

Protection against Heat Stress-Induced Oxidative Damage in Arabidopsis Involves Calcium, Abscisic Acid, Ethylene, and Salicylic Acid

Jane Larkindale* and Marc R. Knight

Department of Plant Sciences, University of Oxford, South Parks Road, Oxford OX1 3RB, United Kingdom

Plants, in common with all organisms, have evolved mechanisms to cope with the problems caused by high temperatures. We examined specifically the involvement of calcium, abscisic acid (ABA), ethylene, and salicylic acid (SA) in the protection against heat-induced oxidative damage in Arabidopsis. Heat caused increased thiobarbituric acid reactive substance levels (an indicator of oxidative damage to membranes) and reduced survival. Both effects required light and were reduced in plants that had acquired thermotolerance through a mild heat pretreatment. Calcium channel blockers and calmodulin inhibitors increased these effects of heating and added calcium reversed them, implying that protection against heat-induced oxidative damage in Arabidopsis requires calcium and calmodulin. Similar to calcium, SA, 1-aminocyclopropane-1-carboxylic acid (a precursor to ethylene), and ABA added to plants protected them from heat-induced oxidative damage. In addition, the ethylene-insensitive mutant *etr-1*, the ABA-insensitive mutant *abi-1*, and a transgenic line expressing *nahG* (consequently inhibited in SA production) showed increased susceptibility to heat. These data suggest that protection against heat-induced oxidative damage in Arabidopsis also involves ethylene, ABA, and SA. Real time measurements of cytosolic calcium levels during heating in Arabidopsis detected no increases in response to heat per se, but showed transient elevations in response to recovery from heating. The magnitude of these calcium peaks was greater in thermotolerant plants, implying that these calcium signals might play a role in mediating the effects of acquired thermotolerance. Calcium channel blockers and calmodulin inhibitors added solely during the recovery phase suggest that this role for calcium is in protecting against oxidative damage specifically during/after recovery.

In nature, plants are subject to changes of temperature, both during changes in season and more rapidly over the course of individual days. The temperature of an individual plant cell can change much more rapidly than other factors that cause stress (e.g. water levels or salt levels). Thus, like other organisms, plants have evolved strategies for preventing damage caused by rapid changes in temperature and for repairing what damage is unavoidable.

Heat stress responses have been well documented in wide range of organisms. In all species studied, heat stress results in the production of specific families of proteins known as heat shock proteins (HSPs; Howarth and Ougham, 1993). These proteins have been classified into a number of families based on their molecular mass, and most have chaperonin function (Jaenicke and Creighton, 1993). All organisms produce HSPs from all of the major families (HSP90s, HSP70s and small HSPs), but plants are unique in the number of different small HSPs that they produce (Jakob and Buchner, 1994). Most studies investigating heat stress in plants have focused on HSPs (Howarth and Ougham, 1993; Sullivan and

Green, 1993; Park et al., 1996; Schoffl et al., 1997; Gurley, 2000).

Despite the ubiquitous nature of the heat shock response, little is known about how the plant senses an increase in temperature or the signaling pathways resulting in HSPs. It is well documented that pretreatment with a mild heating regime allows plants to tolerate higher temperatures than non-pretreated plants. These plants are termed thermotolerant (Howarth and Ougham, 1993; Burke, 2001; Sharkey et al., 2001). It is known that HSPs accumulate during mild heating (Nover et al., 1983) and that their appearance correlates with survival of the plant, but relatively little is known about signaling leading to this event or what other signaling pathways may be involved in the ultimate survival of the plant.

There is considerable evidence that oxidative stress induces pathways resulting in accumulation of some HSPs (Dat et al., 1998; Storozhenko et al., 1998; Schett et al., 1999). Some bacterial HSPs also require an oxidative stress regulator *oxyR* for induction. In addition to oxidative stress-inducing heat shock genes, Gong et al. (1997a, 1997b) presented evidence that heat also induces oxidative stress (as measured using the thiobarbituric acid reactive substances [TBARS] assay used in this study). It has also been shown that thermotolerance can be induced by compounds that induce oxidative bursts (Dat et al., 1998), and that very short heat pulses can induce such bursts of superoxide and/or hydrogen peroxide (Vallelian-

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* Corresponding author; e-mail jane.larkindale@plants.ox.ac.uk; fax 44-1865-275074.

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Bindschedler et al., 1998). This suggests that there is considerable inter-linking between heat and oxidative stress responses.

A wide range of second messengers have been implicated in signaling in response to a variety of stresses. Calcium ions (Sanders et al., 1999; Knight, 2000), salicylic acid (SA; Dat et al., 1998), abscisic acid (ABA; Annamalai and Yanaghiara, 1999; Gong et al., 1998a, 1998b) and ethylene (Foyer et al., 1997) are all involved in several stress responses. As described below any or all of these potential second messengers may be involved in pathways switched on in response to heat stress.

There is some evidence that SA may be involved in heat stress responses in plants. There is limited evidence of any such involvement in animals: SA is known to stabilize the trimers of heat shock transcription factors and to aid them in binding to the heat shock element in the promoter of HSP genes (Jurivich et al., 1992). Despite this, SA does not induce HSP transcription in animal cells (Jurivich et al., 1992). Thermotolerance can be induced in potato plants, however, by treatment with an acetyl-SA spray (Dat et al., 1998), and the induced thermotolerance is extremely long lasting (Lopez-Delgado et al., 1998). There is no evidence in the literature, however, that SA induces HSP gene transcription in plants (although it results in heat shock transcription factor being bound *in vivo* to the transcriptional control elements of HSP70 in animal cells [Jurivich et al., 1992]). SA is well known as an important component of signaling pathways in response to systemic acquired resistance and the hypersensitive response (Kawano et al., 1998). In these systems, it is often linked to oxidative responses and calcium signaling (Kawano et al., 1998).

Calcium transients in response to heat treatment have been detected using the calcium-dependent luminescent protein aequorin in tobacco (Gong et al., 1998b), suggesting that calcium may have a role in heat stress signaling. It has been also shown that calcium signaling inhibitors and calmodulin inhibitors limited survival and increased electrolyte leakage from membranes after heat treatment in maize (*Zea mays*; Gong et al., 1997a). It has also been shown, however, that calcium is not required for HSP production in plants despite the fact that heat stress induces uptake of calcium and the induction of some calmodulin related genes (Gong et al., 1997b). This suggests that some process other than HSP induction is also required for survival of plants after heat stress, and that calcium may be involved in some signaling pathway acting between the perception of heat stress and this process.

It has also been noted that the plant hormone ABA induces thermotolerance in maize (Gong et al., 1998a), a fact also observed in bromegrass (Robertson et al., 1994). This suggests that ABA could also be involved in some pathway resulting in survival of

heat stress in plants. ABA has been shown to induce a limited amount of HSP70 induction at ambient temperature in plants (Wu et al., 1994), but not HSP90 induction (Yabe et al., 1994). ABA appears to induce chimeric genes with a small HSP promoter from sunflower, working synergistically with heat shock transcription factor 3 (Rojas et al., 1999).

The Arabidopsis gene encoding another HSP, *APX1* (defined as such because of the presence of a heat shock transcription factor binding site in its promoter; Storozhenko et al., 1998) is induced by ethephon, a mimic of the plant hormone ethylene (Wu et al., 1994). Ethylene has been implicated in a number of stress-induced pathways, many of which also include molecules such as SA and calcium ions (Foyer et al., 1997).

