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Arabidopsis HSP70-16 is required for flower opening under normal or mild heat stress temperatures

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Abstract

Sepals play important roles in protecting inner floral organs from various stresses and in guaranteeing timely flower opening. However, the exact role of sepals in coordinating interior and exterior signals remains elusive. In this study, we functionally characterized a heat shock protein gene, *Arabidopsis HSP70-16*, in flower opening and mild heat stress response, using combined genetics with anatomic, physiological, chemical, and molecular analyses. We showed that *HSP70-16* is required for flower opening and mild heat response. Mutation of *HSP70-16* led to a significant reduction in seed setting rate under 22°C, which was more severe at 27°C. Mutation of *HSP70-16* also caused postgenital fusion at overlapping tips of two lateral sepals, leading to failed flower opening, abnormal floral organ formation, and impaired fertilization and seed setting. Chemical and anatomic analyses confirmed specific chemical and morphological changes of cuticle property in mutant lateral sepals, and qRT-PCR data indicated that expression levels of different sets of cuticle regulatory and biosynthetic genes were altered in mutants grown at both 22°C and 27°C temperatures. This study provides a link between thermal and developmental perception signals and expands the understanding of the roles of sepal in plant development and heat response.

KEYWORDS

cuticle, postgenital fusion, sepal

1 | INTRODUCTION

Flower opening is one of the most remarkable traits of a reproductive syndrome of flower development. It is regulated by various endogenous and exogenous factors, which have attracted both extensive and intensive studies in the past decades (Scutt & Vandenbussche, 2014; Van Doorn & Kamdee, 2014; Van Doorn & van Meeteren, 2003). Although most of the studies focus on instinctive molecular mechanisms that control almost all aspects of flower development (Ó'Maoiléidigh, Graciet, & Wellmer, 2014), few studies concentrate on external factors that regulate flower opening and closing, much less on their interactions. Flower opening is generally due to orchestrated cell growth of various floral organs; disruption of normal floral organ development, either genetically or environmentally, can often lead to abnormal flower development; in some cases where the postgenital floral organ fusion is induced (Verbeke, 1992), failure of flower opening occurs (Kurdyukov et al., 2006; Shi et al., 2011; Smirnova, Leide, & Riederer, 2013).

The surface of the outermost sepals in a mature *Arabidopsis* flower often contains specialized cells (such as trichomes and giant

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cells) that are perhaps rich in chemical compounds (Roeder, 2010; Smyth, Bowman, & Meyerowitz, 1990); therefore, the sterile and generally green leaf-like sepals are commonly thought to protect developing reproductive structures inside buds from biotic stresses (Roeder, 2010; Van Doorn & Kamdee, 2014; Van Doorn & van Meeteren, 2003). Our current knowledge regarding the protective roles of sepals against abiotic stresses, such as heat stress, is limited. Given that *Arabidopsis* sepals, the direct interfaces between flower buds and the surrounding environment, cover buds at Stage 6 till opening at the end of Stage 12 (Smyth et al., 1990) and that buds usually open in the morning (Van Doorn & van Meeteren, 2003), it is plausible to assume an indispensable role of sepals in flower opening, coordinating interior developmental signals with exterior environmental clues. Nevertheless, the exact role of sepals regarding flower opening and responses to environment remains unclear.

The protective roles of sepals for the developing floral organs from biotic and abiotic environments are maximized by the overlapping margins of adjacent sepals in the way of imbricate aestivation (Roeder, 2010). Previous anatomic studies reveal that in closed *Arabidopsis* buds (before Stage 12), the abaxial sepal tip superimposes that of the adaxial, and the two lateral sepals meet and overlap underneath, with the lateral sepals consistently recurving closest to the inside developing floral organs, followed by the adaxial sepal and then the abaxial sepal (Hill & Lord, 1989; Smyth et al., 1990). When the abaxial and adaxial sepals are detached, the length of lateral sepals is the longest among other floral organs until Stage 12 (Smyth et al., 1990). When a flower blooms at the end of Stage 12, the lengths of the gynoecium and petal overpass those of lateral sepals, which pushes the sepals open (Roeder, 2010; Smyth et al., 1990; Van Doorn & van Meeteren, 2003). Although there is clear evidence that sepals, particularly lateral sepals, are closely associated with flower opening, mainly by observing opening as a phenotype, little is known about underlying cellular and molecular mechanisms.

Heat stress is one of the most common abiotic stresses that affect growth and development of *Arabidopsis*. The heat stress response (HSR) is, in principle, quite conserved among various plants, involving many heat stress transcription factors (HSFs) and various heat shock proteins (HSPs), signalling pathways, and outputs (Fragkostefanakis, Roeth, Schleiff, & Scharf, 2015). Although the HSR–HSP network in *Arabidopsis* is regulated at both transcriptional and posttranscriptional levels in response to biotic or abiotic stresses, increasing studies indicate that HSPs also play important roles in plant development (Oh, Yeung, Babaei-Rad, & Zhao, 2014; Su & Li, 2008), endowing HSPs with dual roles in orchestrating developmental and environmental signals in plants. *HSP70* is one type of highly conserved HSPs, which has 18 members in *Arabidopsis* (Lin et al., 2001). Their properties in cellular localization and spatio-temporal expression pattern imply their additional functions other than stress response, which has also been confirmed experimentally. In addition to heat stress, *HSP70-6/7* is essential for chloroplast development (Latijnhouwers, Xu, & Møller, 2010), and *HSP70-11/12/13* is involved in gametogenesis (Maruyama, Endo, & Nishikawa, 2010; Maruyama, Sugiyama, Endo, & Nishikawa, 2014), whereas *HSP70-14/15* is associated with plant growth and stomatal opening and closing (Jungkunz et al., 2011). In addition, a subclass of cytosolic/nuclear *HSP70* proteins (*HSP70/1/2/3/4/5*) also participates in signalling (Leng et al., 2017). Although a recent study reveals that *HSP90* acts as a hub in networking flowering and is essential for vegetative-to-reproductive phase transition and flower development in *Arabidopsis* (Margaritopoulou et al., 2016), there is no report of plant *HSP70* in flower opening, during which both the interior development and the exterior temperature change are also synchronized.

Here, we present our molecular characterization results of *HSP70-16* in the context of flower opening and mild heat stress (less than 30°C; Groot et al., 2017) signalling. We demonstrated that mutation of *HSP70-16* causes specific modification (chemically, morphologically, and transcriptionally) of cuticle properties in the overlapping tip regions of two lateral sepals, resulting in a local postgenital fusion, which disturbs normal development and function of floral organs (such as stamens and gynoecium), resulting in significant reduction in seed setting rate under both normal and mild heat stress temperatures. This study provides novel insights into the roles of sepal in the harmonization of both developmental and thermal signals in the process of flower development.

2 | MATERIALS AND METHODS

2.1 | Plant materials

Arabidopsis thaliana ecotype Col-0 was used as the wild type (WT). The seeds of *hsp70-16-1* and *hsp70-16-2* T-DNA insertion mutant, corresponding to SALK_028829C and SALK_130998, respectively, were obtained from the Nottingham Arabidopsis Stock Centre. Transformed lines with *hsp70-16-1* background (complementation) were used as well in this study. The seedlings were grown at 22°C with a 16-hr light/8-hr dark photoperiod and moderate humidity. For 27°C treatment, approximately 4-week-old plants were moved from 22°C to growth rooms with controlled temperatures for periods as indicated. For extreme temperature treatment, approximately 5-week-old plants grown at 22°C were subjected to 4°C for 12 hr, or 40°C for 1 hr. Primers for RT-PCR and genotyping of these two T-DNA lines are listed in Table S3.

2.2 | Sequence and promoter analysis

Sequence data from this article can be found in The Arabidopsis Information Resource database under the following accession numbers: *HSP70-16* (AT1G11660), PRS1 (AT2G28610), MYB30 (AT3G28910), MYB106 (AT3G01140), SHN1 (AT1G15360), MAH1 (AT1G57750), CER2 (AT4G24510), WSD1 (AT5G37300); CER10 (AT3G55360); KCS1 (AT1G01120), *HSP70-4* (AT3G12580), *HSP70-1* (AT5G02500), *HSP70-18* (AT1G56410), HsfA1b (AT5G16820), HsfB1 (AT4G36990), and HsfA1a (AT4G17750).

The DNA and protein sequence of 18 *Arabidopsis HSP70* family members were obtained from The Arabidopsis Information Resource (https://www.arabidopsis.org/). *HSP70-16* promoter analysis was

done by PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

2.3 | Phenotype characterization

Flowers and sepals were photographed with a LEICA M205 A microscope. Microscopic observation of each part of the flower organs by semithin sections, transmission electron microscopy (TEM), and scanning electron microscopy (SEM) were performed as previously described (Chen et al., 2011; Xu et al., 2010). For pollen tube in vivo germination, aniline blue staining of pollen tubes in pistils was performed as previously described (Jiang et al., 2005). Photos were photographed with a Nikon ECLIPSE 80i microscope.

