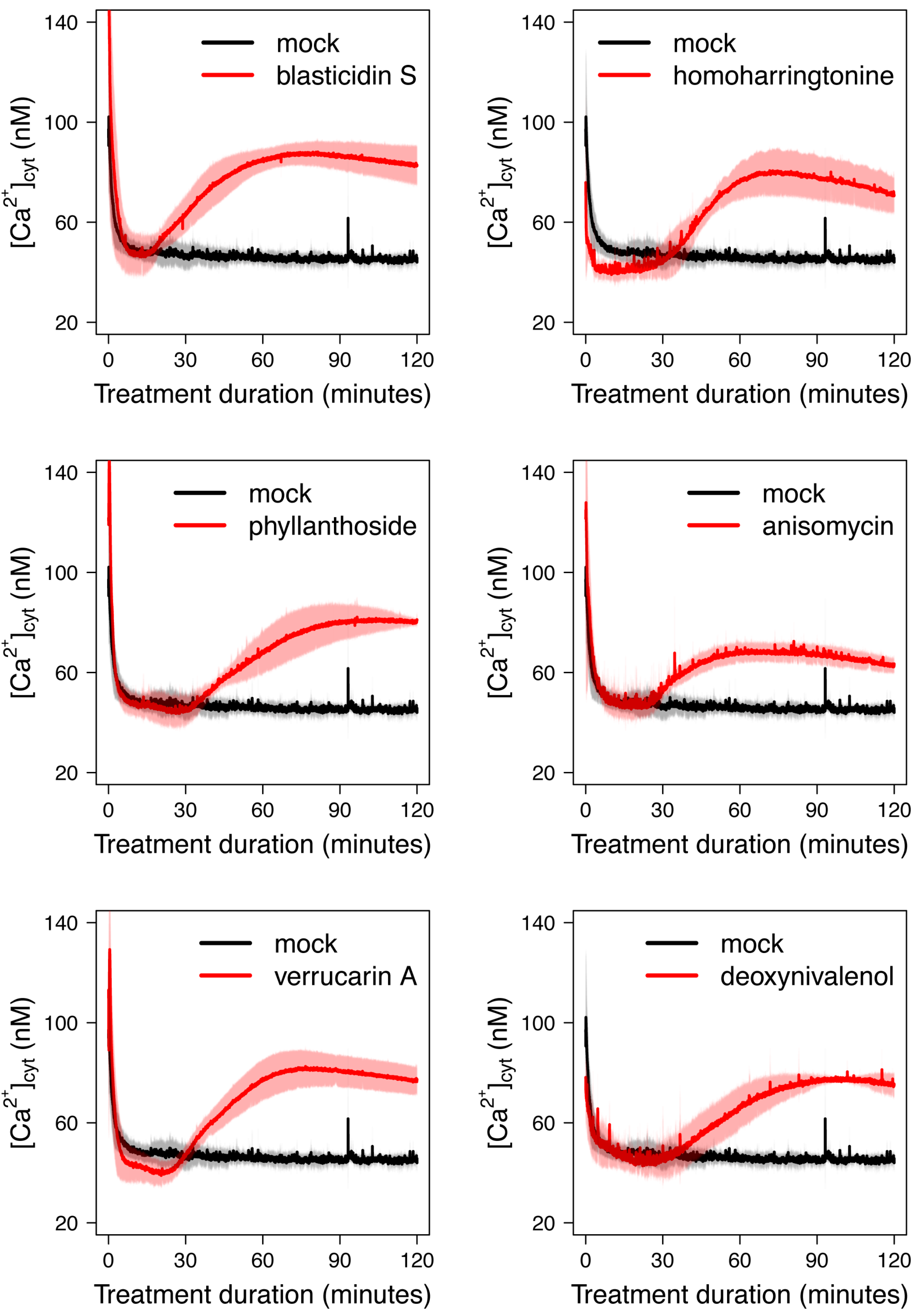


Figure 5.6: Cytosolic calcium signatures induced by inhibitors of translation elongation. *A. thaliana* Col-0 *pCaMV35S::APOAEQUORIN* seedlings were grown in liquid culture for eight days at 20°C with a 12-hour photoperiod and cytosolic free calcium levels were quantified luminometrically during two-hour treatments with 0.1% v/v DMSO (mock control; all graphs have the same mock samples) or inhibitors at 30 µM in deionised water. Cuvettes were placed in the luminometer immediately after the addition of chemicals. Shading indicates standard deviation for three biological replicates, each comprising a cuvette with three seedlings.

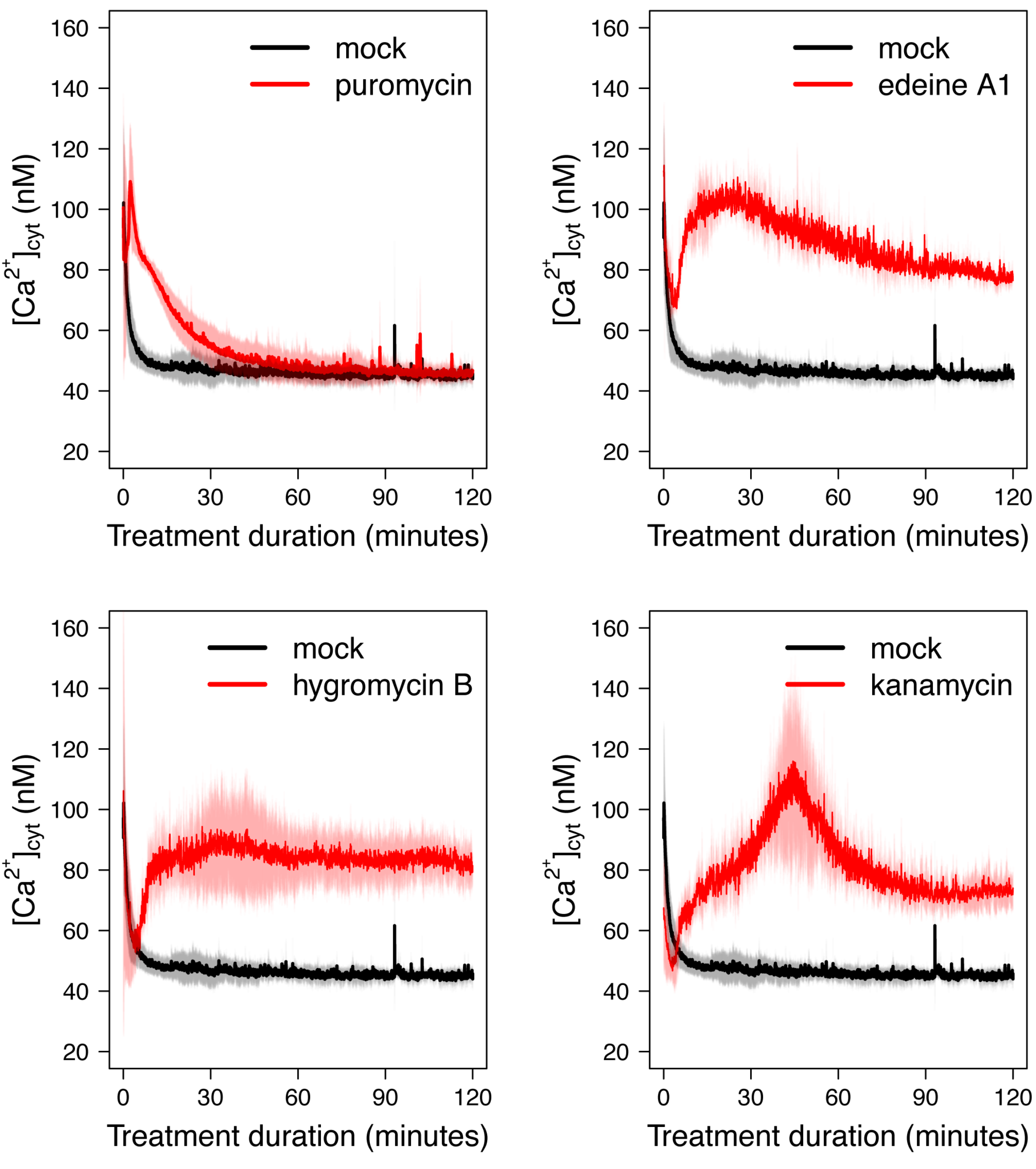


Figure 5.7: Cytosolic calcium signatures induced by various translation inhibitors.
A. thaliana Col-0 *pCaMV35S::APOAEQUORIN* seedlings were grown in liquid culture for eight days at 20˚C with a 12-hour photoperiod and cytosolic free calcium levels were quantified luminometrically during two-hour treatments with 0.1% v/v DMSO (mock control; all graphs have the same mock samples) or inhibitors at 30 μM in deionised water. Cuvettes were placed in the luminometer immediately after the addition of chemicals. Shading indicates standard deviation for three biological replicates, each comprising a cuvette with three seedlings.

