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Research Article

Heat shock protein 101 (HSP101) promotes flowering under nonstress conditions

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Abstract

Heat shock proteins (HSPs) are stress-responsive proteins that are conserved across all organisms. Heat shock protein 101 (HSP101) has an important role in thermotolerance owing to its chaperone activity. However, if and how it functions in development under nonstress conditions is not yet known. By using physiological, molecular, and genetic methods, we investigated the role of HSP101 in the control of flowering in Arabidopsis (*Arabidopsis thaliana* (L.) Heynh.) under nonstress conditions. Knockout and overexpression of *HSP101* cause late and early flowering, respectively. Late flowering can be restored by rescue of *HSP101*. HSP101 regulates the expression of genes involved in the six known flowering pathways; the most negatively regulated genes are *FLOWERING LOCUS C* (*FLC*) and *SHORT VEGETATIVE PHASE* (*SVP*); downstream integrators of the flowering pathways are positively regulated. The late-flowering phenotype of loss-of-*HSP101* mutants is suppressed by both the mutations of *FLC* and *SVP*. The responses of flowering time to exogenous signals do not change in *HSP101* mutants. HSP101 is also found in nonspecific regions according to subcellular localization. We found that HSP101 promotes flowering under nonstress conditions and that this promotion depends on FLC and SVP. Our data suggest that this promotion could occur through a multiple gene regulation mechanism.

Introduction

Heat shock proteins (HSPs) are stress-responsive proteins that are conserved across all organisms. They are composed of a large family classified into several subfamilies (HSP100, HSP90, HSP70, HSP60, and small HSP) on the basis of their molecular weight (Vierling, 1991). They serve as molecular chaperones in cells to prevent aggregation of misfolded proteins induced by stress and assist with refolding in an ATP-dependent manner (Wang et al., 2004). In plants, HSPs have been found to be expressed in response to multiple abiotic stresses, such as heavy metal, drought, and salinity (Zou et al., 2012; Song et al., 2012). Their functions were initially recognized in the response to temperature stress, and they are typically related to thermotolerance (Lindquist, 1986). For example, in plants under temperature stress, small HSPs can form granules associated with a specific subset of mRNAs and provide surfaces to stabilize nonnative proteins and attenuate protein aggregation (Lee et al., 1997; Nover et al., 1989); Specifically, heat shock protein 70 (HSP70) can promote refolding of denatured proteins (Nunes et al.,

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2015). These chaperone activities protect cells from high-temperature damage and consequently increase plant thermotolerance.

Members of the HSP100 subfamily are crucial components of the chaperone system. Its proteins form ring-like oligomers and force unfolding of structured polypeptides of protein aggregates that are threaded through their small central channel (Zolkiewski et al., 2012). These members are widely distributed among prokaryotes and eukaryotes. Arabidopsis (*Arabidopsis thaliana* (L.) Heynh.) HSP100 subfamily has eight homologous proteins, among which HSP101 plays a pivotal role in thermotolerance (Gurley, 2000). Transgenic Arabidopsis plants that show reduced expression of HSP101 are less tolerant to heat shock after mild conditioning pretreatments than wild-type plants, and *HSP101* mutants show inferior thermotolerance (Queitsch et al., 2000). Introduction of Arabidopsis *HSP101* cDNA into rice (*Oryza sativa*), cotton (*Gossypium hirsutum*), and tobacco (*Nicotiana tabacum*) results in significantly improved growth after heat shock and enhanced pollen thermotolerance and boll production under high temperature (Burke and Chen, 2015; Katiyar-Agarwal et al., 2003). Recently, dynamic coordination between protein disaggregation and proteolysis was found after heat shock, and protein disaggregation mediated by HSP101 is important for optimal proteasomal degradation (Mcloughlin et al., 2019).

HSPs are also involved in plant development under nonstress conditions. The expression of *Hsp101* is regulated developmentally in maize (*Zea mays*) and is negatively correlated with the growth rate of primary roots and the development of nodal roots (Nieto-Sotelo et al., 2002; Lopez-Frias et al., 2011; Young et al., 2001). A recent study indicated that, in Arabidopsis, HSP90 is essential for the transition from the vegetative phase to the reproductive phase and for flower morphology (Margaritopoulou et al., 2016; Samakovli et al., 2014). Targeted depletion of *HSP90* represses flower formation and results in flower-like structures under normal growth conditions. The interactions of HSP90 with key flowering regulators (LEAFY (LFY), SUPPRESSOR OF OVEREXPRESSION OF CO1 (SOC1), AGAMOUS-LIKE24 (AGL24)) suggests that it acts as a molecular scaffold to recruit the regulators and coordinate reproductive development (Margaritopoulou et al., 2016). However, knowledge about the function of HSPs in plant development is still limited, and whether and how HSPs play roles under nonstress conditions remain unclear.

To respond to developmental and environmental signals, plants may shift their flowering time to maximize reproductive success through a complex regulation network (Kazan and Lyons, 2016). In Arabidopsis, there are six major pathways regulating flowering time, and there are multiple regulatory genes in each pathway (Figure 1a; Lucas-Reina et al., 2016; Pajoro et al., 2014). The photoperiod pathway receives signals from light and the circadian clock. The *CONSTANS* (*CO*) gene then integrates the external and internal signals, which allows the promotion or inhibition of flowering time (Roden et al., 2002). The vernalization pathway promotes flowering at low temperatures and is governed by a number of genes, such as *VERNALIZATION1* (*VRN1*), *FRIGIDA* (*FRI*), and *FLOWERING LOCUS C* (*FLC*; Choi et al., 2011; Hu et al., 2014). The gibberellin (GA) pathway promotes flowering through degradation of DELLA proteins after GA receptors (e.g., GA INSENSITIVE DWARF1A (GID1A)) receive endogenous and exogenous GA signals (Galvao et al., 2012; Bao et al., 2019). The ambient temperature pathway is affected by the temperature during growth, which leads to early or late flowering through the expression of *FLOWERING LOCUS M* (*FLM*) and *SHORT VEGETATIVE PHASE* (*SVP*; Kinmonth-Schultz et al., 2016; Lee et al., 2013). The autonomous pathway relies primarily on the suppression of *FLC* by means of chromatin and RNA modification to promote flowering (Cheng et al., 2017). In the age pathway, microRNAs and *SQUAMOSA PROMOTER BINDING PROTEIN-LIKEs* (*SPLs*) are core components that function downstream of sugars to determine flowering time (Teotia and Tang, 2015; Wang, 2014; Yu et al., 2015). Among the core components of these pathways, *MADS-box* genes (e.g., *SVP*, *FLC*, and *MADS AFFECTING FLOWERINGs* (*MAFs*)) form complexes with each other to repress flowering (Li et al., 2008; Gu et al., 2013). Flowering signals from all these pathways are ultimately integrated by floral integrators, such as FLOWERING LOCUS T (FT), SUPPRESSOR OF OVEREXPRESSION OF CO1 (SOC1), APETALA1 (AP1), and LEAFY (LFY), thus influencing flowering.

