Heat stress induces an array of physiological adjustments that facilitate continued homeostasis and survival during periods of elevated temperatures. Here, we report that within minutes of a sudden temperature increase, plants deploy specific phospholipids to specific intracellular locations: phospholipase D (PLD) and a phosphatidylinositolphosphate kinase (PIPK) are activated, and phosphatidic acid (PA) and phosphatidylinositol 4,5-bisphosphate (PIP$_2$) rapidly accumulate, with the heat-induced PIP$_2$ localized to the plasma membrane, nuclear envelope, nucleolus and punctate cytoplasmic structures. Increases in the steady-state levels of PA and PIP$_2$ occur within several minutes of temperature increases from ambient levels of 20–25°C to 35°C and above. Similar patterns were observed in heat-stressed Arabidopsis seedlings and rice leaves. The PA that accumulates in response to temperature increases results in large part from the activation of PLD rather than the sequential action of phospholipase C and diacylglycerol kinase, the alternative pathway used to produce this lipid. Pulse-labelling analysis revealed that the PIP$_2$ response is due to the activation of a PIPK rather than inhibition of a lipase or a PIP$_2$ phosphatase. Inhibitor experiments suggest that the PIP$_2$ response requires signalling through a G-protein, as aluminium fluoride blocks heat-induced PIP$_2$ increases. These results are discussed in the context of the diverse cellular roles played by PIP$_2$ and PA, including regulation of ion channels and the cytoskeleton.

**Keywords:** heat stress, phospholipid signaling, phosphatidylinositol 4,5-bisphosphate, phosphatidic acid.
Two perspectives on plant responses to high temperatures have been defined. Basal thermotolerance describes inherent resilience to upward shifts in prevailing temperatures, i.e. intrinsic heat-hardiness that permits a plant to survive temperatures above levels that support optimal growth. In contrast, acquired thermotolerance is a transient physiological adaptation to otherwise lethal high temperatures induced by a brief sub-lethal exposure to high temperatures or alternative inducers such as heavy metals (Larkindale et al., 2005a). Basal and acquired thermotolerance are linked, as events that occur during the early stages of heat stress the mechanisms that enable longer-term protective capacities. The best characterized of these is the dramatic re-setting of transcriptional and translational priorities that allows the synthesis of heat shock proteins (HSPs) that catalyse the refolding of heat-disrupted proteins and protect against thermal denaturation (Kotak et al., 2007; Nieto-Sotelo et al., 1999; Hong and Vierling, 2000, 2001).

The determinants of basal and acquired thermotolerance probably reflect the complex nature of damage caused by heat stress. Neither the details of these mechanisms nor their coordination is understood. However, it is now appreciated that their physiological and biochemical nature is quite diverse, with no single process underlying or controlling the response. A recent survey implicated signalling through ethylene and abscisic acid as critical in the establishment of basal and acquired thermotolerance, respectively. In contrast, salicylic acid, active oxygen species, a DNA repair helicase and heat shock transcription factors are implicated in both aspects of thermotolerance (Larkindale et al., 2005a,b). A further complication is that determinants of thermotolerance vary across developmental stages and show organ-specific differences.

One approach to this problem has been to characterize events that occur immediately after the onset of a heat stress, and thereby identify how the response develops. Heat-induced changes in the level of HSPs (see Larkindale et al., 2004; Liu et al., 2006a,b) develop over periods of several minutes, with perhaps more rapid increases for abscisic acid (Liu et al., 2006a,b). Increases in intracellular concentrations of free Ca$^{2+}$ (Liu et al., 2003) and inositol 1,4,5-triphosphate (IP$_3$) (Liu et al., 2006c) were detected by 1 min after the onset of heat stress. There have also been extensive reports of a role for phospholipid-based signalling in response to a variety of abiotic and biotic stresses, including cold shock, drought and pathogen attack (see Zonia and Munnik, 2006, for review). Remodelling of membrane phospholipids is characteristic of these responses, with changes reported in numerous signalling lipids, notably phosphatidyl inositol 4,5-bisphosphate (PIP$_2$) and phosphatidic acid (PA).

PIP$_2$ performs a variety of key functions well beyond its canonical role as a precursor of the signalling molecules IP$_3$ and diacylglycerol (DAG) (van Leeuwen et al., 2007). These include recruiting cytoskeletal and signalling proteins to the plasma membrane (PM), a role in membrane trafficking, and stimulation of phospholipase D (PLD). PIP$_2$ is thus able to relay upstream signals to effectors that mediate dynamic changes in cell structure and function in response to biotic and abiotic stresses. PA is also an important phospholipid signal (Munnik 2001; Wang et al., 2006), with a growing body of evidence suggesting that PA, rather than DAG, is the key mediator of the PLC signalling pathway in plants (Testerink and Munnik, 2005). The DAG produced by PLC-catalysed hydrolysis of PIP$_2$ is rapidly converted to PA by the action of diacylglycerol kinase (DGK). The PA produced by this pathway mediates a variety of stress responses (for review, see Zonia and Munnik, 2006). Alternative sources of PA are the structural lipids, with PA produced directly by hydrolysis of substrates such as phosphatidylethanolamine catalysed by PLD. PA produced by this route has also been implicated as a mediator of stress responses (for review, see Wang, 2005; Bargmann and Munnik, 2006).