2.4 | Complementation analysis

Genomic DNA of WT Col was used as a template to amplify the 4,621-bp genomic DNA fragment of *HSP70-16*, which contained an 870-bp 5′-upstream region, 3,292-bp genic region (including introns), and 459-bp 3′-downstream region, using a pair of primers CL-F and CL-R (Table S3). The amplified fragment was subcloned into the vector pCAMBIA1300 by restriction endonuclease enzymes *Kpn*I and *Pme*I (NEB, Singapore). The resulting plasmid containing *HSP70-16* pro:*HSP70-16* gDNA was introduced into *hsp70-16-1* mutants by *Agrobacterium tumefaciens*-mediated transformation method.

2.5 | GUS staining

An 870-bp DNA fragment upstream of the transcriptional start codon of *HSP70-16* was amplified as the promoter of *HSP70-16*, using primers *HSP70-16-GUS-F* and *HSP70-16-GUS-R* (Table S3), from genomic DNA and introduced into pCAMBIA1301:GUS by restriction endonuclease enzymes *Eco*RI and *Nco*I. The *HSP70-16* pro:GUS construct was introduced into WT *Arabidopsis* by *A. tumefaciens* transformation. GUS activity was determined by staining inflorescence at different developmental stages of transgenic lines as described previously (Song et al., 2015).

2.6 | Gene expression analysis

Total RNA was isolated from various *Arabidopsis* organs including roots, shoots, rosette and cauline leaves, and flowers at different stages, using the TRIzol reagent (Thermo Fisher Scientific, Waltham). Stages of flowers were defined according to the length of petals and stamens (Smyth et al., 1990). Quality and concentration of total RNA were analysed using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). Reverse transcription of total RNA into cDNA was done with FastQuant RT kit with gDNase (Tiangen, Beijing). A qRT-PCR analysis using gene-specific oligonucleotides was performed on a C1000 CFX96 R-T PCR system (Bio-Rad) with the following programme: 95°C for 2 min, 40 cycles of two-step amplification (95°C for 5 s and 55°C for 35 s), with the Platinum® SYBR SuperMix (Invitrogen) in three biological replicates, each with three technique repeats. Actin was used as an internal control gene (Kang et al., 2016), and expression levels were calculated using a relative quantitation method (comparative CT) to quantify the relative expression level of the target genes. Primers for qRT-PCR are listed in Table S3.

2.7 | Cutin and wax analyses

Sepal cutin and wax analyses with gas chromatography flame ionization detector (GC-FID) and gas chromatography mass spectrometry (GC-MS) were performed as previously described (Chen et al., 2011). Waxes were extracted from the sepals by immersing tissues for 30 s in 1 ml of chloroform containing 10 mg of tetracosane (Fluka) as an internal standard. The extracts were transferred to reactive vials and dried under a gentle stream of nitrogen gas. The dried wax residues were derivatized by adding 20 µl of *N,N*-bis-trimethylsilyltrifluoroacetamide (Macherey-Nagel) and 20 µl of pyridine and incubated for 40 min at 70°C. These derivatized samples were then analysed by GC-FID (Agilent Technologies) and GC-MS (Agilent gas chromatograph coupled to an Agilent 5973N quadrupole mass selective detector). The remaining sepals were delipidated for 2 weeks with methanol and chloroform (vol:vol = 1:1) with daily change of the solution and then dried over silica gels and used to analyse the monomer composition of cutin polyester as described by Franke et al. (2005). Cutin samples were transesterified in 1 ml of 1 N methanolic HCl for 2 hr at 80°C. After the addition of 2 ml of saturated NaCl and 20 mg of dotriacontane (Fluka) as an internal standard, the hydrophobic monomers were subsequently extracted three times with 1 ml of hexane. The organic phases were combined and dried under a stream of nitrogen gas, and the remaining samples were derivatized as described above. GC-MS and GC-FID analyses were performed as for the wax analysis. Results of sepal wax and cutin analyses were expressed relative to dry weight of sepals.

3 | RESULTS

3.1 | Loss of function of *HSP70-16* reduces seed setting rate

During a genetic screening for male sterile mutants, we obtained two T-DNA mutants of *HSP70-16*, SALK_028829C (denoted *hsp70-16-1*), and SALK_130998 (denoted *hsp70-16-2*) from stocks of the Nottingham Arabidopsis Stock Centre. Molecular characterization with PCR results revealed their T-DNA insertions sites at 482 and 871 bp, respectively, downstream of the ATG initiation codon in the first exon of *HSP70-16* and their homozygosity (Figures 1a and S1a). Gene expression analysis using qRT-PCR showed that expression levels of *HSP70-16* in both homozygous mutants were significantly reduced to marginal levels of detection (Figure S1b). Both mutant plants exhibited normal vegetative development but showed significantly reduced seed setting rate at both 22°C and 27°C (Figures 1b–d and S1c,d). Owing to their phenotypic and genetic similarity, we used *hsp70-16-1* for subsequent analyses. All F_1 plants of backcross between the WT and *hsp70-16-1* were fertile, and the F_2 plants showed an approximated 3:1 ratio of phenotypic segregation, normal seed setting

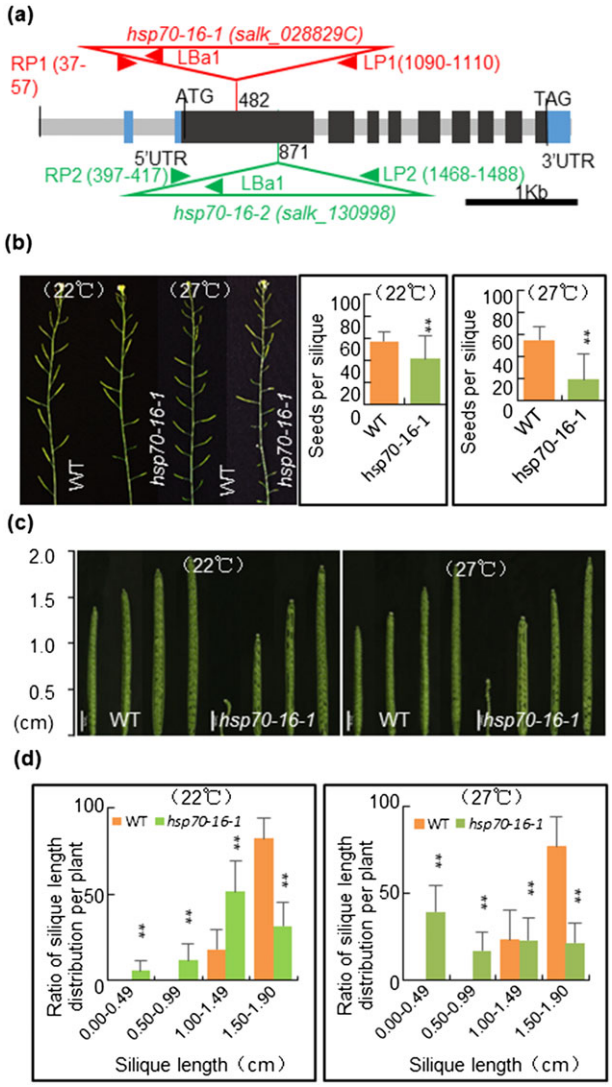

FIGURE 1 Identification and characterization of *HSP70-16* mutants. (a) A schematic representation of the structure of *HSP70-16* gene. T-DNA insertion sites of *hsp70-16-1* (red triangle frame) and *hsp70-16-2* (green triangle frame) are marked with numbers 482 and 871, respectively. Black, grey, and blue boxes represent exon, intron, and untranslated regions (UTR, both 5′ and 3′), respectively. ATG and TAG represent the start codon and termination codon, respectively. Left primer and right primer of *HSP70-16* and the left border primer of the T-DNA used for the identification are indicated as LP, RP, and LBa1, respectively. The localization sites of LP and RP in the first exon of *HSP70-16* are indicated in the corresponding parentheses. (b) Images of the main inflorescence with mature siliques in both wild type (WT) and *hsp70-16-1* plants grown at 22°C and 27°C and their statistical results of the seed number per silique ($^{**}p < 0.01$). (c) Images of silique size of WT and *hsp70-16-1* plants grown at 22°C and 27°C. (d) Silique size distribution patterns in WT and *hsp70-16-1* plants grown at 22°C and 27°C

rate : reduced seed setting rate = 193:64, $\chi^2(0.05) = 0.0013$, $p > 0.05$ (Figure S1f), indicating that *hsp70-16-1* is a single recessive mutation.

