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RESEARCH PAPER

Involvement of the *Arabidopsis* HIT1/AtVPS53 tethering protein homologue in the acclimation of the plasma membrane to heat stress

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Abstract

Arabidopsis thaliana hit1-1 **is a heat-intolerant mutant. The** ***HIT1*** **gene encodes a protein that is homologous to yeast Vps53p, which is a subunit of the Golgi-associated retrograde protein (GARP) complex that is involved in retrograde membrane trafficking to the Golgi. To investigate the correlation between the cellular role of HIT1 and its protective function in heat tolerance in plants, it was verified that HIT1 was co-localized with AtVPS52 and AtVPS54, the other putative subunits of GARP, in the Golgi and post-Golgi compartments in** ***Arabidopsis*** **protoplasts. A bimolecular fluorescence complementation assay showed that HIT1 interacted with AtVPS52 and AtVPS54, which indicated their assembly into a protein complex** ***in vivo*****. Under heat stress conditions, the plasma membrane of** ***hit1-1*** **was less stable than that of the wild type, as determined by an electrolyte leakage assay, and enhanced leakage occurred before peroxidation injury to the membrane. In addition, the ability of** ***hit1-1*** **to survive heat stress was not influenced by exposure to light, which suggested that the heat intolerance of** ***hit-1*** **was a direct outcome of reduced membrane thermostability rather than heat-induced oxidative stress. Furthermore,** ***hit1-1*** **was sensitive to the duration (sustained high temperature stress at 37 °C for 3 d) but not the intensity (heat shock at 44 °C for 30 min) of exposure to heat. Collectively, these results imply that HIT1 functions in the membrane trafficking that is involved in the thermal adaptation of the plasma membrane for tolerance to long-term heat stress in plants.**

Key words: *Arabidopsis thaliana*, GARP complex, heat stress, HIT1, HIT2, membrane trafficking, vesicle-tethering factor, Vps53.

Introduction

Temperature is one of the most variable environmental factors, and heat stress due to elevated temperatures can have a detrimental impact on living organisms. The heat stress that is experienced by an organism can be classified as either sustained high temperature stress or heat shock, depending on the intensity and duration of the increased temperature, and the rate of temperature increase (Sung *et al.*, 2003). Given that plants are immobile and unable to escape from their habitat, they are more vulnerable to high temperature or heat shock than animals, and must evolve appropriate mechanisms of heat tolerance for survival.

The plant cell membrane is a direct and major target of heat stress. High temperatures can alter the physical state of the membrane, and lead to fluidization and disintegration (Los and Murata, 2004). This disruption perturbs the boundary provided by the plasma membrane and causes increased permeability and leakage of ions, which can be measured readily by the efflux of electrolytes (Wahid *et al.*, 2007). Membrane-associated biological processes, such as photosynthesis, can also be disrupted subsequently. As a result of this disruption, high-energy electrons can react with molecular oxygen and yield excess reactive oxygen

Abbreviations: BiFC, bimolecular fluorescence complementation; EMS, ethylmethanesulphonate; ER, endoplasmic reticulum; GARP, Golgi-associated retrograde protein; HIT, heat intolerant; MDA, malondialdehyde; MV, methyl viologen; PVC, pre-vacuolar compartment; ROS, reactive oxygen species; SNARE, soluble *N*-ethylmaleimide-sensitive factor adaptor protein receptor; SYT1, synaptogamin 1; TBA, thiobarbituric acid; TBARS, thiobarbituric acid-reactive substances; TCA, trichloroacetic acid; TGN, trans-Golgi network.

species (ROS), which can attack the cell membrane further; this process manifests as light-dependent, heat-induced oxidative stress (Larkindale and Knight, 2002; Upchurch, 2008). To minimize such effects, prompt repair at the sites where the membrane is damaged is necessary. Recent studies have indicated that plant cells can employ synaptotagmin-mediated repair to maintain the integrity of the plasma membrane under various conditions of stress; possibly by using exocytotic vesicles to seal the site of injury and/or endocytosis to internalize the lesions (Schapire *et al.*, 2008; Yamazaki *et al.*, 2008). It is expected that effective trafficking of vesicles within the endomembrane system is necessary to replenish exocytotic vesicles and retrieve endocytotic vesicles for this process of membrane rejuvenation.

In addition to the above-mentioned repair processes, plant cells can adjust the degree of saturation of lipids in the membrane to enhance membrane thermostability and hence improve the tolerance of the whole plant to heat (Larkindale and Huang, 2004; Benning, 2009; Su *et al.*, 2009). For example, it has been reported that in response to elevated growth temperature the level of saturated fatty acids in plants, including *Arabidopsis*, increases, with a concomitant decrease in unsaturated fatty acids (Pearcy, 1978; Falcone *et al.*, 2004). Mutants and genetically manipulated plants that have a defect in pathways for the formation of unsaturated fatty acids, and, as a result, accumulate a higher proportion of saturated fatty acids, have also been shown to achieve better heat tolerance than wild-type plants (Murakami *et al.*, 2000; Alfonso *et al.*, 2001; Falcone *et al.*, 2004). However, in all plant tissues, the major lipids are synthesized initially using only saturated acyl chains, and unsaturated bonds are introduced later by desaturases (Ohlrogge and Browse, 1995; Benning, 2009). As a consequence, the heat-induced shift in composition to a higher degree of membrane saturation requires *de novo* synthesis of fatty acids and their assembly into lipids. Furthermore, although plant cells contain various membrane-bound organelles, the sites of fatty acid synthesis and lipid assembly are restricted to the plastids and endoplasmic reticulum (ER) (Ohlrogge and Browse, 1995; Jouhet *et al.*, 2007). This indicates that the heat-induced remodelling of extra-plastidic membranes requires the transport of lipids between biogenic and non-biogenic membranes, which is carried out by vesicular trafficking.

Apparently, membrane transport via the trafficking of vesicles plays a crucial role in heat tolerance in plants. Vesicles bud from their donor membrane and then travel to a specific destination where they fuse with the acceptor membrane in a tightly regulated manner (Hwang and Robinson, 2009). One of the major regulatory steps, which affects the effectiveness of the entire trafficking process, is the control of the specificity of the target membrane and the subsequent fusion event (Cai *et al.*, 2007). In this regard, it has been suggested that tethering factors function at the earliest stage to direct vesicles to the correct membrane and promote fusion (Pferrer, 1999; Sztul and Lupashin, 2006). Tethering factors are either long coiled-coil proteins or large multisubunit protein complexes. The tethers formed involve physical links between vesicles and target membranes over considerable distances (Whyte and Munro, 2002; Sztul and Lupashin, 2006). In addition to bringing together transport vesicles and target membranes, many tethering factors have also been demonstrated to interact with soluble *N*-ethylmaleimide-sensitive factor adaptor protein receptors (SNAREs) on the transport vesicle (v-SNAREs) and target membrane (t-SNAREs), and regulate the assembly of the SNARE complex, a four-helix bundle that is responsible for bilayer fusion (Söllner, 2002; Lupashin and Sztul, 2005; Ungermann and Langosch, 2005; Sutter *et al.*, 2006; Cai *et al.*, 2007). A remarkable number of these regulators have been identified in plants through genomic analysis. For example, the genome of *Arabidopsis* contains >50 loci that encode possible homologues of known tethering proteins or subunits of tethering complexes (Latijnhouwers *et al.*, 2005; Zhang *et al.*, 2010). However, it remains unclear which of these proteins are essential for heat tolerance in plants, and the biological roles that they play in this process.

