Calcium signalling in the chloroplast and in the regulation of nuclear gene expression

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"Success is the ability to go from one failure to another with no loss of enthusiasm"

Winston Churchill

"Study hard what interests you the most in the most undisciplined, irreverent and original manner possible"

Richard Feynman

Drawing by Eric Robison

"Ideas come from curiosity"

Walt Disney

Abstract

Calcium is a universal second messenger involved in nearly every aspect of plant physiology and development. In response to a variety of biotic or abiotic stresses, calcium rapidly and transiently increases in the cytosol and in this way triggers the appropriate downstream response. To date, most of the research on calcium has focused on cytosolic calcium signalling, however recent advances have demonstrated that in the chloroplast Ca^{2+} concentrations are also controlled, and that chloroplast calcium signalling is involved in regulating the plant cell physiology.

This thesis describes work investigating both cytosolic and chloroplast calcium signalling, In the first case, I examined how cytosolic Ca^{2+} increases with different kinetics (Ca^{2+}-signatures) can encode specific information, and how this can be translated into appropriate changes in transcript expression. To this aim, a dynamic mathematical model of the SA-mediated pathogen network was developed. Calcium is responsible for activating this defence pathway by a complex regulation of the components of this network. This model was able to predict fold-changes and kinetics of gene expression in response to any given calcium signature, hence it was able to accurately describe how specificity is encoded in plant cells. The properties emerging from this model provided insights into the mechanistic basis of calcium signature decoding.

Work on chloroplast calcium signalling focused on two different aspects. Firstly, the hypothesis that chloroplast calcium might regulate chloroplast gene expression was tested, and it was found to not be the case. Secondly, a new chloroplast-specific calcium response was discovered, in response to heat. Properties of this response were investigated, as well as its possible physiological functions. Finally, by using this calcium response as a readout, I addressed the question of heat-sensing in plants. Using this approach I discovered that there is a prominent role for membrane fluidity in controlling this heat-induced calcium increase.

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Statement of Authorship

I certify that all of 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

The standard scientific conventions for protein and gene naming have been followed: wild type genes and proteins are in capitals and mutants are denoted by lower case, gene names are italicized whereas protein names are not.

Standard scientific abbreviations have been used for units of weight, length, amount, molarity, temperature and time.

Standard chemical element symbols, nucleic acid and amino acid codes are used.

$[Ca^{2+}]$: calcium concentration
ABA: abscisic acid
AC: autoinhibited calcium ATPase
APX: ascorbate peroxidase
ARP: acidic repeat protein
ATP: adenosine triphosphate
AtSR1: *Arabidopsis thaliana* signal responsive 1
BA: benzyl alcohol
BAPTA: 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid
BAPTA-AM: 2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid acetyloxylmethyl ester
BRET: bioluminescence resonance energy transfer
BSO: buthionine sulphoximine
cADPR: Cyclic ADP Ribose
CaM: calmodulin
cAMP: cyclic adenosine monophosphate
CAMTA: calmodulin binding transcription activator
CaS: Ca^{2+}-sensing receptor

CAX: cation exchanger

CBL: Calcineurin B-like protein

CCaMK: Calcium/CaM-dependent protein kinase

cGMP: cyclic guanosine monophosphate

Chl: chloroplast

CIPK: CBL-interacting protein kinase

CNGC: cyclic nucleotide channel

CPK: calcium-dependent protein kinase

Cyt: cytosol

DACC: depolarization-activated calcium channel

DAMP: damage-associated molecular pattern

DBMIB: dibromothymoquinone

DCMU: (3-(3,4-dichlorophenyl)-1,1-dimethylurea)

DMSO: dimethyl sulfoxide

DNQX: 6,7-dinitroquinoxaline-2,3-dione

e[Ca^{2+}]: external calcium

ECA: ER type calcium ATPase

EDS: enhanced disease susceptibility

EGTA: ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid

EGTA-AM: glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid cetyloxylmethyl ester

ER: endoplasmic reticulum

Estr: estradiol

ETI: effector- triggered immunity

EtOH: ethanol

FAD: fatty acid desaturase

FRET: fluorescence resonance energy transfer

GA: gibberellic acid

GFP: green fluorescent probe

GLR: glutamate receptor

GSH: glutathione

GSSG: glutathione disulfide

HACC: hyperpolarization-activated calcium channel

HAMK: heat-activated MAP kinase

HMA1: heavy metal ATPase1

HSR: heat shock response

IAA: indole-3- acetic acid

ICS: isochorismate synthase

IP: inositol phosphate

IR: inverted repeat

IRGA: infrared gas analyser

JA: jasmonic acid

LB: Luria-Bertani

LC/MS: liquid chromatography - mass spectrometry

L-Glu: L-glutammate

LSC: large single copy region

MAMP: microbe- associated molecular pattern

Masto: mastoparan

MDA: malonaldehyde

MLO: Mildew resistance locus o

MS: Murashige and Skoog

MSL: mechanosensitive-like channel

NAADP: Nicotinic acid adenine dinucleotide phosphate

NB-LRR: nucleotide-binding leucine-rich repeat

NEP: nuclear-encoded RNA poymerase

PAMP: pathogen- associated molecular pattern

Parv: parvalbumin

PCR: polymerase chain reaction

PEP: plastid-encoded RNA polymerase

PM: plasma membrane

PTI: PAMP- triggered immunity

RbcL: Rubisco large subunit

RbcS: Rubisco small subunit

RLK: receptor-like kinase

RLP: receptor-like protein

RQ: relative quantitation

RT: relative tran script

SA: salicilic acid

SAMK: stress-activated MAP kinase

SE: standard error

SKOR: Shakerlike stellar K^+ outward rectifier

SSC: small single copy region

TBARS: thioubarbituric acid reactive substances

TPC1: two pore channel 1

TRPM8: transient receptor potential cation channel subfamily M member 8

TRPV1: transient receptor potential cation channel subfamily V member 1

VICC: voltage-independent calcium channel

YFP: yellow fluorescent protein

Acknowledgements

Out of all the pages composing this thesis, this is undoubtedly the most important one.

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Chapter 1

Introduction

1.1 Calcium signalling

One of the best known plant signalling components is calcium. Calcium acts as a universal second messenger for all eukaryotic cells and is involved in nearly every aspect of plant physiology and development (Dodd et al., 2010; Kudla et al., 2010).

Pushed by the need to counterbalance calcium's toxic effect via effects on phosphate (producing insoluble calcium salts), calcium is stored in subcellular compartments or pumped into the apoplast; in order to maintain low resting cytosolic free calcium levels (~100-200 nM) and ensure cell survival (Bush, 1995; Kudla et al., 2010). Calcium is thus accumulated in cellular stores where millimolar concentrations can be reached safely (Dodd et al., 2010; Stael et al., 2012). This energy-consuming situation has been exploited by evolution, and calcium has become used as a second messenger. Thus, in response to a given stimulus, calcium is purposely released from the stores, and this rapid and transient cytosolic increase carries a message able to trigger the appropriate downstream response. This process is supported by a complex machinery composed of Ca^{2+}-permeable channels, that allow calcium release from the stores, Ca^{2+}-binding proteins able to interact with the specific downstream partners, and calcium transporters (i.e. ATPases, antiporters, etc.) responsible for restabilising the basal calcium resting levels (Sanders et al., 1999; Sanders et al., 2002; Kudla et al., 2010).

In order to consider a Ca^{2+}-increase a real calcium signal, some rules must be respected, and they were firstly proposed by Jaffe in 1980 (Jaffe, 1980). These simple rules can be summarised as following:

- A calcium increase should occur in response to a given stimulus (free calcium levels increase), and the two events must be related in time and space.
- Preventing the calcium rise should block the downstream signal.
- Direct artificial calcium induction or a comparable increase in the Ca^{2+}-levels should activate the same response (independently from the stimulus).

1.1.1 Generation of the Ca^{2+}-signal

In order to generate a calcium signal, the ion release from calcium channels must be counteracted by the activation of efflux transporters able to rebalance the calcium basal level, hence to re-establish the calcium homeostasis. These two processes are named calcium influx and efflux and they are mediated by different Ca^{2+}-transporters. A schematic representation of these calcium transporters in an *Arabidopsis* cell is reported in figure 1.1.

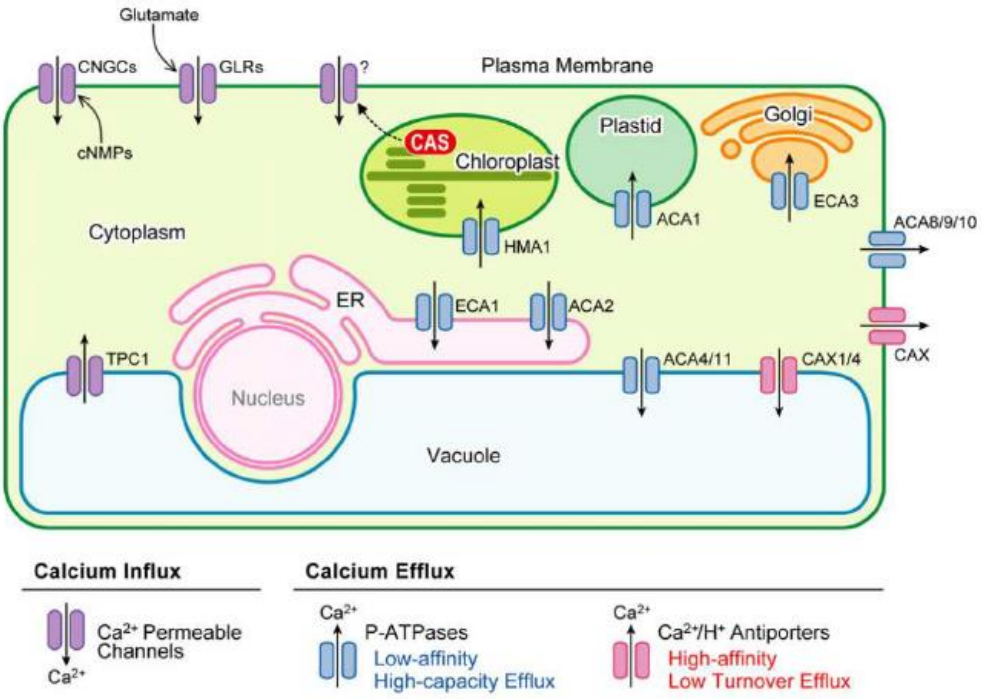


Figure 1.1 From Kudla et al., (2010). Schematic representation of the calcium transporters in an *Arabidopsis* cell. CNGC: cyclic nucleotide channel; GLR: glutamate receptor; TPC1: two pore channel 1; CaS: Ca^{2+}-sensing receptor; ACA: autoinhibited calcium ATPase; ECA: ER type calcium ATPase; HMA1: heavy metal ATPase1; CAX: cation exchanger. For detail see text.

1.1.1.1 Calcium influx

Since free calcium has to be kept low in the cytosol, this causes the generation of strong calcium gradients, as this ion is stored in high concentrations in the apoplast and in internal Ca^{2+}-stores such as the ER and the vacuole. These calcium gradients are sufficient to drive the influx of calcium to the cytosol when Ca^{2+}-channels are open (Swarbreck et al., 2013). There are different types of calcium channels. It is possible to distinguish voltage-activated Ca^{2+}-channels, which subdivide into hyperpolarization-activated calcium channels (HACCs) and depolarization-activated calcium channels

(DACCs) (White et al., 2002). Moreover, there are also voltage-independent calcium channels (VICCs) which are also considered ligand-dependent calcium channels and stretch-activated calcium channels (Cosgrove and Hedrich, 1991; White et al., 2002; White and Broadley, 2003; Dutta and Robinson, 2004; Nakagawa et al., 2007; Kudla et al., 2010). This variety of types of calcium channels enables the plants to translate a wide variety of stimuli, by using a different type of channel and by adjusting the amount of these channels in different cell or tissue types, according to the needs (Demidchik et al., 2002).

1.1.1.1.1 Voltage-dependent calcium channels

Despite the fact that the electrophysiology of HACCs and DACCs has been characterised, the molecular identity of these channels is still debated (Grabov and Blatt, 1998; Thion et al., 1998; Thuleau et al., 1998; Hamilton et al., 2000; Pei et al., 2000; Klusener et al., 2002; White et al., 2002). HACCs operate in guard cell closure, in response to ABA and blue light, plus they contribute to polar growth (Hamilton et al., 2000; Pei et al., 2000; Harada and Shimazaki, 2009; Dodd et al., 2010; Swarbreck et al., 2013), while DACCs are less characterized and their role in plant cells is assume to be linked to short transients Ca^{2+}-increases in response to stimuli like chilling and microbe interaction (Thion et al., 1998; Kudla et al., 2010). Lately there has been emerging evidence of annexins being involved in voltage-dependent and ROS-dependent calcium transport (Demidchik et al., 2002; Mortimer et al., 2008; Swarbreck et al., 2013; Davies, 2014). These ubiquitous proteins can form complexes similar to a pore, which has been demonstrated to have Ca^{2+}-transport activity in a lipid bilayer (Laohavisit et al., 2009; Laohavisit et al., 2010; Swarbreck et al., 2013).

1.1.1.1.2 Mechanosensitive calcium channels

Ca^{2+}-channels are central to mechanosensing in plants, both for developmental processes and for sensing environmental changes. Amongst those, several mechanosensitive-like

channels (MSLs) have been identified both in the plastidial membranes (MLS2 and MLS3) (Stael et al., 2012; Veley et al., 2012), and at the root plasma membranes (MLS9 and MLS10) (Haswell et al., 2008). Other known mechanosensitive channels are MCAs (Mid1-complementing activity), localised at the plasma membrane, and piezo-like proteins, able to form homomeric complexes and create calcium permeable pores in the membranes in response to membrane stretching (Monshausen and Haswell, 2013).

1.1.1.1.3 Ligand-activated calcium channels

The best characterized class of calcium channels is the ligand-activated one. Amongst these there are the cyclic nucleotide gated channels (CNGCs) and glutamate receptors (GLRs). There are twenty characterised CNGCs in *Arabidopsis* (White et al., 2002), and they are involved in cellular homeostasis of cations. These channels are activated by binding of cAMP and cGMP, and have a calmodulin-binding site, which partially overlaps with the ligand-binding site (Kudla et al., 2010). These channels have been implicated in pathogen responses (Balague et al., 2003; Yoshioka et al., 2006), heat response (Saidi et al., 2009), homeostasic regulation during salt stress adaptation (Gobert et al., 2006; Guo et al., 2008), as well as pollen tube growth (Frietsch et al., 2007). GLRs are non-selective cation channels primarily activated by glutamate and glycine, as well as by other amino acids (Qi et al., 2006; Stephens et al., 2008). GLRs are important for plant Ca^{2+} nutrition, as well as for mediating calcium responses such as the cold response (Meyerhoff et al., 2005), systemic signalling in response to wounding (Mousavi et al., 2013) and aphid feeding (Vincent et al., 2017), regulation of ABA levels and stomatal closure (Kang et al., 2004; Cho et al., 2009) and root development and light-mediated hypocotyl elongation (Brenner et al., 2000; Walch-Liu et al., 2006). Furthermore, other ligands have been identified, and they are able to induce a calcium increase in the cytosol, despite the fact that the identity of the relative Ca^{2+}-channels have not been identified yet. These are IP_3 (inositol phosphate 3), cADPR (cyclic ADP ribose) and NAAPD (nicotinic acid adenine dinucleotide phosphate) (Allen et al., 1995; Navazio et al., 2000; Kudla et al., 2010). Finally, two K^+ channels are also able to mediate Ca^{2+} fluxes, namely TPC1

(two pore channel 1) and SKOR (Shakerlike stellar K^+ outward rectifier). SKOR is a ROS-activated channel, which can mediate both a K^+ or a Ca^{2+} inward current (Garcia-Mata et al., 2010). TPC1 is a tonoplast-localised channel, which is involved in the formation of calcium waves and systemic signalling, as well as in the mediation of germination and stomatal movement, aphid signalling, jasmonate-mediated wounding and pathogen response (Peiter et al., 2005; Bonaventure et al., 2007; Bonaventure et al., 2007; Beyhl et al., 2009; Gilroy et al., 2016; Vincent et al., 2017).

1.1.1.2 Calcium efflux

Whilst calcium channels mediate the calcium influx, calcium P-type ATPases and Ca^{2+}/H^+ antiporters are responsible for the calcium efflux, which is a fundamental process as it re-establishes the calcium basal levels in the cytosol. Calcium efflux is an active process, as it goes against the calcium gradient, hence it uses up energy in the form of ATP in the ATPases and proton gradient in the antiporters (Dodd et al., 2010; Kudla et al., 2010). Despite the importance of this process, the role of readjusting the calcium concentrations to a basal level has not been widely as investigated in the terms of its physiological role in the plant. Calcium efflux mediators, as well as the calcium channels, contribute to shaping the "calcium signature". This explains why a range of different proteins mediate similar, possibly redundant functions (Sanders et al., 2002). However, a functional distinction can be made for the calcium efflux mediator proteins; indeed the antiporters are responsible to lowering the calcium levels back to the micromolar range, while the ATPases are important to maintain the nanomolar concentrations measured at the basal level (Kudla et al., 2010).

1.1.1.2.1 Calcium/proton antiporter

Amongst the calcium/proton antiporters the best characterised transporters are named CAX (cation exchangers) (Hirschi et al., 1996; Mäser et al., 2001). There are six CAX

proteins, with reported activity in the vacuole and at the plasma membrane (Kasai and Muto, 1990; Hirschi et al., 2000; Mäser et al., 2001; Cheng et al., 2005; Shigaki et al., 2006), and they have an N-terminal autoregulatory domain that controls their activity (Pittman et al., 2002). Also some Ca^{2+}/Na^{+} antiporters have been identified, and they work similarly to the Ca^{2+}/H^{+} antiporters (Shigaki et al., 2006). The presence of an antiporter activity has also been measured in the chloroplast thylakoid membrane (Ettinger et al., 1999), and the protein responsible of it has recently been identified (Wang et al., 2016).

1.1.1.2.2 Calcium P-ATPases

P-type ATPases are subdivided into three categories: ECAs (ER-type Ca^{2+} ATPases), ACAs (Autoinhibited Ca^{2+} ATPases) and HMA1 (heavy metal transporter 1) (Sze Heven et al., 2000; Kudla et al., 2010). ECAs and ACAs are P-type ATPases belonging to the second (II) subclass, P_{IIA} and P_{IIB}, respectively (Kudla et al., 2010). These ATPases are localised in the ER, Golgi, endosomes, vacuole, plastidial envelope and PM (Huang et al., 1993; Harper et al., 1998; Bonza et al., 2000; Geisler et al., 2000), hence they are able to pump calcium into every cellular store, including the apoplast. ECAs and ACAs are known to be able to pump also heavy metals, as well as HMA1, which is localised in the plastidial envelope (Stael et al., 2012; Nomura and Shiina, 2014). This may suggest that these pumps evolved from a general cell detoxification mechanism, as they are still able to pump away both heavy metals and calcium, which are toxic to the plant cell.

1.1.2 Decoding the Ca^{2+}-signal

The combination of calcium influx and calcium efflux causes cytosolic calcium spiking, but for this to be read as a message by the cell calcium decoders are needed downstream of it.

Decoding the calcium signal leads to a specific cellular response, which mainly occurs through changes in nuclear gene expression. There are several types of calcium sensors, which have been subdivided into two major categories: calcium responders and calcium relays (Sanders et al., 2002). Calcium responders have an intrinsic catalytic activity which is calcium-mediated. Hence they undergo a conformational change in response to Ca^{2+}-binding, and this triggers their activity, which in most cases is a downstream phosphorylation. The classical example is represented by the CPKs (calcium-dependent protein kinases) (Sanders et al., 2002; Kudla et al., 2010). By contrast, calcium relays only undergo a structural change in response to calcium sensing, and this conformational difference is responsible for the downstream interactions of these relays. The best example of this mechanism is the interaction between calmodulin (the calcium relay) and the downstream CAMTA (calmodulin binding transcription activation) transcription factors (Sanders et al., 2002). In addition to calmodulins (CaMs), calmodulin-like proteins (CMLs) are important calcium relays, functioning in plant development and environmental responses (McCormack et al., 2005). Hence calcium can be either translated into a protein-protein interaction or into a phosophorylation event in the downstream response.

1.1.2.1 Calcium controls protein phosphorylation

Phosphorylation is a key controlling mechanism in plant cells, imparting a negative charge which affects protein conformation, interactions and subcellular location (Hunter, 1995; Soderling, 1999; Clapham, 2007; Kudla et al., 2010). In plants, there are three major classes of calcium-dependent kinases: CPKs, CIPKs (CBL-interacting protein kinases) and CCaMKs (Ca^{2+}/CaM-dependent protein kinases) (Sanders et al., 2002; Harper et al., 2004; Das and Pandey, 2010; Kudla et al., 2010). While CPKs and CCaMKs are directly activated by calcium, the CIPKs class is regulated by CBL proteins, which are calcium relays (Kudla et al., 2010). The complex network formed by all these components lead to the variety of calcium signalling outputs observed, however, the way that this specificity is encoded is still under investigation. It is known that different

combinations of 10 CBLs and 25 CIPKs regulate different processes in a specific cellular localization, determined by the CBL moiety localization (Batistic et al., 2008). When there is an increase in cytosolic calcium, the CBL protein binds this ion and undergoes a conformational change, which leads to interaction with a CIPK protein. This CBL/CIPK complex is able to phosphorylate downstream targets, regulating the activity of them. This sort of regulation has been shown to be involved in K^+ homeostasis (Xu et al., 2006). Furthermore it controls responses to plant mineral nutrition, such as nitrate, abiotic stressed (e.g. salt stress) and hormone responses, specifically ABA (Albrecht et al., 2003; Cheong et al., 2003; Pandey et al., 2004; D'Angelo et al., 2006; Ho et al., 2009; Kudla et al., 2010). Interesting, one signalling pathway can activate more than one calcium decoder at the same time, as well as there can be different calcium microdomains at different cellular locations. This is the case for the cold response, where both external calcium and the vacuolar pool are necessary for the response (Knight et al., 1996), and calcium increases are decoded by calmodulins, CIPK/CBL and CPK, leading to the downstream response (changes in nuclear gene expression) via the activation of the CBF transcription factors (Knight and Knight, 2012).

1.1.2.2 Calcium controls transcription factors

Calcium is known to be a major regulator in controlling nuclear gene expression. The best characterised example of calcium-regulated transcription factors is represented by the CAMTA proteins, which are primarily regulated by the binding of a calmodulin, which is a calcium relay. When a calmodulin binds 4 Ca^{2+} ions (two at the C- terminal and two at the N- terminal), this can bind to a CAMTA protein, constituting the active form $4Ca^{2+}$-CaM-CAMTA, which controls downstream gene expression (Liu et al., 2015). In *Arabidopsis* there are seven different calmodulin isoforms, and six different CAMTA proteins, and their combination activate the response to environmental stimuli (Bouche et al., 2002; Yang and Poovaiah, 2002; Kudla et al., 2010; Poovaiah et al., 2013). For example they have a prominent role in the pathogen response (Du et al., 2009; Poovaiah et al., 2013; Zhang et al., 2014), and the cold response (Doherty et al., 2009). In

order to understand how a calcium signal is encoded into information leading to changes in nuclear gene expression, the interaction between Ca^{2+}-calmodulin and CAMTA has been modelled (Liu et al., 2015). One of the key properties that emerged from this model is that the relationship between the calcium concentration and amount decoced through time follows a non-linear relationship (Liu et al., 2015). This model aimed to specifically understand how the amount of $4Ca^{2+}$-CaM-CAMTA active form changes through time to a given "Ca^{2+}-signature" with the final aim of understanding how the information is encoded in the increase in calcium, and read by the cell machinery.

1.1.2.3 The role of calcium in the pathogen response

One of the Ca^{2+}-signalling mediated pathways is the plant immune response. When a pathogen is presented to a plant, some specific microbial compounds, called pathogen, damage- or microbe- associated molecular patterns (PAMPs, DAMPs and MAMPs) are sensed by the plant itself, and recognised through a process called PAMP-triggered immunity (PTI) (Jones and Dangl, 2006; Zipfel and Robatzek, 2010). This process is mainly mediated by receptor-like kinases (RLKs) and receptor-like proteins (RLPs), which are single transmembrane proteins whose activity is regulated by other protein partners. These complexes can bind PAMPs/DAMPs and there is a consequent cytosolic Ca^{2+}-flux via calcium channels (e.g. GLRs), activating downstream defence responses (Seybold et al., 2014).

In many cases pathogens are able to evade this first line of defence, as they can suppress it or pass undetected by the host (Jones and Dangl, 2006; Zipfel and Robatzek, 2010). Hence a second line of defence developed through evolution, called effector- triggered immunity (ETI), which is mediated by receptors able to specifically recognise the attacker using NB-LRR (nucleotide-binding leucine-rich repeat) receptor proteins (Jones and Dangl, 2006; Pieterse et al., 2014). In this case the receptor is pathogen-specific, in a gene-for-gene resistance manner (Dodds and Thrall, 2009).

The induction of plant immune response, hence the transcription of genes involved in plant defence and, in extreme cases, programmed cell death, are mediated by the production of salicylic acid (SA) (Jones and Dangl, 2006; Lu, 2009). This hormone hence controls the downstream responses, but what is mediating its crucial increase? Cytosolic calcium increases are a key factor in triggering this hormonal response (Du et al., 2009; Zhang et al., 2014; Zhang et al., 2014; Zipfel and Oldroyd, 2017). Indeed calcium tightly controls the levels of SA production by positively regulating the *ICS1* gene (At1g74710), which encodes for a key enzyme in the SA synthesis pathway (Seyfferth and Tsuda, 2014). *ICS1* transcription is mediated by the CBP60g transcription factor, whose activation depends on a calmodulin, which is a calcium binding protein (Wang et al., 2009). At the same time, the transcription of *EDS1* (At3g48090), whose product inhibits *ICS1* transcription, is under the control of AtSRD1/CAMTA3, which is a well known calmodulin binding protein. Hence cytosolic calcium increases finely regulate SA production by acting on different elements of the network, which are by themselves interconnected (Zhang et al., 2010; Zhang et al., 2014).

Calcium increases in response to pathogens also control, and are controlled by, levels of ROS (e.g. H_2O_2) and nitric oxide (NO), which are also produced in response to pathogens (Zhang et al., 2010; Seybold et al., 2014). Furthermore, it has been demonstrated in barley that the MLO (Mildew resistance locus o) protein, which suppresses the plant defence response to the powdery mildew fungus, has a calmodulin binding domain necessary for its function (Kim et al., 2002). Moreover, CDPKs and CBLs have been shown to be involved in the plant response to biotic stresses (Poovaiah et al., 2013; Seybold et al., 2014; Zhang et al., 2014).

Cytosolic calcium increases have been measured in response to pathogens and pathogen elicitors (Blume et al., 2000; Lecourieux et al., 2002; Manzoor et al., 2012), and a role for chloroplast and stromal calcium increases in controlling the pathogen response have emerged from recent studies (Manzoor et al., 2012; Nomura et al., 2012). This may be directly linked to the fact that SA production is initiated in the chloroplast itself (Seyfferth and Tsuda, 2014). In any case, this evidence points towards a new role for organellar calcium in regulating the plant immune response (Nomura and Shiina, 2014).

1.1.3 Encoding calcium signalling: the problem of specificity

The calcium ion is employed as a second messenger to convey information about the nature of the stimulus in many different signaling pathways. Since the beginning of calcium signaling studies, a question which has been asked is: how can a single ion encode the message specificity? In 1998, the "Ca^{2+}-signature" hypothesis was formally proposed, and according to it, the specificity is encoded in the kinetics of the calcium increase (McAinsh and Hetherington, 1998). When the hypothesis was formulated, there was already evidence of the fact that in animal cells variations in the calcium oscillations differentially affected gene expression (Dolmetsch et al., 1998). For a calcium increase to be decoded as the right signal, the kinetics in terms of peak height, amplitude, frequency, etc. are important parameters, in which information about the specificity is stored (McAinsh and Hetherington, 1998; McAinsh and Pittman, 2009).

The signature hypothesis is sustained by the fact that different stimulus-induced changes in cytosolic calcium concentrations, actually displaying different kinetics, are observed in many different cell types in response to a diverse range of abiotic and biotic stimuli. Examples include osmotic, salt and drought signals (Knight et al., 1997), oxidative stress (Rentel and Knight, 2004; Evans et al., 2005), cold (Knight et al., 1991; Knight et al., 1996), plant hormones (McAinsh, 1990; Allen et al., 2001) and pathogen elicitors (Manzoor et al., 2012; Nomura et al., 2012) and symbiotic elicitors (Oldroyd and Downie, 2006).

In plants, the most striking evidence of the role of the "Ca^{2+}-signature" comes from the study of calcium in stomatal opening and symbiosis signalling in legumes. Indeed it has been shown that changes in stomatal opening are calcium-dependent, and only the correct kinetics of the calcium oscillations is able to induce a steady-state stomatal closure (Allen et al., 2001). Similarly, the symbiosis signalling in legumes require perinuclear calcium oscillations, and the period and amplitude of these oscillations vary according to the nature of the endosymbiotic organism (inducing nodulation or mycorrhization) (Oldroyd and Downie, 2006; Kosuta et al., 2008). Examples of these signals are reported in figure 1.2. In these specific cases a calcium signature has been directly associated to a

physiological output, however in most cases, the pathway leading from the calcium increase upon stimulus perception to downstream changes is not well characterised. To further corroborate the calcium signature hypothesis, a recent study tested it by electrically inducing different calcium signatures in cells. When either the frequency or amplitude of the calcium oscillations was varied, this caused the expression of a different set of genes in *Arabidopsis* plants (Whalley and Knight, 2013), indicating that the cell is able to read different calcium signatures as different information.

The shape of the calcium signature is not the only way to encode the Ca^{2+} specificity. At the same time, the cell needs to be competent to respond to that specific signal (named "physiological address" of the cell) (McAinsh et al., 1997; McAinsh and Hetherington, 1998), meaning that the appropriate calcium signalling machinery, in term of calcium transporters and downstream Ca^{2+}-binding proteins, must be present. Hence the same kind of stimulus can trigger different calcium signatures in different plant tissues (Kiegle et al., 2000). Furthermore, the subcellular location of the calcium increase is important, as calcium can create local microdomains where millimolar [Ca^{2+}] can be reached. Hence whether the calcium moves from the apoplast rather than from the vacuolar or ER pool is considered as part of the information contributing to the specificity of the calcium signal. Another mechanism contributing to the specificity of the message is the requirement for an additional signal (e.g. second messenger) occurring in parallel to calcium. Thus calcium can be necessary, but by itself not sufficient to start the downstream response (McAinsh and Pittman, 2009).

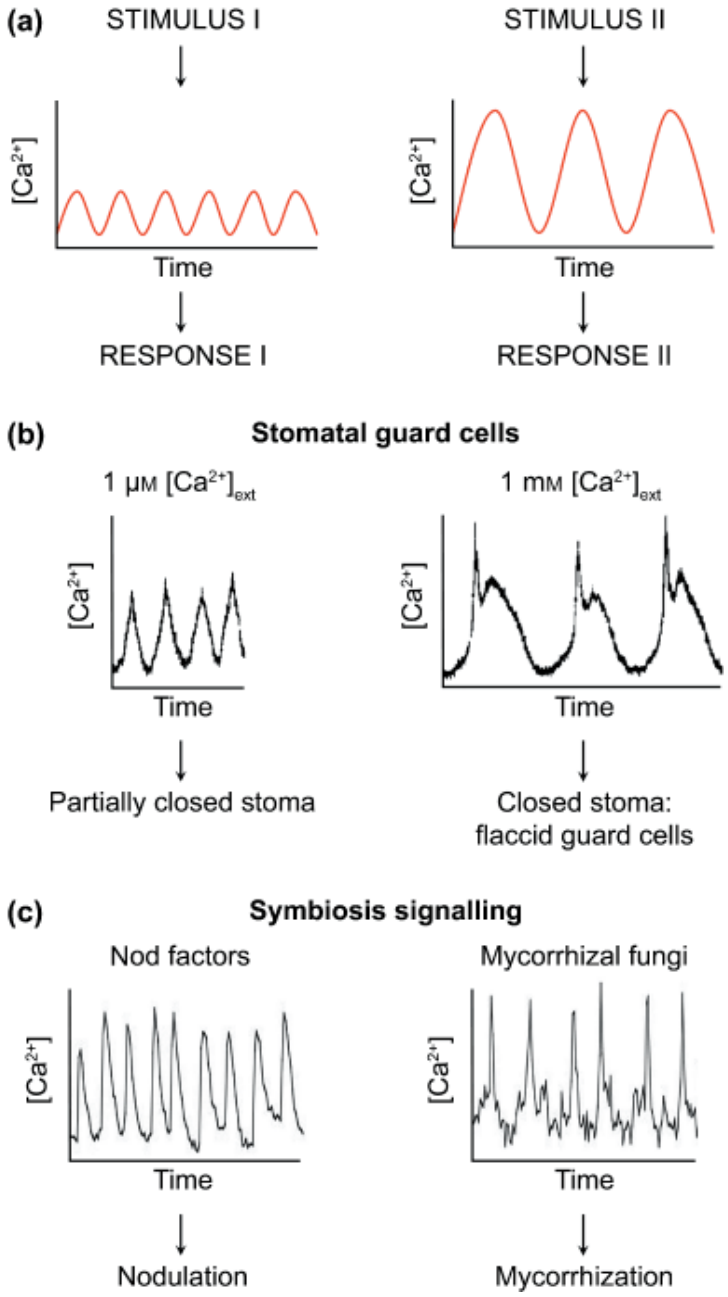


Figure 1.2 Adapted from McAinsh and Pittman, (2009). (a) schematic examples of two different "Ca^{2+}-signatures", (b) correlation of different patterns of calcium oscillations to the state of stomata closure and (c) role of the different calcium kinetics in the communication of the type of symbiotic organism in the symbiosis signalling.

1.1.4 Measuring calcium

A lot of knowledge regarding calcium signalling has been acquired thanks to the existing technologies for measuring calcium inside a cell. Different approaches are available (described below), however, there are general rules relevant for all the Ca^{2+}-probes. Indeed every probe needs to be selective for calcium, and it should form reversible complexes with it. Secondly, the physicochemical characteristics of the free and Ca^{2+}-bound form of the probe must be sufficiently different to allow their relative concentrations to be measured (Rudolf et al., 2003). When using these probes it also has to be taken into account that the presence of these extra Ca^{2+}-binding elements in the cell might have a buffering effect on the cellular calcium concentration, and this may lead to an imbalance in calcium homeostasis. These general rules are valid both for calcium dyes and for protein-based probes. Calcium fluxes can also be measured by using Ca^{2+}-specific electrodes, however this technique is the least favoured as it is invasive, difficult to perform and it only measures calcium in a very specific location of the cell. Despite this, electrodes display some advantages, such as high sensitivity, accuracy, the fact that it is possible to obtain single cell measurements, and transformation is not required. With the advent of non-invasive surface electrodes (Mousavi et al., 2014) this technique may acquire more importance in the future.

While protein-based probes are nowadays the preferred technique for calcium measurements, the field started with the usage of Ca^{2+}-sensitive dyes. In the early 1960 to 1970s the first synthetic indicators emerged, primarily murexide, azo dyes and chlorotetracycline (Tsien, 1980). Then in the late 1970s Roger Tsien designed the first calcium-specific fluorescent dye for intracellular use (Tsien, 1980), and from this a range of other probes were created based on the properties of EGTA, a well known calcium chelator (Tsien, 1980). The general rule used was that conformational changes in the carboxy groups due to calcium binding influenced the chromophore, hence the excitation/emission wavelengths are modified, and calcium concentrations can be

measured. Probes can be divided in ratiometric and non-ratiometric ones, and they are described in detail in Kanchiswamy et al., (2014) (Kanchiswamy et al., 2014). Calcium measurements with ratiometric dyes are based on a ratio between the fluorescent intensities taken at two different wavelengths. The excitation/emission properties of the probes are affected by the calcium binding, hence the ratio is affected. The advantage of ratiometric probes, such as fura-2 and indo-3, is that the amount of dye loaded does not affect the measurements, as the calcium concentration depends on a ratio and not on an absolute concentration (Rudolf et al., 2003). In non-ratiometric probes the measured calcium concentration is solely depending on changes in fluorescence intensity of a single wavelength, thus the amount of probe loaded is a critical influencing parameter. Examples of non-ratiometric probes are fluo dyes and Calcium Green-1. It has to be said that one of the main difficulty of using the calcium dyes is the loading inside the cell, specifically as plant cells are surrounded by a cell wall. These probes acquired popularity when an acetoxmethyl ester group was attached to them, facilitating the loading in living cells, which was previously performed by microinjection (Tsien, 1981).

A major innovation in the field came with the advent of protein-based probes. They derive from the coelenterate *Aequorea victoria*, and they are based either on aequorin or on GPF (green fluorescent protein). In the jellyfish aequorin is able to bind calcium present in the seawater, to change conformation and emit a photon of blue light which stimulates the GFP (Morise et al., 1974). Using their different properties (aequorin is a luminescence-based probe while GFP is a fluorescent one), both these proteins have been used as a tool to measure calcium in the cell.

The calcium-binding chemiluminescent protein aequorin (Shimomura et al., 1962) was the first protein-based calcium reporter used to measure cellular calcium concentrations (Knight et al., 1991). The inactive form apoaequorin is not *per se* able to emit light, but it needs to be bound to the coelenterazine. In this reconstituted conformation, when calcium is bound to the three Ca^{2+}-binding sites, and in presence of bound O_2, the protein undergoes a conformational change, coelenterazine is irreversibly oxidised into coelenteramide, and a photon of blue light is released, as well as CO_2 (Tsuji et al., 1986).

The advantages of using a protein is that the gene can be cloned and recombinantly expressed in a plant cell (Knight et al., 1991). Moreover, it was for the first time possible to target the probe to subcellular domains, hence to measure calcium specifically in different cell compartments (Johnson et al., 1995; Knight et al., 1996). Another advantage of aequorin is that luminescence can be recorded for a long time as it does not undergo photobleaching. The drawbacks of this calcium-measuring technique is that it requires transformation, and that the signal is too low to allow single-cell measurements. Hence aequorin is favourable for cell population experiments.

Single-cell measurements were achieved with GFP-based calcium probes. The three major types of GFP-based probes are named cameleons, camgaroos and pericams (figure 1.3). These chimeric proteins all use calmodulin as the calcium-binding part of the protein, bound to fluorescent proteins at either side of it (or two parts of the same fluorescent protein). The changes in conformation of the calmodulin due to the calcium affects the fluorescent properties of the reporter, and this is used as a proxy to measure intracellular calcium concentrations. In particular, cameleons are FRET-based probes, where the calmodulin (CaM) and the M13 myosin light chain kinase domain (represented as M13 in figure 1.3B) conformational changes bring two fluorescent proteins close enough to allow energy transfer between them, hence the emission wavelength is affected upon calcium binding (Romoser et al., 1997). These probes are ratiometric, non-invasive, and they can be targeted to subcellular compartments. Camgaroos probes were build in a way that they only have one fluorescent protein, and the fluorescence levels are affected by the binding of calcium to the CaM. Indeed the CaM is inserted into the YFP (yellow fluorescent protein) sequence, in this way calcium binding mimics alkalisation, affecting the YFP fluorescent properties (Rudolf et al., 2003). Pericams are, conversely, a circular permutated version of YFP, where the fluorescent protein is put between CaM and the M13 domain of the myosin light chain kinase (Whitaker, 2010). Innovations on the calcium sensing mechanisms (e.g. troponin-based probes), mutated versions of the fluorescent proteins (e.g. brighter YFP) and BRET (bioluminescence resonance energy transfer) approaches combining aequorin luminescence with GFP fluorescence (Baubet et al., 2000; Rogers et al., 2005; Xiong et al., 2014) have advanced the field fluorescent

calcium probes in the last years, and it is still quickly improving (Whitaker, 2010; Matsuda et al., 2013; Perez Koldenkova and Nagai, 2013; Kanchiswamy et al., 2014). In general, these fluorescent-based approaches require expensive instrumentation to be measured and the dynamic range of calcium is relatively limited, plus they are subject to photobleaching.

The choice of the calcium measurement technique must be related to the biological question asked and the requirements of the experimental setup. All these protein-based probes work on the range of 10^{-5}-10^{-7} [Ca^{2+}], which is compatible to the concentrations present in the cell. However, not every cellular compartment can be easily targeted, as in certain organelles, such as ER and vacuole, calcium concentrations are too high to be compatible with calcium measurements (e.g. aequorin luminescence would be completely discharged as soon as it reconstitutes with coelenterazine, hence a modified version of coelenterazine or aequorin has to be used). Furthermore, proteins are affected by changes in the cellular environment such as temperature and pH, and this needs to be taken into account to accurately measure calcium concentrations.

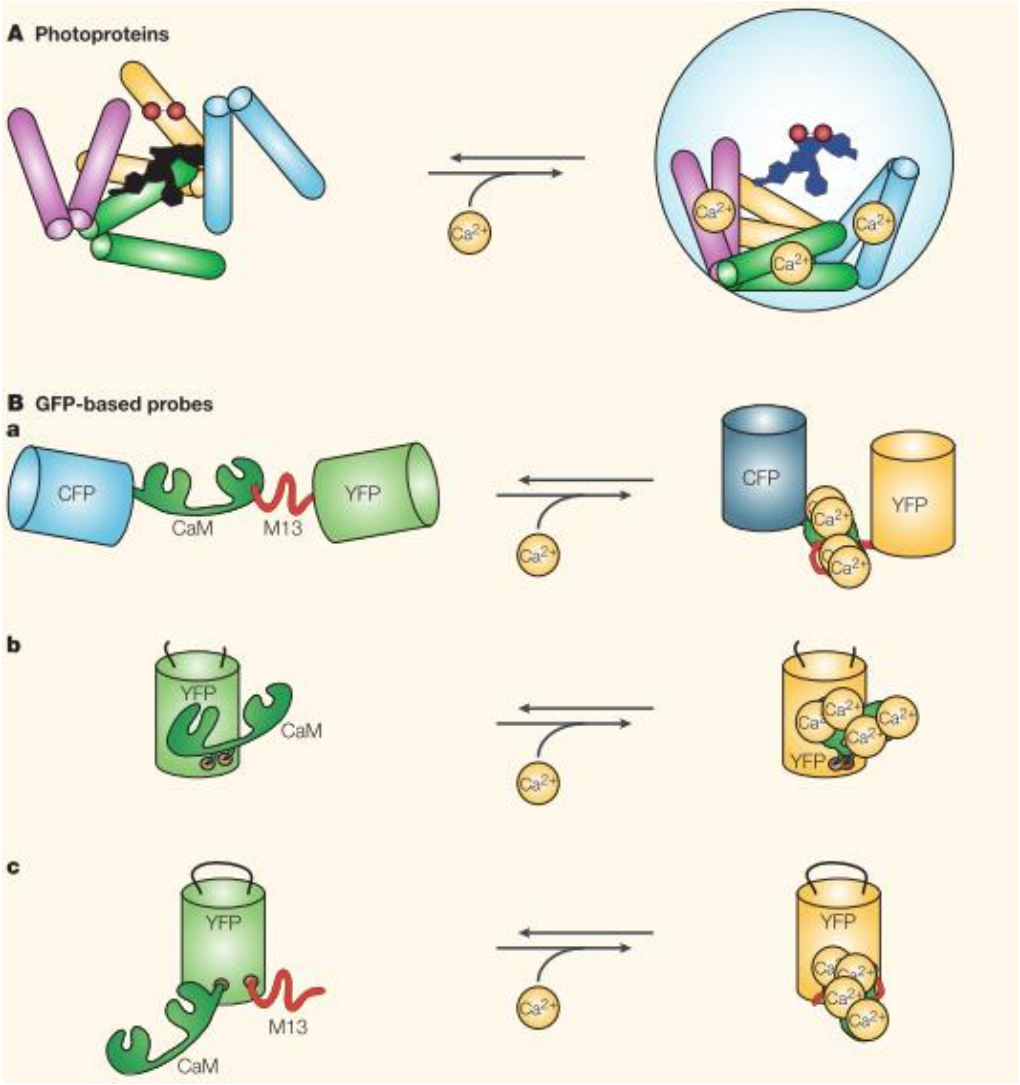


Figure 1.3 From Rudolf et al., (2003). Representation of the different types of protein-based calcium probes. (A) the photoprotein aequorin, (B) GFP-based probes (a) cameleon, (b) camgaroo, (c) pericam.

1.2 Heat sensing

1.2.1 Heat sensing in plants

Temperature is a key environmental cue which strongly affects plant metabolism, development and growth. Land plants are often exposed, daily and seasonally, to temperature fluctuations, as well as to relatively more rapid and unexpected changes, as when clouds shield the sun's heat. Due to the impact on their metabolism, plants have evolved to be able to sense even small temperature fluctuations, anticipate them when possible, and adjust their physiology accordingly (Ruelland and Zachowski, 2010; Saidi et al., 2011; Knight and Knight, 2012; Mittler et al., 2012). The ability to discriminate a cooling from a heating event, as well as the magnitude of it (e.g. chilling and freezing), is essential for survival (Penfield, 2008; Hua, 2009; Thomashow, 2010; Knight and Knight, 2012), especially for sessile organisms like plants, which cannot flee unfavourable temperature conditions.

Despite the major interest in the topic, the mechanisms for temperature sensing, specifically the early events, are still under debate, as the specific thermometers for heat and cold have not been yet identified.
Several classes of biological processes are known to be involved in temperature sensing, as they have the ability to be directly affected by a temperature change, and there is evidence for them to directly control temperature-related downstream responses (Ruelland and Zachowski, 2010). These different mechanisms are represented in figure 1.4, and I will focus on describing the heat response, but analogous mechanisms, but in the opposite direction, are involved in cold responses (e.g. membranes are fluidised by heat, whilst they become more rigid in response to cold).

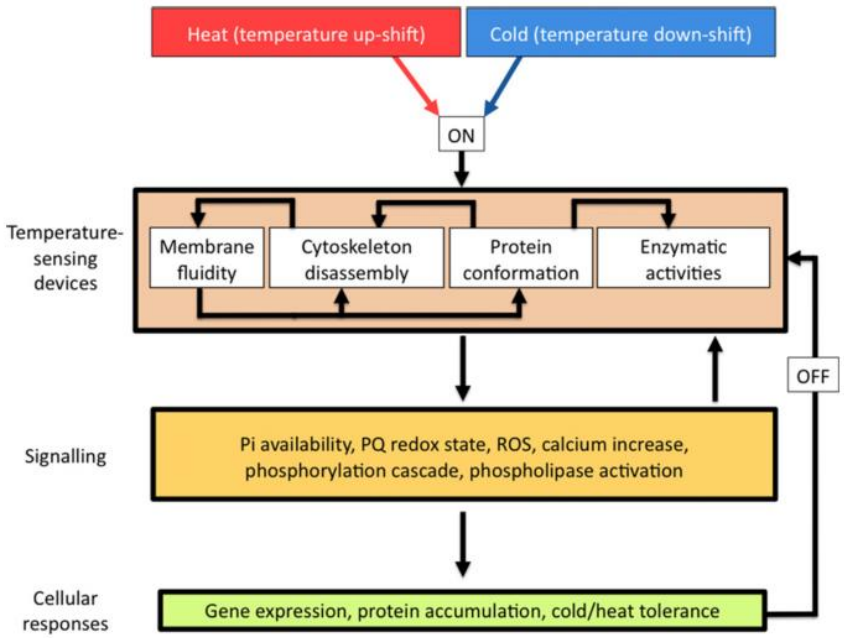


Figure 1.4 From Ruelland and Zachowski (2010). Schematic representation of the mechanisms of temperature sensing and the cellular responses to heat and cold.

These processes are: changes in membrane fluidity, protein unfolding, changes in the catalytic activity of enzymes, cytoskeleton disassembly, and chromatin remodelling (Kumar and Wigge, 2010; Ruelland and Zachowski, 2010; Saidi et al., 2011; Knight and Knight, 2012; Mittler et al., 2012). Furthermore, some of these processes are interconnected.

Temperature upshifts can lead to protein unfolding, and this has for a long time been considered the primary heat-sensing mechanism (Pastore et al., 2007; Voellmy and Boellmann, 2007). It has been shown that heat-induced protein denaturation (e.g. HSP90 denaturation) can participate in the activation of heat stress transcription factors, which are generally maintained in an inactive state in the cell (Yamada et al., 2007). Furthermore, protein unfolding can cause oligomerisation of a thioredoxin and a

thiorexodin-like protein in *Arabidopsis*, and the oligomer has been shown to act as a molecular chaperone (Lee et al., 2009; Park et al., 2009). Interestingly, the activity of the plastidial FAD8 (fatty acid desaturase 8) is reduced at high temperatures (above 27ºC), due to a post-translational heat-induced destabilization of this protein, which causes an indirect saturation of membrane lipids (Matsuda et al., 2005).

As well as protein unfolding, heat is responsible for cytoskeleton disassembly. A temperature increase from ambient to high (42-50ºC) temperature caused microtubule and actin filament disassembly in tobacco cells and *Arabidopsis* plants (Smertenko et al., 1997; Muller et al., 2007; Malerba et al., 2010). Furthermore, is has been shown that accumulation of HSP70 is dependent on cytoskeleton disassembly (Suri and Dhindsa, 2008), hence this temperature-sensing mechanism is required for downstream responses.

Another potential molecular heat-sensing mechanism relies on changes of enzymatic activity. Every metabolic pathway is composed of enzymes, whose activities are temperature-dependent. Specifically, each enzyme has a unique Q_{10}, which is a value representing the activity increase when temperature is shifter upwards of 10 degrees. As each Q_{10} is different for each enzyme, a temperature shift can unbalance the different stages of a metabolic pathway, in terms of creating a new bottleneck, or accumulation of intermediate compounds, which can trigger a downstream response (Ruelland and Zachowski, 2010). This is the case for the photosynthetic pathway, for example, where high temperatures inhibit RUBISCO activity (Salvucci and Crafts-Brandner, 2004), which limits CO_2 fixation, favouring ROS formation (Guy et al., 2008; Hideg et al., 2008; Takahashi and Murata, 2008). This is due to an imbalance in the electron transport chain, affecting the redox state of the plastoquinone pool (Zhang and Sharkey, 2009). ROS and the redox state are then able to trigger downstream responses, and they are known to be involved in retrograde signalling to the nucleus (Leister, 2005; Sun and Guo, 2016).

A more recent mechanism has been identified in the state of the structure of the DNA and RNA. DNA can be more or less accessible, and specifically, the alternative histone H2A.Z wraps DNA more tightly, and the occupancy of H2A.Z decreases with temperature (Kumar and Wigge, 2010), while unfolded mRNA can be more often

detected upon heating, but this is still an open research topic (Ruelland and Zachowski, 2010; Mittler et al., 2012).

The fastest change occurring in a cell in response to heat is membrane fluidisation, which is nearly concomitant with the temperature change, hence this event is likely upstream of all the others. It has been shown that changes in membrane fluidity are responsible for the activation HAMPK (heat-activated MAP kinase) (Sangwan et al., 2002) in plants. Additionally, long-term membrane fluidity modification, where the membrane composition is altered, are also used by plants to acclimate to different temperatures (Murata and Los, 1997; Falcone et al., 2004). Furthermore, as already mentioned, FAD8 is heat-sensitive, causing long-term changes in membrane fluidity after a heat event (Matsuda et al., 2005).

All these different sensing events contribute to the activation of the downstream responses, such as enhancing thermotolerance. These events mostly lead to changes in nuclear gene expression, as represented in figure 1.4.

1.2.2 Heat sensing in mammals

Whilst the primary mechanisms for temperature sensing in plants are still open to discussion, specific channels have been identified in mammals, which are able to read temperature. The mammalian channels are able to detect a wide range of temperatures, indeed TRPA1 is activated by low temperatures (<17ºC, which is considered noxious cold), while TRPV1 and TRPV2 activated by noxious heat (>42ºC and > 52ºC, respectively) (McKemy, 2007; Belvisi et al., 2011). The whole temperature range compatible with life is covered by channels sensing a specific narrow range of temperature (figure 1.5) and the presence of these fine-tuned systems allow mammals to distinguish a pleasant heat from noxious heat, for example (McKemy, 2007).

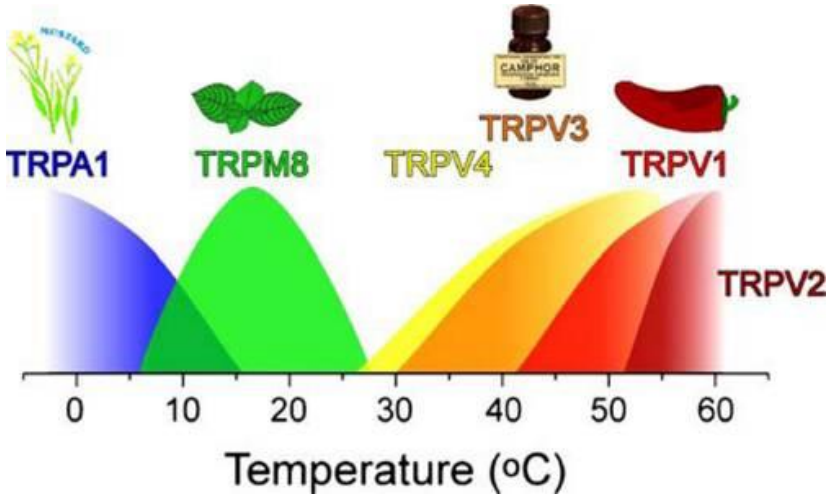


Figure 1.5 From McKemy (2007). Mammalian TRP ion channels and the range of temperature covered according to their *in vitro* properties.

The identification of these channels relied on the fact that some plant-derived compounds, such as the active ingredient of chilli pepper (capsaicin) and menthol were able to mimic the distinct sensation of heat and cold, respectively (Jordt et al., 2003). Indeed, receptor targets of these compounds were identified: TRPV1 (transient receptor potential cation channel subfamily V member 1) for capsaicin (Caterina et al., 1997) and TRPM8 (transient receptor potential cation channel subfamily M member 8) for menthol (McKemy et al., 2002), and found to be involved in temperature sensing.

TRPV1 is considered a noxious heat receptor, and it was the first one to be identified (Caterina et al., 1997). This channel is able to open in response to heat and capsaicin, and it causes a transient cytosolic calcium increase responsible for the activation of the noxious response (Caterina et al., 1997). Interesting, if the TRPV1 channel is consecutively stimulated with either of these two agonists, there is an attenuation of the Ca^{2+}-signal, hence a desensitisation of the channel (Caterina et al., 1997). Furthermore this Ca^{2+} increase can be prevented by adding the capsaicin analogue capsazepine, or the Ca^{2+} channel inhibitor ruthenium red before stimulation (Caterina et al., 1997). It was

further demonstrated that this channel is responsible for the response to noxious pain in mammals (Caterina et al., 2000).

After the identification of the mammalian heat channel, it was then possible to ask questions about the mechanism of heat-induction. To do so, purified TRPV1 channel was expressed in artificial liposome membranes, whose curvature could be controlled. From this work Cao and colleagues concluded that, since the curvature of the membrane had no effect of the TRPV1 function, then this channel must be intrinsically heat sensitive (Cao et al., 2013). Furthermore, they showed that TRPV1 in negatively regulated by a broad range of phosphoinositide species, such as PI, PI3P, PI4P, PI5P and PIP2, and that the determinant of channel sensitivity for phosphoinositides is located at the C-terminus (Cao et al., 2013).

Despite this evidence, there is some literature reporting a role of changes in membrane fluidity in mammalian temperature sensing processes. For example, the membrane fluidiser BA is able to initiate the heat shock protein response in mammals (Balogh et al., 2005), and BA is used to mimic the effect of heat on membranes.

Hence, despite the nature of the channels is clear, work still need to be performed to clarify the mechanism of action of the various temperature-sensing channels in mammals, and more than one molecular mechanism may be acting at the same time (Torok et al., 2014).

1.3 The chloroplast

The chloroplast is the characteristic organelle of photosynthetic organisms, such as plants, mosses and algae. In some exceptional cases chloroplasts have been found in a small number of animals, which process sunlight through symbiosis with algae. The best characterised case is represented by the sea snail *Elysia chlorotica,* which acquires its chloroplasts by ingesting algae (*Vaucheria litorea)* and sequestering the organelles in its intestine (Mujer et al., 1996; Rumpho et al., 2008). Similarly, there are reported cases of protists containing chloroplasts, such as the single cell flagellate *Euglena* (Cramer and Myers, 1952). In these cases autotrophy mediated by chloroplasts is complemented by heterotropic feeding, depending on the food source availability. A further example comes from corals, where the cnidarian partner provides protection with its calcium carbonate skeleton, as well as inorganic nitrogen, phosphorous and carbon, while the dinoflagellate symbiont provides photosynthetically fixed carbon. The limitation of having chloroplasts for organisms is that they have to maintain a transparent surface to allow light to reach the chloroplasts in order to get an advantage from these organelles.

The chloroplast is a highly compartmentalised structure, indeed it is surrounded by two membranes, the outer envelope and the inner envelope. Between these two the inter membrane space is located, and these two membranes form a boundary to the stroma (Taiz, L. and Zeiger, E.). Further compartmentalisation is present, as chloroplasts host thylakoids, and the thylakoid membranes separate the stroma from the thylakoid lumen (Soll, 2002). This last compartment, and specifically the membrane itself, is where the light phase of photosynthesis occurs, as the electron transport chain components are located in the thylakoid membranes (figure 1.6). The thylakoids are further subdivided in two different structures, called grana and stromal lamellae, and the photosynthetic components are preferentially located in either one or the other of these two structures (Armond and Arntzen, 1977). Indeed PSI is found in the stromal lamellae, while PSII is mostly present in the grana (Armond and Arntzen, 1977; Melis and Brown, 1980; Albertsson, 2001).

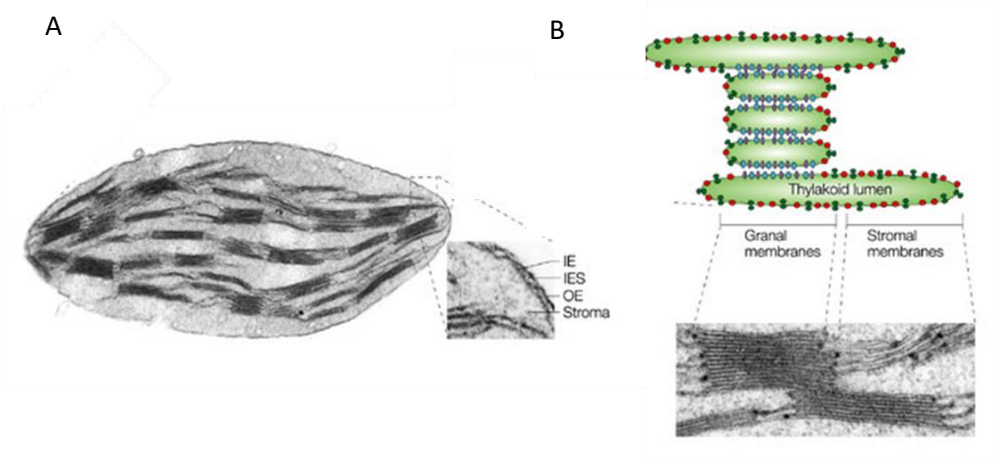


Figure 1.6 Modified from Soll and Schleiff, (2004). Chloroplast structure. (A) Electron microscope image of a chloroplast, where the ultrastructure is visible. IE: inner envelope; IES: intermembrane space; OE: outer envelope. (B) Schematic representation and ultrastructure of the thylakoids, grana and stromal lamellae are shown.

This differential spatial location creates a partial decoupling of the activity of PSII from PSI and *vice versa* (Taiz, L. and Zeiger, E.). This decoupling is convenient as the amount of energy going through either PSII or PSI can be redistributed according to the light/temperature conditions. Indeed in excess light the plant undergoes a process called "state transition" in which the major light antenna complex LHCII (light harvesting complex II) is reversibly phosphorylated, and this causes its movement from PSII to PSI (Haldrup et al., 2001; Bellafiore et al., 2005). This phosphorylation is mediated by the plastidial kinase STN7 (Bellafiore et al., 2005). While the light phase of photosynthesis relies on the spatial distribution of proteins in the thylakoid membranes, the dark phase takes place in the stroma and is mediated by the Rubisco enzyme. This enzyme is able to assimilate both inorganic carbon in the form of CO_2 as well as O_2 (photorespiration) (Taiz, L. and Zeiger, E. Plant physiology. 3rd edition). Moreover it is sensitive to temperature changes and CO_2 levels (Bowes, 1991; Sharkey, 2005) (for details see paragraph 1.3.3 Heat and the chloroplast). Because of its intimate connection with light and the myriad of enzymatic processes taking place in an ordered way in different

subcompartments, chloroplasts are highly sensitive to changes in environmental conditions.

1.3.1 Chloroplast functions

Chloroplasts are defined as the photosynthetic organelle. However, these organelles are capable of performing several other functions which are fundamental in maintaining plant homeostasis. These processes are sulphate and nitrate assimilation (Davidian and Kopriva, 2010; Jensen and Leister, 2014), synthesis of amino acids, fatty acids, carotenoids and chlorophyll (Jensen and Leister, 2014; Bobik and Burch-Smith, 2015). Chloroplasts are also involved in the early steps of synthesis of phytohormones such as SA, ABA and JA (Milborrow, 2001; Turner et al., 2002; Wasternack, 2007; Dempsey et al., 2011; Finkelstein, 2013; Serrano et al., 2013; Bobik and Burch-Smith, 2015). Furthermore, transporters of the hormone precursors/hormones present on the chloroplast membranes, such as EDS5 for SA, are able to control the release of these compounds, creating a regulatory step in the hormone synthesis pathway (Serrano et al., 2013). The production of these compounds and hormones are necessary for basic plant metabolism as well as the control of plant interaction with its environment. Indeed they function in the plant response to biotic stresses like pathogens (mainly SA-mediated) and herbivore-induced wounding (JA-mediated) (Bonaventure et al., 2007; Glauser et al., 2008; Zipfel and Oldroyd, 2017), and they mediate abiotic stress responses such as heat and drought (ABA,SA) (Larkindale and Knight, 2002; Boyce et al., 2003; Finkelstein, 2013). Chloroplasts also directly signal to the nucleus (retrograde signalling) using a multitude of compounds such as ROS, protophorphyrin IX redox state etc. (Sun and Guo, 2016), however the ways in which this signalling is mediated has still many open questions. In addition to all these roles, chloroplasts also mediate their own genome transcription, replication and segregation as well as RNA processing and editing. Hence whilst studying chloroplasts it should always be taken into account that they are responsible for a wide variety of processes and they are responsible for regulating the plant metabolism in several different ways (Pfannschmidt and Yang, 2012).

1.3.2 The chloroplast genome

Despite its relatively small size (120-160 kb) the chloroplast genome, named plastome, constitutes a significant fraction of the total cellular DNA, because of its high number of copies per plastid and the significant number of these organelles per cell (Bock, 2007). In the mesophyll of green leaves the range is between 2000 and 50000 copies of plastome per cell (Kuroiwa et al., 1982; Bendich, 1987; Coleman and Nerozzi, 1999). These copies are organised into spherical structures containing 10-20 plastomes each, named nucleoids (Kuroiwa, 1991), represented in figure 1.7, each being bound to the thylakoid membrane thanks to thylakoid membrane anchoring proteins (Sato, 2001; Sato and Ohta, 2001; Sato et al., 2003; Krupinska et al., 2013). Another structural feature is the presence of sub-nucleoid domains; indeed there is a dense central body composed of DNA and proteins, surrounded by a more loose external layer of DNA and a different set of proteins, and these two different layers have been compared to heterchromatin and euchromatin, respectively (Briat et al., 1982; Hansmann et al., 1985; Sato et al., 2003; Sakai et al., 2004; Krupinska et al., 2013). Nucleoids contain all the enzymes necessary for transcription, replication and segregation of the plastid genome (Krupinska et al., 2013). In addition, mRNA processing and editing, as well as ribosome assembly, take place in association with the nucleoid, suggesting that these processes occur co-transcriptionally. Furthermore, the location of nucleoid bound to the thylakoid membranes favour a rapid sensing of changing conditions in the photosynthetic activity (e.g. redox changes of the plastoquinone pool), reflecting the environmental changes (Pfannschmidt et al., 2009).

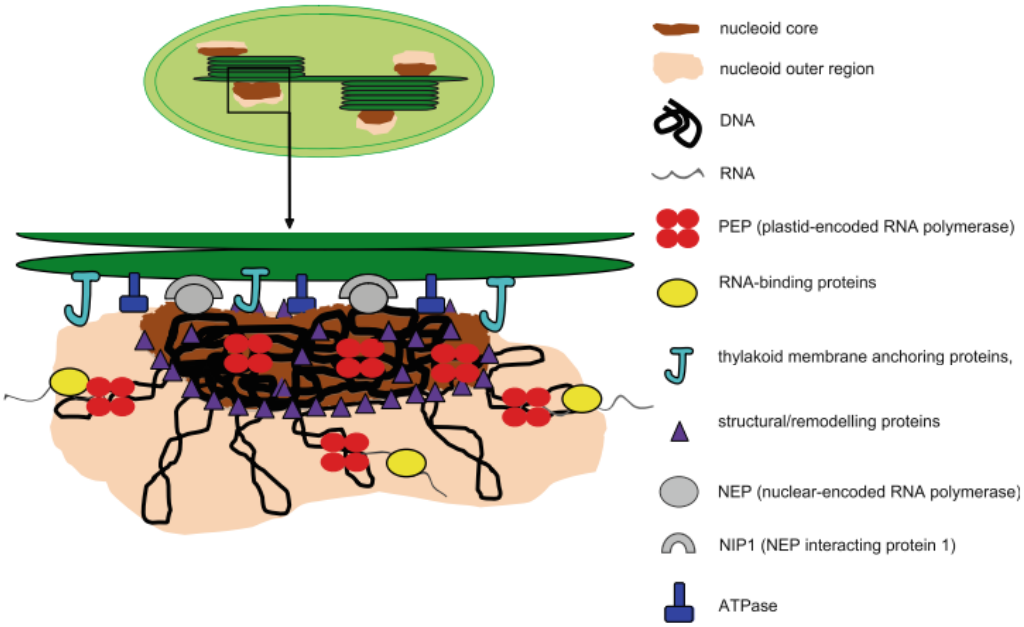


Figure 1.7 Modified from Krupinska et al., (2013). Schematic representation of a nucleoid structure. For details see text.

The chloroplast genome structure is very plastic; it is generally defined as a circular molecule of double stranded DNA, although it has been found in linear conformation and in multiple linked copies, named multimers (Lilly et al., 2001; Oldenburg and Bendich, 2004; Bock, 2007). Each DNA molecule can be divided into four distinctive areas which are a large single copy region (LSC), which is separated from the small single copy region (SSC) by the two inverted repeat regions (IR_A and IR_B) which are identical sequences with opposite orientation, as shown in figure 1.8 (Sato et al., 1999; Bock, 2007).

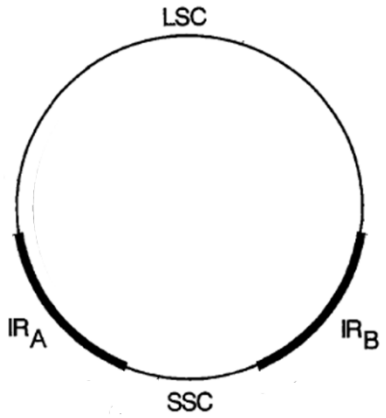


Figure 1. 8 From Khan et al., (2010). Schematic representation of the organization of one copy of the chloroplast genone. LSC: large single copy region; SSC: small single copy region; IR: inverted repeat.