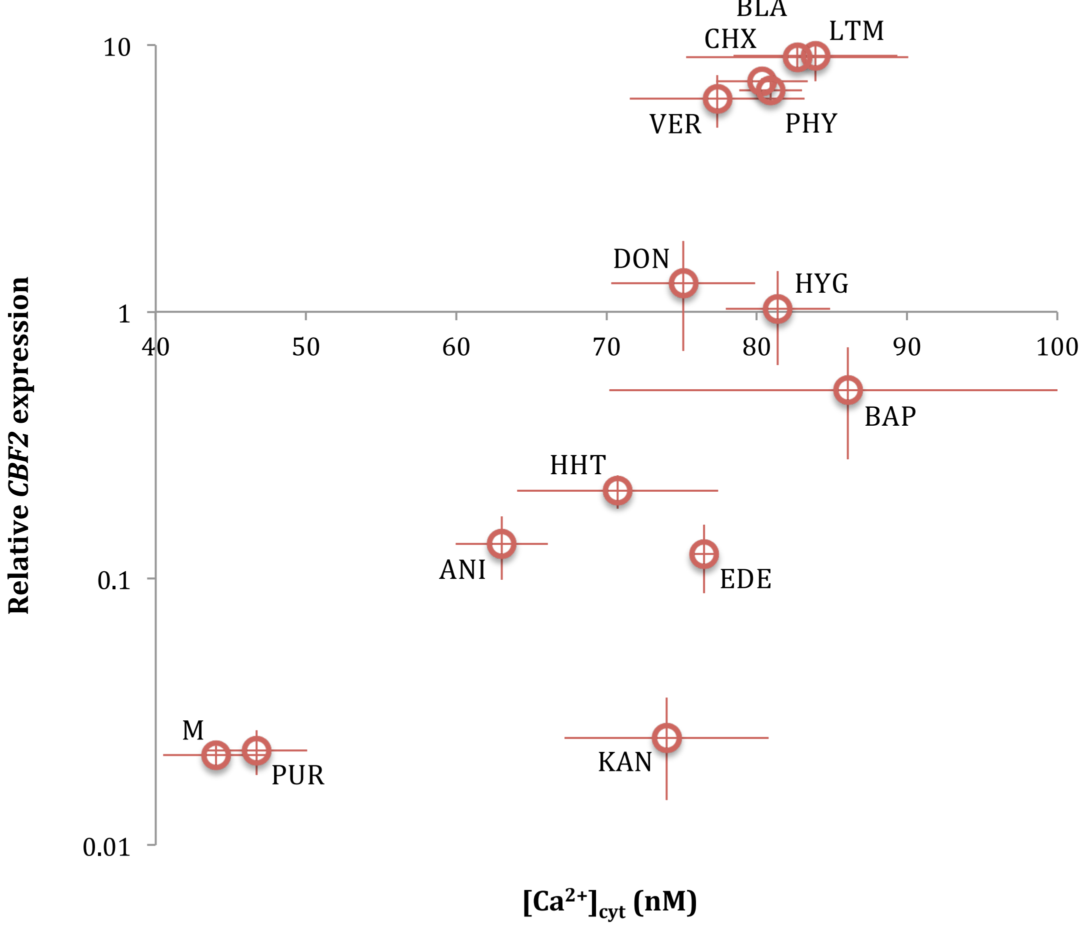


Figure 5.8: Elevations in cytosolic free calcium are not sufficient for strong *CBF2* induction.
For expression analysis, *A. thaliana* Col-0 seedlings were grown in liquid culture for seven days at 21˚C in constant light and treated with inhibitors at 30 µM or 0.1% v/v DMSO (mock control) for two hours. *CBF2* expression was normalised to *PP2A* and *UBC21*. Cytosolic free calcium measurements represent levels after two-hour treatments with inhibitors at 30 µM or 0.1% v/v DMSO in *A. thaliana* Col-0 *pCaMV35S::APOAEQUORIN* seedlings grown in liquid culture for eight days at 20˚C with a 12-hour photoperiod. Lactimidomycin was used at a concentration of 4 µM in all experiments. Error bars represent standard deviation for three biological replicates.
ANI: anisomycin, BAP: BAPTA, BLA: blasticidin S, CHX: cycloheximide, DON: deoxynivalenol, EDE: edeine A1, HHT: homoharringtonine, HYG: hygromycin B, KAN: kanamycin, LTM: lactimidomycin, M: mock (0.1% v/v DMSO), PHY: phyllanthoside, PUR: puromycin, VER: verrucarin A.

cold shock and cycloheximide treatment, though the dominant *los1-1* mutation was not found to affect cold-induced translation repression (*Figure 4.17* and *Figure 4.18*, *Chapter 4*). Although Guo *et al.* (2002) have reported that the *los1-1* mutant is compromised in *de novo* protein synthesis at low temperatures and thus cannot induce the expression of *COR* genes, the precise function of LOS1 in the regulation of early cold genes is unknown.

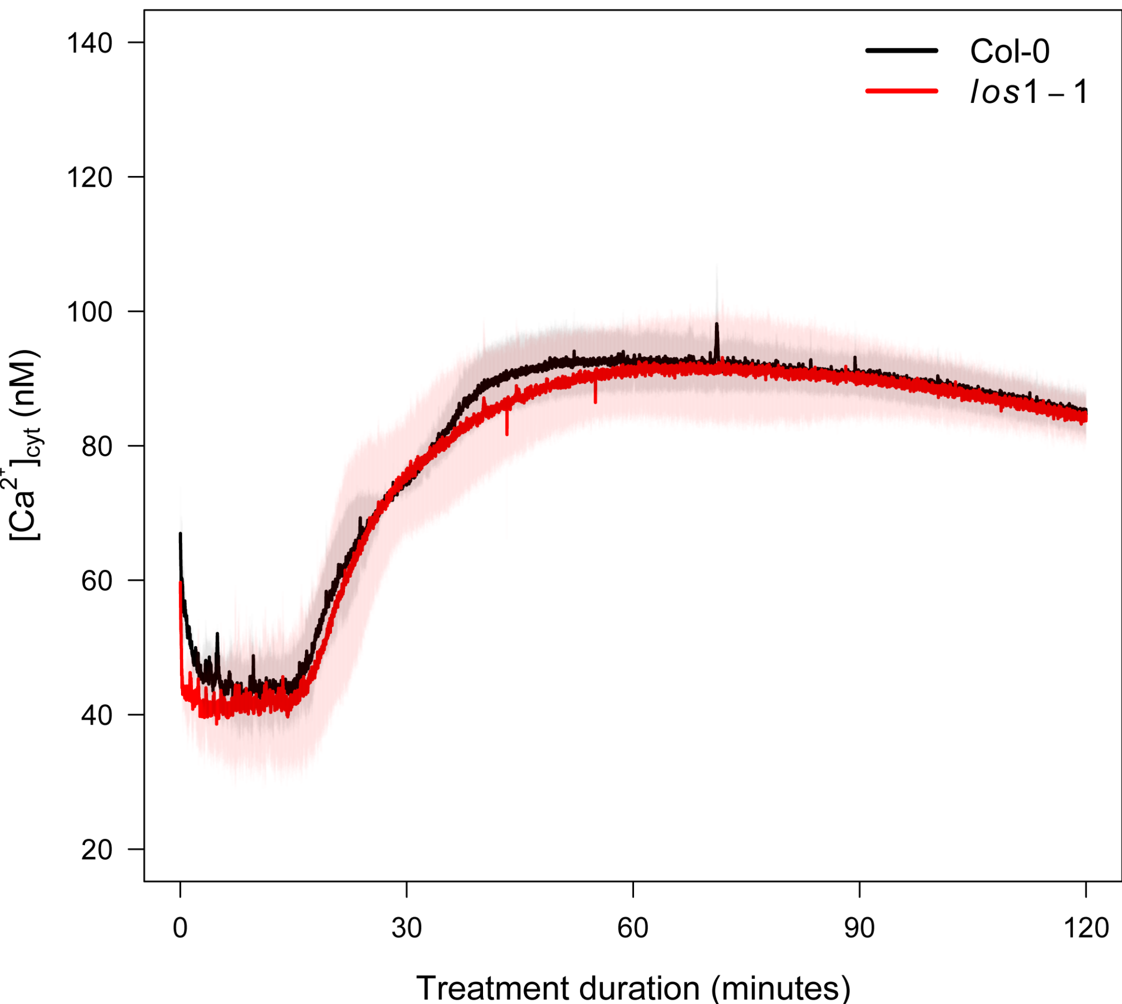


Figure 5.9: The cycloheximide-induced cytosolic calcium signature is not altered in the *los1-1* mutant.
A. thaliana Col-0 *pCaMV35S::APOAEQUORIN* seedlings and Col-0 *pCaMV35S::APOAEQUORIN* × C24 *los1-1* T3 seedlings were grown in liquid culture for eight days at 20°C with a 12-hour photoperiod and cytosolic free calcium levels were quantified luminometrically during two-hour treatments with 30 µM cycloheximide in deionised water. Cuvettes were placed in the luminometer immediately after the addition of chemicals. Shading indicates standard deviation for three biological replicates, each comprising a cuvette with three seedlings.

One possibility is that LOS1 is involved in the upstream signalling pathway that appears to activate *CBF* expression during ribosome stalling. To test whether LOS1 is involved in the cytosolic calcium response to cycloheximide, *A. thaliana* Col-0 plants expressing cytosolic aequorin (*pCaMV35S::APOAEQUORIN*; Xu *et al.*, 2007) were crossed with the *los1-1* mutant. As demonstrated in *Figure 5.9*, however, the cycloheximide-induced calcium signature in the *los1-1* mutant is identical to that in wild-type plants.

5.3 Discussion

5.3.1 Distinct calcium signatures of translation inhibitors

Diverse translation inhibitors were found to trigger increases in cytosolic calcium levels, with different signatures depending on the step of translation that is affected. These inhibitors are likely to affect distinct calcium stores. Calcium influx in response to kanamycin, for example, is expected to involve chloroplasts or mitochondria, as this inhibitor binds to 70S ribosomes but not 80S ribosomes. In mammalian cells, the mitochondrial inhibitors rotenone, myxothiazol and cyanide, which interfere with the mitochondrial electron transport chain, and oligomycin, which blocks mitochondrial ATP synthase, all induce a rapid increase in cytosolic calcium (Wyatt & Buckler, 2004). This response was shown to be dependent on extracellular calcium rather than mitochondrial calcium stores and involves a mitochondrial signalling pathway that triggers plasma membrane depolarisation (Wyatt & Buckler, 2004).

The cytosolic calcium influx triggered by kanamycin may also potentially involve plasma membrane calcium channels activated by stress signals in response to translation inhibition in organelles. Edeine A1 and hygromycin B, which block initiation and translocation, respectively, inhibit 70S as well as 80S ribosomes and also trigger rapid cytosolic calcium responses, though their calcium signatures are not identical to that of kanamycin. Based on their dual inhibitor activity, it is probable that the calcium levels measured in response to hygromycin B or edeine A1 are the sum of multiple calcium signatures, possibly involving similar organellar responses as well as well as other cellular signalling events.

The calcium response to puromycin, in contrast, has been shown to involve calcium leakage from the endoplasmic reticulum (ER) in mammalian cells (Van Coppenolle *et al.*, 2004; Lizák *et al.*, 2006). Ribosome association with the ER translocon causes the pore to dilate up to six times its normal diameter (Hamman *et al.*, 1997,

1998) and this facilitates the translocation of the nascent peptide into the ER lumen. Premature termination of translation by puromycin results in release of the nascent peptide and in mammalian cells this results in ER-bound inactive ribosomes that maintain the translocon pore in its dilated state, thereby allowing ions and other small molecules to leak into the cytosol from the ER lumen (Simon & Blobel, 1991; Heritage & Wonderlin, 2001; Lomax *et al.*, 2002; Lizák *et al.*, 2006).

Calcium leakage from the ER in mammalian cells triggers store-operated calcium entry (SOCE), which involves ER calcium sensors of the STIM (STROMAL INTERACTION MOLECULE) family and the plasma membrane pore-forming protein ORAI1 (named after the Orai 'keepers of the gates of heaven' in Greek mythology) (Van Coppenolle *et al.*, 2004; Ong *et al.*, 2007; Soboloff *et al.*, 2012). During ER store depletion, calcium binding to the EF hands of STIM1 decreases, which triggers its association with ORAI1 at ER-plasma membrane junctions. This interaction brings about a cytosolic influx of calcium ions *via* ORAI1 at the plasma membrane in order to replenish the ER calcium store (Soboloff *et al.*, 2012). SOCE has also been demonstrated upon intracellular calcium store depletion in yeast cells (Hong *et al.*, 2010). In plant cells, however, there are currently no reports of SOCE (Bonza *et al.*, 2013), and thus the mechanism responsible for the puromycin-induced cytosolic calcium transient in *A. thaliana* is not yet clear.