A forward genetic approach has been used to isolate an ethylmethanesuphonate (EMS)-induced *hit1-1* (*heat-intolerant 1*) mutant of *Arabidopsis*, whose growth is more sensitive to inhibition by high temperature than that of the wild type (Wu *et al.*, 2000). The mutated gene was later shown to encode a homologue of yeast Vps53p (Lee *et al.*, 2006), which is a subunit of the Golgi-associated retrograde protein (GARP) tethering complex that is involved in directing the retrograde transport of vesicles to the Golgi (Conibear and Stevens, 2000; Conibear *et al.*, 2003). The yeast *vps53Δ* null mutant also shows reduced thermotolerance, and expression of *HIT1* in this mutant can partially complement the defect, which indicates a conserved biological function for Vps53p and HIT1 (Lee *et al.*, 2006). In the present study, the *hit1-1* mutant was used to investigate the role of HIT1 in the maintenance of cell membrane integrity under high temperatures, and herein information about the causal linkage between vesicular trafficking and heat tolerance in plants is provided.

Materials and methods

Plant materials and growth conditions

The wild-type *A. thaliana* plants used in this study were of the Columbia-0 ecotype. Seeds were obtained from Lehle Seeds Company (Round Rock, TX, USA). The *hit1-1* and *hit2* mutant lines were isolated from the F_2 progeny of EMS-mutagenized plants as described previously (Lee *et al.*, 2006; Wang *et al.*, 2008; Wu *et al.*, 2010). For plants grown in medium, seeds were surface-sterilized in commercial bleach that contained 5% (v/v) sodium hypochlorite and 0.1% (v/v) Triton X-100 solution for 10 min, rinsed five times in sterilized water, and stratified at 4 °C for 2 d in the dark. Seeds were planted on agar plates that contained half-strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962), 2% sucrose (w/v), and 0.8% agar (w/v), buffered to pH 5.7. All seedlings were grown at 23 °C with continuous light at 100 μmol m^{-2} s^{-1} in a growth chamber. For the electrolyte leakage assay, measurement of thiobarbituric acid-reactive substances (TBARS), and treatment with methyl viologen (MV),

plants were grown in soil at 23 °C with a 16/8 h light/dark cycle at 120 μmol m^{-2} s^{-1} before the experiments were carried out.

Cloning of cDNA

The cDNA fragment that encoded the fluorescent protein mCherry was amplified from a clone that was obtained from the Arabidopsis Biological Resources Center (ABRC; Columbus, OH, USA; stock number CD3-967) using primers 5'-GCTCTAGAATGGTGAGCAAGGGCGAGGAG-3' and 5'-CGGGATCCCTTGTACAGCTCGTCCATGCC-3'. The amplified fragment was digested with *Xba*I/*Bam*HI (corresponding restriction sites are underlined in the primer sequences) and cloned into the pLOLA vector (Ferrando *et al.*, 2001) to create pLOLA-mCherry. To clone *HIT1*/*AtVPS53*, *POK*/*AtVPS52*, and *AtVPS54*, DNase-treated RNA that had been isolated from *Arabidopsis* rosette leaves was reverse-transcribed using Moloney murine leukaemia virus HP reverse transcriptase (Epicentre Technologies, Madison, WI, USA) with an oligo(dT) primer to generate first-strand cDNA. The *AtVPS52* cDNA was amplified using primers *AtVPS52-attB1* and *AtVPS52-attB2*, *HIT1* with *HIT1-attB1* and *HIT1-attB2*, and *AtVPS54* with *AtVPS54-attB1* and *AtVPS54-attB2*. The resultant amplicons were re-amplified subsequently with primers *attB1* and *attB2* and cloned into pDONR-221 (Invitrogen, Carlsbad, CA, USA) to create entry clones using BP Clonase (Invitrogen) in accordance with the manufacturer's instructions. Details of the primer sequences are provided in Supplementary Table S1 available at *JXB* online.

Plasmid constructs

For the localization experiment, cDNA that encoded wild-type *Arabidopsis HIT1* was re-amplified from leaf cDNA using the primers 5'- CGGGATCCATGGATAAGTCGAGTGCT-3' and 5'-CGGAATTCTGCGTTGAAGAGTCTCC-3'. The amplicon was introduced into pLOLA-mCherry and inserted in-frame at the 3' end of the mCherry cDNA sequence after digestion with *Bam*HI and *Eco*RI. The resultant mCherry–HIT1 fusion was flanked by a 35S promoter and the nopaline synthase (nos) 3' polyadenylation signal. Meanwhile, *AtVPS52* and *AtVPS54* were fused to enhanced yellow fluorescent protein (EYFP) by recombination of their respective entry clones with the destination vector pEarleygate104 (Earley *et al.*, 2006), using LR Clonase (Invitrogen) to generate the fusions EYFP–AtVPS52 and EYFP–AtVPS54. To generate constructs for the bimolecular fluorescence complementation (BiFC) protein interaction assay, the cDNAs for *HIT1*, *AtVPS52*, and *AtVPS54* were transferred from their respective entry clones into the gateway vector pSAT5-DEST-c(175-end)EYFP-C1(B) (ABRC stock number CD3-1097) or pSAT4-DEST-n(174)EYFP-C1(ABRC stock number CD3-1089), which contained the N-terminal 174 amino acids of EYFP ($EYFP^N$) or the C-terminal 64 amino acids of EYFP ($EYFP^C$), respectively.

Preparation of protoplasts and transient gene expression

Protoplasts were prepared from rosette leaves of 4-week-old plants. The lower epidermis of the leaves was peeled away and the peeled leaves were floated peeled-side down on 5 ml of enzyme solution [0.4 M mannitol, 20 mM KCl, 10 mM $CaCl_2$, 1% (w/v) cellulase R10, 0.25% (w/v) macerozyme R10, 0.1% (w/v) bovine serum albumin (BSA), 5 mM β-mercaptoethanol, 20 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 5.7]. After incubation for 1 h at 25 °C with agitation, protoplasts were harvested in a 50 ml centrifuge tube by centrifugation at 100 *g* for 1 min. Collected protoplasts were washed twice with 10 ml of W5 solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 5 mM glucose, 2 mM MES, pH 5.7). A third wash was performed by re-suspending the protoplasts in 10 ml of W5 solution and incubating on ice for 30 min, after which the protoplasts were pelleted. Washed protoplasts were then re-suspended at 2×10^5 cells ml^{-1} in MMg solution (0.4 M mannitol, 15 mM $MgCl_2$, and 4 mM MES, pH 5.7) for analysis of transient gene expression. Polyethylene glycol (PEG)-mediated transformation was performed as described by Yoo *et al.* (2007). Expression of fluorescent fusion proteins was observed under an Olympus IX71 fluorescence microscope (Center Valley, PA, USA).