In the present study, we demonstrated that HSP101 positively regulates flowering time under normal growth conditions in Arabidopsis. By using physiological, molecular and genetic approaches, we found regulation of HSP101 on flowering depended on FLC and SVP. In part on the basis of its nonspecific subcellular distribution, we proposed a multiple-gene regulation mechanism by which HSP101 affects flowering time. Our results reveal a previously unknown function of HSPs under nonstress conditions and component of flowering time regulation.

Results

Isolation and generation of *HSP101* loss-of-function mutants, transgenic lines and complementation lines

To investigate the biological function of *HSP101* (AT1G74310) systematically, we isolated two knockout (transfer DNA (T-DNA)insertion mutant) Arabidopsis lines (designated *hot1-1* and *hot1-3*) containing a T-DNA insertion within the first and the second exons in the coding region of *HSP101*, respectively (Figure 1b). Genomic DNA from 4-week-old seedlings was extracted, and the homozygosity of each line was verified by the three-primer method (Supplemental Figure S1). The wild type and the two homozygous mutants were transformed with a construct that carried the *HSP101* full-length coding sequence under the control of the 35S promoter to generate a transgenic line (*35S::HSP101-GFP*) and two complementation lines (*hot1-1/35S::HSP101-GFP* and *hot1-3/35S::HSP101-GFP*). No transcripts

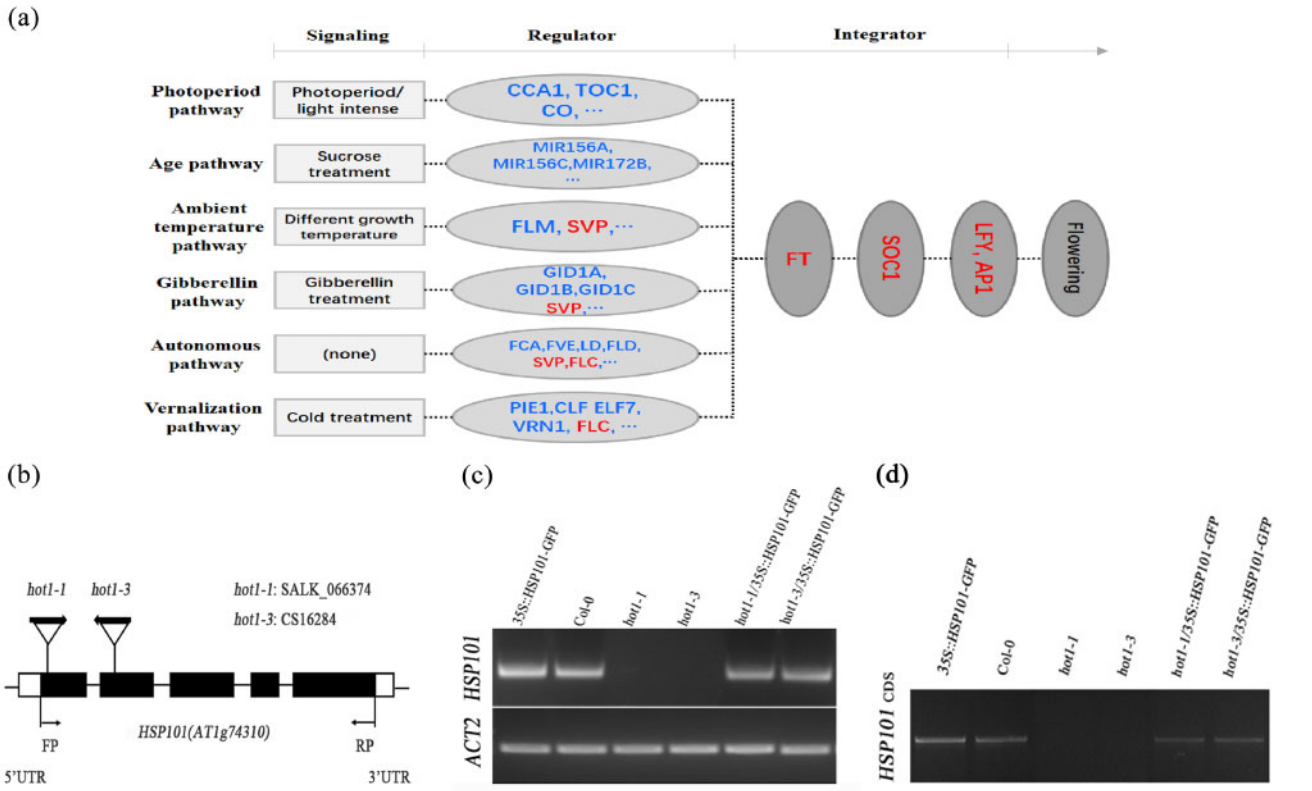

Figure 1 Insertions within the *HEAT SHOCK PROTEIN101* (*HSP101*) gene and detection of *HSP101* in different genotypes. (a) Schematic of flowering network. Signals, regulatory factors measured in this study, and integrators. Regulators and integrators marked red are shared by multiple pathways. (b) Schematic diagram showing sites of transfer DNA insertions within the *HSP101* gene. The black boxes represent *HSP101* exons, the white boxes represent 5′ and 3′ untranslated regions (UTR), and the black line represents introns. (c) Semi reverse transcription quantitative PCR was used to analyze the *HSP101* transcript level in the different genotypes. The *ACTIN2* (*ACT2*) gene was used as a control for constitutive expression. (d) Semi reverse transcription quantitative PCR amplification of the *HSP101* full-length transcript (coding sequence [CDS]) in different genotypes. The selected primers for *HSP101* full-length transcript amplification are indicated in (b) as forward primer (FP) and reverse primer (RP).

of *HSP101* were detected in the *hot1-1* or *hot1-3* mutants, which indicates that both mutants were homozygous loss-of-function mutants. In addition, slightly more *HSP101* transcripts were detected in the *35S::HSP101-GFP* line than in the other lines (Figure 1c). The fragment transcript and full-length transcript of *HSP101* were detected in both homozygous rescue lines (Figure 1c-d).

