The specificity of calcium signature decoding mechanisms in their regulation of transcript and protein expression in Arabidopsis thaliana

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Abstract

Calcium is a secondary messenger involved in many aspects of plant physiology and development. During stress, calcium concentration rapidly increases in the cytosol, triggering appropriate changes in downstream expression. It has been suggested that different calcium signatures regulate particular genes, transcription factors and *cis* elements. The 'calcium signature' hypothesis suggests that the calcium signatures themselves hold the information required for an appropriate response in the form of oscillations and/or other spatial, temporal patterns.

One of the main aims of this study was to investigate calcium signature kinetics and test them against known calcium-regulated *cis* elements. To identify novel calcium signatures, calcium responses were generated from single or combined calcium agonists. Luciferase assays were used to investigate novel calcium signature regulation of specific *cis* elements. Results suggested that individual calcium signatures regulated different *cis* elements specifically, with the expression kinetics changing when two *cis* elements were coupled. Further analysis suggested that some novel signatures occurred in different cell types at different times resulting in subsequent, polyphasic downstream expression kinetics. Furthermore, when a calcium signature was regulated by the circadian clock the downstream expression kinetics were altered depending on subjective time of day.

Investigations to date have concentrated on cytosolic calcium signature specificity in respect to the regulation of genes, with relatively little focus on protein regulation. I identified and characterised proteins whose levels either increased or decreased in response to a cytosolic calcium signature generated by mastoparan. It was seen that the proteins identified varied in function and abundance between timepoints, with a relatively high proportion involved in protein modification.

Contents

Statement of Authorship v

Statement of Copyright v

List of abbreviations vi

Acknowledgements viii

1. Introduction 1

1.1 Calcium signalling in plants 1

1.2 Calcium signatures: the question of specificity 8

1.3 Decoding the calcium signal 12

1.3.1 Calmodulin (CaM) and calmodulin-like proteins (CMLs) 12

1.3.2 Calcineurin-B-like proteins (CBLs) and CBL interacting protein kinases (CIPKs) 13

1.3.3 Calcium-dependent protein kinases (CDPKs) and Calcium CaM regulated kinases (CCaMKs) 14

1.3.4 Calcium regulation of proteins and Postranslational modifications (PTMs) 14

1.3.4 Calcium and osmotic stress 18

1.3.5 Measuring calcium 19

1.4. Calcium regulation of gene expression 21

1.5 Crosstalk 25

1.6 Plant circadian clock regulation 25

1.6.1 The molecular basis of the circadian clock 27

1.7 Proteomic research in plants 28

1.8 Summary 31

1.9 Thesis aims 33

2. Materials and Methods 34

2.1 Materials 34

2.1.1 Chemicals 34

2.1.2 Enzymes 34

2.1.3 Antibiotics/Chromogenic substrates/Inducers 34

2.1.4 Bacterial strains 36

2.1.5 Plant material 36

2.1.6 Oligonucleotides and synthetic genes 36

2.1.7 Plasmid vectors 36

2.1.8 iTRAQ labelling 37

2.2 Methods 37

2.2.1 Bacterial growth 37

2.2.2 Plant Growth 37

2.2.3 Chemical and peptide treatment.....40
2.2.4 Calcium measurements.....42
2.2.5 Luciferase measurements.....43
2.2.6 General molecular techniques.....43
2.2.7 Plant Physiology.....59
2.2.8 Proteomics.....61
3. Is there calcium signature specificity during regulation of transcription?.....63
3.1 Introduction.....63
3.2 Results.....66
3.2.1 Calcium signatures generated from both single and combined calcium agonists.....66
3.2.2 Measuring calcium-regulation of specific *cis* elements.....70
3.2.3 Gene expression in response to specific calcium signatures.....76
.....77
3.2.4 Native gene expression in response to specific calcium signatures.....81
3.2.5 Investigating $[Ca^{2+}]_{cyt}$-dependency of *cis* element responses to calcium agonists.....87
.....89
3.3 Discussion.....91
3.3.1 Calcium signature specificity: the decoding of calcium signatures......91
3.3.2 Calcium signature specificity: transcript regulation.....92
3.4 Conclusion.....93
4.Osmotic Stress and calcium signature specificity.....94
4.1 Introduction.....94
4.2 Results.....96
4.3.1 The influence of calcium signature specificity on chlorophyll levels during osmotic stress......96
4.3.2 Gene expression of *KIN2* and *LT178* genes during osmotic stress.....98
4.3.3 Does changing the concentrations of calcium chloride pre-treatment confer osmotic tolerance to mature *Arabidopsis thaliana*?.....101
4.3.3.1 Does calcium chloride pre-treatment of Arabidopsis seeds lead to osmotic stress tolerance in mature plants?.....102
4.4 Discussion.....106
4.4.1 Do specific calcium signatures display specificity during osmotic stress?.....106
4.4.2 Is calcium chloride induced osmotic stress tolerance age limiting?.....107
4.4.3 Does pre-treatment of calcium chloride at seed level instigate osmotic stress tolerance in mature *Arabidopsis thaliana* plants......108
4.5 Conclusion.....108
5. The biphasic response generated from specific calcium agonists and the influence and importance of circadian regulation......109
5.1 Introduction.....109

5.1.1 The $[Ca^{2+}]_{cyt}$ biphasic response.....109

5.1.2 Circadian regulation in plants......110

5.2 Results.....112

5.2.1. Measuring calcium-regulation of specific *cis* elements in various organs of *Arabidopsis thaliana*.....112

5.2.2 Investigating the circadian regulation of specific $[Ca^{2+}]_{cyt}$ signatures.....117

5.2.3 Determining the influence of circadian regulation on calcium regulated specific cis elements......125

5.3 Discussion.....138

5.3.1 Do ATP or mastoparan/$CaCl_2$ $[Ca^{2+}]_{cyt}$ signature peaks occur in the same cells or in different cells types when regulating specific *cis* elements?.....138

5.3.2 The importance of circadian clock regulation of calcium agonist-generated $[Ca^{2+}]_{cyt}$ signatures......140

5.3.3 Investigating the importance of circadian regulated $[Ca^{2+}]_{cyt}$ signatures on expression kinetics......141

5.4 Conclusion.....143

6. Calcium regulation of combined cis elements.....144

6.1 Introduction.....144

6.2 Results.....145

6.3 Discussion.....155

6.4 Conclusion.....157

Chapter 7 Identifying proteins regulated by mastoparan generated cytosolic calcium signatures.....158

7.1 Introduction.....158

7.2 Results.....161

7.2.1 Proteins regulated by mastoparan generated $[Ca^{2+}]_{cyt}$ signatures.....161

7.2.2 Mastoparan induced gene expression.....167

7.2.3 Investigating four proteins that are regulated by mastoparan generated $[Ca^{2+}]_{cyt}$ signatures......170

7.2.3.1 Protein construct design and experimental process......171

7.2.3.2 Investigation into mastoparan induced calcium regulation of selected proteins: Gene expression.....172

7.3 Discussion.....176

7.3.1 The regulation of proteins by mastoparan generated $[Ca^{2+}]_{cyt}$ signatures and their functions......176

7.3.2 Future investigation of identified proteins of interest.....179

7.4 Conclusion.....180

8. Discussion and conclusions.....181

8.1 Implications of the work.....181

8.2 Calcium signature specificity.....182

8.3 ATP and mastoparan/$CaCl_2$ generated cytosolic calcium biphasic regulation of specific transcription factors 184
8.4 Circadian clock regulation of calcium 185
8.5. Calcium regulation of combined cis elements 189
8.6 The regulation of proteins by a mastoparan generated $[Ca^{2+}]_{cyt}$ signature 190
8.7 Experimental limitations 191
8.8 Alternative approaches to test the roles of calcium signatures 192
8.9 Future work 193
8.9.1 Investigations into calcium signature specificity 193
8.9.2 To complete the investigations into the coupled cis elements 193
8.9.3 Calcium regulation of Proteins 194
8.9 Conclusions 194
9. Bibliography 195
Appendix. A 225
Appendix B 230
Appendix C 233
Appendix D 234
Appendix E 236
Appendix F 237

Statement of Authorship

I certify that all the work described in this thesis is my own original research unless otherwise acknowledged in the text or by references, and has not been previously submitted for a degree in this or any other university.

Statement of Copyright

List of abbreviations

ABA-Abscisic acid

ABRE-ABA responsive element

ACAs-autoinhibited Ca^{2+} ATPases

ATP- adenosine triphosphate

Ca^{2+}- Calcium

$[Ca^{2+}]_{cyt}$ – Cytosolic calcium

$CaCl_2$-Calcium chloride

cADPR- cyclic ADP ribose

CaM-calmodulin

CaMB domain- calmodulin binding domain

cAMP-cyclic adenosine monophosphate

CAX-cation exchangers

CBLs- calcineurin-B-like proteins

CCAMKs-calcium CaM regulated kinases

CDPKs-calcium-dependent protein kinases

Cd^{2+}-cadmium

cGMP- cyclic guanosine monophosphate

CIPKs-CBL interacting protein kinases

CLD- calmodulin-like domain

CMLs-calmodulin-like proteins

CNB domain-cyclic nucleotide binding domain

CNs-cyclic nucleotides

CNGCs-cyclic nucleotide gated channels

CO_2- carbon dioxide

COP1-constititive photomorhogenic 1

DACCs- depolarisation-activated calcium channels

DRE/CRT- dehydration-responsive element (DRE) c-repeat CRT cis element

DNA- deoxyribonucleic acid

DIGE- difference gel electrophoresis

eATP-extracellular adenosine triphosphate

ECAs -ER-type Ca^{2+} ATPases

EE-evening element

EMSA- electrophoresis mobility shift assay

ER-endoplasmic reticulum

GECIs-Genetically encoded calcium indicators

GLRs- glutamate receptors

GST - glutathione-S-transferase

HACCs- hyperpolarisation-activated calcium channels

HMA1s-heavy metal transporter 1

IP3-inositol trisphospate

iTRAQ-isobaric tag for relative and absolute quantification

K+-pottasium

LC-Ms/MS- liquid chromatography and tandem mass spectrometry

MCAs- mid1 complementing activity

Mn^{2+}-maganese

MSLs- mechanosensitive-like channels

NAAPD- nicotinicacid adenine dinucuclotide phosphate

O_2-oxygen

PTMs- Posttranslational modifications

ROSS- Reactive oxygen species

SA-Salicylic acid

SDS-PAGE- SDS polyacrylamide gel electrophoresis

SKOR- shakerlike stellar K+ outward rectifier

SUMO- Small Ubiquitin -Like Modifyer

RNA- ribonucleic acid

RUBisco-ribulose, 1-5 bisphosphate carboxylase

TFs -Transcription factors

TPC1 - two pore channel 1

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1. Introduction

1.1 Calcium signalling in plants

When a plant is exposed to environmental changes it initiates a process of cellular reprograming through a network of signalling events (Galon et al., 2010). These signalling events, initiate an appropriate downstream response, composing of receptors, secondary messengers, transducers and transcription factors (TFs) (Figure 1.1). Generally, receptors are highly specific to a stimulus as are transducers and TFs, which reflects in their high abundance in the plant genome (Sanders et al., 2002). In contrast, secondary messengers are relatively few in numbers (Sanders et al., 2002).

A secondary messenger is an intracellular molecule or chemical element that relays signals received by receptors to the target molecules in the cytosol or nucleus, amplifying the strength, and encoding the specificity of the signal (Bhargava and Sawant, 2013, Chaves et al., 2009, Newton et al., 2016). Calcium is a secondary messenger for all eukaryotic cells and is involved in many aspects of plant development and physiology (Dodd et al., 2010, Kudla et al., 2010). However, high concentrations of calcium are cytotoxic, producing insoluble calcium salts, therefore a minimal level (~100-200 nM) of $[Ca^{2+}]_{cyt}$ is maintained in the cytosol of some unstimulated cells by calcium transporters such as Ca^{2+}-ATPases and H+/Ca^{2+}-antiporters (Bush, 1995, Hirschi, 2001, Kudla et al., 2010, Mahajan and Tuteja, 2005, Sze et al., 2000). High concentrations of $[Ca^{2+}]_{cyt}$ are either removed to the apoplast or the lumen of intracellular organelles, such as the vacuole and endoplasmic reticulum (ER) where calcium accumulation can occur safely (Dodd et al., 2010, Stael et al., 2012).

In response to a stimulus the calcium is released from its stores, this rapid and transient $[Ca^{2+}]_{cyt}$ signal then carries a message which triggers the appropriate downstream response (Sanders et al., 2002, White, 2000). Supporting this process is a complex machinery comprising of Ca^{2+} permeable channels (allows release of calcium from the stores), Ca^{2+}-binding proteins (interacts with downstream partners) and calcium transporters such as ATPases and antiporters (which aid in the return of basal calcium levels) (Kudla et al., 2010, Sanders et al., 2002, White, 2000).

1.1.1 Generating calcium signals

The first stages of calcium signalling comprises of an increase in $[Ca^{2+}]_{cyt}$ and the interaction of free calcium with sensor proteins (Kudla et al., 2010). The formation of calcium signals and their temporal patterns fundamentally depend on the functional collaboration of calcium channels. This process is then counteracted by activation of efflux transporters, which allow a return to the calcium basal level, and the re-establishing of calcium homeostasis (Dodd et al., 2010, Kudla et al., 2010, Pittman and Hirschi, 2016). These two processes are known as influx and efflux and are mediated by different calcium transporters (Figure 1.1).

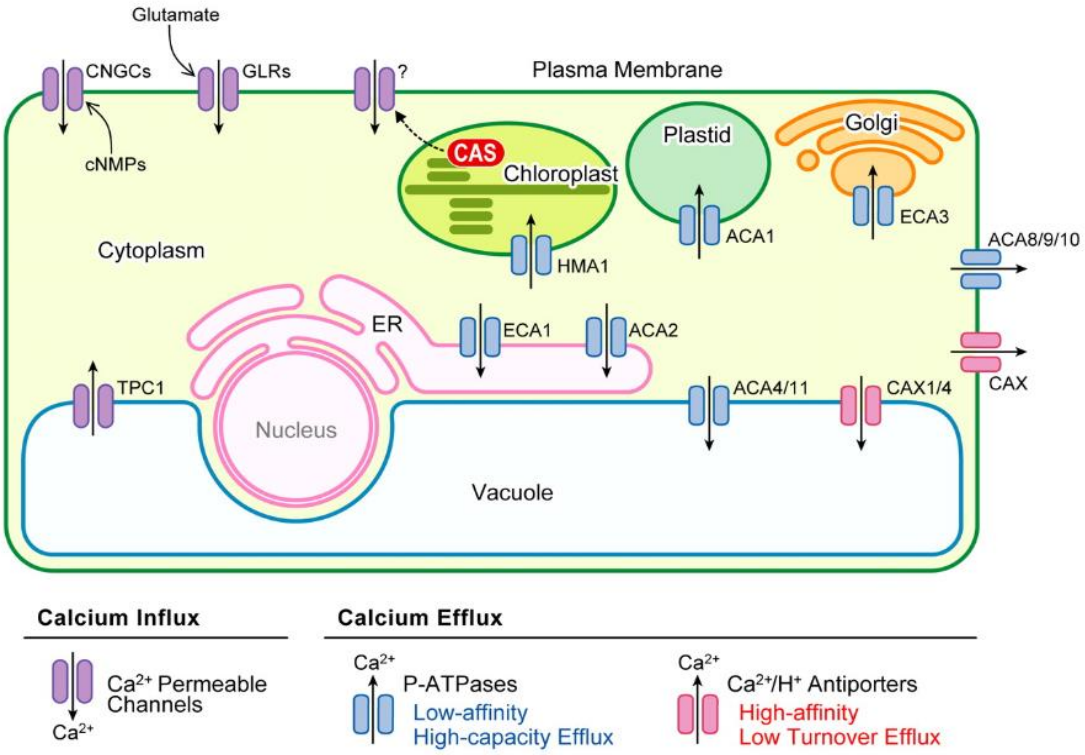


Figure 1.1: The calcium transport systems in Arabidopsis, displaying calcium influx/efflux pathways identified at the molecular level. **CNGC**, cyclic nucleotide channel; **GLR**, glutamate receptor; **TPC1**, two pore channel 1; **CAS**, Ca^{2+}-sensing receptor; **ACA**, autoinhibited calcium **ATPase**; **ECA**, ER type calcium ATPase; **HMA1**, heavy metal ATPase1; **CAX**, cation exchanger. (schematic taken from Kudla et al., 2010).

1.1.1.1 Calcium influx and efflux

Calcium signatures are generated through two opposing reactions: calcium influx through calcium channels and calcium efflux through calcium pumps (Figure 1.2). The differences between low cytosol calcium concentrations and high calcium concentrations found in internal stores, such as the ER, generate strong calcium gradients. When calcium channels are open these gradients drive calcium through the channels into the cytosol (Bush, 1995, DeFalco et al., 2009, Hetherington and Brownlee, 2004, Hirschi, 2001, Hwang et al., 1997, Sanders et al., 2002, Swarbreck et al., 2013, Sze et al., 2000, White and Broadley, 2003). There are a number of different types of calcium channels, these include voltage activated Ca^{2+} channels which are subdivided into hyperpolarisation-activated calcium channels (HACCs) and depolarisation-activated calcium channels (DACCs) (Miedema et al., 2001, White, 2000), ligand-dependent calcium channels and stretch activated channels (Cosgrove and Hedrich, 1991, Dutta and Robinson, 2004, Kudla et al., 2010, White and Broadley, 2003). Due to the diverse array of calcium channels the plant can translate a myriad of stimuli, by utilizing different types of calcium channels, as well as regulating the levels of these channels in the plant's cells.

Calcium efflux in contrast to calcium influx is an active process which returns the cytosol calcium to basal level. Calcium efflux works against the calcium gradient using energy in the form of ATP in the ATPases and proton gradient in the case of proton antiporters (Dodd et al., 2010, Kudla et al., 2010). Calcium ATPases have a high affinity (Km=0.1-2µM) and are low capacity transporters, they are mainly involved in bringing the $[Ca^{2+}]_{cyt}$ signals to an end. Proton-calcium antiporters in contrast, have a low affinity (Km=10-15µM) and are high capacity transporters involved in removing $[Ca^{2+}]_{cyt}$ during high elevations (Sze et al., 2000).

1.1.1.2 Calcium influx channels

1.1.1.2.1 Voltage gated calcium channels

The electrophysiology of both HACCs and DACCs has been characterised, however, the molecular identification of these channels has been extensively debated (Grabov and Blatt, 1998, Hamilton et al., 2000, Hetherington and Brownlee, 2004, Klüsener et al., 2002, Pei et

al., 2000, Thion et al., 1998, Thuleau et al., 1998, White et al., 2002). It has been shown that HACCs are involved in the stomatal closure response to ABA and blue light, as well as contributing to root cell elongation, polar growth, and nutrient acquisition (Dodd et al., 2010, Foreman et al., 2003, Hamilton et al., 2000, Kiegle, 2000, Swarbreck et al., 2013). Unlike HACCs which have been widely characterised, there is limited information in regards to DACCs, however it is believed that their role in plant cells is linked to transient Ca^{2+}-increases in response to cold and microbe interactions (Kudla et al., 2010, Thion et al., 1998).

Annexins are cytosolic proteins that are known to be associated with the membranes of phospholipids and can be calcium-dependent and calcium-independent (Konopka-Postupolska and Clark, 2017, Lizarbe et al., 2013). The presence of a hydrophilic pore at the centre of the molecule is believed to be the structural basis for annexin Ca^{2+} channel activity in the bilayer (Gerke and Moss, 2002, Laohavisit et al., 2010, Laohavisit et al., 2009, Swarbreck et al., 2013). The function of annexins is not fully understood, however some studies have suggested that they are involved in calcium fluxes across the plasma membrane in the form of redox regulated calcium pores (Baucher et al., 2012, Laohavisit et al., 2009). It has also been suggested that annexins are involved in voltage-dependent and ROS-dependent calcium transport (Davies, 2014, Laohavisit et al., 2010, Mortimer et al., 2008).

1.1.1.2.2 Mechanosensitive calcium channels

Calcium channels are important in many plant mechanosensory pathways and are involved in gravity stimulation which is associated with membrane depolarisation (Toyota and Gilroy, 2013), touch stimulus (Fasano et al., 2002) and osmotic stress (Shabala and Lew, 2002). There are several mechanosensitive channels known to be involved with calcium, these include mechanosensitive-like channels (MSLs) which have been found in plastid and root membranes (Haswell et al., 2008, Veley et al., 2012); mid1 complementing activity (MCAs) which have been identified in the plasma membrane and piezo-like proteins, which

create calcium permeable pores in the membranes in response to membrane stretching (Hamilton et al., 2015, Haswell, 2007, Monshausen and Haswell, 2013).

1.1.1.2.3 Ligand activated calcium channels

The most characterised class of calcium channels are ligand activated calcium channels with cyclic nucleotide gated channels (CNGCs) and glutamate receptors (GLRs) being two of these. CNGCs are gated channels mostly found in the plasma membrane (Chin et al., 2009, Ma et al., 2009).There are twenty CNCG gene family members in the Arabidopsis genome, which are divided by phylogenetic relationship into four groups (I-IV) (Mäser et al., 2001b). These channels are activated by the direct binding of cyclic nucleotides (CNs) such as cAMP and cGMP to the CNB domain, and inhibited by calmodulin (CaM) binding to the calmodulin binding (CaMB) domain (Chin et al., 2009, Kudla et al., 2010, Wang et al., 2013). Plant CNCGs are thought to mediate many biological processes from plant development to stress tolerance (Kaplan et al., 2007), including thermal sensing and thermotolerance (Finka et al., 2012), the salt stress response (Gobert et al., 2006) and the pathogen response (Saidi et al., 2009).

GLRs are non-selective cation channels activated by amino acids, particularly glutamate and glycine (Qi et al., 2006, Stephens et al., 2008). In Arabidopsis it has been revealed that there are 20 GLR genes in the genome which are grouped into three clades (Davenport, 2002, Lacombe et al., 2001). GLRs have been shown to be important for plant calcium nutrition, the plant defence response (Kang et al., 2006, Vatsa et al., 2011), calcium response to cold (Meyerhoff et al., 2005) systemic response to wounding and aphid feeding (Kang et al., 2006, Mousavi et al., 2013, Vincent et al., 2017, Wang et al., 2013), stomatal closure and regulation of ABA (Cho et al., 2009, Kang et al., 2004) as well as root development and light mediated hypocotyl elongation (Brenner et al., 2000, Walch-Liu et al., 2006).

There are three other ligands that have been described as calcium inducers in the cytosol, IP_3 (inositol trisphosphate), cADPR (cyclic ADP ribose) and NAAPD (nicotinicacid adenine dinucuclotide phosphate) (Allen et al., 1995, Kudla et al., 2010, Navazio et al., 2000). As well as these ligands it has been shown that are two K^+ channels which also mediate Ca^{2+} fluxes: TPC1 6 (two pore channel 1) and SKOR (shakerlike stellar K^+ outward rectifier). TPC1

6, mediates various biological functions including the formation of calcium waves, induced systemic signalling, jasmonate-mediated wounding and the pathogen response (Beyhl et al., 2009, Bonaventure et al., 2007, Gilroy et al., 2016, Hedrich and Marten, 2011, Hedrich et al., 2018, Vincent et al., 2017). SKOR is a ROS-activated channel, which can mediate both K^+ or a Ca^{2+} current (Garcia-Mata et al., 2010). It must be noted however, that IP_3 receptors have not been found in plants.

1.1.1.3 Calcium efflux transporters

1.1.1.3.1Calcium/proton antiporters (Cation exchangers)

There are six genes that encode for CAX (cation exchangers) proteins in the Arabidopsis genome (Mäser et al., 2001a), these proteins have been reported to display activity in the vacuole, the plasma membrane and the chloroplast (Blumwald and Poole, 1986, Cheng et al., 2005, Ettinger et al., 1999, Hirschi et al., 2000, Mäser et al., 2001a, Wang et al., 2016). It has been noted that CAX activity is controlled by a N-terminal autoregulatory domain (Pittman et al., 2002) and as well as Ca^{2+} transport, CAX are also involved in metal ion transport such as Mn^{2+} and Cd^{2+}; resulting in heavy metal stress tolerance (Hirschi et al., 2000, McAinsh and Pittman, 2009).

1.1.1.3.2 Calcium ATPases

Calcium ATPases are a member of the superfamily of ATPases, they are energised by ATP hydrolysis and are primary transporters in the efflux system (Kudla et al., 2010). P-type ATPases are divided into three categories ECAs (ER-type Ca^{2+} ATPases), ACAs (autoinhibited Ca^{2+} ATPases) and HMA1s (heavy metal transporter 1) (Kudla et al., 2010, Sze et al., 2000). ECAs belong to the second subclass P_{IIA} (Kudla et al., 2010) and are located in the ER (ECA1) (Liang et al., 1997), Golgi (ECA3) (Mills et al., 2008) and the endosomes (ECA3) (Li et al., 2008). ACAs are subcategorised into the P_{IIB} subclass (Kudla et al., 2010) and are found in the ER (ACA2) (Harper et al., 1998), vacuole (ACA4 and ACA11) (Geisler et al., 2000, Lee et al., 2007), plasma membrane (PM) (ACA8, ACA and ACA10) (Bonza et al., 2000, George et

al., 2008, Schiøtt et al., 2004) and the plastid envelope (ACA1)(Huang et al., 1993). Like the HMA1, ACAs and ECAs are heavy metal transporters (Kudla et al., 2010, Moreno et al., 2008), as these pumps can pump both heavy metals and calcium there is an implication that they may have evolved from a general cell detoxification mechanism (Kudla et al., 2010).

1.1.1.3 Calcium agonists

An agonist is a chemical that binds to a receptor and activates the receptor to produce a biological response, in the case of calcium agonists they induce a rise in $[Ca^{2+}]_{cyt}$ (Clark and Roux, 2018). There are several calcium agonists known to induce a rise in $[Ca^{2+}]_{cyt}$ in plants including ATP, $CaCl_2$, glutamate and mastoparan (Choi et al., 2014a, Choi et al., 2014b, Demidchik et al., 2011, Demidchik et al., 2009, Jeter et al., 2004).

ATP for example, has been shown to rapidly induce an increase in $[Ca^{2+}]_{cyt}$ in root and shoot tissue (Demidchik et al., 2003, Jeter et al., 2004). It has been found that Arabidopsis seedlings generate calcium signatures which bind to the DORN1 receptor when treated with eATP (Choi et al., 2014a, Jeter et al., 2004, Nizam et al., 2019); DORN1 is known to be involved in the plant stress response to wounding (Jewell et al., 2019, Tripathi et al., 2018). Extracellular ATP (eATP) has also been linked to PSII protection during salt stress when ran through $[Ca^{2+}]_{cyt}$ as a secondary messenger (Hou et al., 2018) and to wound induced $[Ca^{2+}]_{cyt}$ signalling (Tripathi and Tanaka, 2018, Tripathi et al., 2018). It has been shown that ATP plant responses are dose-dependent, mostly generating a biphasic response with low concentrations generating one response and high concentrations generating multiple responses (Clark et al., 2011, Clark et al., 2010, Deng et al., 2015, Reichler et al., 2009, Roux et al., 2006).

Mastoparan is a peptide isolated from wasp venom (Miles et al., 2004, Takahashi et al., 1998) and has been successfully shown to highly induce $[Ca^{2+}]_{cyt}$ elevations in plants (Lenzoni, 2017, Sun et al., 2007, Whalley et al., 2011). In animal systems mastoparan can activate heterotrimeric G proteins resulting in an ER-mediated calcium increase which is in turn mediated by the production of the IP_3 (inositol trisphospate) intermediate (Mousli et

al., 1990, Ross and Higashijima, 1994, Sukumar et al., 1997). However, Miles et al., (2004) has suggested that mastoparan effects in plants can occur independent of the heterotrimeric G proteins and to date mastoparan mode of action in plant cells has not been defined (Miles et al., 2004).

1.2 Calcium signatures: the question of specificity

One of the most fundamental questions regarding calcium as a secondary messenger is how stimulus specificity is maintained within the highly complex network of calcium signalling in plants (McAinsh and Hetherington, 1998). There could be several factors or combination of factors that determine specificity of response in a plant via calcium. These include cell developmental history, alternative (to calcium) secondary messengers and cellular location of elements involved in calcium homeostasis and/or response, such as calcium channels (Clayton et al., 1999). One of the main contenders and a major focus in research is the hypothesis that the calcium signal itself might contain the information that determines specificity. It has been suggested that this information is known collectively as 'calcium signatures' (Berridge et al., 2000, McAinsh and Hetherington, 1998, McAinsh and Pittman, 2009, Ng and McAinsh, 2003).

It is accepted that in animal cells spatial and temporal heterogenetics in Ca^{2+} play an essential role in the encoding of stimuli specific signals (Berridge et al., 2000). In plants, evidence of specific signalling and decoding originated from research in stomatal guard cells and symbiosis signalling in legumes (Allen et al., 2001, Kosuta et al., 2008, McAinsh and Pittman, 2009). Research conducted by Allen et al., (2001) reported steady state stomatal closure in Arabidopsis only occurred when $[Ca^{2+}]_{cyt}$ oscillations fell into a window of a particular period, number and amplitude. When adopting the ABA insensitive *gca2* mutant, a shorter period of oscillations was observed compared to the wild type, which resulted in the absence of steady state stomatal closure. Allen et al., (2001) also observed

that when exposed to the correct Ca^{2+} signature, this mutant phenotype could be 'rescued'. These data imply that $[Ca^{2+}]_{cyt}$ signatures encode specificity and have a major role in the stomatal closure signalling pathway (Allen et al., 2001, McAinsh and Pittman, 2009). However, in contrast Levchenko et al., (2005) described stomatal closure in the absence of oscillations, suggesting there could be additional mechanisms in the guard cell network (Levchenko et al., 2005).

Within *Medicago truncutula* Ca^{2+} oscillations have been documented in the same cells from both nodulation (nod) factor in response to rhizobia derived nodulation, and in the root hairs in response to arbuscular mycorrhizal fungi (Oldroyd and Downie, 2008). Research employing nod effective mutants *dmi1* and *dmi2* resulted in no detection of nod-factor induced Ca^{2+} oscillations, in response to both rhizobia derived nodulation and arbuscular mycorrhizal fungi, suggesting that both shared similar Ca^{2+} signatures. However, upon further analysis, it was seen that mycorrhizal induced Ca^{2+} oscillations were both shorter and lower in amplitude, compared to that of nod- factor induced Ca^{2+} oscillation (Oldroyd and Downie, 2008). It could be deduced from these results that information from signals could be encoded in both frequency and number of Ca^{2+} oscillations, giving further support that information within Ca^{2+} signatures determines specificity. An example of these signatures can be found in figure 1.2.

Research conducted by Clayton et al., (1999) suggested Ca^{2+} acts as a secondary messenger in the ozone signal transduction pathway. They presented evidence that a biphasic signature event occurs in response to ozone, with the initial spike displaying a relatively higher magnitude, but short duration compared to the second spike, which represented a smaller magnitude lasting over a prolonged period of time. These data suggest there was a direct correlation between the expression of the antioxidant defence enzyme glutathione-S-transferase (*GST*) gene and the second spike. By blocking the phases separately with the calcium channel blocker lanthanum chloride, it was seen that *GST* expression was inhibited in conjunction with the blocking of the second spike only (Clayton et al., 1999). This evidence strongly implied that there was specific information encoded in the $[Ca^{2+}]_{cyt}$ signature. Similar findings were seen in Lecourieux et al., (2005), here a biphasic response was noted after treatment with crytogein and oligosaccharide elicitors, it was also observed

that spike duration and magnitudes varied considerably between the elicitors, suggesting specificity within the Ca^{2+} signatures (Lecourieux et al., 2005).

Transient $[Ca^{2+}]_{cyt}$ signatures can be single (spike), double (biphasic) or multiple (oscillations). Signatures can differ in cellular location, generated from different pools of Ca^{2+} (cytosolic, nuclear, vacuolar etc.) and be tissue specific (Moore et al., 2002). In roots for instance, $[Ca^{2+}]_{cyt}$ signatures can be induced from several abiotic stresses such as salinity, cold stress and osmotic stress but are substantially different between various cell types (Kiegle, 2000). However, some literature has suggested caution when adopting the 'calcium signature hypothesis' to explain specificity in signalling pathways (Lecourieux et al., 2005, Plieth, 2001, Scrase-Field and Knight, 2003). There is the possibility that calcium can also operate as a switch to activate Ca^{2+}-dependent elements, and that other signalling components are required for specificity e.g. in defence signalling pathways it has been shown that Ca^{2+} is only one of a combination of secondary messengers required (Lecourieux et al., 2005).

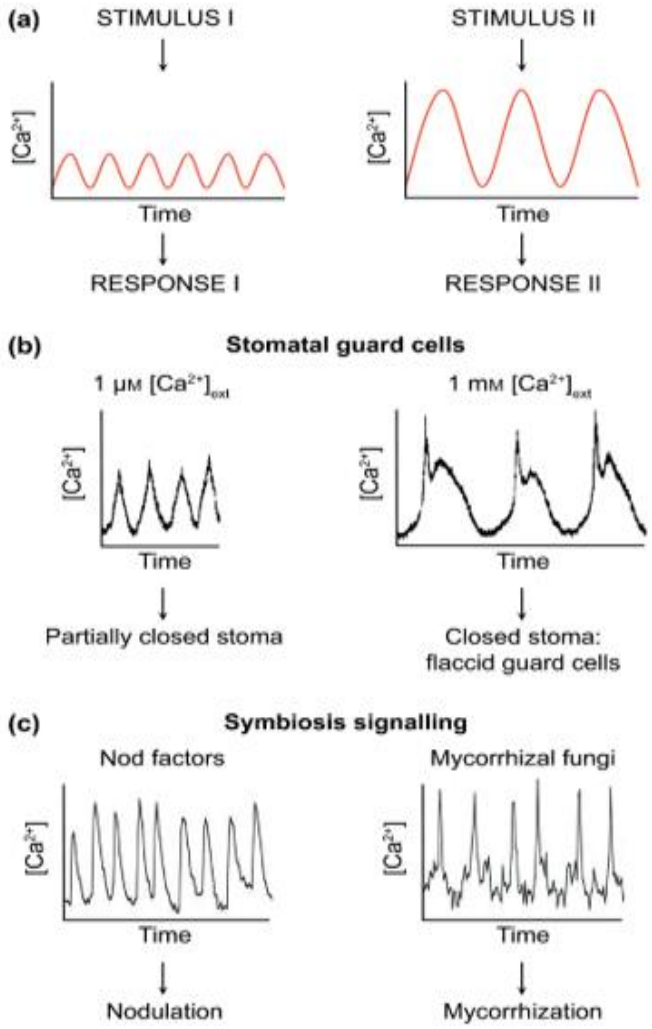


Figure 1.2: (**a**) schematic representation of the encoding of signalling of calcium signatures. (**b**) External $[Ca^{2+}]_{cyt}$ correlation with the calcium signature, which resultants in a steady state stomatal aperture in *Commelina communis* (McAinsh et al., 1995). (**c**) The role of different calcium signatures in symbiotic signalling (Kosuta et al., 2008). Schematic taken from(McAinsh and Pittman, 2009).

1.3 Decoding the calcium signal

The information encoded in calcium signatures is decoded by various Ca^{2+} binding proteins (sensor proteins) leading to a cascade of events eliciting the eventual response to a given stimulus. The importance of Ca^{2+}-binding proteins is reflected by their abundance and diversity, it is believed there are over 250 different binding proteins in Arabidopsis (Day et al., 2002, Kudla et al., 2010). Ca^{2+} - binding proteins are involved in the response to several abiotic and biotic stresses such as plant defence, osmotic stress, drought, and low temperatures (Knight and Knight, 2001). In plants these Ca^{2+} - binding proteins fall into two categories, sensor relays and sensor responders (Reddy and Reddy, 2004a, Sanders et al., 2002). Sensor relays such as, calmodulin (CaM), calmodulin-like proteins (CMLs) and calcineurin-B-like proteins (CBLs) (Bender et al., 2013, Ranty et al., 2016, Sanders et al., 2002), upon Ca^{2+} binding, undergo conformational change and interact with target proteins to elicit enzyme activity or DNA binding. Sensor responders such as CDPKs undergo Ca^{2+} induced conformational change that alters the proteins own enzyme activity and structure (Cheng et al., 2002, Harper et al., 2004, Kudla et al., 2010). Most calcium binding proteins contain EF hands, calcium-binding domains that are involved with the stabilization of the protein, as well as facilitating high affinity to Ca^{2+} binding (Zielinski, 1998).

1.3.1 Calmodulin (CaM) and calmodulin-like proteins (CMLs)

CaM protein sensors are highly conserved and are found in all eukaryotes, in Arabidopsis there are seven genes that encode CaM proteins (Kudla et al., 2018). CaM sensors contain four EF-hand calcium binding domains and are involved in several essential biological responses, such as regulating transcription and enzyme activity (Edel and Kudla, 2015, Galon et al., 2010, Perochon et al., 2011). Unlike CaMs which are found in all eukaryotes, CMLs are restricted to plants. They are however closely related to CaMs but are more diverse in their structure as they can contain 1-6 EF hand motifs. There are 50 CML genes that encode CML proteins in the Arabidopsis genome (Kudla et al., 2018, McCormack et al., 2005, Zhu et al., 2015), with many of their functions already being characterised, for

example, CML24 and CML9 are known to regulate the ABA response and are involved in the ionic stress response (Delk et al., 2005) and CML36 has been shown to regulate Ca^{2+} ATPase activity (Astegno et al., 2017).

1.3.2 Calcineurin-B-like proteins (CBLs) and CBL interacting protein kinases (CIPKs)

Like CMLs, CBLs are only found in plants, there are ten CBL genes in the Arabidopsis genome. There is very little similarity between CaMs and CBLs, suggesting that CBLs diverged from CaM very early in evolution (Batistic and Kudla, 2009, Edel and Kudla, 2015, Weinl and Kudla, 2009). Like CaMs, CBLs contain four EF hands, however, CBLs have a unique first EF hand, with its Ca^{2+} binding loop containing 14 amino acids compared to 12 found in CaMs (Nagae et al., 2003).

CBLs actively interact with protein kinases (CIPKs), this interaction is specific to a particular set of CIPKs (Albrecht et al., 2003, D'Angelo et al., 2007). There are 26 CIPK members in the Arabidopsis proteosome (Shi et al., 1999). CIPKs have a conserved structure containing an N-terminal (serine/threonine) protein kinase domain, a junction domain and a CIPK-specific C-terminal regulating domain (Kudla et al., 2018, Shi et al., 1999). Within the C-terminal regulating domain there is an auto-regulating NAF domain and a phosphatase interaction domain (PPI) (Ohta et al., 2003). During interaction, an active enzyme conformation occurs due to the release of the auto inhibitory NAF domain (Chaves-Sanjuan et al., 2014). CBL-CIPK interaction activates CIPKs towards target phosphorylation and specific cellular targeting (Batistic et al., 2010). For example, CBL12 and CBL3 are S-acylated and targeted to the vacuolar membrane, whereas CBL1 and CBL9 are myristolated and palmitoylated, and are found at the plasma membrane (Batistič and Kudla, 2012, Batistic et al., 2008). CIPKs target membrane locations are specifically governed by their CBL interaction, which allows a CIPK to target different membranes depending on its partner e.g. CIPK24 which regulates SOS1 in conjunction with CBL4/SOS3 is located at the plasma membrane, in contrast when interacting with CBL10, a vacuolar target protein, it is located to the vacuole and is involved in salt stress tolerance (Halfter et al., 2000, Ishitani et al., 2000, Kudla et al., 2018).

1.3.3 Calcium-dependent protein kinases (CDPKs) and Calcium CaM regulated kinases (CCaMKs)

As with CaMs, CDPKs (in Arabidopsis CPKs) also have distinct isoforms that are defined by their highly variable and specific N termini, they also contain a serine/threonine protein kinase domain, a pseudo-substrate segment and four EF-hands which in turn contain a calmodulin-like domain (CLD) (Satterlee and Sussman, 1998, Wernimont et al., 2010). When inactive, the pseudo-substrate binds to the kinase domain (Liese and Romeis, 2013), when Ca^{2+} binds to the kinase domain it triggers a conformational change, activating the enzyme (Liese and Romeis, 2013). CDPKs have been associated with cold acclimation, salt tolerance, drought tolerance and the pathogen response (Saijo et al., 2000, Tähtiharju et al., 1997, Takahashi et al., 1997b, Tuteja and Mahajan, 2007). Characterisation of three isoforms from soybeans, revealed, that each isoform required different calcium thresholds (Lee et al., 1998). From these results it can be surmised that different isoforms of CDPKs relate to specific Ca^{+2} signatures, but, may also be influenced by several other factors for example, signalling pathway modification and downstream target interactions (Sanders et al., 2002). Calcium-CaM regulated kinases (CCaMKs) are plant specific protein kinases, they are jointly regulated by Ca^{2+} and CaM. CCaMKs are particularly involved in the regulation of arbuscular and mycorrhiza, and root nodulation formation (Kistner and Parniske, 2002), there has also been one CCaMK identified in the *Lotus japonicus*, however they are absent from the Arabidopsis genus (Kistner and Parniske, 2002).

1.3.4 Calcium regulation of proteins and Postranslational modifications (PTMs)

Post translational modifications (PTMs) regulate cellular processes and are known to be involved in protein activity, stability, and localisation and are also involved in signalling networks by aiding signal amplification (signal cascades) (Friso and van Wijk, 2015). Hundreds of PTMs have been characterised as early as 1981 (Wold, 1981), however, characterisation of mammalian PTMs is far more advanced compared to plants (Ytterberg

and Jensen, 2010). It is commonly thought that many enzymes and their regulators are subjected to a wide range of PTMs implying that PTMs are involved in changes in osmotic state, stabilisation such as degradation and de-activation as well as promoting metabolic flux (Friso and van Wijk, 2015, Huber and Hardin, 2004).

1.3.4.1 Phosphorylation

Phosphorylation is the most global PTM, playing a major role in plant signal transduction and plant metabolism by regulating protein conformations and protein interactions (Clapham, 2007, Hunter, 1995, Kudla et al., 2010, Kudla et al., 2018, Soderling, 1999). Protein kinases cause phosphorylation by transferring a phosphoryl group to a hydroxyl group (Champion et al., 2004). The specific amino acids serine (Ser), threonine (Thr) and tyrosine (Tyr), and in the case of two component signalling, histidine (His) and aspartic acid (Asp)contain the hydroxyl group (Ghelis, 2011). There are twice as many kinases encoded in the plant genome compared to the mammalian genome (Wang et al., 2014). Arabidopsis, for example encodes 1052 protein kinases and 162 phosphatases (Wang et al., 2014), reflecting the importance of the role of phosphorylation in plants.

1.3.3.2 Ubiquitination

Due to a plants' sessile nature it relies heavily on proteomic plasticity when exposed to environmental changes to allow for adaption and ultimate survival (Miricescu et al., 2018). Therefore, protein alterations by processes such as regulated ubiquitination and proteasome-mediated degradation are essential (Dreher and Callis, 2007). Ubiquitination is conserved across all eukaryotes, for example there is only a difference of three amino acids between yeast and human ubiqutins (Ozkaynak et al., 1984). Ubiquitination labels proteins to be degraded by the proteosome (usually through the 26s proteosome system) thereby regulating the stability of proteins (Sharma et al., 2016, Thrower et al., 2000).

The ubiquitination/proteosome system (UPS) targets proteins for degradation by the attachment of a 76 amino acid polypeptide ubiquitin to a degradative substrate (Dreher

and Callis, 2007, Miricescu et al., 2018). This process is catalysed by three components: a ubiquitin activating enzyme (E1), a ubiquitin conjugating enzyme (E2) and a ubiquitin ligase (E3). There is over 6% of the Arabidopsis genome that encodes UPS proteins (Downes and Vierstra, 2005) with E3s being the most abundant (Lee and Kim, 2011). The Arabidopsis proteome for example, contains two E1s, 37 E2s and 1300 E3s, suggesting E3s are the main specificity components for this system (Moon et al., 2004, Schwechheimer and Schwager, 2004, Smalle and Vierstra, 2004, Sullivan et al., 2003). This high abundance of different E3s stresses the importance of the ubiquitination system in plants compared to other eukaryotes such as humans or yeasts for example, which encode 600 and 100 E3s respectively (Finley et al., 2012). It is important to note that there is a fourth enzyme the ubiquitin elongating enzyme E4 which contributes to poly-ubiquitination (Ferreira et al., 2015, Huang et al., 2014). Components of ubiquitination are implicated in myriad processes including light signalling, circadian clock regulation, hormonal signalling, growth, abiotic stress, embryogenesis and senescence and organ initiation and patterning (Han et al., 2004, Hoecker, 2005, Imaizumi et al., 2005, Samach et al., 1999, Sharma et al., 2016, Shen et al., 2002).

Ubiquitination is involved in target degradation of proteins that effect downstream regulation of hormone response genes such as auxin (Schwechheimer and Schwager, 2004, Woodward and Bartel, 2005), gibberlin (Fleet and Sun, 2005, Sun and Gubler, 2004), ABA signalling pathway (Himmelbach et al., 2003), ethylene and Jasmonate signalling pathways (Devoto and Turner, 2005, Lorenzo and Solano, 2005, Turner et al., 2002) as well as the plant defence mechanism(Dangl and Jones, 2001, Nürnberger et al., 2004, Schulze-Lefert and Bieri, 2005). Calcium plays a major role in the auxin based signalling pathway and efflux system (Shih et al., 2015), it is involved in stomatal closure in response to ABA (Hamilton et al., 2000) and plant and pathogen defence response (Saidi et al., 2009).

Calcium is known to affect ubiquitination either directly by binding molecules of the ubiquitin cascade (Meng et al., 1999, Wang et al., 2010) or by indirectly regulating calcium channels and pumps (Chen et al., 2011, Rauh et al., 2005). Ubiquitination in turn influences calcium homeostasis by regulating the activity and protein concentrations of calcium channels and pumps (Mukherjee et al., 2017). Ubiquitination has been shown to be

involved in Ca^{2+}/CaM mediated regulation of the plant immune response via the Cullin 3-based ubiquitin/proteosome pathway (Zhang et al., 2014). Cullin-3-based E3s contain the Bric-a-Brac/Tramtrack/Broad complex (BTB) domain proteins which target proteins for ubiquitination and degradation (Hua and Vierstra, 2011). It has been shown that Cullin 3s are involved in the regulation of the SA signalling pathway (Spoel et al., 2009), suggesting possible crosstalk between the calcium signalling pathway, the SA signalling pathway and ubiquitination/proteosome pathway.

1.3.4.2 SUMOylation

SUMOylation involves the attachment of SUMO (Small Ubiquitin -Like Modifyer) protein to lysine residues on target substrates (Kurepa et al., 2003a). This process is like ubiquitination, however, SUMOylation is believed to be involved in different biological processes such as intercellular transport, the cell cycle, DNA repair, RNA metabolism and cell signalling (Baczyk et al., 2017, Nair et al., 2017, Qiu et al., 2017, Wei et al., 2017). Research on SUMO began as early as 1999 looking at a novel tomato SUMO which was found to inhibit EI X-induced (ethylene-induced xylanase) cell death (Hanania et al., 1999). This was followed by several studies identifying Arabidopsis SUMO E3 encoding genes, which initiated the drive to a greater understanding of SUMOylations role in the regulation of varies plant processes (Huang et al., 2009, Ishida et al., 2012, Kwak et al., 2016, Miura et al., 2005, Tomanov et al., 2014).

Models have implied that SUMOylation and ubiquitination interact in three ways: they are antagonists, as they compete for the same lysine on target proteins(Benlloch and Lois, 2018), they show synergism during some feedback loops e.g. SUMO E3 SIZ1 may enhance transubiquitination activity of ubiquitin E3 ligase COP1 (Constititive photomorhogenic 1), while protein levels and stability of SUMO E3 SIZ1 itself are modified by this E3 ligase (Lin et al., 2016); finally, SUMOlaytion mediates ubiquitin-dependent degradation by the proteosome (Miteva et al., 2010, Praefcke et al., 2012). It has been shown that SUMOylation promotes plant immunity, controls stress signalling and is involved in many plant stress responses such as salt tolerance, for example SUMOylatinon of NPR1 leads to

an increase in downstream salt stress-dependent gene transcription (Gong et al., 2020, Srivastava et al., 2016). Ubiqutins are known to regulate calcium channels (Mukherjee et al., 2017) and we know ubiquitination and SUMOylation interact, implying possible crosstalk between SUMOylation pathways and calcium signalling pathways, however, there is no current literature which supports this theory directly.

1.3.4 Calcium and osmotic stress

Plants are exposed to numerous environmental stresses, affecting both growth and development. It has been shown that a rise in $[Ca^{2+}]_{cyt}$ occurs in response to these environmental stresses (Sanders et al., 1999) Drought stress is considered one of the most important stresses and is responsible for the highest decrease in crop productivity compared to any other stress (Rollins et al., 2013). Drought devastates the plant by attacking it multidimensionally, mainly by inducing ROS production (Ashraf and Harris, 2013). Drought influences reduction in leaf growth, reduced cell elongation and increased stomatal closure (Avramova et al., 2015, Geiger et al., 2011, Potopová et al., 2016) and also has a major effect on photosynthetic processes by inhibiting chlorophyll synthesis leading to a decline in chlorophyll content and a decline in ribulose, 1-5 bisphosphate carboxylase (RUBisco) (Ashraf and Harris, 2013, Carmo-Silva et al., 2015, Jaleel et al., 2008).

Calcium is an important secondary messenger in osmotic and drought stress signaling (Chinnusamy et al., 2004). It has been shown that calcium fluxes generated from osmotic stress display differences in both kinetics and magnitude, suggesting calcium signature specificity to both the stress and the cell type (Takahashi et al., 1997a). Studies by Knight et al., (1997) found that drought stress on Arabidopsis seedlings containing constitutionally expressing aequorin resulted in a single $[Ca^{2+}]_{cyt}$ peak.

All environmental stresses induce gene expression, with each stress leading to the regulation of specific gene sets. During drought stress there are two groups of genes that are up regulated, the first, encodes for proteins involved in transduction e.g. TFs, the second regulates genes that encode for enzymes involved in osmolyte biosynthesis

(Shinozaki et al., 2003). When ABA is also involved in the drought stress response further genes are expressed involving two *cis* acting elements, the ABA responsive element (ABRE) and the MYB and MYC (MYBR/MYCR) recognition sites. ABRE has been shown to activate the stress response gene *LTI78*, by binding the ABRE motiff in response to drought (Knight et al., 1997a). *LTI78* expression promotes protection against water deprivation, osmotic stress, and mannitol induced stress (Dodd et al., 2006, Knight et al., 1997a, Xiong et al., 2002a). MYBR/MYCR leads to the expression of *RD22* (Abe et al., 2003) which promotes the suppression of chlorophyll degradation during drought stress (Harshavardhan et al., 2014). ABA-independent expression in contrast, involves the expression of the dehydration-responsive element (DRE) c-repeat CRT *cis* element. DRE motifs have been reported in promoter regions in numerous osmotic stress regulating genes (Sakuma et al., 2006), including *KIN1*, *KIN2* and *RD17*. It is known that calcium regulates ABRE and DRE, implying that calcium could have direct downstream effects on the regulation of osmotic-stress induced genes. Drought stress has been shown to cause increased expression *LTI78* genes through an ABA-regulated signal transduction (Nordin et al., 1991), which regulates the mechanisms involved in osmotic stress tolerance (Lång and Palva, 1992). Experiments by Knight et al., (1997) showed that calcium was required for expression of these genes, supporting the idea that the initial $[Ca^{2+}]_{cyt}$ increase in response to drought stress is required for the expression of specific drought-stress induced genes, implying $[Ca^{2+}]_{cyt}$ signature specificity (Knight et al., 1997b).

1.3.5 Measuring calcium

Understanding the role of calcium regulation in plant signalling has significantly improved in the last two decades, this is due to the development of more reliable measuring techniques. Early techniques relied on microelectrodes, later followed by microinjected fluorescent dyes. Both these methods held technical problems, with measurements being limited to individual or groups of cells in protoplasts and the potential to physically damage the plant (Knight et al., 1991, Knight et al., 1993). This led to the development of a more effective measurement technique, utilizing aequorin; a calcium sensitive luminescent

protein derived from the jellyfish *Aequorea victoria*. Aequorin is formed from apoaequorin and coelenterazine in the presence of O_2. Binding of calcium leads to the release of CO_2 and the emittance of blue light (Knight et al., 1991), the intensity of the light is directly correlated to $[Ca^{2+}]$. Initially aequorin was used successfully in animal cells but was limited to large cells in plants (Knight et al., 1991). Genetic transformation of plants with the apoaequorin gene strived to eliminate earlier issues. Once the transformed plants were introduced to coelenterazine, calcium levels could be measured from all the tissues in the intact plant (Knight et al., 1991). Although this method has previously shown success in bacteria, yeast, and human cells as well as plants (Knight et al., 1991), there was an issue with quantification. However, using dual wavelength coelenterazines, Knight et al., (1993) were able to quantify the response, thereby, eliminating the quantification issue (Knight et al., 1993). More research has allowed targeting of apoaequorin to individual organelles resulting in evidence which suggests that different calcium signals are generated from different pools of $[Ca^{2+}]_{cyt}$ (Trewavas et al., 1996).

1.3.5.1 The advantages and disadvantages of genetically encoded fluorescents versus bioluminescence reporters

Early images of calcium activity involved the use of small molecular calcium indicators such as furaptra and rhod-2 (Suzuki et al., 2016, Takahashi et al., 1998). Although easy to deliver into the cell they have a distinct disadvantage in that they cannot be selectively localised to specific target organelles and are often seen to be retained in the cytoplasm (Suzuki et al., 2016). Genetically encoded calcium indicators (GECIs) such as aequorin on the other hand can be selectively expressed and retained in the target organelle due to the presence of a target specific sequence fused to it (Mehlmer et al., 2012). It has been seen that aequorin one of the first GECIs have been successful in detecting calcium activity in a variety of organelles including the ER, nucleus, and the plasma membrane (Bonora et al., 2013, Logan and Knight, 2003). The main advantage to this system is that light is not required for the calcium measurements, however, as the luminescence is irreversible the duration of the measurements is limited (Suzuki et al., 2016). Some GECIs are based on fluorescence proteins (FP), the main advantage of these proteins is that their reaction is

reversable and does not require cofactors. Some FPs are based on the principles of FRET, here calcium binds to a responsive element initiating a conformational change in the indicator which then alters FRET efficiency between the two FP, examples of this are FIP-CB_{SM} (Romoser et al., 1997) and cameleon (Miyawaki et al., 1997). FIP-CB_{SM} comprised of the M13 peptide is located between two fluorescent proteins, BGFP and RGFP. For the cameleon FP, two FPs were fused to calmodulin and the M13 peptide. Further improvements in GECIs led to the development of single FPs calcium sensors e.g., GCaMP (Nakai et al., 2001). Single FPs initiate a conformational change of the indicators altering the state of the fluorescence, they have the advantage of a narrow range of excitation and emission wavelength as well as a higher dynamic range compared to the FRET FP (Suzuki et al., 2016).

For the work conducted in this study I used the calcium sensitive bioluminescent protein aequorin to measure $[Ca^{2+}]_{cyt}$ elevations. Although single FP have several advantages, they do require some time dedicated in the design of the constructs and transformation into the plant, as I already possessed transgenic *A. thaliana* lines expressing aequorin this eliminated the design and cloning processes. Finally, my work involved the identification of novel $[Ca^{2+}]_{cyt}$ signatures which followed on from studies conducted by Lenzoni et al., (2018), therefore it was prudent to use the same calcium reporter as described in this study to be able to make a direct comparison.

1.4. Calcium regulation of gene expression

It has been known for some time that Ca^{2+} has an effect on gene expression in plants (Bickerton, 2012)and therefore feasibly that responses mediated by stimulus induced Ca^{2+} signals are generated transcriptionally. The process involved in transcriptional regulation by Ca^{2+} can occur in either the cytosol or the nucleus or both (Galon et al., 2010). It is well documented that calcium signalling pathways regulate expression in both the cytosol and the nucleus, for instance, van der Luit et al., (1999) described that wind – induced

expression of a specific CaM isoform in tobacco was regulated by a nuclear calcium ($[Ca^{2+}]_{nuc}$) elevation, whereas expression of another tobacco CaM isoform in response to cold shock was regulated by a $[Ca^{2+}]_{cyt}$ elevation (van Der Luit et al., 1999). It is worth noting that stimulus- induced $[Ca^{2+}]_{cyt}$ signatures can both activate and repress Ca^{2+} dependent gene activity. Protein kinases, phosphatases and CaM interaction with target proteins can lead to activation or repression of downstream genes (Galon et al., 2010). Recent studies have identified Ca^{2+} regulated genes, Ca^{2+} - regulated TFs and *cis* elements are differentially expressed in response to elevated $[Ca^{2+}]_{cyt}$ (Whalley et al., 2011). Several Ca^{2+} regulated TFs are associated with CaM, CaM binding transcription factors (CAMTAs) being the most characterised (Bickerton and Pittman). CAMTAs are located in the nucleus, they contain a specific DNA binding domain and are associated with low temperatures and plant innate immunity (Bickerton and Pittman, Whalley et al., 2011). Early work researching genes involved in Ca^{2+} regulated gene expression focussed on one or two genes, which limited the overall understanding of Ca^{2+} gene expression (Whalley et al., 2011). The discovery of the abscisic acid responsive element (ABRE) as described by Kaplan et al., (2006) has implicated that Ca^{2+}may regulate 100s if not 1,000s of genes in the Arabidopsis genome (Kaplan et al., 2006). The introduction of transcriptomic, bioinformatic and modelling techniques have allowed for a broader insight into the role of Ca^{2+} regulation of gene expression (Kaplan et al., 2006, Whalley et al., 2011). It has been described by Whalley et al., (2011) that four distinct promoter motifs have been discovered in the Arabidopsis genome in response to artificially induced $[Ca^{2+}]_{cyt}$ elevations, the previously documented ABRE motif, CRT/DRE, CaM box and Site II. Whalley et al., (2011) compared genes that were induced in response to three different $[Ca^{2+}]_{cyt}$ elevations; transient, prolonged and oscillating (Figure 1.4), revealing differences in both number and identity of genes. Combining two of these elevations (transient and oscillation) allowed for identification of *cis* elements that responded to a wider range of elevation. This work is further supported by research conducted by Whalley and Knight (2013) which analyzed the Arabidopsis transcriptome using different calcium signatures, here it was seen that more genes were induced allowing a direct comparison to the 2011 work. It was found that the genes induced from each signature exhibited higher frequencies of different promoter elements (Whalley and Knight, 2013). Work by Whalley et al., (2011) and Whalley and Knight, (2013) has

increased the understanding of stimulus response $[Ca^{2+}]_{cyt}$ elevation regulated gene expression, however, the precise molecular pathways that regulate interactions between transcription factors and these motifs will need to be further examined.