The plastome is gene-dense, with about 80-150 genes in its 120-160 kb genome. Genes can be mainly divided into three groups according to their function (Barkan, 2011). The smallest class of genes is constituted by highly conserved ORF name ycf (hypothetical chloroplast reading frame), involved in various functions e.g. chlorophyll biosynthesis (Peter et al., 2011). A second group consists of photosynthetic genes. Although the chloroplast photosynthetic apparatus derives from the original cyanobacterium ancestor, and most of the proteins are still localised in the plastome, none of the complexes involved in the main activity of this organelle is entirely composed by chloroplast-encoded subunits (Bock, 2007). Thus, cross-talk between plastome and nuclear genome is required to supply the right protein subunit in the correct stoichiometric amount. The classic example is the Rubisco enzyme, which is involved in the Calvin-Benson cycle and it is the most abundant protein in plant cells; its correct assembly requires nuclear encoded small subunits (RbcS) and plastidial large subunits (RbcL) in a 1:1 ratio (8 RbcL and 8 RbcS) (Wostrikoff and Stern, 2007). The same kind of relationship between the nucleus and the plastome is seen in the third group of genes, which are involved in the gene expression machinery (Bock, 2007; Barkan, 2011). This is the largest group of

genes and includes tRNA, rRNA, ribosomal proteins and others. These components are responsible of transcribing and translating the plastidial DNA inside the chloroplast itself, hence the chloroplast is able to transcribe, translate and replicate its own genetic material autonomously. An interesting member is represented by the plastid-encoded RNA polymerase: PEP (Shiina et al., 2005; Lerbs-Mache, 2011). This *E. coli*-like RNA polymerase recognises PEP promoters which contain eubacterial-like consensus sequences (-35 and -10 sequences), moreover it promotes the transcription of operons (Bock, 2007). Even if the prokaryotic organisation of the genome seems to be conserved, the nuclear intervention is required both for promoter recognition (through 6 σ-factors) (Liere et al., 2011), and for the synthesis of the PEP protein. In fact, 3 out of 4 subunits of PEP are part of the rpoBC operon. These subunits are named a, b, b′ and b″, and they are encoded by *RPOA, RPOB, RPOC*1 and *RPOC2*. The rpoBC operon is under the control of a NEP promoter, where NEP is a nuclear encoded plastid RNA polymerase (Ellis and Hartley, 1971). Most of the genes in the plastome are under the control of multiple promoters (PEP and NEP promoters) and they seem to be activated at different developmental stages (Swiatecka-Hagenbruch et al., 2007; Zoschke et al., 2007; Lerbs-Mache, 2011). However, a division in labour can be made between the two transcription systems: PEP-transcribed genes are mostly linked to photosynthesis, while NEP-transcribed genes cover more the housekeeping functions, such as encoding for parts of the transcription machinery (Emanuel et al., 2004). This evidence suggests that there is an interplay between the nuclear gene expression and the plastidial one, and they synergistically act to ensure the correct functioning of the plastid itself. Furthermore, PEP sigma-factors modifications (e.g. through phosphorylation) are responsible for changes in the plastome transcription (Tiller and Link, 1993; Baginsky et al., 1997).

Despite the relative large amount of knowledge about the chloroplast genome and its components, the way the transcription and translation are regulated is still partially an open question. It is thought that the plastidial location of these genes is linked to their specific functions, and this may give some insight on the regulation mechanisms. Indeed, even though most of the plastidial genome has been transferred to the nuclear

compartment during evolution following the first endosymbiotic event (endosymbiotic gene transfer), part of the genome is preserved inside the chloroplast. Therefore there must be a selective pressure favouring the conservation of specific genes in the organelle (Timmis *et al.*, 2004). At the moment the most credible hypothesis to explain why certain genes remain in the chloroplast is the CORR regulation (co-location of genes and gene products for redox regulation of gene expression; Allen, 1993; Pfannschmidt *et al.*, 1999; Timmis *et al.*, 2004). If this hypothesis is correct, the localisation of the genome in proximity to the electron transport chain would favour a quick response to changes in the redox state of the chloroplast environment (e.g. alteration in the reduction levels of the plastoquinone pool; Pfannschmidt *et al.*, 1999), while the plastomes localise in the proximity of the inner membrane would be able sense stimuli coming from the cytosol. The other hypothesis is that hydrophobic proteins are imported into the organelle with difficulties, hence it is convenient for the chloroplast to produce them in the organelle itself. (Timmis et al., 2004). In any case, the chloroplast preserves part of its own genome (further explained in the paragraph X), and despite its partial similarity with its cyanobacterium ancestor, evolutionarily it has evolved independently, hence it has its own functions and regulation.

1.3.3 Heat and the chloroplast

Temperature shifts strongly affect chloroplast activity. Exposure to sub- or super-optimal temperatures can irreversibly damage photosynthesis, leading to photoinhibition (Berry and Bjorkman, 1980; Mathur et al., 2014). Indeed photosynthesis is one of the first amongst the plant cell functions to be inhibited by excessive heat, and specifically the most sensitive part is PSII (Berry and Björkman, 1980; Aro et al., 1993; Tikkanen et al., 2012).

High temperature stress is often associated with high light intensity stress, which increases the chances of photoinhibiton and ROS production. Photoinhbition occurs when there is an overload of energy on the photosystems, and there is an excessive amount of energy that cannot be completely transferred photochemically because of lack of $NADP^+$

as the final acceptor (Murata et al., 2007; Mathur et al., 2014). The unused energy is transferred to the nearest pigments and amino acids or it interacts with atmospheric triplet oxygen, forming singlet oxygen, thus damaging the photosystems (Mathur et al., 2014). PSII is the most heat-sensitive complex, for several reasons. One is that it contains the D1 protein, which, when damaged, takes a long time to get repaired or substituted, thus impairing photosynthesis (Santarius, 1976; Santarius and Muller, 1979; Berry and Bjorkman, 1980; Enami et al., 1994; Nijo et al., 2011). Another heat-induced problem of PSII is the degradation of the oxygen evolving complex (Enami et al., 1994). It is interesting to note that PSII is damaged at relatively high temperatures (> 45^{o}C), whilst moderate heat stress affects reactions downstream of PSII (Sharkey, 2005). Furthermore, PSII stability is affected by any degree of heating because of the fast changes in the thylakoid membrane fluidity which causes dislodging of PSII complexes (Takeuchi and Thornber, 1994; Havaux et al., 1996; Mathur et al., 2014). Changes in the structure of the thylakoid membranes upon heating were observed (Armond et al., 1980; Gounaris et al., 1984), and this has been associated with zeaxathin production, which counteracts the increase in fluidity and leakiness of the thylakoid membranes (Havaux and Gruszecki, 1993; Havaux et al., 1996).

Mild heat is also responsible for the increasing the cyclic electron flow (CEF) around PSI, which is more thermotolerant than PSII (Havaux et al., 1996; Bukhov et al., 1998; Bukhov and Carpentier, 2000; Bukhov et al., 2000; Bukhov et al., 2001; Egorova and Bukhov, 2002; Sharkey, 2005). This process involves PSI, cytb6f complex, one ferredoxin and one plastocyanin and it is still able to produce ATP, even if less efficiently than the linear electron flow (Joliot and Joliot, 2002).

State transition is another mechanism that helps the photosynthetic process to cope with an increase in temperature. In this case, the antenna complex of PSII, namely light harvesting complex II (LHCII), is phosphorylated under heat stress, and this causes a physical movement of this antennae towards PSI (Haldrup et al., 2001; Nellaepalli et al., 2011). In *Arabidopsis* this phosphorylation is dependent upon the STN7 kinase causing

movement of LHCII towards the stroma lamellae of the thylakoid membranes, thus enhancing absorption and emission around PSI (Chow et al., 1991; Allen, 1992; Nellaepalli et al., 2011; Mathur et al., 2014).

Virtually every component of the electron transport chain is affected by heat. Indeed, the plastoquinone pool is reduced upon heating, and this reducing power is efficiently used for the scavenging of singlet oxygen (Kruk and Trebst, 2008). Moreover the chlorophyll biosynthesis is impaired (Dutta et al., 2009). Another component affected is the usage of the energy captured from the sun, indeed some of the excess light is dissipated through NPQ (non photochemical quenching) (Müller et al., 2001).

Furthermore, heat increases the photorespiratory activity of Rubisco, as well as the ratio between dark respiration and photosynthesis (Jordan and Ogren, 1984; Salvucci and Crafts-Brandner, 2004; Mathur et al., 2014). Rubisco is the enzyme in charge of CO_2 fixation, however it is a peculiar enzyme since it is able to catalyse both photosynthesis and photorespiration (it has both carboxylase and oxygenase activities). When the temperature increases, the solubility of CO_2 decreases more than the solubility of O_2 (CO_2/O_2 ratio is affected), hence less CO_2 and more O_2 is available, proportionally.

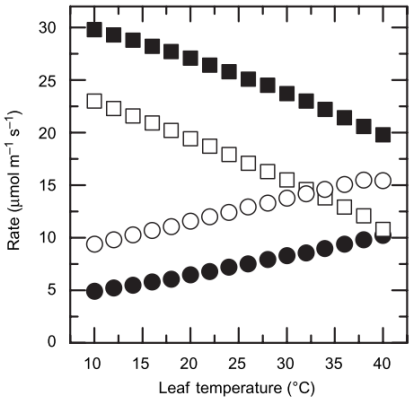


Figure 1.9 from Shakey et al., (2005). Rates of CO_2 assimilation (squares) and oxygenation (circles) of Rubisco were modelled as a function of temperature. Open symbols (white) are data assuming 190 p.p.m. CO_2., and filled symbols (black) are data assuming 370 p.p.m. CO_2.

Moreover, Rubisco affinity for CO_2 decreases with increasing temperatures, and these two factors together favour photorespiration (represented in figure 1.9) (Laing et al., 1974; Berry and Bjorkman, 1980; Monson et al., 1982; Jordan and Ogren, 1984; Salvucci and Crafts-Brandner, 2004; Sharkey, 2005).

Higher temperatures are perceived by plants as a problem even when photosynthesis is not occurring. Indeed, the amount of starch accumulated by plants to survive the night period takes into consideration a non-stressed rate of energy consumption (Graf and Smith, 2011; Scialdone and Howard, 2015). When metabolic activities are increased by heat, then plants experience energetic stress throughout the dark period and they initiate processes such as autophagy and protein degradation in order to survive (Caldana et al., 2011).

Not only photosynthesis is affected in the chloroplast by increasing temperature. Several signallling molecules such as ROS, NO, and Mg-protoX have been identified upon chloroplast heat sensing, and for this evidence of retrograde signalling to the nucleus have been reported (Pogson et al.; Sun and Guo, 2016). From the nucleus there is a production of proteins to improve the chloroplast heat tolerance such as heat shock proteins (e.g. Hsp70) and antioxidants such as ascorbate peroxidises that aim to improve chloroplasts heat tolerance (Horemans et al., 2000; Lee et al., 2007). Furthermore, upon heating, swelling of chloroplasts have been reported, as well as plastoglobule formation, which are lipoprotein particles inside the chloroplasts (Austin et al., 2006; Brehelin et al., 2007; Zhang et al., 2010). Hence heat causes both a functional and a physical rearrangement of chloroplasts, affecting primarily photosynthesis. Due to its characteristics and functions, the chloroplast organelle is considered a heat-sensitive target in the plant cell.

1.3.4 Calcium and the chloroplast

Recently, attention has been focused on understanding calcium signalling in chloroplasts. This endosymbiotic organelle is known to act as a calcium store (Roh et al., 1998;

Nomura and Shiina, 2014), and at the same time its Ca^{2+} levels are tightly regulated to allow biological processes to take place. Moreover, chloroplasts revealed the ability to generate their own specific Ca^{2+} signals (Johnson et al., 1995; McAinsh and Pittman, 2009; Nomura et al., 2012; Rocha and Vothknecht, 2012) leading to new insights into these previously underestimated organelles.

1.3.4.1 Calcium compartmentalisation and transport across chloroplast membranes

Calcium signalling derived from the need of cells to compartmentalise this ion and keep its concentration low in the cytosol, to prevent its toxicity. A similar concept can be applied to the chloroplast stroma. Calcium is necessary in the chloroplasts, but its levels must be tightly controlled in the stromal compartment to allow processes to occur (see paragraph 1.5.3 role of calcium in the chloroplast). Indeed, high chloroplastic total calcium levels have been estimated, up to 15 mM or higher (Stael et al., 2012; Nomura and Shiina, 2014), and these levels increase during daytime (Nobel et al., 1966; Kreimer et al., 1985; Roh et al., 1998), but very little free calcium resides in the stroma. The first measurement of stromal Ca^{2+}-levels come from Johnson and colleagues (Johnson et al., 1995) using transgenic tobacco where aequorin was targeted to the stroma. In their work, they also measured a strong and chloroplast-specific calcium increase in the stroma in response to the light-to-dark transition, suggesting that calcium increases in the photosynthetic organelle at the onset of darkness to release excessive calcium accumulated during the daytime, as well as to signal to shut down the photosynthetic apparatus. Low resting calcium concentrations were confirmed by further work, and in different species as well (e.g. *Arabidopsis*), and have been estimated to be around 150 nM, similar to cytosolic resting levels (Sai and Johnson, 2002; Sanders et al., 2002; Dodd et al., 2010; Kudla et al., 2010; Manzoor et al., 2012; Nomura et al., 2012). Low resting calcium concentrations in the stroma can be achieved if calcium is stored elsewhere, however, for the chloroplasts, the identity of this store is still unknown. Different

hypothesis have been made, and are discussed later in this paragraph. In order to understand where calcium can be stored it is relevant to know the Ca^{2+}-permeability of the different plastidial membranes.

The chloroplast is surrounded by the outer envelope and the inner envelope. Whilst the inner membrane is unequivocally considered to be impermeable to calcium (Stael et al., 2012), the most accredited idea is that the outer envelope is calcium permeable, hence it does not act as any kind of selection filter for ions (Bolter and Soll, 2001; Uehlein et al., 2008; Borisova et al., 2012; Stael et al., 2012). There is also are more internal set of membranes which form the thylakoid compartment, where Ca^{2+}-trafficking is controlled since these membranes are impermeable to it (Stael et al., 2012). Different hypothesis regarding the chloroplast calcium store envisage the possibility that this ion is accumulated in the thylakoid lumen (this idea is supported by the presence of Ca^{2+}-transporters on the thylakoid membranes, see below), or bound to the thylakoid membranes, which are known to host proteins which are negatively charged upon light by SNT7 and STN8 (Wunder et al., 2013). Another valid hypothesis foresees the presence of high-capacity calcium binding proteins in the stromal compartment able to buffer the ion levels (Kreimer et al., 1987). However, the exact location of the millimolar concentrations of Ca^{2+} have not been identified yet, and there are still other possibilities opened such as connections to the ER via stromules (Schattat et al., 2011).

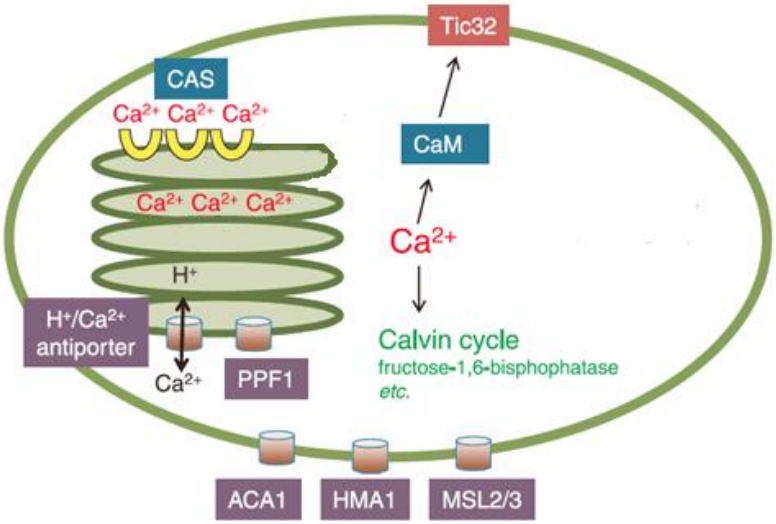


Figure 1.10 Modified from Nomura and Shiina, (2014). Representation of the identified and putative chloroplast Ca^{2+}-transporters located in the inner envelope (ACA1, HMA1, MLS2/3) and in the thylakoid membrane (Ca^{2+}/H^{+} antiporter, PPF1). CaS is a thylakoid-localised calcium sensing protein, while calcium-binding proteins such as CaM are represented, as their activity has been measured (e.g. they control Tic32). Furthermore, stromal calcium increases affect the Calvin-Benson cycle. For details see text.

The first site of Ca^{2+}- flux regulation is the inner membrane that separates the intermembrane space from the stroma. To explain the measured increased chloroplast Ca^{2+} levels upon illumination (Nobel et al., 1966; Kreimer et al., 1985; Roh et al., 1998), calcium transporters must be present on the inner envelope and/or on the thylakoid membrane. Some candidates have been identified (represented in figure 1.10), which are for the inner envelope AtACA1, which is a Ca^{2+}-ATPase, AtHMA1, a putative heavy metal ATPase (Seigneurin-Berny et al., 2006), MSL2 and MSL3, which are homologues to bacterial mechanosenstitive channels (Haswell and Meyerowitz, 2006). Furthermore a glutamate receptor (AtGLR3.4), known to have non-selective cation transport activity, has been hypothesised to reside in the chloroplast (Teardo et al., 2011). The thylakoid membrane hosts the Ca^{2+}/H^{+} antiporter, whose activity was measured by Ettinger and

colleagues, and it has been recently identified (Ettinger et al., 1999; Wang et al., 2016). Thus, the proton-motive force created by light will drive the storage of the calcium imported upon chloroplast illumination. Moreover, another Ca^{2+} transporter is present on the thylakoid membrane named post-floral specific gene 1 (PFF1) (Wang et al., 2003). The presence of these putative transporters, specially the Ca^{2+}/H^{+} antiporter, on the thylakoids make of this compartment an even more attractive calcium store.

1.3.4.2 CaS

The best characterised chloroplastic calcium-binding protein is the calcium-sensing receptor (CaS, represented in figure 1.11). Despite the initial mislocalisation of this protein to the plasma membrane of onion epidermis cells (Han et al., 2003). This 37.8 kDa protein is now known to be localized in the thylakoid membrane (Nomura et al., 2008; Vainonen et al., 2008). CaS has a rhodanese homology domain at the C-terminal as well as a phosporylation site on a threonine in position 380 (the total length is 387 aa). It also has one motif of interaction with the 14-3-3 domain and one with the FHA domain ("forkhead-associated" domain), while the transmembrane domain is in the middle of the protein (Vainonen et al., 2008). The N-terminal contains a chloroplast transit peptide and it is the calcium-sensing part of the protein (Han et al., 2003). Despite the knowledge on the structure, a consensus has not yet been reached about the subcellular location of the C- and N-terminus. The C-terminal hosts the phosphothreonine, the rhodanese-like domain and two protein-interacting motifs known for signalling to proteins with 14-3-3 and FHA domains, which are interacting with the protein kinase STN8, stress-associated proteins and 14-3-3 proteins, respectively, which are located in the chloroplast stroma (Vainonen et al., 2008). According to this fact, the C-terminus is more likely to be located in the stroma. The N-terminus side of the protein, hence the Ca^{2+}-sensing part, has been either localised in the stromal side (Nomura 2008) or in the lumen, and there is still not an agreement on its location.

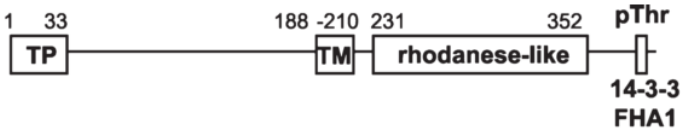


Figure 1.11 Modified from Vainonen et al., (2008). CaS structure and domains are represented.

CaS is a plant specific protein, it has holomogues in higher plants (*Arabidopsis thaliana*, *Oryza sativa*, *Medicago truncatula*), *Chlamydomonas reinhardtii* and *Ostreococcus tauri*, where it exists without a transmembrane domain, while no homologues were detected in cyanobacteria (Vainonen et al., 2008), moreover it is present as a single copy in the genome, and no redundancy has been observed. CaS levels and phosphorylation are affected mainly by light conditions (Vainonen et al., 2008), and throughout development and senescence (Nomura et al., 2008).

Evidence of CaS sensing calcium come from the very first paper about CaS (Han et al., 2003) where it was shown that CaS possesses low affinity-high capacity calcium binding properties. Since its discovery, this protein has been extensively studied and it has been found to take part to a wide range of chloroplast functions. CaS is involved in pathogen responses (Nomura et al., 2012), stomatal closure (Nomura et al., 2008; Weinl et al., 2008; Wang et al., 2014), and photoprotection (Vainonen et al., 2008; Petroutsos et al., 2011). Moreover, it is responsible for long-lasting Ca^{2+} mobilization in the chloroplast, and it is involved in ROS signalling and in altering the electron flow (Nomura et al., 2012; Nomura and Shiina, 2014; Wang et al., 2014). Developmentally, *cas* mutants displayed growth defects (Vainonen et al., 2008) and antisense lines showed late bolting in response to reduced calcium supplies (Han et al., 2003). CaS can regulate concentrations of inositol 1,4,5-trisphosphate (IP_3), which in turn directs release of Ca^{2+} from internal stores, regulating circadian rhythms (Tang et al., 2007). Furthermore, in *Chlamydomonas* CaS is required for photoacclimation to high light (Petroutsos et al., 2011). Hence phosphorylation by STN8 and control of photoacclimation state a role of CaS in the regulation of the photosynthetic process. Despite its involvement in a large

number of processes, the CaS receptor is still not fully characterised and further analysis are required to elucidate its role in calcium signalling.

1.3.4.3 Role of calcium in the chloroplast

Calcium plays both a regulatory and structural role in the chloroplast. The stromal compartment, which hosts the chloroplast genome, has a low calcium concentration (Manzoor et al., 2012; Nomura et al., 2012). Although toxic, calcium's presence is a requirement to guarantee the right conditions for chloroplastic biological activity. Ca^{2+} is necessary for the correct assembly, functioning and repair after photodamage of PSII, plus it is a cofactor of the oxygen evolving complex (Becker et al., 1985; Mattoo et al., 1989; Miller and Brudvig, 1989; Yocum, 1991; Grove and Brudvig, 1998; Ferreira et al., 2004). Furthermore, the light-regulated NAD kinase, which is responsible for providing reducible substrate to the photosynthetic chain, is activated by calcium (Muto and Miyachi, 1981; Jarrett et al., 1982).

However, if the stromal calcium concentration increases, photosynthesis is impaired. High Ca^{2+} concentrations are known to block the Calvin-Benson cycle acting on the fructose-1,6-bisphophatase and sedoheptulose bisphosphatase enzymes (Charles and Halliwell, 1980; Kreimer et al., 1988), thus affecting the CO_2 fixation step. Calcium uptake into the chloroplast upon illumination has been measured (Nobel et al., 1966; Kreimer et al., 1985; Roh et al., 1998). Therefore, when calcium enters the chloroplast stroma compartment, it has to be removed and stored to avoid chloroplast damage. Although the location of such storage is still unknown, some candidates have been identified. Thylakoids are an attractive possibility because of the presence of a Ca^{2+}/H^{+} antiporter and PFF1 on their membranes (Ettinger et al., 1999; Wang et al., 2003; Wang et al., 2016).. Another valid hypothesis foresees the presence of high-capacity calcium binding proteins in the stromal compartment able to buffer the ion levels (Kreimer et al., 1987). To discover the biological identity of the storage compartment further studies are needed, without discarding the idea of an alternative, still unknown Ca^{2+}-storing location.

To summarise, in the chloroplasts low resting calcium concentration in the stroma, calcium stores and transporters are present; the final requirement for calcium signalling in this organelle is the presence of calcium-sensing proteins.

So far, no calmodulins have been identified in the chloroplast, but several calmodulin-binding proteins are known to be involved in specific processes (Rocha and Vothknecht, 2012; Nomura and Shiina, 2014). For example, the NAD kinase, that provide NAD for photosynthetic reduction, is a calmodulin-binding protein, as well as Tic32, which is a component of the protein import complex (Chigri et al., 2005; Chigri et al., 2006). Thus, there are good reasons to postulate the existence of a Ca^{2+}/calmodulin signalling pathway in the chloroplast. In addition, other calcium binding proteins have been identified in the chloroplasts. Some EF-hand proteins are known to be present, such as RelA/SpoT, which is responsible for the production of a small signalling nucleotide (Magnusson et al., 2005; Masuda et al., 2008). Moreover, the small nucleotide ppGpp (guanosine 5'-disphosphate 3'-disphosphate) acts as a signalling molecule regulating the activity of the PEP protein (Sato et al., 2009). Interestingly, the production of ppGpp is under the control of RelA/SpoT, which is a calcium binding protein, and its levels are up-regulated in response to wounding, jasmonic acid treatment and after the transition from light to dark (Takahashi et al., 2004; Nomura and Shiina, 2014).

1.3.4.4 Evidence of calcium signalling

Not only does the chloroplast have the potential for calcium signalling, but there is already evidence of this ion acting as a second messenger. Johnson and colleagues (Johnson et al., 1995) recorded a calcium peak in the stromal compartment 20 minutes after the light to dark transition of tobacco seedlings previously kept in constant light. Furthermore, a proportional relationship between hours of LL and peak size was measured, and this calcium increase has a circadian recurrence in DD (Sai and Johnson, 2002). These results together suggest a role for calcium in sensing the transition from light to dark and possibly in stopping the photosynthetic machinery. Recently, chloroplast

calcium increases have been reported in response to cold, salt and hyperosmotic stresses (Nomura et al., 2012) as well as pathogen response elicitors (Manzoor et al., 2012; Nomura et al., 2012), with different kinetics compared to the cytosolic calcium counterparts. However, to date, no other chloroplast-specific (i.e. not cytosolic calcium-inducing) stimuli have been identified.

1.4 Summary

Calcium signalling plays a pivotal role in all eukaryotic organisms. It acts as a universal second messenger, mediating a wide range of signals going from biotic and abiotic stress responses to developmental processes. Hence, in plants, it is fundamental to both sense the surrounding environment and adjust to it and to accomplish plant physiological functions such as pollen tube elongation, stomatal closure etc (Holdaway-Clarke et al., 1997; Sanders et al., 1999; Allen et al., 2001; Kudla et al., 2010). Despite the abundant research on calcium signalling, some questions remained open, such as the way plant cells are able to encode the specificity of the signal, and what is the cellular basis for the wide range of fold change in nuclear gene expression induced by a linear cytosolic calcium increase. Another relatively new area of research is looking at the role of organelles such as mitochondria and chloroplasts in contributing to the cellular calcium homeostasis, as well as to their ability to generate their own independent calcium signalling (Stael et al., 2012). Specifically, chloroplast calcium is emerging as an interesting field, as calcium controls photosynthetic activity, and there is evidence of chloroplast-specific calcium signalling (Johnson et al., 1995). Finally, looking at the organellar calcium may help to give a new insight to unanswered scientific questions, such as the identification of plant thermometers, by addressing the problem using a different angle of research.

1.5 Thesis aims

The aim of this study was to:

- To create a dynamic mathematical model to understand how a calcium signature is translated into changes in nuclear gene expression, by using a known biological network as a template (Chapter 3).
- To answer the question: is chloroplast gene expression regulated by calcium increases in the stromal compartment? Hence does calcium in the chloroplasts have an analogous function to the one that cytosolic calcium has on controlling nuclear gene expression (Chapter 4)?
- To investigate the level of independence of the chloroplast organelle in controlling its own calcium signalling, by looking at a newly identified chloroplast-specific calcium heat response (Chapter 5).
- To investigate the possible physiological significance of the chloroplast-specific heat-induced calcium response identified (Chapter 6).
- To further understand heat sensing in plants investigating the chloroplast heat response as well as the animal sensing thermometer TRVP1 in the model organism *Arabidopsis thaliana* (Chapter 7).

Chapter 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: Melford Laboratories Ltd (Ipswich, UK), Fisher Scientific UK Ltd (Loughborough, UK), Bioline (London, UK), Duchelfa Biochemie BV (Haarlem, NL), Sigma-Aldrich Ltd (Poole, UK).

2.1.2 Plant material

Arabidopsis (*A. thaliana*) ecotype Columbia (Col-0) seeds were obtained from Lehle seeds (Round Rock, Texas, USA). These lines were used to create transformants (Col-0, *cas SALK* and *cas GABI* expressing stromal aequorin). *Arabidopsis thaliana* lines constitutively expressing 35S::apoaequorin either in the cytosol (pMAQ2, Col-0 ecotype (Knight et al., 1991)) or in the chloroplast (pMAQ6, Ws-0 ecotype, kind gift from Prof William F. Ettinger, Gonzaga University, Spokane, WA, USA) were available in the lab. Lines expressing aequorin linked to YFP (Mehlmer et al., 2012), targeted either to the cytosol, chloroplast or mitochondria were previously created by floral dipping in the lab (see 2.2.7.2). The two *cas* (At5g23060) mutant lines (*cas SALK* and *cas GABI*) were obtained as a kind gift from Prof Eva-Mari Aro (Turku University, Finland). TRPV1 expressing lines were previously obtained in the lab by dipping (see 2.2.7.2) pMAQ2

plants with a construct expressing the rat sequence of TRPV1 (Caterina et al., 1997) under an estradiol-inducible promoter (pMDC7 plasmid) (Curtis and Grossniklaus, 2003). Tobacco (*Nicotiana benthamiana*) seeds were available in the Department of Biosciences, Durham University.

2.1.3 Bacterial strains

Escherichia coli (E. coli) α-select silver cells were obtained from Bioline (London, UK). TOP10 Chemically Competent *E. coli* cells were obtained from Life Technologies (Paisley, UK). *Agrobacterium tumefaciens* (*A. tumefaciens*) strains C58C1 (Holsters et al., 1978) and GV3103 were used for stable and transient transformation of *Arabidopsis*, respectively, and were propagated in house (see 2.2.1.3).

2.1.4 Enzymes

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

2.1.5 Oligonucleotides and synthetic genes

Oligonucletotides were purchased from Life Technologies (Paisley, UK), and Integrated DNA Technologies (IDT, Leuven, Belgium). All synthetic genes were purchased from IDT (Leuven, Belgium).

2.1.6 Plasmid vectors

Vectors used were the commercially available pENTR/D-TOPO entry vector (Thermo Fisher Scientific, Loughborough, UK), and expression vectors coming from the Ghent

collection of GATEWAY vectors (Karimi et al., 2002), specifically pB7WG2 and pK7WG2.

2.1.7 Antibiotics

All antibiotics were purchased from Melford Laboratories Ltd (Ipswich, UK). Antibiotics were filter sterilized using a 0.22 μm filter (Millipore Corporation, Bedford, USA) attached to a syringe (VWR International Ltd, Lutterworth, UK), to make stocks, conserved at -20ºC, or directly prior to addition to liquid media. The concentrations used are listed in table 2.1.

Antibiotic	**Stock concentration (mg/ml)**	**Working concentration (μg/ml)**	**Stock solvent**
Spectinomycin	50	50	water
Kanamycin	100	for bacteria: 100 for plants: 50	water
Rifampicin	50	100	DMSO
Timentin	200	200	water
Glufosinate	NA	50	water

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

2.2 Methods

2.2.1 Bacterial growth

2.2.1.1 Bacterial growth media

E. coli and *A. tumefaciens* were grown either on solid agar plates consisting of 2 % (w/v) Luria-Bertani (LB) medium (Sigma-Aldrich) 1.5% (w/v) bacto agar and or liquid media made from 2% (w/v) LB. Before usage, all growth media was sterilised by autoclaving at 121 °C, 10^5 Pa pressure for 20 min and allowed to cool to 50 °C prior to the addition of appropriate antibiotics.

2.2.1.2 Bacterial growth conditions

Bacteria were incubated either at 37 °C *(E. coli)* or 28 °C *(A. tumefaciens).* Solid agar plates were incubated statically whilst liquid media cultures were shaken at 200 rpm for *E. coli* and 150 rpm for *A. tumefaciens*.

2.2.1.3 *A. tumefaciens* competent cells production

To obtain competent *A. tumefaciens* cells, cultures containing 5 ml of LB supplemented with appropriate antibiotics were inoculated with a single *Agrobacterium* colony (from a fresh LB plate). This was grown overnight at 28 °C in a shaking incubator. The next day, 4 ml of the overnight culture was added to 100 ml of LB in a sterile 500 ml flask and this was shaken vigorously (250 rpm) at 28 °C until the culture reached an OD_{600} of 0.5 to 1.0. The culture was chilled on ice and the cells were centrifuged at 3500 *g* for 5 min at 4 °C. The supernatant was discarded and the cells were resuspended in 2 ml of ice-cold 20 mM $CaCl_2$ solution. The cells were then dispensed into 0.1 ml aliquots in 1.5 ml pre-chilled microfuge tubes, flash frozen in liquid nitrogen and stored at -80 °C until used.

2.2.2 Plant growth

2.2.2.1 Seeds sterilization methods

Arabidopsis seeds were sterilized by shaking (Labnet Vortex Mixer, Labnet International Inc., Woodbridge, New Jersey, USA) in a 1.5 ml microfuge tube with 70% ethanol (v/v) for 5 min. Seeds were then transferred to sterile filter paper (WhatmanTM International Ltd, Kent, UK) and air dried in a sterile laminar flow hood before being sprinkled onto solid MS agar medium (see 2.2.2.2). *Arabidopsis* seeds collected from plants dipped in *A. tumefaciens* solution were first surface sterilized with ethanol as described above. They were then shaken in a solution containing 10% (v/v) sodium hypochlorite (NaOCl) and 0.25% (w/v) sodium dodecyl sulphate (SDS) for 10 min. The seeds were then washed six times in sterile water (10 min per wash), spread directly onto MS agar plates.

2.2.2.2 Plant growth media

Arabidopsis seeds were grown on solid 1 X MS medium agar plates (Murashige and Skoog, 1962). This comprised of 0.8% (w/v) plant tissue culture grade agar (Sigma-Aldrich, Poole, UK) and 1 X Murashige and Skoog salts (Duchefa Biochemie BV, Haarlem, Netherlands). All growth media was sterilized by autoclaving at 121 °C, 10^5 Pa pressure for 20 min. The pH was adjusted to 5.8 using 0.1 M KOH before autoclaving. If required, appropriate antibiotics were added to the liquid medium after autoclaving and cooling to 50 °C.

2.2.2.3 Plant growth conditions

Seeds were vernalised for a minimum of 48 h at 4ºC before growing them in a Percival (CU-36L5D, CLF plant climatics, Emersacker, Germany) at 20ºC with a 16/8 h photoperiod at a light intensity of 150 μmol m^{-2} s^{-1}. Imaging experiments were performed on 8-day-old seedlings (see 2.2.4.2); aequorin reconstitution was performed on 7-day-old seedlings (see 2.2.41). For *Agrobacterium tumefaciens*-mediated transformation, seedlings were transferred onto hydrated 44 mm peat plugs (Jiffy Products International AS, Moerdijk, Norway) and grown at 20ºC with a photoperiod of 12/12 h until they bolted, and 16/8 h after *Agrobacterium*-mediated transformation (light intensity 150 μmol m^{-2} sec^{-1}); for seed collection. Individual seedlings werethen (after selection) grown on hydrated 41 mm peat plugs (Jiffy Products International AS, Moerdijk, Norway) and grown at 20ºC in a 16/8 h photoperiod. Peat plugs were watered with 100 mg/L intercept (Everris International, Ipswich, UK) and Baby Bio (Bayer Garden, Cambridge, UK), following the manufacturer's instructions, until the siliques had developed. If seeds needed to be collected from individual plants, the Aracon system (Beta tech, Ghent, Belgium) was used to contain each plant.

Nicotiana benthamiana plants were grown on soil at 27ºC for 4 weeks with a 16/8 h photoperiod at 250-300 μmol m^{-2} sec^{-1}.

2.2.3 Temperature and chemical treatments of plants

2.2.3.1 Temperature treatment

Fast changes in temperature were performed on a Peltier (IPD-PC) cooling element. *Arabidopsis* seedlings were laid down on the cooling element on wet filter paper, and covered with cling film, whilst detached tobacco leaves were flattened with a transparent glass slide. The Peltier element allow changes in temperature from -20ºC to 50ºC, at different rates. Acclimation temperature treatments were performed by floating coelenterazine-reconstituted plants in water for 12 to 24 h in the dark at 15ºC, 20ºC or

30ºC. Changes in temperature upon F_v/F_m measurements (see 2.2.8.3) were also performed with the Peltier element. Longer temperature treatments, such as the ones performed prior to gene expression measurements, HPLC or LC/MS (see 2.2.8.1 and 2.2.8.3) were performed by putting seedlings in water overnight, and then transferring them into a heat block at the appropriate temperature, always in liquid. Temperature treatments prior to TBARS measurements (see 2.2.8.4) were also performed in liquid for 1 h either at 40ºC or 20ºC in darkness, then plants were left to recover in the light for 3 days on MS agar plates (see 2.2.2.2).

2.2.3.2 Light treatment

For light/dark treatments, plants were floated for 30 minutes, or as otherwise reported, in water in the light (light intensity 150 μmol m-2 s-1) or dark at 20ºC. To test the effect of light upon heating, a light of the same intensity illuminated the Peltier element hosting *Arabidopsis* seedlings on wet filter paper (see 2.2.4.2.2).

2.2.3.2 Chemical treatment

Chemical pre-treatments were performed by floating plants in water plus the chemical for 30 minutes unless otherwise stated. Appropriate controls were applied by adding an equal concentration of the solvent to a control set of plants.

To induce TRPV1 expression, estradiol-pretreatment was applied to *Arabidopsis* seedlings overnight by adding 5 μM estradiol or 0.1% (v/v) DMSO as a control.

To measure calcium concentration upon a specific treatment (in the luminometer, see 2.2.4.2.1), chemicals were directly applied to the plants by injection with a syringe inside the luminometer cuvette, where the plant was contained. As the plant was in 0.5 ml of water, 0.5 ml of chemical at double concentration was added in each case. For longer chemical treatments, plants were floated in water including the chemical.

High CO_2 concentrations were achieved by attaching a IRGA (infrared gas analyser, Elite Scientific Instruments SDN BHD, Selangor Darul Ehsan, Malaysia) to a air-tight plastic transparent chamber containing the plants. CO_2 concentrations were controlled by the IRGA. The chamber was placed inside the camera light-tight box, allowing calcium measurements (see 2.2.4.2.2).

All chemicals were supplied by one of the companies listed in Chemicals (see 2.2.1), except for DNQX (Calbochem, Merk, Nottingham, UK).

2.2.4 Calcium measurements

2.2.4.1 Reconstitution of aequorin

Aequorin reconstitution was performed by floating *Arabidopsis* seedlings on water containing 10 μM coelenterazine (Biosynth Srl, Staad, Switzerland) 1% (v/v) methanol. Reconstitution of tobacco plants was performed by infiltrating with a syringe the aequorin-expressing area with a 50 μM coelenterazine solution, 1% (v/v) methanol 24 h after aequorin binary vector infiltration. All plants were left in the dark from 12 to 24 h at 20ºC before calcium measurements.

2.2.4.2 Calcium dependent luminescence measurements

2.2.4.2.1 Luminometer

Arabidopsis seedlings were individually transferred to a cuvette containing 0.5 ml of water (Sarstedt, Nümbrecht, Germany). Following a 30 min resting period, the cuvettes were individually inserted into the luminometer chamber and luminescence levels were recorded every 1 sec using a digital chemioluminometer with discriminator and cooled housing unit (Electron Tubes Limited, Middlesex, UK) in order to reduce background

noise (Knight et al., 1991; Knight et al., 1996). Luminescence was recorded for 10 sec before injection of any chemical. Discharge was performed at the end of the experiment by injection of an equal volume of 2 M $CaCl_2$, 20% (v/v) ethanol (final concentration 1 M $CaCl_2$, 10% (v/v) ethanol).

2.2.4.2.2 Photon-counting camera

For Ca^{2+} imaging during temperature and CO_2 treatments, aequorin luminescence was recorded under a plate-intensified charge-coupled CCD camera (Photek 216; Photek, East Sussex, UK). The camera was housed in a light-tight box into which samples could be placed. Thanks to the intensifier plates single photons could be collected, and their spatial position was recorded. Data were analysed with the Photek IFS32 software. Total aequorin for calibration was measured by decreasing the temperature to -15ºC for 5 min, and then back to room temperature to discharge the remaining aequorin.

2.2.4.3 Aequorin luminescence calibration

Calibration was performed as previously described (Knight et al., 1996), following this logarithmic equation: pCa = 0.332588(-logk) + 5.5593, where k = counts luminescence counts per second/ total remaining counts. The number of total counts is calculated as the amount of data collected from the beginning of the experiment to the end of the discharge.

2.2.5 Basic techniques for DNA and RNA

2.2.5.1 DNA isolation

Bacterial plasmid DNA extraction was performed using the Wizard Plus SV Minipreps DNA Purification System (Promega, Southampton, UK), according to the manufacturer's instructions.

2.2.5.2 PCR

For general PCR reactions either BioTaq or BioTaq Red Taq polymerases (Bioline, London, UK) were used. For high-fidelity applications, a proof reading Phusion DNA polymerase was used (Finnzymes, Keileranta, Finland). Reaction mixes were made up according the manufacturer's instructions using the buffers and $MgCl_2$ provided.

Primers were designed to satisfy the cloning purposes. A full list of oligonucleotides used for PCR can be found in appendix A.

PCR was performed using a 96 well Px2 thermocycler (Thermo Electron Corporation, Waltham, Massachusetts, USA). PCR conditions for the two different enzymes are listed in table 2.2. The annealing temperature for new templates and primer pairs were optimized before use. Typically, annealing temperatures were chosen to be 5 °C lower than the melting temperature of the lowest from the primer pair. The resulting PCR products were analysed using gel electrophoresis (see 2.2.5.3).

Cycle step	Time and temperature		No. of cycles
	BioTaq	**Phusion**	
Initial denaturation	95ºC, 5'	98ºC, 30"	1
Deanturation	95ºC, 30"	98ºC, 10"	25-35
Annealing	50-60ºC, 30"	50-60ºC, 20"	
Extension	72ºC, 2'	72ºC, 30"	
Final extension	72ºC, 2'	72ºC, 10'	1

Table 2.2 PCR conditions for different commercial polymerases.

2.2.5.3 Gel electrophoresis

DNA was separated by size using agarose gel electrophoresis. Gels were prepared by melting 1% (w/v) electrophoresis grade agarose (Sigma, Poole, UK) in 0.5 X TBE buffer (0.11 M Tris, 90 mM borate, 2.5 mM EDTA, pH 8.0) in a microwave oven. After cooling to ~50 °C, Midori Green (NIPPON Genetics EUROPE, Dueren, Germany) was added to a final concentration of 5 μg/ml. The molten gel was poured into a gel tank containing a comb. When solid, the comb was removed and the tank filled with the running buffer TBE (0.5 X). Before loading into wells, 5 X DNA sample-loading buffer (Bioline, London, UK) was added to DNA samples. Gels were run at 35 mA for approximately 1 h. Nucleic acid bands were visualized using a UV trans-illuminator (Uvitec, Cambridge, UK). Fragment size was determined by comparing to a 1 kb molecular size standard (Bioline, London, UK, Hyperladder 1).

2.2.5.4 DNA purification from gel

DNA fragments separated by gel electrophoresis (see 2.2.5.3) were visualized with a blue light trans-illuminator (Syngene, Cambridge, UK), and the desired band was excised from

the gel using a scalpel blade. The DNA was then purified using a QIAquick gel extraction kit (Qiagen, Crawley, UK), according to the manufacturer's instructions

2.2.5.5 Sequencing

For sequencing of plasmids containing cloned fragments, DNA was isolated using the Miniprep method (see 2.2.5.1). All sequencing reactions were carried out by the DNA sequencing laboratory (Department of Biosciences, Durham University). Analysis of chromatograms was carried out using SnapGene (www.snapgene.com). Sequences were aligned using Clustal W (www.ebi.ac.uk/Tools /msa/clustalw2/).

2.2.5.6 RNA isolation

The RNeasy Plant Total RNA kit (Qiagen, Crawley, UK) and ReliaPrep RNA Miniprep Systems (Promega, Southampton, UK) were used to extract total plant RNA, for *Arabidopsis* tissue, according to the manufacturer's instructions.

2.2.5.7 DNA and RNA quantification (nanodrop)

DNA and RNA concentrations were determined using a ND-1000 UV-Vis Spetrophotomer (Labtech, East Sussex, UK), according to the manifacturer's instructions.

2.2.5.8 cDNA synthesis

cDNA was produced from RNA using the Applied Biosystems High Capacity cDNA synthesis kit again according to the manufacturer's instructions. A total volume of 10 µl was made up with 2 µg total RNA and nuclease-free water. To this, 10 µl of master mix containing 2 µl 10 x RT buffer, dNTP Mix (100 mM), 1 µl MultiscribeTM Reverse Transcriptase and 4.2 µl of nuclease free water was added, to give a total volume of 20 µl.

Controls with no RNA and no reverse transcriptase enzyme were also set up in parallel. Hence cDNA was synthesised from these samples by treating them at 25 °C for 10 min, 37 °C for 120 min and then 85 °C for 5 s in a thermocycler. The resulting cDNA was diluted 1:50 with nuclease free water before use in RT-PCR (see 2.2.5.9) and then stored at -20^{o}C until needed.

2.2.5.9 RT-PCR

The relative transcript (RT) level of genes of interest was determined by RT-PCR using the Applied Biosytems 7300 real time PCR machine and Go Taq qPCR master mix (Promega, Southampton, UK). Diluted cDNA (5 µl) (see 2.2.5.8) was added to 10 µl of SYBR green master mix containing (per reaction): 7.5 µl of 2 X GoTaq qPCR master mix, 0.9 µl of each forward and reverse primer (primer stock 5 µM) and 0.7 µl of nuclease free water. To account for optical differences between wells, The GoTaq qPCR master mix is supplied with ROX as a reference dye. The diluted cDNA and master mix were added to wells of a 96-well plate (STARLAB UK, Milton Keynes, UK). For each sample tested, three replicate wells were set up to give three technical replicates. In addition, at least three biological replicates were also carried out for each experiment. *PEX4* (At5g25760) was used as an endogenous housekeeping control when testing *Arabidopsis* gene expression (Moffat et al., 2012). A full list of RT-PCR primers can be found in appendix A. All RT-PCR primers were designed using Primer3 (http://primer3.ut.ee) with an amplicon size of 80-120 bp, and when possible covering an intron. Relative quantification was performed by the $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001), the values obtained representing the relative quantitation (RQ) estimates, and the error bars, representing RQ_{MAX} and $RQ_{MIN,}$ were calculated as described previously (Knight et al., 2009).

2.2.6 Cloning

2.2.6.1 Restriction digest

Restriction digests were carried out to obtain fragments either for diagnosis or for cloning. Digests were carried out using compatible NEB restriction enzymes and buffers and these were incubated for a minimum of 2 h at the temperature recommended by the manufacturer (usually 37 °C). Double digestions were carried out in a way that the enzyme volume never exceed 10% (v/v) of the total reaction volume. Digests were then run on a gel to determine the size of the insert (see 2.2.5.3).

2.2.6.2 Ligation

DNA fragments were ligated into a linearized vector using T4 DNA ligase (Promega, Southampton, UK) in the supplied buffer. A 1:3 linearised vector: insert molar ratio was used, in a final volume of 10 µl. As a control, linearised vector alone was ligated and water was added instead of DNA. Ligation reactions were incubated overnight at 16 °C.

2.2.6.3 Gateway cloning

Phusion Taq Polymerase (see 2.2.5.2) was used to amplify the PCR product of interest from the DNA. Primers were designed to make products compatible with Gateway cloning. This product was visualized using gel electrophoresis (see 2.2.5.3) and extracted from the gel (see 2.2.5.4). The fragment was then ligated into an entry vector (TOPO vector) using the p-ENTR/D-TOPO cloning kit according to the manufacturer's instructions (Life Technologies, Paisley, UK). Gateway recombination using the LR Clonase II Enzyme Mix (Life Technologies, Paisley, UK) was used according to the manufacturer's instructions to create final binary vector constructs. One Shot TOP10

Chemically Competent *E. coli* cells (Life Technologies, Paisley, UK) were then transformed with the resulting plasmids (see 2.2.7.1). Plasmids were checked for the incorporation of the correct insert by restriction digestion (see 2.246.1) and then sequenced (see 2.2.5.5).

2.2.6.4 Gibson cloning

The DNA fragment to be cloned was ordered as a synthetic gene from IDT (Leuven, Belgium. The fragment was cloned into a linearized vector using a Gibson Assembly Cloning Kit (NEB) according to the manufacturer's instructions. *E. coli* competent cells were transformed with 2 µl of the reaction product (see 2.2.7.1).

2.2.7 Plant and bacterial transformation

2.2.7.1 Transformation of *E. coli* and *A. tumefaciens* competent cells

Aliquots (25 µl) of either One Shot TOP10 Chemically Competent *E. coli* cells (Life Technologies, Paisley, UK) or α-select silver cells (Bioline, London, UK) were transformed with plasmid DNA. DNA (2 µl) was added to thawed cells on ice and incubated for a minimum of 20 min. The cells were then heat shocked by putting them at 42 °C for 30 s, and then quickly back on ice for at least 2 min. SOC media (250 µl) (Life Technologies, Paisley, UK) was added to the cells and they were then incubated with shaking at 220 rpm for 1 hr at 37 °C to allow antibiotic resistance to develop in the transformed cells.. Cells were then plated onto LB containing the appropriate antibiotics (see table 2.1) and incubated overnight at 37 °C.

Aliquots containing 100 µl of C58C1 or GV3103 *A. tumefaciens* cells were incubated on ice for at least 30 min after the addition of 1 µg of plasmid DNA. Cells were then heat-shocked for 5 min at 37 °C before returning them on ice for at least 2 min. Liquid LB

media (1 ml) was then added to each aliquot and the cells were incubated with shaking for 4 hr at 28 °C to allow antibiotic resistance to develop. The cells were then briefly spun down in a microcentrifuge and the cell pellet was resuspended in 100 µl of LB before being spread on LB agar containing the appropriate antibiotics for selection (see table 2.1). Plates were incubated at 28 °C for 48 hr to allow colonies to develop.

2.2.7.2 *Arabidopsis* transformation with floral dip

Wild-type *Arabidopsis* seeds were grown on MS media (see 2.2.2.2) for seven days. Large (44 mm) peat plugs were set up with three seedlings per plug, and grown in a 12/12 h light/dark photoperiod. When the plants began to flower, the bolts were clipped to allow multiple secondary stems to develop. The final clipping was carried out seven days before transformation, producing an abundance of stems with flowers. Overnight cultures containing 5 ml of LB media with *A. tumefaciens* containing the correct plasmid and appropriate antibiotics were set up (see 2.2.1). An aliquot (1 ml) of the 5 ml overnight culture was then added to a 200 ml overnight culture, again with the appropriate antibiotics for selection. The culture was incubated overnight at 28 °C with agitation at 150 rpm. The *A. tumefaciens* cells were then pelleted by centrifugation at 3500 *g* for 20 min at room temperature. The bacterial pellet was then resuspended in 200 ml of 5% sucrose solution (w/v) and 0.05% (v/v) Silwet L-77. The flower stems of each *Arabidopsis* plant were dipped in the sucrose solution before being placed on their side in a tray lined with tissue paper to blot the excess of bacterial culture (Clough and Bent, 1998). The tray was covered in cling film and returned to the growth chamber with a 16/8 h light/dark photoperiod. The following day the plants were transferred to a fresh tray, stood upright and grown until seeds were obtained.

2.2.7.3 Primary transformant screening

Seeds (T1) were collected from the transformed *Arabidopsis* plants. These seeds were bleach sterilized (see 2.2.2.1) before germinating on large MS agar plates containing kanamycin or glufosinate and timentin antibiotics (see table 2.1 for antibiotic concentrations). The plates were put in the light for 6-8 h, wrapped in foil and left in the dark for 48 h (Harrison et al., 2006). The plates were then kept in the light (16/8 h photoperiod) until transformed seedlings could be identified by the presence of dark green leaves and roots which were able to penetrate the agar (usually after 5-6 days) (Harrison et al., 2006). Primary transformants (T1) surviving the selection were transferred to peat plugs (42 mm) and grown to maturity under normal conditions in the growth room. The seeds from this generation (T2) were harvested separately. For selection of aequorin-containing lines, the total amount of aequorin (total counts) was measured by discharging the plants (see 2.2.4.2) and lines with aequorin expression comparable to their relative control (pMAQ2 or pMAQ6) were selected.

2.2.7.4 Tobacco infiltrations

Infiltration of tobacco with *A. tumefaciens* GV3103 strain was performed for transient expression of proteins in a plant system. To do so, the bacteria containing the construct of interest was grown on LB media supplied with the appropriate antibiotics (see 2.2.1.2) for 24 h. The liquid culture was centrifugated at 3000 *g* for 15 minutes at room temperature and resuspended in 5 ml of autoclaved 10 mM $MgCl_2$ twice. The optical density (OD at 600 nm) was adjusted to 0.6 and this bacterial concentration was used to infiltrate tobacco expanding leaves with a syringe (VWR International Ltd, Lutterworth, UK). Leaves transiently expressing the ectopic protein were used at 48-72 h after infiltration.

2.2.8 End-point assays

2.2.8.1 HPLC

Amino acid levels were quantified using UPLC (Waters Acquity H-Class UPLC® system with fluorescence (FLR) and photodiode array (PDA) detectors) (Waters, Elstree, UK). Leaf tissue was ground in liquid nitrogen and lyophilized overnight. Lyophilized tissue (0.04 g) was extracted in 1.5 ml of 0.1 N HCl by grinding and then centrifugation at 17000 *g* for 20 min at 4 °C after leaving the samples for 30 min on ice. The extracts were then sequentially derivatised with OPA (o-Phthaldialdehyde) reagent (Sigma). OPA reagent consists of 260 mM N-Isobutyryl-L-cysteine (IBLC) (Sigma) and 170 mM OPA (Sigma) in 1 M potassium borate buffer (pH 10.4). The following reactions were set up in a HPLC vial: 10 µl sample, 10 µl OPA reagent, and 80 µl of 100% methanol. Separations were performed on a Cortecs C18 column, 100 mm x 2.1 mm, 1.6 M µM column (Waters, Elstree, UK) and elution was achieved at 40 °C. Mobile phase A was made up of 20 mM sodium acetate, pH 6.0. Mobile phase B was made up of acetonitrile: methanol: water in a 45:45:10 (v/v/v) ratio. A flow rate of 400 µl/min was used. Automated HPLC injection added 3 µl of the sample for analysis and samples were run for 20 min. For OPA detection the excitation and emission wavelengths were 340 nm and 455 nm respectively. For each amino acid, standard amino acids were run to obtain the retention times (Leu: 11.400 min, Ile: 10.838 min, Tyr: 6.697 min, Lys: 15.557 min).

2.2.8.2 LC/MS

Sample preparation was performed following Forcat *et al*., 2008. *Arabidopsis* samples (100 mg of fresh weight) were frozen in liquid nitrogen and then ground in 400 µl of a buffer solution of 10% (v/v) methanol containing 1% (v/v) acetic acid. For hormone extraction, samples were left in this buffer for 30 min on ice to allow extraction, followed by 10 min centrifugation at 13000 *g* at 4°C. The supernatant was carefully extracted, and

400 µl of buffer solution were re-added to the pellet for a second extraction cycle. The two lots of supernatant were pooled together before measurements. A control with no plant material was also performed. Compounds of interest were separated and detected by LC-MS. The separation was performed by using a LC system (Shimadzu Nexera UHPLC system), with a Waters HSS T3 C18 1.7 µm 1 mm x 100 mm column (Waters, Elstree, UK), at a flow rate of 100 µL/min (elution was achieved at 40°C, sample temperature 4°C). Mobile phase A was made up 0.1% formic acid in water. Mobile phase B was made up of acetonitrile (initial 5% B, held for 2 minutes, with a linear gradient to 95% at 9 minutes, held for 3 minutes). A flow rate of 400 µl/min was used Automated LC injection added 2 µl of the sample for analysis. Compounds were identified by multiple-reaction-monitoring (MRM) analysis in the negative mode, using transitions optimised by infusion of standards where available (ABA, JA, JA-Ile, GA3, IAA and SA) and using published transitions for JA-Ile. Samples were injected with blank runs in between. Two transitions for each compound were used for MRM analysis, with the most suitable being used for quantification based upon external standards. Transitions used were as follows; ABA – 262.9→153.0; GA3 – 344.9 → 143.0; IAA – 174.0 → 129.6; JA – 209.0 → 58.9; JA-Ile – 321.9 → 129.6; SA – 136.9 → 64.8.

2.2.8.3 F_v/F_m

Stress-induced damage can modify the status of the electron transport chain and their components, causing a reduced ratio of variable fluorescence (F_v) to maximal fluorescence (F_m) of plant photosystems (Maxwell and Johnson, 2000; Oxborough, 2004). This was measured in seedlings using a FluorCam 700mf (Photon Systems instruments, Brno, Czech Republic) on the F0, Fm and Kautsky effect setting. Plants were dark-adapted for at least 30 min prior to measurements. Heat treatments were performed on plants prior to the F_v/F_m measurement, using the Peltier element as described in 2.2.3.1.

2.2.8.4 TBARS assay

To perform the TBARS assay, plants were heat shocked as described in 2.2.3.1, and left to recover in the light at 20ºC for 3 days on MS plates. After recovery, 0.25 g of seedlings were frozen in liquid nitrogen, ground in 0.5 mL of a solution made by mixing equal volumes of 0.5% (w/v) thiobarbituric acid in 20% (w/v) trichloroacteic acid, and buffer 175mm NaCl in 50mm Tris-HCl, pH 8. After piercing a hole in the eppendorf tubes, samples were then heated to 95°C for 25 min and then spun down in a microcentrifuge at max speed for 20 min. The absorbance of the supernatant was measured at 532 nm, with a reading at 600 nm subtracted from it to account for non specific turbidity. The amount of malonaldehyde (MDA) was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1} (Larkindale and Knight, 2002).

Chapter 3

Decoding the Ca^{2+}-signatures: control of nuclear gene expression

3.1 Introduction

Calcium is a universal second messenger involved in a variety of plant responses, ranging from responses to stresses to plant development (Sanders et al., 2002; Kudla et al., 2010). In responses to stresses such as cold, drought, and oxidative stresses a transient cytosolic calcium increase occurs, and this is responsible for changes in the transcriptome, placing this second messenger as an intermediate between stimulus perception and nuclear gene expression (Knight et al., 1996, 1997; Rentel and Knight, 2004; Whalley et al., 2011). However, how different stimuli are decoded as different messages by using the same ion is still unclear. The most credible hypothesis is that the kinetics, spatial location and the different characteristics of the calcium elevation, termed "calcium signatures", are encoding the specificity (Webb et al., 1996; McAinsh and Hetherington, 1998; Dodd et al., 2010; Whalley and Knight, 2013). This hypothesis is supported by recent work in which it was demonstrated that different calcium signatures were able to differentially induce specific sets of genes (Whalley and Knight, 2013).

Understanding how different calcium signatures encode the specificity of the signal also requires an understanding of how components downstream of calcium, i.e. the calcium-binding proteins, are affected by the different signatures. Indeed, the way in which plant cells read and transduce the calcium signal has still not yet been clarified.

An initial step has been taken in this direction, by investigating at the affinity of calcium binding to the calmodulin binding transcription activators (CAMTAs), containing calmodulin binding domains (Liu et al., 2015). Hence a model of Ca^{2+}–CaM–CAMTA

binding and gene expression responses was developed following thermodynamic and kinetic principles (Liu et al., 2015), and it is represented in figure 3.1. Further explanation of this "cube" will be found in the discussion section of this chapter.

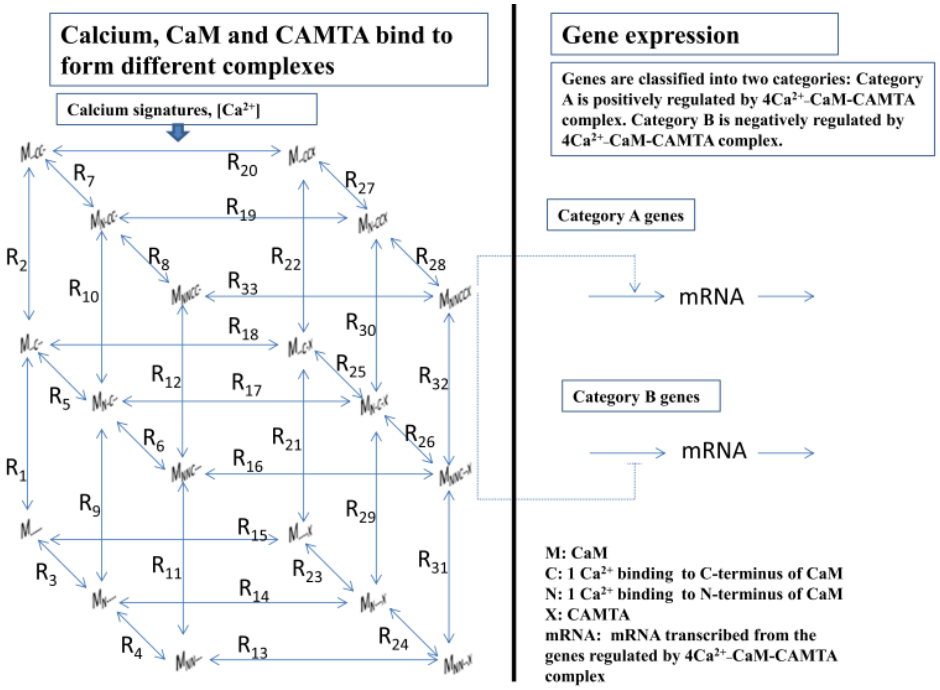


Figure 3.1. From Liu et al., (2015). A dynamic model that describes the information flow from calcium signatures to calmodulin-binding transcription activator (CAMTA)-regulated gene expression in *Arabidopsis thaliana*. Left panel: Ca^{2+}, calmodulin (CaM) and CAMTA bind to form different complexes. When [Ca^{2+}] changes, this binding process responds following thermodynamic principles. Right panel: gene expression is regulated by the active complex $4Ca^{2+}$–CaM–CAMTA (M_{NNCCX}). This figure illustrates a generic model for studying the information flow from calcium signatures to CAMTA-regulated gene expression.

To further investigate how this calcium-encoded specificity can lead to changes in nuclear gene expression we decided to stimulate *Arabidopsis* plants with different calcium agonists, inducing different signatures, and then measure changes in nuclear gene expression of specific targeted genes. Hence a mathematical model was created, which is not just able to explain how a calcium signature is decoded, but can also predict changes in gene expression of this network to any given calcium signature. For this work, a known calcium-regulated pathway was used. Hence the salicylic acid (SA)-mediated pathogen-induced pathway was chosen (figure 3.2). This network is calcium-mediated, it contains elements that have already been modelled, such as CaM and CAMTAs (Liu et al., 2015) and the effects in terms of gene expression are known (Zhang et al., 2014). As an output, two genes involved in the SA synthesis, *EDS1* (At3g48090) and *ICS1* (At1g74710), were chosen, and the relationship between them and the calcium-regulated elements is also shown in figure 3.2 (Zhang et al., 2014).

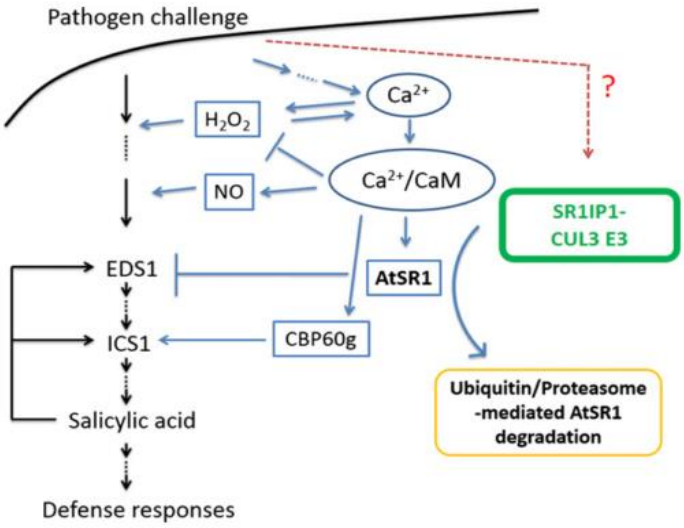


Figure 3.2 From Zhang et al., (2014). Proposed model the plant defence response pathway. Upon pathogen challenge, calcium increases in the cytosol. This binds to the calmodulin (CaM), and it regulates two transcription factors: AtSR1 (*Arabidopsis thaliana* signal responsive 1, also called CAMTA3) and CBP60g. These two transcription factors regulate the expression of *EDS1* and *ICS1*, which are key enzymes in the synthesis of salicylic acid. SA production is necessary to activate the defence response, and it also regulates its own synthesis by controlling the expression of *EDS1* and *ICS1*.

Aims of the research described in this chapter:

- Find four calcium agonists able to trigger different signatures.
- Correlate different calcium signatures induced by known agonists to differential gene expression of *ICS1* and *EDS1*.
- Model the calcium response on the plant immune pathway according to the collected data for gene expression and cytosolic calcium increases (fitting the model parameters to the lab data).
- Test if the model is able to predict the measured changes in gene expression (output), giving the cytosolic calcium increases as an input.

3.2 Results

3.2.1 Gene expression measurements in response to selected Ca^{2+}-signatures

3.2.1.1 Selection of calcium agonists to induce different Ca^{2+}-signatures

We set out to measure calcium elevations in *Arabidopsis* seedlings expressing cytosolic aequorin (pMAQ2). The idea was to induce cytosolic calcium elevations with different kinetics by using Ca^{2+}-agonists, as they do not, as much as possible, mimic a specific signal that plants perceive as a natural stress or as a development signal. To this aim, four different calcium agonists were chosen and for each of them a dose-response curve was produced. In each case, five pMAQ2 *Arabidopsis* 8-day-old seedlings were used in each luminometer cuvette, hence for each measurement, and data from at least three different measurements were averaged to get the final calcium concentrations for the dose-response curves. These curves are shown in figure 3.3 (ATP: adenosine triphosphate), figure 3.4 (L-Glu: L-glutamate), figure 3.5 (e[Ca^{2+}]: external calcium) and figure 3.6 (masto: mastoparan). For each calcium agonist a range of concentrations was tested, in order to find the most suitable condition to induce a significant calcium signature.

Mastoparan is a peptide occurring in the wasp venom. While in animals it is capable of activating G proteins, and this causes an ER-mediated calcium increase mediated by the creation of the IP_3 (inositol trisphospate) intermediate (Mousli et al., 1990), its mode of action in plants is still under debate. Indeed, the G protein pathway has not yet been identified, as there are no IP_3R calcium transporters in *Arabidopsis* (Edel et al., 2017), but there are resported cases of phopsolypases activaded by mastoparan, whose activity produce IP_3 (Takahashi et al., 1998; Himbergen et al., 1999) . In any case mastoparan has been widely and successfully used as a calcium agonist (Sun et al., 2007; Whalley et al., 2011).