HSP101 promotes flowering under normal long day conditions

To test whether HSP101 affects plant development under nonstress conditions, plants of three genotypes (Col-0, *hot1-1*, and *35S::HSP101-GFP*) were grown side-by-side under 22°C (long days), and their morphology and development were compared. No difference in morphology was found between the genotypes at the juvenile or adult stages, including shape and size of whole plant, inflorescences and infructescences, flowers and siliques (Supplemental Figure S2). This data indicates that HSP101 does not affect plant morphology under nonstress conditions.

We compared the reproductive development of the three genotypes by observing their flowering phenotype (Figure 2a) and counting their rosette leaves at the onset of flowering (Figure 2b), and the days to flowering (Figure 2c). In comparison with the flowering time of Col-0, that of *hot1-1* occurred markedly later, and that of *35S::HSP101-GFP* occurred earlier due to slight up-regulation of *HSP101* expression (Figure 2a and 1c). Furthermore, the numbers of rosette leaves at the onset of flowering (*35S::HSP101-GFP*, 8.3 ± 0.5; Col-0, 9.8 ± 0.9; *hot1-1*, 15.9 ± 1.1) were significantly different (Figure 2b), which was consistent with the difference in the days to flowering time (*35S::HSP101-GFP*, 26.1 ± 2.1; Col-0, 28.0 ± 3.1; *hot1-1*, 38.4 ± 2.8; Figure 2c). These results show that loss of HSP101 delays flowering time in Arabidopsis and suggest that HSP101 promotes flowering under normal growth conditions.

HSP101 complementation rescues the late-flowering phenotype of *hot1-1* and *hot1-3* mutants

To verify that HSP101 promotes flowering under nonstress conditions, we rescued *HSP101* in the two *HSP101*-knockout

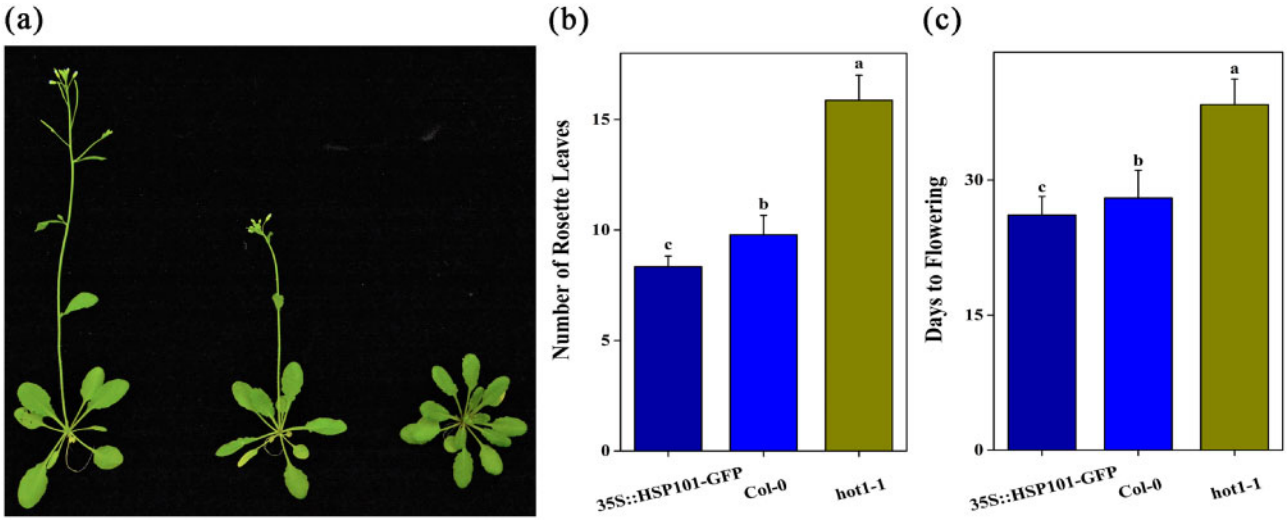

Figure 2 Flowering times vary among *35S::HSP101-GFP*, wild-type (Col-0), and *hot1-1* plants grown under long days. (a) Flowering phenotypes of *35S::HSP101-GFP*, wild-type (Col-0), and *hot1-1*. (b) Number of rosette leaves at flowering in *35S::HSP101-GFP*, wild-type (Col-0), and *hot1-1*. (c) Days to flowering for *35S::HSP101-GFP*, wild-type (Col-0), and *hot1-1*. More than 40 plants ($N > 40$) grown under long days were used for each experiment, and the average value was used. The error bars indicate the standard deviations of three independent experiments. The different letters above the bars indicate significant differences ($P \leqslant 0.01$, Least Significant Difference [LSD]). The experiments were performed at least three times, each yielding similar results.