Conversely to puromycin, inhibitors of 80S ribosomal translation elongation cause ribosomes to stall on ER translocon pores without releasing the nascent peptide, and the elongation inhibitor anisomycin has been shown to block puromycin-triggered calcium leakage from the ER (Van Coppenolle *et al.*, 2004; Lizák *et al.*, 2006). Mechanisms of calcium mobilisation triggered by elongation inhibitors are therefore likely to be distinct from those associated with responses to kanamycin or puromycin.

Cycloheximide was previously reported to trigger a rapid decrease in abundance of the auxin transporter PIN2 (PIN-FORMED 2) at the plasma membrane in *A. thaliana* (Jásik *et al.*, 2013). Similar effects of cycloheximide were demonstrated for the epidermal growth factor receptor in mammalian cells (Oksvold *et al.*, 2012) and for various amino acid transporters in yeast (Lin *et al.*, 2008; MacGurn *et al.*, 2011). The cytosolic calcium increase in response to inhibitors of translation elongation could therefore be a consequence of internalisation of a regulator of ion homeostasis, such as calcium exporters. Progressive loss of plasma membrane localisation may account for the gradual elevation in cytosolic calcium in response to these inhibitors.

As demonstrated in *Chapter 4*, anisomycin is a weaker translation inhibitor than cycloheximide in *A. thaliana* seedlings, likely because the conformation of ribosomes

stalled by anisomycin are substrates for the ribosome quality control (RQC) rescue pathway (Lareau *et al.*, 2014; Matsuo *et al.*, 2017). While the temporal dynamics of the cytosolic calcium increase triggered by anisomycin are similar to those caused by cycloheximide, the maximum calcium levels attained are lower in the case of anisomycin, consistent with lower levels of protein synthesis inhibition.

5.3.2 Involvement of calcium in cold gene induction

Lanthanum ions, which non-specifically block ion channels, and BAPTA, which chelates calcium ions, are widely-used inhibitors of calcium signalling and were both found to reduce the induction of *CBF2* expression in response to low temperature or cycloheximide treatments. Similar effects of calcium signalling inhibitors on the expression of other cold-inducible genes in plants have previously been reported (Monroy *et al.*, 1993; Knight *et al.*, 1996; Polisensky & Braam, 1996; Berberich & Kusano, 1997; Tähtiharju *et al.*, 1997). These results must be treated with caution because of the high millimolar concentrations of inhibitors required to bring about decreases in the expression of these genes. These inhibitors may bring about pleiotropic effects, for example disruptions to ion homeostasis caused by these inhibitors could have more general effects on the transcriptional capacity of cells. In addition, lanthanides have been shown to stimulate endocytosis (Wang *et al.*, 2014) and can enter plant cells (Quiquampoix *et al.*, 1990), where they may bind and either activate or repress calcium sensors (Lepšík & Field, 2007) or calcium-regulated enzymes (Zimmerman & Schlaepfer, 1988; Kemple *et al.*, 1990). Nevertheless, the fact that mechanistically distinct inhibitors cause comparable effects on *CBF* gene induction supports the involvement of calcium in this transcriptional response. This involvement is supported by the fact that temperature or chemical treatments that induce *CBF* expression also trigger cytosolic calcium responses (Plieth *et al.*, 1999). Furthermore, these treatments activate the expression of many genes containing calcium-responsive promoter motifs (Whalley *et al.*, 2011), and strong induction of the *CBF2* gene by cold shock or cycloheximide requires calmodulin-binding transcriptional regulators of the CAMTA family (Kim *et al.*, 2013).

Although the effects of different cooling rates on cold-induced cytosolic calcium signatures in *A. thaliana* could not studied in the current study, Plieth *et al.* (1999) demonstrated that moderate cooling at 3˚C per minute causes a gradual increase in cytosolic calcium, unlike instantaneous cooling, which triggers a short-lived high-amplitude calcium transient. Although calcium levels were measured over a shorter

timeframe in the experiments performed by Plieth and colleagues, cycloheximide was also found to trigger a gradual increase in cytosolic calcium. As discussed above, the cytosolic calcium response to cycloheximide is potentially a consequence of decreased plasma membrane association of calcium signalling components. Given their comparable effects on *CBF2* expression, it is possible that the increases in cytosolic calcium triggered by moderate cooling or cycloheximide are mediated *via* similar mechanisms.

In support of this hypothesis, a rapid decrease in protein synthesis is detected in *A. thaliana* seedlings during cold shock, as demonstrated in *Chapter 4*. In addition, Jásik *et al.* (2013) have shown that similarly to cycloheximide treatment, low temperatures reduce the abundance of the auxin transporter PIN2 at the plasma membrane in *A. thaliana*. Furthermore, indirect inhibition of protein synthesis using actinomycin D, a transcriptional inhibitor that blocks the supply of mRNA or tRNAs to the ribosome (Cooper & Braverman, 1977; Schneider-Poetsch *et al.*, 2010), also decreases PIN2 abundance at the plasma membrane, though with slower kinetics than cycloheximide (Jásik *et al.*, 2013). Therefore, it is possible that translational repression by low temperature or cycloheximide may alter the plasma membrane abundance of certain proteins that are prone to membrane cycling, such as transporters and receptor kinases. These may include calcium exporters of the ACA (AUTOINHIBITED CA^{2+}-ATPASE) or CAX (CATION EXCHANGER) families, some members of which localise to the plasma membrane (Kasai & Muto, 1990; Bonza *et al.*, 2000; Schiøtt *et al.*, 2004). Possibly as a result of negative feedback upon reduced exporter activity, genes encoding ACA8 and CAX1 are transcriptionally upregulated at low temperatures (Catalá *et al.*, 2003; Schiøtt & Palmgren, 2005).

While the results presented suggest that calcium is required for *CBF2* induction by low temperature or cycloheximide treatments, prolonged increases in cytosolic calcium *per se* are insufficient to trigger high expression of *CBF2*. This is demonstrated by treatments with kanamycin, edeine A1 or BAPTA, which rapidly cause elevations in cytosolic calcium to the same or higher levels than those measured during incubations with cycloheximide. Levels of *CBF2* expression remain low after two-hour treatments with these chemicals.

It is therefore likely that at least two pathways regulate the expression of early cold-inducible genes, both of which are required for strong *CBF2* induction. One pathway may involve a prolonged elevation in cytosolic calcium caused by translation repression associated with cold shock or chemical treatments, which activates the CAMTA regulators. As discussed in *Chapter 4*, an abundance of stalled ribosomes may

trigger *CBF* expression in a pathway that involves the elongation factor eEF2 protein LOS1, which is required for full *CBF2* induction but neither for cold-associated translation repression nor for calcium elevations in response to cycloheximide treatments. Inhibitors that cause a sustained increase in cytosolic calcium but do not block elongating 80S ribosomes, such as kanamycin and edeine A1, are incapable of inducing *CBF2* expression to the levels observed during cold shock or cycloheximide treatment. Similarly, *CBF2* expression levels remain low during treatments with anisomycin, which causes an increase in cytosolic calcium levels but stalls ribosomes in a conformation that can be rescued by the RQC pathway (Lareau *et al.*, 2014; Matsuo *et al.*, 2017). Conversely, inhibitors that cause global ribosome stalling, for example cycloheximide and blasticidin S, cannot trigger *CBF2* induction in the presence of calcium signalling inhibitors lanthanum or BAPTA. Based on these findings, a model is proposed whereby strong induction of *CBF2* expression requires both global ribosome stalling and an increase in cytosolic free calcium induced by translation repression (*Figure 5.10*).

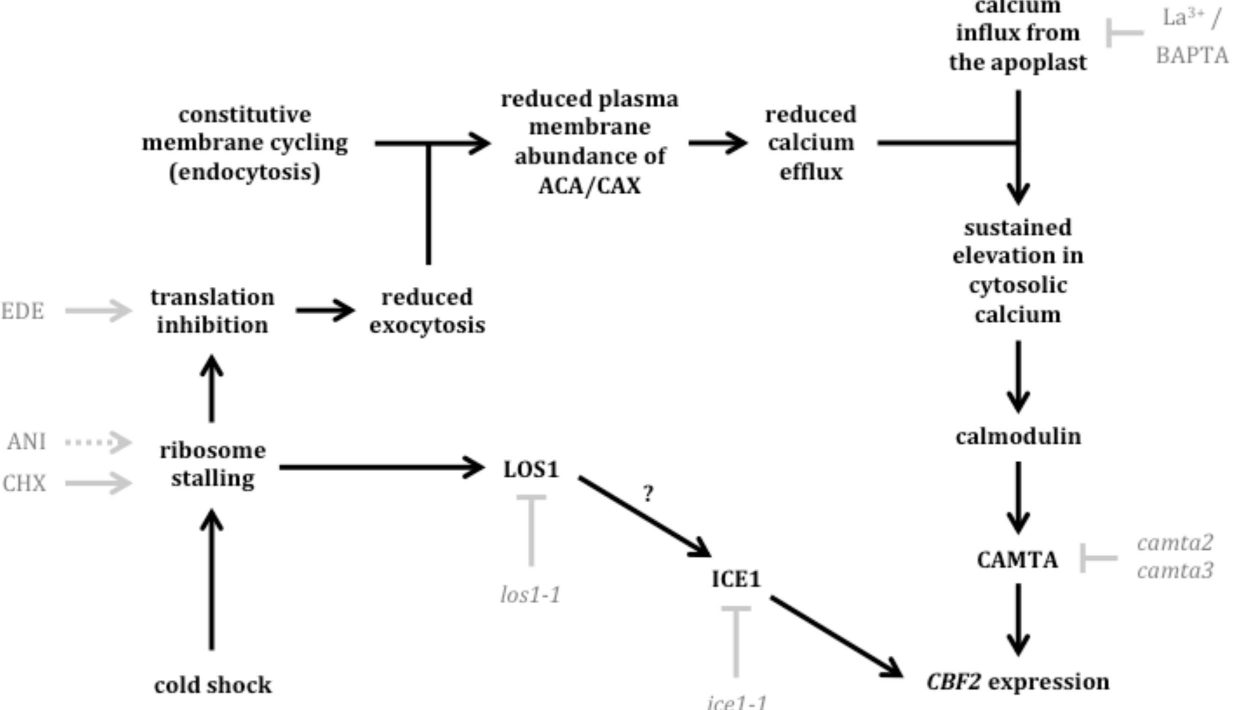


Figure 5.10: Proposed cellular events regulating *CBF* induction in response to translation inhibitors or cold shock. ANI: anisomycin, CHX: cycloheximide, EDE: edeine A1, La^{3+}: lanthanum. The dashed line indicates the weaker effects of anisomycin on translation inhibition. A hypothetical interaction between LOS1 and ICE1 (INDUCER OF *CBF* EXPRESSION 1) is indicated with a question mark.

