

Hsa32, a phosphosulfolactate synthase-related heat-shock protein, is not involved in sulfolipid biosynthesis in *Arabidopsis*

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ABSTRACT. Hsa32 is a novel heat-shock protein (Hsp) mainly found in land plants. Recently, it was shown to be essential for acquired thermotolerance following a long recovery after acclimation heat-shock (HS) treatment. Without Hsa32, *Arabidopsis* mutant plants become more sensitive to severe HS than wild-type plants due to faster decay of a previously acquired protection. Sequence homology showed Hsa32 to be a phosphosulfolactate synthase-related protein, and it was proposed to be involved in the biosynthesis of sulfoquinovosyl diacylglycerol (SQDG), one of the major sulfur-containing glycolipids in the chloroplast thylakoid membrane. Currently, Sqd1 and Sqd2 are known to catalyze the consecutive reactions in the biosynthetic pathway of the sulfolipid. In this study, we examine Hsa32's possible involvement in an alternative pathway that bypasses Sqd1. Our analysis of the wild type and *Hsa32* T-DNA knockout mutant plants revealed no significant differences in SQDG accumulation. In addition, the *Arabidopsis* mutant with a disrupted *Sqd1* gene did not synthesize SQDG, which discounts the existence of an alternative pathway. The *Sqd1* and *Sqd2* knockout mutants, both lacking SQDG, did not show the same defect in acquired thermotolerance as did the *Hsa32* null mutant, which suggests that the sulfolipid level is not related to the HS-sensitive phenotype. Our data suggest that Hsa32 is not involved in SQDG biosynthesis.

Keywords: *Arabidopsis*; Sulfolipid; Thermotolerance.

Abbreviations: HS, Heat-shock; UDP-SQ, uridine diphosphate-sulfoquinovose; SQDG, sulfoquinovosyl diacylglycerol.

INTRODUCTION

Heat-shock proteins (Hsps) are transiently induced by elevated temperature to confer protection against the detrimental effect of heat stress, a phenomenon known as heat shock (HS) response (Lindquist, 1986). Conserved Hsps, such as Hsp100, Hsp90, Hsp70, Hsp60, and the small Hsps, have been well characterized as molecular chaperones, responsible for the acquisition of thermotolerance by maintaining the homeostasis of protein folding in cells (Vierling, 1991; Parsell and Lindquist, 1993). With the progress of genomic and functional genomic research, novel Hsps have been identified from various species (Gasch et al., 2000; Helmann et al., 2001; Shockley et al., 2003; Pysz et al., 2004; Rizhsky et al., 2004; Busch et al., 2005). However, the functions of many of these novel Hsps are unknown, thus hindering a further understanding of this important physiological process.

In an attempt to discover the unique features in the plant HS response, we have previously identified and

characterized a novel Hsp gene, *Hsa32*, which is highly conserved in land plants but not present in most other organisms (Liu et al., 2006). Suppression of *Hsa32* expression by T-DNA insertion or RNAi transgene led to significant defects in acquired thermotolerance during a long recovery period after acclimation, which suggests an important role for the protein in a protection mechanism needed in plants (Charng et al., 2006). How Hsa32 exerts its function is currently unknown. The gene encodes an HS-induced 32-kD protein dissimilar to any well-characterized Hsps but weakly similar (about 35%) to the bacterial (2R)-phospho-3-sulfolactate (PSL) synthase (Graham et al., 2002). The structural homology to PSL synthase provides a possible clue.

In archaeal methanogens, PSL synthase or CoM catalyzes the first step in the biosynthetic pathway of coenzyme M (CoM), which serves as a cofactor for methane production (Graham et al., 2002). In this reaction, PSL is formed by the stereospecific addition of sulfite to phosphoenolpyruvate. Since plants do not have the other CoM biosynthesis genes, it was suggested that the plant PSL synthase-related protein is involved in the biosynthesis of sulfoquinovosyl diacylglycerol (SQDG) (Graham et al.,

