Jasmonates act with salicylic acid to confer basal thermotolerance in *Arabidopsis thaliana*

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Summary

- The *cpr5-1 Arabidopsis thaliana* mutant exhibits constitutive activation of salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) signalling pathways and displays enhanced tolerance of heat stress (HS).
- *cpr5-1* crossed with *jar1-1* (a JA-amino acid synthetase) was compromised in basal thermotolerance, as were the mutants *opr3* (mutated in OPDA reductase3) and *coi1-1* (affected in an E3 ubiquitin ligase F-box; a key JA-signalling component). In addition, heating wild-type *Arabidopsis* led to the accumulation of a range of jasmonates: JA, 12-oxophytodienoic acid (OPDA) and a JA-isoleucine (JA-Ile) conjugate. Exogenous application of methyl jasmonate protected wild-type *Arabidopsis* from HS.
- Ethylene was rapidly produced during HS, with levels being modulated by both JA and SA. By contrast, the ethylene mutant *ein2-1* conferred greater thermotolerance.
- These data suggest that JA acts with SA, conferring basal thermotolerance while ET may act to promote cell death.

Introduction

Plants are sessile organisms and their ability to cope with exposure to temperatures above optimal growth conditions is crucial for survival. Plants have a natural capacity to ameliorate the effects of heat shock (HS) via a 'basal' thermotolerance mechanism (Hong & Vierling, 2000). In addition, lesser increases in temperature can acclimatize plants against HS via a process known as 'acquired' thermotolerance. The production of heat shock proteins (HSPs) plays an important role in protecting proteins against irreversible heat-induced damage by preventing denaturation and facilitating the refolding of damaged proteins (Boston *et al.*, 1996; Hong & Vierling, 2000; Hong *et al.*, 2003). In addition, a number of signalling pathways have been implicated in thermotolerance including redox, hormonal and calcium-based (Foyer *et al.*, 1997; Dat *et al.*, 1998a,b; Larkindale & Knight, 2002; Pastori & Foyer, 2002; Clarke *et al.*, 2004; Larkindale *et al.*, 2005).

Our studies using *Arabidopsis thaliana* mutants indicate that salicylic acid (SA)-signalling pathways, which were originally defined as being involved in antipathogenic responses and in systemic-acquired resistance (SAR; Dong, 2001) also promote basal thermotolerance but were dispensable for the acquired form (Clarke *et al.*, 2004). Heat shock was found to induce SA-regulated pathogenesis-related 1 (*PR1*) transcripts and the ability of the nonexpresser of PR1 protein (*npr1-1*) mutant, which is involved in SA-signal transduction, to recover from heat stress was impaired. Correspondingly, the constitutive expresser of PR1 protein (*cpr5-1*) mutant displayed an enhanced basal thermotolerant phenotype. Although the deduced CPR5 sequence revealed no significant homology to any other gene, it has features of a signal transduction protein with a putative nuclear localization signal and five putative transmembrane domains (Clarke *et al.*, 2000).

In addition to activation of the SA pathway in *cpr5-1*, the jasmonic acid (JA)-inducible genes *PDF1.2* and *THI2.1* were also found to be constitutively expressed (Bowling *et al.*, 1997; Clarke *et al.*, 1998). It may therefore be that the enhanced thermotolerant phenotype observed in the *cpr5-1* mutant also involves the JA-signalling pathway. Jasmonates regulate or coregulate a wide range of developmental processes in plants and responses to biotic and abiotic stresses (Wasternack,

2006, 2007; Balbi & Devoto, 2008). Wounding and pathogen infection or exposure to ozone cause endogenous JA accumulation and the expression of JA-responsive genes, for example, proteinase inhibitors and antifungal proteins such as thionins and defensins (Vijayan, *et al.*, 1998; Rao *et al.*, 2000; Kanna *et al.*, 2003; Howe, 2004; Wasternack, 2006). Defects in JA biosynthesis or signalling pathways have been shown to increase the sensitivity to stresses such as pathogen infection or ozone exposure (McConn *et al.*, 1997; Thomma *et al.*, 1998; Overmyer *et al.*, 2000; Stintzi, *et al.*, 2001; Kanna *et al.*, 2003).

The *coi1-1 Arabidopsis* mutant is defective in most of the known JA-dependent responses, indicating that COI1 has a central role in JA signalling (Xie *et al.*, 1998; Reymond *et al.*, 2000, 2004). COI1 is an E3 ubiquitin ligase which tags proteins with ubiquitin as a signal for their proteolytic destruction in the proteosome. There are also several other *Arabidopsis* mutants that show reduced sensitivity to JA, although they are not defective in all the JA responses; for example, the mutants of *jar1/jin4/jai2* allelic series (encoding a JA-amino acid synthetase; Staswick *et al.*, 1992, 2002, Berger *et al.*, 1996; Lorenzo *et al.*, 2004; Staswick & Tiryaki, 2004) and *jin1/jai1* alleles (encoding a nuclear-localised bHLHzip transcription factor, AtMYC2; Lorenzo *et al.*, 2004). Recent work has provided a more comprehensive view of the JA signalling cascade (Katsir *et al.*, 2008; Staswick, 2008). COI1 has been shown to target a class of putative repressor proteins known as JAZ (jasmonate ZIM-domain), one of which interacts with AtMYC2. The COI1–JAZ complex is stabilized by interaction with JA-isoleucine (JA-Ile), the product of JAR1 (Chini *et al.*, 2007; Thines *et al.*, 2007; Yan *et al.*, 2007; Katsir *et al.*, 2008).

The relationship between the JA- and SA- signalling has often been shown to be antagonistic. This antagonism may reflect SA-mediated suppression of JA biosynthesis (Pěna-Cortes *et al.*, 1993; Doares *et al.*, 1995) by reducing the expression of genes encoding JA-biosynthetic enzymes (Laudert & Weiler, 1998; Spoel *et al.*, 2003). However, *Arabidopsis* mutants *cpr5*, *cpr6, cet* (*constitutive expression of thionin*), *cpr22* and *hrl1* (*hypersensitive response-like lesions1*) (Clarke *et al.*, 1998; Hilpert *et al.*, 2001; Yoshioka *et al.*, 2001; Devadas *et al.*, 2002) show simultaneous expression of SA- and JA- marker genes. Recently, Mur *et al.* (2006) suggested that the reciprocal SA and JA antagonism of gene expression was dose dependent.

In *cpr5*, JA-mediated effects on plant pathogen resistance were influenced by ethylene (ET, Clarke *et al.*, 2000). Similarly, resistance to necrotrophic pathogens (Penninckx *et al.*, 1998) and CaMV (Love *et al.*, 2005) involved ET–JA interactions. In some necrotrophic interactions, for example, between *Cochliobolus victoriae* and oats (Navarre & Wolpert, 1999), ET will contribute to the elicitation of cell death. However, within the context of thermotolerance, ET may act mainly in basal thermotolerance as this was increased on treatment with the ET precursor 1-aminocyclopropane-1-carboxylic acid (ACC; Larkindale & Knight, 2002). Further, basal rather than acquired thermotolerance was most compromised in ET receptor mutant *etr1* and in *ein2-1*, which is mutated within membrane-associated metal-ion transporter that is essential for ethylene signalling (Alonso *et al.*, 1999).