Chapter 6

Discussion

6.1 Small molecules as tools to study the heat shock response in plants

The chemical genomics method has proven invaluable to investigate dynamic biological processes such as membrane trafficking (Zouhar *et al.*, 2004; Norambuena *et al.*, 2008) that are difficult to study using classical genetics because of the limitations of gene redundancy and mutant lethality. Biological thermosensors and components of signalling pathways rely on specific structural changes and cellular interactions for their activity. Because of the immense diversity and complexity of molecules, the latter are promising tools to identify regulators of temperature signalling pathways in plants.

In this thesis, a chemical screen was carried out in collaboration with Syngenta to identify activators and repressors of the heat-inducible *HSP70* (*HEAT SHOCK PROTEIN 70*) promoter. From over 3000 molecules, five were identified that rapidly induce the expression of *HSP70* in *Arabidopsis thaliana*, while fifteen blocked *HSP70* induction during heat shock. Chemical effects were characterised using transcriptomics and physiological and molecular assays, and candidates for potential cellular targets were predicted using cheminformatic searches to identify chemicals with similar structures and known biological interactions.

Based on these analyses, the *HSP70*-activators are predicted to have diverse modes of action: S04A100314B may stimulate the production of reactive oxygen species (ROS) by inhibiting chloroplast or mitochondrial carbonic anhydrases; S01A052378F and S01A052379R may inhibit the enzymes of the chloroplast plastoquinone (HST; homogentisate solanesyl transferase) or mitochondrial ubiquinone (PPT; 4-hydroxybenzoate polyprenyl transferase) pathways, leading to an accumulation of metabolites and activation of retrograde signalling pathways; and S01A463859Y and S01E974935C may activate or repress components of abiotic stress signalling pathways involving sphingosine 1-phosphate (S1P) and G-proteins. In contrast, among *HSP70*-repressors, S03A109616K was identified as the translation inhibitor cycloheximide, S01E932874G is predicted to be a kinase inhibitor, S01A305483C may be a weak reducing agent and twelve other chemicals are likely to be protonophores that cause uncoupling between electron transport chains and ATP synthesis in chloroplasts and

mitochondria. Although these predicted modes of action are largely speculative, they provide a direction for future work and can be tested experimentally.

Chemical effects can be tested in *A. thaliana* or *Saccharomyces cerevisiae* lines overexpressing putative target proteins, for example mitochondrial (AT1G19580, AT1G47260) or chloroplast (AT4G20990, AT3G01500) carbonic anhydrases, or chloroplast HST (AT3G11945) or mitochondrial PPT (AT4G23660) enzymes, in the case of S04A100314B and S01A052378F, respectively. Overexpression lines or null mutants of sphingosine kinases (AT4G21540, AT2G46090, AT5G23450) or S1P phosphatases/lyases (AT3G58490, AT1G27980), or of G-proteins such as GPA1 (G-PROTEIN ALPHA SUBUNIT 1; AT2G26300), AGB1 (ARABIDOPSIS G-PROTEIN BETA SUBUNIT 1; AT4G34460) or GCR1 (G-PROTEIN-COUPLED RECEPTOR 1; AT1G48270), could be tested for altered responses to S01E974935C and S01A463859Y, respectively. In addition, the involvement of S01E974935C in sphingolipid signalling could be assessed by expression analyses of *HSP70* during treatments of seedlings with S1P or sphingosine kinase inhibitors.

A large number of compounds identified from previous chemical genomics screens in plant or yeast cells have uncharacterised biological targets, because of the difficulties associated with identifying the cellular components with which they interact (Robert *et al.*, 2009). From the literature, typical approaches to identify interacting proteins include proteomics, *via* immunoprecipitation or fractionation with tagged or radiolabelled compounds, or classical genetics to isolate mutants with altered responses to chemicals. Screening a mutagenised population of *A. thaliana* plants for altered *HSP70* responses to candidate compounds would be time-consuming and may not identify any relevant genes, because the compounds may affect cellular components that are not specifically encoded by a single gene, as is the case for protonophores, or they may target proteins for which resistant mutants cannot be obtained owing to redundancy or lethality. In addition, screens for mutants with altered responses to cytotoxic chemicals may identify proteins with which they do not directly interact, for example screens in budding yeast for resistance to the translation inhibitor cycloheximide identified transcription factors regulating the expression of multidrug-resistance transporters and stress-responsive genes as well as the ribosomal protein that is bound by this compound (Huang *et al.*, 2013). Because of cellular similarities between unicellular algae such as *Chlamydomonas reinhardtii* and higher plants, the former may potentially be used for rapid high-throughput forward genetic screens to identify mutants resistant to the candidate compounds, though additional tests in algae would be required to firstly verify conserved responses to these chemicals.

From the twenty chemicals that were identified as *HSP70*-activators or *HSP70*-repressors, only S01E974935C would be amenable to simple tagging using 'click chemistry' (*i.e.*, simple reactions that join two molecules containing particular chemical groups): S01E974935C contains a tetrazine moiety, which can react with an alkene or isonitrile group. Prior to undertaking such modifications, though, chemical analogues would have to be screened for their ability to induce or repress *HSP70* expression, in order to identify chemical groups that are essential for the activity of the compound. Radiolabelling of compounds could circumvent these limitations and provides the additional benefit of being able to detect the compound in cellular fractions with very high sensitivity. Several other label-free methods of detecting protein interactors have been developed in the last decade, all based on the property of ligands to stabilise peptides in one of several native states, which reduces transient protein unfolding and refolding, known as 'breathing'. This feature increases the resistance of interacting proteins to protease digestion, thermal unfolding or oxidation and potentially allows targets to be identified by mass spectrometry. These denaturing treatments form the basis for target identification in the methods known as DARTS (drug affinity responsive target stability) (Lomenick *et al.*, 2009), TPP (thermal proteome profiling) (Savitski *et al.*, 2014) and SPROX (stability of proteins from rates of oxidation) (West *et al.*, 2008), respectively. The success of such techniques, however, relies on protein targets being expressed at levels sufficient for detection by mass spectrometry, and methods such as dialysis-enrichment of samples and gel-free separation of peptides may enhance the detection of lowly expressed proteins (Lomenick *et al.*, 2011). Finally, whole proteome analyses may also provide useful information into potential protein modifications that occur as a result of chemical activity.

The screen for molecules affecting warm temperature signalling in plants could be improved in several ways. Given that the composition of the Syngenta library was based in large part on predicted hydrophobicity to maximise cell permeability (Mikael Courbot, Syngenta, personal communication), it is not surprising that many candidates from the screen have cytotoxic effects and are predicted to interfere with the function of organellar membranes. Because all assays were performed on liquid-grown seedlings, which have less cuticle deposition than seedlings grown on agar and are thus more permeable, molecules with lower hydrophobicity would have been preferential for this screen, as this would have minimised potential protonophore activity. A secondary screen for chemical lethality could have been used to select from the ninety-eight candidates of the primary screens only those that have no cytotoxic effects.

Given the permeability of liquid-grown seedlings, larger and more complex molecules, such as those typically found to inhibit HSP90 (West *et al.*, 2012), could have been included in the library. A larger library of compounds would increase the chances of identifying molecules targeting temperature-specific rather than simply stress-related pathways. Screens for regulators of human HSF1 (HEAT SHOCK FACTOR 1) have employed chemical libraries up to 100-times larger than the Syngenta library used in this study (Zaarur *et al.*, 2006; Neef *et al.*, 2010; Santagata *et al.*, 2012, 2013; Rye *et al.*, 2016). Screening at lower concentrations would be likely to select for compounds with single potent interactions with fewer pleiotropic effects. In addition, a dual-reporter assay, for example using a constitutively-expressed Renilla luciferase reporter as a positive control, would allow for rapid elimination of chemicals that have non-specific effects on gene expression.

Finally, rather than using the *HSP70* promoter to screen for molecules affecting temperature signalling, a more specific promoter could be utilised. From the RNA-sequencing dataset generated in this study, over a hundred genes are induced specifically by mild heat shock but not by any of the chemicals tested, of which two genes (*AT3G13470/CPNB2* [*CHAPERONIN 60 BETA 2*], *AT4G21310/DEAL2* [*DESIGUAL 2*]) are particularly promising for screening purposes, as they are upregulated by warm temperatures but not by any other biotic or abiotic stress represented in the AtGenExpress dataset (Kilian *et al.*, 2007).

6.2 Role of chloroplasts and mitochondria in heat signalling

The heat shock response (HSR) in yeast and mammalian cells is known to be mediated by interactions between HSF1-type transcription factors and chaperones of the HSP70 and HSP90 families, whereby an accumulation of misfolded proteins during heat shock titrates these chaperones away from HSF1, thereby allowing for its activation (Zheng *et al.*, 2016). This model is supported by genetic and biochemical evidence, for example these chaperones physically interact with HSF1 *in vivo* (Zou *et al.*, 1998; Bonner *et al.*, 2000; Zheng *et al.*, 2016), and enhanced HSF1 activity is detected in yeast mutants of *HSP70* and *HSP90* genes (Halladay & Craig, 1995; Duina *et al.*, 1998; Liu *et al.*, 1999; Bonner *et al.*, 2000). Furthermore, inhibitors of HSP90 promote HSF1 activity in yeast and mammalian cells (Murakami *et al.*, 1991; Millson & Piper, 2014).

Transcription factors of the HSFA1 family in plants have also been shown to interact with HSP70 and HSP90 chaperones (Kim & Schöffl, 2002; Hahn *et al.*, 2011). In addition, increased HSFA1 activity is detected in *A. thaliana* during genetic or chemical

inhibition of chaperone activity (Lee & Schöffl, 1996; Yamada *et al.*, 2007; Qian *et al.*, 2014), indicating a conserved HSR mediated by HSP70/90 chaperones in plants.

There is evidence that HSF1 in yeast and mammalian cells is also regulated directly by heat (Bulman & Nelson, 2005; Hentze *et al.*, 2016). Elevated temperatures favour oligomerisation of human HSF1, by preventing the interaction between the inhibitory HR-C domain and the HR-A/B regions of the oligomerisation domain that occurs at low temperatures (Hentze *et al.*, 2016). In addition, Liu *et al.* (2013a) have demonstrated that high temperatures stimulate the oligomerisation and DNA binding of *Arabidopsis* HSFA1a *in vitro*. Thus, HSF1-type transcription factors may respond directly to temperature changes as well as *via* their interactions with HSP70 and HSP90 chaperones.

