Title: Increases in absolute temperature stimulate free calcium concentration elevations in the chloroplast

Running head: Heat increases chloroplast calcium concentration

Corresponding authors (joint):

- M.R. Knight, Department of Biosciences, Durham University, South Road, Durham DH1 3LE, UK. Tel: +44 191 334 1224; Fax: +44 191 334 1224; Email: m.r.knight@durham.ac.uk
- G. Lenzoni, School of Pharmaceutical Sciences, University of Geneva, Rue Michel-Servet 1, CH-1211 Geneva, Switzerland.

Subject area: environmental and stress responses

Number of black and white figures: 2
Number of colour figures: 4
Number of tables: 0
Type and number of supplementary material: 3 supplementary figures

# Increases in absolute temperature stimulate free calcium concentration elevations in the chloroplast

Running head: Heat increases chloroplast calcium concentration

Gioia Lenzoni[1]* and Marc R. Knight*

*Joint corresponding authors, m.r.knight@durham.ac.uk, Gioia.Lenzoni@unige.ch

Abbreviations:

$[Ca^{2+}]_{chl}$ – chloroplastic calcium concentration

$[Ca^{2+}]_{cyt}$ – cytosolic calcium concentration

BA – benzyl alcohol

$Ca^{2+}$ - calcium

chl - chloroplastic

CNGCs - cyclic nucleotide-gated channels

Col-0 – Columbia-0

cyt - cytosolic

HSR – heat shock response

n – number

SE – standard error

Ws-0 – Wassilewskija-0

wt – wild type

Footnotes: [1]Current address: School of Pharmaceutical Sciences, University of Geneva, Rue Michel-Servet 1, CH-1211 Geneva, Switzerland.

**Abstract**

Plants need to sense increases in temperature to be able to adapt their physiology and development to survive; however, the mechanisms of heat perception are currently relatively poorly understood. Here we demonstrate that in response to elevated temperature the free calcium concentration of the stroma of chloroplasts increases. This response is specific to the chloroplast, as no corresponding increase in calcium is seen in the cytosol. The chloroplast calcium response is dose-dependent above a threshold. The magnitude of this calcium response is dependent upon absolute temperature, not rate of heating. This response is dynamic: repeated stimulation leads to rapid attenuation of the response, which can be overcome by sensitisation at a higher temperature. More long-term acclimation to different temperatures resets the basal sensitivity of the system, such that plants acclimated to lower temperatures are more sensitive than those acclimated to higher temperatures. The heat-induced chloroplast calcium response was partially dependent upon the calcium-sensing receptor CAS which has been shown previously to regulate other chloroplast calcium signalling responses. Taken together our data demonstrate the ability of chloroplasts to sense absolute high temperature and produce commensurately quantitative stromal calcium response, the magnitude of which is a function of both current temperature and stress history.

**Keywords**

Arabidopsis, calcium, CAS, chloroplast, heat stress, temperature.

## Introduction

Temperature is one of the key environmental parameters affecting all living organisms. Fluctuations in temperature occur seasonally, daily as well as more 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 (Knight and Knight 2012; Mittler et al. 2012; Ruelland and Zachowski 2010; Saidi et al. 2011). 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 (Hua 2009; Knight and Knight 2012; Penfield 2008; Thomashow 2010). 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 thermometers for heat and cold have not been yet identified. Amongst the 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. Knight and Knight 2012; Mittler et al. 2012; Ruelland and Zachowski 2010; Saidi et al. 2011). Membrane rigidification/fluidisation occur nearly concomitantly with the temperature variation, hence these events are likely 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 (Falcone et al. 2004; Murata and Los 1997).

A widely studied plant second messenger is calcium ($Ca^{2+}$), which is involved in nearly every aspect of cell physiology and development (Batistic and Kudla 2012; Kudla et al. 2010; Kudla et al. 2018). Alterations in the cytosolic calcium concentration ($[Ca^{2+}]_{cyt}$) have been reported in response to a variety of environmental stimuli (e.g. cold, pathogens etc.) and they are considered crucial early events in stress response pathways (Batistic and Kudla 2012; Kudla et al. 2018; Sanders et al. 1999). Specific information regarding the nature and the magnitude of the stress is achieved by using

different spatio-temporal calcium elevations, called "$Ca^{2+}$ signatures" (McAinsh and Hetherington 1998), which differ in parameters such as amplitude, duration, frequency, and sub-location of the calcium increase (Allen et al. 2001; Miwa et al. 2006; Whalley and Knight 2013).

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. 1996; Knight et al. 1991), 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 (Finka and Goloubinoff 2014; Saidi et al. 2009; Saidi et al. 2010).

Recently, attention has been focused on understanding calcium signalling in the chloroplast. This organelle not only functions as a calcium store (Costa et al. 2018; Nomura and Shiina 2014; Roh et al. 1998; Stael et al. 2012b), but also has the ability to generate its own specific $Ca^{2+}$ signals in response to stresses, hence to contribute to downstream signalling responses (Johnson et al. 1995; Kmiecik et al. 2016; Loro et al. 2016; Manzoor et al. 2012; Nomura et al. 2012; Sai and Johnson 2002; Sello et al. 2018; Sello et al. 2016). Calcium plays both a regulatory and structural role in the chloroplast (Sello et al. 2016; Stael et al. 2012b). Importantly, $Ca^{2+}$ is required for photosystem II (PSII) assembly, photoprotection and recovery after photoinhibition (Grove and Brudvig 1998; Mattoo et al. 1989; Miller and Brudvig 1989; Yang et al. 2015), but high calcium levels are able to inhibit photosynthesis, by acting on the Calvin-Benson cycle (Charles and Halliwell 1980; Kreimer et al. 1988). Furthermore, PSII has been recently identified as the site of action of the small chloroplast-localised heat shock protein 21 (Chen et al. 2017). 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 (Nomura and Shiina 2014; Stael et al. 2012b). Chloroplast calcium increases have been reported in response to cold, salt and hyperosmotic stresses (Nomura et al. 2012; Sello et al. 2016) as well as pathogen elicitor molecules (Manzoor et al. 2012; Nomura et al. 2012; Sello et al. 2016), with different kinetics compared to the cytosolic calcium counterparts. In the case of response to elicitors and the light-dark transition, the chloroplast-localised calcium sensing receptor CAS (Han et al. 2003; Nomura et al. 2008; Stael et al. 2012a; Vainonen et al. 2008; Wang et al. 2012) has been shown to be necessary for the full chloroplast calcium response