We here demonstrated that SA and JA both act to confer thermotolerance; however, in our experimental approach, ET acts to increase HS-associated cell death.

Materials and Methods

All chemicals were obtained from Sigma -Aldrich Company Ltd (Gillingham, UK) unless stated otherwise.

Plant materials and growth conditions

Arabidopsis thaliana (L.) Heynh. ecotype Columbia (Col-0) was used as the wild-type control. The mutant lines, *cpr5-1*, *npr1-1*, *jar1-1*, *ein2-1*, *cpr5-1 npr1-1* and *cpr5-1 jar1-1* (from Xinnian Dong, Duke University, Durham, NC, USA), *HSP17.3B-GUS* (Clarke *et al.*, 2004), *coi1-1* (from Prof. John Turner, University of East Anglia, Norwich, UK) and 35S-NahG line 10, from Scott Uknes (Ciba-Geigy Corp, Greensboro, NC, USA; currently Cropsolution Inc., Morrisville, NC, USA), were in this ecotype background. The *PDF1.2-GUS* line (from Willem Broekaert, Katholieke Universiteit Leuven, Belgium; Manners *et al.*, 1998) was in the C24 ecotype background. The *opr3* line (from John Browse and Annick Stinzi, Pullman, Washington State University) was in the WS-0 background.

Arabidopsis plants were grown in 9-cm Petri dishes on Murashige and Skoog (MS; Duchefa, Haarlem, the Netherlands) medium (0.8% (w : v) plant agar, 3.0% (w : v) sucrose, pH 5.8) at 22°C with a 16-h photoperiod (65 μmol m^{-2} s^{-1}). Stratified seeds were surface-sterilized in 20% (v : v) bleach for 5 min, rinsed twice in sterile H_2O (Millipore Milli-Q water purification system; Millipore Australia Pty. Ltd, North Ryde, Australia) before plating onto MS agar plates and grown for 3 wk to principal growth stage 1.08 (Boyes *et al.*, 2001).

Heat treatment of plants

All heat treatments were carried out in the dark to ensure that cell death was a result of increased temperature and not photo-oxidative stress. Unless otherwise specified, Petri dishes containing 3-wk-old plants on MS medium were heated at the appropriate temperature and specified time in a Sanyo MLR-350 environmental test chamber, without light. When electrolyte leakage was measured during the heat treatment, the aerial parts of 3-wk-old plants were placed individually in glass scintillation vials containing 5 ml H_2O and placed in the environmental chamber without light (< 1 μmol m^{-2} s^{-1}) at the appropriate temperature.

For the acquired thermotolerance assay, Petri dishes containing 3-wk-old plants grown on MS medium were exposed to a 2 h 45°C HS with (acclimatized) or without (nonaccli-

matized) a pre-treatment. The pretreatment consisted of 90 min at 38°C followed by a 2-h recovery period at 22°C before the HS. Following the 45°C HS, the plants were returned to 22°C.

The hypocotyl elongation assay was carried out as described by Queitsch *et al.* (2000). Stratified seeds were surface-sterilized and plated in rows on MS medium. Plates were covered with foil and placed in a vertical position at 22°C for 2.5 d. The plates were then either kept at 22°C or heat shocked at 45°C with (acclimatized) or without (nonacclimatized) a 90-min pretreatment at 38°C. Hypocotyl elongation was examined after an additional 2.5 d.

Electrolyte leakage measurements

Heat-induced changes in cell death were quantified by measuring ion leakage with a Horiba Twin Cond B-173 conductivity meter (HORIBA Ltd, Kyoto, Japan). For analysis of electrolyte leakage following a 16-h heat treatment, aerial parts of 3-wk-old plants were placed in glass scintillation vials containing 5 ml H_2O and were incubated at 22°C in the light (65 μmol m^{-2} s^{-1}), unless otherwise stated. The conductance of the H_2O was measured at intervals and calculated per mg fresh weight. Boiling plants of each genotype to determine total ion content established that this did not differ among the plant lines used in this study (data not shown).

Chemical treatment of plants

For experiments where MeJA was applied to the medium for electrolyte leakage analysis, 3 ml at the appropriate concentration in H_2O was filter sterilized and pipetted around the plants 24 h before heat treatment. For gene expression analysis, plants were sprayed with either 0 or 5 μM MeJA. The aerial part of the plant was harvested after 30 min, surface moisture gently removed and immediately frozen in liquid N and stored at −80°C.

Histochemical GUS staining

Plants were placed into staining solution containing 1 mM 5-bromo-4-chloro-3-indolyl glucuronide (first dissolved in dimethyl formamide at 1 mg ml^{-1}) in 50 mM $NaPO_4$ pH 7.0 and 0.1% Triton X-100. The samples were then placed under vacuum twice for 2 min each time to infiltrate the samples and then incubated at 37°C overnight. The chlorophyll was removed by sequential changes in 30, 75 and 95% ethanol. For histochemical staining of heat-induced GUS activity, plants were returned to 22°C following the heat treatment for 24 h before staining.

RNA extraction

The aerial part of the plants were harvested following the appropriate treatment and immediately frozen in liquid nitrogen. Total RNA was prepared from samples kept at −80°C using Plant RNA Purification reagent (Invitrogen, Carlsbad, CA, USA) as per manufactures instructions and quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The integrity of RNA samples was also assessed by agarose gel electrophoresis.

Northern blot analysis

Total RNA was separated on a denaturing formaldehyde agarose gel, and transferred to Hybond N membrane (Amersham International Ltd, GE Healthcare Life Sciences, Little Chalfont, UK) as in Draper *et al.* (1998). Hybridization was carried out as in Warner *et al.* (1992) and the cDNA probe was labelled using the Invitrogen Random Primers DNA Labelling System. The *AtHSP17.6* clone GenBank No. X16076 (stock number CD3-5) was obtained from the *Arabidopsis* Biological Resource Center. The *PDF1.2* cDNA was obtained by polymerase chain reaction (PCR) using the primers 5′-CACCCTTA-TCTTCGCTGCTC-3′ and 5′-GTTGCATGATCCATG-TTTGG-3′.

cDNA synthesis

To eliminate residual genomic DNA, the RNA was treated with RNase free DNase 1 (Roche Diagnostics, Mannheim, Germany). First-strand cDNA was synthesized by reverse transcribing 1 μg of total RNA using Transcriptor Reverse Transcriptase (Roche Diagnostics, Mannheim, Germany) and 3.2 μg of random hexamer primers (Roche Diagnostics) in a 20-μl reaction as per manufacturers instructions. A two-step incubation was performed to allow efficient annealing of the primers (10 min at 25°C, followed by 30 min at 55°C). Following heat inactivation (85°C for 5 min) of the reverse transcriptase, cDNA samples were stored at −20°C.