Plant cells can be exposed to large temperature fluctuations, unlike most mammalian cells, and it is therefore not surprising that the transcriptional response to heat shock in plants is highly complex, with distinct patterns of gene expression depending on the time of day and rate of temperature change as well as the duration and absolute temperature of heat treatment (Larkindale & Vierling, 2008; Kumar & Wigge, 2010; Cortijo *et al.*, 2017). This complexity is a result of numerous HSFA1-interacting proteins, which include transcriptional repressors (Hsu *et al.*, 2010), cyclin-dependent kinases (Reindl *et al.*, 1997), mitogen-activated protein kinases (Evrard *et al.*, 2013) and calcium-dependent kinases and phosphatases (Liu *et al.*, 2007a, 2008).

Interestingly, many of the compounds identified as activators or repressors of *HSP70* expression in this thesis are predicted to affect chloroplasts and mitochondria. S04A100314B, which is structurally similar to the inhibitor of carbonic anhydrases benzothiazole-sulfonamide, decreases seedling viability and triggers oxidative stress-associated induction of *HSP70*, similar to phenotypes reported for mutants of chloroplast or mitochondrial carbonic anhydrases (Yi *et al.*, 2005; Ferreira *et al.*, 2008; Wang *et al.*, 2012a; Soto *et al.*, 2015; Córdoba *et al.*, 2016; Fromm *et al.*, 2016; Rudenko *et al.*, 2017, 2018; Zhang *et al.*, 2018). S01A052378F and S01A052379R cause seedling chlorosis, consistent with effects on chlorophyll biosynthesis in chloroplasts. These compounds are structurally similar to haloxydine, which inhibits the plastoquinone biosynthetic enzyme HST (Sadre *et al.*, 2010) and likely causes an accumulation of the upstream metabolite methylerythritol cyclodiphosphate (MEcPP), known to activate nuclear stress-associated genes (Xiao *et al.*, 2012). Lastly, S01A289354A, S04A104014F and S01A109956R are confirmed protonophores, and S04A259155K, S04A104169N, S04A020355G, S01E528454W, S01E342213Q, S01A264569E, S01A240600W, S01A051686M and S01A022136M are also predicted to be protonophores based on

their structures. Such compounds disrupt the proton gradient across the thylakoid membranes of chloroplasts and the inner membrane of mitochondria, thereby reducing the activity of ATP synthase. These uncoupling agents were all found to block the heat-induction of *HSP70* expression.

Inhibitors of the electron transport chain in chloroplast or mitochondria can also affect the expression of heat-inducible genes in *A. thaliana*. Methyl viologen, for example, disrupts electron transport primarily in chloroplasts by acting as an electron acceptor at PSI, and upregulates the expression of *HSP70* by triggering the production of superoxide radicals (Scarpeci *et al.*, 2008). 2,5-Dibromo-6-isopropyl-3-methyl-1,4-benzoquinone (DBMIB) prevents the transfer of electrons from plastoquinols to the cytochrome b6f complex in chloroplasts and triggers an increase in hydrogen peroxide levels in chloroplasts, presumably by increasing the plastoquinol-to-plastoquinone ratio (Mühlenbock *et al.*, 2008; Wang *et al.*, 2016d). Interactions between plastoquinones and plastoquinols can produce plastosemiquinone radicals that react with molecular oxygen to generate superoxide radicals, and plastoquinols in turn react with superoxides to generate hydrogen peroxide (Mubarakshina & Ivanov, 2010). Increased hydrogen peroxide levels in chloroplasts correlate with an induction of *HSP70* expression by DBMIB (Mühlenbock *et al.*, 2008; Dickinson *et al.*, 2018). In contrast, *HSP70* expression is reduced by 3-(3,4-Dichlorophenyl)-1,1-dimethylurea (DCMU), which prevents the transfer of electrons from PSII to plastoquinone, thereby reducing the plastoquinol-to-plastoquinone ratio and diminishing chloroplast levels of hydrogen peroxide (Mühlenbock *et al.*, 2008; Dickinson *et al.*, 2018). DBMIB was also found to upregulate *HSP70* expression in yeast cells, consistent with its comparable effects on the electron transport chain in mitochondria (Hauska *et al.*, 1983). In addition, certain molecules produced by chloroplasts, including heme, hemin and Mg-protoporphyrinIX (von Gromoff *et al.*, 2008; Voss *et al.*, 2011) as well as the aforementioned metabolite MEcPP (Xiao *et al.*, 2012), are capable of triggering the expression of a subset of stress-associated nuclear genes.

In a forward classical genetics screen for *A. thaliana* mutants with altered *HSP70* expression, Dickinson *et al.* (2018) identified a large number of chloroplast genes that regulate heat-inducible genes. Furthermore, both the induction of *HSP70* expression by mild heat shock and the basal level of thermotolerance are higher during the day, when plants are actively engaged in photosynthesis (Cortijo *et al.*, 2017; Dickinson *et al.*, 2018). The above findings suggest that chloroplasts and mitochondria are key regulators of the HSR.

These organelles are extremely sensitive to elevated temperatures, which cause dysfunction of the electron transport chain, and are primary sites of ROS production in cells (Sun and Guo, 2016). It is therefore possible that they contribute to the perception of warm temperatures, for example *via* ROS signalling to activate HSFA1 transcription factors. Chloroplast stress has been shown to cause an accumulation of hydrogen peroxide, which rapidly diffuses out of chloroplasts (Mubarakshina *et al.*, 2010) or can be channelled to the nucleus in structures known as stromules (Brunkard *et al.*, 2015; Caplan *et al.*, 2015). Hydrogen peroxide levels also increase during heat stress (Zhou *et al.*, 2012) and exogenous treatments with hydrogen peroxide induce the expression of plant *HEAT SHOCK PROTEIN* (*HSP*) genes (Lee *et al.*, 2000; Desikan *et al.*, 2001). Chemical treatments that quench ROS or attenuate ROS production have been shown to reduce the heat-induction of *HSP* genes in *A. thaliana* (Volkov *et al.*, 2006). Whereas hydrogen peroxide promotes the DNA-binding ability of HSFA1a *in vitro*, reducing agents DTT and NADPH prevent its DNA association during heat or hydrogen peroxide treatments (Liu *et al.*, 2013a). Furthermore, treatments with hydrogen peroxide or DBMIB promote HSFA1a binding to target genes in the absence of heat shock (Dickinson *et al.*, 2018).

Two cysteine residues in the HSP70 protein SSA1 in budding yeast play a key role in perceiving oxidative stress (Wang *et al.*, 2012b). While mutational analyses of cysteine residues in HSP70 proteins have not been carried out in *A. thaliana*, six of these proteins contain cysteine residues in regions homologous to those in SSA1, suggesting they may also potentially function as sensors of oxidative stress. In addition, mutations in two cysteine residues in mammalian HSF1 block its activation in response to heat and hydrogen peroxide (Ahn & Thiele, 2003). Similar mutations in *A. thaliana* HSFA1d diminish the induction of genes associated with redox homeostasis during high light stress (Jung *et al.*, 2013b). In addition to direct activation of the HSR *via* redox modifications of HSP70 and HSF1 proteins, ROS signalling may be mediated *via* oxidised metabolites such as β-cyclocitral, which is formed upon singlet oxygen reaction with carotenes and triggers the induction of nuclear stress-associated genes (Ramel *et al.*, 2012).

Retrograde signalling from chloroplasts or mitochondria during heat stress may also involve the accumulation of metabolites as a result of disruptions to organellar biosynthetic pathways and their subsequent diffusion or transport to the nucleus. As mentioned above, certain chloroplast-derived tetrapyrroles, isoprenoids or phosphonucleotides affect stress-responsive gene expression in plants and algae and have been proposed to affect chromatin architecture (Xiao *et al.*, 2012), mRNA stability

(Estavillo *et al.*, 2011) or the activity of chaperones or signalling proteins (Kindgren *et al.*, 2012; Ramel *et al.*, 2012). Plant mitochondria have also been proposed to regulate the HSR *via* calcium signalling as a result of heat-induced alterations in membrane potential of the inner mitochondrial membrane (Pyatrikas *et al.*, 2014; Rikhvanov *et al.*, 2014). Furthermore, heat-associated retrograde signalling could also be mediated by mobile organellar proteins. The chloroplast outer membrane-associated protein PTM, for example, is proteolytically cleaved during chloroplast stress, allowing it to re-localise to the nucleus, where it binds to methylated histones and regulates gene expression (Sun *et al.*, 2011). Based on the predicted modes of action of the *HSP70*-activators and *HSP70*-repressors identified in this thesis, it is likely that ROS, ion or metabolite signalling from chloroplasts and mitochondria contribute to the HSR in plants. Further analyses will be required to elucidate the mechanisms by which these signals are generated and transduced in plant cells upon heat shock.

6.3 Regulation of temperature responses by translational machinery

Cycloheximide is one of the compounds in the Syngenta chemical library that was identified as a repressor of heat-inducible genes. Unlike other *HSP70*-repressors, this molecule also rapidly induced the expression of early cold-responsive genes, such as *CBF1*, *CBF2* and *CBF3* (*C-REPEAT BINDING FACTOR 1/2/3*). Transcriptome changes brought about by cycloheximide treatment were compared with those triggered by various abiotic stresses from public microarray datasets, and positive correlations were found between genes induced by cycloheximide and those activated by cold shock or repressed by heat shock, whereas a negative correlation was found with heat-inducible genes. Weaker correlations were observed with other abiotic stress treatments, indicating that cycloheximide affects temperature signalling pathways rather than general stress responses.

The induction of *CBF2* expression by cycloheximide is mediated at the transcriptional rather than post-transcriptional level (Zarka *et al.*, 2003) and requires the binding of this molecule to the E-site of 80S ribosomes, as demonstrated in this thesis by the abolished *CBF2* response in plants producing mutated cycloheximide-insensitive ribosomes. The transcriptional response of early cold genes to cycloheximide was found to be mediated at least in part by the known cold signalling regulators ICE1 (INDUCER OF *CBF* EXPRESSION 1), CAMTA2 and CAMTA3 (CALMODULIN-BINDING TRANSCRIPTION ACTIVATOR 2/3), as well as by other regulators such as the WRKY (Tryptophan-Arginine-Lysine-Tyrosine domain) transcription factors, which have

received relatively little attention with respect to low temperature responses in *A. thaliana*. In addition, it was demonstrated that *CBF2* induction by cycloheximide is controlled by the circadian clock, similarly to induction by cold shock (Fowler *et al.*, 2005). These findings indicate that cycloheximide activates cold signalling while repressing responses to heat shock in plants.