The evidence implicating phosphoinositide signalling in the early events following the onset of heat stress, along with the rapidly growing literature on phospholipid signalling as a key component of stress responses, suggests that changes in the levels of signalling lipids are among the early consequences of heat stress. To test this possibility, we monitored membrane phospholipid levels in response to increases in temperature. We report here that heat stress leads to dramatic increases in PIP$_2$ and PA within 2 min after a heat stress, and that these changes are mediated by PIPK and PLD.

RESULTS

**PIP$_2$ and PA levels increase in response to heat stress at 40°C**

To determine whether phospholipid-based signalling patterns respond to heat stress, BY-2 cells were pre-labelled with $^{32}$P-orthophosphate for 3 h at 20°C prior to temperature treatments, thereby generating radioactive pools of ATP, PIP$_2$ and its phospholipid precursors that facilitate determination of changes in PIP$_2$ levels. Cells were transferred to a 40°C water bath and sampled at intervals of up to 40 min prior to extracting the lipids for analysis by TLC and phosphoimaging. Levels of the major structural phospholipids, including phosphatidylglycerol (PG), phosphatidylethanolamine (PE) and phosphatidylcholine (PC), as well as most of the lower-abundance phospholipids, remained unchanged over the time course (Figure 1a). Against this constant background, a dramatic increase in PIP$_2$ of more than five-fold is evident (Figure 1b). This was accompanied by a heat-induced increase in the level of PA as well. Steady-state levels of PA in BY-2 cells were quite variable, with the culture used in the experiment illustrated in Figure 1(a) containing relatively high levels under control conditions. Nonetheless, significant increases in relative levels of PA in response to...
heat stress were detected upon quantification of the autoradiograph (Figure 1b). Due to high pre-stress PA levels, the relative increase in this experiment was limited to approximately 0.2. Other experiments performed using cells with lower pre-stress PA levels show significantly greater fold increases in PA in response to heat (Figure 1c, and see Figure 3 below).

Given this response, we determined the temperature required to elicit increases in PIP$_2$ levels. After pre-incubation as described above, cells were treated at 25, 30, 35, 40 or 45°C for 20 min prior to extracting the lipids for analysis by TLC and phosphoimaging. Despite differences between experiments in terms of the absolute extent of the changes observed, the levels of PIP$_2$ expressed as a proportion of the total phospholipids always increased in response to the temperature increase. Whereas levels of this lipid remained unchanged in cells incubated at 30°C, increases were typically observed in treatments at 35°C and above (Figure 2). Changes in PIP$_2$ across the three typical experiments shown in Figure 2 range from about 3–6-fold after 20 min at 45°C.

Heat stress-induced PA is generated by phospholipase D

Experiments were performed to determine which of the two distinct routes that generate PA in plants is activated by heat stress. One occurs through the sequential activity of PLC, which cleaves PIP$_2$ to yield IP$_3$ and DAG, and DAG kinase, which phosphorylates the DAG generated in the previous step to generate PA at the expense of ATP. The other generates PA in one enzymatic step through PLD-catalysed transfer of the phosphatidyl group of a structural phospholipid to water, generating a free fatty acid and PA. PLD can also use primary alcohols as alternatives to water as acceptors of phosphatidyl groups, and such substrates can be included in cell incubation media as a means to monitor PLD activity. Therefore, 1-butanol was added so that changes in PLD activity could be monitored by assay of the non-biological lipid phosphatidylbutanol (PBut).

Figure 1. Heat stress triggers the rapid accumulation of PA and PIP$_2$ in suspension-cultured tobacco BY-2 cells.
(a) Cells were pre-labelled with $^{32}$P-O$_4$ for 3 h, and then incubated for the indicated times at 22 or 40°C. Lipids were then extracted and separated by alkaline TLC. An autoradiograph of a typical experiment is shown. (b) Quantification of PIP$_2$ and PA levels by densitometry of the autoradiograph shown in (a). The fold change was calculated relative to levels present at $t = 0$. (c) Summary of two experiments similar to that described in (a) after 30 min at 22 or 40°C (open and closed bars, respectively).