(Nomura et al. 2012). In the case of response to elicitors, the attenuation of chloroplast calcium response lead to reduced pathogen-related gene expression (Nomura et al. 2012). All the primary stimuli tested to date, apart from light-to-dark transition increase both cytosolic and chloroplast calcium. To date, no other chloroplast-specific (i.e. not cytosolic calcium-inducing) stimuli have been identified. In this paper we report a second instance of a chloroplast-specific calcium increase, which occurs in response to heat. We describe its characteristics and discuss the significance of this ability of chloroplasts to sense increases in temperature.

## Results

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

To examine the role of calcium in chloroplast signalling, we 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 (Fig. 1). *Arabidopsis* seedlings were heated on a Peltier element positioned under a photon counting camera and calcium-dependent luminescence was collected before and during the heating event. As shown in Fig. 1, *Arabidopsis* seedlings were kept at 20$^{o}$C for 2 minutes and then heated at 40$^{o}$C for 7 minutes before dropping the temperature back to 20$^{o}$C. The 40$^{o}$C 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 Fig. 1, the temperature drop from 40$^{o}$C to 20$^{o}$C 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). This cold response was also detected in the chloroplast, leading to the modest increase in stromal calcium previously reported (Nomura et al. 2012).

In order to test the dose-dependency of the calcium heat response, a series of temperatures was applied to *Arabidopsis* seedlings, ranging from mild heat (30$^{o}$C) to just sub-lethal temperatures (45$^{o}$C), with an interval of 2.5$^{o}$C. Each temperature above 30$^{o}$C caused a stromal calcium increase (Fig. 2A), and the kinetics of the calcium were dependent on the temperature sensed, in a dose-dependent manner. For instance, the peak height increased linearly with increasing temperature (Fig. 2B). Conversely, peak time decreased with increasing temperature following a logarithmic relationship (Fig. 2C). Statistical significance of each temperature compared to another is represented in Fig. 2D

which shows peak height and Fig. 2E which shows time at which peak occurs. Interestingly, giving plants a 30°C heat stimulus did not cause a stromal calcium increase, defining this temperature as the threshold for the chloroplast calcium heat response under these conditions. Cytosolic calcium increases were monitored for each of the temperatures tested in Fig. 2A, and results are reported in Fig. S1, as showing little or no increase.

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. Supplementary Fig. S2 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 Fig. 2A with Fig. S2). The calcium heat response is also conserved amongst the *Arabidopsis* ecotypes Col-0 and Ws-0, whose traces are almost identical (Fig. S3).

**Characteristics of the chloroplast heat response: attenuation and sensitisation**

Attenuation is a property observed when an organism is repeatedly exposed to a stimulus of the same magnitude within a relatively short time: the size of the response decreasing each time as a consequence of the previous experience. This was found to be the case for the chloroplast heat response; where seedlings were consecutively exposed to 4 minutes 40°C heat pulses every 5 minutes they showed a reduced calcium response upon each subsequent stimulation (Fig. 3A). A stimulation at 45°C following such three 40°C heat pulses was able to re-establish the stromal calcium increase, and the magnitude of this elevation was significantly greater than the one recorded upon the first 40°C heating pulse (Fig. 3A and Fig. 3B). This property is known as sensitisation and it was able to overcome attenuation.

**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 20°C to 40°C at rates of either 0.4, 0.2, 0.15 or 0.1 °C/s. In Fig. 4A 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 is no correlation between the rate of heating and the magnitude of the calcium peak. These data indicated that the magnitude of response could be fundamentally dependent upon

either absolute temperature, or upon the absolute change in temperature (ΔT) that the plant experienced. To discriminate between these two cases, *Arabidopsis* plants were heated up to 40°C starting from different initial temperatures namely, 15°C, 20°C and 25°C (Fig. 4B). In this case the ΔT varies (25°C, 20°C and 15°C, respectively), but the final absolute temperature (40°C) remains the same. Fig. 4B shows that the calcium peak values were similar in response to the different ΔT (the horizontal regression line indicates no correlation, $R^2$=0.0921). On the other hand, when plants were exposed to different absolute temperatures, but the same ΔT of 20°C (heat regimes applied were from 15°C to 35°C, from 20°C to 40°C and to 25°C to 45°C) maintained a different pattern emerged. In this case (Fig. 4C) $Ca^{2+}$ peak values were significantly different from each other and linearly proportional to the magnitude of absolute temperature (Fig. 4C). These data clearly demonstrate, therefore, that absolute temperature is the primary parameter regulating the magnitude of the chloroplast heat response.

**Acclimation to different temperatures regimes alters the heat-induced chloroplast calcium response**

To test the effect of growth-history upon the heat-induced chloroplast calcium response, plants were treated overnight either at 15°C, 20°C or 30°C. The $Ca^{2+}$ response of these three sets of plants to the same heat stimulus (40°C for 7 minutes) was compared (Fig. 5A). Plants acclimated at different temperatures produced a larger (15°C pre-treatment) or smaller (30°C pre-treatment) stromal calcium response to heat compared to the control (20°C pre-treatment). The concentration of calcium at the peak is inversely proportional to the acclimation temperature, with 15°C pre-treatment showing the biggest calcium response (Fig. 5B). As a control, plants were treated for 30 minutes at the same acclimation temperatures (15 °C, 20 °C and 30 °C) before the 40 °C heat treatment. At this timescale (30 min) acclimation would not be expected to occur. Indeed, 30 min acclimation was not sufficient to affect the heat response, as can be seen by comparing Fig. 5C and Fig. 5D. Differences in baseline calcium levels were not detected at the different acclimation temperatures, suggesting that the steady state levels of stromal calcium is kept at the same level in response to the different acclimation temperatures.