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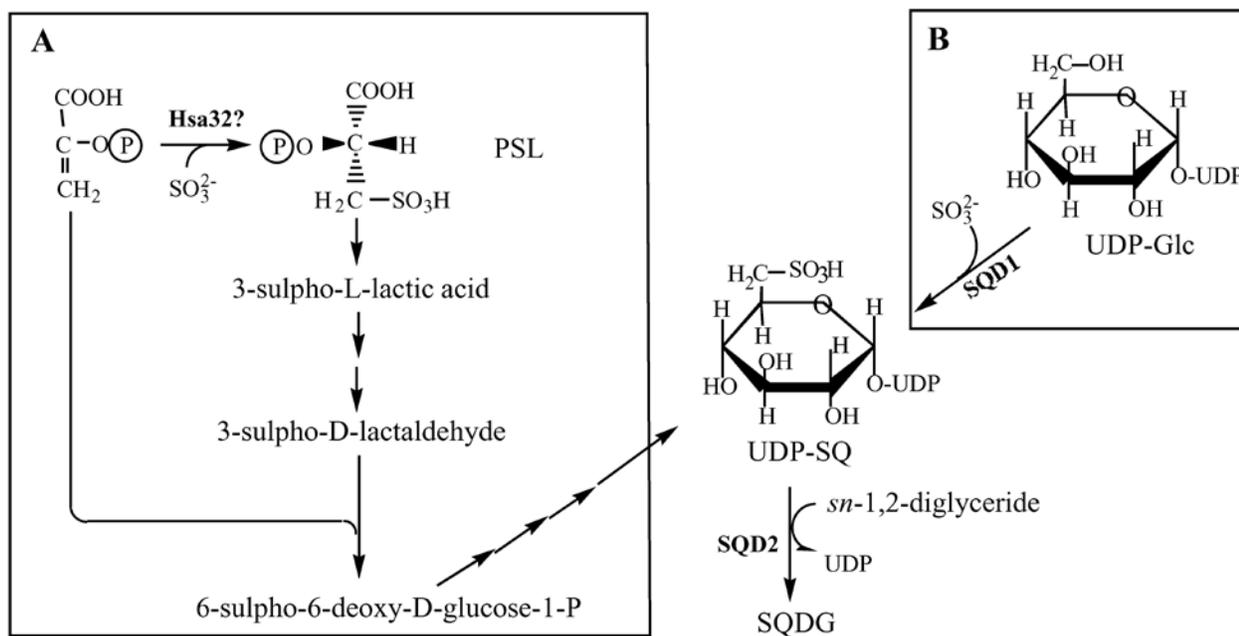


Figure 1. Schematic pathways of SQDG biosynthesis. Two distinct pathways have been proposed for the synthesis of UDP-SQ, the precursor of the sulfolipid head group. The sulfoglycolytic pathway (A) was drawn according to Davis et al. (1966) with modification. The enzymes for this pathway have not been identified and the step possibly catalyzed by Hsa32 is shown. The sugar-nucleotide pathway (B) was drawn according to Benning (1998).

2002). SQDG is a sulfur-containing glycolipid specifically associated with the photosynthetic membranes of plants and most photosynthetic bacteria (Benning, 1998). The major function of SQDG is the substitution of anionic phospholipids under phosphate-limited growth conditions (Yu et al., 2002).

Recently, Benning (1998) demonstrated a 2-step biosynthetic reaction sequence of SQDG, the sugar-nucleotide pathway (Figure 1): uridine diphosphate-SQ (UDP-SQ) is formed from UDP-Glc and sulfite and catalyzed by Sqd1, the UDP-SQ synthase (Essigmann et al., 1998; Sanda et al., 2001), then SQ is transferred from UDP-SQ onto DG, and catalyzed by Sqd2, the SQDG synthase (Yu et al., 2002). Insertion of an *Agrobacterium* T-DNA into *Sqd2* in *Arabidopsis* inhibits sulfolipid formation in the knockout mutant (Yu et al., 2002). Although a deletion mutant of *Sqd1* of the unicellular alga *Chlamydomonas reinhardtii* was shown to be devoid of SQDG (Riekhof et al., 2003), the existence of an alternative pathway for UDP-SQ synthesis in higher plants cannot be excluded. Actually, about 40 years ago, Davies et al. (1966) proposed that phosphosulfolactate was one of the intermediates of SQDG biosynthesis in the so-called sulfoglycolytic pathway (Figure 1). Recent reports revealed that SQDG accumulation increases in leaves of drought-resistant plants under high temperature (Taran et al., 2000) and that SQDG is involved in the structural integrity and heat tolerance of photosystem II (Sato et al., 2003), which suggests that synthesis of SQDG might be increased to confer protection in plants under heat stress. Therefore, it is of interest to know whether the plant PSL synthase-related

protein (i.e. Hsa32) participates in an HS-inducible SQDG biosynthetic pathway.