Inhibitors of translation elongation that cause widespread ribosome stalling were able to trigger high levels of *CBF2* expression, unlike inhibitors blocking translation initiation or causing premature termination of translation, which reduce the occupancy of ribosomes on mRNA. It is therefore proposed that global ribosome stalling triggered by chemical inhibitors or cold shock may be a cellular signal for the induction of *CBF2* gene expression. In support of this hypothesis, cold shock was found to cause rapid translation repression in *A. thaliana* seedlings and previous studies have demonstrated that low temperatures enhance ribosome occupancy on mRNAs (Wang *et al.*, 2017b) and trigger the upregulation of genes encoding translational machinery (Usadel *et al.*, 2008; Beine-Golovchuk *et al.*, 2018), consistent with the potential effects of cold shock on ribosome stalling. Based on previous knowledge that a lower proportion of ribosomes are engaged in active translation in the dark relative to the light (Juntawong & Bailey-Serres, 2012), the effects of cycloheximide on ribosome stalling are expected to be less severe during the night than during the day. Accordingly, *CBF2* induction by either cold shock or cycloheximide is lowest after dusk and highest after dawn.

The possibility that low temperature signalling in plants may be mediated *via* ribosomes could explain why no thermosensors were identified from previous classical genetic screens for cold signalling mutants (Ishitani *et al.*, 1997; Chinnusamy *et al.*, 2003; Guan *et al.*, 2013a), as ribosomes are large ribonucleoprotein complexes encoded by essential and often multiple genes. The ribosome would be a suitable candidate as a biological thermosensor, given its abundance in cells and the inherent ability of ambient temperature to influence translation by affecting RNA structure or enzyme activity.

In contrast, the ability of plant cells to induce the HSR appears to require active translation, which may reflect the available cellular energy to bring about this energy-intensive process. Consistent with this model, Santagata *et al.* (2013) demonstrated that genes positively regulated by mammalian HSF1 are downregulated by translation inhibition and vice versa for genes that are negatively regulated by HSF1. The authors showed that the transcriptome of cells with RNAi-silenced HSF1 positively correlates to the transcriptomes of wild-type cells treated with translation inhibitors. In addition, the global occupancy of HSF1 but neither that of RNA polymerase II nor the cellular

abundance of HSF1 protein was diminished by cycloheximide treatment (Santagata *et al.*, 2013), suggesting that translation inhibition negatively regulates that activity of HSF1-type transcription factors. Furthermore, in *A. thaliana*, the higher levels of ribosome activity in the light (Juntawong & Bailey-Serres, 2012) correlate with the greater induction of *HSP70* expression by mild heat shock during the day relative to the night (Cortijo *et al.*, 2017).

6.4 Translation-associated activation of cold signalling pathways

The mechanisms by which translation regulates temperature signalling pathways in plants are currently unclear. The translation-associated kinase TORC1 (TARGET OF RAPAMYCIN COMPLEX 1) is known to positively regulate HSF1 activity in mammalian cells (Chou *et al.*, 2012), however its activity is promoted rather than repressed by cycloheximide (Kimball *et al.*, 2008; Binda *et al.*, 2009) and thus cannot account for the reduced HSF1 activity observed during translation inhibition. Numerous components of translational machinery have been found to contribute to gene expression in an extra-ribosomal context, by interacting with specific RNA or DNA templates or with proteins such as transcription factors or kinases (Liu *et al.*, 2002; Hanbauer *et al.*, 2003; McClatchy *et al.*, 2006; Schrader *et al.*, 2006; Amiri *et al.*, 2007; Bluem *et al.*, 2007; Liu & Gelli, 2008; Rosorius *et al.*, 1999; Mingot *et al.*, 2013; Vera *et al.*, 2014; Samra *et al.*, 2015). In human cells, for example, elongation factor eEF1A binds to HSF1 in the nucleus and regulates its heat-dependent activation of *HSP70* expression (Vera *et al.*, 2014). There is evidence that eEF1A also regulates the HSR in plant cells (Momčilović *et al.*, 2016) and it is possible that ribosome stalling triggered by cycloheximide may perturb eEF1A-dependent activation of HSF1 target genes. The eEF2-type elongation factor LOS1 (LOW EXPRESSION OF OSMOTICALLY-RESPONSIVE GENES 1) was found to contribute to the induction of *CBF2* expression during cold shock or cycloheximide treatment, though no effects on cold-induced translation repression were detected in the *los1-1* mutant, suggesting that LOS1 acts downstream of ribosome stalling. Similarly to eEF1A in mammalian cells, LOS1 may potentially regulate the activity of cold signalling regulators such as the ICE1 or CAMTA transcription factors in *A. thaliana*.

Despite the clear effects of the dominant *los1-1* allele on *CBF2* induction by cold or cycloheximide treatments, the effect of this mutation on the activity of the LOS1 protein is unknown. Similarly, the mechanism by which the *ice1-1* mutation affects the

function of ICE1 in cold signalling is also unknown. The lack of null mutants for these genes limits our understanding of their exact roles in cold signalling pathways. In addition, while T-DNA insertion mutations in most other elongation factor genes were not found to affect *CBF2* induction by cycloheximide, redundancy between paralogues and the recessive nature of these mutations may have masked any phenotypes in these plants. In the future it will be of interest to obtain a null allele of *LOS1* using CRISPR/Cas9-mediated genome editing, if viable, or to generate an inducible mutant, for example by RNA interference. It will be key to characterise the extra-translational roles of LOS1, including its localisation patterns, potential interactions with signalling proteins and effects on the expression of other cold-responsive genes, in order to elucidate its exact role in cold responses. It will be interesting to test the effects of combined *los1-1* and *ice1-1* or *camta123* (*camta1 camta2 camta3*) mutations on *CBF2* induction by cold shock or cycloheximide treatment, to assess whether LOS1 and ICE1 or CAMTAs act concertedly or in separate pathways. Similar experiments could be performed in plants overexpressing ICE1 or CAMTAs in the *los1-1* mutant or LOS1 in the *ice1-1* and *camta123* mutants. Furthermore, as cycloheximide-induced *CBF2* expression is compromised in *ice1-1* and *camta123* mutants, the increased binding of ICE1 and CAMTAs at the *CBF2* promoter during cycloheximide treatments can be confirmed using chromatin immunoprecipitation.

An additional mechanism by which translation may regulate temperature responses in plants is *via* calcium signalling. In this thesis, inhibitors of translation elongation were found to trigger a gradual increase in cytosolic calcium levels. This calcium response could potentially be a result of reduced occupancy of calcium exporters at the plasma membrane during translation inhibition. Such effects of cycloheximide have been demonstrated in yeast, mammalian and plant cells for certain proteins that are prone to membrane cycling, such as receptor kinases or amino acid or hormone transporters (Lin *et al.*, 2008; MacGurn *et al.*, 2011; Oksvold *et al.*, 2012; Jásik *et al.*, 2013). Reduced plasma membrane occupancy of ACA (AUTOINHIBITED CA^{2+}-ATPASE) or CAX (CATION EXCHANGER) exporters would be likely to cause an increase in the cytosolic concentration of calcium ions as a result of passive influx through calcium-permeable channels combined with a diminished capacity for calcium extrusion. A similar response may occur during cold shock, given that low temperatures trigger rapid translation repression in *A. thaliana* seedlings and also reduce the abundance of certain transporters at the plasma membrane (Jásik *et al.*, 2013).

There appear to be at least two different cytosolic calcium responses to cold shock. Instantaneous cooling at rates of 0.5˚C to 20˚C per second triggers an immediate

high-magnitude short-lived calcium signature, whereas moderate cooling at no more than 0.05˚C per second, representative of cold treatments used for *CBF2* expression analyses in this thesis, brings about a gradual low-amplitude sustained calcium signature (Plieth *et al.*, 1999). Very low rates of cooling, for example at 0.005˚C per second, cause smaller changes in *CBF2* expression than moderate cooling (Kidokoro *et al.*, 2017) and may perhaps produce a distinct cytosolic calcium signature. While the calcium response to instantaneous cooling is mediated at least partly by mechanosensitive calcium-permeable channels of the MCA (MID1-COMPLEMENTING ACTIVITY 1/2) family (Mori *et al.*, 2018), it is possible that the gradual increase in cytosolic calcium during moderate cooling is mediated at least in part by translation inhibition-associated reduction in plasma membrane occupancy of calcium exporters. This model would account for the fact that only minor differences in the cold-induction of *CBF* genes were detected in the double *mca1 mca2* mutant, even though a 50% reduction in calcium influx was reported upon instantaneous cooling to 3˚C (Mori *et al.*, 2018). It could also account for the desensitisation of *CBF* gene induction when seedlings are subjected to repeated cold treatments (Zarka *et al.*, 2003), as these plants would presumably require a certain amount of time to replenish the plasma membrane pool of calcium exporters before a similar response can be provoked.

In yeast cells, cycloheximide-induced internalisation of amino acid transporters was reported to involve the TORC1 kinase, the activity of which is promoted by cycloheximide (Kimball *et al.*, 2008; Binda *et al.*, 2009). The yeast TORC1 kinase inhibits the negative regulator of endocytosis NPR1 (NITROGEN PERMEASE REACTIVATOR 1) (MacGurn *et al.*, 2011), thereby allowing the membrane association of ART1 (ARRESTIN 1), a ubiquitin ligase adaptor protein that targets membrane proteins for ubiquitin-mediated endocytosis (Lin *et al.*, 2008). In *A. thaliana*, however, cold shock has been reported to repress rather than activate TORC1 activity (Wang *et al.*, 2017b), suggesting that TORC1 signalling is not involved in the internalisation of membrane transporters during cold shock in plants (Jásik *et al.*, 2013). Instead, this may be a combined effect of constitutive membrane cycling, causing endocytosis of calcium transporters, and reduced flux through the protein export pathway as a result of translation repression, which presumably prevents exocytosis required for the delivery of new or recycled transporters to the plasma membrane. This model is supported by quantitative analyses of PIN2 (PIN-FORMED 2) auxin transporter occupancy at the plasma membrane during cold treatment, which appears to block exocytosis but not endocytosis in *A. thaliana* (Jásik *et al.*, 2013).

Constitutive membrane cycling can regulate the localisation of ion channels as well transporters (Royle & Murrell-Lagnado, 2003), and thus the localisation of calcium influx channels may also be affected by translation inhibition. In either case, altered membrane occupancy of proteins involved in calcium mobilisation would be expected to alter the electrochemical properties of cellular membranes, which could account for a gradual increase in cytosolic calcium. In *Physcomitrella patens*, for example, elimination of the calcium channel CNGCb (CYCLIC NUCLEOTIDE-GATED CALCIUM CHANNEL b) can enhance the activation of other channels (Finka *et al.*, 2012). Alternatively, low temperatures may simply alter the activity of specific calcium channels or transporters, for example *via* post-translational modifications or binding of regulatory proteins, both of which affect ACA and CAX activity (Hwang *et al.*, 2000; Cheng & Hirschi, 2003; Cheng *et al.*, 2004; Bose *et al.*, 2011), or, in the case of annexins, by increased membrane integration, as has been demonstrated during cold shock in wheat (Breton *et al.*, 2000). To address these possibilities, it will be essential to study the localisation patterns of candidate calcium channels or transporters, including the plasma membrane-associated exporters ACA8, ACA10 and ACA12 (Bonza *et al.*, 2000; George *et al.*, 2008; Limonta *et al.*, 2014), during low temperature or cycloheximide treatments. In addition, as the gradual increase in cytosolic calcium during such treatments is hypothesised to be a consequence of reduced exocytosis caused by translation repression, it will be interesting to test whether inhibitors of endocytosis can block these calcium responses and whether inhibitors of exocytosis bring about similar calcium signatures.