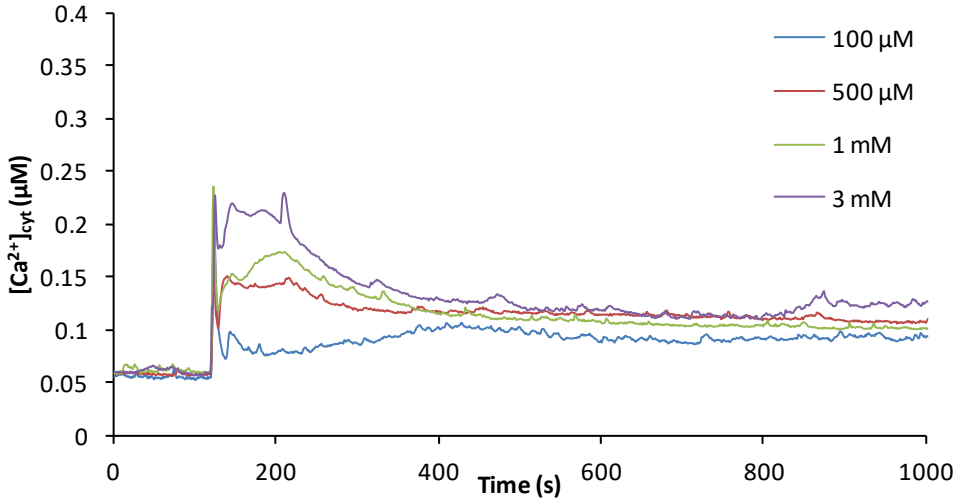


Figure 3.3 Cytosolic calcium traces in response to 100 µM, 500 µM, 1 mM and 3 mM ATP. ATP was injected after 120 seconds, and the signal was collected for 1020 seconds. Each trace is an average of 3 or more measurements, and each measurement represents the signal from 5 seedlings.

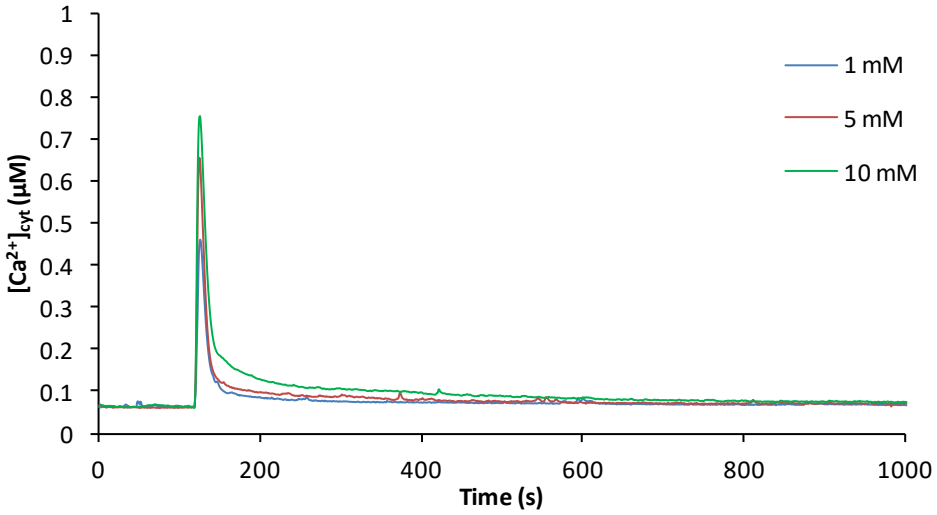


Figure 3.4 Cytosolic calcium traces in response to 1 mM, 5 mM and 10 mM L-Glu. L-Glu was injected after 120 seconds, and the signal was collected for 1020 seconds. Each trace is an average of 3 or more measurements, and each measurement represents the signal from 5 seedlings.

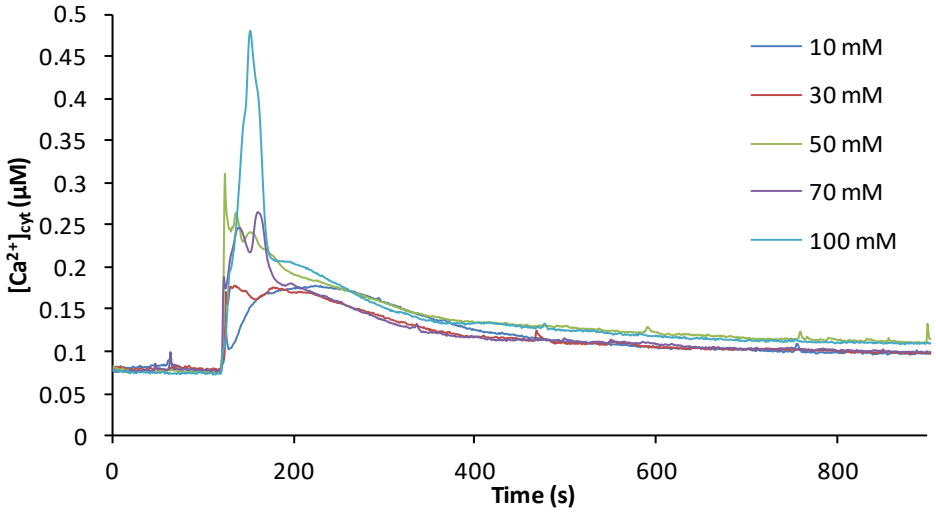


Figure 3.5 Cytosolic calcium traces in response to 10 mM, 30 mM, 50 mM, 70 mM and 100 mM e[Ca^{2+}]. e[Ca^{2+}] was injected after 120 seconds, and the signal was collected for 1020 seconds. Each trace is an average of 3 or more measurements, and it represents the signal from 5 seedlings.

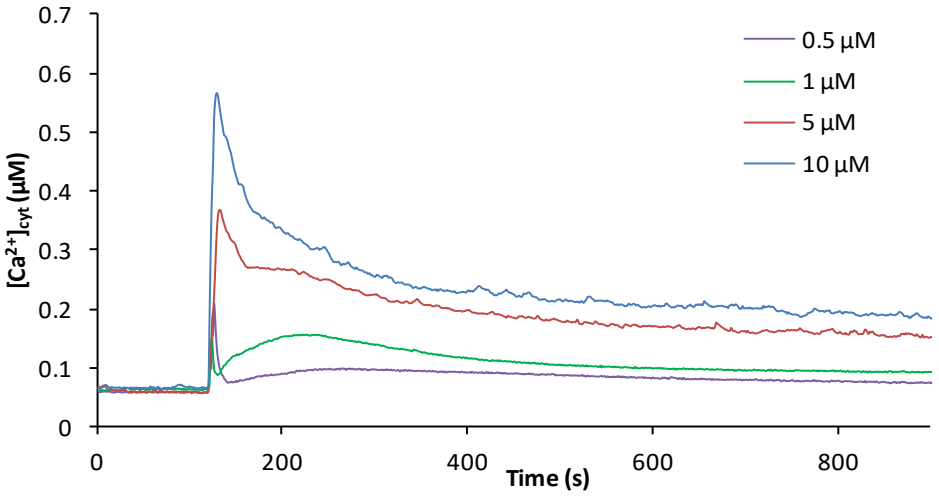


Figure 3.6 Cytosolic calcium traces in response to 0.5 µM, 1 µM, 5 µM and10 µM masto. Masto was injected after 120 seconds, and the signal was collected for 1020 seconds. Each trace is an average of 3 or more measurements, and it represents the signal from 5 seedlings.

From these four dose-response curves, the final concentration of agonists were chosen as following: 500 μM ATP as it is the lowest concentration causing a robust calcium increase (it is preferable to use low ATP concentrations as it can become toxic if too high, but a robust calcium increase is required). Similarly 1 mM L-Glu was chosen, which was the lowest concentration, as higher concentrations did not significantly increase the calcium release. The intermediate concentration of 50 mM e[Ca^{2+}] was picked as the calcium response is robust and the kinetics do not resemble the ones of ATP or L-Glu; while the highest concentration of mastoparan was preferred (10 μM) in order to maximise the stimulation of the calcium downstream pathway.

These concentrations were further used to create very robust calcium signatures. Calcium measurements were recorded on pMAQ2 *Arabidopsis* plants for a total of six times for each stimulus, over three different days using a different batch of plants each time, and collecting data at different times of the day. Additionally to the four calcium agonists, I also collected the signal coming from non-treated plants, which gives the calcium baseline value. These calcium traces are shown in figures 3.7A and 3.7B. These traces were used as the calcium signatures given as input into the mathematical model.

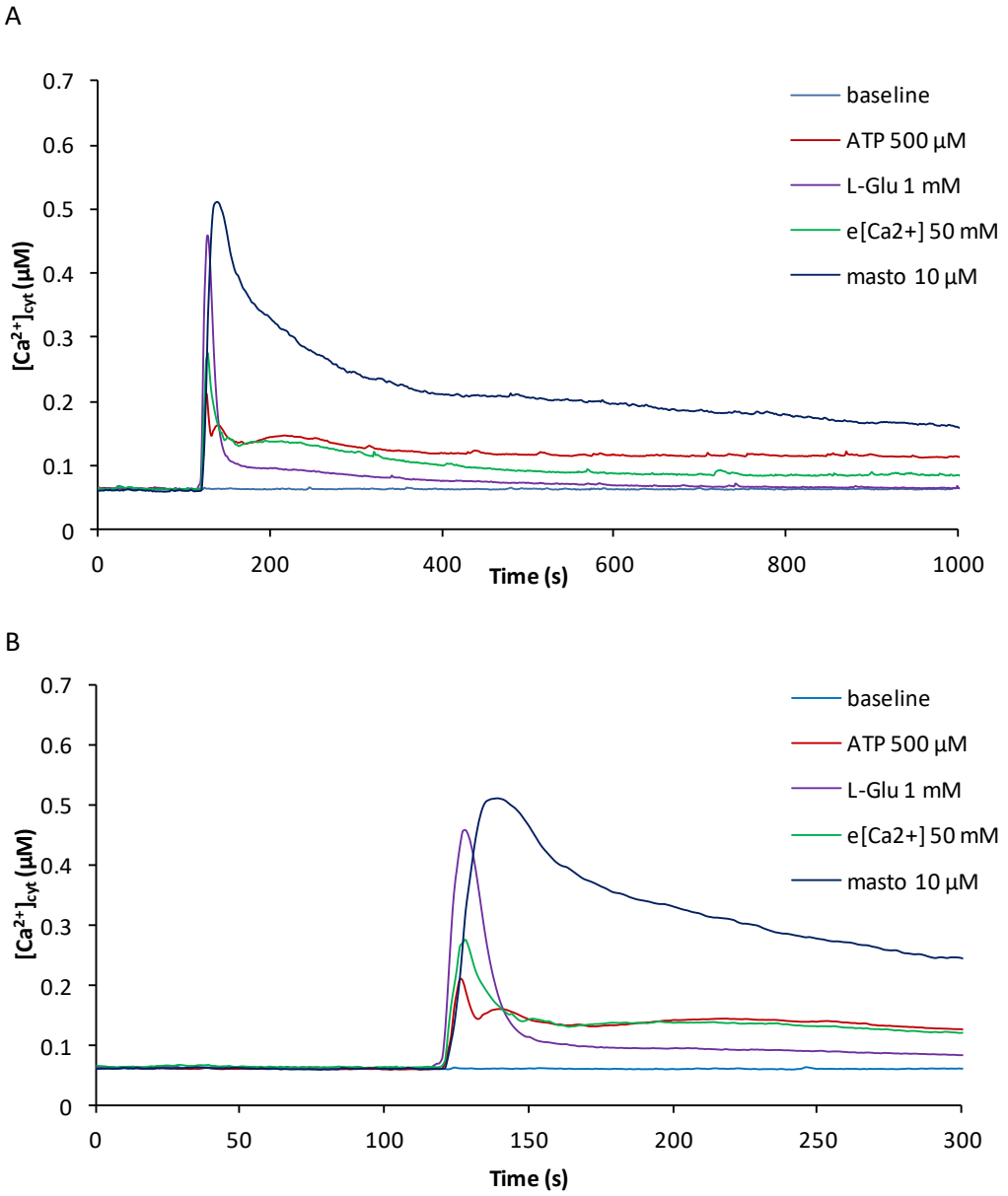


Figure 3.7 Calcium curves showing the kinetics of cytosolic-targeted aequorin lines exposed to 500 µM ATP, 1 mM L-Glu, 50 mM e[Ca^{2+}] and 10 µM masto. Stimuli were injected after 120 seconds (except for the baseline). (A) Full run of 1020 seconds and (B) enlargement of the first 300 seconds. Each trace was obtained by averaging the signal of n = 30 8-day-old *Arabidopsis* seedlings (as determined in the text).

3.2.1.2 Nuclear gene expression in response to the calcium agonists

In order to model the calcium-dependent, SA-mediated immune response, the other biological data needed was a measurement of changes in gene expression. Specifically, levels of *ICS1* and *EDS1* transcripts were measured upon stimulation with the calcium agonists after 1 h, 3 h, 6 h and 9 h. These two genes are responsible for the synthesis of salicylic acid, and EDS1 positively regulates *ICS1* synthesis, as represented in the network in the introduction (figure 3.2). Hence the level of these genes was assessed for each of the stimuli at each timepoint on 15 seedlings collected over 3 days (to randomise the conditions of the experiment). The agonist treatments were performed using the same conditions that were used to measure $[Ca^{2+}]_{cyt}$, so it is legitimate to assume that the calcium increase was taking place in the seedlings as previously measured (figure 3.7). The whole experiment was performed twice and each sample had quadruple RT-PCR technical replicates. Therefore each had 8 datapoints, with data coming from two independent experiments and 4 technical replicates. For modelling purposes the ratio with the baseline is the most relevant data regarding changes in gene expression. Therefore, results shown in figure 3.8 and figure 3.9 are shown as the ratio between the relative transcript abundance. This ratio compares levels of either *EDS1* (figure 3.8) or *ICS1* (figure 3.9) in response to the agonist, with the transcript abundance of the same gene in the control treatment (baseline, untreated plants) for that specific timepoint.

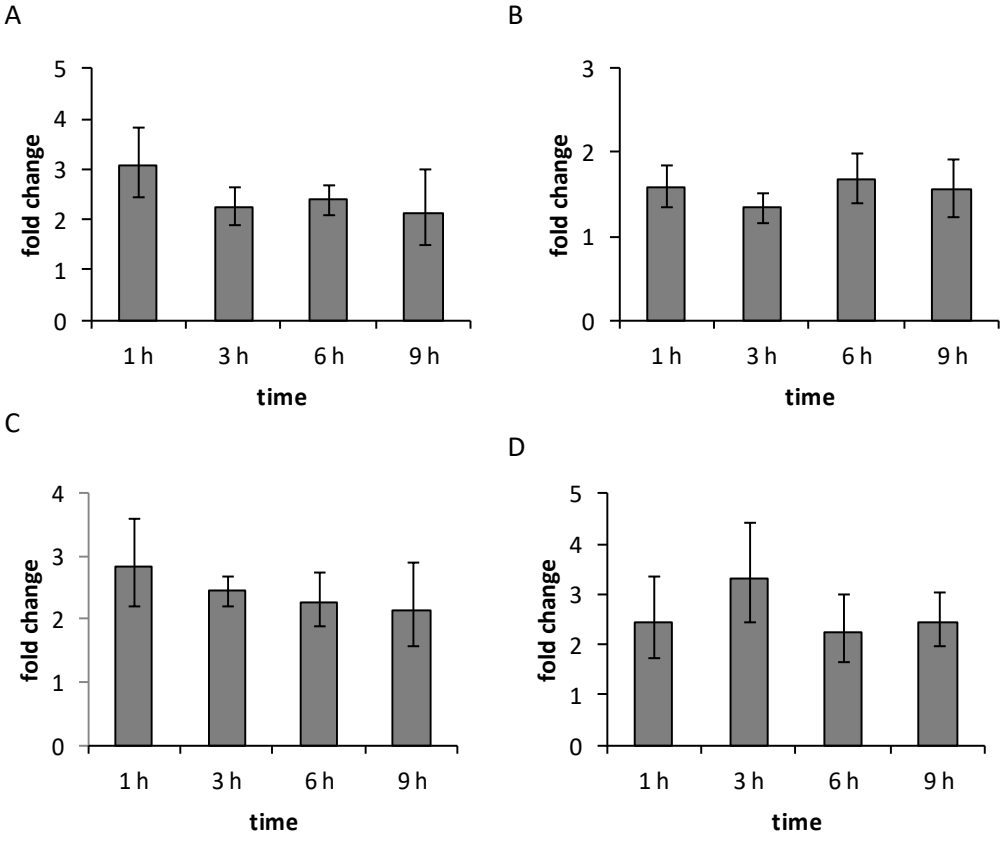


Figure 3.8 relative fold change in *EDS1* expression levels in response to (A) 500 µM ATP (B) 1 mM L-Glu, (C) 50 mM e[Ca^{2+}] and (D) 10 µM masto. Error bars represent SE

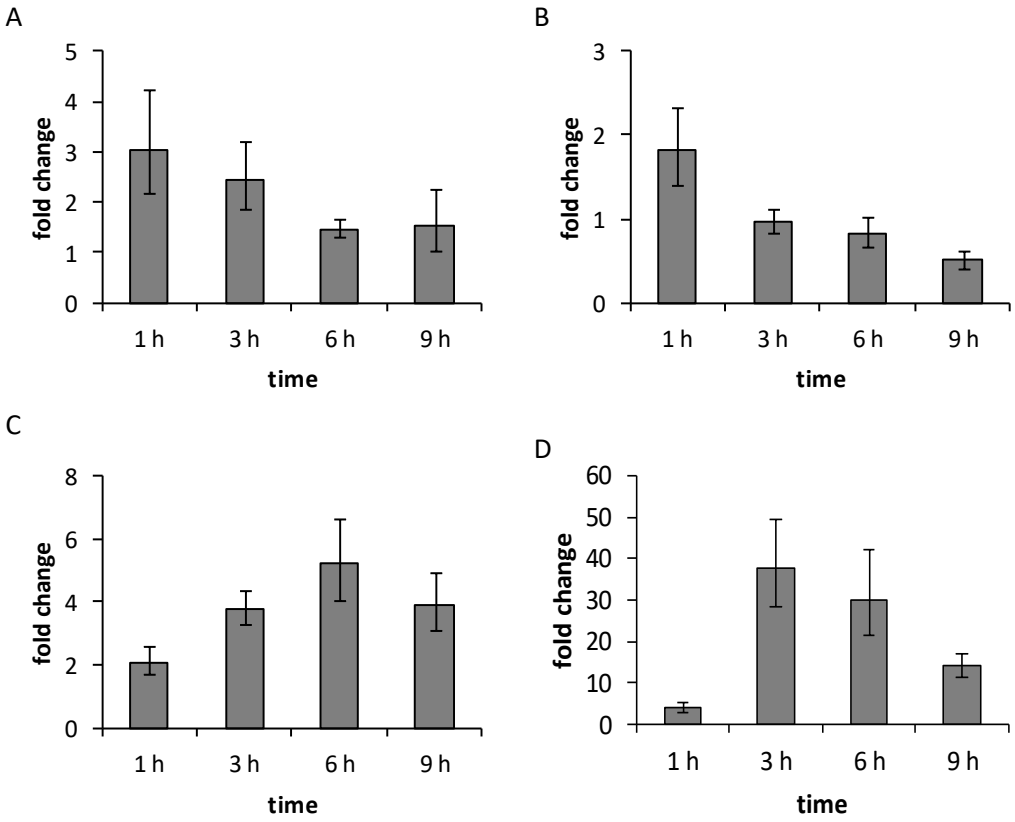


Figure 3.9 relative fold change in *ICS1* expression levels in response to (A) 500 µM ATP (B) 1 mM L-Glu, (C) 50 mM e[Ca^{2+}] and (D) 10 µM masto. Error bars represent SE.

From figures 3.8 and 3.9 it is clear that the treatment inducing the bigger differences in term of fold difference in gene expression was mastoparan; and that changes in *ICS1* expression were bigger than changes in expression of *EDS1*. These measured biological parameters were necessary to develop a mathematical model (described below).

3.2.2 Using gene expression and calcium data to model a specific signalling pathway that leads to nuclear gene expression

3.2.2.1 Mathematical model of the biological network

As the experimental data needed were available, the next step was to create a network whose parameters could be used to generate a mathematical dynamic model able to predict the gene expression output. Basing the network on figure 3.2 (Zhang et al., 2014), figure 3.10 was created, which represents the regulatory network. The upper part (upstream of *EDS1*) can be simplified as a simple process regulated by a Ca^{2+}-signal. This contains the calcium itself, its binding to the calmodulin (CaM) in its four Ca^{2+}-binding sites, and the binding of the active complex $4Ca^{2+}$-CaM to downstream components such as CAMTA3 (AtSR1), CBP60g, and other components not belonging to this network i.e. all the other calmodulin-binding proteins. Only the active complexes $4Ca^{2+}$-CaM-CAMTA3 and $4Ca^{2+}$-CaM-CBP60g will be directly regulating expression of *EDS1* and *ICS1*, while the total of active complexes is three, as also $4Ca^{2+}$-CaM needs to be included.

The second part of the network describes the relationship of the 3 active complexes and the *EDS1* and *ICS1* genes, the positive control of EDS1 on *ICS1* expression and the role of both genes on the downstream response (DR, simplified as an unique element for the sake of modelling). The DR in itself creates a feedback loop on gene expression regulation, which is taken into account.

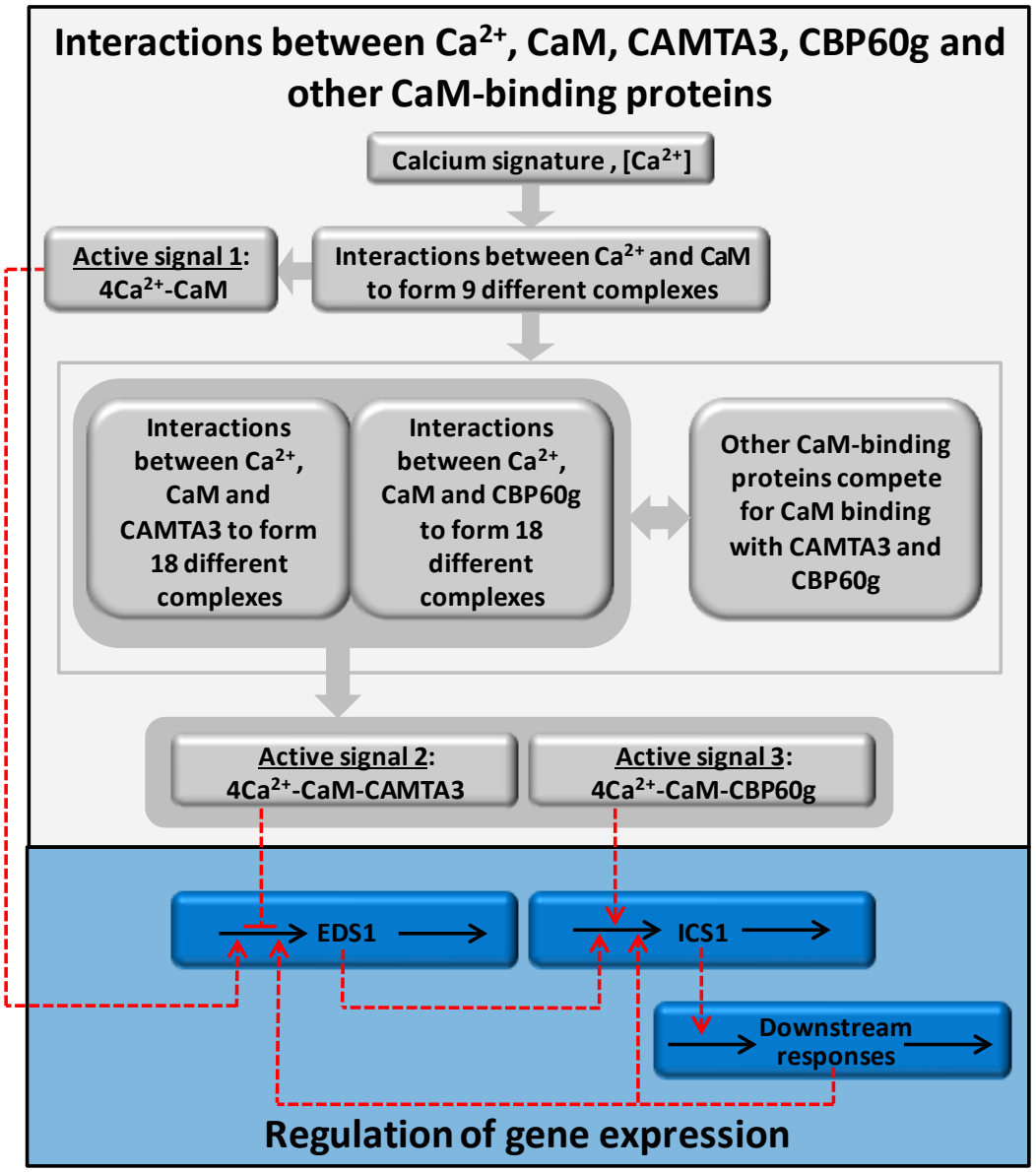


Figure 3.10 From Marc R Knight. Dynamic model to explain how plants decode calcium signatures to generate specific dynamic gene expression responses in plant immunity.

3.2.2.2 The model is able to predict changes in nuclear gene expression in response to specific Ca^{2+}-signatures

The idea behind building this model was to understand the mechanism by which calcium signatures encode the information leading to the correct changes in gene expression. To satisfy this aim, the final model should be able to predict, given the calcium signature as an input, the correct change in gene expression for *EDS1* and *ICS1* as output. Out of the four calcium signatures produced, mastoparan was used to fit and adjust the parameters of the model, knowing both the starting point (calcium signature) and the end point (changes in gene expression). This is represented in figure 3.11, where the calcium signature through time is converted into the amount of concentration of the three active complexes (M_{CCNNb} which corresponds to $4Ca^{2+}$-CaM, M_{CCNNX} which represents $4Ca^{2+}$-CaM-CAMTA3 and M_{CCNNY} which represents $4Ca^{2+}$-CaM-CBP60g). As an output, gene expression changes were reproduced, in terms of fold change of expression of *EDS1* (figure 3.11.E) and *ICS1* (figure 3.11F). The different curves throughout each step represent variations of predicted parameters, such as the time required for transient elevation of calcium concentration to re-establish a steady state (from bottom to top time to reach the steady state was set to 1000s, 3700s, 7300s, 11800s, 15400s, respectively) .

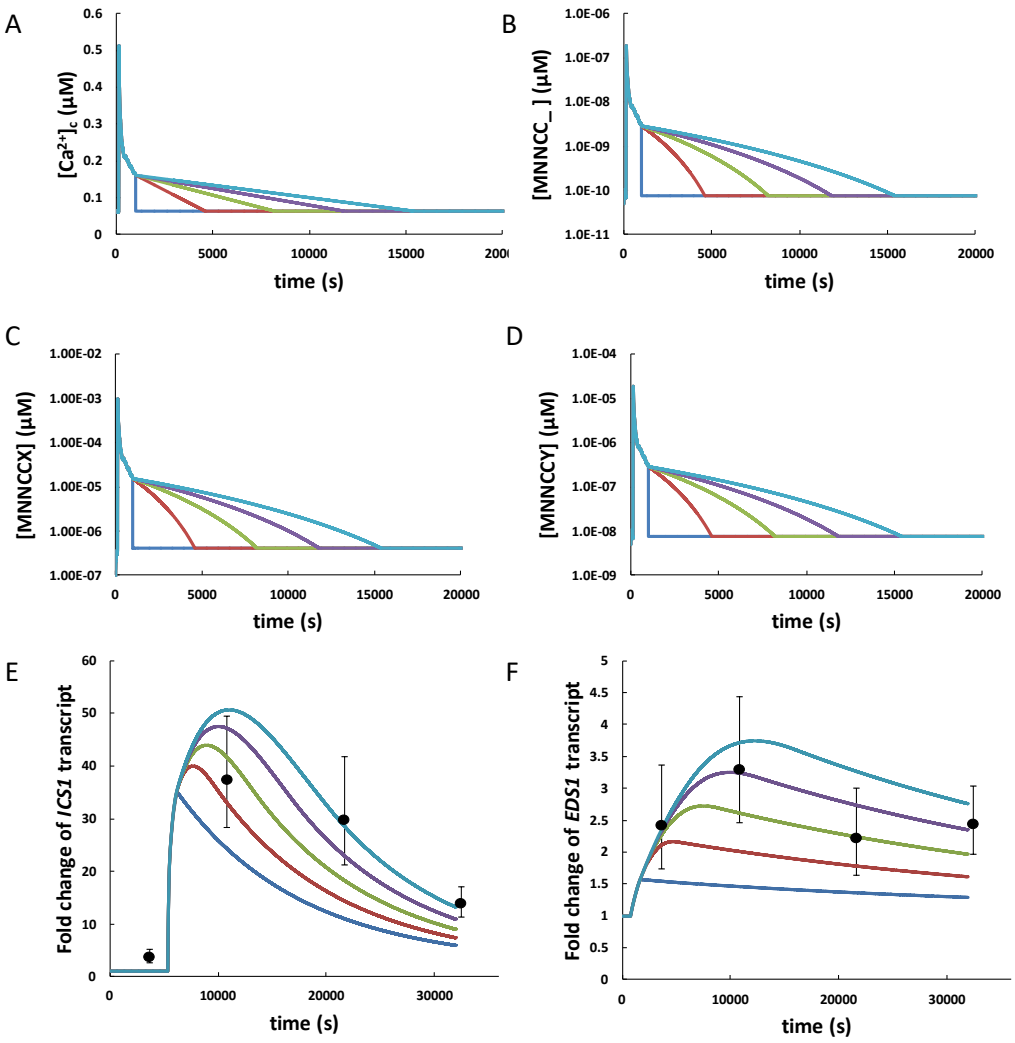


Figure 3.11 Fitting the dynamic model to the experimentally measured transcript fold changes for both *EDS1* and *ICS1* genes in response to the calcium signature induced by 10 µM mastoparan. Each sub-graph in figure 5 has 5 curves, corresponding to different values of the time required for transient elevation of calcium concentration to re-establish a steady state. From bottom to top time to reach the steady state was set to 1000s, 3700s, 7300s, 11800s, 15400s, respectively. (A) calcium signature induced by 10 µM mastoparan and how it approaches its steady state, (B) active signal $4Ca^{2+}$-CaM-CAMTA3, (C) active signal $4Ca^{2+}$-CaM-CBP60g, (D) active signal $4Ca^{2+}$-CaM. (E) Comparison of modelled fold change of *ICS1* transcript with experimental data. (F) Comparison of modelled fold change of *EDS1* transcript with experimental data.

Once the dynamic model had been fitted, then we used the other three calcium signatures as input to see if the mathematical model was able to correctly predict changes in gene expression. As shown in figure 3.12, 3.13 and 3.14, for different parameter sets which we searched and tested, modelling results could always reproduce experimental observations for transcript fold change for both *EDS1* and *ICS1* genes. Since the dynamic model in figure 3.10 can reproduce the experimental data, we consider that the model has captured the main features for the information flow from calcium signatures to *EDS1* and *ICS1* gene expression.

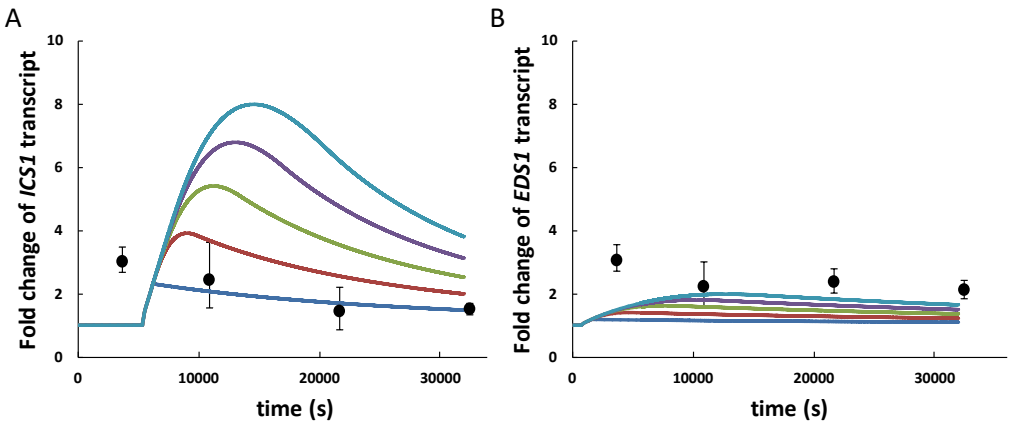


Figure 3.12 Gene expression predictions deriving from the mathematical model for the calcium signature in response to 500 μM ATP. (A) predictions gene expression levels of *ICS1* and (B) *EDS1*, the different curves derive from adjustments of how calcium approaches the steady state (same as in figure 3.11), but they all represent the output in terms of gene expression in response to the same input in terms of calcium signature. Measured gene expression data are represented by the error bars, which correspond to the ones reported in figures 3.8A (*EDS1*) and 3.9A (*ICS1*).

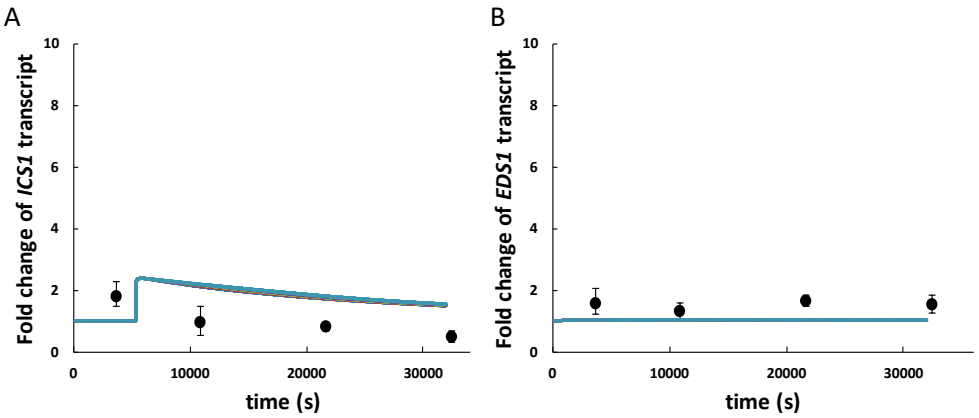


Figure 3.13 Gene expression predictions deriving from the mathematical model for the calcium signature in response to 1 mM L-Glu. (A) predictions gene expression levels of *ICS1* and (B) *EDS1*, the different curves derive from adjustments calcium approaches the steady state (same as in figure 3.11), but they all represent the output in terms of gene expression in response to the same input in terms of calcium signature. Measured gene expression data are represented by the error bars, which correspond to the ones reported in figures 3.8B (*EDS1*) and 3.9B (*ICS1*).

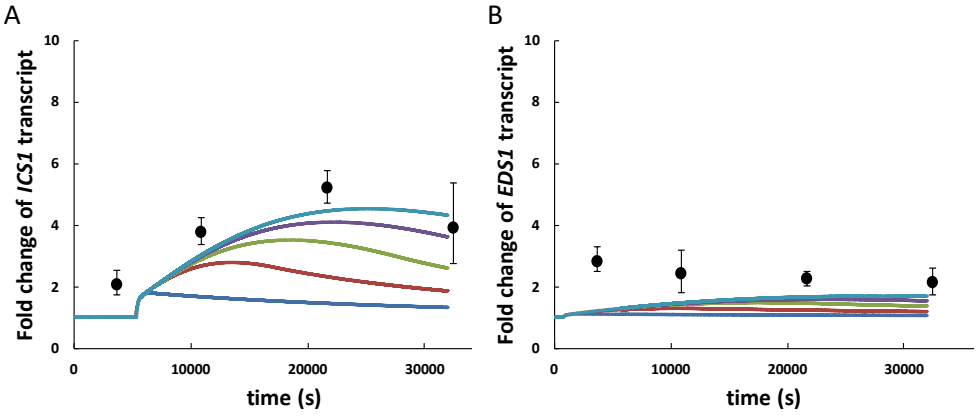


Figure 3.14 Gene expression predictions deriving from the mathematical model for the calcium signature in response to 50 mM e[Ca^{2+}]. (A) predictions gene expression levels of *ICS1* and (B) *EDS1*, the different curves derive from adjustments calcium approaches the steady state (same as in figure 3.11), but they all represent the output in terms of gene expression in response to the same input in terms of calcium signature. Measured gene expression data are represented by the error bars, which correspond to the ones reported in figures 3.8C (*EDS1*) and 3.9C (*ICS1*).

3.3 Discussion

3.3.1 The discriminative power of the calcium signature regulates gene expression

3.3.1.1 Decoding the calcium signature into active signals

Calcium is a universal second messenger, hence it is involved in a multitude of responses that regulate plant physiology and development. From stimulus perception to the plant response the information regarding the nature of the information is conserved. However, transient calcium increases occur in response to a multitude of stimuli such as cold, drought and pathogens, for example, and these calcium increases trigger the appropriate downstream response (Knight et al., 1996, 1997; Wais et al., 2000; Allen et al., 2001; Rentel and Knight, 2004). Hence calcium is a convergence point for a variety of plant responses, but how can a single ion encode all this information and maintain the specificity? To answer this question, in the late 90s the "calcium signature hypothesis" was developed (McAinsh and Hetherington, 1998). This idea envisages a role for the kinetics of the calcium increase in decoding the information about the stimulus sensed, hence the specificity. This hypothesis is supported by factual evidence coming from calcium measurements: for different natural stimuli the calcium kinetics are characteristic (Knight et al., 1996, 1997; Wais et al., 2000; Allen et al., 2001; Rentel and Knight, 2004; Dodd et al., 2010). Furthermore, it has been demonstrated in some cases, e.g. in guard cell closure (Allen et al., 2001) and in the nodulation response of legume root hairs (Oldroyd and Downie, 2006), that cells "decode" these specific calcium signatures which lead to the appropriate downstream pathway. The relationship between calcium signature and changes in gene expression was formally tested by imposing different increases in calcium. Hence Whalley and collaborators demonstrated the the ability of different signatures to control non-overlapping set of genes, which are regulated by different transcription factors (Whalley and Knight, 2013). Thus, calcium signatures are thought to encode specificity concerning the primary stimulus, thus relying that information in the

end response via calcium-binding proteins (the "decoders") (Hashimoto and Kudla, 2011). However, how the Ca^{2+}-signal is decoded by the cell is still an open question.
In the work describe in this chapter, we developed a novel integrated experimental and modelling study in which a wide range of biological knowledge in the literature was integrated with experimental data. This model aims to establish information flow from the calcium increase to the expression of the two SA biosynthesis-related genes *EDS1* and *ICS1*, by using the pathogen SA-mediated pathway as a starting point. The biological knowledge accumulated over many years in the literature was abstracted into a dynamical model (figure 3.10). The model was parameterised by using experimentally measured parameters in the literature (Liu et al., 2015) and by fitting the model to the experimentally measured transcript fold changes for both *EDS1* and *ICS1* genes in response to the calcium signature induced by 10 µM mastoparan (figure 3.7). We further demonstrated that the model developed in this study was always able not only to reproduce experimental observations (figure 3.11), but also to make predictions that are experimentally validated (figures 3.12, 3.13, 3.14). Therefore, a combined experimental and modelling study was able to reveal how different calcium signatures are decoded to generate specific response of *EDS1* and *ICS1* expression responses. Relationships between calcium signatures and responses of *EDS1* and *ICS1* gene expression can thus be elucidated and predicted.

The first step to obtain good predictions from the model was to create highly reliable data as an input, hence the initial calcium signatures must be representative of the true behavior of plants in response to that agonist. In order to get calcium signatures as reproducible as possible, hence to avoid any influence from any other variable parameter such as light conditions, growth medium and circadian/diurnal effects, calcium data were recorded from *Arabidopsis* seedlings over different days using independent batches of plants of the same age, and taking into account of randomising the time of the day. Furthermore, calcium agonists rather than natural stimuli were preferred, as the aim of this model is to understand how a calcium signature is decoded, independently from the nature of the stimulus given. A similar approach was adopted with the generation of

electrically-induced stimulus-independent calcium signatures (Whalley and Knight, 2013; Liu et al., 2015). The same randomisation in the sample collection was adopted while generating material for gene expression measurements further used for creating the model.

In order to understand the dynamic model, it is necessary to have in mind how it is possible to predict, by giving a calcium concentration through time, the amount of the active form of $4Ca^{2+}$-calmodulin-CAMTA. This non-linear relationship has been mathematically modelled by Liu and colleagues (Liu et al., 2015), and it is represented by "the cube" (figure 3.1).

This cube represents all the possible transitions that the calmodulin protein can undergo to reach the active state, and all of them have a different, but finite probability to exist. These transition probabilities were either found in the literature, or they were hypothesized, and they are all reported in Liu et al., 2015. Amongst all these states, only the active form, represented by M_{CCNNX}, is able to cause changes in gene expression (Pifl et al., 1984). In the SA-dependent calcium-mediated pathway hereby modelled, there are three cubes, represented in figure 3.10. The first one is exactly the one modelled previously (Liu et al., 2015), as AtSR1 is also called CAMTA3, and it binds the calmodulin. The second cube is analogous to the first one, but as an output it gives the amount of the active form of $4Ca^{2+}$-CaM-CBP60g, which is another transcription factor whose activation is calmodulin-dependent. CAMTA and CBP60g are well characterised Ca^{2+}/calmodulin-regulated transcription factors, and both have a CaM-binding domain (Finkler et al., 2007; Galon et al., 2008; Kim et al., 2009; Wang et al., 2009; Zhang et al., 2010; Reddy et al., 2011; Bickerton and Pittman, 2012; Poovaiah et al., 2013). Since both CAMTA3 and CBP60g have CaM binding domains, it has been proposed that Ca^{2+} signals regulate the network of *EDS1* and *ICS1* expression and their downstream response (Zhang et al., 2014).

In figure 3.10, we consider that CAMTA3 has one calmodulin binding site. Since CaM has two pairs of Ca^{2+}-binding EF-hand domains located at the N-and C-terminus respectively, interactions of Ca^{2+}-CaM and CAMTA3 generate 18 different binding

complexes via 33 elementary binding processes (Liu et al. 2015). Similarly, interactions of Ca^{2+}-CaM and CBP60g also generate 18 different binding complexes, 9 of which are Ca^{2+}-CaM only complexes and are analogous to those involved in interactions of Ca^{2+}-CaM and CAMTA3. For each of other CaM binding proteins existing in the cell, an additional 9 binding complexes are generated. Thus, for *n* CaM binding proteins or transcriptional factors, there are $9(n+1)$ binding complexes. As described above, experimental measurement has shown that $4Ca^{2+}$- CaM is the active CaM -Ca^{2+} binding complex (Pifl et al., 1984). Therefore, this work assumes that the $4Ca^{2+}$-CaM-TF complex is the active complex for gene expression response (Liu et al. 2015). For CAMTA3 and CBP60g, the active complexes for gene expression response are $4Ca^{2+}$-CaM-CAMTA3 and $4Ca^{2+}$-CaM-CBP60g, respectively.

For the reason of simplicity, in this model the fact that any other protein rather than calmodulins can bind calcium, such as calcium-dependent protein kinases, calmodulin-like proteins and calcineurin B-like proteins (Sanders et al., 1999; Sanders et al., 2002; Dodd et al., 2010; Kudla et al., 2010; Hashimoto and Kudla, 2011), has not been taken into account, as the starting assumption is that the majority of the calcium controlling gene expression of this network will be acting through calmodulin binding.
Moreover, in a cell, other proteins are also able to bind calmodulin. As a consequence, less calmodulin is available to be bound by CAMTA3 and CBP60g, hence to take part to the immune response pathway. In the model this is represented in the third cube.

3.3.1.2 Predicting changes in nuclear gene expression

Expression levels of *EDS1* and *ICS1* were quantified in response to the different agonist-induced calcium signatures, by using real-time PCR as described previously (Hemsley et al., 2014), and results are shown in figure 3.8 and figure 3.9.
Firstly, the imposed calcium elevations from the four different treatments were in certain cases capable of inducing quite different responses e.g. *ICS1* expression in response to

mastoparan compared to the other three agonists (figure 3.9). In this case mastoparan treatment induced *ICS1* gene expression at 3 hours by approximately 37 folds whereas the other three calcium signatures could only induce much smaller fold changes in *ICS1* gene expression (ca. maximum 4 folds at 3 hours). Secondly, in other cases the imposed calcium elevations resulted in relatively similar temporal trends in gene expression responses e.g. *ICS1* expression in response to ATP or glutamate (figure 3.9), and *EDS1* expression in response to ATP or external calcium (figure 3.8). Thirdly, different imposed calcium signatures caused a qualitatively similar, but quantitatively different temporal gene expression response e.g. *ICS1* expression in response to ATP and glutamate, the kinetics of expression being similar in the two but ATP producing a greater magnitude of gene expression (figure 3.9). These experimental data suggest that plant cells can respond to different calcium signatures to generate specific *EDS1* and *ICS1* expression responses.

The regulatory network upstream of *EDS1* gene is composed of many components, which are regulated by Ca^{2+} signals (Zhang et al. 2014). For modelling development, we simplified the upstream of *EDS1* gene expression into a simple process that is activated by the Ca^{2+} signal. Since experimental measurement has shown that $4Ca^{2+}$- CaM is the active CaM -Ca^{2+} binding complex (Pifl et al., 1984), we assume $4Ca^{2+}$- CaM complex is the active Ca^{2+} signal that regulates *EDS1* gene expression from its upstream. In addition, we simplify *ICS1* downstream into a single response component, DR. The transcription factor CAMTA3 inhibits *EDS1* gene expression, and DR activates *EDS1* gene expression (Zhang et al., 2014). The expression of *ICS1* is positively regulated by *EDS1*, CBP60g transcription factor and DR (Zhang et al., 2014). Thus, the interaction of *EDS1*, *ICS1* and DR forms a regulatory network, as seen in figure 3.2. They regulate mutually via feedbacks and feedforwards, i.e. upstream and downstream components can mutually regulate each other.

In this dynamic model, certain parameter such as the time calcium required to reach the steady state, the time delay between the calcium increase and the onset of changes in gene

expression, or the constant of binding of the active complexes to the DNA were not known. To solve this problem, we tried to fit the parameters in the model to appropriately describe the biological network by using the Ca^{2+}-signature measured in response to 10 μM mastoparan as an input, and the measured *EDS1* and *ICS1* changes in gene expression as an output (figure 3.11). After fitting this model to the real data, then this mathematical network was used to predict changes in *EDS1* and *ICS1* levels in time given as input any calcium signature. To this aim, the cytosolic calcium increases in response to ATP, L-Glu and e[Ca^{2+}] shown in figure 3.7 were used. As a result, the model was able to predict the trend in the gene expression levels of *EDS1* and *ICS1*, and by comparison with the measured values, the predictions resulted correct. The different curves represented in figures 3.12, 3.13 and 3.14 indicate different predictions, specifically outputs varied by taking into account the possible variability of the unknown parameters (specifically the time calcium takes to reach the steady state).

3.3.2 Emerging properties of the calcium signalling response

Many of the properties required to understand the model already emerged from the model of the $4Ca^{2+}$-CaM-CAMTA. Indeed Liu and colleagues (2015), reported that the binding of the four calcium ions to the calmodulin may follow different binding mechanisms, hence they can bind to the four calcium binding sited of calmodulin in a differential order (Galon et al., 2008; Kim et al., 2009; Bickerton and Pittman, 2012), all according to the thermodynamics. This is a non-linear process, which allows a non-linear amplification of the calcium signal. This kind of amplification needs to be incorporated in the model to explain the ability of one single ion to induce a wide range of changes in nuclear gene expression. Indeed, small changes in cytosolic calcium levels can induce relatively large fold changes in gene expression (Liu et al., 2015).

Another important variable is the binding constant of the active complexes to the DNA. These predicted parameters are fundamental to explain the large fold changes in gene expression, as if the binding and release is too fast then the active complexes cannot accumulate on the DNA. On the contrary if the DNA binding constants are too slow, then

the DNA is bound most of the time, hence the dynamic range for changes in the levels of gene expression is saturated at low calcium concentrations.

Another property that emerged is that the fold ratio in the calcium concentration in respect to the Ca^{2+}-baseline is more important than the change in absolute calcium values in the cell. Additionally, the gene expression response depends on the calcium history and it accumulates all the information during the lifetime of a calcium signature. This is because the calcium is able to affect the amount of active complex at a determined time, but the starting amount of active complex at each timepoint also depends upon calcium. In this way the calcium signal can induce accumulation of active complex over time. Thus, it was demonstrated that the calcium history and kinetics are very important to regulate the gene response (Liu et al., 2015).

Another relevant parameter in controlling changes in gene expression is the time delay between the calcium increase and changes in gene expression, indicating that the cell machinery must have evolved to adjust the timing between these two events to allow the appropriate changes in gene expression to occur, maintaining a wide range in fold-change in gene expression.

Calcium signatures are generally relatively short lived increases in calcium concentration. As a dynamically transient signal, a calcium signature generally tends to return to its original steady state. Traditionally, much attention has been paid to the characteristics of a calcium signature within a relatively short period after initiation. How a calcium signature returns to its steady state is largely ignored. This work shows that the time required for a calcium signature to return to its steady state is a factor which quantitatively affects the subsequent Ca^{2+}-regulated gene expression response. By considering that a plant cellular system is a dynamical system, the emergence of a calcium signature is the result of perturbation to a steady state. How the transient calcium concentration elevation returns to its steady state is determined by the properties of the dynamical system (Murray 2002). Thus, it is not necessary that a higher calcium

concentration elevation will take longer time to return its steady state, and *vice versa*. Thus, experimental measurement of the "tail" of a calcium signature (i.e. how a calcium signature approaches its steady state) will in the future be of importance for quantitatively studying the relationship between gene expression and a calcium signature.

3.3.3 Conclusions

The results presented here have demonstrated that a novel integrated experimental and modelling study, in which a wide range of biological knowledge in the literature was integrated with my experimental data, can elucidate and predict the response of *EDS1* and *ICS1* gene expression to different calcium signatures. The novelty of this work does not only rely on the fact that this model is "cracking the code" of the calcium signalling, by mathematically explaining how the specificity can be encoded by a biological system, and the mechanism by which this can be achieved. This model is also able to produce rather accurate predictions of gene expression changes, hence it can predict the behaviour of a biological system, which is one of the major aims of system biology.

Chapter 4

The role of calcium in regulating chloroplast gene expression

4.1 Introduction

In a plant cell, one of the key signals able to regulate nuclear gene expression and affect the transcriptome is the second messenger calcium (Whalley et al., 2011; Whalley and Knight, 2013). Indeed, in response to a series of biotic and abiotic stresses, cytosolic calcium increases with stimulus-specific kinetics, named "Ca^{2+}- signatures" (Webb et al., 1996; McAinsh and Hetherington, 1998), are able to trigger the appropriate downstream signalling pathway, which leads to a reprogramming of the set of nuclear genes required in those stress conditions (this concept has been extensively explained in chapter 3 and in the Introduction).

In the plant cell specifically there is another source of genetic material, which comes from the chloroplast (in addition to the mitochondrial one). This photosynthetic organelle maintains its own genome named plastome, located in the chloroplast stroma (Krupinska et al., 2013) and is able to autonomously transcribe its own set of genes, that are mainly involved in photosynthesis and gene expression (Sugiura, 1995). Since the endosymbiotic event from which originated the first photosynthetic cell, there has been a movement of genes from the plastome to the plant nucleus, called gene transfer, a process which is still taking place. However, despite the costs of keeping additional transcription and translation machinery, the chloroplast maintained its own genetic material. Consequently, this must come with evolutionary advantages (described in the Introduction); one possible

explanation being the possibility of quickly changing the expression levels of transcripts as a consequence of fast changes in chloroplast physiology (e.g. redox potential) to rapidly adjust to the new conditions (Allen, 1993; Pfannschmidt et al., 1999). As calcium is a fast-acting second messenger which would be suitable to achieve the aim of rapidly affecting gene expression *in situ* in the chloroplast, the biological question leading this research project was whether, similar to the nucleus, chloroplast gene expression is regulated by calcium increases in the stromal compartment.

Recently, evidence of chloroplast stromal calcium increases have been emerging in the literature (Johnson et al., 1995; Sai and Johnson, 2002; Manzoor et al., 2012; Nomura et al., 2012), as well as some knowledge about calcium transporters in this organelle (Nomura and Shiina, 2014), offering a solid starting point to answer this biological question.

Aims of the research described in this chapter:

- To find suitable conditions to controllably increase calcium levels in the chloroplast stroma, and to block this increase.
- To quantify chloroplast gene expression upon controlled changes in the stromal calcium levels.
- To measure if any calcium increase is occurring in the stroma upon stimulation with treatments that are known to induce changes in the chloroplast transcriptome; the final aim is to see if calcium signalling is involved in controlling chloroplast gene expression.

4.2 Results

4.2.1 Measuring chloroplast gene expression upon controlled stromal calcium increase

4.2.1.1 Validation of the RT-PCR technique on chloroplast genes

In order to test for the ability of the RT-PCR technique to detect chloroplast-encoded mRNAs we performed an experiment where 8 day old *Arabidopsis* plants were kept in the light for 0 h, 12 h, 24 h and 48 h, and control plants were kept in the dark for 0 h and 48 h. These samples were used for total RNA extraction and cDNA synthesis using random nucleotides as described in chapter 2 Material and Methods. Dilutions (1: 50) of the original cDNA were used for RT-PCR, and the efficacy if this technique tested on two Rubisco subunits: RBCS1A and RBCL. *RBCS1A* (At1g67090) encodes for the Rubisco small subunit, it is nuclear encoded, while the gene for the Rubisco large subunit (*RBCL*, Atcg00490) is chloroplast encoded, and the levels of these two genes are known to be affected by long exposure to light (Chun et al., 2001; Feng et al., 2014). Results of the light treatment are shown in figure 4.1, where *RBCS1A* levels increase proportionally to the length of time of light exposure (figure 4.1A), with a peak of expression at 48 h, while in the control kept in the dark there is no corresponding transcript increase for the same timepoint. *RBCL* levels also changed depending on the light conditions (figure 4.1B), but the fold change is two orders of magnitude smaller than the one measured for *RBSC1A*, and the peak does not coincide (24 h rather than the 48 h measured for the gene encoding the nuclear small subunit). *PEX4* (At5g25670) was used as an housekeeping gene. These results are an indication of the ability of RT-PCR as a technique to detect fold changes in chloroplast genes, hence it can be used for this purpose.

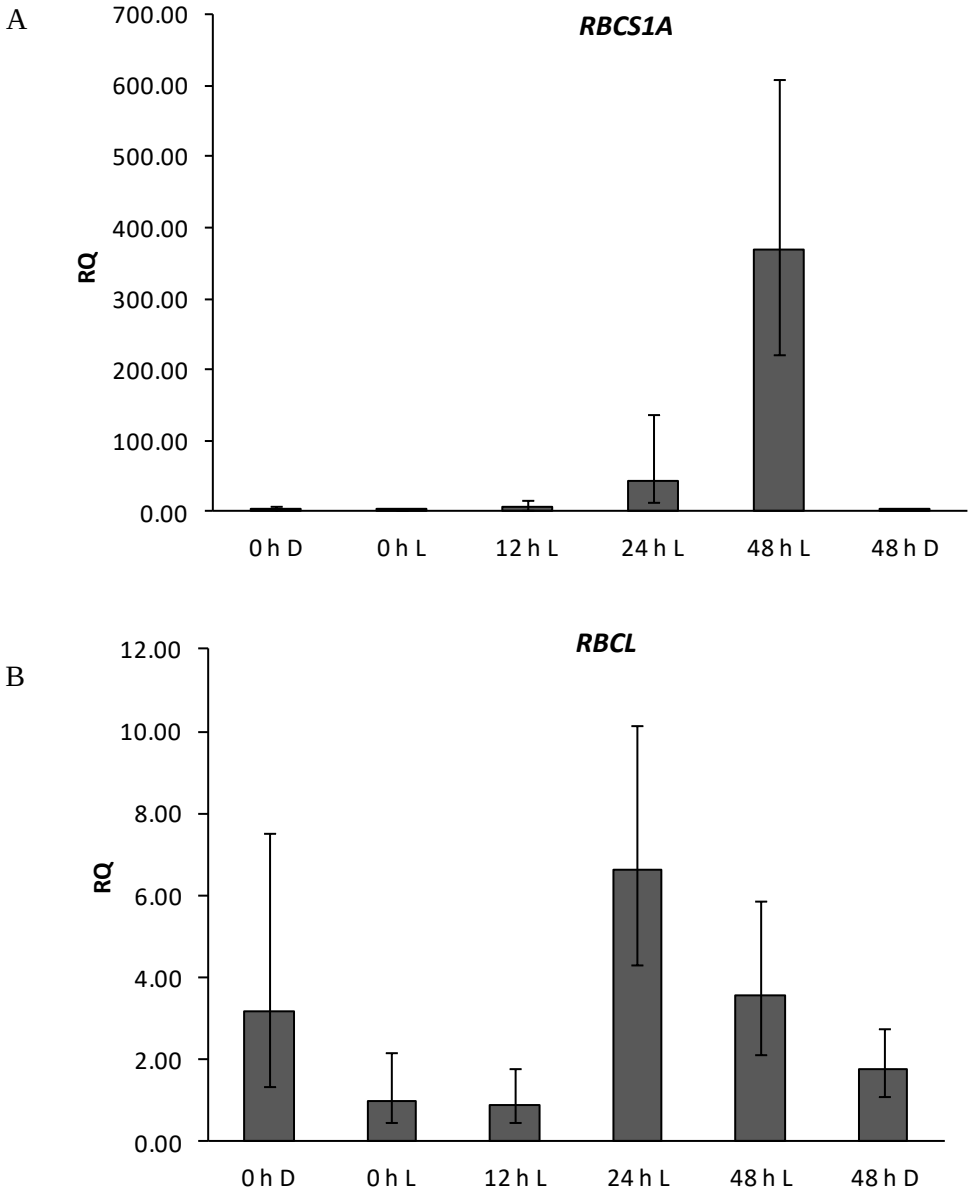


Figure 4.1 Relative transcript abundance of the nuclear encoded gene *RBCS1A* (A) and the chloroplast encoded gene *RBCL* (B) in response to 0 h, 12 h, 24 h or 48 h of constant light (L), or 0 h or 48 h of darkness (D). Error bars = standard error (SE).

4.2.1.2 Stromal calcium increases in response to agonists

In order to answer the biological question "is calcium regulating chloroplast gene expression?" the first step required is to find a way to controllably increase Ca^{2+}-levels in the stroma. For this purpose, a chemical known to increase cytosolic calcium, named mastoparan (Takahashi et al., 1998), was tested on *Arabidopsis* seedlings expressing the bioluminescent calcium reporter aequorin targeted to the chloroplast stroma (pMAQ6 plants). As a positive control the same chemical was tested on pMAQ2 plants, where aequorin is targeted to the cytosol. A dose-response curve of pMAQ2 and pMAQ6 plants to mastoparan 10 μM, 25 μM, 50 μM and 100 μM is represented in figure 4.2 (figure 4.2A and 4.2B represent pMAQ2 and pMAQ6, respectively). It is possible to see that mastoparan is able to induce a calcium increase in both the cytosolic (fig 4.2A, as expected from the literature, Takahashi et al., 1998) and stromal (figure 4.2B) compartments, with different kinetics. By looking at the pMAQ6 dose-response curve (figure 4.2B) it can be seen that at 50 μM mastoparan the calcium response reaches a plateau, and increasing the mastoparan concentration did not result in an increase in the calcium levels. The same observation is not valid for pMAQ2 (figure 4.2A), where at 100 μM mastoparan calcium is still increasing, but since the focus of this experiment was to find a condition to control stromal calcium, the 50 μM concentration of mastoparan was chosen for further experiments.

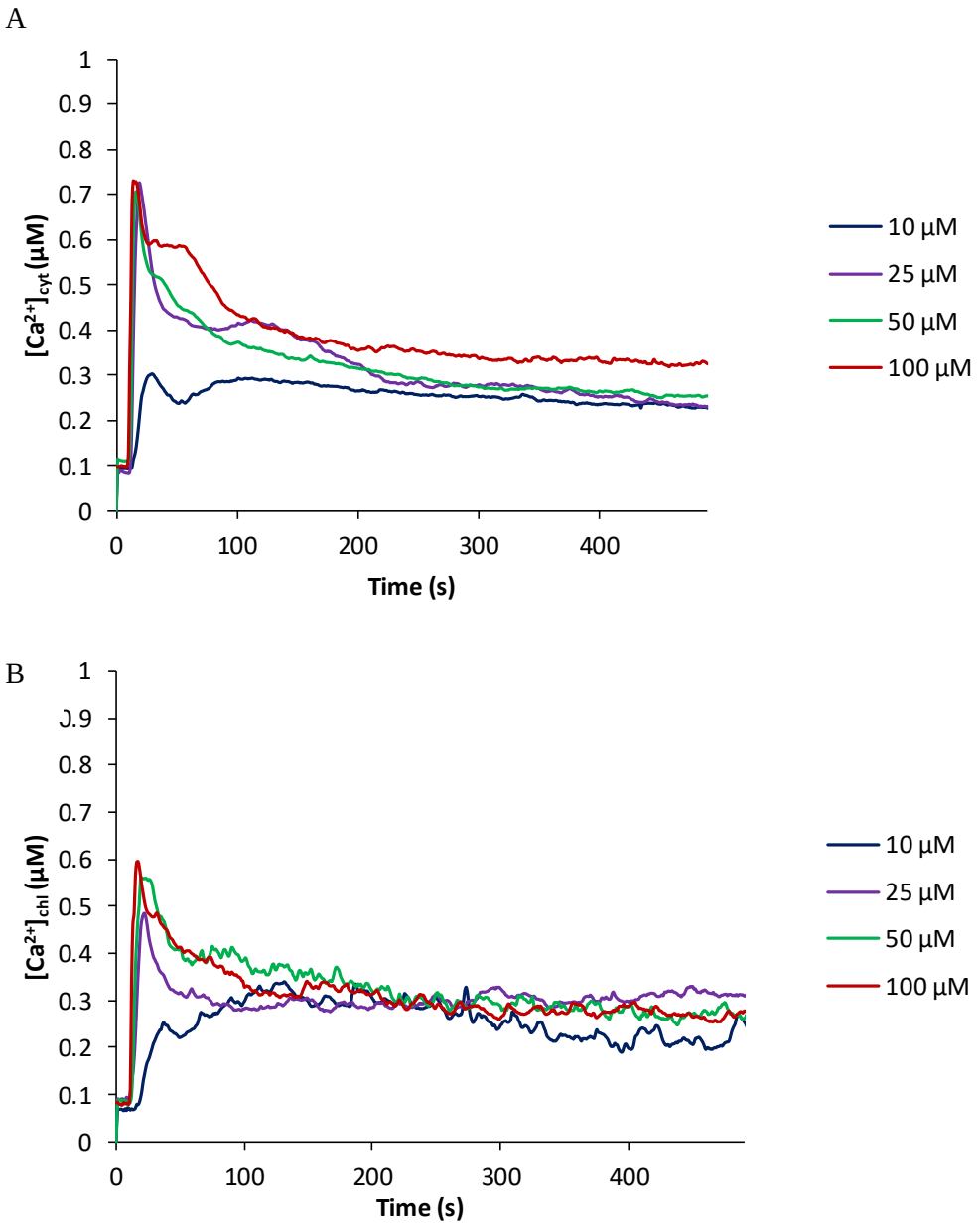
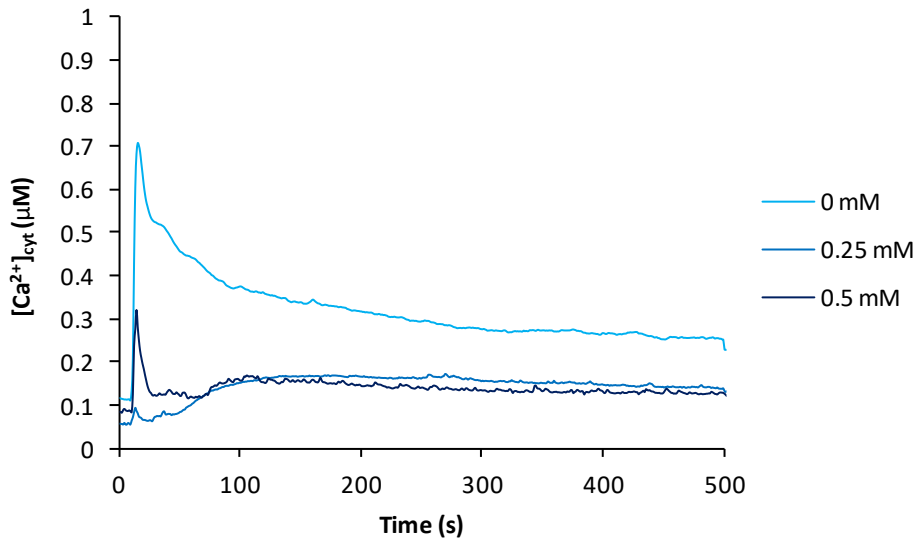


Figure 4.2 Calcium traces of *Arabidopsis* seedlings treated with different concentrations of mastoparan. (A) Cytosolic (pMAQ2) and (B) stromal (pMAQ6) calcium curves are represented. Injection time = 10 seconds.

4.2.1.3 Inhibition of the agonist-induced stromal calcium increase

In order to understand if calcium is able to affect chloroplast gene expression it is necessary to find the conditions to switch the gene expression on by inducing a calcium increase with an agonist (in this case mastoparan 50 μM). However, this evidence by itself is not sufficient. To prove that calcium is triggering changes in transcript expression we need a way to block the mastoparan-induced calcium signal, and as a consequence, changes in gene expression should not occur (Jaffe, 1980). Hence, the next step was to find a way to antagonise the calcium increase induced by the agonist mastoparan. For this purpose, pMAQ2 and pMAQ6 plants were pre-incubated with lanthanum chloride ($LaCl_3$) at 0.25 mM and 0.5 mM for 30 minutes before adding mastoparan 50 μM. Calcium traces representing this dose-response experiment are reported in figure 4.3. In figure 4.3A, the positive control pMAQ2 is represented, where $LaCl_3$ is able to block the mastoparan-induced calcium increase, confirming the calcium inhibitor activity of lanthanum (Knight et al., 1996). Figure 4.3B represents the stromal response after the lanthanum chloride pre-incubation, where it is evident that the mastoparan-induced stromal calcium increase can be blocked by the pre-treatment. Specifically, the higher concentration of $LaCl_3$ (0.5 mM) is responsible for an almost total inhibition of the calcium response, while the lower concentration (0.25 mM) was less efficient in blocking the signal. To further test chloroplast gene expression upon calcium increase, the conditions chosen were therefore mastoparan 50 μM to induce the calcium increase and 30 minutes pre-incubation with $LaCl_3$ 0.5 mM to block the effect of this calcium agonist.

A



B

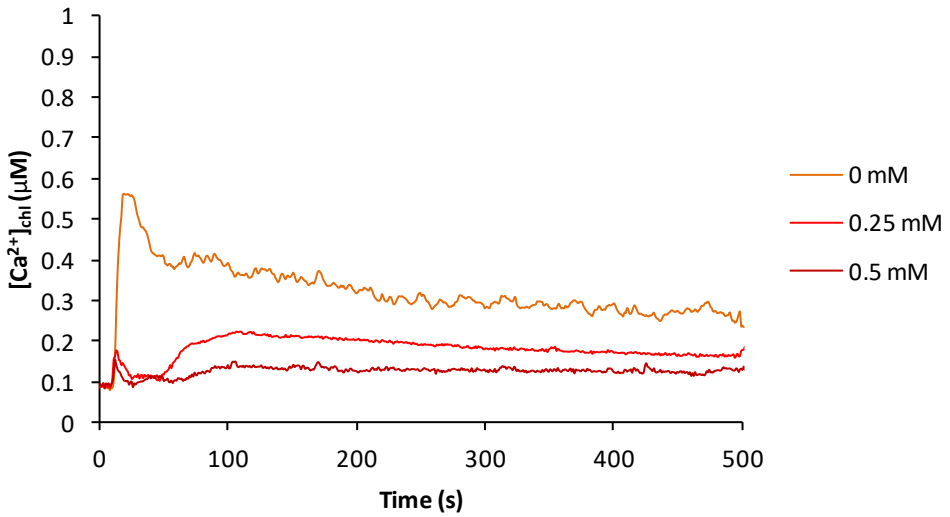


Figure 4.3 Calcium traces of *Arabidopsis* seedlings upon stimulation with mastoparan 50 µM, pre-treated for 30 minutes with different concentrations of lanthanum chloride. (A) Cytosolic (pMAQ2) and (B) stromal (pMAQ6) calcium curves are represented. Injection time = 10 seconds.