Figure 2. Heat stress induces accumulation of PIP$_2$ at temperatures of 35°C and above. BY-2 cells were labelled with $^{32}$P-O$_4$ for 3 h at ambient room temperature (about 21°C) prior to temperature treatments. After this pre-incubation, cells were treated at 25, 30, 35, 40 or 45°C for 20 min, before stopping the labelling using perchloric acid and extracting the lipids for analysis by TLC and phosphoimaging. Using phosphoimaging, the proportion of radioactivity in $^{32}$P-PIP$_2$ relative to that in all phospholipids was calculated. These proportions are expressed relative to that in cells maintained at 25°C. The results of three separate experiments are shown.
Immediately prior to their transfer to 20 or 40°C, 1-butanol (0.5%) was added to pre-labelled BY-2 cells in order to assay for PLD activity during heat stress. Under these conditions, increases in levels of PIP<sub>2</sub> and PA occur as observed in the absence of 1-butanol (Figure 3a,c). Although cells incubated at 20°C exhibit only trace amounts of PBut, the levels of this lipid increased several-fold within 2.5 min at 40°C (Figure 3b,c). Levels increased dramatically during subsequent incubation at 40°C, demonstrating that acute heat stress leads to rapid PA accumulation in cellular membranes, at least in part through activation of a PLD pathway.

**Heat stress generates PIP<sub>2</sub> through activation of PIPK**

Two paths potentially contribute to the increase in PIP<sub>2</sub> observed with the onset of heat stress – decreased turnover due to inhibition of PLC or phosphatase activity, and increased synthesis resulting from increased PIPK activity. We assayed the contribution of PIPK using a pulse–chase labelling procedure (Munnik et al., 1994). The 3 h labelling experiments described above label pools of ATP and structural phospholipids with radioactive phosphate, thus permitting detection of new lipid species through either processing of a labelled lipid precursor or transfer of 32P-phosphate from labelled ATP to lipid acceptors through the action of a lipid kinase. Here, we pulse-labelled BY-2 cells for 2 min with 32P<sub>P</sub>, and followed by a chase in the presence of excess unlabelled phosphate at either 20 or 40°C. During the 2 min pulse, 32P<sub>P</sub> is mainly incorporated into the ATP pool, thereby increasing during the subsequent chase period the proportion of lipids labelled by direct kinase activity at the expense of those generated by the inter-conversion of previously labelled lipids. For example, there was a high ratio of labelled PIP (which gains its phosphate through the action of PI kinase) to PI in the pulse–chase experiment (Figure 4a); in contrast, these lipids display comparable levels of labelling after a 3 h pre-incubation with 32P-phosphate (see Figure 3a). Thus, while some labelling of structural lipids occurs during the pulse and subsequent chase periods, lipids that acquire phosphate through the action of a kinase represent a significantly greater proportion of the labelled lipids than they do after longer-term labellings.

As shown in Figure 4, PIP<sub>2</sub> levels increase dramatically in heat-stressed, 2 min-labelled cells despite the absence of large-scale synthesis of structural lipids (compare the labelling extent of PG, PE, PC and PI in Figures 3a and 4a). PIP<sub>2</sub> levels are clearly higher after 2 min at 40°C, with an increase of more than 7.5-fold after 8 min at the elevated temperature (Figure 4b). These data strongly indicate activation of a PIP kinase as an early response to heat stress.

Figure 4(a) also shows the synthesis of structural lipids, including PG, PE, PC and PI during the chase period. As cytoplasmic pools of nucleoside triphosphates become labelled during the pulse, the accumulation of 32P in these species probably represents the normal functioning of lipid biosynthetic pathways. Note the similarity in the rate at which radioactivity accumulates in these lipids at 20 and 40°C (Figure 4a). It thus appears that general aspects of lipid metabolism are largely unaffected by the acute temperature stress. We have also observed that cell viability during a 30 min incubation at 40°C remains unchanged as monitored by labelling with fluorescein diacetate (data not shown).

Significant levels of PA are labelled during the 2 min pulse, presumably through the sequential action of PLC and DAG kinase. In unstrained cells, and in contrast to the steady

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**Figure 3.** Phospholipase D activity increases within 5 min of the onset of temperature stress. BY-2 cells were incubated as described in Figure 1, and then maintained either at 20 or 40°C for 30 min. At the indicated time points, incubations were stopped, and the lipids were extracted and analysed by TLC and phosphoimaging.

(a) Alkaline TLC profile.

(b) EtAc TLC profile showing PLD activity in response to temperature stress as assayed by the accumulation of phosphatidylbutanol (PBut).

(c) Quantification of PIP<sub>2</sub>, PA and PBut levels in the control- and temperature-stressed cells.

For definitions of abbreviations, see legend to Figure 1.
increase in levels of structural lipids, the PA pool exhibits significant turnover during the subsequent 32 min, with a substantially diminished proportion of the initial radioactivity present at the conclusion of the chase (Figure 4a). It thus appears that rapid turnover of PA contributes to its low levels in unstressed cells. In contrast, the amount of labelled PA is more stable in cells incubated at 40°C. Although the relative contributions of changes in synthesis (through PLC and DAG kinase) and turnover rates that lead to the observed stability cannot be distinguished by these experiments, it is clear that the dynamics of PA metabolism are dramatically influenced by heat stress.