In this study, we examined the role of Hsa32 by genetic and biochemical analysis of the *Arabidopsis Sqd1* and *Hsa32* T-DNA insertion mutants. The *Arabidopsis Sqd1* knockout mutant did not accumulate a measurable amount of SQDG under normal and heat stress conditions. The sulfolipid level in wild-type and *hsa32* knockout mutant plants was also not affected by HS treatment, which suggests no alternative pathway for UDP-SQ synthesis under normal or heat stress conditions and that Hsa32 is not involved in the biosynthesis of sulfolipids in higher plants. Moreover, the *Arabidopsis* plants without SQDG accumulation did not exhibit an HS-sensitive phenotype as did the *Hsa32* knockout plants, which rules out the hypothesized role of Hsa32 as a sulfolipid biosynthesis-related enzyme.

MATERIALS AND METHODS

Sequence alignment

The amino acid sequences of *Arabidopsis* Hsa32 (NP_567623) and the PSL synthase of *Methanococcus jannaschii* (Q57703) were aligned by use of the AlignX (InforMax) program with the default setting (Figure 2).

Plant materials and growth conditions

The *Arabidopsis* T-DNA insertion lines of *Sqd1*, *Sqd1-KO* (SALK_016799), and *Sqd2*, *Sqd2-KO* (SALK_105492), generated in a Col-0 background

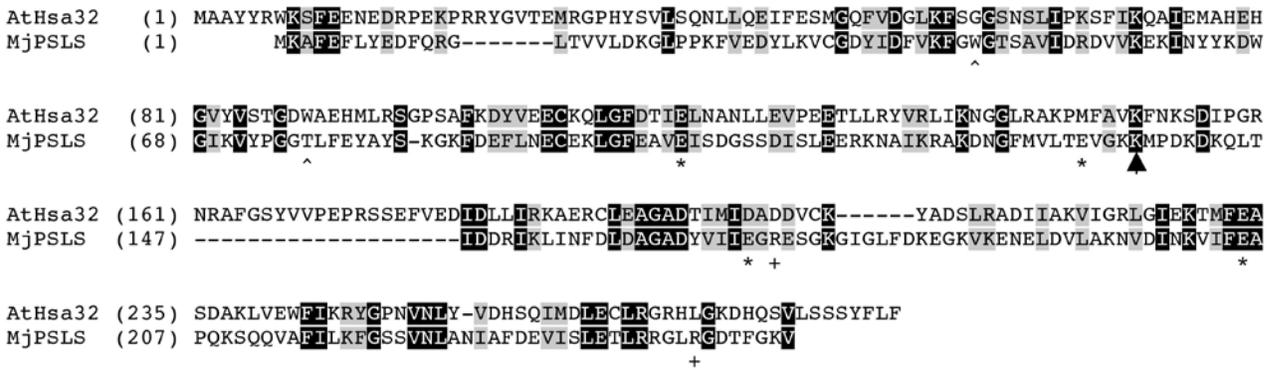


Figure 2. Alignment of the deduced amino acid sequences of *Arabidopsis* Hsa32 (AtHsa32) and *M. jannaschii* PSL synthase (MjPSLS). Residues that are identical between the two sequences are shown in white letters with a black background; chemically conserved residues are shown with a gray background. Gaps (indicated by hyphens) have been introduced to give a better alignment. Based on the three dimensional structure and sequence of the *M. jannaschii* enzyme (Wise et al., 2003), the catalytic lysine residue (K137) is indicated by an arrow. The proposed binding residues for PEP (W46 and T76), sulfite (R170 and R244'), and magnesium (E103, E133, E168, and E205) are indicated by ^, +, and *, respectively, underneath the alignment.