Increased levels of cytosolic calcium are likely to activate *CBF2* gene expression *via* calmodulin-regulated transcription factors of the CAMTA and potentially WRKY families (Li *et al.*, 2011a), binding sites of which are enriched in the promoters of genes upregulated by cycloheximide treatment. In contrast, this calcium response may repress the activity of HSFA1 transcription factors, in accordance with the reduced activity of HSF1 activity in mammalian cells during cycloheximide treatment (Santagata *et al.*, 2013) or high calcium levels (Soncin *et al.*, 2000). It is likely that translation regulates temperature signalling *via* both calcium-dependent and calcium-independent pathways, as it was demonstrated that elevated levels of cytosolic calcium are necessary but not sufficient for inducing the expression of *CBF2* to levels observed during cold shock or cycloheximide treatment. Furthermore, the *los1-1* mutation compromised *CBF2* induction during these treatments but affected neither translation repression during cold shock nor the cytosolic calcium response to cycloheximide.

A key limitation in this thesis was the inability to quantify cytosolic calcium dynamics in real time in response to different rates of cooling, as performed by Plieth *et*

al. (1999). In addition, measurements of cytosolic calcium levels using the aequorin reporter in whole plants may mask calcium dynamics occurring at the single-cell level, such as oscillatory behaviour, as well as localised calcium mobilisation events, for example calcium influx from internal and external stores. In the future, it will be of interest to quantify calcium responses to cycloheximide or to moderate cooling in single cells of *A. thaliana*, for example using suspension cultures with high-sensitivity calcium reporters such as GECOs (genetically-encoded calcium indicators for optical imaging) or Cameleons (Koldenkova & Nagai, 2013), as well as using reporters localised to different membranes or subcellular compartments (Krebs *et al.*, 2012; Mehlmer *et al.*, 2012). It will also be essential to confirm that the cytosolic calcium response to cycloheximide is abolished in plants expressing cycloheximide-insensitive ribosomes, as is the induction of *CBF2* expression by this inhibitor.

Another potential limitation in this thesis is the sensitivity of the SUnSET (surface sensing of translation) assay used to measure the extent of protein synthesis *in vivo*. This method is based on the incorporation of the aminoacyl-tRNA analogue puromycin into *de novo* synthesised proteins and their subsequent detection using a puromycin antibody (Schmidt *et al.*, 2009). While this assay is a straightforward way of measuring translation in cells, it is less sensitive than traditional methods using radiolabelled amino acids, it relies on constant rates of puromycin uptake by cells and it assumes that there are no pleiotropic cellular effects emanating from puromycin-associated protein misfolding. Because of the time required for cellular uptake and ribosomal incorporation of puromycin, it is challenging to study rapid translational responses to cold shock. The relatively short incubation period in puromycin limited the dynamic range of translation measurements. In addition, Marciano *et al.* (2018) recently demonstrated that there may be differences in the accuracy of the SUnSET assay between different cell types and under certain conditions such as energy deprivation. The results obtained using the SUnSET assay in this thesis will therefore need to be confirmed using more accurate methods, for example radiolabelling or AHA labelling, in which the synthetic methionine analogue L-azidohomoalanine (AHA) is incorporated into nascent peptides and can be modified by click chemistry to allow subsequent detection (Marciano *et al.*, 2018).

As ambient temperature was proposed to regulate translation in *A. thaliana* seedlings, it will be essential to test this hypothesis *in vitro*, for example by comparing the effects of different incubation temperatures on the accumulation of a reporter protein such as luciferase or the extent of puromycin incorporation using a cell-free SUnSET assay. Polysome profiling and ribosome profiling will be useful to study the

mechanisms by which cold shock causes translation repression, as ribosome stalling caused by an inherent reduction in ribosomal activity and that triggered by RNA secondary structures are likely to produce different patterns of ribosome footprints on mRNA. Additionally, the involvement of ribosome stalling in the activation of *CBF2* expression can be tested by triggering stalling *in vivo*, for example by inducibly overexpressing or microinjecting mRNAs containing strong stalling motifs, such as pseudoknots (Kontos *et al.*, 2001) or inhibitory uORFs (Gogala *et al.*, 2014).

6.5 Concluding remarks

Although the chemical genomics approach is limited by the difficulties associated with identifying *in vivo* targets of bioactive compounds, this thesis has demonstrated that small molecules are useful tools to study temperature signalling in plants. Such dynamic processes can be chemically perturbed in a rapid, dose-dependent and often reversible manner, with temporal control over chemical effects, thereby overcoming the problems of gene redundancy or lethal mutants associated with classical genetics.

A key outcome of this thesis is the identification of translational machinery as a potential regulator of temperature responses in plants, with translation repression blocking the activation of heat-responsive genes while triggering the expression of early cold-inducible genes in *A. thaliana*. As ribosomal processivity is influenced by the ambient growth temperature as well as by rapid temperature changes (Farewell & Neidhardt, 1998), with an increased likelihood for stalling during cold shock, ribosomes are candidates for transducing environmental temperature signals into biological responses. Though further analyses are required to elucidate the molecular mechanisms by which translational machinery influences temperature responses in plants, such regulation has been proposed in other organisms (VanBogelen & Neidhardt, 1990; Vera *et al.*, 2010; Santagata *et al.*, 2013). Thus, while the process of translation is generally regarded as being downstream of RNA synthesis, experimental evidence suggests that translational machinery can feed back to regulate transcriptional responses. This work highlights the value of using small molecules to study temperature signalling in plants, because in most cases proteins associated with translational regulation are essential or are encoded by multiple genes and findings from forward classical genetics screens in plants have not previously implicated translational machinery in temperature perception.

This thesis has also identified molecules that implicate chloroplasts and mitochondria in the regulation of temperature responses in plants. Although additional experiments are necessary to understand the mechanisms involved, chemicals affecting ROS, metabolite or ion homeostasis in these organelles were found to induce or block the heat shock response. Such regulation would presumably be evolutionarily favourable for plants, given the exceptional sensitivity of these organelles to temperature changes (Sun & Guo, 2016) and their control over cellular energy levels *via* photosynthesis and respiration, which influence biological processes such as protein synthesis (Juntawong & Bailey-Serres, 2012).

In addition to identifying regulators of temperature signalling pathways, this work has demonstrated the potential for small molecules as enhancers of plant growth in unfavourable climatic conditions. Heat stress produces deleterious effects on plant growth and reproduction, ultimately causing a decline in productivity by decreasing the viability of the stigma, pollen and seeds and reducing the duration of seed filling (Prasad *et al.*, 1999, 2003; Boote *et al.*, 2005; Gan *et al.*, 2004; Morrison & Stewart, 2002; Egli *et al.*, 2005). Small increases in seasonal temperatures can have devastating effects on crop yields (Battisti & Naylor, 2009), and given that the mean surface temperature of the earth is predicted to increase by up to 5.8°C during this century (Cubasch *et al.*, 2001), global warming can be expected to have considerable impacts on food security. In this thesis, three molecules were found to increase thermotolerance in *A. thaliana* seedlings with no detectable reductions in plant health in the absence of heat stress. These include two publicly available chemicals, DBMIB, which elevates chloroplast levels of hydrogen peroxide, and 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG), which inhibits HSP90 ATPase activity. The third thermoprotectant discovered is S01A463859Y, a compound from the Syngenta library that has an unknown mode of action but may be involved in stress signalling pathways, based on structural similarities to known activators of G-protein signalling in human cells. It will be essential to identify and characterise the biological targets of this molecule in plant cells in the future.

Given their effects on the survival of *A. thaliana* seedlings during severe heat shock, these three chemicals are promising candidates for enhancing crop growth or yields during high seasonal temperatures. Given the crosstalk between stress responses in plants, for example the improved heat resilience observed upon prior osmotic or heavy metal stress and the increased drought tolerance of heat-acclimated plants (Bonham-Smith *et al.*, 1987), such compounds could potentially also be used to enhance crop performance in the presence of other abiotic stresses. While thermotolerance tests have been carried out on young *A. thaliana* seedlings, it will be of interest to test these

compounds for enhanced thermotolerance in the vegetative and reproductive phases of plants grown on soil, in conditions that replicate those in agricultural contexts. It will also be key to test these molecules for thermotolerance in monocot species, as these are more representative of the major food crops than the model plant *A. thaliana*. Finally, given that many medical conditions are caused by misregulation of the heat shock response, including neurodegenerative illnesses and cancer (West *et al.*, 2012), and that 17-DMAG has been established as a potent anti-tumour drug (Smith *et al.*, 2005), such molecules are also of great interest in the medical field.