**PIP2 and PA levels increase in Arabidopsis and rice seedlings in response to heat stress**

To determine whether the response to heat stress observed in cultured tobacco BY-2 cells also occurs in plant tissue, experiments similar to those described above were performed in Arabidopsis seedlings and rice leaves. Material was labelled overnight (16 h) with 32P-orthophosphate, and processed further as described for the BY-2 cells. Consistent with the results for the cell culture system, both Arabidopsis seedlings and rice leaves responded to heat stress with statistically significant increases in both PIP2 and PA after 30 min at 40°C (Figure 5a,b). A longer exposure of the TLC provides a clearer image of the extent of the PIP2 increase that occurs upon the onset of heat stress (Figure 5a, lower panel).

**YFP–PHPLC1 re-localizes in response to heat stress in tobacco BY-2 cells**

The experiments described above demonstrate that plant cells respond to heat stress with increases in PIP2 levels. As we have recently shown that PIP2 can be imaged in living tobacco BY-2 cells stably expressing the PIP2-specific biosensor YFP–PHPLC1 (van Leeuwen et al., 2007), we used these cells to monitor the effect of heat stress on the localization of YFP–PHPLC1. BY-2 cells expressing YFP alone served as controls. To highlight the PM, FM4-64 was added to a final concentration of 2 μM immediately after the heat treatment. As shown in Figure 6, there was no change in the subcellular localization of YFP after 15, 30, 45 or 60 min of incubation at 40°C. In contrast, in the cells expressing YFP–PHPLC1, we observed a clear translocation of the PIP2 marker to various membranes (Figure 7). As soon as 15 min after incubation at 40°C, YFP–PHPLC1 fluorescence was observed at the PM (Figure 7, white arrow) and on punctate structures in the cytosol. Although the number of YFP–PHPLC1-labelled punctate structures increased between 15 and 60 min at 40°C, the labelling of the PM appeared transient, with its highest intensity during the first 30 min. Interestingly, after 30 min, we also observed YFP–PHPLC1 labelling of the nuclear envelope (yellow arrow in Figure 7d,e), accompanied by an increase in fluorescence in the nucleolus after 45 min of treatment (asterisk in Figure 7e).

**GTPase activity is required for heat-activated PIP2 increases**

PIP2 has been implicated in vesicular trafficking and organization of the actin cytoskeleton (Di Paolo and De Camilli, 2006; for review). As such, its synthesis and turnover must be tightly regulated both spatially and temporally. In animal cells, the PI and PIP kinases required for PIP2 synthesis are recruited to the PM and activated by a mechanism mediated by members of the Arf family of small GTPases (Martin, 2001). Aluminium fluoride (AlF3) has been widely used to provide initial evidence for the participation of GTPases in various cellular processes (Li, 2003; for review). Here, we tested its effect on heat-induced PIP2 increases. When applied as 100 μM AlCl3 plus 30 mM NaF to pre-labelled BY-2 cells, 30 min prior to heat stress, subsequent
increases of PIP2 levels at 40°C are completely inhibited (Figure 8a). AlF3 was also found to reduce the level of PA induced during temperature stress. Inhibition of the PIP2 and PA response required the presence of NaF, as AlCl3 on its own has no effect. In contrast, NaF without added AlCl3 was found to behave similarly to the NaF/AlCl3 combination, most likely as a result of contaminating aluminium ions (see Discussion). These data suggest that heat-
induced PIP2 and PA increases are mediated by a GTPase-requiring mechanism.

DISCUSSION

Here we demonstrate that heat stress elicits rapid deployment of well-characterized components of phospholipid-based signalling pathways. Through the activation of PLD and PIPK, heat stress induces rapid increases in the levels of PA and PIP2, with the PIP2 accumulating at the PM, nuclear envelope, nucleolus and punctate cytoplasmic structures. Although downstream consequences of these events remain to be determined, PA and PIP2 are known to

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**Figure 7.** Effect of heat stress on the localization of YFP–PHPLC d1.

Tobacco BY-2 cells stably expressing YFP–PHPLC d1 were incubated and imaged at room temperature (a), and after 15 (b), 30 (c), 45 (d) and 60 min (e) at 40°C. To resolve the PM, 2 μM FM4-64 was added immediately after the treatment. YFP fluorescence is shown in green, FM4-64 in red, and differential interference contrast imaging in grey. Arrowheads indicate YFP–PHPLC d1-labelled punctate structures, white arrows show PM labelling, yellow arrows indicate nuclear envelope labelling, and the asterisks indicate the nucleolus. Scale bars = 10 μm.