(Alonso et al., 2003), were obtained from the *Arabidopsis* Biological Resource Center (Columbus, OH). The *Hsa32* T-DNA insertion mutant *hsa32-1* was obtained as previously described (Charg et al., 2006). The location of the T-DNA insertion was confirmed by PCR and DNA sequencing. Seeds of the mutants and wild type *Arabidopsis thaliana* (Col-0) were sown on 0.8% (w/v) agar-solidified MS medium supplemented with 1% (w/v) sucrose and kept at 4°C for at least 3 days. Plants were grown in a growth chamber with cool white fluorescent light of 130 $\mu\text{mol m}^{-2}\text{s}^{-1}$ under 14-h light/10-h dark and 23°C/18°C cycles. For the acquired thermotolerance test, *Arabidopsis* seedlings were grown at 24°C with 16-h light (130 $\mu\text{mol m}^{-2}\text{s}^{-1}$) in a Petri dish (90 × 15 mm) with 25 mL of solid medium (25 mL of 0.5 × MS containing 0.8% agar and 1% sucrose). The plate containing 3-d old seedlings was submerged in a water bath for HS treatment as described in the figure legends. During recovery from each HS treatment, the plate was removed from the water bath and kept at the previous growth conditions under the same light/dark cycles until photographed.

RNA isolation and analysis

Total RNA was isolated from frozen samples with use of TRIZOL reagent (Invitrogen). The expression of *Hsa32* was determined by northern blot analysis as previously described (Li et al., 2003). The expression of *Sqd1* was detected by RT-PCR with the forward primer 5'-GCGCATCTACTTTCAGCTTCATGCCCTTC-3' and reverse primer 5'-TTATGTGGTCATGGACTTAGTCTTGACGC-3', and that of *Sqd2* with the forward primer 5'-GCTTCATCTCCCGGAGTTATG-3' and reverse primer 5'-TCCAATGAAAGCAATCCGAGC-3'. RT-PCR experiments were performed as described previously (Wang et al., 2001). All RT-PCR products were confirmed by direct sequencing.

Immunoblot analysis

The total protein of plant samples was extracted by use of Tris-HCl buffer (60 mM, pH 8.5, containing 2% SDS, 2.5% glycerol, 0.13 mM EDTA, and 1% protease inhibitor cocktail). The protein amount was measured by use of DC Protein Assay reagents (Bio-Rad), with bovine serum albumin used as a standard. For immunoblot analysis, protein was separated on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with a precast minigel assembly (NuPAGE 4-12% Bis-Tris gel + MOPS SDS running buffer; Invitrogen) and transferred onto a nitrocellulose membrane for antibody probing. The polyclonal antibody against *Arabidopsis* Hsa32 was prepared by immunizing rabbits with purified recombinant protein (Charg et al., 2006). The polyclonal antibody against rice class-I sHsp was kindly provided by Dr. Chu-Yung Lin (National Taiwan Univ.). The amount of antigen was detected by use of the Super Signal West Dura Extended Duration Substrate system (Pierce). Following chemiluminescence detection, the membrane was stained with 0.1% (w/v) amido black to ensure equal loading of protein.

Lipid analysis

The ³⁵S-labeled SQDG in the leaves of the *Arabidopsis* wild-type and mutant plants were analyzed by thin layer chromatography (TLC) as previously described (Yu et al., 2002).

RESULTS AND DISCUSSION

Hsa32 is similar to PSL synthase in primary structure

To elucidate the molecular function of Hsa32, we started from a structure comparison approach. Hsa32 is not

homologous to any previously identified Hsps. The plant protein is most similar to a PSL synthase as previously reported (Graham et al., 2002). Alignment of PSL synthase and Hsa32 revealed conserved residues spanning the entire protein, including the lysine residue of the catalytic site (Figure 2). However, the degree of homology is low. The two proteins share only 18% identity and 38% similarity. Nevertheless, the sequence homology has led to a postulation that Hsa32 contributes to sulfolipid biosynthesis (Graham et al., 2002) in the sulfoglycolytic pathway (Figure 1).