Thus, the literature suggests that these second messengers/plant growth regulators might be involved in aspects of plant (and thus possibly Arabidopsis) heat shock signaling pathways. They do not all, however, necessarily appear to be involved in the classic heat shock response, i.e. the induction of HSPs. The majority of data obtained to date has been through the addition of compounds, showing that these compounds can endow greater thermotolerance. This does not answer the question as to whether these compounds are actually used by the plants themselves in nature for this purpose. In this paper we describe experiments examining the role of these second messengers/plant growth regulators specifically in protection against heat-induced oxidative damage in Arabidopsis.

RESULTS

Heat Stress Induces Oxidative Damage in Arabidopsis Seedlings

When Arabidopsis seedlings were treated at 40°C for 1 h and returned to normal growth temperatures on agar plates, they visibly became progressively bleached over a period of days after the heat treatment, even though they were no longer subject to heating. Three days after this treatment, very few of the seedlings had survived (Fig. 1). Bleaching suggested that damage caused by heating may have been due to oxidative stress occurring during the recovery phase. This hypothesis was tested by measuring lipid peroxidation in plants after heating. This was measured using the TBARS assay, which is a common assay for oxidative damage to membranes (Heath and Packer, 1968). TBARS are the product of lipid peroxidation, and thus higher levels of these substances are found in plants that are subject to higher levels of oxidative stress. As shown in Figure 1a, the 40°C treatment caused a significant (greater than 3-fold compared with unheated controls) increase in lipid peroxidation after 2 d that increased even further after 3 d (up to more than 6-fold compared with unheated controls). In contrast, seedlings treated in

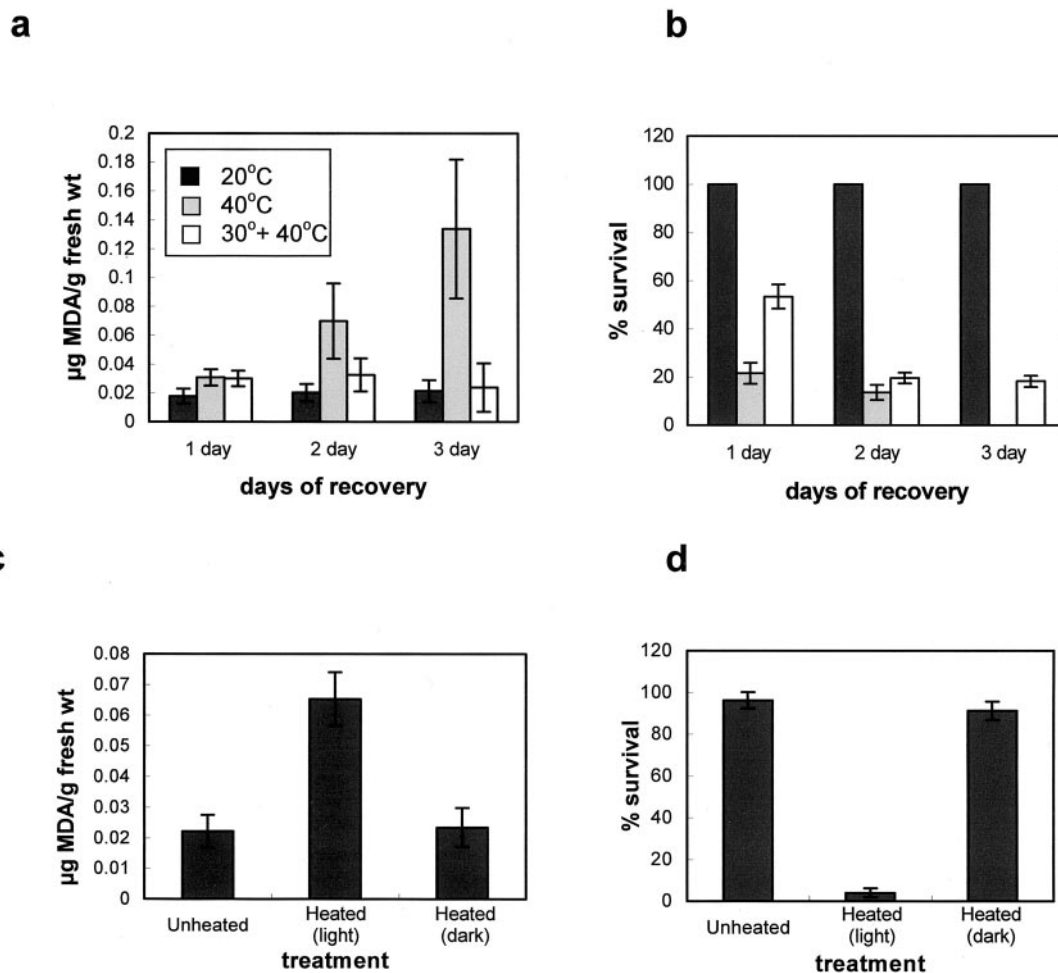


Figure 1. Oxidative damage and survival in response to heating in *Arabidopsis* seedlings is affected by light and heat pretreatment. Graphs showing levels of TBARS (a and c) and survival (b and d). *Arabidopsis* plants were treated at different temperatures and conditions. In a and b, plants were treated 1 h at 40°C either with (white bars) or without (light-gray bars) a 1-h pretreatment at 30°C and compared with plants treated throughout at 20°C (dark-gray bars). TBARS/survival data is shown for 1, 2, and 3 d after end of 40°C treatment. In c and d, plants were treated for 40°C for 1 h and allowed to recover either in the dark or in the light, and TBARS/survival was compared with control plants treated at 20°C throughout, 3 d after the end of the 40°C treatment. Data shown are averages of five replicates. Error bars represent SE of mean for these five replicates.

the same way but subject to a prior treatment at 30°C for 1 h showed no significant increase in lipid peroxidation over 3 d (relative to unheated controls; Fig. 1a). When survival was assessed, it was found that the 30°C pretreatment partially ameliorated survival (roughly 20% survival after 3 d, compared with 0%, in this particular experiment, with no pretreatment). Clearly, however, the majority of plants did not survive even with this pretreatment.

The TBARS assay measures oxidative damage to membranes, so it seemed possible that the damage seen was due to photooxidative stress, caused indirectly by the breakdown of the photosynthetic machinery. To test this hypothesis, plants were heated to 40°C as before and allowed to recover for 3 d, either in the dark or in the light. As can be seen in Figure 1, c and d, in light conditions there were significant

increases in membrane peroxidation and decreases in survival; but in dark conditions, these parameters were comparable with unheated control plants.

Effect of Calcium Channel Blockers and Calmodulin Inhibitors on Levels of Oxidative Damage and Survival of Plants after Heat Treatment

Plants were treated with a number of different calcium channel blockers, and inhibitors of calmodulin before and during a 1-h 35°C heat treatment (a treatment causing no increase in TBARS levels and allowing 100% survival in the wild type). This temperature was used specifically so that any increased oxidative stress due to the chemical treatments could be observed (as a higher temperature, i.e. 40°C, would have given a very high background levels of

TBARS and mortality). The calcium channel blockers used were lanthanum (III) chloride, nifedipine, and verapamil. The calmodulin inhibitors used were hydrochloride *N*-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide (W7) and trifluoperazine (TFP). The plants were removed from the inhibitor after heating and allowed to recover in the light. The levels of TBARS and survival were measured after 3 d. All of the inhibitors tested increased heat-induced levels of TBARS in the plant as compared with control plants, and reduced survival when assessed after 3 d (Fig. 2). The effects on survival were quite varied, with TFP, W7, verapamil, lanthanum, and nifedipine reducing survival to approximately 80%, 70%, 40%, 0%, and 10%, respectively (Fig. 2b). The effects of TFP, W7, verapamil, and nifedipine on TBARS were quite similar, with all four increasing TBARS about 4-fold after heating at 35°C. Lanthanum had the greatest effect, increasing TBARS about 8-fold after heating at 35°C (Fig. 2a).