Primer designing and real-time PCR analysis

To facilitate the real-time PCR analysis of all the investigated genes under same reaction conditions, primers were designed using OligoPerfect Designer (Invitrogen) under default parameters. The primers were designed spanning an intron to detect any genomic DNA contamination. Real-time PCR was performed on the Roche LightCycler 2.0 using LightCycler Faststart DNA Master^{plus} Sybrgreen1 (Roche Diagnostics) as per manufactures instructions. Each 10-μl reaction contained 1 μl of a 1 : 10 dilution of cDNA and 250 nM of each gene-specific primer. *Actin 2* (At3g18780) and the cap-binding protein (*AtCBP20*; At5g44200) were used as the reference gene internal controls for comparison of the target gene transcripts amplified per cDNA sample. The following primers were used for reference gene amplification: *Actin 2*, 5′-CGCTCTTTC-TTTCCAAGCTCAT-3′ and 5′-TCCTGCAAATCCAG-CCTTC-3′; *AtCBP20*, 5′-ATCGCTATGGCCGCGATA-3′

and 5′-AGTCACCGCTCTCACGGAAT-3′. The following primers were used for target gene amplification: *PDF1.2* (At5g44420), 5′-CACCCTTATCTTCGCTGCTC-3′ and 5′-GTTGCATGATCCATGTTTGG-3′; *THI2.1* (At1g72260), 5′-TTCCAAGGGAAGGTGTATGC-3′ and 5′-AGAGTG-TTCATGGCACCACA-3′; *VSP1* (At5g24780), 5′-TTTT-ACGCCAAAGGACTTGC-3′ and 5′-AATCCCGAG-TTCCAAGAGGT3′. LightCycler Software 4 (Roche Diagnostics) relative quantification analysis module was used to determine the relative fold expression changes between treatments. This software compares the difference between the amplification of target genes to that obtained for the reference genes for each cDNA treatment sample and normalizes to a value of 1 based on the treatment used as the calibrator (0 min heat stressed or 0 μM MeJA treated plants) and takes into account the efficiency of the primer pair. Standard curves were obtained for each primer pair and the following efficiencies and errors, respectively, were obtained; *Actin 2* (1.96; 0.007), *CAB2* (1.97; 0.009), *PDF1.2* (1.84; 0.002), *THI2.1* (1.88; 0.006) and *VSP1* (1.88; 0.002). All the PCRs were performed under the following conditions: 10 min at 95°C followed by 45 cycles of 5 s at 95°C, 5 s at 60°C and 8 s at 72°C in LightCycler capillaries (Roche Diagnostics). The specificity of amplicons was verified by melting curve analysis (65–95°C) after 45 cycles and agarose gel electrophoresis. At least three biological replicates for each sample were used for real-time PCR analysis and at least two technical replicates were analysed for each biological replicate.

Measurements of JA, 12-oxo-phytodienoic acid (OPDA) and JA-Ile levels

The plant material was extracted and analysed on a Micromass LCT ESI-MS (Waters Ltd, Manchester, UK) as described in Clarke *et al.* (2004). The JA levels were calculated from the recovery of JA against an added d_6-SA standard as described in Allwood *et al.* (2006). In addition, JA, OPDA and JA-Ile were extracted and quantified as described elsewhere (Hause *et al.*, 2000; Miersch *et al.*, 2008) using ($^2H_6$)JA, ($^2H_5$)OPDA and JA-($^2H_3$)Leu as standards and a Polaris Q instrument (Thermo-Finnigan; Thermo Fisher Scientific Inc, Waltham, MA, USA) for gas chromatography–mass spectrometry (GC-MS) analysis.

Photoacoustic measurements of ET production

Ethylene production was measured using a sensitive laser-based ET detector (type ETD-300; Sensor Sense BV, Nijmegen, the Netherlands) in combination with a gas handling system. The ETD-300 is a state-of-the art ethylene detector based on laser photoacoustic spectroscopy (Harren *et al.*, 2000) that is able to detect on-line *c*. 300 pptv (parts-per-trillion volume, $1{:}10^{12}$) of ethylene within a 5-s time scale. The gas handling was performed by a valve control box (type VC-6, Sensor Sense B.V., Nijmegen, the Netherlands), designed for measuring up to six sampling cuvettes per experiment. The valve control box allowed automated sampling of ethylene production at a flow rate of 3 l h^{-1} and its transport to the ETD-300 alternately, in succession for 15 min for each cuvette. In this study, four glass cuvettes were used per experiment. One contained a Petri dish with agar and the other three cuvettes contained a Petri dish with 12 3-wk-old *Arabidopsis* seedlings grown on MS medium supplemented with 3% sucrose. Two cuvettes were incubated in a Sanyo MLR-350 environmental test chamber, without light; gas-lines were fed directly from the chamber through the VC-6 to the ETD-300 allowing prolonged monitoring of ethylene production without opening the chamber. The third cuvette was maintained at 22°C and wrapped in aluminium foil to maintain plants in the dark. For a better overview the ethylene emission rate is displayed every 1 h. Each experiment was repeated giving similar results.

Results

NPR1- and JAR1-dependent pathways are required for the enhanced basal thermotolerant phenotype observed for cpr5-1

To examine the contribution of the SA and JA pathways in the elevated thermotolerance seen with *cpr5-1* electrolyte leakage was measured in the double mutants *cpr5-1 npr1-1* and *cpr5-1 jar1-1* and the corresponding single mutants. Electrolyte leakage represents a rapid, sensitive and quantitative method to assess stress on plant cells. We have previously used electrolyte leakage to assess basal thermotolerance in the SA-signalling mutants *npr1-1* and *cpr5-1* (Clarke *et al.*, 2004). In agreement with Clarke *et al.* (2004), the *cpr5-1* mutant exhibited reduced electrolyte leakage compared with Col-0 during heat stress and the *npr1-1* mutant had an increase in electrolyte leakage (Fig. 1a). However, a similar level of electrolyte leakage was observed for *cpr5-1 npr1-1* and Col-0 suggesting that the enhanced basal thermotolerant phenotype observed for *cpr5-1* is dependent on NPR1. A similar result was shown following heat stress where the electrolyte leakage levels during heat stress were restored to wild-type levels in the *cpr5-1 npr1-1* mutant compared with the reduction detected for *cpr5-1* (Fig. 1b).

Wild-type levels of electrolyte leakage were detected for *jar1-1* both during heat stress and subsequent recovery (Fig. 1c,d). Although this may suggest that JAR1 is not involved in thermotolerance, electrolyte leakage levels were restored to wild-type levels in the *cpr5-1 jar1-1* double mutant. Significantly less electrolyte leakage was detected for *cpr5-1* compared with that observed for the double mutant *cpr5-1 jar1-1* during heat stress after 5 h ($P < 0.054$) and 7 h ($P < 0.051$; Fig. 1c, and 3 h ($P < 0.043$) and 5 h ($P < 0.049$) post heat stress (Fig. 1d). This could indicate that the JA pathway is required for the enhanced thermotolerance of *cpr5-1*.