These data by Whalley et al., (2011) and Whalley and Knight, (2013) have indicated that Arabidopsis can decode different $[Ca^{2+}]_{cyt}$ signatures leading to a specific gene expression response. More recent work by Liu et al., (2015) created a dynamic model of Ca^{2+}–CaM–CAMTA binding and gene expression responses suggesting that $[Ca^{2+}]_{cyt}$ signals were amplified enabling them to be decoded to give specific CAMTA-regulated gene expression responses (Liu et al., 2015). In 2018 a further mathematical model of the SA-mediated plant immunity was designed, which explored the decoding of calcium signatures in the expression of plant immunity genes *EDS1* and *ICS1* (Lenzoni et al., 2018). Here it was found that calcium, calmodulin, calmodulin-binding transcription activators CAMTA3 and calmodulin binding protein 60g (CBP60g) amplified each calcium signature into three active signals, which simultaneously regulated expression, producing a unique and specific response (Lenzoni et al., 2018).

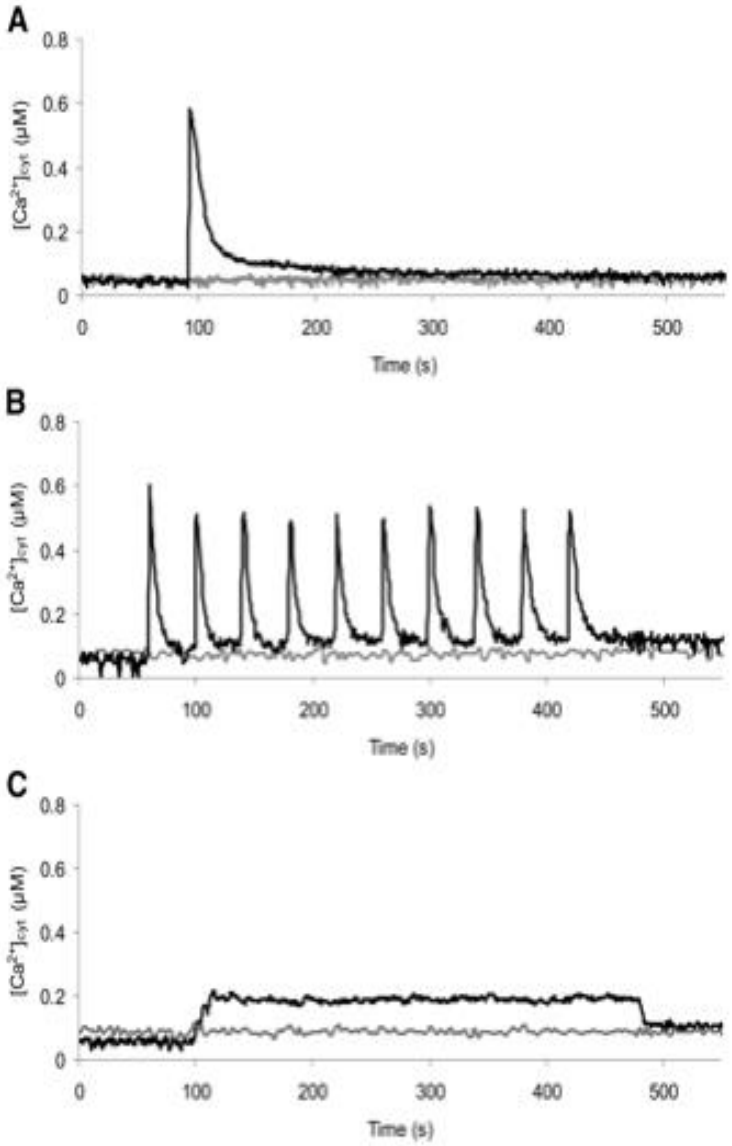


Figure 1.3: Three artificial induced calcium elevations. (A) Single transient (B) Repeated transients (C) Prolonged $[Ca^{2+}]_{cyt}$ elevation The graph depicts the average $[Ca^{2+}]_{cyt}$ response for control (grey) and Arabidopsis seedlings stimulated by voltage (Black).

(From Whalley et al., 2011)

1.5 Crosstalk

For simplicity individual stress signalling is generally studied in isolation (Knight and Knight, 2001). In a natural environment, however a plant can realistically be exposed to several stimuli simultaneously, to accommodate for this, crosstalk may occur between two or more different signalling pathways (Genoud and Métraux, 1999). Crosstalk can be defined as a combined signal from more than one pathway producing a unique response compared to each single pathway (Farmer and Ryan, 1992). It has been seen that some genes involved in calcium signalling, ABA signalling and nucleic acid pathways are up-regulated in response to both cold and drought stresses suggesting possible crosstalk (An et al., 2012, Fu et al., 2016, Turyagyenda et al., 2013). Most abiotic stresses initiate a rise in $[Ca^{2+}]_{cyt}$ and involve MAPKs and CDPKS which are themselves regulated by calcium (Jonak et al., 2002). Although plants produce specific $[Ca^{2+}]_{cyt}$ elevations in response to a particular stress, these can be substantially altered after previous stress exposure (Knight, 2000), suggesting possible crosstalk between abiotic stress signal transduction pathways (Knight and Knight, 2001). As $[Ca^{2+}]_{cyt}$ elevations occur in most abiotic and biotic stress responses this implies a wider complex network of different signalling pathways and crosstalk.

1.6 Plant circadian clock regulation

Due to the 24h rotation of the earth, most organisms have evolved an internal circadian clock (McClung, 2006b). The circadian clock regulates metabolism, physiology, growth, and behaviour of the plant which can vary substantially between day and night (McClung, 2006a, Wood et al., 2001b). The term 'circadian' comes from two Latin words *circa* (around) and *diem* (day) and was first coined by Franz Haberg in the late 1950s (Wood et al., 2001a). Plant circadian rhythms promote many aspects of plant activity, for example photosynthetic activity, flowering, pollination, leaf movement, growth, germination, stomatal gas exchange, enzyme activity, and fragrance emission(Altenburger and Matile, 1988, Fenske and Imaizumi, 2016, Yakir et al., 2007). Plants whose circadian clocks are in

unison with the environment have displayed higher biomass accumulation, enhanced photosynthesis, and increased chlorophyll content (Dodd et al., 2005, Kim et al., 2017).

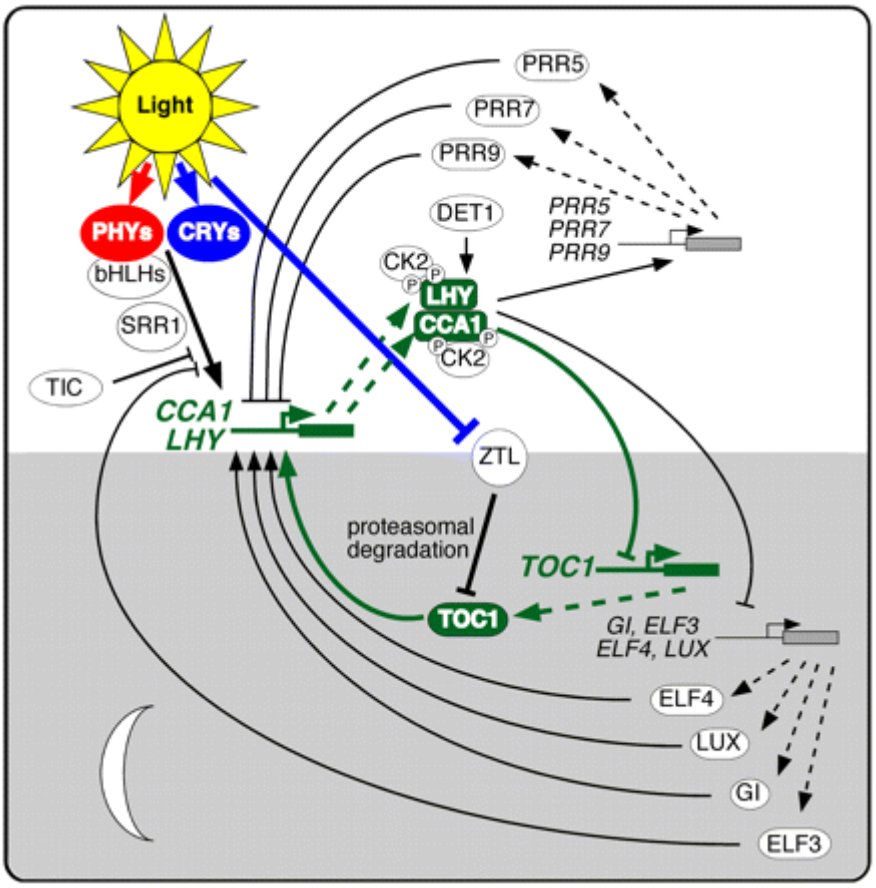
It is known that the circadian system in plants include several conceptual components (Wood et al., 2001b). Firstly, the entrainment pathway adjusts the circadian oscillator to match the environmental phase, by changing the circadian phase in response to environmental cues. Secondly, the circadian oscillator which is a complex molecular network estimates the time of day (Harmer et al., 2000, Wood et al., 2001b, Hardin, 2004, Hardin et al., 1990, Wood et al., 2001a). In plants, the circadian oscillator consists of an interconnected network of genes and processes arranged in feedback loops (Hardin, 2004, Hardin et al., 1990, Wood et al., 2001a). Next, the output pathway communicates the time of day generated by the oscillator to the clock regulated processes within the cells of the plant (Hardin, 2004, Wood et al., 2001a). Finally, all signalling pathways have the propensity to be circadian regulated, therefore, their response to a specific stimulus is dependent upon the time of day (Gómez and Simón, 1995, Trewavas, 1999, Webb, 2003, Wood et al., 2001a). This process is known as circadian gating of signal transduction (Hotta et al., 2007, Yakir et al., 2009)

A considerable proportion of circadian clock regulated genes are expressed during abiotic stress and have been confirmed in Arabidopsis, soyabean and rice (Covington et al., 2008, Duan et al., 2014, Kolmos et al., 2014, Kreps et al., 2002a, Zeilinger et al., 2006b). Environmental stimuli signal the clock to prepare the plant for the oncoming stress by optimising internal rhythmic expression of appropriate genes. Circadian regulated stress genes for example are found in the response to dehydration (*ERD10*; *ERD7*), the *RD29A* gene is expressed in response to water deficiency and osmotic stress and *COR15B* and *COR15A* are expressed during cold stress, (Fowler and Thomashow, 2002b, Liu et al., 1998a, Mizuno and Yamashino, 2008).

Basal calcium levels are rhythmic in a circadian manner (Johnson et al., 1995). Cytosolic calcium is known to play a prominent regulatory role in the transduction of many signals in plants (Gilroy et al., 1990, Neuhaus et al., 1993, Wood et al., 2001a) including those involved in the circadian clock i.e. red light (which is often used to entrain circadian rhythms) transiently elevates $[Ca^{2+}]_{cyt}$ (Ermolayeva et al., 1996, Shacklock et al., 1992) and

$[Ca^{2+}]_{cyt}$ signatures through Calmodulin-Like24 modulate the circadian clock itself (Dodd et al., 2007, Frank et al., 2018, Martí Ruiz et al., 2018).

1.6.1 The molecular basis of the circadian clock



(McClung, 2006b)

Figure 1.5: A molecular model of the *A. thaliana* oscillator taken from McClung (2006), the model highlights both gene and protein regulation during both subjective day and subjective evening.

Proteins are depicted as oval and oblong shapes; transcription and translation as dashed lines; positive protein activity solid lines with arrowheads; negative protein activity solid lines with perpendicular dashes; core CCA1/LHY/TOC1 are coloured green; a circled PS represent phosphorylation of LHY and CCA1 by CK2; subjective evening is shaded grey and subjective morning is white.

Early models of the plant circadian clock described a single feedback loop consisting of CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPERCOTYL (LHY*)* genes (which are highly expressed in the morning) repressed TIMING OF CAB EXPRESSION 1

(TOC1) by binding to the Evening Element (EE) which is found in the TOC1 promoter (Harmer and Kay, 2005). TOC1 (expression peaks in the evening) promotes the expression of *CCA2* and *LHY* through the binding TF CCA1 (Alabadí et al., 2001, Pruneda-Paz et al., 2009). However, this model was deemed oversimplistic and later studies indeed confirmed that it was incomplete(Green and Tobin, 1999, Mizoguchi et al., 2002). In fact, analysis of varying members of the PRR family resulted in the proposal of a new feedback loop subsequently named the 'morning loop' (Matsushika et al., 2002), this loop known as the TRANSCRIPTIONAL AND TRANSLATIONAL FEEDBACK LOOP (TTFL) involved CCA1 and LHY binding to the promoters and activating *PRR9* and *PRR7* . Further studies in 2006 (Locke et al., 2006, Zeilinger et al., 2006a) proposed a further third loop called the evening loop which was composed of TOC1 and GIGANTEA (*GI*) genes. With the discovery of the REVEILLE 4 (RV4), REVEILLE 6 (RV6) and the REVEILLE 8 (RF8) TFs (Nakamichi et al., 2012, Rugnone et al., 2013) which activate evening genes, a fourth model was proposed consisting of a ring of four repressors with RVEs and LNKs as activators (Millar, 2016). It is the general consensus that the core oscillator is composed of interconnecting feedback loops comprised of transcriptional interactions where regulation is attained through both positive and negative regulators. For a molecular model of the circadian oscillator see figure 1.5.

1.7 Proteomic research in plants

1.7.1 Quantitative techniques

The proteomic field of study is now over thirty years old with the first decade dominated by two-dimensional gel electrophoresis (SDS polyacrylamide gel electrophoresis (SDS-PAGE)) and traditional protein staining techniques such as Coomassie blue (Lilley and Dupree, 2006, Thelen and Peck, 2007). SDS-PAGE coupled with staining has been, and remains, the initial key method for protein identification for most biological systems,

however, due to limitations in this method such as sensitivity, throughput and reproducibility the need for powerful and complementary techniques has increased.

1.7.1.1 SDS-PAGE and Difference gel electrophoresis (DIGE)

During SDS-PAGE the proteins are separated firstly by isoelectric charge and then by separation based on molecular weight, the proteins are then visualised by staining the gels with stains such as Coomassie blue, Sypro ruby and deep purple (Lilley and Dupree, 2006). From these results proteins of interest are normally digested to peptides and further analysed through mass spectrometry analysis. This system has several limitations, for example, it is normally required to analyse several gels when comparing proteins, which can be both tedious and time consuming, there can be cases of sample overload, the system itself has limited sensitivity and the ability to reproduce the results is poor due to gel-to-gel variation.

DIGE aims to address both the issue of sensitivity and gel variability. DIGE which was first described by Joe Minden (Ünlü et al., 1997), it involves the labelling of samples with one of three fluorescent CyDyes (Cy2, Cy3 and Cy5) allowing for multi samples to be analysed at the same time on the same polyacrylamide gel, therefore eliminating gel to gel variation. There are two types of CyDye labelling: minimal labelling which involves low stoichiometry labelling of the lysine side chains; and saturated labelling which leads to stoichiometric labelling of cysteine residues (Shaw et al., 2003), both can be used to quantify protein abundance in the solution. To date there have been several studies adopting DIGE analysis in plant science, for example the identification of glycosylphosphatidylinositol-anchored proteins in Arabidopsis (Borner et al., 2003), investigating detergent resistant membranes in Arabidopsis (Borner et al., 2005), investigating the *ppi1* mutant and photosynthetic proteins (Kubis et al., 2004), the identification of seed proteins in barley (Maeda et al., 2004) and analysing changes in the extracellular matrix of Arabidopsis cell cultures when induced by fungal elicitors (Ndimba et al., 2003). The system is limited in that some proteins are poorly represented on the gels such as those with extreme pIs or molecular weight (particularly low abundance proteins), and hydrophobic membrane proteins. Analysis of

membrane proteins are a particular problem in this system even after treating the proteins with detergent such as amidosulphobetine 14 (ASB14). Membrane proteins are poorly represented (Santoni et al., 2000), due to their precipitation during isoelectric focusing. Because of these limitations quantitative non 2D gel-based techniques are becoming more popular due to their potential to identify proteins missed by the 2D-DIGE approach (Lilley and Dupree, 2006).

1.7.1.2 iTRAQ labelling

Non-2D gel-based technologies fall into two categories, isotope labelling, mainly followed by liquid chromatography (LC) and tandem mass spectrometry (MS-MS) (Gygi et al., 1999) or no pre-labelling, measuring peptide peak intensity by mass spectrometry (Chelius and Bondarenko, 2002). iTRAQ labelling is one of the methods that is becoming a popular isotope labelling tool in proteomic analysis. Known for its relative and absolute quantification of proteins, the iTRAQ system uses isobaric reagents to label the primary amines of peptides and proteins (Chong et al., 2006, Gan et al., 2007, Ross et al., 2004). It consists of a N-methyl piperazin reporter group, a balance group (carbonyl group) and a N-hydroxy succinimide ester group which reacts with the primary amines on the proteins (Ernoult et al., 2008). The function of the balance groups is to make the labelled peptides isobaric, quantification from tandem mass spectrometry is then analysed from the reporter groups that have been generated from fragmentation during liquid chromatography (LC-LS) (Ernoult et al., 2008).The nature of the iTRAQ system allows for multiple samples to be pooled for easy and concise comparative analysis. There are several advantages to this system compared to 2D-gel and 2D-DIGE methods these include enhanced sensitivity, which allows for the detection of small novel proteins with low peptide numbers and low magnitudes (Ow et al., 2009), it has the ability to detect post-translational modifications and is a relatively quick method compared to DIGE analysis. iTRAQ has been used in several more recent studies and investigations into the plant proteome for example focusing on programmed cell death in Arabidopsis (Smith et al., 2015).

1.7.1.2 LC- MS/MS analysis

Combining the power of liquid chromatography and the selective and sensitive mass analysis from mass spectrometry (LC-MS/MS) creates a powerful tool for proteomic analysis. This method is highly desirable in many fields of research e.g. fuel, chemical, pharmaceutical, safety assessment and breeding (Fernie et al., 2004, Fiehn et al., 2000, Hall et al., 2008, Last et al., 2007, Milo and Last, 2012, Patti et al., 2012, Saito and Matsuda, 2010, Schauer and Fernie, 2006, Sumner et al., 2003, Surh, 2003, Weckwerth, 2003). This coupling of LC and MS was limited for many years due to the incompatibility with MS ion sources and a continuous liquid stream combined with high expenditure (Pitt, 2009), however by the 1990s the price and performance of the system had vastly improved (Pitt, 2009).

LC is a separation process, which isolates each component in a sample, mass transferring the sample through a polar mobile phase and then a non-polar stationary phase. MS ionises atoms and molecules, which aids in their separation and detection via their charge and molecular mass (mass to charge ratio) (Pitt, 2009). Combining LC-MS/MS improves accuracy and minimises experimental error. It is generally accepted that this system is preferred when quantitating small molecules, metabolites etc.(Coskun, 2016, Pitt, 2009)

1.8 Summary

Calcium plays an essential role in all eukaryotes. In plants calcium acts as a secondary messenger mediating an extensive range of signals involved in many processes, from growth to abiotic stress tolerance. Therefore, calcium is fundamental to the plant's response to various environmental stimuli as well as regulating physiological functions such as stomatal closure (Kudla et al., 2010, Sanders et al., 1999).

Despite over 30 years of extensive research on calcium signalling the question of calcium specificity is still a matter of contention. Although the 'calcium signature' hypothesis is now generally accepted, it still needs substantial research to truly support the idea. It has been

shown that calcium regulates gene expression in plants (Bickerton, 2012) and there has been extensive research conducted to identify these genes. Recent work by Whalley et al., (2011) and Whalley and Knight (2013) have gone one step further and identified conserved sequences in the promoter regions of these calcium regulated genes suggesting that these genes, TFs and *cis* elements responded to different calcium signatures, however this is only theoretical and has yet to be confirmed. Studies by Lenzoni et al., (2018) described four $[Ca^{2+}]_{cyt}$ signatures which displayed unique novel kinetics which produced different downstream gene expression responses, suggesting specificity, however, this study was very limited and a more extensive study on several $[Ca^{2+}]_{cyt}$ signatures would be required to truly ascertain $[Ca^{2+}]_{cyt}$ signature specificity.

One area of research that is relatively new to the calcium field is the identification of proteins that are specifically regulated through posttranslational mechanisms. With new proteomic technology such as LC-MS/MS, identifying and understanding of calcium regulated proteins could be achieved relatively quickly and efficiently. Identifying these proteins could give a better understanding in the role of calcium and protein regulation, as well as possible avenues of investigation into novel stress proteins for future plant engineering towards stress tolerance.

1.9 Thesis aims

The aim of this study was to:

- To increase the repertoire of novel $[Ca^{2+}]_{cyt}$ signatures initially identified in Lenzoni et al. (2018) and to test specificity against known calcium regulated *cis* elements. (Chapter 3)
- To determine if specific $[Ca^{2+}]_{cyt}$ signatures regulate osmotic stress induced genes leading to osmotic stress tolerance, and if the osmotic stress tolerance is age limiting. (Chapter 4)
- To determine if biphasic kinetics observed in ATP or mastoparan/$CaCl_2$ generated $[Ca^{2+}]_{cyt}$ signatures are due to the response occurring in different cells or the same cells at different times. (Chapter 5)
- To investigate calcium agonist generated $[Ca^{2+}]_{cyt}$ signature regulation of combined calcium regulated *cis* elements. Does the expression kinetics differ between the combined *cis* elements and the single *cis* elements? (Chapter 6)
- To identify and ascertain the biological and molecular functions of proteins regulated by a mastoparan generated $[Ca^{2+}]_{cyt}$ signature.

2. Materials and Methods

2.1 Materials

2.1.1 Chemicals

All chemicals and media were supplied by one of the following companies unless otherwise stated:

Bioline (London, UK),

Duchefa Biochemic BV (Harlem, NL),

Thermo Fisher Scientific (Loughborough, UK),

Melford Laboratories Ltd (Ipswich, UK),

Sigma-Aldrich Ltd (Poole, UK).

2.1.2 Enzymes

All DNA and RNA modifying enzymes were purchased from Applied Biosystems (Forster City, USA), Bioline (London, UK), Fisher Scientific UK Ltd (Loughborough, Uk), New England Biolabs (Hitchin, Uk), Promega (Southampton, UK) or Qiagen (Crawly, UK).

2.1.3 Antibiotics/Chromogenic substrates/Inducers

All antibiotics/chromogenic substrates and inducers were purchased from Melford laboratories LTD (Ipswich, UK); concentrations are listed in (Table 2.1).

Table 2.1 Concentrations of reagents used for both bacterial and plant culture plates

Antibiotic/Reagent	**Stock concentration (mg/mL)**	**Working Concentration (µg/mL))**	**Stock Solvent**
Ampicillin	100	100	H_2O
Kanamycin	100	Bacteria: 100 Plants: 50	H_2O
Rifampicin	50	50	H_2O
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	238	24	H_2O
X-Gal	20	20	DMF
Timentin	200	200	H_2O
Spectinomycin	50	50	Water
Glufosinate-ammonium	NA	50	Water

2.1.4 Bacterial strains

Escherichia coli (*E. coli*) strains α select silver cells were obtained from Bioline (London, UK).

Agrobacterium tumefaciens (*A. tumefaciens*) strain C58C1 (Holsters et al., 1978) which were propagated in house were used for stable transformation of *Arabidopsis thaliana*.

2.1.5 Plant material

A. thaliana ecotype Columbia (Col-0) seeds were purchased from Lehle seeds (Round Rock, Texas, USA). *A. thaliana* lines constitutively expressing 35S::apoaequorin in the cytosol (pMAQ2 Col-0 ecotype) (Knight et al., 1991) or lines containing chimeric concatemer constructs expressing luciferase (Whalley et al., 2011) were available in house.

2.1.6 Oligonucleotides and synthetic genes

All oligonucleotides and synthetic genes were designed with SnapGene software (from Insightful Science; available at snapgene.com) or Primer 3 (http://primer3.ut.ee/) and were purchased from Integrated DNA Technologies (IDT) (Leuven, Belgium).

2.1.7 Plasmid vectors

A modified pDH51 vector (Pietrzak et al., 1986) containing a *LUC*+ coding region as described by Whalley et al., (2011) was available in house (Map found in appendix B.1). The binary vector pBIN19 (Bevan, 1984) (Appendix B.2) was supplied in house. Vectors adopted for Gateway cloning were available commercially; entry vector pENTR-D-TOPO; (Thermo Fisher Scientific, Loughborough, UK). Expression vectors pK7WG2, pK7WGF2, pK7FWG2 and pB7WG2 (Karimi and Depicker, 2002).

2.1.8 iTRAQ labelling

iTRAQ® Reagent - 8PLEX Multiplex Kit was supplied by Sigma-Aldritch LTD (Poole, UK).

2.2 Methods

2.2.1 Bacterial growth

2.2.1.1 Bacterial growth media

All bacteria were grown on either solid agar plates made from 1.5% (w/v) bactoagar and 2% (w/v) Luria-Bertani (LB) media (Sigma-Aldrich) or 2% (w/v) liquid LB.

2.2.1.2 Bacterial growth conditions

Bacteria were incubated at 37 ^{o}C or 28 ^{o}C for *E. coli* and *A. tumefaciens*, respectively. Liquid media was subject to agitation during incubation at 250 rpm (*E. coli*) or 150 rpm (*A. tumefaciens*).

2.2.2 Plant Growth

2.2.2.1 Plant growth media

Arabidopsis seeds were grown on solid 1x MS medium agar plates (Murashige T, 1962), which comprised of 1x Murashige and Skoog salts (Duchefa Biochemie BV, Haarlem, Netherlands) and 0.8% (w/v) plant tissue culture grade agar (Sigma-Aldrich, Pool, UK). The pH was adjusted to 5.8 with 0.1M KOH before autoclaving at 121 ^{o}C, 10^{5} Pa pressure for 20 min. When cooled to 50 ^{o}C appropriate antibiotics were added if required, before pouring onto plates.

2.2.2.2 Seed sterilisation (ethanol method)

Arabidopsis seeds were added to a 1.5mL microfuge Eppendorf tube containing 500µl of 70% ethanol (v/v). The tube was subsequently agitated on a Labnet vortex mixer (Labnet International Inc. Woodbridge, New Jersey, USA) for 5 min. Seeds were then transferred to a 9cm diameter sterile filter paper (Whatman™ International LTD, Kent, UK) and air dried in a sterile laminar flow hood before dispersing onto petri dishes containing solid agar (2.2.2.1).

2.2.2.3 Seed sterilisation (bleach method)

Arabidopsis seeds harvested from plants previously dipped in *A. tumefaciens* were initially surface sterilised using the ethanol method (described in 2.2.2.2). Seeds were then transferred to a 50mL Falcon tube containing 10% (v/v) sodium hypochorite (NaOCl) and 0.25% (w/v) sodium dodecyl sulphate (SDS) and shaken for 10min. The seeds were then washed with sterile water for 10min, the washing step was repeated 6 times to ensure complete removal of the SDS and NaOCl. The seeds were spread directly onto solid Murashige and Skoog (MS)/agar plates (2.2.2.1).

2.2.2.4 Standard Plant growth conditions

Plates containing Arabidopsis seeds were stratified at 4 ^{o}C in the dark for 48h (at least 72h for transformants) to attain uniform germination. Next, they were transferred to a Percival cabinet (CU-36L5D, CLF Plant Climatics, Emmersaker, Germany) set at 20±1 °C. with a photoperiod of 16/8h and a light intensity of 150 µmol^{-2} s^{-1}

Seedlings required to grow to maturity were individually transferred to hydrated 41mm diameter Jiffy peat plugs (LBS Horticulture, Colne, UK) or in the case of seedlings for *A. tumefaciens* transformation, three seedlings were transferred to a single 44mm hydrated

Jiffy peat plug (LBS Horticulture, Colne, UK). All peat plugs were placed in trays and covered with cling film, all trays were placed in a plant growth room set at 20 ^{o}C, a 16/8h photoperiod and a light intensity of 150 µmol^{-2} s^{-1}. After 48h, holes were pierced in the cling film and after 72 hours the cling film was removed altogether. Seedlings required for *A. tumefaciens* transformation were grown to flowering stage and then dipped (2.2.6.13.1). Plants required for seeds were grown adopting the Aracon system (Beta tech, Ghent, Belgium) for containment.

Imaging experiments were conducted on 10d old seedlings and/or 3w old leaves with aequorin reconstitution (2.2.4.1) and luciferase discharge performed on 9d old seedlings (2.2.5.1).

2.2.2.5 Circadian plant growth conditions

For the investigation of circadian clock control a different set of growth conditions as those described in 2.2.2.4 were adopted. The initial stratification period was the same as 2.2.2.4, whereby the sterilised seeds were stratified at 4°C for 48h in darkness to encourage uniformed germination. The seeds were then transferred and grown in a Percival (CU-36L5D, CLF plant climatics, Emersacker, Germany) for 48h, with a photoperiod of 16/8h, a light intensity of 150µmol m^{-2} s^{-1}, and a temperature of 20±1 °C. The seeds were then moved into one of two MLR-351 Environmental test chambers (Sanyo, Panasonic, Osaka City, Japan) for 7 days. The first chamber was set to a photoperiod of 12L/12D, a light intensity of 150µmol m^{-2} s^{-1}, and a temperature of 20±1°C (forward cycle). The second chamber was set to the same light intensity and temperature as the first chamber, but with a photoperiod of 12D/12L; representing the reverse light cycle. After 7d the seedlings were transferred to a further MLR-351 Environmental test chamber (Sanyo, Panasonic, Osaka City, Japan) for 24h. The chamber was set to the same light intensity as the previous chambers but with constant light for 24h. Imaging experiments were conducted as described in 2.2.4 and 2.2.5 for luminescence and luciferase measurements, respectively.

2.2.3 Chemical and peptide treatment

2.2.3.1 For luminometry

To measure cytosolic calcium concentration upon a specific treatment, chemicals and/or peptides were applied directly to individual seedlings or leaves by syringe injection into a luminometer cuvette, where the plants where contained. Each cuvette contained 0.5mL of water so it was necessary to inject 0.5mL of chemical and/or peptide at double the required final concentration (2.2.4.3).

2.2.3.2 For photon camera luminescence imaging

Chemicals and/or peptides were added to cooled liquid agar (consisting of 0.8% (w/v) plant tissue culture grade agar (Sigma-Aldrich, Pool, UK) made with water, the agar was then poured into a petri dish (2.2.4.4). An 9cm diameter sterile filter paper (Whatman™ International LTD, Kent, UK) was divided into five sections and moistened with 1mL of water. Five transgenic seedlings were individually placed in one section of the paper, this was repeated with a further 4 different transgenic lines in each section. The paper was then placed on top of an agar plate containing the specific chemical and/or peptide in the agar. For luminescent measurements of double *cis* element transgenic seedlings, two plates were required, one containing the chemical/peptide and one without as a control and both were placed into the photon camera simultaneously; only transgenics containing the double *cis* element, its single *cis* element counter parts and the minimal control were measured, rather than all the single *cis* elements from the earlier experiments (2.2.4.2).

2.2.3.3 For Osmopriming

For pre-treatment of seedlings or leaves for osmotic stress assays, chemicals and/or peptides were applied for 1h to 15/20 seedlings or 3 leaves, which were floating in 5mL of

water contained in a 15mL well. For the controls water was used as a substitute for the chemicals and/or peptides (2.2.7.1).

For pre-treatment of seeds to encourage osmopriming, the chemical was added to a 15mL Falcon tube containing Arabidopsis seeds and water, and then agitated for 24h in the dark.

For mannitol induced osmotic stress assays, mannitol was added to previously pre-treated seedlings/leaves which were floating in 5mL of water in 15mL wells for a 10d period (2.2.7.1).

2.2.3.4 For gene expression

Chemicals and/or peptides were added to 15mL wells in a 6 well plate containing 15/20 seedlings or 3 leaves floating in 5mL of water per well. The plant material was removed at various time points and frozen in liquid nitrogen for subsequent RNA extraction (2.2.6.7).

2.2.3.5 To inhibit cytosolic calcium

Fifteen A. thaliana seedlings from 4 transgenic lines (ABRE, SITEII, CRT or a minimal promotor control) were added to separate 15ml wells. Each well contained either 5mL water or 5mL of an overall concentration of 1mM lanthanum chloride (LaCl3) in water, the seedlings were left for 30 min as described in Whalley et al., (2011) and then transferred into new 15ml wells. Each well contained either 5mL of water or 5mL of water plus one of three calcium agonists (overall concentrations of 0.5mM ATP, 50mM CaCl2 or 1mM L-glutamate). Seedlings were removed at various time points and flash frozen with liquid N2 before RNA extraction (2.2.6.7), cDNA synthesis (2.2.6.9) and RT-qPCR (2.2.6.10).

2.2.3.6 Chemical or peptide treatment for protein extraction

Fifteen, 10d old *A. thaliana* WT seedlings were placed into a well containing 5ml of water and an overall concentration of 10µL Mastoparan or water as a control. After 1h the seedlings were removed, flash frozen in liquid nitrogen and stored at -80 ^{o}C until required.

2.2.4 Calcium measurements

2.2.4.1 Aequorin reconstitution

For aequorin reconstitution Arabidopsis seedlings or leaves were floated on water containing 10µM coelenterazine (Biosynth Srl, Staad, Switzerland), 1% (v/v) methanol. The plant material was left 12 to 24h in the dark at 20^{o}C before calcium measurements commenced.

2.2.4.2 Calcium dependent luminescence measurements (luminometer).

Individual Arabidopsis seedlings or leaves were transferred into a 3mL luminometer cuvette (Röhren Tubes, Sarstedt, Germany), which contained 0.5mL of water. To minimise undue stress the seedlings or leaves were left to rest for 30 min, the cuvettes containing the plant material were then individually inserted into a luminometer chamber. Luminescence levels were recorded every second via a digital chemioluminometer with discriminater and cooled housing unit (Electron Tubes LTD, Middlesex, UK), to reduce background noise (Knight et al., 1991; Knight, 1996). Before injecting any chemical and /or peptide, or water luminescent levels were recorded for 60s. After injection luminescence levels were recorded for a further 1000s. To end the experiment a 300s discharge was performed by injecting an equal (1mL) volume of 2M $CaCl_2$, 20% (v/v) ethanol, giving a final concentration of 1M $CaCl_2$, 10% (v/v) ethanol.

2.2.4.3 Aequorin luminescence calibration

Calibration was completed to give calcium concentration as a final output, and calibration was performed as previously described in Knight et al., (1996), following the logarithmic equation: pCa = 0.332588(-logk) + 5.5593, where k = luminescence counts per second/ total remaining counts. The number of total counts were calculated as the amount of data collected from the beginning of the experiment to the end of the discharge.

2.2.5 Luciferase measurements

2.2.5.1 Luciferase discharge

Luciferase discharge was performed on seedlings floating in 5mL of water containing 5mM of luciferin (potassium salt). The seedlings were left overnight in the dark at room temperature for the luciferin to discharge any luciferase already present before calcium measurements could take place.

2.2.5.2 Luminescence measurements (photon Camera)

Chemical and/or peptide agar plates containing specific transgenic seedlings (2.2.3.2) were placed into a light tight box containing a plate-intensified charge-coupled CCD camera (Photek 216; Photek, East Sussex, UK). Single photons were collected, and their spatial position were recorded. Data was analysed with the Photek IFS32 software.

2.2.6 General molecular techniques

2.2.6.1 gDNA extraction

This gDNA isolation method was adapted from Edwards *et al*., (1991). In short, 15 7d old transgenic Arabidopsis seedlings were transferred to a 1.5mL Eppendorf tube and flash frozen in liquid N_2. Once frozen the sample was ground in 400µl of Edwards extraction buffer (200mM Tris-HCL, pH 7.5; 250mM NaCl; 25mM EDTA, pH 8.0; 0.5% (w/v) SDS). The

tube was centrifuged for 1 min at full speed (16000 *g*). After centrifuging, 300µl of the supernatant was transferred to a fresh tube containing 300µl of isopropanol, the sample was gently pipetted to mix and then incubated at RT for 2 min. Following centrifuging at maximum speed (15000 *g*), the supernatant was discarded, and the pellet dried using a vacuum desiccator (5031 Eppendorf UK Ltd, Stevenage, UK), and then resuspended in 50µl of TE buffer (10mM Tris, pH 8.0; 1mM EDTA).

2.2.6.2 Plasmid DNA isolation

Bacterial plasmid DNA was extracted using the Wizard Plus SV Minipreps DNA Purification system (Promega, Southampton, UK), adhering to manufacturer's instructions. Briefly, 1-1.5mL of overnight bacterial culture was centrifuged (Progen centrifuge 24D, Progen Biotecknic, GmbH, Heidleburg, Germany) at 10000 *g* for 5 min, the supernatant was discarded, and the pellet was resuspended and lysed via alkaline phosphatase. Following centrifugation at 11000 *g* for 10 min, the supernatant was transferred to a DNA column supplied from the kit, further centrifugation at 16000 *g* removed the supernatant and bound the DNA to the column. The column was subsequently washed using ethanol-based buffer and DNA was eluted in nuclease free water.

2.2.6.3 PCR analysis

For PCR reactions, either a T4 DNA polymerase (New England Biolabs ® INC. Hitchin, UK) or a Phusion high fidelity (H/F) DNA polymerase (New England Biolabs ® INC. Hitchin, Uk) were used. Reaction mixes were made according to the manufacturer's instructions. PCR was performed using a Px2 Thermocycler (Thermo Fisher Scientific, Waltham, Massachusetts, USA), PCR conditions can be found in Table 2.2. The PCR product was analysed using gel electrophoresis (2.2.6.4)

Table 2.2 PCR conditions for T4 and Phusion High fidelity (H/F) DNA polymerases

Step	T4 Polymerase		Phusion H/F Polymerase		No. of cycles
	Temp (^{o}C)	Time (s)	Temp (^{o}C)	Time (s)	
Initial denaturation	95	5	98	30	1
Denaturation	95	30	98	10	25-35
Annealing	50-60	30	45-72	20	
Extension	72	2	72	30	
Final Extension	72	2	72	10	1
Hold	4	_	4	_	_

2.2.6.4 Gel electrophoresis

Using gel electrophoresis DNA was separated by size. Gels were prepared by melting 1% (w/v) electrophoresis grade agarose (Sigma Aldrich, Poole, UK) in 0.5 x TBE buffer (0.11M Tris, 90mM borate, 2.5mM EDTA, pH8.0). To allow for the detection of the DNA, Midori Green (NIPPON Genetics EUROPE, Dueren, Germany) was added to the cooled liquid gel (50 ^{o}C). The cooled gel was then poured into a gel tank (Bio-Rad, Hercules, California, USA) containing a comb. Once the gel was solid the comb was removed, and the tank was filled with 0.5 x TBE running buffer. Before loading the samples into the gel 5 x DNA sample loading buffer (Bioline, London, UK) was added to each sample, the samples were then loaded into the gel as well as a 1Kb hyperladder 1, molecular DNA ladder (Bioline, London, Uk,). Gels were run at 35 mA constant amperage for approximately 1h, nucleic bands were visualised using a trans-illuminator (Vitax, Cambridge, UK), the DNA fragments size was identified by comparing the fragments to the molecular ladder. To determine construct and vector concentration, bands were also compared to the molecular ladder.

2.2.6.5 DNA gel extraction

Fragments of DNA which had been separated by gel electrophoresis (2.2.6.4) were visualised using a UV blue light trans-illuminator (Syngene, Cambridge, Uk). Using a scalpel blade the DNA band of interest was excised from the gel. The DNA band was purified using a QIAquick gel extraction kit (Qiagen, Crawley, UK) following the manufactures instructions. To briefly summarise, the gel slices were dissolved in QG buffer at 50 ^{o}C for around 10 min with intermittent vortexing. The dissolved gel was then applied to a column containing a silica membrane which allowed the DNA to be adsorbed, all other material was washed out of the column. The DNA was then eluted into a low-salt buffer.

2.2.6.6 DNA sequencing

All Sanger sequencing was performed by the Durham University Biosciences sequencing suite (Department of Biosciences, Durham University, Durham, UK). A list of primers used for sequencing can be found in Appendix: A.1

2.2.6.7 RNA extraction

The ReliaPrep™ RNA Tissue Miniprep System (Promega, Southampton, UK) was used to extract total RNA from treated or untreated Arabidopsis seedlings or leaves according to the manufacturer's instructions. The tissue was ground in liquid N_2, and then homogenised using a micro pestle (Pellet Pestle motor, New Jersey, USA). RNase free DNase (Promega, Southampton, UK) was used to digest genomic DNA, and then RNA was eluted into 50µl of nuclease free water and stored at -80°C.

2.2.6.8 DNA and RNA Quantification

DNA and RNA concentrations were determined by measuring optical density of the samples at 260nm, using a ND-1000 spectrophotometer (Nanodrop Technologies, Deleware, USA), with water or elution buffer used as a zero reference.

2.2.6.9 cDNA synthesis

Using the Applied Biosystems High-Capacity cDNA synthesis kit and following the manufacturer's instructions cDNA was produced from RNA. Briefly, a total volume of 10µl was made up with 2µg total RNA and nuclease free water. Ten microliters of master mix (see Table 2.3) was aliquoted for each diluted RNA sample, giving a final volume of 20µl. Two controls were set up in parallel, one, was lacking an RNA template, the second with no reverse transcriptase enzyme. The samples were mixed by gently pipetting and then they were transferred to a Px2 thermocycler (Thermo Fisher Scientific, Waltham, Massachusetts, USA). cDNA was synthesised by running on the program described in Table

2.4. The subsequent cDNA was diluted to a ratio 1:50 with nuclease free water and stored at -20^{o}C until required.

Table 2.3: cDNA master mix reagents

Component	**Volume in one reaction (µl)**
10x RT buffer	2.0
25x dNTP Mix (100mM)	0.8
10x RT Random Primers	2.0
Nuclease-free water	4.2
Multiscribe™ Reverse Transcriptase	1.0 (but not in NRT control)
Total per reaction	**10.0**

Table 2.4: Px2 thermocycler programme conditions (Thermo Fisher Scientific)

	Step 1	**Step 2**	**Step 3**	**Step 4**
Temperature	25°C	37°C	85°C	4°C
Time	10 min	120 min	5 sec	∞

2.2.6.10 Real time quantitative PCR analysis (RT-qPCR)

The relative transcript (RT) levels for the genes of interest were determined by RT-qPCR using the 7300 real time PCR machine (Applied Biosystems, Thermo Fisher Scientific, Bleiswijk, Netherlands), and GO Taq qPCR master mix (Promega, Southampton, UK). Diluted cDNA (5µl per reaction) (see 2.2.6.9) was added to 10µl of SYBR green master mix (Table 2.5) in each well of a 96 well qPCR plate (STARLAB, Milton Keynes, UK), making a total volume of 15µl. The GoTag PCR mastermix was supplied with ROX as a reference dye, which would allow accountability for the optical differences between the wells. For each different sample three replicate wells were used to produce technical repeats. *PEX4* (At5g25760) was utilised as an endogenous housekeeping control (Wathugala et al., 2012).

All RT-qPCR primers were designed using Primer3 (http://primer3.ut.ee/) or SnapGene software (snapgene.com), with an amplicon between 80-120bp and spanning an intron where possible. A full list of RT-qPCR primers can be found in Appendix A.1. The ΔΔCt method (Livak and Schmittgen, 2001) was used to produce relative quantification (RQ), and values obtained represented the RQ estimates. The error bars generated represented RQ_{MAX} and RQ_{MIN}, and were calculated as described by Knight et al., (2009). All the qRT-PCR runs were performed under identical thermal conditions (Table 2.6). All experiments were repeated 3-5 times to produce biological replicates.

Table 2.5 SYBR green master mix reaction components

Component	Volume in one reaction (µl)
2 x SYBR mix (ROX)	7.5
Forward Primer (5 µM)	0.9
Reverse Primer (5 µM)	0.9
Water	0.7
Total per reaction	**10.0**

Table 2.6 RT-qPCR cycling conditions

Cycle steps	Time and temperature	No. of cycles
Stage 1	50 °C; 2 min	1
Stage 2	95 °C; 10 min	1
Stage 3	95 °C; 15s	40
Stage 4	60°C; 1min	*Holding*

2.2.6.11 Cloning

2.2.6.11.1 Constructs, Plasmids and Vectors

All constructs, plasmids and vectors can be found in Appendix B, including vector maps, construct schematic diagrams and construct sequences.

2.2.6.11.2 Restriction Digests

Restriction digests were carried out to procure fragments for diagnosis or for cloning. The digests were carried out using compatible NEB enzymes and buffers which were incubated in line with the manufacturer's instructions. The enzymes for all the digests whether it be single, double, or multiple did not exceed a maximum of 10% (v/v) of the total reaction volume. To determine the size of the fragments produced, the digests were subjected to gel electrophoresis (see 2.2.6.4).

2.2.6.11.3 Gibson Assembly

All DNA constructs to be cloned were ordered as gBlock synthetic genes (Integrated DNA Technologies (IDT) Leuvan, Belgium). The DNA fragment of interest was cloned into a linearized vector using a Gibson Assembly® Cloning Kit (NEB, Cat. No. E5510S) according to the manufacturers recommended instructions. In short, a DNA fragment containing overlapping ends, a linearized vector and Gibson Assembly master mix were incubated in a single reaction. In the mastermix a 5' exonuclease activity produces long overhangs; the gaps of the annealed single strand are subsequently filled in by a polymerase and the nicks sealed with a DNA ligase. A molar ratio of 3:1 insert to vector was used. *E. coli* cells were transformed with 2µl of the reaction (2.2.6.12.1.1). The DNA of interest was confirmed by restriction digests (2.2.6.11.2) and sequencing (2.2.6.6).

2.2.6.11.4 Gateway cloning

The DNA to be cloned was initially inserted into a p-ENTR-D-TOPO vector by Gibson Assembly (2.2.6.11.3), which was linearized by cutting the vector with NotI and AscI enzymes (see 2.2.6.11.2 for methods). Following the manufacturer's instructions, Gateway recombination using LR Clonase II Enzyme Mix (Life Technologies, Paisley, UK) was used to create the final binary vector constructs. *A tumefaciens* cells (C58C1) were transformed (2.2.5.11.1.2), the DNA of interest was confirmed by restriction digest (see 2.2.6.9.2) the plasmid DNA was extracted (2.2.6.2) and then sent to sequencing for further confirmation (2.2.5.4).

2.2.6.12 Bacteria and plant transformation

2.2.6.12.1 Bacterial transformation

2.2.6.12.1.1 Transformation of E. coli

An aliquot of α-select silver cells (Bioline, London, UK) were transformed with plasmid DNA. The DNA (2 to 2.5µl) was added to the cells whilst on ice and incubated for 30 min. The cells were heat shocked at 42 ^{o}C for 30s and then returned to the ice for a further 2 min. SOC media (950µl) (Thermo Fisher Scientific, Loughborough, UK) was added to the cells, the cells were then incubated at 37^{o}C whilst being shaken at 250rpm for 1h. The cells (100µl) were pipetted onto previously warmed LB plates containing appropriate antibiotics (see Table 2.1 for concentrations) and incubated at 37^{o}C overnight.

2.2.6.12.1.2 Transformation of A. tumefaciens

2.2.6.12.1.2.1 Competent cell production

A 5mL overnight bottle of LB containing the appropriate antibiotic was inoculated with a single Agrobacterium colony (from a fresh LB plate supplemented with an appropriate antibiotic; usually rifampicin) using a sterile loop. The culture was grown overnight at 28 ^{o}C in a N-Biotek shaking incubator NB-205 (N-Biotek, Gueonggi, Korea) at 150rpm. The following day, 4mL of the culture was added to a 500mL conical flask containing 100mL Lb. The flask was shaken at 250rpm at 28 ^{o}C until the culture reached an OD600 of 0.5 to 1.0

(this normally takes 4 to 8h). The culture was then chilled on ice before centrifuging at 3000 g for 5 min at 4 ^{o}C. The supernatant was removed and discarded; the pellet was then resuspended in 2mL of ice-cold 20mM $CaCl_2$ solution. Next, 0.1mL of the cells were dispensed into ice-cold 1.5mL Eppendorf tubes. The cells were frozen in liquid N_2 before storing at -80 ^{o}C.

2.2.6.12.1.2 Transformation

Aliquots of 100µl of competent C58C1 *A. tumefaciens* cells were thawed on ice. One µg of plasmid DNA was added to the cells and incubated on ice for 30 min. The cells were heat shocked at 37 ^{o}C for 5 min and then returned to the ice for a further 2 min. One mL of liquid LB was added to the cells and then the cells were incubated at 28 ^{o}C whilst shaking at 150rpm for 4h. The cells were briefly centrifuged, and the pellet was then resuspended in 100µl of LB before spreading onto LB plates containing the appropriate antibiotics (see Table 2.1.) The plates were incubated for 72h at 28 ^{o}C.

2.2.6.12.1.3 Blue white selection of bacterial transformants.

To confirm the production of a binary construct and transformation into the binary vector pBIN19 the blue/white selection method was adopted. Briefly, the multiple cloning site (MCS) of the plasmid vectors used for cloning are found within the *lac*Z sequence. When the plasmid takes up foreign DNA it interrupts the lacZ and prevents the production of β-galactosidase. Plasmids were incubated on LB plates containing X-Gal, if β-galactosidase is produced X-Gal is hydrolysed which produces an insoluble blue pigment. The recombinant colonies are therefore white, and the non-recombinant appear blue in colour. The white colonies were individually lifted off the plate, and further incubated overnight in liquid LB and appropriate antibiotics (Table 1), the plasmids were then extracted using the mini prep method (2.2.6.2) and sent for sequencing (2.2.6.6).

2.2.6.13 Arabidopsis transformation: the floral dip method

2.2.6.13.1 Plant transformation

Wild type *A. thaliana* seeds were grown on MS media (see 2.2.2.1) for one week. The seedlings were transferred to a single large (44mm) Jiffy hydrated peat plug (3 seedlings per plug) and grown at 20±1 ^{o}C in a 16/8h light/dark photoperiod. Once the plants began to flower the bolts were clipped to encourage the growth of multiple secondary bolts. The final clipping occurred no less than 7 days before transformation. A 5mL LB culture consisting of *A. tumefaciens* containing the plasmid of interest, liquid LB media and the appropriate antibiotics (see Table 2.1 for antibiotic list) was incubated at 28 ^{o}C, shaken at 150rpm overnight. The next day 1mL of this culture was added to 200mL of liquid LB with the appropriate antibiotics and incubated overnight under the same conditions as the 5mL overnight culture. The following day the *A. tumefaciens* cells were centrifuged at 3500 *g* for 20 min at RT. After disposing of the supernatant, the pellet was resuspended in 200mL of 5% sucrose solution (w/v) and 0.05% Silwet L-77 (v/v). The flower stems of each Arabidopsis plant were dipped in the sucrose/Silwet solution, and then the plants were placed on their sides in a tissue-lined tray to absorb any excess bacterial culture (Clough and Bent, 1998). The tray was sealed with cling film and moved to a growth chamber set at 20±1 ^{o}C and a 16/8h day/night photoperiod for 24h. After 24h the plants were transferred to a new tray in an upright position and left to grow for 5/7d when they were removed and re-dipped, following the procedure previously described. After the plants were transferred to a fresh tray and placed upright a second time the plants were left to grow until seed stage.

2.2.6.13.3 Primary transformant screening

Seeds (T1) from transgenic Arabidopsis plants were collected and bleach sterilised (see 2.2.2.3). The seeds were then dispersed onto large MS plates containing either kanamycin, spectinomycin or glufosinate ammonium along with timentin (see Table 2.1 for concentrations). The seeds were left in light for 6-8h and then covered in foil and placed in the dark at 4 ^{o}C for 72h. After 72h the plates were transferred to a Percival set at the

conditions described in 2.2.2.4, until transformed seedlings could be identified. Seedlings that displayed dark green leaves and long roots that had penetrated the agar were deemed to be transformants: this usually occurred around the 5th or 6th day of growth (Harrison et al., 2006). The seedlings (T1) that survived the selection process were transferred to hydrated Jiffy peat plugs (42mm) and grown to maturity under normal conditions (see 2.2.2.4), the seeds were then harvested (T2).

To confirm the presence of the foreign DNA further selection was conducted on the T2 seeds. Seeds were sterilised using the ethanol method (2.2.2.2) and grown on MS plates for 7d. To confirm whether transformants contained a luciferase sequence, candidates were sprayed with 1mM of luciferin and left in the dark at RT for 4h. The seedlings were then placed under the photon camera to determine if there was luciferase activity, which would indicate that the plants were indeed transformed. To determine the presence of the DNA of interest, DNA was extracted from the transformants (see 2.2.6.1) followed by PCR (2.2.6.3) and gel electrophoresis (2.2.6.5), the DNA was further sent for sequencing (2.2.6.6) for a final confirmation. For T2 seedlings containing genes that encoded for other proteins of interest, RNA was extracted (2.2.6.7), synthesised to cDNA (2.2.6.9) and then gene expression was measured using RT-qPCR (2.2.6.10). DNA and RT-qPCR primers used for selection can be found in Appendix A.1

2.2.6.14 Protein extraction

The protein extraction method used was adapted from Méchin *et al*., (2007). Briefly, pre-treated WT 10d old Arabidopsis seedlings (2.2.3.5) were placed into 2mL Eppendorf tubes containing ice cold 10% TCA in acetone (1mL per 0.1g of tissue), the samples were then left overnight at -20 ^{o}C. The next day the samples were centrifuged at 16000 *g* for 15min, after centrifugation the supernatant was decanted. The sample was then washed by adding ice cold acetone (1mL/0.1g of tissue), vortexed briefly and then centrifuged for a further 15min at 16000 *g*, the supernatant was decanted, and this step was repeated 6 times, or until all chlorophyll was removed from the sample. The supernatant was decanted, and the pellet

air dried at RT before resuspension in resolubilisation buffer (7M urea; 2M thiourea; 30mM Tris base; protease inhibitor (10µl/mL) and 2% CHAPs pH 8.0).

2.2.6.15 Protein Quantification: Bradford assay

The following Bradford assay was adapted from the recommended Bio-Rad protocol (Bio-Rad, Hercules, California, USA). Eppendorf (1.5mL) tubes were set out to accommodate two sets of BSA standards (ranging from 0-50mg of BSA) and 10 protein samples. Each tube contained 80µl of dH_2O; 10µl of 0.1mM HCl; resolubilisation buffer (2.2.6.14), pH 8.0 and protein sample (buffer plus the sample make a total of 10µl, the buffer equates to the amount of sample i.e., 5µl of sample would require 5µl of buffer, in the case of the BSA standards each tube would contain 1µl of standard and 9µl of buffer). The Bradford assay supplied by Bio-Rad was diluted to a ratio of 1:4, 1-part assay 4 parts dH_2O, 900µl of the diluted assay was added to each tube. The samples were left at RT to incubate for 1h and absorbance was measured at 595nm with a Beoco spectrophotometer (Beoco, Germany). Protein concentrations were determined by a typical Bradford standard curve as described in Bradford, (1976).

2.2.6.16 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Using SDS-PAGE, proteins were separated by size. The SDS gel electrophoresis system is divided into two gels: separating and stacking gels. A 10mL separating gel was made (12% acrylamide) consisting of H_2O, acrylamide/bis-acrylamide (30%/0.8% w/v), 1.5M Tris pH 8.0, 10% (w/v) SDS, 10% (w/v) ammonium persulfate (APS) and TEMED (10µl). Both APS and TEMED were added just before pipetting, the gel solution was pipetted into a gel cast, and to ensure the separating gel was horizontal a layer of isopropanol was added. Once the gel was set the isopropanol was washed off with water. A 5mL stacking gel (5% acrylamide) containing H_2O, 0.5M Tris-HCl, pH 6.8, 10% (w/v) SDS, acrylamide/bis-acrylamide (30%/0.8% w/v), 10% APS and TEMED (0.005mL) was pipetted on top of the separating gel and a comb was added. The gel was wrapped in cling film and left overnight at 4 ^{o}C to

polymerise. The next day the cling film was removed, and the gel was secured into a gel tank, the comb was removed, and the tank was filled with 1 x SDS running buffer (25mM Tris-HCl, pH 8.3; 200mM glycine; 0.1% (w/v) SDS).

For protein sample preparation 10µg of protein was added to 4µl of 5 x SDS loading dye (10% (w/v) SDS; 10mM beta mercaptoethanol; 20% (v/v) glycerol; 0.2M Tris-HCl, pH 6.8; 0.05% (w/v) bromophenol blue) and made up to 20µl with dH_2O. The samples were briefly vortexed, and a hole was pierced in the lid of each tube before placing into a heat block for 10 min which was set at 100 ^oC. Following this the samples were briefly centrifuged at 14000 *g* for around 2 min. A protein marker (Bioline, London, UK) was loaded into the first lane of the gel followed by the samples in the subsequent lanes. The gel was run at 75V 35mA for 35mins and then 35A, 150V for 1.5h.

Once the gel run was finished the gel was removed and placed into a poly vinyl chloride staining dish, rinsed with dH_2O, and then fixed with 10% methanol 7% acetic acid and water for 30m. The fixative was rinsed off with dH_2O and SYPRO Ruby (Sigma-Aldrich, Poole, UK) was poured onto the gel until the gel was completely covered, the dish was covered in foil and gently agitated at 150rpm on a shaker for 3h. After 3h the gel was transferred to a clean dish and rinsed with water for 30 min, the gel was then placed into a Typhoon 9400 Variable Mode Imager (GE Healthcare, Little Chalfont, UK) and scanned to confirm the presence of protein bands.

2.2.6.17 iTRAQ labelling

The iTRAQ labelling protocol used was a modification of the protocol supplied in the iTRAQ labelling kit (Sigma Aldrich LTD, Poole, UK). A reaction was set up for each sample containing 20µg of protein made up to 100µl with milliQ water, 5µl of 1.5M Tris-Cl, pH 8.8 and 400µl of acetone. The reaction was left to stand for 35 min to 1h at RT and then centrifuged for 10 min at 16000 *g*. The supernatant was decanted and discarded, the pellet was dried in a sterile flume hood for 2 to 2.5 min and 2% (w/v) SDS was added to the pellet which was gently pipetted to ensure the SDS was mixed thoroughly with the pellet. The samples were transferred to a heat block set at 60 ^oC for 1h, the pellet was then

resuspended in 36.6 µl resuspension buffer (supplied in the iTRAQ ® Reagent-8PLEX Multiplex kit, Sigma Aldrich LTD), vortexed and shaken at RT for 20 min. After centrifuging briefly for 2s, (12000 *g*) 2µl of iTRAQ Kit reducing agent was added to the samples and incubated for 1h at 60 °C. iTRAQ blocking agent (1µl) was added to the samples and left to incubate for 10 min at RT. Following incubation 20µl of Trypsin buffer (100µg Trypsin Gold (Promega, Southampton, UK) and 200µl of buffer consisting of 0.115M HCl; 100% acetonitrile; MQ water) was added to the samples and incubated overnight at 37 °C.