Many amino acid residues in the substrate binding sites of PSL synthase from *M. jannaschii* (Wise et al., 2003) are not conserved in the Hsa32 sequence. Of the eight amino acid residues proposed to interact with the substrates phosphoenolpyruvate, sulfite, and magnesium in PSL synthase, only two, E103 and E205, which bind to magnesium ions, are conserved in the plant sequence (Figure 2). The W46 and T76 residues, proposed to bind phosphoenolpyruvate, are also not conserved in Hsa32. Thus, Hsa32 might not necessarily have the same catalytic activity as the *M. jannaschii* enzyme. No direct evidence to date suggests that Hsa32 can synthesize PSL *in vitro* or *in vivo*.

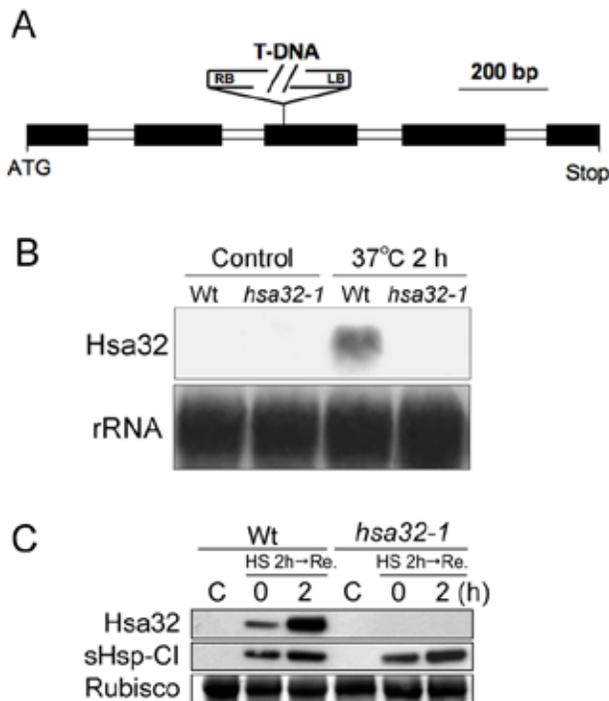


Figure 3. Disruption of *Arabidopsis* Hsa32 by T-DNA insertion led to a null mutation. The location of the T-DNA insertion in the *hsa32-1* (GABI-Kat 268A08) mutant is indicated schematically (A). Black bars indicate exons. The transcription and translation of Hsa32 in response to heat stress (37°C, 2 h) in *hsa32-1* and wild-type (Wt) were revealed by northern (B) and western blotting (C). The level of class I small Hsp (sHsp-Cl) was shown as a HS positive control. Rubisco large subunit stained by amido black was shown to ensure equal loading.

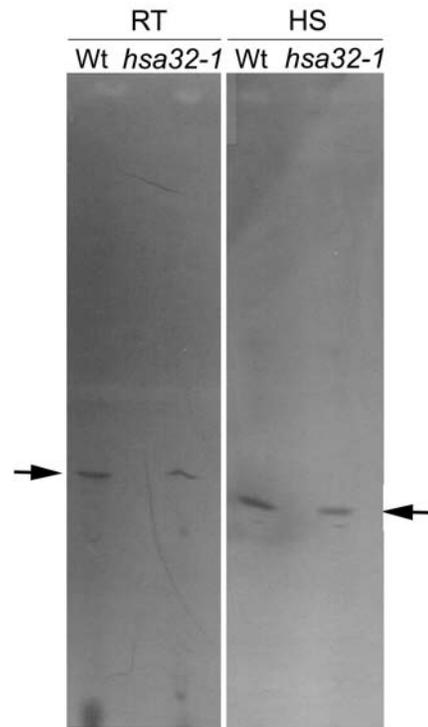


Figure 4. TLC analysis of sulfolipid in *hsa32-1* leaves. Lipids were extracted from leaves treated with [^{35}S]SO $_4^{2-}$ of the wild-type (Wt) and *hsa32-1* plants without (kept at room temperature, RT) or with HS treatment (37°C for 2 h), then separated on a TLC plate, and [^{35}S]-labeled SQDG was visualized by autoradiography. The SQDG bands are indicated by arrows.