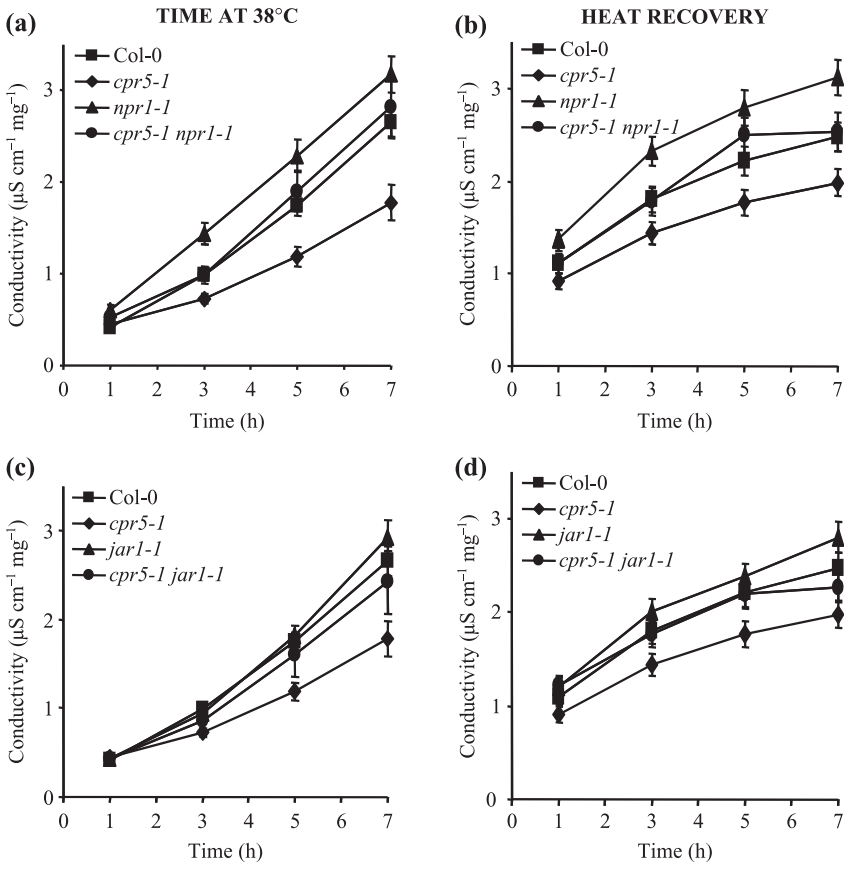

Fig. 1 *JAR1* and *NPR1* are required for the enhanced thermotolerant phenotype of *cpr5-1*. (a,c) Electrolyte leakage in *Arabidopsis thaliana* wild-type Col-0 and the mutants *cpr5-1, npr1-1, jar1-1* and the double mutants *cpr5-1 npr1-1* and *cpr5-1 jar1-1* during heat treatment. The aerial parts of the plants were incubated in H_2O at 38°C and the conductivity of the solution was monitored. Data are means (± SE) of six experiments each with five plants per treatment ($n = 6$). (b,d) Electrolyte leakage in wild-type Col-0 and the mutants *cpr5-1, npr1-1, jar1-1* and the double mutants *cpr5-1 npr1-1* and *cpr5-1 jar1-1* during recovery from heat treatment. Following heat treatment (16 h at 38°C) the aerial parts of the plants were placed in H_2O at 22°C and the conductivity of the solution was monitored. Data are means (± SE) of six experiments each with five plants per treatment ($n = 6$).

When acquired thermotolerance was assessed in these genotypes, all exhibited this effect to levels equivalent to wild type controls (see the Supporting Information, Fig. S1).

Heat stress induces the JA signal transduction pathway

Given the equivocal data obtained with the *cpr5-1 jar1-1* double mutant we sought to further investigate the possible role of jasmonates in thermotolerance. Thus, we investigated thermotolerance in *coi1-1*, which plays a more central role in the signalling mechanism (Staswick, 2008). A clear increase in electrolyte leakage was observed for *coi1-1* compared with that observed for Col-0 both during the 38°C heat treatment and subsequent recovery from a 16-h 38°C heat treatment (Fig. 2a,b). It was also noted that unlike any other mutant genotype, *coi1-1* plants were found to have severe chlorosis beginning from the base of the petioles (Fig. 2c).

We sought further evidence for the involvement of the jasmonates in heat stress in wild-type plants. The JA-inducible genes *PDF1.2* (At5g44420), *THI2.1* (At1g72260) and *VSP1* (At5g24780) were analysed in wild-type (Col-0) plants following a 38°C heat treatment. Heat-induced expression was initially assessed by Northern analysis but while *PDF1.2* transcript accumulation was noted no expression was observed for *THI2.1* or *VSP1* (data not shown). To confirm this result, real-time PCR was employed. A threefold increase in *PDF1.2* transcript was detected following a 30-min heat treatment at 38°C and this remained relatively constant during the 360-min heat treatment (Fig. 3a). By contrast, transcript levels at only 45% and 37% those of controls were detected with *THI2.1* and *VSP1*, respectively, following a 30-min heat stress. Following 360 min of heat stress both *THI2.1* and *VSP1* transcript levels were approx. 10% those of nonHS plants.

In investigating the reason for this differential response of jasmonate marker genes we examined whether they exhibited differing sensitivities to jasmonate. *PDF1.2* displayed a greater response to exogenous application of 5 µM MeJA where a 300-fold increase over controls was detected, compared with a 3- and 36-fold increase for *THI2.1* and *VSP1* transcripts, respectively (Fig. 3b).

Plants transformed with *GUS* driven by the *PDF1.2* promoter were employed to investigate the spatial patterns of heat-induced *PDF1.2* expression. Although basal level of GUS expression was detected throughout the plant, an increase in GUS expression was detected following 2 h of heat stress (Fig. 3c). The intensity of the heat-induced GUS expression increased following 6 h and 12 h of heat stress and appeared to be localized to leaf tissue with little upregulation in root or stem tissue.

Further support for a protective role of jasmonate during heat stress was shown when 3-wk-old Col-0 plants were subjected to a 38°C heat treatment following exogenous application

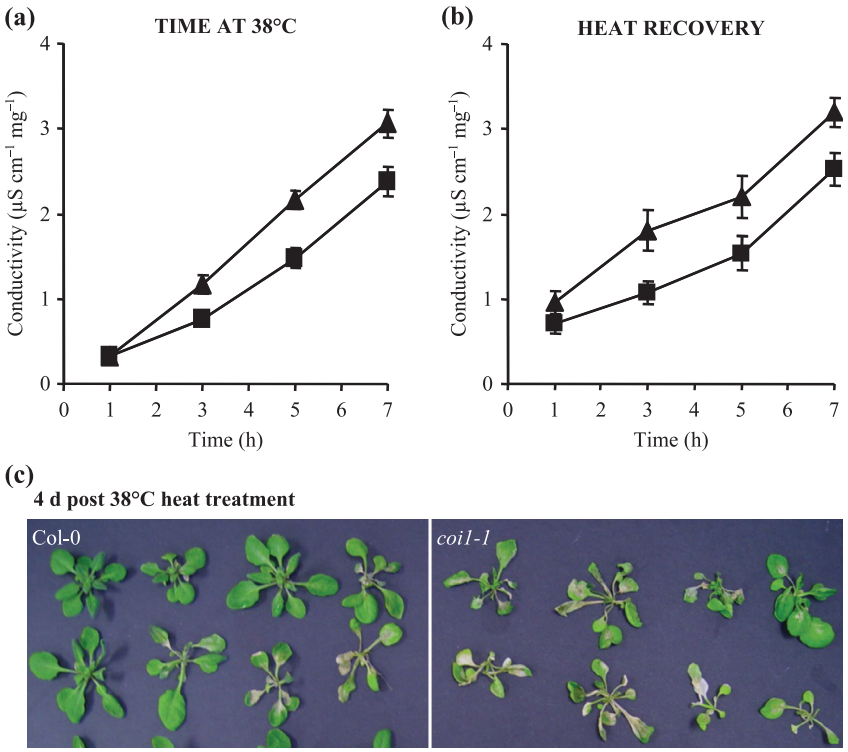

Fig. 2 *coi1-1* is more susceptible to heat stress. (a) Electrolyte leakage during heat treatment. The aerial parts of *Arabidopsis thaliana* Col-0 (squares) and *coi1-1* (triangles) were incubated in H_2O at 38°C and the conductivity of the solution was monitored. Data are means (± SE) of four experiments each with five plants per treatment ($n = 4$). (b) Electrolyte leakage during recovery from heat treatment. Following heat treatment (16 h; 38°C) the aerial parts of Col-0 (squares) and *coi1-1* (triangles) were placed in H_2O at 22°C and the conductivity of the solution was monitored. Data are means (± SE) of five experiments each with five plants per treatment ($n = 5$). (c) Col-0 (left) and *coi1-1* (right) 3-wk-old plants on Murashige and Skoog (MS) medium were exposed to 38°C for 16 h, returned to 22°C and photographed 4 d later.