Measurement of electrolyte leakage

Electrolyte leakage induced by treatment at a sustained high temperature was measured using plants grown in soil. For the heat stress treatment, pots that contained plants with eight rosette leaves were transferred to a growth chamber at 37 °C with continuous illumination (120 μmol m^{-2} s^{-1}). The pots were placed in a tray that was covered with a transparent plastic roof, and sufficient water was supplied by keeping the tray flooded. After heating at 37 °C for various times, the fifth, sixth, and seventh rosette leaves were harvested and placed in 5 ml of deionized distilled water in 15 ml centrifuge tubes, and shaken at 100 rpm at 25 °C for 3 h. The conductivity of the bathing solution (C_1) was measured using a conductivity meter (CyberScan 1500; Eutech Instruments, Singapore). Samples were autoclaved at 121 °C for 15 min to release the total electrolytes, and the conductance (C_2) was measured again. The percentage leakage of electrolytes was calculated as the ratio C_1:C_2. At least three individual plants were measured for each time point, and each data point represented the average from the total replicates.

TBARS assay

Products of lipid peroxidation such as malondialdehyde (MDA) form adducts with thiobarbituric acid (TBA) that can be measured by fluorometry. Analysis of heat-induced TBARS was performed in parallel with the measurement of electrolyte leakage. The fifth, sixth, and seventh rosette leaves of plants grown in soil, which weighed ~0.1 g, were harvested and homogenized in liquid nitrogen immediately after heat stress treatment. The homogenized samples were added to 0.5 ml of 5% (w/v) trichloroacetic acid (TCA) and centrifuged at 12 000 *g* for 15 min. An aliquot of 200 μl of supernatant was mixed with 800 μl of 0.5% (w/v) TBA in 20% (w/v) TCA. The mixtures were heated to 95 °C for 30 min and cooled quickly in an ice bath for 15 min to stop the reaction. The mixtures were centrifuged a second time, and the absorbance of the supernatants was read at 532 nm and 600 nm using a spectrophotometer (UV-VIS SP-8001; Metertech Inc., Taipei, Taiwan). The non-specific absorbance at 600 nm was subtracted from the absorbance at 532 nm, and the difference was used to calculate the amount of MDA equivalents using an extinction coefficient of 155 mM^{-1} cm^{-1}.

Treatment with MV

To analyse the sensitivity of the whole plant to the inducer of photo-oxidation MV, seeds were sown directly on potting soil, with each pot containing seeds of *hit1-1*, *hit2*, and wild-type *Arabidopsis*. After growth at 23 °C for 4 weeks, the plants in each pot were sprayed directly with 10 μM MV (~1 ml per three plants) once daily for three consecutive days. Control plants were sprayed with water. After treatment, the plants were placed at 23 °C with continuous illumination at 180 μmol m^{-2} s^{-1} for a further 3 d before photographs were taken to assess MV-induced foliar lesions. The percentage necrotic area in the whole plant leaf was measured using imaging software (Photoshop CS5; Adobe Systems, San Jose, CA, USA). To analyse the sensitivity of leaf discs to MV, at least three discs (diameter, 6 mm) were punched from different plants and placed adaxial side up on MS agar plates that contained various concentrations of MV. The samples were incubated at 23 °C for 3 d with continuous illumination at 180 μmol m^{-2} s^{-1}. MV-induced damage was examined by

observing phenotypic changes that occurred as compared with the controls, and quantified by measuring chlorophyll content.

Determination of chlorophyll content

Ten leaf discs (~0.06 g) that had been treated with MV were homogenized in 1 ml of 80% (v/v) acetone and incubated at 4 °C in the dark for 30 min. After centrifugation to precipitate the debris, the optical density of the supernatant was read at 663 nm and 646 nm (chlorophyll *a* and *b*) using a spectrophotometer (UV-VIS SP-8001; Metertech Inc.). At least three replicates were performed for each treatment. The chlorophyll content was expressed as a percentage of that of wild-type controls that had not been treated with MV.

Sustained high temperature and heat shock

Seedlings grown in medium were used for all analyses of heat stress. Freshly autoclaved medium (33 ml) was poured into Petri dishes (90 mm in diameter, 20 mm in depth; Viogene, Sijhih, Taiwan). For sustained high temperature treatment, Petri dishes that contained 10-day-old seedlings were transferred to a growth chamber set at 37 °C, and incubated for 3 d with continuous illumination at 100 μmol m^{-2} s^{-1}. After treatment, the plates were returned to 23 °C for a further 4 d for observation. During the entire process, the plates were sealed with one layer of cling film and then a layer of Parafilm to prevent dehydration (Wu *et al.*, 2010). For heat shock, seeds were sown on agar plates and allowed to grow at 23 °C to the stage at which the first leaf pair had just emerged. The plates were sealed tightly with Parafilm and completely submerged in a water bath set at 44 °C for 30 min. After treatment, the plates were returned to 23 °C for recovery and observation.

Results

HIT1 is co-localized intracellularly with other subunits of the putative Arabidopsis *GARP complex*

The *Arabidopsis* genome contains three genes that encode homologues of subunits of the GARP complex: *AtVPS52* (At1g71270, also known as *POK*), *HIT1/AtVPS53* (At1g50500), and *AtVPS54* (At4g19490) (Lobstein *et al.*, 2004; Latijnhouwers *et al.*, 2005; Guermonprez *et al.*, 2008). To verify that HIT1 is associated with the putative plant GARP complex, and thus gain more insight into its cellular function, experiments were carried out to determine whether these homologues were co-localized subcellularly. Reporter constructs were created by in-frame fusion of the C-terminal end of the red fluorescence protein mCherry to the N-terminal end of HIT1, and the C-terminal end of EYFP to the N-terminal end of AtVPS52 or AtVPS54, under the control of the cauliflower mosaic virus 35S promoter. Protoplasts derived from *Arabidopsis* cell suspensions were transformed for transient co-expression of mCherry–HIT1 with EYFP–AtVPS52 or mCherry–HIT1 with EYFP–AtVPS54, and examined under an epifluorescence microscope. In addition, because AtVPS52 has previously been shown to accumulate in vesicle-like structures and localize in the Golgi apparatus and post-Golgi compartments (Guermonprez *et al.*, 2008), AtVPS52 also served as an ideal compartmental marker in this experiment. It was shown that fluorescently tagged HIT1, AtVPS52, and AtVPS54 all gave rise to a punctate pattern of fluorescence that was distributed homogeneously in the cytoplasm, and yielded no detectable fluorescent signal in the plasma membrane (Fig. 1). Clear co-localization of HIT1 with AtVPS52 and AtVPS54 was indicated by the perfect overlay of the fluorescent signals of mCherry and EYFP when mCherry–HIT1 was co-expressed with the EYFP–AtVPS52 or EYFP–AtVPS54 fusion protein (Fig. 1).