4.2.1.4 Changes in chloroplast gene expression upon agonist-induced calcium increase

Since the system to induce and block stromal calcium had now been established, it was possible to test the starting hypothesis, which was: can calcium directly control chloroplast gene expression? To do so, the first step was to measure chloroplast gene expression upon mastoparan treatment. *Arabidopsis* seedlings were treated with mastoparan 50 μM, or a water control, for a series of timepoints ranging from 30 minutes to 48 hours (30 min, 1h, 3 h, 6 h, 12 h, 24 h, 48 h), total RNA was extracted from these samples and the cDNA was then tested for differential gene expression.

Before testing for chloroplast genes, we tested if the mastoparan treatment was effective by using two nuclear genes which have been shown to be up-regulated in response to mastoparan (Whalley et al., 2011): *RAP2.6* (At1g43160) and *ABR1* (At5g64750). From Fig 4.4A it is evident that the mastoparan treatment was effective as it induced the expression of *RAP2.6*, with a peak between 1 h and 6 h. For convenience, only this range of timepoints was used on *ABR1*, whose expression is indeed also up-regulated in response to the mastoparan treatment, as expected from previous evidence (figure 4.4B).

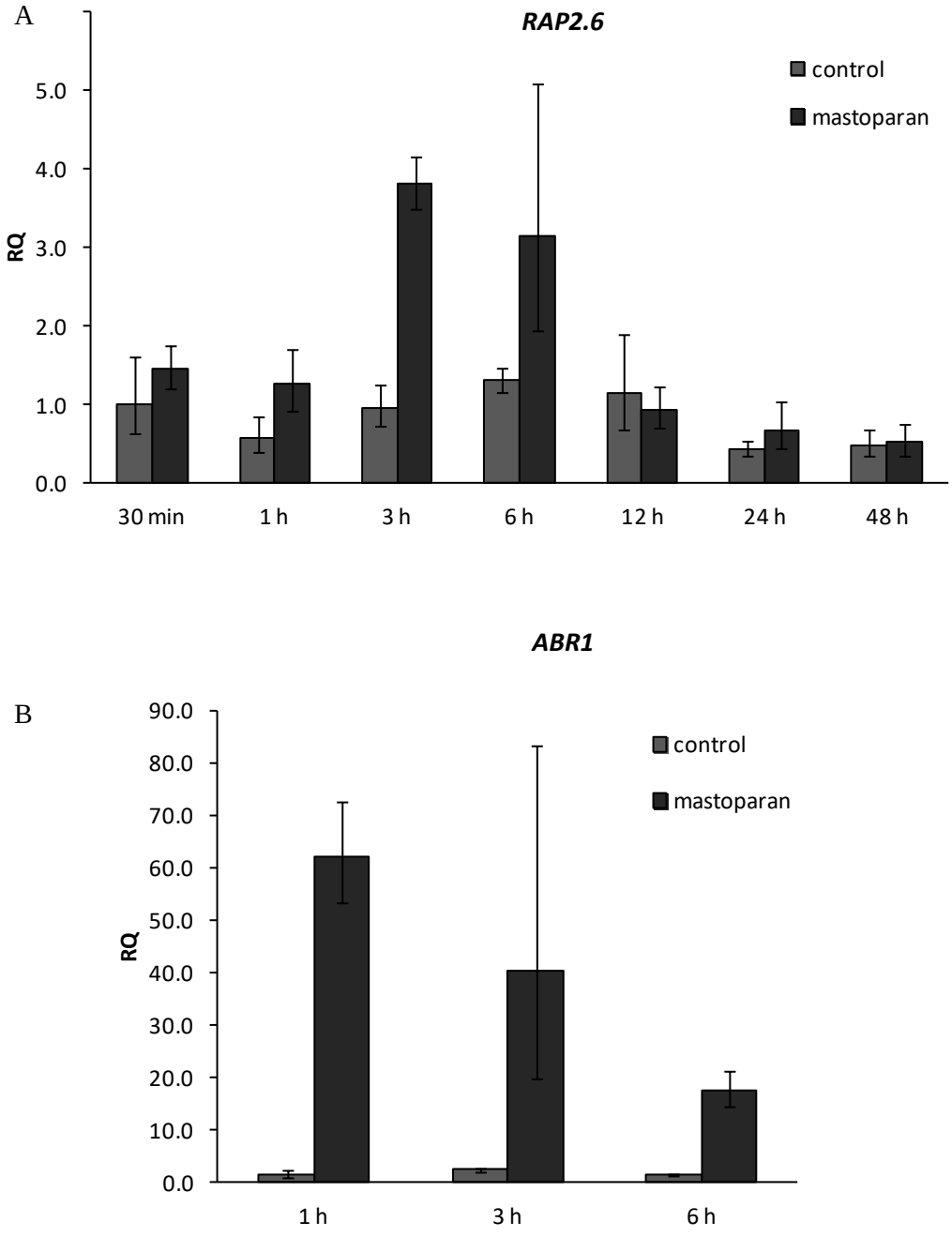


Figure 4.4 Relative gene expression of *RAP2.6* (A) and *ABR1* (B) of *Arabidopsis* seedlings in response to mastoparan treatment and water control at different timepoints. Error bars = SE.

Hence, the level of chloroplast encoded genes was tested upon mastoparan treatment, starting from *PSBA* (Atcg00020), which encodes for the photosystem II reaction centre protein A, and *RPOC1* (Atcg00180), gene for the RNA polymerase beta' subunit. These genes were tested for all the available timepoints, and results are represented in figure 4.5. None of these first two candidates showed any significant difference in the level of transcripts for any of the timepoints tested in plants treated with mastoparan rather than water.

More chloroplast genes were tested on a limited range of timepoints (1 h, 3 h and 6 h) to see if any of these are affected by the mastoparan treatment. These genes are *PSAA* (Atcg00350), *PETB* (Atcg00720), *PSBD* (Atcg00270) and *PSBB* (Atcg00680) and they all belong to different operons. Results of the RT-PCR experiments are reported in figure 4.6, and it is clear that the mastoparan treatment is not affecting the expression of these genes, since there were no significant differences for any gene at any timepoint compared to the water control.

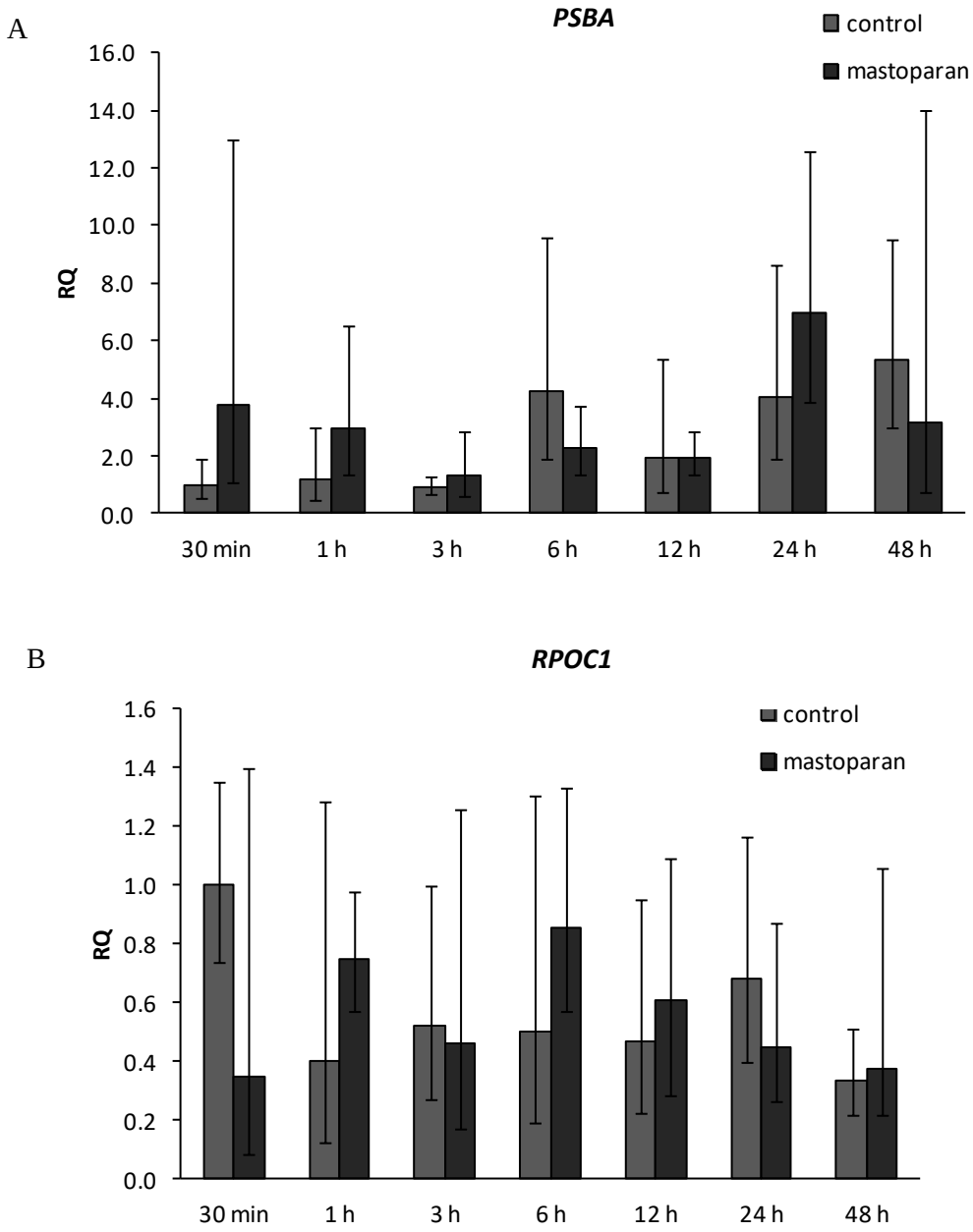


Figure 4.5 Relative gene expression of the chloroplast-encoded genes *PSBA* (A) and *RPOC1* (B) of *Arabidopsis* seedlings in response to mastoparan treatment and water control at different timepoints. Error bars = SE.

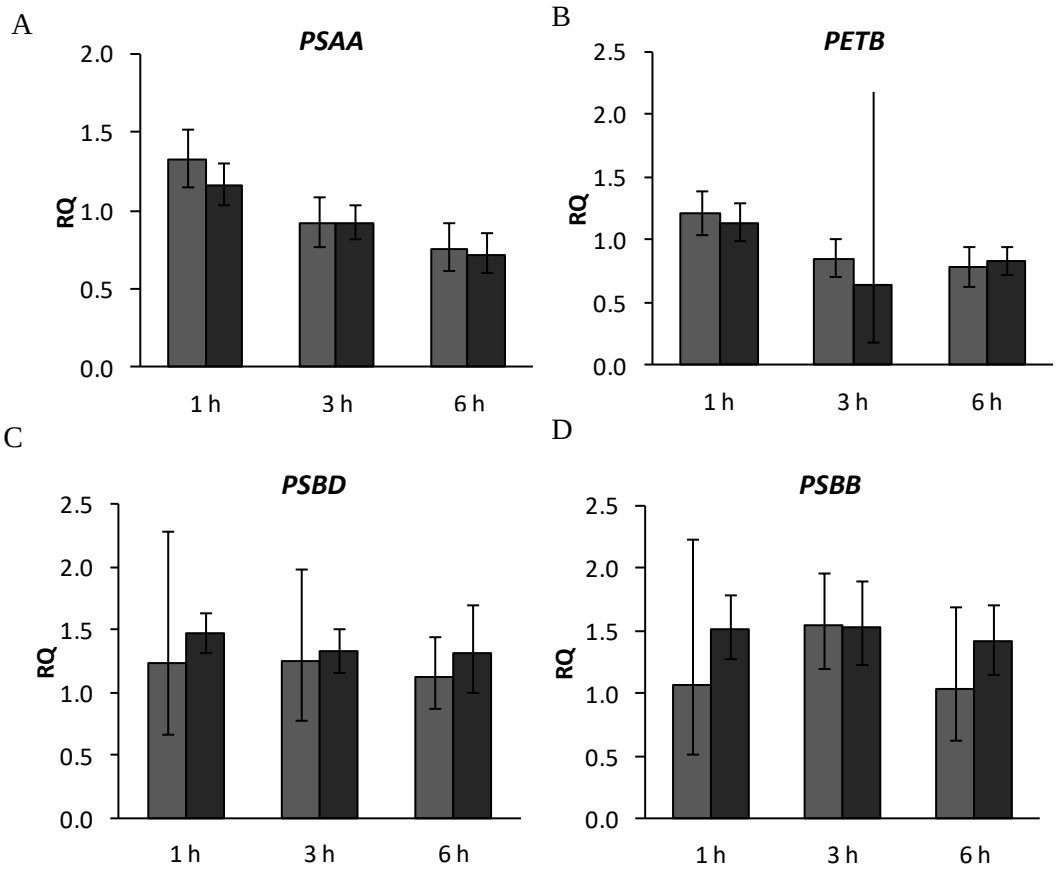


Figure 4.6 Relative transcript abundance of four chloroplast genes in response to mastoparan treatment in *Arabidopsis* seedling**.** Grey bars represent water control, black bars mastoparan treatment. Error bars = SE.

4.2.2 Measuring stromal calcium increases in response to selected stimuli that affect chloroplast gene expression

4.2.2.1 Identification of stimuli that affect chloroplast gene expression

The first approach adopted to answer the biological question "does calcium regulate chloroplast gene expression?" was to look at changes in the expression of chloroplast genes upon treatment with the calcium agonist mastoparan. This first approach was not successful in showing a role for calcium in affecting the plastome transcripts, however this approach by itself is not a conclusive evidence against the role of calcium in the process. Indeed calcium can be necessary, but by itself not sufficient to induce the pathway leading to changes in chloroplast gene expression. Hence, a second approach was taken, where a series of treatments for which there is evidence of differential chloroplast gene expression was shortlisted, and subsequently tested for the ability of these treatments to induce stromal calcium. If that was the case, this would indicate that stromal Ca^{2+} signalling might be involved in the process, then by inhibiting the signal the changes in the plastidial transcriptome should be lost. This would establish a role for calcium in this process, giving an affirmative answer to the initial research question.

To undertake this second approach, a list of treatments that affect chloroplast gene expression was produced using the program Genevestigator (Hruz et al., 2008), by searching all the transcriptomic data available for *Arabidopsis* for all the treatments that affect the 80 chloroplast genes available on Genevestigator. The complete result of the search is available on appendix B. A set of treatments was chosen amongst the ones listed both for down- and up-regulation of chloroplast genes, and the list was ranked on the gene that showed the biggest fold changes, which is *PSBA* (Atcg00020). This list of stimuli was screened for feasibility of measuring calcium in these conditions, as well as for their ability to detectably change chloroplast gene expression. Therefore the second step was to look for any evidence in the literature of stromal calcium signalling linked to

this stimulus, and if not, the third step was to directly measure chloroplast calcium upon direct stimulation. These decisions are reported, stimulus by stimulus, in table 4.1.

Treatment	**Decision/evidence**
Elevated CO_2	Measure Ca^{2+}
Iron deficiency	Not tested
Salicylic acid	Not tested
Infection with *Heterodera schachtii*	Not tested
Primisulforon-methyl	Not tested
Dexamethasone	Not tested
Heat	Measure Ca^{2+}
6-Benzylaminopurine	Measure Ca^{2+}
Low light	Not tested
1-Naphthaleneacetic acid	Not tested
Lyncomycin	Not tested
Norflurazon	Not tested
Cold	Nomura et al., 2012
Germination	Not tested
Salt	Nomura et al., 2012
Hypoxia	Not tested
ABA	Measure Ca^{2+}

Table 4.1 List of treatments that affect chloroplast gene expression, and decision whether to measure calcium in response to them (Measure Ca^{2+}), or not (Not tested). If evidence of stromal calcium signalling in response to one stimulus was available in literature, the paper is reported.

4.2.2.2 Measuring stromal calcium increases in response to stimuli that affect chloroplast gene expression

The stimuli shortlisted according to the above listed criteria were: elevated CO_2, heat, 6-benzylaminopurine (BAP), cold, salt and ABA.

Results for elevated CO_2 are reported in chapter 6, figures 6.12, and no calcium increase was measured in response to treatment with CO_2 1000 ppm. Heat-induced calcium increase was measured and fully described in chapter 5, specifically in figures 5.1and 5.2. Stromal calcium responses to BAP 100 μM, cold given as four pulses at 10ºC, separated by 1 minute at 20ºC, NaCl 250 mM and ABA 25 μM and 100 μM are reported in figure 4.7.

Results in figure 4.7 show that all these four stimuli tested were able to increase calcium in the chloroplast stroma (as well as heat, shown in chapter 5 figure 5.1), and they produce different "calcium signatures" (McAinsh and Hetherington, 1998), hence the kinetics of the calcium increase are different, suggesting the possibility that they may encode a specific message (i.e. stimulus-specificity). In many cases these signatures are strongly similar in shape and kinetics to the ones observed in response to the same stimuli in the cytosol, but of smaller amplitude and with a short delay (data not shown), indicating that these calcium responses are not completely independent from the cytosolic counterpart. In order to test this blocking the calcium increase in the stroma is needed.

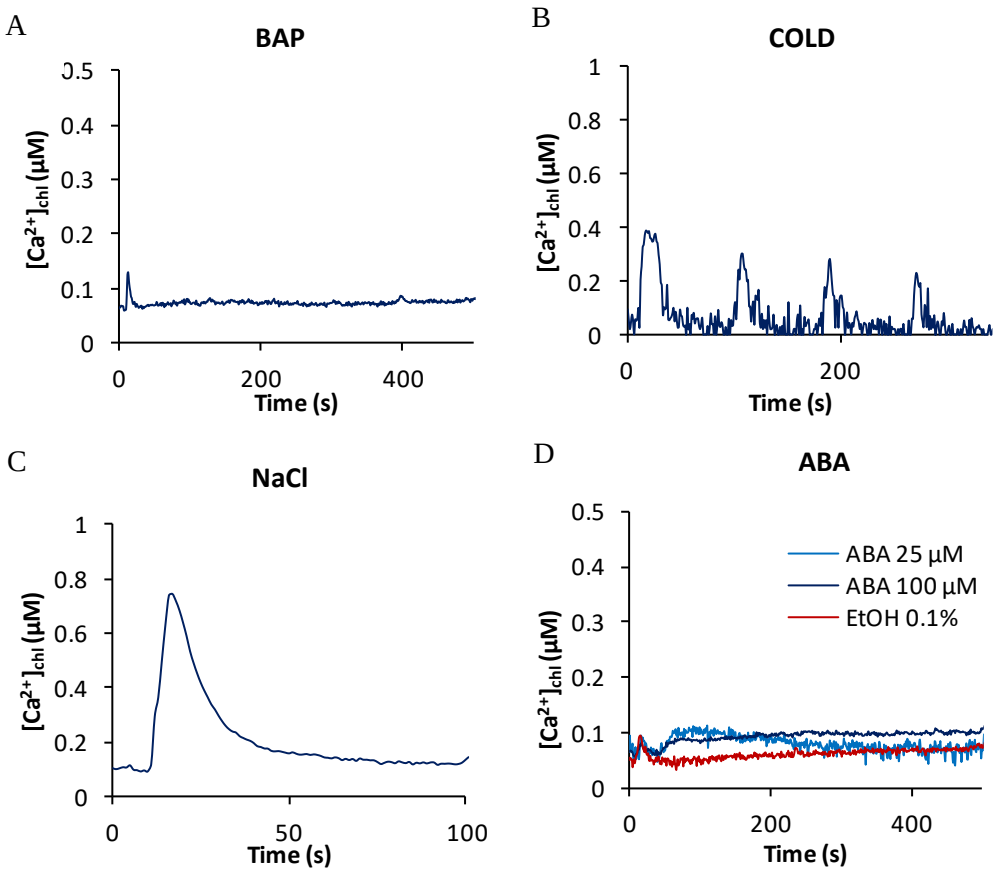


Figure 4.7 Stromal calcium levels upon treatments with BAP 100 µM (A), four stimuli at 4ºC (cold, B), NaCl 250 mM (C) and ABA 25 µM, 100 µM and EtOH control for ABA 100 µM (D). Stimuli were injected after 10 seconds, except for cold, which was applied starting after 10 seconds, as four stimuli at 4ºC of 30 seconds with intervals of 60 seconds at 20ºC between each other.

Amongst these five treatments able to induce stromal calcium, we were not able to block heat (see chapter 6), while cold and NaCl, despite being able to induce the stromal calcium increase, are not suitable for the aim of this project, because in order to induce changes in chloroplast gene expression these two treatments need to be applied for a long time (two weeks according to the conditions used on Genevestigator), which are not the same conditions we are testing for calcium measurements. Hence the only two stimuli left to try to block were ABA and BAP. To inhibit the calcium we applied a 30 minutes pre-incubation with $LaCl_3$ 5 mM, which was effective in inhibiting the mastoparan-induced stromal calcium increase (paragraph 4.2.1.3). As represented in figure 4.8, the lanthanum pre-treatment did not inhibit the calcium increase upon stimulation with ABA 100 µM and BAP 100 µM, but on the contrary the calcium levels were already higher at the beginning of the experiments.

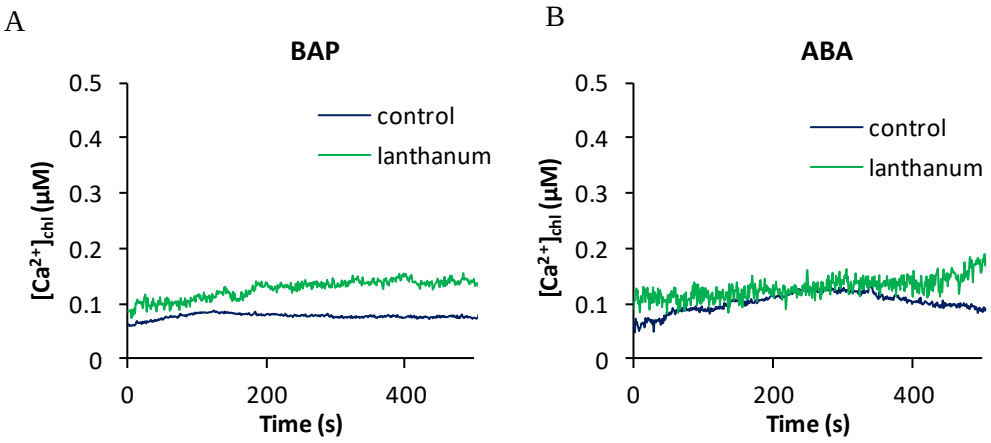


Figure 4.8 Calcium curves after 30 minutes of $LaCl_3$ pre-treatment (5 mM, lanthanum) in response to BAP 100 µM (A) and ABA 100 µM (B). Control plants were pre-treated with water (control). Stimuli were injected after 10 seconds.

4.3 Discussion

4.3.1 Validation of a system to increase and decrease stromal calcium levels

The first approach adopted to answer the biological question "does calcium control chloroplast gene expression?" was to controllably increase stromal calcium levels, and subsequently measure transcript levels. If the working hypothesis is true then the calcium increase should trigger changes in chloroplast gene expression. This cause-effect relationship is the first step to prove the calcium dependency of an event (Jaffe, 1980). The other condition that must be true is that when the calcium signal is prevented from happening, then the previously measured change should be lost (Jaffe, 1980). Hence, to do so, a system was needed where it was possible to controllably increase stromal calcium, and at the same time, it has to be possible to block it.

We achieved this by using mastoparan as a calcium agonist and lanthanum chloride ($LaCl_3$) to block the mastoparan-induced calcium increase. Indeed, mastoparan was able to induce an increase in the stromal calcium, measured with the calcium reporter aequorin (for details see chapter 1 Introduction) targeted to this compartment (figure 4.2). The increase in the cytosol was monitored at the same time as a positive control for the effect of the calcium agonist. A dose-response curve was obtained for mastoparan, and the aim was to maximize the concentration of calcium induced in the stroma, which was achieved at the concentration of 50 μM.

Increasing the calcium concentration to a maximum gave us more confidence that the signalling pathway leading to an eventual change in chloroplast transcript levels has been activated and possibly saturated. On the contrary, for the lanthanum treatment, the aim of the dose-response analysis (figure 4.3) was to find the smallest concentration of the chemical still able to block the mastoparan-induced calcium increase, which was 0.5 mM. Lower concentrations were not as effective in the inhibition. Lanthanum by itself is able to affect gene expression (Rentel and Knight, 2004), hence lowering the concentration

minimizes this side effect. In any case, a control with $LaCl_3$ only is needed to confirm that any change in the chloroplast gene expression levels are not dependent on this chemical. It is interesting to note that the 30 minutes pre-treatment with $LaCl_3$ did affect the baseline in the cytosolic positive control (figure 4.3A), but it did not change the initial calcium levels of the stromal compartment (figure 4.3B).

By using these two chemicals we created an optimal system to induce a controlled stromal calcium increase in the chloroplast stroma, and to inhibit it, which was the starting point of the gene expression analysis. The downside of this system is that to increase the calcium levels we are not using a natural "Ca^{2+}-signature", so we do not know whether these induced calcium kinetics are read by the chloroplast as a signal to induce gene expression. Furthermore, calcium may be a necessary component, but not sufficient by itself to trigger the appropriate pathway leading to those changes. However, mastoparan-induced nuclear gene expression modifications have been recorded (Takahashi et al., 1998; Whalley et al., 2011), giving confidence that this system may be suitable for the aim of this research project.

4.3.2 Gene expression in the chloroplast is not regulated by stromal calcium

In my research, chloroplast gene expression was tested in response to controlled Ca^{2+}-increase in the stromal compartment. The increase was triggered by incubation with mastoparan and $[Ca^{2+}]_{chl}$ of 0.6 μM could be reached within seconds (figure 4.2B). This increase was blockable by pre-incubation with the calcium channel blocker lanthanum (figure 4.3B).

Firstly, the success of the mastoparan treatment was tested by looking at nuclear genes that were known from previous evidence to be mastoparan-induced, which are *RAP2.6* and *ABR1* (figure 4.4). These genes were indeed up-regulated in the treated plants, indicating that the mastoparan incubation was effective, hence chloroplast genes could be tested on the same cDNA samples.

Before testing if the level of chloroplast genes was affected by any treatment (and even before starting to set up the whole project) the applicability of the RT-PCR as a technique to detect chloroplast genes was assessed. To do so, we measured by RT-PCR relative levels of the nuclear gene *RBCS1A* encoding one of the small subunit of the Rubisco enzyme, and levels of the chloroplast gene *RBCL*, coding for the large subunit of Rubisco. The treatment applied was constant light, and a control was kept in the dark for the same length of time. For both of these genes, we measured changes in the mRNA levels in response to 48 h constant light (figure 4.1). However, for *RBCL*, the peak was measured after 24 h of constant light, while *RBCS1A* mRNA levels reach a maximum at 48 h. This lack of synchronism in the peaks of two genes encoding different subunits of the same complex can be due to different reasons. One of the explanations is that other control mechanisms can affect the amount of the final product, like post-translational modifications, protein import etc. Indeed, levels of mRNA and protein detected for *RBCS1A* for the same light conditions are not equal, and this has already been reported in the literature (Lu et al., 2005; Feng et al., 2014). However, for the sake of validating the RT-PCR technique on chloroplast-encoded genes, this asynchronism was not considered a problem, and the experiment was considered technically valid. Furthermore, RT-PCR has been previously used to measure chloroplast genes (Weihe, 2014; Kikkawa et al., 2016), so we decided to adopt it to answer our initial biological question.

RT-PCR experiments on mastoparan-treated *Arabidopsis* seedlings did not show any significant alteration in gene expression between the treatment and the control for the two chloroplast genes investigated: *PSBA* and *RPOC1* (figure 4.5). These specific genes were chosen as representatives of two different plastidial functions. *PSBA* is one of the photosynthetic genes, in fact it encodes the reaction centre core protein of PSII. *RPOC1* is part of the *RPOBC* operon, which codes for one of the core subunit of the plastidial encoded RNA polymerase (PEP), hence it covers the other big class of chloroplast genes, which are the ones involved in the transcription machinery (Hudson et al., 1988; Pfannschmidt et al., 1999). Moreover, *PSBA* is under the control of the PEP polymerase, which is an *E. coli*-like RNA polymerase and is encoded in the chloroplast genome itself,

whilst *RPOC1* is under the control of the second polymerase acting on the plastome, the nuclear encoded RNA polymerase (NEP), a phage-type polymerase (Liere et al., 2011; Finster et al., 2013). Furthermore, while looking at available microarray data and relevant papers, *PSBA* was identified as a gene that experiences relatively large changes in transcription levels in response to CO_2, hormones, light and biotic perturbations (Pfannschmidt et al., 1999; van Leeuwen et al., 2007; Dinneny et al., 2008; Rugnone et al., 2013; Markelz et al., 2014a; Markelz et al., 2014b). Thus, these two genes were purposely chosen to give higher probability to detect a response to a given perturbation.
Further chloroplast genes were tested to see if any difference was detectable in their expression in response to mastoparan. None of the genes tested (*PSAA, PETB, PSBD, PSBB*) was significantly affected in its expression level in response to mastoparan (figure 4.6). This lack of changes in the transcript levels in response to mastoparan-induced stromal calcium increase may indicate that calcium is not involved in this regulation. However, an alternative explanation is that calcium *per se* is not sufficient to induce the transcriptional response, and another messenger must be involved, e.g. ROS or pH. Furthermore, there is no evidence that the mastoparan-induced calcium increase mimics any "Ca^{2+}-signature" that the plant can associate with natural perturbations (McAinsh and Hetherington, 1998).

Through this first approach of inducing changes in chloroplast gene expression by controllably increasing stromal calcium no significant differences emerged in response to mastoparan treatment for the chloroplast-encoded genes tested. Since the criteria of choice of these genes were based on covering different operons, different functional classes of genes and different plastidial types of polymerases, we decided not to further persevere with this approach. However, according to the collected evidence it is still not possible to exclude a role of calcium in controlling gene expression in the photosynthetic organelles, since this ion may act synergistically with other stimuli. Therefore, a second approach was taken. The idea behind it is that if calcium is needed, but not sufficient, then stimuli that do affect chloroplast gene expression shall induce a stromal calcium

increase when applied to the plants; and blocking this calcium shall prevent the transcriptional changes.

First of all, a list of stimuli that up- or down-regulate the plastidial gene expression was produced with Genevestigator. The 80 chloroplast gene list was used to search the whole available data on the Genevestgator database for *Arabidopsis*, and a list of stimuli was made by ranking the treatments based on the gene whose levels were changing the most: *PSBA*. The complete list is available in appendix B. From this list a series of treatments was selected for their feasibility (table 4.1). Both treatments that up- and down- regulate the plastidial transcriptome were selected, as the effects of the calcium on regulating chloroplast gene expression, either promoting or repressing it, are not known yet. From this list, literature was searched for evidence of calcium signaling in the stroma in response to them, and this was only available for cold and salt (Nomura et al., 2012). The other treatments were either chosen or excluded based on feasibility of measuring calcium upon them with the instrumentation available. For example, long treatments like iron deficiency or hypoxia were excluded; while lyncomycin and norflurazon were avoided since they act as chloroplast protein synthesis inhibitors, hence they do not mimic any natural stimulus (Beisel et al., 2011; Kerchev et al., 2011). Even if based on strong criteria, the shortlisted stimuli chosen to test the possible involvement of calcium was partially subject to arbitrary judgment and chemical availability, and in case of some positive results, it would have been possible to go back to it and select more treatments to test from table 4.1.

I decided to start from elevated CO_2, heat, 6-benzylaminopurine (BAP), cold, NaCl and ABA. Elevated CO_2 did not displayed any stromal calcium increase (figure 6.12 chapter 6), while the heat treatment was able to specifically induce stromal calcium increase (figure 5.1 chapter 5), but none of the approaches tested succeeded in blocking this heat-induced stromal calcium, making it unsuitable for testing it for causality of gene expression. Despite having literature evidence and direct measurements of stromal calcium increases in response to cold and salt treatments, the conditions used in

experiments available on Genevestigator, hence the ones inducing the gene expression changes, were long treatments (3 weeks) with either cold or NaCl, while calcium responses is measured upon a fast stimulation with those treatments (figures 4.7 B and C). Therefore, these two stimuli were excluded from the list. BAP and ABA produced a very small stromal calcium increase (figures 4.7 A and D), however, we decided to try to inhibit these responses by 30 minutes pre-incubation with $LaCl_3$. This attempt was unsuccessful for both treatments, since in both cases the baseline was strongly affected by the pre-treatment, causing an even higher calcium signal in the $LaCl_3$-reated *Arabidopsis* samples (figure 4.8). From this data we were not able to prove that calcium is a regulator of chloroplast gene expression; on the contrary, the evidence collected strongly suggest that this is not the case, and other regulators or different regulatory mechanisms may act to control the levels of chloroplast-encoded transcripts.

4.3.3 Conclusions

From the analysis in this chapter it is possible to conclude that there is no obvious evidence that regulation of chloroplast gene expression is mediated by calcium. It is possible that in some specific cases this second messenger may have a role in controlling the plastomic transcriptome, however this study suggests that calcium does not belong to a major mechanism of regulation of chloroplast gene expression unlike for the nuclear counterpart. It also suggests that, in the conditions tested, levels of chloroplast transcripts are not majorly affected, suggesting that there must be alternative primary ways of controlling amounts of chloroplast-encoded proteins. Testing for a possible involvement of calcium in other mechanisms to control protein levels such as alternative splicing or post-transcriptional modifications was not the aim of this study, hence these results are conclusive for the biological question we aimed to answer.

Chapter 5

Heat and the chloroplast

5.1 Introduction

The compartmentalisation of calcium into Ca^{2+}-stores to avoid toxicity, and the consequent low concentration (100 nM) in the cytosolic compartment is the first condition necessary to allow cellular calcium signalling (Sanders et al., 1999). It has emerged that even at the organellar level there can be calcium signalling. Indeed mitochondria and chloroplasts show the ability to control their calcium levels in the different sub-compartments (Stael et al., 2012). Specifically, the chloroplast not only functions as a calcium store (Roh et al., 1998; Stael et al., 2012; Nomura and Shiina, 2014), but it also revealed the ability to generate its own specific Ca^{2+} signals as the levels are kept low in the stromal compartment, and increase in response to specific stimului (Johnson et al., 1995; Sai and Johnson, 2002; Manzoor et al., 2012; Nomura et al., 2012). Calcium plays both a regulatory and structural role in the chloroplast. Notably, Ca^{2+} is required for photosystem II assembly and recovery after photoinhibition (Mattoo et al., 1989; Miller and Brudvig, 1989; Grove and Brudvig, 1998), but high calcium levels are able to inhibit photosynthesis, by acting on the Calvin-Benson cycle (Charles and Halliwell, 1980; Kreimer et al., 1988). Hence, calcium entering the chloroplast has to be stored, and the main candidates are the thylakoid compartment, as thylakoids contain a Ca^{2+}/H^{+} antiporter (Ettinger et al., 1999; Wang et al., 2016), and high-capacity calcium binding proteins able to buffer calcium in the stroma.

In 1995 (Johnson et al., 1995) a chloroplast-specific Ca^{2+}-increase was measured in response to the light-to-dark transition, initiating the field of chloroplast calcium

signalling. More than 20 years later, putative calcium channels and transporters have been identified both in the inner envelope and on the thylakoid membranes (Stael et al., 2012; Nomura and Shiina, 2014). To date, no other chloroplast-specific (i.e. not cytosolic calcium-inducing) stimuli have been identified. Chloroplast calcium increases have been reported in response to cold, salt and hyperosmotic stresses (Nomura et al., 2012) as well as elicitors of the pathogen response (Manzoor et al., 2012; Nomura et al., 2012), with different kinetics compared to the cytosolic calcium counterparts.

Temperature is one of the key environmental parameters affecting all living organisms. Fluctuations in temperature occur seasonally, daily as well as rapidly and unexpectedly, such as when clouds shield the sun's heat. Plants have evolved to be able to sense these events, anticipate them when possible, and adjust their physiology accordingly (Ruelland and Zachowski, 2010; Saidi et al., 2011; Knight and Knight, 2012; Mittler et al., 2012). The ability to discriminate a cooling from a heating event, as well as its magnitude (e.g. chilling and freezing), is essential for survival (Penfield, 2008; Hua, 2009; Thomashow, 2010; Knight and Knight, 2012).

Temperature shifts strongly affect chloroplast activity. Exposure to sub- or super-optimal temperatures can irreversibly damage photosynthesis, leading to photoinhibition (Berry and Bjorkman, 1980; Mathur et al., 2014). Furthermore, heat increases the photorespiratory activity of Rubisco, as well as the ratio between dark respiration and photosynthesis (Jordan and Ogren, 1984; Salvucci and Crafts-Brandner, 2004; Mathur et al., 2014). Higher temperatures are perceived by plants as a problem even when photosynthesis is not occurring. Indeed, the amount of starch accumulated by plants to survive the night period assumes a non-stressed rate of energy consumption (Graf and Smith, 2011; Scialdone and Howard, 2015). When metabolic activities are increased by heat, then plants experience energetic stress throughout the dark period and they initiate processes such as autophagy and protein degradation in order to survive (Caldana et al., 2011).

In this chapter I report the second case of a chloroplast-specific calcium increase, which occurs in response to heat.

Aims of the research described in this chapter:

- To describe a chloroplast-specific calcium response, observed in response to heat.
- To investigate the properties of this heat-induced calcium response, in order to obtain information about the plant heat thermometer.
- To understand the role of light in this heat response.
- To study the role of mitochondria in controlling the chloroplast-specific heat response.
- To determine a possible cross-talk between ROS and heat in the regulation of this calcium increase.

5.2 Results

5.2.1 Heat induces a chloroplast-specific calcium increase

5.2.1.1 Heat increases Ca^{2+} concentration in the chloroplast, but not in the cytosol

To examine the role of calcium in chloroplast signalling, I treated *Arabidopsis thaliana* seedlings expressing aequorin targeted to the cytosol (pMAQ2) or stromal compartment (pMAQ6) with a range of stimuli known to induce abiotic stress responses in plants. A chloroplast-specific calcium increase was observed in response to heat (figure 5.1). *Arabidopsis* seedlings were heated on a Peltier element positioned under a photon counting camera and luminescence was collected before and during the heating event. As shown in figure 5.1, *Arabidopsis* seedlings were kept at 20ºC for 2 minutes and then heated at 40ºC for 7 minutes before dropping the temperature back to 20ºC. The 40ºC pulse caused a transient increase in the stromal calcium levels, up to concentrations of around 0.4-0.5 μM. In contrast, the cytosol did not display any calcium increase during the same heat stimulus, however, as can be seen in figure 5.1, the temperature drop from 40ºC to 20ºC was sensed by the plants as a cold shock, which is known to cause a rapid calcium peak in the cytosol (Knight et al., 1996; Larkindale and Knight, 2002). A cold response was also detected in the chloroplast, leading to the relatively modest increase in stromal calcium previously reported (Nomura et al., 2012).

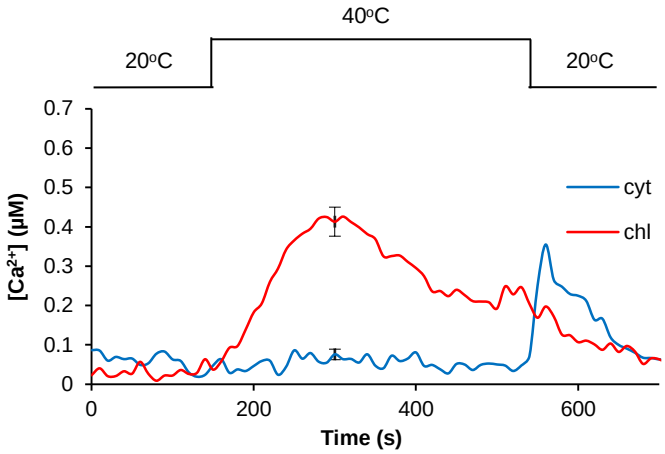


Figure 5.1. Plants respond to heat with a chloroplast-specific calcium increase. Calcium elevations in response to heating (20ºC to 40ºC) and cooling (40ºC to 20ºC) events in the cytosol (pMAQ2) and chloroplast (pMAQ6) plotted through time. Each trace was obtained by averaging the signal recorded from n = 6 pMAQ2 and n = 5 pMAQ6 8-day-old *Arabidopsis* seedlings. Error bars represent standard error (SE) at the chloroplast calcium peak.

5.2.1.2 The kinetics and magnitude of the Ca^{2+}-increase are temperature-dependent

In order to test the dose-dependency of the calcium heat response, a series of temperatures was tested on *Arabidopsis* seedlings, ranging from mild heat (30ºC) up to just sub-lethal temperatures (45ºC), with an interval of 2.5ºC. Each temperature above 30ºC caused a stromal calcium increase (figure 5.2A), and the kinetics of the calcium were dependent on the temperature sensed, in a dose-dependent manner. Indeed, the peak height increased with increasing temperature (figure 5.2B) almost linearly, as well as the time of peak, which was shorter with increasing temperature (figure 5.2C). Interestingly, giving plants a 30ºC heat stimulus did not cause a stromal calcium increase, defining this temperature as the threshold for the chloroplast calcium heat response. Cytosolic calcium increases were monitored for each of the temperatures tested in figure 5.2, and results are reported in figure 5.3, showing little or no increases. These figures demonstrate again the

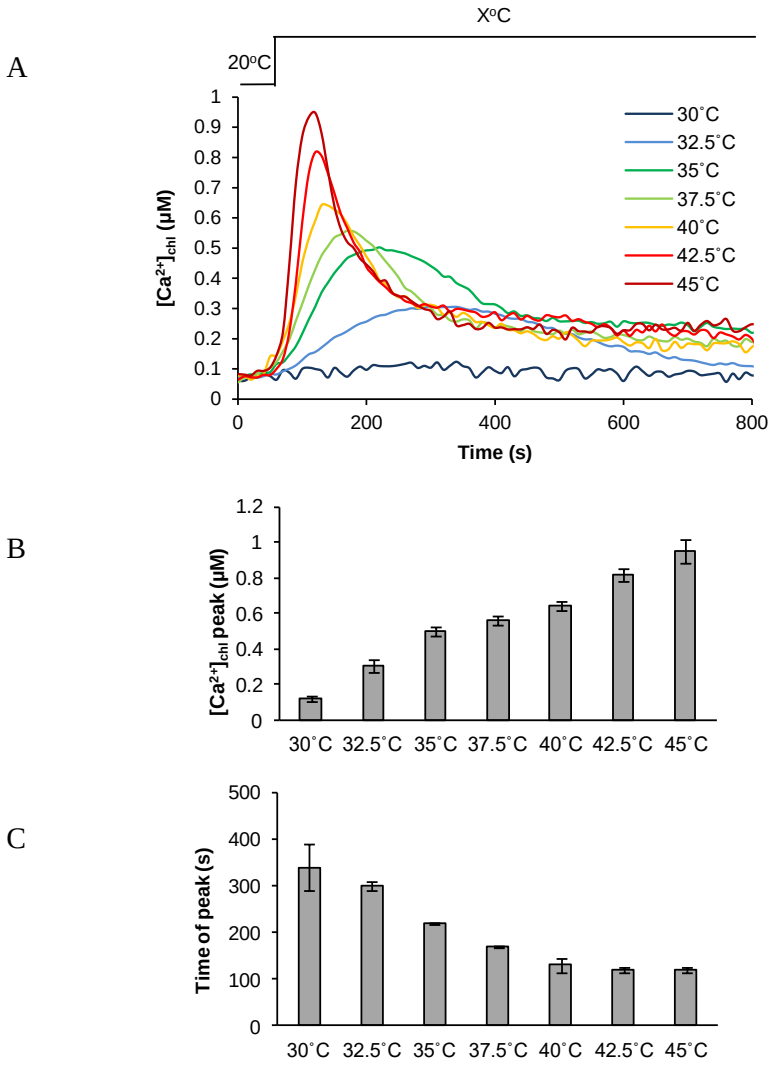


Figure 5.2 The kinetics and magnitude of the heat-induced Ca^{2+} increase are temperature-dependent. Chloroplast-targeted aequorin seedlings were exposed to a series of temperatures ranging from 30ºC to 45ºC, at intervals of 2.5ºC. (A) Kinetics of the calcium increase upon heating and (B) average chloroplastic calcium concentration peak heights and (C) peak times.

Data were obtained by averaging n = 4 8-day-old *Arabidopsis* seedlings, and for each temperature a different set of plants was used. Error bars = SE.

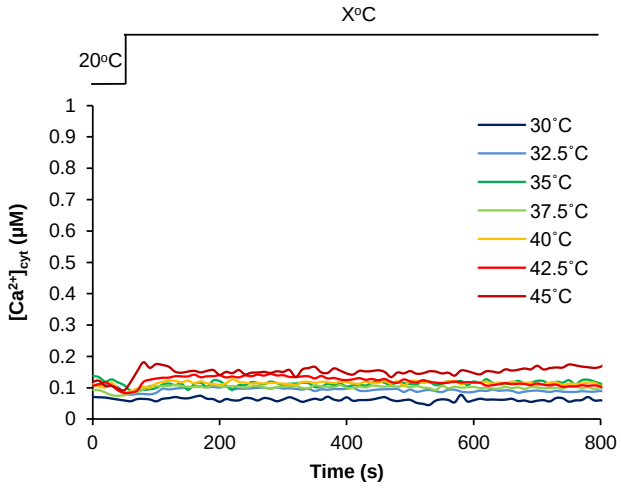


Figure 5.3 The cytosolic calcium does not increase in response to heat. Calcium curves representing the kinetics of cytosolic-targeted aequorin lines exposed to the same series of temperatures as the chloroplast lines shown in figure 2 (from 30ºC to 45ºC, with intervals of 2.5ºC). Each trace was obtained by averaging the signal of n = 4 8-day-old *Arabidopsis* seedlings.

specificity of the chloroplast calcium increase, relative to the cytosol, and that the luminescence measured is due to *bona fide* calcium increases in the stroma: the effect of temperature upon aequorin activity can be considered negligible.

5.2.1.3 The calcium heat response is conserved amongst species and ecotypes

We then tested whether the heat-induced chloroplast calcium response was specific to *Arabidopsis*, or might be conserved amongst plant species. In order to test this, stromal and cytosolic aequorin were transiently expressed in *Nicotiana benthamiana*, and calcium was measured 48 hours after infiltration. figure 5.4 shows that tobacco is also capable of responding to heat with a transient stromal calcium increase; however the magnitude of the response is lower for the equivalent temperature compared to *Arabidopsis* (compare

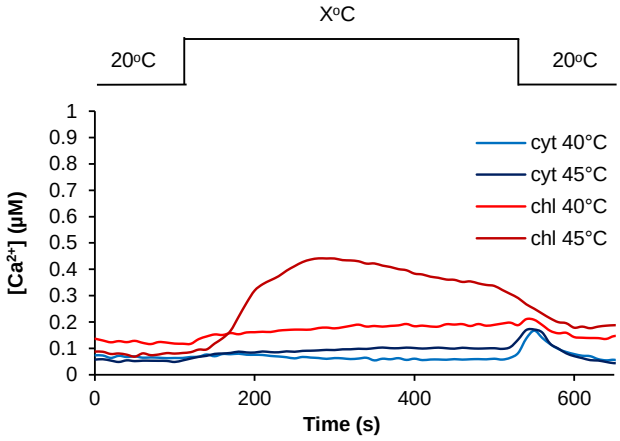


Figure 5.4 The chloroplast calcium heat response is conserved in tobacco. Calcium elevations in response to heating (40ºC and 45ºC) in the cytosol (pMAQ2 infiltrated tobacco leaves, "cyt") and chloroplast (pMAQ6 infiltration, "chl") are represented through time. Each trace is an average of calcium traces of n = 3 *Nicotiana benthamiana* infiltrated leaves.

figure 5.2A with figure 5.4). The calcium heat response was also conserved amongst the *Arabidopsis* ecotypes Col-0 and Ws-0, whose traces are almost identical (figure 5.5).

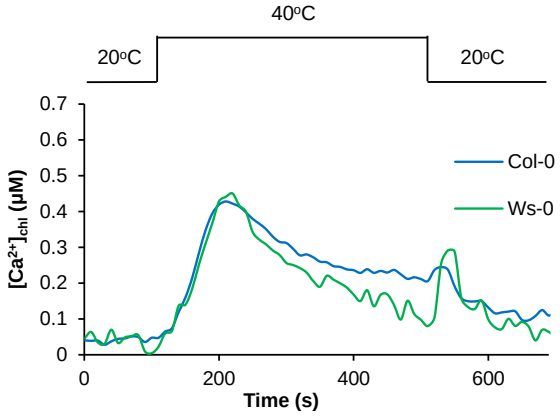


Figure 5.5 The chloroplast calcium heat response is conserved amongst at least two *Arabidopsis* ecotypes. Calcium kinetics upon heating (40ºC for 7 min) of Col-0 and Ws-0 *Arabidopsis thaliana* ecotypes constitutively expressing chloroplast-targeted aequorin (pMAQ6). Each trace was calculated by averaging data from n = 4 seedlings.

5.2.1.4 Does the heat treatment affect plant survival?

When *Arabidopsis* plants were stimulated at 50ºC for 7 minutes, this treatment was lethal. Indeed differences are already noticeable from the calcium signature observed at 50ºC (figure 5.6), where this temperature does not follow the dose-response trend observed in figure 5.2A, but resulted in a first fast peak, followed by another relatively large one, which can be observed when plants are purposely treated with a lethal stimulus (e.g discharge at -15ºC, see chapter 2 Materials and Methods). The kinetics of the calcium curve of plants treated at 50ºC are considerably different compared to the curves observed when stimulating at 40ºC and 45ºC (figure 5.6), suggesting that this higher temperature may be lethal for plants.

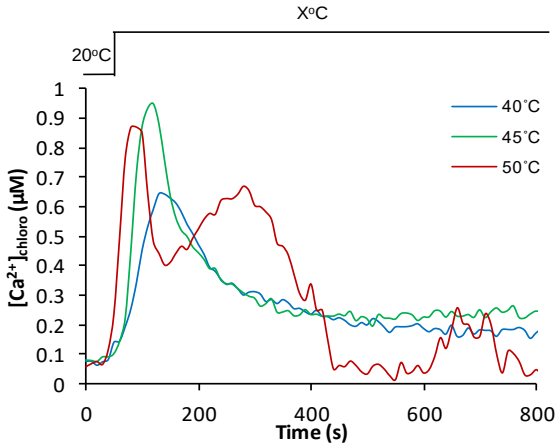


Figure 5.6 The chloroplast calcium heat response is temperature-dependent. Calcium kinetics upon heating (indicated temperature for 7 min) in *Arabidopsis* plants constitutively expressing chloroplast-targeted aequorin (pMAQ6). Each trace was calculated by averaging data from n = 4 seedlings.

5.2.2 Properties of the heat response

5.2.2.1 Characteristics of the chloroplast heat response

Attenuation is a property observed when an organism is repeatedly exposed to a stimulus of the same magnitude within a relatively short time; and the size of the response decreases each time as a consequence of the previous experience. This was found to be the case for the chloroplast heat response; where seedlings consecutively exposed to 4 minutes 40ºC heat pulses every 5 minutes showed a reduced calcium response upon each subsequent stimulation (figure 5.7). A stimulation at 45ºC following three such 40ºC heat pulses was able to re-establish the stromal calcium increase, and the magnitude of this elevation was greater than the one recorded upon the first 40ºC heating pulse (figure 5.7). This property is known as sensitisation and it is able to overcome attenuation.

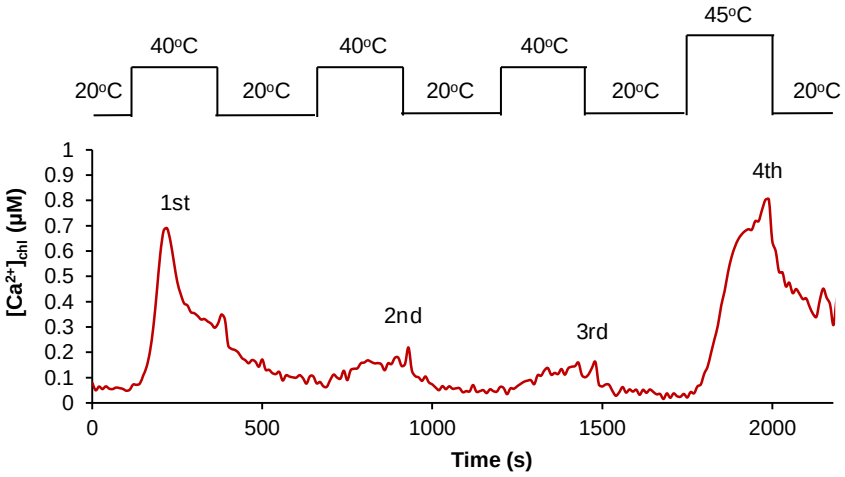


Figure 5.7 The chloroplast calcium heat response displays attenuation and sensitisation. $[Ca^{2+}]_{chl}$ response to 3 consecutive heat pulses of the same magnitude (40ºC for 4 min) followed by a fourth pulse at a higher absolute temperature (45ºC for 4 min). Each heat pulse was separated by a 5 min resting period at 20ºC. Data represent an average of n = 7 *Arabidopsis* seedlings.

5.2.2.2 Heat sensing is mainly dependent upon absolute temperature

In order to investigate whether the rate at which the temperature increase is given is a key parameter of the chloroplast heat response, plants were treated to an increase from 20ºC to 40ºC at rates of 0.4, 0.2, 0.15 or 0.1 ºC/s. In figure 5.8 the chloroplast calcium concentration at the peak was plotted against the rate of temperature increase, and all the data fit a horizontal line (R^2= 0.0133), which indicated that there was no correlation between the rate of heating and the magnitude of the calcium peak. These data demonstrate that absolute temperature is the primary parameter regulating the magnitude of this response.

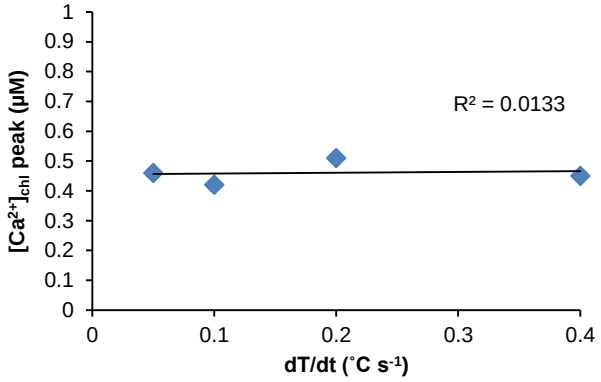


Figure 5.8 Rate has no effect upon heat-induced chloroplast calcium response. The $[Ca^{2+}]_{chl}$ at the peak is not regulated by the heating rate, but by absolute temperature. Each data point represents an average of the value reached at the $[Ca^{2+}]_{chl}$ peak obtained from n = 4 *Arabidopsis* seedlings exposed to a temperature shift from 20ºC to 40ºC at a specific rate. For each rate a different set of plants was used. Rates tested: 0.4, 0.2, 0.15 and 0.1 ºC s^{-1}. Data points represent experimental data, interpolated by a regression line (R^2 = 0.0133).

5.2.3 Cross-talk between light and heat in the chloroplasts

One of the major processes affected by heat in plants is photosynthesis, specifically the electron transport chain (Berry and Björkman, 1980; Weis and Berry, 1988; Havaux et al., 1996). To test the effects of light and dark on the heat induced chloroplast calcium response, *Arabidopsis* seedlings were pre-incubated for 30 minutes, either in the dark or in the light, before measuring calcium levels upon heating. Plants pre-treated in the light (figure 5.9 and figure 5.10) responded to the 40ºC heat pulse with a consistently delayed calcium increase compared to the dark adapted plants.

This delay could be partially rescued by adding during the light pre-incubation either DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea, figures 5.9A and 5.9B) or DBMIB (2,5-dibromo-3-methyl-6-isopropylbenzoquinone, figures 5.10A and 5.10B), two photosynthesis inhibitors that impair electron transport chain by acting on different targets.

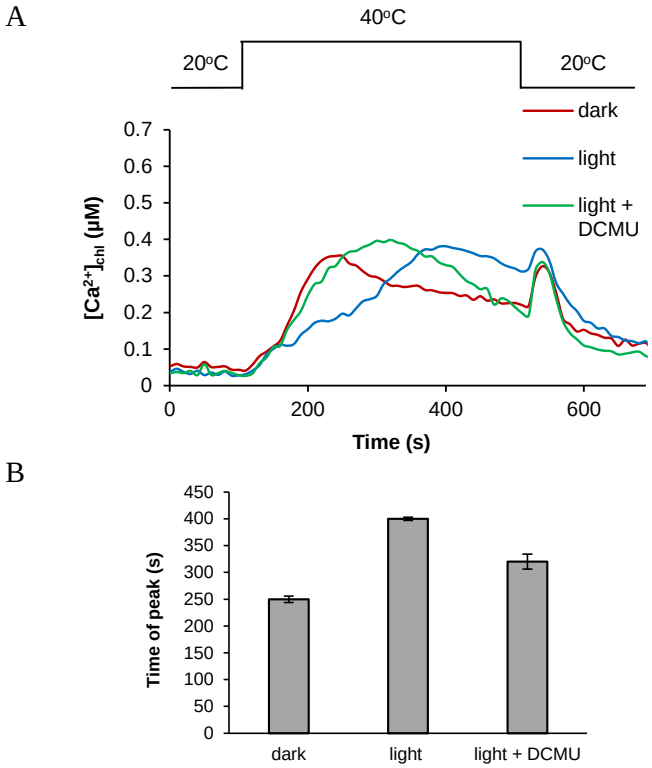


Figure 5.9 Effect of light and DCMU on the calcium heat response. *Arabidopsis* transgenic pMAQ6 lines were pre-incubated for 30 min either in the dark, light, light+ DCMU (10 µM), then calcium was measured concomitantly with heat stimulation (40°C for 7 min) in the dark. (A) average kinetics of the calcium increase upon heating, (B) average chloroplastic calcium peak time of dark (n = 10), light (n = 10) and light + DCMU (n = 9) treated plants. Error bars = SE.

The experiments described above point towards a role of light as a negative regulator of the calcium heat response. However, the limitation of working with a luminescence marker such as aequorin is that it is technically impossible to measure calcium levels in the light.

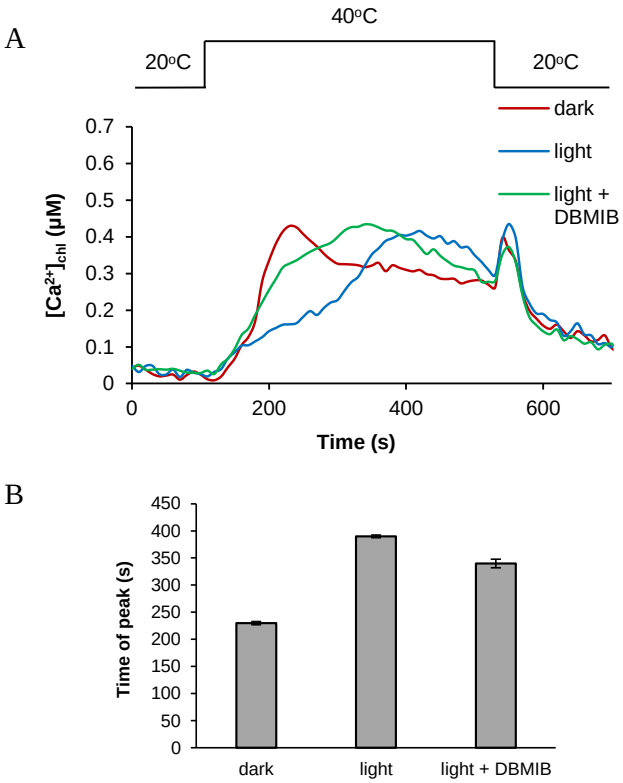


Figure 5.10 Effect of light and DMBIB on the calcium heat response. *Arabidopsis* transgenic pMAQ6 lines were pre-incubated for 30 min either in the dark, light, light + DBMIB (7 µM), then calcium was measured concomitantly with heat stimulation (40°C for 7 min) in the dark. (A) average $[Ca^{2+}]_{chl}$ traces and (B) average time of the calcium peaks in response to heat following a dark (n = 7), light (n = 5) or light + DMBIB (n = 10) pre-treatment. Error bars = SE.

To circumvent the problem two different additional approaches were taken, in order to indirectly measure calcium during the heating event in the light.

The first approach taken exploited aequorin as a marker for calcium increase by measuring the consumption. The idea is that any calcium increase will bind aequorin, and, as the pool of protein is limited, then there will be less of this luminescent calcium

reporter available for the discharge. The higher the calcium, the less aequorin will be left, hence the signal coming from the discharge can be used as a proxy to measure calcium increases previously occurred. This approach was tested on plants treated in the dark, and indeed while measuring total counts of aequorin after heating at 40ºC the amount of aequorin left was significantly different compared to the control kept at 20ºC (figure 5.11, significance was confirmed by a t-test with confidence level of 98%). The same approach was used on light-treated plants during the heating event at 40ºC for 7 minutes, and the difference in aequorin consumption measured for the dark-heated plants was lost, suggesting that in the light the calcium increase is negatively regulated (figure 5.11).

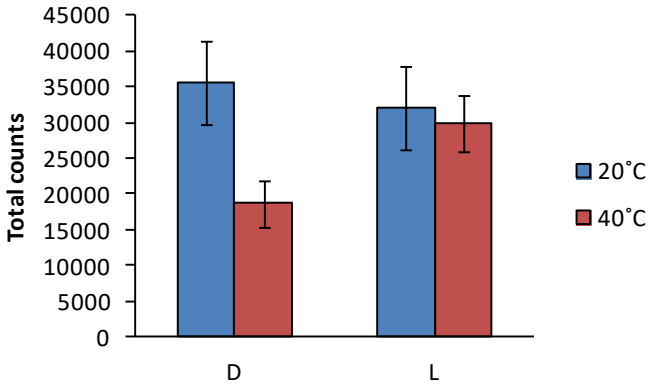


Figure 5.11 Light is a negative regulator of the heat-induced calcium increase. Aequorin consumption was measured by recording the luminescence total counts during the discharge of a pool of plants treated either at 20ºC or 40ºC in the light (L) or dark (D). Data represent an average of n = 16 *Arabidopsis* seedlings, error bars = SE.

To further confirm these data, a second experiment was performed in which plants were heated for 7 minutes at 40ºC, and of those 7 minutes, in the 3 initial minutes of heating plants were stimulated with light, then lights were turned off and calcium was recorded in the remaining 4 minutes of heating. If the calcium increase happens in the light, then at the onset of luminescence measurement Ca^{2+} levels should be already high. As shown in figure 5.12, the calcium increase corresponds with the light-off event, since the levels of

calcium were low at the onset of measurement. These results taken together confirm the negative regulation of the heat-induced calcium increase by light.

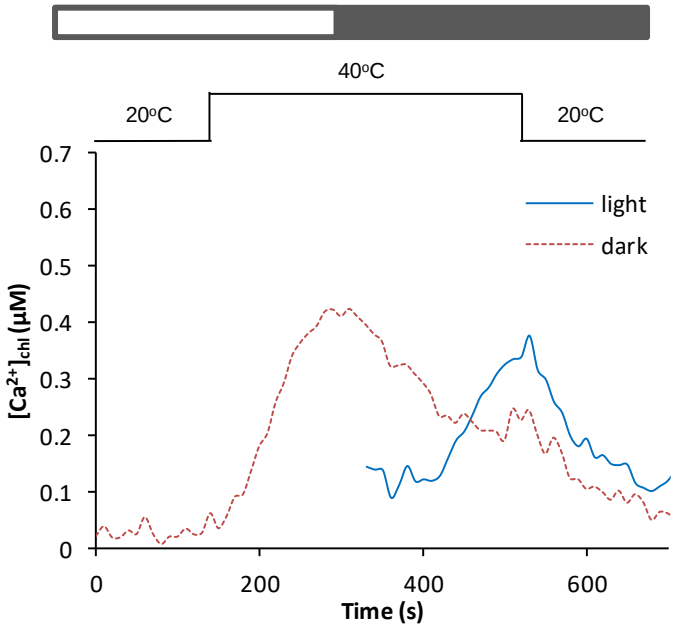


Figure 5.12 Effect of light on the chloroplast calcium heat response. *Arabidopsis* transgenic pMAQ6 lines were heated at 40ºC for 7 minutes, plants were kept in the light for the first 3 min of the heating event (white bar) and then lights were turned off and calcium was measured (grey bar). The traces represent the calcium curve recorded in the light, compared to the dashed line, representing the Ca^{2+}-kinetics in the dark. Traces represent an average of n = 8 *Arabidopsis* seedlings.

The next question that arose from this evidence was: what happens if the light pre-treatment is longer than 30 minutes? Hence light treatments were applied to *Arabidopsis* seedlings for 1 h, 2 h or 6 h before measuring calcium response in the chloroplasts upon heating at 40ºC. For the same incubation times a control was kept in the dark. From figure 5.13A it is clear that a longer light pre-incubation corresponded to a higher calcium

increase, while no difference in the calcium kinetics were observed for dark-adapted plants (figure 5.13B).

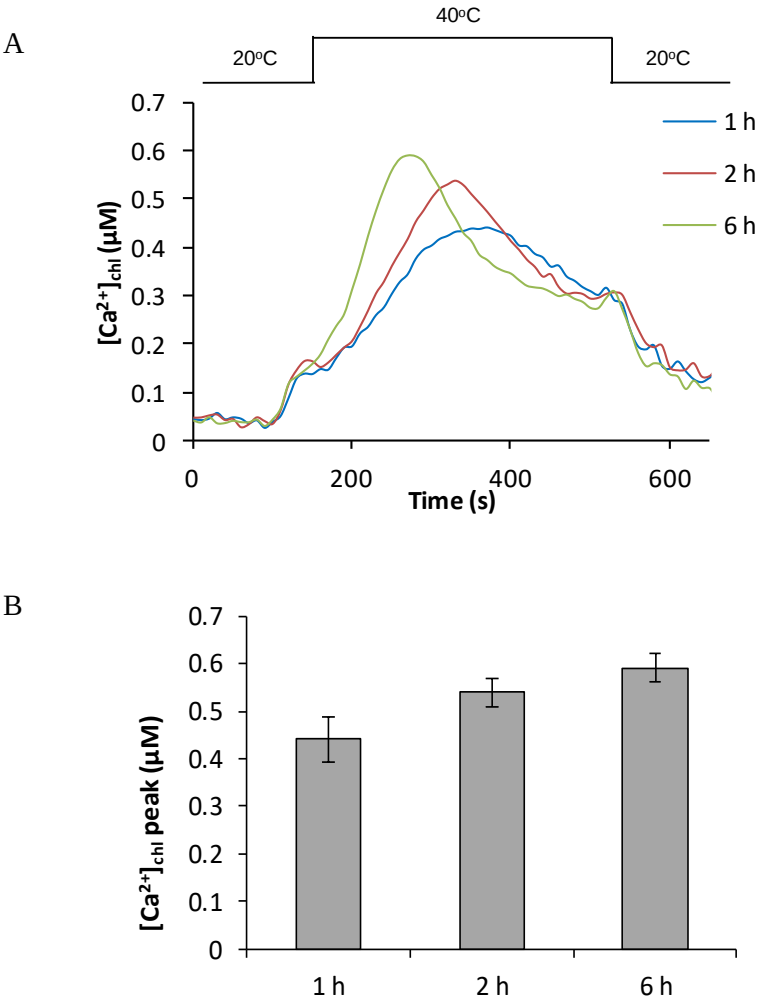


Figure 5.13 Effect of light pre-incubation for different time periods on the heat-induced chloroplast calcium increase. (A) Calcium kinetics upon heating at 40°C in *Arabidopsis* plants constitutively expressing chloroplast-targeted aequorin (pMAQ6) pre-incubated for 1 h, 2 h or 6 in the light (B) and average chloroplastic calcium concentration at the peak. Each trace was calculated by averaging data from n = 5 seedlings. Error bars = SE.