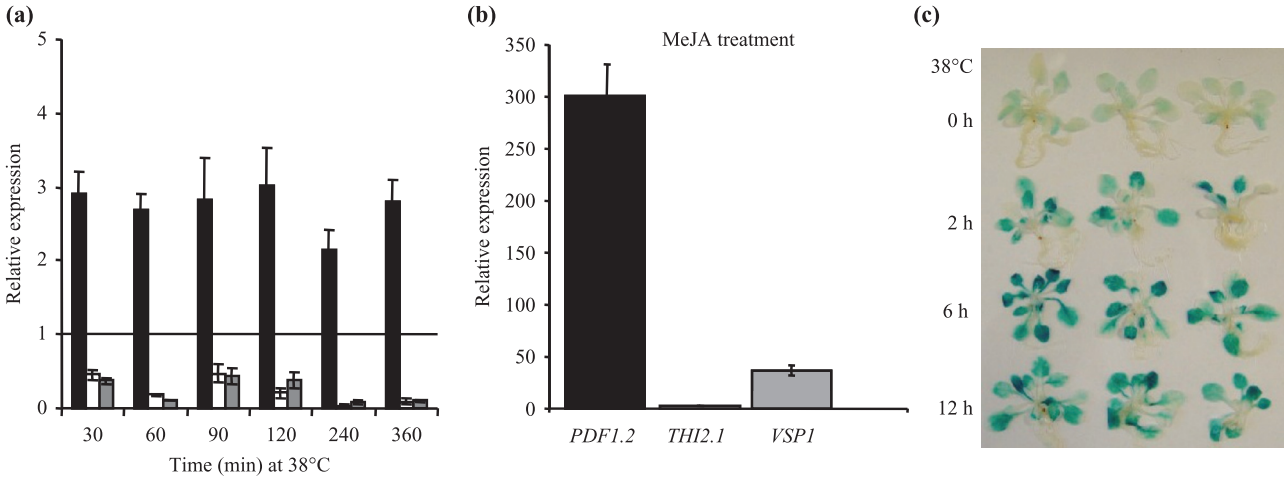

Fig. 3 Heat induces *PDF1.2* expression. (a) *PDF1.2* (closed bars), *THI2.1* (open bars) and *VSP1* (tinted bars) transcript accumulation in *Arabidopsis thaliana* Col-0 during heat stress. Real-time polymerase chain reaction (PCR) was performed on cDNA synthesised from total RNA extracted from the aerial parts of 3-wk-old Col-0 exposed to a 38°C heat treatment for the specified time. Transcript expression was normalized to the amount detected for nonheat stressed tissue. Data are means (± SE) of at least five experiments ($n \geq 5$). (b) Induction of *PDF1.2*, *THI2.1* and *VSP1* transcripts in Col-0 following methyl jasmonate (MeJA) treatment. Real-time PCR performed on cDNA synthesized from total RNA extracted from the aerial parts of 3-wk-old Col-0 sprayed with 5 μM methyl jasmonate (MeJA). Transcript expression was normalized to the amount detected for 0 μM MeJA treated tissue. Data are means (± SE) of at least five experiments ($n \geq 5$). (c) *PDF1.2-GUS* expression during heat stress: 3-wk-old *PDF1.2-GUS* plants were incubated at 38°C for 0, 2, 6 and 12 h, and returned to 22°C for 24 h before staining.

of MeJA 24 h before heat stress. Electrolyte leakage measurements indicated that treatment with 1 μM MeJA did not provide any protection from heat stress but 5 μM MeJA significantly ($P < 0.013$) reduced electrolyte leakage compared with the control plants (Fig. 4a). However, further increases in MeJA concentration had the reverse effect, suggesting that MeJA protection from heat stress is concentration specific. To confirm that exogenous application of MeJA via the media was inducing

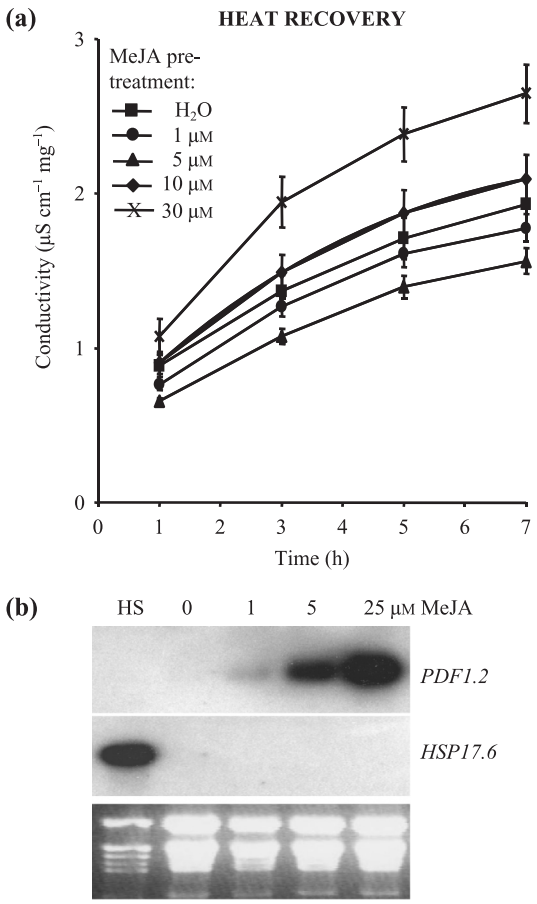

Fig. 4 Methyl jasmonate (MeJA) protects *Arabidopsis thaliana* Col-0 from heat stress. (a) H_2O (0 μM MeJA), 1 μM MeJA, 5 μM MeJA, 10 μM MeJA and 30 μM MeJA was applied to the Murashige and Skoog (MS) media 24 h before heat treatment (16 h for 38°C). Following heat treatment the aerial parts were placed in H_2O at 22°C and the conductivity of the solution was monitored. Data are means (± SE) of four experiments each with five plants per treatment ($n = 4$). (b) Northern analysis of *PDF1.2* and *HSP17.6* transcripts in 3-wk-old Col-0 seedlings exposed to the various concentrations (μM) of MeJA indicated by supplementing the culture media. Northern blots of separated RNA (10 μg) were hybridized with ^{32}P-labelled *PDF1.2* or *HSP17.6* cDNA. RNA (2 μg) from Col-0 seedlings exposed to a heat treatment (38°C for 60 min) was included as a positive control for *HSP17.6* transcripts. The ethidium bromide-stained gel is shown below.

the JA pathway, the expression of *PDF1.2* was assessed by Northern analysis. This indicated that exogenous application of MeJA induced *PDF1.2*.