**Figure 8.** G-protein signalling is required for heat-induced PIP2 and PA increases.

BY-2 cells were pre-labelled for 3 h with [32P]PO4, and then treated at 22 or 40°C for 30 min in the presence of AlF, AlCl3 or NaF. Note the substantial increase in levels of PIP2 and PA after 30 min at 40°C in control and AlCl3-treated cells, and that the G-protein stabilizer AlFx blocks these increases. The similar inhibition seen in the presence of NaF is attributed to trace levels of aluminium in the samples (see text for discussion). AlFx refers to the mixture of AlF complexes that form upon mixing of AlCl3 and NaF in aqueous solutions.

For definitions of abbreviations, see legend to Figure 1.
act as key mediators of signalling pathways, membrane dynamics and cytoskeletal organization (for review, see Testerink and Munnik, 2005; Di Paolo and De Camilli, 2006; Wang et al., 2006).

Heat thus joins several other stresses, including osmotic, salt (Meijer et al., 2002), cold (Arisz et al., 2003), pathogen (William et al., 2005) and wounding (Lee et al., 1997; Wang et al., 2000), in activating these fundamental components (Meijer and Munnik, 2003; de Jong et al., 2004). Zhu (2002) warned that phospholipid signalling is a ‘double-edged sword’, with low levels of signalling molecules suggestive of specific responses, but high levels implying more generalized stress-induced damage. The heat-induced responses that we report are evident within 2 min of the onset of a sudden rise in temperature. Moreover, as they occur at temperatures as low as 35°C and without effects on cell viability, the phenomena most likely represent regulated early responses with specific downstream consequences rather than non-specific heat-induced damage. Although not thoroughly documented, heat-induced increases in PIP2 also occur in yeast (Desrivières et al., 1998; Audhya and Emr, 2003).

The hallmark of the heat response described here is a rapid increase in PIP2 and PA levels, accompanied by increases in IP3K and PLD activities. Other environmental factors such as cold (Welti et al., 2002; see Wang et al., 2006; for review), fungal pathogens (van der Luit et al., 2000) and rhizobial symbionts (den Hartog et al., 2001, 2003) also induce phospholipid signalling, but the particular characteristics of these responses vary widely. Although some of these differences may be due to the experimental protocols employed, this variation reflects at least in part modulation of signalling pathways to the particular needs of the plant in a stress specific manner. At present, it is too early to speculate about the nature of these differences, but it is noteworthy that distinct programs of lipid changes are induced by different abiotic and biotic stresses.

A sizable proportion of the PA that accumulates following the onset of heat stress results from the activation of PLD. This was demonstrated directly through the transphosphatidylation reaction with 1-butanol (Figure 3). Furthermore, the increase in radioactive PA levels in cells that were pulse-labelled for 2 min is dramatically less than that observed in cells that had been pre-labelled for 3 h. This discrepancy can be explained if the PA that accumulates during heat stress arises predominantly through the action of PLD. PLD generates PA from pools of structural phospholipids and these do not acquire significant levels of radioactivity during the brief labelling interval used in the pulse-chase procedure. The dynamics of the labelled PA pools in the pulse-labelled cells are distinct in the control and heat-shocked cell populations. Whereas PA synthesized during the 2 min pulse is turned over at a significant level at 20°C, the level of radioactivity in PA remains relatively constant at 40°C. This difference may reflect contributions to the PA pool from the PLC–DGK pathway or a decline in the turnover rate of PA at 40°C. The latter appears less likely as changes in the level of diacylglycerolpyrophosphate (DGPP), a metabolic product of PA (Munnik et al., 1998; van der Luit et al., 2000; van Schooten et al., 2006), were not seen during TLC. In either case, however, our data demonstrate that PA rapidly accumulates in the membranes of heat-stressed cells. The elevated PA levels may serve a variety of functions, including regulating membrane traffic and protein recruitment (Testerink and Munnik, 2005; Wang et al., 2006), cytoskeletal dynamics (Staiger and Blanchon, 2006), or the oxidative burst that follows pathogen attack and other stresses (de Jong et al., 2004; Park et al., 2004; Zhang et al., 2005; Chen et al., 2007).

The increase in PIP2 that occurs at the PM and nuclear envelope in heat-stressed cells suggests that heat stress has an impact on membrane dynamics. Indeed, we observed that heat stress reduces the extent of cytoplasmic streaming (data not shown), an effect that is potentially caused by lipid-induced re-organization of the actin cytoskeleton. Dramatic changes in membrane dynamics are further supported by the YFP–PHPLC51 imaging of PIP2 during the course of a heat-stress response (Figure 7). Within 15 min of the onset of exposure to 40°C, PIP2 appears at the PM and associated with as yet unidentified punctate structures in the cytoplasm. After 30–45 min, PIP2 predominates at the nuclear envelope and nucleolus, continues its association with cytoplasmic punctate structures, and shows a diminished presence at the PM. Our data do not enable us to distinguish whether these spatially distinct PIP2 pools arise through membrane trafficking or by site-specific synthesis.