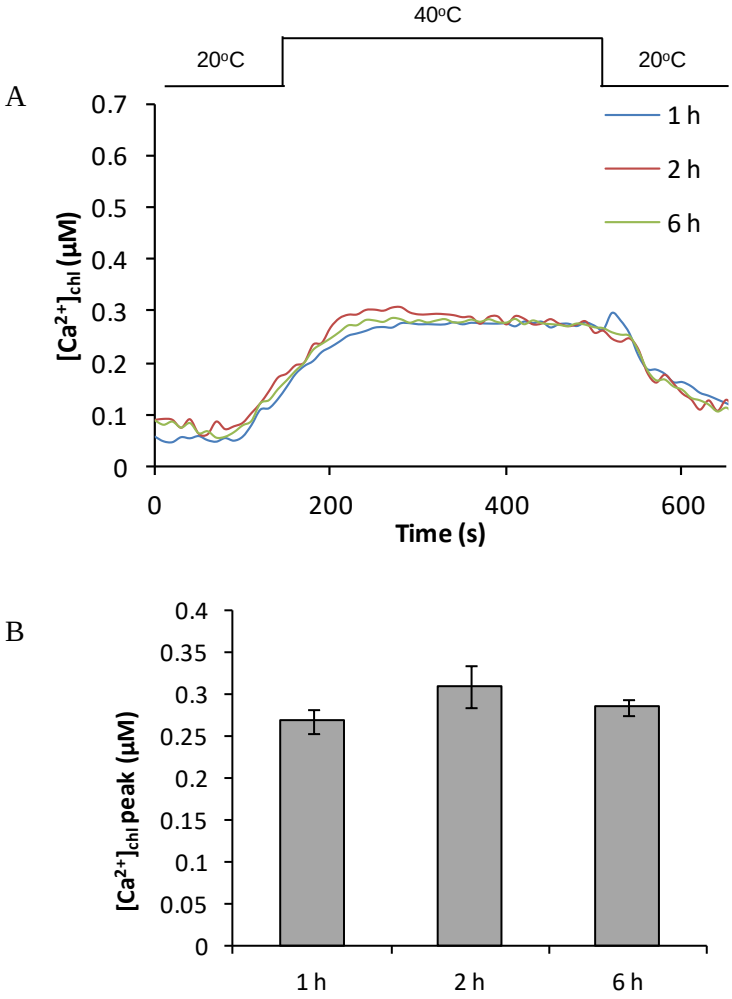


Figure 5.14 Effect of dark pre-incubation for different time periods on the heat-induced chloroplast calcium increase. (A) Calcium kinetics upon heating at 40°C in *Arabidopsis* plants constitutively expressing chloroplast-targeted aequorin (pMAQ6) pre-incubated for 1 h, 2 h or 6 h in the dark (B) and average chloroplastic calcium concentration at the peak. Each trace was calculated by averaging data from n = 5 seedlings. Error bars = SE.

5.2.4 Mitochondria and chloroplast cross-talk

Cross-talk between mitochondria and chloroplasts is essential to balance the metabolic activities of these two energy-handling organelles, in both normal and stress conditions (Raghavendra and Padmasree, 2003; Blanco et al., 2014; Ng et al., 2014; Rurek, 2014; Bailleul et al., 2015; Bobik and Burch-Smith, 2015; Sun and Guo, 2016).

In order to investigate the possible invovlement of mitochondria in the chloroplast heat response I pre-treated *Arabidopsis* seedlings with two mitochondrial respiration inhibitors: rotenone and myxothiazol. Pre-treatment of plants with either rotenone or myxothiazol caused an increase in the magnitude of the stromal calcium increase in response to heat (figure 5.15A), and the result of the action of the two inhibitors on the calcium increase was comparable (figure 5.15B).

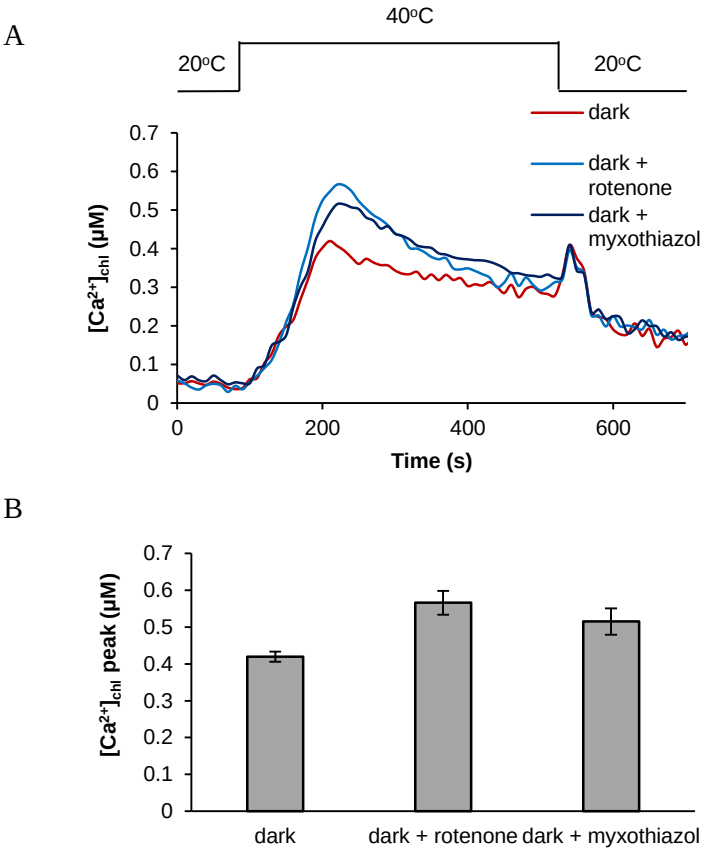


Figure 5.15 Mitochondrial respiration inhibitors affect the chloroplast calcium heat response. Pre-incubation of *Arabidopsis* seedlings with dark, dark + rotenone (50 µM) or dark + myxothiazol (20 µM) for 30 min was performed before measuring chloroplastic calcium upon heating (40°C for 7 min). (A) average $[Ca^{2+}]_{chl}$ kinetics of rotenone (n = 8) and myxothiazol (n = 9) treated plants, plus dark control (n = 10) and (B) average chloroplastic calcium concentration peak heights. Error bars = SE.

When plants were pre-treated with these inhibitors in the light for 30 minutes, the pronounced effects of mitochondria inhibition were partially (myxothiazol) or totally (rotenone) rescued by the presence of light, indeed the calcium traces of light plus mitochondrial inhibitors do not differ much from the control one (light only), as shown in figure 5.16.

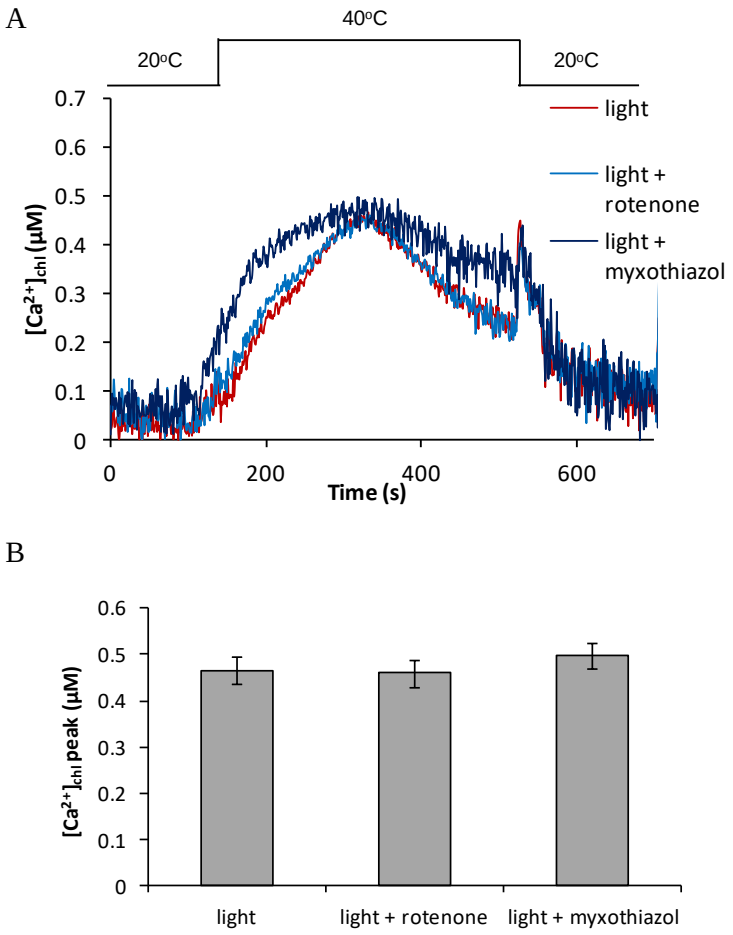


Figure 5.16 Mitochondrial respiration inhibitors affect the chloroplast calcium heat response in the dark, but not in the light. Pre-incubation of *Arabidopsis* seedlings with light, light + rotenone (50 µM) or light + myxothiazol (20 µM) for 30 min was performed before measuring chloroplastic calcium upon heating (40°C for 7 min). (A) Average $[Ca^{2+}]_{chl}$ kinetics of rotenone (n = 10) and myxothiazol (n = 9) treated plants, plus light control (n = 10) (B) and average chloroplast calcium concentrations at the peak are represented. Error bars = SE.

Hence the next step was to test if there is any calcium increase in the mitochondria upon heating at 40°C. Plants with YFP-aequorin targeted to the mitochondria were available, and they were tested in comparison with YFP-aequorin in the cytosol and targeted to the

chloroplast stroma, like pMAQ6 (Mehlmer et al., 2012). The calcium kinetics measured for the cytosol and chloroplast were comparable to the ones seen previously (figure 5.1), with a calcium increase happening specifically in the chloroplast, while despite the higher baseline, no significant calcium increase was detected in the mitochondria in response to the heat pulse (figure 5.17).

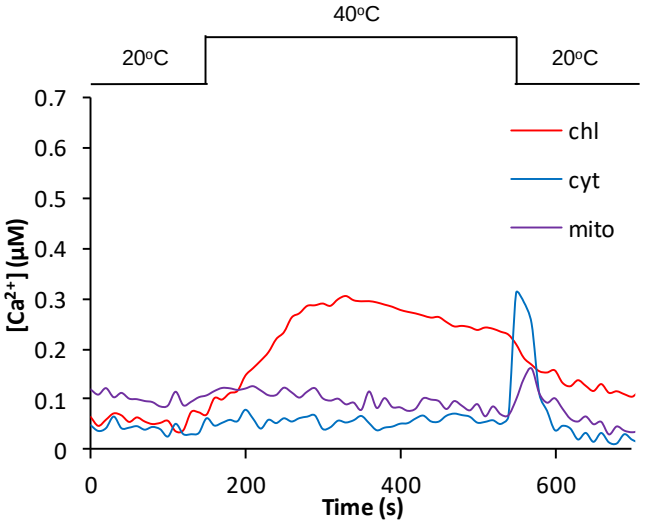


Figure 5.17 Heat does not induce mitochondrial calcium concentration. Calcium kinetics upon heating at 40ºC in *Arabidopsis* plants constitutively expressing aequorin targeted either to the cytosol (cyt), chloroplast (chl) or mitochondria (mito). Each trace represents an average of n = 4 seedlings.

5.2.5 Involvement of ROS in the heat response

The involvement of ROS was tested by pre-incubating plants with or without H_2O_2 for 30 minutes before measuring calcium in the chloroplast upon heating. This experiment was performed both in the light or dark, and in both cases the response was enhanced by the

hydrogen peroxide pre-incubation (figure 5.18). This indicates that ROS can affect calcium kinetics upon heating in the chloroplast stroma.

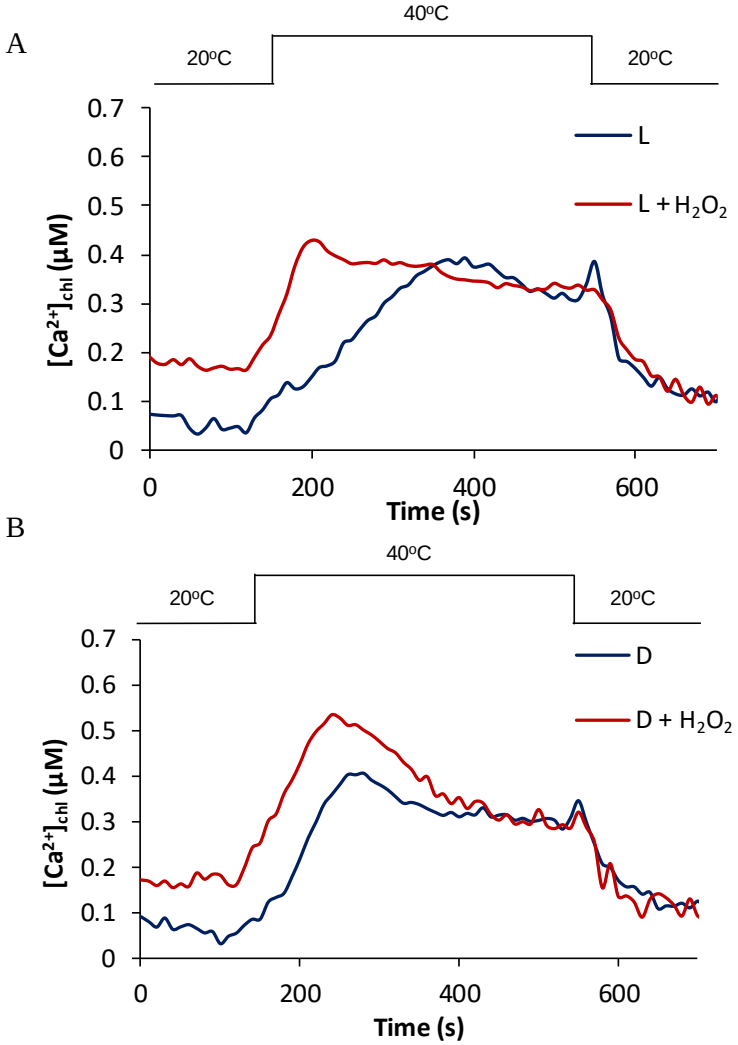


Figure 5.18 Hydrogen peroxide pre-incubation affects the chloroplast-specific heat response. Calcium kinetics upon heating at 40ºC in *Arabidopsis* plants constitutively expressing aequorin targeted to chloroplast pre-incubated either in the (A) light or (B) in the dark for 30 minutes with or without 10 mM H_2O_2 before measuring calcium upon heating in the dark. Each trace represents an average of n = 4 seedlings.

5.3 Discussion

5.3.1 Heat triggers a chloroplast-specific calcium response

In this study, a chloroplast-specific calcium signal was identified in response to heat. I demonstrated that this response occurs uniquely in the chloroplastic compartment and that it is dependent upon the magnitude of the absolute temperature applied, not the rate.

Evidence of calcium signalling in the chloroplasts has been previously reported in response to pathogens (Manzoor et al., 2012; Nomura et al., 2012) and abiotic stress (Nomura et al., 2012); and these stimuli are able to cause both a cytosolic and a stromal calcium increase. However, the only other reported case of a chloroplast-specific calcium increase was discovered by Johnson and colleagues in 1995 in response to a light-to-dark transition (Johnson et al., 1995).

Heat and calcium have previously been linked in the literature. It has been shown that calcium is able to confer protection against heat stress, specifically preventing oxidative damage, and that it is involved in the acquisition of long term thermotolerance (Gong et al., 1997; Gong et al., 1998a; Larkindale and Knight, 2002). Moreover, in moss, specific calcium cyclic nucleotide-gated channels (CNGCs) located in the plasma membrane have been shown to regulate the thermosensory response (Saidi et al., 2009; Finka and Goloubinoff, 2014). Further evidence of a possible role for calcium in heat response pathways comes from the study of unicellular prokaryotic cyanobacteria, where a calcium increase analogous to the one presented in this study (figure 5.1) was reported in response to heat shock (Torrecilla et al., 2000). The presence of a similar mechanism in prokaryotes might suggest that such responses were developed before the endosymbiotic event leading to chloroplasts in eukaryotes, and then conserved in the chloroplast throughout subsequent evolution.

The heat-induced calcium response was consistent between different *Arabidopsis* ecotypes (Col-0 and Ws-0, figure 5.5) and species (tobacco and *Arabidopsis*, figures 5.4 and 5.2), suggesting that there may be a common signalling mechanism in higher plants. However, differences in the magnitude of the calcium increase were observed when different species were stimulated by the same temperature. Indeed, comparable stromal calcium concentrations were detected in *Arabidopsis* when stimulated at 40ºC as in tobacco at 45ºC (compare figures 5.1 and 5.4). These differences could be either attributed to genetic differences in the thermometer between the two species, or to the different growth temperature regime applied before the heat treatment (consistent with data shown in figure 7.2, chapter 7). Regardless, in both species the relationship between higher temperatures causing a larger calcium increase was observed. This relationship is clearly demonstrated in figures 5.2A and 5.2B, where the kinetics of the calcium responses, as well as peak heights, change progressively with increasing temperature, i.e. follow a dose-response relationship. Such differences in calcium kinetics can be detected by plant cells as unique "Ca^{2+}-signatures" (McAinsh and Hetherington, 1998), which are crucial to encode different cellular messages. Therefore, the different calcium signatures seen at different temperatures might be used by plants to discern one temperature from another, acting as a cellular "thermometer".

The dose-response relationship between calcium signature and heat increase was lost at 50ºC (figure 5.6); for this temperature the first expected calcium peak was followed by another two peaks, the first of which was of very big magnitude (up to 0.63 μM). Furthermore, the aequorin left in the discharge part of the experiment was reduced compared to the amount measured for lower temperatures (data not shown). Taken together these two evidences pointed towards the possibility that treating plants at 50ºC for 7 minutes was lethal to them. According to this experiment, the 40ºC treatment was chosen for further experiments, as it did not kill the plants, hence allowing to further investigate plant physiology upon heating.

One interesting property of the chloroplast calcium heat response is that its amplitude attenuates when plants are exposed to consecutive heat stimulation of the same magnitude (figure 5.7). This characteristic is termed attenuation and it has been previously demonstrated for the cytosolic calcium cold response (Plieth et al., 1999). Attenuation is most probably attributable to the activity of channels, which are desensitised by the consecutive stimulations. The possibility that the reduction in the signal may be due to lack of calcium available in the stores was excluded by the data shown in figure 5.7, where a higher absolute temperature stimulation restored the calcium increase. This property (overcoming attenuation) is known as sensitisation and has also been observed for the cold response (Plieth et al., 1999).

Another very important feature observed in the cold-induced cytosolic calcium increase is its dependence upon the cooling rate (dT/dt, Plieth et al., 1999), rather than absolute temperature. Hence, I tested the effect of rate upon the chloroplast heat response, and it emerged (figure 5.8) that high temperature sensing in plants is mostly dependent upon absolute temperature, rather than rate. Indeed, for the range of rates tested, the value obtained for the calcium peak height was highly similar. This lack of correlation between rate and peak height is an indication that the absolute temperature reached at the end of the heating regime (40^{o}C for all the samples) is the major parameter controlling the calcium increase.

This major difference in the behaviour of the cold response compared to the heat response reported here is indicative of the fact that two distinct thermometers must be present in plants for sensing increases and decreases in temperatures.

5.3.2 Cross-talk between light, ROS mitochondria and heat in the chloroplasts

The major role of chloroplasts is to transform CO_2 and water into a source of chemical energy via photosynthesis, requiring light energy. Hence, a main regulatory signal that

controls chloroplast physiology is light. In order to investigate the effect of light on the heat-induced stromal calcium increase, plants were either dark- or light-adapted before the heating event. As shown in figures 5.9 and 5.10, light has a profound effect on the heat response, which is manifested as a delay in the start of the calcium response. Unfortunately, the usage of a luminescent reporter (aequorin) constrains measurements to be taken in the dark, hence direct measurements of calcium in the light are technically not possible. Nevertheless, from figures 5.9 and 5.10 there is evidence of a strong regulation of the heat response by the lighting conditions. It is possible to conclude from these data that, in the light, the stromal calcium increase is either not occurring, or the release is counterbalanced by calcium uptake. To explain the mechanism, it is tempting to speculate a role for the Ca^{2+}/H^{+} antiporter (Ettinger et al., 1999; Wang et al., 2016) in the thylakoid membrane in controlling this response. Supporting evidence comes from the effect of DCMU and DMBIB on the light pre-treatment as these photosynthetic inhibitors act to impair the accumulation of the proton gradient, affecting the electron flow (Karpinski et al., 1997; Pfannschmidt et al., 2001). When applied during the light treatment both these inhibitors caused a partial rescue of the light-induced calcium delay (figures 5.9 and 5.10). To further confirm what was observed in the light/dark experiment (figures 5.9 and 5.10) and to find a way to measure what happens to the calcium response concomitantly with light, an aequorin consumption experiment was performed. In figure 5.11 it is possible to see that this approach could report the dark heating event, which indeed showed a significant reduction of the amount of aequorin during the discharge. This difference was not recorded when plants were heated in the light (figure 5.11), confirming the data obtained before (figure 5.9 and figure 5.10). A third experiment consolidated these two previous observations about heating, light and calcium; and is reported in figure 5.12. If heat caused a stromal calcium increase in the light, then at the onset of lights off calcium should have been already high. However, that was not the case (figure 5.12), on the contrary, there was a gradual and delayed calcium increase as expected if the calcium signal was dark-specific. Taken together, these data indicate that light is a negative regulator of the calcium heat response.

All this evidence can indeed be explained by stating a role for the proton gradient, and possibly the Ca^{2+}/H^{+}-antiporter, in regulating the heat-induced calcium response, whose activity can be gradually overcome by heat and darkness and lead to an increase in stromal calcium concentration. However, this hypothesis cannot explain the data shown in figure 5.15. In this case, plants were light- or dark-treated for longer time periods (1 h, 2 h and 6 h), hence the chloroplasts could gradually accumulate a proton gradient. Hence, one could expect that after such longer light exposure times the heat response could be even more strongly counterbalanced by the Ca^{2+}/H^{+} antiporter. However, the stromal calcium response was enhanced by light pre-incubation, i.e. the longer the exposure the bigger and faster the calcium increase (figure 5.13). This kind of relationship between chloroplast calcium signalling and light had already been observed in response to the light-to dark transition (Sai and Johnson, 2002), where it was shown that the calcium peak height and the integrated luminescence were directly proportional to the length of the light exposure. These differences were not present when plants were kept in the dark for different time periods (figure 5.14).
For these data we can propose two explanations; the first one being that our hypothesis about the proton gradient mechanism is incorrect, and a completely different mechanism is involved. The second option envisages the presence of more than one component controlling the calcium heat response in the light, and these can explain the data observed. The relatively limited knowledge on calcium signalling components in the chloroplast does not allow to discern between these two hypothesis, yet.

Since evidence are pointing towards a role of the chloroplast calcium heat response in darkness, our reasoning was to try to possibly link the response to the plant energy balance. Indeed during heat the plant faces high energy requirements, If this happens concomitantly with heat, then the plant is facing a starvation situation (Caldana et al., 2011). Hence we tried to investigate a possible role for mitochondria in this response. Our data show that inhibition of mitochondrial respiration leads to enhancement of the heat induced chloroplast calcium response (figure 5.15). Rotenone affects the activity of Complex I of the mitochondrial electron transport chain, while myxothiazol, acts

specifically on Complex III (Meinhardt and Crofts, 1982; von Jagow et al., 1984; Schwarzländer et al., 2009). When the mitochondrial electron transport chain was inhibited by rotenone or myxothiazol pre-treatment, the magnitude of the stromal calcium response to heat was increased, and the extent of the change was comparable for the two inhibitors (figure 5.15). When the same experiment was performed by illuminating during the mitochondria inhibition treatments, light was able to partially (myxothiazol) or totally (rotenone) revert the mitochondrial inhibitor effect observed in the darkness. Indeed, in the dark plants are energetically stressed by the lack of photosynthesis occurring concomitantly with heat, which is a cause for starvation. These results indicate that mitochondria are able to cross-talk with chloroplasts during the heating process, and that the energetic status of mitochondria might be tuning the chloroplast-specific calcium response.

In order to test if the calcium increase upon heating is chloroplast-specific, or if heating increases the mitochondrial calcium as well, lines expressing YFP::aequorin targeted to the mitochondria, chloroplasts and cytosol were used. The cytosolic and stromal lines confirmed the results observed with *Arabidopsis* lines expressing aequorin without YFP (compare figure 5.1 with figure 5.17). Differences in the magnitude and kinetics of the responses are due to the usage of two different aequorin isoforms in these two set of lines. By contrast, the mitochondrial lines displayed a higher baseline, as expected since mitochondrial basal calcium levels are known to be higher compared to cytosolic ones (Logan and Knight, 2003). However, upon heating there was no mitochondrial calcium increase, indicating that the calcium signal upon heating is chloroplast-specific, and that organelles are able to control their calcium fluxes independently. Indeed, mitochondria and chloroplasts are located very close to each other in a plant cell (Bobik and Burch-Smith, 2015), hence not seeing a reduced calcium increase in the mitochondria upon the heat-induced chloroplast shows that these two organelles are able to tightly control their calcium levels.

Since the mitochondrial status is able to affect the chloroplast-specific heat response, but calcium itself is not the second messenger involved in this organellar cross-talk (figure 5.17), I hypothesized that ROS might be involved. To test this I affected the ROS status of the plants by pre-incubating them for 30 minutes with H_2O_2 before measuring calcium in response to heat. From figure 5.18 it is possible to see that H_2O_2 pre-incubation both in light or dark enhances the chloroplast heat response. However, at the onset of measurement, the baseline was already higher, indicating that H_2O_2 by itself is able to trigger a chloroplast Ca^{2+}-increase. Taken as they are, these observations are not strong enough to state a role for ROS in this heat response, and further experiments with ROS inhibitors or ROS mutant lines would be required.

5.3.3 Physiological significance of the chloroplast calcium heat response

Regarding the physiological role of the heat-induced chloroplast calcium response I speculate that there might be two possible roles: one to switch off photosynthesis under high temperature conditions or, the second, to act as a signal of reduced energy status due to heating. In support of the first possibility is the fact that the only other chloroplast-specific calcium increase reported in the literature is in response to the light-to-dark transition (Johnson et al., 1995). It is interesting to note that both in their study and in our recent findings these chloroplast-specific Ca^{2+}-responses seem to be specifically associated with darkness. The suggested biological significance for the light-to-dark transition is to signal the onset of darkness and to set the photosynthetic apparatus off for the night (Sai and Johnson, 2002). Therefore, if stromal calcium increases are responsible for blocking photosynthesis as a physiological daily signal, then the same system might be exploited by the plant in a heat stress condition, when activation of photosynthesis can be damaging for the organism. Hence this heat-induced calcium release may act as a signal for pre-setting the photosynthetic apparatus in the off mode in the darkness, preventing photosynthesis if light later occurs concomitantly with heat.

The second possibility is that the chloroplast calcium signal conveys information regarding energy status i.e. the lower the energy status, the higher the calcium response. This would be consistent with an increase in stromal calcium in response to darkness when photosynthesis is stopped (Johnson et al., 1995), and also in response to heat which itself provokes energy stress when presented in the dark (Caldana et al., 2011). At night (in darkness), plants have a limited amount of resources (mainly accumulated as starch), which are carefully calibrated to get through the period of darkness (Graf et al., 2010; Graf and Smith, 2011; Scialdone et al., 2013; Scialdone and Howard, 2015). However, when heat occurs, plants face a situation of even higher energy requirements, and processes such as protein degradation and autophagy are initiated (Heckathorn et al., 1996; Caldana et al., 2011) to produce sufficient carbon skeletons to support the required level of energy through mitochondrial respiration. The possibility that the stromal calcium response is a signal for energy stress is consistent with 3 observations (1) light inhibition of the heat induced stromal calcium increase (as under photosynthetic conditions energy status will be improved); (2) that this light inhibition of the heat-induced stromal calcium increase can be overcome by inhibitors of photosynthesis (as energy produced through photosynthesis in the light will be inhibited) and; (3) inhibiting mitochondrial respiration enhances the stromal calcium response (as inhibition of respiration will enhance the level of energy stress). Future research will be aimed at determining whether either, or both, of these possibilities are indeed physiological roles of the heat-induced chloroplast calcium response.

5.3.4 Conclusions

In this chapter a novel calcium signature was identified in response to elevated temperature, and it is chloroplast-specific. This chloroplast calcium response is dose-dependent above a threshold of 30°C and is conserved amongst species and ecotypes. The calcium response is dependent upon absolute temperature, not rate of heating. The relationship between heat-induced increases in chloroplast calcium with both light and the status of the mitochondria was examined. It was found that light acts as a negative

regulator of the heat-induced calcium response, and that this negative regulation was dependent upon active photosynthesis via the electron transport chain. Inhibiting mitochondrial function enhances the chloroplast heat induced calcium response, suggesting that the chloroplast is able to assess mitochondrial energy status, and increase its response to heat accordingly.

Chapter 6

Biological significance of the chloroplast calcium heat response

6.1 Introduction

Calcium is a universal second messenger involved in nearly every aspect of plant cell physiology. In response to a calcium increase, the plant cell is able to decode the information contained in the "calcium signature" and trigger the appropriate downstream response (for details see chapter 3). The transient calcium elevation contains a message, which can be read only if the cell has the downstream components which are capable of binding calcium and continuing the downstream response. Hence, to understand the significance of a calcium event it is important to understand the downstream physiological response controlled by it. This chapter is aimed at finding what the biological significance of the chloroplast-specific heat-induced calcium response analysed extensively in chapter 5 is. As mentioned in the introduction (chapter 1), in order to prove that a signal is mediated by calcium, a calcium increase should occur in response to it, and these events must be correlated in time and space (Jaffe, 1980). The second law mentioned in Jaffe's paper is very relevant for this chapter, and it is that blocking the calcium increase must prevent the downstream response. Indeed the first approach taken in order to understand the biological significance of the heat response was to try to block the chloroplast calcium increase. Finally, the third characteristic defining a calcium signal is that an artificial calcium increase comparable in size to the one caused by the stimulus should be able to activate the response even in absence of the stimulus itself (Jaffe, 1980). This third rule is not always valid, as for some downstream responses

to be triggered another messenger may be required as well as calcium. Hence calcium can be necessary, but not *per se* sufficient to cause the physiological change. For this reason the approach taken tested both possibilities (see results section in this chapter).

Aims of the research described in this chapter:

- To identify a way to block the heat-induced chloroplast calcium increase.
- To identify cellular components of the chloroplast involved in this response (specifically the role of CaS).
- To identify the physiological output of this calcium increase in response to heat.

6.2 Results

6.2.1 Blocking the heat-induced chloroplast calcium increase

Blocking the calcium response is a fundamental step to investigate the biological significance of the heat response. To do so, several approaches were taken, from the more classical ones, where calcium channel inhibitors or chelators were added prior to the heating event, to the usage of stromal-target calcium sponge proteins to buffer the calcium increase. Finally, mutants for the thylakoid-bound calcium sensing protein CaS were investigated, as the heat-induced chloroplast calcium response was partially inhibited in this genetic background.

6.2.1.1 Attempting to block the heat-induced chloroplast calcium increase by chemical means

One of the most common approaches to affect calcium signalling is to use chemical inhibitors. These can be divided into calcium channel blockers, with different specificities (e.g. DNQX acts on glutamate receptors specifically), calcium chelators, and chemicals that impair the calcium uptake (e.g. by blocking the Ca^{2+}-ATPases). Furthermore, the cellular location at which they act can be different, as not all of them are able to penetrate the plasma membrane. For example, the chelators BAPTA and EGTA have limited ability to penetrate cell membranes, hence a version was made, called BAPTA-AM and EGTA-AM, where the chelators were bound to an acetyloxymethyl ester group. This addition changes the characteristics of the chelators, which are then able to freely permeate membranes, plus as they do, cellular esterases cleave the –AM group, making the chelator loading permanent. In this work, a range of calcium blockers was applied for 30 minutes on *Arabidopsis* pMAQ6 plants (except for ruthenium red, which was added for 3 h), then

chloroplast calcium was measured upon heating. These chemicals and their effect on the calcium response are reported in table 6.1.

Inhibitor	Concentration	Mode of action	Effect of the calcium heat response	Reference
Ruthenium red	100 μM	calcium channel blocker	none	(Rentel and Knight, 2004)
BAPTA-AM	326.9 μM	chelator	none	(Siegel et al., 2009)
EGTA-AM	299.1 μM	chelator	none	(Wu et al., 1997)
Nifedipine	50 μM	calcium channel blocker	none	(Larkindale and Knight, 2002)
DNQX	500 μM	GLR-type calcium channel blocker	none	(Ni et al., 2016)
$LaCl_3$	5 mM	L-type calcium channel blocker	increased	(Knight et al., 1997)
$GdCl_3$	10 mM	stretch- and voltage-activated calcium channel blocker	increased	(Knight et al., 1997)
Verapamil	1 mM	calcium channel blocker	increased	(Larkindale and Knight, 2002)

Table 6.1 List of calcium inhibitors, effect on the heat-induced calcium response measured in the chloroplast stroma of *Arabidopsis* in response 30 min pre-incubation with the inhibitor (except for ruthenium red, which was applied for 3 h) and then subjected to 40°C heating.

None of the chemicals used was able to inhibit the stromal calcium increase upon heating. However, three of them caused the opposite effect, meaning that they increased the calcium response upon heating. These chemicals were lanthanum ($LaCl_3$), gadolinium

($GdCl_3$) and verapamil, and the effect of 30 min pre-incubation with these chemicals at 5 mM, 10 mM and 1 mM, respectively, are reported in figure 6.1.

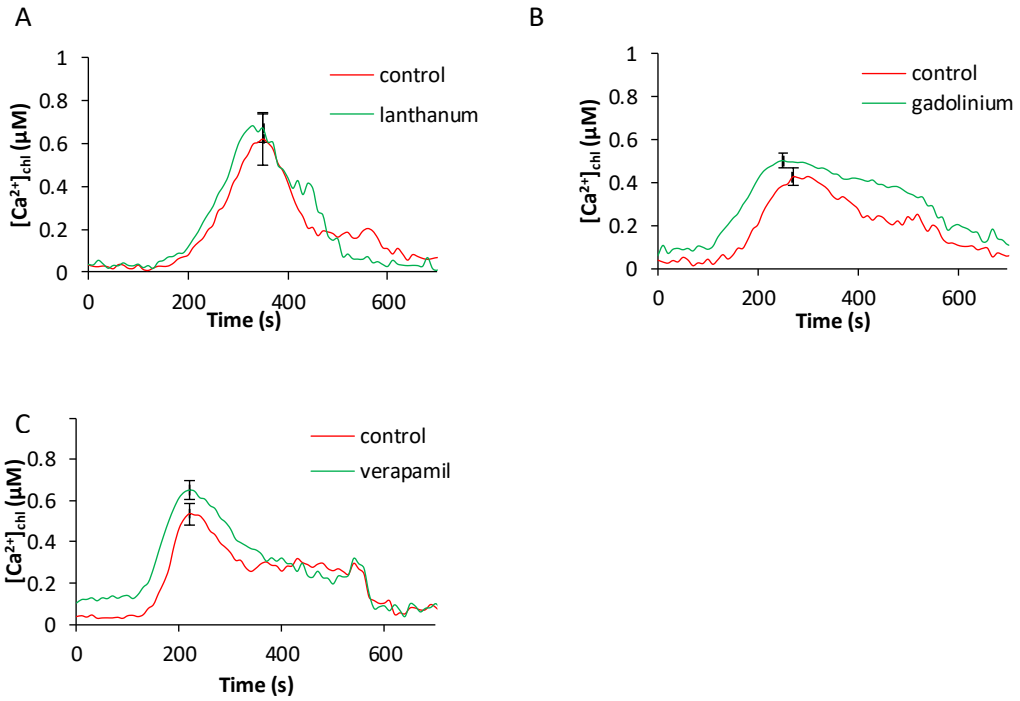


Figure 6.1 Calcium traces of pMAQ6 plants in response to heat upon 30 minutes pre-incubation with (A) 5 mM lanthanum, (B) 10 mM gadolinium or (C) 1 mM verapamil, compared to the control. Error bars represent SE at the calcium peak, with n = 6 plants.

As can be seen in figure 6.1, the usage of gadolinium and verapamil causes a significant increase in the stromal calcium levels already before heating (first 120 seconds), and consequently the heat response was increased upon the heat pulse itself. Lanthanum in contrast did not increase the baseline, but it did affect the calcium response to heat, whose peak was higher and reached more rapidly compared to the control (figure 6.1A), but this difference was not significative.

6.2.1.2 Attempting to block the heat-induced calcium increase by expressing Ca^{2+}-buffering proteins

As the calcium channel blockers and chelators did not succeed in inhibiting the heat-induced calcium increase, then a different approach was taken. The idea behind this was to express a calcium-binding protein able to locally buffer this ion when its levels increased in the stroma, hence the downstream response would be inhibited. To do so, three calcium-binding proteins were targeted to the stromal compartment: ARP, Parv and a modified version of aequorin.

ARP is a calcium-binding acidic repeat protein identified in *Euglena gracilis* (Gumpel and Smith, 1992), while Parv (parvalbumin) is a non canonical, archetypal EF-hand protein able to bind calcium with high affinity (Wang et al., 2013). According to the literature, these two proteins are able to bind calcium and buffer it, hence they were targeted to the chloroplast stroma with the RbcS targeting sequence. Another protein was expressed in order to try to bind calcium locally in the stroma, namely aequorin. Since it was shown that in pMAQ6 lines aequorin is functioning in the chloroplast stroma, we reasoned that this protein must be able to bind calcium, as we could measure changes in luminescence. Hence a modified versions of aequorin was used: it was codon-optimised for *Arabidopsis* (aequorin derives from the coelenterate *Aequorea victoria*) to try increase the buffering effect by increasing the aequorin amount. Furthermore, lysine in position 9 was removed as it is a predicted ubiquitination site, and only the calcium-binding part was kept, while the coelenterazine-binding domain was removed.

However, when any of these three calcium buffering proteins was transiently expressed in tobacco, as well as stromal aequorin, none of them was able to chelate the heat-induced calcium increase in the stroma, as represented in figure 6.2. Indeed calcium curves obtained in leaves expressing the buffering proteins were comparable to the results obtained for the control plants when stimulated a 40°C.

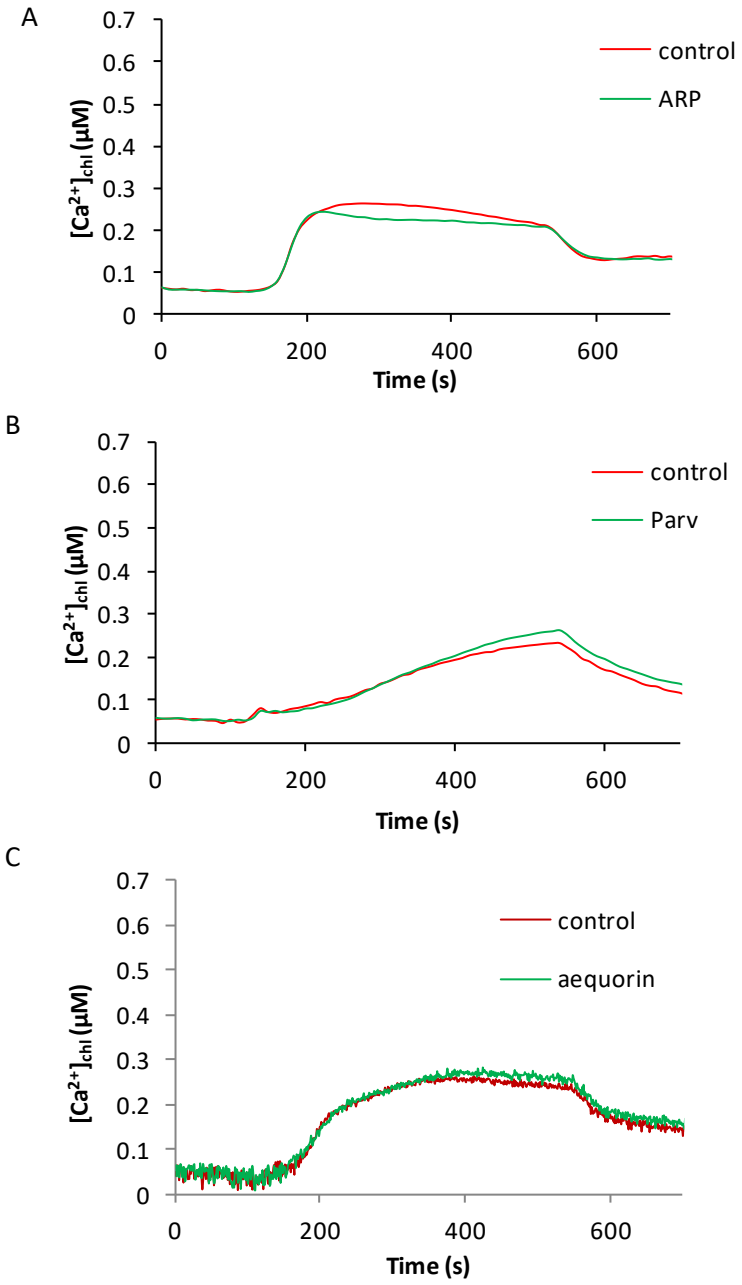


Figure 6.2 Calcium traces obtained by stimulating at 40ºC tobacco leaves transiently expressing stromal aequorin and (A) the ARP protein, (B) Parv protein and (C) a truncated version of aequorin, compared to the control (same vector expressing the non-calcium binding protein GFP).

6.2.1.3 Attempting to block the heat-induced calcium increase by targeting the upstream response

After trying to block the heat-induced calcium increase by using chemicals or with Ca^{2+}-buffering proteins, without any positive result, the third approach adopted aimed to target a different step of the heat-induced calcium increase, which was the putative upstream components. I reasoned that, by blocking any signal upstream of the calcium increase, then it could have been possible to inhibit the calcium elevation itself. However, the pathway components are not known, and I had to work with hypothesis. The first idea was to try to see if the redox state of the glutathione (GSH) pool was affecting the calcium heat response. To test this, plants were pre-incubated for 6 h in 10 mM BSO (buthionine sulphoximide), a chemical which impairs GSH synthesis, and switches the guthatione pool towards the oxidised form of GSSG (glutathione disulfide) (Griffith and Meister, 1979; Rentel and Knight, 2004). The state of the GSH/GSSG pool did not, however, affect the stromal calcium response upon heating, as shown in figure 6.3.

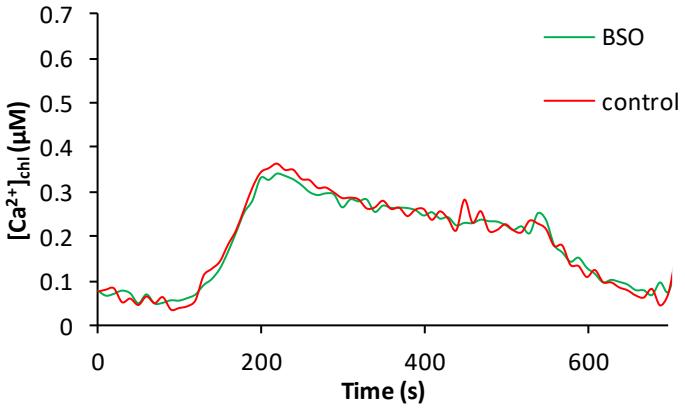


Figure 6.3 Representative calcium traces obtained by pre-incubating pMAQ6 *Arabidopsis* plants treated with BSO or water control for 6 h, an then heated at 40°C. The stromal calcium response to heat is represented.

An alternative approach adopted to try to block this calcium increase was to deplete the calcium stores. To do so, plants were pre-incubated with 1 µM thapsigargin or 10 mM erythrosin B for 30 minutes in order to deplete ER calcium, as the reticulum is a major calcium store and it is physically linked to chloroplasts via specialized structures called stromules (Natesan et al., 2005). Thapsigargin and erythrosin B are able to block the ER Ca^{2+}-ATPases, hence the calcium uptake in the subcellular compartment (Lytton et al., 1991; Thomson et al., 1993). Depleting the calcium from the ER did affect the stromal calcium increase. Indeed, as it is shown is figure 6.4, pre-incubation with erythrosin B increased the stromal calcium response to heat, and it affected the basal level of calcium in the stroma (figure 6.4), while thapsigargin did not affect the calcium response. In any case, none of these two chemicals were able to inhibit the chloroplast calcium signal.

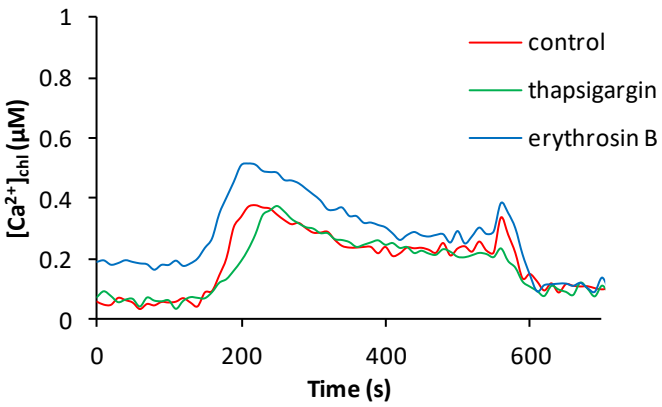


Figure 6.4 Representative calcium traces of the stromal heat response of *Arabidopsis* plants pre-treated for 30 minutes with 1 µM thapsigargin, 10 mM erythrosin B or DMSO control before heating at 40ºC.

6.2.1.4 The heat-induced calcium response is impaired in the *cas* mutant

As none of the previous approaches succeeded in blocking the calcium response, I lastly decided to see if it was possible to identify a mutant for the stromal calcium machinery in which the response was affected. Amongst the possible candidates the CaS protein was chosen as it is a calcium-binding protein (Han et al., 2003), and it has been shown to be involved in stromal calcium responses (Nomura et al., 2012). Two independent *cas* mutant lines were obtained as a kind gift from Eva-Mari Aro (Turku University, Finland), a SALK line (*cas SALK*) and GABI line (*cas GABI*) (Vainonen et al., 2008). In these lines, expression levels of *CaS* (At5g23060) were tested to confirm that the gene was not expressed, and this was the case (shown in figure 6.5).

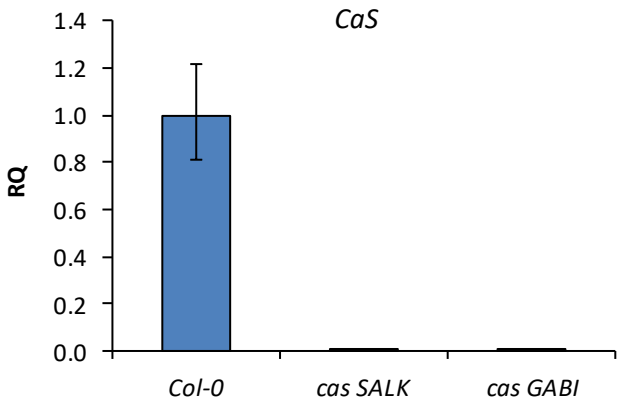


Figure 6.5 Transcript levels of the *CaS* gene (At5g23060) in Col-0, *cas SALK* and *cas GABI* background. Data were obtained by RT-PCR, as describes in Materials and Methods (chapter 2), error bars represent SE.

These *Arabidopsis* lines were transformed to express stromal aequorin, and lines with aequorin levels comparable to pMAQ6 were selected amongst the transformants (see chapter 2 Material and Methods for details). Before testing the *cas* lines for the heat response a control was performed on them, to test if the activity of the aequorin was

affected in the *cas* mutant background. Hence these lines were stimulated with 10 µM mastoparan, and all the different genotypes responded in a comparable manner, as shown in figure 6.6 (traces are superimposable). This confirms that the activity of the aequorin enzyme is not affected in the *cas* lines.

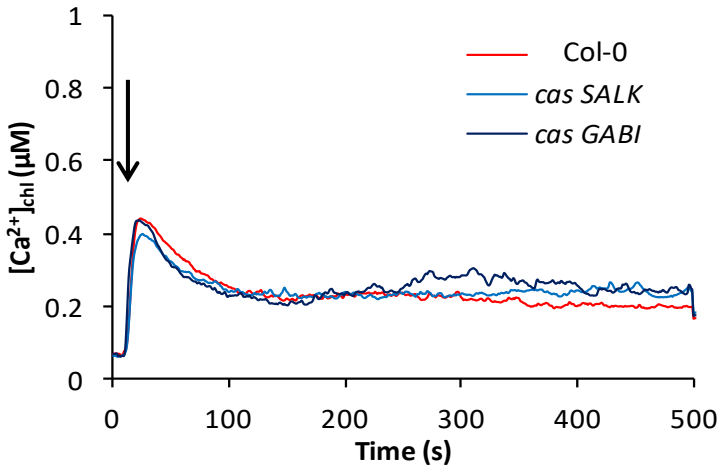


Figure 6.6 Average chloroplast calcium traces of *Arabidopsis* Col-0, *cas SALK* and *cas GABI* lines in response to 10 µM mastoparan. Mastoparan was injected after 10 seconds (injection represented by the arrow).

Subsequently the *cas* mutant lines containing stromal aequorin were tested for the heat response. As shown in figure 6.7, the heat-induced chloroplast calcium increase is partially, but significantly, inhibited in both the *cas* mutant lines, suggesting a role for this protein in the chloroplast heat response. Furthermore, these lines represent a precious tool to further investigate the biological significance of the calcium heat response in the chloroplast, as there is an inhibition of the calcium increase upon heating.

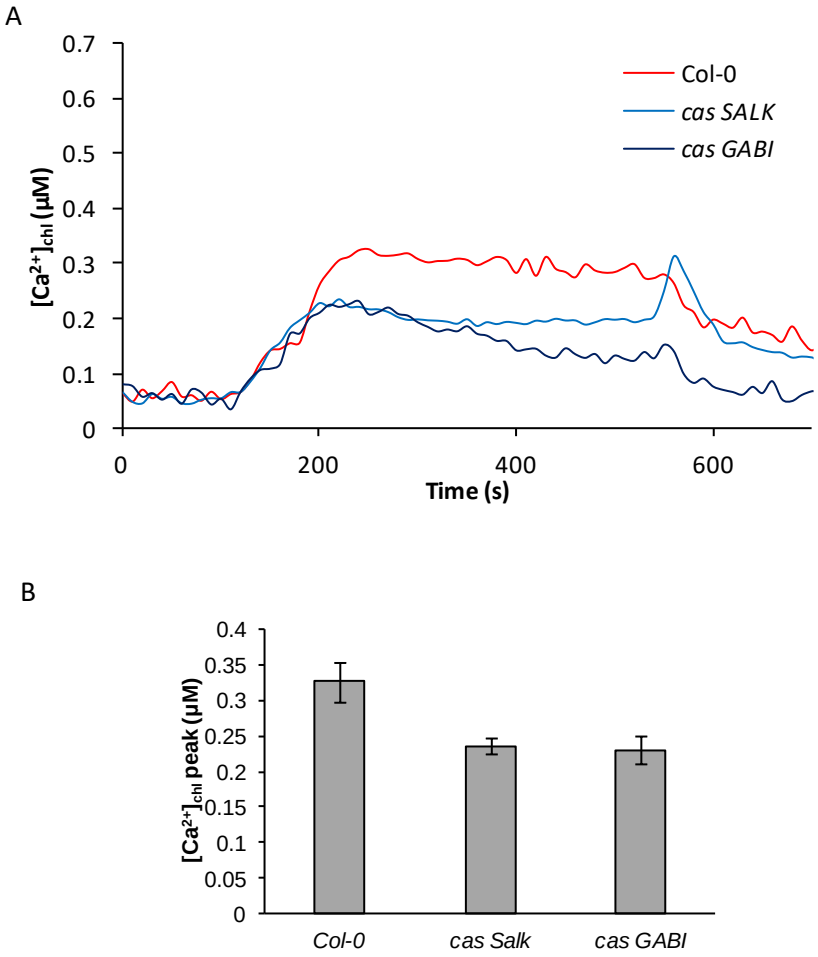


Figure 6.7 (A) Representative calcium traces of *Arabidopsis* wt Col-0 , *cas SALK* and *cas GABI* lines in response to a 40ºC heat pulse, and (B) average chloroplastic calcium concentration peak heights. Data were obtained by averaging n = 4 8-day-old *Arabidopsis* seedlings, and for each temperature a different set of plants was used. Error bars = SE.

As a result of all these different approaches taken to block the heat response I was able to identify a reduced calcium increase to heating (in the *cas* mutants, figure 6.7), and pre-incubation conditions able to increase the calcium response upon heating (5 mM lanthanum, 10 mM gadolinium and 1 mM verapamil, figure 6.1). Another chemical able

to increase the calcium response upon pre-incubation was identified in chapter 5, figure 5.18, namely 10 mM H_2O_2. Finally, mastoparan is able to cause a stromal calcium increase in absence of heating (chapter 4, figure 4.2B), hence at this point we had a complete set of conditions we could exploit to investigate the physiological significance of the heat-induced chloroplast calcium response.

6.2.2 Linking the chloroplast calcium increase in response to heat to the plant physiological response

6.2.2.1 Changes in photosynthesis during heating are not dependent on the chloroplast calcium increase

High stromal calcium concentrations have been shown to inhibit photosynthesis, by acting on the Calvin-Benson cycle. Specifically, the fructose-1,6-bisphospatase and sedohepthulose 7-phospatase activities are inhibited by high calcium concentrations (Charles and Halliwell, 1980; Kreimer et al., 1988). In 1995 the first chloroplast-specific calcium response was identified, in response to the light-to-dark transition (Johnson et al., 1995). One of the predicted outputs of this response was to set the photosynthetic apparatus off for the night, hence the calcium increase would block and prevent photosynthesis from happening (Sai and Johnson, 2002). Given this previous identified link between stromal calcium and photosynthesis, a possible role of the heat-induced calcium increase in controlling the photosynthetic apparatus was investigated. Hence I measured F_v/F_m in response to the 40^{o}C heat stimulus in Col-0 and the two *cas* mutant lines at different timepoints during the heat pulse (2 min, 5 min, 8 min and 12 min, as represented in figure 6.8).

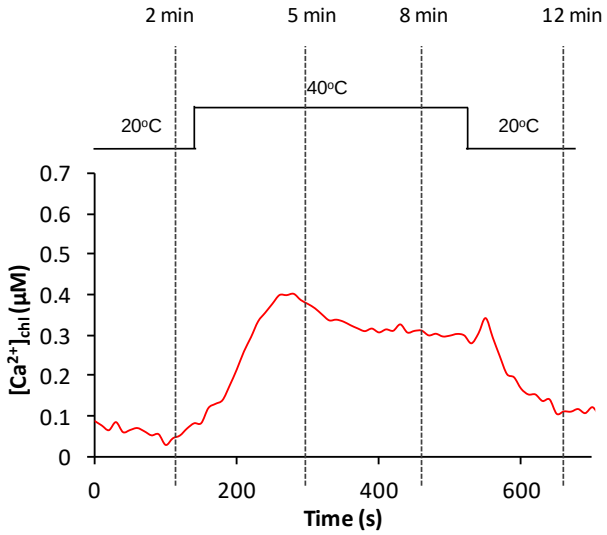


Figure 6.8 Representative calcium curve upon heating at 40°C for 7 minutes, and relative sampling timepoints at 2 min (20°C), 5 min (40°C), 8 min (40°C) and 12 min (20°C).

These different timepoints represent the basal status of the plants before heating (2 min), the early part of the heating event (5 min), a longer heating timepoint (8 min) and recovery after heating (12 min), hence if any difference in F_v/F_m occurs at any stage, this setup shall be able to capture it. Figure 6.9 shows the response of the Col-0 and the two *cas* mutant lines to the heating pulse in terms of F_v/F_m measured on a pool of seedlings. It is possible to see that F_v/F_m decreased with temperature in the control (8 min and 12 min), and this decrease was reduced in the two independent *cas* mutants at 8 min compared to the control (figure 6.9). These data are in agreement with the role of calcium in blocking the photosynthetic apparatus, as a reduced calcium increase caused a reduction of the inhibition effect.

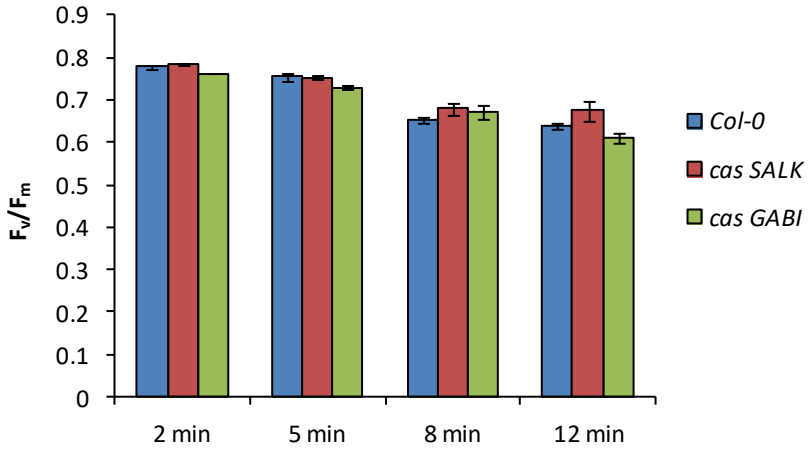


Figure 6.9 F_v/F_m measured over four different timepoints throughout the heating event (represented in figure 6.8) in Col-0, *cas SALK* and *cas GABI* lines. Error bars represent the SE obtained by averaging the signal of n=4 pools of *Arabidopsis* seedlings each one being an independent experiment.

The results presented in figure 6.9 show that it is possible that calcium may be blocking the photosynthetic apparatus, and that CaS is involved in this process. However, a previous link between CaS and photosynthesis has been made (Vainonen et al., 2008), hence the role of CaS in controlling F_v/F_m may be calcium-independent.

To further test this idea, we applied different conditions able to increase the chloroplast-specific calcium response to heat. Specifically plants were pre-incubated for 30 min with 10 mM gadolinium, 1 mM verapamil or 10 mM H_2O_2. All these chemicals have been shown to increase the calcium response to heat (figures 6.1B and 6.1C this chapter and figure 5.18 chapter 5), hence if the starting hypothesis is true, the inhibition of photosynthesis should be more marked in plants pre-treated with these chemicals. This was true for the gadolinium pre-treatment (figure 6.10A) and H_2O_2 (figure 6.10C) at 8 minutes and 12 minutes, but not for the verapamil (figure 6.10B). Furthermore, these differences are very small in terms of absolute value and significant (error bars: SE) only for the case of lanthanum (figure 6.10B), while for gadolinium and verapamil, the differences are not significant (figures 6.10A and 6.10C). This lack of consistency

between the data, hence the different behaviour of F_v/F_m in response to three pre-treatments which all increase the calcium response, points towards the evidence that the photosynthetic changes in F_v/F_m upon heating are not calcium-dependent. To further test this I tried to see if inducing a calcium increase in the stroma did affect F_v/F_m by itself by adding 50 μM mastoparan, but the levels of F_v/F_m were unaffected for all the different timepoints tested (data not shown), confirming that calcium may not have a role in controlling F_v/F_m changes upon heating.

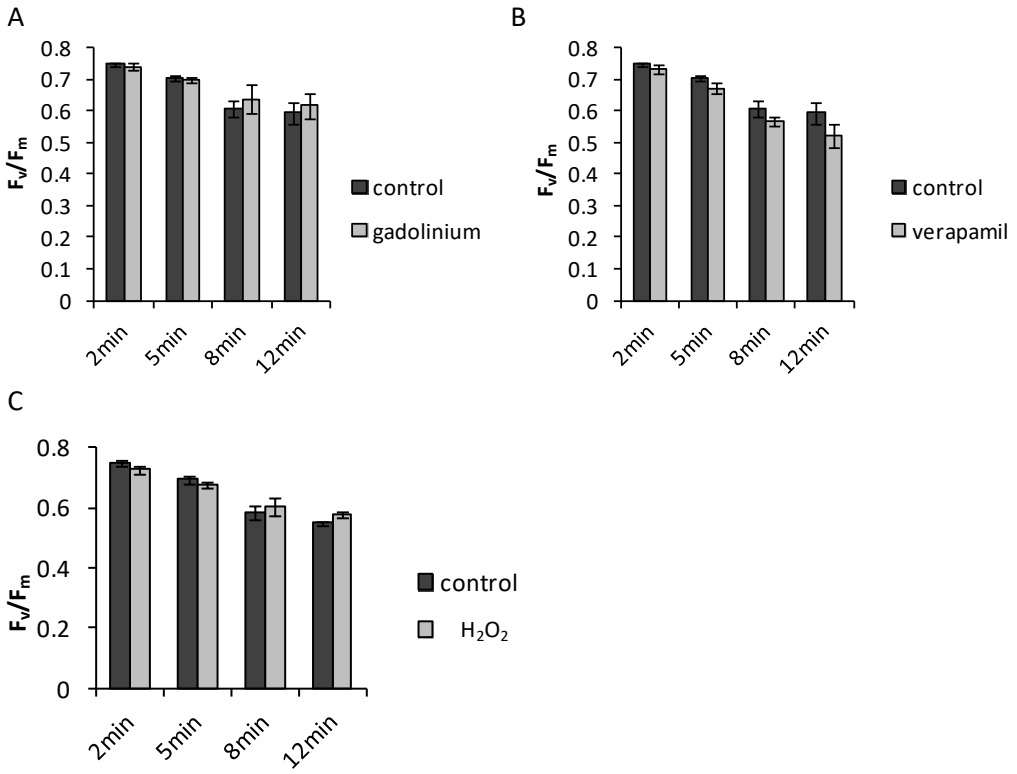


Figure 6.10 F_v/F_m of Col-0 *Arabidopsis* seedlings at different timepoints upon heating. Plants were pre-treated for 30 minutes before heating with (A) 10 mM gadolinium, (B) 1 mM verapamil, or (C) 10 mM H_2O_2, and a water control was added. Error bars represent SE of n=4 pools of seedlings.

6.2.2.2 Chloroplast calcium does not control the starvation response to heat

Another possible role for the chloroplast calcium heat response was already speculated in chapter 5, and it is the possibility that calcium is signalling the energy status of the plant. Evidence supporting this hypothesis is reported in the discussion of chapter 5. To further test if the calcium can be signalling the plant carbon balance, I initially decided to test whether the CO_2 levels by themselves are able to cause a stromal calcium increase, as increasing atmospheric CO_2 should affect the plant carbon balance. To do so, calcium levels were recorded in *Arabidopsis* seedlings expressing cytosolic (cyt, pMAQ2) or stromal (chl, pMAQ6) aequorin in response to 20 mM $NaHCO_3$. Results in figure 6.11 show that there was no calcium increase neither in the cytosol nor in the chloroplast in response to $NaHCO_3$. In the cytosolic aequorin line a calcium peak was recorded, as it is an unavoidable result of the injection, and it is considered a "touch response", which is calcium mediated (Knight et al., 1991).

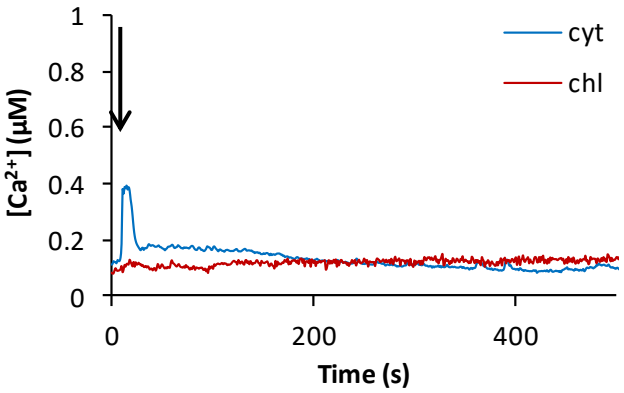


Figure 6.11 Average calcium traces recorded in the cytosol (cyt) or chloroplast (chl) of *Arabidopsis* plants in response to 20 mM $NaHCO_3$. The arrow represents the injection point (after 10 seconds since the beginning of data collection).

A second approach was adopted to further test the idea that CO_2 may be sensed by a stromal calcium increase. In this case, tobacco leaves were transiently transformed with

cytosolic or stromal aequorin, and then put in a chamber where levels of CO_2 could be controlled at the same time as recording luminescence levels (then converted into calcium concentrations). CO_2 levels were adjusted from atmospheric levels (around 400 ppm) to 1000 ppm while recording calcium, and the results are shown in figure 6.12. Changes in CO_2 concentration in the air did not increase calcium in the plants in either of the two compartments tested. The evidence obtained in two different organisms (tobacco and *Arabidopsis*) show that CO_2 levels are not directly affecting cytosolic or stromal calcium levels.

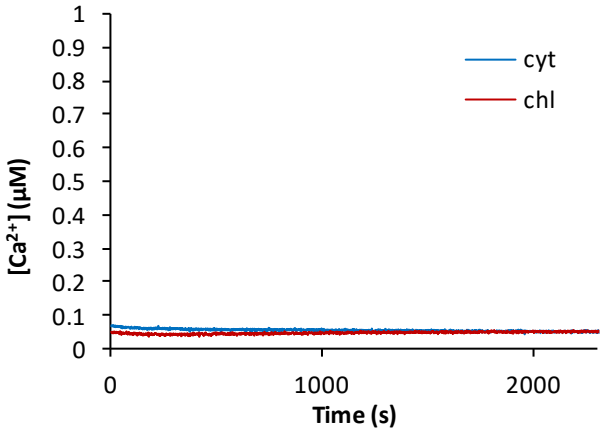


Figure 6.12 Average calcium traces recorded in the cytosol (cyt) or chloroplast (chl) of tobacco leaves in response to 1000 ppm CO_2.

To further investigate the possibility of calcium being involved in sensing the energy status, but not directly CO_2 levels, the next step was to try to change the energy balance of the plant in the dark, to ideally decrease the stromal calcium signal in response to heat. In order to do so *Arabidopsis* pMAQ6 seedlings were pre-incubated for 30 minutes with 0.5 mM or 1 mM ATP, or alternatively for 8 h with 50 mM D-glucose, and for each experiment a water control was included. The aim was to try to supply the plant with an additional source of energy, to reduce the calcium increase upon heating. ATP pre-incubation caused either no difference (0.5 mM) or an increase in the calcium basal level

in the stroma (1 mM, figure 6.13), and this could have been expected as ATP is a known calcium agonist (as shown in chapter 3 figure 3.3). The increase in the basal calcium levels due to 1 mM ATP also increased the stromal calcium response to heat (figure 6.13), as previously observed for other chemicals (e.g. H_2O_2, erythrosin B, verapamil).