HIT1 interacts with other subunits of the putative Arabidopsis *GARP complex* in vivo

To corroborate the association of HIT1, AtVPS52, and AtVPS54 in a GARP complex, the protein–protein interactions between these subunit homologues were studied further, using the BiFC assay. This assay is based on the restoration of a functional fluorophore by bringing together the two non-fluorescent halves of the molecule through the association of two interacting proteins in living plant cells (Bracha-Drori *et al.*, 2004; Walter *et al.*, 2004). BiFC vectors were constructed in which full-length HIT1, AtVPS52, or AtVPS54 was fused with $EYFP^N$ or $EYFP^C$. Pairs of vectors that contained potentially interacting protein partners fused with $EYFP^N$ and $EYFP^C$, respectively, were co-transformed into *Arabidopsis* protoplasts, and the reconstitution of fluorescent EYFP was examined under an epifluorescence microscope. As shown in Fig. 2, co-expression of $EYFP^C$–HIT1 and $EYFP^N$–AtVPS52 or $EYFP^C$–HIT1 and $EYFP^N$–AtVPS54 generated BiFC fluorescence. However, a BiFC signal was not detected for any possible pairwise combination of fusions of the split EYFP halves with AtVPS52 and AtVPS54 (data not shown). This suggested that AtVPS52 and AtVPS54 did not interact with each other, or that in these pairs of fusion proteins, the fluorophore halves were in a steric orientation that was unfavourable for the complementation of EYFP. Nevertheless, these results indicated that HIT1 did interact with AtVPS52 and AtVPS54 *in vivo*, and the intracellular distribution of the BiFC signals resembled that observed in the co-localization experiments, which suggested that these proteins were assembled into the plant GARP complex.

hit1-1 *shows decreased thermostability of the plasma membrane*

The *hit1-1* mutant was identified originally by its inability to tolerate high temperature stress, and HIT1 was shown to be a mediator of endomembrane transport. Thus, it was reasonable to postulate that the heat intolerance of *hit1-1* is due to impairment of the membrane transport that is required for repair or acclimation of the plasma membrane. To investigate this possibility, the integrity of the plasma membrane in *hit1-1* and wild-type leaves at high temperature was analysed by an electrolyte leakage assay. Figure 3A demonstrates that *hit1-1* and wild-type leaves showed a similar level of electrolyte leakage under non-stressed conditions (23 °C), and for the first 20 h following the shift to high temperature (37 °C). However, after

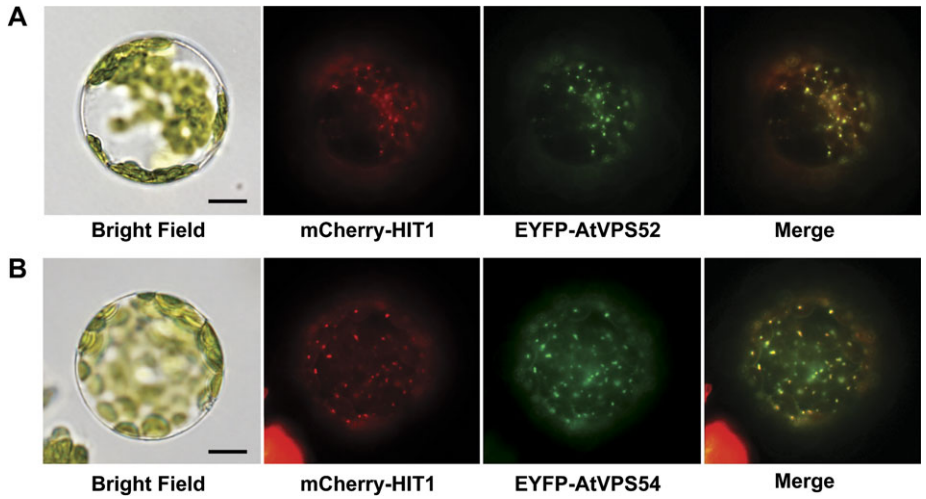

Fig. 1. HIT1 was co-localized intracellularly with other putative subunits of the *Arabidopsis* GARP complex. Protoplasts from *Arabidopsis* leaf tissue were co-transformed with mCherry–HIT1 and EYFP–AtVPS52 (A) or mCherry–HIT1 and EYFP–AtVPS54 (B). At 16 h after transformation by PEG-mediated transfection, each fluorescent signal was observed with a fluorescent microscope. The signals of EYFP-tagged AtVPS52 acted as a marker of the Golgi and post-Golgi compartments. Panels in column 1 show bright-field images. Panels in column 2 show the signal from mCherry. Panels in column 3 show the signals from EYFP. Panels in column 4 show merged signals of mCherry and EYFP. Bar=10 μm.

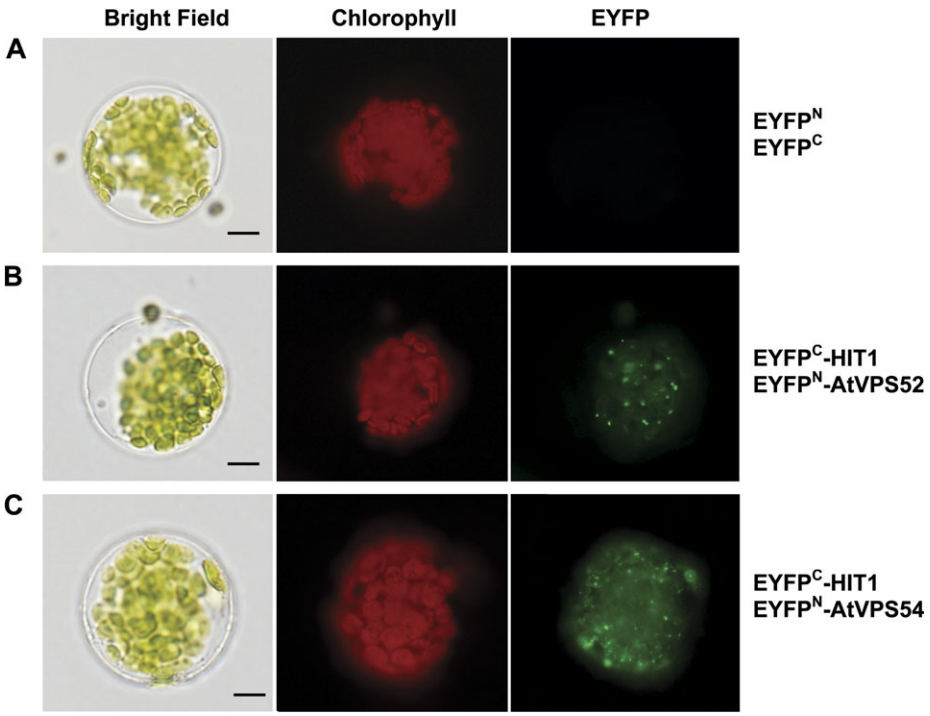

Fig. 2. Interaction of HIT1 with other putative subunits of the *Arabidopsis* GARP complex as examined by BiFC assay in *Arabidopsis* protoplasts. Protoplasts from *Arabidopsis* leaf tissue were co-transformed with (A) 35S-EYFP^{N} and 35S-EYFP^{C}; (B) EYFP^{C}–HIT1 and EYFP^{N}–AtVPS52; or (C) EYFP^{C}–HIT1 and EYFP^{N}–AtVPS54. Panels in column 1 show bright-field images. Panels in column 2 show chlorophyll autofluorescence. Panels in column 3 show the signals from EYFP. Bar=10 μm.