When we assessed the expression of *HSPs* that are well-established markers for thermotolerance (Clarke *et al.*, 2004), MeJA did not induce expression of *HSP17.6* (At5g12020; Fig. 4b); neither was *HSP101* (At1g74310) detected (data not shown). No GUS expression was detected in HSP17.3-GUS plants following exogenous application whereby MeJA was either pipetted onto the media or the plant was submerged in a solution of MeJA (data not shown). Examination of publicly available array data using GENEVESTIGATOR (Zimmermann *et al.*, 2004) also did not indicate that MeJA induced any class of HSP (Fig. S2). Hence, we can find no evidence that MeJA-conferred thermotolerance is associated with HSP gene expression.

Next, we analysed the endogenous free JA levels in Col-0 during heat stress (Fig. 5a). The JA levels were significantly ($P \leq 0.001$) increased over levels at $t = 0$ at 1 h of 38°C heat treatment and continued to rise afterwards. To indicate the contribution of *de novo* JA biosynthesis to thermotolerance, heat tolerance in the OPDA reductase 3 mutant, *opr3* was examined. During heating no significant difference was observed between Col-0 and *opr3* (Fig. 5b) but during the recovery phase, *opr3* was found to be compromised, exhibiting greater electrolyte leakage (Fig. 5c).

To relate these *opr3* responses with jasmonates; levels of JA, OPDA and JA-Ile were determined (Fig. 5d–f). Significant accumulation of all jasmonates was observed in response to heating to 38°C by 8 h. In the case of JA and OPDA levels were significantly reduced at 8 h following heating in *opr3*. The JA-Ile levels in *opr3* were significantly reduced before and following heating to 38°C until 12 h (Fig. 5f).

Heat stress initiates ethylene production

The expression of *PDF1.2* is influenced by both JA and ET (Pré *et al.*, 2008), hence the significant elevation of its expression during HS could indicate a contribution by ET. Larkindale *et al.* (2005) demonstrated that following heating to 45°C for 60 min, the *ein2-1* mutant was compromised in its tolerance to heat. By contrast, with our gentler heating regime dramatically different responses were observed with the *ein2-1* mutant. Electrolyte leakage was decreased in *ein2-1* compared with Col-0 following heat stress (Fig. 6a) but not during heating (Fig. 6b) suggesting that ET contributed to the poststress loss in viability.

To investigate this further, ET production in 2-wk-old agar-grown seedlings was measured by laser photoacoustic detection (Fig. 6c). Since the actual ET concentration levels were far below 1 ppbv (parts-per-billion volume, $1{:}10^9$) this technique had to be employed instead of the traditional gas chromatography. Incubation of Col-0 at 38°C resulted in a rapid increase in ET production that persisted for at least 24 h. Ethylene production from the agar was negligible. The possible contribution of JA and SA to ET production was assessed using *opr3* and a line harbouring the SA hydroxylase *NahG* transgene that are unable to accumulate SA (Delaney *et al.*, 1994). When *opr3* mutants were incubated at 38°C, ET production was reduced compared with wild-type (*Ws-0)* plants (Fig. 6d). Examination of ET production in NahG plants at 38°C was also reduced compared with wild type (Col-0) controls (Fig. 6e). Taking these data together it appears that both JA and SA augment ET production in response to heating. However, ET is likely to be exerting a negative effect on plant cell survival.

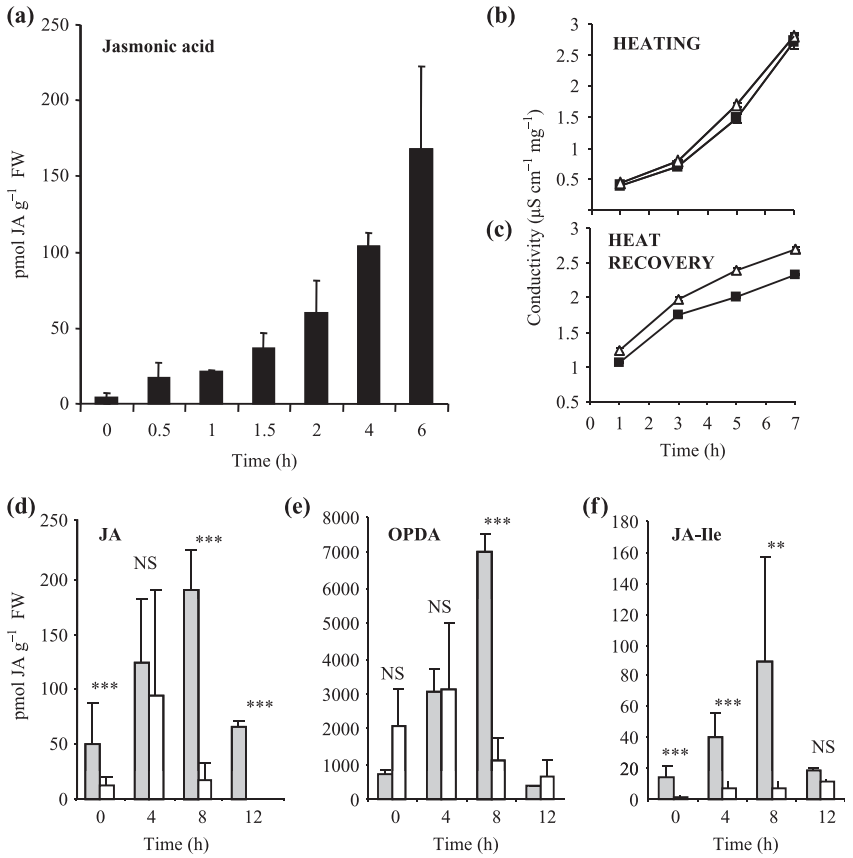

Fig. 5 Levels of jasmonic acid (JA), 12-oxophytodienoic acid (OPDA) and JA-isoleucine conjugate (JA-Ile) rise during heat stress. (a) Endogenous levels of JA in Col-0 plant tissue exposed to 38°C for the indicated time. Data are means (± SE) of at least three replicates. (b) Electrolyte leakage during heat treatment. The aerial parts of the plants were incubated in H_2O at 38°C and the conductivity of the solution was monitored. Data are means (± SE) of six experiments each with five plants per treatment ($n = 6$). (c) Electrolyte leakage during recovery from heat treatment of Ws-0 (squares) and *opr3* (triangles) mutant. Following heat treatment (16 h for 38°C) the aerial parts of the plants were placed in H_2O at 22°C and the conductivity of the solution was monitored. Data are means (± SE) of six experiments each with five plants per treatment ($n = 6$). (d–f) Levels of JA, OPDA and JA-Ile in wild type (Ws-0; tinted bars) and *opr3* (open bars) plants. Data are three replicate plants per treatment. Error bars are + SE. Significance: **, $P < 0.01$; ***, $P < 0.001$; ns, not significant.