Effect of Calcium, SA, 1-Aminocyclopropane-1-Carboxylic Acid (ACC), and ABA on Levels of Oxidative Damage and Survival of Plants after Heat Treatment

The calcium/calmodulin inhibitor data (Fig. 2) suggested that calcium might act as a second messenger in some signaling pathway limiting heat-induced oxidative damage. This hypothesis was further tested by the addition of exogenous calcium chloride to plants for 1 h before heating and during the heat treatment. These plants were heated to 40°C for 1 h, a temperature treatment at which most plants fail to survive (Fig. 1; Fig. 3b). This calcium treatment en-

hanced survival of 40°C by more than 3-fold (Fig. 3b) and reduced levels of TBARS in heated plants by about 50% (Fig. 3a). In the same way, the effect of pretreating plants with either SA, ACC (a precursor of ethylene), or ABA was tested (Fig. 3). All three of these caused both enhanced survival and reduced levels of TBARS after a 40°C treatment. The effects on survival were quite varied, with SA, ACC, and ABA enhancing survival by approximately 5-fold, 3-fold, and 2-fold, respectively (Fig. 3b). The effects of all three messengers/plant growth regulators on TBARS was quite similar, with all three reducing TBARS to about 50% of control after heating at 40°C.

These data (Fig. 3) suggested that SA, ACC (ethylene), and ABA, when added exogenously, could protect Arabidopsis plants against heat-induced oxidative damage as measured by TBARS and survival. Therefore, we subsequently tested whether Arabidopsis actually uses SA, ethylene, and ABA in vivo to protect itself against heat-induced oxidative damage. To do this we tested effects of heating on an ethylene-insensitive mutant, an ABA-insensitive mutant, and a transgenic line with reduced levels of SA. TBARS and survival were measured in all these lines after heating for 1 h at 37°C. This temperature was used specifically so that any increased oxidative stress due to the chemical treatments could be observed (as a higher temperature, i.e. 40°C, would have given a very high background levels of TBARS and mortality). The *etr-1* mutant is defective in an ethylene receptor subunit and consequently is insensitive to ethylene (Sopory and Munshi, 1998). After a 37°C treatment, *etr-1* showed increased TBARS (more than 3-fold greater; Fig. 4a), and reduced survival (approximately 50% of levels of control; Fig. 4b) com-

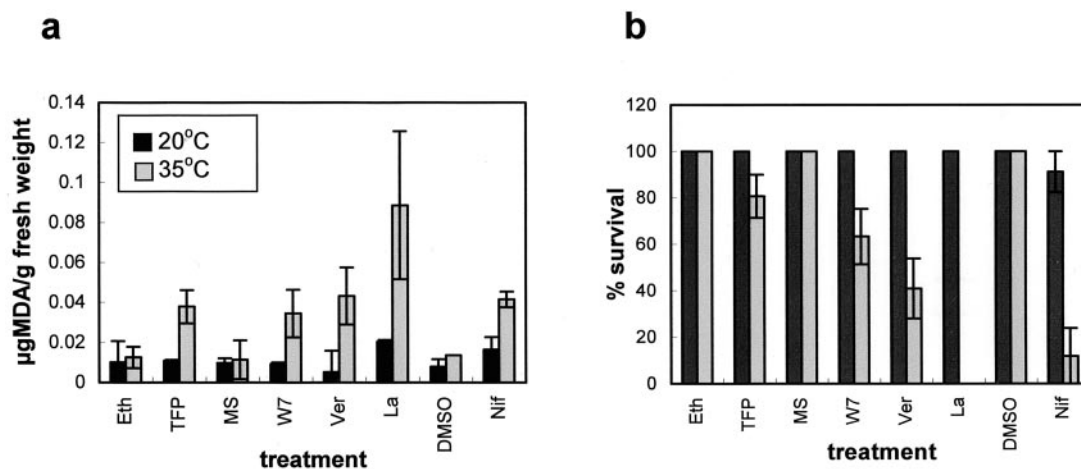


Figure 2. Effect of calcium channel blockers and calmodulin inhibitors on oxidative damage and survival in response to heating in Arabidopsis seedlings. Plants were pretreated in calcium channel blockers (TFP, verapamil, lanthanum chloride, or nifedipine) or calmodulin inhibitors (TFP or W7) before a 35°C treatment for 1 h. Graphs indicate TBARS levels (a) and survival (b) 3 d after the end of the 35°C treatment. Samples treated at 35°C (gray bars) were compared with controls kept at 20°C throughout (black bars). TFP was dissolved in ethanol and nifedipine in DMSO, whereas all other inhibitors were dissolved in water in Murashige and Skoog medium, and appropriate controls are presented. Data shown are averages of five replicates. Error bars represent SE of mean for these five replicates.

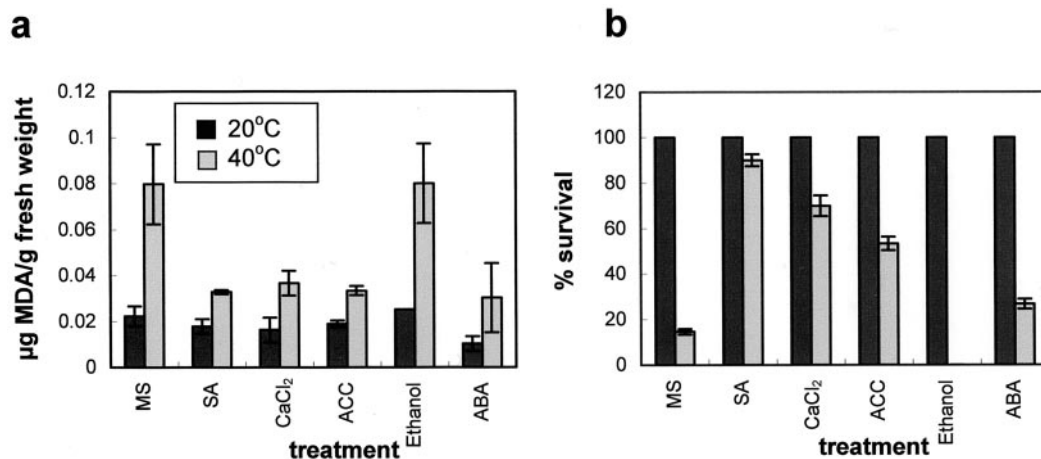


Figure 3. Effect of calcium, SA, ACC, and ABA on oxidative damage and survival in response to heating in Arabidopsis seedlings. Plants were pretreated with calcium, SA, ACC, or ABA before a 40°C treatment for 1 h. Graphs indicate TBARS levels (a) and survival (b) 3 d after the end of the 40°C treatment. Samples treated at 40°C (gray bars) were compared with controls kept at 20°C throughout (black bars). ABA was dissolved in ethanol, all other compounds were dissolved in water in Murashige and Skoog medium, and the appropriate control is presented. Data shown are averages of five replicates. Error bars represent SE of mean for these five replicates.

pared with Columbia wild type. *abi-1* carries a mutation in a protein phosphatase required for sensing ABA and, thus, is insensitive to ABA (Meyer et al., 1994). After a 37°C treatment, *abi-1* showed increased TBARS (approximately 2-fold greater; Fig. 4c), and reduced survival (reducing survival to 0%, from a 40% control value; Fig. 4d) compared with Landsberg wild type. It can also be seen in Figure 4 that the Landsberg ecotype of Arabidopsis is more sensitive to the 37°C treatment than Columbia in terms of survival and levels of TBARS. Transgenic lines expressing *nahG* were also tested. These lines express bacterial salicylate hydroxylase, an enzyme that breaks down SA as soon as it is formed. Thus these plants show substantially reduced levels of SA (Rao and Davis, 1999). In this series of experiments, the 37°C treatment did not measurably affect TBARS levels (Fig. 5a), but reduced survival to around 80% of unheated control. In comparison, after a 37°C treatment, *nahG* plants showed increased TBARS (approximately 4-fold greater; Fig. 5a), and reduced survival (reducing survival to 0%, compared with around 80% for Columbia control; Fig. 5b).