Synthesis of SQDG is not affected in the Hsa32 knockout mutant

To test whether Hsa32 is involved in the biosynthesis of sulfolipids (Figure 1), a genetic approach was used to directly examine SQDG in a knockout mutant of Hsa32, *hsa32-1*, caused by a T-DNA insertion at the third exon (Figure 3A). The *hsa32-1* mutant did not respond to HS treatment at either the transcriptional (Figure 3B) or translational (Figure 3C) levels as did the wild type, which indicates that the T-DNA insertion results in a null mutant by disrupting the integrity of the gene.

Since Hsa32 is a single-copy gene in the *Arabidopsis* genome, we thought the SQDG level would be affected if Hsa32 contributed significantly to the biosynthesis of the lipid under heat stress. Therefore, we examined the SQDG synthesis in the mutant under normal conditions and after HS treatment by TLC analysis of the lipids extracted from detached leaves fed with ^{35}S isotope-labeled sulfate. In the wild-type leaf, ^{35}S -labeled sulfate was converted into ^{35}S -labeled SQDG (Figure 4) as was previously reported (Yu et al., 2002). However, the level of SQDG was not significantly affected by HS treatment in the wild type. The level of SQDG was likely maintained at a steady level under heat stress by up-regulating the sulfoglycolytic pathway with the suppression of another pathway. If so, then the

level of SQDG in *hsa32-1* should decline. However, the sulfolipid level did not significantly change before, during, or after HS treatment, and no significant difference between the wild-type and the mutant plants was observed either (Figure 4).

Knockout mutant of *Sqd1* does not synthesize SQDG under normal and HS conditions

To further confirm our results, we analyzed the *Arabidopsis Sqd1* knockout mutant to determine the existence of an alternative pathway of SQDG biosynthesis. Because *Sqd1* is a single-copy gene in the *Arabidopsis* genome, the *Sqd1* knockout mutant plant should not synthesize SQDG if the sugar-nucleotide pathway (Figure 1) is the sole pathway. To test this hypothesis, we obtained and confirmed a T-DNA knockout line with a disrupted *Sqd1* gene (Figure 5A). A T-DNA knockout mutant of *Sqd2* essential for SQDG biosynthesis (Yu et al., 2002) was employed here as a control. RT-PCR analysis showed that the T-DNA insertion lines *Sqd1-KO* and *Sqd2-KO* did not accumulate corresponding transcripts (Figure 5B), which confirmed the disruption of the genes. Neither mutant showed any significant phenotypic difference as compared with the wild type (data not shown), a result consistent with a previous report that the absence of SQDG did not result in any defect in development or growth under normal conditions (Yu et al., 2002). Then, we analysed the level of SQDG in each of these mutants

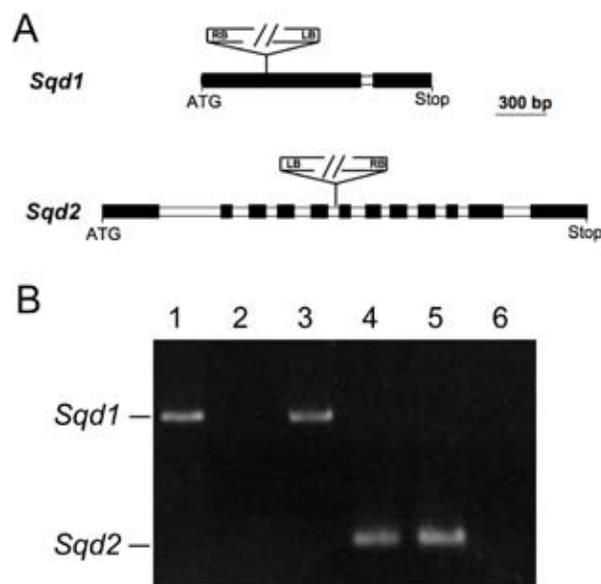


Figure 5. Disruption of *Arabidopsis Sqd1* and *Sqd2* by T-DNA insertion. The locations of T-DNA insertion in the *Sqd1-KO* (SALK_016799) and *Sqd2-KO* (SALK_105492) mutants are indicated schematically (A). Black bars indicate exons. The transcript levels of *Sqd1* (lane 1-3) and *Sqd2* (lane 4-6) in the wild-type (lane 1 and 4), *Sqd1-KO* (lane 2 and 5), and *Sqd2-KO* (lane 3 and 6) plants were revealed by RT-PCR (B). The bands representing RT-PCR products of *Sqd1* (1,431 bp) and *Sqd2* (444 bp) are indicated.