exposure to 37 °C for 24 h, significantly more electrolytes began to be lost from *hit1-1* leaves than from wild-type leaves (Fig. 3A). The heat-enhanced leakage of electrolytes from *hit1-1* leaves remained higher than that from the wild-type leaves throughout the 36 h period of heat stress. Meanwhile, no significant difference in appearance was observed between the *hit1-1* and wild-type plants immediately after the exposure to heat stress for 36 h. However, the *hit1-1* leaves shrivelled thereafter, even when the plants were returned to 23 °C (Supplementary Fig. S1 at *JXB* online), which suggested that the injury to the plasma membrane in *hit1-1* leaves had reached a substantial level, from which it

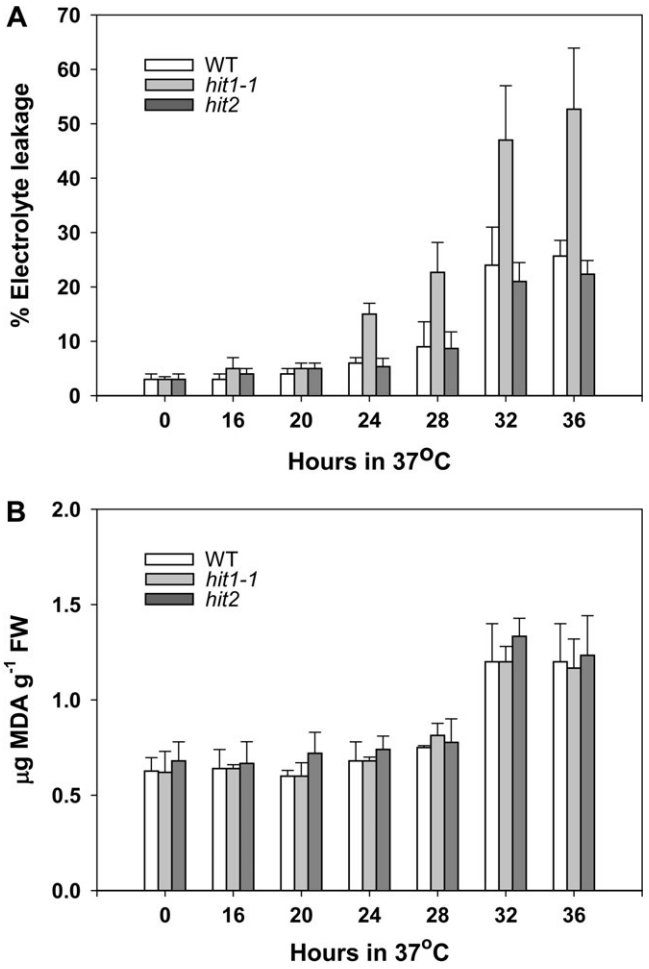

Fig. 3. Involvement of HIT1 in the maintenance of plasma membrane integrity under sustained high temperature conditions. (A) The integrity of plasma membrane was analysed by measurement of electrolyte leakage. Four-week-old *hit1-1*, *hit2*, and wild-type (WT) plants were heat stressed at 37 °C for various times under continuous illumination. After heat treatment, the percentage leakage of electrolytes from the leaves of each plant was measured. Data are the means of at least three independent assays. Error bars represent the SD for all experiments. (B) Heat-induced oxidative damage was analysed in terms of TBARS. Plants and heat treatments were the same as for the electrolyte leakage assay. The MDA–TBA adduct produced was measured, and TBARS were expressed as μg MDA (g fresh weight)$^{-1}$. Data shown are the average of three independent assays, and error bars represent the SD of these three assays.

was not easy to recover. These results indicated that heat-induced damage of the plasma membrane occurred faster and was more severe over time in *hit1-1* plants than in the wild-type plants.

Heat can induce overproduction of ROS, and membrane lipids are a major target of ROS damage. Therefore, the enhanced electrolyte leakage that was observed from heat-treated *hit1-1* leaves might have resulted from heat-induced oxidative injury. To test this possibility, the heat-induced electrolyte leakage of *hit1-1* was compared with that of another heat-intolerant mutant *hit2*. This mutant was chosen because its hypersensitivity to heat has been demonstrated to be due in part to reduced adaptability to heat-induced oxidative stress (Wu *et al.*, 2010). The first time point at which a significant rise in electrolyte leakage from *hit2* could be detected was after 28 h, which was the same as for the wild type (Fig. 3A).

To clarify the cause of the observed heat-induced disruption of the plasma membrane, a TBARS assay was performed, which reflects the degree of oxidative damage to lipid membranes. As shown in Fig. 3B, the trend of an increase in TBARS in *hit1-1* in response to treatment at 37 °C was similar to that found in wild-type and *hit2* plants, with a significant rise in TBARS being detected after 28 h. This time point was close to that for the increase in electrolyte leakage that occurred in wild-type and *hit2* plants, but was later than that for the increase in electrolyte leakage in *hit1-1*. This finding indicated that, in the *hit1-1* mutant, heat-enhanced electrolyte leakage occurred before the effects of heat-induced oxidative stress, suggesting that enhanced electrolyte leakage was a direct outcome of the reduced thermostability of the plasma membrane, rather than the result of secondary oxidative injuries.

Heat-induced lethality in hit1-1 *does not result from heat-induced oxidative stress*

In *hit1-1*, lipid peroxidation occurs after the enhanced leakage of electrolytes, but it is still uncertain whether the cause of heat-induced lethality of *hit1-1* is primarily from the loss of integrity of the plasma membrane or from the subsequent oxidative injury. To clarify this, the ability of *hit1-1* seedlings to survive heat in the light and in the dark was compared. Light is known to exacerbate heat-induced oxidative stress (Larkindale and Knight, 2002); therefore, keeping heat-stressed plants in the dark can minimize the effect of oxidative injury. Plates that contained 10-day-old seedlings were incubated at 37 °C for 3 d. After treatment, the plates were returned to 23 °C for a further 4 d for observation. Dark conditions were achieved by wrapping the plates in aluminium foil to avoid exposure to light throughout the heat stress and the subsequent recovery period. Figure 4 shows that, although the viability of *hit2* seedlings was increased dramatically in the dark as reported previously (Wu *et al.*, 2010), the viability of *hit1-1* seedlings was not increased. Most *hit1-1* seedlings that were heat stressed and allowed to recover in the dark eventually became bleached and were considered to be dead.