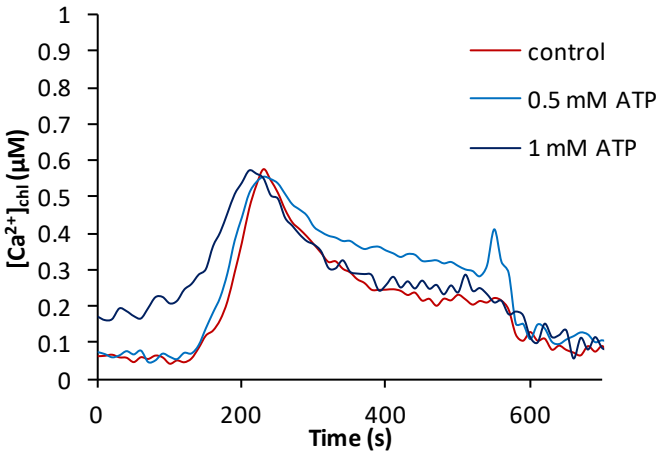


Figure 6.13 Representative calcium traces of the stromal heat response of *Arabidopsis* plants pre-treated for 30 minutes with 0.5 mM or 1 mM ATP, plus a water control, before heating at 40ºC.

The 8 h pre-treatment with 10 mM D-glucose had a little effect on the heat-induced chloroplast calcium response (figure 6.14), showing a reduced increase in the chloroplast calcium increase upon heating. To possibly correlate this response to a physiological effect of the glucose, a control for osmolarity was needed. Hence a control of 8 h pre-incubation with 10 mM mannitol was performed, and it showed a similar if not more pronounced inhibition of the response (figure 6.15). Furthermore, the mannitol *per se* is known to cause a calcium increase, and this could be seen from the total amount of aequorin left to do this experiment (data not shown). Unfortunately these pre-incubation experiments resulted to be not very informative on the possible physiological role of the calcium increase upon heating.

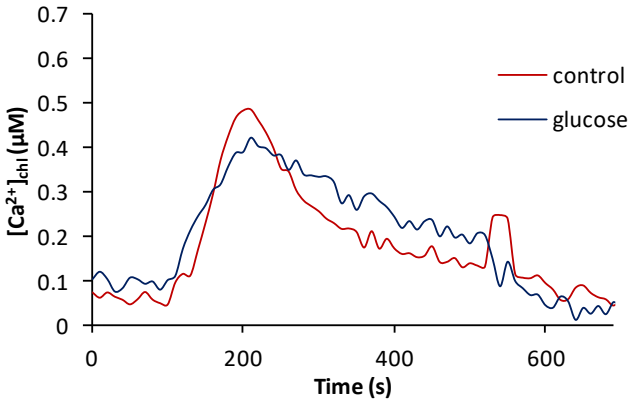


Figure 6.14 Representative calcium traces of the stromal heat response of *Arabidopsis* plants pre-treated for 8 h with 10 mM D-glucose, or water control, before heating at 40ºC.

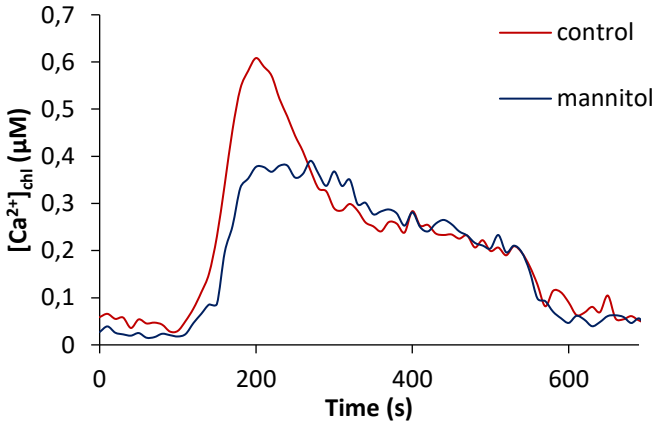
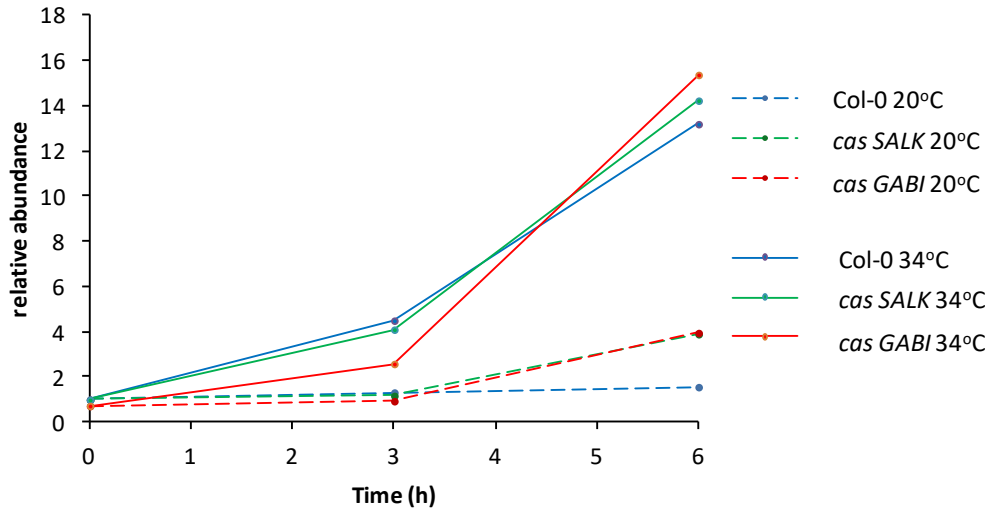


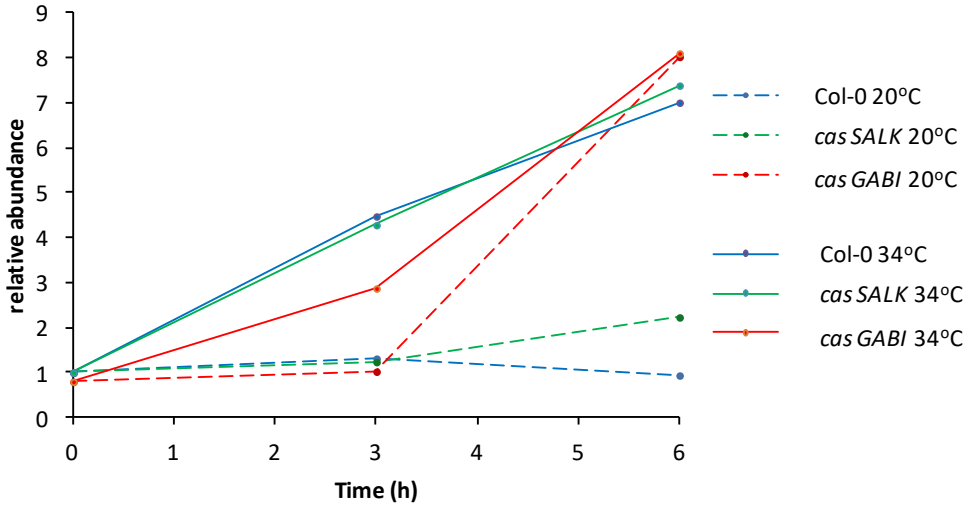
Figure 6.15 Representative calcium traces of the stromal heat response of *Arabidopsis* plants pre-treated for 8 h with 10 mM mannitol, or water control, before heating at 40ºC.

In a previous study Caldana and colleagues investigated the effect of temperature and light on *Arabidopsis* plants. They specifically investigated how changing light/darkness and temperature conditions (4ºC, 21ºC, 32ºC) affected plant physiology by undertaking a transcriptomic and metabolomic approach (Caldana et al., 2011). Out of the eight environmental conditions tested, the 32ºC darkness represents a similar situation to the one in which we measure the chloroplast heat response. In this condition it emerged that plants undergo starvation, and processes such as autophagy and protein degradation are taking place (Caldana et al., 2011). Specifically, they identified some amino acids whose levels were affected upon heating in the dark, and specifically leucine (Leu), isoleucine (Ile), tyrosine (Tyr) and lysine (Lys) levels were increased (Caldana et al., 2011). Hence I decided to measure amino acid levels with a HPLC approach in Col-0, *cas SALK* and *cas GABI* lines at 20ºC and 34ºC. The zero timepoint was taken only at one temperature, while samples at 20ºC and 34ºC were collected for each genotype at 3 h and 6 h, as in Caldana's work autophagy occurred prevalently in this timeframe (Caldana et al., 2011). For each amino acid, standard amino acids were run to obtain the retention times (Leu: 11.400 min, Ile: 10.838 min, Tyr: 6.697 min, Lys: 15.557 min). Results are shown in figure 6.16. The general trend for all the amino acids is that at 34ºC the levels were higher than at 20ºC, as expected. Furthermore, specifically at the 3 h timepoint, some differences were visible between the genotypes, however, *cas SALK* often behaves like the Col-0 wt, while the *cas GABI* showed a different behaviour. As the two *cas* mutants did now show the same results in terms of amino acid levels upon heating in the dark we could not conclude anything about the role of calcium in controlling this response.

A


relative abundance
Time (h)
Col-0 20°C
cas SALK 20°C
cas GABI 20°C
Col-0 34°C
cas SALK 34°C
cas GABI 34°C

B


relative abundance
Time (h)
Col-0 20°C
cas SALK 20°C
cas GABI 20°C
Col-0 34°C
cas SALK 34°C
cas GABI 34°C

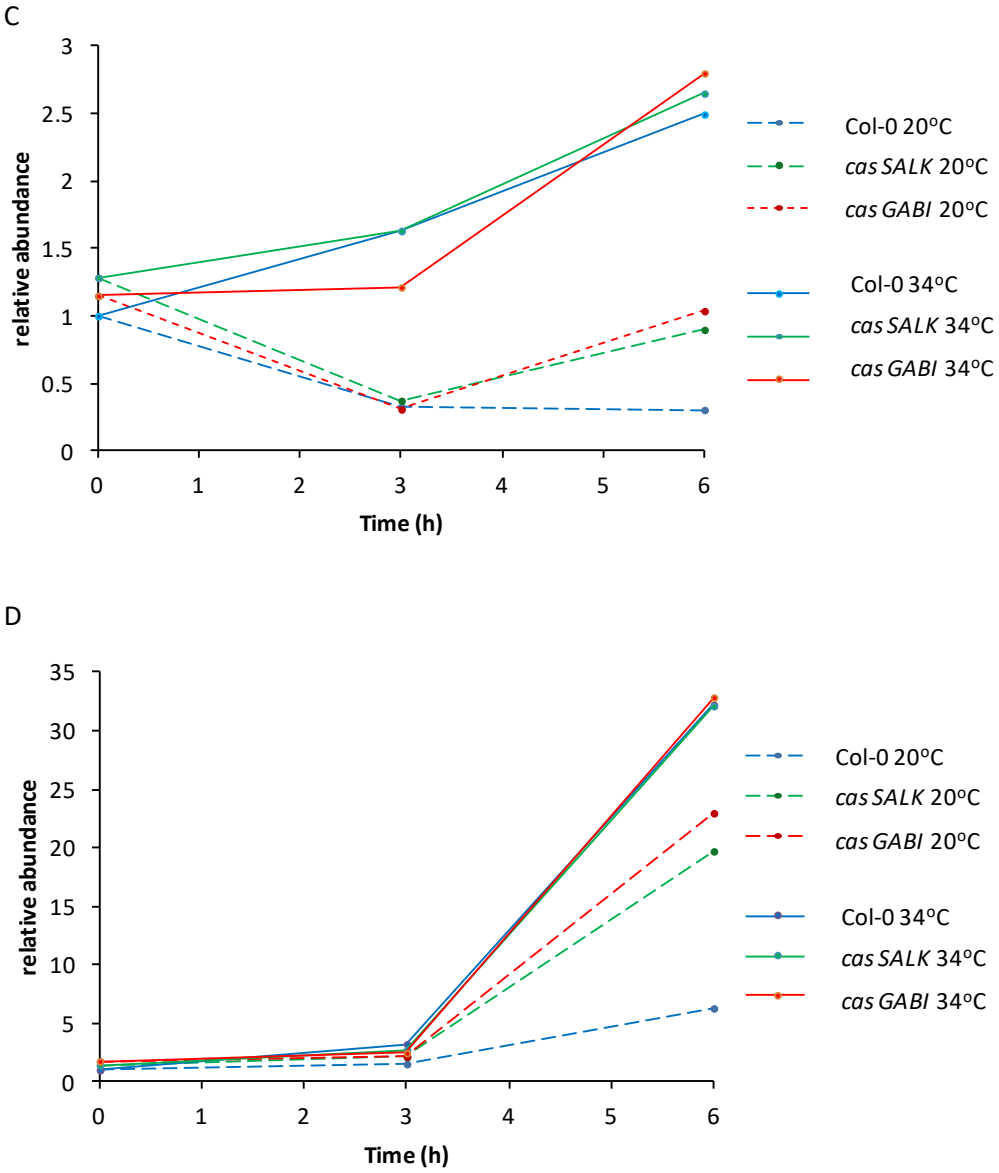


Figure 6.16 Relative abundance of amino acid levels in Col-0, *cas SALK* and *cas GABI* lines treated at 20°C or 34°C for 0 h, 3 h and 6 h. (A) leucine (B), isoleucine (C), tyrosine (D) lysine levels.

The last experiment left was to see if stromal calcium increases were able *per se* to induce amino acid degradation. To do so, Col-0 *Arabidopsis* plants were treated with 10 µM mastoparan for 3 h or 6 h, and amino acid levels were tested as described before (HPLC). From figure 6.17 it is possible to see that the relative amino acid abundance increased after 6 h of liquid treatment (also in the water control), but there was not a consistent trend in terms of the mastoparan treatment increasing the relative amino acid content more than the water control. We therefore concluded that the stromal calcium levels are not controlling the degradation of amino acids upon heating in the dark.

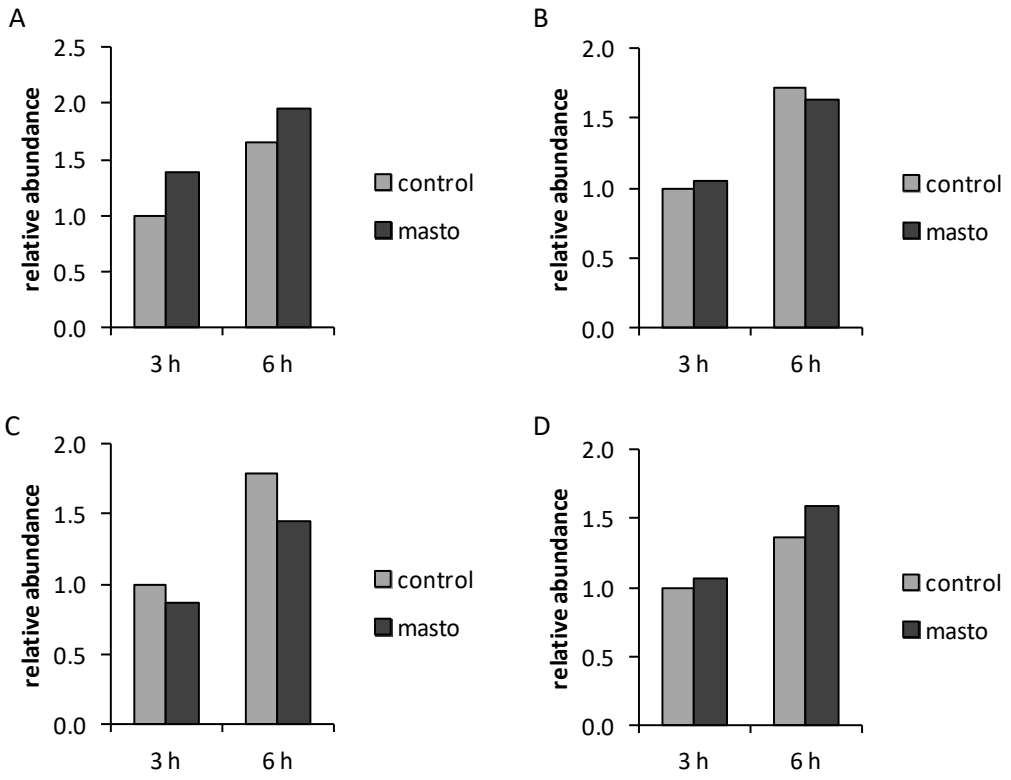


Figure 6.17 Relative abundance of amino acid levels in Col-0 treated with 10 µM mastoparan (masto) for 3 h and 6 h. (A) leucine (B), isoleucine (C), tyrosine (D) lysine levels.

6.2.2.3 The heat-induced chloroplast calcium increase does not regulate specific phytohormones levels

Amongst the major components regulating plant physiology there are plant hormones. Indeed, their levels are affecting virtually every aspect of plant development and physiology. It has been previously demonstrated that hormones such as salicylic acid (SA), ethylene and abscisic acid (ABA) play a role in the plant heat response (Larkindale and Knight, 2002; Larkindale et al., 2005). Interestingly, plastids are the initial site of synthesis for several plant hormones, such as SA, JA and ABA (Dempsey et al., 2011; Chauvin et al., 2013; Finkelstein, 2013). Hence it is possible that a direct way of controlling phytohormone synthesis in response to heat is to increase chloroplast calcium, and that the chloroplast-specific calcium response measured upon heating is a direct and localised way to regulate hormone synthesis. I specifically focused my attention on SA and ABA. For SA, first of all the levels of expression of two key genes controlling SA biosynthesis and export from chloroplast was tested upon heating (Strawn et al., 2007; Dempsey et al., 2011; Serrano et al., 2013). These genes are *ICS1* (At1g74710) and *EDS5* (At4g39030). Col-0 *Arabidopsis* plants were heated at 20^{o}C or 40^{o}C for 3 h, 6 h, 12 h or 24 h, and gene expression levels were tested for *ICS1* and *EDS5* in these samples. Results are shown in figure 6.18, where it was clear that these two genes are heat-induced. Specifically, the 6 h timepoint was chosen to further test whether the heat-induce gene expression changes are calcium-dependent. Hence the same experiment was repeated on Col-0 and the two *cas* mutant lines, where the chloroplast-specific calcium heat response is partially inhibited (figure 6.7).

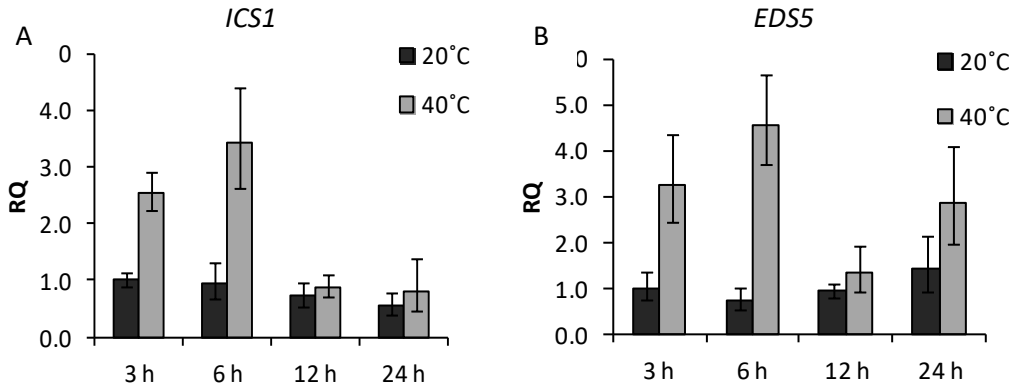


Figure 6.18 Expression levels of (A) *ICS1* (At1g74710) and (B) *EDS5* (At4g39030) at 20ºC and 40ºC for 3 h, 6 h, 12 h and 24 h in *Arabidopsis* Col-0 lines. Error bars = SE.

As it is shown in figure 6.19, there was no significant difference between the three genotypes (Col-0, *cas SALK* and *cas GABI*) after 6 h of treatment at 40ºC or 20ºC. This behaviour was consistent between *ICS1* and *EDS5*. This evidence indicates that calcium may not be involved in controlling gene expression of these two genes controlling the SA biosynthetic pathway. However, we cannot exclude that this ion may be regulating the SA biosynthesis locally in the chloroplast, e.g controlling the activity of specific enzymes rather than their production.

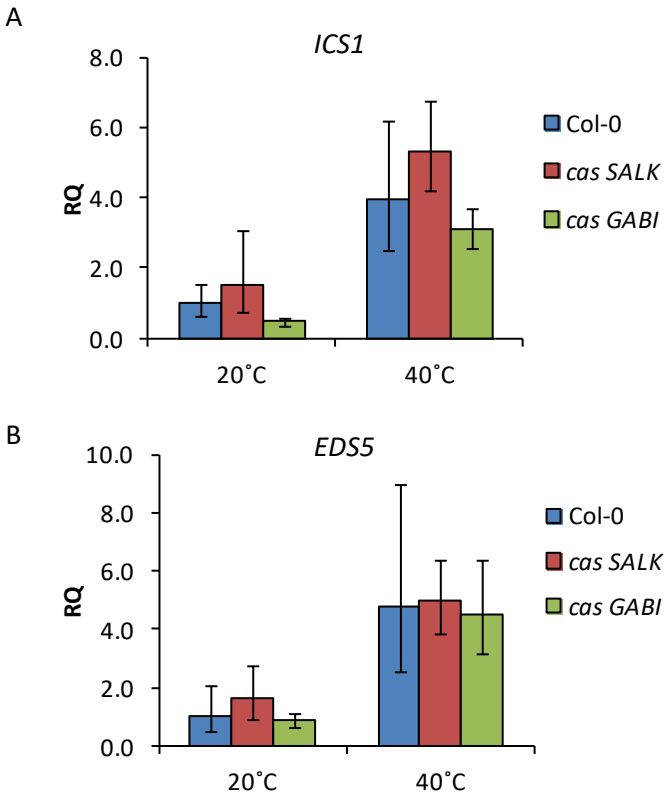


Figure 6.19 Expression levels of (A) *ICS1* (At1g74710) and (B) *EDS5* (At4g39030) at 20ºC and 40ºC 6 h in Col-0, *cas SALK* and *cas GABI* backgrounds. Error bars = SE.

A similar approach was taken for testing the role of chloroplast calcium in controlling ABA synthesis. Levels of an ABA-regulated gene were tested in response to heating at 32ºC for 1 h or 40ºC for 3 h in Col-0, *cas SALK* and *cas GABI*. The gene selected was *APX2* (ascorbate peroxidise 2, At3g09640), whose levels increase in response to ABA, and decreases when ABA levels decrease (Fryer et al., 2003). As it is shown in figure 6.20, levels of *APX2* were increased in response to heat, and for both temperatures the two *cas* mutants consistently shown higher mRNA levels compared to the Col-0 control upon heating. Despite the fact that there is a lack of significance in the differences (error bars representing SE are overlapping), the trend is consistent between temperatures (*APX2* gene levels are always higher in response to heat in the two *cas* mutans). This may

indicate that calcium can negatively regulate the ABA production upon heating, while some other cellular components are responsible of inducing it.

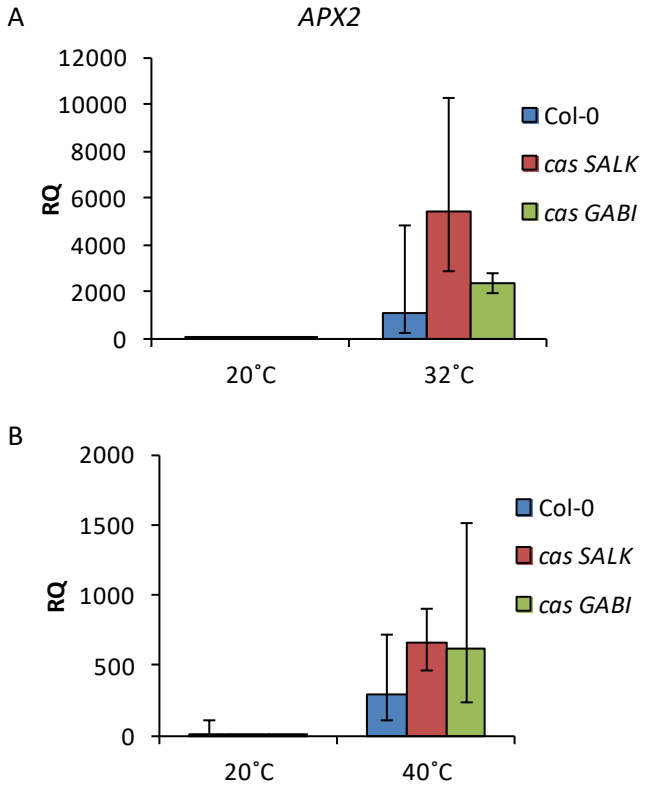


Figure 6.20 Expression levels of *APX2* (At3g09640) in Col-0, *cas SALK* and *cas GABI* in response to (A) 32ºC for 1 h or (B) 40ºC for 3 h. Error bars = SE.

To further determine if the hormone levels are increased upon the heat-induced calcium increase in the chloroplast, hormone levels were directly measured upon heating in Col-0, *cas SALK* and *cas GABI* by using a LC/MS approach (see Materials and Methods, chapter 2) (Forcat et al., 2008). To investigate the levels of SA, ABA, GA3 (gibberellic acid 3), JA (jasmonic acid), Ile-JA (conjugated form of JA with isoleucine, which represents the biologically active form) and IAA (auxin), plants were heated at 20ºC, 35ºC or 40ºC before extracting the hormones, which were analysed according to transition values

obtained with internal standards. Levels of SA and ABA are shown in figure 6.21 and 6.22, respectively. The levels for the other hormones are reported in appendix C.

Despite the variable levels of the hormones in the different timepoints at different temperature, it was not possible to define a consistent pattern for the two *cas* mutants which differed from the one observed in Col-0 for any of the tested hormone. Thus we were not able to conclude that the chloroplast calcium increase upon heating is able to control hormonal levels.

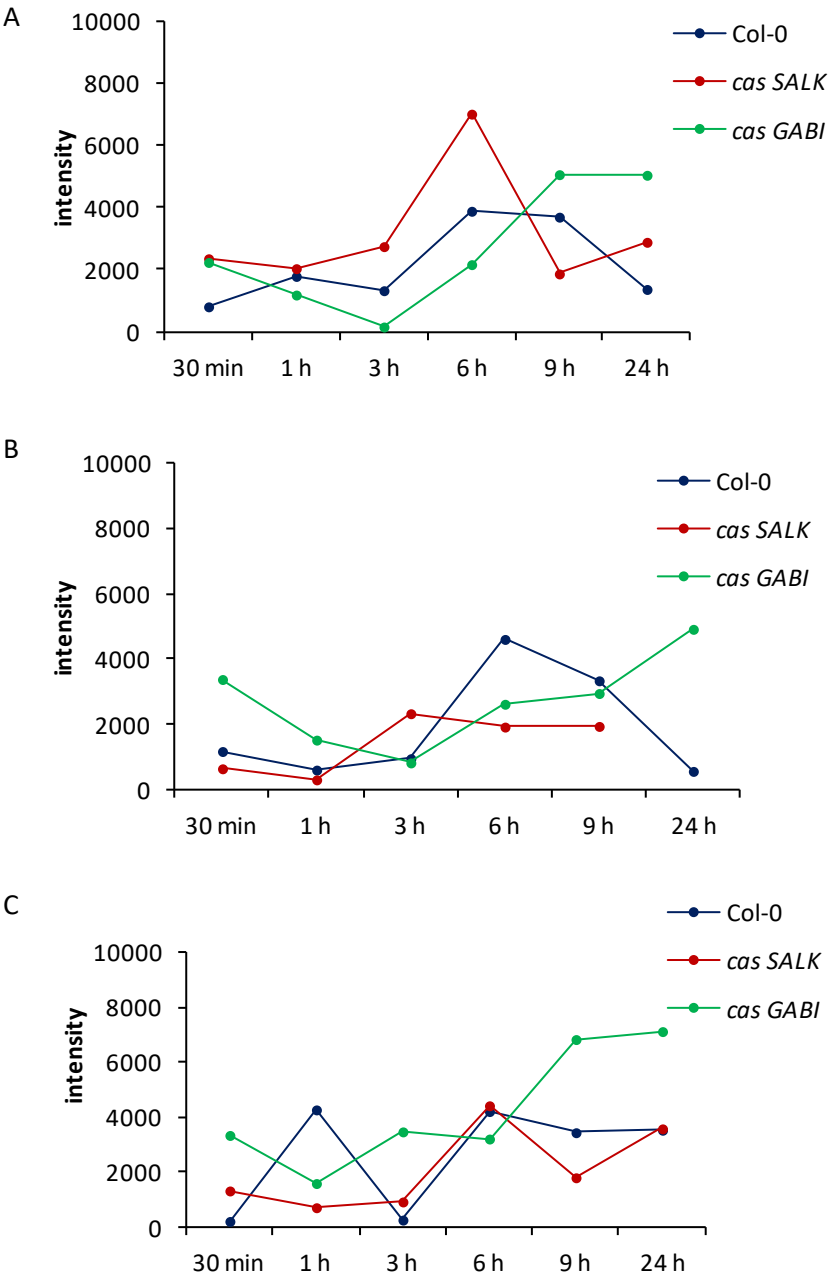


Figure 6.21 Intensity levels of SA peaks in a MS analysis. The three genotypes Col-0, *cas SALK* and *cas GABI* were analysed ad 30 min, 1 h, 3 h, 6 h, 12 h and 24 h in response to (A) 20ºC (B) 35ºC or (C) 40ºC.

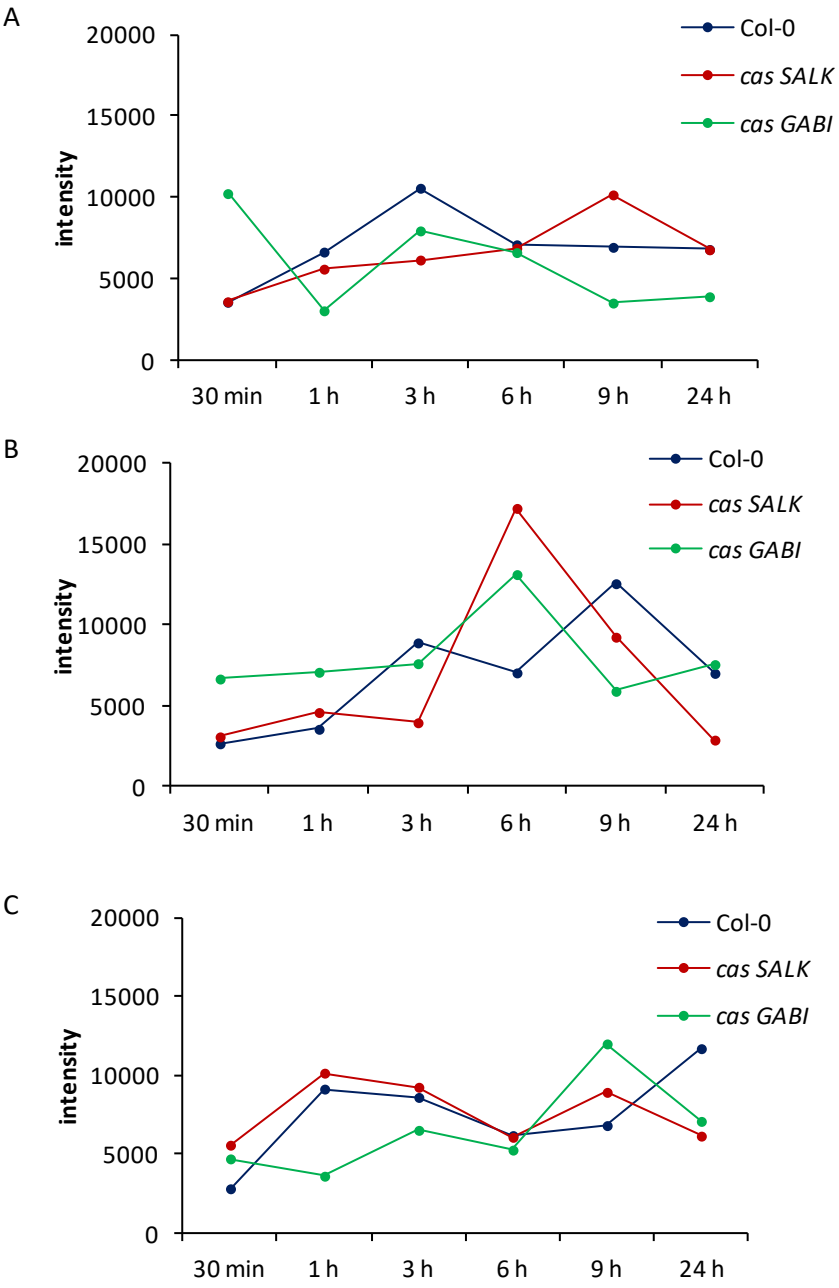


Figure 6.22 Intensity levels of ABA peaks in a MS analysis. The three genotypes Col-0, *cas SALK* and *cas GABI* were analysed ad 30 min, 1 h, 3 h, 6 h, 12 h and 24 h in response to (A) 20ºC (B) 35ºC or (C) 40ºC.

6.3 Discussion

6.3.1 Blocking the chloroplast-specific calcium increase

After describing the properties of the chloroplast-specific calcium mediated heat response in chapter 5, we decided to further investigate what the biological significance of this calcium increase was in terms of changes in plant physiology. To achieve this, it was important to identify a condition in which the stromal calcium signal was inhibited upon heating. In this way only would it have been possible to investigate causality of any biological output identified with the calcium signal. The problem was addressed with different approaches, reported in detail below. One general consideration that has to be taken into account regarding blocking the calcium increase is that the nature of the calcium source was not known. The most probable hypothesis is that calcium comes directly from the organelle itself, and in that case it could be stored either in the thylakoid lumen or bound to high-capacity calcium binding proteins (Kreimer et al., 1987; Stael et al., 2012). However, another hypothesis is that the calcium is coming from outside the organelle, this means that there can be a localised calcium increase in the cytosol that aequorin was not able to measure for technical reasons, and this calcium would then enter the chloroplast. Alternatively calcium may be released very locally from other calcium stores such as the ER (connected to the chloroplasts via a specific plastidial emergence called stromules). Since no clear information was available about the source of this calcium increase, the problem of blocking calcium had to be investigated with a shotgun approach, rather than a more targeted one.

6.3.1.1 Blocking the chloroplast-specific calcium increase with chemicals

The first idea to try to block the stromal calcium was to add chemical inhibitors which have been reported in the literature to be able to affect calcium responses. The list of them and their specific function is reported in table 6.1. In general, calcium channel inhibitors

with more general (e.g. $LaCl_3$) or more specific targets (e.g. DNQX) were used Finally, calcium chelators were used such as EGTA-AM and BAPTA-AM, in order to locally buffer the stromal calcium increase upon heating. Specifically, as we were aiming to target the stromal compartment, which is surrounded by the inner envelope, outer envelope and at the cell level also by the plasma membrane, the –AM version of these chemicals was used, to facilitate the membrane penetration. Despite this precaution, we did not have any means to check if the chelators reached the stromal compartment. In any case, the fact that they were not able to inhibit the response made them of no interest to our work. While none of the treatments applied in table 6.1 was able to block the calcium, three of them had an opposite effect and they are reported in figure 6.1. Pre-incubation with 5 mM lanthanum, 10 mM gadolinium or 1 mM verapamil caused a potentiation of the stromal calcium response upon heating (figure 6.1). Specifically, gadolinium (figure 6.1B) and verapamil (figure 6.1C) caused a significant increase in the stromal calcium basal levels, which can explain the higher calcium response upon heating. Lanthanum instead only increased the calcium upon heating, and basal levels were not affected (figure 6.1A), and it has to be taken into account that this increase is not statistically significant. This difference is difficult to explain, as they inhibit the same cellular components (same calcium channels). Furthermore, not much is known in terms of chloroplast calcium transporters. However, the fact that these chemicals can increase the stromal calcium heat response can be used as a tool to artificially increase the response, hence to correlate a possible biological output to the magnitude of the calcium response.

6.3.1.2 Blocking the chloroplast-specific calcium increase with sponge proteins

As the components involved in the chloroplast heat response are not known, it was not possible to target them. Therefore the idea of using calcium chelators such as EGTA-AM and BAPTA-AM was adopted, as chelators should act directly on the calcium increase itself, independently of how the signal was created. However, one of the doubts about the

functioning of those chelators in the chloroplast heat response was related to correct targeting. Ideally, if it was possible to target a chelator directly in the stroma, this should be able to block the calcium increase upon heating. This is possible if the chelator is a protein, as targeting to the stroma can easily be achieved. Hence we decided to try to target three proteins to the chloroplast: ARP, Parv and a truncated version of aequorin. These proteins are able to bind calcium in the range of micormolar concentrations, which correspond to the ones reached in response to natural stimuli. ARP is a calcium binding protein identified in *Euglena gracilis* (Gumpel and Smith, 1992). Similarly, Parv is an archetypal type of EF-hand protein, which contains high affinity calcium binding domains (Wang et al., 2013). Hence this was another good candidate to use in order to buffer stromal calcium increase, and it was targeted to the chloroplast stroma with the RbcS targeting sequence. The last protein used was a truncated form of aequorin, which only contained the calcium binding domains, while the coelenterazine binding domain was deleted. When these three calcium buffering proteins were transiently expressed in tobacco it was not possible to see any difference between the lines expressing one of these three proteins and the control (figure 6.2). One possibility to explain these data is that in the 48 to 72 hours in which the proteins are expressed in tobacco, plant are rebalancing their internal calcium levels in a way that the calcium signal is consistently not changing in terms of absolute levels. It is reasonable to think this as calcium is very tightly regulated in the cell, as it is a required element for plant physiology, but higher levels are toxic for the plant. Since tobacco did not give any positive result in terms of blocking the calcium signal upon heating, stable expression of these buffering proteins was not attempted in *Arabidopsis*.

6.3.1.3 Blocking the chloroplast-specific calcium increase by affecting the upstream components

All the approaches tried so far to inhibit the calcium response did not succeed, hence we decided to try to target processes upstream of the calcium response itself. However, as the components involved in this signalling pathway were not known, the targets had to be guessed at. Firstly we reasoned that, in the chloroplast, one of the important parameters controlling the chloroplast physiology is the redox state, which is likely to be affected during heat. If changes in the redox state are upstream of the heat-induced chloroplast calcium increase, then depleting the glutathione pool by pre-incubation with BSO (it inhibits the first enzyme of GSH synthesis) might affect the heat calcium signal (Marquez-Garcia et al., 2014). As shown in figure 6.3, the BSO pretreatment had no effect on the chloroplast heat response, indicating that probably the glutathione pool is not involved in this response, and definitely is not upstream of the calcium increase.

The second approach taken was based on the idea that, if the calcium is coming from external stores, such as the ER, then depleting these stores will impair the calcium increase upon heating. This was tested by pre-incubating the plants with thapsigargin and erythrosin B before heating at 40ºC (figure 6.4). While thapsigargin had no effect compared to the control, erythrosin B greatly affected the stromal calcium basal levels (figure 6.4), and consequently the response was enhanced upon heating. This treatment was not able to block the calcium increase, but it may be indicating that, if calcium levels are affected in other cellular compartments, this can change the chloroplast calcium balance. This might also mean that ER normally acts to take up Ca^{2+} from chloroplasts as the donor phase of the calcium response (to heat). Another possible idea was to try to deplete the unknown chloroplast calcium reservoir, by stimulating the plant with stimuli able to increase stromal calcium (e.g. flagellin 22) (Nomura et al., 2012), however this was not investigated.

6.3.1.4 The chloroplast-specific calcium increase is affected in the *cas* mutant

CaS is a calcium sensing receptor localised to the thylakoid membrane (Nomura et al., 2008; Vainonen et al., 2008). Calcium binding activity has been reported for this protein (Han et al., 2003), and it is known to be involved in a variety of processes ranging from the pathogen response (Nomura et al., 2012), stomatal closure (Nomura et al., 2008; Weinl et al., 2008; Wang et al., 2014) and photoprotection (Vainonen et al., 2008; Petroutsos et al., 2011). Hence CaS was a good candidate to be involved in the chloroplast calcium heat response. We kindly obtained two independent insertional mutant lines form Prof Eva- Mari Aro (Turku University, Finland), which have been characterised in the literature (Vainonen et al., 2008). These two lines, named *cas SALK* and *cas GABI*, were firstly checked for their mutant nature, and indeed levels of the *cas* transcript were strongly downregulated in both the mutants (figure 6.5). These lines were then stably transformed with stromal aequorin, screened, selected, and these lines were tested for the heat response. Before testing them for the heat response, an additional control was performed, in which the activity of the aequorin was checked upon mastoparan treatment. The idea behind this was that aequorin is an enzyme, hence its activity is subject to the condition of the cellular environment in which it operates. Mutation of the CaS protein may affect the cellular conditions e.g. pH. Hence if plants respond to the mastoparan stimulus with the same kinetics, as we do not expect CaS to be specifically involved in the calcium increase mediated by this agonist, then we can say that the aequorin activity is not affected in the *cas* mutant. This was indeed the case, as shown in figure 6.6. It was then finally possible to test the activity of the calcium mutants upon heating, and it is possible to see in figure 6.7 that both *cas SALK* and *cas GABI* responded to the 40^{o}C heat pulse with a reduced stromal calcium increase compared to the Col-0 control. This evidence is pointing towards an involvement of the CaS protein in the heat-induced chloroplast response, as well as giving us a genetic tool to further test the biological significance of this response, as the calcium response was inhibited.

6.3.2 Investigation of the significance of the chloroplast calcium heat response to plant physiology

Heat is reported to have many physiological effects on chloroplasts. Several of them were tested in Col-0 and the two *cas* mutant lines upon heating at 40ºC, and indeed as expected there were effects on the chloroplast response, but no differences were recorded between the three genotypes. Only some of these responses are reported in this chapter. However, for completeness, I would like to mention that other physiological outputs were investigated, such as levels of lipid peroxidation with a TBARS assay (Larkindale and Knight, 2002), expression of genes such as *SARD1* (At1g73805), *WRKY28* (At4g18170) and *WRKY46* (At2g46400), which are heat-induced and whose levels had already been indentified to change in response to pathogens in the *cas* mutant (Nomura et al., 2012). Furthermore, an electrolyte leakage assay was performed upon heating on these three different genotypes, and again leakage levels increased upon heating, but with no differences between the lines, and chloroplast swelling upon heating was measured (Zhang et al., 2010), with similar results. The most interesting cases of chloroplast physiology upon heating are discussed below.

6.3.2.1 The photosynthetic process is not controlled by the heat-induced calcium increase

One of the known effect of increases in stromal calcium is to block photosynthesis, mainly acting on the Calvin-Benson cycle (Charles and Halliwell, 1980; Kreimer et al., 1988). Heat strongly affects the photosynthetic process, because if a heating event occurs concomitantly with light, the photosynthetic chain can be damaged (Sharkey, 2005). Hence this calcium increase in darkness upon heating may act as a preventive mechanism to block the photosynthetic process, and avoid damages at the onset of light. In order to test this, F_v/F_m in Col-0, *cas SALK* and *cas GABI* plants was measured at different

timepoints during the heating process. This approach was adopted both to have an idea of the behaviour of F_v/F_m in Col-0 plants upon heating, and because we do not know how long the time delay between the stromal calcium increase and photosynthesis inhibition is. So four different timepoints were selected during the course of the heating event, and they are represented in figure 6.8. Results are reported in figure 6.9, where it is possible to see that in the Col-0 line the F_v/F_m decreased while temperature increases, with a bit of a delay (down at 8 min and 12 min). In the same figure the behaviour of the two *cas* mutant lines is reported. It is possible to see that already at the first timepoint (2 min) F_v/F_m is affected in the *cas* mutants (lower), and specifically at 8 minutes, the response is different from the Col-0 wt line and consistent between the two mutant lines. Indeed the reduction in F_v/F_m induced by heat is less pronounced in these two mutants. This may be interpreted as the calcium having a protective effect on F_v/F_m upon heating, and that deceasing the calcium signal (as it happens in the *cas* mutants) decreases the protection. However, it is hard to draw conclusion solely about the role of calcium by using *cas* mutants, as roles for the CaS protein in controlling the photosynthetic process have already been identified (Vainonen et al., 2008; Petroutsos et al., 2011; Wang et al., 2016). Indeed F_v/F_m was already altered before heating the plants (2 min timepoint), suggesting that other controls are needed to associate the effect of calcium to the changes in F_v/F_m. If the role of calcium is to protect the photosynthetic process, then less calcium as in the *cas* mutants will result in a decreased reduction of F_v/F_m upon heating (as seen in figure 6.9, 8 min) and the opposite effect would be expected upon higher calcium increase. To this aim, pre-incubation with chemicals able to increase the stromal calcium response upon heating were used, specifically verapamil, gadolinium and H_2O_2. Results of this experiment are reported in figure 6.10, where verapamil indeed behave as expected, hence giving a bigger inhibition of F_v/F_m after 8 min (figure 6.10B), while gadolinium and H_2O_2 showed the same trend as the *cas* mutants (compare figures 6.10A and 6.10C with figure 6.9). As the calcium response upon heating was higher upon all the pre-incubation treatments, but the F_v/F_m behaviour is not consistently following the same trend, we could not conclude that calcium has an effect on this physiological response.

6.3.2.2 Starvation during heat is not regulated by stromal calcium

As the chloroplast-specific calcium increase was measured when heat occurred concomitantly with darkness, and the calcium increase was negatively regulated by light (chapter 5 figures 5.9 and 5.10), I speculated that the calcium response may be indicating that the plant is starving. Another evidence supporting this idea is represented by the mitochondria inhibitor experiments. Indeed, when the energy status was affected by impairing the mitochondrial activity with rotenone or mixothiazol, this caused an increase in the heat-induced chloroplast calcium response, in accordance with the idea that the calcium levels may be proportionally higher with the degree of energy stress (chapter 5 figure 5.15). When light was added during the mitochondria inhibitor pre-incubation, the increase in the chloroplast calcium response to heat was either partially (myxothiazol) or totally (rotenone) inhibited (chapter 5 figure 5.16). Furthermore, higher temperatures cause a proportional increase in the magnitude of the calcium increase (chapter 5 figure 5.2B), once again adding evidence towards a possible role of calcium in indicating the energy status in a way that the higher the stromal calcium, represents the magnitude of energy stress the plant is experiencing. This idea was tested further, and the first experiment performed was to check whether CO_2 itself can be sensed by plants with a cytosolic or stromal calcium increase. This was performed both by changing atmospheric CO_2 concentrations to 1000 ppm in a controlled chamber (figure 6.12) or adding $NaHCO_3$ in solution (figure 6.11). None of these treatments caused any significant calcium increase in the cytosol or in the stroma (figures 6.11 and 6.12), while $NaHCO_3$ injection caused an non specific cytosolic touch response, already reported in the literature (Knight et al., 1991).

The follow up idea was to try to mimic the effect of light (chapter 5 figure 5.9.), hence to add energy back to the system either by adding ATP or D-glucose. Ideally, by treating plants with a source of energy should affect the energy balance, and decrease the chloroplast calcium response upon heating. Unfortunately ATP is a calcium agonist itself, hence 30 minutes pre-incubation with already increased the basal calcium concentrations if given at 1 mM, or had no effect at lower concentrations (figure 6.13). Hence ATP was

not a good candidate to test this idea. Therefore, 10 mM D-glucose pre-treatment was tested instead, and was able to reduce the calcium response upon heating (figure 6.14). These data are consistent with our hypothesis. As an osmotic control for this response, 10 mM mannitol was used to pre-incubate plants before heating, and in response to this treatment the inhibition of the response was even bigger (figure 6.15). Thus it was not possible to exclude that the glucose might partially rescue the plant starvation condition, however the partial reduction of the calcium stromal response was mostly probably due to an osmotic effect.

The last approach taken to test the idea of this calcium response signalling plant energy balance was derived from the data obtained by Caldana and colleagues (Caldana et al., 2011). In their paper they obtained data combining a metabolomic and transcriptomic approach and they showed that dark concomitantly with heating at 32ºC caused autophagy and protein degradation in *Arabidopsis*. Specifically, levels of leucine, isoleucine, tyrosine and lysine increased in these conditions (Caldana et al., 2011). Hence we decided to measure levels of these amino acids upon heating in Col-0, *cas SALK* and *cas GABI* plants at 34ºC, which is a temperature close to the 32ºC tested by Caldana and colleagues, but able to induce a bigger stromal calcium signal compared to 32ºC (chapter 5 figure 5.2). Control plants were kept at 20ºC, and timepoints were collected at 0 h, 3 h and 6 h, as they are consistent with timepoints in which changes were observed (Caldana et al., 2011). As shown in figure 6.16, for all the four amino acid tested there was an increase in their level upon heating, specifically at 3 h and 6 h, but it was not possible to define a trend for the two *cas* mutant lines which differ from the Col-0 one. On the contrary, often the trend observed was similar for Col-0 and *cas SALK*, while *cas GABI* had a different behavior (figure 6.16). These inconsistent results did not allow us to conclude that calcium is able to control protein degradation upon heating. To have more evidence for the same hypothesis we also decided to test if stromal calcium increases induced by mastoparan were able to affect amino acid levels without any heating involved. Hence we were investigating the possibility that increases in stromal calcium *per se* is sufficient to cause protein degradation. Figure 6.17 shows that this hypothesis is not true either, as for any change measured in response to the mastoparan treatment at 3 h

and 6 h, the trend of treated plants compared to untreated ones was not consistent between amino acids. If calcium was controlling it, then the mastoparan-treated plants should have shown a higher level of all four amino acids, but this was not the case (figure 6.17). Taken together, all this evidence was not able to demonstrate a possible role for this heat-induced chloroplast calcium increase in sensing and signaling plant energy balance in the cell, hence the physiological significance of this calcium response is still under investigation.

6.3.2.3 ABA and SA are not affected by stromal calcium increases in response to heat

The last hypothesis we decided to test to investigate the physiological output of the chloroplast calcium response upon heating was the possibility that this calcium increase controls ABA or SA levels. In support of this idea there was already evidence for these hormones being involved in the heat response (Larkindale and Knight, 2002). Furthermore, a link between CaS and both SA and ABA had already been established in literature, as CaS is involved in the SA-mediated pathogen response (Nomura et al., 2012), and it controls stomata closure, which is an ABA-controlled process (Han et al., 2003; Nomura et al., 2008). Moreover, for both SA and ABA the initial site of synthesis of these hormones resides in the plastids. Hence we reasoned that it was possible for the calcium to be a mechanism for regulating the amount of SA and/or ABA produced upon a heating event. First of all, levels of expression of *ICS1* and *EDS5* genes were tested upon heating at 40ºC, to check if these two genes whose products control SA production are heat-induced. Therefore Col-0 *Arabidopsis* plants were treated at 20ºC or 40ºC for 3, 6, 12 or 24 h and expression levels of *ICS1* and *EDS5* were measured, and they are represented in figure 6.18 and 6. 19, respectively. The data showed that both these genes are indeed heat-induced, and the 6 h timepoint was further used to test if there is any difference in the genes levels upon heating in Col-0, *cas SALK* and *cas GABI* plants. Figure 6.19 showed that there was no significant differences in the levels of *ICS1* and

EDS5 in the different genotypes, indicating that the nuclear regulation of these two genes is not dependent on the stromal calcium increase. These results do not exclude the possibility that locally, at the chloroplast level, the SA synthesis may be calcium-regulated, as this ion can directly affect the activity of enzymes involved in the hormone biosynthesis. A similar approach was taken to test ABA levels. Firstly, the level of expression of a ABA-induced nuclear gene (*APX2*) was tested upon heating at two different temperatures, as well as at a control temperature (20ºC or 32ºC for 1 h or 20ºC or 40ºC for 3 h) in Col-0, *cas SALK* and *cas GABI* plants. The results shown this gene to be heat-induced, and its transcript levels were consistently higher in the two *cas* mutants at both temperatures (figure 6.20). Hence there is reasonable evidence for ABA levels to be calcium-regulated.

In order to directly test SA and ABA levels in Col-0, *cas SALK* and *cas GABI*, plants were heated at 35ºC or 40ºC for 30min, 1 h, 3 h, 6 h, 9 h and 24 h and a LC/MS approach was used (Forcat et al., 2008). In the same measurement, levels of other plant hormones were also recorded, and they are reported in appendix C. As it is possible to see in figure 6.21 for SA and 6.22 for ABA, the levels of these hormones did not follow a specific trend, and it was very difficult to draw any conclusion from these data. Furthermore, this experiment was only repeated once, so it was impossible to statistically evaluate the significance of the differences measured. With the data available, we concluded that we cannot attribute a role for the chloroplast stroma calcium increase upon heating in controlling plant hormone biosynthesis.

6.3.3 Conclusions

Despite the considerable amount of work invested into this chapter, we did not succeed in identifying what the physiological significance of the heat-induced chloroplast specific calcium increase is. Furthermore, many different approaches were taken to try to block the calcium response, and all the orthodox ones did not have any effect on this response. All these data are illustrating the fact that we are probably dealing with an atypical type

of calcium response, and some novel components may be involved in generating the calcium signal. One interesting result of this chapter is the discovery of the role of the CaS protein in the generation of the stromal calcium response, as it is a first step towards identifying the cellular components of this newly identified biological pathway.

Chapter 7

Calcium and temperature sensing in plants

7.1 Introduction

Temperature is continuously changing in the natural environment, and photosynthetic organisms are exposed to these fast and long term alterations. Sensing temperature changes and responding accordingly is essential to ensure plant adaptation and survival (Penfield, 2008; Hua, 2009; Thomashow, 2010; Saidi et al., 2011; Horvath et al., 2012; Knight and Knight, 2012). Whilst cellular events downstream of temperature changes are well described, the mechanisms for temperature sensing, specifically the early events, are still an open research topic. Indeed, in plants, the specific cellular thermometers for heat and cold have not been yet identified. Amongst putative temperature sensing mechanisms, several classes of biological processes have been shortlisted as possible primary sensors. These candidates are not only able to respond to temperature changes directly, but they also activate downstream response pathways (Ruelland and Zachowski, 2010). These processes include protein unfolding, changes in the catalytic activity of enzymes, cytoskeleton disassembly, changes in membrane fluidity and chromatin remodelling (explained in detail in several reviews e.g. (Ruelland and Zachowski, 2010; Saidi et al., 2011; Knight and Knight, 2012; Mittler et al., 2012). Membrane rigidification/fluidisation occur nearly concomitantly with the temperature variation, hence these events are likely to be upstream of the others. In *Synechocystis*, cold sensing is dependent on a histidine kinase (Hik33) whose activation relies on the cold-induced physical rigidification of the membrane (Mikami et al., 2002), whilst altering membrane fluidity by chemical means to mimic heat caused *de novo* synthesis of heat shock proteins

(Horvath et al., 1998). Furthermore, in plants, opposite changes in membrane fluidity are responsible for the activation HAMPK (heat) and SAMK (cold) MAP kinases (Sangwan et al., 2002), and Orvar and colleagues (2000) showed that changes in membrane rigidification act upstream of cytoskeleton remodelling in response to cold. Long term membrane fluidity modification, where the membrane composition is altered, are also used by plants to acclimate to different temperatures (Murata and Los, 1997; Falcone et al., 2004).

Relationships between temperature sensing, specifically membrane fluidity, and calcium signalling have already been reported. The cold response in plants is strongly dependent on a fast and transient cytosolic calcium increase (Knight et al., 1991; Knight et al., 1996), and membrane fluidity changes affect the magnitude of these calcium elevations (Orvar et al., 2000). In *Physcomitrella patens*, heat is responsible for an increase in cytosolic calcium levels, leading to activation of the heat shock response (HSR, Saidi et al., 2009), and the extent of the calcium heat response (and, consequently, of the HSR) is strongly dependent on membrane fluidity (Saidi et al., 2009; Saidi et al., 2010; Finka and Goloubinoff, 2014).

As stated before, very little information is available about temperature sensing in plants; in contrast, in mammals there are several temperature-sensing channels that are known for sensing temperatures from around 10^{o}C to 60^{o}C (Belvisi et al., 2011). Amongst these channels, TRPV1 (transient receptor potential cation channel subfamily V member 1) is considered the heat sensor (Bromberg et al., 2013). This channel was identified by Caterina and colleagues in 1997 as the nociceptor channel. Indeed TRPV1 is activated both by high temperature (the threshold for activation is 42-43^{o}C) and by capsaicin, which is the main "pungent ingredient" of hot chili peppers (Caterina et al., 1997; Szallasi et al., 2007; Belvisi et al., 2011). Evidence from the literature show that this channel is intrinsically activated by heat, and when opened, it releases calcium in the cytosol (Caterina et al., 1997; Cao et al., 2013; Torok et al., 2014). In plants, no homologues of the TRPV1 channel were identified, and the identity of the heat thermometer is still unknown.

To further understand temperature sensing in plants, I decided to express TRPV1 in *Arabidopsis* plants, as well as to study the effect of membrane fluidity on the heat response observed in chapter 5.

Aims on this chapter:

- To understand the role of membrane fluidity as part of the plant heat thermometer.
- To functionally express the animal heat channel TRPV1 in *Arabidopsis* plants.
- To investigate the behaviour of TRPV1-expressing plants in order to better understand heat sensing in *Arabidopsis*.
- To try to improve plant heat tolerance by expressing the mammalian TRPV1 heat channel.

7.2 Results

7.2.1 Long and short term changes in membrane fluidity regulate the stromal calcium increase

Rapid biophysical changes in membrane fluidity have been proposed to act as temperature-sensing mechanisms in plant cells, rigidification conveying cold, and fluidization conveying heat (Orvar et al., 2000; Sangwan et al., 2002). In order to test the ability of plant cells to respond to these fast changes in membrane fluidity, and specifically if the chloroplast calcium response is regulated by them, I chemically fluidised the membranes by the addition of benzyl alcohol (BA), a known membrane fluidizer (Horvath et al., 1998; Orvar et al., 2000; Sangwan et al., 2002; Balogh et al., 2005; Finka and Goloubinoff, 2014). Benzyl alcohol is a non-polar molecule with an aromatic ring, hence it affects membrane fluidity by inserting itself in between the phospholipids, disrupting the order in the lipid bilayer and its activity as a membrane fluidiser has been measured in Medicago sativa (Orvar et al., 2000). BA is known to induce calcium increases in the cytosol, and indeed this was visible in figure 7.1, confirming that the treatment is working. Results shown in figure 7.1 demonstrated that also chloroplasts are able to respond to BA-induced membrane fluidisation with a stromal calcium increase, and that the kinetics of the calcium elevation was similar to the one reported in response to heat (chapter 5, figure 5.1).

Altering the gross long term fluidity of the membrane by chemical or biological means consequently affects temperature sensing of plant cells, and allows the testing of a role for membrane fluidity in thermosensing (Murata and Los, 1997; Saidi et al., 2010). One way in which the gross fluidity of membranes can be altered is by long term temperature acclimation treatments. When treated for some time at lower temperatures, the level of saturation of fatty acids in the membranes is decreased in order to maintain the same degree of fluidity at this lower temperature (Wilson and Crawford, 1974; Graham and

Patterson, 1982; Quinn et al., 1989). Conversely, membranes fatty acids are highly saturated to counteract the effect of higher temperatures (Pearcy, 1978).

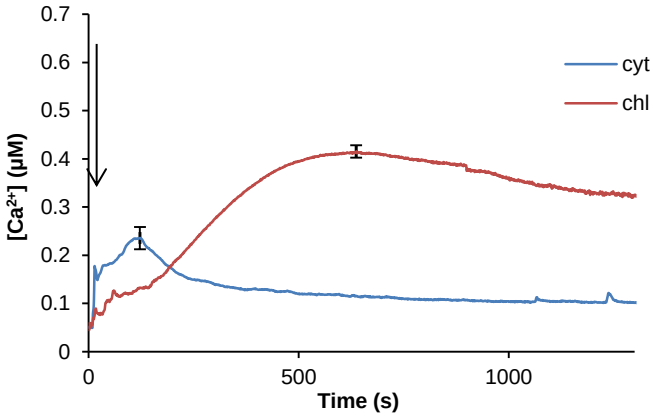


Figure 7.1 Fast changes in membrane fluidity induce calcium elevations in both the cytosol and the chloroplast stroma, with different kinetics. Cytosolic and chloroplast calcium traces in response to 50 mM BA. The time of injection is represented by an arrow. For each trace, 3 *Arabidopsis* seedlings were used, and their calcium response to BA was averaged. Error bars represent SE at the cytosol and chloroplast calcium peaks.

We therefore tested the effect of both low and high temperature pre-treatment upon the heat-induced chloroplast calcium response. Plants were treated overnight either at 15ºC, 20ºC or 30ºC. The Ca^{2+} response of these three sets of plants to the same heat stimulus (40ºC for 7 minutes) was compared (figure 7.2A). Plants acclimated at different temperatures produced a larger (15ºC pre-treatment) or smaller (30ºC pre-treatment) stromal calcium response to heat compared to the control (20ºC pre-treatment). The concentration of calcium at the peak is inversely proportional to the acclimation temperature, with 15ºC pre-treatment showing the biggest calcium response (figure 7.2B). Differences in baseline calcium levels were not detected suggesting that the steady state levels of stromal calcium is kept at the same levels in response to the different acclimation temperatures.

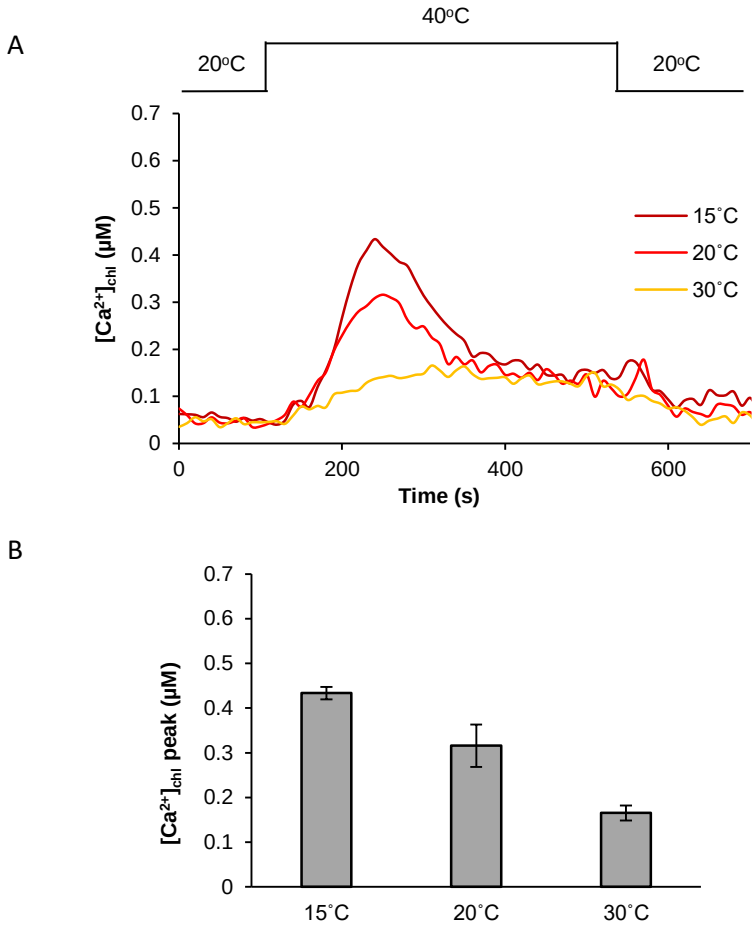


Figure 7.2 Temperature-induced long term changes in membrane fluidity affect the chloroplast calcium response to heat. Chloroplast-targeted *Arabidopsis* aequorin lines were acclimated overnight at 15ºC, 20ºC or 30ºC, then stimulated at 40ºC for 7 min. (A) Calcium kinetics upon heating of the different pre-acclimated lines and (B) average chloroplastic calcium concentration peak heights. Data were obtained by averaging traces of n = 6, 4, 5 *Arabidopsis* seedlings for the 15ºC, 20ºC and 30ºC pre-treatments, respectively. Error bars = SE.

To further test the idea that the membrane fluidity is responsible for regulating the heat induced calcium response, the chloroplast-specific fatty acid desaturase 7 (Iba et al.,

1993), FAD7, was transiently over-expressed in tobacco. Results shown in figures 7.3A and 7.3B corroborate those seen in the temperature pre-treatment experiment (respectively, figures 7.2A and 7.2B); the FAD7 expressing tobacco, which are predicted to have less saturated membrane fatty acids, responded to the heat pulse with a significantly higher calcium increase compared to the control.

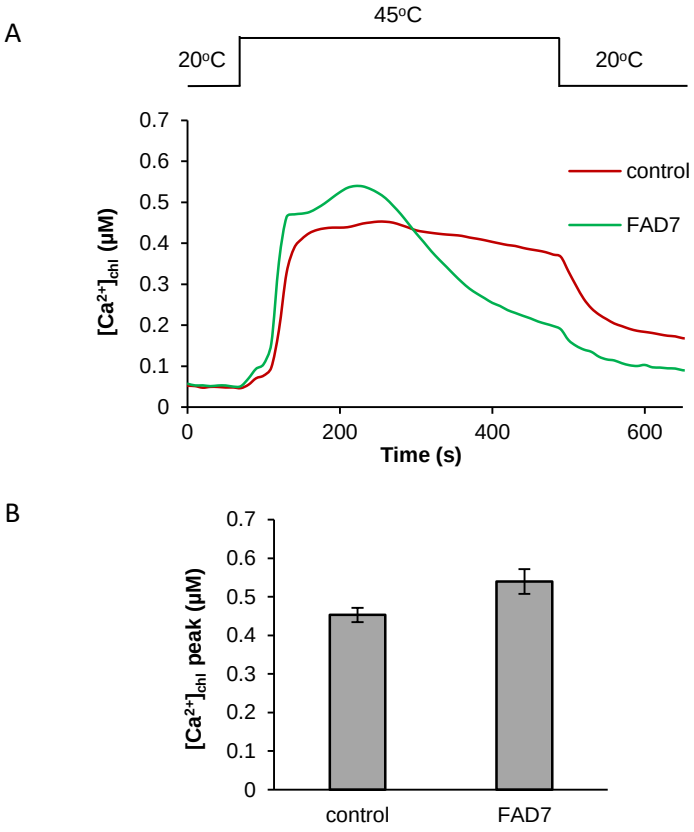


Figure 7.3 Overexpression of FAD7 enhances the calcium response to heat. The $[Ca^{2+}]_{chl}$ response to heat (45ºC for 7 min) is greater in magnitude in tobacco leaves overexpressing the plastidial-specific fatty acid desaturase FAD7, compared to control leaves. (A) Calcium kinetics upon heating of FAD7 overexpressors and control lines (expressing GFP instead of FAD7) and (B) average chloroplastic calcium concentration peak heights. Tobacco leaves were also transformed to express chloroplastic aequorin (pMAQ6). Data were obtained by averaging the signal of n = 5 *Nicotiana benthamiana* leaves. Error bars = SE.

7.2.2 The mammalian TRPV1 heat channel is functional when expressed in plants

7.2.2.1 The TRPV1 channel can be expressed in plants and responds to capsaicin

To test whether it was possible to reconstitute the active mammalian TRPV1 heat receptor in plant cells, we genetically transformed *Arabidopsis thaliana* to inducibly express the full length coding region for TRPV1 from rat(Caterina et al., 1997). TRPV1 is a calcium channel gated by high temperature and capsaicin (Caterina et al., 1997). In order to assess the activity of TRPV1 in plants, we measured cytosolic free calcium in these transgenic plants using the luminescent calcium reporter, aequorin, expressed in the cytosol as described previously (Knight et al., 1991; Knight et al., 1996). The animal heat channel TRPV1 was ectopically expressed in *Arabidopsis thaliana* under the control of an estradiol-inducible promoter. In this way plants stably express the construct, but TRPV1 could be induced when necessary, to avoid toxicity due to constitutive expression. TRPV1 in plants is thought to be targeted to the plasma membrane (as it is in mammals), however, its precise location is not known as the GFP::TRPV1 overexpressing tobacco plants were not viable, and no experiments could be performed on them. Levels of expression of the TRPV1 gene in *Arabidopsis* were tested by RT-PCR on 6 independent transformants, and three lines were selected to cover a range of expression levels. As it is shown in figure 7.4, all the overexpressing lines express TRPV1 and at different levels, while the level is close to zero in the control (pMAQ2), as expected as this gene is completely absent there. TRPV A was chosen as the high expressor, TRPV B showed a middle level of expression of TRPV1 and the lowest level

was represented by TRPV C. DMSO control (i.e. non estradiol-induced lines) did not show any TRPV1 expression, as seen in figure 7.4.