The protein samples were freeze dried with liquid nitrogen for 3-4h, the pellet was resuspended in 40µl of MQ water. Labels in the iTRAQ kit were diluted with 180µl of Isopropanol, vortexed and briefly centrifuged at 8000 *g*. To each sample 40µl of one of 8 different iTRAQ labels were added before vortexing and incubating at RT for a minimum of 2h. All the labelled samples (both controls and treated) were mixed together in one tube and then freeze dried for 2-3h with liquid N_2. The samples were then subjected to Mass Spectrometry analysis.

2.2.7 Plant Physiology

2.2.7.1 Osmotic stress assays

Ten-day old Arabidopsis seedlings were initially pre-treated with an overall concentration of either 50µl mastoparan, 250mM $CaCl_2$, 0.5mM ATP, 1mM L-glutamate or combination of mastoparan/$CaCl_2$, mastoparan/ATP or mastoparan/L-glutamate or water, as described in 2.2.3.3. After pre-treatment, the seedlings were placed in 5ml of water containing an overall concentration of 300mM mannitol. Plant material was subjected to mannitol induced osmotic stress for 10 days. For RNA extraction (2.2.6.7) and gene expression measurements (2.2.6.10) the plant material was harvested at 1, 3, 6, and 24h. After 10d of osmotic stress chlorophyll extraction was performed (2.2.7.2) to analyse bleaching in response to mannitol-induced osmotic stress. Further assays concentrated on pre-treatment of $CaCl_2$, mastoparan/$CaCl_2$ or water for both seedlings and leaves. For seed pre-treatment for osmotic stress assays, Arabidopsis seeds were pre-treated with either 5mM,

50mM, 250mM or 500mM $CaCl_2$ or water (2.2.3.3) and then grown under normal growth conditions to maturity (2.2.2.4). Once at maturity the leaves were subjected to a mannitol-induced osmotic stress assay as described above.

2.2.7.2 Photosynthetic activity measurements: chlorophyll concentrations

To quantitatively measure bleaching in Arabidopsis in response to mannitol-induced osmotic stress (2.2.7.1), a chlorophyll assay was carried out. Treated seedlings/leaves were blotted dry with tissue and transferred to a 1.5mL Eppendorf microfuge tubes, 1mL of acetone was added to the tubes, and the tubes were incubated overnight in the dark at RT. The following day the samples were homogenised using a mini pestle (Pellet Pestle motor, New Jersey, USA) and vortexed to resuspend the material. To separate the chlorophyll from the plant material the samples were centrifuged at 2000 *g*. The supernatant containing the chlorophyll was removed and set aside, the pellet was resuspended in 400µl of acetone, and the process was repeated for further chlorophyll extraction. This process was repeated until 1.2mL of acetone had been used. The chlorophyll supernatant was pooled and made up to 1.5mL with distilled water, giving an overall acetone concentration of 80% (v/v). Using a spectrophotometer, chlorophyll content was measure at an OD of 663 and OD 645 nm, with 80% (v/v) acetone used for a blank. The following equation was adopted to calculate overall chlorophyll concentration (mg/g) (Hipkins and Baker, 1986).

$$Chlorophyll\ (mg/g) = \frac{(20 \times A645) + (6.02 \times A643) \times V}{FW}$$

V= Volume of 80% acetone

2.2.8 Proteomics

2.2.8.1 Mass Spectrometry analysis: Liquid chromatography mass spectrometry (LC-MS/MS) analysis

Using data dependent LC-MS/MS (see 1.9.2), peptides were analysed on a TripleToF 6600 mass spectrometer (Sciex, Warrington, UK) linked to an Eksigent 425 liquid chromatography system via a Sciex Duospray source. To separate peptides by micro flow chromatography a trap and elute method was adopted with a YMC TriArt (YMC, Kyoto, Japan) C18 trap column, 5µm, 0.5 x 5mm, and a YMC TriArt C18 resolving column (3µm, 0.3 x 150mm). Peptide samples were applied to the trap column within a 5µl/min for 5 min gradient. Subsequent linear gradients of 3 to 30% were ran in formic acid/ACN buffer containing 0.1% (v/v) formic acid in acetronitrite (ACN) for 60 min, 30%-40% in formic acid/ACN buffer over 17 min and 40%-80% in formic acid/ACN buffer over 2 min, followed by a 3 min column wash with 2 x 2mL of binding solution (85% CAN, 30mM ammonium formate, pH 3.0). The samples were returned to 3% of acid/ACN buffer for a minimum of 2 min, followed by re-equilibration of the columns in the acid/ACN buffer for 6 min.

Data-dependent top-30 MS-MS acquisition from the labelled peptides started immediately upon gradient initiation and lasted for 85min. Throughout this period, precursor-ion scans (400 to 1600 m/z) of 250 ms enabled selection of up to 30 multiply charged ions of >500 cps intensity for CID fragmentation and MS/MS spectrum acquisition (m/z 100-1500) for 33 ms. Collision energy was increased for efficient fragmentation of iTRAQ-labelled peptides using an integral setting in Sciex control software Analyst 1.7.1, and a rolling precursor exclusion of 15s was applied throughout to limit multiple fragmentation of the same peptide.

Protein identification and relative quantification used ProteinPilot 5.0.1 (Sciex), and results were exported for calculation of the significance of observed fold-changes between control and treated samples.

2.2.8.2 Protein analysis: Microsoft Excel

Protein data exported from mass spectrometry analysis was further analysed using Microsoft Excel. All peptides <95% confident were removed and single peptides were also disregarded as possible one-off anomalies. Fold difference between the controls and the treatments were calculated by finding the ratios (which involved dividing each treated sample by the mean of the controls for each protein) and then adding all four ratios and dividing by four, anything less than 1.2%-fold difference was discarded. P-values were calculated using a two-tailed T-test, the proteins with a p-value > 0.5 were classed as insignificant and removed. From these calculations a significant list from different time points was generated, which allowed analysis for comparison and overlap between the time points. Using this information, the coding genes for each protein were identified, either from TAIR data base (https:/www.arabidopsis.org/) or UniProt (https:/www.uniprot.org/), and molecular and biological functions were noted.

3. Is there calcium signature specificity during regulation of transcription?

3.1 Introduction

Due to a plant's sessile nature it must be able to adapt to a changing environment (Reddy et al., 2011). The majority of a plant's defence against stress is through changes in gene expression, it is therefore vital that the plant receives correct information to produce specific proteins to respond to a specific stimulus. Calcium is a ubiquitous secondary messenger in all eukaryotes and an intermediate between stimulus perception and plant response (Galon et al., 2010, Kudla et al., 2010, McAinsh and Hetherington, 1998, Reddy and Reddy, 2004b, Sanders et al., 2002, Short et al., 2012). It is generally accepted that cytosolic calcium ($[Ca^{2+}]_{cyt}$) elevations (Ca^{2+} signals) mediate a complex signal transduction pathway that leads to a given response to a specific stimulus (Allen et al., 2001, Whalley and Knight, 2013, Zipfel and Oldroyd, 2017). It has been suggested that the calcium signal itself might contain the information that determines specificity, and that this information is conveyed in the form of oscillations and/or other spatial, temporal patterns known collectively as 'calcium signatures' (Berridge et al., 2000, McAinsh and Hetherington, 1998, McAinsh and Pittman, 2009, Ng and McAinsh, 2003). The 'calcium signature' hypothesis implies that the plant needs the correct signature to initiate an appropriate response to a given stimulus, without the correct signature the plant will not be able to adapt to the stimulus, therefore reducing its ability to survive.

It has been known for some time that calcium regulates gene expression in plants (Bickerton, 2012); therefore, it is feasible that responses mediated by stimulus-induced calcium signals are generated transcriptionally. Early work researching genes involved in calcium regulated gene expression focussed on one or two genes, which limited a more global understanding (Whalley et al., 2011). The discovery that the abscisic acid responsive element (ABRE) was calcium-regulated (Kaplan et al., 2006) has implicated that calcium may regulate hundreds, if not thousands, of genes in the Arabidopsis genome. Recent

studies by Whalley et al., (2011) and Whalley and Knight (2013) have identified that calcium-regulated genes, could be differentially expressed in response to elevated $[Ca^{2+}]_{cyt.}$ Whalley et al., (2011) describes that four distinct promoter motifs have been discovered in the Arabidopsis genome that respond to artificially-induced $[Ca^{2+}]_{cyt}$ elevations, the previously identified ABRE motif (Kaplan et al., 2006), and a further three motifs: C-Repeat/Drought-Responsive Element (CRT/DRE), SITE II and CaM box. This work compared genes that were induced in response to three different $[Ca^{2+}]_{cyt}$ elevations: transient, prolonged and oscillating, revealing differences in both number and identity of genes induced/repressed. This work has identified the genes, TFs and *cis* elements regulated by calcium (Whalley et al., 2011) and has suggested that they respond to different calcium signatures (Whalley and Knight, 2013), but, this theory has yet to be tested.

Recent studies by Lenzoni et al., (2018) involved inducing $[Ca^{2+}]_{cyt}$ elevations with different kinetics using four different calcium agonists: mastoparan, $CaCl_2$, adenosine triphosphate (ATP) and L-glutamate. This work showed that mastoparan induced large fold changes in the expression of *EDS1* and *ICS1* Arabidopsis genes, compared to a small fold difference induced by the other three agonists. However, using only four agonists gives a relatively limited repertoire of signatures, and to fully elucidate specificity would require a more extensive collection of different signatures.

It is important to note that mastoparan can induce both $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{nuc}$ signatures (Huang et al., 2017, Pauly et al., 2001), and that mastoparan $[Ca^{2+}]_{cyt}$ signature peaks can differ in seconds to $[Ca^{2+}]_{nuc}$ signature peaks (Pauly et al., 2001). In this study I have investigated mastoparan induced $[Ca^{2+}]_{cyt}$ signatures and compared them to other calcium agonists induced $[Ca^{2+}]_{cyt}$ signatures to determine if signature kinetics differ between agonists. From this work I further investigated the regulation of known calcium regulated *cis* elements by these signatures, to determine if a specific calcium signature regulates different cis elements when compared to other calcium signatures. In regards to mastoparan induced calcium regulation of these *cis* elements I cannot say with complete confidence that the regulation is due to $[Ca^{2+}]_{cyt}$ or $[Ca^{2+}]_{nuc}$ signatures, however, I can suggest that mastoparan induced calcium signatures may hold specific information that leads to the regulation of particular downstream elements. To eliminate this uncertainty

further research could try to determine which signature is regulating these elements. This could entail conducting the calcium signature measurements using transgenic plants expressing aequorin, targeted to either the cytosol or the nucleus. Which could determine if indeed both $[Ca^{2+}]_{nuc}$ and $[Ca^{2+}]_{cyt}$ signatures were induced by mastoparan. The next step would be to block either the cytosol or nuclear calcium signals by introducing expressing proteins (in either the cytosol or nucleus) that chelate calcium (mimicking EDTA) in the transgenic plants containing the over expressing *cis* elements. This would help to determine which signature is regulating the elements or in fact if both signatures are required for regulation of these particular *cis* elements.

Questions to be addressed in this chapter:

- Does combining calcium agonists lead to calcium signatures which are novel, compared to single treatments?
- Do the four calcium-regulated promoter motifs respond specifically to different calcium signatures

3.2 Results

3.2.1 Calcium signatures generated from both single and combined calcium agonists

To investigate calcium signature kinetics and specificity, initial work from Lenzoni et al., (2018) was repeated, namely inducing $[Ca^{2+}]_{cyt}$ elevations using four different calcium agonists: mastoparan, $CaCl_2$, ATP or L-glutamate. Each agonist had been previously shown to cause different calcium kinetics and it is believed that they do not mimic a specific natural calcium signal in the plant, such as signals produced during abiotic and biotic stress (Dennison and Spalding, 2000, Lenzoni et al., 2018, Sun et al., 2010, Whalley et al., 2011).

Individual 10 day old seedlings expressing cytosolic aequorin (pMAQ2) were treated with one of the four agonists and $[Ca^{2+}]_{cyt}$ traces were generated from the average of 10 seedlings (see 2.2.4.2 for detailed methods). To generate a larger repertoire of artificial calcium signatures investigations were conducted to measure $[Ca^{2+}]_{cyt}$ traces induced from combined agonist treatments: mastoparan/$CaCl_2$, mastoparan/ATP or mastoparan/L-glutamate. These results were then compared to the single agonist treatments to determine if a novel signature had been induced (Figures 3.1 to 3.3).

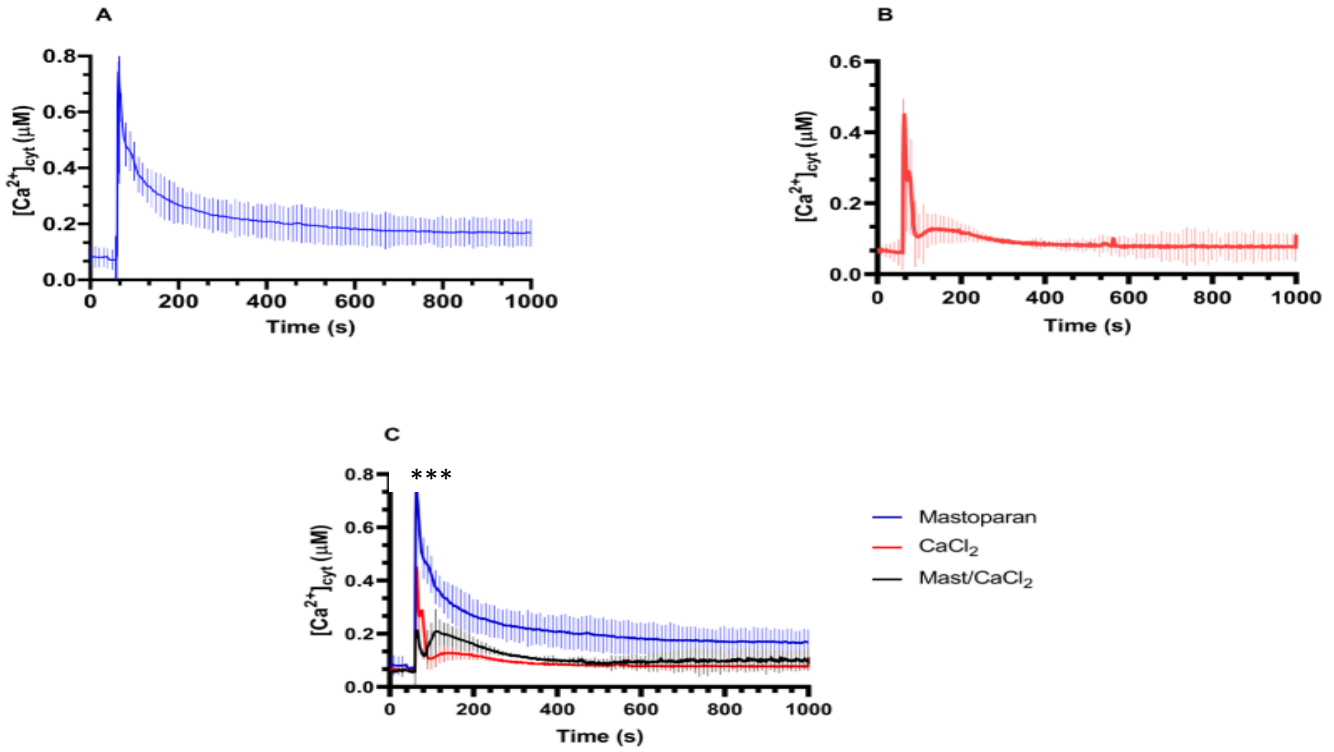


Figure 3.1: Calcium signature traces generated from single or combined calcium agonist treatments. Each trace depicts the average signals from 10 seedlings injected with an agonist treatment at 60 s and measured for 1000 s. Figure 3.1: A, a calcium trace generated in response to an overall concentration of 10µM mastoparan; 3.1: B, an overall concentration of 50mM $CaCl_2$; 3.1: C, a combination of 10µM mastoparan and 50mM $CaCl_2$. Error bars represent ±SEM (n=10 treated seedlings). One-way ANOVA of peak at 60s between 3 calcium agonist generated signatures **** (P≤0.0001), *** (P≤0.001), ** (P≤0.01) * (P≤0.05) and Tukey *post hoc.*

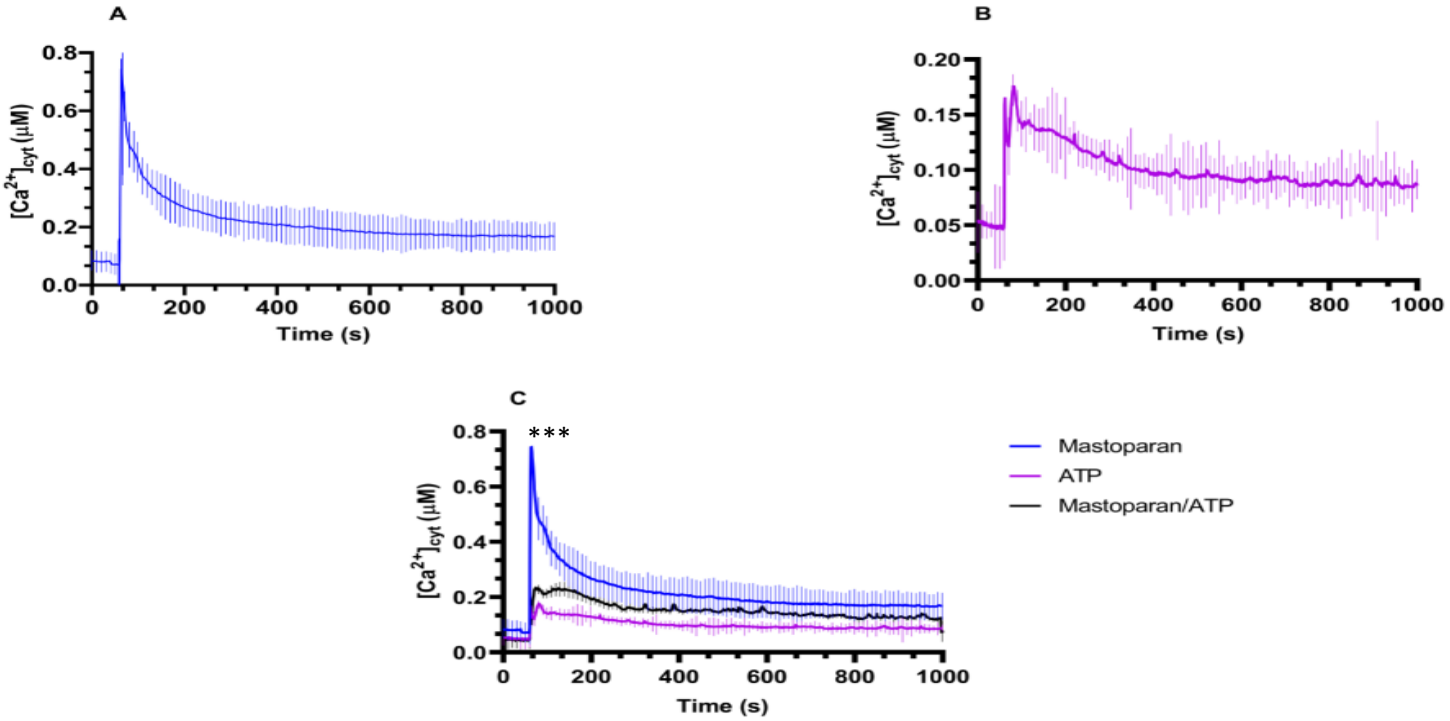


Figure 3.2: Calcium signature traces generated from single or combined calcium agonist treatments. Each trace depicts the average signals from 10 seedlings injected with an agonist treatment at 60 s and measured for 1000 s. Figure 3.2: A, a calcium trace generated from an overall concentration of 10µM mastoparan; 3.2: B, an overall concentration of 0.5mM ATP; 3.2: C, a combined treatment of 10µM mastoparan and 0.5mM ATP. Error bars represent ±SEM (n=10 treated seedlings). One-way ANOVA of peak at 60s between 3 calcium agonist generated signatures **** (P≤0.0001), *** (P≤0.001), ** (P≤0.01) * (P≤0.05) and Tukey *post hoc*.

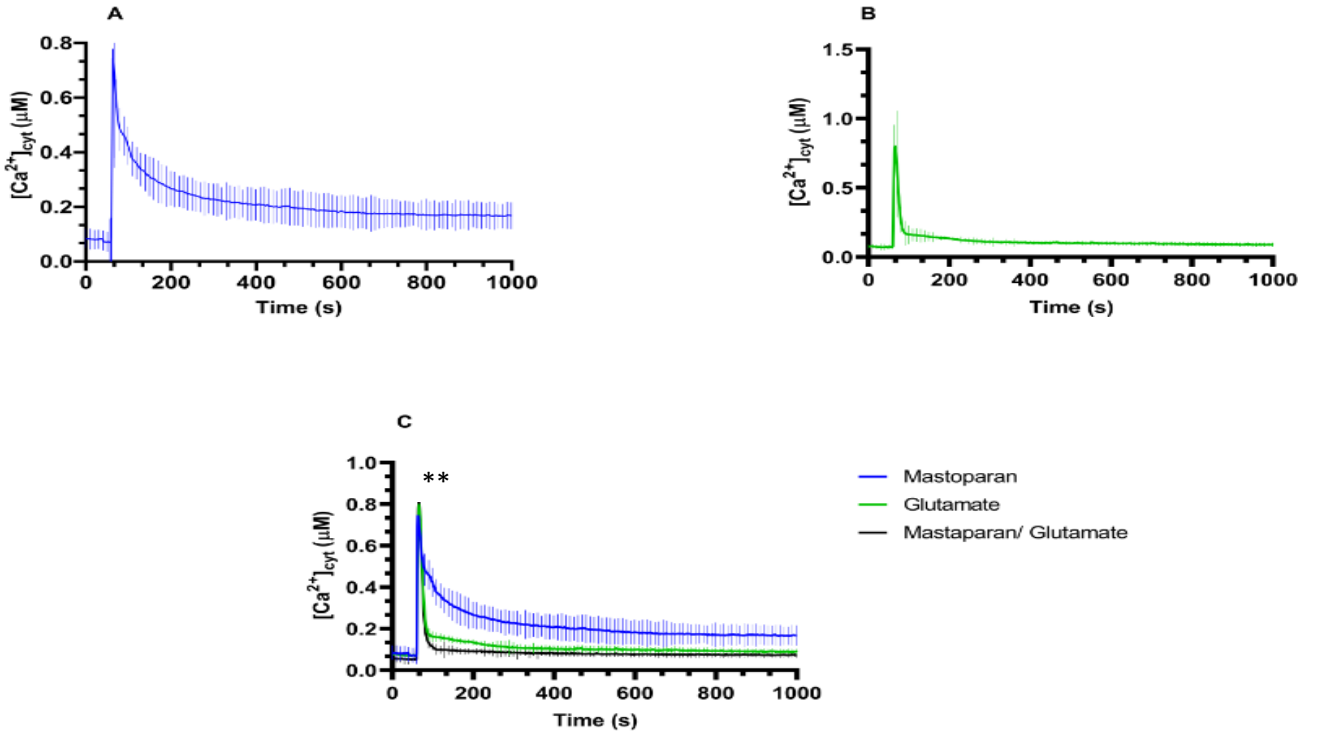


Figure 3.3: Calcium signature traces generated from single or combined calcium agonist treatments. Each trace depicts the average signals from 10 seedlings injected with an agonist treatment at 60 s and measured for 1000 s. Figure 3.3: A, a calcium trace generated from an overall concentration of 10µM mastoparan; 3.3: B, an overall concentration of 1mM L-glutamate; 3.3: C a combination of 10µM mastoparan and 1mM L-glutamate. Error bars represent ±SEM (n=10 treated seedlings). One-way ANOVA of peaks at 60s between 3 calcium agonist generated signatures **** (P≤0.0001), *** (P≤0.001), ** (P≤0.01) * (P≤0.05) and Tukey *post hoc*.

The initial results from the single treatments (Figures 3.1 to 3.3: A-B) support the findings described in Lenzoni et al. (2018), in that each calcium agonist induces a signature with distinctive kinetics. The traces generated from the combined treatments, displayed different kinetics again when compared to that of the single treatments; in terms of peak height, overall response (Figures 3.1-3.3: C) and number of peaks (Figure 3.3: C). A one-way ANOVA and *post hoc* statistical test of the peaks generated probabilities of $p<0.001$ to $p<0.003$, suggesting that the calcium signatures were indeed significantly different.

3.2.2 Measuring calcium-regulation of specific *cis* elements

As discussed in 3.1, Whalley et al. (2011) identified four different promoter *cis* elements that were regulated by calcium. These were proven by the authors to be calcium regulated, but no information as to their specific response to a particular calcium signature was obtained. Therefore, to investigate the response of these *cis* elements to specific calcium signatures, 10-day old transgenic Arabidopsis seedlings expressing constructs consisting of one of the *cis* elements repeated 4 times fused to a firefly luciferase (*LUC+*) coding region sequence, or a control where the *LUC+* coding region was fused to a minimal promoter (lacking the *cis* element repeats) were treated with one of four calcium agonists, or a combination of agonists as described in 2.2.3.2. Luminescence was recorded using a photon camera (see methods 2.2.5), and an overall average was taken from 5 seedlings for each construct (Figures 3.4 to 3.6). A control experiment treated with water was also recorded (Appendix C.1).

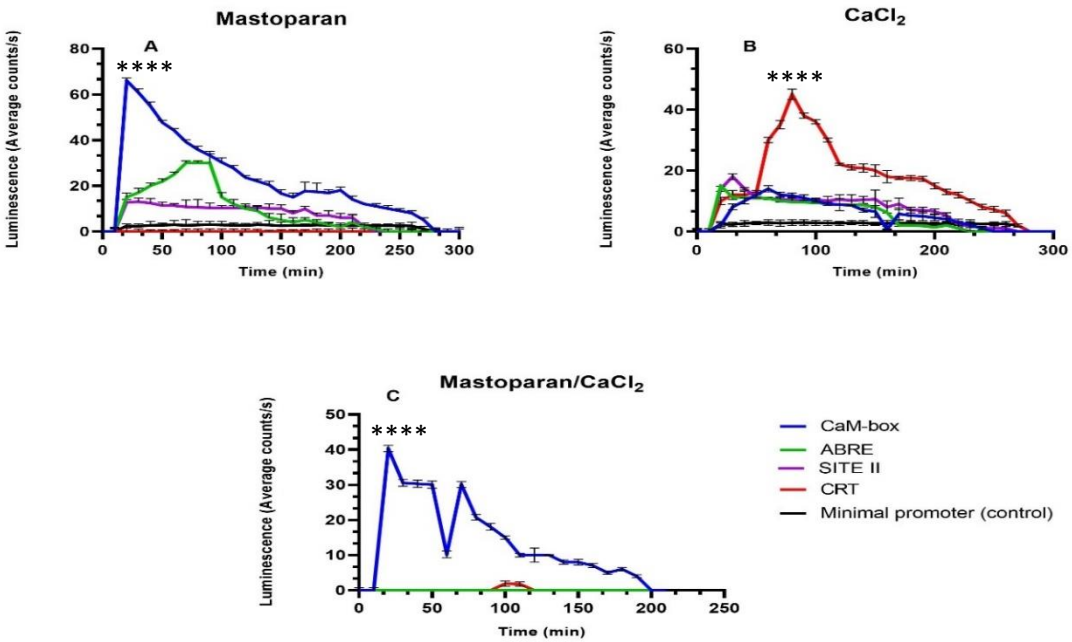


Figure 3.4: Luminescence average counts per second, each line represents an average of five 10-day old Arabidopsis seedlings containing a construct expressing *LUC+* through 1 of 4 calcium regulated *cis* elements, or a minimal promoter control. Figure 3.4: A, displays results from seedlings treated with an overall concentration of 10µM mastoparan over time, statistical analysis of peaks at 30 mins; 3.4: B, 50mM $CaCl_2$, statistical analysis of peaks at 80 mins; 3.4: C, a combination 10µM mastoparan and 50mM $CaCl_2$, statistical analysis of peaks at 30 min. Error bars represent ±SD (n=5 replicates of 5 treated seedlings). Two-way ANOVA between peaks of CaM, CRT, SITEII and ABRE *cis* elements **** (P≤0.0001), *** (P≤0.001), ** (P≤0.01) * (P≤0.05) and Tukey *post hoc*.

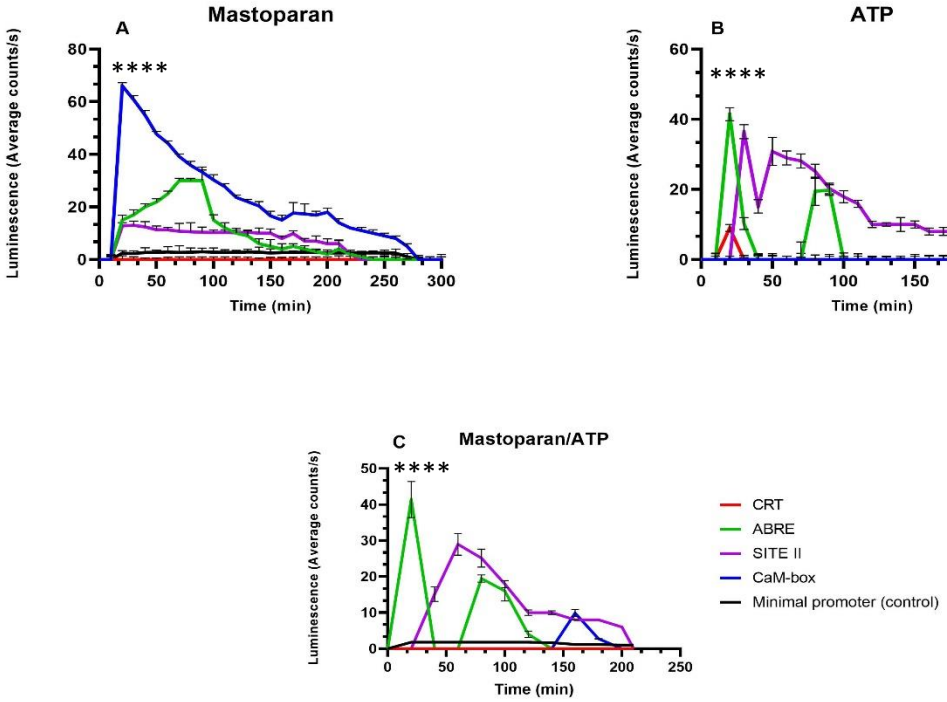


Figure 3.5: Luminescence average counts per second, each line represents an average of five 10-day old Arabidopsis seedlings containing a construct expressing *LUC+* through 1 of 4 calcium regulated *cis* elements or a minimal promoter control. Figure 3.5: A, displays results from seedlings treated with an overall concentration of 10µM mastoparan over time; 3.5: B, 0.5mM ATP; 3.5:C, a combination 10µM mastoparan and 0.5mM ATP. Error bars represent ±SD (n=5 replicates of 5 treated seedlings). Two-way ANOVA between peaks of CaM, CRT, SITEII and ABRE *cis* elements at 30 min **** (P≤0.0001), *** (P≤0.001), ** (P≤0.01) * (P≤0.05) (p>0.0001) and Tukey *post hoc*.

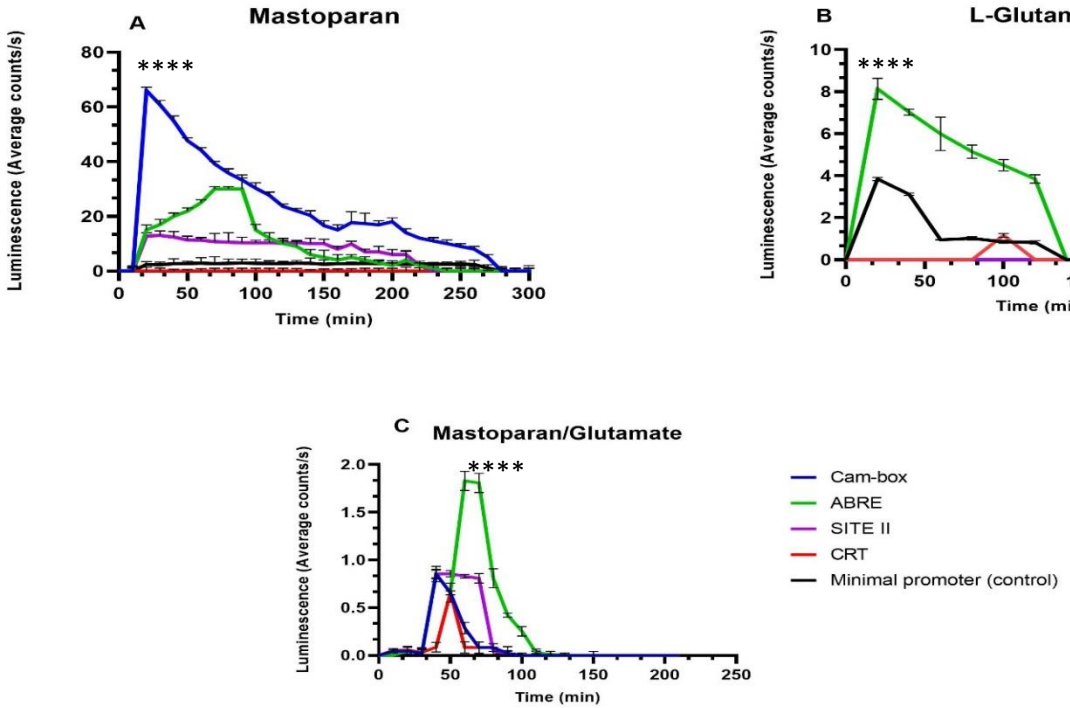


Figure 3.6: Luminescence average counts per second, each line represents an average of five 10-day old Arabidopsis seedlings containing a construct expressing *LUC+* through 1 of 4 calcium regulated cis elements or a minimal promoter control. Figure 3.6: A, displays results from seedlings treated with an overall concentration of 10µM mastoparan over time; 3.6: B, 50mM L-glutamate; 3.6: C, a combination 10µM mastoparan and 50mM L-glutamate. Error bars represent ±SD (n=5 replicates of treated seedlings). Two-way ANOVA between peaks of CaM, CRT, SITEII and ABRE *cis* elements at either 30 min or 70 min **** (P≤0.0001), *** (P≤0.001), ** (P≤0.01) * (P≤0.05) (p>0.0001) and Tukey *post hoc*.

When treated with mastoparan, seedlings containing the CaM box *cis* element recorded the highest luminescence activity >60 counts/s. Luminescent activity was recorded from all transgenic lines, but, the levels displayed were one third less (<40 counts/s) than those recorded from the CaM box line (Figures 3.4: A, 3.5: A and 3.6: A). When seedlings were treated with $CaCl_2$ a different response was generated (Figure 3.4: B), seedlings containing the CRT *cis* element displayed the highest luminescent activity ≥50 counts/s with all other transgenic lines showing a relatively low response <20 counts/s. A combined treatment of mastoparan and $CaCl_2$ resulted in a novel response (Figure 3.4: C) when compared to the two single treatments. Seedlings containing the CaM box *cis* element recorded luminescent levels >40 counts/s, resembling the results recorded in the single mastoparan treatment, however, the kinetics were different, in that a biphasic response was seen with peaks generated at 20 min and 70 min. All other transgenic lines showed low or no luminescence in the combined treatment, this was particularly evident in the CRT line which had displayed a high activity in the single $CaCl_2$ treatment but only recorded ≤2 counts/s with the combined treatment.

ATP treatment of the transgenic seedlings recorded high luminescence activity in both the ABRE and SITE II lines, 45 and 38 counts/s respectively (Figure 3.5: B). Interestingly both lines also recorded a biphasic response albeit with different kinetics e.g., different peak times and duration of peaks. Seedlings containing the CRT *cis* element also recorded some luminescent activity (12 counts/s), but this was low in comparison to both the ABRE and SITE II lines. When the ATP treatment was combined with mastoparan (Figure 3.5 C) a novel response was generated. The ABRE line showed similar results in both the ATP single treatment and the combined treatment, in that the luminescent count was the same and a biphasic response was recorded, yet the peaks themselves differed both temporally and spatially between the two treatments. The SITE II line recorded a lower luminescence activity in the combined treatment (28 counts/s) compared to the single ATP treatment. The SITE II biphasic response was lost in the combined treatment and the single peak displayed a gradual incline compared to a steep incline that was recorded in the single ATP treatment. The CRT line was completely suppressed in the combined treatment, however, the CaM box line which was supressed in the ATP treatment showed luminescence activity

in the combined treatment, but at a much lower level and later in time than that seen in single mastoparan treatment (Figure 3.5: A and 3.5: C).

The L-glutamate treatment displayed highest activity with seedlings containing the ABRE *cis* element, however, the overall response was low with luminescence counts <10 counts/s, when combined with mastoparan the counts were reduced to 2 counts/s. This could indicate that this calcium signature may not specifically regulate this *cis* element.

Results this far have indicated that specific calcium signatures regulate specific *cis* elements; this is further supported by statistical analysis suggesting that each response is statistically significant with $p < 0.0001$ (statistical analysis was conducted by measuring differences between the peaks generated from each $[Ca^{2+}]_{cyt}$ signature trace or cis element response).

To gain a more accurate insight to actual gene expression it will be necessary to perform RT-qPCR on transgenic plants (containing the specific promoter motifs of interest) which have been treated with one of the four agonists.

3.2.3 Gene expression in response to specific calcium signatures

To support the previous results recorded from the luciferase assays (3.2.2), gene expression was measured using RT-qPCR (see 2.2.6.10 for methods). Transgenic seedlings containing the constructs described in 3.2.2 were treated with one of the previously described calcium agonists, a combination of these agonist or water (methods 2.2.3.4), and RNA was extracted at various time points (2.2.6.7). Using RT-qPCR, *LUC+* gene expression was measured.

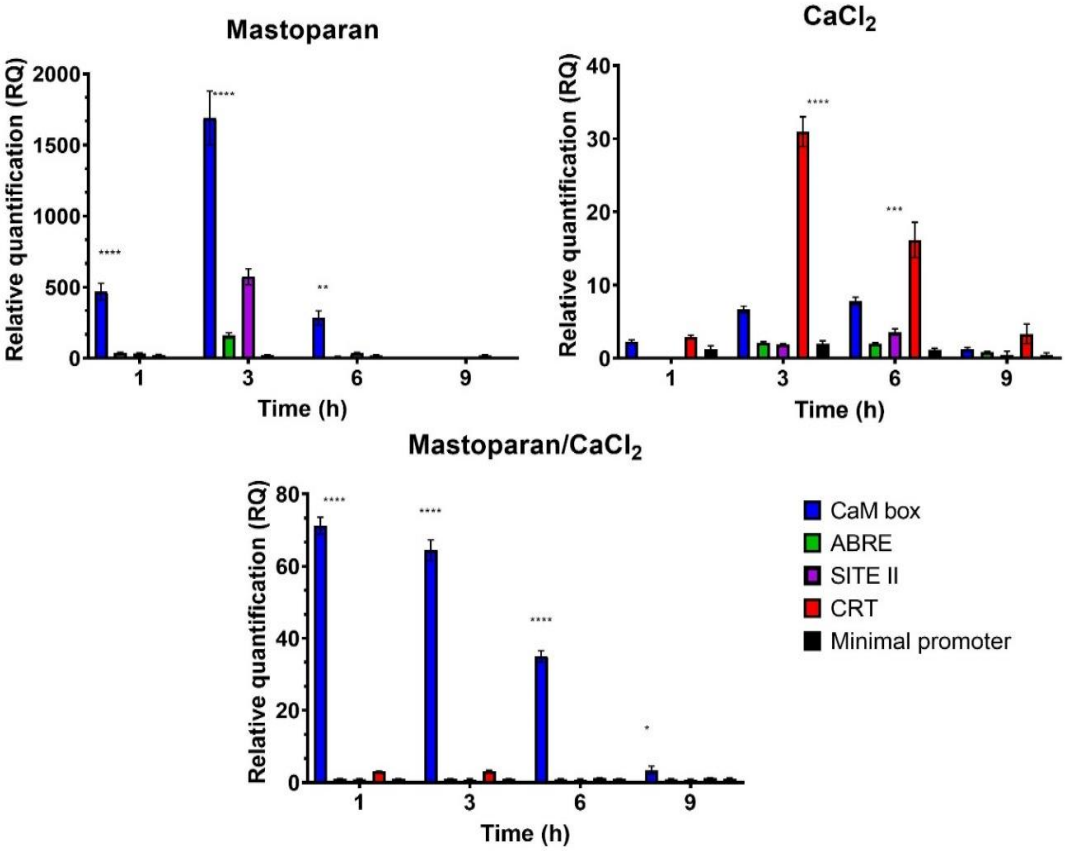


Figure 3.7: *LUC+* gene expression over time, measurements attained from 10-day old transgenic Arabidopsis seedlings containing constructs consisting of 1 of 4 calcium regulated *cis* elements and a *LUC+* sequence, or a control lacking a repeat *cis* element sequence (minimal promoter construct). Seedlings were treated with either an overall concentration of 10µM mastoparan, 50mM $CaCl_2$ or a combination of these agonists. Error bars represent ±SD (n=5 biological replicates). Two-way ANOVA, multiple comparison of gene expression of between 4 different *cis* elements and a control at various time points **** (P≤0.0001), *** (P≤0.001), ** (P≤0.01) * (P≤0.05) Tukey *post hoc*.

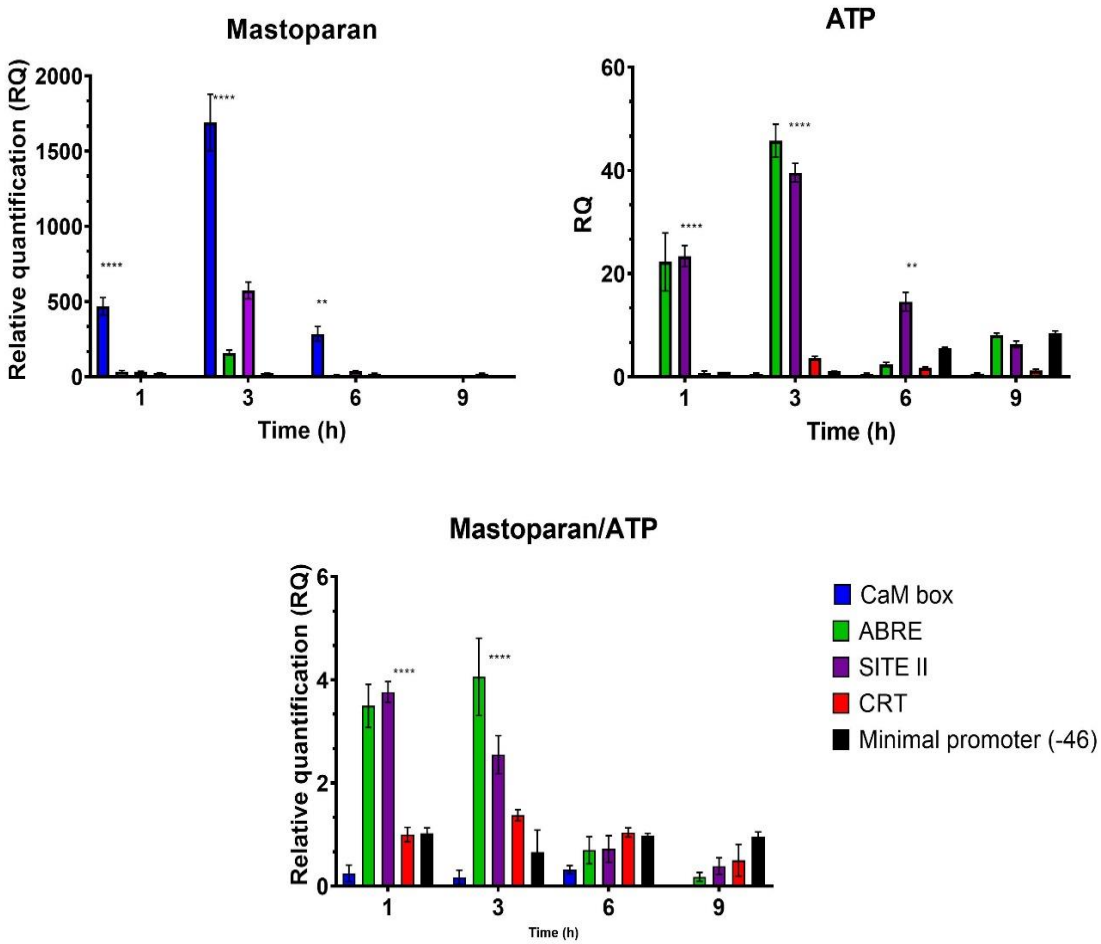


Figure 3.8: *LUC+* gene expression over time, measurements attained from 10 day old transgenic Arabidopsis seedlings containing constructs consisting of 1 of 4 calcium regulated cis elements and a *LUC+* sequence, or a control lacking a repeat cis element sequence. Seedlings were treated with either an overall concentration of 10µM Mastoparan 0.5mM ATPor a combination of these agonists. Error bars represent ±SD n=5 biological replicates). Two-way ANOVA, multiple comparison of gene expression of between 4 different *cis* elements and a control at various time points **** (P≤0.0001), *** (P≤0.001), ** (P≤0.01) * (P≤0.05) Tukey *post hoc*.

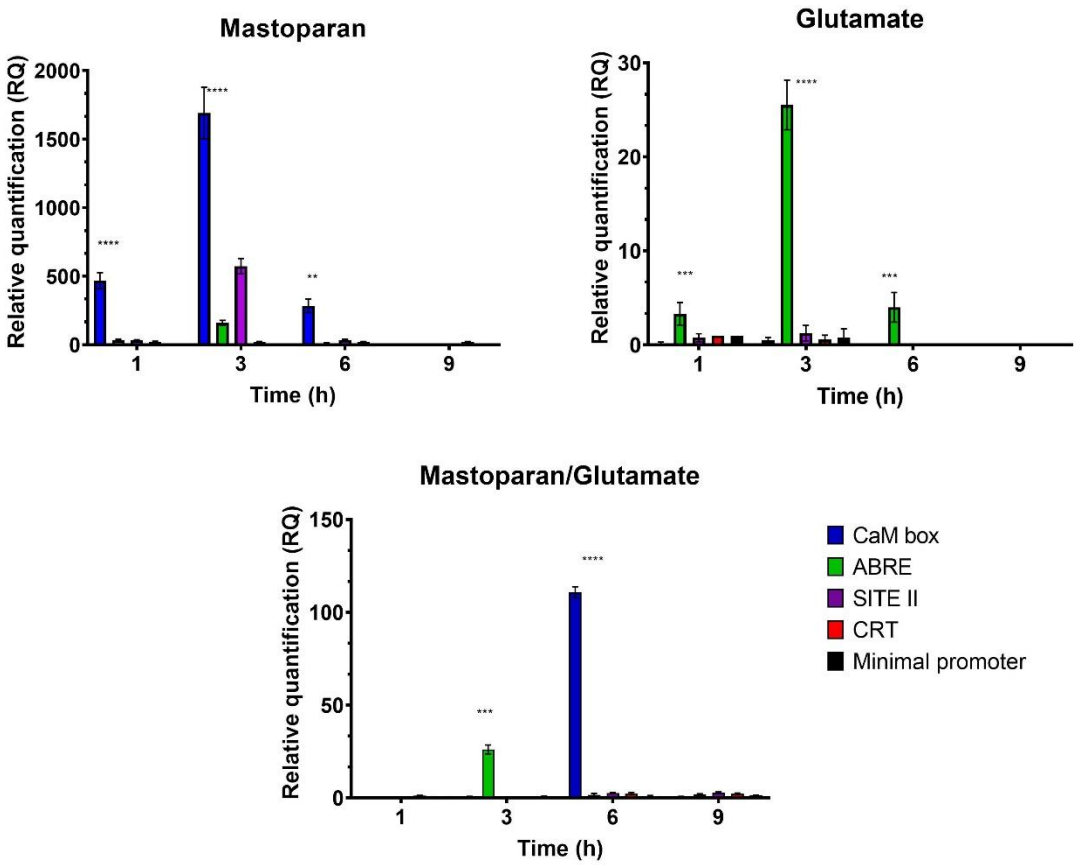


Figure 3.9: *LUC+* gene expression over time, measurements attained from 10 day old transgenic Arabidopsis seedlings containing constructs consisting of 1 of 4 calcium regulated cis elements and a *LUC+* sequence, or a control lacking a repeat cis element sequence. Seedlings were treated with either an overall concentration of 10µM mastoparan, 50mM L-glutamate or a combination of these agonists. Error bars represent SD± (n=5 biological replicates). Two-way ANOVA, multiple comparison of gene expression of between 4 different *cis* elements and a control at various time points **** (P≤0.0001), *** (P≤0.001), ** (P≤0.01) * (P≤0.05) Tukey *post hoc*.

When treated with mastoparan, seedlings containing the CaM box *cis* element displayed the highest gene expression, with maximal expression recorded at 3h. At 3h expression was also seen from ABRE and SITE II *cis* elements, however, this was relatively low when compared to CaM box at the same time point, as confirmed by statistical analysis showing a p-value <0.0001 (Figure 3.7). By comparison, $CaCl_2$ treatment yielded a high *LUC+* gene expression from seedlings containing the CRT *cis* element, as in the mastoparan treatment, maximal expression was observed at 3h (Figure 3.7). When the treatments were combined, a novel response was observed: *LUC+* gene expression was highest with CaM box, like that of the individual mastoparan treatment, however, expression patterns were different, in that maximal expression was recorded earlier at 1h compared to 3h which was seen in the single treatment. *LUC+* expression from CRT was downregulated relative to the expression measured with the single $CaCl_2$ treatment (Figure 3.7).

ATP treatment resulted in expression measured from *LUC+* driven by ABRE and SITE II, with the highest expression levels recorded at 3h. When ATP was combined with mastoparan similar expression patterns to the single ATP treatment were seen, however, CaM box, which was highly expressed in the singular mastoparan treatment, was down regulated in the combined treatment (Figure 3.8).

The L-glutamate treatment resulted in expression of *LUC+* genes driven by the ABRE *cis* element with negligible expression measured from the other *cis* elements. Expression was predominately recorded at 3h, considerably declining by 6h. The combined treatment of mastoparan and L-glutamate resulted in a novel response, initially ABRE driven-expression was measured at 3h, but by 6h CaM box was expressed and ABRE was down regulated, these data were supported by a two-way ANOVA yielding a p<0.001 (Figure 3.8).

These data suggest that different calcium signatures regulate different *cis* elements. When calcium agonists were combined, different gene expression patterns were evident as compared to single treatments, suggesting different calcium signatures could hold the information to initiate a substantially different response *via* downstream gene regulation. To determine if the same response is seen naturally, gene expression will need to be

observed from WT Arabidopsis genes that have the same *cis* elements as the synthetic concatemers and subjected to the same conditions.

3.2.4 Native gene expression in response to specific calcium signatures

Ten-day old WT Arabidopsis seedlings were treated with either an overall concentration of 10µM mastoparan, 50mM $CaCl_2$, 0.5 mM ATP, 50mM L-glutamate or a combination of these treatments (mastoparan/ATP, mastoparan/$CaCl_2$, mastoparan/L-glutamate) or water (see 2.2.3.4 for methods). RNA was extracted at three different time points and gene expression was measured using RT-qPCR (methods 2.2.6.10) of native genes whose promoters contained either CaM box, CRT, SITE II or ABRE, respectively.

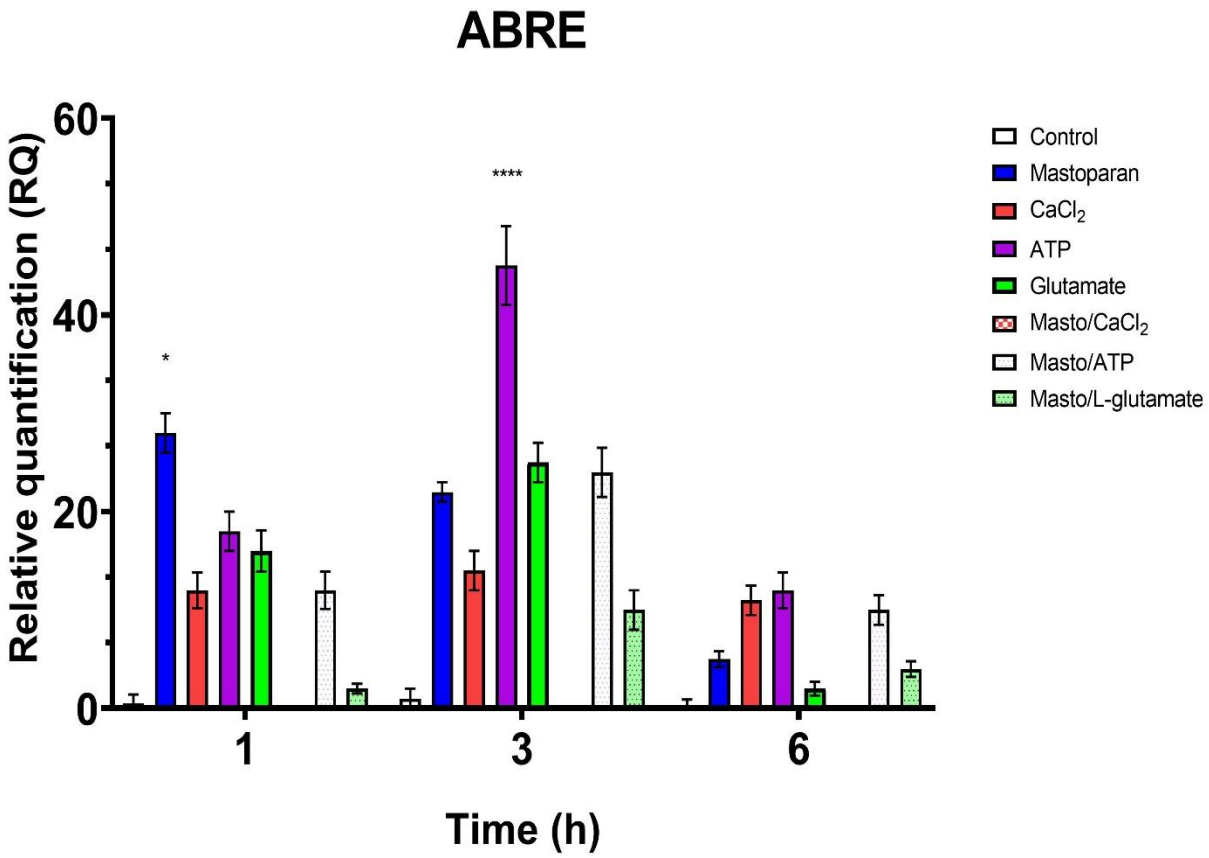


Figure 3.10: ABRE native gene expression measured at 3 specific time points. Measurements were obtained from 10-day old WT Arabidopsis seedlings treated with either an overall concentration of 10µM mastoparan, 50mM $CaCl_2$, 0.5mM ATP, 50mM L-glutamate or dH_2O. Error bars represent ±SD (n=5 biological replicates). A multiple comparison two-way ANOVA between calcium agonist treatments at 3 different time points**** (P≤0.0001), *** (P≤***(P≤0.001), ** (P≤0.01), * (P≤0.05) Tukey *post hoc*.