To further determine whether the observed seed setting rate reduction phenotype is caused by the loss of function of *HSP70-16* in *hsp70-16-1*, a 4,621-bp genomic DNA fragment of AT1G11660, containing an 870-bp 5′-upstream region of ATG, 3-292-bp encoding region (including introns), and 459-bp 3′-downstream region, was cloned into the vector pCAMBIA1300, and the resulting plasmid was introduced into *hsp70-16-1* homozygous plants by agrobacterium-mediated transformation method (Clough & Bent, 1998). Transgenic plants of *hsp70-16-1* carrying the WT *HSP70-16* genomic DNA fragment recovered the expression level of *HSP70-16* (Figure S1b) and restored the seed setting rate of mutant plants (Figure S1c,d), which corroborated that *AT1G11660* is *HSP70-16*.

3.2 | *HSP70-16* is more sensitive to mild heat stress

To characterize the seed setting rate reduction phenotype of *hsp70-16-1* in depth, we examined silique length and seed number per silique (n = 400) and found that the reduced seed setting rate of *hsp70-16-1* under 27°C, a mild heat stress temperature (Groot et al., 2017), was more severe than that under 22°C. Under 22°C, the average seed number per silique in WT was 58 whereas that in *hsp70-16-1* was 41 (Figure 1b), and the ratio of the siliques longer than 1.0 cm was 100% in WT whereas that in *hsp70-16-1* was around 80% (Figure 1c,d). Under 27°C, the average seed number per silique in WT plants was 55, closely similar to that of WT under 22°C, but that in *hsp70-16-1* was 20, which was significantly lower than that of *hsp70-16-1* at 22°C (Figure 1b), and the ratio of siliques longer than 1.0 cm was 100% in WT, but that in *hsp70-16-1* was about 40% (Figure 1c,d). These results indicated that loss of function of *HSP70-16* affects fertility by affecting seed setting rate and that this effect is more obvious under a mild heat stress condition.

3.3 | Flower opening in *HSP70-16* is impaired

To further investigate into the causes of semisterility in *HSP70-16* plants, we comparatively examined the pollen development process via semisection analysis (Figure S2a), mature pollen viability and pistil fertility via reciprocal cross (Figure S2b), and mature pollen morphology via SEM (Figure S2c). Our results demonstrated that neither anther and pollen development nor mature pollen viability and pistil fertility were affected in *hsp70-16-1*, indicating that the reduction of fertility in *hsp70-16-1* plants does not result from the developmental and functional aspects of anthers and pollens.

We then focused closely on morphological abnormality along the process of flower development, starting from Stage 9 when WT buds begin to plump to Stage 13 when WT petals spread out and flowers open. No obvious morphological difference along flower development was observed between WT and *hsp70-16-1* from Stages 9 to 11 (Figure S3a,b); however, unopened buds and flowers with abnormal structures and atypical abscission patterns were witnessed in *hsp70-16-1* inflorescences at Stage 12 and afterwards, which clearly distinguished *hsp70-16-1* from WT (Figure 2a,b,d,e). Notably, no unopened flowers or buds were observed in the inflorescences of *hsp70-16-1* plants complemented by *HSP70-16* genomic sequence (Figure 2c). These results demonstrated that *HSP70-16* participates in normal flower opening.

The observed phenotypes of fused buds and flowers were quite subtle, but the phenotype was server grown at 27°C than that at 22°C (Figure S1e), which indicated again the higher susceptibility of *HSP70-16* to mild heat stress.

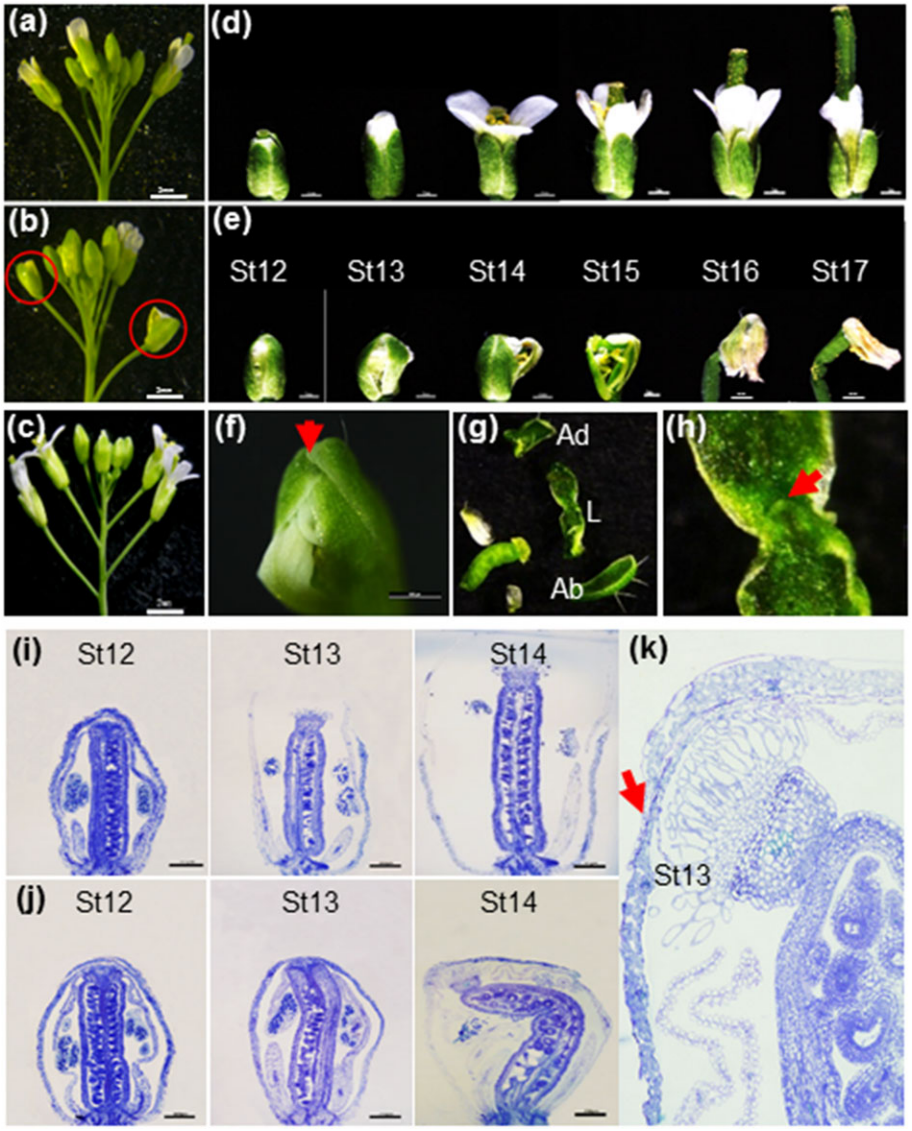

FIGURE 2 Morphological characterization of *HSP70-16* buds and flowers grown at 27°C. (a–c) Inflorescence images of wild type (WT), *hsp70-16-1*, and *hsp70-16-1* rescued by the supplementation with *HSP70-16* genomic DNA, respectively. Fused buds are marked with red circles in Panel b. (d and e) Typical images of WT and *hsp70-16-1* buds or flowers at different developmental stages, respectively. (f) Magnified image of a fused *hsp70-16-1* bud at Stage 14. The red arrow points to the imbricate aestivation formed between the abaxial (Ab) and adaxial (Ad) sepals. (g) Images of dissected (cutting just above the abscission zone) floral organs from a fused *hsp70-16-1* bud, showing the separation of the Ab and the sepals and the fusion between the two lateral sepals (L). (h) Magnified image of the fusion between two lateral sepals in a fused *hsp70-16-1* bud. The red arrow points to the fusion region. (i and j) Semisection images of WT and *hsp70-16-1* buds or flowers at different developmental stages, respectively. (k) Magnified semisection image of an *hsp70-16-1* flower at Stage 14, showing the overlapping and fusion of the two lateral sepals. The red arrow points to the fusion region

3.4 | *HSP70-16* shows postgenital fusion in tips of overlapping lateral sepals

Because the observed morphological phenotype is very similar to a previous study of a cuticle-defective mutant that causes postgenital fusion in floral organs (Shi et al., 2011), we further dissected those fused buds and flowers by cutting the buds at the bottom just above the abscission zone. This study revealed that since Stage 12 of flower development, sepals in *hsp70-16-1* buds or flowers did not open as its WT ones (Figure 2f), not due to the overlapping that occurred between the abaxial and adaxial sepals but due to the postgenital fusion that occurred between the tips of two overlapping lateral sepals (Figure 2g,h). There were no morphological differences that were detected in both abaxial and adaxial sepals between *hsp70-16-1* and WT flowers (Figures 2g and S3c–f); however, distinguishable morphological differences were observed in lateral sepals, petals, stamens, and gynoecium (Figure 3a–h). The tips of those fused lateral sepals of unopened *hsp70-16-1* flowers were sharper and longer as compared with WT ones (Figures 3a,b and 5a,b). Stamens, gynoecium, and petals in those fused mutant flowers did not extend up normally and grew twisted or curved eventually (Figure 3c–h). Because most of the mature and viable pollens released by functional stamens could not touch the stigma properly due to restricted spaces within unopened buds and most of the papilla cells on the stigma of *hsp70-16-1*