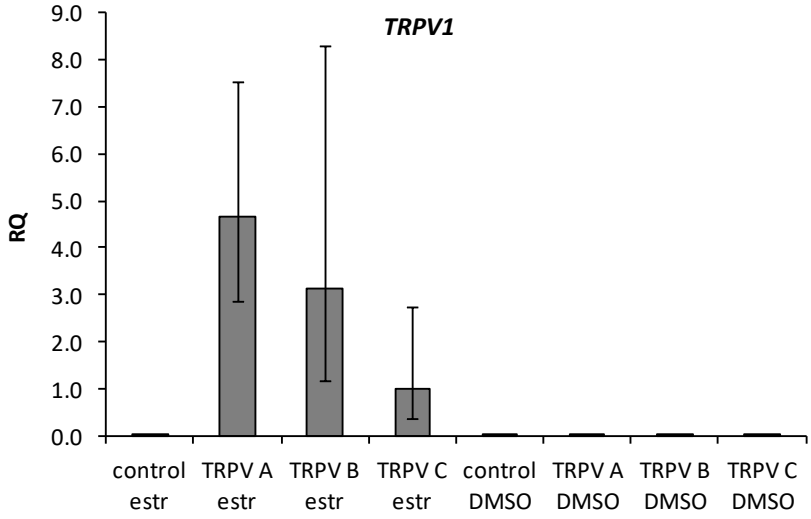


Figure 7.4 Levels of *TRVP1* mRNA expression in stable transformants of *Arabidopsis*. Relative values are compared to *TRPV1* levels in the TRPV C overexpressor treated with estradiol (estr). A control treated with DMSO is also represented. Error bars represent RQ_{MIN} and RQ_{MAX} and constitute the acceptable error level for a 95% confidence level according to the Student's t-test.

In order to test if this channel was functional in plants, plants were treated with the TRPV1 agonist capsaicin (Caterina et al., 1997). As seen in figure 7.5A, addition of capsaicin at 0.6 μM increased cytosolic calcium in the TRPV1 expressing lines, and it had no effect on the control. Our data show that this effect was clearly dependent upon capsaicin specifically, as treatment with equivalent concentration of ethanol (EtOH, solvent for capsaicin) did not elicit this response (figure 7.5B). It could be seen that this capsaicin-induced calcium elevation was specific to the activity of TRPV1 not only because controls not expressing TRPV1 did not show capsaicin-induced calcium elevations (figure 7.5A), but the magnitude of calcium response to capsaicin in the 3 different lines expressing TRPV1 correlated to the levels of *TRPV1* transcript expression as measured by RT-PCR (figure 7.4). The EtOH control represented in figure 7.5B did

cause a calcium increase whose magnitude is significantly lower than the one induced by the capsaicin (figure 7.5A), and this can be due to the known effect of EtOH on increasing membrane fluidity (Patra et al., 2006).

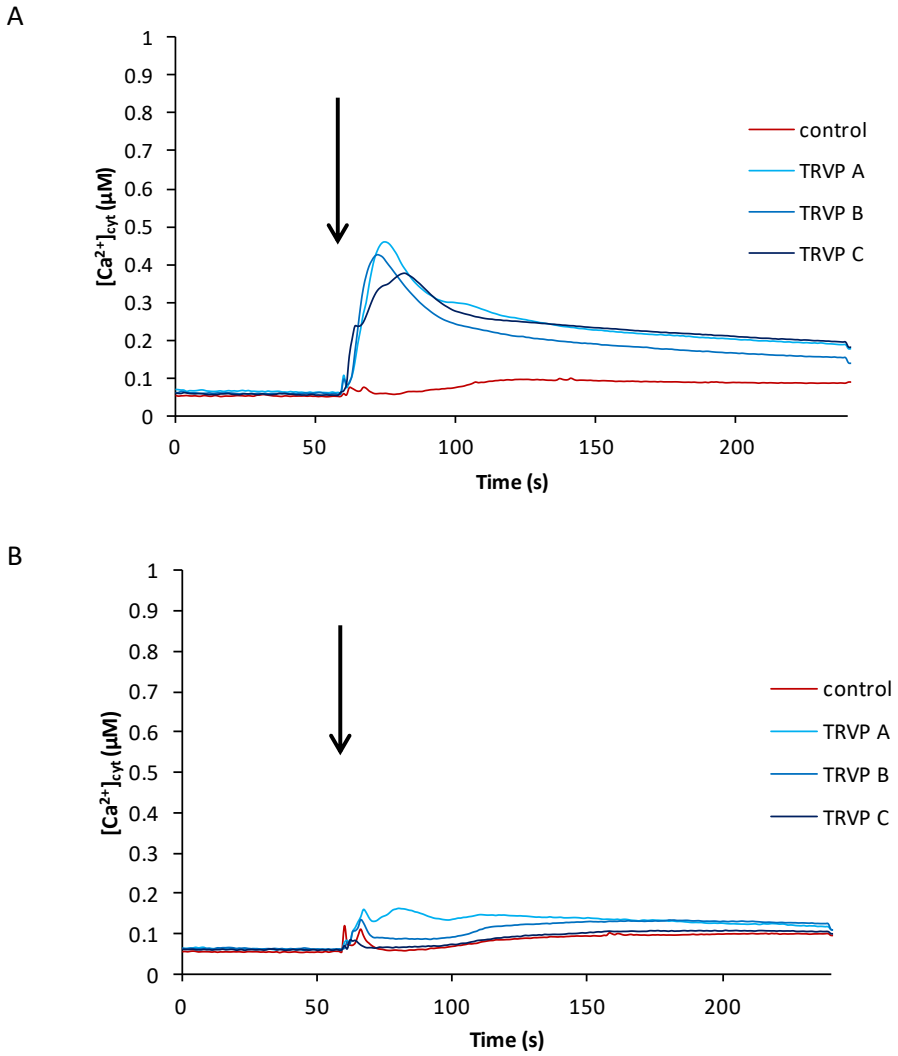


Figure 7.5 The TRPV1 channel is functional in plants and responds specifically to capsaicin. Calcium traces in response to (A) capsaicin 0.6 µM or (B) EtOH 0.05% (v/v) of three *Arabidopsis* lines expressing *TRPV1* as well as the calcium reporter aequorin in the cytosol, and a control line where TRPV1 is not present. The arrow represents the injection time of (A) capsaicin (B) or EtOH.

7.2.2.2 Plants expressing TRPV1 display a novel heat-induced elevation in cytosolic free calcium

When transgenic plants expressing *TRPV1* were treated to change in temperature from 20°C to 40°C, increases in cytosolic free calcium concentration could be observed (figure 7.6). In contrast the increases in calcium in response to the same heat treatment in the controls not expressing *TRPV1* were modest or non-existent. It is possible to notice in figure 7.6 that in the *TRPV1*-expressing lines the calcium levels are already elevated compared to the control, indicating that the channel is already partially opened and calcium is entering the cytosol before heating. Despite this difference, the amount of calcium released in response to the heat stimulus is bigger in the TRPV1-expressing lines compared to the control, as shown in figure 7.6B, where the difference in the calcium level at the peak compared to the calcium level at the baseline for each *Arabidopsis* line is shown. Again it can be seen that the magnitude of the calcium response and of the baseline elevation correlated with the levels of expression of *TRPV1* transcript (compare Ca^{2+}-responses in figure 7.6 with gene expression levels in figure 7.4).

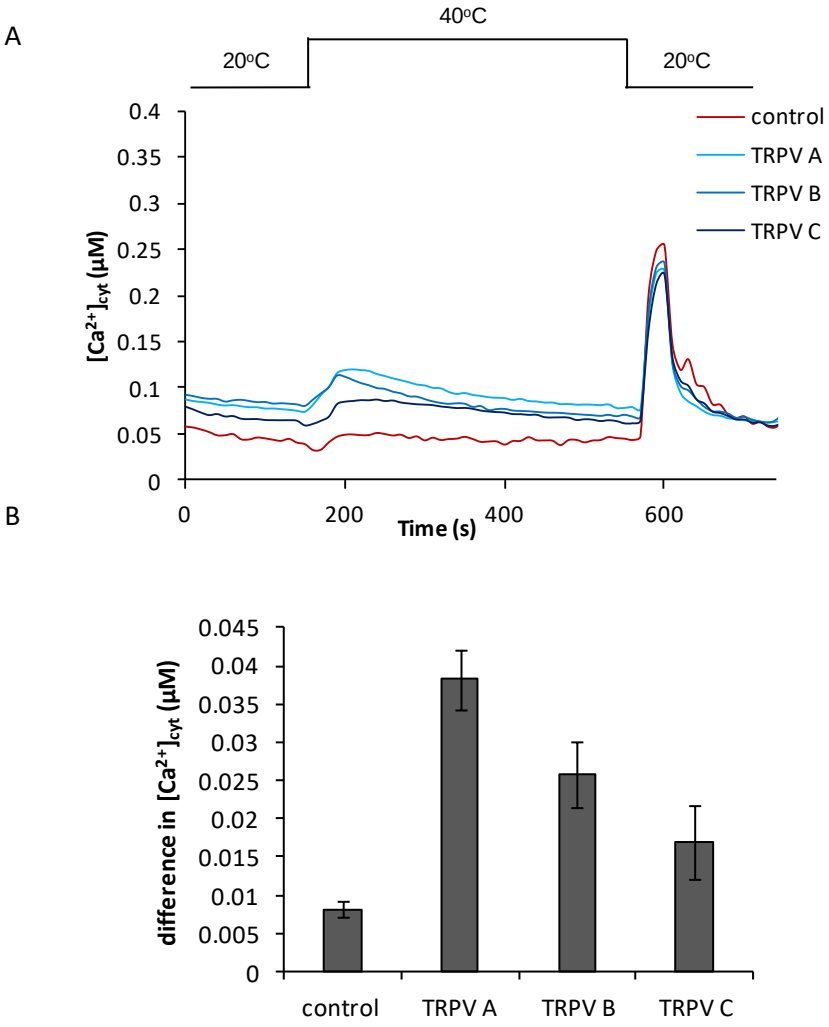


Figure 7.6 The TRPV1 channel is responding to heat. (A) Calcium traces of *Arabidopsis* lines expressing TRPV1 in response to a heat stimulus at 40°C for 7 min and (B) relative increase in the calcium levels comparing for each line the calcium peak in respect to the starting baseline. Error bars represent the standard error.

7.2.2.3 Plants expressing TRPV1 display an enhanced benzyl alcohol-induced elevation in cytosolic free calcium

To test whether plants expressing TRPV1 could respond to biophysical changes in the membrane specifically, we treated the plants with a known fluidiser of membranes, benzyl alcohol (BA), whose effect is to mimic the effect of heat upon membranes (Horvath et al., 1998; Balogh et al., 2005; de Marco et al., 2005; Saidi et al., 2009). As can be seen in figure 7.7, BA stimulates an increase in cytosolic free calcium even in control plants. However, the magnitude of the response was higher in plants expressing TRPV1. Again, the magnitude of the response was correlated with the levels of expression of *TRPV1* transcript (compare figure 7.7 with expression levels shown in figure 7.4).

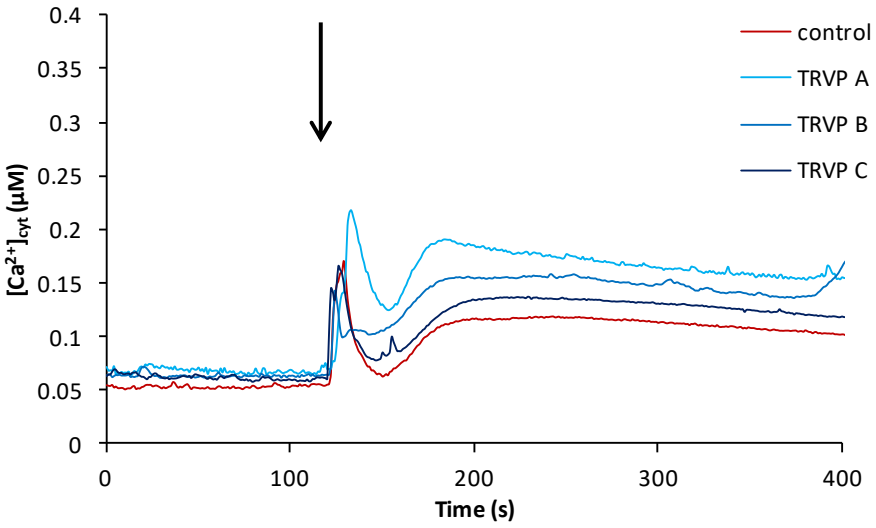


Figure 7.7 BA induces a cytosolic calcium increase in TRPV1-expressing plants. Calcium traces in response to 10 mM BA of the *Arabidopsis* lines expressing TRPV1. The arrow represents the injection time.

7.2.2.4 Long term alterations in membrane fluidity affect TRVP1-dependent heat response

It is well established that plants change the saturation status of the fatty acids in their membranes in response to prolonged changes in ambient temperature (Murata and Los, 1997; Falcone et al., 2004). This phenomenon occurs in order to maintain similar levels of fluidity irrespective of ambient conditions, and is call homeoviscous adaptation (Sinensky, 1974; Ernst et al., 2016). To test the effect that adapting plants to higher (30°C) or lower (15°C) than control growing conditions (20°C) upon the TRPV1-dependent heat-induced calcium response we pretreated control (not expressing TRPV1) plants and plants expressing TRPV1 to either 15°C, 20°C or 30°C overnight. A similar treatment was applied on plants to test for the chloroplast-specific heat response in chapter 7, figure 7.2. We then heated to 45°C and measuring cytosolic calcium to compare responses. As can be seen in figure 7.8, pre-treatment at 15°C enhanced the level of the calcium response to 45°C, whereas conversely, pre-treatment at 30°C reduced the level of the calcium response to 45°C. This suggests that the biophysical context of the membrane in which the TRPV1 channel resides is important in its ability to read temperature in plant cells.

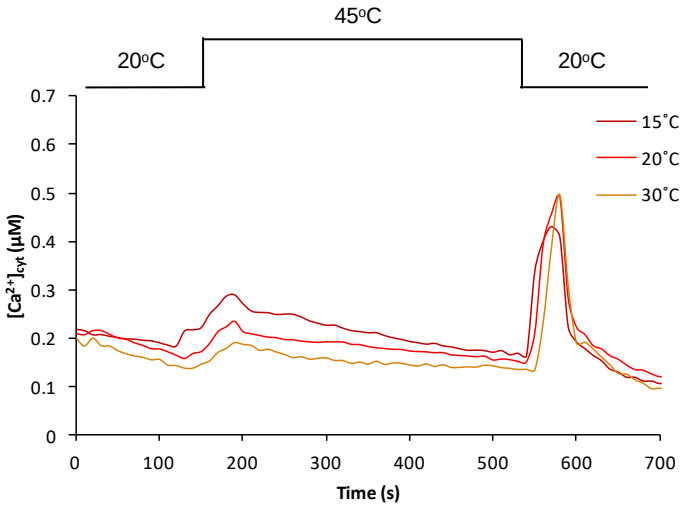


Figure 7.8 The TRPV1 channel response is affected by membrane fluidity. Calcium response of the TRPV B line in response to a heat stimulus at 45ºC for 7 min. Plants were pre-acclimated either at 20ºC, 15ºC or 30ºC overnight before heat stimulation. Each trace is an average of n= 5 *Arabidopsis* seedlings.

7.2.3 Effects of the TRPV1-induced cytosolic calcium increase in response to heat

7.2.3.1 Plant physiology is not consistently affected by TRPV1 in response to heat

One of the aims of expressing the TRPV1 channel in plants was to try to improve plant heat tolerance. Indeed, cytosolic calcium in response to heat has previously been linked to heat tolerance (Gong et al., 1997; Gong et al., 1998; Gong et al., 1998; Larkindale and Knight, 2002; Liu et al., 2005). The first way to test for this altered tolerance was to measure the level of lipid peroxidation in plants after heating, by using the TBARS assay (thioubarbituric acid reactive substances). This spectrophotometric-based analysis measure the level of oxidative damage of membranes, which is a proxy for measuring

plant heat stress tolerance (Heath and Packer, 1968) and it is explained in details in chapter 2, Material and Methods. Hence we performed TBARS analysis on TRPV1-expressing plants treated either at 20ºC or 40ºC for 1 h in the dark, after recovery for 3 days on MS plates in the light at 20ºC. Peroxidation levels were assessed and are shown in figure 7.9, where it is clear that the heat treatment induces a higher level of peroxidation compared to the 20ºC treatment, however, there was no significant differences between the control and the TRPV1-expressing lines.

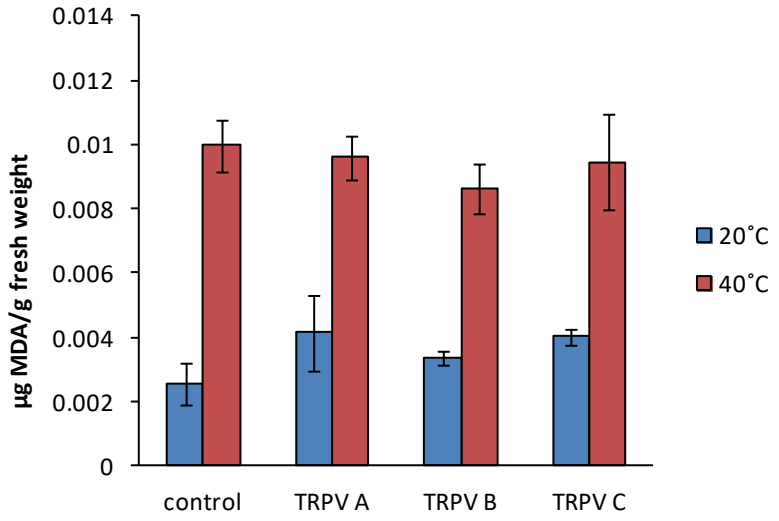


Figure 7.9 The TRPV1 channel does not affect plant peroxidation levels in response to heat. Graphs showing TBARS representing µg of malonadehyde (MDA) per gram of fresh weight. *Arabidopsis* plants were treated either at 20ºC or 40ºC for 1 h and allowed to recover for 3 days before measurements. Data represent an average of four replicates. Error bars represent the standard error.

7.2.3.2 TRPV1 plants differentially express heat responsive genes

The TBARS analysis did not show any significant difference in physiology in response to heat in plants expressing TRPV1 compared to control plants. However, there is the

possibility that by looking at this level subtle differences may be lost and that the TRPV1 channel may still have an effect on plants. Hence we hypothesized that as there is evidence of heat-induced calcium-dependent changes in gene expression (Liu et al., 2005), then the levels of these genes might be higher in the *TRPV1*-expressing lines. As no calcium increase was measured in the cytosol in response to heat (see chapter 5), it is possible that calcium does increase in localised microdomains, from where the response can be triggered. The presence of TRPV1 in plants might create localised calcium increases, in a way that might induce the calcium-dependent heat responses observed (Liu et al., 2005). Hence, TRPV1-expressing plants and a control line were treated at 20ºC, 35ºC and 40ºC for 30 or 90 minutes before testing them for changes in gene expression. Expression levels of a selection of genes for which there was evidence of both heat- and calcium-induction of expression were tested (Liu et al., 2005; Whalley et al., 2011) (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE6154). These genes were *HSP18.2* (At5g59720), *CAM7* (At3g43810), *CAM3* (At3g56800), *HSP17.6AII* (At5g12030), *HSP17.8* (At1g07400*), SUI1* (At5g54940) and *HSP70.2* (At5g02490). Results for *HSP18.2* are shown in this result section, and all the three TRPV1-expressing lines were tested. Results for the other genes are reported in appendix D, where for *CAM3* and *CAM7* all the TRPV1 lines have been tested, while for the other genes levels of expression are shown for TRPV A and control only (TRPV A is the higher TRPV1 expressing line, as reported in figure 7.4). Figure 7.10 shows that levels of expression of *HSP18.2* were indeed heat-induced, and that in the TRPV1 lines the response is bigger compared to the control both after 30 and 90 minutes of treatment. At 90 minutes there is also a visible trend that correlates the amount of calcium (associated with TRPV1 expression) with *HSP18.2* expression levels (figure 7.10B).

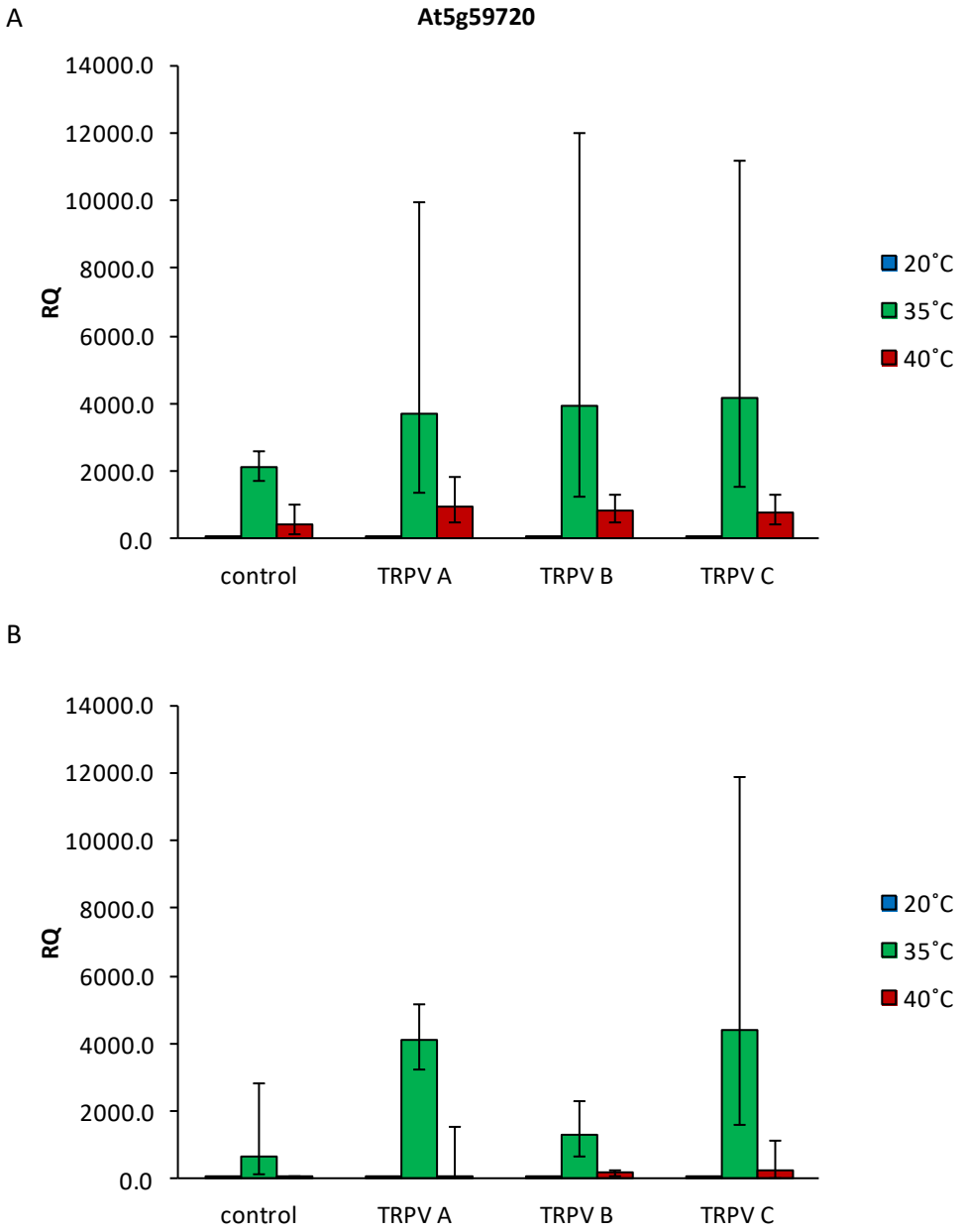


Figure 7.10 Expression levels of *HSP18.2* (At5g59720) at 20ºC, 35ºC and 40ºC for (A) 30 min or (B) 90 min in TRPV1 A, TRPV B, TRPV C and control line. Error bars represent RQ_{MIN} and RQ_{MAX} and constitute the acceptable error level for a 95% confidence level according to the Student's t-test.

7.3 Discussion

7.3.1 The cellular heat thermometer is able to read membrane fluidity

When plants are exposed to any temperature changes compatible with plant survival, they are able to adjust the fluidity of their membranes to the new conditions through acclimation, which is a long term process that involves modifications of the level of saturation of fatty acids (Wilson and Crawford, 1974; Pearcy, 1978; Graham and Patterson, 1982; Murata and Los, 1997). This is to maintain the functioning of membrane-resident processes in the face of long term changes in temperature. As well as these biologically-derived changes in membrane fluidity used by plants to perform long term acclimation to the new temperature regime, rapid changes in membrane fluidity occur as a basic biophysical property of the membranes themselves when the temperature is suddenly modified (Dynlacht and Fox, 1992; Mejia et al., 1995; Horvath et al., 1998; Saidi et al., 2009). It is thought that these rapid changes in membrane fluidity are used for temperature sensing by plant cells (Orvar et al., 2000; Sangwan et al., 2002).

In this study we show that acclimated plants respond differently to a heat stimulus depending upon the temperature they have experienced previously. Indeed plants pre-treated at 15^{o}C, whose membrane will be more fluid due to a higher level of desaturation of the fatty acids, responded to the same heat stimulus by producing a bigger calcium response compared to the control pretreated at 20^{o}C (figure 7.2). Conversely saturating the membrane fatty acids by pre-acclimating plants at 30^{o}C caused a decreased stromal calcium response to heat (figure 7.2). This behaviour recalls the one observed when plants were exposed at different heating temperatures (chapter 5, figure 5.2). These data indicate that the cellular thermometer is able to reset according to the temperature plants have been experienced before the experimental heating event, and this process occurs likely due to long term changes in membrane fluidity. It also points the possibility of rapid changes in membrane fluidity being the primary temperature-sensing event.

Major players in regulating fluidity desaturating fatty acids are fatty acid desaturases, FADs (Somerville and Browse, 1991; Falcone et al., 1994). Amongst these enzymes, FAD7 has been specifically attributed to chloroplast, and it is known to be involved in acclimation to cold (Kodama et al., 1995), where the production of polyunsaturated fatty acid enhances chilling tolerance, and a *fad7* mutant displayed increased tolerance to high temperatures (Murakami et al., 2000).

To further test the hypothesis that long term changes in membrane fluidity are able to reset the heat thermometer, FAD7 was overexpressed in tobacco. In figure 7.3 it can be shown that tobacco where membranes were desaturated by overexpression of FAD7 responded to heat by producing a higher calcium peak compared to the control. The difference in the response of FAD7-expressing tobacco correlates well with the response of *Arabidopsis* seedlings pre-acclimated at 15ºC (figure 7.2), as in both cases membranes are less saturated. Together, these experiments provide further strong evidence for a role for membrane fluidity in sensing heat.

The role of membrane fluidity in sensing temperature has for a long time been postulated in plants (Murata and Los, 1997; Sangwan et al., 2002; Saidi et al., 2010; Mittler et al., 2012). In *Physcomitrella patens* the heat shock response is dependent on calcium release due to opening of CNGCs located in the plasma membrane (Saidi et al., 2009; Finka and Goloubinoff, 2014). A similar role for these channels has recently been reported in *Arabidopsis* (Gao et al., 2012). These CNGCs have been shown to respond to changes in the fluidity of the plasma membrane, specifically by pre-treatment with the membrane fluidizer BA as a surrogate for heat (Finka and Goloubinoff, 2014).

Furthermore, in *Synechocystis*, changes in the membrane fluidity by acclimation or BA treatment affected the transcription profile of heat shock inducible genes, and the effects could be specifically attributed to the thylakoid membranes (Horvath et al., 1998). These membranes also host the susceptible targets of thermal damage in plants (i.e. photosystem II) (Pearcy et al., 1977; Berry and Björkman, 1980; Weis and Berry, 1988; Havaux et al., 1991). Treatment of chloroplast-targeted aequorin lines with BA resulted in a stromal calcium increase, whose signature resembles the one measured in response to a heat pulse

(figure 7.1). This suggests that the heat-induced chloroplast calcium response might occur via a rapid fluidization of one or more chloroplast membranes.

Taken together, our data show increases in stromal calcium induced by both increased temperature and BA, as well as long term modifications of membrane fluidity caused by acclimation and FAD7 overexpression affecting the heat-induced calcium response, demonstrate a fundamental role of membranes and their fluidity in sensing temperature. It is tempting to speculate from this evidence that the still unknown plant heat thermometer may be able to read these changes, and that this message is further communicated as an increase in the stromal calcium. This would point towards a chloroplast location of the thermometer. In the simplest scenario the thermometer would be a calcium channel responding to changes in membrane fluidity. Members of the CGNC family have been identified in chloroplasts (Witters et al., 2004; Witters et al., 2005), as well as other putative calcium transporters (Stael et al., 2012; Nomura and Shiina, 2014). Specifically, two mechanosensitive-like channels, named MLS2 and MLS3 have been localised on the chloroplast inner envelope (Haswell and Meyerowitz, 2006), and recently these channels have been shown to be Ca^{2+}-permeable in bacteria (Cox et al., 2013). For these reasons they would be suitable candidates for temperature sensing components in plants.

7.3.2 TRPV1 is functional when expressed in plants and it senses changes in plant membrane fluidity

The way animal cells sense changes in temperature is well documented. Specifically, increases in temperature are read by the TRPV1 channel, which is a calcium channel active above the threshold of 42^{o}C (Belvisi et al., 2011). In order to better understand the plant heat thermometer, the TRPV1 channel from rat was ectopically expressed in cytosolic aequorin-expressing (pMAQ2) *Arabidopsis* plants, under the control of an estradiol inducible promoter. The pMAQ2 background was chosen as my interest was to further measure calcium in the TRPV1 transformants. The estradiol-induced promoter as

chosen since plants constitutively expressing TRPV1 were not viable. The nature of this toxicity can be explained by the fact that TRPV1 increases cytosolic calcium levels, and this continuous increase in basal calcium was lethal to plants. Evidence of this higher baseline calcium levels are shown in figure 7.6A, and these calcium baselines correlate with the TRPV1 expression levels (compare figure 7.6A with figure 7.4). When the TRPV1 channel was only expressed for a short time period plants survived and were physiologically healthy.

In order to test whether the TRPV1 channel was functional in plants, and not just expressed (figure 7.4), the cytosolic calcium levels of TPRV A, TRPV B, TRPV C and pMAQ2 control plants were monitored in response to the natural channel agonist capsaicin (Caterina et al., 1997). Capsaicin was able to trigger a specific calcium increase (figure 7.5A), which was significantly bigger than the one measured in response to the ethanol (EtOH) control (figure 7.5B). In both cases, the amount of calcium released was proportional to the amount of TRPV1 channel expressed (compare figure 7.5 with figure 7.4), indicating that also the EtOH may have a significant effect on this response. To explain so, one way is to consider the effect of ethanol on membrane fluidity (Goldstein, 1986; Patra et al., 2006), and membrane fluidisation is able to initiate the TRPV1 calcium response, as shown in figure 7.7.

TRVP1 is a heat-induced channel. According to the literature (Cao et al., 2013), heat directly activates the channel, causing a cytosolic calcium increase. We applied a 40^{o}C heat stimulus on *Arabidopsis* plants, and this was able to trigger a cytosolic Ca^{2+}-increase in TRPV1 expressing plants (figure 7.6), and once more there was a correlation between calcium and TRPV1 expression levels (compare figure 7.6 with figure 7.4). As stated before, the channel is known to open in mammals above 42^{o}C, however animal cells are kept at a temperature around 37^{o}C, whilst *Arabidopsis* is grown at 20^{o}C. Hence there can be two explanations, one being that the properties of the channel are different in *Arabidopsis*, and this can change its opening threshold, or the second option is that more likely the surrounding environment in which the channel is set is somehow affecting the

threshold. This second possibility is supported by the ethanol treatment data (figure 7.5B), as well as by the effect of direct changes in plant membrane fluidity (figure 7.7 and figure 7.8). Both BA-induced fast changes in membrane fluidity (figure 7.7) and more long term changes as the ones induced by overnight acclimation at different temperatures (figure 7.8) were able to consistently affect the TRPV1 calcium response. These observation do not strictly demonstrate that the TRPV1 channel is regulated by membrane fluidity directly, but in plants it seems as if the fluidity is somehow controlling the activity of this channel. It is interesting to note that during the heating period, the calcium increase decreases at the end (e.g. figure 7.6 and figure 7.8), indicating that there must be a sort of regulation on the channel, which is closing it. We cannot exclude that there might be a conserved mechanism of channel closure between animal and plants, despite the lack of homologues of TRPV1 in plants, but it is more likely that the membrane fluidity itself regulates the TRPV1 channel activity in this context.

A parallel can be made between the stromal calcium response to heat and its behaviour in response to both long and short term changes in membrane fluidity, and the TRPV1 heat-heat induced response in plants. Indeed by comparing figure 7.1 with figure 7.7 (BA treatment), and figure 7.2 with figure 7.8 (membrane acclimation treatment) it is clear that the regulation of the two responses is similar, and the simplest possible explanation is to attribute a role to membrane fluidity in temperature sensing in plants. Unfortunately, conclusive evidence on the topic would require the molecular identity of the plant heat thermometer, which is till now still unknown.

7.3.3 An attempt to use TRPV1-dependent cytosolic calcium elevations to enhance heat tolerance in plants

One of the aims of expressing TRPV1 in plants was to try to decipher the plant cell's ability to measure temperature. The second aim was to try to test whether the presence of this ectopic calcium channel could improve plant heat tolerance. Indeed, there are reports

of a role of calcium in the plant heat response. Larkindale and colleagues showed that a cytosolic calcium increase can be measured after a heat pulse, when the temperature drops back to a lower one, and that this calcium "recovery" peak is necessary to start the heat-induced oxidative damage response (Larkindale and Knight, 2002). A cytosolic calcium response had already been observed in tobacco in 1998, and this ion had already been associated with plant thermotolerance (Gong et al., 1998). Furthermore, despite the fact that we were not able to measure a calcium increase in the cytosol in response to heat (chapter 5), we cannot exclude that this may be due to the limits of the technique used. Indeed aequorin is only able to measure calcium responses coming from cell populations, and in this case specifically the signal measured originated from whole plants. It is possible that only a subset of cells/tissues are primed to respond to heat by inducing a cytosolic calcium increase or that even within cells that signalling event occurs in specific localised microdomains. If that was the case, calcium would still be a relevant second messenger, but the signal-to-noise ratio would be too low to be detected with aequorin. Detection of such signals would require the usage of a different calcium reporter (e.g. cameleon). Cytosolic calcium increases in response to heat have been observed other organisms such as *Physcomitrella patens* (Saidi et al., 2009) and cyanobacteria (Torrecilla et al., 2000). Furthermore, in *Arabidopsis*, there are evidence of a role of calcium in the heat response, specifically in controlling the expression of heat shock proteins (Liu et al., 2005), and this cements a role for calcium as a messenger in the plant heat response.

The first approach to check whether the presence of TRPV1 could improve plant thermotolerance was to test the physiological state of the plant after heating by looking at the level of oxidative damage to the membranes. A direct measurement of this can be obtained by measuring the lipid peroxidation level using the TBARS assay (Heath and Packer, 1968; Larkindale and Knight, 2002). This assay requires light and gives an indication of the physiological state of the plant during heating and above all during the heat recovery phase. The TBARS assay measures the amount of MDA present in the sample such that the higher the oxidative stress, the higher the MDA and the higher the

TBARS value is. Results in figure 7.9 show that even if the oxidative level is a bit higher in plants expressing TRPV1 at 20ºC, no significative differences were measured upon heating at 40ºC and recovery between the different lines, showing that the presence of this rat channel is not sufficient to decrease plant oxidative damage in response to heat.

Then we decided to investigate the possible role of TRPV1, and consequently of the induced cytosolic calcium, in controlling plant gene expression in response to heat. The set of genes investigated was either coming from the literature (Liu et al., 2005), or obtained searching for the best candidates amongst genes which are both calcium and heat induced (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE6154) (Whalley et al., 2011). The complete list of genes is reported in figure 7.10 and appendix D. Genes were either tested in all the TRPV1 lines (*HSP18.2*, *CAM3* and *CAM7*), or only in the higher expressor and the control lines (TRPV A and pMAQ2, all the other genes). The temperatures tested were 20ºC, 35ºC and 40ºC for 30 or 90 minutes. The data show that when a gene is heat induced (*CAM3* and *CAM7* were not), then there are differences in the transcript amount in the TRPV1 lines compared to the control, specifically at 35ºC. Figure 7.10 explains it. Indeed both after 30 minutes (figure 7.10A) and even more clearly after 90 minutes (figure 7.10B) the *HSP18.2* gene was heat induced and the amount of transcript was relatively higher in the TRPV1 lines. It is difficult to state a proportionality between *TRPV1* expression and *HSP18.2* expression in response to heat, but in TRVP A the levels are significantly higher, and that is the higher expressor. For the other genes (available in appendix D) the general trend was the same, but more or less marked for some of them and there are differences in the heat-inducibility and the time of peak (30 or 90 minutes). However, all of them show higher expression at 35ºC rather than 40ºC. The fact that the TRPV1 lines were able to affect the level of expression of some heat-induced genes confirms a role of calcium as a second messenger in the plant heat response. However, the difference in the amount of transcripts in the TRPV1 expressing lines, despite promising, may not be sufficient to improve plant thermotolerance, as observed from the TBARS experiment (figure 7.9).

7.3.4 Conclusions

In this chapter the role of membrane fluidity in heat sensing was investigated in plants. Two approaches were taken. The first approach used the chloroplast-specific heat response observed in chapter 5 as a proxy to directly investigate the effect of long and short term changes in membrane fluidity in heat sensing. The second approach was exploiting the rat heat sensing calcium channel TRPV1 to further study the properties of the plant heat thermometer. These two different approaches showed similar results, pointing towards a strong role of membrane fluidity in the regulation of the plant heat sensing machinery. We finally tried to test these biotechnically engineered TRPV1 plants for improved plant heat thermotolerance, and some promising results emerged from gene expression experiments. Until the present day how plants sense temperature is still unknown and the new evidence collected in this chapter may help research to address the problem in the future, highlighting the role of membrane fluidity in this process.

Chapter 8

General discussion

8.1 Implications of the work

Calcium signalling is a fundamental aspect of plant physiology and development. Because of its relevance, this topic has been object of research for more than two decades, and still not all aspects of calcium signalling have been completely clarified. For example, the problem concerning how the specificity of the calcium signature is encoded in a plant cell still represents an open question. Furthermore, the investigation of organellar calcium signalling has only recently started, and is giving new insights into the functioning of cell signalling machinery. The research work presented in this thesis was aimed to further understand some of these still unexplored aspects of calcium signalling in plants. This chapter will briefly recap the results of this work and provide suggestions for future investigations.

8.2 Specificity of calcium signatures

As measuring intracellular calcium concentrations had become easier to perform thanks to the advent of new technologies such as the protein-based calcium reporter aequorin (Knight et al., 1991); it became clear that transient cytosolic calcium increases occurred in response to a wide variety of stimuli. Hence the problem of specificity needed to be addressed, namely: how can a single ion encode the information regarding the nature of the stimulus? In the late 90s the calcium signature hypothesis was developed (McAinsh and Hetherington, 1998), according to which such specificity is encoded in the different

kinetics and spatial location of the calcium elevation. Indeed, in nature, stimulus-induced changes in cytosolic calcium concentrations with differential kinetics are observed in many different cell types in response to a diverse range of abiotic and biotic stimuli (Knight et al., 1991; Knight et al., 1996, 1997; Allen et al., 2001; Rentel and Knight, 2004; Evans et al., 2005; Manzoor et al., 2012). This hypothesis was also supported by recent work in which it was demonstrated that when different calcium signatures (with variable amplitude and frequency) were imposed to the plants, these were able to differentially induce specific sets of genes, with very little overlap between the different signatures (Whalley and Knight, 2013). This evidence supports the idea that different signatures can encode different information, however, the way in which this process is performed in a cell, hence how the specificity is encoded, how it is then decoded into a physiological response and what components are involved in both processes are still open questions. The work presented in chapter 3 was aimed at answering these questions, by addressing the problem with the support of a mathematical dynamic model. The SA-mediated pathogen response pathway (Zhang et al., 2014) was selected as a biological pathway to model, as it is calcium-mediated and the components involved and interaction between them are known (figure 3.2). Furthermore, the main calcium decoder is a calmodulin (CaM), which is able to bind the transcription factors CAMTA3 and CBP60g in presence of calcium; and the interaction between $4Ca^{2+}$-CaM-CAMTA had already been modelled (Liu et al., 2015)(figure 3.1). Starting from this, a dynamic model of the pathway was created, taking into account all the interactions between components of the pathway, and it is represented in figure 3.10. In this model, the binding of Ca^{2+}-CaM either to CAMTA3 or CBP60g is represented by two "cubes", which are able to give as an output the amount of active form ($4Ca^{2+}$-CaM-CAMTA3 or $4Ca^{2+}$-CaM-CBP60g) through time given any calcium signature as an input. A third "cube" represents all the other CaM binding proteins not involved in the network, but competing for the pool of CaM. In this pathway calcium controls the transcript expression of *EDS1* and *ICS1* both directly and by changing the amount of the active transcription factors (CAMTA3 and CBP60g), EDS1 itself regulates *ICS1* levels, plus these two genes control the downstream response (SA production), which is then itself regulating the levels of these two genes.

The idea was to try to see whether this model was able to predict changes in *EDS1* and *ICS1* expression levels for a given calcium signature. If so, then the model would have been able to describe for the first time how the calcium specificity was encoded in a plant cell. Hence 4 different calcium signatures were produced experimentally, and changes in levels of *EDS1* and *ICS1* transcripts were measured in response to these signatures. To fit the model parameters, one of the signatures (mastoparan) and the relative transcript levels were provided as an input to the model. Then the model was tested by providing it with a different calcium signature (completely new for the model) and as an output we looked at the predicted levels of transcripts of *EDS1* and *ICS1* over time. As seen in figures 3.12, 3.13 and 3.14, the model was able to correctly predict the trends in terms of changes in transcript levels of both genes (compare model prediction with measured levels, represented by the error bars). Hence this model is not only descriptive of the network, but it can also predict its outputs from any calcium signature given as an input. Moreover, this mathematical model was correctly able to describe how a cell can encode the calcium specificity, as the predictions were accurate. The specificity in the message encoded is due to all the components of the network, and the interaction between them, which allow a non-linear amplification of the calcium signal upon time. Interesting, this model can be applied to any eukaryotic system, with some modification, hence it can have a wider use in systems biology.

8.3 Chloroplast gene expression and calcium

In plants, increases in cytosolic calcium upon biotic or abiotic stress mainly lead to changes in nuclear gene expression, as they ensure plant adaptation to the new conditions, hence survival. Lately, it has become evident that there is also strong regulation of chloroplast calcium concentrations, specifically that stromal calcium levels need to be kept low to ensure correct organellar functioning (Stael et al., 2012; Nomura and Shiina, 2014). Furthermore, evidence of chloroplast calcium acting as a signal has been emerging (Johnson et al., 1995; Sai and Johnson, 2002; Manzoor et al., 2012; Nomura et al., 2012).

Thus we decided to test whether increases in chloroplast calcium regulate plastidial gene expression in terms of changing its trascriptome, in an analogous way to the cell nucleus. To do so, two approaches were taken. In the first case we set up a system to artificially increase stromal calcium concentrations upon mastoparan addition (figure 4.2), and to block this induction by lanthanum pre-incubation (figure 4.3). In this way we were able to tightly control calcium elevations in the chloroplast stroma, and if any change in chloroplast transcript gene expression was due to the treatment with the calcium agonist (mastoparan), then by blocking the calcium with the antagonist (lanthanum) the change should be lost. For all the chloroplast genes tested, no differences in transcript levels were recorded in response to the mastoparan treatment (figures 4.7 and 4.8), suggesting that calcium may not be controlling chloroplast gene expression.

Consequently, a second approach was taken, in which we looked at which stimuli are able to affect chloroplast gene expression at the transcript level (appendix B), and stromal calcium was measured in response to them, to establish a correlation. As shown in figure 4.10 and 4.11, this approach did not give any strong evidence in support of a role for calcium in controlling chloroplast gene expression, as in response to most of the stimuli identified no stromal calcium increase was recorded.

From all this analysis we concluded that there is no evidence in support of a role for stromal calcium in controlling changes in the chloroplast transcriptome, and that this ion probably does not constitute a major regulative mechanism in this organelle, as it does in the nucleus. Testing for specific calcium-controlled cases, or for a possible involvement of calcium in other mechanisms to control protein levels such as alternative splicing or post-transcriptional modifications was not the aim of this study, hence these results are conclusive for the biological question we aimed to answer and I would not recommend any future work in this direction, unless the biological question was re-addressed.

8.4 Chloroplast calcium signalling in response to heat

In this thesis work, a new chloroplast-calcium response was identified, which occurs specifically in response to heat (chapter 5). Using transgenic *Arabidopsis thaliana* seedlings in which the calcium reporter aequorin was targeted to the stroma, chloroplast-specific free calcium increases were detected in response to mild (from 32.5^{o}C) heating (figure 5.2). This response is chloroplast-specific i.e. it does not occur in the cytosol (figure 5.3) nor in the mitochondria (figure 5.17), and it is conserved amongst ecotypes (*Arabidopsis* Col and WS, figure 5.4) and different plant species (*Arabidopsis thaliana* and *Nicotiana benthamiana*, figure 5.4). The heat response was dependent upon absolute temperature (figures 5.2 and 5.8) and displayed the properties of acclimation and sensitisation. An additional component interacting with this calcium signal was light: the heat-induced calcium response was delayed in the dark when preceded by a light treatment. This light-dependent delay could be partially recovered by adding the electron transport chain uncouplers DCMU (figure 5.9) and DBMIB (figure 5.10). This suggested that this chloroplast-specific heat response is associated with darkness. Furthermore, a crosstalk between mitochondria, heat and light was identified, as adding a mitochondrial respiration inhibitor such as rotenone or myxothiazol affected the heat calcium response in the chloroplast, specifically in darkness (figures 5.15 and 5.16). This specific link with darkness and respiration was further investigated when searching for biological significance (chapter 6) as it suggests that this heat-induced chloroplast calcium increase may be indicating the energetic stress of the plant cell.

While chapter 5 mostly described the properties of this newly identified heat response, the entirety of chapter 6 was dedicated to attempt to find the physiological significance of this response. Initially, we unsuccessfully tried to block the chloroplast calcium increase upon heating with chemical incubation with inhibitors (table 6.1), and by expressing calcium sponge proteins inside the chloroplast stroma (figure 6.2). We also tried to target possible upstream unknown components of the pathway, by targeting the glutathione pool

(figure 6.3) or the ER calcium store (figure 6.4), but still we did not succeed in blocking the chloroplast calcium increase upon heating. Despite these difficulties in blocking the signal, reduced calcium increases upon heating were finally observed in two independent mutant lines for a thylakoid membrane calcium sensor protein: CaS (figure 6.7). The calcium-response difference between the *cas* mutants and wild-type was lost upon light pre-treatment (data not shown), suggesting once more a specific role for this response in darkness. The partial inhibition of the calcium response upon heating in the *cas* mutants state a role for this protein in the response. Since the calcium increase is impaired in the *cas* mutants, it is reasonable to assume that CaS is involved in establishing the stromal calcium increase, probably by regulating some chloroplast components (e.g. Ca^{2+} channels). As calcium initially increases, and the inhibition occurs only after this initial peak (figure 6.7), there may be a calcium-induced calcium release regulation on the stromal heat response, and CaS may be involved in this process.

The biological significance was then investigated by using these *cas* mutant lines, hence differences in F_v/F_m upon heating (figures 6.9 and 6.10), correlation of the calcium increase with signalling the energy status of the plant, such as testing for amino acid levels upon protein degradation (figure 6.16); and hormone levels (figures 6.21 and 6.22) were tested upon heating, as all these responses are known to be affected by heat and darkness (specific reasons are extensively given in chapter 6). However, it was not possible to clearly identify a function for the heat-induced chloroplast calcium increase, hence its physiological significance is still an open question. Future work will be discussed in the paragraph 8.7.2.

8.5 Identifying the plant heat thermometer

Temperature is a key environmental cue which strongly affects plant metabolism, development and growth. Despite the major interest in understanding how plants sense temperatures, up to now neither the heat nor the cold thermometers have been identified

in plants. In contrast, the receptors involved in temperature-sensing have been widely investigated in animals (described in the Introduction), and the heat-sensing receptor, namely TRPV1, is a calcium channel (Caterina et al., 1997). No homologues of these channels have been identified in plants. Despite the lack of identity of the plant heat thermometer, there is evidence of biological processes which are directly affected by heat and are known to control downstream responses, such as protein unfolding, changes in the enzymatic activity and cytoskeleton disassembly (Ruelland and Zachowski, 2010), which may be candidates. However, on a time scale, the fastest event occurring in response to a heating event is a fluidization of cell membranes (Ruelland and Zachowski, 2010). Hence we decided to further investigate the possible role of membrane fluidity in heat sensing by using as a proxy the chloroplast-specific calcium heat response. The idea behind this was that, if the chloroplast calcium response is affected by changes in membrane fluidity, then this biological process must be involved in heat sensing. To test this idea, membranes were fluidized by adding benzyl alcohol (a known membrane fluidiser). Alternatively, the fluidity of membranes was affected in the long-term by acclimating plants at different temperatures (which changes the composition of the fatty acids in a way that the higher the acclimation temperature the more rigid the membrane is), or by specifically fluidising the plastidial membranes by overexpressing FAD7 (a plastidial-specific fatty acid desaturase). Direct fluidisation of membranes by BA caused a chloroplast calcium increase similar in its kinetics to the one measured upon heating (compare figure 5.1 with figure 7.1), while changing membrane fluidity by changing the fatty acid composition affected the chloroplast calcium heat response (figures 7.2 and 7.3). These data together point towards a role for membrane fluidity in sensing heat. Moreover, a possible specific role of chloroplasts can be stated, as FAD7 is plastidial specific. Furthermore, one of the cellular components which is highly damaged by heat and whose functioning is affected by it is the chloroplast. It would then be evolutionarily convenient to place the heat thermometer in the chloroplast compartment

To further understand the role of membrane fluidity in plant temperature sensing, *Arabidopsis* TRPV1 overexpressing lines were investigated upon heating. The correct

expression of TRPV1 in plants was assessed both by adding the TRPV1 agonist capsaicin (figure 7.5) (Caterina et al., 1997), or by heating (figure 7.6) (Caterina et al., 1997), and consequently measuring cytosolic calcium (TRPV1 is expressed in the plasma membrane). Affecting plant membrane fluidity by BA or by acclimation at different temperatures affected the TRPV1-mediated calcium increase (figures 7.7 and 7.8), and the kinetics were similar to the ones observed in the chloroplast in response to the same treatments. Despite evidence in the literature that TRPV1 in animals is intrinsically heat activatable (Cao et al., 2013), the data obtained in this work suggest that, at least in plants, TRPV1 activity is influenced by membrane fluidity.

8.6 Limitations of the approach taken

Different types of work have been described in this thesis, and very different approaches have been taken to answer a range of scientific questions, from wet lab work to the use of a mathematical model. Each different approach has limitations. For the modelling part, the major limitations in describing a biological system is that the amount of data available to build the model in terms of binding affinities, time delays and other parameters. To overcome this limitation, it is necessary to either measure those parameters directly, or to refine the model by fitting the unknown parameters to experimental data provided as input. Another limitation is that any component of a network also interacts with other cellular components, and to consider all of them is nearly impossible, hence approximations need to be considered, and predictions will be adequate, but not perfect.

For the chloroplast calcium heat response, one limitation is that it is impossible to simultaneously measure aequorin and shine light on the plants, hence alternative approaches had to be taken to understand the relationship of heat response with light. Furthermore, aequorin is a good reporter for indicating the behaviour of a population of cells, but it is possible that there is a more detailed sub-location of the calcium increases in a specific set of plant cells, tissues etc., whose level of detail cannot be resolved using this reporter. In the same way, we did not detect any cytosolic calcium increase upon

heating, however, calcium may be increasing in localised cell microdomains, and again a different calcium reporter would be needed to visualise them (e.g GFP-based calcium reporter can visualise calcium dynamics in single cells, see introduction).

8.7 Future work

This section will propose the direction I would take if I had to carry on working on these research topics, and some of these approaches have been initiated, but not concluded because of time limitations. A discussion of future work relative to the research carried on in chapter 4 was not included as the initial question was answered and new different questions should be addressed to continue this research line.

8.7.1 Modelling

As stated in paragraph 8.2, the work described in chapter 3 was able to create a mathematical model of a calcium-mediated biological network. This model was predictive, as it was able to give as an output the correct change in term of gene expression fold difference, given any calcium signature as an input. This powerful mathematical tool described in chapter 3 can have many future applications, as the components of the network in terms of calcium-binding proteins, transcription factors, genes and downstream responses could represent virtually any calcium-mediated eukaryotic network. By changing the parameters of the different components (e.g. the calcium binding kinetics differ in different calcium binding proteins) and the components themselves, alternative networks can be modelled, and different ways of encoding specificity in the same organism (e.g. different pathways in *Arabidopsis thaliana*), or completely different ones (e.g. comparing animals and plants) can be investigated. Furthermore, another advantage of modelling a network is the fact than the importance of some specific elements, otherwise understated, can emerge (emerging properties). For example, in the specific case represented in chapter 3, it was found that an important

parameter controlling gene expression fold differences is the time taken for the cytosolic calcium levels to go back to resting concentrations.
Moreover, it would be interesting to test whether the model is able to discriminate a pathogen-induced signature from a signature which is not activating the pathway itself (e.g. a symbiotic signature or salt, cold etc.), hence to see if the information encoded in the calcium kinetics is sufficient to discriminate for the right pathway.

8.7.2 The heat response pathway in the chloroplast and its biological significance

Despite the extensive descriptive work of the properties of the chloroplast-specific heat response shown in chapter 5, and the attempts to identify its physiological significance reported in chapter 6, two major questions still remain open. The first question to address in future work is to investigate which components of the chloroplast, and in general of the plant cell, are involved in this response, both upstream (e.g. channels or calcium-binding proteins responsible for the calcium increase) and downstream (e.g. calcium-binding proteins) of the chloroplast calcium increase. Ideally, a mutant screening would be performed to identify components in which the chloroplast calcium heat response is affected, however, this would be a long and difficult approach, and it would not identify the components downstream of calcium. Some educated guesses could be made, considering that there are some putative calcium transporters in the chloroplasts (Stael et al., 2012; Nomura and Shiina, 2014). Mutants for these transporters could be transformed with stromal aequorin and tested for chloroplast calcium increase upon heating. One of the pathway components has already being identified using a similar approach: the CaS protein. Indeed in the *cas* mutants the heat-induced calcium response is partially inhibited (figure 6.7), suggesting that CaS is a component of the machinery responsible for the calcium increase, however, as the inhibition is only partial, therefore other proteins can be involved (the alternative hypothesis is that these mutations only cause a partial phenotype).

Identifying the downstream components would be a challenge because the physiological output is not known. Because of this reason, it would be necessary to take a shotgun approach. Hence, a reasonable experiment to perform would be to see if, in the presence of the heat-induced calcium, there is any change in the proteome of the chloroplast, and even more specifically in the phosphorylation state of plastidial proteins (e.g. CaS itself). Furthermore, some general approaches such as genomic or metabolomic should be performed, as educated guesses were not able to direct us towards the significance of this chloroplast-calcium response upon heating. To this aim, Col-0 and the *cas* mutants, where the calcium increase is partially inhibited, could be heat-treated and for example a RNA seq analysis could be performed on them. From a comparison with a non-heated control (a light/darkness control could be used too, if possible, as this response is specific for darkness), a set of up- and down-regulated genes should emerge in the *cas* mutants upon heating, indicating the processes affected by the chloroplast-specific calcium increase upon heating.

8.7.3 Heat sensing in plants

Despite the significant interest of the scientific community in understanding temperature sensing in plants, and identifying the specific components involved, there is still no evidence for the specific identity of the heat and cold thermometers. In this study we investigated the role of membrane fluidity in heat sensing in plants, by reading as an output the chloroplast-specific calcium response identified in chapter 5. The novelty of this work is that this calcium response upon heating was adopted as a readout, and this could easily be used to screen for plant cell components involved in heat sensing. Ideally, to identify components of the plant heat-sensing pathway, if not the thermometer itself, it would be interesting to screen for a pMAQ6 (stromal aequorin, (Johnson et al., 1995)) mutant population by using either temperature itself or membrane fluidisers, such as BA. To test the potential of this idea, I tested if the *cas* mutants, which are known to be involved in the heat response, displayed a different response to BA, and they did (data not shown). Hence there are good reasons to think that this approach may be successful. At

the same time, directly targeting pMAQ6 in mutants for chloroplast calcium channels (as suggested in the paragraph 8.7.2) can be efficient in identifying possible components involved in the plant heat response. Specifically, members of the CNGC family have been and associated with heat sensing in moss (Saidi et al., 2009; Finka and Goloubinoff, 2014), and they have been recently identified in chloroplasts (Witters et al., 2004; Witters et al., 2005), as well as other putative calcium transporters (Stael et al., 2012; Nomura and Shiina, 2014). Two mechanosensitive-like channels, named MLS2 and MLS3 have also been localised on the chloroplast inner envelope (Haswell and Meyerowitz, 2006), and recently these channels have been shown to be Ca^{2+}-permeable in bacteria (Cox et al., 2013). These would be good candidates to test for a role in heat sensing in plants.

8.8 Conclusions

In conclusion, in this work calcium signatures have been analysed as outputs of the plant cell thermometer, in the chloroplast-specific calcium heat response, and the problem of how specificity is encoded has been approached from a mathematical point of view.
With the studies proposed in this thesis, a little step forward has been made in the understanding of calcium signaling in plants, and future work may extend it to other eukaryotes, or towards the identification of the plant heat thermometer.

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Appendix A

Oligonucleotides

Primer name	Sequence 5'> 3'	AGI code
RbcL Fw	TCGGTGGAGGAACTTTAGGC	Atcg00490
RbcL Rev	TGCAAGATCACGTCCCTCAT	Atcg00490
AtRBCS1A Fw	AATTTCCGGACTTAACGTTTGTTT	At1g67090
AtRBCS1A Rev	CATCAGACAGTTGAGAATCCGATAGA	At1g67090
PEX4_SYBR_Fw	TCATAGCATTGATGGCTCATCCT	At5g25670
PEX4_SYBR_Rev	ACCCTCTCACATCACCAGATCTTAG	At5g25670
PsaA Fw	GCCAAGAAATCCTGAATGGA	Atcg00350
PsaA Rev	CATCTTGGAACCAAGCCAAT	Atcg00350
PetB Fw	ATTGGGCGGTCAAAATTGTA	Atcg00720
PetB Rev	AGACGGCCGTAAGAAGAGGT	Atcg00720
PsbD Fw	CACAAATCTTTGGGGTTGCT	Atcg00270
PsbD Rev	CCATCCAAGCACGAATACCT	Atcg00270
PsbB Fw	CGTGCGACTTTGAAATCTGA	Atcg00680
PsbB Rev	TAGCACCATGCCAAATGTGT	Atcg00680
ABR1 Fw	CTGCACTTCGGTTTAGAGGC	At5g64750
ABR1 Rev	CAGGTTGTGCTTCGGTTGAA	At5g64750
RAP2.6 Fw	GCAACACAAAACTGGCGAAC	At1g43160
RAP2.6 Rev	TGCGGTGGTAGACAAGTTGT	At1g43160
psbA-for	GAGCAGCAATGAATGCGATA	Atcg00020
psbA-rev	CCTATGGGGTCGCTTCTGTA	Atcg00020
rpoC1 Fw	TCGGATACGAAGATATCAAATGG	Atcg00180
rpoC1 Rev	TTAGTTATGGGCCTAGCAAAAGA	Atcg00180
ICS1 Fw	CAAATCTCAACCTCCGTCGT	At1g74710
ICS1 Rev	AATCAATTGCTCCGATTTGC	At1g74710
EDS5 Fw	CTCTTGGACCGGGAACAGTA	At4g39030
EDS5 Rev	GTTGCGCTTCTTTCTTGTCC	At4g39030

CaS Fw	TCATTGTTGTTGCTGCTGGT	At5g23060
CaS Rev	CGGCGTAAGATCACCTTTGT	At5g23060
EDS1 Fw	ACCTAACCGAGCGCTATCAC	At3g48090
EDS1 Rev	TTGTCCGGATCGAAGAAATC	At3g48090
TRPV1 Fw	AGCGAGTTCAAAGCCCAGA	NA
TRPV1 Rev	GTGTCATTCTGCCCATTGTG	NA
Hsp18.2 RT Fw	TCG TGA TGT GGC AGC GTT TA	At5g59720
Hsp18.2 RT Rev	AAG TCC GCT TTG AAC ACA TGT G	At5g59720
CaM3 RT Fw	GGA CTC GAG GTA TGT TTT CTG CTT	At3g56800
CaM3 RT Rev	TGT TCA GAC GCA AAA TAG AGC ATA A	At3g56800
CaM7 RT Fw	TTT GTT GGT CGT GAT TTT TTG G	At3g43810
CaM7 RT Rev	CGT CAC GGA CAA TAA CAA AAC C	At3g43810
Hsp17.6AII_RT_F	CTGAGCCAAAGAAACCAAAG	At5g12030
Hsp17.6AII_RT_R	TAGTTGCTTATCGATTACAT	At5g12030
Hsp17.8_RT_F	AAGCTGCCGGAGAATGTGAA	At1g07400
Hsp17.8_RT_R	TCCACTTTAGGCACCGTCAC	At1g07400
SUI1_RT_F	TTTTTCCCATTCCTCTGCTG	At5g54940
SUI1_RT_R	TTCCATCAGCATACGATCCA	At5g54940
Hsp70-2_RT_F	CAGCGATCTCACGCATCTTA	At5g02490
Hsp70-2_RT_R	CTCCGGAACTGCAGAGAAAC	At5g02490
APX2 Fw	ATTGCCGTTAGGCTTCTTGA	At3g09640
APX2 Rev	ATGGAATCTCTGGTCCTCCA	At3g09640
M13 Fw	GTAAAACGACGGCCAGT	NA
M13 Rev	CAGGAAACAGCTATGAC	NA
AscI-Aeq-Rev	GGCGCGCCTTAGGGGACAGCTCCACCGT	NA
PSPOMI-pMAQ2-Fw	CGCGGGGCCCCACCATGACCAGCGAACAATACTCA	NA
PSPOMI_pMAQ6-Fw	CGCGGGGCCCCACCATGGCTTCTATGATATCCTCT	NA
ARP-Fw	GAGTAAAGTGCATGATAGAGGCCAAAGACAGTGACGGC	NA
Asc1-ARP-Rev	CGCGGGCGCGCCTCATGGGTCGGTGCTGGC	NA
35S promoter Fw	GAACCTCCCTCGGATTCCAT	NA
35S terminator Rev	GCAGGTCACTGGATTTTGGT	NA
NotI- CaS Fw	CGCGGCGGCCGCATGGCTATGGCGGAAATG	At5g23060
AscI- CaS Rev	CGCGGGCGCGCCTCAGTCGGAGCTAGGAAGGA	At5g23060
CAS-MID-SEQ	ACAGTAACAGATGTAGCACAACA	At5g23060

Appendix B

List of treatments affecting chloroplast gene expression

These treatments were obtained by searching Genevestigator for conditions able to up- or down- regulating the chloroplast genome (for details see chapter 4). The treatments are ranked based on the score of *PsbA* (Atcg00020). Only the value of fold difference of *PsbA* is reported, but the research was performed using all the chloroplast genes.