As described above, to minimize the effect of heat-induced oxidative damage, the ability of the plants to survive heat in the dark was examined. Conversely, to minimize the effect of heat and establish the antioxidative capability of the plants, the inducer of photo-oxidation, MV, was applied to plant leaves. As shown in Fig. 5A, although detached leaf discs from wild-type and *hit1-1* plants maintained a relatively normal shade of green after 3 d on medium that contained 0.2 μM MV, those from *hit2* plants showed more severe chlorosis and lost more chlorophyll content (Fig. 5B). In addition, MV was applied as

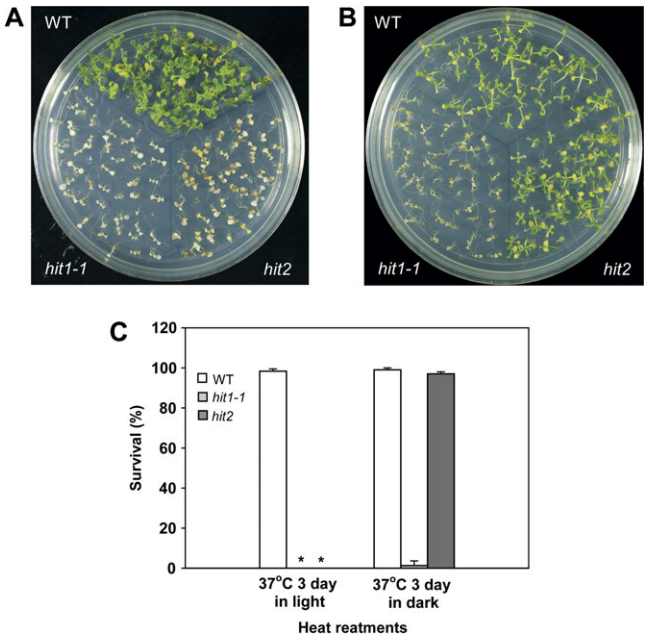

Fig. 4. Survival of *hit1-1* seedlings under sustained heat stress was not affected by exposure to light. Phenotypes of 10-day-old wild-type (WT), *hit1-1*, and *hit2* plants after incubation at 37 °C for 3 d in the light (A) or in the dark (B). The numbers of plants that were alive before and after treatment were counted to determine the survival rate (C). Data shown are the means of three replicate values. Error bars represent the SD. Asterisks indicate zero survival rates.

a direct spray to 4-week-old plants grown in soil. Figure 5C shows that, after spraying for 3 d with 10 μM MV solution, necrotic lesions were obvious on *hit2* plants but were minor on wild-type and *hit1-1* plants. These results imply that heat-induced lethality in *hit1-1* was derived directly from the effect of reduced membrane thermostability, rather than indirectly from heat-induced oxidative stress.

hit1-1 *is specifically defective in tolerance to sustained high temperature stress*

hit1-1 was identified originally as being intolerant to sustained high temperature stress (Wu *et al.*, 2000). Its ability to tolerate transitory heat shock has yet to be determined. In addition to their inherent ability to tolerate high temperatures without pre-acclimation, which is known as basal thermotolerance, plants also have an ability to survive otherwise lethal high temperatures if pre-exposed briefly to a sublethal temperature, which is known as acquired thermotolerance. Different protective mechanisms might contribute to the survival of plants during different types of heat stress. Therefore, characterization of the responses of *hit1-1* to these various forms of heat stress can provide additional information for the elucidation of the role of HIT1 in heat tolerance in plants. For the sudden heat shock test, seedlings grown at 23 °C were heated directly to 44 °C for 30 min and returned to room temperature for recovery. *hit2* was again used as a control because it has been demonstrated to be sensitive to heat shock treatment (Wu *et al.*, 2010). After recovery for 8 d, although the leaves of *hit2* seedlings were bleached completely, the leaves of the *hit1-1* and wild-type seedlings remained green in colour and exhibited visible expansion (Fig. 6A). For the acquired thermotolerance test, seedlings were pre-treated at 37 °C for 60 min before being subjected to 44 °C for 45 min. *hit1-1* was able to acquire tolerance to severe heat shock to the same extent as the wild type (Fig. 6B). Thus, the ability to acquire thermotolerance was also unaffected in the *hit1-1* mutant. This implied that the protective role of *HIT1* in heat tolerance in plants was a response more to the duration rather than the intensity of the heat exposure.

Discussion

The plasma membrane plays many vital roles in plant cells, from forming a selective barrier so that a cell can be defined, to providing a suitable environment in which integral proteins can function. Potentially detrimental growth conditions, such as high temperatures, can damage the plasma membrane, which leads to the escape of essential cytoplasmic constituents and ultimately causes cell death (Wahid *et al.*, 2007). Maintenance of the integrity of the plasma membrane under such harmful conditions depends on prompt repair and/or remodelling of the membrane (Upchurch, 2008), both of which necessitate the effective transport of membrane components to and from the plasma membrane via directed intracellular trafficking of vesicles (Levine, 2002). Accumulating evidence has suggested that tethering (bringing a vesicle into close proximity with the target membrane), which is mediated by tethering factors, is central to efficient and accurate vesicular trafficking (Sutter *et al.*, 2006; Cai *et al.*, 2007). However, no experimental data have clarified the protective role of tethering factors on the thermostability of the plasma membrane and its effects on heat tolerance in plants. The characterization of the *hit1-1* mutant described herein does shed light on this issue.

The GARP tethering complex was identified originally from yeast. It consists of four subunits (Vps51p–Vps54p) and mediates the retrograde transport of membrane proteins from the endosome/pre-vacuolar compartment (PVC) to the Golgi (Conibear and Stevens, 2000; Conibear *et al.*, 2003). Later, genomic analysis revealed that the presence of this complex is widespread among eukaryotes (Koumandou *et al.*, 2007). In yeast, Vps52p–Vsp54p form a stable vesicle-associated complex and Vps51p mediates the association of this complex with the t-SNARE Tlg1p (Conibear *et al.*, 2003). Deletion or point mutations that ablate the Vps51p–Tlg1p interaction do not result in any functional phenotype in yeast (Fridmann-Sirkis, 2006). Furthermore, the mammalian GARP complex, which lacks the Vps51p subunit, is sufficient to promote tethering and support retrograde transport from endosomes to the *trans*-Golgi network (TGN) (Pérez-Victoria *et al.*, 2008; Pérez-Victoria and Bonifacino, 2009), which suggests that the involvement of Vps51p is unnecessary for this trafficking route. In