Discussion

This study developed observations made in our previous study (Clarke *et al*., 2004) where the *Arabidopsis* mutant *cpr5-1* displayed exceptional thermotolerance. Electrolyte leakage, as a rapidly measured parameter, enabled us to establish that the enhanced thermotolerant phenotype observed for *cpr5-1 Arabidopsis* plants was compromised in the *cpr5-1 npr1-1* double mutant. This indicated that NPR1 is required for the enhanced basal thermotolerance observed for *cpr5-1*. Studies into induced systemic resistance (ISR) have indicated that NPR1 also acts to integrate defence signalling by SA, JA and ET (Pieterse & van Loon, 2004). We have previously indicated that SA plays a role in basal thermotolerance but the *npr1* data could indicate contributions by JA or ET. Crucially, however, an NPR1-independent pathway must also exist because the electrolyte leakage detected for the double mutant was similar to that observed for Col-0 and not *npr1-1*, which exhibited a higher level of electrolyte leakage.

Clarke *et al*. (2000) established that the *cpr5-1* phenotype in biotic stress responses is partially influenced by the JA-signalling pathway, so we first examined if this could also be contributing to the thermotolerance pathway. To investigate this possibility we employed *jar1-1* and the *cpr5-1 jar1-1* double mutant. The single *jar1-1* mutant was not compromised in basal thermotolerance suggesting that JAR1 is not required for tolerance during heating. However, the enhanced thermotolerance observed in *cpr5-1* was not seen with the *cpr5-1 jar1-1* double mutant implying a requirement for jasmonates. Based on these somewhat equivocal observations, we tested the role of JA in thermotolerance within wild-type *Arabidopsis*. Electrolyte leakage experiments demonstrated that exogenous application of 5 µM MeJA helped to maintain cell viability in heat-stressed plants. However, increasing the concentration to 10 µM had no protective affect and a further increase to 30 µM resulted in increased membrane damage. Given that this experiment supplemented endogenous JA production, this suggested a threshold for jasmonate-conferred thermotolerance. Further, jasmonate increases occur at the same time as SA production, this could be an example of the reduced viability seen with high concentration SA–JA interactions (Mur *et al*., 2006). However, when the expression of JA-inducible genes *PDF1.2*, *THI2.1* and *VSP1* during heat stress were monitored a threefold induction of only *PDF1.2* transcript was detected whereas *THI2.1* and *VSP1* transcripts decreased during heat stress. One likely explanation is that both *THI2.1* and *VSP1* are less sensitive to MeJA, as only a 3- and 36-fold increase in transcript was found, respectively, following MeJA treatment compared with a 300-fold increase for *PDF1.2*. The endogenous level of JA detected during heat stress may therefore be

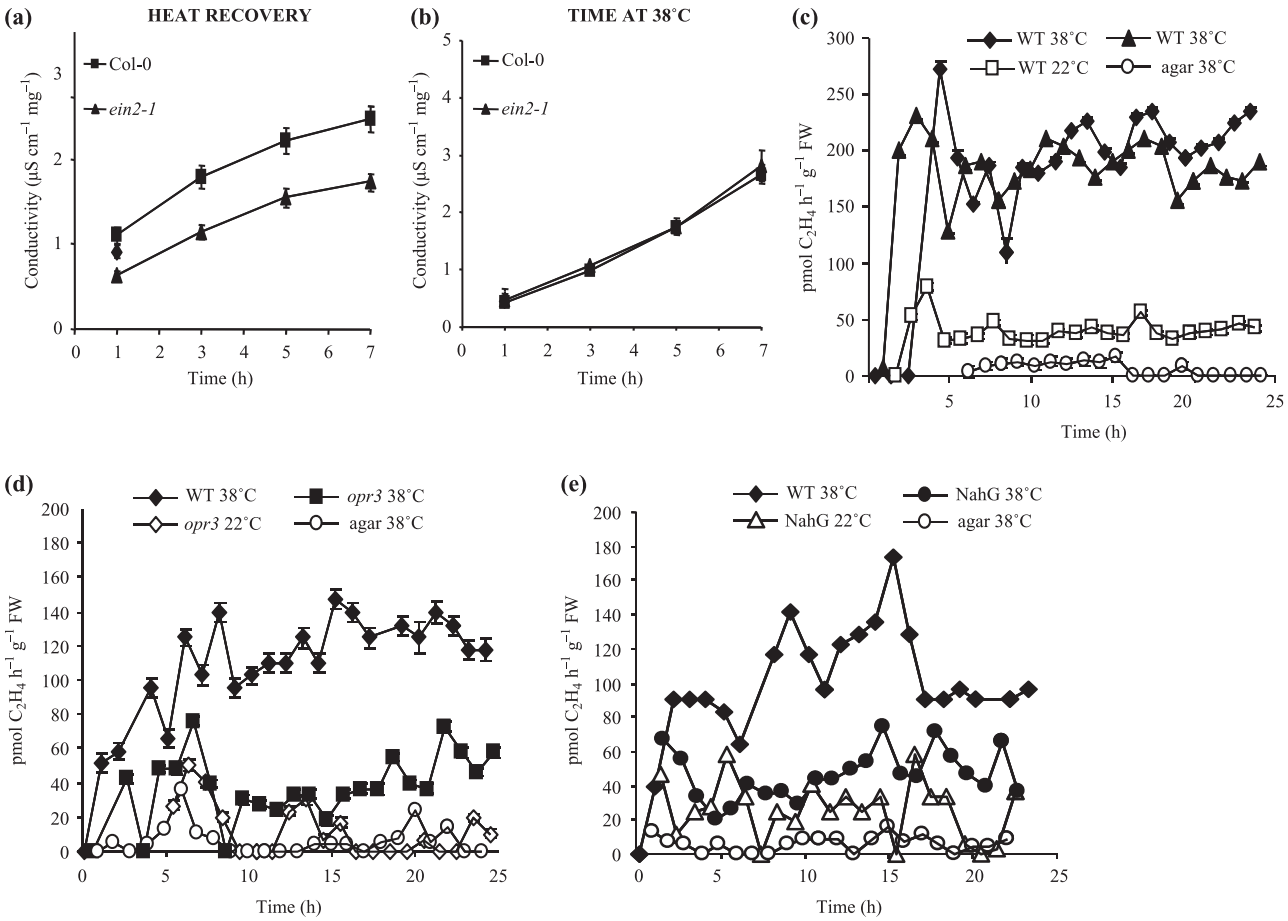

Fig. 6 Analysis of heat-treated *ein2-1* and ethylene production in *Arabidopsis thaliana* wild type, *opr3* and NahG *Arabidopsis* plants during heating to 38°C. (a) Electrolyte leakage during recovery from heat treatment. Following heat treatment (16 h for 38°C) the aerial parts of the plants were placed in H_2O at 22°C and the conductivity of the solution was monitored. Data are means (± SE) of six experiments each with five plants per treatment ($n = 6$). (b) Electrolyte leakage during heat treatment. The aerial parts of the plants were incubated in H_2O at 38°C and the conductivity of the solution was monitored. Data are means (± SE) of six experiments each with five plants per treatment ($n = 6$). (c–e) C_2H_4 production from 12 2-wk-old *Arabidopsis* seedlings, grown on Murashige and Skoog (MS) medium + 3% sucrose following heating to 38°C and measured using laser photoacoustic detection. (c) C_2H_4 produced from Col-0 incubated at 38°C from two separate experiments (diamonds, triangles); C_2H_4 from Col-0 incubated at 22°C (squares) and from Petri-dishes with MS salts heated to 38°C (circles). (d) C_2H_4 produced from *Ws-0* incubated at 38°C (closed diamonds); C_2H_4 from *opr3* incubated at 38°C from two separate experiment (open diamonds, squares) and from Petri-dishes with MS salts heated to 38°C (circles). (e) C_2H_4 produced from Col-0 incubated at 38°C (diamonds); C_2H_4 from NahG incubated at 38°C from two separate experiment (closed circles, triangles) and from Petri dishes with MS salts heated to 38°C (open circles). Seedlings were maintained in the dark for all measurements.