**The heat-induced chloroplast calcium response is partially dependent on CAS**

The calcium sensing receptor CAS has previously been identified as a thylakoid membrane-resident protein postulated to be a calcium sensor (Han et al. 2003; Nomura et al. 2008; Vainonen et al. 2008). It has been shown previously to be necessary for full chloroplast calcium responses to pathogen elicitors and the light to dark transition (Nomura et al. 2012). Therefore the effect of heating upon chloroplast calcium was tested in two independent mutant alleles of the CAS protein (At5g23060)

(Vainonen et al. 2008). As can be seen in Fig. 6 whilst the mutants were qualitatively responsive to the heat stimulus, the magnitude of response was significantly reduced to around 50% of the wild type level.

## Discussion

In this study, a chloroplast-specific calcium signal was identified in response to heat. We demonstrated that this response occurs uniquely in the chloroplastic compartment and that it is dependent upon the magnitude of the 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; Sello et al. 2016) and abiotic stress (Nomura et al. 2012; Sello et al. 2016); 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; Sai and Johnson 2002).

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. 1998; 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 (Finka and Goloubinoff 2014; Saidi et al. 2009). 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 (Fig. 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) and it could be observed in different plant species (tobacco and *Arabidopsis*), suggesting that there may be a common signalling mechanism in higher plants. However, differences in the magnitude of the calcium increase were observed in *Arabidopsis* itself and between different species. Each of the two *Arabidopsis* ecotypes tested, when stimulated at 40$^{o}$C, responded with a $Ca^{2+}$ increase whose

magnitude ranged from 0.3 µM to 0.7 µM on different days, most probably depending on slight uncontrollable differences in the growth conditions. For this reason, only experiments conducted on the same day were directly compared to each other, and each of them was replicated at least twice to confirm the results. When different species were stimulated by heating, differences were observed in terms of sensitivity. Indeed, comparable stromal calcium concentrations were detected in *Arabidopsis* when stimulated at 40°C as in tobacco at 45°C (compare Fig. 1 and Fig. S2), while at 40°C there is no distinguishable calcium peak in tobacco (only a slight $Ca^{2+}$ increase was observed). These differences can be either attributed to a genetic factor distinguishing the thermometer between the two species, or to the different growth temperature regime applied before the heat treatment (consistent with data shown in Fig. 5). In both species the relationship between higher temperatures causing a larger calcium increase was observed. This relationship is clearly demonstrated in Fig. 2A and Fig. 2B, where the kinetics of the calcium curves, as well as peak heights, change progressively with increasing temperature, following a dose-response relationship. Such differences in the calcium kinetics are able to 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".

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 (Fig. 3). 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 Fig. 3, 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, we tested the effect of rate upon the chloroplast heat response, and it emerged (Fig. 4A) 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°C for all the samples) is the major parameter controlling the calcium increase, in stark contrast to the cytosolic calcium response to cold.

Fig. 4A suggested the importance of absolute temperature, but it did not formally distinguish if the response to heat is mainly dependent on absolute temperature or relative temperature change ($\Delta$T). To discriminate between these two options, two experiments were performed, one in which $\Delta$T was varied, whilst absolute T was not (Fig. 4B) and, conversely, in the second, $\Delta$T was fixed to 20°C, but the final absolute T reached was varied (Fig. 4C). While in the first experiment there was no observed correlation between the magnitude of $[Ca^{2+}]_{chl}$ at the peak at different $\Delta$T applied (Fig. 4B), a strong linear dependency was observed for the second case, where the change was in absolute final temperature (Fig. 4C). Notably, results in Fig. 4C are comparable with the ones shown in Fig. 2B the major difference in the behaviour of the cold response (dependent of rate of cooling) 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, respectively. It is interesting that in plants cold receptor leading to calcium elevation appears to be in the plasma membrane (Plieth et al. 1999) whereas heat receptor leading to calcium elevation is in the chloroplast. In the case of mammals both cold and heat receptors (themselves calcium channels) located in the plasma membrane (Caterina et al. 1997; McKemy et al. 2002).

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 (Graham and Patterson 1982; Murata and Los 1997; Pearcy 1978; Wilson and Crawford 1974). 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; Horvath et al. 1998; Mejia et al. 1995; 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 according to the temperature they have experienced previously. Indeed plants pre-treated overnight at 15°C, whose membrane will be more fluid due to a higher level of desaturation of the fatty acids, were responding to the same heat stimulus by producing a bigger calcium response compared to the control pre-treated at 20°C (Fig. 5A and Fig. 5B). Conversely saturating the membrane fatty acids by pre-acclimating plants at 30°C overnight caused a decreased stromal calcium response to heat (Fig. 5A and Fig. 5B). Additionally, if the pre-treatments at 15°C,20°C and 30°C were reduced to 30 min only, these differences were abolished (Fig. 5C and Fig. 5D), confirming the results obtained

in Fig. 4B. These results are consistent with the idea that acclimation leading to changes in membrane fluidity, which is a long-term process, may be responsible for the differences observed in Fig. 5A and Fig. 5B. Additionally, these data indicate that the cellular thermometer involved is able to reset according to the temperature plants have been experiencing before the experimental heating event. Therefore it might be that rapid changes in membrane fluidity are the primary temperature-sensing event leading to elevations in chloroplast free calcium concentration, a theory we will test in the future.