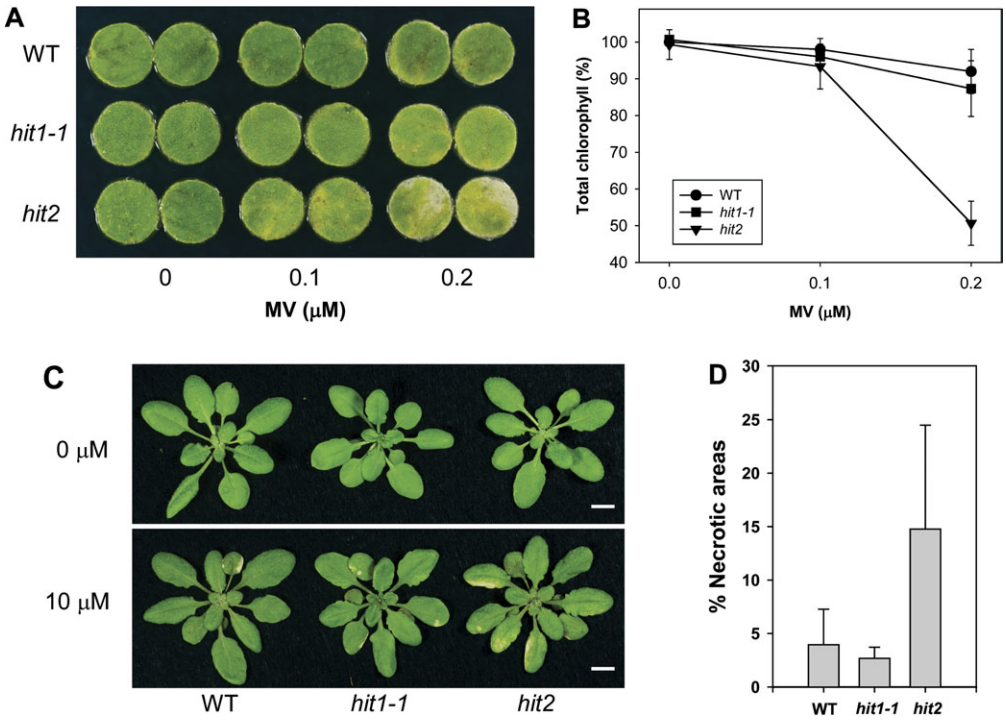

Fig. 5. *hit1-1* was not sensitive to MV-induced photo-oxidative stress. (A) Leaf discs 6 mm in diameter were punched from rosette leaves of wild-type (WT), *hit1-1*, and *hit2* plants, and placed adaxial-side up on agar plates that contained various concentrations of MV, with continuous illumination. After treatment for 3 d, the discs were rearranged for photography. (B) The sensitivity of leaf discs from (A) to MV treatment was quantified by measurement of chlorophyll content. The chlorophyll content was expressed as a percentage of that of MV-untreated wild-type controls. Data represent the averages of three replicates. Error bars represent the SD. (C) Susceptibility of whole plants to MV-induced oxidative stress. Four-week-old wild-type, *hit1-1*, and *hit2* plants grown in soil were sprayed on their aerial parts with 10 μM MV once daily for three consecutive days. Plants were illuminated continuously after the first spray and photographs were taken at 3 d after the third spray. Bar=1 cm. (D) MV-induced oxidative lesions on plants from (C) were quantified by using image analysis software. Necrosis was measured as the percentage of the total leaf area of each plant. Error bars represent the SE (n=3).

Arabidopsis, genes that encode homologues of the Vps52p, 53p, and 54p subunits (*POK*/*AtVPS52*, *HIT1*/*AtVPS53*, and *AtVPS54*, respectively), but not the Vps51p subunit, have been identified (Lobstein *et al.*, 2004). In addition to the characterization of HIT1, which can partially complement the function of Vps53p in yeast (Lee *et al.*, 2006), it has been shown that AtVPS52 is associated with membranes, belongs to a large protein complex, accumulates in vesicle-like structures, and resides in the Golgi and several post-Golgi compartments, including the PVC (Guermonprez *et al.*, 2008). These characteristics are all consistent with those of the aforementioned GARP complex. Nevertheless, there has been no direct evidence that links HIT1, AtVPS52, and AtVPS54. In the present study, transient expression of various fusions with fluorescent protein tags has demonstrated that these homologues are co-localized, and that HIT1 can interact with AtVPS52 and AtVPS54. These findings confirm that HIT1, AtVPS52, and AtVPS54 constitute at least part of the *Arabidopsis* GARP complex.

The *Arabidopsis hit2* mutant is similar to *hit1-1* in that it cannot tolerate sustained high temperature stress (37 °C for 3 d). The role of *HIT2* in heat tolerance is partly to mediate the protection of cells against heat-induced, light-dependent oxidative stress (Wu *et al.*, 2010). Membrane thermostability is a key factor that influences the heat-induced generation of ROS, and the membrane itself is a prime target of attack by ROS. Therefore, the comparison of heat stress responses between *hit1-1* and *hit2* will provide valuable information about the causal relationships between the putative function of HIT1, a regulator of membrane trafficking, and heat tolerance in plants. In the present study, the first increase in the level of TBARS, a measure of lipid peroxidation, was detected at a similar time in *hit1-1* and in *hit2*, but the heat-induced leakage of electrolytes from *hit1-1* occurred earlier than that from *hit2*. These results indicated that *HIT1* did play a role in the maintenance of plasma membrane integrity under high temperature conditions, and that the enhanced electrolyte leakage from *hit1-1* was the result of reduced plasma membrane thermostability. Although neither the *hit1-1* nor the *hit2* mutant could tolerate sustained high temperature stress, *hit1-1*, but not *hit2*, retained the ability to survive sudden heat shock treatment (44 °C for 30 min). This indicates that HIT1 is involved more in the process of membrane acclimation, by which the thermostability of the plasma membrane can be enhanced and its normal biological processes can be sustained, than in the repair of heat-damaged membranes.

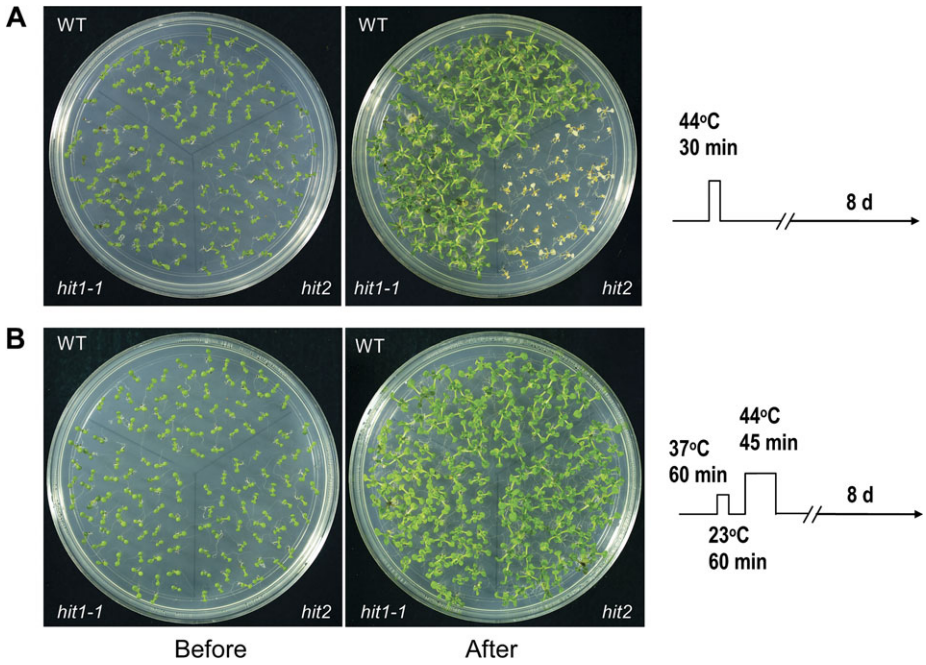

Fig. 6. The Arabidopsis *hit1-1* mutant was not defective in tolerance to heat shock and acquired thermotolerance. Seven-day-old wild-type (WT), *hit1-1*, and *hit2* seedlings that had been grown on the same plates were subjected to the different heating regimens indicated. Other than during the heat treatment, plants were incubated at 23 °C with continuous illumination. Photographs were taken before treatment and at 8 d after the final heat treatment.