inadequate to induce *THI2.1* and *VSP1* transcription. Contrasting expression of *PDF1.2* compared with both *THI2.1* and *VSP1* genes has also been seen in response to JA via the transcription factor AtMYC2 which upregulated wound-response genes *VSP* and *THI2* but repressed *PDF1.2* (Lorenzo *et al.*, 2004). However, microarray analysis of MeJA-treated *Arabidopsis* seedlings grown on MS-agar failed to exhibit significant *PDF1.2* expression. This was in contrast to both *VSP1* and *THI2.1* which were readily induced by MeJA (Devoto *et al.*, 2005). Instead, high-level *PDF1.2* expression was dependent on both jasmonates and ET (Penninckx *et al.*, 1998). Hence, the rapid induction of *PDF1.2* on heating (Fig. 3a) may be a reflection of both jasmonate (Fig. 5) and ET production (Fig. 6).

Although, exogenous application of MeJA protects membranes from heat stress, categorical proof of a role for jasmonates in thermotolerance required a JA mutant to demonstrate reduced heat-resistance. Unlike *jar1-1*, *coi1-1* is defective in many JA-dependent responses (Xie *et al.*, 1998, Farmer *et al.*, 2003) and, crucially, we found *coi1-1* to be thermosensitive to heat stress. The *COI1-1* gene encodes an F-box protein, part of the SCF^{COI} (Skip-cullin-F-box) ubiquitin ligase complex, providing the first indication that ubiquitin-mediated protein degradation is involved in JA signalling (Xie *et al.*, 1998; Xu

et al., 2002; Devoto *et al.*, 2003; Feng *et al.*, 2003). Temperature stress, both high and low, has also been shown to induce transcript of other E3 ubiquitin ligases (Yan *et al.*, 2003). In an attempt to generate plants with increased stress tolerance, Yan *et al.* (2003) overexpressed the *AtCHIP* gene (encoding a protein with a U-box domain and E3 ubiquitin ligase activity) in *Arabidopsis*. However, this rendered the plant more sensitive to both low- and high-temperature treatment, with an increase in electrolyte leakage observed (Yan *et al.*, 2003); this suggests that expression of this E3 ubiquitin ligase at least, is under tight regulation and any disturbances lead to serious consequences in temperature stress tolerance. This is highlighted by our finding that the *coi1-1* plants, impaired in the ubiquitin-mediated degradation pathway, also show increased heat-induced membrane damage.

A significant finding of our study was the discrete roles for particular jasmonates in thermotolerance. MeJA/JA could protect plants during both heating and recovery phases (Fig. 2a,b), but not confer acquired thermolerance. However, only recovery following stress (Fig. 5b) was compromised with the *opr3* (OPDA reductase) mutant but not leakage during heating (Fig. 5a). This suggests the OPDA was sufficient for basal thermotolerance but downstream jasmonates are required for the recovery phase. Significantly, in a comparison of OPDA versus MeJA induced gene expression, only OPDA proved to be sufficient to induce a range of heat shock proteins (Taki *et al.*, 2005).

An additional signalling interaction within *cpr5-1* was that between SA and ET. In contrast to the findings of Larkindale *et al.* (2005), we found that *ein2-1* plants were less susceptible to heat stress. The discrepancy observed may be caused by the difference in heat treatment and age of the plants; Larkindale *et al.* (2005) subjected 7-d-old seedlings to a 60-min 45°C heat treatment to assess basal thermotolerance compared with our milder experimental condition of 38°C heat treatment for 16°h using 3-wk-old plants. One interpretation based on evidence from the literature is that ET is a response to stress and an indicator of cell death. Ethylene has been linked to the initiation of cell death associated with, for example, ozone (Vahala *et al.*, 2003), disease (Cohn & Martin, 2005) and in roots (He *et al.*, 1996). Further, this matches our own observations of reduced cell death in *ein2-1*. Moreover, with NahG and *opr3*, where heat tolerance is reduced and cell death increased (Clarke *et al.*, 2004; Fig. 5c), ET levels are lower (Fig. 6). Hence, the ET may simply reflect the levels of cell death at 38°C but given the data of Larkindale *et al.* (2005) ET may act to confer thermotolerance at higher temperatures. This latter effect may reflect differential ET interactions with SA and JA. This important aspect requires further exploration. It is interesting to note that *ein2-1* and *coi1-1* mutations appear to have opposite affects on thermotolerance in 3-wk-old plants. Jasmonic acid and ET can either cooperate or act as antagonists in the regulation of different stress responses. In the case of pathogen attack, both hormones appear to synergize in the activation of defence gene expression (Xu *et al.*, 1994; Penninckx *et al.*, 1998; Lorenzo *et al.*, 2003). However, an antagonistic interaction between JA and ET has been described in response to ozone exposure where JA protects tissues from ozone stress and ET enhances ozone-induced cell death (Overmyer *et al.*, 2000; Tamaoki *et al.*, 2003; Tuominen *et al.*, 2004). In addition the *jar1-1 Arabidopsis* mutant was found to be ozone sensitive in contrast to *ein2-1*, which is ozone tolerant (Tuominen *et al.*, 2004). Such ET–JA antagonism may effect at the transcriptional level as MYC2-mediated induction of *VSP1* is suppressed by ET (Dombrecht *et al.*, 2007). This contrasts with the positive contribution made by JA to *PDF1.2* expression acting through the ERF1 and ORA59 transcription factors which were first characterized as mediating ET effects (Pré *et al.*, 2008). An important task for future work is to establish the mechanism(s) for JA-conferred thermotolerance, given that HSP apparently play no role (Fig. 4b, Fig. S2). Examination of transcriptome data for heat and MeJA responsive genes via GENEVESTIGATOR (Zimmermann *et al.*, 2004) indicated a preponderance of genes associated with protein translation and photosynthetic electron transport, which could represent features associated with cellular recovery following HS (Fig. S3).

Through a network of signalling pathways plants are able to tolerate both biotic and abiotic stresses where selection of the specific response that is appropriate for each stress stimulus seems to be determined by the type of interaction established between the signalling pathways. Our studies (this and Clarke *et al.*, 2004) and those of Larkindale & Knight (2002) and Larkindale *et al.* (2005) shed some light on crosstalk between the hormonal, redox and calcium-activated signalling pathways which play a role in thermotolerance to ensure a rapid response which is crucial for survival against temperature stress.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 An unaltered acquired thermotolerance in *npr1-1*, *jar1-1* and *ein2-1*.

Fig. S2 Assessing methyl jasmonate and ethylene effects on heat shock protein gene expression within publicly available transcriptomic databases.

Fig. S3 Genes exhibiting the highest upregulation in response to methyl jasmonate treatment and to heat shock.

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