The calcium-sensing thylakoid protein CAS has been shown in previous studies to be necessary for the chloroplast calcium responses to elicitors and the light-dark transition (Nomura et al. 2012). We show that CAS mutants displayed a similarly significant reduction in response to heat (Fig. 6). In the case of elicitors, a reduced chloroplast calcium response was correlated to reduced expression of salicylic acid-dependent pathogen gene expression and the production of salicylic acid itself (Nomura et al. 2012). This demonstrates that changes in chloroplast free calcium concentration can act as signals regulating downstream processes. Therefore it is quite possible that the heat-induced chloroplast free calcium increases we report here regulate an as yet unidentified downstream response to heating. It will be interesting to identify what these responses are in future work.

In conclusion, we discovered a chloroplast-specific absolute temperature-dependent calcium response to heat. This suggest that a plant heat thermometer may be located in the chloroplast. This thermometer is dependent upon CAS protein function and stress history. Determining the nature of this thermometer would be an important target for future work.

## Materials and Methods

### Plant material and growth conditions

The majority of the experiments were conducted on *Arabidopsis thaliana* lines constitutively expressing 35S::apoaequorin either in the cytosol (pMAQ2, Col-0 ecotype (Knight et al., 1991)) or in the chloroplast (pMAQ6, both Col-0 and Ws-0 ecotypes (Ws-0 was a kind gift from Dr William F. Ettinger, Gonzaga University, Spokane, WA, USA)), and for plant transformation, wild type (wt) Col-0 seeds were used. Two homozygous *cas* (At5g23060) mutants lines 665G12 (from GABI-KAT collection) and SALK 070416 (from Salk collection) were a kind gift from Prof Eva-Mari Aro (Turku University, Finland). Seeds were ethanol-sterilised, sown on 1 X Murashige and Skoog (MS, Duchefa Biochemie)

medium (Murashige and Skoog 1962) 0.8% (w/v) agar (Sigma-Aldrich) on Petri dishes, vernalised for a minimum of 48 h at 4°C before growing them at 20°C 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; aequorin reconstitution was performed on 7-day-old seedlings. For *Agrobacterium tumefaciencs*-mediated transformation, seedlings were transferred onto 44 mm peat plugs (Jiffy Products International) and grown at 20°C with a photoperiod of 12/12 h untill bolting, and 16/8 h after *Agrobacterium*-mediated transformation (light intensity 150 µmol $m^{-2}$ $sec^{-1}$); for seed collection individual seedlings were grown on 41 mm peat plugs (Jiffy Products International) and grown at 20°C in a 16/8 h photoperiod (light intensity 150 µmol $m^{-2}$ $sec^{-1}$). *Nicotiana benthamiana* plants were grown on soil at 27°C for 4 weeks with a 16/8 h photoperiod at 250-300 µmol $m^{-2}$ $sec^{-1}$.

**Plant transformation**

Plant genetic transformation was performed on *Arabidopsis thaliana* Col-0 wt plants with the binary construct pMAQ6 (Johnson et al. 1995) using the floral dip method (Clough and Bent 1998). Selection of the primary transformants was performed on MS medium containing kanamycin (50 mg/L), and successfully transformed plants were grown to seed as described above. Similarly, the *cas* mutants were transformed using the pMAQ6 construct in the binary vector pB7WG2 (Karimi et al. 2002) and selected on BASTA. Aequorin-based selection was performed by using a photon-counting camera (for details see temperature and chemical treatments of plants and *in vivo* reconstitution of aequorin and $Ca^{2+}$-dependent luminescence measurements below), and the total amount of aequorin was measured by changing the temperature to -15°C for 5 min, followed by 2 min at 20°C. Lines with levels of aequorin closest to the average expression were chosen for further experiments.

**Temperature and chemical treatments of plants**

Fast changes in temperature were performed on a Pelter cooling element (Photek 5.0 and TCS1.0; Photek). *Arabidopsis* seedlings were laid down on the cooling element on wet filter paper, and covered with cling film, whilst tobacco detached leaves were flattened with a thin transparent glass plate. Acclimation temperature treatments were performed as following: 48 h before performing the measurements plants were coelenterazine-reconstituted at 20°C overnight in the dark. The following day plants were left at 20°C in light for 8 h, then transferred for 12 h in the darkness at either 15°C 20°C, or 30°C until calcium was measured. When the pre-acclimation treatment was reduced to 30 min, plants were colelenterazine-reconstituted overnight at 20°C in darkness, the next day treated for 30 min at either 15°C 20°C, or 30°C for 30 minutes in darkness and used for calcium measurements.

**In vivo reconstitution of aequorin and $Ca^{2+}$-dependent luminescence measurements**

Aequorin reconstitution was performed by floating *Arabidopsis* seedlings on water containing 10 µM coelenterazine in 1% [v/v] methanol (Biosynth). Reconstitution of tobacco plants was performed by infiltrating with a syringe the aequorin-expressing area with a 50 µM coelenterazine solution, in 1% [v/v] methanol 24 h after infiltration. All plants were left in the dark from 12 to 24 h at 20°C before calcium measurements. For $Ca^{2+}$ imaging during temperature treatments, aequorin luminescence was recorded under a plate-intensified charge-coupled camera (Photek 216; Photek). Total aequorin for calibration was measured by decreasing the temperature to -15°C for 5 min, and then back to room temperature to discharge the remaining aequorin. This freezing treatment ruptures all cellular membranes including the chloroplast and allows excess calcium from the cell to saturate the aequorin and fully discharge it. Subsequent to this treatment, there is no remaining reconstituted stromal aequorin as previously described (Mehlmer et al. 2012). Calibration was performed as previously described (Knight et al. 1996). Statistical analysis of data involved unpaired t-test for the comparison of two conditions, one way ANOVA for the comparison of three or more conditions. Subsequently, ANOVA tests were followed by post-hoc tests, either by a Tukey's multiple comparisons test for comparison of each mean with every other mean or by Dunnett's multiple comparison test for comparing every mean to a control mean. All statistical tests were performed with GraphPad Prism (GraphPad Software, Inc.).