Changes in Intracellular Calcium Levels during Heating and Recovery

Calcium and calmodulin antagonists added before heating caused decreases in survival and increases in oxidative damage, and addition of exogenous calcium chloride before heating increased survival and reduced oxidative damage (Figs. 2 and 3). This implies a role for calcium as a signaling molecule in some pathway induced in response to heat stress. This was further investigated by using plants expressing the recombinant protein apoaequorin, as a luminescent cytosolic free calcium concentration

([Ca²⁺]_{cyt}) reporter in vivo (Knight et al., 1991). After reconstitution with the luminophore (coelenterazine) to form the active luminescent aequorin protein, this emits light in the presence of calcium ions.

Traces showing aequorin luminescence from individual whole plants subject to heat treatments are shown in Figure 6. Figure 6a shows a representative plant heated to 40°C for 1 h, and then cooled to 20°C. Figure 6b shows the trace for an identical representative plant heated first to 30°C for 1 h, then to 40°C for 1 h, followed by recovery at 20°C. In both cases, no significant increase in [Ca²⁺]_{cyt} was observed during the heat treatments themselves, either at 30°C or at 40°C. A transient [Ca²⁺]_{cyt} increase was seen, however, after 3,600 s (Fig. 6a) and 7,200 s (Fig. 6b), which correlates exactly to the end of heating. At this point the plants were cooled from 40°C to 20°C, and the increase in [Ca²⁺]_{cyt} occurred seconds after the initiation of cooling.

The height of this [Ca²⁺]_{cyt} peak appeared to be significantly greater in thermotolerant (pretreated at 30°C) plants, as compared with plants that have undergone no pretreatment (compare Fig. 6, a with b). This was confirmed by examining the average [Ca²⁺]_{cyt} responses of a number of pretreated and non-pretreated plants. Figure 6c shows average results from 10 thermosensitive (non-pretreated) and 10 thermotolerant (pretreated) plants. Peak heights are shown, which have been calibrated to normalize for the amount of active aequorin (Knight and Knight, 1995) thus giving a value that relates to the total calcium response (Knight and Knight, 2000). Thermotolerant (pretreated) plants generated significantly larger calcium peaks on initiation of recovery than thermosensitive plants (Fig. 6c). This suggests that whereas calcium signaling may play a role in

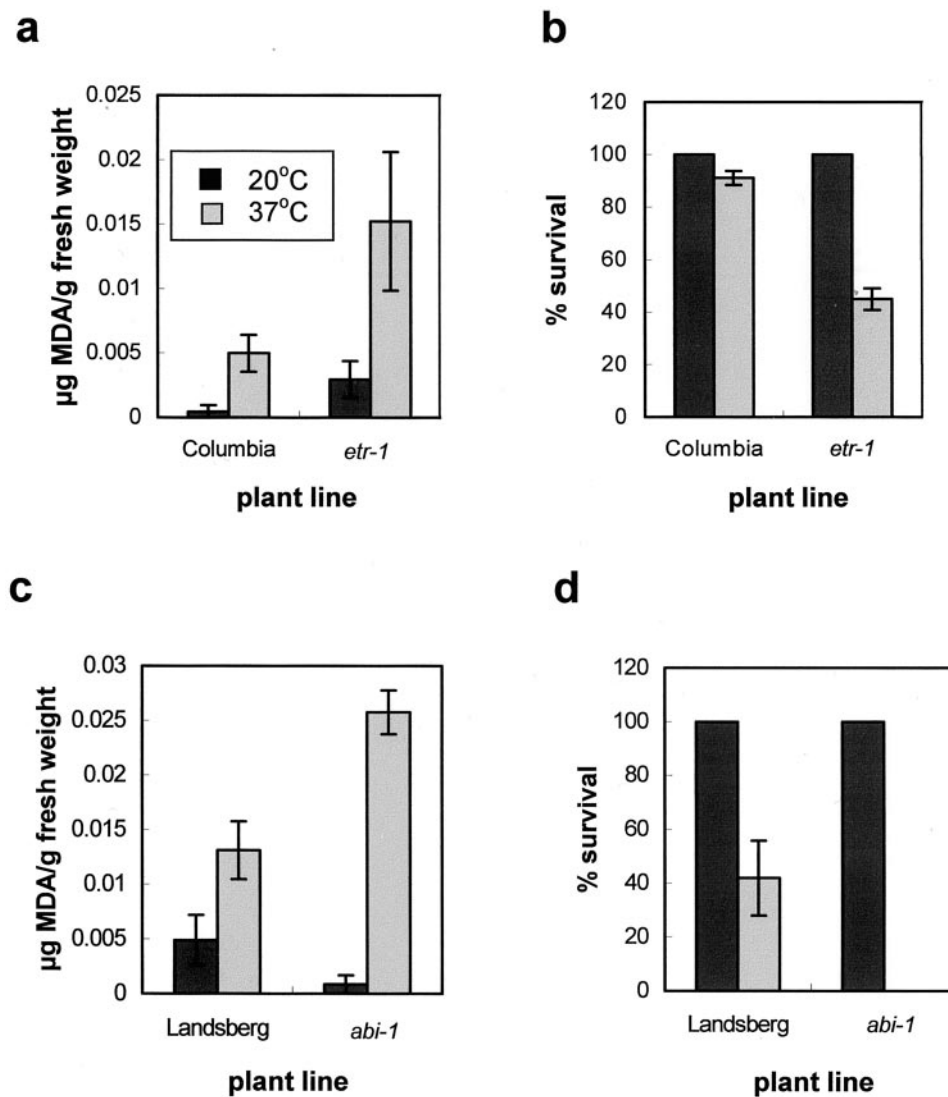


Figure 4. Effect of the *etr-1* and *abi-1* mutations on oxidative damage and survival in response to heating in Arabidopsis seedlings. Graphs showing levels of TBARS (a and c) and survival (b and d). Plants were given a 37°C treatment for 1 h. Graphs indicate TBARS levels (a and c) and survival (b and d) 3 d after the end of the 37°C treatment. Samples treated at 37°C (gray bars) were compared with controls kept at 20°C throughout (black bars). The ethylene-insensitive mutant, *etr-1*, was compared with its background ecotype, Columbia (a and c), and the ABA-insensitive mutant, *abi-1*, was compared with its background ecotype, Landsberg *erecta* (b and d). Data shown are averages of five replicates. Error bars represent SE of mean for these five replicates.

recovery from heating, rather than acting in response to heat itself, it is nonetheless affected by events that occur during heating.