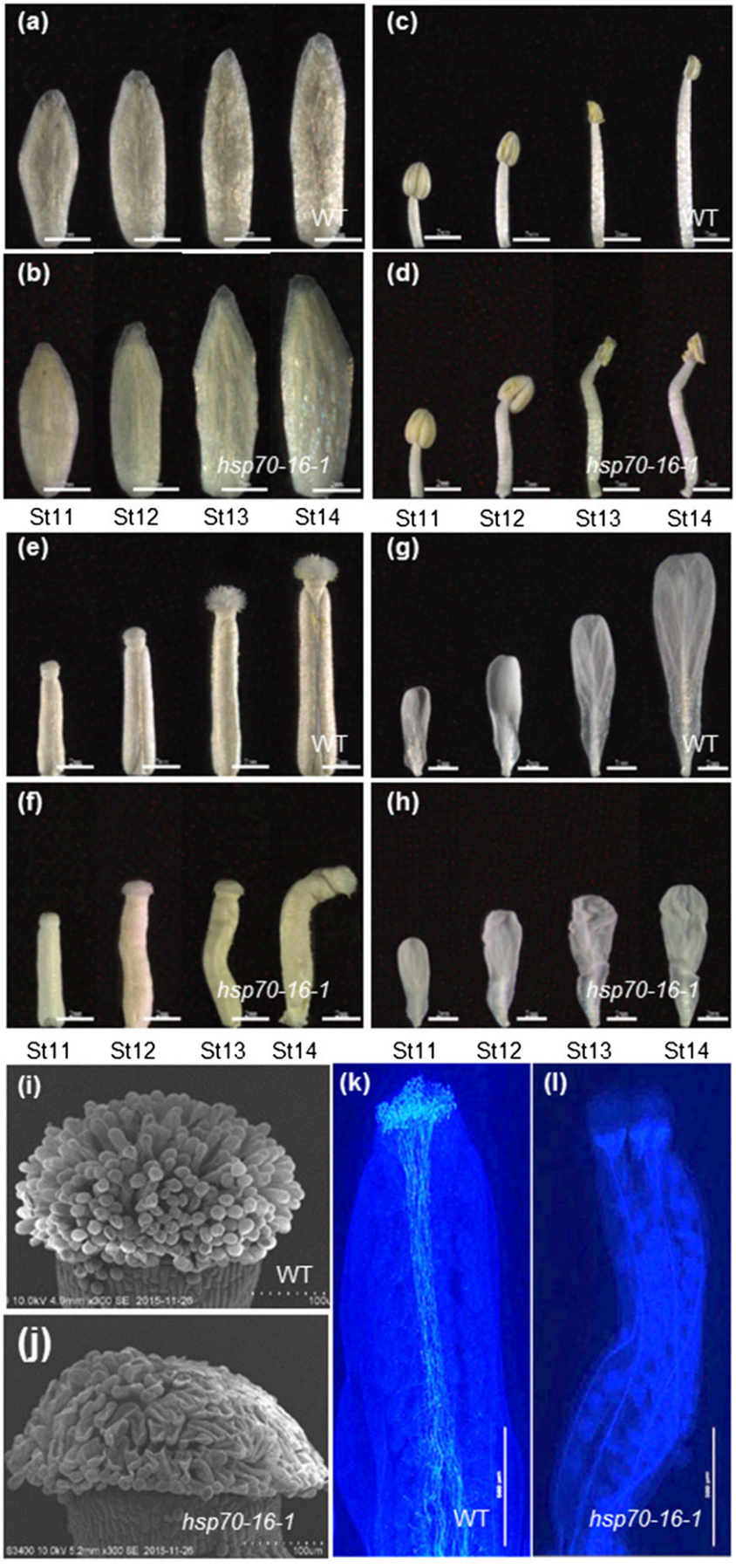

FIGURE 3 Morphological characterization of *hsp70-16-1* floral organs grown at 27°C. (a and b) Scanning electron microscopy (SEM) images of wild type (WT) and *hsp70-16-1* lateral sepals, respectively, showing elongated and sharper sepal tips in *hsp70-16-1*. The length/width of WT and *HSP70-16* lateral sepals are 1.64 ± 0.11 cm/0.50 ± 0.04 cm and 1.78 ± 0.09 cm/0.54 ± 0.03 cm, respectively. (c and d) SEM images of WT and *hsp70-16-1* stamens, respectively, showing curved filaments in *hsp70-16-1*. (e and f) SEM images of WT and *hsp70-16-1* gynoecium, respectively, showing twisted gynoecium in *hsp70-16-1*. (g and h) SEM images of WT and *hsp70-16-1* petals, respectively, showing misshaped petals in *hsp70-16-1*. (i and j) Magnified SEM images of WT and *hsp70-16-1* gynoecium, respectively, showing squeezed papilla cells with much less pollens on top of the twisted gynoecium in *hsp70-16-1*. (k and l) Aniline blue staining images of pollinated WT and *hsp70-16-1* gynoecium, respectively, showing the absence or much less pollen tubes in *hsp70-16-1*

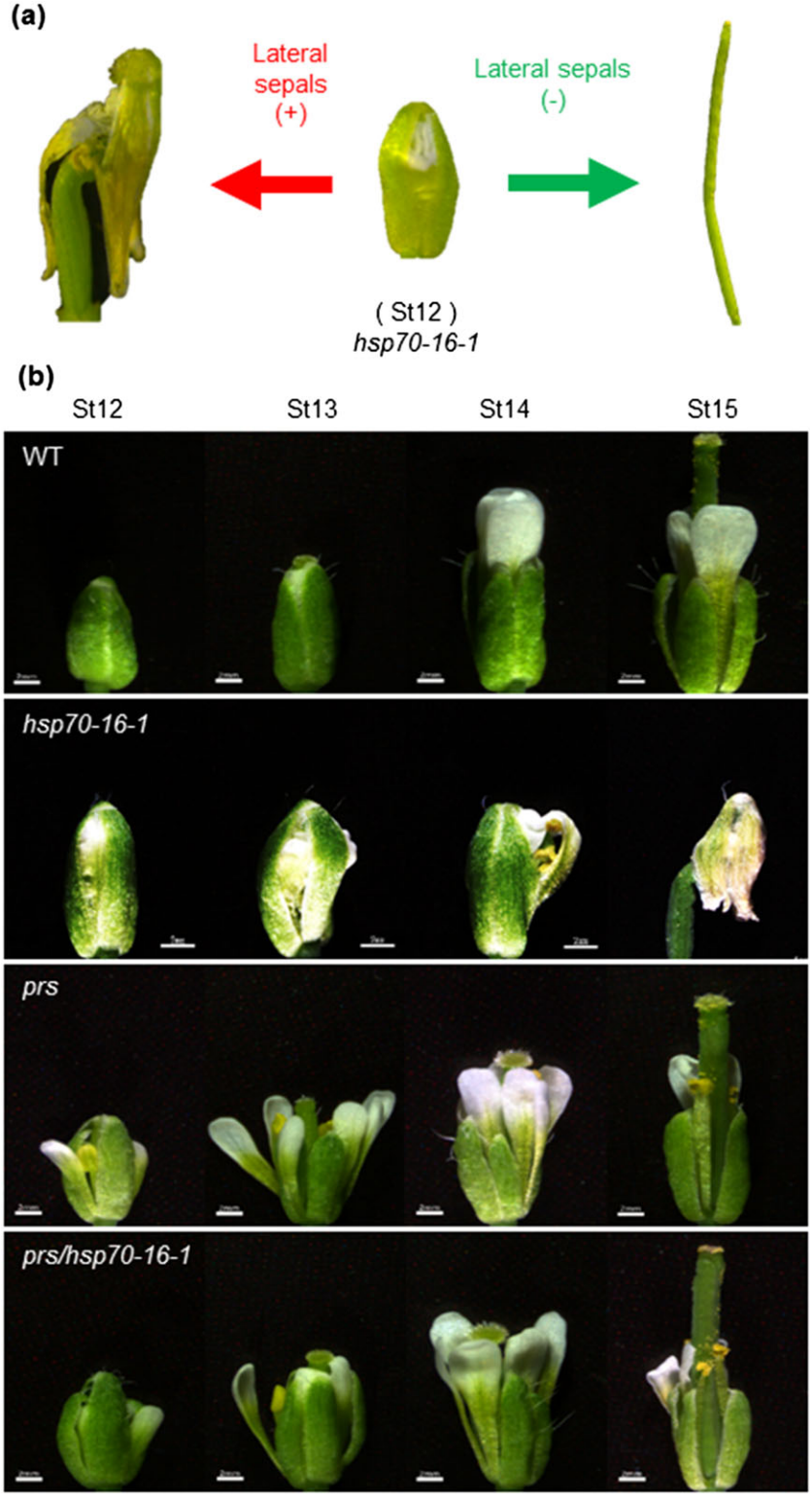

FIGURE 4 Genetic analysis of the function of lateral sepals grown at 27°C. (a) Effect of the removal of lateral sepals on silique development in *hsp70-16-1*. (b) Morphological characterization of flower development in wild type, *hsp70-16-1*, *prs*, and *prs*/*hsp70-16-1*, respectively

gynoecium were abnormal (Figure 3i,j), normal pollination in *hsp70-16-1* was significantly reduced (Figure 3k,l). The above results indicated that it is the postgenital fusion that occurred between the tips of two overlapping lateral sepals that causes failed flower opening in *hsp70-16-1*, leading to subsequent seed setting rate reduction.