**Funding**

The authors acknowledge financial support from the EU Marie Curie project CALIPSO GA 2013–607607 (GL)

**Disclosures**

None

**Acknowledgments**

The authors acknowledge Dr Willian F. Ettinger (Gonzaga University, Spokane, WA, USA) for the pMAQ6 Ws-0 seeds, Eva-Mari Aro (Department of Biology, Plant Physiology and Molecular Biology, University of Turku, Finland) for the *cas* mutant seeds, Heather Knight (Durham University, UK), Véronique Larosa (University of Liège, Belgium) and Ute Vothknecht (University of Bonn, Germany) for critical suggestions. We thank Beccy Manning excellent technical assistance and support.

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**Legends to figures**

**Fig. 1. .** Plants respond to heat with a chloroplast-specific calcium increase. Calcium elevations in response to heating (20°C to 40°C) and cooling (40°C to 20°C) events in the cytosol (cyt) and chloroplast (chl) are represented through time. Each trace was obtained by averaging the signal recorded from n = 6 for cyt and n = 5 for chl 8-day-old *Arabidopsis s*eedlings. Error bars represent standard deviation (SD). To mark where the chloroplast calcium concentration is significantly different from the cytosolic one, the p-value (grey line) was calculated through time with an unpaired t-test.

**Fig. 2.** The kinetics of the heat-induced chloroplast $Ca^{2+}$ increase is temperature-dependent. Chloroplast-targeted aequorin seedlings were exposed to a series of temperatures ranging from 30°C to 45°C, at intervals of 2.5°C. (A) kinetics of the calcium increase upon heating. (B) Average relative chloroplastic calcium concentration peak height and a linear regression line ($R^2$=0.9799) interpolating the peaks are represented. (C) average chloroplastic calcium concentration peak times a logaritmic regression line ($R^2$=0.9416) interpolating the peaks are represented. (D) Statistical significance of the calcium concentration peak height at different temperatures and (E) of peak times were calculated with one way ANOVA followed by a Tukey's multiple comparisons test, *p≤0.1, **p≤0.01, ***p≤0.001, **** p≤0.0001, ns= not significant. 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 = SD.

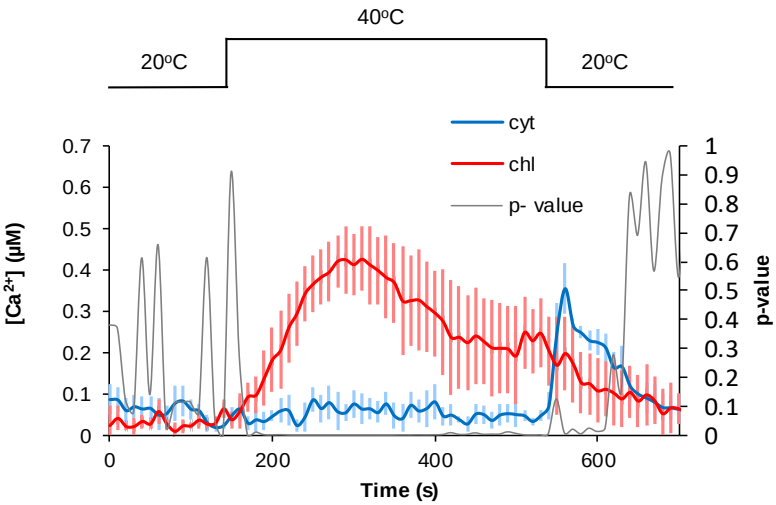
**Fig. 3.** The chloroplast calcium heat response displays attenuation and sensitisation. (A) $[Ca^{2+}]_{chl}$ response to 3 consecutive heat pulses of the same magnitude (40°C for 4 min) followed by a fourth pulse at a higher absolute temperature (45°C for 4 min). Each heat pulse was separated by a 5 min resting period at 20°C. (B) Relative $[Ca^{2+}]_{chl}$ peak heights of the 4 individual peaks. Data represent an average of n = 7 *Arabidopsis* seedlings. Error bars = SD, ****p≤0.0001 calculated by one way ANOVA followed by Dunnett's multiple comparison test using the 1st peak as a control reference.

**Fig. 4.** The peak level of the heat-induced $[Ca^{2+}]_{chl}$ response is regulated by absolute temperature not by the heating rate. Each data point represents an average of the value reached at the $[Ca^{2+}]_{chl}$ peak obtained from: (A) n = 4 *Arabidopsis* seedlings exposed to a temperature shift from 20°C to 40°C at different rates For each rate a different set of plants was used. Rates tested: 0.4, 0.2, 0.15 and 0.1 °C $s^{-1}$; (B) n=7 *Arabidopsis* seedlings exposed to a temperature shift from 15°C, 20°C and 25°C to 40°C at the same rate and (C) n=7 *Arabidopsis* seedlings exposed to the temperature shift of 20°C ( from 15°C to 35°C, from 20°C to 40°C and from 25°C to 45°C) at the same rate. Data points represent experimental data, interpolated by a regression line, error bars = SD. Statistical significance was were calculated with one way ANOVA followed by a Tukey's multiple comparisons test, ns= not significant ****p≤0.0001.

**Fig. 5.** Acclimation to high or low temperature affects the subsequent calcium response to heat. Chloroplast-targeted *Arabidopsis* aequorin lines were acclimated overnight (A and B) or for 30 min (C and D) at 15°C, 20°C or 30°C, then stimulated at 40°C for 7 min. (A) Chloroplast calcium kinetics upon heating of the different overnight pre-acclimated lines and (B) average chloroplastic calcium concentration peak heights. (C) Chloroplast calcium kinetics upon heating of the lines pre-acclimated

for 30 min and (D) respective average chloroplastic calcium concentration peak heights. Data were obtained by averaging traces of n = 5 *Arabidopsis s*eedlings for overnight acclimation and n= 8 seedlings for 30 minutes acclimation. Error bars = SD, p values are represented (**p≤0.01, ****p≤0.0001, ns= not significant) and were calculated with one way ANOVA followed by a Turkey multiple comparison test.