Effect of Exogenous Calcium, Calcium Channel Blockers, and Calmodulin Inhibitors during Recovery from Heating

Calcium transients are seen in plants upon the onset of recovery from heat, but not during heat treatments themselves (Fig. 6). This suggests an important role for calcium specifically in the recovery period after heating. This hypothesis was further tested by repeating the TBARS and survival experiments described in Fig-

ure 2 but by only adding the treatment (inhibitor or calcium chloride) 15 min before the end of heating to specifically gauge the effect on the recovery. The plants then remained in the solution (or Murashige and Skoog medium for controls) for the first 6 h of recovery at 20°C phase. They were removed from the treatment and allowed to recover for 3 d on Murashige and Skoog plates, as before. The calcium and calmodulin inhibitors all reduced survival and increased oxidative damage at 35°C in these samples (Fig. 7, a and b). This temperature was used specifically so that any increased oxidative stress due to the chemical treatments could be observed (as a higher temperature, i.e. 40°C would have given a very high background levels

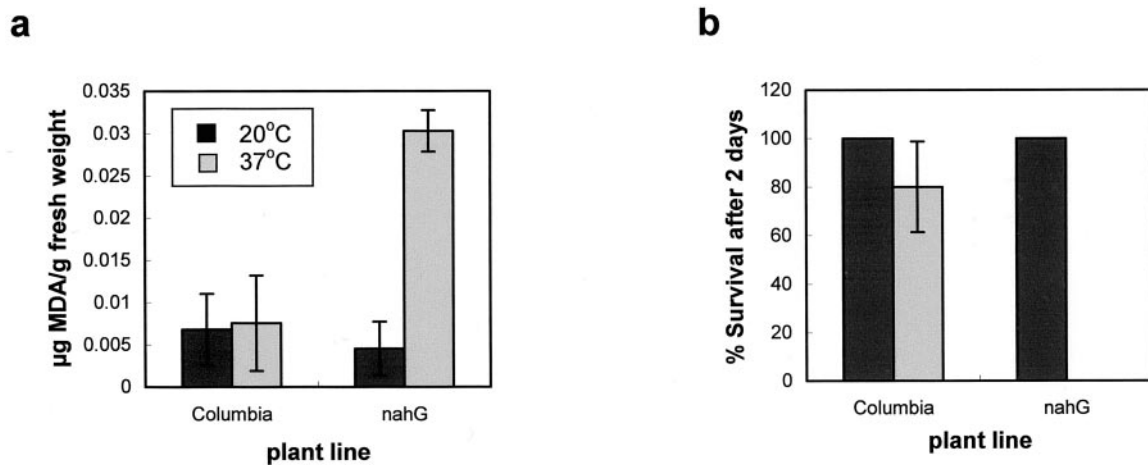


Figure 5. Effect of *nahG* expression on oxidative damage and survival in response to heating in Arabidopsis seedlings. Plants were given a 37°C treatment for 1 h. Graphs indicate TBARS levels (a) and survival (b) 3 d after the end of the 37°C treatment. Samples treated at 37°C (gray bars) were compared with controls kept at 20°C throughout (black bars). The transgenic Arabidopsis line *nahG* was compared with its background ecotype. Data shown are averages of five replicates. Error bars represent SE of mean for these five replicates.

of TBARS and mortality). Addition of exogenous calcium chloride increased survival and decreased TBARS (Fig. 7, c and d). This gives further credence to the concept that calcium is involved in pathways switched on during recovery from heat.

DISCUSSION

Our data shows that oxidative damage occurs in Arabidopsis plants after heating, and that the levels of damage increases over 3 d post heating (Fig. 1a). This concurs with work described by Gong et al. (1998a) in which they used the same assay in maize.

Pretreatment with moderate temperature before a higher temperature treatment has been shown to induce thermotolerance in a number of species (e.g. Howarth and Ougham, 1993; Dat et al., 1998; Lopez-Delgado et al., 1998). We found that thermotolerant Arabidopsis plants (plants that had undergone a 30°C pretreatment) experienced lower levels of oxidative damage during recovery from heating at 40°C as compared with thermosensitive (non-pretreated plants; Fig. 1a). This correlated also with a greater survival rate for these plants (Fig. 1b). This suggests that at least one aspect of the development of the thermotolerant phenotype in Arabidopsis is an increased ability to either prevent or repair heat-induced oxidative damage.

Heat-induced oxidative damage in Arabidopsis is light-mediated (Fig. 1c). Photooxidative damage has been observed after a wide range of stresses (e.g. Foyer et al., 1994; Harndahl et al., 1998; Lu and Zhang, 1999). In particular, oxidative damage has been previously observed in heat-treated maize seedlings (Gong et al., 1998a), with treatments undertaken in light conditions. Reduced levels of photosynthesis in stressed pea plants has been shown to

result in light absorption by antenna molecules in excess of that which can be dissipated by electron transport, which results in photoinhibition and damage to the electron transport system (Havaux et al., 1991). Even under optimal conditions, damaging active oxygen species are synthesized at very high rates from electron transport chains involved in respiration and photosynthesis (Noctor and Foyer, 1998). Once damage is done to the photosystems, the production of these potentially damaging molecules increases (Noctor and Foyer, 1998), and these are the likely cause of the light-dependent, heat-induced oxidative damage that we observed.

Levels of oxidative damage measured generally correlated with the ultimate survival of the plants. The two variables are not completely linked, however. Although in all experiments death occurred in plants that had higher levels of TBARS than in plants that survived, the percentage survival did not always vary exactly with the level of TBARS. In Figure 1, for example, the survival rate after 1 d for pretreated plants was more than double that for non-pretreated plants, but the level of TBARS was identical in both sets of plants. This suggests that although survival after heat stress requires an ability to tolerate or repair oxidative damage, it also requires an ability to tolerate or minimize other kinds of heat-induced damage. Heat is known to damage most parts of the cell, and it affects most cellular processes (Munro and Pelham, 1985; Karim et al., 1999). Photosynthesis is severely affected by temperature changes (Karim et al., 1999), as are transcription and translation (Bond, 1988; Bendena et al., 1989; Munro and Pelham, 1985). Some of this damage is likely to be caused directly by the temperature changes itself, as opposed to being caused by heat-induced oxidative stress.