PIP2 localization patterns adjust rapidly in response to a variety of stimuli. In guard cells, white light leads to PIP2 accumulation at the PM, but without the intense accumulation observed at the nuclear envelope and other intracellular sites (Lee et al., 2007). PIP2 is also re-distributed in response to osmotic stress, with an appearance at the PM 15 min after the onset of stress, followed by association with clathrin-coated vesicles (König et al., 2008). Despite considerable effort, these authors were unable to demonstrate any association of stress-induced PIP2 with the nucleus.

While nuclear-localized PI kinases have been reported in plants (e.g. Bunney et al., 1995, 2000), it is in yeast and animal cells that a nuclear PIP2 pathway has been documented in detail (for review, see Bunce et al., 2006; Irvine, 2006) and implicated in regulating the expression of genes involved in stress responses (Jones et al., 2006; Mellman et al., 2008). The PIP2 that we observed to accumulate in the nuclear envelope and nucleolus after a heat stress may thus be part of the machinery that serves to integrate transcriptional and post-transcriptional responses to heat stress.

It is now also known that IP3 generated in the nucleus by PLC-catalysed hydrolysis of nuclear PIP2 is metabolized to
inositol polyphosphates, and that these poly-anions play key roles in regulating gene expression in yeast, animals (Alcázar-Román and Wente, 2008), and perhaps plants, where IP6 is a co-factor of the auxin receptor TIR1 (Tan et al., 2007). In addition, nuclear phosphoinositide signalling probably plays a role in overall cellular integration. For example, cycling of a PI4P 5-kinase between the nucleus and the cytosol in yeast is essential to its role as a regulator of the cytoskeleton (Audhya and Emr, 2003). The complex temporal and spatial changes that we find in PIP2 in response to heat stress may be mediated in plants by a similar between nuclear and cytoplasmic compartments.

The inhibitor data reported in Figure 8 suggest that active cycling of a G protein is necessary to transduce the heat-initiated signal required for PIP2 and PA accumulation. AlF4− binds GDP-occupied Gs subunits of heterotrimeric G proteins and small monomeric G proteins mimicking a GTP γ-phosphate. In doing so, it stabilizes the G protein in an active, signalling-competent conformation. AlF4− thereby prevents both GDP release and continued cycling of the G protein (Li, 2003). The fact that AlF4− blocks the heat-induced PIP2 increase suggests that a downstream consequence of G protein cycling is required to activate or localize a PIPK appropriately during heat stress. A possible role for G proteins working in concert with membrane lipids in assembling functional modules at specific cell-surface locations such as pollen tubes and root hair tips (Kost et al., 1999; Dowd et al., 2006; Preuss et al., 2006) is an area of considerable current interest (for discussion, see Di Paolo and De Camilli, 2006; Kost, 2008).

A role for G protein-based signalling as a component of the response to heat stress is suggested by the finding by Misra et al. (2007) that over-expression of the two Gα subunits and the Gβ subunit of the heterotrimeric G protein of pea confers a degree of tolerance to elevated temperatures. Over-expression studies also support a role for a small G protein in salt and osmotic stress responses (Mazel et al., 2004). The increased G protein levels in these lines could support greater overall cycling rates. Our data show that it is the on-going cycling of a G protein and not simply the degree of activation that is required for the lipid remodelling that occurs in the wake of heat stress. As AlF4− stabilizes the active conformation of both monomeric small G proteins and the Gβ subunits of heterotrimeric G protein, it is not yet possible to distinguish which of these pathways mediates the increase in PIP2 that occurs during the onset of heat stress.

The observation that NaF is as active as AlF4− (Figure 8) is most likely a consequence of trace amounts of aluminium contamination in glassware or other sources. The exceptionally high affinity of Al for F suggests that active complexes of AlF4− form in the presence of trace amounts of the metal (Strunecká et al., 2002). When AlF4− is used in 2 min pulse-chase experiments, the structural lipid PIP accumulates label at rates similar to those for untreated controls (data not shown). AlFx appears to act selectively, as another nucleoside triphosphate-dependent reaction, the transfer of phosphate to PI catalysed by PK, continues unabated. Long-term incubation of plant cells with AlCl3 at the concentration used here (100 μM) induces cell death by activating apoptotic pathways, but these effects occur after 24 h in contact with the metal (Yakimova et al., 2007), rather than the 30 min pre-incubations employed here. Furthermore, the inhibition that we observe requires the presence of fluoride, and thus is not likely to be a secondary consequence of apoptotic pathway activity or effects such as aluminium inhibition of shaker-type K+ channels (Liu et al., 2005). We emphasize that the pharmacological effects reported here only suggest a role for G-protein signalling in heat-mediated activation of PIPK; alternative approaches are required to confirm this possibility.