**Fig. 6.** Chloroplast-specific calcium increases are partially CAS-dependent. (A) Representative calcium traces of *Arabidopsis* wt Col-0, *cas* SALK and *cas* GABI lines in response to a 40$^{o}$C 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 = SD. Asterisks represent statistical significance compared to the Col-0 control, **p≤0.01, ***p≤0.001 analysed with one way ANOVA followed by Dunnett's multiple comparisons test.

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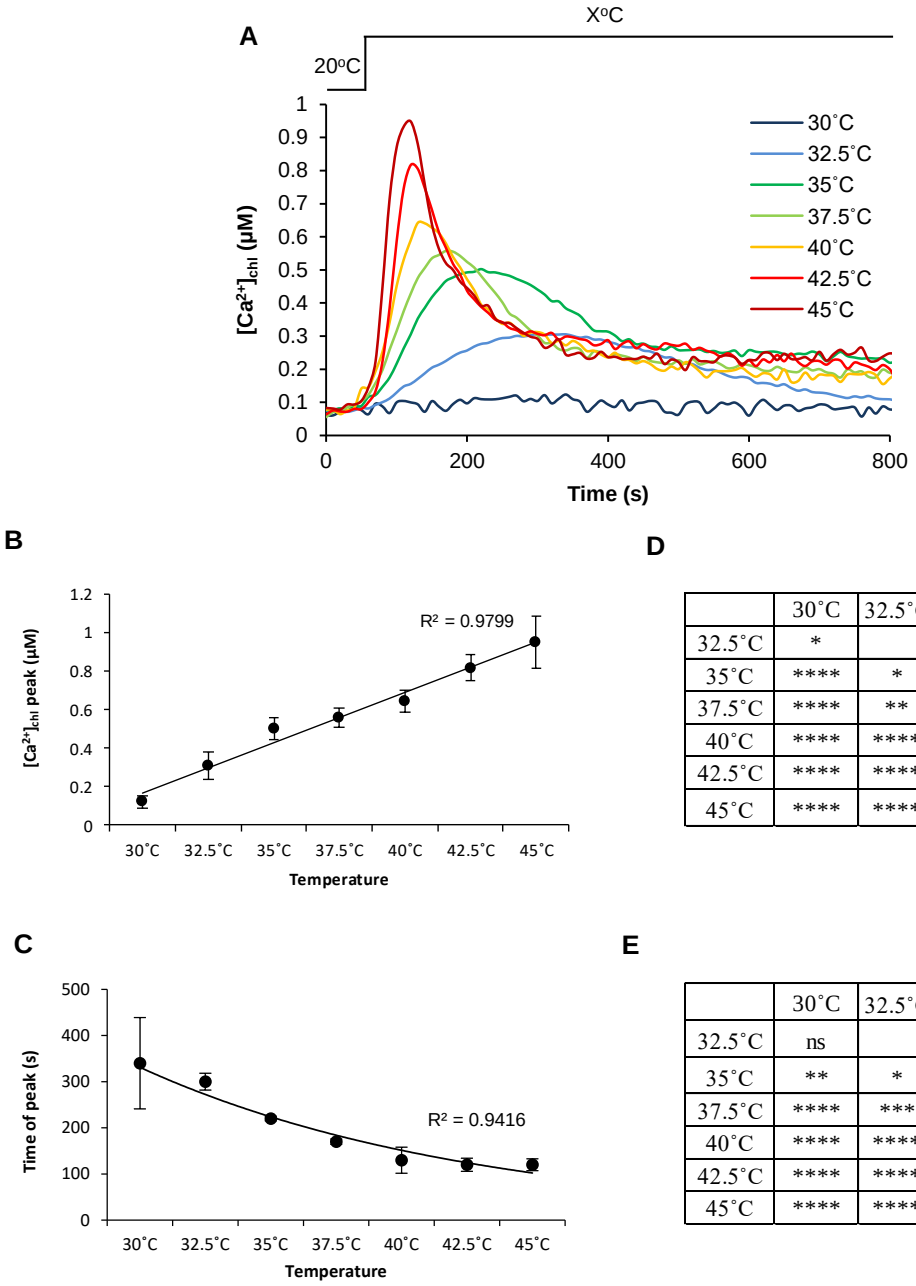


D

| | 30˚C | 32.5˚C | 35˚C | 37.5˚C | 40˚C | 42.5˚C |
|---|---|---|---|---|---|---|
| 32.5˚C | * | | | | | |
| 35˚C | **** | * | | | | |
| 37.5˚C | **** | ** | ns | | | |
| 40˚C | **** | **** | * | ns | | |
| 42.5˚C | **** | **** | **** | *** | * | |
| 45˚C | **** | **** | **** | **** | *** | ns |

E

| | 30˚C | 32.5˚C | 35˚C | 37.5˚C | 40˚C | 42.5˚C |
|---|---|---|---|---|---|---|
| 32.5˚C | ns | | | | | |
| 35˚C | ** | * | | | | |
| 37.5˚C | **** | *** | ns | | | |
| 40˚C | **** | **** | ns | ns | | |
| 42.5˚C | **** | **** | * | ns | ns | |
| 45˚C | **** | **** | * | ns | ns | ns |