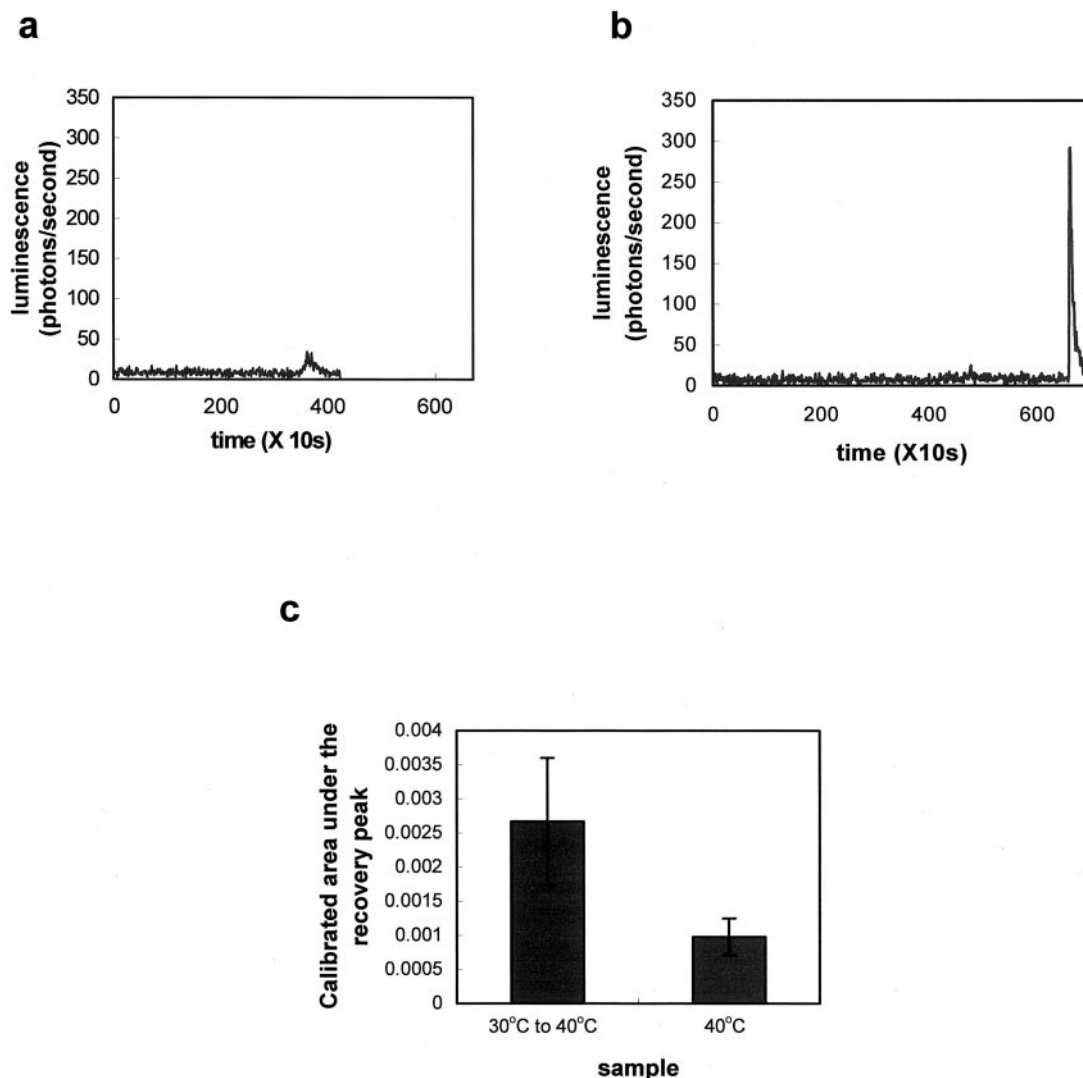


Figure 6. Cytosolic calcium responses of Arabidopsis seedlings in response to heating and recovery from heating. Photon counts emitted by individual plants heated either straight to 40°C for 1 h from 20°C (a) or to 40°C for 1 h after 30°C for 1 h (b). Time = 0 represents start time for heating (40°C [a] or 30°C [b]). Return to 20°C after heating occurred at 3,600 s (a) and 7,200 s (b). Traces are representative of the two different types of responses observed. The amount of reconstituted aequorin in each seedling was then measured and used to calibrate these measurements, and averages are presented (c) as total area under the calibrated peak, which is proportional to the $[Ca^{2+}]_{cyt}$ response in each case. Error bars represent SE of mean over the 20 seedlings in each replicate.

Evidence has previously been presented for links between calcium, membrane leakage of electrolytes, and thermotolerance in maize coleoptiles (Gong et al., 1998a). Calcium/calmodulin inhibitors reduced the survival of Arabidopsis plants after a mild heat treatment and increased the oxidative damage caused by the heat (Fig. 2). The development of thermotolerance (in terms of reduced TBARS levels and increased survival) in plants pretreated in calcium chloride before heating (Fig. 3) reinforced the idea that calcium might be required in some signaling pathway in Arabidopsis leading to improved survival. This suggests that a flux of calcium ions is required to switch on some mechanism by which

plants prevent or repair oxidative damage caused by heating, and thus a calcium flux is required for the plant to survive even mild increases in temperature. We examined the nature of this calcium flux (Fig. 6) as discussed below.

This calcium-dependent pathway is presumed to act through calmodulin, as calmodulin inhibitors also reduced survival and increased oxidative damage after heating (Fig. 2). A role for calmodulin is supported by the fact that higher levels of calmodulin have been observed in thermotolerant maize cells than in those that are more sensitive to heat (Gong et al., 1997a). Higher calmodulin levels have also been linked to lower levels of heat-induced membrane