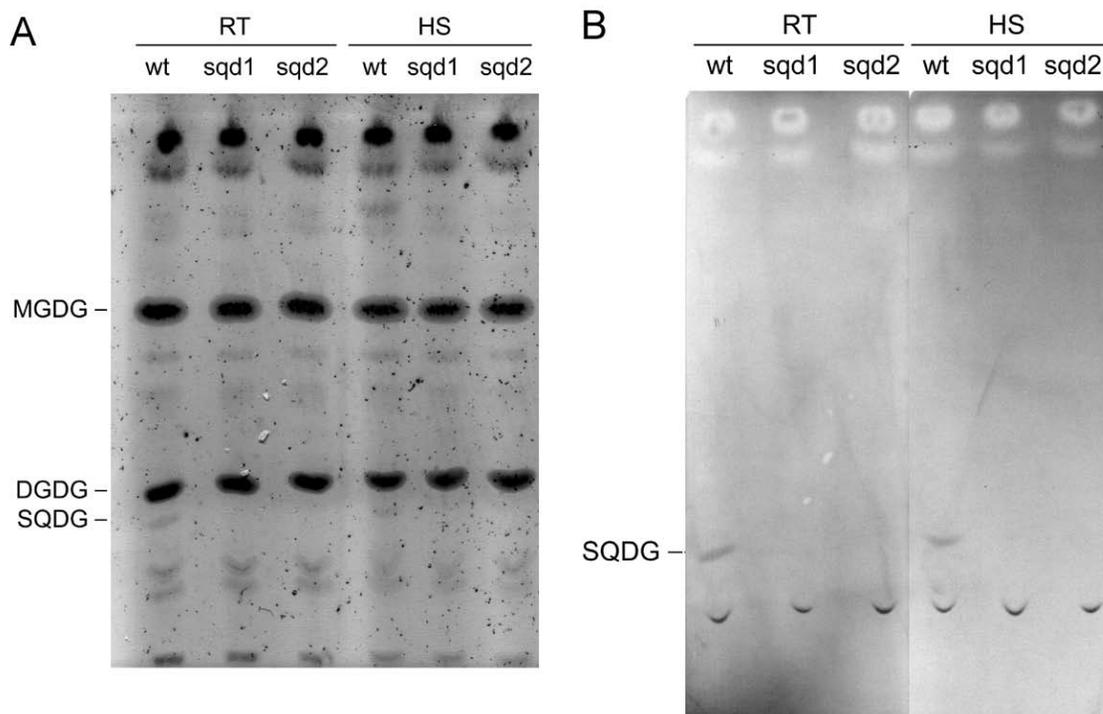


Figure 6. TLC analysis of sulfolipid in *Sqd1* knockout mutant. Lipids were extracted from leaves fed with $[^{35}\text{S}]\text{SO}_4^{2-}$ of the wild-type (Wt), *Sqd1-KO* (sqd1), and *Sqd2-KO* (sqd2) mutants without (RT) or with HS (37°C for 2 h) treatment, then separated on a TLC plate and visualized by use of α -naphthol, which primarily stains glycolipids (A), or by autoradiography to show $[^{35}\text{S}]$ -labeled SQDG (B).

by TLC. Under normal and HS conditions SQDG was accumulated in the wild-type *Arabidopsis* but not in the *Sqd1-KO* or *Sqd2-KO* mutant leaves (Figure 6). Thus, evidence of an alternative pathway is lacking, and *Sqd1* is apparently involved in the major if not the sole pathway of SQDG biosynthesis. A complementation experiment is needed to ensure that the absence of SQDG in the *Sqd1-KO* mutant was not caused by a secondary mutation. However, this is beyond the scope of our study and hence was not done.