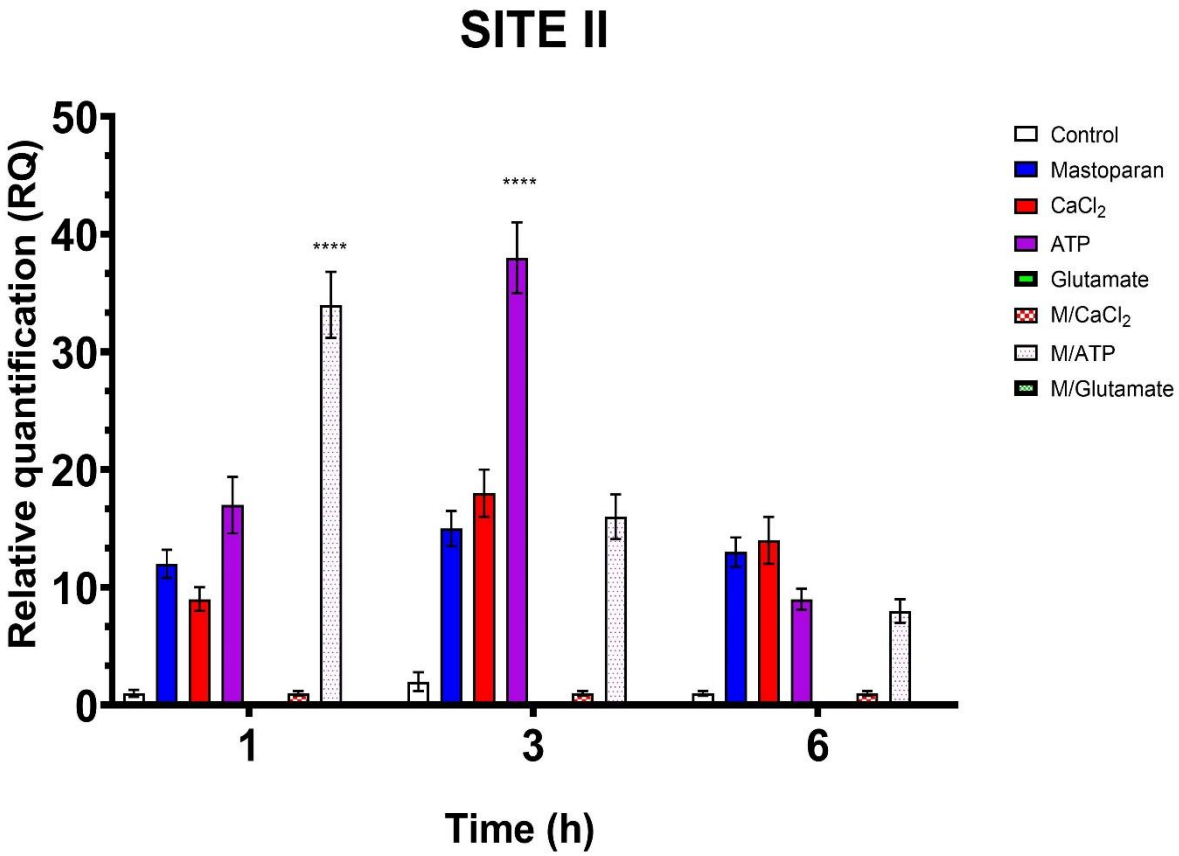


Figure 3.11: SITE II native gene expression measured at 3 specific time points. Measurements were obtained from 10-day old WT Arabidopsis seedlings pre-treated with either an overall concentration of 10µM mastoparan, 50mM CaCl2, 0.5mM ATP, 50mM L-glutamate or dH_2O. Error bars represent ±SD (n=5 biological replicates). A multiple comparison two-way ANOVA between calcium agonist treatments at 3 different time points**** (P≤0.0001), *** (P≤***(P≤0.001), ** (P≤0.01), * (P≤0.05) Tukey *post hoc*

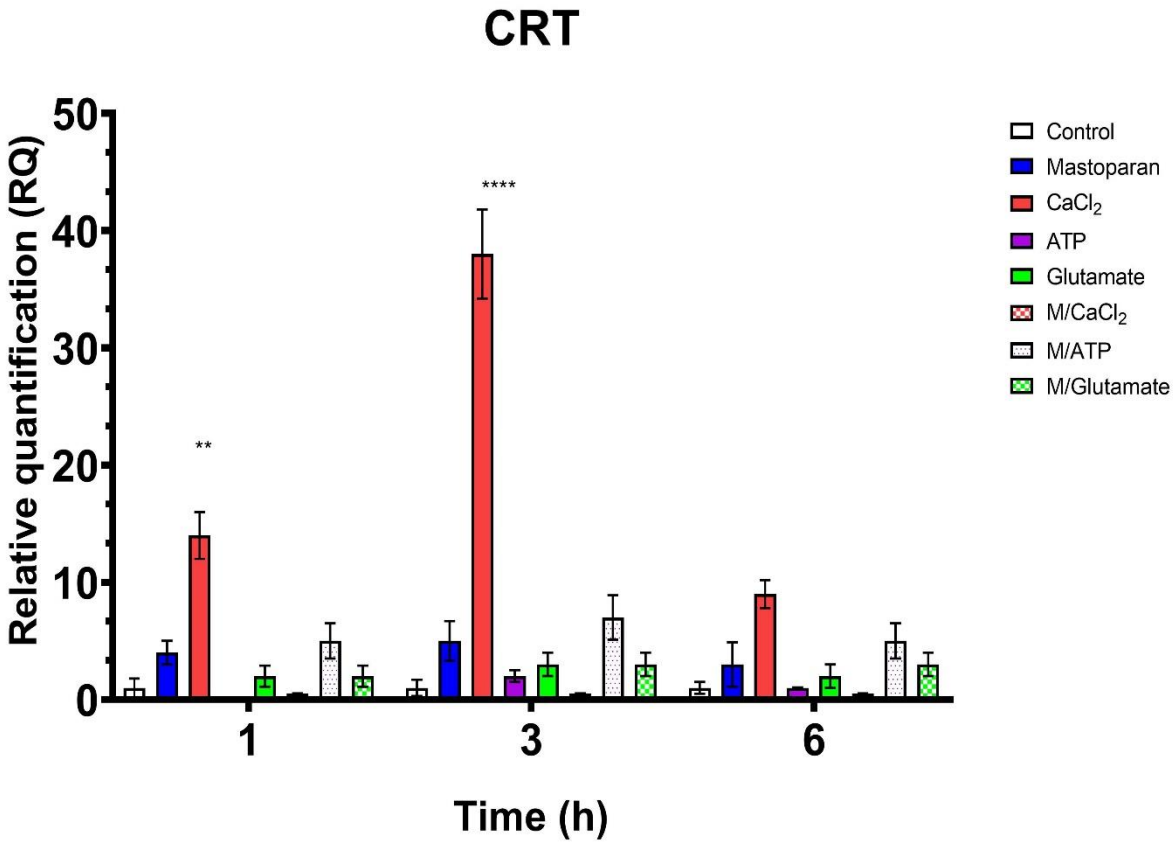


Figure 3.12: CRT native gene expression measured at 3 specific time points. Measurements were obtained from10 day old WT Arabidopsis seedlings pre-treated with either an overall concentration of 10µM mastoparan, 50mM CaCl2, 0.5mM ATP, 50mM L-glutamate or dH_2O. Error bars represent ±SD (n=5 biological replicates). A multiple comparison two-way ANOVA between calcium agonist treatments at 3 different time points**** (P≤0.0001), *** (P≤***(P≤0.001), ** (P≤0.01), * (P≤0.05) Tukey *post hoc*

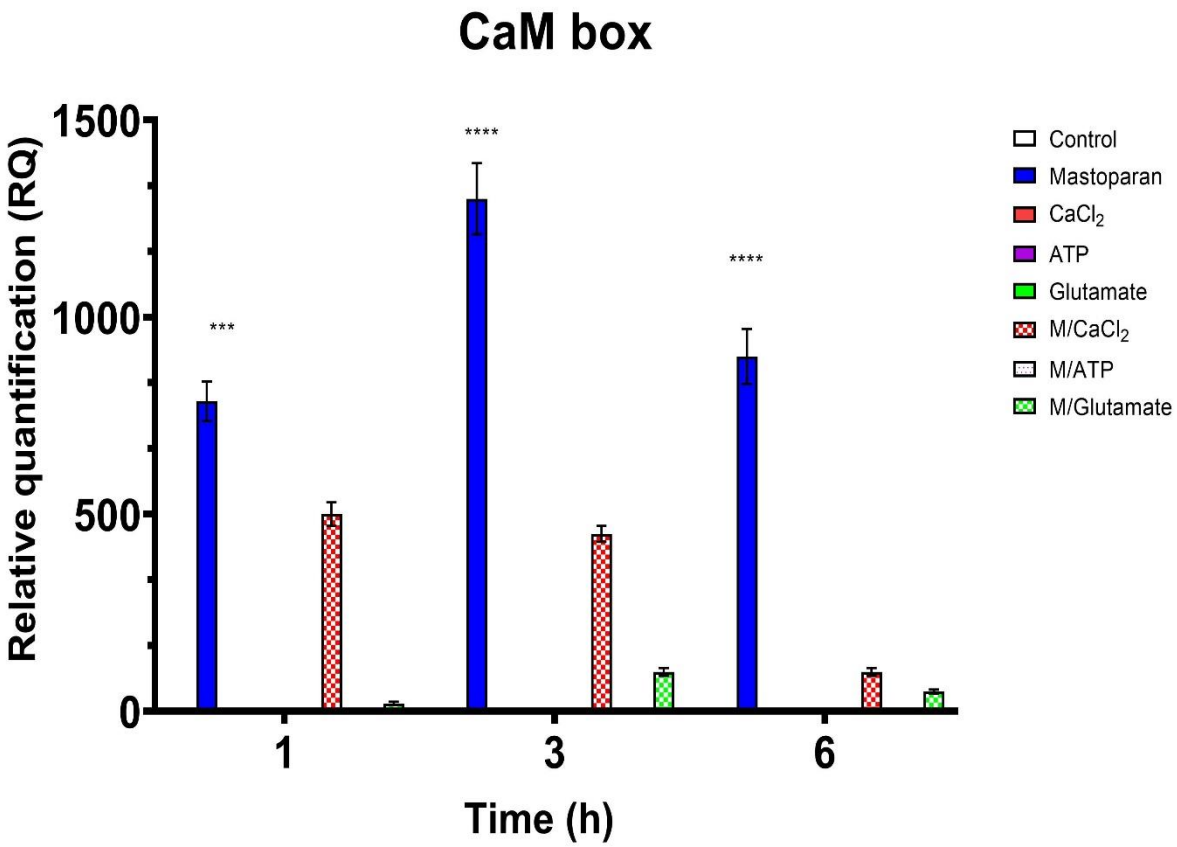


Figure 3.13: CaM box native gene expression measured at 3 specific time points. Measurements were obtained from10-day old WT Arabidopsis seedlings pre-treated with either an overall concentration of 10µM mastoparan, 50mM $CaCl_2$, 0.5mM ATP, 50mM L-glutamate or dH_2O. Error bars represent ±SD (n=5 biological replicates). A multiple comparison two-way ANOVA between calcium agonist treatments at 3 different time points**** (P≤0.0001), *** (P≤***(P≤0.001), ** (P≤0.01), * (P≤0.05) Tukey *post hoc*

The native genes displayed a gene expression pattern similar to the synthetic genes. The ABRE and SITE II genes recorded their highest expression levels in the ATP treatment with maximal expression measured at 3h (Figure 3.10; Figure 3.11). ABRE was also expressed in the L-glutamate treatment and a low expression of ABRE and SITE II was measured in the mastoparan and $CaCl_2$ treatments (Figure 3.10; Figure 3.11). The combination of mastoparan and ATP yielded a relatively high expression of ABRE and SITE II like the single ATP treatment. The mastoparan/L-glutamate treatment, however, yielded a relatively lower expression of ABRE and a minimal expression of SITEII compared to the single treatment of L glutamate. ABRE and SITEII showed no expression with the mastoparan/$CaCl_2$ treatment (Figure 3.10; Figure 3.11;) compared to the recorded expression in both single treatments. Statistical analysis confirmed that ABRE and SITE II gene expression were significantly different between calcium agonist treatments ($p<0.0001$).

The mastoparan treatment resulted in the highest gene expression level from the CaM box native gene with relatively low expression recorded from the other native genes (Figure 3.13). The $CaCl_2$ treatment resulted in a high gene expression measurement from the CRT native gene and low or no expression from the remaining native genes (Figure 3.12). A combination of mastoparan and $CaCl_2$ resulted in a high expression of the CaM box gene but relatively low expression of the CRT gene, compared to the single $CaCl_2$ treatment (Figure 3.12; Figure 3.13).

The native gene data supported the observations recorded from the synthetic genes displaying similar expression traits, therefore suggesting that calcium signatures were specific and regulated specific genes *via* the four *cis* elements in both a synthetic and natural context.

3.2.5 Investigating $[Ca^{2+}]_{cyt}$-dependency of *cis* element responses to calcium agonists

The results so far suggest that the *cis* elements are responsive to specific $[Ca^{2+}]_{cyt}$ elevations. To confirm that the responses were truly due to $[Ca^{2+}]_{cyt}$ signatures and not calcium-independent effects of the calcium agonists it was necessary to block the effects of the $[Ca^{2+}]_{cyt}$ in a controlled experiment. To do this I used lanthanum chloride ($LaCl_3$) as a calcium channel blocker, $LaCl_3$ has previously been shown to inhibit the action of calcium channels (Knight et al., 1996, Knight et al., 1997b, Knight et al., 1992). The optimal concentration of 1mM was determined experimentally as described by Whalley et al., (2011).

Ten-day old transgenic seedlings were either pre-treated with $LaCl_3$ or water, these seedlings were subsequently treated with 1 of 3 calcium agonist ($CaCl_2$; ATP or L-glutamate) or a water control gene expression was measured at the 3h time point (2.2.6.10). It was not necessary to treat the transgenics with mastoparan as Whalley et al., (2011) had previously shown that *cis* element responses to mastoparan were $[Ca^{2+}]_{cyt}$ dependent.

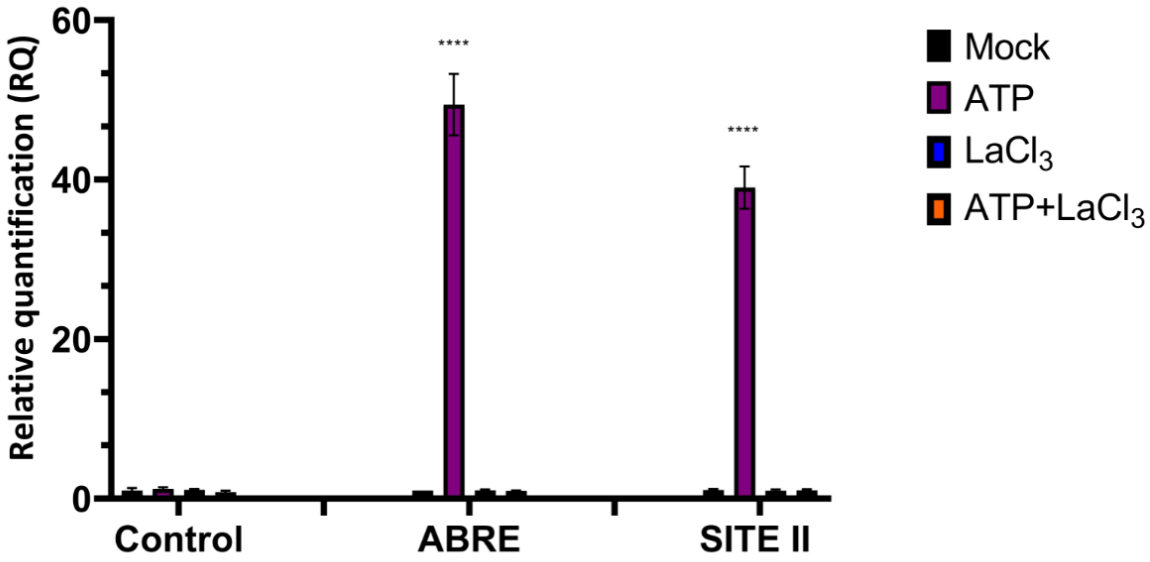


Figure 3.14: Arabidopsis seedlings expressing *LUC+* under the control of either a minimal promoter only (control) or four repeats of the cis element sequences ABRE or SITE II. Seedlings were pre-treated with either 1mM $LaCl_3$ or water for 30 min before adding ATP to a final concentration of 0.5mM. Tissue was harvested at 3h and *LUC+* gene expression was measured using RT-qPCR. Each value is the mean of 3 biological replicates. A multiple comparison two-way ANOVA of gene expression of either ABRE, SITE II or a control between treatments **** (P≤0.0001), *** (P≤0.001), **(P≤0.01), * (P≤0.05) *post hoc* Tukey.

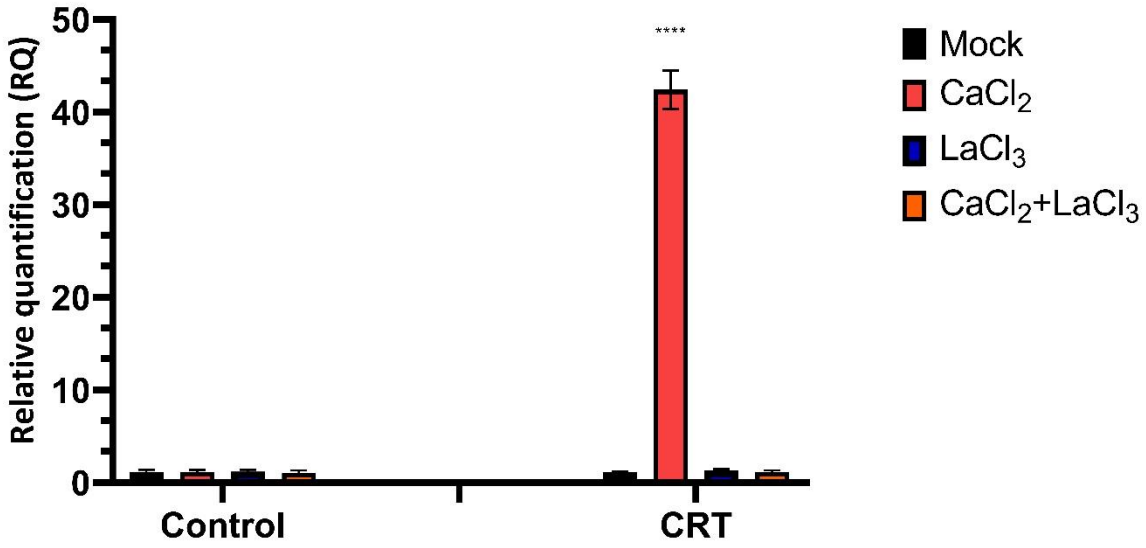


Figure 3.15 Arabidopsis seedlings expressing *LUC+* under the control of either a minimal promoter only (control) or four repeats of the cis element sequence CRT. Seedlings were pre-treated with either 1mM $LaCl_3$ or water for 30 min before adding $CaCl_2$ to a final concentration of 250mM. Tissue was harvested at 3h and *LUC+* gene expression was measured using qPCR. Each value is the mean of 3 biological replicates. A multiple comparison two-way ANOVA of gene expression of either CRT or a control between treatments **** (P≤0.0001), *** (P≤0.001), **(P≤0.01), * (P≤0.05) *post hoc* Tukey.

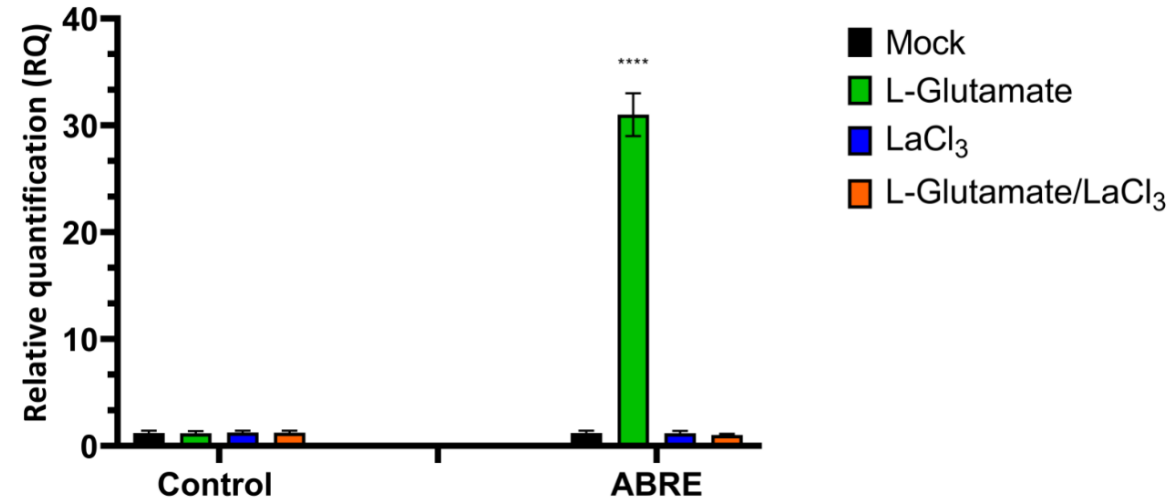


Figure 3.16: Arabidopsis seedlings expressing *LUC+* under the control of either a minimal promoter only (control) or four repeats of the *cis* element sequence ABRE. Seedlings were pre-treated with either 1mM $LaCl_3$ or water for 30 min before adding L-glutamate to a final concentration of 50mM. Tissue was harvested at 3h and *LUC+* gene expression was measured using qPCR. Each value is the mean of 3 biological replicates. A multiple comparison two-way ANOVA of gene expression of either ABRE or a control between treatments **** (P≤0.0001), *** (P≤0.001), **(P≤0.01), * (P≤0.05) *post hoc* Tukey.

Results show that $CaCl_2$, ATP and L glutamate driven expression of the promotor motifs was inhibited when samples were pre-treated with $LaCl_3$ (figures 14, 15 and 16). These results suggest that *cis* element responses to all three calcium agonists were calcium dependent. This was further supported by statistical analysis showing that indeed, expression between $LaCl_3$ pre-treated samples and water samples that were subsequently treated with one of the calcium agonists were strongly significantly different.

3.3 Discussion

3.3.1 Calcium signature specificity: the decoding of calcium signatures.

It has been shown that calcium is an intermediate from stimulus perception to plant response and the nature of this information is conserved (Galon et al., 2010), however, transient calcium elevations occur in response to a myriad of different stimuli both abiotic and biotic such as the response to cold, drought, heat and pathogens, initiating an appropriate downstream response (Allen et al., 2001, Knight et al., 1996, Knight et al., 1997b, Larkindale and Knight, 2002, Rentel and Knight, 2004, Wais et al., 2000). How does the plant distinguish between calcium signals and then initiate an appropriate response? For several decades the scientific community has tried to answer this question.

In 1998 MCAnish and Hetherington (McAinsh and Hetherington, 1998) developed a theory known as the "calcium signature hypothesis" which suggested that the calcium signature itself may contain the information that determines specificity. It is accepted that in animal cells spatial and temporal heterogenetics in calcium play an essential role in the encoding of stimuli specific signals (Berridge et al., 2000), so it would seem feasible that this response could be mirrored in plants. The "calcium signature hypothesis" has been supported by evidence of specific signalling and decoding from research in stomatal guard cells (Allen et al., 2001) and symbiosis signalling in legumes (McAinsh and Pittman, 2009). More recent research by Whalley and Knight (2013) and Lenzoni et al., (2018) has suggested that indeed different calcium signatures do in fact differ kinetically and regulate specific genes, however, the range of signatures in their work was limiting.

Data acquired in this work has supported initial findings by Lenzoni et al., (2018) but has taken their findings one step further to a greater understanding of signature specificity. By combining two single calcium agonists novel signatures were generated, the kinetics of the new signatures were different to that of the single signatures further supporting the earlier work by Lenzoni et al., (2018), and suggesting that each signature was indeed specific. It would be interesting to determine if further novel signatures could be generated with different calcium agonist combinations such as ATP/ $CaCl_2$ for instance. In this work I decided to combine each agonist with mastoparan as mastoparan had shown the highest

response in the single treatments, but ideally several different combinations would give a broader result.

3.3.2 Calcium signature specificity: transcript regulation

Data from the luciferase assays (3.2.2) suggests that known calcium signatures with particular signature kinetics (3.2.1) regulate specific *cis* elements. This work has shown that combined calcium agonists which have been shown to induce novel signatures (3.2.1) specifically regulate different TFs or combinations of TFs. The CaM box *cis* element seemed to be predominately regulated by mastoparan with little activity recorded from any of the other *cis* elements. When combined with $CaCl_2$ which predominately regulates the CRT *cis* element, CaM box is still relatively highly expressed, and CRT seems to be suppressed. This suggests that the response has prioritised the mastoparan part of the signature, however, the kinetics from both signature and luciferase activity are very different to the single treatments. The fact that the mastoparan/$CaCl_2$ treatment had induced a biphasic response could indicate that the signature is occurring at different times within different cell types.

The ATP treatment generated a different response to that seen with the mastoparan, with ABRE and SITE II *cis* elements recording high levels of activity, interestingly both *cis* element responses displayed a biphasic response. Literature has indicated that ATP often generates a biphasic response (Clark and Roux, 2018) however this literature was not looking at the response at gene level, it would be interesting to determine if the biphasic response recorded in my work was found within the root itself or between different cells. It is important to note when ATP is combined with mastoparan this biphasic response is lost from the SITE II kinetics, which is very different from the mastoparn/$CaCl_2$ treatment where a biphasic response was generated, further supporting the specificity of the signatures and how they specifically regulate responses.

Although we can be confident that the results recorded from the luciferase assays were induced by calcium it was important that I confirmed that luciferase activity was not induced from a calcium-independent effect of the agonist. Blocking the calcium channels with $LaCl_3$ confirmed that indeed the *cis* elements were calcium-dependent, supporting work by Whalley et al., (2011).

The luciferase assays, however, are limiting in that they measure luciferase activity rather than gene expression, qPCR enabled a different assessment of specific calcium signature downstream regulation. These data support the initial findings recorded from the luciferase assays, in that all the signatures seemed to specifically regulate specific *cis* elements. Furthermore, the L-glutamate results indicated that indeed, this signature specifically regulated ABRE: ABRE activity had been seen with the luciferase assays but at a very low luminescence level. From the luciferase assays I could not conclude that L-glutamate specifically regulated any of the target *cis* elements, but from the qPCR I can with some confidence suggest that indeed L-glutamate specifically regulates ABRE, this is further supported by statistical analysis.

3.4 Conclusion

Calcium signatures display specific novel kinetics, and these individual signatures regulate different *cis* elements specifically; both in a synthetic and natural environment. These results have reinforced findings by Whalley *et al*., (2011), Whalley and Knight (2013) and Lenzoni et al., (2018) and supported my hypothesis.

The aim of this work was to increase the repertoire of novel signatures from the initial findings of Lenzoni *et al*., (2018) and to test specificity against known calcium regulated *cis* elements identified by Whalley et al., (2011). Further investigations will be required, firstly, to ascertain if any of the signatures generated from the single treatments or the combined treatments regulate the *COR* genes *KIN2* and *LT178* during osmotic stress, and if so, is the response specific? (Chapter 4). Secondly, to look at the biphasic response in more detail to determine if the signature is occurring in different cells, or in the same cells at different times (Chapter 5). Finally, to investigate responses generated by specific calcium signatures when regulating a combination of two *cis* elements (Chapter 6). It has been shown previously that genes often have two or more *cis* elements, for example, the Arabidopsis gene *RD29A* has at least two *cis* acting elements, one involved in the ABA response to dehydration (ABRE) and the other (DRE) is induced by fluctuations in osmotic potential and is ABA-independent (Yamaguchi-Shinozaki and Shinozaki, 1994).

4.Osmotic Stress and calcium signature specificity

4.1 Introduction

The data from chapter three suggest that specific agonists generate novel signatures which regulate particular genes and TFs. Leading on from this it would be interesting to determine if these specific signatures can regulate stress genes, leading to an improved tolerance to that particular stress.

In a natural environment a plant can be exposed to a myriad of environmental stresses and the ultimate response from the plant determines the plant's survival (Rizhsky et al., 2004). One of the plant's main response against stress is through changes in gene expression, so it is vital that a plant receives the correct information to respond to a specific stimulus. Previous literature has highlighted the role of calcium in the plant stress response (Bickerton, 2012, Hetherington and Brownlee, 2004, Knight, 2000, Knight et al., 1996, Knight et al., 1997a, Rudd and Franklin-Tong, 1999, Sanders et al., 2002), indicating that when a plant senses a stress it responds with a rise in intracellular calcium which leads to a change in gene expression. Literature has suggested that because each stimulus is unique, and requires the appropriate response needed for survival, that each calcium signal differs both spatially and temporally; termed as calcium signatures (McAinsh and Hetherington, 1998, Ng and McAinsh, 2003).

It has been suggested that calcium signatures are specific and regulate particular genes and TFs (Kaplan et al., 2006, Lenzoni, 2017, Whalley and Knight, 2013, Whalley et al., 2011). The work in the previous chapter has supported the hypothesis put forward by Whalley et al., (2011) and Whalley and Knight (2013), suggesting that one or more genes are particularly regulated by a specific calcium signal, and in fact several genes can be regulated by a specific calcium signature. However, it is not known if the expression of these genes leads to enhanced stress tolerance through increased expression of proteins.

Osmotic stress which occurs during drought, heat, cold and high salinity can cause changes in the metabolism, growth and physiology of the plant (Monclus et al., 2006) . Osmotic

stress can lead to a reduction in turgor, photosynthesis, stomatal conductance, and increase in cell damage (Soares-Cordeiro et al., 2009, Xu et al., 2013, Xu et al., 2008). Plants employ several mechanisms to respond and adapt to osmotic stress conditions (Diaz-Espejo et al., 2007) including changes in gene expression (Knight et al., 1997a, Xiong et al., 2002b). It has been demonstrated that calcium deficiencies in plants increase susceptibility to osmotic stress (Xiong et al., 2002b). It has also been demonstrated that particular stress genes, *KIN2* and *LT178* for example, are regulated by calcium during osmotic stress (Knight et al., 1997a, Xiong et al., 2002b).

Questions to be addressed in this chapter

- Do specific calcium signatures regulate osmotic stress-induced genes which may subsequently lead to osmotic stress tolerance?
- If there is specificity in the response, is it affected by the age of the plants?

4.2 Results

4.3.1 The influence of calcium signature specificity on chlorophyll levels during osmotic stress.

Osmotic stress often induces cell damage which may lead to chlorophyll loss (Jagtap et al., 1998). To determine calcium signature specificity during osmotic stress I have measured the effect of osmotic stress and tolerance physiology in Arabidopsis plants. Ten-day old Arabidopsis seedlings were pre-treated for 1h with an overall concentration of either 0.5mM ATP, 50mM $CaCl_2$, 1mM L-glutamate, 10µM mastoparan or a combination of these treatments with mastoparan (mastoparan/ATP, mastoparan/$CaCl_2$ or mastoparan/L-glutamate) or water. After 1hr the seedlings were removed from the pre-treatment and further treated with an overall concentration of 300mM mannitol, to induce osmotic stress, or water for a control. After 10 days chlorophyll was extracted and measured as described in chapter two (2.2.7.2).

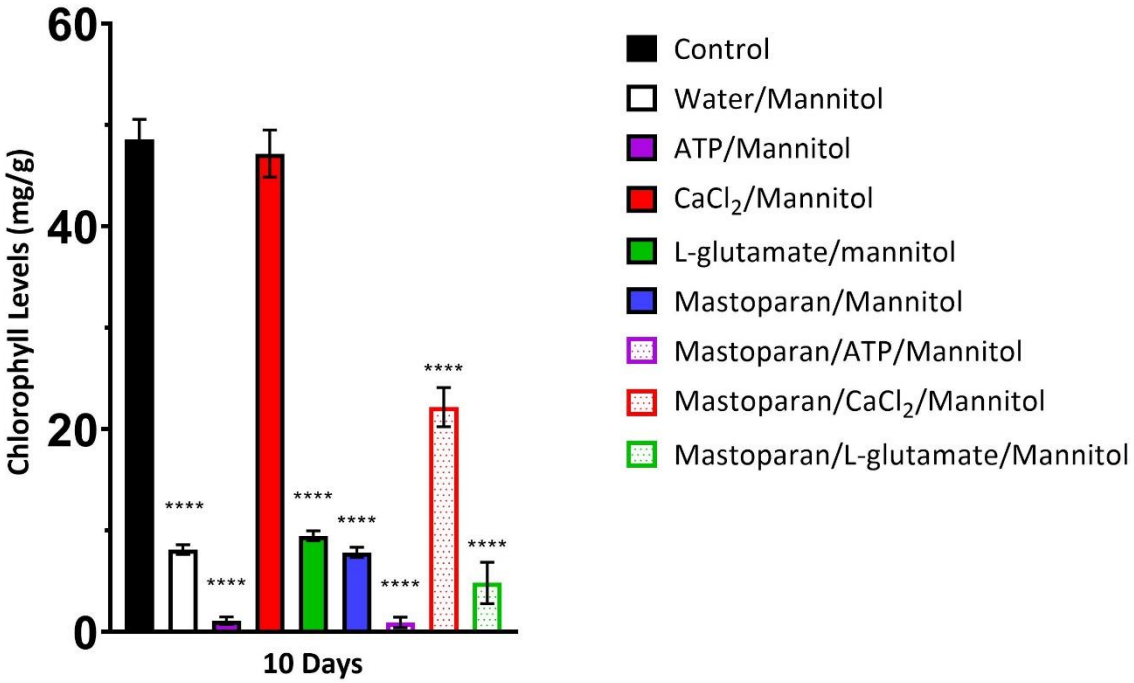


Figure 4.1: Chlorophyll levels measured from 10d old Arabidopsis seedlings subjected to 10 days of osmotic stress (induced by an overall concentration of 300mM mannitol) or water (control). Seedlings were pre-treated with either water or an overall concentration of 0.5mM ATP, 50mM $CaCl_2$, 1mM L-glutamate, 10µM mastoparan or combination of these treatments. Error bars represent ± SD (n=3 pseudo-biological replicates). Student Two tailed T Tests **** (P≤0.0001), *** (P≤***(P≤0.001), ** (P≤0.01), * (P≤0.05) between control and one treatment.

Following osmotic stress, seedlings pre-treated with $CaCl_2$ displayed chlorophyll levels of 46mg/g of fresh weight (FW), which was insignificantly different to that of the non-stressed control levels of 48mg/g (Figure 4.1). All other pre-treated seedlings displayed chlorophyll levels <10mg/g (FW) apart from the mastoparan/$CaCl_2$-treated seedlings which registered a level of 22mg/g (FW) (Figure 4.1). Statistical analysis from two tailed T tests indicated that all treated seedlings except those treated with the single $CaCl_2$ treatment were significantly different to the control. These data suggest that pre-treatment of $CaCl_2$ may confer osmotic stress tolerance to Arabidopsis seedlings. To investigate further it was necessary to repeat the conditions, and measure gene expression from *COR* genes that are known to be both expressed during osmotic stress and are calcium-regulated (Knight et al., 1997b).

4.3.2 Gene expression of *KIN2* and *LT178* genes during osmotic stress

Ten-day old Arabidopsis seedlings were treated as previously described in 4.3.1, RNA was extracted at 3h, 6h and 24h (see 2.2.6.7 for detailed methods) and gene expression was measured from the *COR* genes *KIN2* and *LT178*. Chlorophyll levels were also measured after 10 days as described in chapter 2 (2.2.7.2). To determine if $CaCl_2$-induced stress tolerance was not age limited I ran a mirrored stress experiment replacing the seedlings with 4-week-old mature Arabidopsis leaves, measurements of both gene expression and chlorophyll levels were recorded at the same time points as the seedling experiments described in 4.3.1.

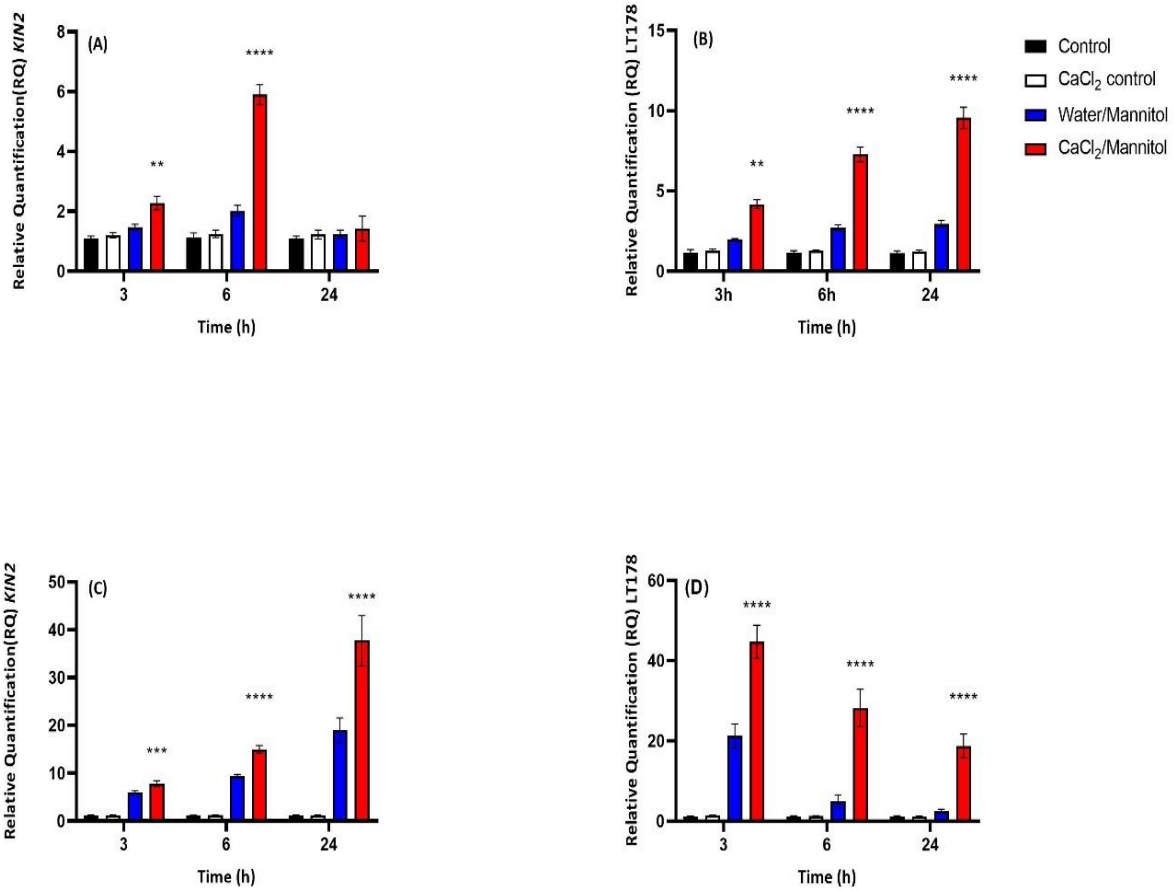


Figure 4.2: *COR* gene expression at three specific time points. Measurements were obtained from either 10d old Arabidopsis seedlings or leaves from 4-week-old Arabidopsis plants. Seedlings and leaves were pre-treated with an overall concentration of 50mM $CaCl_2$ or water followed by a treatment of 300mM of mannitol or water.

Figures 4.2: A and B: gene expression from *KIN2* and *LT178* genes respectively from seedlings.

Figures 4.2: C and D: gene expression from *KIN2* and *LT178* genes respectively from mature Arabidopsis leaves.

Error bars represent ±SD (n=3 biological replicates). Two-way ANOVA between treatments at 3 different time points **** (P≤0.0001), *** (P≤***(P≤0.001), ** (P≤0.01), * (P≤0.05) Tukey *post hoc*.

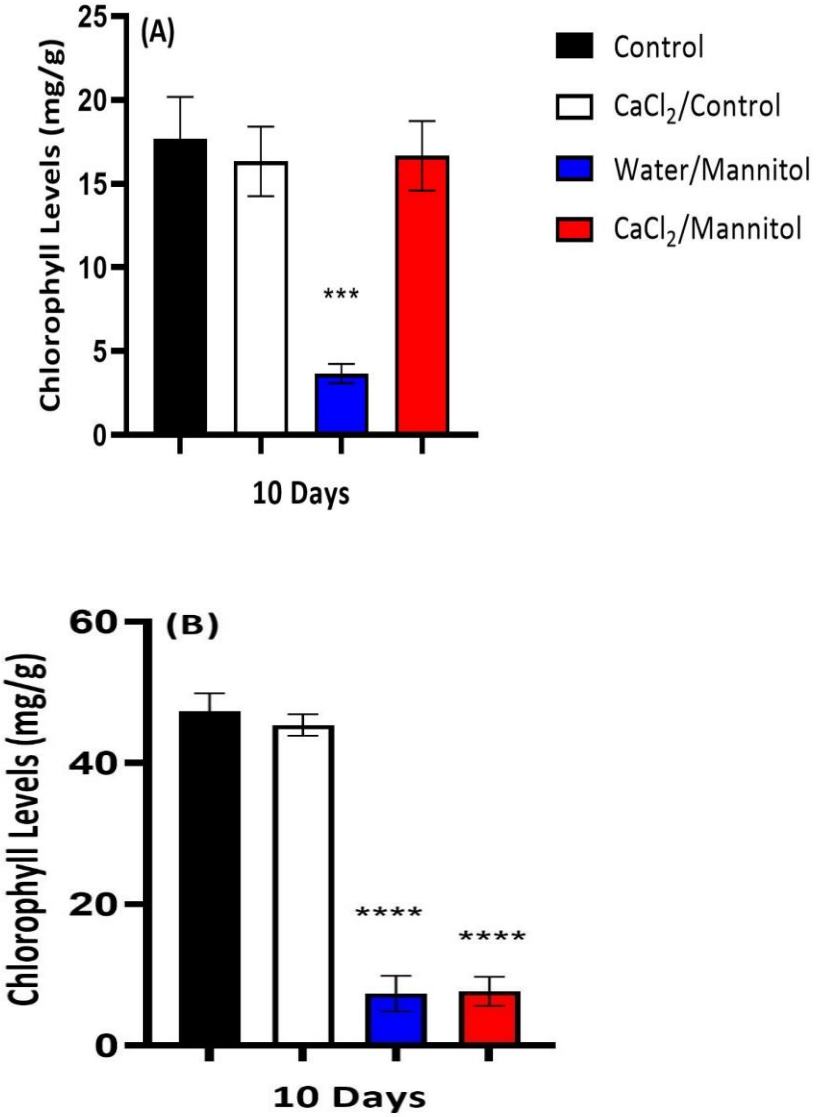


Figure 4.3: Chlorophyll levels from Arabidopsis seedlings or leaves over 10 days. Plant tissue has been pre-treated with an overall concentration of 50mM of $CaCl_2$ or water and then treated with 300mM mannitol or water as a control.

Figure 4.3: A. Ten-day old Arabidopsis seedlings.

Figure 4.3: B. Four-week-old Arabidopsis leaves.

Error bars represent ± SD (n=3 pseudo-biological replicates) Two tailed T Tests **** (P≤0.0001), *** (P≤***(P≤0.001), ** (P≤0.01), * (P≤0.05) between control and one treatment.

Seedlings pre-treated with $CaCl_2$ recorded the highest *KIN2* gene expression (Figure 4.2: A). The highest levels were recorded at the 6h timepoint, with seedlings that were pre-treated with $CaCl_2$ registering >5 fold and 3.5-fold difference compared to both controls and the water/mannitol seedlings, respectively (Figure 4.2: A). In the mature leaf assays, $CaCl_2$ pre-treated leaves registered the highest levels of Chlorophyll at 24h with a level of 38mg/g compared to 1mg/g and 12mg/g in the controls and water/mannitol treatments, respectively (figure 4.2: C).

Gene expression of *LT178* was seen to be most prevalent in $CaCl_2$ pre-treated seedlings, with the highest expression measured at the 24h timepoint; 6 times that of the water control and the $CaCl_2$ control (Figure 4.3: B). In the mature leaf assays *LT178* expression was again the highest in leaves pre-treated with $CaCl_2$ (Figure 4.3: D), however, the highest expression was recorded earlier at the 3h timepoint measuring 48mg/g.

Chlorophyll levels from seedlings measured after 10 days of osmotic stress indicated that there were similarities between the water control, the $CaCl_2$ control and the $CaCl_2$ pre-treated seedlings (Figure 4.3: A). These data supported the earlier findings in 4.3.1 (Figure 4.1), suggesting that $CaCl_2$ pre-treatment could prime the seedlings towards osmotic stress tolerance. Chlorophyll levels measured from the leaf assays, however, did not indicate osmotic stress tolerance in leaves pre-treated with $CaCl_2$ (Figure 4.3: B). Chlorophyll levels decreased in the mature plant assays, with the $CaCl_2$ pre-treated leaves during osmotic stress displaying similar levels of chlorophyll to the water/mannitol leaves of 10mg/g (FW). Student T Tests suggested there was a significant difference between the $CaCl_2$/mannitol treatment and both controls (Figure 4.3: B).

To further investigate, I will test various concentrations of $CaCl_2$, to try to find an optimum concentration for the mature leaves to display osmotic tolerance

4.3.3 Does changing the concentrations of calcium chloride pre-treatment confer osmotic tolerance to mature *Arabidopsis thaliana*?

To establish an optimal concentration of $CaCl_2$ for successful osmopriming in mature Arabidopsis plants, leaves from 3-week old plants expressing cytosolic aequorin (pMAQ2) were treated with three different concentrations of $CaCl_2$ (250mM, 300mM or 400mM) or

water and $[Ca^{2+}]_{cyt}$ responses to these additions were measured from an average of 5 leaves (detailed methods 2.2.4.2). From the subsequent calcium signatures, I could visualise individual responses generated from each treatment. Although the signature patterns from the 250mM, 300mM and the 400mM were similar, the magnitude of increase after the injection was much greater and the basal levels remained higher in the 400mM signature (Appendix D1.). From these traces it was determined that the optimum concentration of $CaCl_2$ was 400mM (Appendix D.1).

Going forward with this concentration a further osmotic stress assay was conducted as previously described in 4.3.2, however, it was seen that by day three all plant tissue had undergone cell death and no chlorophyll was detected by day ten (Appendix D.2), implying that the plants could not tolerate high concentrations of $CaCl_2$. It is possible that the high concentrations of calcium may in fact cause further ionic and osmotic stress to the plants.

4.3.3.1 Does calcium chloride pre-treatment of Arabidopsis seeds lead to osmotic stress tolerance in mature plants?

As applying high concentrations of $CaCl_2$ directly to mature leaves proved to be lethal, I decided to approach the pre-treatment differently and apply the $CaCl_2$ at the seed stage, which has been shown previously to be successful for osmopriming in maize (Khalil et al., 2014), wheat (Farooq et al., 2017) and barley (Kaczmarek et al., 2016). Seeds were pre-treated with four different concentrations of $CaCl_2$ (5mM, 50mM, 250mM or 500mM) or water and grown for four weeks (methods described in detail for chemical preparation in 2.2.3.3 and growth conditions in 2.2.2). A total of 3 leaves from each treatment was subjected to mannitol-induced osmotic stress or water for 10 days (as described in 2.2.7.1). RNA was extracted at three different time points: 3, 6 and 24h (methods in 2.2.6.7), and gene expression was measured from *COR* genes *KIN2* and *LT178* (detailed methods 2.2.6.10).

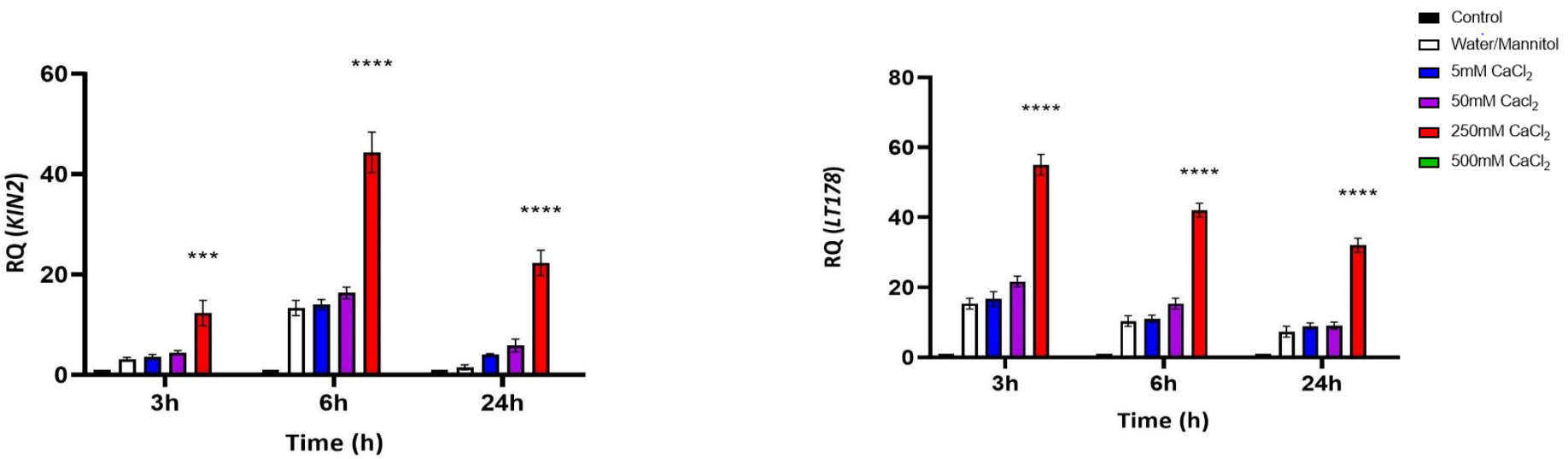


Figure 4.4: *COR* gene expression at three specific time points. Measurements were obtained from 4-week-old Arabidopsis leaves which had been pre-treated at seed stage with either 5mM, 50mM, 250mM or 500mM of $CaCl_2$ or water. All leaves had been further treated by 300mM of mannitol to induce osmotic stress or water.

Figure 4.4 (A). *KIN2* gene expression at 3, 6 and 24h.

Figure 4.4 (B). *LT178* gene expression at 3, 6 and 24h.

Error bars represent ±SD (n=3 biological replicates). Two-way ANOVA between treatments at 3 different time points **** (P≤0.0001), *** (P≤***(P≤0.001), ** (P≤0.01), * (P≤0.05) Tukey *post hoc.*

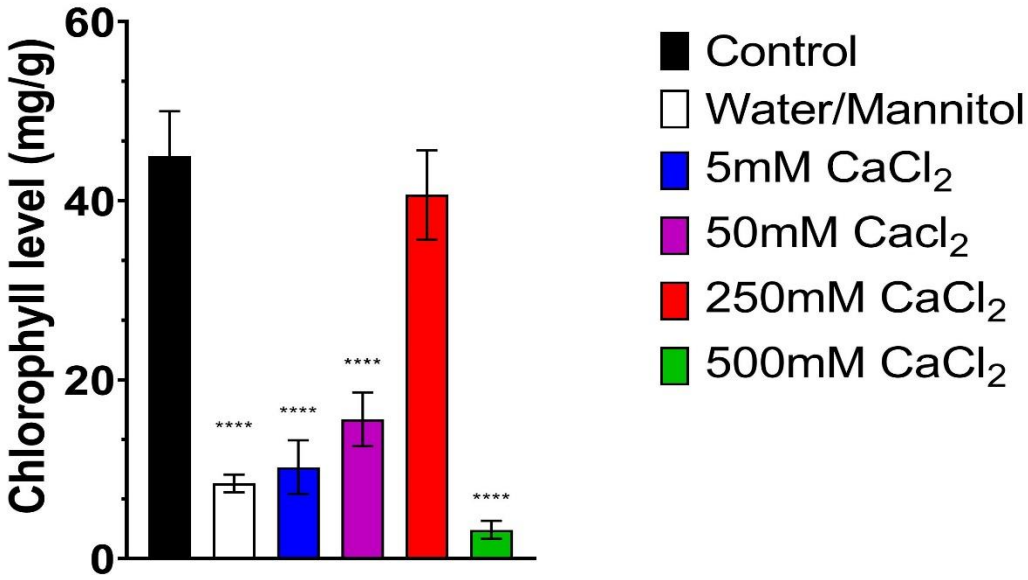


Figure 4.5: Chlorophyll concentration from 4-week-old Arabidopsis leaves over 10 days. Plant tissue has been pre-treated at seed stage with either 5mM, 50mM, 250mM or 500mM of $CaCl_2$ or water and then treated with 300mM mannitol or water (control) for 10d.

Error bars represent ±SD (n=3 biological replicates). Two tailed T Tests **** (P≤0.0001), *** (P≤***(P≤0.001), ** (P≤0.01), * (P≤0.05) between control and one treatment.

Both *KIN2* and *LT178* displayed their highest expression from the leaves that had been pre-treated with 250mM of $CaCl_2$, albeit with different expression patterns (Figure 4.4: A and B). *KIN2* measured highest expression at 6h (Figure 4.4: A) whereas *LT178* measured the highest expression at 3h (Figure 4.4: B). However, at all three time points with both genes the 250mM samples displayed significantly higher expression compared to all of the other treated samples and the controls. The expression levels of *COR* genes in plants treated with the 500mM concentration was lower than what was seen in the control, this is evident by the lack of representation on the graph (Figure 4.4). These data compared favourably to what was seen in the earlier gene expression measurements from mature leaves in 4.3.2.

After 10d of osmotic stress, plants that had been pre-treated at the seed stage with 250mM of $CaCl_2$ displayed a chlorophyll level of 43 mg/g (FW) which was similar to the control concentration of 45mg/g (FW). Two tailed T tests between the control and the 250mM samples suggest that there was no significant difference between these two samples (Figure 4.5). The 500mM concentration yielded a low level of chlorophyll suggesting that this level of $CaCl_2$ could be lethal to the plant.

Both the gene expression and the chlorophyll level data suggest that pre-treatment with 250mM of $CaCl_2$ at the seed stage, seems to confer osmotic stress tolerance to mature Arabidopsis plants.

4.4 Discussion

4.4.1 Do specific calcium signatures display specificity during osmotic stress?

The pre-treatment of $CaCl_2$ yielded the highest chlorophyll levels comparing favourably to the control, suggesting that $CaCl_2$ treatment confers osmotic stress tolerance to *Arabidopsis* seedlings. This would seem plausible as we know that the *CRT* element is activated during both cold and drought stress, and data from chapter 3 suggests that the $CaCl_2$ signature induces *CRT* specifically (Chapter 3, 3.2.2). The combined treatment of mastoparan/$CaCl_2$ also yielded a significantly higher concentration of chlorophyll (22mg/g (FW)) compared to the other pre-treatments (Figure 4.1), however, this was relatively low in comparison to both the single $CaCl_2$ treatment and the control (Figure 4.1). As suggested in chapter 3 each calcium signature is specific, therefore, it would be expected that the response to calcium alone and calcium with mastoparan would be different.

The ATP and the mastoparan/ATP pre-treatment displayed a dramatic effect, in that they resulted in a concentration of <1mg/g (FW) of chlorophyll, suggesting cell death had occurred. We know from previous literature that extracellular ATP regulates plant viability, growth, gravitropism and stress response (Chivasa et al., 2005, Jeter et al., 2004, Kim et al., 2006, Roux and Steinebrunner, 2007, Wu et al., 2007). ATP also acts as a suppressor of cell death and can reverse cell death via extracellular application (Chivasa et al., 2005, Demidchik et al., 2009). It has been suggested that too much ATP arrests growth by possibly impairing the auxin transport system (Demidchik et al., 2009, Kim et al., 2006, Roux and Steinebrunner, 2007, Tang et al., 2003, Wu et al., 2007), and it has also been shown by Chivasa et al., (2005) that osmotic stress leads to the release of ATP from cells, inducing ROS, leading to cell death. As previously described in chapter 3 different calcium agonists produce novel signatures which regulate different *cis* elements, it would therefore seem feasible that the ATP signature in this instance, was not regulating the required genes for osmotic stress tolerance but was instead inducing ROS and subsequent cell death.

The mastoparan, L-glutamate and their combinations did not seem to confer osmotic stress tolerance to seedlings, as deduced from their low chlorophyll concentrations (Figure 4.1) which were similar to the water/mannitol treated seedlings. This suggests that these signatures did not initiate the response required for osmotic stress tolerance.

4.4.2 Is calcium chloride induced osmotic stress tolerance age limiting?

To further investigate the $CaCl_2$-induced osmotic stress tolerance, both gene expression and chlorophyll levels were measured after osmotic stress treatments for both seedlings and mature plants (4.3.2). It was seen that genes that are known to be expressed during osmotic stress and are regulated by calcium were highly expressed in seedlings and leaves that had been pre-treated with $CaCl_2$ (Figure 4.3: A-D). This suggested that $CaCl_2$ pre-treatment conferred stress tolerance to *Arabidopsis*, which supported the data from the initial chlorophyll extractions from seedlings (Figure 4.1). However, the chlorophyll levels observed from $CaCl_2$ pre-treated mature leaves were low (Figure 4.3: B), similar to the levels after treating with water/mannitol rather than the untreated control, suggesting that pre-treatment of $CaCl_2$, did not in fact confer stress tolerance to mature plants, and osmopriming with $CaCl_2$ was age limited.

These gene expression data (Figure 4.2: C and D) and the chlorophyll extractions (Figure 4.3: B) are conflicting, therefore it cannot be deduced with confidence that $CaCl_2$ pre-treatment of mature plants confers osmotic stress tolerance. The chlorophyll assays for the mature plants seem to indicate that $CaCl_2$ pre-treatment leading to enhanced osmotolerance is age-limiting, but the gene expression data suggests that stress genes were indeed expressed at a high level in the mature plants. It could be argued in this case that the mature plants were more efficient at expelling calcium than the seedlings, which is reflected by the chlorophyll levels. We know from previous literature that mature plants have a much higher concentration of calcium channels and pumps (White, 2000, White and Broadley, 2003) therefore are more efficient at expelling calcium than seedlings. However, because the mature plants measured high levels of *COR* gene expression, a more likely explanation could be that indeed the calcium was penetrating the outer cells but not reaching the deeper tissues/cells of the mature leaves. Therefore, the whole leaf would not be osmotically tolerant and would not show physiological resistance, which concurs with these data.

A quick method to increase calcium uptake by the plant is to determine optimum $CaCl_2$ concentrations and then repeat the experiments with the stronger concentration. From calcium signature traces I was able to determine the optimum concentration of $CaCl_2$ at

400mM. However, it was shown during osmotic stress, leaves had reached cell death by day three, further chlorophyll measurements at day 10 confirmed no chlorophyll was present in the leaves. From these results it can be assumed that high doses of $CaCl_2$ over a 1h period followed by osmotic stress is lethal for mature *Arabidopsis* leaves.

There has been some success with pre-treatment of $CaCl_2$ applied at seed level in various mature species e.g. spring barley (Kaczmarek et al., 2016), maize (Khalil et al., 2014), wheat (Farooq et al., 2017) and rice (Hussain et al., 2016), I decided that this avenue could also be a way forward to confirm if $CaCl_2$ pre-treatment conferred osmotic stress tolerance to mature *Arabidopsis* plants.

4.4.3 Does pre-treatment of calcium chloride at seed level instigate osmotic stress tolerance in mature *Arabidopsis thaliana* plants.

Repeats of the stress assays with mature *Arabidopsis* leaves from plants which had been pre-treated with $CaCl_2$ at the seed stage (detailed methods 2.2.3.3 and 2.2.7.1), yielded relatively high *KIN2* and *LT178* expression levels (Figure 4.4: A and B) and supported what had been observed earlier when pre-treatment was applied at the leaf stage. The chlorophyll levels were seen to be high also after this treatment and compared favourably with the control (Figure 4.5), unlike the earlier data recorded with plants pre-treated at the leaf stage (Figure 4.3: B). These data suggest that pre-treatment of $CaCl_2$ at seed level induces an osmotic stress tolerance in subsequently mature Arabidopsis plants.

4.5 Conclusion

The aim of this work was to determine if calcium signatures show specificity during osmotic stress. Data so far implies that indeed, pre-treatment of $CaCl_2$ seems to initiate a response that leads to osmotic stress tolerance in both seedlings and mature *Arabidopsis* plants. Thus, suggesting that a novel signature does show specificity during osmotic stress.

5. The biphasic response generated from specific calcium agonists and the influence and importance of circadian regulation.

5.1 Introduction

5.1.1 The $[Ca^{2+}]_{cyt}$ biphasic response

Data from chapter three indicated that novel calcium signatures generated from either ATP (3.2.2, Figure 3.5:C) or a combined treatment of mastoparan/$CaCl_2$ (3.2.2, Figure 3.4:C) regulated specific TFs via a biphasic or triphasic response. It would be interesting to determine if the different phases of the signatures generated, were due to the calcium responses occurring in different cells types or the same cell type but at different times, or both.

Calcium enters the cell through Ca^{2+}-permeable ion channels located in the plasma membrane or from internal stores (White, 2000). To prevent high concentrations of $[Ca^{2+}]_{cyt}$ accumulating leading to toxicity, Ca^{2+} ATPases and H^+Ca^{2+} antiporters remove the $[Ca^{2+}]_{cyt}$ to either the apoplast or the inside of intracellular organelles, such as the vacuole or the endoplasmic reticulum (ER) (Hirschi, 2001, Sze et al., 2000). Proteins termed 'protein sensors' change conformation or catalytic activity when binding to Ca^{2+}, for example, calmodulin (CaM), calcineurin B-like proteins (CBLs) and Ca^{2+}-dependent protein kinases (CDPKs). Transient increases in $[Ca^{2+}]_{cyt}$ can differ in their amplitude, repetitiveness or duration, therefore location of the Ca^{2+} channels is essential (Evans et al., 2001).