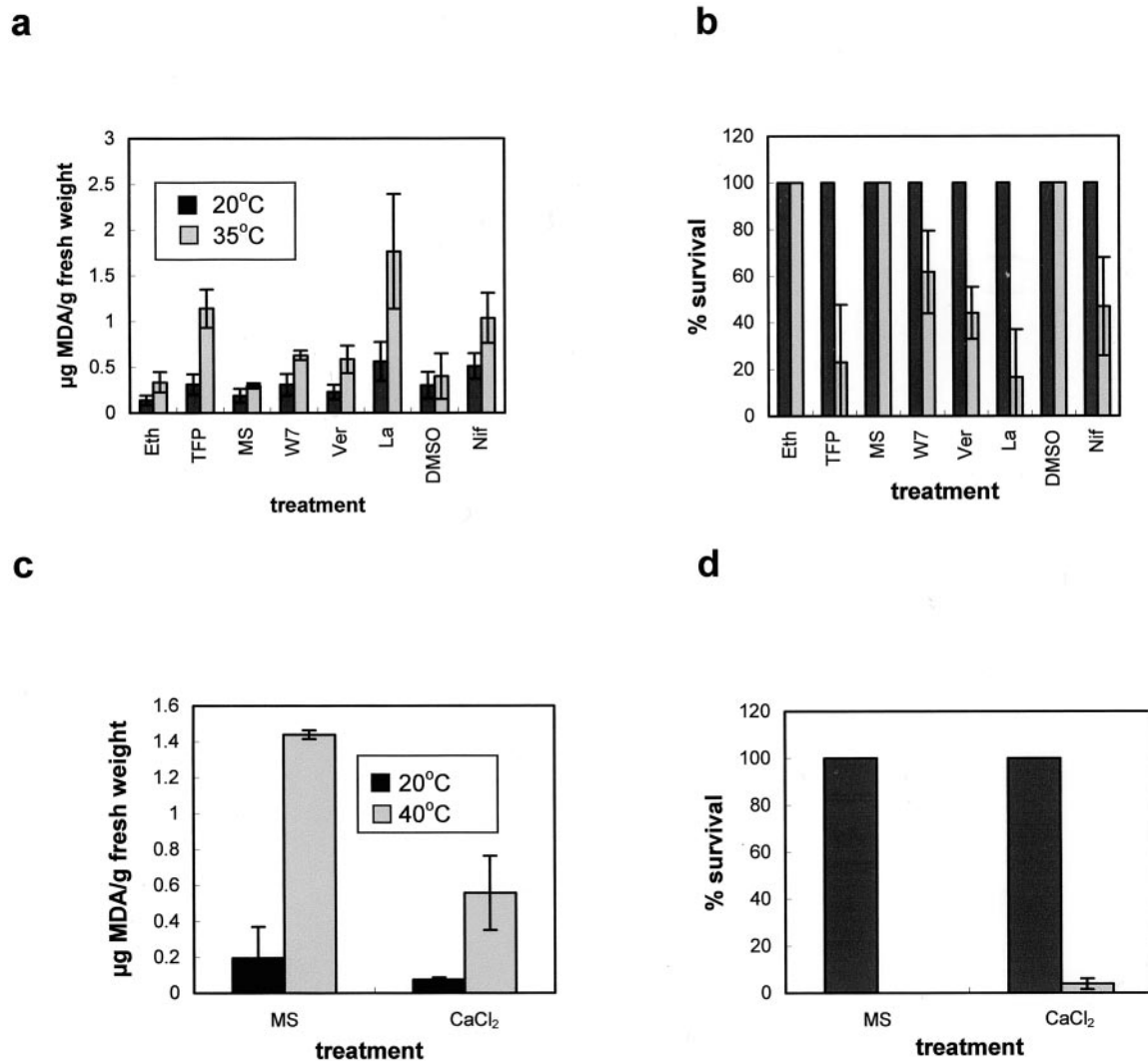


Figure 7. Effect of calcium, calcium channel blockers, and calmodulin inhibitors during recovery to heating. In a and b, plants were treated with calcium channel blockers (TFP, verapamil, lanthanum chloride, or nifedipine) or calmodulin inhibitors (TFP or W7) 15 min before return to 20°C after a 1-h 35°C treatment. Graphs indicate TBARS levels (a) and survival (b) 3 d after the end of the 35°C treatment. Plants were left exposed to the antagonists for 6 h after the end of heating. Samples treated at 35°C (gray bars) were compared with controls kept at 20°C throughout (black bars). TFP was dissolved in ethanol and nifedipine in DMSO, whereas all other inhibitors were dissolved in water in Murashige and Skoog medium, and appropriate controls are presented. In c and d, plants were pretreated with calcium chloride 15 min before return to 20°C after a 40°C treatment. Graphs indicate TBARS levels (c) and survival (d) 3 d after the end of the 40°C treatment. Samples treated at 40°C (gray bars) were compared with controls kept at 20°C throughout (black bars). Data shown are averages of five replicates. Error bars represent SE of mean for these five replicates.

damage in maize (Gong et al., 1997a). These experiments showed that pretreatment with calmodulin inhibitors increased levels of oxidative damage after heating in maize seedlings, as was shown here for Arabidopsis.

In addition to calcium, ABA, SA, and ACC (a precursor of ethylene) all also induced some degree of thermotolerance (Fig. 3). Thus plants given these pretreatments showed reduced oxidative damage in recovery from heating (Fig. 3a) and a greater survival rate at 40°C (Fig. 3b) than plants given no pretreatment. This suggests that these molecules may switch

on pathways that result in prevention of oxidative damage or repair of that damage.

To determine whether or not these pathways were actually used by Arabidopsis in vivo to protect themselves against heat-induced damage, we examined the response of *etr1* mutant and transgenic plants. We used the *etr1* mutant, which is unable to perceive ethylene (Sopory and Munshi, 1998), the *abi1* mutant, which is insensitive to ABA (Leung et al., 1994), and transgenic plants (*nahG*) expressing the bacterial salicylate hydroxylase gene, which causes break down of SA as soon as it is formed (Delaney et al., 1994). All

three plant lines showed increased TBARS and reduced survival at 37°C as compared with wild-type plants (Figs. 4 and 5), providing genetic evidence to support the hypothesis that ethylene, ABA, and SA are truly used by Arabidopsis plants to mediate protection against, or repair of, heat-induced oxidative stress.

It is worthy to note that the Arabidopsis ecotype Landsberg *erecta*, in which the *abi1* mutation is resident, is less thermotolerant than either Columbia or RLD1. At 37°C, typically 80% of wild-type Columbia (Fig. 4b) or RLD1 (data not shown) plants survived, whereas in Landsberg *erecta* the value is closer to 40% (Fig. 4c). Landsberg plants also show higher levels of TBARS after heat stress than Columbia (compare Fig. 4, a with c, experiments performed at the same time) and RLD1 (data not shown). Thus, it is possible that the antioxidant systems in these plants do not up-regulate to the same extent as in other ecotypes, resulting in a lower level of thermotolerance.

As shown in Figure 6, no significant cytosolic calcium elevation was seen at the initiation of or during 40°C treatment. This is in contrast to the work of Gong et al. (1998b) in which aequorin-expressing tobacco cotyledons were shown to luminesce during heat stress. Several explanations for this disparity are possible. It is possible that tobacco plants respond to heat stress in a different manner to Arabidopsis, especially as very young tobacco seedlings were used by Gong et al. (1998b). This seems unlikely because all of the heat stress responses characterized so far (predominantly the expression of HSPs) have been highly conserved between even highly divergent species (Lindquist, 1980). The second possibility relates to the way in which $[Ca^{2+}]_{cyt}$ was measured by Gong et al. (1998b). These authors heated plants in cuvettes in a water bath, then took them out of the water bath periodically to place in a luminometer to measure $[Ca^{2+}]_{cyt}$ at room temperature. This means that $[Ca^{2+}]_{cyt}$ was measured during/after cooling from the temperature in the water bath down to room temperature. This cooling will have profound effects on $[Ca^{2+}]_{cyt}$ as described below, which would lead to the erroneous conclusion that heat causes increase in $[Ca^{2+}]_{cyt}$ levels. A third explanation could be that heating does induce some calcium flux, but that the calcium flux is below the background level in our experiments, or very localized (cellularly or subcellular).

Although no peak in luminescence was observed during heating, a significant calcium transient was detected immediately upon initiation of cooling (Fig. 6, a and b). This peak was reliably detected within seconds of the initiation of cooling, and its magnitude was affected by events before heating, namely the peak was higher in thermotolerant plants as compared with those that received no pretreatment (Fig. 6). This suggests a potential role for calcium signal-

ing not during heating itself, but in triggering some process that occurs immediately upon initiation of recovery. This also provides a possible explanation for the tobacco result (Gong et al., 1998b). The increases in $[Ca^{2+}]_{cyt}$ -dependent luminescence above background may well have been due to cooling from high temperatures, i.e. the recovery $[Ca^{2+}]_{cyt}$ peak we observe (Fig. 6), rather than because of heat itself.

Price et al. (1996) have reported links between calcium signaling and oxidative stress in tobacco plants—oxidative stress induces calcium transients. Thus the cooling-induced calcium peak may be involved in prevention of oxidative damage during recovery from heating. Adding calcium chloride or calcium/calmodulin signaling antagonists to plants just before the recovery phase but not throughout the heating phase itself increased survival and decreased TBARS, whereas the antagonists all increased TBARS and reduced survival (Fig. 7). This supports the hypothesis that calcium signaling plays at least some role in limiting heat-induced oxidative damage during the recovery phase from heating specifically.

The increase in survival due to addition of calcium chloride during recovery only was not as great as when calcium was added before heating (compare Fig. 3a with 7c). That is, the increase in survival in recovery only samples was only just significant, whereas the increase was around 4-fold in samples treated before initiation of heating. There are two possible explanations for this. It is possible that the calcium chloride had not penetrated the cells of the plant as thoroughly in the recovery only samples as it had in those plants soaked throughout heating and for 1 h before heating. Thus, added calcium may not be present at the site required in the cell to protect against oxidative damage, when presented to the plants for a shorter time. The difference between soaking for 2 h as compared with merely the last 15 min of heating makes this hypothesis plausible. Alternatively, there may also be a role for calcium during the heating process itself, although no visible calcium transient was seen in this time. A role for calcium during the heating phase is supported by the fact that calcium/calmodulin antagonists had a greater effect when presented before heating, than when presented at recovery (compare Fig. 2 with 7). However, this could again be due to accessibility, meaning that a greater effective dose is achieved when the antagonists are added before heating, having a greater effect on the recovery processes. As discussed above, there may be a $[Ca^{2+}]_{cyt}$ transient so small or so localized that it was invisible using the recombinant aequorin technique, but still causing significant effects on survival.

Taken together, the data presented here suggest that there are roles for ethylene, ABA, SA, and calcium in protection against, or repair of, heat-induced oxidative damage in Arabidopsis. Both inhibitor data and genetic data suggest that ethylene,

SA, and ABA are actually used by *Arabidopsis* in vivo to protect against heat-induced oxidative stress. Evidence is presented for the hypothesis that calcium signaling plays an important role in pathways induced during recovery from heat shock. Inhibitor data suggests that this requirement for calcium acts through calmodulin. The future challenge will be to determine what signaling pathways these four components are involved in, and identify other signaling components in these pathways leading to protection against heat-induced oxidative stress in *Arabidopsis*.