To make sure that the seed setting rate reduction was derived directly from the lateral sepal fusion, we first removed the fused lateral sepals of a bud from an *hsp70-16-1* plant just before anthesis (at Stage 12); this silique developed to full length to that of WT although it looked curved, whereas the silique from another bud with

fused lateral sepals developed much shorter with fused floral debris attached on its tip (Figure 4a). This lateral sepal removal experiment verified that lateral sepal fusion impedes silique development and seed setting rate. We validated the above results again by crossing *hsp70-16-1* with a reported mutant, pressed flower (*prs*), which lacks or has smaller lateral sepals (Matsumoto & Okada, 2001). The flowers of the *prs*/*hsp70-16-1* double mutant opened as normal as that of the *prs* single mutant (Figure 4b) with normal silique development and seed setting rate (Figure S4c,d). Altogether, the abovementioned data demonstrated that the loss of function of *HSP70-16* induces postgenital fusion between lateral sepals, which adversely affects floral organ development and reduces subsequent pollination, causing seed setting rate reduction.

3.5 | Loss of function of *HSP70-16* alters sepal cuticle properties and cuticular lipid profiles

Previous studies have shown that a defective cuticle often causes postgenital fusion (Kurdyukov et al., 2006; Shi et al., 2011; Smirnova et al., 2013). To test whether loss of function of *HSP70-16* changes cuticle properties and chemical profiles in floral organs, we performed floral organ surface characterization using SEM and TEM and sepal wax and cutin composition assays using GC-MS and GC-FID. Because mutant plants grown at 27°C had more fused buds and flowers, these assays were performed on sepals grown at this mild heat stress condition. Surprisingly, we did not find any significant changes of cuticle properties in various floral organs except two lateral sepals by SEM, in which typical spaghetti-like cutin decoration patterns in surfaces of petals, the nonfused part of lateral sepals, and adaxial and abaxial sepals in *hsp70-16-1* were hardly different from those in WT ones (Figure S5a–j). However, SEM observation on the overlapping regions on surfaces of fused lateral sepals revealed that spaghetti-like cutin decoration patterns in this region of two lateral sepals in *hsp70-16-1* were much looser or more unevenly distributed, different from that of WT (Figures 5c,d and S5k,l), which was confirmed by TEM observation (Figure 5e,f). Likely, this altered spaghetti-like cutin decoration patterns on surfaces of regions of overlapping lateral sepals caused lateral sepal fusion, which disturbed flower opening and subsequent pollination and fertility.

To further prove whether altered sepal cutin distribution is the consequence of reduced sepal cutin deposition in *hsp70-16-1*, we identified and quantified sepal cuticular waxes and cutin monomers in WT and *hsp70-16-1* plants. Surprisingly, the determined total amount of cutin monomers in *hsp70-16-1* sepals was very similar to that of WT (Figure S5m), indicating that altered sepal cutin distribution is not accompanied by reduced sepal cutin deposition in *hsp70-16-1* and that postgenital fusion of lateral sepals in *hsp70-16-1* is unlikely the consequence of altered cutin distribution. On the other hand, however, the amount of total wax constituents determined in *hsp70-16-1* sepals was significantly lower than that of WT (Figure 5g and Table S1), in which dominant reduction in C29:0 alkane contributed significantly to the reduced sepal waxes in *hsp70-16-1* (Figure 5h). We performed sepal wax analysis in plants grown at 22°C as well. Although the total wax in *hsp70-16-1* was lower than that of WT,

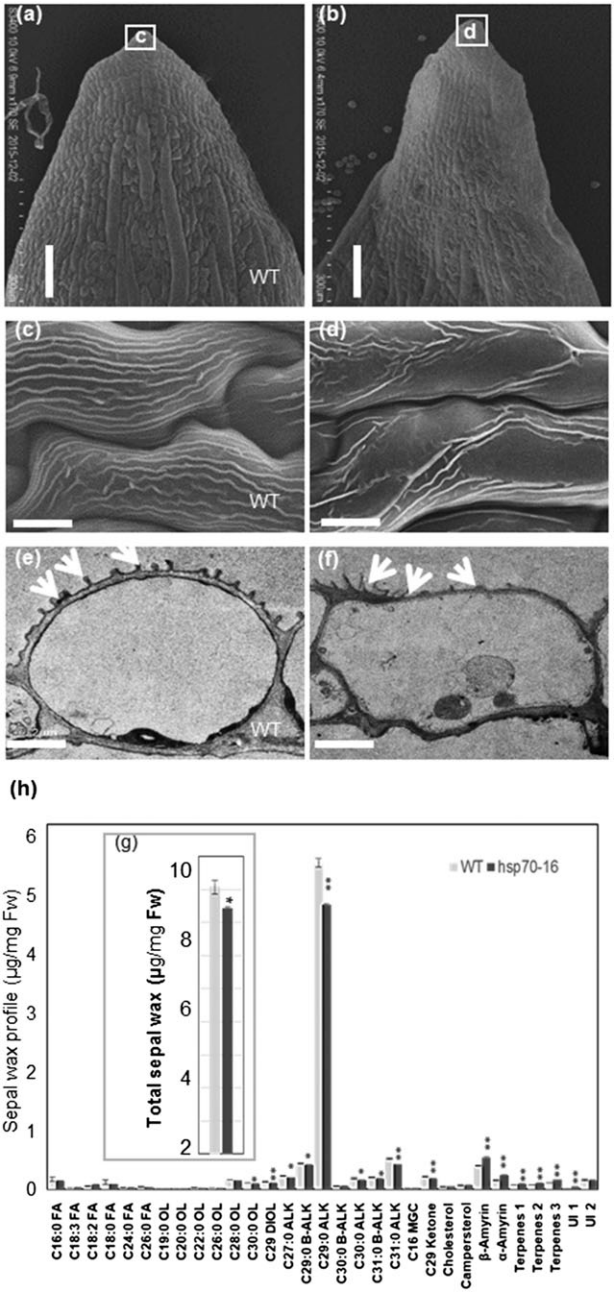

FIGURE 5 Cuticle properties and chemical profiles of *hsp70-16-1* sepals grown at 27°C. (a and b) Scanning electron microscopy (SEM) images of a lateral sepal in wild type (WT) and *hsp70-16-1*, respectively, showing longer and sharper tip in *hsp70-16-1*. Bar scales: 300 μm. (c and d) Magnified SEM images of the tip region of lateral sepals in WT and *hsp70-16-1*, observed in Panels a and b, respectively, showing less and uneven distributed spaghetti-like cutin decoration in *hsp70-16-1*. Bar scales: 5 μm. (e and f) Magnified transmission electron microscopy images of the tip region of lateral sepals in WT and *hsp70-16-1*, observed in Panels a and b, respectively, showing evenly and randomly distributed cuticular nanoridges (white arrows), respectively. Bar scales: 2 μm. (g) Total wax amounts of WT (light grey bar) and *hsp70-16-1* (black bar) sepals. (h) Wax profiles of WT and *hsp70-16-1* sepals. FA: fatty acids; ALK: alkanes; HFA: hydroxy fatty acids; DFA: double fatty acids; MGC: monoglyceride; OL: alcohols. Data represent mean plus/minus standard deviation (n = 5). Student's t test ($^{*}p < 0.05$, $^{**}p < 0.01$)

the difference was not significant (Figure S6). Taken together, SEM, TEM, and chemical analysis data indicated that loss of function of *HSP70-16* reduces sepal wax biosynthesis, exerting profound effects on flower development and fertility.