Plant tissue is comprised of many different cell types that may have varying abilities to generate $[Ca^{2+}]_{cyt}$ signatures. For instance, $[Ca^{2+}]_{cyt}$ signatures generated by mechanical means, salinity, osmotic stress, cold shock or slow cooling are different between cell types (Kiegle, 2000) . It has been shown that shoot cells display a biphasic $[Ca^{2+}]_{cyt}$ response during anoxia, but in contrast, a slow monophasic increase in $[Ca^{2+}]_{cyt}$ can be observed in the root cells during this process (Plieth, 2001, Sedbrook et al., 1996). It has also been shown that cells of the same type can generate different $[Ca^{2+}]_{cyt}$ responses, for example, two guard

cells of a stomate have been seen to frequently generate different $[Ca^{2+}]_{cyt}$ responses to a specific stimulus (Allen et al., 1999a).

The type of response is not just determined by the $[Ca^{2+}]_{cyt}$ signature alone but also the expression of the $[Ca^{2+}]_{cyt}$ sensors, their abundance, activity and their affinity for Ca^{2+} (Gilroy and Trewavas, 2001). As different cell types and individuals of the same cell type have differing transcript, protein and enzyme profiles this could result in an individual $[Ca^{2+}]_{cyt}$ response, which in turn could contribute to phenotypic plasticity (Gilroy and Trewavas, 2001).

5.1.2 Circadian regulation in plants.

The earth rotates approximately every 24h, to adapt to this rotation most organisms have evolved an internal circadian clock (McClung, 2006b). These circadian clocks drive daily rhythms in behaviour, metabolism and growth (Gorton et al., 1989, McClung, 2006b, Wood et al., 2001a). Circadian rhythms regulate the daily phase of biological processes, organising the daily timings of the transcriptome, which enables cellular processes to occur at a given time in a coordinated manner (Harmer et al., 2000, McClung, 2006b).

It is considered that the circadian system in plants include several conceptual components (Wood et al., 2001a). Firstly, the entrainment pathway adjusts the circadian oscillator to match the environmental phase, by changing the circadian phase in response to environmental cues. Secondly, the circadian oscillator which is a complex molecular network estimates the time of day (Wood et al., 2001a). In plants, the circadian oscillator consists of an interconnected network of genes and processes arranged in feedback loops (Wood et al., 2001a). Next, the output pathway communicates the time of day generated by the oscillator to the clock regulated processes within the cells of the plant (Wood et al., 2001a). Finally, signalling pathways that entrain the circadian clock or are involved in environmental responses are often themselves circadian regulated. Therefore, their response to a specific stimulus is dependent upon the time of day (Gómez and Simón, 1995, Trewavas, 1999, Webb, 2003, Wood et al., 2001a). This process is known as circadian gating of signal transduction (Hotta et al., 2007, Yakir et al., 2009).

Cytosolic calcium is an important secondary messenger in plant cells and is known to play a prominent regulatory role in the transduction of many signals (Gilroy et al., 1990, Neuhaus et al., 1993, Wood et al., 2001a). It has been demonstrated that red light, which is often used to entrain circadian rhythms transiently elevates $[Ca^{2+}]_{cyt}$ (Ermolayeva et al., 1996, Shacklock et al., 1992) and that $[Ca^{2+}]_{cyt}$ signatures may be transduced through Calmodulin-Like24 to modulate the circadian clock (Dodd et al., 2007, Frank et al., 2018, Martí Ruiz et al., 2018).

In regards to $[Ca^{2+}]_{cyt}$ signatures specifically generated from mastoparan, $CaCl_2$, ATP, L-glutamate or combinations of these calcium agonists it is not known if they are circadian regulated. It would be informative if we could determine if these calcium signatures are regulated by the circadian clock, and if so, does this reflect in a subsequent change in expression kinetics, which may suggest that the clock could be affecting specific calcium channels.

The questions to be addressed in this chapter

- Are the biphasic responses generated from ATP or mastoparan/$CaCl_2$ $[Ca^{2+}]_{cyt}$ signatures due to responses from different cell types or produced in the same cells?
- Are the $[Ca^{2+}]_{cyt}$ signatures generated in response to calcium agonists regulated by the circadian clock?
- Does circadian regulation of specific $[Ca^{2+}]_{cyt}$ signatures change subsequent gene expression kinetics?

5.2 Results

5.2.1. Measuring calcium-regulation of specific *cis* elements in various organs of *Arabidopsis thaliana*

To investigate the effect of the biphasic calcium signatures measured from ATP or mastoparan/$CaCl_2$ generated $[Ca^{2+}]_{cyt}$, 10 d old transgenic Arabidopsis seedlings, containing a construct consisting of a *LUC+* coding region driven by either ABRE or SITE II were treated with an overall concentration of 0.5mM ATP (methods 2.2.3.2). Luminescence of whole seedlings, cotyledons and roots was recorded using a photon camera (methods found in 2.2.5.2). This process was repeated with CaM box driven luciferase transgenic seedlings treated with a combined overall concentration of 10µM mastoparan and 50mM $CaCl_2$ (methods 2.2.3.2). I limited the transgenics to ABRE, SITE II or CaM box as data from chapter three indicated these *cis* elements displayed a polyphasic response when treated with ATP or a combination of mastoparan/$CaCl_2$ (all results can be seen in figures 5.1 to 5.3). As controls, measurements from seedlings containing a minimal promoter fused to *LUC+* were recorded from both agonist treatments (Appendix E.1).

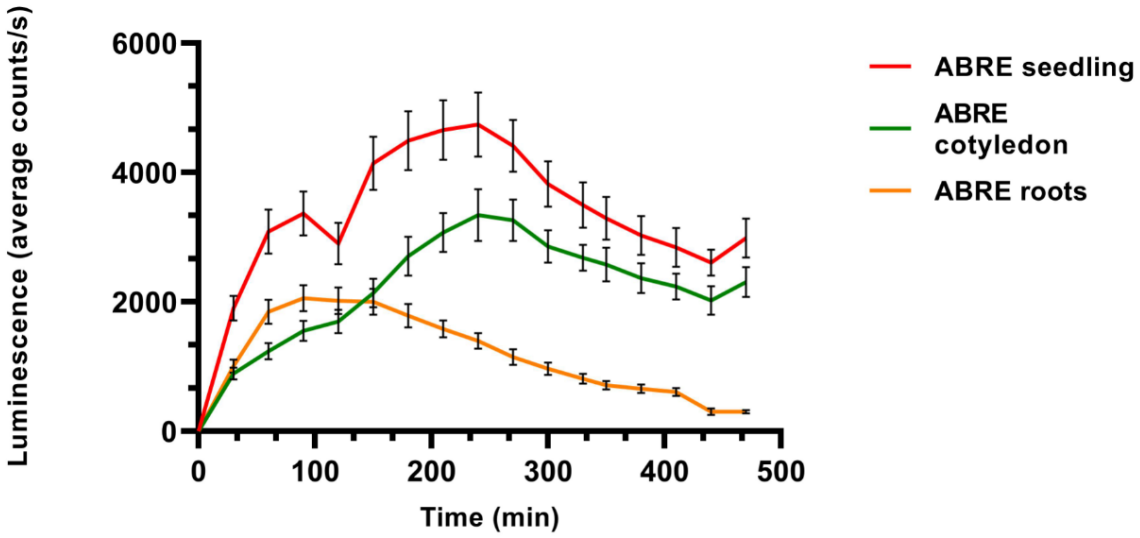


Figure 5.1: Luminescence average counts per second from *A. thaliana* seedlings containing a construct expressing *LUC+* through ABRE. Each line represents an average of 5 seedlings measuring from either the whole seedling, cotyledons or the roots treated with an overall concentration of 0.5mM ATP.

Error bars represent ± SEM (n=5 replicates of 5 treated seedlings).

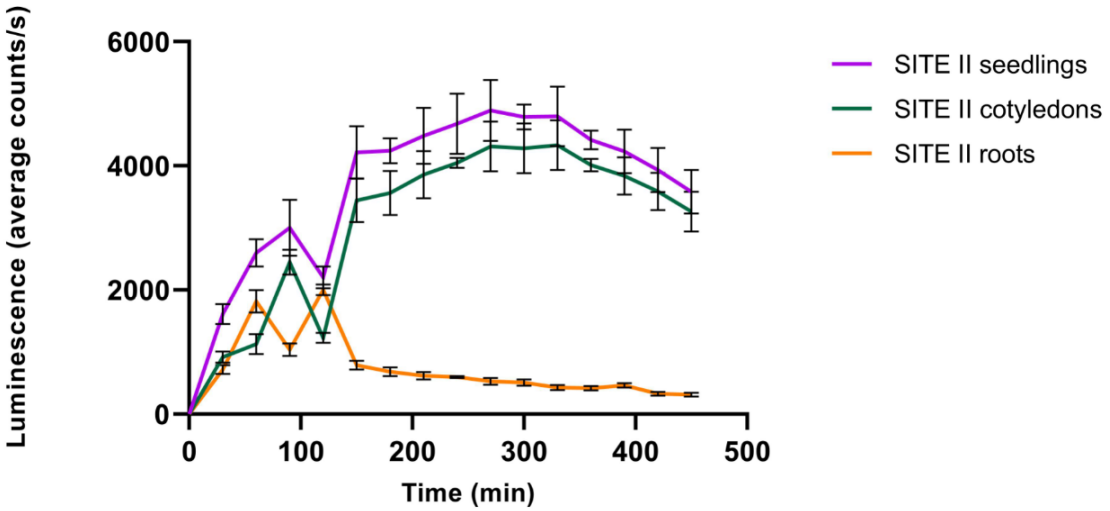


Figure 5.2: Luminescence average counts per second from Arabidopsis seedlings containing a construct expressing *LUC+* through SITE II. Each line represents an average of 5 seedlings measuring from either the whole seedling, cotyledons or the roots treated with an overall concentration of 0.5mM ATP.

Error bars represent ±SE M (n=5 replicates of 5 treated seedlings).

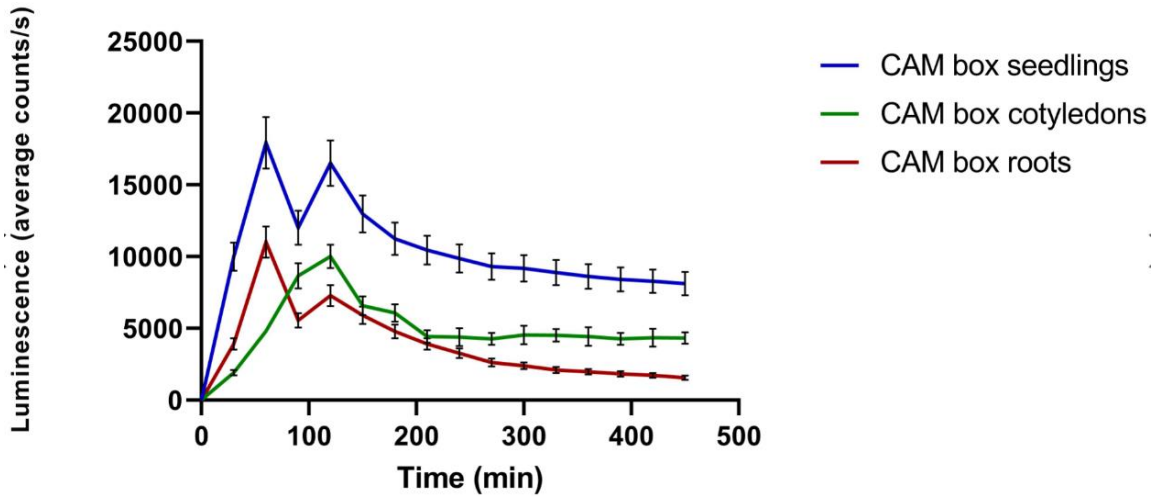


Figure 5.3: Luminescence average counts per second from Arabidopsis seedlings containing a construct expressing *LUC+* through CaM box. Each line represents an average of 5 seedlings measuring from either the whole seedling, cotyledons or the roots treated with an overall concentration of 10µM mastoparan and 50mM $CaCl_2$.

Error bars represent ±SE M (n=5 replicates of 5 treated seedlings).

From Arabidopsis seedlings treated with ATP containing *LUC+* constructs driven by ABRE, a biphasic response was recorded (Figure 5.1), with luminescence peaks measured at 90 min and 120 min. The second peak displayed a further gradual rise reaching maximum luminescence counts at 240 min, followed by slow gradual decline over time (Figure 5.1). Measurements taken from the cotyledons and roots displayed a monophasic response, with peaks recorded at 240 mins and 90 mins respectively (Figure 5.1).

ATP treated seedlings containing *LUC +* driven by SITE II displayed a biphasic response, with luminescence peaks at 90 min and 150 min (Figure 5.2). The first peak demonstrated a rapid increase followed by an immediate sharp decrease; the second response was initially sharp but plateaued followed by a gradual decrease in luminescence counts. Measurements from the cotyledons of the SITE II driven seedlings showed a biphasic response, which mirrored what was seen in the whole seedling, with peaks at 90 min and 150 min but, with a lower luminescence count (Figure 5.2). The roots from the SITE II driven seedlings recorded a biphasic response at 60 and 120 min, but with different kinetics to that of the cotyledons (Figure 5.2).

Seedlings treated with a combination of mastoparan/$CaCl_2$ containing *LUC+* constructs driven by CaM box displayed a biphasic response, with defined peaks at 60 min and 120 min (Figure 5.3). Cotyledons from the CaM box driven seedlings measured a monophasic response with a peak at 120 min, the peak displayed a rapid decline which plateaued at 220 min (Figure 5.3). As in the whole seedling a biphasic response was recorded within the roots, with peaks displayed at 60 min and 120 mins (Figure 5.3).

5.2.2 Investigating the circadian regulation of specific $[Ca^{2+}]_{cyt}$ signatures

During the investigation of the biphasic calcium response (5.2.1) it was noted that repetitions recorded after 5pm differed in kinetics to those measured at an earlier time. It has been suggested in literature that the circadian clock regulates gene expression e.g. in cold stress, the expression of cold response genes is higher in the morning compared to the evening (Bieniawska et al., 2008). Data from several studies has also stressed the importance of ionic signalling for circadian time keeping in both mammals and plants (Bieniawska et al., 2008, Feeney et al., 2016, Harrisingh et al., 2007, Martí Ruiz et al., 2018). To determine if the different kinetics observed at the later time points were due to circadian influence it was important to determine if any of the calcium agonist driven $[Ca^{2+}]_{cyt}$ signatures were truly circadian regulated.

Seedlings expressing cytosolic aequorin (pMAQ2) were entrained in one of two light cycles; a forward cycle of 12L:12D or a reverse cycle of 12D:12L (methods found in 2.2.2.5). Following 24h of constant light, seedlings were treated with one of four calcium agonists (mastoparan; $CaCl_2$; ATP or L-glutamate) or a combination of these agonists (mastoparan/$CaCl_2$; mastoparan/ATP or mastoparan/L-glutamate), and $[Ca^{2+}]_{cyt}$ traces were generated from an average of 10 seedlings (methods 2.2.4). The calcium agonist induced changes in $[Ca^{2+}]_{cyt}$ were tested at the same time (8am, which relates to the light/dark switch over time).

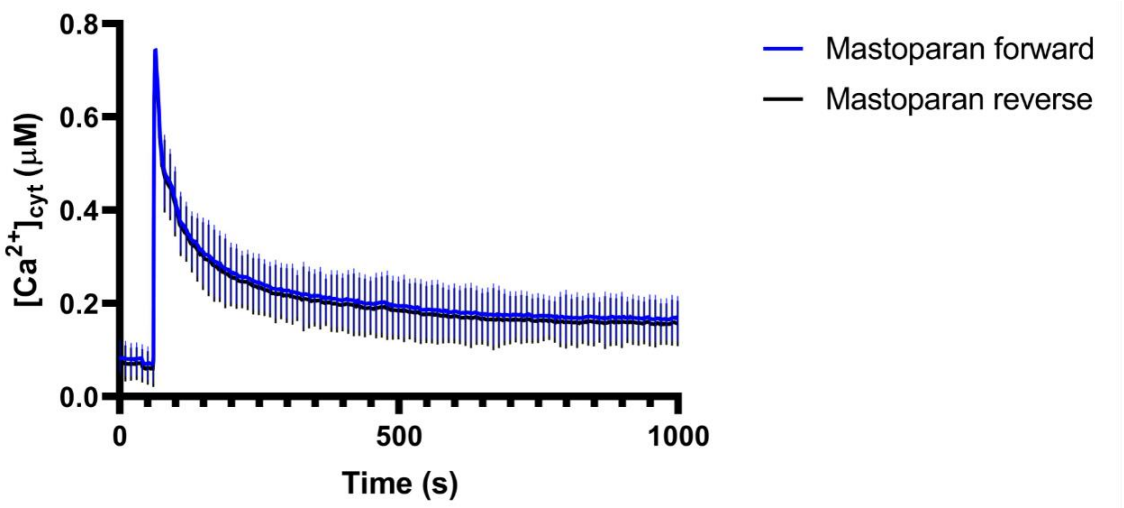


Figure 5.4: Calcium signature traces generated from an overall concentration of 10µM mastoparan. Each trace represents the average signals from 10 transgenic Arabidopsis seedlings injected with at 60s and measured for 1000s. The mastoparan forward trace depicts measurements from seedlings grown in a 12L/12D light cycle, the mastoparan reverse displays measurements from seedlings grown in a 12D/12L light cycle.

Error bars represent ± SEM (n=10 seedlings) One-way ANOVA of peaks at 60s of mastoparan generated calcium signatures in either forward or reverse light cycles**** (P≤0.0001), *** (P≤0.001), ** (P≤0.01) * (P≤0.05) and Tukey *post hoc.*

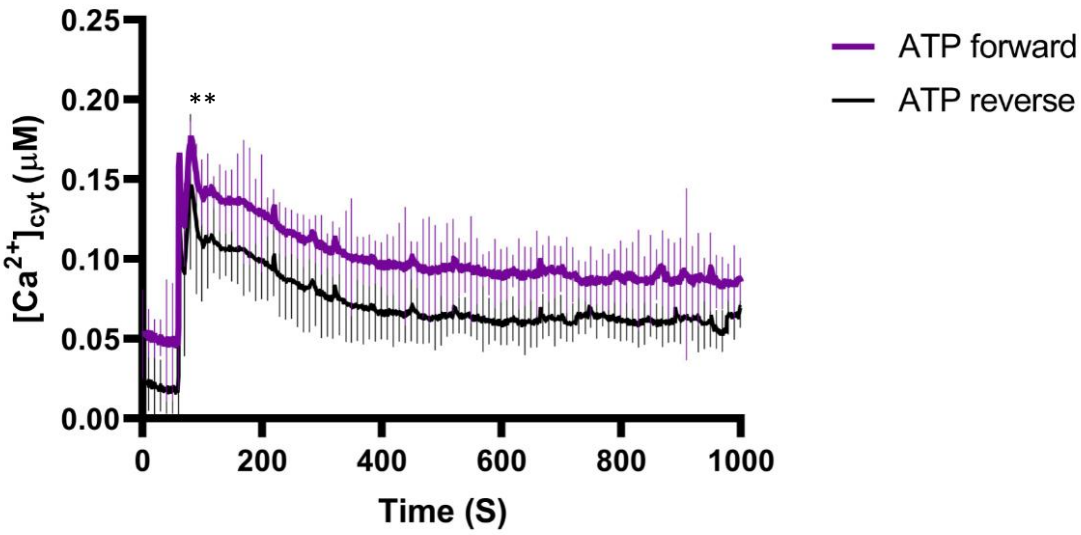


Figure 5.5: Calcium signature traces generated from an overall concentration of 0.5mM ATP. Each trace represents the average signals from 10 transgenic Arabidopsis seedlings injected with at 60s and measured for 1000s. The ATP forward trace depicts measurements from seedlings grown in a 12L/12D light cycle, the ATP reverse displays measurements from seedlings grown in a 12D/12L Light cycle.

Error bars represent ± SEM (n=10 seedlings) One-way ANOVA of peaks at 60s of ATP generated calcium signatures in either forward or reverse light cycles**** (P≤0.0001), *** (P≤0.001), ** (P≤0.01) * (P≤0.05) and Tukey *post hoc*

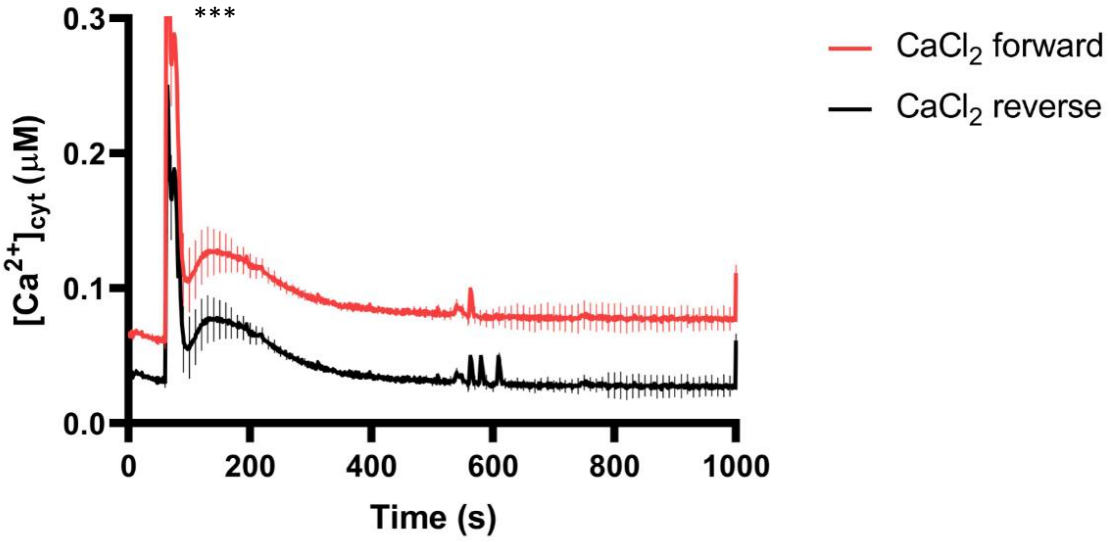


Figure 5.6: Calcium signature traces generated from an overall concentration of 50mM $CaCl_2$. Each trace represents the average signals from 10 transgenic Arabidopsis seedlings injected with at 60s and measured for 1000s. The $CaCl_2$ forward trace depicts measurements from seedlings grown in a 12L/12D light cycle, the $CaCl_2$ reverse displays measurements from seedlings grown in a 12D/12L light cycle.

Error bars represent ± SEM (n=10 seedlings) One-way ANOVA of peaks at 60s of $CaCl_2$ generated calcium signatures in either forward or reverse light cycles**** (P≤0.0001), *** (P≤0.001), ** (P≤0.01) * (P≤0.05) and Tukey *post hoc*

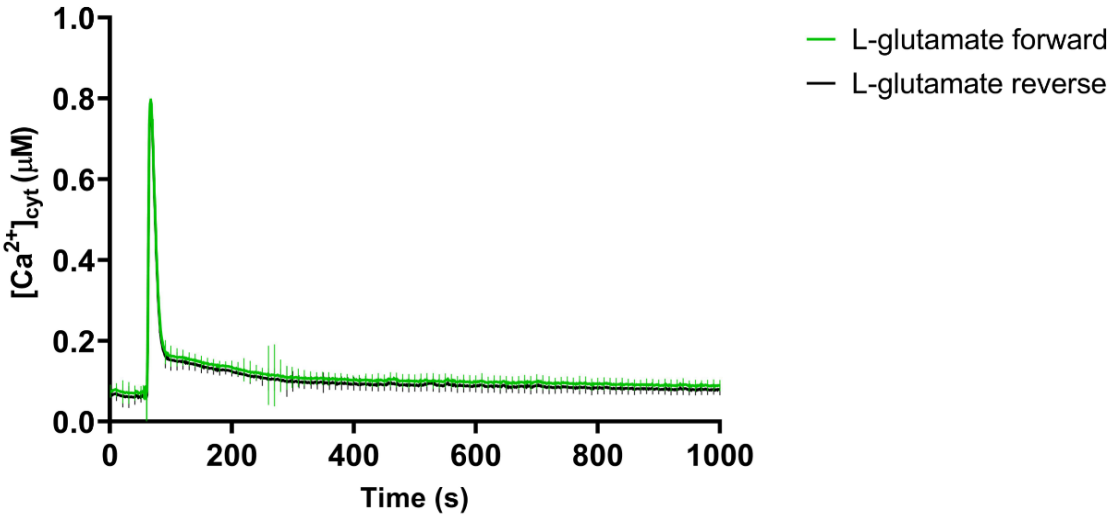


Figure 5.7: Calcium signature traces generated from an overall concentration of 1mM L-glutamate. Each trace represents the average signals from 10 transgenic Arabidopsis seedlings injected with at 60s and measured for 1000s. The L-glutamate forward trace depicts measurements from seedlings grown in a 12L/12D light cycle, the L-glutamate reverse displays measurements from seedlings grown in a 12D/12L light cycle.

Error bars represent ± SEM (n=10 seedlings) One-way ANOVA of peaks at 60s of L-glutamate generated calcium signatures in either forward or reverse light cycles **** (P≤0.0001), *** (P≤0.001), ** (P≤0.01) * (P≤0.05) and Tukey *post hoc*

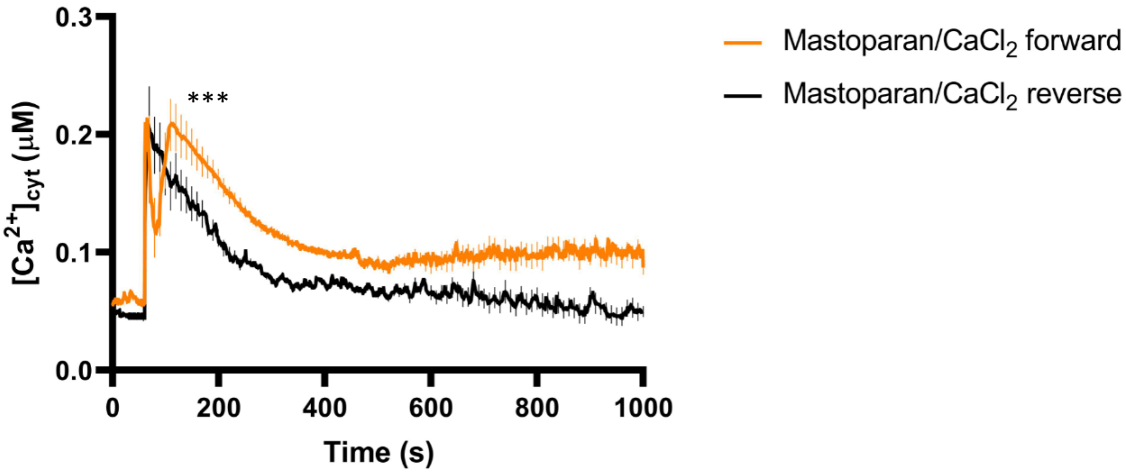


Figure 5.8: Calcium signature traces generated from an overall concentration of 10µM mastoparan and 50mM $CaCl_2$. Each trace represents the average signals from 10 transgenic Arabidopsis seedlings injected at 60s and measured for 1000s. The mastoparan/$CaCl_2$ forward trace depicts measurements from seedlings grown in a 12L/12D light cycle, the mastoparan/$CaCl_2$ reverse displays measurements from seedlings grown in a 12D/12L light cycle.

Error bars represent ± SEM (n=10 seedlings) One-way ANOVA of peaks at 60s and 150s of mastoparan/$CaCl_2$ generated calcium signatures in either forward or reverse light cycles**** (P≤0.0001), *** (P≤0.001), ** (P≤0.01) * (P≤0.05) and Tukey *post hoc*

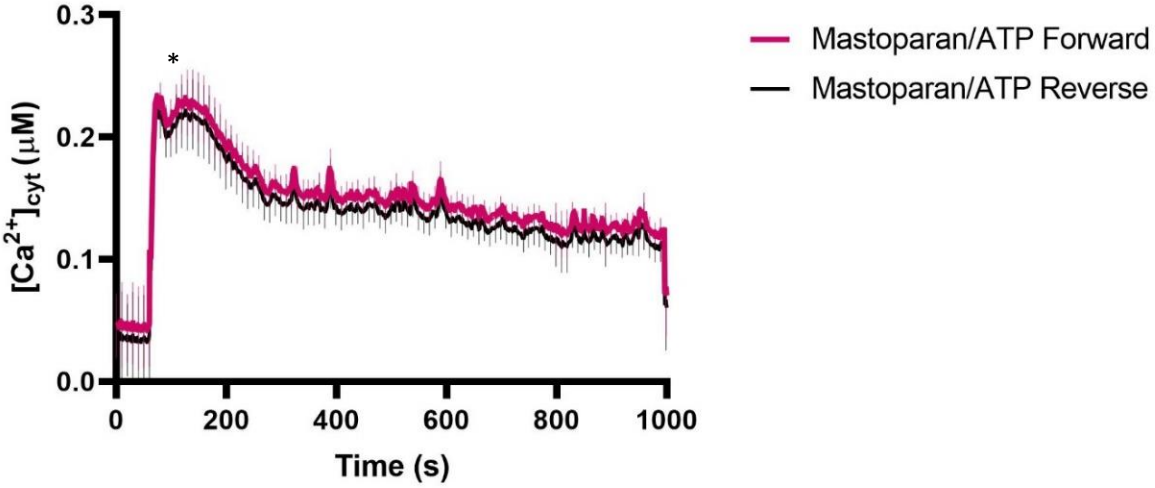


Figure 5.9: Calcium signature traces generated from an overall concentration of 10µM mastoparan and 0.1mM ATP. Each trace represents the average signals from 10 transgenic Arabidopsis seedlings injected at 60s and measured for 1000s. The mastoparan/ATP forward trace depicts measurements from seedlings grown in a 12L/12D light cycle, the mastoparan/ATP reverse displays measurements from seedlings grown in a 12D/12L light cycle.

Error bars represent ± SEM (n=10 seedlings) One-way ANOVA of peaks at 60s of mastoparan/ATP generated calcium signatures in either forward or reverse light cycles**** (P≤0.0001), *** (P≤0.001), ** (P≤0.01) * (P≤0.05) and Tukey *post hoc*

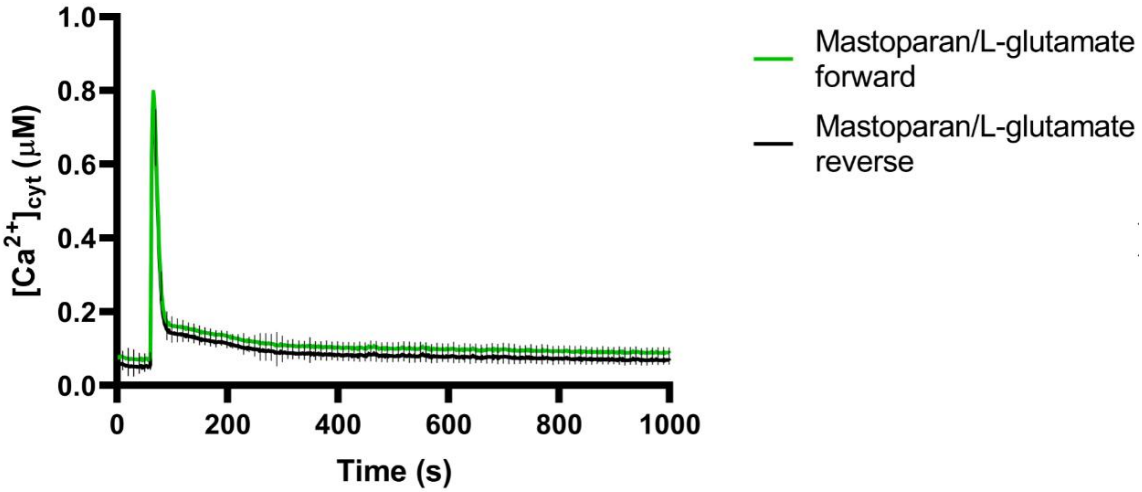


Figure 5.10: Calcium signature traces generated from an overall concentration of 10µM mastoparan/1mM L-glutamate. Each trace represents the average signals from 10 transgenic Arabidopsis seedlings injected at 60s and measured for 1000s. The mastoparan/ATP forward trace depicts measurements from seedlings grown in a 12L/12D light cycle, the mastoparan/ATP reverse displays measurements from seedlings grown in a 12D/12L light cycle.

Error bars represent ± SEM (n=10 seedlings) One-way ANOVA of peaks at 60s of mastoparan/L-glutamate generated calcium signatures in either forward or reverse light cycles**** (P≤0.0001), *** (P≤0.001), ** (P≤0.01) * (P≤0.05) and Tukey *post hoc* .

Cytosolic calcium signatures generated from seedlings entrained in the reverse cycle (12D:12L) and treated with either mastoparan, L-glutamate or a combination of mastoparan/L-glutamate, when compared with $[Ca^{2+}]_{cyt}$ signatures generated from seedlings entrained in the forward light cycle(12L:12D) displayed the same kinetics; in terms of peak and overall response (Figures 5.4; 5.7; 5.10). Statistical analysis revealed p values >0.74-0.79, implying an insignificant difference between the signatures generated in the two different light cycles.

With ATP-generated $[Ca^{2+}]_{cyt}$ signatures it can be seen that the response is reduced in the reverse cycle (12D:12L), in which 08:00 represents a time of subjective evening (Figure 5.5). Although the actual patterns of both signatures are similar, the response is lower both at basal level (which is a well-known phenomenon indicating successful entrainment) and throughout the whole signature (Figure 5.5). Subtle differences between signatures generated from a combination of mastoparan/ATP can be seen between the forward and reverse cycles (Figure 5.9). Statistical analysis between the peaks measured a p value of <0.05 suggesting a statistical difference.

Calcium chloride generated $[Ca^{2+}]_{cyt}$ signatures displayed a reduced response in the reverse cycle (12D:12L), where 08:00 represents subjective evening (Figure 5.6) compared to the forward cycle (12L:12D) in both basal level and overall response. Cytosolic calcium signatures generated from the combined treatment of mastoparan/$CaCl_2$ displayed different kinetics between the two light cycles (Figure 5.8). The initial peak at 60s was the same in both signatures, however, the signature generated in the reverse light cycle was monophasic, whereas the forward light cycle produced a biphasic $[Ca^{2+}]_{cyt}$ signature. The reverse $[Ca^{2+}]_{cyt}$ signature displayed a lower basal level and lower overall response over time (Figure 5.8).

5.2.3 Determining the influence of circadian regulation on calcium regulated specific cis elements.

The data from section 5.2.2 implied that specific calcium signatures were regulated by the circadian clock. To investigate the importance of these changes regarding *cis* element regulation, I repeated the experiments conducted in chapter 3 (3.2.2), with seedlings entrained in either a 12L:12D (forward) or 12D:12L (reverse) light cycle. Therefore to

investigate the response of these *cis* elements, 10-day old transgenic Arabidopsis seedlings expressing constructs consisting of one of the *cis* elements ABRE, SITE II, CaM box, CRT or a minimal promotor (control) as described in chapter 3 (3.2.2), were treated with either a single calcium agonist or a combination of two calcium agonists whose $[Ca^{2+}]_{cyt}$ signatures had been identified in 5.2.2 as possibly circadian regulated (ATP, $CaCl_2$, mastoparan/$CaCl_2$ or mastoparan/ATP). Luminescence was recorded using a photon camera (see methods 2.2.5), and an overall average was taken from 3 seedlings for each construct from both light cycles.

The initial results (Appendix F.1) displayed differences between the two light cycles in the regulation of ABRE, SITE II and CaM box by ATP, CRT by $CaCl_2$, CaM box by mastoparan/$CaCl_2$ and ABRE and SITE II by mastoparan/ATP. From these data (Appendix F.1) the experiments were repeated with the seedlings containing the *cis* elements of interest and luminescence was measured in the whole seedlings, the cotyledons and the roots from both light cycles.

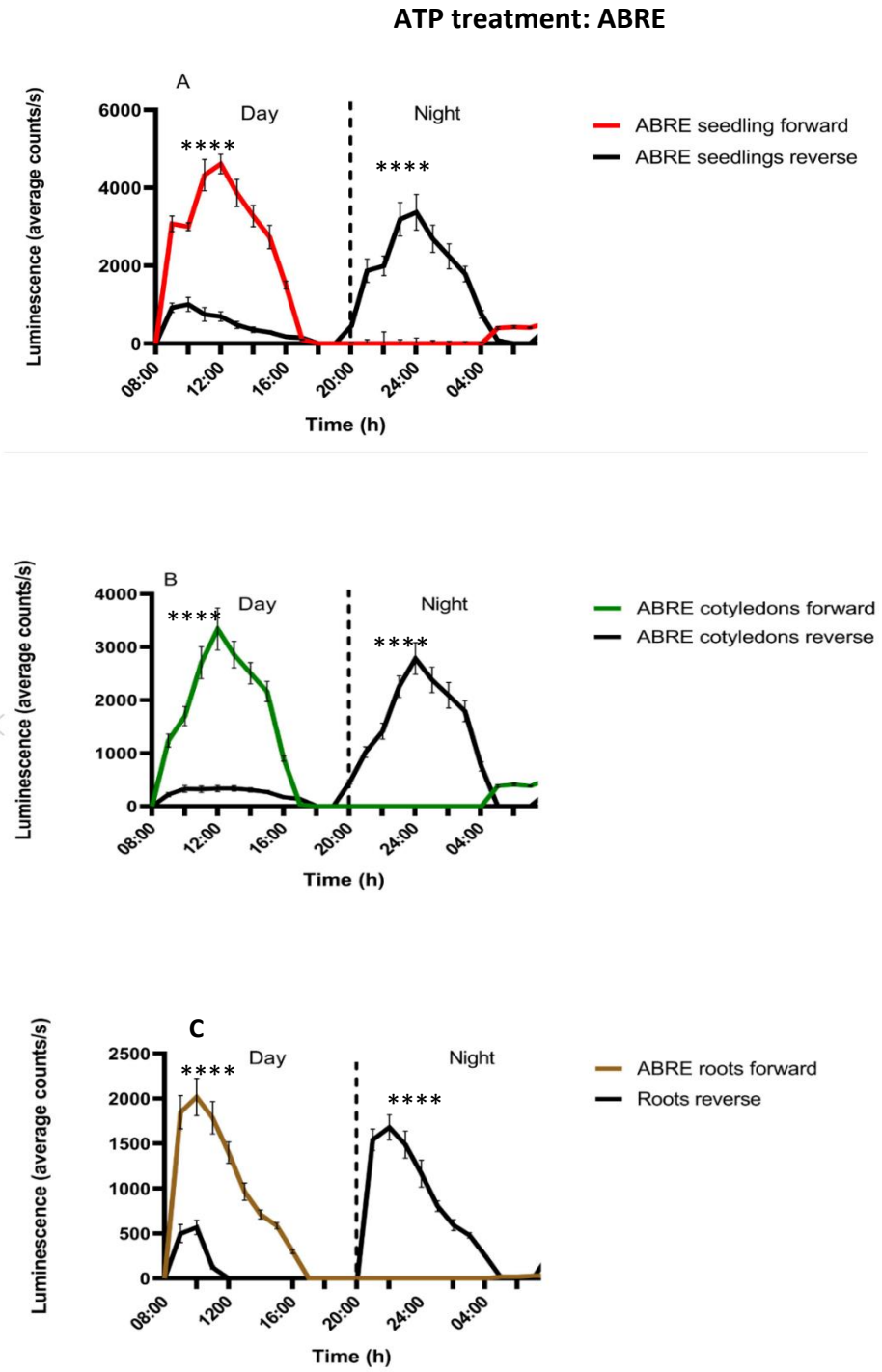


Figure 5.11: Luminescence average counts per second over 24h from Arabidopsis seedlings containing a construct expressing *LUC+* through ABRE. Each line represents an average of 3 seedlings treated with an overall concentration of 0.5mM ATP. All seedlings were grown in a photoperiod 16L/8D for 24h followed by 7d of entrainment in either 12L:12D (forward) or 12D:12L (reverse) and a further 24h of constant light. Luminescence levels were recorded over 24h. **A:** Seedlings, **B:** Cotyledons, **C:** Roots

Error bars represent ±SEM (n=3 replicates of 3 treated seedlings). One-way ANOVA of peaks between forward and reverse cycles**** (P≤0.0001), *** (P≤0.001), ** (P≤0.01) * (P≤0.05) and Tukey *post hoc*.

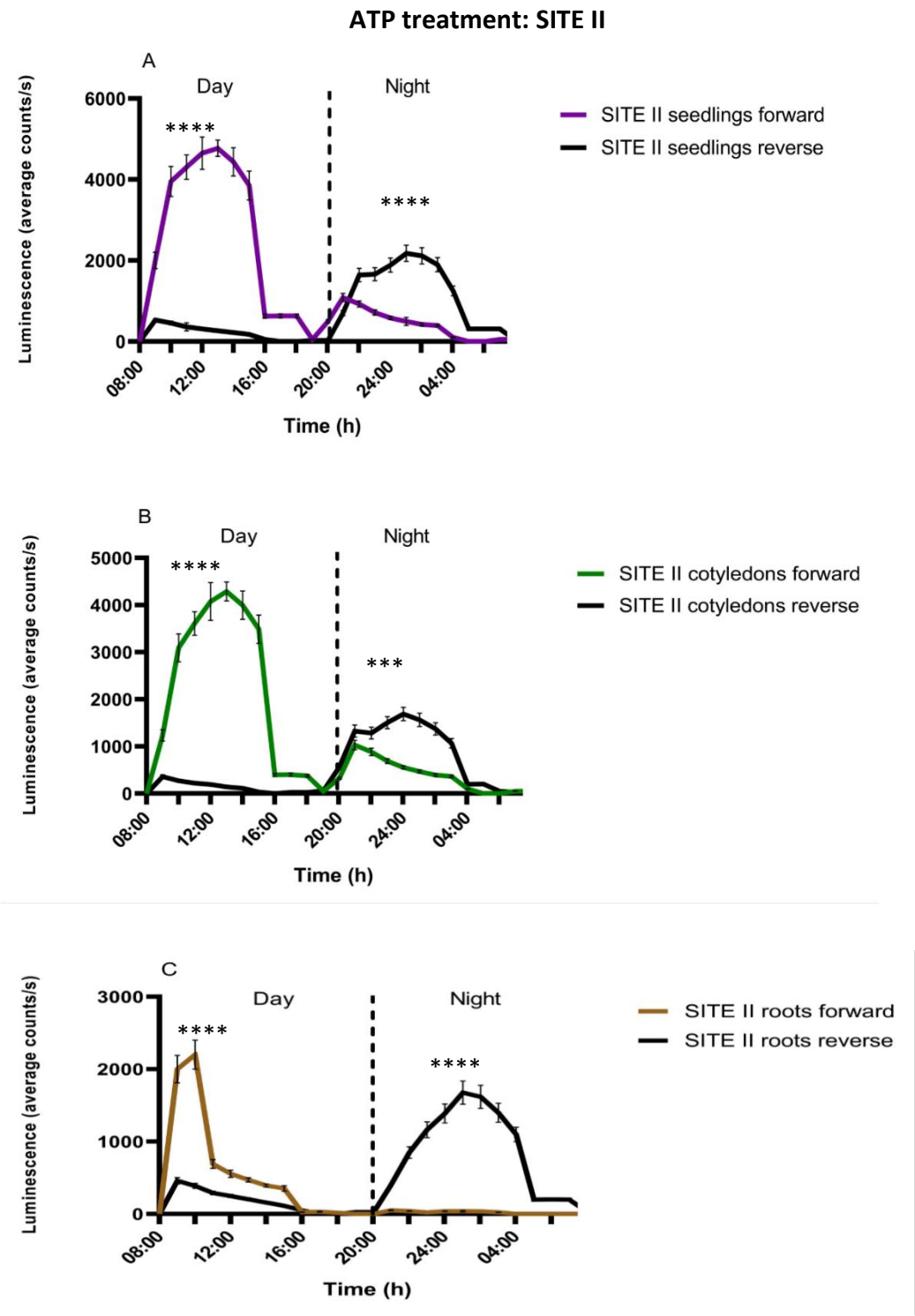


Figure 5.12: Luminescence average counts per second over 24h from Arabidopsis seedlings containing a construct expressing *LUC+* through SITE II. Each line represents an average of 3 seedlings treated with an overall concentration of 0.5mM ATP. All seedlings were grown in a photoperiod 16L/8D for 24h followed by 7d of entrainment in either 12L:12D (forward) or 12D:12L (reverse) and a further 24h of constant light. Luminescence levels were recorded over 24h. **A:** Seedlings, **B:** Cotyledons, **C:** Roots

Error bars represent ±SEM (n=3 replicates of 3 treated seedlings). One-way ANOVA of peaks between forward and reverse light cycles **** (P≤0.0001), *** (P≤0.001), ** (P≤0.01) *

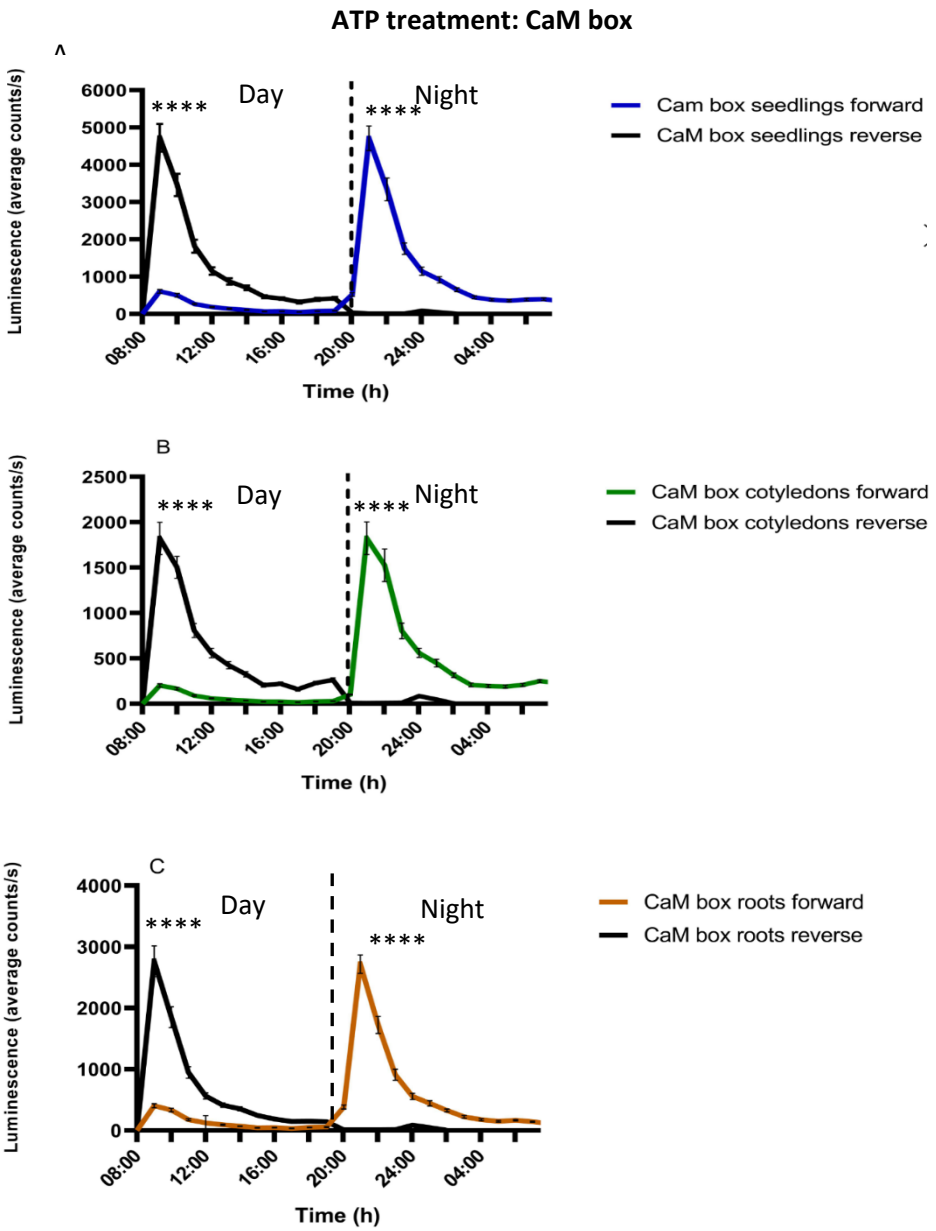


Figure 5.13: Luminescence average counts per second over 24h from Arabidopsis seedlings containing a construct expressing *LUC+* through CaM box. Each line represents an average of 3 seedlings treated with an overall concentration of 0.5mM ATP. All seedlings were grown in a photoperiod 16L/8D for 24h followed by 7d of entrainment in either 12L:12D (forward) or 12D:12L (reverse) and a further 24h of constant light. Luminescence levels were recorded over 24h. **A:** Seedlings, **B:** Cotyledons, **C:** Roots

Error bars represent ±SEM (n=3 replicates of 3 treated seedlings). One-way ANOVA of peaks between forward and reverse light cycles **** (P≤0.0001), *** (P≤0.001), ** (P≤0.01) *

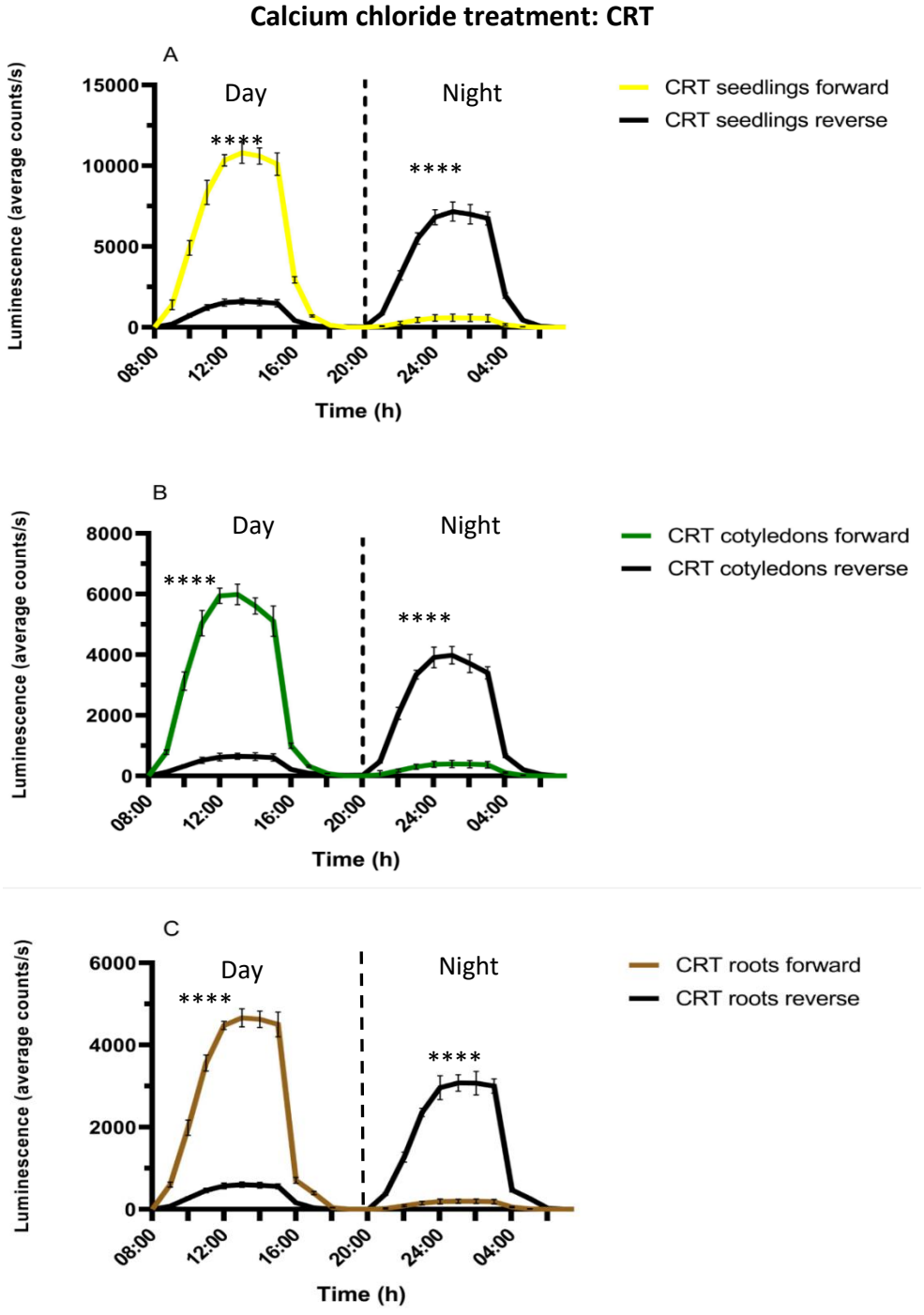


Figure 5.14: Luminescence average counts per second over 24h from Arabidopsis seedlings containing a construct expressing *LUC+* through CRT. Each line represents an average of 3 seedlings treated with an overall concentration of 50mM $CaCl_2$. All seedlings were grown in a photoperiod 16L/8D for 24h followed by 7d of entrainment in either 12L:12D (forward) or 12D:12L (reverse) and a further 24h of constant light. Luminescence levels were recorded over 24h. **A:** Seedlings, **B:** Cotyledons, **C:** Roots

Error bars represent ±SEM (n=3 replicates of 3 treated seedlings). One-way ANOVA of peaks between forward and reverse light cycles **** (P≤0.0001), *** (P≤0.001), ** (P≤0.01) *

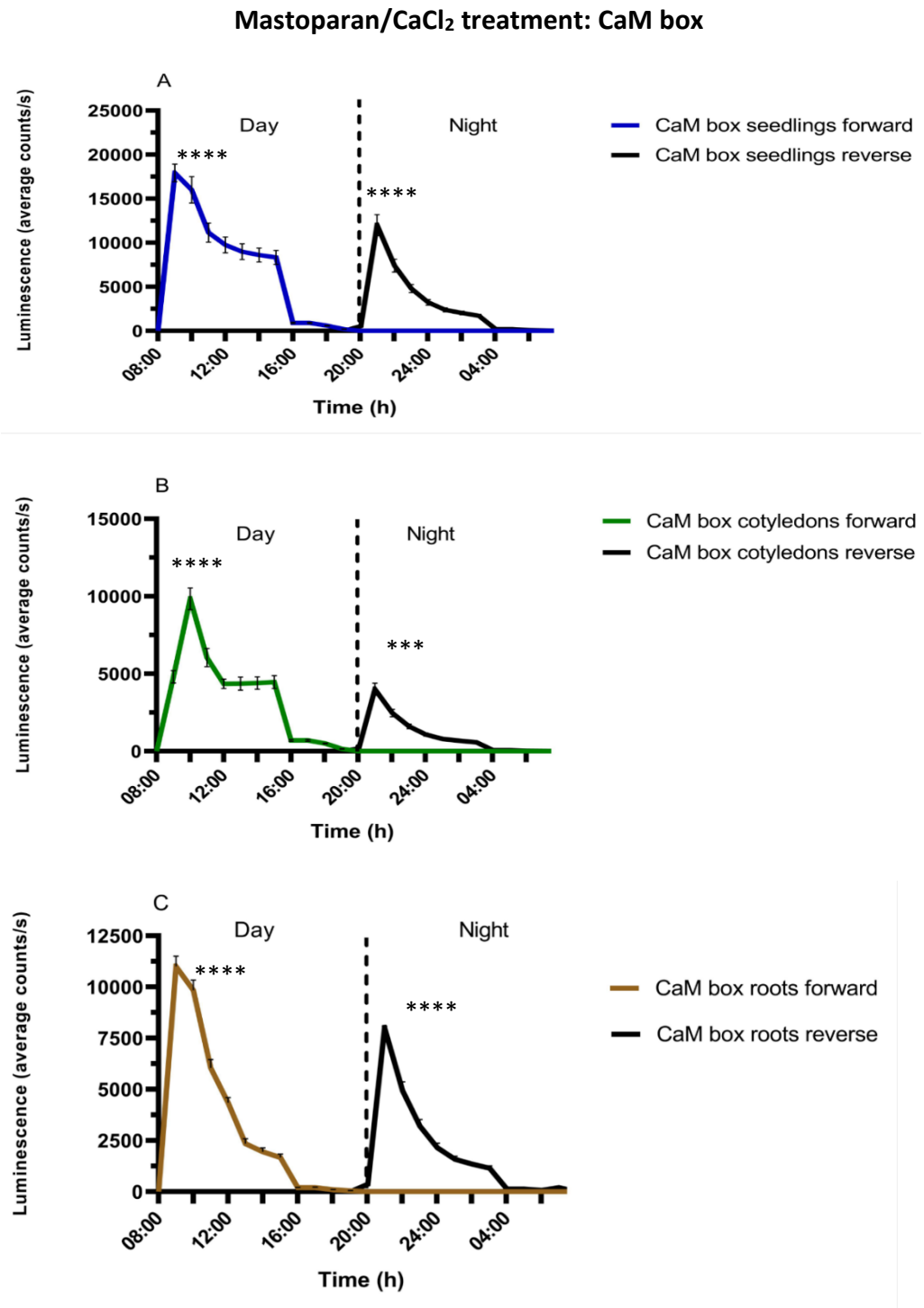


Figure 5.15: Luminescence average counts per second over 24h from Arabidopsis seedlings containing a construct expressing *LUC+* through CaM box. Each line represents an average of 3 seedlings treated with an overall concentration of 10µM mastoparan and 50mM $CaCl_2$. All seedlings were grown in a photoperiod 16L/8D for 24h followed by 7d of entrainment in either 12L:12D (forward) or 12D:12L (reverse) and a further 24h of constant light. Luminescence levels were recorded over 24h. **A:** Seedlings, **B:** Cotyledons, **C:** Roots

Error bars represent ±SEM (n=3 replicates of 3 treated seedlings). One-way ANOVA of peaks between forward and reverse light cycles **** (P≤0.0001), *** (P≤0.001), ** (P≤0.01) *

Mastoparan/ATP treatment: ABRE

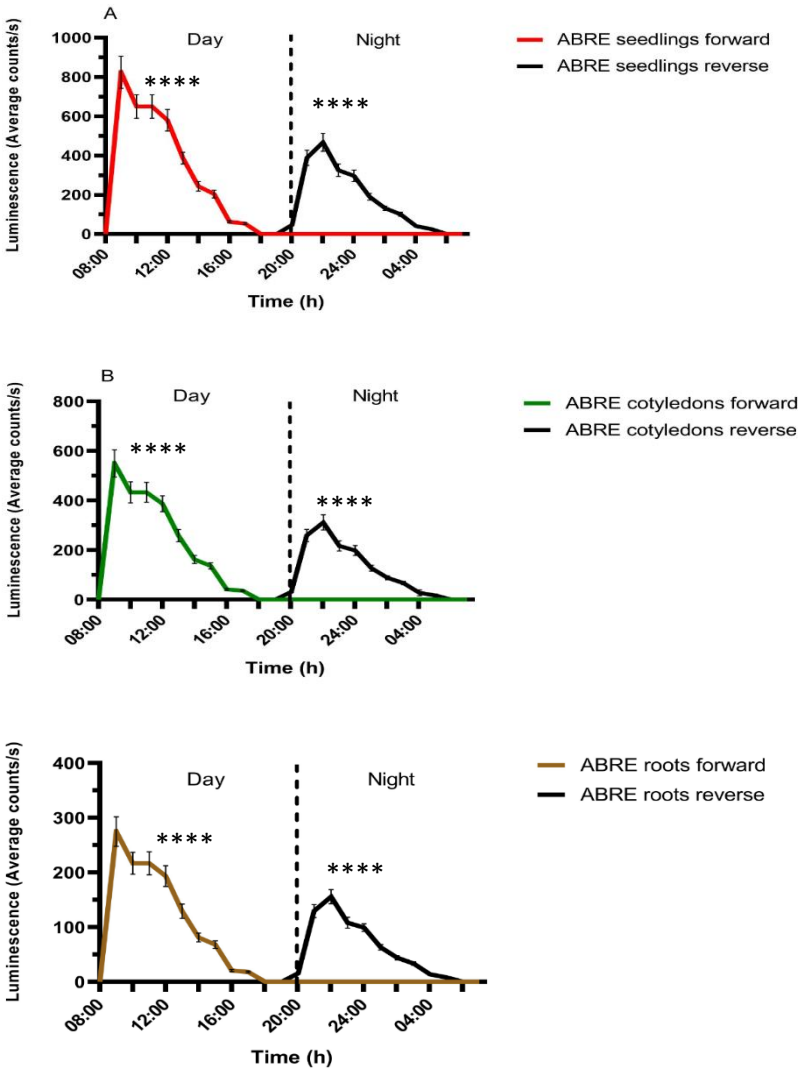


Figure 5.16: Luminescence average counts per second over 24h from Arabidopsis seedlings containing a construct expressing *LUC+* through ABRE. Each line represents an average of 3 seedlings treated with an overall concentration of 10µM mastoparan and 0.5mM ATP. All seedlings were grown in a photoperiod 16L/8D for 24h followed by 7d of entrainment in either 12L:12D (forward) or 12D:12L (reverse) and a further 24h of constant light. Luminescence levels were recorded over 24h. **A:** Seedlings, **B:** Cotyledons, **C:** Roots

Error bars represent ±SEM (n=3 replicates of 3 treated seedlings).). One-way ANOVA of peaks between forward and reverse light cycles **** (P≤0.0001), *** (P≤0.001), ** (P≤0.01) *

Mastoparan/ATP treatment: SITE II

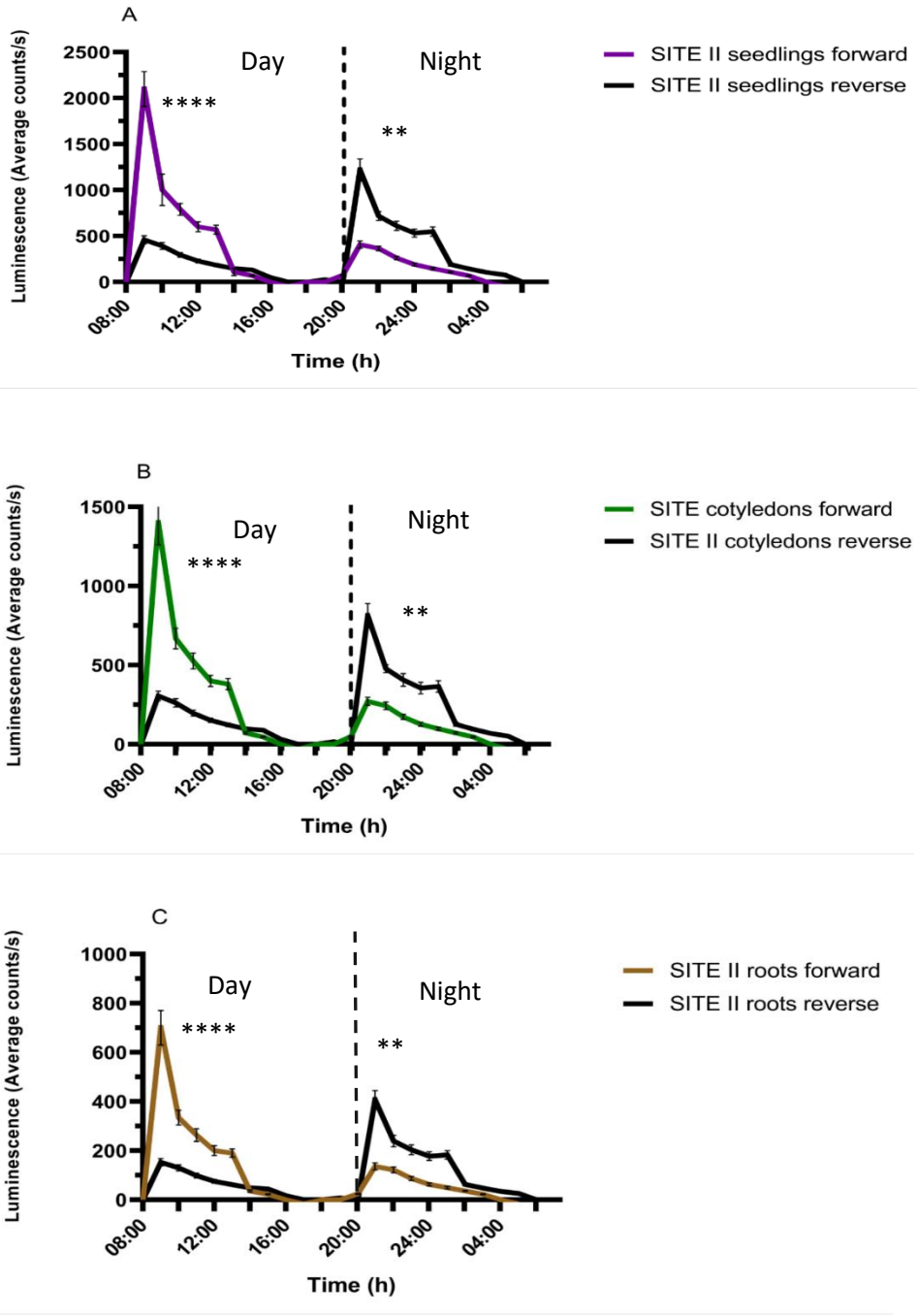


Figure 5.17: Luminescence average counts per second over 24h from Arabidopsis seedlings containing a construct expressing *LUC+* through SITE II. Each line represents an average of 3 seedlings treated with an overall concentration of 10µM mastoparan and 0.5mM ATP. All seedlings were grown in a photoperiod 16L/8D for 24h followed by 7d of entrainment in either 12L:12D (forward) or 12D:12L (reverse) and a further 24h of constant light. Luminescence levels were recorded over 24h. **A:** Seedlings, **B:** Cotyledons, **C:** Roots

Error bars represent ±SEM (n=3 replicates of 3 treated seedlings). One-way ANOVA of peaks between forward and reverse light cycles **** (P≤0.0001), *** (P≤0.001), ** (P≤0.01) *

Table 5.1: Summary of results from transgenic Arabidopsis seedlings treated with calcium agonists whose signatures have been identified as circadian regulated.