As discussed above, the pulse–chase experiment suggests that, in addition to the participation of PLD, PLC is also implicated in early responses to heat stress. In mouse embryonic fibroblasts, a role for PLC-γ1 has been reported in heat-stress responses (Bai et al., 2002). Furthermore, we have found that the PLC inhibitor U73122 blocks the heat-induced increase in PIP2 levels (M.M. and T.M., unpublished results). Although non-specific effects of U73122 need to be ruled out, these results suggest that heat stress induces what appears to be futile cycling of PIP2, in which increases in its synthesis require its turnover catalysed by PLC. It has often been noted that what appear to be a futile cycle may in fact be a means to obtain greater control and specificity in dynamically regulated systems, especially for spatio-temporal control. Indeed, Balla (2005) has commented that the apparent futile cycling of the PIP2 phosphomonoester groups (the phosphates at positions 4 and 5) may be critical to their role in regulating the organization of signalling complexes at the PM. Furthermore, recent in vivo imaging coupled with computational analysis in an animal cell system concluded that, in order to generate sufficient levels of signalling metabolites, PIP2 synthesis and hydrolysis must occur together upon hormonal stimulation (Loew, 2007).

Sudden activation of phospholipid-based signalling pathways is consistent with reports on the role of calcium ions in heat-stress responses (Gong et al., 1998; Larkindale and Knight, 2002; Liu et al., 2006a,b,c). Heat shock releases calcium into the cytoplasm, where it appears to stimulate accumulation of heat shock proteins through a calmodulin-dependent mechanism (Liu et al., 2003, 2007). Although heat-dependent activation of PLC and the subsequent liberation of IP3 may provide a mechanism for calcium release, the signalling roles of PLC and PIP2 in vascular plants may differ in critical respects from the canonical pathway characterized in animal cells. For example, the 10–100-fold lower levels of PIP2 in plant membranes relative to those of
animals, compounded by the low turnover rate of PIP₂, argue strongly that this lipid is not capable of sustaining the IP₃ synthesis rates that are necessary for rapid mobilization of calcium stores (see van Leeuwen et al., 2007, for a detailed discussion). Furthermore, candidates for such central players in the pathway as the IP₃ receptor (see Krinke et al., 2007; for recent review) and protein kinase C, have not yet been identified in plants (Zonia and Munnik, 2006; van Leeuwen et al., 2007). However, IP₃ can be quickly converted into IP₆, and it is the latter compound that appears to be responsible for the release of Ca²⁺ from an intracellular store, rather than IP₃ (Lemtiri-Chlieh et al., 2003). The canonical IP₃ pathway is also absent from yeast, which lacks an IP₃ receptor and in which PLC-generated IP₃ is rapidly metabolized to IP₆ (Michell et al., 2003; Perera et al., 2004). In yeast, IP₆ signalling is not related to Ca²⁺, but directly regulates gene transcription and mRNA export from the nucleus. The pathway involves PLC and two inositol polyphosphate multi-kinases that can stepwise phosphorylate IP₃ to IP₆ (York et al., 1999, 2001; Odom et al., 2000).

Our data demonstrate that heat sensing in plants leads to rapid and specific changes in the spectrum of signalling phospholipids, and that these changes occur in both the cytoplasm and nucleus. As heat probably has immediate effects on the osmotic status of cells, and osmotic status has been shown to have highly selective effects on lipid signalling, osmotic effects may play a role in the early events that lead to the changes in levels and localization patterns of the signalling lipids we report here (see Zonia and Munnik, 2006; for a review, and Kung, 2005, for a discussion of the possible inter-relationships of heat stress, osmotic adjustment and ion channels).

Heat stress sets in motion a complex set of interconnected signalling responses. The data reported here demonstrate that increases in PIPK and PLD activities, accompanied by increases in IP₃ and PA levels, are among the earliest events following the onset of temperature stress. The particular members of the PIPK and PLD multi-gene families (11 and 12 isoenzymes, respectively, in Arabidopsis) that participate in the heat-stress response remain to be determined. More generally, the events that occur downstream of these lipid responses and their relationship to the heat shock protein apparatus are critical next questions.

**EXPERIMENTAL PROCEDURES**

**Plant material**

Suspension-cultured tobacco (*Nicotiana tabacum*) Bright Yellow-2 (BY-2) cells were grown in Murashige and Skoog medium supplemented with Gamborg vitamins, 3% w/v sucrose and 4.5 µM 2,4-dichlorophenoxyacetic acid. Cells were cultured in a rotary shaker (125 rpm) in the dark at 25°C, and used 4–6 days after sub-culturing. BY-2 cells expressing either YFP or YFP–PH-PLCᵢα were obtained as described previously (van Leeuwen et al., 2007).