3.6 | Expression patterns of *HSP70-16*

To further reveal a possible relationship between gene expression and gene function of *HSP70-16*, we carried out a series of expression analyses of *HSP70-16* using different approaches. qRT-PCR results using RNAs derived from both vegetative and reproductive tissues, including roots, stems, rosette leaves, cauline leaves, and flowers, showed that *HSP70-16* was constitutively expressed with the highest expression in stems. Notably, its expression increased along the early flower development initiating from Stage 10 and peaked at Stage 12 (Figure 6a). qRT-PCR results using RNAs derived from dissected floral organs of Stages 10–12 buds grown at 22°C indicated that *HSP70-16* was also ubiquitously expressed in all tested floral organs including lateral sepals with the highest expression in stamens and pistils (Figure 6b). The abovementioned qRT-PCR results confirmed the high expression of *HSP70-16* in mature pollens and Stage 12 flowers as revealed by in silico analyses based on online available microarray data (Figure S7a) and pointed out that *HSP70-16* is a ubiquitously expressed gene with a particular expression pattern in developing flowers. The spatial and temporal expressions of *HSP70-16* were further confirmed by GUS staining analysis using transgenic plants expressing the β-glucuronidase marker protein (GUS) driven by the *HSP70-16* promoter (*HSP70-16* pro:GUS), in which GUS signals were detectable in vegetative organs (such as leaves, stems, and roots; Figure S7b) and in reproductive organs (such as anthers and sepals; Figures 6c–f and S7b). The high GUS signals were detected in developing anthers (Figure 6c–e).

Because *HSP70-16* belongs to the *HSP110/SSE* subfamily of *Arabidopsis HSP70* family (Lin et al., 2001) and the seed setting rate reduction phenotype of *hsp70-16-1* was more severe when growth temperature was increased from 22°C to 27°C, we then inspected the inducibility of *HSP70-16* to altered growth temperatures. qRT-PCR result revealed that either a low temperature (4°C for 12 hr) or an elevated temperature (27°C for a whole cycle or 40°C for 60 min) could induce the expression of *HSP70-16* in early developing flowers (Figure 6g). The induction of *HSP70-16* by elevated temperatures was also confirmed by increased GUS signals in vegetative organs including leaves, stems, and roots (Figure S7b), sepals in transgenic plants grown at 27°C (Figure 6f), and younger anthers (Figure 6d). Higher GUS signals could be detected as well in 4°C treated (1 hr) anthers (Figure 6e).

To better understand the expression patterns of *HSP70-16*, we performed in silico analysis on cis-elements sitting in the promoter of *HSP70-16*, which uncovered that many stress- and hormone-responsive elements are present in the promoter region of *HSP70-16* (Figure 6h). These elements included three TC-rich repeats (cis-acting element involved in defence and stress responses), two HSE (cis-acting element involved in HSR), two LTR (cis-acting element involved in low-temperature response), one TATC-box (gibberellin

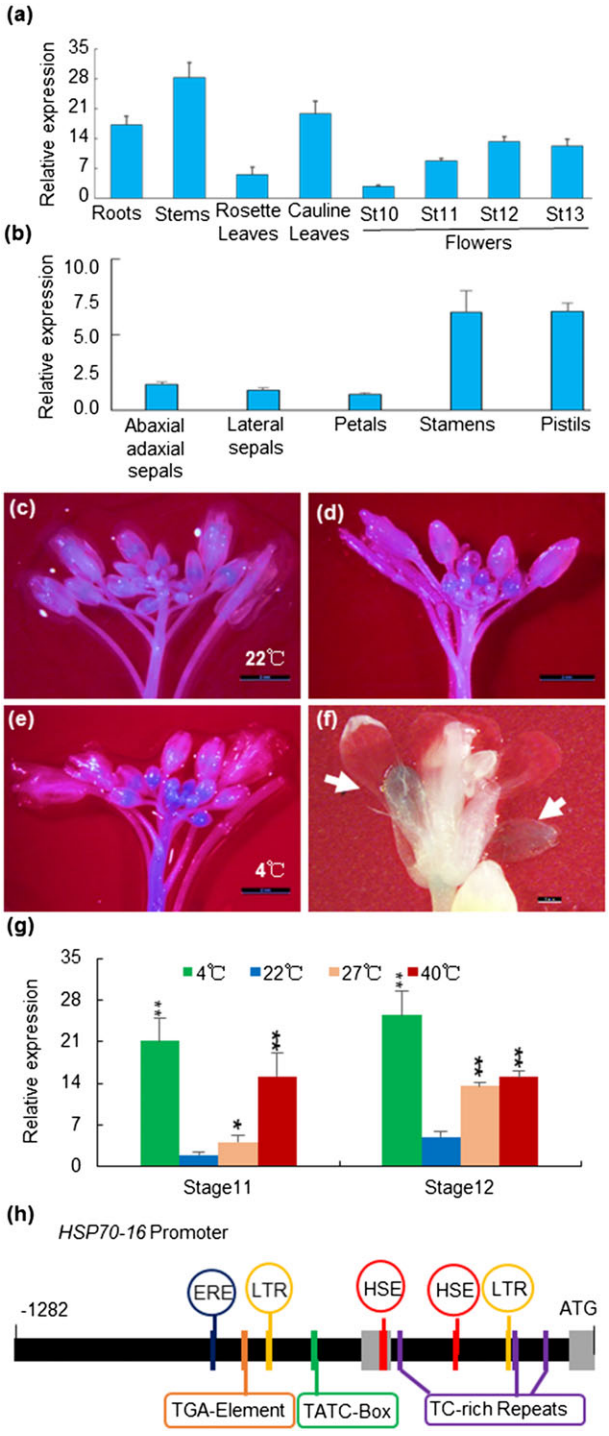

FIGURE 6 Expression pattern of *HSP70-16*. (a) Spatial and temporal expression patterns of *HSP70-16* determined by qRT-PCR. Results are presented as mean plus/minus standard deviation (N = 3). (b) Expression levels of *HSP70-16* in dissected floral organs of flowers at Stages 10–12 grown at 22°C. Results are presented as mean plus/minus standard deviation (N = 3). (c–e) Images of GUS-stained inflorescences of transgenic plants containing *HSP70-16* pro:GUS grown at 22°C, 27°C, and 4°C, respectively. (f) An image of GUS signals detected in lateral sepals grown at 27°C. White arrow points to the GUS signal detected. (g) The induction of *HSP70-16* expression by altered temperatures revealed by qRT-PCR. Results are presented as mean plus/minus standard deviation (N = 3). Student's t test ($^{*}p < 0.05$, $^{**}p < 0.01$). (h) The simplified promoter region of *HSP 70-16* gene showing various stress- and hormone-responsive elements

cis-regulatory elements), one TGA element (auxin-responsive element), and one ERE (ethylene-responsive element; Table S2). The presence of DNA elements associated with various hormones (ethylene, auxin, and gibberellin) and abiotic stressors (high and low temperatures and high salt) in the analysed promoter region (1,000 bp upstream of ATG) of *HSP70-16* correlated well with the abovementioned expression pattern of *HSP70-16*. Altogether, these expression analyses indicated that the expression of *HSP70-16* can be regulated by both developmental and environmental clues, pinpointing that it likely functions as an indispensable element linking flower development and stress response.

3.7 | Expression levels of genes associated with sepal cuticle property and heat shock response networks are altered in *HSP70-16*

To understand the molecular mechanisms underlying the roles of *HSP70-16* in floral organ development and mild HSR as shown above, we carried out qRT-PCR analysis using sepals of mixed buds (Stages 11 and 12) in WT and *hsp70-16-1* plants grown under 22°C and 27°C, respectively, focusing on eight known cuticle metabolic genes or regulators. The results showed that expression levels of *At1g15360/SHN1*, *At1g01120/KCS1*, and *At1g57750/MAH1* were remarkably lower in *hsp70-16-1* sepals than those in WT sepals grown at 22°C (Figure 7), implying that loss of function of *HSP70-16* transcriptionally represses cuticle formation in sepals grown at normal temperature. Because *KCS1* and *MAH1* are involved in wax biosynthesis (Greer et al., 2007; Todd, Post-Beittenmiller, & Jaworski, 1999) and *SHN1* is a regulator of wax biosynthesis (Aharoni et al., 2004), the reduced expression of these three genes likely contributes to observed wax reduction in *hsp70-16-1* sepals. Our results also revealed that the expression levels of three cuticle biosynthesis regulators, *At3g28910/MYB30*, *At3g01140/MYB106*, and *At1g15360/SHN1*, and three cuticle biosynthetic genes, *At4g24510/CER2*, *At1g57750/MAH1*, and *At5g37300/WSD1* (Li et al., 2008), were significantly induced by mild heat stress in WT sepals, which, however, was significantly repressed in *hsp701-16-1* sepals (Figure 7a,b). This result indicated that the cuticle biosynthesis pathway is required to respond to mild heat stress in *Arabidopsis* sepals and that the disruption of cuticle biosynthesis resulting from the loss of function of *HSP70-16* could impair sepals' responses to mild heat stress.