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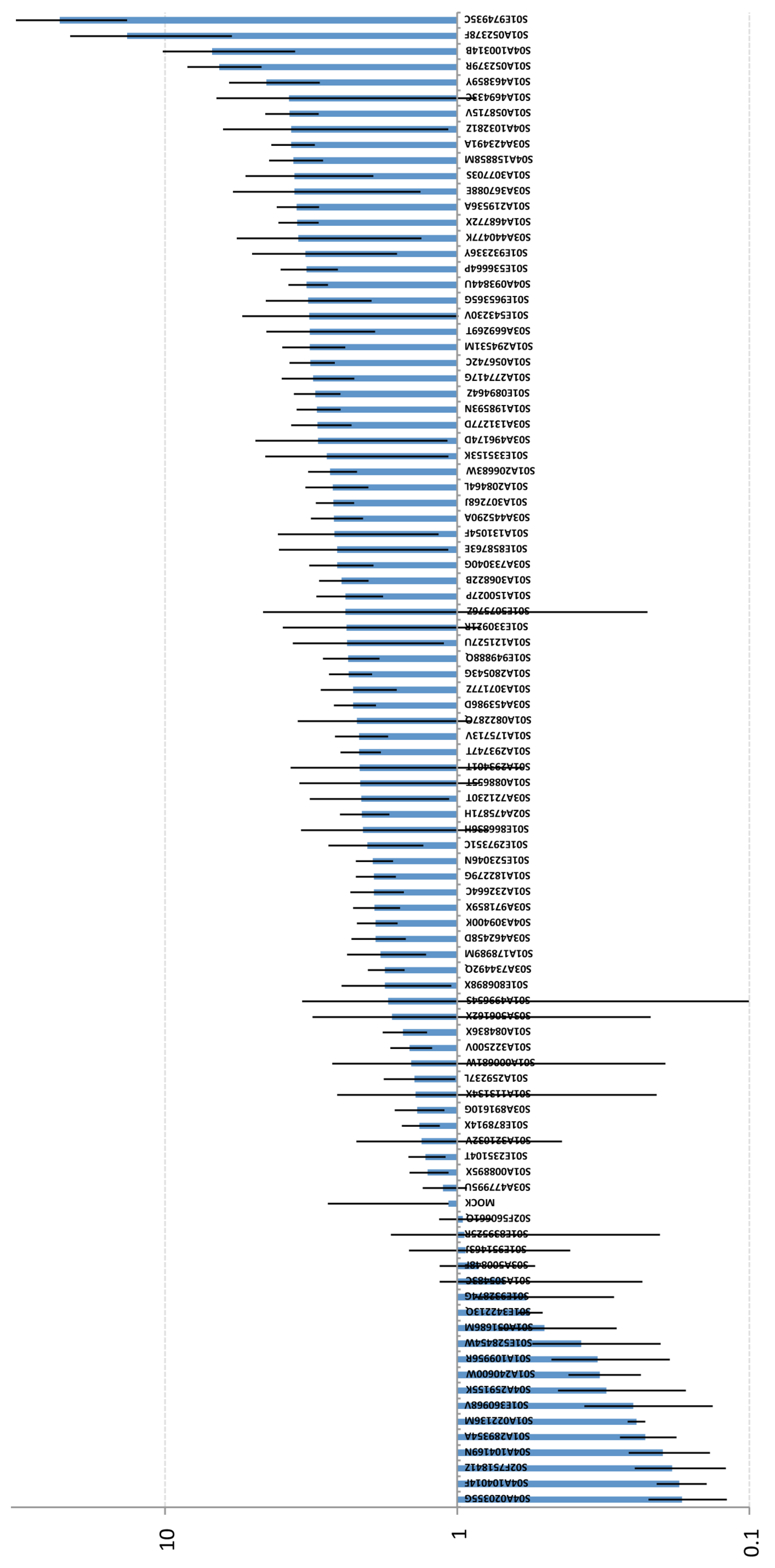
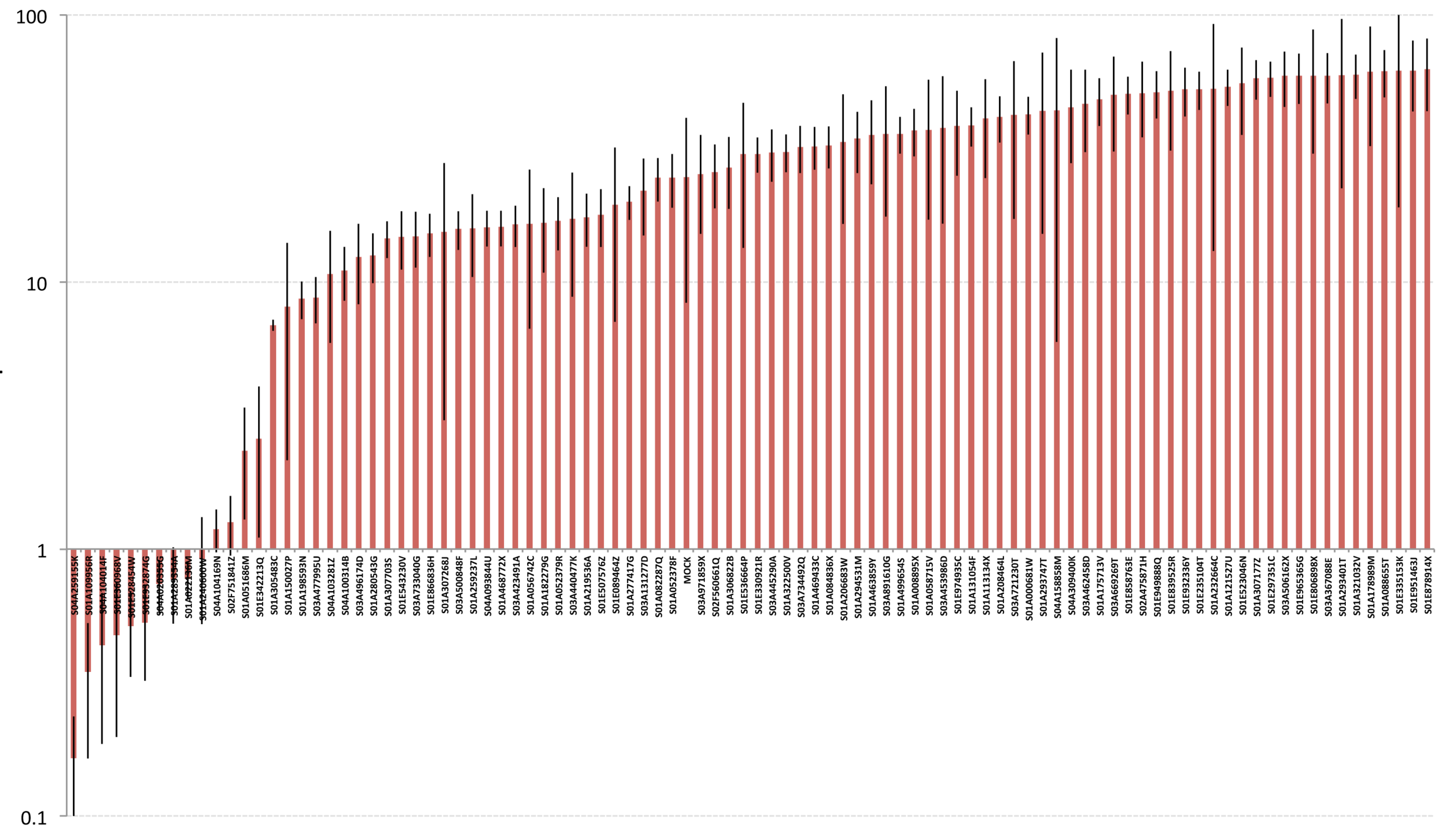
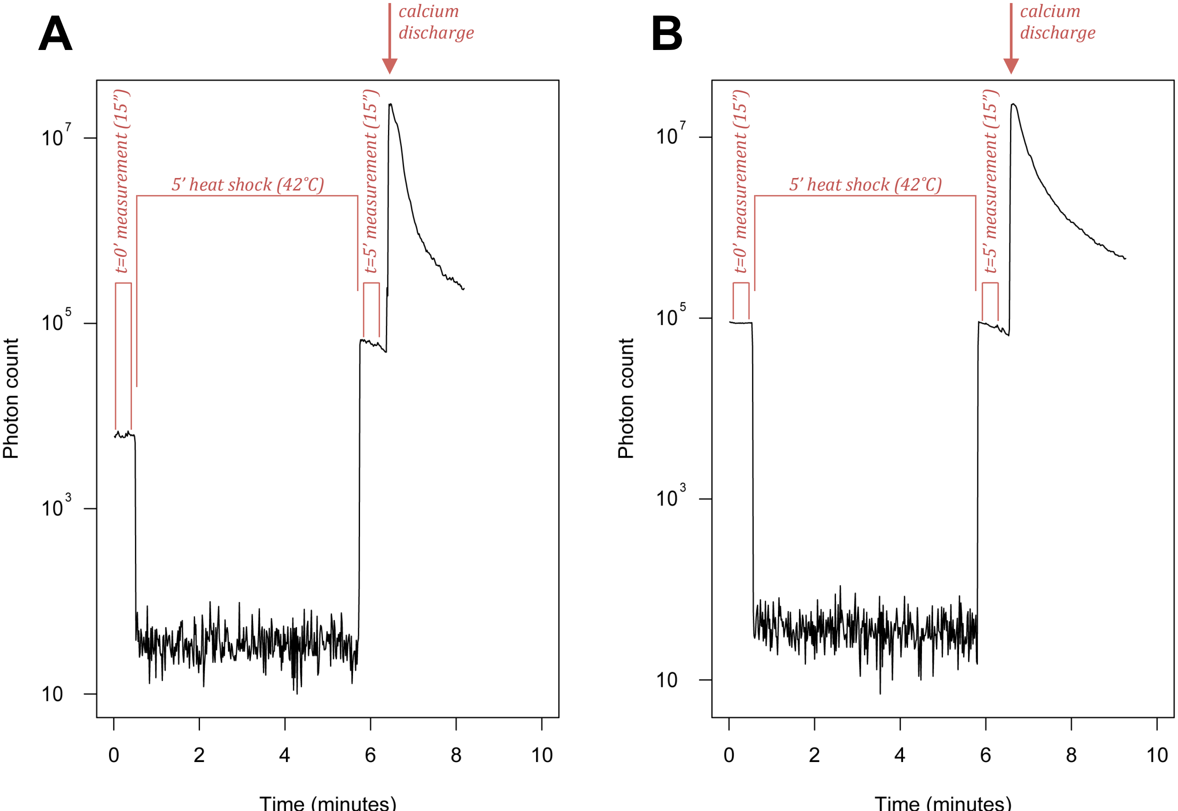
Appendix



Supplementary Figure 1: Chemical effects on basal *HSP70* expression.
Expression of *HSP70* in *A. thaliana* Col-0 seedlings treated with chemicals (10 ppm) identified from the primary screens (mock control: 0.1% v/v DMSO) for one hour at 22°C. Expression was normalised to *PP2A* and *UBC21* levels. Error bars represent standard deviation for at least two biological replicates, with 10-15 seedlings per replicate.



Supplementary Figure 2: Chemical effects on heat-induced *HSP70* expression.
Expression of *HSP70* in *A. thaliana* Col-0 seedlings treated with chemicals (10 ppm) identified from the primary screens (mock control: 0.1% v/v DMSO) for one hour at 22˚C with a 30-minute heat treatment at 30˚C. Expression was normalised to *PP2A* and *UBC21* levels. Error bars represent standard deviation for at least two biological replicates, with 10-15 seedlings per replicate.



Supplementary Figure 3: Example bioluminescent traces from aequorin-expressing *A. thaliana* seedlings treated with chemicals for one hour and exposed to moderate heat shock.

A. thaliana Col-0 *p35SCaMV::APOAEQUORIN* seedlings were grown on ½×MS agar for 12 days at 20°C and, following aequorin reconstitution, were treated for one hour at 20°C with chemicals at 10 ppm in deionised water (**A**: mock control = 0.1% v/v DMSO; **B**: cycloheximide). After the one-hour treatments, cuvettes containing single seedlings in 500 µl chemical solutions were placed in the luminometer for approximately 30 seconds, incubated in a water bath at 42°C for five minutes and immediately returned to the luminometer. After 30 seconds, 1 ml discharge solution (final concentrations 1M $CaCl_2$ and 10% v/v ethanol) was added to the cuvette in the luminometer (indicated with an arrow). Luminescence levels are given in black and experimental details are annotated in red. Given traces are representative of those obtained during mock or cycloheximide treatments.