Treatments name	Details	Levels of PsbA
Ws > Ws-2	Ws-2 / Col-0	3.25
Ws > Ws-2	Ws-2 / Col-0	3.154
Other > elevated CO_2 study 3 (mature leaf 10)	elevated CO_2 study 3 (mature leaf 10) / elevated CO_2 study 3 (leaf 10 primordia)	3.121
Other > ambient CO2 (mature leaf 10)	ambient CO_2 (mature leaf 10) / ambient CO_2 (leaf 10 primordia)	3.088
Other > iron deficiency / protoplasting	iron deficiency / protoplasting / iron deficiency study 8 (24h)	2.857
Hormone > salicylic acid study 4 (Van-0)	salicylic acid study 4 (Van-0) / silwet L77 treated Van-0 leaf samples (4h)	2.681
Biotic > H. schachtii	H. schachtii / non-infected root samples	2.61
Other > non-polysomal RNA study 2 (Col-0)	non-polysomal RNA study 2 (Col-0) / total RNA study 3 (Col-0)	2.462
Other > non-polysomal mRNA (Ws)	non-polysomal mRNA (Ws) / total RNA study 2 (Ws)	2.405
Chemical > primisulfuron-methyl (24h)	primisulfuron-methyl (24h) / mock treated leaf samples (24h)	2.389

Chemical > dexamethasone study 10 (Col)	dexamethasone study 10 (Col) / untreated seedling samples (Col)	2.312
Other > elevated CO2 study 3 (expanding leaf 10)	elevated CO_2 study 3 (expanding leaf 10) / elevated CO_2 study 3 (leaf 10 primordia)	2.287
Photoperiod > circadian clock study 9 (17h dark+1h light)	circadian clock study 9 (17h dark+1h light) / circadian clock study 9 (18h dark)	2.263
Hormone > salicylic acid study 6 (Tsu-1)	salicylic acid study 6 (Tsu-1) / silwet L77 treated Tsu-1 leaf samples (52h)	2.161
Stress > heat study 3	heat study 3 / dark grown Col-0 seedling samples	1.997
Hormone > BA (Col-0)	BA (Col-0) / vehicle treated shoot samples (Col-0)	1.988
Light intensity > shift low to high light (Col-0)	shift low to high light (Col-0) / low light study 3 (Col-0)	1.983
Chemical > dexamethasone study 11 (Col)	dexamethasone study 11 (Col) / untreated seedling samples (Col)	1.981
Light intensity > low light / 4°C (220-280min)	low light / 4°C (220-280min) / low light / 21°C (220-280min)	1.978
Biotic > P. syringae pv. tomato study 9 (DC3118 Cor-hrpS)	P. syringae pv. tomato study 9 (DC3118 Cor-hrpS) / mock inoculated leaf samples	1.862
C24	C24 / Col	1.828
Light intensity > low light / 4°C (300-360min)	low light / 4°C (300-360min) / low light / 21°C (300-360min)	1.815
Hormone > NAA study 3 (2d)	NAA study 3 (2d) / mock treated inflorescence stem internode starch sheath cell samples (2d)	1.788
Other > ambient CO_2 (expanding leaf 10)	ambient CO_2 (expanding leaf 10) / ambient CO_2 (leaf 10 primordia)	1.764
C24	C24 / Col	1.758
Other > shift to pH 4.6 (LZ4)	shift to pH 4.6 (LZ4) / mock treated root samples (LZ4)	1.753
Light intensity > low light / 4°C (140-200min)	low light / 4°C (140-200min) / low light / 21°C (140-200min)	1.623
Stress > hypoxia study 12 (Col-0)	hypoxia study 12 (Col-0) / mock treated Col-0 root samples	1.575
Light intensity > low light / 4°C (640 and 1280min)	low light / 4°C (640 and 1280min) / low light / 21°C (640 and 1280min)	1.546

Chemical > H_2O_2 study 3 (Col-0)	H_2O_2 study 3 (Col-0) / untreated seedlings (Col-0)	1.54
Hormone > salicylic acid study 4 (Est)	salicylic acid study 4 (Est) / silwet L77 treated Est leaf samples (4h)	1.533
Rsch > Rsch-0	Rsch-0 / Col-0	1.511
Nutrient > sucrose study 5 (Col-0)	sucrose study 5 (Col-0) / mock treated Col-0 guard cell samples	1.498
Other > shoot regeneration study 2 (Ws)	shoot regeneration study 2 (Ws) / callus formation study 5 (Ws)	1.444
Stress > heat (roots)	heat (roots) / untreated root samples (early)	1.429
Hormone > salicylic acid study 5 (Van-0)	salicylic acid study 5 (Van-0) / silwet L77 treated Van-0 leaf samples (28h)	1.394
Fei-0	Fei-0 / Col-0	1.372
Nutrient > KNO_3 study 4 (15min)	KNO_3 study 4 (15min) / mock treated root samples (15min)	1.354
Other > ambient CO_2 (mature leaf 10)	ambient CO_2 (mature leaf 10) / ambient CO_2 (expanding leaf 10)	1.323
Other > shift etiolated seedlings to light (late)	shift etiolated seedlings to light (late) / shift etiolated seedlings to light (early)	1.317
Nutrient > nitrate(0mM) / sucrose(30mM)	nitrate(0mM) / sucrose(30mM) / root samples (N-free/suc-free)	1.283
Rsch > Rsch-0	Rsch-0 / Col-0	1.28
Light quality > red study 4 (18h)	red study 4 (18h) / dark study 5 (18h)	1.272
Rsch > Rsch-0	Rsch-0 / Col-0	1.258
Temperature > 10°C / long day (Ler-0)	10°C / long day (Ler-0) / long day study 2 (Ler-0)	1.236
Light intensity > dark / 4°C (60-120min)	dark / 4°C (60-120min) / dark / 21°C (60-120min)	1.216
Light quality > red study 3 (45h)	red study 3 (45h) / dark grown seedlings (Col-0)	1.212
Photoperiod > circadian clock study 9 (17h dark+1h light)	circadian clock study 9 (17h dark+1h light) / circadian clock study 9 (5h dark+1h light)	1.206
C24	C24 / Col-0	1.203
Other > stratification (1h)	stratification (1h) / seed desiccation	1.193

Hormone > salicylic acid study 6 (Cvi-1)	salicylic acid study 6 (Cvi-1) / silwet L77 treated Cvi-1 leaf samples (52h)	1.178
Hormone > zeatin study 2 (Col-0)	zeatin study 2 (Col-0) / solvent treated aerial parts (Col-0)	1.168
Bur-0	Bur-0 / Col-0	1.166
Light intensity > shift dark to light study 2 (polys. RNA)	shift dark to light study 2 (polys. RNA) / dark study 6 (polys. RNA)	1.153
Biotic > E. coli (TUV86-2 fliC)	E. coli (TUV86-2 fliC) / E. coli (O157:H7)	1.144
SQ-8	SQ-8 / Bur-0	1.128
Biotic > *P. parasitica* (2.5h)	*P. parasitica* (2.5h) / non-infected root samples (Col-0)	1.127
Stress > cold study 22 (Col)	cold study 22 (Col) / untreated rosette leaf samples (Col)	1.094
Light quality > red study 5 (Ler)	red study 5 (Ler) / dark study 10 (Ler)	1.086
Nutrient > mannitol study 2 (Col-0)	mannitol study 2 (Col-0) / mock treated Col-0 guard cell samples	1.084
Nutrient > sulfur deficiency study 2 (48h)	sulfur deficiency study 2 (48h) / mock treated root samples	1.073
Hormone > BL/H3BO3 (10d)	BL/H3BO3 (10d) / untreated cell culture samples	1.073
Nutrient > sulfur deficiency	sulfur deficiency / Seeds of Col-0 grown with sufficient sulfur	1.071
Light intensity > low light / 21°C (640 and 1280min)	low light / 21°C (640 and 1280min) / moderate light / 21°C (640 and 1280min)	1.05
Light intensity > light study 6 (Col-0)	light study 6 (Col-0) / dark grown Col-0 seedlings	1.049
Biotic > G. orontii study 6 (Col-0)	G. orontii study 6 (Col-0) / untreated rosette leaf samples (Col-0)	1.046
Hormone > ABA study 2	ABA study 2 / untreated embryo endosperm samples	1.043
Tamm-2	Tamm-2 / Got-22	1.032
Other > shoot regeneration (Ws)	shoot regeneration (Ws) / callus formation study 5 (Ws)	1.03
Sha	Sha / Col-0	1.026
Stress > osmotic study 2 (late)	osmotic study 2 (late) / untreated root samples (late)	0.997

Rsch > Rsch-0	Rsch-0 / Col-0	0.984
Hormone > ABA study 8 (Col-0)	ABA study 8 (Col-0) / solvent treated leaf samples (Col-0)	0.981
Biotic > *P. syringae* pv. tomato study 4 (DC3000 hrpA-)	*P. syringae* pv. tomato study 4 (DC3000 hrpA-) / P. syringae pv. tomato study 4 (DC3000 avrRpm1)	0.968
Biotic > *P. syringae* pv. tomato study 9 (DC3118 Cor-)	*P. syringae* pv. tomato study 9 (DC3118 Cor-) / mock inoculated leaf samples	0.968
Stress > anoxia study 2	anoxia study 2 / dark grown Col-0 seedling samples	0.966
Chemical > cloransulam-methyl (24h)	cloransulam-methyl (24h) / mock treated leaf samples (24h)	0.964
Chemical > MSB (3h)	MSB (3h) / mock treated rosette leaf samples (3h)	0.945
Chemical > dexamethasone study 12 (Col)	dexamethasone study 12 (Col) / untreated seedling samples (Col)	0.932
SQ-8	SQ-8 / CIBC-17	0.927
Stress > cold study 3 (7d)	cold study 3 (7d) / untreated seedlings	0.923
Te-0	Te-0 / Col-0	0.918
Light intensity > dark / 21°C (640 and 1280min)	dark / 21°C (640 and 1280min) / moderate light / 21°C (640 and 1280min)	0.902
Stress > osmotic study 2 (early)	osmotic study 2 (early) / untreated root samples (early)	0.896
Stress > cold study 3 (24h)	cold study 3 (24h) / untreated seedlings	0.891
Hormone > BL/H3BO3 (8d)	BL/H3BO3 (8d) / untreated cell culture samples	0.888
Light quality > shift R+B 0.5µmol m-2 s-1 to R+B 60µmol m-2 s-1 (1h)	shift R+B 0.5µmol m-2 s-1 to R+B 60µmol m-2 s-1 (1h) / R+B (0.5µmol m-2 s-1)	0.886
Biotic > *H. pilosella* (Col-0)	*H. pilosella* (Col-0) / untreated Col-0 root samples	0.876
Stress > genotoxic study 2 (late)	genotoxic study 2 (late) / untreated root samples (late)	0.868
Bl-1	Bl-1 / Bay-0 parent	0.848
SQ-8	SQ-8 / Knox-18	0.844
Hormone > 2,4-D + trichostatin A (Ler)	2,4-D + trichostatin A (Ler) / 2,4-D study 2 (Ler)	0.844

Nutrient > sulfur deficiency study 3 (LZ3)	sulfur deficiency study 3 (LZ3) / sulfur deficiency study 3 (LZ2)	0.843
Other > elevated CO_2 study 3 (mature leaf 10)	elevated CO_2 study 3 (mature leaf 10) / elevated CO_2 study 3 (expanding leaf 10)	0.834
Stress > heat study 7	heat study 7 / untreated cell suspension samples	0.833
Hormone > NAA study 2 (2d)	NAA study 2 (2d) / untreated inflorescence stem internode cell samples	0.821
Nutrient > sulfur deficiency study 2 (3h)	sulfur deficiency study 2 (3h) / mock treated root samples	0.812
Stress > cold study 2 (early)	cold study 2 (early) / untreated root samples (early)	0.797
Stress > genotoxic study 2 (early)	genotoxic study 2 (early) / untreated root samples (early)	0.784
Nutrient > KNO_3 study 3	KNO_3 study 3 / NH4NO3 (Col-0)	0.784
Biotic > *P. syringae* pv. tomato study 8 (DC3000)	*P. syringae* pv. tomato study 8 (DC3000) / mock inoculated leaf samples	0.777
Nutrient > KNO_3 study 3	KNO_3 study 3 / (NH4)2SO4	0.776
Sha > Sha parent	Sha parent / Bay-0 parent	0.775
Ag-0	Ag-0 / Got-22	0.774
NFA-10	NFA-10 / Knox-18	0.77
Light intensity > dark / 32°C (60-120min)	dark / 32°C (60-120min) / dark / 21°C (60-120min)	0.767
Bur-0	Bur-0 / Col-0	0.759
Fei-0	Fei-0 / Col-0	0.751
Chemical > fosmidomycin	fosmidomycin / solvent treated seedlings	0.743
Light intensity > light study 2	light study 2 / dark grown Col-0 seedlings	0.743
Biotic > *P. syringae* pv. tomato study 11 (Ler)	*P. syringae* pv. tomato study 11 (Ler) / untreated leaf disc samples (Ler)	0.742
Hh-0	Hh-0 / Bla-1/Hh-0	0.735
Temperature > 27°C (total RNA)	27°C (total RNA) / 17°C (total RNA)	0.733
Hormone > IAA study 10 (1h)	IAA study 10 (1h) / mock treated root samples (1h)	0.727
Bl-1	Bl-1 / Col-0	0.726

Nutrient > nitrate(45mM) / sucrose(90mM)	nitrate(45mM) / sucrose(90mM) / root samples (N-free/suc-free)	0.721
Stress > cold study 20 (Col-0)	cold study 20 (Col-0) / untreated Col-0 rosette samples	0.703
Chemical > fenclorim (24h)	fenclorim (24h) / solvent treated root culture samples (24h)	0.687
Hormone > IAA study 10 (2h)	IAA study 10 (2h) / mock treated root samples (2h)	0.686
Fei-0	Fei-0 / Col-0	0.682
Bay-0 > Bay-0 parent	Bay-0 parent / Col-0	0.678
Bl-1	Bl-1 / Bay-0 parent	0.671
SQ-8	SQ-8 / Knox-18	0.669
NFA-10	NFA-10 / Bur-0	0.664
HR > HR-5	HR-5 / Bur-0	0.663
Biotic > G. orontii study 2 (Col-0)	G. orontii study 2 (Col-0) / untreated rosette leaf samples (Col-0)	0.661
Bur-0	Bur-0 / Col-0	0.652
Sha	Sha / Col-0	0.642
Bla > Bla-1	Bla-1 / Bla-1/Hh-0	0.641
Sha	Sha / Col-0	0.638
Ws	Ws / Col-0	0.63
Nutrient > Cs-137 (root)	Cs-137 (root) / untreated seedlings (root)	0.625
Hormone > BL/H3BO3 (6d)	BL/H3BO3 (6d) / untreated cell culture samples	0.623
Other > shift to pH 4.6 (12h)	shift to pH 4.6 (12h) / mock treated root samples (12h)	0.619
Fei-0	Fei-0 / Col-0	0.617
Chemical > MSB (6h)	MSB (6h) / mock treated rosette leaf samples (6h)	0.607
Te-0	Te-0 / Col-0	0.606
Other > shift to pH 4.6 (48h)	shift to pH 4.6 (48h) / mock treated root samples (48h)	0.602
SQ-8	SQ-8 / Ts-1	0.586
Biotic > *G. cichoracearum* study 2 (36h)	*G. cichoracearum* study 2 (36h) / non-infected whole rosette samples (Col-0)	0.552

Stress > hypoxia study 5 (2h)	hypoxia study 5 (2h) / 21% O2 treated root samples (2h)	0.509
Light intensity > moderate light / 32°C (60-120min)	moderate light / 32°C (60-120min) / moderate light / 21°C (60-120min)	0.488
Temperature > 17°C / 4SU (total RNA)	17°C / 4SU (total RNA) / 17°C (total RNA)	0.391
Light intensity > moderate light / 32°C (140-200min)	moderate light / 32°C (140-200min) / moderate light / 21°C (140-200min)	0.368
Stress > shift cold to freezing (Col-0)	shift cold to freezing (Col-0) / cold study 23 (Col-0)	0.341
Stress > oxidative study 2 (late)	oxidative study 2 (late) / untreated root samples (late)	0.323
Hormone > IAA study 10 (0.5h)	IAA study 10 (0.5h) / mock treated root samples (0.5h)	0.215
Stress > hypoxia	hypoxia / untreated seedling samples (low light)	-0.22
Stress > hypoxia study 3 (0.5h)	hypoxia study 3 (0.5h) / 21% O2 treated root samples (0.5h)	-0.393
Stress > hypoxia study 4 (0.5h)	hypoxia study 4 (0.5h) / 21% O2 treated root samples (0.5h)	-0.432
Hormone > IAA study 10 (8h)	IAA study 10 (8h) / mock treated root samples (8h)	-0.455
Hormone > IAA study 10 (12h)	IAA study 10 (12h) / mock treated root samples (12h)	-0.477
Biotic > P. parasitica (30h)	P. parasitica (30h) / non-infected root samples (Col-0)	-0.482
Nutrient > iron deficiency study 2 (intermediate)	iron deficiency study 2 (intermediate) / mock treated root samples	-0.535
Ull2-5	Ull2-5 / Bil-5	-0.548
Stress > cold study 5 (8°C)	cold study 5 (8°C) / 20°C treated rosette samples (78h)	-0.549
Biotic > *P. syringae* pv. tomato study 10 (DC3000)	*P. syringae* pv. tomato study 10 (DC3000) / mock inoculated leaf samples	-0.557
Stress > cold study 6 (Can)	cold study 6 (Can) / 20°C/18°C treated rosette samples (Can)	-0.57
Col > Col-2	Col-2 / Knox-18	-0.579

Photoperiod > shift SD to LD study 2 (5d)	shift SD to LD study 2 (5d) / short day shoot apex samples at 16°C (Ler)	-0.588
Temperature > shift 16°C to 22°C (Ler-0)	shift 16°C to 22°C (Ler-0) / 22°C grown Ler-0 plants (4h/20h)	-0.589
Bay-0 > Bay-0 parent	Bay-0 parent / C24	-0.593
Chemical > benzothiadiazole study 3 (Col-0)	benzothiadiazole study 3 (Col-0) / untreated (Col-0) plant samples	-0.599
Nutrient > iron deficiency (LZ4)	iron deficiency (LZ4) / mock treated root samples (LZ4)	-0.61
Other > pollen-pistil interaction (3.5 hap)	pollen-pistil interaction (3.5 hap) / unpollinated pistil samples	-0.619
Stress > submergence (7h)	submergence (7h) / root samples of Col-0 shifted to darkness (7h)	-0.622
Nutrient > P deficiency study 5 (24h)	P deficiency study 5 (24h) / mock treated root samples (24h)	-0.626
Chemical > Cd study 3 (Col-0)	Cd study 3 (Col-0) / untreated root samples (Col-0)	-0.629
Ag-0	Ag-0 / Eden-1	-0.63
Omo2-3	Omo2-3 / Bil-5	-0.639
Other > volatiles of herbivore-damaged *P. lunatus* (48h)	volatiles of herbivore-*damaged P. lunatus* (48h) / volatiles of healthy *P. lunatus* (48h)	-0.641
Light intensity > low light / 21°C (5-40min)	low light / 21°C (5-40min) / moderate light / 21°C (5-40min)	-0.645
Bl-1	Bl-1 / Fei-0	-0.646
Knox-18	Knox-18 / Ws-2	-0.651
Tsu-1	Tsu-1 / Kas-1	-0.654
Col > Col-2	Col-2 / Ler-1	-0.654
Ler > Ler-1	Ler-1 / HR-5	-0.655
Omo2-3	Omo2-3 / Eden-1	-0.655
Biotic > *S. sclerotiorum* (Col-0)	*S. sclerotiorum* (Col-0) / mock inoculated rosette leaf samples (Col-0)	-0.668
Col > Col-2	Col-2 / Ts-1	-0.678
Bay-0 > Bay-0 parent	Bay-0 parent / Fei-0	-0.694
Ag-0	Ag-0 / Eden-1	-0.701
Other > callus formation (48h)	callus formation (48h) / untreated root samples	-0.714

Other > IVT-E RNA labeling (root elongation zone)	IVT-E RNA labeling (root elongation zone) / one-cycle RNA labeling (root elongation zone)	-0.728
Elicitor > EF-Tu (elf18) study 4 (Col-0)	EF-Tu (elf18) study 4 (Col-0) / mock treated seedling samples (Col-0)	-0.729
Knox-18	Knox-18 / HR-5	-0.729
Hormone > salicylic acid study 6 (Est)	salicylic acid study 6 (Est) / silwet L77 treated Est leaf samples (52h)	-0.742
Tsu-1	Tsu-1 / Kas-1	-0.745
Tsu-1	Tsu-1 / Kas-1	-0.755
Elicitor > HrpZ (4h)	HrpZ (4h) / H2O treated leaf samples (4h)	-0.778
Ull2-5	Ull2-5 / Eden-1	-0.781
Chemical > benzyladenine	benzyladenine / untreated seedlings	-0.786
Stress > cold study 17 (C24)	cold study 17 (C24) / untreated C24 rosette leaf samples	-0.792
Light intensity > dark study 6 (polys. RNA)	dark study 6 (polys. RNA) / dark study 6 (tot. RNA)	-0.793
Col > Col-2	Col-2 / HR-5	-0.799
Col > Col-2	Col-2 / NFA-10	-0.799
Hormone > MeJa study 5 (Ler)	MeJa study 5 (Ler) / untreated leaf disc samples (Ler)	-0.823
Bay-0 > Bay-0 parent	Bay-0 parent / Fei-0	-0.837
Chemical > rotenone (12h)	rotenone (12h) / solvent treated cell culture samples (12h)	-0.858
Bay-0 > Bay-0 parent	Bay-0 parent / Sha	-0.863
Omo2-3	Omo2-3 / Eden-1	-0.871
Biotic > *P. syringae* pv. tomato study 10 (DC3000 hrpAfliC)	*P. syringae* pv. tomato study 10 (DC3000 hrpAfliC) / mock inoculated leaf samples	-0.878
Photoperiod > shift SD to LD study 2 (9d)	shift SD to LD study 2 (9d) / short day shoot apex samples at 16°C (Ler)	-0.893
Stress > cold study 10 (7d)	cold study 10 (7d) / untreated seedling samples (soil)	-0.893
Stress > drought study 12 (Bur-0)	drought study 12 (Bur-0) / mock treated Bur-0 rosette leaf samples	-0.9
Other > decapitation	decapitation / axillary bud samples (Col-0)	-0.904

Te-0	Te-0 / Rsch-0	-0.905
Other > stratification (48h)	stratification (48h) / seed desiccation	-0.906
Stress > heat (green)	heat (green) / untreated green tissue samples (early)	-0.912
Stress > drought study 13 (Got-22)	drought study 13 (Got-22) / mock treated Got-22 rosette leaf samples	-0.93
Col > Col-2	Col-2 / CIBC-17	-0.948
Ler > Ler-1	Ler-1 / SQ-8	-0.952
Bay-0 > Bay-0 parent	Bay-0 parent / Bur-0	-0.964
Biotic > *S. sclerotiorum* study 2 (Col-0)	*S. sclerotiorum* study 2 (Col-0) / mock inoculated rosette leaf samples (Col-0)	-0.991
Bay-0 > Bay-0 parent	Bay-0 parent / Fei-0	-0.994
Te-0	Te-0 / Rsch-0	-1.003
Other > germination (1h)	germination (1h) / stratification (48h)	-1.003
NFA-10	NFA-10 / Ws-2	-1.004
HR > HR-5	HR-5 / Ws-2	-1.005
Nutrient > iron deficiency study 14 (Col-0)	iron deficiency study 14 (Col-0) / untreated Col-0 root samples	-1.024
Chemical > norflurazon	norflurazon / untreated seedlings	-1.033
Biotic > P. syringae pv. syringae study 2 (Col-0)	P. syringae pv. syringae study 2 (Col-0) / P. syringae pv. syringae (Col-0)	-1.054
Chemical > syringolin study 4 (early)	syringolin study 4 (early) / solvent treated leaf samples (Col-0; early inf.)	-1.056
Photoperiod > shift SD to LD study 8 (Col-0)	shift SD to LD study 8 (Col-0) / short day study 4 (Col-0)	-1.063
Biotic > *P. syringae* pv. tomato study 10 (DC3000 hrpA)	*P. syringae* pv. tomato study 10 (DC3000 hrpA) / mock inoculated leaf samples	-1.068
Other > callus formation study 2 (96h)	callus formation study 2 (96h) / untreated shoot samples	-1.073
Biotic > *P. infestans* (24h)	*P. infestans* (24h) / mock treated leaf samples (24h)	-1.092
Nutrient > sucrose study 6 (dark)	sucrose study 6 (dark) / dark study 13	-1.11
Col > Col-2	Col-2 / Ts-1	-1.123
Ts-1	Ts-1 / Ws-2	-1.126

Chemical > sulfamethoxazole + sucrose (dark)	sulfamethoxazole + sucrose (dark) / dark study 13	-1.135
Biotic > *E. coli* (O157:H7)	*E. coli* (O157:H7) / mock inoculated leaf samples	-1.135
Te-0	Te-0 / Rsch-0	-1.149
Col > Col-2	Col-2 / Bur-0	-1.152
Stress > cold (early)	cold (early) / untreated green tissue samples (early)	-1.162
Nutrient > mannitol (4h)	mannitol (4h) / untreated seedlings	-1.173
Hormone > ABA study 3 (Col-0)	ABA study 3 (Col-0) / untreated seed samples	-1.229
Col > Col-2	Col-2 / Ws-2	-1.231
Got-22	Got-22 / Bil-5	-1.243
Col > Col-2	Col-2 / SQ-8	-1.248
Chemical > lincomycin (Col-0)	lincomycin (Col-0) / untreated seedling samples (Col-0)	-1.254
Col > Col-2	Col-2 / SQ-8	-1.264
Nutrient > nitrate starvation	nitrate starvation / untreated seedlings	-1.29
Col > Col-2	Col-2 / HR-5	-1.309
Nutrient > mannitol (2h)	mannitol (2h) / untreated seedlings	-1.312
Light quality > shift lincomycin+R+B 0.5µmol m-2 s-1 to lincomycin+R+B 60µmol m-2 s-1 (24h)	shift lincomycin+R+B 0.5µmol m-2 s-1 to lincomycin+R+B 60µmol m-2 s-1 (24h) / shift R+B 0.5µmol m-2 s-1 to R+B 60µmol m-2 s-1 (24h)	-1.334
Nutrient > iron deficiency study 2 (late)	iron deficiency study 2 (late) / mock treated root samples	-1.335
Light intensity > shift dark to light (polys. RNA)	shift dark to light (polys. RNA) / shift dark to light (tot. RNA)	-1.335
Col > Col-2	Col-2 / NFA-10	-1.349
Chemical > imazapyr (24h)	imazapyr (24h) / mock treated leaf samples (24h)	-1.35
Knox-18	Knox-18 / Ws-2	-1.384
Chemical > 5-AC	5-AC / solvent treated seedling samples (Ws)	-1.396
Photoperiod > circadian clock study 9 (5h dark+1h light)	circadian clock study 9 (5h dark+1h light) / circadian clock study 9 (6h dark)	-1.455
Hormone > salicylic acid study 5 (Cvi-1)	salicylic acid study 5 (Cvi-1) / silwet L77 treated Cvi-1 leaf samples (28h)	-1.457

CIBC-17	CIBC-17 / Ws-2	-1.467
Got-22	Got-22 / Eden-1	-1.476
Hormone > salicylic acid study 5 (Est)	salicylic acid study 5 (Est) / silwet L77 treated Est leaf samples (28h)	-1.485
Ler > Ler-1	Ler-1 / Ws-2	-1.492
Chemical > phenanthrene	phenanthrene / untreated Col plant samples	-1.53
Hormone > NAA study 2 (5d)	NAA study 2 (5d) / untreated inflorescence stem internode cell samples	-1.543
Light quality > lincomycin+R+B study 2 (0.5µmol m-2 s-1)	lincomycin+R+B study 2 (0.5µmol m-2 s-1) / R+B study 2 (0.5µmol m-2 s-1)	-1.582
Stress > hypoxia study 6 (Col-0)	hypoxia study 6 (Col-0) / untreated plant samples (Col-0)	-1.617
Stress > drought study 5 (pre-dawn)	drought study 5 (pre-dawn) / untreated Col-0 rosette samples (pre-dawn)	-1.622
Biotic > *P. infestans* (12h)	*P. infestans* (12h) / mock treated leaf samples (12h)	-1.643
Stress > cold study 10 (1h)	cold study 10 (1h) / untreated seedling samples (soil)	-1.646
Other > callus formation study 3 (35d + 1d)	callus formation study 3 (35d + 1d) / untreated hypocotyl samples (35d)	-1.648
Chemical > sulfometuron methyl (24h)	sulfometuron methyl (24h) / mock treated leaf samples (24h)	-1.655
Bur-0	Bur-0 / Ws-2	-1.669
Hormone > salicylic acid study 3	salicylic acid study 3 / mock treated seedlings	-1.687
Stress > drought study 18 (Col-0)	drought study 18 (Col-0) / untreated rosette leaf samples (Col-0)	-1.727
Other > shift to pH 4.6 / protoplasting	shift to pH 4.6 / protoplasting / shift to pH 4.6 (24h)	-1.73
Biotic > CaLCuV	CaLCuV / non-infected rosette leaf samples	-1.754
Other > callus formation study 3 (7d + 1d)	callus formation study 3 (7d + 1d) / untreated hypocotyl samples (7d)	-1.79
Col > Col-2	Col-2 / Ws-2	-1.804
Biotic > *R. solani* (AG2-1)	*R. solani* (AG2-1) / mock inoculated whole plant samples	-1.862

Other > germination (1h)	germination (1h) / seed desiccation	-1.909
Other > germination (6h)	germination (6h) / stratification (48h)	-1.913
Nutrient > sucrose study 3 (Col-7)	sucrose study 3 (Col-7) / untreated seedlings (Col-7)	-1.93
Nutrient > glucose (4h)	glucose (4h) / untreated seedlings	-1.964
Other > germination (24h)	germination (24h) / stratification (48h)	-1.993
Other > germination (48h)	germination (48h) / stratification (48h)	-2.028
Stress > salt study 4 (Ws)	salt study 4 (Ws) / Hoagland solution watered Ws leaf samples	-2.046
Light quality > shift lincomycin+R+B 0.5µmol m-2 s-1 to lincomycin+R+B 60µmol m-2 s-1 (4h)	shift lincomycin+R+B 0.5µmol m-2 s-1 to lincomycin+R+B 60µmol m-2 s-1 (4h) / shift R+B 0.5µmol m-2 s-1 to R+B 60µmol m-2 s-1 (4h)	-2.096
Chemical > syringolin study 3 (early)	syringolin study 3 (early) / solvent treated leaf samples (Col-0; early)	-2.208
Photoperiod > circadian clock study 9 (18h dark)	circadian clock study 9 (18h dark) / circadian clock study 9 (6h dark)	-2.512
Other > germination (12h)	germination (12h) / stratification (48h)	-2.659
Other > germination (6h)	germination (6h) / seed desiccation	-2.819
Other > germination (24h)	germination (24h) / seed desiccation	-2.898
Stress > salt study 4 (Col-0)	salt study 4 (Col-0) / Hoagland solution watered Col-0 leaf samples	-2.902
Other > germination (48h)	germination (48h) / seed desiccation	-2.934
Chemical > norflurazon study 2 (Col-0)	norflurazon study 2 (Col-0) / untreated seedlings (Col-0)	-2.996
Light quality > lincomycin+R+B (0.5µmol m-2 s-1)	lincomycin+R+B (0.5µmol m-2 s-1) / R+B (0.5µmol m-2 s-1)	-3.162
Light quality > shift lincomycin+R+B 0.5µmol m-2 s-1 to lincomycin+R+B 60µmol m-2 s-1 (0.5h)	shift lincomycin+R+B 0.5µmol m-2 s-1 to lincomycin+R+B 60µmol m-2 s-1 (0.5h) / shift R+B 0.5µmol m-2 s-1 to R+B 60µmol m-2 s-1 (0.5h)	-3.313
Chemical > mefenpyr + isoxadifen (Col-0)	mefenpyr + isoxadifen (Col-0) / mock treated rosette leaf samples (Col-0)	-3.484
Other > germination (12h)	germination (12h) / seed desiccation	-3.565

Light quality > shift lincomycin+R+B 0.5µmol m-2 s-1 to lincomycin+R+B 60µmol m-2 s-1 (1h)	shift lincomycin+R+B 0.5µmol m-2 s-1 to lincomycin+R+B 60µmol m-2 s-1 (1h) / shift R+B 0.5µmol m-2 s-1 to R+B 60µmol m-2 s-1 (1h)	-4.046

Appendix C

Hormone levels in Col-0, *cas* SALK and *cas* GABI lines upon heating

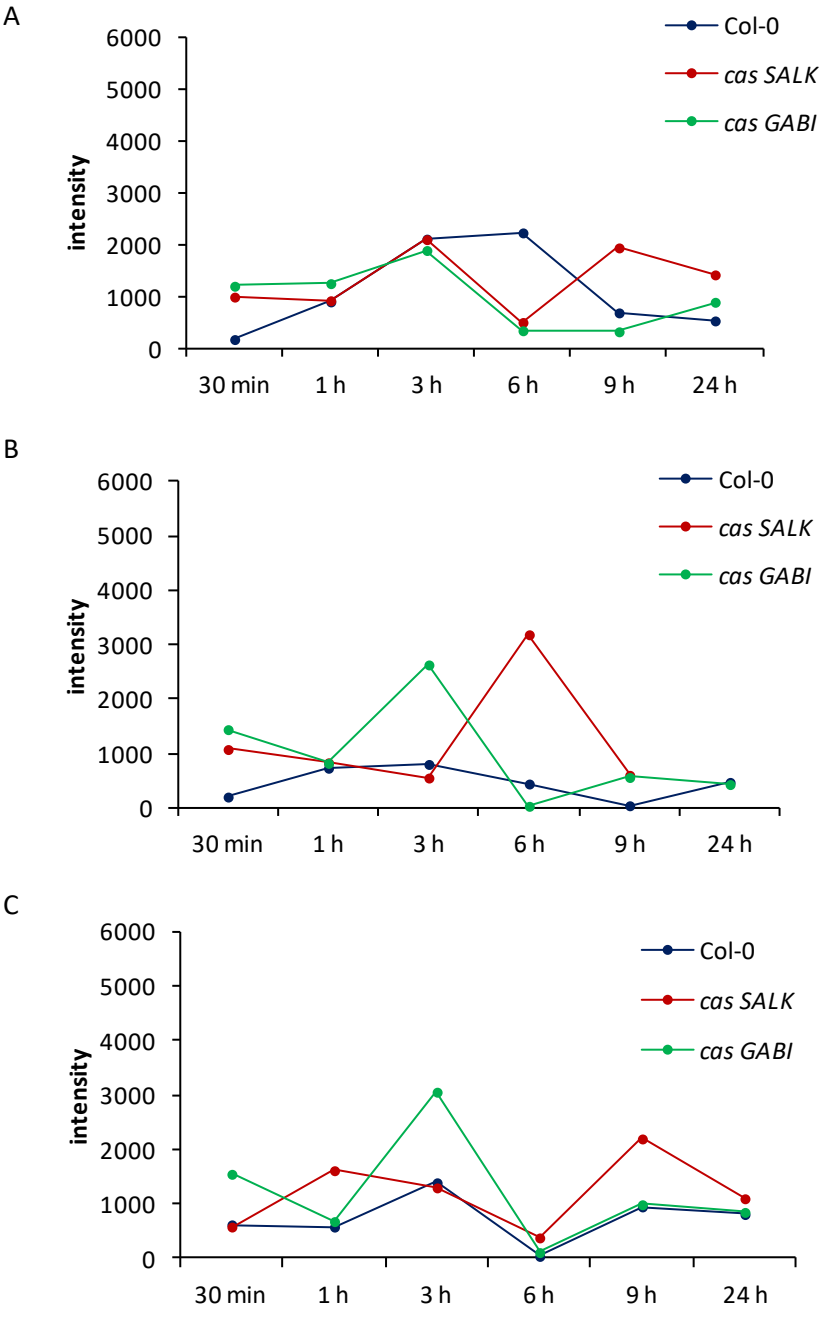

Figure C.1 Intensity levels of IAA peaks in a MS analysis. The three genotypes Col-0, *cas SALK* and *cas GABI* were analysed ad 30 min, 1 h, 3 h, 6 h, 12 h and 24 h in response to (A) 20°C (B) 35°C or (C) 40°C.

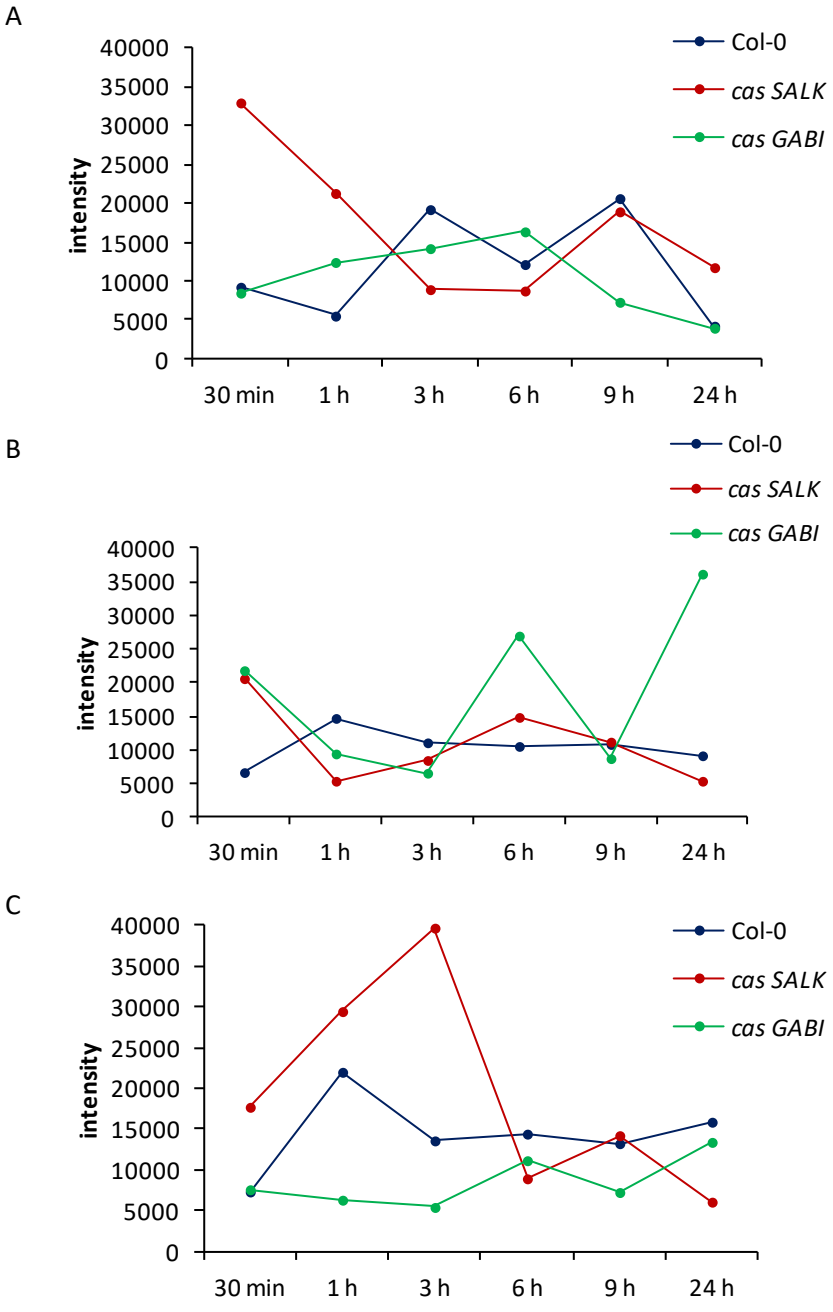

Figure C.2 Intensity levels of JA peaks in a MS analysis. The three genotypes Col-0, *cas SALK* and *cas GABI* were analysed ad 30 min, 1 h, 3 h, 6 h, 12 h and 24 h in response to (A) 20°C (B) 35°C or (C) 40°C.

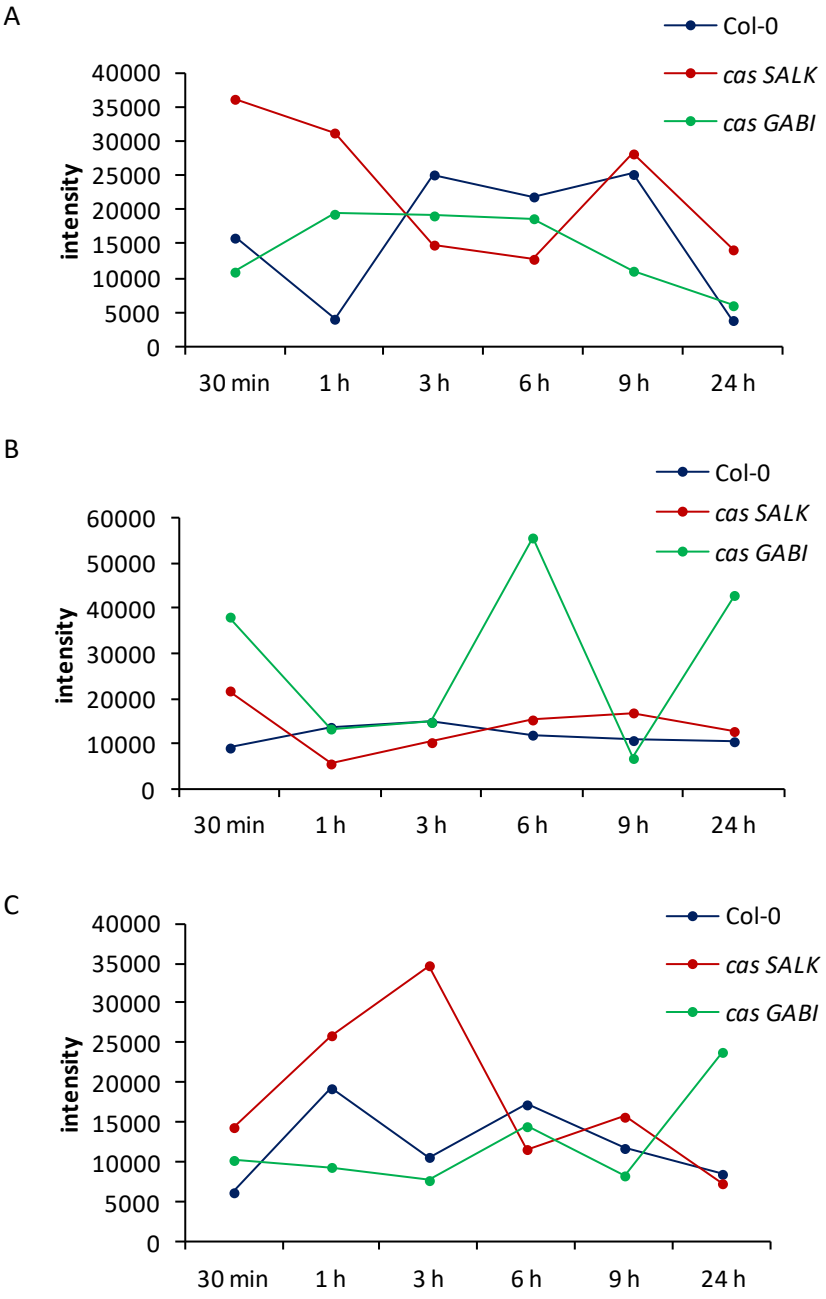

Figure C.3 Intensity levels of JA-Ile peaks in a MS analysis. The three genotypes Col-0, *cas SALK* and *cas GABI* were analysed ad 30 min, 1 h, 3 h, 6 h, 12 h and 24 h in response to (A) 20°C (B) 35°C or (C) 40°C.

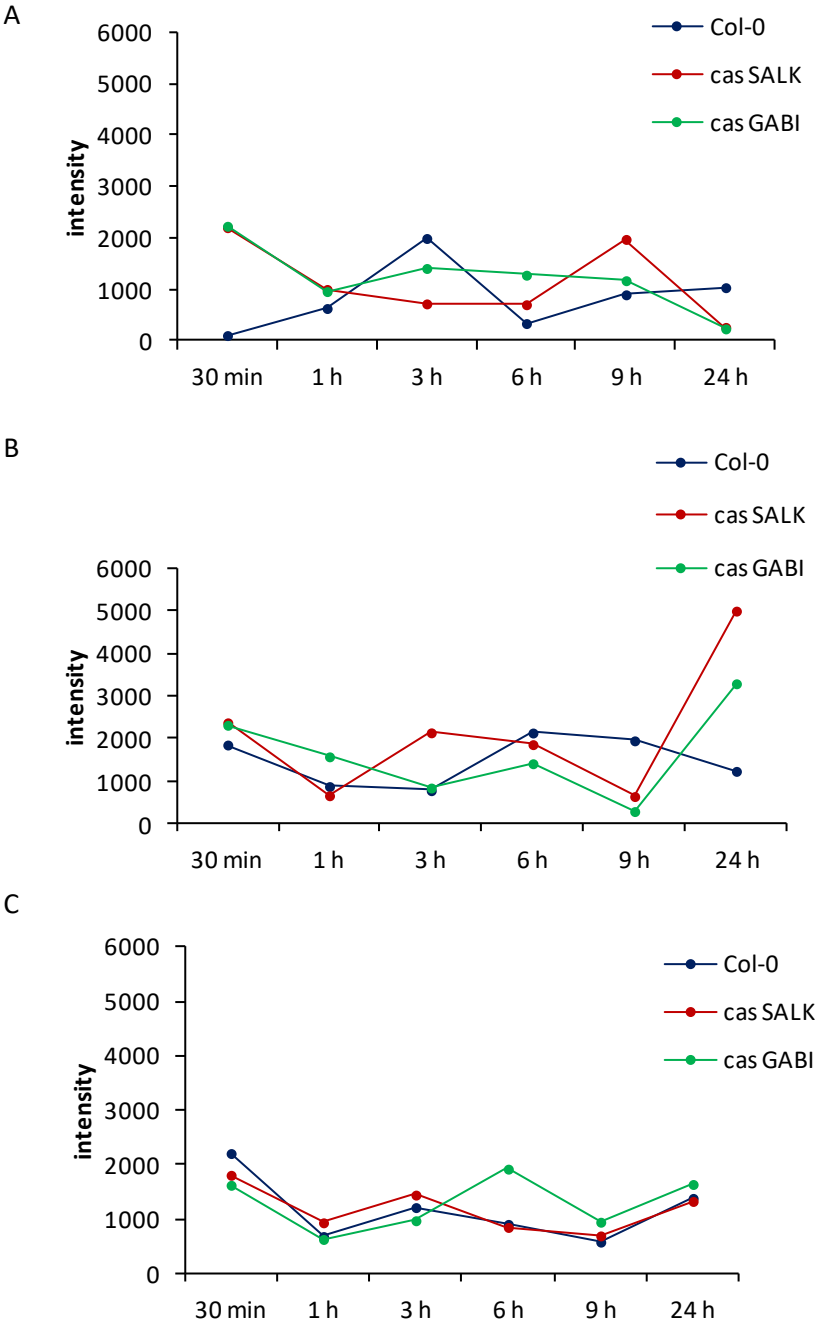

Figure C.4 Intensity levels of GA3 peaks in a MS analysis. The three genotypes Col-0, *cas SALK* and *cas GABI* were analysed ad 30 min, 1 h, 3 h, 6 h, 12 h and 24 h in response to (A) 20°C (B) 35°C or (C) 40°C

Appendix D

Gene expression of calcium-regulated heat-induced genes in control and TRPV1 expressing lines

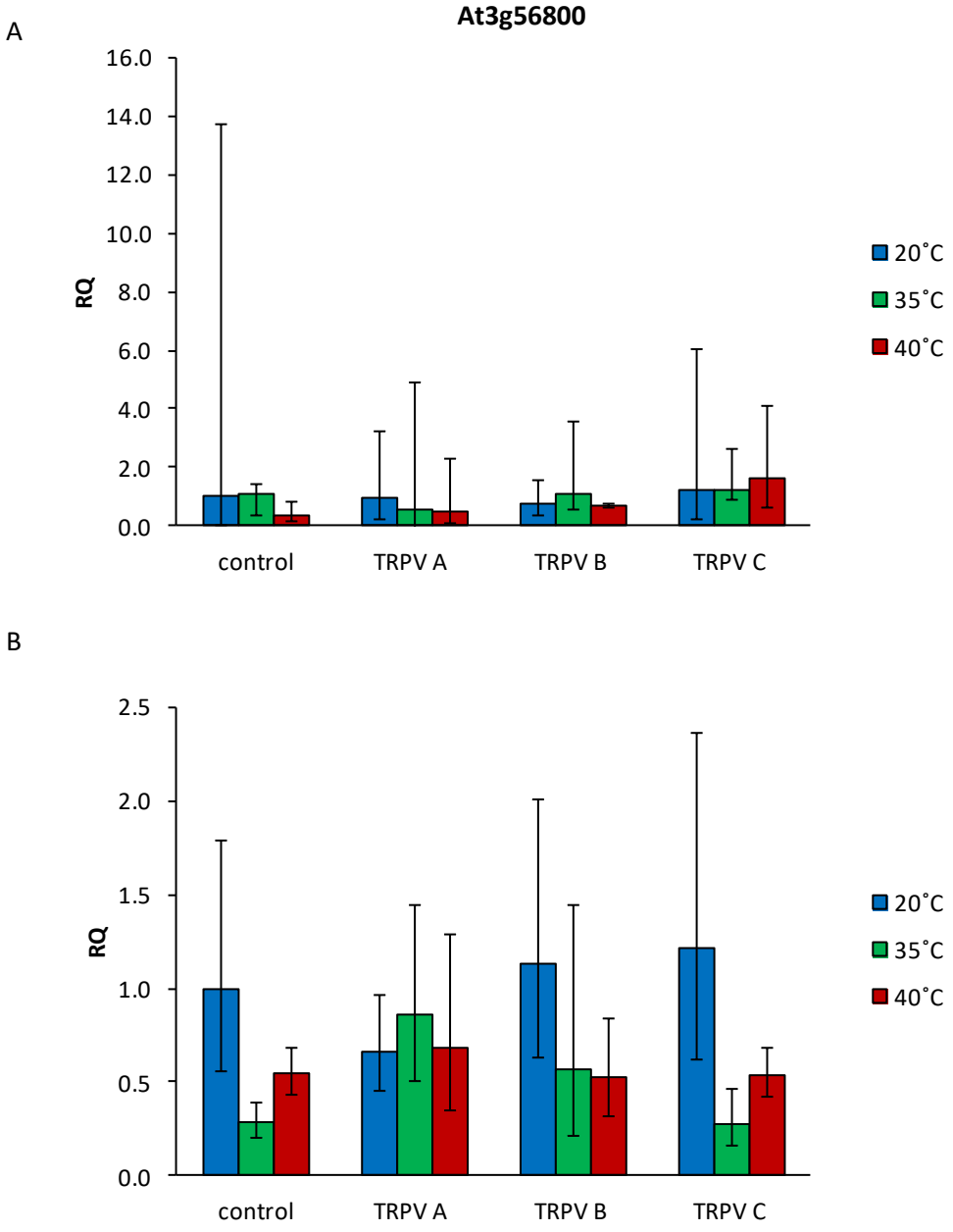

Figure D.1 Expression levels of *CAM3*(At3g56800) at 20^{o}C, 35^{o}C and 40^{o}C for 30 min (A) 90 min (B) in TRPV1 A, TRPV B, TRPV C and control line. Error bars = SE.

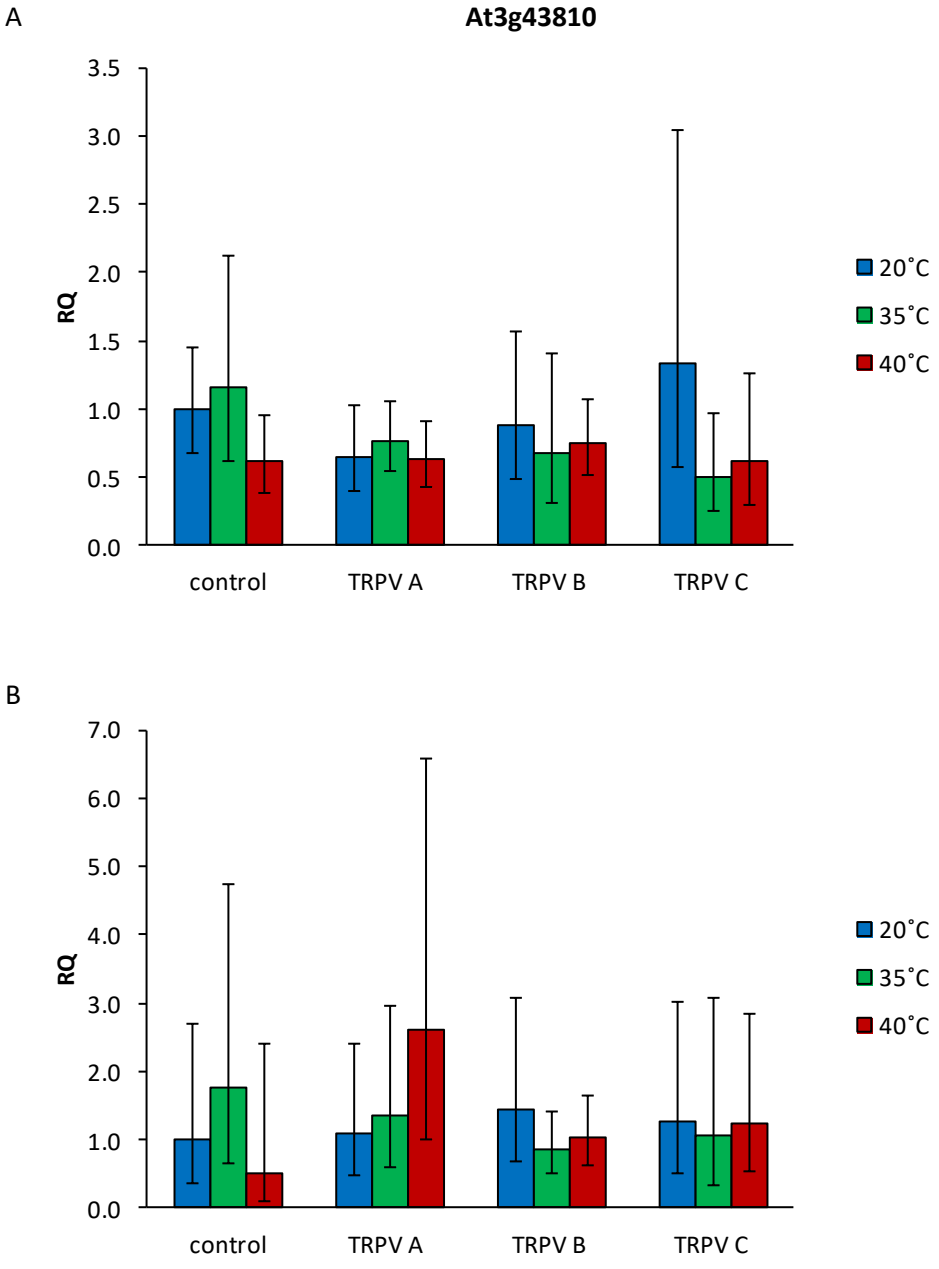

Figure D.2 Expression levels of *CAM7*(At3g43810) at 20^{o}C, 35^{o}C and 40^{o}C for 30 min (A) 90 min (B) in TRPV1 A, TRPV B, TRPV C and control line. Error bars = SE.

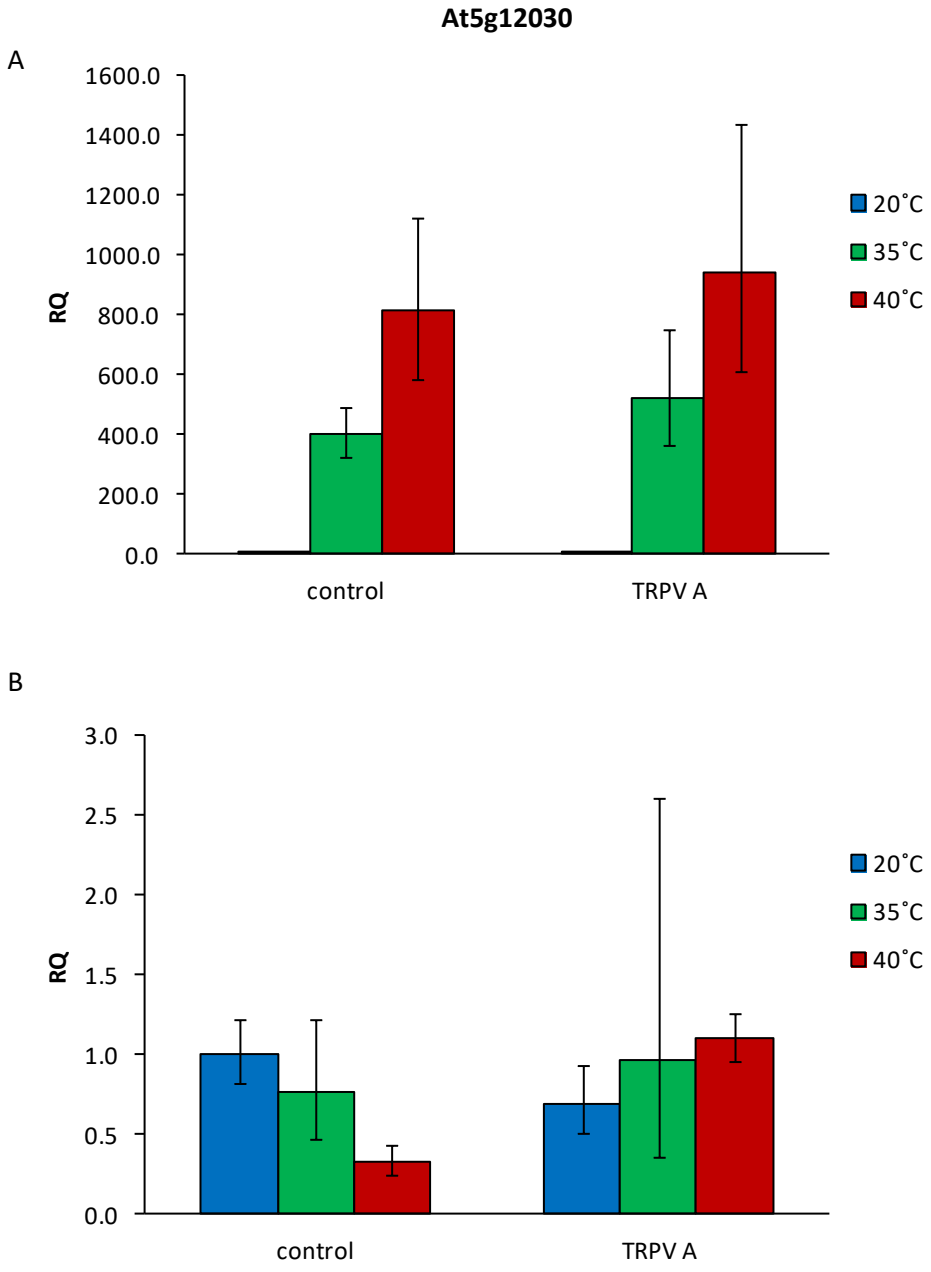

Figure D.3 Expression levels of *HSP17.6AII* (At5g12030)at 20°C, 35°C and 40°C for 30 min (A) or 90 min (B) in TRPV1 A and control line. Error bars = SE.

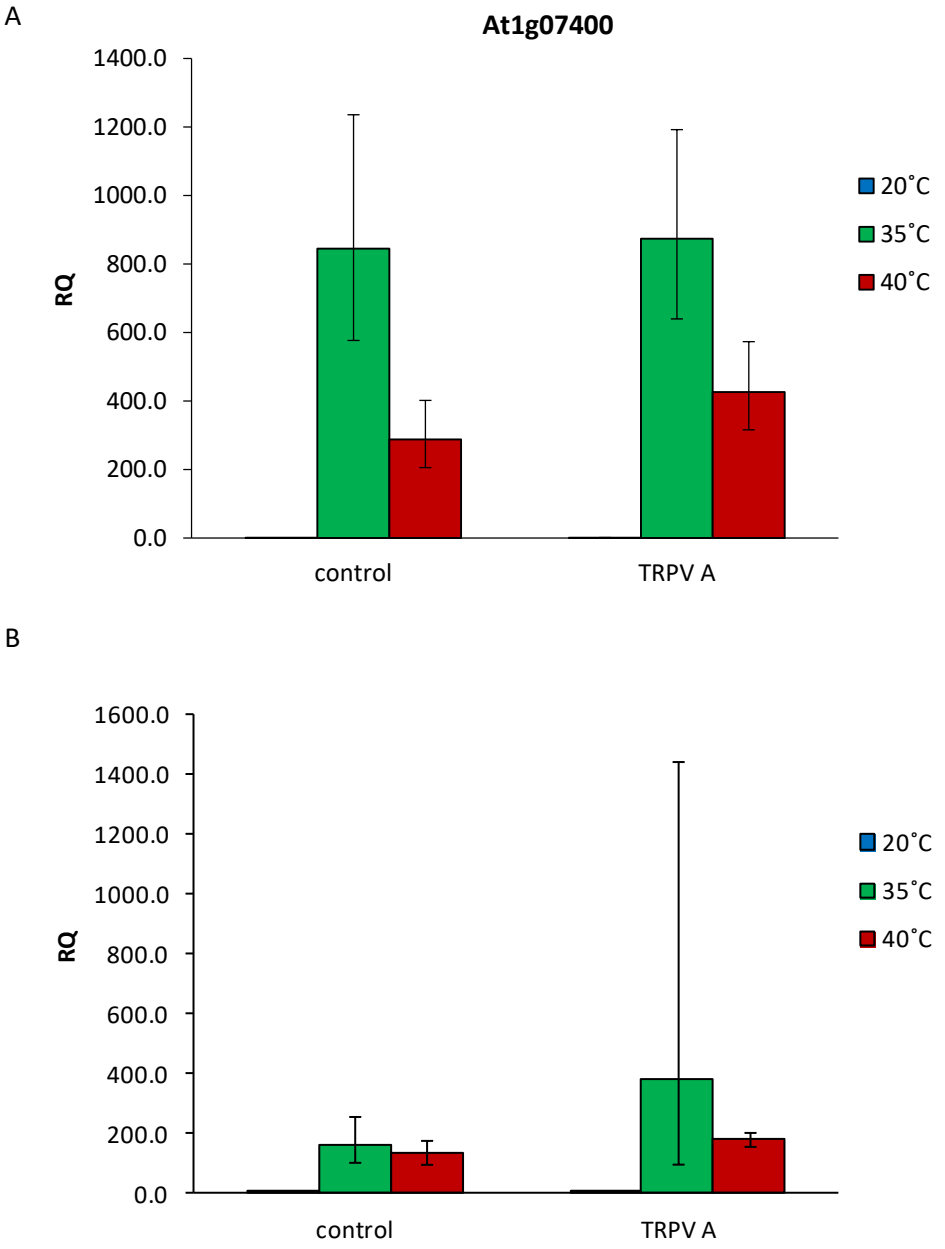

Figure D.4 Expression levels of *HSP17.8* (At1g07400) at 20°C, 35°C and 40°C for 30 min (A) or 90 min (B) in TRPV1 A and control line. Error bars = SE.

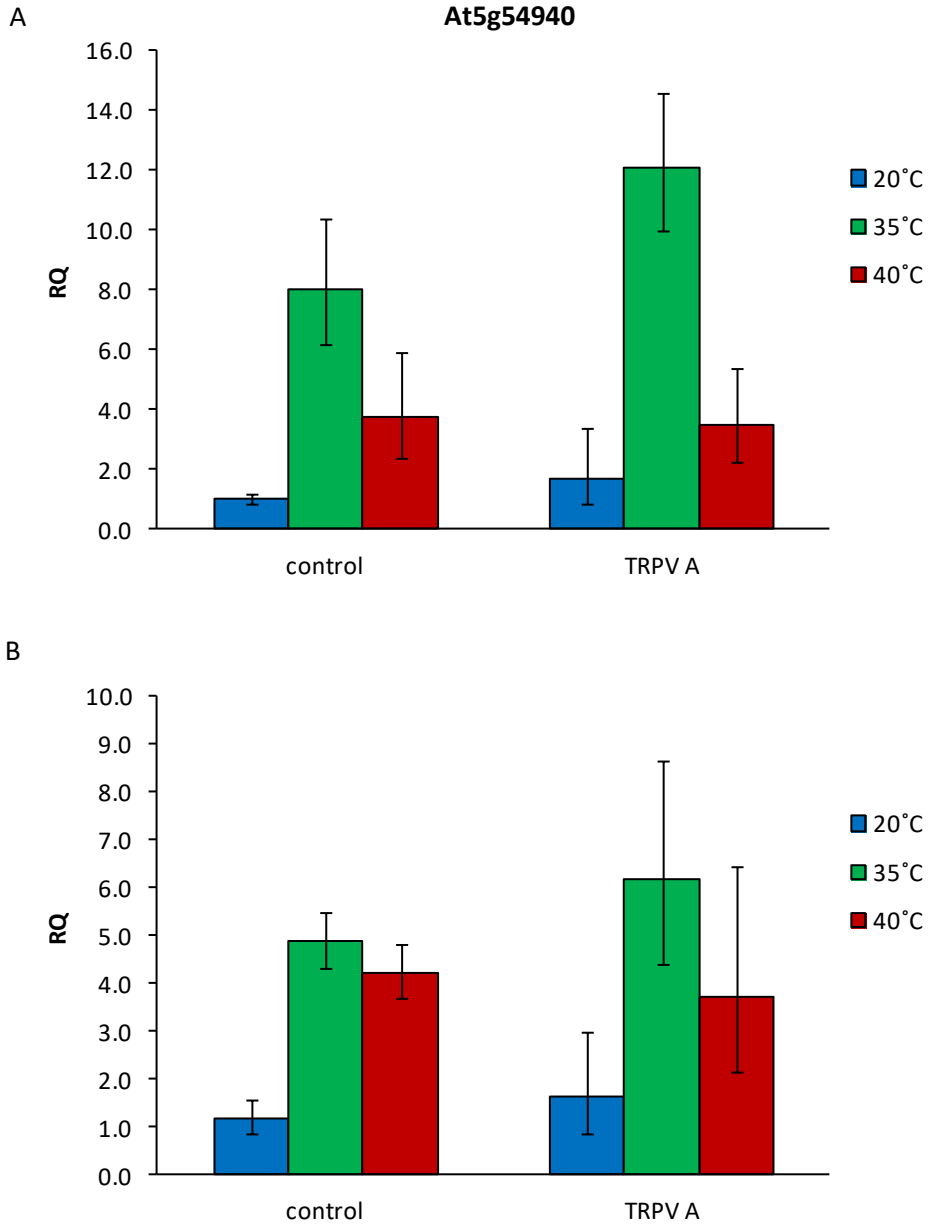

Figure D.5 Expression levels of *SUI1* (At5g54940) at 20°C, 35°C and 40°C for 30 min (A) or 90 min (B) in TRPV1 A and control line. Error bars = SE.

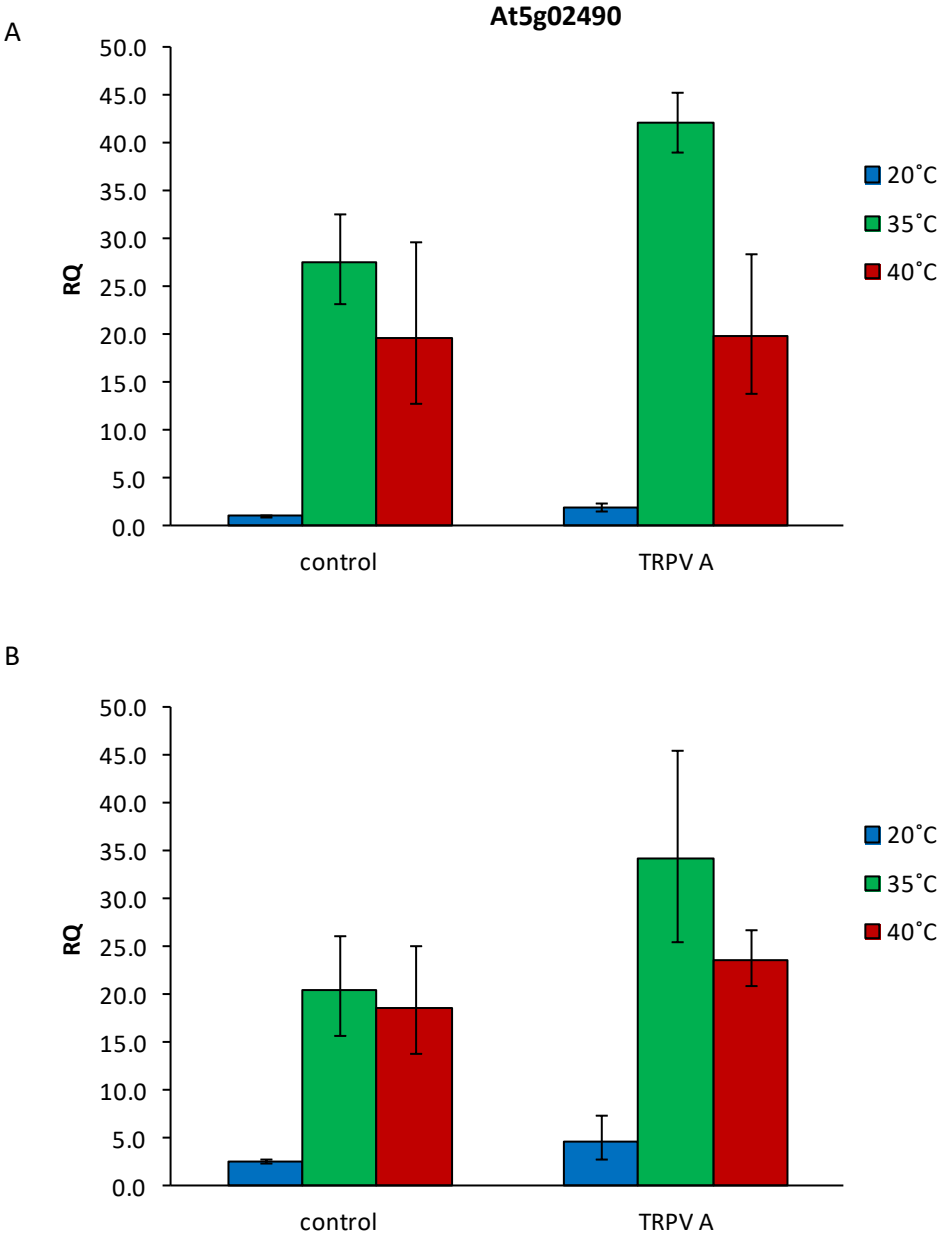

Figure D.6 Expression levels of *HSP70.2* (At5g02490) at 20°C, 35°C and 40°C for 30 min (A) or 90 min (B) in TRPV1 A and control line. Error bars = SE.