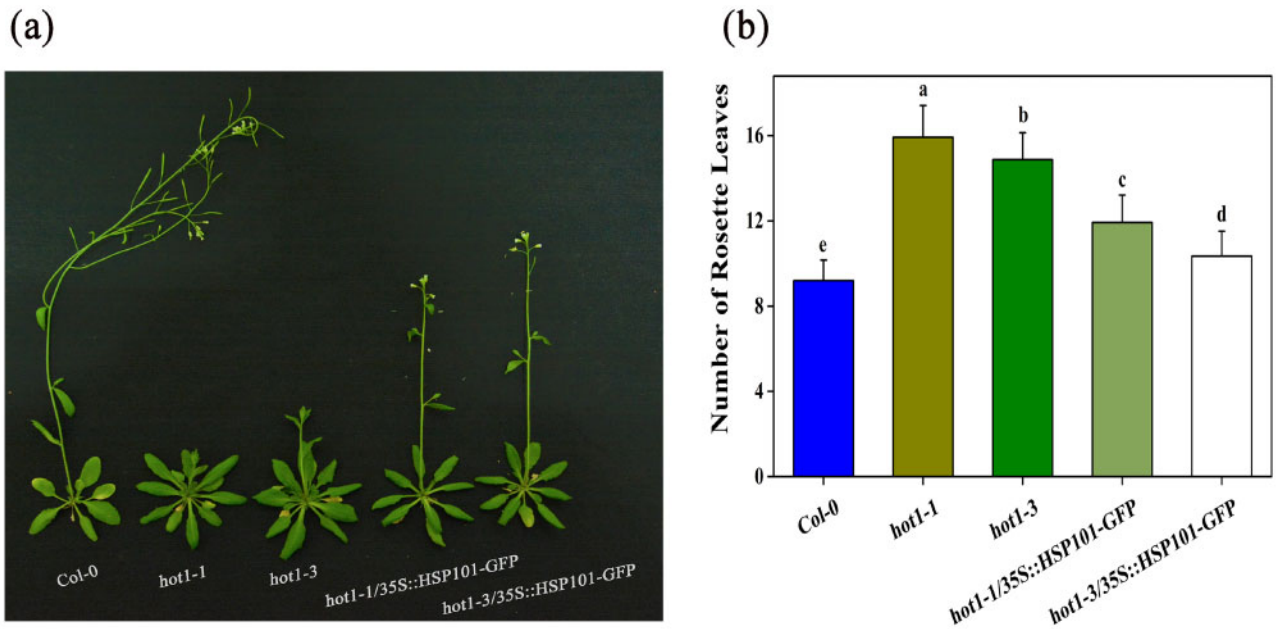

Figure 3 *HSP101* complementation rescues the late-flowering phenotype of *hot1-1* and *hot1-3* mutants under long days. (a) Flowering phenotypes of rescued lines (*hot1-1/35S::HSP101-GFP* and *hot1-3/35S::HSP101-GFP*), the wild type (Col-0) and *hot1-1* and *hot1-3* mutants. (b) Number of rosette leaves of the rescued lines and Col-0, *hot1-1* and *hot1-3*. More than 40 plants ($N > 40$) grown under long days were used for each experiment, and the average value was used. The error bars indicate the standard deviations of three independent experiments. The different letters above the bars indicate significant differences ($P \leqslant 0.01$, Least Significant Difference [LSD]). The experiments were performed at least three times, each yielding similar results.

lines (*hot1-1 and hot1-3*) and compared their flowering time with that of Col-0 under normal growth conditions (Figure 3a-b). Col-0 flowered when it had 9.2 ± 1.0 rosette leaves. Both *hot1-1* and *hot1-3* mutants showed significantly late-flowering phenotypes, flowering when they had 15.9 ± 1.5 and 14.9 ± 1.3 rosette leaves, respectively. However, the delayed flowering of their corresponding *hot1-1/35S::HSP101-GFP* and *hot1-3/35S::HSP101-GFP* complementation lines was

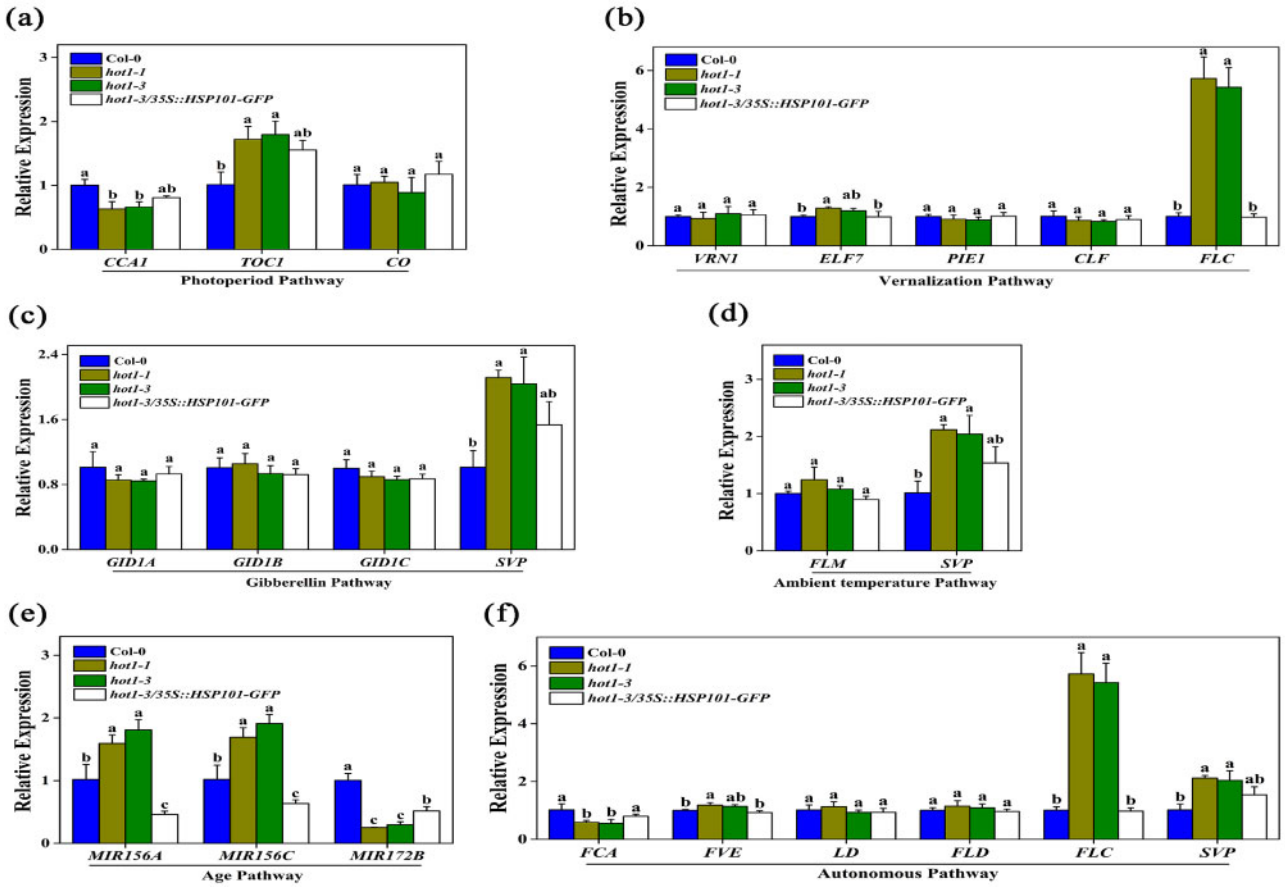

Figure 4 Regulation of flowering time-related genes by HSP101 in Arabidopsis. Reverse transcription quantitative PCR analysis of transcript levels of genes involved in the photoperiod pathway (a), vernalization pathway (b), gibberellin pathway (c), ambient temperature pathway (d), age pathway (e) and autonomous pathway (f) in Col-0, *hot1-1*, *hot1-3*, and *hot1-3/35S::HSP101-GFP* plants. The value of *FLC* is shared in (b), and (f). The value of *SVP* is shared in (c), (d) and (f). Seedlings were grown under long days for 2 weeks, and then the total RNA was extracted for Reverse transcription quantitative PCR analysis. Gene expression was normalized by comparison with the abundance of *ACTIN2* (*ACT2*) transcripts (endogenous control). The error bars indicate the standard deviations of three measurements. The different letters above the bars indicate significant differences ($P \leqslant 0.01$, Least Significant Difference [LSD]). The experiments were performed three times, each yielding similar results. A repeat of these analyses is presented in Supplemental Figure S3.

significantly reduced, as these plants flowered when there were 11.9 ± 1.3 and 10.4 ± 1.2 rosette leaves, respectively. Taken together, these results confirm that HSP101 promotes the onset of flowering under normal growth conditions.