Relation between SQDG level and HS sensitivity

To further confirm that the thermotolerance defect of *hsa32-1* is not due to any minor change in sulfolipid level, we compared the thermotolerance of the *Sqd1-KO*, *Sqd2-KO*, and *hsa32-1* mutants. As reported earlier, *hsa32-1* plants became less tolerant than the wild type to severe HS challenge after a long recovery, despite containing normal levels of SQDG; however, the *Sqd1-KO* and *Sqd2-KO* plants, which did not accumulate measurable SQDG, still remained tolerant to the HS treatment (Figure 7). Thus, the sulfolipid level was not associated with the defective phenotype in the acquired thermotolerance of *hsa32-1*.

Taken together, our data indicate that *Arabidopsis* Hsa32 is not involved in the biosynthesis of SQDG.

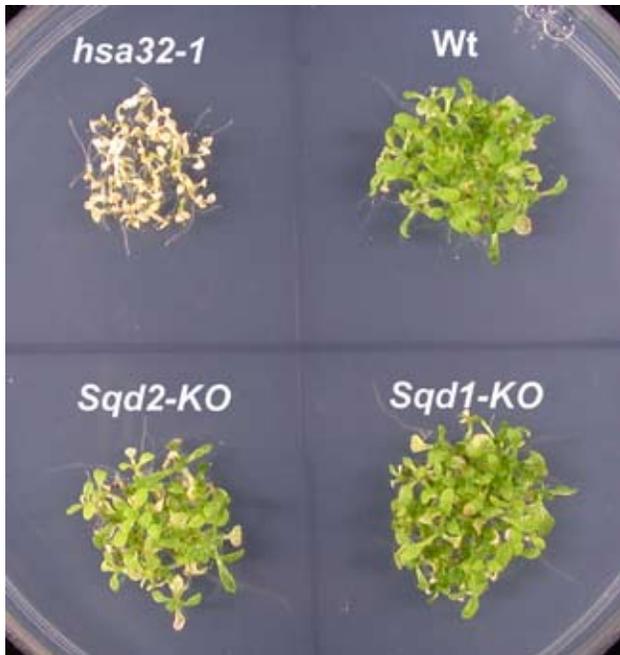


Figure 7. SQDG level is not associated with the HS-sensitive phenotype of *hsa32-1*. The acquired thermotolerance level of the wild-type, *hsa32-1*, *Sqd1-KO* and *Sqd2-KO* plants was determined as follows: 3-d-old seedlings grown on the same plate were acclimated at 37°C for 1 h then recovered for 48 h before a severe HS challenge at 44°C for 45 min. The plants were incubated under normal growth conditions afterward. The photograph was taken 7 d after the severe HS treatment.

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一個與 2-磷酸 3-磺基乳酸合成酶相關的熱休克蛋白 Hsa32 並不參與阿拉伯芥硫脂之生合成

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Hsa32 是一種主要發現於植物的新穎熱休克蛋白。最近的證據顯示 Hsa32 在高溫馴化後之長恢復期間的後天耐熱性具有必要性。缺少 Hsa32 的阿拉伯芥突變株在該段期間的後天耐熱性衰退得較野生株為快，因而對於嚴峻的高溫處理較野生株為敏感。Hsa32 的氨基酸序列與 2-磷酸 3-磺基乳酸合成酶 (phosphosulfolactate synthase) 類似，因此其功能被認為與葉綠體類囊體膜主要糖脂成份之一的硫脂 (sulfolipid) 的生合成有關。然而，目前已知的植物硫脂生合成係經由 Sqd1 與 Sqd2 兩酵素的作用。本實驗的目的，在檢驗 Hsa32 是否具有參與取代 Sqd1 的替代合成途徑之可能性。我們發現阿拉伯芥 *Hsa32* T-DNA 插入突變株與野生株在硫脂 sulfoquinovosyl diacylglycerol (SQDG) 含量上並沒有明顯差異。此外，阿拉伯芥的 *Sqd1* 缺失突變株無法合成 SQDG，因此不支持硫脂合成有替代途徑的假說。*Sqd1* 與 *Sqd2* 插入突變株雖缺乏 SQDG，但卻不會像 *Hsa32* 的突變株對高溫具有敏感性。這個結果顯示，*Hsa32* 突變株對於高溫的敏感性並不是肇因於 SQDG 含量之變化，而 Hsa32 也不參與硫脂的生合成。

關鍵詞：阿拉伯芥；硫脂；耐熱性。