Furthermore, our qRT-PCR data showed that expression levels of two additional cuticle biosynthetic genes, *At5g55360/CER10* and *At1g01120/KCS1*, were significantly reduced in *hsp70-16-1* sepals grown at 27°C although both of them were not induced by mild heat stress in WT sepals (Figure 7c). This could reflect the altered upstream regulatory factors of these two cuticle genes in *hsp70-16-1* sepals. The above gene expression analysis result indicated that loss of function of *HSP70-16* alters expression levels of sepal cuticle metabolism-associated genes in *HSP70-16* sepals grown at both normal and mild heat stress temperatures, implying that cuticle metabolism plays important roles in *HSP70-16*-mediated plant development and stress response.

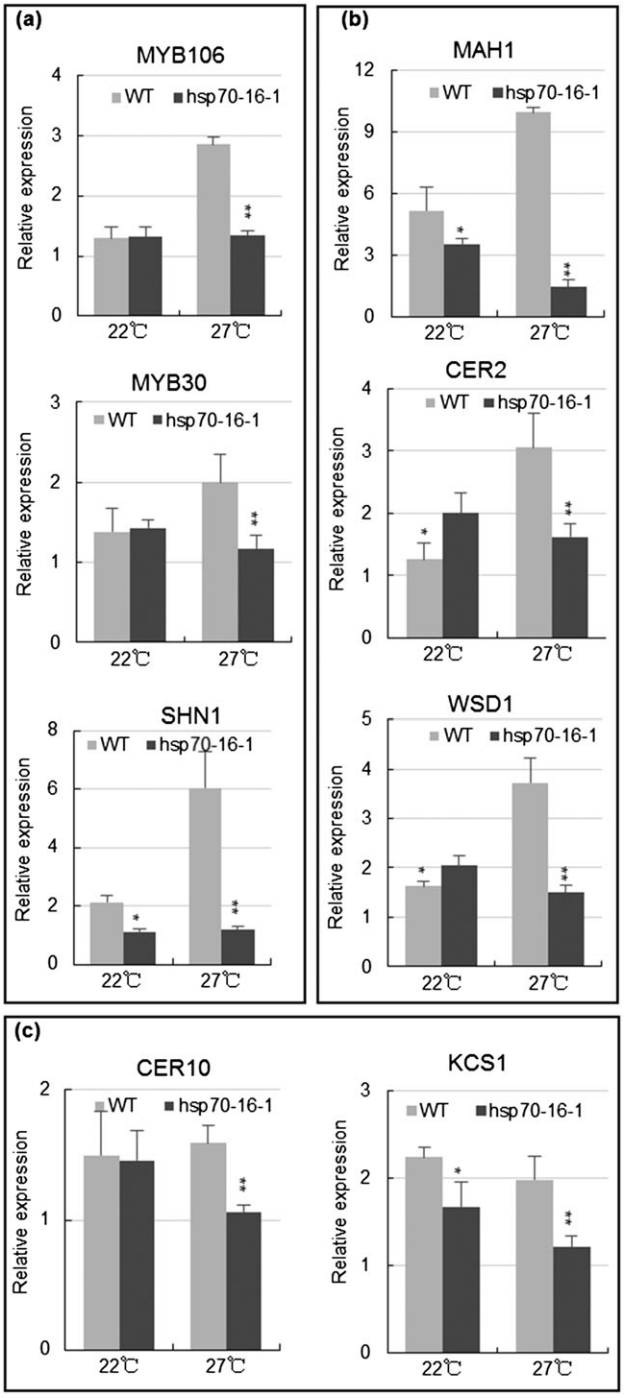

FIGURE 7 Expression analysis of known cuticle regulators and biosynthetic genes. (a) Expression patterns of three known cuticle regulator genes determined by qRT-PCR. Results are presented as mean plus/minus standard deviation (N = 3). (b) Expression patterns of three known cuticle biosynthetic genes determined by qRT-PCR, whose expression are inducible by elevated growth temperature. Results are presented as mean plus/minus standard deviation (N = 3). (c) Expression patterns of two additional known cuticle biosynthetic genes determined by qRT-PCR, whose expression are not inducible by elevated growth temperature. Results are presented as mean plus/minus standard deviation (N = 3)

Although numerous studies have indicated the importance of HSPs in thermo-tolerance, the specific functions and targets of HSPs remain largely unknown. To understand whether the loss of function

of *HSP70-16* affects heat shock response network in sepals, we performed qRT-PCR to further examine expression level changes of additional three *HSP70* genes and three heat shock factors (Figure S8). Interestingly, the loss of function of *HSP70-16* significantly repressed the expression of *HSP70-4* in sepals grown at 22°C; however, this suppression was lost in sepals grown at 27°C (Figure S8a). The same effect of loss of function of *HSP70-16* was seen on *HsfB1* (Figure S8b). *HSP70-4* is reported to be induced strongly in seedlings with increasing ambient temperatures (Kumar & Wigge, 2010), whereas *HsfB1* is a transcriptional repressor, which negatively regulates the expression of heat-inducible *HSFs* and several *HSP* genes (Ikeda, Mitsuda, & Ohme-Takagi, 2011). Although expression levels of other *HSP* genes and *HSFs* detected were not significantly affected as for *HSP70-4* and *HsfB1*, this result indicated that loss of function of *HSP70-16* does affect heat shock response networks in plants.

4 | DISCUSSION

Sepals, the outermost organs of flowers, are the first floral organs formed, which protect other floral organs developed subsequently from the very beginning of flower development (Stage 3), guaranteeing a normal early flower development in *Arabidopsis*. On the other hand, timely flower opening (at the end of Stage 12) that breaks the imbricate aestivation formed between overlapping margins of neighbouring sepals is also vital for satisfied fertility (Roeder, 2010; Smyth et al., 1990). The separation of crosslinked sepals is a complex process that involves cell expansion and osmotic status changes in sepals, which is regulated by both interior and exterior factors (Van Doorn & Kamdee, 2014; Van Doorn & van Meeteren, 2003). Nevertheless, the molecular mechanisms underlying the involvement of sepals in the coordination of developmental and environmental signals remain to be elucidated. In this study, we characterized a loss-of-function mutant of *HSP70-16* and demonstrated that *HSP70-16*, a cold- and heat-responsive gene, is required for flower opening under both normal and mild heat stress conditions. Results obtained in this study shed new light on sepal functions in flower development and interaction with surrounding thermal conditions.

4.1 | *HSP70-16* is required for flower opening

Although *HSP70* genes are originally regarded as the most abundant genes induced by heat stress, increasing evidence shows that *HSP70* genes are also essential for normal plant development and responsive to many other forms of stresses (Jiang et al., 2014; Kim & Hwang, 2014; Lin et al., 2001). Previous studies in *Arabidopsis* clearly demonstrated that *HSP70* genes respond to other biotic and abiotic stresses rather than heat stress (Jungkunz et al., 2011; Leng et al., 2017) and that *HSP70* genes are also essential/required for normal plant development, including chloroplast development (Latijnhouwers et al., 2010), gametogenesis (Maruyama et al., 2010, 2014), and stomatal opening and closing (Jungkunz et al., 2011). These studies indicated that there are probably multiple functions for a given *HSP70* gene. *HSP70-16*, together with *HSP70-14*, *HSP70-15*, and *HSP-17*, belongs to *HSP110/SSE* subfamily (Lin et al., 2001). Among them, all four genes are induced by heat stress (Kim, Cho, Lee, & Yoo, 2017; Lin et al., 2001) and only has *HSP70-14/HSP70-15* being functionally characterized (Jungkunz et al., 2011). Loss of function of *HSP70-16*, as evidenced in this study, reduced fertility under normal growth temperature (Figure 1b–d), assigning an essential role of *HSP70-16* for reproductive development in *Arabidopsis*. The reduced fertility in *HSP70-16* was not the consequence of defectiveness of anther and pollen development (Figure S2a,c), neither was it the consequence of the defectiveness of pollen viability and pistil functionality (Figure S2b), but it was the consequence of disrupted lateral sepal opening that impairs normal floral organ development and subsequent pollination process (Figure 3i–l). Therefore, *HSP70-16* is, in fact, required for normal flower opening. This finding is quite intriguing, as this is the first report, to our knowledge, on an *HSP70* gene that is associated with flower development. The qRT-PCR detected expression pattern of *HSP70-16* in flowers along early developmental stages and in various floral organs, together with those revealed by GUS staining assay (Figure 6), corresponding well with its observed function in flower development. An early study reported that the GUS signals of *Arabidopsis HSP18.2* are detectable in sepals, filaments, and styles but not in petals grown under 22°C, which indicates a possible role of *HSP* genes in flower development (Takahashi, Naito, & Komeda, 1992). Recently, it is reported that *HSP90* is recruited during flowering and that *HSP90* consolidates a molecular scaffold to facilitate interactions among key flowering genes (Margaritopoulou et al., 2016). Notably, the unique phenotype of *HSP70-16* in flower opening is different from the abolishment of flowering of *HSP90* mutants (Margaritopoulou et al., 2016); therefore, it is interesting to investigate the exact role of *HSP70-16* in flower development.