**Arabidopsis thaliana cv.** Columbia seedlings were grown under a 16 h/8 h light/dark cycle on 0.5x MS plates in a growth chamber at 25°C. Typically, 5-day-old seedlings were used in the experiments. Rice plants (Sakha 102; Faculty of Agriculture, Cairo, Egypt) were grown on soil in a growth chamber under a 14 h light/10 h dark cycle at 28°C, until 4 weeks old.

**32P-phospholipid labelling and lipid analysis**

Labelling of BY-2 cells was performed directly in the medium in which they had been growing. Briefly, 85 µl of cell suspension, adjusted to 100 µCi ml⁻¹[^2]PO₄³⁻ (carrier-free; Amersham, http://www1.gelifesciences.com/), was added to 2 ml Eppendorf ‘safe-lock’ tubes (http://www.eppendorf.com). After 3 h, 85 µl of cell-free medium was added, and the tubes placed in temperature-adjusted water baths and incubated for the times indicated. Added reagents were prepared at twice their working concentrations in the 85 µl of cell-free medium that were added just prior to the temperature treatments. AlF₄⁻ was supplied as a mixture of 60 µM NaF and 100 µM AlCl₃, as described by Radhakrishna et al. (1996). For assay of PLD activity, the cell-free medium was adjusted to 1% v/v 1-butanol prior to its addition to labelled cell suspensions.

Pulse-chase experiments (Munnik et al., 1994) were performed as described above, but with the following modifications. Two-minute pulse labellings were initiated by mixing 50 µl of cell suspension with 50 µl of cell-free medium containing 5 µCi of[^2]PO₄³⁻. Chase periods were initiated by adding 100 µl of cell-free medium containing 10 mm potassium phosphate, pH 5.4. Tubes were then immediately transferred to pre-adjusted water baths for temperature treatments as indicated.

Arabidopsis seedlings (three per tube) were labelled overnight in 2 ml Eppendorf tubes containing 400 µl MES buffer [2-(N-morpholino)ethanesulfuric acid, pH 5.7 (KOH)] at 100 µCi ml⁻¹[^3]PO₄³⁻. For rice, pieces (~5 mm) were cut from the leaves of 4-week-old plants and metabolically labelled by floating them overnight on 200 µl 5 mM MES buffer supplemented with 5 µCi carrier-free PO₄³⁻ in a 2 ml Eppendorf microcentrifuge tube. Tubes were transferred to temperature-adjusted water baths as described above.

Incubations were stopped at the indicated times by adding 0.2 volumes of 50% perchloric acid prior to lipid extraction and analysis. To extract lipids from the cell suspensions and seedlings, 3.75 volumes of CHCl₃/MeOH/25% NH₄OH/H₂O (90:70:4:16, by volume) were added, the samples vigorously mixed, and a two-phase system was induced by adding 3.75 volumes CHCl₃ and 1 volume of NaCl (0.9% w/v). The samples were then processed for TLC as described previously (Munnik et al., 1996; Zonia and Munnik, 2004).

Lipids were separated on Silica-60 TLC plates (Merck, http://www.merck-chemicals.nl) using either an alkaline solvent system (CHCl₃/MeOH/25% NH₄OH/H₂O, 90:70:4:16, by volume) or the upper organic phase of an ethyl acetate system (EtAciso-octane/HCOOH/H₂O, 12:2:3:10, by volume). Structural phospholipids were separated in the alkaline system, and the ethyl acetate separations were used to visualize and quantify PA and PBut levels. Radioactivity was visualized and quantified by Storm phosphoimaging (Molecular Dynamics, http://www5.gelifesciences.com).

**Confocal microscopy**

For microscopy, BY-2 cells expressing either YFP or YFP–PH-PLCᵢα were imaged 4-5 days after culturing, using an eight-chambered coverslide (Nalge Nunc International, http://www.nalgenunc.com). Fluorescence microscopy of YFP- and YFP–PH-PLCᵢα-expressing cells co-labelled with FM4-64 (2 µM) was performed using a Zeiss LSM

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REFERENCES

Ratory-based methods for assessing seedling thermotolerance in pear 
Jones, D.R., Bultsma, Y., Keune, W.J., Halstead, J.R., Elouarrot, D., Moham-
Krinke, O., Novotna, Z., Valenta, O. and Martinez, J. (2007) Inositol tris-
phosphate is important for stomatal opening. Plant J., 52, 993–998.
van Leeuwen, W., Vermeer, J.E.M., Gadella, T.W.J. and Munnik, T. (2007) Visualisation of phosphatidylinositol 4,5-bisphosphate in the plasma membrane of suspension-cultured tobacco BY-2 cells and whole Arabid-
Heat stress activates phospholipid-based signalling