Agonist	***Cis* element**	**Results**	
		Subjective Day	**Subjective Evening**
ATP	ABRE (Figure 15.11)	Biphasic response across different cells at different times	Overall diminished monophasic response in comparison to subjective day
	SITE II (Figure 15.12)	Biphasic response seen in both different cells and the same cells at different times	Overall diminished monophasic response in seedlings
	CaM box (Figure 15.13)	Negligible response	A monophasic response recorded in seedlings
$CaCl_2$	CRT (Figure 15.14)	Monophasic response	Diminished prolonged response with no defined peak
Mastoparan/$CaCl_2$	CaM box (Figure 15.15)	A biphasic response recorded in the same cell type (roots)	Negligible response
Mastoparan/ATP	ABRE (Figure 15.16)	Monophasic response	Negligible response
	SITE II (Figure 15.17)	Biphasic response, with each response occurring in different cell types at different times	Monophasic, lower prolonged response

Arabidopsis seedlings expressing *LUC+* driven by ABRE and which were entrained in the forward light cycle (12L:12D) and treated with 0.5mM ATP displayed a biphasic response (Figure 5.11: A) during subjective day (08:00-20:00), with luminescence peaks recorded at 9:30 and 12:00 (Figure 5.11: A). Measurements recorded from the cotyledons and roots resulted in a monophasic response at 12:00 and 11:00, respectively (Figure 5.11: B; C). At subjective evening (20:00-08:00), the seedlings and cotyledons displayed a monophasic response with peaks at 04:00 (Figure 5.11 A; B), however, the response recorded from the roots was negligible as reflected in Figure 5.11: C.

Arabidopsis seedlings expressing *LUC+* driven by ABRE and which were entrained in the reverse light cycle (12D:12L) and treated with ATP, measured a monophasic luminescence

response between 08:00 and 20:00 (subjective evening), with a peak recorded at 09:30 (Figure 5.11: A). A peak at 09.30 was also measured from the cotyledons and the roots (Figure 5.11: B; C), however the roots displayed a sharp incline peaking at 09:30 followed by an immediate sharp decline, in contrast, the cotyledons displayed a more prolonged response plateauing over time (Figure 5.11: B; C). Luminescence counts measured between 20:00 and 08:00 (subjective morning) yielded a biphasic response in the whole seedlings with luminescence peaks recorded at 20:30 and 24:00 (Figure 5.11: A). A monophasic response was displayed in both cotyledons and the roots with peaks generated at 24:00 and 20:30, respectively (Figure 5.11: B; C).

Arabidopsis seedlings expressing *LUC+* driven by SITE II and treated with 0.5mM of ATP which were entrained in the forward light cycle (12L:12D) resulted in a biphasic luminescence response when measured between 08:00-20:00 (subjective day) (Figure 5.12: A). Whole seedlings and cotyledons recorded peaks at 09:00 and 11:00 with similar kinetics (Figure 5.12: A; B;). Measurements recorded from the roots also displayed a biphasic response with peaks at 09:00 and 10:00, but, with a steeper decline and a lower luminescent count to that seen in the whole seedlings and cotyledons (Figure 5.12: C). Between 08:00 and 20:00 seedlings entrained in the reverse cycle (subjective evening) displayed a monophasic luminescence response with a peak generated at 09:00 (Figure 5.12: A), with both cotyledons and roots displaying the same patterns (Figure 15.12: B; C). Between 20:00-08:00 (subjective evening), seedlings entrained in the forward light cycle expressing *LUC+* driven by SITE II displayed a monophasic luminescence response with a peak recorded at 21:00 (Figure 15.12: A), this response was also recorded in both the cotyledons and roots (Figure 5.12: B; C). In comparison, seedlings entrained in the reverse photoperiod after 20:00 (subjective morning) displayed a biphasic luminescent response with peaks recorded at 21:00 and 24:00 (Figure 12: A), this response was also recorded in the cotyledon measurements (Figure 5.12; B), however, in contrast, data recorded from the roots displayed a monophasic response with a peak at 01:00 (Figure 5.12: C).

Arabidopsis seedlings expressing *LUC+* driven by CaM box and treated with 0.5mM ATP which had been entrained in the forward light cycle (12L:12D) displayed a minimal response between 08:00-20:00 (Figure 5:13 A). The response in the whole seedlings, cotyledons and roots was monophasic, with a peak at 09:00 (Figure 5:13 A; B; C). During subjective evening (20:00-08:00) seedlings recorded high luminescence counts, generating a monophasic

response with a peak recorded at 21:00 (Figure 5.13: A). Measurements from cotyledons and roots mirrored what was recorded in the whole seedlings displaying a single peak at 21:00, but with lower luminescence counts (Figure 5.13: B; C).

Seedlings, entrained in the reverse photoperiod (12D:12L) expressing *LUC+* which were driven by CaM box and treated with ATP recorded a monophasic response between 08:00-20:00 (subjective evening), with a peak recorded at 09:00 (Figure 5.13: A). Luminescence measurements from both cotyledons and roots displayed the same kinetics as those recorded in the seedlings but with lower luminescence counts (Figure 5.13: B; C). The response from the reverse entrained seedlings after 20:00 (subjective morning) produced a minimal luminescence count with a single peak recorded at 21:00 (Figure 5.13 A), both cotyledons and roots displayed the same kinetics recording a peak at 21:00 albeit, with lower luminescence counts (Figure 5.13 B; C).

Arabidopsis seedlings expressing *LUC+* and driven by CRT which had been entrained in a forward light cycle (12L:12D) and treated with an overall concentration of 50mM $CaCl_2$, generated a monophasic luminescence response between 08:00-20:00 (Figure 5.14: A). The response displayed a gradual increase of counts which peaked at 12:00, followed by a sharp decline at 16:00 (15.14: A). Luminescence measurements recorded from both cotyledons and roots displayed patterns in line with those recorded in the whole seedlings (Figure 5.14: B; C). During subjective evening (20:00-08:00) these seedlings yielded a low response, recording a prolonged slope over time with no defined peaks (Figure 5.14: A), this result was also reflected in the subsequent cotyledon and root measurements (Figure 5.14: B; C).

Arabidopsis seedlings expressing *LUC+* which were driven by CRT, entrained in a reverse light cycle (12D:12L) and treated with $CaCl_2$ recorded a low luminescence count when measured between 08:00 and 20:00 (subjective evening) (Figure 5.14: A). A low prolonged response was recorded over time with no distinctive peaks (Figure 5.14: A), measurements recorded from both cotyledons and roots displayed the same kinetic patterns as those seen in the seedlings (Figure 5.14: B; C). During subjective day (20:00-08:00) seedlings entrained in the reverse light cycle (12D:12L) displayed a monophasic result, with a prolonged response peaking at 01:00 followed by a sharp decline at 03:00 (Figure 5.14: A). Luminescence measurements from cotyledons and roots displayed the same patterns generating a gradual monophasic response with a peak at 01:00 (Figure 5.14: B; C).

Arabidopsis seedlings expressing *LUC+* which were driven by CaM box, entrained in a forward light cycle (12L:12D) and treated with an overall concentration of 10µM mastoparan/50mM $CaCl_2$ generated a biphasic luminescence response, with peaks recorded at 09:00 and 10:00 (Figure 5.15: A). Cotyledon measurements recorded a monophasic response with a single peak displayed at 10:00 (Figure 5.15: B), and roots displayed a biphasic response reflecting the kinetics observed from the whole seedlings with peaks at 9:00 and 10:00 (Figure 5.15: C). After 20:00 the luminescence count was reduced to negligible amount in whole seedlings, cotyledons, and roots (Figure 5.15; A; B; C).

Arabidopsis seedlings expressing *LUC+* which were driven by CaM box, entrained in a reverse light cycle (12D:12L) and treated with a combination of mastoparan and $CaCl_2$, displayed low levels of luminescence (Figure 5.15: A) between 08:00 and 20:00 (subjective evening). After 20:00 (subjective morning) these seedlings generated a biphasic response, with peaks measured at 21:00 and 22:00 (Figure 5.15: A). The root measurements generated similar kinetics to that of the seedlings with peaks displayed at 21:00 and 22:00 (Figure 5.15: C). Luminescence counts recorded from the cotyledons displayed a monophasic response with a peak generated at 22:00 (Figure 5.15: B).

Arabidopsis seedlings expressing *LUC+* which were driven by ABRE, entrained in a forward light cycle (12L:12D) and treated with a combination of 10µM mastoparan 0.5mM ATP displayed monophasic kinetics between 08:00-22:00 (Figure 5.16 A). The initial response recorded a sharp incline with a peak at 09:00, followed by an immediate decline until 10:00 with a plateau for 1.5h followed by a further decline (Figure 5.16: A). Measurements from both the cotyledons and roots displayed the same kinetics as those recorded with the whole seedlings (Figure 5.16: B; C).

Arabidopsis seedlings expressing *LUC+* which were driven by ABRE entrained in a reverse light cycle (12D:12L) and treated with a combination of mastoparan and ATP displayed a low luminescence level before 20:00 (subjective evening), which is reflected by the lack of representation on the graphs (Figure 5.16: A; B; C). After 20:00 (subjective morning) a monophasic response was recorded displaying an initial sharp incline with a peak at 22:00, followed by a decline, a plateau for 1.5h and a subsequent decline over time (Figure 5.16:

A). Both the cotyledons and the roots recorded favourable kinetics to that of the seedlings (Figure 5.16: B; C).

Arabidopsis seedlings expressing *LUC+* which were driven by SITE II, entrained in a forward light cycle (12L:12D) and treated with a combination of mastoparan and ATP generate a biphasic luminescence response when measured before 20:00 (Figure 5.17: A). A single peak was recorded at 09:00 which was followed by a rapid decline and recorded in the seedlings, cotyledons, and roots (Figure 5.17: A; C-E). In subjective evening (20:00-08:00) a lower luminescence response was recorded with a peak at 21:00 and a gradual prolonged decline in the seedlings, cotyledons, and the roots (Figure 5.17: A; C-E).

Between 09:00 and 20:00 Arabidopsis seedlings expressing *LUC+* which were driven by SITE II, entrained in a reverse light cycle (12D:12L) and treated with a combination of mastoparan and ATP recoded a monophasic prolonged response, with the seedlings, cotyledons and roots recording a peak at 09:00 (subjective evening) (Figure 5.17: A; B; C). From 20:00 (subjective morning) seedlings entrained in the reverse light cycle generated a biphasic response with a defined peak at 21:00 and a small peak at 01:00 (Figure 5.17; A). Both cotyledons and roots recorded similar kinetic patterns as those displayed in the whole seedlings (Figure 5.17: B; C).

5.3 Discussion

5.3.1 Do ATP or mastoparan/$CaCl_2$ $[Ca^{2+}]_{cyt}$ signature peaks occur in the same cells or in different cells types when regulating specific *cis* elements?

ATP $[Ca^{2+}]_{cyt}$ signatures regulating Arabidopsis seedlings containing a construct consisting of a *LUC+* coding region driven by ABRE displayed a biphasic expression response (Figure 5.1), which supports the data recorded in chapter three. When analysing cotyledons and roots separately it can be seen that there are differing kinetics between the different plant tissues. The cotyledons and roots recorded a biphasic response at 240 min and 90 min respectively (Figure 5.1), which correspond to the peaks recorded in the whole seedlings.

These data suggests that the regulation of ABRE by ATP $[Ca^{2+}]_{cyt}$ signatures occurs in different cells at different times.

Previous literature has indicated that the P2K1 extracellular ATP (eATP) receptor is expressed during all major stages of plant development and growth (Cho et al., 2017, Jewell et al., 2019), therefore by inference, suggesting that ATP is involved in numerous physiological processes including growth, abiotic and biotic stress responses (Balagué et al., 2017, Bouwmeester et al., 2011, Cho et al., 2017, Tripathi and Tanaka, 2018). This is interesting because we know that ABRE has been linked to abiotic and biotic stress responses (Assmann, 2003, Cutler et al., 2010, Hubbard et al., 2010, Lee and Luan, 2012, Lim et al., 2014, Wilkinson and Davies, 2010) and my data thus far suggests that ATP regulates ABRE. ATP data from both chapter 3 and 5 suggest that both ATP-generated $[Ca^{2+}]_{cyt}$ signatures and subsequent ABRE expression display polyphasic kinetics. It has been shown that the ABRE promoter region is possibly regulated by $[Ca^{2+}]_{cyt}$ (Kaplan et al., 2006) and therefore may regulate thousands of genes, it would then seem feasible that ATP generated $[Ca^{2+}]_{cyt}$ signatures could be regulating these genes in varying cells at different times.

ATP treatment produced a biphasic expression response from the SITE II *cis* element (Figure 5.2), which supports earlier data from chapter 3 section 3.3.2. Again, the cotyledons displayed a similar expression pattern to that of the whole seedlings, with luminescence peaks at 90 and 150 min, but, unlike the ABRE, the roots from SITE II seedlings demonstrated a biphasic response, with peaks at 60 min and 120 min. These data suggest that ATP $[Ca^{2+}]_{cyt}$ signatures regulate SITE II in different cells and the same cells at different times. It has been suggested that ATP generates a calcium cascade within the plant root (Demidchik et al., 2009) which supports the data highlighted in this chapter. Work by Mattheus et al., (2019) describes eATP application to specific plant tissues e.g. the sub apex of the root, but they have not been able to conclude if the $[Ca^{2+}]_{cyt}$ signature induced by eATP results in a systemic transcriptional response. They have suggested that the DORN1 eATP receptor is mainly active in the root, but in contrast several different receptors may be involved in leaf perception of the eATP signal, which could explain why a biphasic response was seen in the cotyledons in my data.

The combined treatment of mastoparan and $CaCl_2$ resulted in a biphasic expression response from CaM box driven seedlings, which supports the data described earlier in chapter 3 (3.2.2.); suggesting that a biphasic response occurs both with the generated calcium signature and the subsequent gene expression. When analysing data from cotyledons and roots subjected to this treatment, a biphasic response occurs in the roots only (Figure 5.3) at 60 min and 120 min, which coincides with the biphasic response in the whole seedlings. The cotyledons only present a single luminescence peak at 120 min, suggesting that the overall biphasic response is occurring in the same cell types at different times.

As described in chapter 1, mastoparan is a G protein agonist and in animal systems can activate heterotrimeric G proteins which in turn induce calcium changes (Ross and Higashijima, 1994, Sukumar et al., 1997). However, it has been suggested that mastoparan effects in plants can occur independent of the heterotrimeric G proteins and to date the target of mastoparan in plant cells has not been defined (Miles et al., 2004). In regard to a combination of mastoparan and $CaCl_2$ generated $[Ca^{2+}]_{cyt}$ signatures and their effect upon subsequent gene regulation, there is no published information. From this we can surmise that mastoparan/$CaCl_2$ $[Ca^{2+}]_{cyt}$ signatures generate a biphasic response in CaM box driven seedlings, however, it is difficult to determine the reason for this.

5.3.2 The importance of circadian clock regulation of calcium agonist-generated $[Ca^{2+}]_{cyt}$ signatures.

Results in this chapter suggest that novel agonist generated $[Ca^{2+}]_{cyt}$ signatures may be regulated by the circadian clock. These data have recorded differences between signals generated in seedlings entrained in a forward light cycle (12L:12D) to those entrained in a reverse light cycle (12D:12L). ATP (Figure 5.5), $CaCl_2$ (Figure 5.6) and a combination of Mastoparan/$CaCl_2$ (Figure 5.8) and mastoparan/ATP (Figure 5.9) display distinct differences in their signal kinetics between the two different light cycles, which is further supported by statistical analysis of p values <0.005 to 0.001. These data have also shown that some $[Ca^{2+}]_{cyt}$ signatures may not be regulated by the circadian clock. Mastoparan (Figure 5.4), L-glutamate (Figure 5.5) and a combination of mastoparan and L-glutamate (Figure 5.7)

recorded no significant difference between signals generated in seedlings entrained in a forward light cycle to those entrained in a reverse light cycle.

Previous literature has suggested that $[Ca^{2+}]_{cyt}$ responses can be gated by the circadian clock (Martí Ruiz et al., 2018, Trewavas, 1999, Webb, 2003, Wood et al., 2001a) but there has been no investigation in regards to the specificity of these signatures in terms of downstream effects. In chapter 3 it was suggested that $[Ca^{2+}]_{cyt}$ signatures are specific and carry distinct information that leads to a change in gene expression, and subsequent expression of appropriate proteins as a response to a stimulus. If this hypothesis is correct, then it would be feasible that circadian regulation of $[Ca^{2+}]_{cyt}$ may also be specific, that there may be consequences to the differences in the calcium signatures in response to the different agonists when applied at subjective morning versus subjective evening, as seen in these data . Further evidence for possible specificity can be seen with results recorded from the combined agonists, e.g., mastoparan by itself does not seem to be circadian regulated but when combined with $CaCl_2$ or ATP, circadian regulation seems to be apparent, thus, further supporting the earlier data in chapter three, showing the potential for specificity.

5.3.3 Investigating the importance of circadian regulated $[Ca^{2+}]_{cyt}$ signatures on expression kinetics.

In section 5.2.2 data indicated that novel $[Ca^{2+}]_{cyt}$ signatures may be regulated by the circadian clock, therefore the next logical step was to determine if circadian regulation of these signatures resulted in changes in subsequent expression kinetics. Repeats of experiments conducted in chapter 3 to determine specific $[Ca^{2+}]_{cyt}$ signature regulation of previously identified calcium regulated TFs (Kaplan et al., 2006, Whalley et al., 2011), was conducted on transgenic Arabidopsis seedlings entrained either in a forward light cycle (12L:12D) or a reverse light cycle (12D:12L). These seedlings were treated with an agonist which generated a calcium signal identified as circadian regulated in 5.2.2. It was seen that ATP and a combination of mastoparan/ATP predominantly regulated ABRE and SITE II TFs during subjective morning, supporting the findings from chapter 3 (Figure 3.5), however the expression kinetics at subjective evening differed, here ABRE and SITE II displayed a

diminished response and CaM box was predominately expressed (Appendix A.7). Following on from this, further investigations identified that ATP treatment led to biphasic expression kinetics from both ABRE and SITE II TFs (Figure 5.12) during subjective day which supports earlier data in chapter 3 and chapter 5 (Figure 5.1), but this response was lost at subjective evening, which could explain the anomalies reported in some of the repeats conducted in 5.2.1. Similar results were seen from SITE II expression kinetics (Figure 5.12) with the biphasic response lost during subjective evening. CaM box expression was low during subjective morning with ATP treatment, which supported the findings in chapter 3, however, during subjective evening where ABRE and SITE II expression kinetics were reduced CaM box luminescence counts were high (Figure 5:13). ABRE and SITE II kinetics differed between light cycles with the combined treatment (Figure 5.16; 5:17), in the case of ABRE, this manifested in a reduced expression rather than a change in number of peaks (Figure 5:16). The SITE II TF, data however, suggests that at subjective evening there was a possible biphasic response compared to a monophasic response during subjective morning (Figure 5:17).

The ABA responsive element (ABRE) is known to be an enriched promotor of cold induced genes (Kreps et al., 2002b). It has been acknowledged that cold genes are regulated by the circadian clock (Bieniawska et al., 2008) so it is feasible that ABRE TFs could also be circadian regulated which concurs with these data in this chapter. SITE II TFs have been linked to diurnal changes in organelle protein abundances (Giraud et al., 2010) suggesting circadian regulation, which supports these data in this chapter. CaM box TFs have been suggested as promotor regions found in the circadian oscillator and involved in the negative feedback loop which generates circadian rhythms (Yang et al., 2018), which could possibly explain why CaM box is regulated at night by circadian controlled $[Ca^{2+}]_{cyt}$ signatures in these data.

Work conducted with $CaCl_2$-generated $[Ca^{2+}]_{cyt}$ signatures recorded high expression kinetics, with a monophasic response from CRT driven seedlings during subjective morning (Figure 5:14), which supports data from chapter 3 (Figure 3.4). During subjective evening, the luminescence counts were reduced, displaying a prolonged response with no distinctive peak (Figure 5:14). This suggests that $CaCl_2$-generated signatures are regulated by the circadian clock, which has a direct effect on subsequent expression kinetics. As with ABRE it is known that CRT is linked to drought and the cold response therefore, could

possibly be circadian regulated (Fowler et al., 2005, Kreps et al., 2002b). As data in chapter 3 and 5 suggest $[Ca^{2+}]_{cyt}$ signatures generated from $CaCl_2$ regulate CRT, it is therefore reasonable to propose that circadian regulation of $[Ca^{2+}]_{cyt}$ signatures generated from $CaCl_2$ result in a change in expression kinetics of CRT.

Cytosolic calcium signatures generated from a combination of mastoparan and $CaCl_2$ displayed high luminescence counts and a biphasic expression response from CaM box driven seedlings in subjective morning (Figure 5:15), supporting earlier data in chapters 3 and chapter 5 (5.2.1). However, data from subjective evening suggested that CaM box had been suppressed, implying that the circadian regulated $[Ca^{2+}]_{cyt}$ signatures generated from mastoparan/$CaCl_2$ were in fact inducing changes in the expression kinetics of CaM driven seedlings (5.15).

5.4 Conclusion

The objective of this chapter was to answer three specific questions:

I. Are biphasic expression patterns due to $[Ca^{2+}]_{cyt}$ signatures occurring in different cell types or the same cell types at different times ?
II. Are particular $[Ca^{2+}]_{cyt}$ signatures regulated by the circadian clock?
III. If there is circadian regulation of calcium signatures does this reflect in changes in expression kinetics?

The data presented in this chapter seems to suggest that some specific $[Ca^{2+}]_{cyt}$ signatures do in fact generate biphasic expression kinetics. These biphasic responses can be due to calcium signatures occurring in different cell types or in the same cell types at different times. Data from this chapter also implies that specific $[Ca^{2+}]_{cyt}$ signatures can be regulated by the circadian clock, and this regulation may produce significant changes in the transcriptional activity of particular calcium-regulated TFs.

6. Calcium regulation of combined cis elements

6.1 Introduction

It is known that when a plant encounters an abiotic stress it responds by triggering a cascade of events. These events begin with stimuli perception, which initiates a signalling pathway, which then relays information to instigate changes including downstream gene expression, imparting conveying stress tolerance to the plant (Allen et al., 2001, Kim et al., 2011, Whalley and Knight, 2013, Zipfel and Oldroyd, 2017). It has been shown in literature that a complicated transcriptional regulatory network is associated with these stress genes, conserved specific *cis*-acting elements have been identified in their promoter regions, as well as interacting TFs (Bartels and Sunkar, 2005, Medina et al., 2011, Nakashima et al., 2009) .

It is known that plants can be exposed to several stress stimuli at a single given time, and therefore by inference to many signal transduction pathways which could cross talk at several steps (Roychoudhury et al., 2013). Abscisic acid (ABA) for instance is involved with many responses including abiotic stresses such as osmotic and cold stress (Agawal and Jha, 2010, Kang et al., 2002, Kim et al., 2004, Roychoudhury et al., 2013). During osmotic stress ABA triggers the expression of osmotic stress response genes (OR) which may contain a single copy or multiple copies of the abscisic acid-response (ABRE) *cis* element in their promoters (Hobo et al., 1999, Marcotte et al., 1989, Yamaguchi-Shinozaki and Shinozaki, 2006). It has been shown that expression of ABA-responsive genes require multiple copies of ABREs or an ABRE with a coupling element (Hobo et al., 1999, Marcotte et al., 1989, Yamaguchi-Shinozaki and Shinozaki, 2006).

The dehydration-responsive element (DRE/CRT) is another example of a *cis* element that is involved in both osmotic and cold stress responsive transcription (Agawal and Jha, 2010, Nakashima et al., 2009) and is part of the ABA-independent pathway (Shinozaki and Yamaguchi-Shinozaki, 2000). Knight and Knight, (2001) suggested that several abiotic stress pathways share common elements and therefore, could potentially cross talk (Knight and Knight, 2001). The *RD29A* gene for instance, contains both DRE/CRT and ABRE repeats and

therefore integrates input stimuli from cold, drought, high salinity, and ABA signalling pathways. Early studies have indicated that *aba1* mutations only partially block osmotic induction of *RD29A* implying that ABA-independent and ABA dependent regulation is occurring (Yamaguchi-Shinozaki and Shinozaki, 1994).

Both in chapter 1 and chapter 3 I discussed that Whalley et al., (2011) discovered that there were a high frequency of four conserved *cis* element sequences (ABRE, CaM box, DRE/CRT and SITE II) were found in the promoter regions of genes regulated by calcium. Using constructs designed for this study I suggested that these *cis* elements were regulated by different $[Ca^{2+}]_{cyt}$ signatures, implying specificity. It has been shown that in a natural environment plant gene may contain more than one type of these conserved *cis* regulatory sequences, therefore, to reflect this I have decided to repeat the experiments from chapter 3 in regard to the regulation of these *cis* elements, but with new constructs containing a combination of these sequences.

Question to be addressed in this chapter

I. When combined, do calcium-regulated *cis* elements respond to agonist generated $[Ca^{2+}]_{cyt}$ signatures?
II. Does this response generate different expression kinetics to that seen with the single *cis* elements?

6.2 Results

To investigate calcium agonist-generated $[Ca^{2+}]_{cyt}$ signature regulation of multiple *cis* elements, I modified the constructs designed in Whalley at el., (2011). Seven constructs were designed (See Appendix A.1 for sequences), 6 containing 2 alternating repeats of 2 different calcium-regulated *cis* elements (either ABRE/SITE II; CRT/ABRE; CaM box/ABRE; CaM box/SITEII; CRT/CaM box) and the 7th a minimal promoter sequence of 90bp. Each construct drove expression of a *LUC+* coding sequence and contained a 35Ss transcriptional terminator as described in Whalley et al., (2011). The *LUC+* had been previously cloned into pDH51 (Pietrzak et al., 1986) using *SmaI* and *XbaI* restriction sites to produce the construct

pDHLC+1, which contained the LUC+ coding region between the 35S promoter and terminator. *NcoI* was used to delete the region between the start of the 35S promoter and the start codon of *LUC+* (within the NcoI restriction site). This resulted in a promoter less *LUC+* construct, pDHLC+2, into which *NcoI-NcoI* promoter fragments could be cloned. The synthesised concatemer with the minimal promoter was then cloned into the modified pDH51 (Whalley et al., 2011) plasmid vector (plasmid map Appendix B.1) adopting the Gibson assembly method, followed by further cloning into the binary vector pBIN19 (Vector map Appendix B.2) (for full methods see 2.2.6.11.3). The pBIN19 vectors containing the construct were transformed into *A. tumefaciens* (full methods found at *2.2.6.12.1.2*) and then transformed into *A. thaliana* ecotype Columbia (Col-0) via the floral dip method (method found in chapter 2: *2.2.6.13.1*). After selection (chapter 2: *2.2.6.13.3*) the seeds were grown as described in chapter 2: 2.2.2.4 for 10d. Ten-day old transgenic seedlings were treated with one of the calcium agonists or water and luminescence was measured over 8h (see chapter 2: 2.2.3.2 for full methods). Due to the impact of Covid 19 I was only able to record ATP generated $[Ca^{2+}]_{cyt}$ signature regulation of all the combined *cis* elements, mastoparan $[Ca^{2+}]_{cyt}$ signature regulation of the combined *cis* elements CRT/CaM box and a water control for all of the transgenic plants.

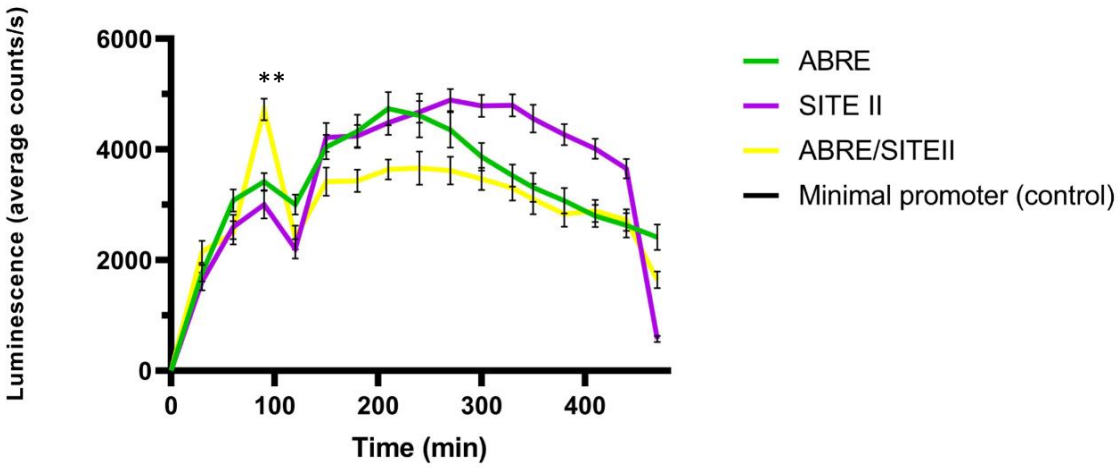


Figure 6.1: Average luminescence counts per second from five 10d old transgenic Arabidopsis seedlings. Each line represents seedlings containing a construct expressing *LUC+*, driven by either ABRE, SITE II or a combination of these regulatory *cis* elements, or a minimal promotor control. Seedlings have been treated with 0.5mM ATP over 480 min.

Error bars represent ±SD (n=3replicates of 5 treated seedlings).

Two-way ANOVA of peaks at 90 min between *cis* elements **** (P≤0.0001), *** (P≤0.001), ** (P≤0.01) * (P≤0.05)

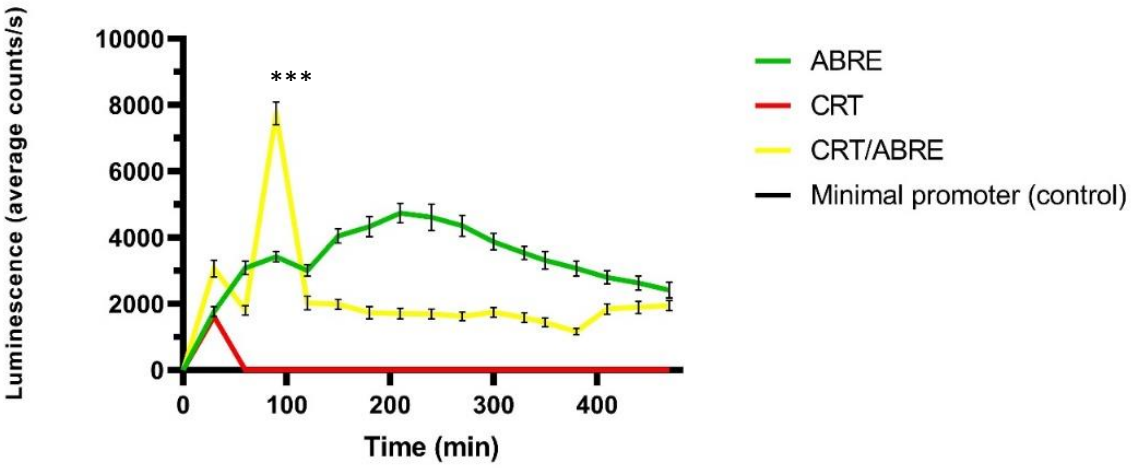


Figure 6.2: Average luminescence counts per second from five 10d old transgenic Arabidopsis seedlings. Each line represents seedlings containing a construct expressing *LUC+,* driven by either CRT, ABRE or a combination of these regulatory *cis* elements, or a minimal promotor control. Seedlings have been treated with 0.5mM ATP over 480 min.

Error bars represent ±SD (n=3 replicates of 5 treated seedlings).

Two-way ANOVA of peaks at 90 min between *cis* elements **** (P≤0.0001), *** (P≤0.001), ** (P≤0.01) * (P≤0.05)

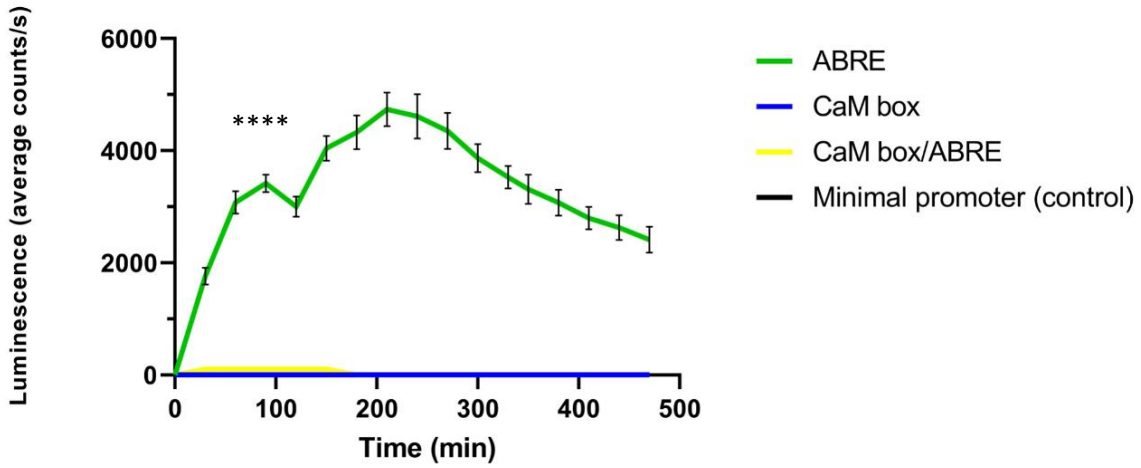


Figure 6.3: Average luminescence counts per second from five 10d old transgenic Arabidopsis seedlings. Each line represents seedlings containing a construct expressing *LUC+,* driven by either ABRE, CaM or a combination of these regulatory *cis* elements, or a minimal promotor control. Seedlings have been treated with 0.5mM ATP over 480 min.

Error bars represent ±SD (n=3 replicates of 5 treated seedlings).

Two-way ANOVA of peaks at 90 min between *cis* elements **** (P≤0.0001), *** (P≤0.001), ** (P≤0.01) * (P≤0.05)

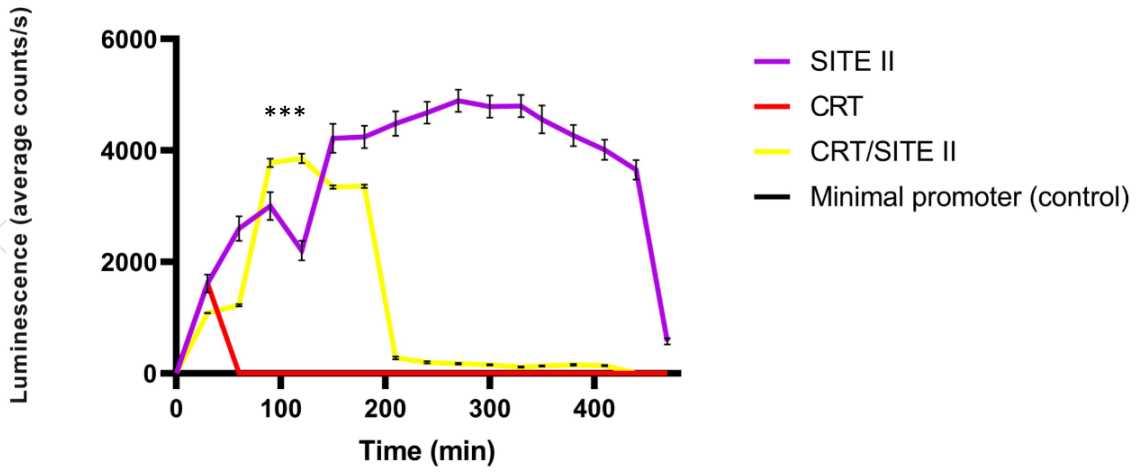


Figure 6.4: Average luminescence counts per second from five 10d old transgenic Arabidopsis seedlings. Each line represents seedlings containing a construct expressing *LUC+,* driven by either SITE II, CRT or a combination of these regulatory *cis* elements, or a minimal promotor control. Seedlings have been treated with 0.5mM ATP over 480 min.

Error bars represent ±SD (n=3 replicates of 5 treated seedlings).

Two-way ANOVA of peaks at 90 min between *cis* elements **** (P≤0.0001), *** (P≤0.001), ** (P≤0.01) * (P≤0.05)

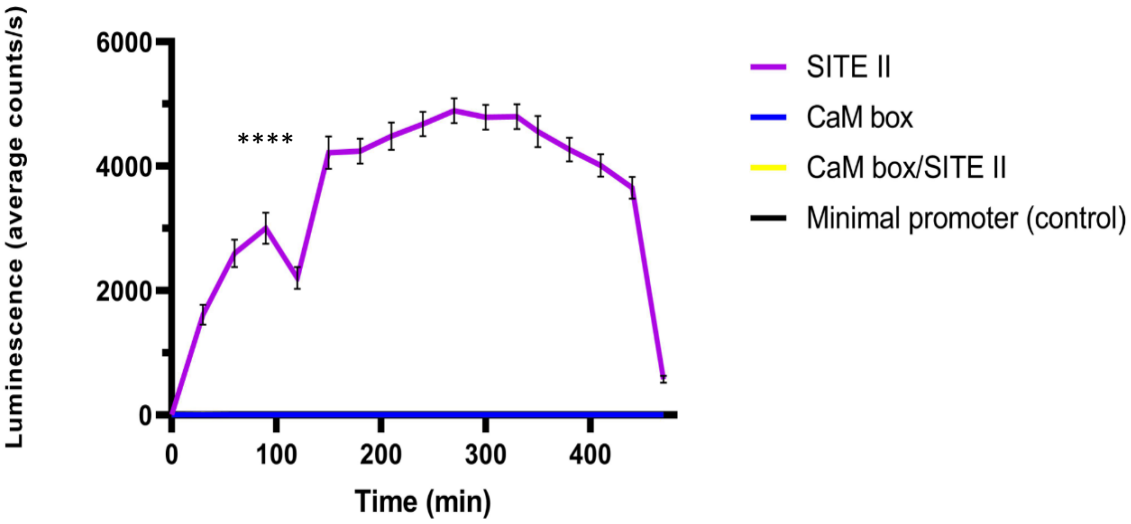


Figure 6.5: Average luminescence counts per second from five 10d old transgenic Arabidopsis seedlings. Each line represents seedlings containing a construct expressing *LUC+,* driven by either SITE II, CaM box or a combination of these regulatory *cis* elements, or a minimal promotor control. Seedlings have been treated with 0.5mM ATP over 480 min.

Error bars represent ±SD (n=3 replicates of 5 treated seedlings).

Two-way ANOVA of peaks at 90 min between *cis* elements **** (P≤0.0001), *** (P≤0.001), ** (P≤0.01) * (P≤0.05)

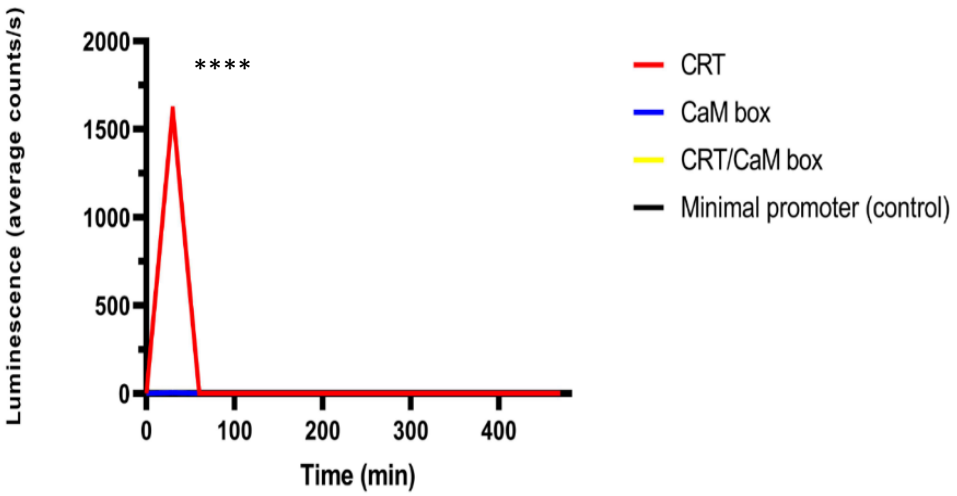


Figure 6.6: Average luminescence counts per second from five 10d old transgenic Arabidopsis seedlings. Each line represents seedlings containing a construct expressing *LUC+,* driven by either CRT, CaM box or a combination of these regulatory *cis* elements, or a minimal promotor control. Seedlings have been treated with 0.5mM ATP over 480 min.

Error bars represent ±SD (n=3 replicates of 5 treated seedlings).

Two-way ANOVA of peaks at 30 min between *cis* elements **** (P≤0.0001), *** (P≤0.001), ** (P≤0.01) * (P≤0.05)

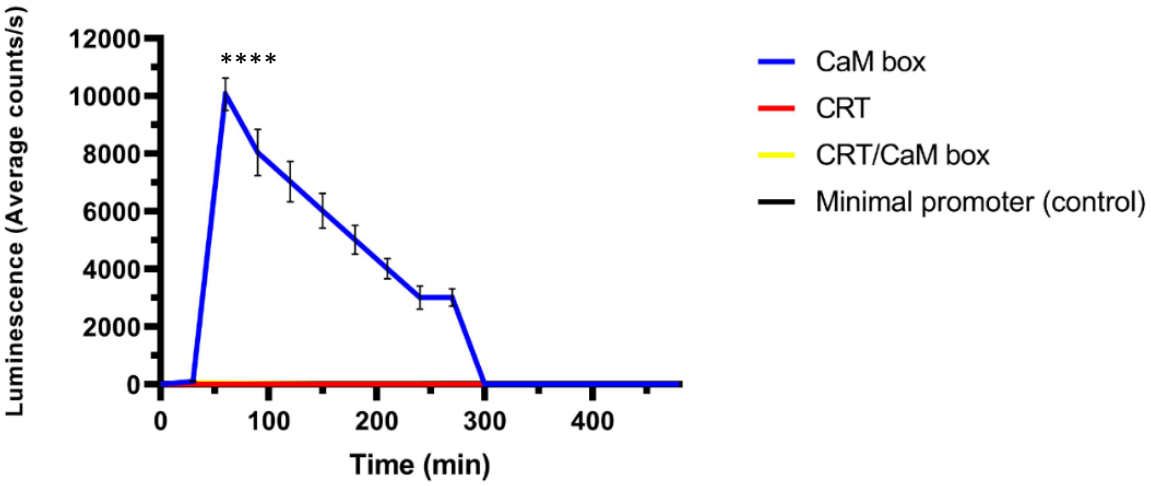


Figure 6.7: Average luminescence counts per second from five 10d old transgenic Arabidopsis seedlings. Each line represents seedlings containing a construct expressing *LUC+,* driven by either CaM box or CRT or a combination of these regulatory *cis* elements, or a minimal promotor control. Seedlings have been treated with an overall concentration of 10µM mastoparan over 480 min.

Error bars represent ±SD (n=3 replicates of 5 treated seedlings). Two-way ANOVA of peaks at 60 min between *cis* elements **** (P≤0.0001), *** (P≤0.001), ** (P≤0.01) * (P≤0.05)

Seedlings expressing *LUC+* driven by either ABRE or SITE II and treated with 0.5mM ATP displayed biphasic responses with peaks recorded at 90 min and 120 for ABRE, and 90 min and 150 min by SITE II (Figure 6.1-6.5), which supports earlier data from both chapter 3 (Figure 3.5: B) and chapter 5 (Figures 5.1; 5.2), displaying the same kinetics. Data from seedlings driven by the two combined *cis* elements ABRE and SITE II treated with 0.5mM ATP also produced a biphasic response (Figure 6.1). The response initially displayed a sharp incline which peaked at 90 min, followed by an immediate decline. The second peak was

generated at 150 min which was prolonged until 450 min where it displayed a rapid decline (Figure 6.1).

Seedlings expressing *LUC+* driven by a combination of CRT/ABRE and treated with 0.5mM ATP generated a triphasic response (Figure 6.2). The first peak was measured at 30 min followed by a peak at 90 min which declined sharply until 120 min where a low prolonged response was recorded until the third peak at 410 min (Figure 6.2). Seedlings expressing *LUC +* driven by CRT and treated with 0.5mM ATP displayed a single peak at 30 min (Figures 6.2;6.4; 6.6), which supports earlier data in chapter 3 (Figure 3.5: B), which also measured a peak at 30 min.

Seedlings expressing *LUC+* driven by a combination of CaM box and ABRE *cis* elements and treated with 0.5mM ATP recorded a response like that of the minimal promoter control (Figure 6.3). Seedlings expressing *LUC+* driven by CaM box and treated with 0.5mM ATP recorded no response (Figures 6.3 and 6.5), which corresponds to data in chapter 3 (Figure 3.5: B).

Seedlings expressing *LUC+* driven by combined *cis* elements CRT/SITE II and treated with 0.5mM ATP recorded a biphasic response (Figure 6.4). Peaks were generated at 30 min and 90 mins which preceded a decline and a plateau followed by a gradual increase to 120 mins (Figure 6.3). A further decline in the response was recorded until 140 min, which was followed by a plateau for 50 min and then a decline from a 190 min to 210 min, the response then gradually declined until 410 min (Figure 6.3).

Seedlings expressing *LUC+* driven by a combination of *cis* elements CRT and CaM box and treated with 0.5mM ATP or 10µM of mastoparan displayed no response (Figures 6.6 and 6.7 respectively). The lines containing the single CaM box *cis* element treated with 10µM mastoparan recorded a monophasic response with a peak at 60 min followed by a gradual decline (Figure 6.7), which reflects the results recorded in chapter 3 (Figure 3.4). Seedlings containing the MP control displayed a very low response which is reflected by their lack of representation on all the graphs.

6.3 Discussion

Results from chapter 3 suggested that ATP-generated $[Ca^{2+}]_{cyt}$ signatures may specifically regulate ABRE and SITE II *cis* elements , and to a lesser extent the CRT *cis* element (Figure 3.5: B). From initial analysis, the data displayed in figure 6.1 indicates that when combined, the ABRE and SITE II *cis* elements also seem to be regulated by the ATP-generated $[Ca^{2+}]_{cyt}$ signature (Figure 6.1). The kinetic expression patterns recorded from the ABRE/SITE II combination is similar to the expression patterns displayed from the single ABRE, with a peak at 90 min and a second prolonged peak, albeit with different luminescence counts (Figure 6.1). It could be possible that the close proximity of the *cis* element sequences may be affecting the response, in a natural environment you would expect to find the sequences separated across the promoter region (Shinozaki and Yamaguchi-Shinozaki, 2000) . It could also be argued that *cis* element order may affect the expression kinetics, however, this is unlikely as many TFs work as dimers, one on each side of the element. It has been suggested by Whalley et al., (2011) that SITE II is rarely coupled with the ABRE *cis* element in calcium regulated genes, therefore, as the expression kinetics between the single and double *cis* elements are similar, may represent the response from a single ABRE *cis* element rather than the two combined *cis* elements.

Work by Whalley et al., (2011) suggested that the CRT *cis* element was most likely to be coupled to a second *cis* element in calcium regulated genes. Their research concluded more than half of the genes regulated by calcium containing a CRT also contained a second *cis* element, particularly ABREs or SITE IIs, however, it was uncommon to find CRT coupled with CaM box. Results recorded in figures 6.2 and 6.4, support the conclusions by Whalley et al., (2011), both CRT/ABRE (Figure 6.2) and CRT/SITE II (Figure 6.4) in response to ATP generated $[Ca^{2+}]_{cyt}$ signatures displaying novel expression kinetics compared to their single counterparts. It can be seen that CRT/CaM box (Figure 6.6), CaM box/ABRE (Figure 6.3) and CaM box/SITE II (Figure 6.5) recorded a very low or no response from ATP generated $[Ca^{2+}]_{cyt}$ signatures. Previous results from chapter 3 (Figure 3.5: B), suggest that ATP-generated $[Ca^{2+}]_{cyt}$ signatures do not regulate CaM box, this was also reflected in the single CaM box expression kinetics in figures 6.3; 6.5 and 6.6. It could be possible that the positioning of the *cis* elements is affecting expression kinetics, that the CaM box in some way is affecting the Ca^{2+} signature regulating the second cis element. This could occur if the TCP TFs were blocking the binding of the other TFs by steric hinderance. The results from

mastoparan generated $[Ca^{2+}]_{cyt}$ signatures displayed a high CaM box expression (Figure 6.7) which supports what was seen in chapter 3 (Figure 3.4: A), however when CaM box is combined with CRT this expression is lost. Analysing the results from mastoparan generated signatures it would seem CaM box, when coupled to a second *cis* element is no longer calcium regulated, suggesting the CaM box itself is blocked. However, DREB CBFs are small and the CaM box is large so it would be unlikely that the CaM box would be blocked, but, there is a possibility that the CRT itself may be altering the DNA, this could be confirmed using an electrophoretic shift assay (EMSA). There is also the possibility that the two TFs could be communicating in a different way, perhaps on a biochemical level, but this seems less likely. As the investigations into mastoparan $[Ca^{2+}]_{cyt}$ generated signature regulation of combined *cis* element was incomplete, we can only guess the possible scenarios. In chapter 3 mastoparan mainly regulated CaM box (Figure 3.4: A) but there was some response from ABRE, SITE II and CRT. However, the response was low, so I assume that there would be a low or no response from the remaining combinations, reflecting what was recorded with the ATP generated signatures with cis elements that were not highly regulated in their single form by that particular $[Ca^{2+}]_{cy}$t signature.

As no investigations were conducted on $CaCl_2$ or L-glutamate we can only surmise about possible findings. I would expect some novel double *cis* element expression responses from $CaCl_2$ generated signatures. Previous data in chapter 3 (Figure 3.4: B,) recorded high expression from CRT *cis* elements from $CaCl_2$ generated $[Ca^{2+}]_{cyt}$ signatures, and as both the data so far in this chapter and previous literature suggest, CRT is often coupled with other *cis* elements (Nakashima et al., 2009, Roychoudhury et al., 2013, Whalley et al., 2011). Therefore, it would seem feasible to expect novel expression responses from some of the combined *cis* elements in response to $CaCl_2$. In regards to L-glutamate $[Ca^{2+}]_{cyt}$ signature, in chapter 3 (Figure 3.6: B) it was observed that both ABRE and SITE II were expressed, but the response was relatively low in comparison to what was seen in the ATP response and I could not conclude with certainty that L-glutamate signatures were regulating these cis-elements. However, for the sake of argument if we say L-glutamate $[Ca^{2+}]_{cyt}$ signatures were regulating ABRE and SITE II , then it would be reasonable to assume that a combination of these *cis* elements may display novel expression kinetics reflecting what was seen in the ATP responses (Figures 6.1; 6.2).

As these data are incomplete it will be necessary in the future to continue investigating all 4-calcium agonist generated signatures against all the new combined *cis* element transgenic plants to definitive conclusions.

It would be interesting to investigate the biphasic response displayed in the combined *cis* element kinetics, to determine if these responses were due to $[Ca^{2+}]_{cyt}$ signatures occurring in different cells, or in the same cells at different times, and to compare these results to what was seen in the single *cis* elements from chapter five.

It was seen in chapter five that ATP, $CaCl_2$, mastoparan/ATP and mastoparan/$CaCl_2$ generated $[Ca^{2+}]_{cyt}$ signatures were regulated by the circadian clock and this was also reflected in subsequent *cis* element regulation. It would be interesting to investigate circadian regulation of the double cis elements, I would expect some regulation in regard to ABRE/SITE II combinations when treated with ATP or mastoparan/ATP as both of these *cis* elements were seen to be down regulated at night with ATP treatment. However, I feel the CaM box combined elements would be the most intriguing due to the negative expression effect caused when CaM box is combined with a second *cis* element.