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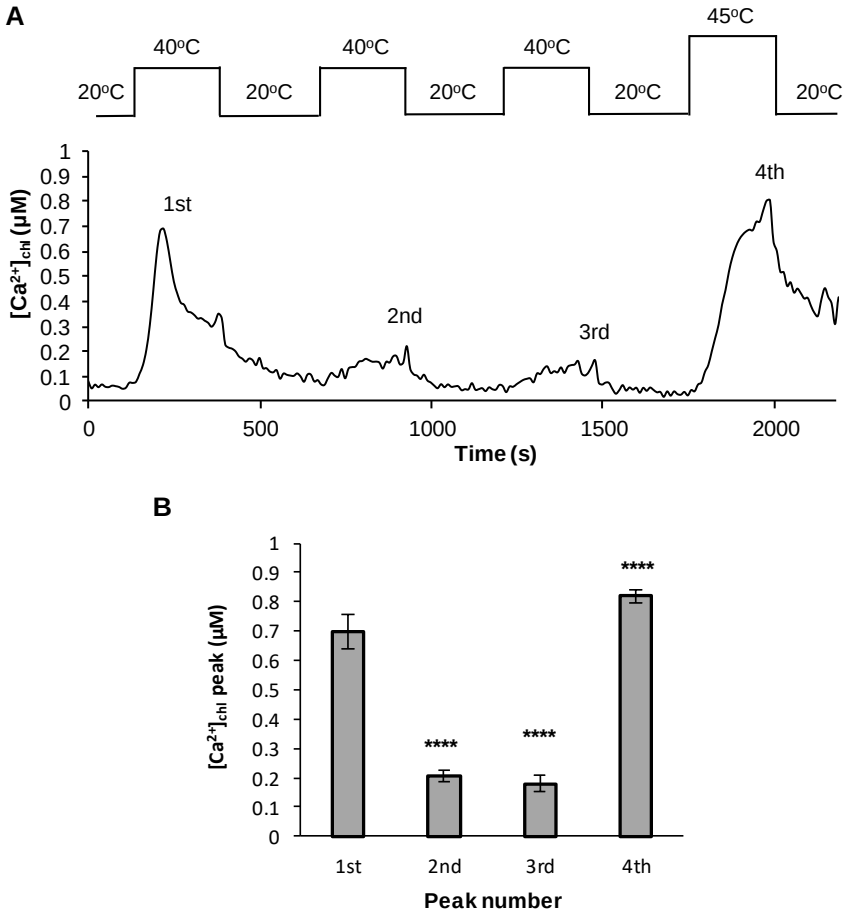


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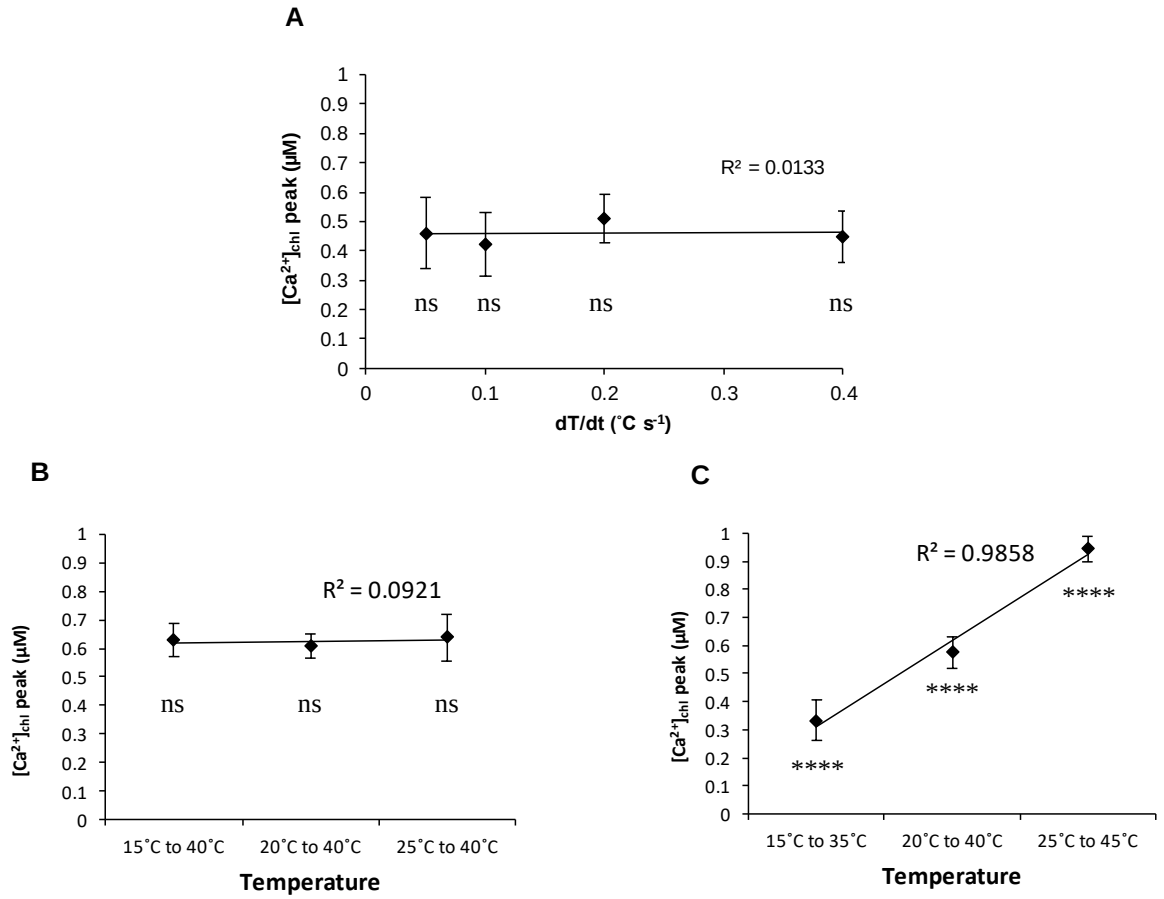