HSP101 regulates the expression of multiple genes in flowering pathways

To explore the mechanism by which HSP101 regulates flowering time, we investigated the expression of 20 key genes in 6 known flowering pathways (Figure 1a; Wellmer and Riechmann, 2010; Fornara et al., 2010) in *hot1-1* and *hot1-3* plants and compared them with those in Col-0 and *hot1-3/35S::HSP101-GFP* plants (Figure 4 and Supplemental Figure S3). Among these genes, *SVP* was shared by three pathways, and *FLC* was shared by two pathways (Figure 1a). We found that 12 genes (60% of the total amount of detected genes) showed no change and that 8 genes (40% of the total amount of detected genes) were dysregulated (the expression in both *hot1-1* and *hot1-3* plants significantly differed from that in Col-0). The dysregulated genes were distributed in all pathways, meaning that HSP101 widely affect pathways of the flowering regulation network. Of the 8 genes whose expression significantly changed (40%), 5 (25%) were upregulated, and 3 genes (15%) were downregulated. In detail, 5 genes (*CIRCADIAN CLOCK ASSOCIATED1* (*CCA1*), *TIMING OF CAB EXPRESSION1* (*TOC1*), *microRNA156A* (*MIR156A*), *microRNA156C* (*MIR156C*), and *FLOWERING CONTROL LOCUS A* (*FCA*)) involved in 3 pathways showed slight but significant changes ($<$ 2-fold); 3 genes (*FLC*, *SVP*, and *microRNA172B* (*MIR172B*)) involved in 5 pathways showed marked differences ($>$ 2-fold; Figure 4). The rhythmic expression of *CCA1* and *TOC1* were also detected (Supplemental Figure S4). Notably, the expression of *FLC* in the *hot1-1* and *hot1-3* plants was approximately 5-fold higher than that in the Col-0 plants (Figure 4) and *FLC* is

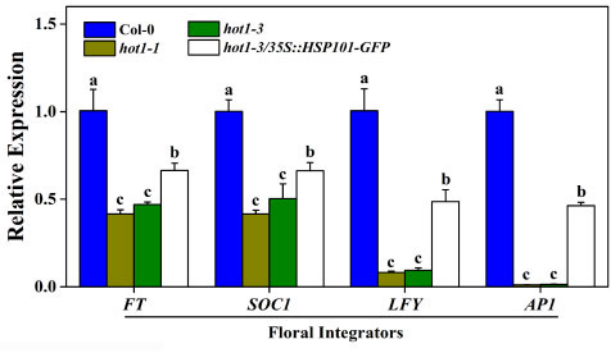

Figure 5 Regulation of floral integrators by HSP101 in Arabidopsis. Reverse transcription quantitative PCR analysis of transcript levels of floral integrators of plants with different genotypes (Col-0, *hot1-1*, *hot1-3*, and *hot1-3/35S::HSP101-GFP*). Seedlings were grown under long days for 2 weeks, and then the total RNA was extracted for Reverse transcription quantitative PCR analysis. Gene expression was normalized by comparison with the abundance of *ACTIN2* (*ACT2*) transcripts (endogenous control). The error bars indicate the standard deviations of three measurements. The different letters above the bars indicate significant differences ($P \leqslant 0.01$, Least Significant Difference [LSD]). The experiments were performed three times, each yielding similar results. A repeat of these analyses is presented in Supplemental Figure S5.

revealed as a regulator of the natural variation of flowering time in Arabidopsis (Mendez-Vigo et al., 2016), it could be the most important contributor to the effects of *HSP101* on flowering time. The expression changes of all dysregulated genes in the *hot1-3* plants were rescued completely or to a certain extent in the *hot1-3/35S::HSP101-GFP* plants (Figure 4). The HSP101-regulated genes varied from pathway to pathway, the expression change varied from upregulation to downregulation, and the change intensity varied from small to large. Together, the data indicate that HSP101 influences flowering time of Arabidopsis and suggests that its regulation of flowering occurs through a multiple-gene regulation mechanism. If this is true, the question raised here is how this multiple-gene regulation mechanism leads to a clear and substantial promotion of flowering time.

HSP101 negatively regulates the expression of downstream integrators in the flowering network

According to a known flowering regulatory network, the signals from the 6 flowering pathways are integrated by downstream floral integrators (FT, SOC1, LFY, and AP1; Figure 1a; Fornara et al., 2010; Wellmer and Riechmann, 2010) that positively regulate flowering. The question raised here is whether the effect of HSP101 on flowering time occurs through the integrators. We therefore sought to answer this question by comparing the expression of the integrators in *hot1-1* and *hot1-3* plants with those in Col-0 and *hot1-3/35S::HSP101-GFP* plants (Figure 5 and Supplemental Figure S5). The expression of *FT*, *SOC1*, *LFY*, and *AP1* in the *hot1-1* and *hot1-3* plants was significantly lower than that in the Col-0 plants, and the reduced expression in *hot1-3* plants was rescued, at least partly, in the *hot1-3/35S::HSP101-GFP* plants. In addition, these expression patterns were consistent with the late-flowering phenotypes of *hot1-1*, *hot1-3*, and *hot1-3/35S::HSP101-GFP* (Figure 3), in which flowering time was delayed for the *hot1-1* and *hot1-3* plants, and the delay was partly restored in the *hot1-3/35S::HSP101-GFP* plants. These results indicate that the HSP101 effects indeed occur through the integrators of the flowering network. Together, the above gene expression data indicate that the mechanism by which HSP101 regulates flowering time can be completely mapped onto the flowering network.