6.4 Conclusion

From these data it would seem that ATP generated $[Ca^{2+}]_{cyt}$ signatures regulate a combination of various *cis* elements (ABRE/SITE II; CRT/ABRE; CRT/SITE II), displaying novel expression kinetics compared to the single *cis* elements. There is also evidence that when CaM box is coupled to another cis element it may no longer be regulated by calcium signatures, however, these data is limited to only two calcium agonists and further investigation would be needed to come to this conclusion with conviction.

Chapter 7 Identifying proteins regulated by mastoparan generated cytosolic calcium signatures

7.1 Introduction

Calcium mediates gene expression by regulating the activity of specific nuclear proteins. Calcium may regulate these proteins through mechanisms that include gene expression, regulating specific protein transport or by regulating protein stability, though posttranslational mechanisms.

Plants possess several groups of Ca^{2+} sensors, which include CaM and CaM-like proteins (CMLs), Ca^{2+}-dependent protein kinases (CDPKs), Ca^{2+}-and Ca^{2+}/CaM-dependent protein kinases (CCaMKs) and calcineurin B-like proteins (CBLs) (Dodd et al., 2010, Galon et al., 2010, Sanders et al., 2002, Steinhorst and Kudla, 2014). A specific $[Ca^{2+}]_{cyt}$ signature which has been generated in response to a given stimuli may initiate the activation or repression activity of Ca^{2+} responding proteins, such as a kinase or phosphatase (Galon et al., 2010, Kim et al., 2009). Cytosolic calcium signatures interact with Ca^{2+} sensors such as CaM and can also increase or prevent binding to TFs, resulting in the activation or repression of target genes (Kim et al., 2009). Some Ca^{2+} regulated TFs target specific genes that are also transcription activators or repressors themselves, which results in the expression of many other downstream genes(Danquah et al., 2014, Kim et al., 2009).

Cytosolic Ca^{2+} signatures may also regulate proteins post-translationally. Post translational modifications are among the earliest and fastest plant responses to environmental stimuli and are implicated in a myriad of processes such as protein activity, stability and localisation, signalling and amplification (cascades), and removal e.g. reversible phosphorylation and kinase/phosphatase networks (Vierstra, 1996). Posttranslational modifications fall into many different categories, the most well studied being phosphorylation, proteolysis, ubiquitination and SUMOylation. Phosphorylation is reversable and is controlled by a balance of phosphatases and kinases (Schulze et al., 2010) with 4-5% of the Arabidopsis genome encoding various kinases, double of that found in mammals (Schulze et al., 2010). Phosphorylation has been found to be involved in regulating most of the metabolic and physiological pathways found in plants including defence (Jones and Dangl, 2006, Nühse et al., 2007), RNA metabolism (De La Fuente van

Bentem et al., 2006), carbon metabolism (Wu et al., 2014) and root growth (Zhang et al., 2016, Zhang et al., 2013).

Proteolysis is an irreversible process which is involved in the breakdown of proteins into smaller polypeptides or amino acids by hydrolysis of peptide bonds with a protease (Rogers and Overall, 2013). The ability to quickly switch from one state to another to adapt to a new environmental change often requires the breakdown of the existing regulatory networks, a process that heavily depends on proteolysis. Examples of this process can be found in progression of the cell cycle and the initiation of various signal transduction pathways e.g. leaf senescence (Diaz-Mendoza et al., 2016, Girondé et al., 2015), seed germination and seedling growth (Müntz, 2007).

Ubiquitination involves the conjugation of multiple ubiquitin molecules with short lived proteins which are then subsequently broken down by the 26s proteosome (Ciechanover, 1994, Vierstra, 1993). Ubiquitination occurs in regulation of hormone signalling, ethylene and jasmonic signalling, and the plant defence mechanism (Dangl and Jones, 2001, Devoto and Turner, 2005, Fleet and Sun, 2005, Lorenzo and Solano, 2005, Nürnberger et al., 2004, Schulze-Lefert and Bieri, 2005, Schwechheimer et al., 2001).

SUMOylation involves the attachment of SUMO (Small Ubiquitin -Like Modifyer) protein to lysine residues on target substrates (Kurepa et al., 2003b) and is involved in DNA repair, RNA metabolism, cell signalling and the plant stress response (Baczyk et al., 2017, Castro et al., 2012, Lois, 2010, Nair et al., 2017, Qiu et al., 2017, Verma et al., 2018, Wei et al., 2017).

My data thus far has suggested that different Ca^{2+} agonist generated $[Ca^{2+}]_{cyt}$ signatures produce a novel response and they regulate different TFs, implying possible $[Ca^{2+}]_{cyt}$ signature specificity. This data however has been directed towards downstream gene effects and has not investigated $[Ca^{2+}]_{cyt}$ signature regulation at a protein level, particularly post translational regulation.

Questions to be answered in this chapter:

- Which proteins are regulated by a mastoparan generated $[Ca^{2+}]_{cyt}$ signatures?
- To identify which of these proteins are regulated post translationally, and to ascertain their biological and molecular functions.

7.2 Results

7.2.1 Proteins regulated by mastoparan generated $[Ca^{2+}]_{cyt}$ signatures

To identify proteins that are regulated by mastoparan generated $[Ca^{2+}]$cyt signatures 10d old WT Arabidopsis seedlings were treated with an overall concentration of 10µM mastoparan or water (methods found in 2.2.3.6). Seedlings were removed from the treatment at both 3h and 6h, and proteins were extracted using the TCA/Acetone method, as described by Damerval et al., (1996) (see 2.2.6.14 and 2.2.6.15 for detailed methods). Once extracted, the protein samples were quantified adopting a modified Bradford assay method (2.2.6.15) and visualised by running the samples through an SDS/PAGE gel, and then further imaged with a Typhoon Imager (methods in 2.2.6.16). The protein samples were labelled with iTRAQ tags using a modified version of the manufacturer's instructions (methods in 2.2.6.17). Following iTRAQ labelling the protein samples were subjected to liquid chromatography and mass spectrometry (LC-MS/Ms) analysis (methods found in 2.2.8). From this analysis a protein list was generated based on identify of up or downregulated proteins between mastoparan treated samples and untreated controls. Further analysis was conducted using Microsoft excel to eliminate all proteins with a less than 95% confidence level in identity for up or down regulation to the control and any proteins that registered less than two peptides (methods 2.2.8.2). From this, cross over of protein expression between the two time points could be ascertained (figure 7.1), followed by further analysis to determine significant changes in protein expression between controls and treated samples as well as determining protein function (figure 7.2, table 7.1 and table 7.2).

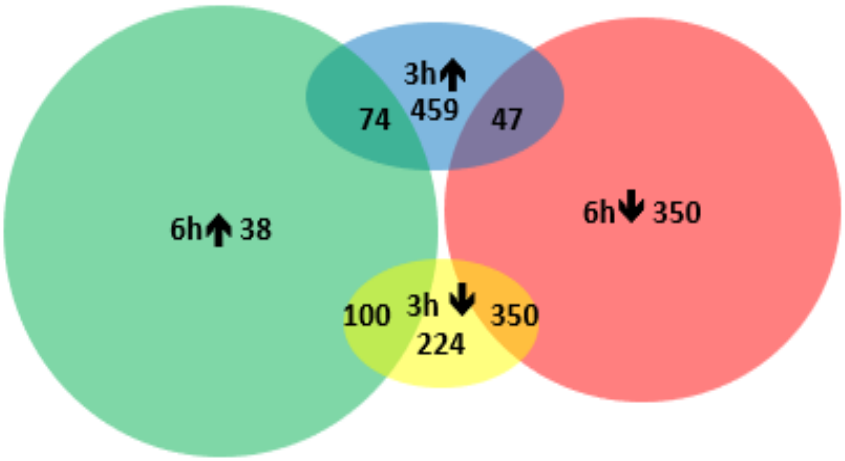


Figure 7.1: Venn diagram depicting proteins identified from a treatment of 10µM mastoparan at both 3h and 6h. All proteins have a confidence identity level of ≥ 95% for up and down regulated from mastoparan treated to the control and ≥ 2 peptides. Proteins have been categorised by up or down regulation and the cross over between the two time points.

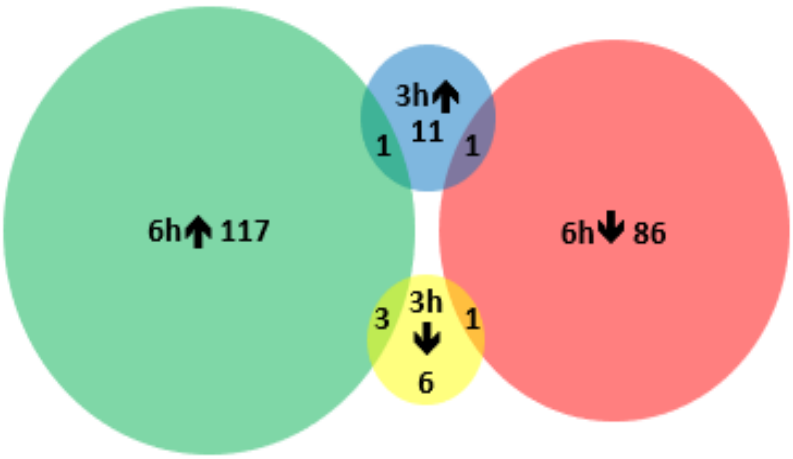


Figure 7.2: Venn diagram depicting up and down regulated proteins generated from a treatment of 10µM mastoparan at both 3h and 6h. All proteins have been identified as significant, with p- values < 0.05 and a fold difference ≥ 1.2, between controls and treated samples. Proteins have been categorised by up or down regulation and the cross over between the 3h and 6h time points.

Table 7.1: Twenty three proteins regulated by mastoparan induced $[Ca^{2+}]_{cyt}$ identified at 3h, all proteins are statistically different in confidence identity between up and down regulation to the control with p value ≤0.05 and a fold difference of ≥1.2 . Each identified protein has been categorised by percentage of molecular and/or biological function, and numbers directly involvement in the plant stress response. (Information sourced from uniprot and TAIR).

Function	Proteins (%)	Up and Down regulated proteins per function		Number of proteins involved in the plant stress response	
		Up (%)	Down (%)	Up	Down
Photosynthesis	7.1	50	50	0	0
ATP synthesis and electron transport system	10.7	100	0	0	0
Protein modification/Poteolysis, protease/lisase	21.4	66.7	33.3	0	0
Translation	25	86	14	1	0
Glycolysis/Gluconeogenesis	7.1	100	0	1	0
Lipid biosynthesis	3.6	100	0	0	0
Metal/Heme binding	14.3	75	25	1	0
Methionine reductase	3.6	0	100	0	1
Amino acid biosynthesis	3.6	0	100	0	0
Phosphoenolypyruvate carboxalase activity	3.6	100	0	1	0

Table 7.2: Two hundred and nine proteins regulated by mastoparan induced $[Ca^{2+}]_{cyt}$ identified at 6h, all proteins are statistically different in confidence identity between up and down regulation to the control with p value ≤0.05 and a fold difference of ≥1.2. Each identified protein has been categorised by percentage of molecular and/or biological function, and involvement in the plant stress response. (Information sourced from uniprot and TAIR)

Function	Proteins (%)	Up and Down regulated proteins per function		Number of proteins involved in the plant stress response	
		Up (%)	Down (%)	Up	Down
RNA/DNA/Translation	11.4	75	25	8	2
Glycolysis/Gluconegenesis	3.4	87.5	12.5	0	0
Other	7.3	72.2	27.8	8	4
Photosynthesis	7.7	52.6	47.4	3	1
Protein modification and transport (Proteolysis/Protease/Hydrolase/Lysase)	23.9	69	31	7	12
Lipid biosynthesis	4	50	50	1	1
Heme/Metal binding	14.2	48.6	51.4	15	4
Growth and development	2.4	50	50	0	0
Carbohydrate binding	3.7	33.3	66.7	1	1
Ion transport	4	22.2	77.8	1	1
Amino acid biosynthesis	2.4	60	40	1	0
ABA response	1.8	66.7	33.3	1	2
ATP synthesis and electron transport system	13.8	44.1	55.9	9	2

From the mass spectrometry analysis, 2,025 and 2,860 proteins were identified from the 3h and 6h time points, respectively. All proteins with a <95% confidence level of identity and < 2 peptides were removed, leaving 1266 and 959 proteins from the 3h and 6h timepoints, respectively (tables found in supplementary disc, Table S.1 and S.2). From the 3h protein list (supplementary S.1), it was seen that 580 proteins were up-regulated and 674 down-regulated relative to the control. From those proteins up-regulated at 3h, 74 were also up-regulated at 6h (Figure 7.1) and a further 47 were down-regulated at the 6h time point (Figure 7.1). Of the 674 down-regulated proteins at 3h, 350 were also down-regulated at 6h and a further 100 up regulated at the 6h timepoint (Figure 7.1). From the 959 proteins identified at the 6h timepoint, 212 were up-regulated and 747 were down-regulated (Supplementary S.2). From the proteins up-regulated at 6h, 174 overlapped with the 3h time point, with 74 up-regulated and 100 down-regulated (Figure 7.1). An overlap of 397 proteins was identified between the 6h down-regulated proteins and the 3h proteins, of these, 350 were down-regulated and 47 were up-regulated (Figure 5.1).

All proteins that were statistically insignificant (p value >0.05) and recorded <1.2-fold difference between the mastoparan treated and water controls were removed to generate two significant lists (Supplementry S.3 and S.4). From the 3h significant list 23 proteins were identified, 13 up-regulated and 10 down-regulated (Supplementry S.3), of these 18 were also found at the 6h timepoint; however only 6 of the 18 proteins were statistically significant at 6h (Supplementry S.3). From the 13 up-regulated proteins at 3h, 1 of these was significantly up-regulated and 1 down-regulated at 6h (Figure 7.2), from the 10 down-regulated proteins, 1 was down-regulated and 3 up-regulated at 6h (Figure 7.2). From the 6h significant list 209 proteins were identified, 121 up-regulated and 88 down-regulated (Supplementry S.4). One hundred and sixty-two proteins overlapped between the 6h and 3h significant lists, however, only 9 of the proteins were statistically significant (Supplementry S.4). From 121 up-regulated proteins at 6h, 1 was up-regulated and 3 down-regulated at 3h, and from the 88 down-regulated proteins at 6h there was an overlap of 1 down-regulated and 1 up-regulated proteins at the 3h timepoint (Figure 7.2).

From the 3h significant list 25% of the proteins identified were involved in protein coding and translation (Table 7.1), of these, 86% were up regulated and 14% down regulated, with 1 up regulated protein involved in the response to ABA and drought specifically (Table 7.1: Supplementary S3). Twenty-one-point four percent of the identified proteins were found

to be involved in protein modification (Table 7.1), which included protein folding and unfolding, ubiquitination and proteolysis; 66.4% of the proteins were up regulated and 33.3% were down regulated, none of these proteins was seen to be involved in the plant stress response (Table 7.1; Supplementary 3). Proteins categorised in the heme and metal binding category consisted of 14.3% of the proteins identified at 3h (Table 7.1; Supplementary 3), of these 75% were up regulated and 25% downregulated with one up regulated protein directly involved in the defence response to fungi. All of the remaining proteins were categorised in several different functions (Table 7.1; Supplementary S3), out of these remaining proteins one was seen to be directly involved in the plants stress response to oxidative stress and was categorised in the L-methionine reductase activity, one from the glycolysis category and was involved in the cold response and one from the phosphoenolypyruvate carboxylase activity which was directly involved in the salt stress response (Table 7.1; Supplementary S.3).

The proteins identified in the 6h significant list was found to be involved in several processes, with protein modification being the most abundant at 23.9%, of these 69% were up regulated and 31% down regulated (Table 7.2; Supplementary S.4). The second most abundant category was heme and metal binding at 14.2%, followed by ATP synthesis/electron transport and translation at 13.8% and 11.4%, respectively (Table 7.2). The remaining proteins fell into varying categories including photosynthesis, glycolysis/gluconeogenesis, lipid biosynthesis and carbohydrate biosynthesis (Table 7.2). Several of the proteins identified from 6h significant list was involved in either abiotic or biotic stress responses, including but not limited to, cold, nematode response, high salinity, oxidative stress, bacteria defence response, response to fungi, and response to heat (supplementary 3). The largest category, protein modification, returned the most varied list of stress responses, 7 up regulated and 12 down regulated (Supplementary 4; Table 7.1). The heme and metal binding category showed an involvement predominantly in oxidative and cold stress, particularly in the up regulated proteins (Supplementary 4).

7.2.2 Mastoparan induced gene expression

When comparing the genes that encode for the proteins in the protein lists where fold difference was ≥1.2 I chose four of these genes that have been shown to be calcium

regulated (Whalley and Knight, 2013, Whalley et al., 2011) AT2G39730, AT4G30920 from the significant list (Supplementary data S3 and S4) and AT5G47210 and AT1G54270 (Supplementary data S1 and S2) to measure gene expression from samples of Arabidopsis that had been subjected to the same conditions as that of the protein experiments. This was to determine if the gene expression was the same or different to that of the protein levels. Ten-day old Arabidopsis seedlings were treated with 10µM mastoparan or water as described in 7.2.2 (methods found in chapter 2.2.3.6). RNA was extracted at 1h, 3h and 6h (methods 2.2.6.7), synthesised to cDNA (methods 2.2.6.9) and gene expression was measured using RT-qPCR (methods 2.2.6.10).

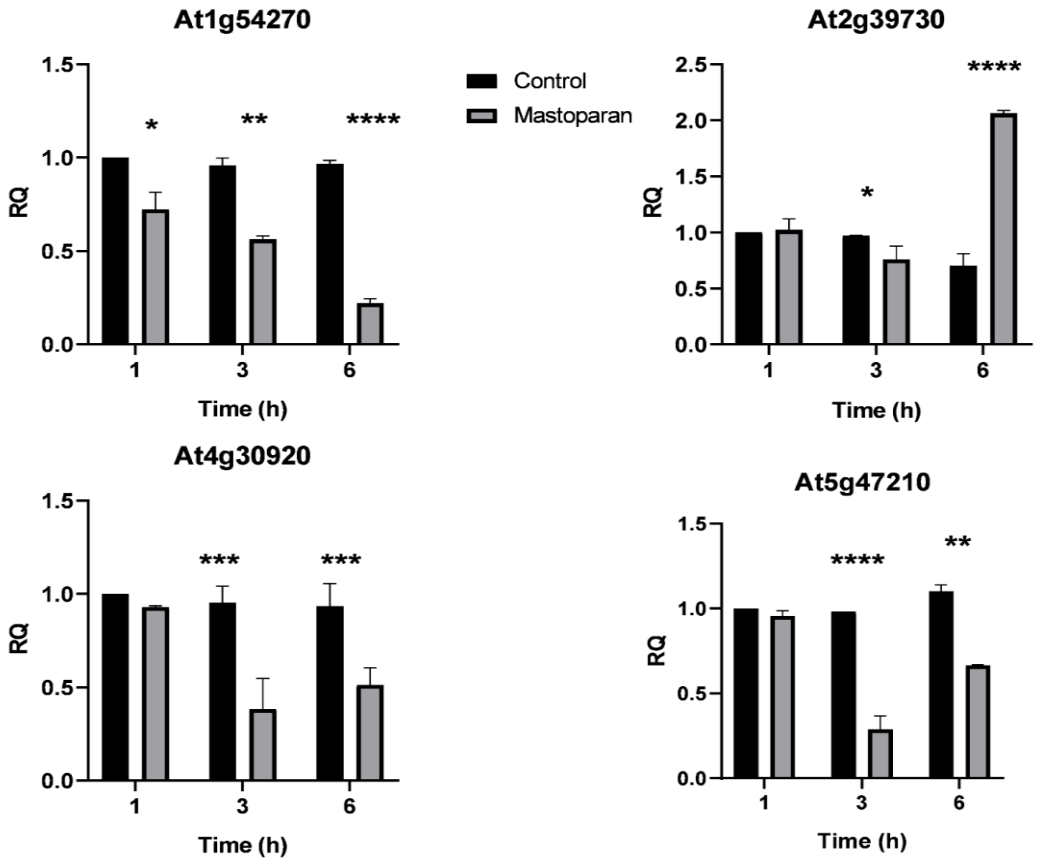


Figure 7.3: Mastoparan gene expression of selected genes. Ten-day old Arabidopsis seedlings were treated with either an overall concentration of 10µM mastoparan or water, seedlings were harvested after 1, 3 and 6h. RQ values for expression were measured using qPCR. Expression was normalised to expression of PEX4 (endogenous control). Error bars represent ±SD (n=3 biological replicates). One-way ANOVA between mastoparan treated and control at 3 different time points **** (P≤0.0001), *** (P≤0.001), ** (P≤0.01) * (P≤0.05)

From the significant lists (Supplementary S.3 and S.4) the gene At1g54270 recorded down-regulated expression over time, when compared to the untreated control (Figure 7.3). The greatest significant difference between the mastoparan treated samples and the controls samples was measured at 6h, recording a statistically significant p-value of ≤0.0001 which concurs with protein levels recorded at this time point. Initially the gene At2g39730 measured down-regulated expression at 3h, but at 6h was up-regulated, recording double of that seen with the untreated control, which was supported by the p-value of ≤0.0001, suggesting a significant difference (Figure 7.3). The genes At4g3092 and At5g47210 chosen from the supplementary data (S1 and S2) measured significant down-regulation at 3h, recording p-values of 0.001 and 0.0001, respectively (Figure 7.3). The genes also measured down-regulated expression at 6h, with AT4g3092 recording a p<0.001 and At5g47210 a p value of 0.01 (Figure 7.3). From these investigations it could be seen that gene expression correlated with the protein results; At2g3970 up regulated at 6h, At4g30920 down regulated at 3h, At5g47210 down regulated at 3h and At1g54270 down regulated at 6h (Figure 7.3; Supplementary S.1, S.2, S.3 and S.4).

7.2.3 Investigating four proteins that are regulated by mastoparan generated $[Ca^{2+}]_{cyt}$ signatures.

I chose four proteins from the significant lists to further investigate which were not described as calcium regulated at gene level in Whalley et al., (2011) or Whalley and Knight (2013). Glycerol-3-phosphate acyltransferase (At1g32200), Peroxidase 62 (At5g39580), Nucleotide diphosphate kinase II (At5g63310) and Proteasome subunit alpha type 2-A (At1g16470) (Supplementary S4). Peroxidase 62 which is located in the golgi and cell wall of plants and Nucleotide diphosphate kinase II (NDK II) (found in the chloroplast) have been shown to be involved in abiotic or biotic stress responses through ROS signalling regulation(Abu-Taha et al., 2018, Liszkay et al., 2003, Luzarowski et al., 2017, McInnis et al., 2006). Peroxidase 62 was down-regulated at 6h with a fold difference of 1.85 and a significant p-value of 0.0145 (Supplementry: S.4), and NDK-II was also down-regulated at 6h with a fold change of 2.07 and a p value of 0.0093 (Supplementry:S.4), NDK-II was also down regulated at the 3h timepoint but was insignificant with a fold difference <1.2 and a p-value >0.05 (Appendix, K-2). Glycerol-3-phosphate acyltransferase (G-3-P acyltransferase) which is located in the chloroplast and is an enzyme involved in the

alteration of membrane fatty acids in response to chilling (Li et al., 2000, Murata, 1983). G-3-P acyltransferase was up-regulated at six hours with a fold difference of 2.67 and a p-value of 0.0078 (Supplementary S.4) The proteasome subunit alpha type 2 (PS2) is located in the chloroplast, nucleus and cytosol and is involved in regulating protein levels by protein degradation, it is a protein that has drawn considerable attention in current literature (Gallois et al., 2009, Gladman et al., 2016, Grimmer et al., 2020, Kurepa et al., 2003a, Smalle et al., 2003). The PS2 protein measured a fold difference of 3.3 and p-value of 0.014 suggesting a significant difference (a full list of functions can be found in the Supplementary data, S.3 and S.4).

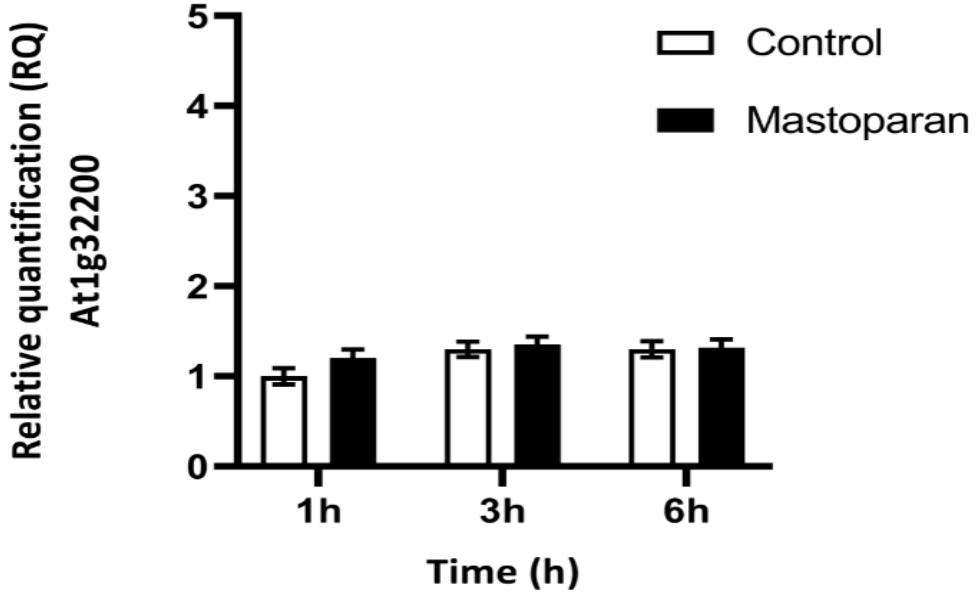
7.2.3.1 Protein construct design and experimental process.

Three different constructs were needed for expression in plants, one for untagged protein expression, one with a GFP at the N-terminus, and one with a GFP at the C-terminus. To accommodate this, I designed two versions of the coding sequence for each of the four proteins, one with and one without a stop codon (protein coding sequences can be found in Appendix A). For the untagged proteins, I used the version with a stop codon as there is no stop codon in the destination vectors. For GFP attached at the N-terminus, I also used this version because a stop codon was needed at the end downstream of the GFP-PROTEIN fusions. For the GFP attached at the C-terminus I used the version without the stop codon, this is because the GFP has a stop codon in the destination vector and I needed the protein to translate all the way through a PROTEIN-GFP fusion. Once designed the protein sequences were sent to IDT to be synthesised. After synthesis they were cloned into a linearized (double digest of *NotI* and *Asc I* (methods, 2.2.6.11.2) pENTR™/D-TOPO® vector (map can be found in Appendix A.2) using the Gibson assembly method (2.2.6.11.3). Using the Gateway cloning method (2.2.6.11.4) the vector containing the protein sequence was cloned into one of 3 destination vectors: pB7GW2 (for overexpression without GFP), pK7WGF2 (for adding GFP to N terminus) and pK7FWG2 (for adding GFP to C terminus and used to clone without a stop codon). The vectors containing the protein sequences were then cloned into Arabidopsis plants using the floral dip method (methods: 2.2.6.13). To date I have confirmed the presence of the sequences by primary screening (2.2.6.13.3) followed by RT-PCR (methods: 2.2.6.10, Primers: Appendix A.1).

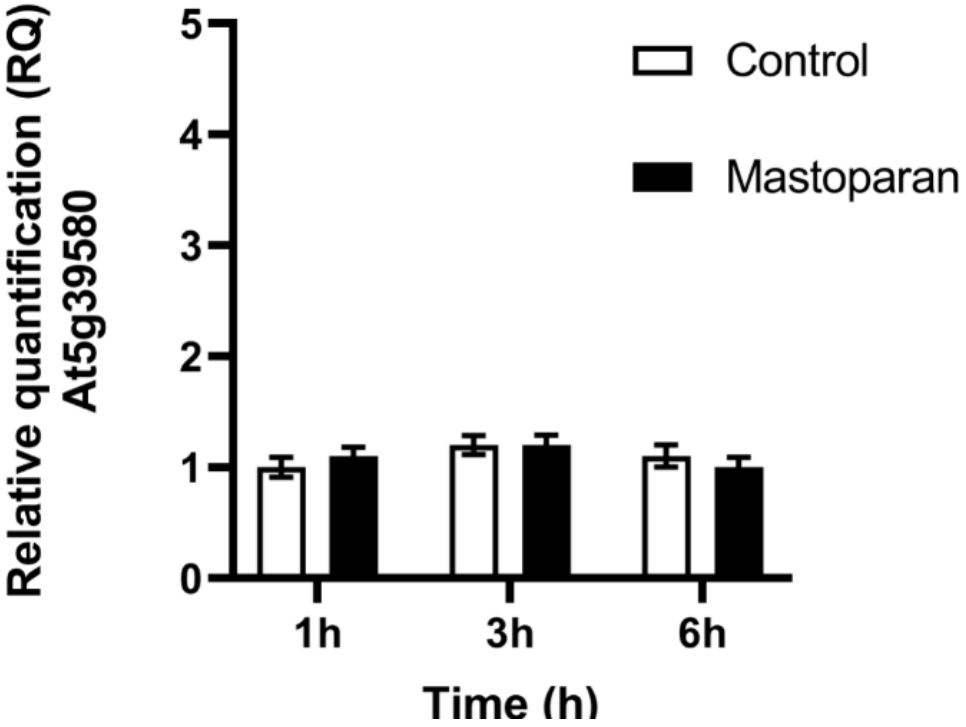
7.2.3.2 Investigation into mastoparan induced calcium regulation of selected proteins: Gene expression

To confirm that the four chosen proteins of interest were not regulated by calcium at a gene level, gene expression was measured using RT-PCR. Transgenic 10d old Arabidopsis seedlings containing constructs which consisted of one of the encoding genes for the four chosen proteins were treated with an overall concentration of 10µM mastoparan or water as a control. RNA was extracted at 1h, 3h and 6h (methods 2.2.6.7), synthesised to cDNA (methods 2.2.6.9) and gene expression was measured using RT-PCR (method 2.2.6.10).

A


Relative quantification (RQ)
At1g32200
5
4
3
2
1
0
Control
Mastoparan
1h
3h
6h
Time (h)

B


Relative quantification (RQ)
At5g39580
5
4
3
2
1
0
Control
Mastoparan
1h
3h
6h
Time (h)

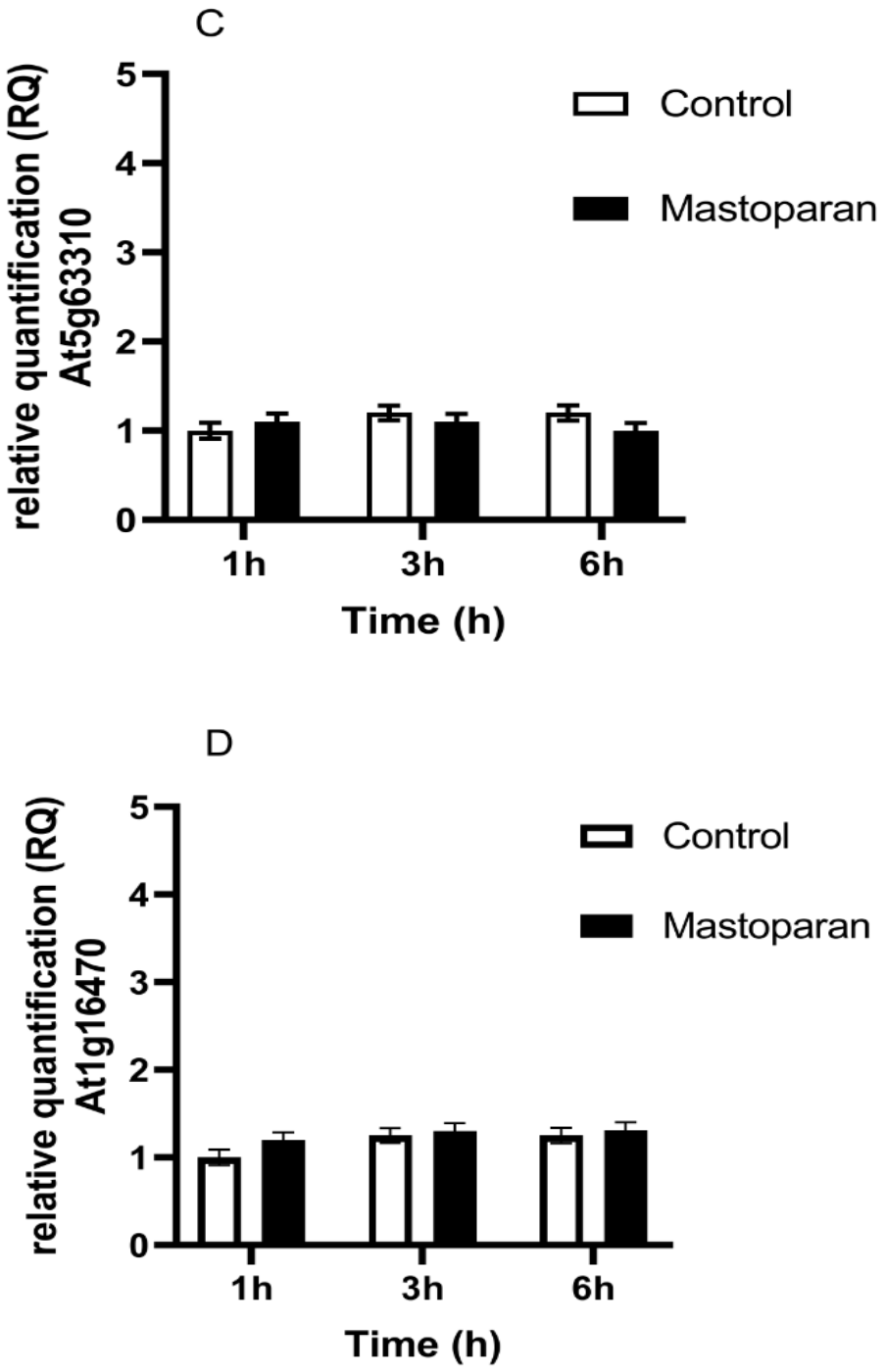


Figure 7.4: Mastoparan-induced gene expression of selected genes which encode proteins of interest. Ten-day old Arabidopsis seedlings were treated with either an overall concentration of 10µM mastoparan or water, seedlings were harvested after 1, 3 and 6h. RQ values for expression were measured using qPCR. Expression was normalised to expression to PEX4 (endogenous control). Error bars represent ±SD (n=3 biological replicates). Two tailed ŦTest p> 0.05. (A)At1g32200 (Glycerol-3-phosphate acyltransferase, (B) At5g39580 (Peŗroxidase 62), (C) At5g63310 (Nucleotide diphosphate kinase II), (D) At1g16470 (Proteosome subunit alpha type 2-A).

All four genes which encoded for the chosen proteins of interest, At1g32200 (Glycerol-3-phosphate, At5g39580 (peroxidase 62), At5g63310 (Nucleotide diphosphate Kinase II) and At1g16470 (proteosome subunit alpha type 2-A) measured low expression levels in both control and the mastoparan treated samples (Figure 7.4: A-D). Statistical analysis recorded p values above 0.5 suggesting there was no statistical difference between the controls and the mastoparan treated samples at all time points (Figure 7.4: A-D).

7.3 Discussion

7.3.1 The regulation of proteins by mastoparan generated $[Ca^{2+}]_{cyt}$ signatures and their functions.

The initial results from the proteomic experiments identified above 2,000 proteins for each time point, however, further elimination of proteins by confidence level and peptide number revealed that the 3h timepoint yielded more proteins than the 6h timepoint, 1266 and 959, respectively (Supplementary S.1, S.2). However, on further investigation, employing a threshold of a fold difference > 1.2 and a P-value of <0.05 there was nine times more significant proteins in the 6h timepoint to that of the 3h timepoint; 209 proteins to 23, respectively.

By comparing the encoding genes of the proteins to the genes identified as calcium regulated by Whalley et al., (2011) and Whalley and Knight (2013), it was found that 11 of these matched with the proteins identified in these data. By investigating gene expression of four of these calcium regulated genes from plants treated with identical conditions to that of the protein samples, it could be seen that the gene expression correlated with the protein expression; At2g3970 up regulated at 6h, At4g30920 down regulated at 3h, At5g47210 down regulated at 3h and At1g54270 down regulated at 6h (Figure 7.3). The low number of encoding genes regulated by calcium in these results could imply that some of the proteins identified were possibly regulated post transcriptionally, but I cannot say this with certainty without further investigation. One way to investigate this would be to transform *A. thaliana* plants to over express the identified proteins and then test protein-protein interactions using fluorescence resonance energy transfer (FRET).

If we assume some of the proteins identified were regulated post translationally by mastoparan induced $[Ca^{2+}]_{cyt}$ signatures it could explain why the most abundant protein category included protein modification, which involves both phosphorylation and proteolysis at a post translational level (Schulze et al., 2010). These data from the 6h timepoint (Supplementary S.4) does in fact concur with this assumption, showing that protein modification contained the highest abundancy of proteins at 23.9% (Table 7.2; Supplementary S.4), with 69% of these being up regulated.

It is surprising that proteins involved with translation were found to be relatively abundant, particularly in the 3h timepoint at 25% (Table 7.1; 7.2; Supplementary S.3, S.4). It has been shown in previous literature that the pool of translating mRNAs decrease during the plant stress response, for example, it has been shown that 50% of translating mRNAs were down regulated during heat stress (Yángüez et al., 2013) and up to 77% during hypoxia(Branco-Price et al., 2005), leaving only those mRNAs encoding proteins involved in translation and stress responses; suggesting they are selectively translated during stress. On closer inspection of the 3h data it can be seen that a third of the proteins that were categorised into the translation category, were indeed involved in either the stress response directly, or metal binding (Supplementary S.4) which is known to be heavily involved in the oxidative stress response (Stohs and Bagchi, 1995). The 6h time point revealed that a lower percentage of proteins were involved in translation at 11.4% (Table 7.2) of these 75% were upregulated, with 40% of these up regulated genes involved with the plant stress response, or regulation of plant cell death; it has been shown that plant cell death is particularly associated with ROS (Gechev and Hille, 2005), and a large quantity of the proteins regulated in this data were involved either directly or indirectly with ROS and oxidative stress (Supplementary S.3, S.4). However, even though translation may go down in stress I cannot discount the effect of translation on the proteins, one way to investigate the effect of translation would be to inhibit translation by using cytlochexamide (Ma et al., 2013) and then inhibit protein degradation via MG132 (Speranza et al., 2001).

Both in the 3h and 6h timepoints, the heme and heavy metal category displayed the second highest protein abundancy at 14.3% and 14.2%, respectively (Table 7.1; 7.2). However, at 3h, only one of the proteins was involved in the plant stress response, being involved with the plant's defence response to fungi and ROS. In contrast at 6h, 19 proteins were involved either directly or indirectly with the plant stress response; 15 up regulated and 4 down regulated, with 8 of the up regulated proteins being involved with oxidative stress directly, and 2 proteins being involved with cell redox homeostasis and auxin signalling (Table 7.2), which have been linked to heavy metal stress and oxidative stress (De Smet et al., 2015, Foyer, 2015, Vanstraelen and Benková, 2012, Wagner et al., 2004). It is known that the presence of heavy metals impose damage on a number of plant mechanisms, and it is believed that this often results in the production of ROS which induces oxidative stress (Stohs and Bagchi, 1995). Therefore, it is not surprising that proteins involved with heavy metal binding are also linked to oxidative stress, as seen in these data. It has been shown

by Zhao et al., (2015) by maintaining auxin homeostasis via Ca^{2+} regulation, the toxic effects of cadmium are mitigated, indicating a cross talk between signalling pathways to alleviate heavy metal stress (Zhao et al., 2015). These data have indicated protein involvement in both metal binding of cadmium and in auxin homeostasis as well as the ethylene signalling pathway (Supplementary S.4). It has been shown that ethylene directly effects the response to heavy metal stresses by modulating auxin homeostasis (Potters et al., 2007), suggesting further complex cross talk between signalling pathways. These data reported here also support possible cross talk between signalling pathways, given the fact that several different proteins which have been expressed and regulated by the mastoparan generated $[Ca^{2+}]_{cyt}$ are also involved with other signalling pathways, such as ABA, auxin and ethylene (Supplementary S.4).

In both the 3h and 6h time points proteins involved with photosynthesis and photosynthetic activity were of similar abundance at 7.1% and 7.7%, respectively (Table 7.1; 7.2), and they also displayed similar ratios of about 50:50 between up and down regulation. This is surprising, as you would expect photosynthesis to be down regulated during plant stress, as stress can affect the photosynthetic system in many ways, from stomatal conductance (Schroeder et al., 2001) to carbon assimilation (Gururani et al., 2015). Photosynthesis affected by drought (Chaves, 1991) or by salinity (Munns et al., 2006) for example, can lead to changes in the photosynthetic system (Lawlor and Cornic, 2002) or can lead to secondary effects such as oxidative stress. Oxidative stress is most prevalent under multiple stress conditions (Chaves and Oliveira, 2004) and has been shown to have a serious detrimental effect on leaf photosynthetic processes (Ort, 2001). It has also been reported by Allen et al., (1999) that mastoparan itself leads to a down regulation of photosynthetic activity (Allen et al., 1999b) , which contradicts what was found in these data (Table 7.1; 7.2), however, this is only one paper and the role of mastoparan and its interaction with plants is not completely understood. These data have shown that the proteins regulated by mastoparan induced $[Ca^{2+}]_{cyt}$ show a high abundance in regards to association with oxidative stress or components involved in the oxidative stress response (Supplementary S.4). The up regulated photosynthetic proteins in these data have also been shown to be involved in heavy metal binding, so it could be feasible that these particular photosynthetic proteins are up regulated due to their association with metal binding.

It was interesting to find that the protein serine hydroxymethyltransferase 1, mitochondrial (Supplementry S.4) which was found in the 6h timepoint and was down regulated, was involved with circadian regulation. As previously discussed in chapter 5, I suggested that mastoparan was not regulated by the circadian clock and the fact that a circadian protein was down regulated by mastoparan induced $[Ca^{2+}]_{cyt}$ signatures may give strength to this suggestion, however, one protein is not enough to fully support the theory.

Preliminary results from the transgenic Arabidopsis plants containing one of four chosen proteins from the six-hour significant list suggests that these proteins may be regulated post translationally (Figure 7.4: A-D). Gene expression from these transgenic plants treated with mastoparan measured low gene expression and showed no significant difference to those treated with a water control.

From these data it would seem that mastoparan generated $[Ca^{2+}]_{cyt}$ signatures regulated a myriad of proteins across the two timepoints, with many of these proteins found to be directly involved in the plant stress response. It could be possible that some of the proteins at both time points may be regulated at a post translational level. This theory is supported by the preliminary results from the transgenic plants containing genes that encode four of these proteins, which indicated indeed, that calcium did not regulate these proteins at a gene level, however, further investigations would be needed to explore this possibility. Post translational modifications would also explain the high abundance of proteins categorised in the protein modification category. It would be interesting to repeat these experiments over a 24h period to gain greater insight of mastoparan generated protein regulation in Arabidopsis. Further experiments could also involve the investigation of proteins regulated by $CaCl_2$, ATP and L-glutamate generated $[Ca^{2+}]_{cyt}$ signatures, to determine if they regulate different proteins to that of the mastoparan. I would anticipate they would regulate a different set of proteins which would reflect what was seen in the transcriptomics in the earlier chapters and would support the calcium signature hypothesis.

7.3.2 Future investigation of identified proteins of interest

As discussed in 7.2.3.1, I have designed and cloned constructs into Arabidopsis plants containing a gene coding sequence for of one of four proteins of interest selected from the 6h timepoint (Supplementry A.4). I have successfully confirmed the presence of these

constructs in the transgenic plants and preliminary investigated calcium regulation of these proteins at gene level, indicating that these four proteins may be regulated at a post translational level. On reflection I think it may be necessary to clone these constructs into *Nicotiana tobacum* for transient expression, which will allow me to identify the location of each protein of interest. It would also be interesting to treat the Arabidopsis transgenic plants with other calcium agonists to determine if different $[Ca^{2+}]_{cyt}$ signatures regulate these proteins. If the $[Ca^{2+}]_{cyt}$ signatures are truly novel and carry specific information I would expect that that most of the mastoparan generated $[Ca^{2+}]_{cyt}$ signature regulated proteins would not be regulated by the other calcium signatures.

7.4 Conclusion

The aim of this work was to identify proteins regulated by mastoparan generated $[Ca^{2+}]_{cyt}$ signatures, to categorise their functions and determine if they were regulated post translationally. It was seen that the majority of the proteins varied in both function and abundance. Preliminary work on four of these proteins indicated that calcium did not regulate these proteins at gene level, suggesting possible posttranslational regulation. However, further investigations will be needed to be able to say with confidence that these proteins are generally regulated by calcium at a post translational level.

The highest abundance of proteins was categorised in the protein modification category with many involved with the plant stress response. Proteins with heavy metal binding activity were also high in abundance and were in general directly involved in oxidative stress or ROS. The result across the board indicated cross talk between signalling pathways with regulation of ABA and auxin by calcium, which was reflected in the variation of stress response proteins identified. As far as I know there has been little or no investigation into mastoparan $[Ca^{2+}]_{cyt}$ signature regulation on a proteomic level to date, and in general very little is understood about the mastoparan mode of action in plants.

Future investigation could look at other novel $[Ca^{2+}]_{cyt}$ signatures and determine if these signatures regulate a specific set of proteins, which would further indicate signature specificity.

8. Discussion and conclusions

8.1 Implications of the work

Over the last thirty years it has been recognised that calcium signalling is a fundamental aspect of plant physiology and development. Due to its importance, calcium signalling has commanded a considerable interest as a field of study, however, not all aspects of calcium signalling are fully understood. Although the calcium 'signature hypothesis' which suggests that the signature is specific and holds specific information which leads to an appropriate response to a given stimuli is generally accepted, there is relatively limited research to concretely support this. Furthermore, most research conducted in this field concerns calcium signature specificity and gene regulation, guard cell responses and nodulation in legumes very little focus has been directed towards calcium specificity and protein regulation particularly at post translational level. The aim of this study was to further investigate calcium signature specificity, to deepen the understanding of specificity during calcium regulation of TFs, and to determine the effect of circadian regulation on these calcium signatures. Finally, I aimed to explore and identify proteins regulated by a specific calcium signature.

8.2 Calcium signature specificity

Due to advances in calcium measurement techniques such as the protein based calcium reporter aequorin (Knight et al., 1991) it has become evident that transient $[Ca^{2+}]_{cyt}$ signals occur in response to various individual and multiple environmental stimuli. This leaves the conundrum as to how these signals initiate an appropriate response specific to each stimulus. To address the question of calcium signal specificity, in 1998 the 'calcium signature' hypothesis was proposed by Martin McAinsh and Alistair Hetherington (McAinsh and Hetherington, 1998); suggesting specificity was encoded in the different kinetics and location of the calcium elevation. Indeed, there is a great deal of evidence which suggests that $[Ca^{2+}]_{cyt}$ elevations induced by stimuli exhibit different kinetics in response to a wide range of both abiotic and biotic stresses (Allen et al., 2001, Knight et al., 1996, Knight et al., 1997b, Knight et al., 1991, Manzoor et al., 2012, Rentel and Knight, 2004). More recent studies conducted by Whalley et al., (2011) and Whalley and Knight, (2013) demonstrated that different calcium signatures differentially induced specific sets of genes with very little overlap between the different signatures. They also identified a higher frequency of four conserved sequences within the promotors of these genes, namely the *cis* elements ABRE, CaM box, CRT/DRE and SITE II. Although this work has identified the genes, TFs and *cis* elements regulated by calcium (Whalley et al., 2011) and has suggested that they respond to different calcium signatures (Whalley and Knight, 2013), more practical evidence is needed to support this theory. In 2018 Lenzoni et al., described four chemical agonist induced signatures which displayed different kinetics and only one of these signatures induced large fold changes in expression of the genes *EDS1* and *ICS1*, suggesting specificity, however, to truly conclude signature specificity a wider repertoire of signatures would be needed.

The data recorded in chapter three aimed to support the work by Whalley et al., (2011) and Lenzoni et al., (2018). To do this I identified a wider repertoire of novel calcium signatures and then investigated if these signatures regulated previously identified calcium-regulated promoter motifs ABRE, SITE II, CRT and CaM box, (Whalley et al., 2011) specifically. Firstly, I replicated the work presented by Lenzoni et al., (2018) in that I measured $[Ca^{2+}]_{cyt}$ signatures generated by four different calcium agonists: mastoparan, $CaCl_2$, ATP and L-

glutamate, from this it could be ascertained that each separate signature indeed displayed different kinetics. To try to increase the range of novel signatures I combined mastoparan with either $CaCl_2$, ATP or L-glutamate. Each generated signature displayed novel kinetics to that seen compared to the single agonist generated signatures. The next step was to determine if different calcium-regulated promoter motifs responded specifically to both the single and combined calcium agonist generated novel signatures. To do this I treated transgenic Arabidopsis seedlings (containing a concatemer consisting of *LUC+* driven by either ABRE, SITE II, CaM box or CRT) with one of the agonists or agonist combinations. By measuring luciferase activity it could be seen that mastoparan generated $[Ca^{2+}]_{cyt}$ signatures predominately regulated the CaM box *cis* element, however, the other *cis* elements did show some activity. When mastoparan was combined with $CaCl_2$, it could be seen, like the single mastoparan treatment, that the CaM box *cis* element was again most highly expressed. Unlike the single mastoparan treatment, however, all the other *cis* elements seemed to be supressed including the CRT *cis* element, which had recorded a high expression when treated with the single $CaCl_2$ generated $[Ca^{2+}]_{cyt}$ signature. It could also be seen that the kinetics of expression measured from the luciferase activity were different in the combined treatment to both the single counterparts individually, with the combined treatment generating a biphasic response. In contrast, the ATP generated $[Ca^{2+}]_{cyt}$ signature regulated the expression of both ABRE and SITE II *cis* elements in a biphasic manner, when combined with mastoparan the $[Ca^{2+}]_{cyt}$ signature again regulated both the ABRE and SITE II *cis* elements but with different kinetic patterns. These data supported the 'calcium signature 'hypotheses, in that, different signatures appeared to regulate different *cis* elements. The L-glutamate generated $[Ca^{2+}]_{cyt}$ signature seemed to regulate both the ABRE and SITE II *cis* elements, however, the response was low. Subsequent measurements of gene expression via RT-PCR of the transgenic seedlings treated under the same conditions supported what was seen in the luciferase assays, and also indicated that indeed, L-glutamate generated signatures regulated both ABRE and SITE II, and the expression patterns differed to that seen with ATP and mastoparan/ATP generated $[Ca^{2+}]_{cyt}$ signatures. It was also seen that mastoparan/L-glutamate generated $[Ca^{2+}]_{cyt}$ signatures displayed novel expression patterns regulating ABRE and SITE II initially, but at 6h expression from the CaM box gene was seen to be induced. These data suggest that different calcium signatures may regulate different *cis* elements or regulate the same *cis* elements but with different expression patterns. When calcium agonists were combined, different gene

expression patterns were evident as compared to single agonist treatments, suggesting different calcium signatures have the potential to hold the information to initiate a substantially different response *via* downstream gene regulation. Furthermore, gene expression measured from native genes of WT Arabidopsis plants treated with the same calcium agonists reflected what was recorded from the reporter genes, therefore suggesting that calcium signatures were indeed specific and regulated specific genes *via* the four *cis* elements in both a synthetic and natural context.

The data presented in chapter four aimed to determine if any or all of these calcium agonist-generated calcium signatures could regulate stress genes, thus conferring stress tolerance to the plant. Osmotic stress can cause changes in plant metabolism, growth and physiology (Monclus et al., 2006), and it has been demonstrated that the stress genes *KIN2* and *LT178* are regulated by calcium during osmotic stress (Knight et al., 1997a, Xiong et al., 2002b). Therefore, Arabidopsis seedlings that had been pre-treated with one of the calcium agonists or combination of these agonists were subjected to mannitol-induced osmotic stress, and *KIN2* and *LT178* gene expression was measured at specific time points. Relatively high levels of gene expression were recorded in the $CaCl_2$ pre-treated samples compared to all other samples; both calcium agonist treated (ATP, mastoparan, L-glutamate or combinations of mastoparan/$CaCl_2$, mastoparan/ATP or mastoparan/L-glutamate) and controls. After 10d of osmotic stress it was seen that chlorophyll concentrations were depleted in all the seedlings except those pre-treated with $CaCl_2$, which exhibited a chlorophyll concentration similar to the water control. These data imply that pre-treatment of $CaCl_2$ seems to initiate a response that leads to osmotic stress tolerance in Arabidopsis, thus, suggesting that a novel signature does show specificity during osmotic stress.

8.3 ATP and mastoparan/$CaCl_2$ generated cytosolic calcium biphasic regulation of specific transcription factors

Data from chapter three indicated that both ATP and a combination of mastoparan/$CaCl_2$ generated $[Ca^{2+}]_{cyt}$ signatures which regulated specific TFs via a polyphasic response. There are many examples in previous literature of different $[Ca^{2+}]_{cyt}$ signatures occurring in different cells in response to various stimuli such as osmotic stress or cold stress (Kiegle,

2000) or in the same cells as described by Allen et al., (1999) when two cells of a stomate generate different $[Ca^{2+}]_{cyt}$ signatures in response to the same stimulus. The data in chapter five aimed to determine if the polyphasic responses described in chapter three were due to $[Ca^{2+}]_{cyt}$ elevations occurring in different cells or the same cell types at different times.

Luciferase activity was measured from the whole seedlings, cotyledons and roots of transgenic Arabidopsis. ATP $[Ca^{2+}]_{cyt}$ signatures regulating ABRE or SITE II *cis* elements displayed a biphasic expression response in the whole seedlings, however where the ABRE displayed a biphasic response in the cotyledons and roots at same time, the SITE II registered a biphasic response in the cotyledons and roots at different times. These results suggested that ATP generated $[Ca^{2+}]_{cyt}$ signatures regulated ABRE in various cell types and SITE II in different cell types as well as the same cells at different times**.** However, it was found with seedlings treated with mastoparan/$CaCl_2$ that the biphasic event was limited to the roots only, suggesting that this event was occurring in the same cell types at different times. The ATP calcium signature generated results are not surprising as we know that the ATP receptor P2K1 is expressed during all major stages of the plants development and growth (Cho et al., 2017, Jewell et al., 2019) inferring that ATP could be involved in a myriad of plant processes including abiotic and biotic stresses (Balagué et al., 2017, Bouwmeester et al., 2011, Cho et al., 2017, Tripathi and Tanaka, 2018). Previous work by Kaplan et al., (2006) and Whalley et al (2011) has demonstrated that the ABRE promotor region is regulated by calcium, inferring that calcium may regulate possibly thousands of genes in the Arabidopsis genome, it is therefore, reasonable to surmise that ATP generated $[Ca^{2+}]_{cyt}$ signatures could be regulating these genes in varied cell types at differing times. The root response seen in the SITE II regulation by ATP could be possibly explained by the findings of Demidchick (2009) who described calcium cascade effect in the roots from plants treated with eATP. It has been suggested by Mattheus et al., (2019), that there are many different ATP receptors in the leaf, which could explain the biphasic responses in the cotyledons recorded in this work.

8.4 Circadian clock regulation of calcium

Most organisms have internal circadian clocks with plants being no exception (McClung, 2019). The circadian clock regulates metabolism, physiology, growth, and behaviour, which

varies between day and night (McClung, 2006b, Wood et al., 2001a). In plants the circadian system is governed by several components consisting of feedback loops and gene regulation (Millar, 2016, Wood et al., 2001a). Most signalling pathways within the plant can be subjected to circadian regulation, therefore the response to a specific stimulus is dependent on time of day (Gómez and Simón, 1995, Trewavas, 1999, Webb, 2003, Wood et al., 2001a), this is known as circadian gating of signal transduction (Hotta et al., 2007, Yakir et al., 2009). It has been determined that many genes in plants are circadian regulated during abiotic and biotic stress (Covington et al., 2008, Hubbard et al., 2010, Kolmos et al., 2014, Kreps et al., 2002b, Zeilinger et al., 2006b), in abiotic stress for instance *ERD10* and *ERD7* are circadian regulated during dehydration, *RD29A* in response to water deficiency and *COR15* and *COR15B* in response to cold stress, (Fowler and Thomashow, 2002a, Liu et al., 1998b, Mizuno and Yamashino, 2008). Therefore, circadian regulation effects the responses to stresses causing different outcomes at particular circadian time (circadian gating).

Whilst investigating the biphasic response generated by $[Ca^{2+}]_{cyt}$ signatures regulating specific *cis* elements (chapter 5), it was seen that different kinetic patterns were presented when experiments occurred later in the day. The second half of chapter five was dedicated to investigating possible circadian regulation of these $[Ca^{2+}]_{cyt}$ signatures and determining if this regulation affected subsequent gene expression kinetics. Transgenic seedlings containing the calcium reporter aequorin following entrainment in either a forward cycle (12L:12D) or a reverse cycle (12D:12L) and then a 24h period of constant light were treated with one of the four calcium agonists or a combination of the agonists and calcium traces were generated. The data revealed that ATP, $CaCl_2$ and combinations of mastoparan/ATP and mastoparan/$CaCl_2$ displayed different signature kinetics between the circadian regimes. In contrast mastoparan, L-glutamate and a combination of mastoparan/L-glutamate displayed no significant difference between the traces generated from the forward and reverse entrained seedlings. Previous literature has indicated that $[Ca^{2+}]_{cyt}$ responses are gated by the circadian clock (Martí Ruiz et al., 2018, Trewavas, 1999, Webb, 2003, Wood et al., 2001a), but specificity of these signatures and the downstream effect has not been investigated. I presented data from chapters three and four supporting the 'calcium signature' hypothesis, if this is correct then there is a possibility that $[Ca^{2+}]_{cyt}$ signatures regulated by the circadian clock may also be specific

To determine if circadian regulation of $[Ca^{2+}]_{cyt}$ signatures resulted in changes in expression kinetics, luciferase assays were repeated on the transgenic plants (containing a construct consisting of *LUC+* driven by one of the calcium regulated *cis* elements) which had been entrained in either a forward or reverse light cycle followed by 24h of continuous light and then treated with a calcium agonist identified as circadian regulated, namely, ATP, $CaCl_2$, mastoparan/ATP or mastoparan/$CaCl_2$. It was seen that all of the $[Ca^{2+}]_{cyt}$ signatures were different in particular circadian times and this led to differences in gene expression kinetics. In the case of ATP, ABRE and SITE II recorded a reduction in expression and CaM box displayed increased expression kinetics during subjective evening. With a combination of mastoparan/ATP similar effects were seen in that ABRE and SITE II displayed a reduction in expression during subjective evening, with SITE II recording a biphasic response. It is not surprising that this data implies that ABRE TFs are circadian regulated, as previous literature has acknowledged that cold genes are circadian regulated (Bieniawska et al., 2008) and we know that ABRE is known to be an enriched promotor of cold induced genes (Kreps et al., 2003; Suzuki et al., 2005).