Adjustment of membrane fluidity by increasing the levels of saturated fatty acids is a well-recognized mechanism for the acclimatization of membranes to heat (Alfonso *et al.*, 2001; Larkindale and Huang, 2004; Su *et al.*, 2009). However, because existing fatty acids in membranes cannot be modified, restructuring through the *de novo* synthesis and turnover of membrane lipids must occur. Indeed, temporal evaluation of heat-induced alterations in the membranes of *Arabidopsis* leaf cells has shown that changes in the composition of fatty acids cannot be detected for many hours (Falcone *et al.*, 2004). Furthermore, this period is consistent with the time that is required for the synthesis of new fatty acids, as established by time-course radiotracer labelling studies (Browse *et al.*, 1986). These results suggest that the modification of levels of lipid saturation is more significant for the tolerance of plants to longer term heat stress than for heat shock (Falcone *et al.*, 2004), and the observed *hit1-1* heat-sensitive phenotype correlates with this notion. Given that plant plastids are the major site for fatty acid synthesis and lipid assembly, and that HIT1 is localized intracellularly to the Golgi and post-Golgi compartments, these findings suggest that HIT1 functions in vesicular trafficking that it is involved in the remodelling of the plasma membrane. Such remodelling enables the membrane to withstand conditions of elevated temperature, and thus circumvent the lethal effects due to heat-induced damage of the plasma membrane.

Many stressful environmental conditions, such as salinity and hyperosmolarity, are also known to cause disruption to the plasma membrane. Therefore, it is plausible that HIT1 has a role in the tolerance of plants to other causes of stress. Indeed, it has been demonstrated previously that the development of *hit1-1* seedlings is sensitive to osmotic stress imposed by exogenous mannitol (Wu *et al.*, 2000; Lee *et al.*, 2006). In the course of the present study, it was found that the development of *hit1-1* seedlings was inhibited by NaCl (Supplementary Fig. S2 at *JXB* online). Recently, a mechanism to reseal the plasma membrane that employs a synaptotagmin homologue (SYT1) has been demonstrated in *Arabidopsis* (Schapire *et al.*, 2008; Yamazaki *et al.*, 2008). SYT1 is localized in the plasma membrane, and it has been implicated in the promotion of endocytosis or exocytosis at wound sites. Loss of SYT1 results in decreased fitness of plants under conditions of salt and osmotic stress (Schapire *et al.*, 2008; Yamazaki *et al.*, 2008). It is conceivable that HIT1 is part of the logistic network that supports the function of SYT1. However, the fact that HIT1 is involved more in acclimation than in repair of the plasma membrane suggests that HIT1 and SYT1 are involved in different mechanisms for the maintenance of plasma membrane integrity.

In addition to the transport of membrane lipids, vesicles are also responsible for shuttling membrane proteins and secreted macromolecules between the Golgi and plasma membrane. For example, pectin, a major plant cell wall polysaccharide, is synthesized in the Golgi and then passes through the TGN and Golgi-derived vesicles to the plasma membrane for secretion. In contrast, cellulose is synthesized at the plasma membrane, and cellulose synthase is either delivered from the Golgi to the plasma membrane to perform its function or it is transported from the plasma membrane to the TGN for recycling (Micheli, 2001;

Wightman and Turner, 2010). In plants, the content and properties of the cell wall are modified in response to environmental cues, and such modification is required for the maintenance of plasma membrane integrity that confers thermotolerance in plants (Wu *et al.*, 2010). As a consequence, disruption of HIT1-mediated vesicle tethering events might also interrupt the recycling of biomolecules other than membrane lipids between the Golgi and plasma membrane. This in turn would reduce plasma membrane thermostability leading to the heat-intolerant phenotypes observed in the *hit1-1* mutant. In the course of the present study, electrophoretic patterns of plasma membrane proteins from heat-treated plants were also analysed, and the result revealed that wild-type and *hit1-1* plants have different plasma membrane protein profiles (Supplementary Fig. S3 at *JXB* online).

Another protein complex, which is known as the retromer, has been identified in many organisms, including *Arabidopsis* (Oliviusson *et al.*, 2006). The retromer mediates the trafficking of vesicles from the endosome/PVC to the TGN, which is similar to the GARP complex. Studies in yeast have shown that GARP and the retromer can function synergistically during certain stages of cell growth (Morishita *et al.*, 2007). However, it remains unclear whether the *Arabidopsis* retromer participates in the tolerance of plants to stress along with HIT1. Further studies are necessary to answer these intriguing yet important questions, and continued characterization and analysis of the *hit1-1* mutant should provide invaluable information to elucidate the highly dynamic yet finely regulated membrane trafficking that occurs during plant stress responses.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Progression of phenotypes of soil-grown wild-type (WT) and *hit1-1* plants after heat exposure at 37 °C for 36 h. Pots that contained 4-week-old plants were transferred to a growth chamber at 37 °C with continuous illumination (120 μmol m^{-2} s^{-1}). After heat treatment for 36 h, plants were returned to 23 °C for recovery and to take photographs. Although the leaves of WT plants maintained a normal appearance with respect to shape, the leaves of the *hit1-1* plants shrivelled 2 d after the heat treatment.

Figure S2. Seedling development and root elongation in *hit1-1* were more sensitive to NaCl inhibition than in the wild type. (A) Seeds were sown on agar plates that contained 0 mM or 100 mM NaCl. Photographs were taken after germination and development for 10 d at 23 °C. (B) Root elongation of *hit1-1* and wild-type seedlings was measured by transferring 5-day-old seedlings grown in medium onto the surface of vertical agar plates supplemented or not with 100 mM NaCl. Each plate contained four mutant and four wild-type seedlings. Three replicate plates were used for the treatments. Increases in root length were measured every day for 6 d. Each point represents the mean (n=12) and the error bars represent the SD.

Figure S3. Electrophoretic banding patterns of wild-type (WT) and *hit1-1* plasma membrane proteins after heat exposure at 37 °C for 32 h. Plasma membrane proteins were prepared as previously described (Santoni, 2007) and then separated on a 12.5% SDS–polyacrylamide gel. Detection of proteins was accomplished by silver staining of the gel. Equal counts of proteins were loaded in each lane. Arrows indicate visualized electrophoretic bands that exhibit differences in staining intensity showing that these proteins were present in different amounts in the wild-type and *hit1-1* plasma membranes. Molecular mass markers are shown on the left.

Table S1. Oligonucleotides used for cloning *HIT1*, *AtVPS52*, and *AtVPS54* cDNA.

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