The flowering promotion of HSP101 is dependent on both FLC and SVP

Floral integrators are negatively regulated by FLC and SVP (Jang et al., 2009; Michaels et al., 2005). Therefore, their decreased expression (Figure 5) may be resulted from elevated expression of *FLC* and *SVP* (Figure 4). However, HSP101 may directly influence the expression of integrators. If this is the case, the late flowering of the *hot1-1* and *hot1-3* mutants should be independent of FLC and SVP. To test this possibility, we introduced loss-of-function mutations of *FLC* (*flc-31*) and *SVP* (*svp-41*) into *hot1-1* and *hot1-3* mutants through cross-pollination and then compared the flowering time of these double mutants with that of Col-0 and single mutants (Figure 6, Supplemental Table S1). The *flc-31* and *svp-41* mutants flowered earlier than the Col-0 plants, similar with previous reports (Gregis et al., 2006; Michaels and Amasino, 1999). The flowering time of the *flc-31/hot1-1* and *flc-31/hot1-3* double mutants occurred earlier than Col-0 and was the same as that of the *flc-31* mutant (Figure 6a-b). This means that the late-flowering phenotypes of the *hot1-1* and *hot1-3* mutants were suppressed by the *flc-31* mutation. Similarly, the late-flowering phenotypes of the *hot1-1* and *hot1-3* mutants were suppressed by the *svp-41* mutation in the *svp-41/hot1-1* and *svp-41/hot1-3* double mutants (Figure 6c-d). These genetic data demonstrate that the effect of HSP101 on flowering time depends on both FLC and SVP. This is also consistent with the fact that FLC and SVP form a complex to negatively regulate flowering (Li et al., 2008) and supports that HSP101 regulates flowering through FLC and SVP (Figure 4f and Result 6).

HSP101 mutants respond to exogenous flowering signals

Considering that the genes affected by HSP101 exist in multiple flowering pathways (Figure 4) and that the pathways have their own specific exogenous signals for flowering (Figure 1a), we went onto test whether the HSP101 affects the flowering responses to the exogenous signals. We applied five pathway-specific treatments (Figure 1a) and then examined whether flowering time was affected in *hot1-1* plants in comparison with that in Col-0 and *35S::HSP101-GFP* plants (Figure 7 and Supplemental Table S2). For the photoperiod pathway, we grew plants under different day-length and light intensities. We found that the flowering

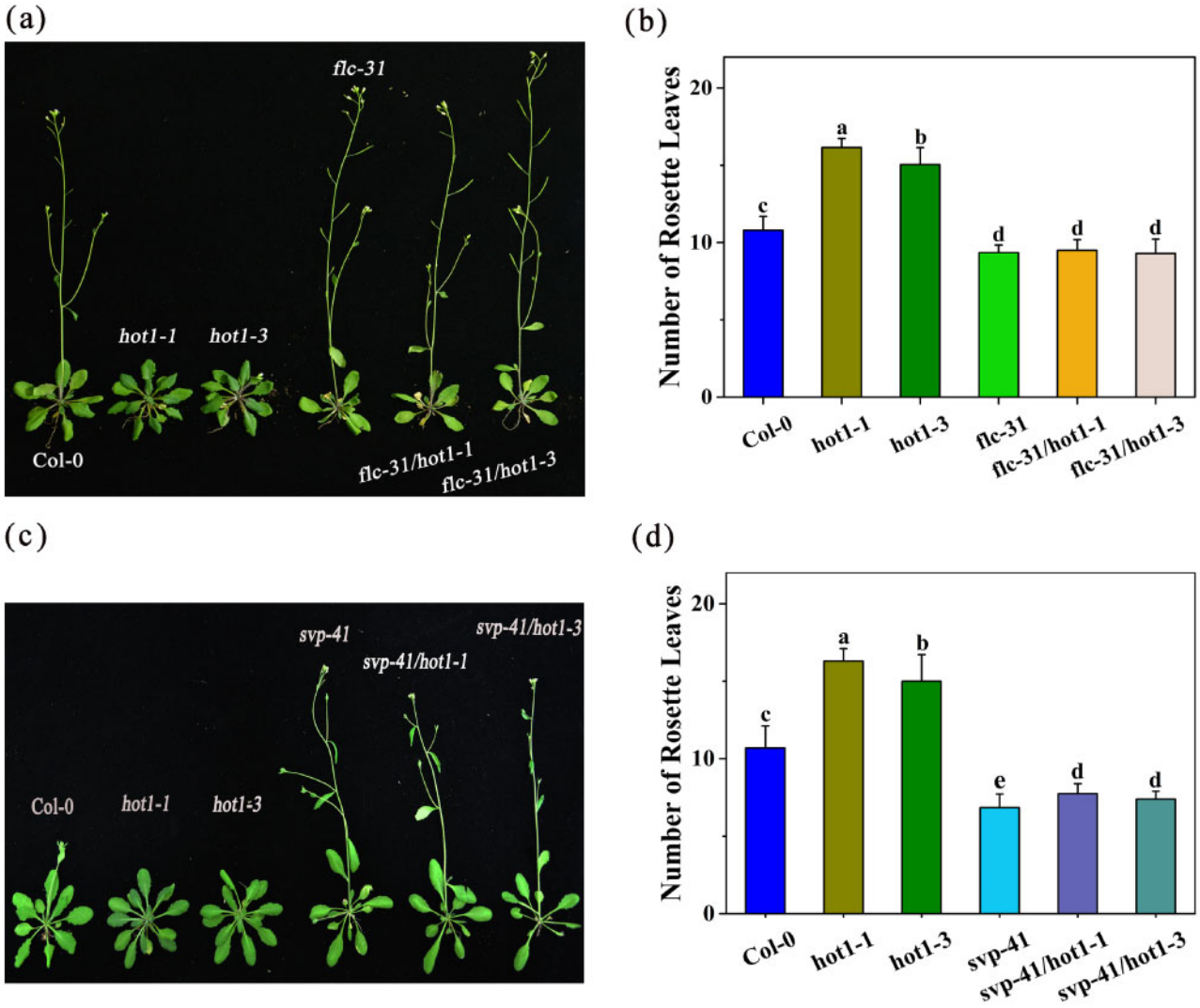

Figure 6 HSP101 promotes flowering through the regulation of *FLC* and *SVP*. (a, b) Flowering phenotypes and rosette leaf numbers at flowering of Col-0, *hot1-1*, *hot1-3*, *flc-31*, *flc-31/hot1-1*, and *flc-31/hot1-3* plants. (c, d) Flowering phenotypes and rosette leaf number at flowering of Col-0, *hot1-1*, *hot1-3*, *svp-41*, *svp-41/hot1-1*, and *svp-41/hot1-3*. For phenotypic observations and flowering time statistics, plants were grown under long days . More than 30 plants ($N > 30$) were used for each experiment, and the average values were calculated. The error bars indicate the standard deviations of three independent experiments. The different letters above the bars indicate significant differences ($P \leqslant 0.01$, Least Significant Difference [LSD]). The experiments were performed at least three times, each yielding similar results.

time of Col-0 plants became significantly delayed as the day length decreased (Figure 7a) or significantly early as the light intensity increased (Figure 7b), similar to previous reports (Munir et al., 2004; Onouchi and Coupland, 1998). Moreover, the flowering time in *35S::HSP101-GFP* and *hot1-1* plants changed in the same way. Thus, the presence or absence of HSP101 did not affect the flowering response induced by the photoperiod or light intensity.