4.2 | *HSP70-16* is involved in sepal cuticle formation and patterning

In *Arabidopsis*, sepal identity is long time known to be determined by APETALA1 (AP1) and APETALA2 (AP2), two A class genes (Jofuku, Den Boer, Van Montagu, & Okamuro, 1994; Mandel, Gustafson-Brown, Savidge, & Yanofsky, 1992); however, knowledge on sepal's late development and its function, in the context of flower development and stress response, is quite limited. In addition, although plant cuticle is known to be involved in plant defence to biotic stresses such as bacterial and fungal pathogens (Bourdenx et al., 2011; Reina-Pinto & Yephremov, 2009; Shi et al., 2013) and abiotic stresses such as drought and osmotic stresses (Aharoni et al., 2004; Bourdenx et al., 2011; Wang et al., 2011), the involvement of plant cuticle in heat stress is largely unknown. A recent study found that *Arabidopsis* CER9, together with HRD1A/1B, regulates plant thermo-tolerance via its regulation of expression of cytosolic protein response and the unfolded protein response (Li, Lü, & Li, 2017). CER9 is previously reported to be involved in cuticle formation and water status maintenance (Lu et al., 2012). Plant cuticle is essential for plant interaction with surrounding environments; it regulates epidermal permeability and nonstomatal water loss and mediates pathogen infection (Shi et al., 2013; Sieber et al., 2000). Plant cuticle is also vital for plant growth and development; it prevents or promotes postgenital organ fusion and mediates interactions between pollens and pistils (Lolle,

Hsu, & Pruitt, 1998). Previous studies have shown that disruption of cuticle formation or patterning in plants affects not only vegetative and reproductive development but also responses to biotic or abiotic stresses (Aharoni et al., 2004; Kurdyukov et al., 2006; Lolle et al., 1998; Panikashvili et al., 2007, 2010; Sela et al., 2013; Shi et al., 2011, 2013; Sieber et al., 2000; Smirnova et al., 2013). Among them, defective-cuticle-induced postgenital fusion between vegetative and reproductive organs or between floral organs often results in defective growth and development and abnormal stress response. The observed floral organ fusion phenotype in *hsp70-16-1* is very similar to that of *35S:miR-SHN1/2/3*, a mutant of three AP2 transcription factors that regulate floral organ cuticle formation and patterning (Shi et al., 2011), which promoted us to explore the role of *HSP70-16* in cuticle formation and patterning. Because the lateral sepal fusion phenotypes in *hsp70-16-1* sepals grown at 22°C and 27°C were identical and there were more fused sepals in mutant grown at 27°C, we did cuticular lipid profiling in plants grown at 27°C. The significantly reduced total waxes in *hsp70-16-1* (Figure 5g,h) demonstrated that *HSP70-16* is indispensable for proper sepal wax formation under mild heat stress temperature. Although the total sepal cutin had no significant difference between *hsp70-16-1* and WT (Figure S5m), obvious changes in cutin patterning on surfaces of overlapping lateral sepal tips were observed in *hsp70-16-1* (Figures 5c–f and S5k,l); this result reflected the function of *HSP70-16* on cuticle patterning. We also measured waxes of sepals grown at 22°C and did not find significant difference between WTs and mutants although the total amount of waxes in mutants was lower than that of WTs (Figure S6). This could be explained by the dilution effect of much fewer fused sepals in examined samples as compared with that in WT, because there were only 5% fused buds in plants grown at 22°C (Figure S1e). Nevertheless, *HSP70-16* is also indispensable for proper sepal wax formation under normal growth temperature, because the expressions of *MAH1* and *KCS1*, two wax biosynthetic genes (Greer et al., 2007; Li et al., 2008), and *SHN1*, a wax inducer (Aharoni et al., 2004), were mild but significantly down-regulate in *hsp70-16-1* sepals at 22°C (Figure 7). The altered cutin distribution could be derived from the down-regulation of *SHN1*, a known floral organ cutin patterning regulator (Kannangara et al., 2007; Shi et al., 2011). To our knowledge, this is the first report on the involvement of wax in floral organ postgenital fusion albeit the alteration in cutin distribution pattern. However, the exact molecular network of *HSP70*-protein-mediated cuticle-related floral organ development remains to be further investigated.

4.3 | *HSP70-16* is likely an important element of sepal mild heat stress sensory pathway

Sessile plants are highly sensitive to temperature and can perceive a temperature difference of as little as 1°C, initiating multiple downstream responses. How temperature is sensed and integrated in plant growth and development is largely unknown. Evolutionarily conserved network, revealed in *Arabidopsis* (Schramm et al., 2008) and maize (Qin et al., 2007), consisted of dehydration-responsive element-binding protein (DREB), HSFs, and HSPs, which play important roles in HSR. Previous studies in *Arabidopsis* seedlings revealed that *HSP70-4* acts as a component of the nonstress ambient temperature (12°C to 27°C) sensory pathway (Kumar & Wigge, 2010), and its temperature-regulated expression is mediated by cell cycle transcription factors E2F2 (Zhou, Sun, Zheng, Li, & Zhang, 2014). The presence of several hormone- and stress-responsive cis-elements, particularly two elements each responsive to heat stress and low temperature, respectively, in the promoter region of *HSP70-16* gene (Figure 6h), and its induction to both low and elevated temperatures (Figure 6g), revealed in this study, indicated that *HSP70-16* is likely a thermal sensor as well in sepal. We, however, do not know currently what temperature sensory pathway *HSP70-16* belongs to. Further investigations into the nature and components of this sensory pathway would facilitate our understanding of plant HSR in general and of HSP functions in plant development in particular.

A recent study found that the heat-inducible expression of *HSP70-3*, *HSP70-4*, or *HSP70-16* in *Arabidopsis* seedlings is pre-mRNA splicing activity independent (Kim et al., 2017), implying that *HSP70-16* may be in the same temperature sensory pathway as *HSP70-4*. In *Arabidopsis* sepals, however, 27°C, the nonstress temperature to *Arabidopsis* seedlings, is likely no longer a nonstress temperature, because plant reproductive development is more vulnerable to high temperature (Giorno, Wolters-Arts, Mariani, & Rieu, 2015; Lavania, Dhingra, Siddiqui, Al-Whaibi, & Grover, 2015). Actually, in *Arabidopsis* sepals, 27°C induced expression of several stress markers, such as *SHN1* and *MYB30* (Figure 7a). Thus, *HSP70-16* and *HSP70-4* are unlikely in the same temperature sensory pathway. The observed different changes in expression patterns of *HSP70-4* in sepals of *HSP70-16* grown at 22°C and 27°C (Figure S8) supported this hypothesis. The different expression patterns of *HsfB1* in sepals of *HSP70-16* grown at 22°C and 27°C (Figure 7d) suggested, on the other hand, that loss of function of *HSP70-16* activates additional *HSFs* and *HSPs* at 22°C, which does not occur at 27°C. It is noteworthy to mention that altered cutin patterning in *hsp70-16-1* sepals at 27°C could be a consequence of simultaneous down-regulation of two more cutin patterning regulators *MYB30* and *MYB106*, whereas reduction in wax components in *hsp70-16-1* sepals at 27°C could be a consequence of simultaneous down-regulation of four more wax biosynthetic genes *CER2*, *CER10*, *WSD1*, and *KCS1* (Figure 7). This could explain the more severe seed setting rate reduction phenotype of *hsp70-16-1* grown at 27°C than grown at 22°C. *MYB30* is a hypersensitive response regulator and a wax biosynthesis regulator as well putatively targeting wax genes such as *CER2* and *CER10* (Raffaele et al., 2008). *MYB106* acts upstream of *SHN1* and directly promotes cutin and wax biosynthesis probably through cotarget wax biosynthetic genes *CER2* and *KCS1* (Oshima et al., 2013). Clearly, such a mild temperature response cassette centred on *MYB106-SHN1* and their targeted wax genes induced by mild heat stress temperature in WT sepals is blocked in *hsp70-16-1* sepals, which indicated that *HSP70-16* is indispensable for establishing such a mild HSR network. Whether this mild HSR network holds true under higher temperature heat stress and whether the posttranscriptional regulation is involved in this process merit further investigations.

In summary, we functionally characterized an HSP gene, *HSP70-16*, which provided a link between thermal and developmental perception signals and expanded our understanding of the roles of sepal in

plant development and heat response. This study also brought in one question: whether this temperature sensory pathway is conserved in other plant species.

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CONFLICT OF INTEREST

No conflict of interest declared.

AUTHOR CONTRIBUTION

J. X. and J. S. designed experiments, analysed data, and revised the manuscript. X. C. carried out most of experiments and wrote the draft, L. S. performed all reviewer-required additional experiments, Y. Q. C., D. S. Z., and L. Z. helped in some experiments, and S. X. and A. A. participated in project discussion and data analysis. And all authors reviewed and commented on the manuscript.

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SUPPORTING INFORMATION

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