Calcium chloride generated $[Ca^{2+}]_{cyt}$ signatures which regulate CRT also displayed reduced expression kinetics during subjective evening. Again we know that CRT is also linked to drought and the cold response and we know cold genes are circadian regulated (Fowler et al., 2005, Kreps et al., 2002b). These data in this report has suggested $CaCl_2$ regulates CRT, so it could be feasible that that circadian regulation of $[Ca^{2+}]_{cyt}$ signatures generated from $CaCl_2$ results in a change in expression kinetics of CRT. In contrast, when $CaCl_2$ is combined with mastoparan expression levels of CaM box are reduced during subjective evening, suggesting that circadian regulated $[Ca^{2+}]_{cyt}$ signatures generated from mastoparan/$CaCl_2$ were inducing changes in the expression kinetics of CaM box driven seedlings.

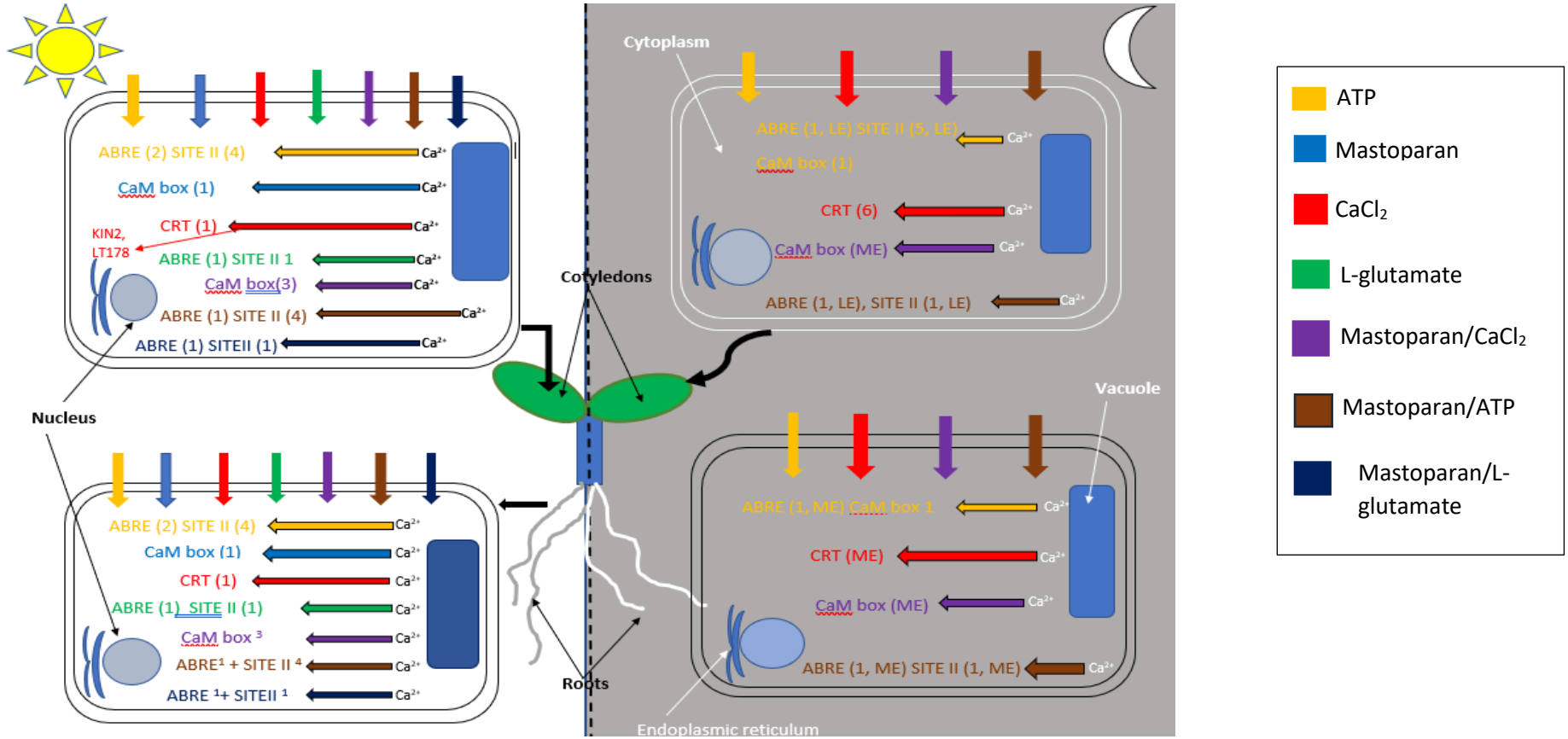


Figure 8.1: A schematic diagram of the results attained from chapter three, four and five. Plant cells from both cotyledons and roots are depicted both at subjective day and subjective night. Calcium agonists are represented by different colours as shown in the figure key, these colours subsequently match with the corresponding Ca^{2+} signatures. *Cis* elements are displayed in the colours of the Ca^{2+} signature that is up regulating them. The expression kinetics are denoted with numbers or letters next to the relevant cis elements: 1. Monophasic response throughout the plant; 2. Monophasic response in the cotyledons and biphasic in the roots; 3. Biphasic in the cotyledons, monophasic in the roots; 4. Biphasic in the roots and cotyledons; LE low response; minimal or no expression. Expression of KIN2 and LT178 during drought stress and pre-treatment of $CaCl_2$ is also represented.

8.5. Calcium regulation of combined cis elements

It is accepted that a plant may be exposed to several stresses at any given time, thereby suggesting that many single signal transduction pathways could be involved in crosstalk at several steps (Roychoudhury et al., 2013). In fact, Knight and Knight, (2001) suggested that several abiotic stress pathways share common elements and therefore, could potentially cross talk. The *RD29A* gene for instance, contains both DRE/CRT and ABRE repeats integrating input stimuli from cold, drought, high salinity, and ABA signalling pathways. Work in this thesis has analysed data taken from the regulation of single specific cis elements regulated by calcium, to mirror a more natural environment I designed six constructs containing two alternating repeats of two different calcium-regulated *cis* elements (either ABRE/SITE II; CRT/ABRE; CaM box/ABRE; CaM box/SITEII; CRT/CaM box) and a seventh as a control containing a minimal promotor only and a *LUC+* sequence (Chapter 6).

Transgenic plants containing these new concatemers were treated with ATP and luciferase activity was measured. It was seen that when ABRE and SITE II were combined and regulated by ATP-generated $[Ca^{2+}]_{cyt}$ signatures they displayed similar expression kinetics to that seen by ATP regulation of the single ABRE *cis* element, suggesting there could be a possibility that only the ABRE was being regulated. There could be several reasons for this e.g. the proximity of the *cis* element could be inhibiting the other, as we know in a natural environment *cis* elements may be separated by several base pairs (Yamaguchi-Shinozaki and Shinozaki, 2006). Furthermore, evidence discovered by Whalley et al., (2011) suggests that ABRE and SITE II are rarely found coupled together naturally in calcium-regulated genes. When either ABRE or SITE II were coupled with the CRT *cis* element and regulated by ATP generated $[Ca^{2+}]_{cyt}$ signatures they expressed novel expression kinetics when compared to their single counter parts. This supports the data described by Whalley et al., (2011) who suggested that CRT was the most likely element to be coupled to a second element in calcium regulated genes. When the CaM box *cis* element was coupled to any other *cis* element and regulated by ATP generated $[Ca^{2+}]_{cyt}$ there was very low levels of expression recorded. The data in chapter 3 suggested that CaM box was not regulated by ATP generated $[Ca^{2+}]_{cyt}$ signatures so it could be possible that the CaM box *cis* element was

blocking calcium signature regulation of the second element (steric hindrance), which supports the evidence by Whalley et al., (2011) suggesting that the CaM box *cis* element is rarely coupled to any other *cis* element in calcium regulated genes.

Finally, when seedlings containing the combined *cis* elements CRT/CaM box were treated with mastoparan, the results suggested that when CaM box was coupled to a second *cis* element it was no longer regulated by calcium. As the CRT TF is small in comparison to the CaM box it would seem unlikely that the CaM box was being blocked, but some other aspect such as the CRT itself could be alternating the DNA, this could be easily confirmed by EMSA.

8.6 The regulation of proteins by a mastoparan generated $[Ca^{2+}]_{cyt}$ signature

The data provided in chapters three, four, five and six has described downstream gene effects regulated by different $[Ca^{2+}]_{cyt}$ signatures, but has not investigated $[Ca^{2+}]_{cyt}$ signature regulation at a protein level. Calcium mediates gene expression by regulating specific proteins, calcium regulates these proteins in several ways including gene expression, protein transport and protein stability via posttranslational modification. The aim of chapter seven in this thesis was to identify proteins regulated by a mastoparan generated $[Ca^{2+}]_{cyt}$ signature, and to determine their biological and molecular functions.

To identify levels of proteins regulated by a mastoparan generated $[Ca^{2+}]_{cyt}$ signature WT Arabidopsis seedlings were treated with mastoparan and proteins were extracted at 3h and 6h. After iTRAQ labelling the proteins were identified through mass spectrometry analysis and further elimination by peptide abundance and confidence of identification. From these proteins the encoding genes were compared to calcium regulated genes identified by Whalley et al., (2011) and Whalley and Knight (2013). Only 11 encoding genes were identified as calcium regulated from the protein lists and four of these genes (At2g3970, At4g30920, At5g47210, and At1g54270) were taken forward to determine if gene expression compared favourably to protein expression. It was indeed seen that all four encoding genes reflected the same expression patterns to that seen with the proteins. Due to the low number of identified proteins whose encoding genes were regulated by calcium in these data it could be suggested that some of the proteins may be regulated post

transcriptionally, however, to confirm this further investigation would be required e.g., testing protein-protein interactions via FRET.

Further elimination of the proteins removed all proteins with a fold difference less than 1.2 to the control and P values above 0.05, generating a significant list for both time points. From these significant lists it could be seen that mastoparan generated $[Ca^{2+}]_{cyt}$ signatures regulated myriad proteins across the two timepoints, with many of these proteins found to be directly involved in the plant stress response. Four of these proteins Glycerol-3-phosphate, peroxidase 62, Nucleotide diphosphate Kinase II and proteosome subunit alpha type 2-A were chosen to take forward, and their encoding genes were designed into constructs to be expressed in Arabidopsis. These transgenic seedlings were then treated with mastoparan or water and gene expression was measured at various timepoints. Preliminary results from the transgenic Arabidopsis plants suggests that these proteins may be regulated post translationally as a low gene expression was recorded from all four transgenic plants measuring no significant difference to those treated with a water control.

8.7 Experimental limitations

The approach undertaken in this thesis has taken a step towards the understanding of calcium signature specificity by identifying novel signatures and how they regulate specific TFs, genes and proteins. However, the experimental procedures are not without their limitations. Firstly, although we identified several more novel signatures than those described in earlier literature (Lenzoni et al., 2018) to truly conclude signature specificity would require a much more extensive study with a wider repertoire of novel calcium signatures. Secondly all the work described in this thesis was conducted under strict experimental condition. The mannitol induced osmotic stress experiment (chapter 4) for instance only represented one stress, we know however, that in a natural environment a plant is exposed to many stimuli both abiotic and biotic which in itself could represent crosstalk between many signal transduction pathways. I have suggested that $CaCl_2$ generated $[Ca^{2+}]_{cyt}$ signatures regulate stress genes involved in osmotic stress which ultimately confer osmotic stress tolerance to Arabidopsis, however, I cannot say for sure that this would occur in this manner if the plant was exposed to several stimuli at the same time. A major limitation to this study was the design of the constructs containing two *cis* elements (chapter 6). It could be possible that the close proximity of the *cis* element

sequences may have affected the response when regulated by $[Ca^{2+}]_{cyt}$ signatures, in a natural environment it is normal to find the sequences separated across the promoter region (Shinozaki and Yamaguchi-Shinozaki, 2000).

8.8 Alternative approaches to test the roles of calcium signatures

In this thesis calcium signature kinetics have been measured, and their roles determined using the bioluminescence reporters aequorin and luciferase. An alternative approach to determine the role of specific calcium signatures could be the use of knockdown or knockout mutants. As described in the general introduction calcium sensors can be specific to a particular calcium signature, therefore, one way to study the role of the calcium signature would be to modify these sensors by knockout or knockdown. These mutant plants could then be compared to wild type plants when treated with various calcium agonists. However, although good in theory, practically it would be difficult to implement this approach as we have not yet identified all the sensors involved in specific signature decoding. One possible alternative to calcium sensor modification would be to modify calcium pumps or channels. As we know calcium homeostasis is controlled by both influx calcium channels and efflux calcium pumps (Kudla et al., 2010), therefore modification of these pumps could allow investigation of the roles of specific calcium signatures. Previous literature has described the use of calcium channel mutants in several studies including $[Ca^{2+}]_{cyt}$ heat induced elevations (Finka et al., 2012, Gao et al., 2012)and the touch response (Kurusu et al., 2013, Nakagawa et al., 2007), therefore it could be feasible to use this approach to investigate the role of the calcium signatures identified in this thesis as well as other calcium signatures in general. If I were to implement this approach, I would mutate random calcium pumps and then test these against different calcium agonists comparing the results with WT plants subjected to the same treatments.

8.9 Future work

8.9.1 Investigations into calcium signature specificity

To present a more complete analysis of calcium signature specificity it would be beneficial to identify a wider range of novel $[Ca^{2+}]_{cyt}$ signatures. To do this I would combine ATP and $CaCl_2$, ATP and L-glutamate, and $CaCl_2$ and L-glutamate. Due to the success of combining mastoparan with a second calcium agonist (chapter 3) I surmise that I could possibly identify several novel signatures from these new combinations. If indeed more novel calcium signatures were identified I would then continue with the work described in chapter 3 analysing the possible regulation by these novel signatures of the *cis* elements identified by Whalley et al., (2011). If the results showed specificity during *cis* element regulation, then I could repeat the work conducted in chapter 4 in relation to signature specificity and osmotic stress. However, it must be noted that it is well documented that $CaCl_2$ pre-treatment confers osmotic stress tolerance to plants and work in chapter 4 seemed to indicate that $CaCl_2$ when combined with another agonist did not produce these results. This suggested that the signature produced by $CaCl_2$ was the only signature that contained the required information to lead to osmotic stress tolerance. Therefore, I would expect that any new novel signatures would not lead to osmotic stress tolerance, but I cannot be completely confident in this without conducting these suggested experiments.

8.9.2 To complete the investigations into the coupled cis elements

The work conducted on transgenic Arabidopsis containing concatemers which consisted of two calcium regulated *cis* elements was incomplete. Due to the Covid 19 pandemic and the subsequent lock down restrictions I was unable to continue experiments with these plants, investigating calcium signature specificity in respect to the regulation of two coupled cis elements. If I was to continue with this work I would treat these plants with all the identified novel $[Ca^{2+}]_{cyt}$ signatures allowing for a direct comparison between single and double *cis* elements when regulated by these novel $[Ca^{2+}]_{cyt}$ signatures. I would also like to repeat the work conducted in chapter 5 on these new transgenic plants, examining any biphasic responses to determine if $[Ca^{2+}]_{cyt}$ signatures are occurring in different cells or in the same cells at different times. I would also like to determine if circadian regulated

$[Ca^{2+}]_{cyt}$ signatures resulted in changes in downstream expression when two *cis* elements are coupled.

8.9.3 Calcium regulation of Proteins

Again, this work was incomplete due to time restrictions. To determine $[Ca^{2+}]_{cyt}$ signature specificity during protein regulation it would be necessary to treat the Arabidopsis plants containing the protein constructs with other calcium agonists to measure protein expression levels, allowing a direct comparison to mastoparan $[Ca^{2+}]_{cyt}$ signature generated protein regulation. Also, if I were to continue with this work, I would clone the constructs containing the four proteins of interest into *Nicotiana tobacum* for transient expression, and determine the location of these proteins by visualising GFP expression under a microscope.

8.9 Conclusions

In conclusion, this thesis has gone a short way to support the 'calcium signature' hypothesis first put forward by McAinsh and Hetherington in 1998. I have identified a range of novel $[Ca^{2+}]_{cyt}$ signatures and have noted that these novel signatures regulate different *cis* elements, suggesting specificity. I have also suggested that when these $[Ca^{2+}]_{cyt}$ signatures are regulated by the circadian clock this has a direct effect on downstream expression patterns. Furthermore, we have provided evidence that downstream expression regulated by a specific calcium signature changes when two *cis* elements are coupled. Finally, I have identified and characterised proteins that are regulated by one of these novel signatures, which as far as I know has not been described in any current literature to date.

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Appendix. A

A.1 Primer, gene and construct sequences

F-At1G54270	CCGGAAGGAACACAATTTGATACG
R-At1G54270	CTCCACGTTCACGTAGAATTGCTTG
F-At5G47210	TCTCACTGCCGCATCTTTTCTTAGG
R-At5G47210	GTCCCGGTTGTATCCACCATTACC
F-At4G30920	CTCCAGCCAATGTTGTCACTCCTG
R-At4G30920	GCGACAATAAAGTGCACCTCAACAC
F-At2G39730	CAAGGCCTTAGGCAGTACAACTTGG
R-At2G38730	CTCACGGTACCTCTGACGGATAAGC
CtermGFP	TGAACAGCTCCTCGCCCTTG
NtermGFP	GCATGGACGAGCTGTACAAG
P35S-FOR	ATTTACTATTCTAGTCGACC
T35S-REV	TTTTGCGGACTCTAGCATGG
KIN2 For	TCAGGCCGCTGGCAAA
KIN 2 Rev	TGCCGCATCCGATATACTCTT
LT178 For	GCACCCAGAAGAAGTTGAACA
LT178 Rev	TCATGCTCATTGCTTTGTCC
LUC For	GCCCGCGAACGACATTTA
LUC Rev	CTGCGAAATGCCCATACTGTT
pBIN19 For	CCGGGTACCGAGCTCGACTGGATTTTGGTTTTAGGA
pBIN19 Rev	AAAACGACGGCCAGTGAACGACGGCCAGTGAATTCC
CRT For	CGGTATGAACCAGCATTCCT
CRT Rev	TTGGGTTCTCTTGCTCGTTC
CaM Box For	GAAGTTACTGGTGGCCCTGA
CaM Box Rev	CCCTTGGTAGCATCAGGAAG
ABRE For (AT1G43160-F)	TCTTTGCCTCCTCAACCATT
ABRE Rev (AT1G43160-R)	CCTCTCCAAGGACATTGAGC
SITE II For	CAGTTCATGTTGGGAAT
SITE II Rev	TCAGAAGCAAGACGCTCA
PEX4 For	TCATAGCATTGATGGCTCATCCT
PEX4 Rev	ACCCTCTCACATCACCAGATCTTAG

Glycerol-3-phosphate-stop with flanking sequence	TGTACAAAAAAGCAGGCTCCGCGGCCGCATAACAATGACTCTCACGTTTTCCTCCTCC GCCGCAACCGTTGCCGTTGCTGCTGCAACCGTAACCTCCTCCGCTAGGGTTCCGGTTT ATCCACTCGCTTCGTCGACTCTTCGTGGATTAGTATCTTTCAGATTAACCGCGAAGAAG CTGTTTCTGCCGCCTCTTCGTTCTCGCGGCGGCGTTAGTGTGAGAGCCATGTCTGAGC TTGTTCAGGATAAAGAATCGTCCGTCGCGGCGAGCATTGCTTTCAATGAAGCCGCCG GTGAGACGCCGAGTGAGCTTAGTCATTCCCGTACTTTCTTGGATGCGCGAAGTGAACA AGATCTTTTATCTGGTATCAAGAAGGAAGCTGAAGCTGGAAGGTTGCCAGCAAATGT TGCAGCAGGAATGGAAGAATTGTATTGGAACTACAAAAATGCAGTTTTAAGTAGTGG AGCTTCCAGGGCAGATGAAACTGTTGTATCAAACATGTCTGTTGCTTTTGATCGCATG CTTCTTGGTGTGGAGGATCCTTATACTTTTAATCCATATCATAAAGCAGTCAGAGAACC ATTTGACTACTACATGTTTGTCCATACATACATCCGTCCTCTTATTGATTTCAAAAATTC GTACGTTGGAAATGCTTCTATATTCTCTGAGCTGGAAGACAAGATTCGACAGGGACAC AATATCGTGTTGATATCAAACCATCAAAGTGAAGCTGATCCGGCTGTCATTTCTCTATT GCTTGAAGCACAATCTCCTTTCATAGGAGAGAACATTAAATGTGTGGCTGGTGATCGA GTCATCACTGATCCTCTTTGTAAGCCGTTCAGTATGGGAAGGAACCTCATATGTGTTTA CTCGAAAAAGCACATGAATGATGATCCTGAGCTTGTTGACATGAAAAGAAAAGCAAA CACACGAAGCTTAAAGGAGATGGCTACAATGCTAAGGTCTGGCGGTCAACTTATATG GATTGCACCAAGCGGTGGAAGGGACCGCCCGAATCCTTCTACTGGGGAATGGTTTCC TGCACCCTTTGATGCTTCTTCGGTAGACAACATGAGAAGACTGGTTGAACATTCTGGC GCTCCTGGACATATATATCCAATGTCTTTGCTTTGCTATGACATCATGCCCCCTCCACCC CAGGTTGAGAAAGAAATCGGAGAGAAAAGATTAGTTGGGTTTCACGGTACTGGACTA TCAATTGCTCCTGAAATCAACTTCTCAGACGTCACAGCAGACTGCGAGAGCCCTAATG AGGCGAAAGAAGCATACAGCCAAGCTTTGTACAAGTCGGTGAATGAACAATACGAGA TCTTAAACTCTGCGATTAAACACAGAAGAGGAGTAGAAGCATCAACTTCAAGGGTCTC TTTGTCACAACCTTGGAATTAGGGCGCGCCGACCCAGCTTTCTTGTACAA
Glcerol-3-phosphate-nostop with flanking seq.	TGTACAAAAAAGCAGGCTCCGCGGCCGCATAACAATGGTGGGAGCGACTGTAGTTAG TAAATGGACTCCCCTATGTGTGGCTTCGCCGCCGGAGAGAAACTCGGCAAGTCTCAAT CCACACTGTTCTCCAGCCAGGGTTAATTTTAGAACAGCGTTGGCCGCATTTCGTCCTCA GTTCCGTCTTTTCTCTCGCAATTCCGCGTCTCGCCGCCGTCTTCGCGCTTCCAGCTCCGC CGAATCGGGAATCTTCCTTCCTCACCTTGTAGCTTCTATGGAGGACGTTGAGGAGACT TACATTATGGTGAAACCTGATGGCATACAACGAGGCCTTGTAGGAGAAATCATTTCTC GCTTTGAGAAAAAGGGGTTTAAACTTATTGGGCTCAAGATGTTTCAGTGCCCAAAAG AATTGGCTGAGGAGCATTATAAGGATCTTAGTGCTAAATCATTCTTTCCTAACCTGATT GAGTACATCACTTCAGGTCCAGTTGTGTGTATGGCTTGGGAAGGTGTTGGTGTTGTTG CTTCAGCCAGGAAGCTAATAGGGAAAACAGATCCGCTTCAAGCTGAACCTGGTACTA TAAGAGGAGATCTTGCTGTGCAAACCGGAAGGAACATTGTGCATGGTAGTGACAGCC CTGAAAACGGCAAGCGGATTGGTCTGTGGTTCAAAGAGGGCGAGCTATGCAAGTGG GATTCGGCTCTAGCTACATGGCTAAGGGAGTGAGGCGCGCCGACCCAGCTTTCTTGT ACAA
Nucleoside diphosphate kinase II nostop-flanking seg.	TGTACAAAAAAGCAGGCTCCGCGGCCGCATAACAATGGGCTTGGTCCGATCATTTGCT TTGGTCATAGTCTTCCTTAGTTGTCTCATCGCCGTTTATGGCCAAGGTACGAGGATCG GGTTCTACTCGACTACGTGCCCTAATGCCGAGACAATTGTTCGGACCACTGTGGCATC TCACTTTGGTTCAGATCCAAAGGTTGCACCCGGGTTACTGAGAATGCACAACCATGAT TGCTTTGTCCAAGGTTGTGATGGTTCGGTGCTTTTATCGGGACCTAACTCTGAAAGAA CCGCTGGCGCAAACGTTAACCTCCATGGTTTTGAGGTCATTGACGATGCCAAGAGGC AGCTCGAGGCAGCATGTCCTGGTGTTGTCTCTTGTGCTGATATCTTGGCCTTAGCGGC TCGTGATTCGGTTTCTCTCACAAACGGACAAAGTTGGCAAGTTCCAACAGGACGTAGA GATGGAAGAGTTTCCTTGGCATCGAACGTTAACAATCTTCCTTCTCCAAGTGACTCTCT AGCCATTCAACAAAGGAAATTCTCCGCTTTTCGCCTCAACACTCGCGATCTCGTCACTC TTGTTGGAGGAGGACACACGATCGGAACAGCTGCATGTGGGTTTATCACGAACAGGA TATTCAACTCGAGCGGAAACACAGCAGATCCAACAATGGACCAAACATTTGTACCACA ACTTCAAAGACTTTGTCCCCAAAACGGCGACGGATCAGCACGTGTTGATCTTGACACC GGAAGTGGAAACACTTTTGACACATCTTATTTCATCAATCTCAGCCGTAACAGAGGAA TTCTTCAATCCGATCACGTTCTTTGGACTAGTCCGGCCACAAGATCCATAGTGCAAGA

	GTTTATGGCACCTAGAGGCAACTTCAATGTCCAGTTCGCAAGGTCAATGGTTAAAATG AGTAATATTGGTGTGAAGACGGGGACAAATGGAGAAATTCGTAGGGTTTGCTCTGCG GTTTAAGGCGCGCCGACCCAGCTTTCTTGTACAA
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Peroxidase 62sNostop with flanking seq.	TGTACAAAAAAGCAGGCTCCGCGGCCGCATAACAATGGGAGATAGTCAGTACTCGTT TTCTCTCACCACTTTCAGCCCATCTGGTAAGCTGGTGCAGATAGAACATGCCCTTACAG CTGTTGGATCTGGCCAAACATCTTTAGGGATTAAAGCTTCTAATGGAGTTGTCATTGC AACTGAAAAGAAGTTGCCTTCTATTCTGGTTGATGAAGCATCTGTTCAAAAAATTCAG CATTTGACTCCTAATATTGGAGTTGTTTACAGTGGCATGGGTCCTGATTTTCGAGTTCT TGTTAGGAAGAGTAGGAAACAGGCTGAGCAATATCTTCGTCTGTACAAAGAACCCAT CCCTGTTACCCAACTTGTAAGGGAAACCGCTACTGTTATGCAAGAGTTTACTCAATCG GGAGGTGTTAGGCCTTTCGGGGTTTCCTTGCTGGTGGCTGGATATGATGACAAGGGT CCACAATTGTATCAGGTGGATCCATCTGGCTCTTATTTCTCCTGGAAAGCTTCAGCCAT GGGGAAGAACGTTTCTAATGCAAAAACCTTCCTTGAGAAAAGGTACACAGAAGACAT GGAACTTGACGATGCCATTCACACAGCGATACTGACATTGAAAGAAGGCTTTGAGGG AGAGATCTCAAGCAAAAATATTGAAATTGGCAAAATCGGTGCTGACAAAGTTTTCAG GGTACTAACACCAGCAGAGATCGATGATTACCTTGCAGTCGAGTAAGGCGCGCCGAC CCAGCTTTCTTGTACAA
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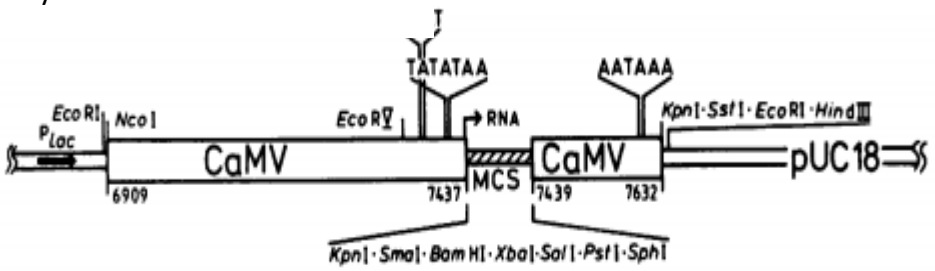
Proteosome subunit alpha type 2A- nostop with flanking seq.	TGTACAAAAAAGCAGGCTCCGCGGCCGCATAACAATCGGAGATAGTCAGTACTCGTT TTCTCTCACTTTCAGCCCATCTGGTAAGCTGGTGCAGATAGAACATGCCCTTACAGCTG TTGGATCTGGCCAAACATCTTTAGGGATTAAAGCTTCTAATGGAGTTGTCATTGCAAC TGAAAAGAAGTTGCCTTCTATTCTCGTTGATGAAGCATCTGTTCAAAAAATTCAGCATT TGACTCCTAATATTGGAGTTGTTTACAGTGGCATGGGTCCTGATTTTCGAGTTCTTGTT AGGAAGAGTAGGAAACAGGCTGAGCAATATCTTCGTCTGTACAAAGAACCCATCCCT GTTACCCAACTTGTAAGGGAAACCGCTACTGTTATGCAAGAGTTTACTCAATCGGGAG GTGTTAGGCCTTTCGGGGTTTCCTTGCTGGTGGCTGGATATGATGACAAGGGTCCACA ATTGTATCAGGTGGATCCATCTGGCTCTTATTTCTCCTGGAAAGCTTCAGCCATGGGG AAGAACGTTTCTAATGCAAAAACCTTCCTTGAGAAAAGGTACACAGAAGACATGGAA CTTGACGATGCCATTCACACAGCGATACTGACATTGAAAGAAGGCTTTGAGGGAGAG ATCTCAAGCAAAAATATTGAAATTGGCAAAATCGGTGCTGACAAAGTTTTCAGGGTAC TAACACCAGCAGAGATCGATGATTACCTTGCTGAAGTCGAGGGCGCGCCGACCCAGC TTTCTTGTACAA
Proteasome subunit alpha type 2a stop with flanking seq.	TGTACAAAAAAGCAGGCTCCGCGGCCGCATAACAATGGGCTTGGTCCGATCATTTGCT TTGGTCATAGTCTTCCTTAGTTGTCTCATCGCCGTTTATGGCCAAGGTACGAGGATCG GGTTCTACTCGACTACGTGCCCTAATGCCGAGACAATTGTTCGGACCACTGTGGCATC TCACTTTGGTTCAGATCCAAAGGTTGCACCCGGGTTACTGAGAATGCACAACCATGAT TGCTTTGTCCAAGGTTGTGATGGTTCGGTGCTTTTATCGGGACCTAACTCTGAAAGAA CCGCTGGCGCAAACGTTAACCTCCATGGTTTTGAGGTCATTGACGATGCCAAGAGGC AGCTCGAGGCAGCATGTCCTGGTGTTGTCTCTTGTGCTGATATCTTGGCCTTAGCGGC TCGTGATTCGGTTTCTCTCACAAACGGACAAAGTTGGCAAGTTCCAACAGGACGTAGA GATGGAAGAGTTTCCTTGGCATCGAACGTTAACAATCTTCCTTCTCCAAGTGACTCTCT AGCCATTCAACAAAGGAAATTCTCCGCTTTTCGCCTCAACACTCGCGATCTCGTCACTC TTGTTGGAGGAGGACACACGATCGGAACAGCTGCATGTGGGTTTATCACGAACAGGA TATTCAACTCGAGCGGAAACACAGCAGATCCAACAATGGACCAAACATTTGTACCACA ACTTCAAAGACTTTGTCCCCAAAACGGCGACGGATCAGCACGTGTTGATCTTGACACC GGAAGTGGAAACACTTTTGACACATCTTATTTCATCAATCTCAGCCGTAACAGAGGAA TTCTTCAATCCGATCACGTTCTTTGGACTAGTCCGGCCACAAGATCCATAGTGCAAGA GTTTATGGCACCTAGAGGCAACTTCAATGTCCAGTTCGCAAGGTCAATGGTTAAAATG AGTAATATTGGTGTGAAGACGGGGACAAATGGAGAAATTCGTAGGGTTTGCTCTGCG GTTAATTAAGGCGCGCCGACCCAGCTTTCTTGTACAA
***Cis* elements**	
>90+CaM box with Gibson flanks	TGACCATGATTACGAATTCCCATGGAACGCGTCAAACGCGTCAAACGCGTCAAACGC GTCAGATATCTCCACTGACGTAAGGGATGACGCACAATCCCACTATCCTTCGCAAGAC CCTTCCTCTATATAAGGAAGTTCATTTCATTTGGAGAGGACACGGTACCCGGGCTCGA GATCTGCGATCTAAGTAAGCTTGGCATTCCGGTACTGTTGGTAAAGCCACCATGGAAG ACGCCAAAAACATAAA
>90+ ABRE with Gibson flanks	TGACCATGATTACGAATTCCCATGGACACGTGTAACACGTGTAACACGTGTAACACGT GTAGATATCTCCACTGACGTAAGGGATGACGCACAATCCCACTATCCTTCGCAAGACC CTTCCTCTATATAAGGAAGTTCATTTCATTTGGAGAGGACACGGTACCCGGGCTCGAG ATCTGCGATCTAAGTAAGCTTGGCATTCCGGTACTGTTGGTAAAGCCACCATGGAAGA CGCCAAAAACATA
>90 + SITE II with Gibson flanks	GACCATGATTACGAATTCCCATGGTATGGGCCTTATGGGCCTTATGGGCCTTATGGGC CT GATATCTCCACTGACGTAAGGGATGACGCACAATCCCACTATCCTTCGCAAGACCCTT CCTCTATATAAGGAAGTTCATTTCATTTGGAGAGGACACGGTACCCGGGCTCGAGATC TGCGATCTAAGTAAGCTTGGCATTCCGGTACTGTTGGTAAAGCCACCATGGAAGACG CCAAAAACATAAA
>90 + CRT with gibson flanks	TGACCATGATTACGAATTCCCATGGTACCGACATTACCGACATTACCGACATTACCGA CATGATATCTCCACTGACGTAAGGGATGACGCACAATCCCACTATCCTTCGCAAGACC CTTCCTCTATATAAGGAAGTTCATTTCATTTGGAGAGGACACGGTACCCGGGCTCGAG ATCTGCGATCTAAGTAAGCTTGGCATTCCGGTACTGTTGGTAAAGCCACCATGGAAGA CGCCAAAAACATAAA

>90+ with gibson flanks	TGACCATGATTACGAATTCCCATGGGATATCTCCACTGACGTAAGGGATGACGCACAA TCCCACTATCCTTCGCAAGACCCTTCCTCTATATAAGGAAGTTCATTTCATTTGGAGAG GACACGGTACCCGGGCTCGCGATCTGCGATCTAAGTAAGCTTGGCATTCCGGTACTGT TGGTAAAGCCACCATGGAAGACGCCAAAAACATAAA
Combined *cis* elements	
>-90 + CRT+CAM BOX with Gibson flanks	TGACCATGATTACGAATTCCCATGGTACCGACATTTACGCGTAATACCGACATTTACGC GTAAGATATCTCCACTGACGTAAGGGATGACGCACAATCCCACTATCCTTCGCAAGAC CCTTCCTCTATATAAGGAAGTTCATTTCATTTGGAGAGGACACGGTACCCGGGCTCGA GATCTGCGATCTAAGTAAGCTTGGCATTCCGGTACTGTTGGTAAAGCCACCATGGAAG ACGCCAAAAACATAAA
>-90+CRT+SITE II with Gibson flanks	TGACCATGATTACGAATTCCCATGGTTACCGACATTATGGGCCTTTACGGACATTATG GGCCTTGATATCTCCACTGACGTAAGGGATGACGCACAATCCCACTATCCTTCGCAAG ACCCTTCCTCTATATAAGGAAGTTCATTTCATTTGGAGAGGACACGGTACCCGGGCTC GAGATCTGCGATCTAAGTAAGCTTGGCATTCCGGTACTGTTGGTAAAGCCACCATGGA AGACGCCAAAAACATAAA
>90+CRT+ABRE with Gibson flanks	TGACCATGATTACGAATTCCCATGGTACCGACATACACGTGTATACCGACATACACGT GTAGATATCTCCACTGACGTAAGGGATGACGCACAATCCCACTATCCTTCGCAAGACC CTTCCTCTATATAAGGAAGTTCATTTTTGGAGAGGACACGGTACCCGGGCTCGAGATC TGCGATCTAAGTAAGCTTGGCATTCCGCTACTGTTGGTAAAGCCACCATGGAAGACGC CAAAAACATAAA
>90+CAM BOX+SITEII with Gibson flanks	TGACCATGATTACGAATTCCCATGGTTACGCGTAATATGGGCCTTTTACGCGTAATAT GGGCCTTGATATCTCCACTGACGTAAGGGATGACGCACAATCCCACTATCCTTCGCAA GACCCTTCCTCTATATAAGGAAGTTCATTTCATTTGGAGAGGACACGGTACCCGGGCT CGAGATCTGCGATCTAAGTAAGCTTGGCATTCCGGTACTGTTGGTAAAGCCACCATGG AAGACGCCAAAAACATAAA
>90+CAM BOX+ABRE with Gibson flanks	TGACCATGATTACGAATTCCCATGGTTACGCGTAAACACGTCTATTACGCGTAAACAC GTGTAGATATCTCCACTGACGTAAGGGATGACGCACAATCCCACTATCCTTCGCAAGA CCCTTCCTCTATATAAGGAAGTTCATTTCATTTGGAGAGGACACGGTACCCGGGCTCG AGATCTGCGATCTAAGTAAGCTTGCCATTCCGGTACTGTTGGTAAAGCCACCATGGAA GACGCCAAAAACATAAA
>90+ABRE+SITEII with Gibson flanks	TGACCATGATTACGAATTCCCATGGACACGTGTATATGGGCCTTACACGTCTATATGG GCCTTGATATCTCCACTGACGTAAGGGATGACGCACAATCCCACTATCCTTCGCAAGA CCCTTCCTCTATATAAGGAAGTTCATTTCATTTGGAGAGGACACGGTACCCGGGCTCG AGATCTGCGATCTAAGTAAGCTTGGCATTCCGGTACTGTTGGTAAAGCCACCATGGAA GACGCCAAAAACATAAA
>90 with Gibson flanks	TGACCATGATTACGAATTCCCATGGGATATCTCCACTGACGTAAGGGATGACGCACAA TCCCACTATCCTTCGCAAGACCCTTCCTCTATATAAGGAAGTTCATTTCATTTGGAGAG GACACGGTACCCGGGCTCGCGATCTGCGATCTAAGTAAGCTTGGCATTCCGGTACTGT TGGTAAAGCCACCATGGAAGACGCCAAAAACATAAA

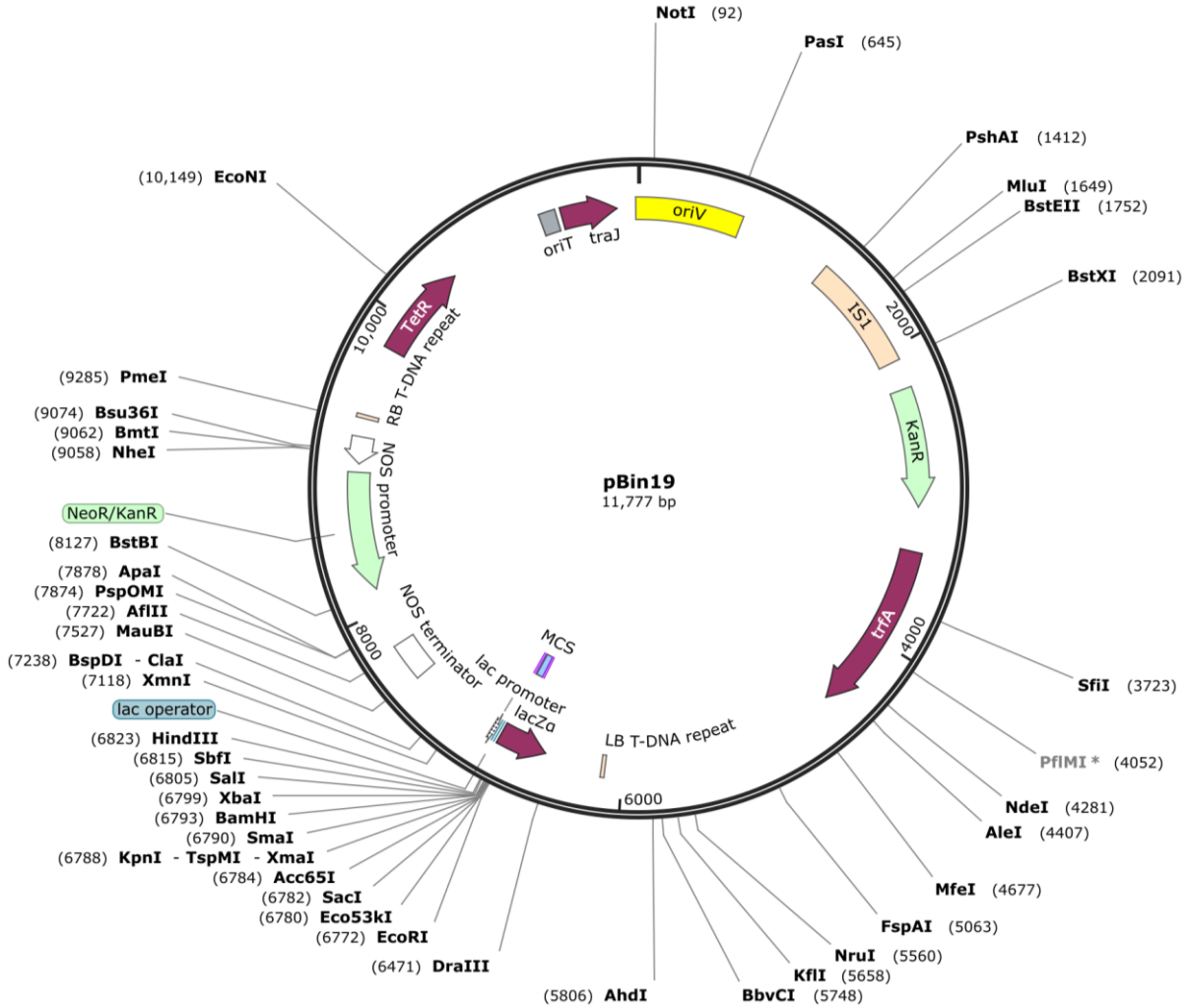
Appendix B

B.1: pDH51 plasmid for Gibson assembly of cis element constructs

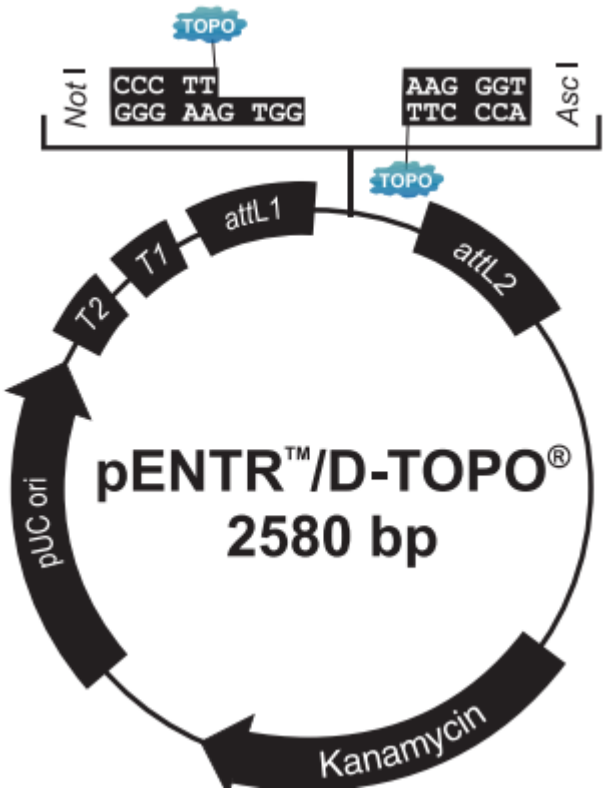


Pietrzak et al., 1986

B.2 pBIN19 Binary vector for transformation of Gibson cis-element constructs.

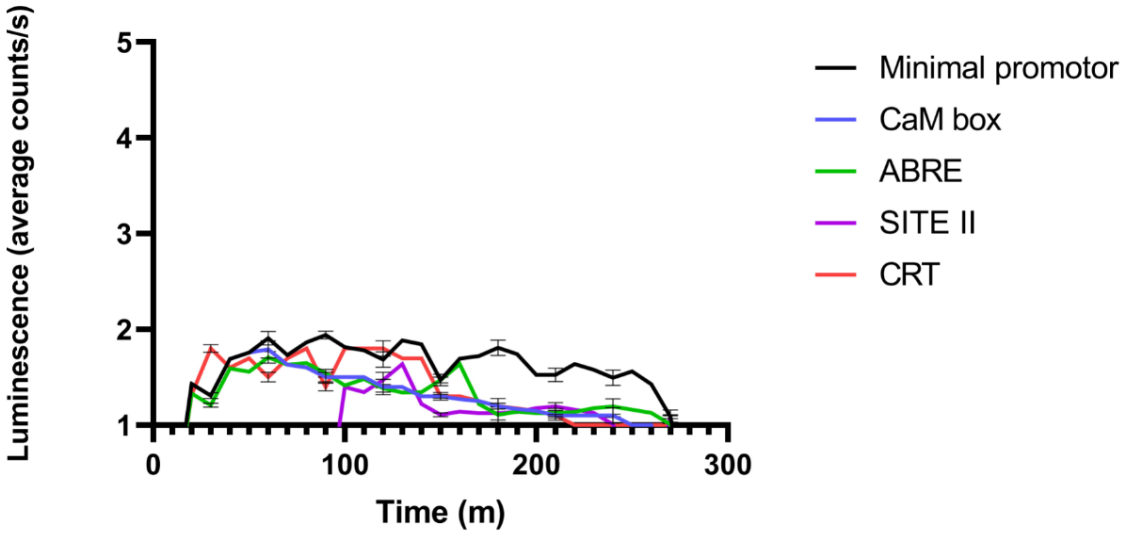


B.3 pENTRD-TOPO vector for protein constructs



Appendix C

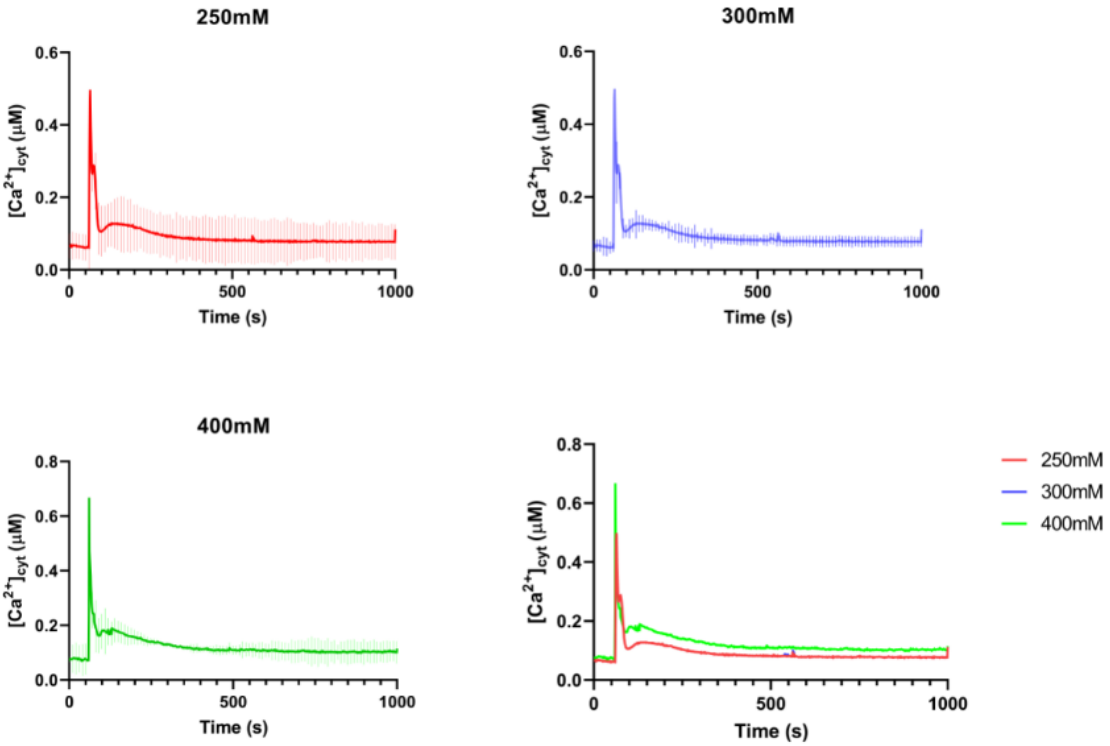
C.1: Water control.



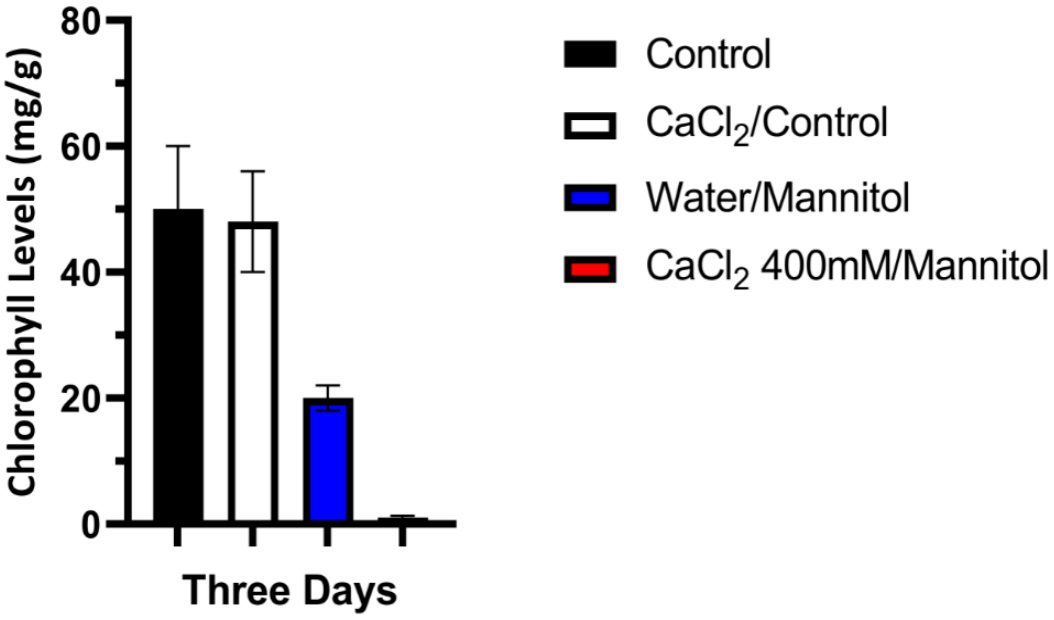
Luminescence average counts per second, each line represents an average of five 10-day old Arabidopsis seedlings containing a construct expressing *LUC+* through 1 of 4 calcium regulated cis elements, or a minimal promoter control.

Appendix D

D.1 Calcium signature traces generated from three different concentrations of $CaCl_2$



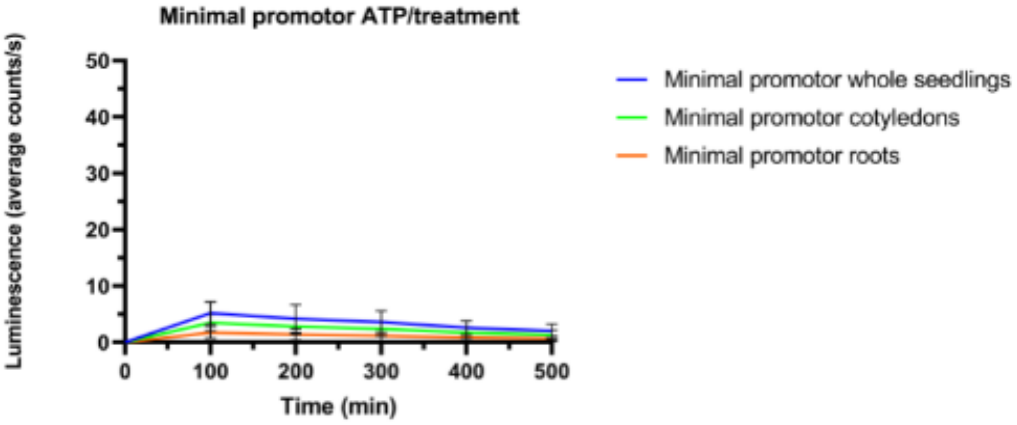
D.2 Chlorophyll levels measured after three days of osmotic stress

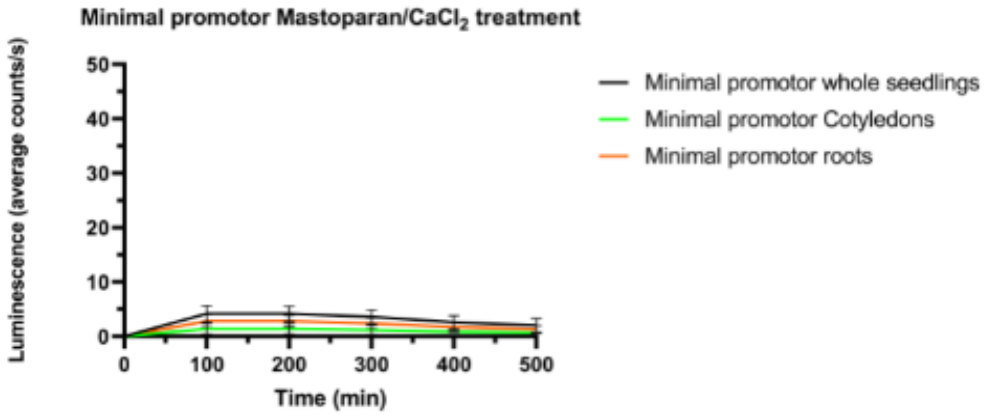


Chlorophyll levels from Arabidopsis leaves over 3 days. Plant tissue has been pre-treated with an overall concentration of 400mM of $CaCl_2$ or water and then treated with 300mM mannitol or water as a control.

Appendix E

E.1 Water control experiments for combined *cis* elements

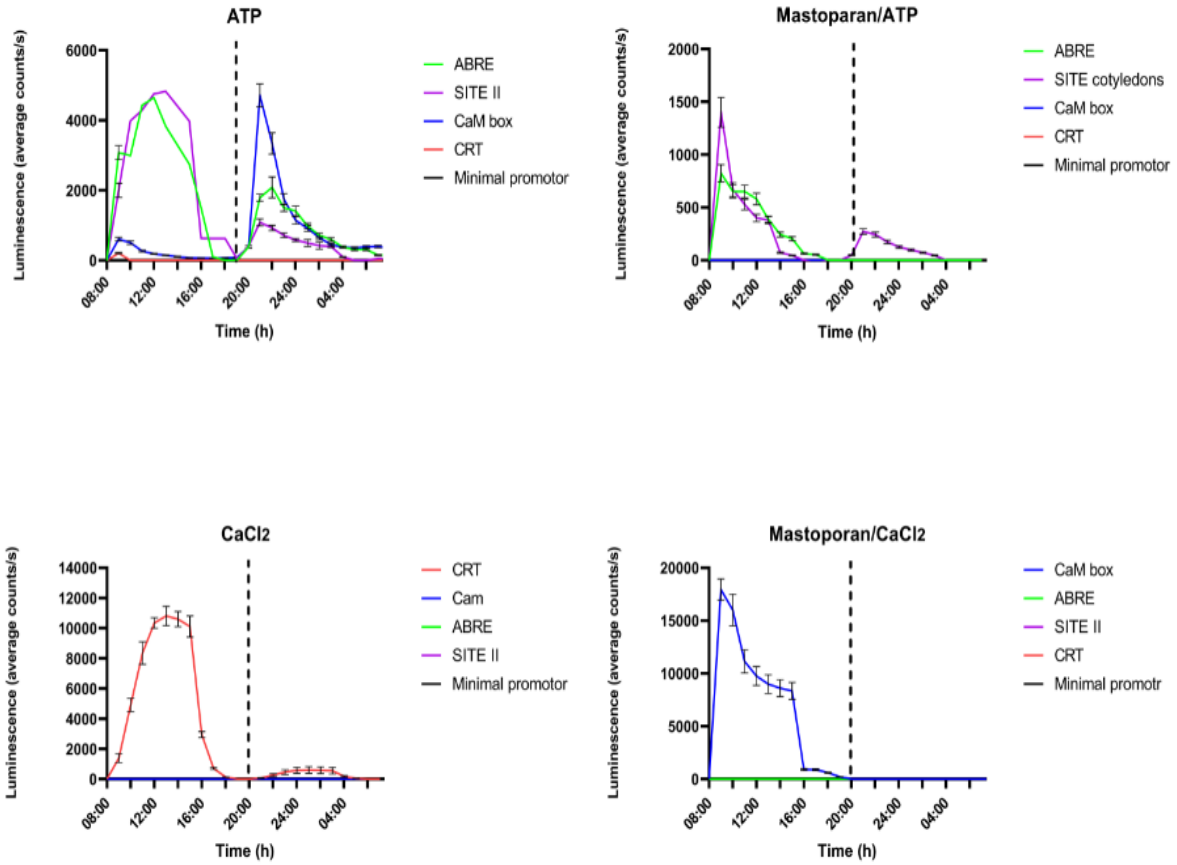




Luminescence average counts per second from Arabidopsis seedlings containing a construct expressing *LUC+*. Each line represents an average of 5 seedlings measuring from either the whole seedling, cotyledons or the roots treated with an overall concentration of 0.5mM ATP or 50µM of mastoparan and 250mM $CaCl_2$.

Appendix F

F.1



Luminescence average counts per second over 24h from Arabidopsis seedlings containing a construct expressing *LUC+* through either CaM box, CRT, ABRE, SITE II or a minimal promotor control. Each line represents an average of 3 seedlings treated with an overall concentration of either 0.5mm ATP, 10µM mastoparan and 0.5mM ATP, 250mM $CaCl_2$ or 10µM mastoparn and 250mM $CaCl_2$. All seedlings were grown in a photoperiod 16L/8D for 24h followed by 7d of entrainment in either 12L:12D (forward) or 12D:12L (reverse) and a further 24h of constant light. Luminescence levels were recorded over 24h