For the vernalization and gibberellin pathway, the *35S::HSP101-GFP*, Col-0, and *hot1-1* plants were subjected to corresponding vernalization (4°C) and GA_3 treatment. The flowering time of Col-0 plants was promoted in all the treatments, being consistent with previous reports (Wang et al., 2012), and the flowering time of *35S::HSP101-GFP* and *hot1-1* plants were also promoted (Figure 7c-d). The results suggest that *HSP101* mutants respond to vernalization and GA_3. Notably, detailed analysis of rosette leaf number showed that the promotion of flowering time in *hot1-1* plants were significantly greater than that in Col-0 (Supplemental Table S3). In other words, *hot1-1* is more sensitive than Col-0 to vernalization and GA_3 induced flowering.

For the ambient temperature and age pathway, the *35S::HSP101-GFP*, Col-0, and *hot1-1* plants were treated with increasing temperature during growth, and exogenous sucrose respectively, and their flowering times were examined. The flowering time of Col-0 plants became significant early in all the treatments, as previously reported (Balasubramanian et al., 2006; Roldan et al., 1999), and same

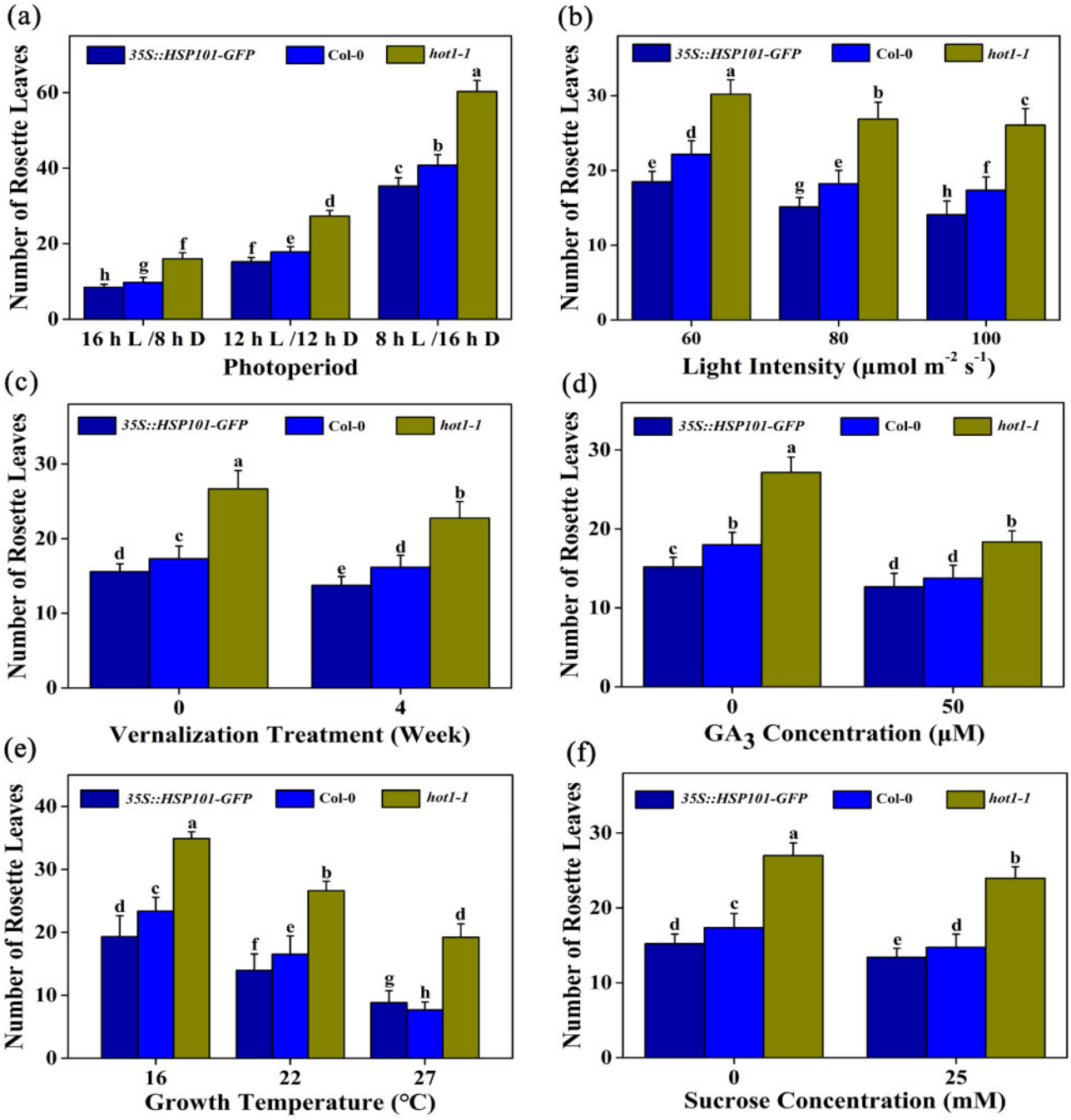

Figure 7 Number of rosette leaves at flowering in *35S::HSP101-GFP*, wild-type (Col-0), and *hot1-1* mutant plants under various treatments. (a) Photoperiod treatment with long days (16 h light/8 h dark), moderate days (12 h light/12 h dark), and short days (8 h light/16 h dark). L-light, D-dark. (b) Light intensity of 60, 80, and 100 μmol m^{-2} s^{-1}. (c) Vernalization treatment of 4°C for 4 weeks. (d) Gibberellin treatment. (e) Ambient temperatures of 16, 22, and 27 °C. (f) Sucrose treatment. More than 40 plants ($N > 40$) grown under moderate days, 90 μmol m^{-2} s^{-1} light intensity were used for each experiment (except for the photoperiod experiment in (a) and the light intensity in (b)), and the average values were calculated. The error bars indicate the standard deviations of three independent experiments. The different letters above the bars indicate significant differences ($P \leqslant 0.01$, Least Significant Difference [LSD]). The experiments were performed at least three times, each yielding similar results.

phenotype happened to *35S::HSP101-GFP* and *hot1-1* plants (Figure 7e-f). This means that HSP101 does not affect the ambient temperature- or sucrose-induced flowering responses.