MATERIALS AND METHODS

All chemicals used were obtained from Sigma (St. Louis), except for coelenterazine, which was obtained from Molecular Probes (Eugene, OR).

Plant Material and Growth Conditions

For most experiments, plants used were *Arabidopsis* ecotype RLD1 (Lehle Seeds, Roundrock, TX). For calcium measurements, the transgenic line RLD1.1 (Polisensky and Braam, 1996) constitutively expressing aequorin (Knight et al., 1991) was used. The ethylene mutant *etr-1* and the *nahG* transgenic line are in the Columbia background, so experiments involving these used wild-type Columbia controls. The ABA mutant *abi-1* is in the background Landsberg *erecta*, and so experiments involving this mutant used wild-type Landsberg *erecta* control. Seedlings were grown on Murashige and Skoog medium (0.8% [w/v] agar) at 20°C with a 16-h photoperiod as described previously (Knight et al., 1999). Seeds were sterilized in 70% (v/v) ethanol for 5 min, dried on filter paper, and vernalized on Murashige and Skoog plates at 4°C for 2 d before growth for 10 d.

Heat and Chemical Treatment of Plants

For most experiments, seedlings were heated in 1 mL of Murashige and Skoog medium (or pharmacological inhibitor/second messenger in Murashige and Skoog medium) in a heat block. This ensured constant exposure to inhibitors/messengers and prevented dehydration during heating. In detail, 0.5 g of seedlings were removed from Murashige and Skoog plates and placed in 1 mL of Murashige and Skoog medium in a 1.5-mL microfuge tube 1 h before the experiment. The plants were returned to the growth cabinet during this time. Pharmacological inhibitor/second messenger was added and then added in a 10- μ L volume, to make up the appropriate final concentration, as required. Final concentrations used were 10 mM lanthanum (III) chloride, 200 μ M nifedipine, 10 mM verapamil, 200 μ M W7, 200 μ M TFP, 10 mM calcium (II) chloride, 100 μ M ACC, 100 μ M ABA, and 10 μ M SA. All stock solutions were made up in water apart from TFP and ABA (ethanol) and nifedipine (DMSO). Controls consisted of plants to which 10 μ L of the appropriate solvent was added and treated in the

same way as test plants. The plants were then placed in the growth room again for another hour before heating. For heating, the tubes were placed in a heat block set to the appropriate temperatures. After heating, plants were carefully removed from the microfuge tubes and placed on Murashige and Skoog plates. These were returned to the growth cabinet and allowed to recover for up to 3 d. Four different heating regimes were used for different experiments. For experiments examining the acquisition of thermotolerance by a heat pretreatment plants were heated at 40°C for 1 h, after a pretreatment for 1 h at either 30°C or 20°C. These temperature regimes were also used for calcium measurements (see below). To examine the effects of SA, calcium, ABA, and ACC on thermotolerance, plants treated with these compounds (as described above) were treated for 1 h at 40°C or 20°C (as control). To investigate the effect of pharmacological inhibitors or gene mutations/*nahG* plants were either treated for 1 h at 35°C/37°C or 20°C (as control). These temperatures (lower than 40°C) were used to allow some survival (as there was none at 40°C) and obtain lower TBARS (as these were high at 40°C) in the controls, so that the effects of the inhibitors or mutations/*nahG* on these parameters could be measured.

In experiments examining the effect of pharmacological inhibitor/second messengers specifically on the recovery phase, no chemical treatments were applied before heating. Instead 10 μ L of the appropriate solution (as described above) was added 15 min before the end of the heating period, and the plants were left in the microfuge tube in this solution for 6 h in the growth room after heating. Solvent controls were set up as described above, and treated in the same way as test samples. The plants were then removed from solution and placed on Murashige and Skoog plates, which were then placed in the growth room to complete the recovery period. After heat and chemical treatments, TBARS and survival assays were performed.

TBARS Assays

TBARS assays were performed on 0.5 g of seedlings following the method of Heath and Packer (1968). Seedlings were frozen in liquid nitrogen, ground in a 1.5-mL microfuge tube using a micropestle and then 0.5 mL of 0.5% (w/v) thiobarbituric acid in 20% (w/v) trichloroacetic acid and 0.5 mL of buffer (175 mM NaCl in 50 mM Tris-HCl, pH 8) was added. The samples were then heated to 95°C for 25 min in a 1.5-mL microfuge tube and then spun down in a microcentrifuge at full 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 was calculated using an extinction coefficient of 155 $\text{mM}^{-1}\text{cm}^{-1}$, in agreement with a standard curve relating malonaldehyde concentrations to absorbance (data not shown).

Plant Survival

Percentage survival was counted for samples treated as described above. Seedlings on Murashige and Skoog recovery plates were assessed after up to 3 d of recovery.

In Vivo Reconstitution of Aequorin and Calcium-Dependent Luminescence Measurements

Aequorin measurements were performed using an intensified CCD camera (model EDC-02; Campbell et al., 1996), with camera control unit (HRPCS-2) and image acquisition and processing software (IFS216), all from Photek (St. Leonards-on-Sea, UK) as described previously (Knight and Knight, 2000). Reconstitution of the calcium-sensitive photoprotein aequorin was performed *in vivo* by taking twenty 10-d-old seedlings from Murashige and Skoog plates, and placing them each in different wells of a 24-well plate in 1 mL of distilled water. Ten microliters of 1 mM coelenterazine in methanol was added to each well, and the plants were kept at 20°C in the dark overnight. After this treatment, the plants were placed in individual wells of another 24-well plate containing 0.5 mL of Murashige and Skoog medium, which was placed inside a light-tight box connected to the photon counting camera. Real-time measurements of aequorin luminescence were then taken. The camera dark box contained a Peltier element (IPD-PC) in its base, directly under the 24-well plate, the plate fitting directly onto the element, and was used to change the temperature of the wells. A thermocouple probe was placed in one of the wells to ensure that the temperature changes recorded by the Peltier element was that experienced by the plants. The plants were allowed to rest for 5 min at 20°C after placing in the dark box. Then the Peltier element was set to the appropriate temperature, and the plants remained under the camera throughout the heating regime. Plants were either heated directly to 40°C for 1 h or heated to 30°C for 1 h and then the temperature was increased to 40°C for a further hour. At the end of the heat treatment, the Peltier element temperature was reduced to 20°C and the plants were left for a further 15 min. The seedlings remained in the dark box with the photon counting camera recording emitted photons throughout the experiments.

To calibrate the heights of the peaks to the amount of reconstituted aequorin left in the plant, remaining aequorin was discharged by adding 1 mL of 2 M CaCl₂, 20% (v/v) ethanol to each plant, after removal from the camera, in a luminometer cuvette containing 1 mL of distilled water (Knight and Knight, 1995). The rate constant of aequorin *i.e.* the rate divided by the maximum rate is proportional to calcium concentration (Knight and Knight, 1995). Calculation of rates of luminescence divided by total possible luminescence provides this rate constant, and this calculation can be used to compare differences in levels of free calcium (Knight and Knight, 2000). Total luminescence was measured using a digital chemiluminometer consisting of a 9829A photomultiplier tube with a 1.5-kV potential from a FACT50 air-cooled thermoelectric housing and an AD2 amplifier/discriminator (all from Thorn EMI, Ruislip, UK)

to produce a numerical output that was stored on a personal computer. Area under the peaks produced from *in vivo* calcium measurement experiments was divided by the discharge value to correct for potential differences in aequorin levels and allow comparison of calcium responses between treatments.

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