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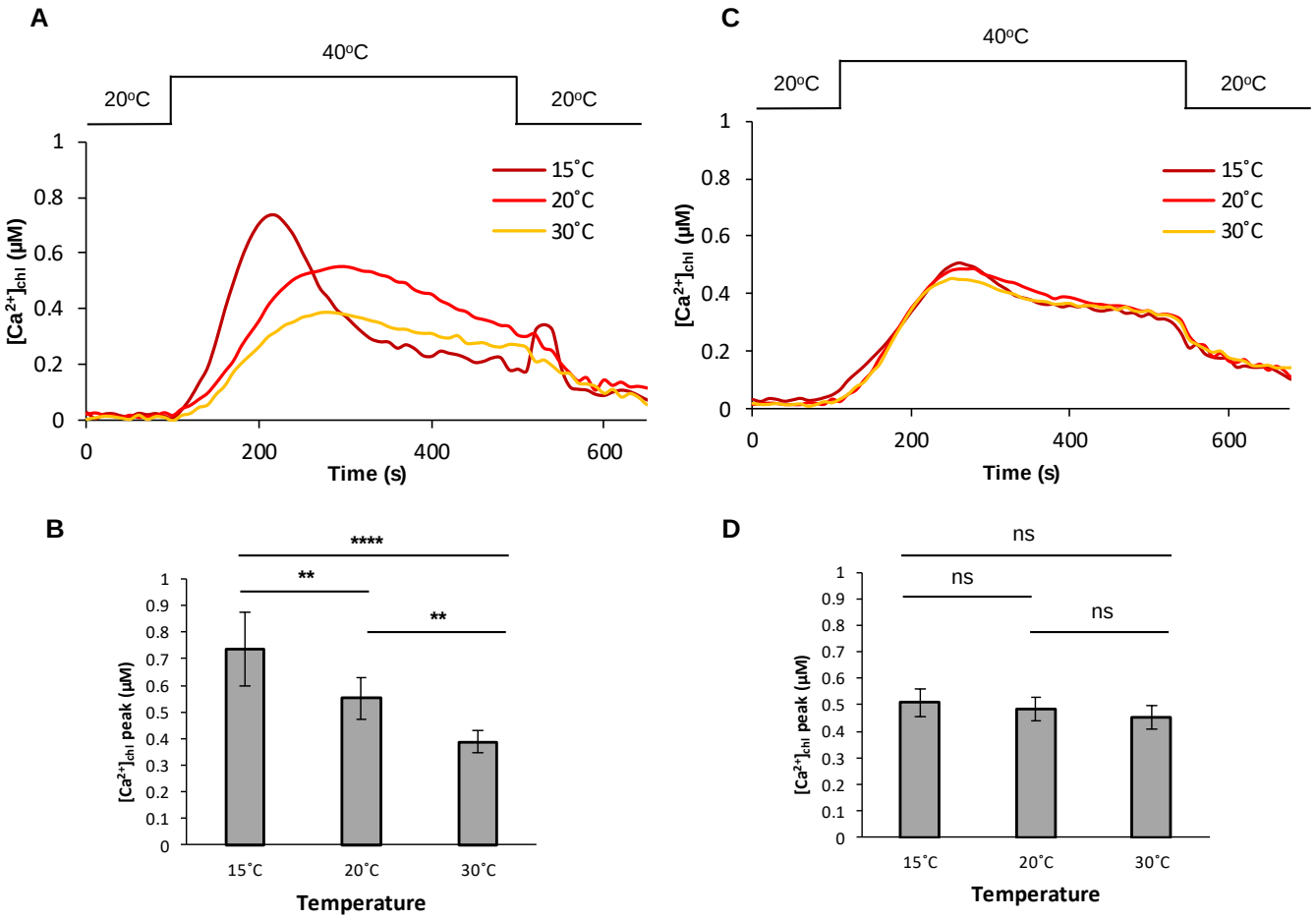


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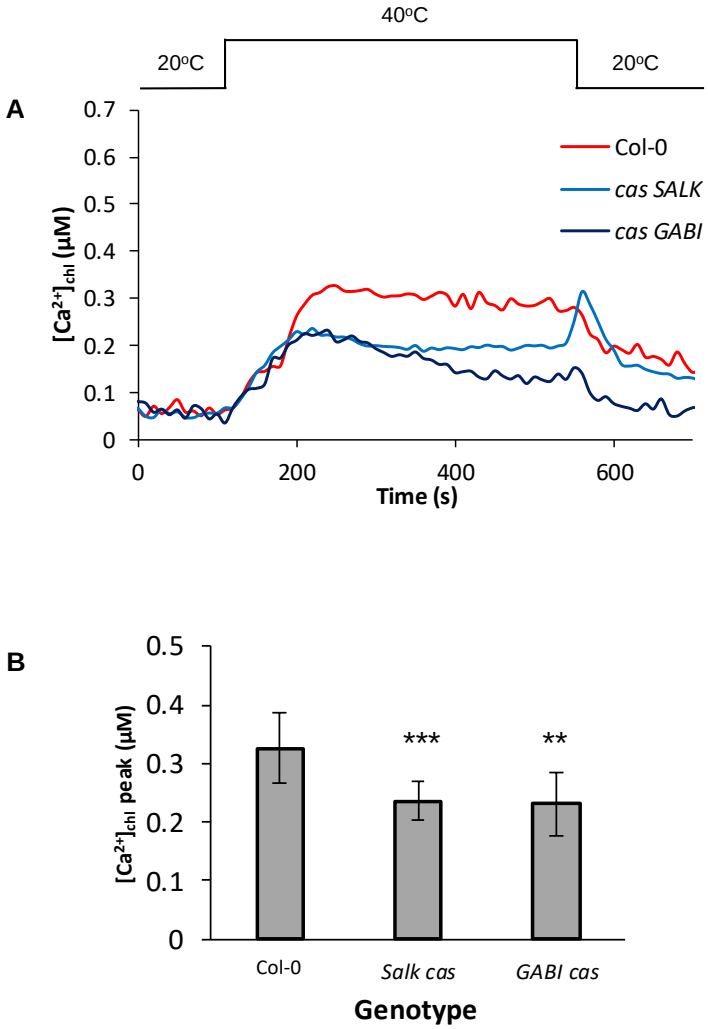


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