HSP101 shows a nonspecific subcellular distribution and relatively concentrated in the nucleus

The biological function of a protein is closely associated with its subcellular localization. To further test our

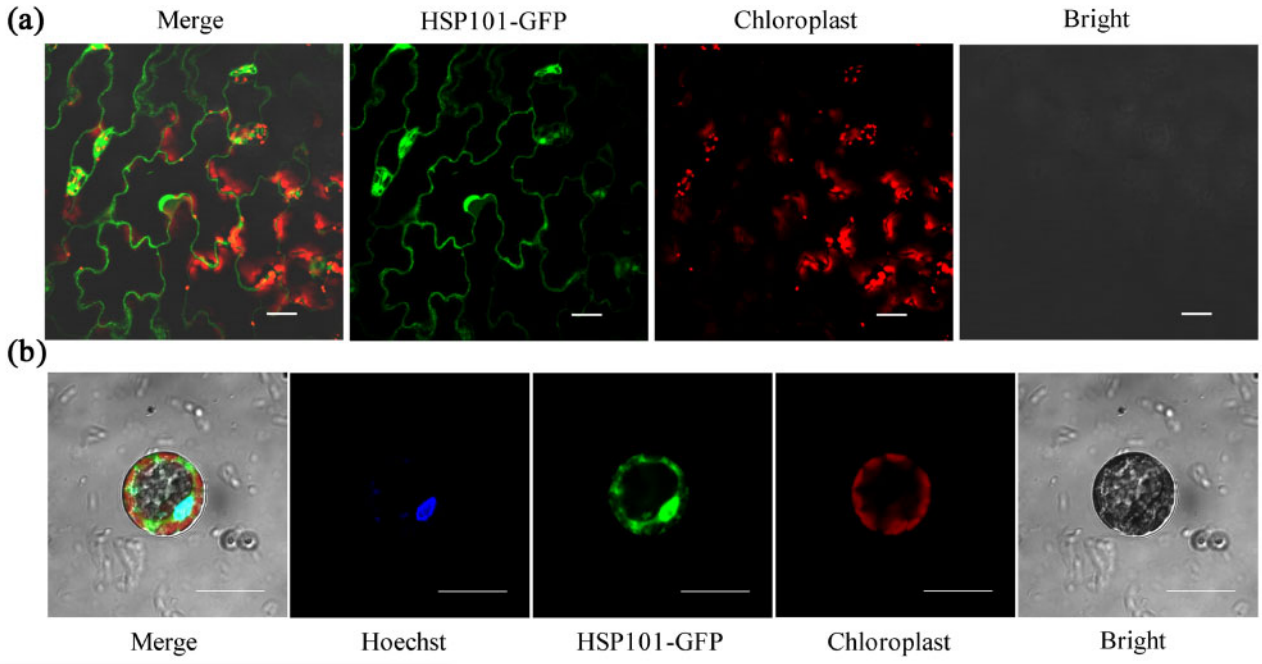

Figure 8 Subcellular localization of the HSP101-GFP fusion protein under nonstress conditions. Lower epidermis of *Nicotiana benthamiana* leaves injected with *Agrobacterium tumefaciens* strain *GV3101* (transformed with a 35S::HSP101-GFP fusion protein) (a) and protoplasts from *35S::HSP101-GFP* transgenic Arabidopsis seedlings (b) were observed with a 60 × oil objective by Confocal Laser Scanning Microscopy using an Olympus FV 1000 system equipped with argon as an excitation source. HSP101-GFP was localized in the nucleus, cytoplasm, and plasma membrane based on GFP fluorescence. Hoechst 33342 nuclear dye was used as a reference for the nuclear localization in (b). The experiments were performed at least three times, each yielding similar results. Bar = 20 μm.

hypothesis that the regulatory effects of HSP101 on flowering time involve a multiple-gene regulation mechanism, we used two approaches to examine HSP101 subcellular localization under nonstress conditions. One approach was to inject *Agrobacterium tumefaciens* strain *GV3101* (transformed with a 35S::HSP101-GFP fusion protein) into the abaxial leaf epidermis of *Nicotiana benthamiana* (Figure 8a), the other was to isolate protoplasts from *35S::HSP101-GFP* transgenic Arabidopsis seedlings (Figure 8b). We found GFP signals in the nucleus, cytoplasm, and plasma membrane in both epidermal cells and protoplasts. The results demonstrate that HSP101 does not have a specific subcellular localization under nonstress conditions. This is in agreement with its subcellular localization under heat stress (Mcloughlin et al., 2016). However, HSP101 was relatively concentrated in the nucleus under nonstress conditions, which was different from relatively concentrated in the cytoplasm under stress. Moreover, the proteins encoded by flowering-related genes, particularly the HSP101-affected genes, were nearly completely localized in the nucleus (Supplemental Table S4). This provides more support for HSP101 to regulate flowering time through a multiple-gene regulation mechanism.

Discussion

HSPs serve as molecular chaperons to protect proteins from denaturation damage under stress (Wang et al., 2004). HSPs are also suggested to perform functions under nonstress conditions (Young et al., 2001). However, there are few cases revealing the roles of HSPs in development under nonstress conditions at the physiological, transcriptional and genetic levels. The results of the present study showed that HSP101 plays a role in promoting flowering but does not affect the morphology or structure of Arabidopsis under normal growth conditions (Supplemental Figure S2; Figs. 2-3). HSP101 largely participates in the flowering network by regulating chosen genes of the 6 flowering pathways (Figure 4). In the present study, the regulation by HSP101 at the transcriptional level is widely distributed in all pathways, ultimately exerting positive effects through integrators of the flowering network (Figs. 4 and 5). The promotion of flowering occurs mainly through SVP and FLC (Figs. 4 and 6). Based on various regulated genes and the "nonspecific but relatively concentrated in the nucleus" subcellular localization of HSP101, our data suggest that the promotive effect of HSP101 on flowering time could involve a multiple-gene regulation mechanism and mainly results from the effect on SVP and FLC.

The implication of HSPs in flowering has been previously reported for HSP70 and HSP90. HSP70 presents chaperone activity in preventing aggregation and assisting protein refolding (Sung et al., 2001). The expression of *HSP70* is suggested to be positively associated with flowering time under

temperatures ranging from 12°C to 27°C (Kumar and Wigge, 2010). The HSP90 subfamily has 7 members in Arabidopsis; these members are localized in the cytosol, chloroplast, mitochondrion, and endoplasmic reticulum (Krishna and Gloor, 2001). RNAi lines with depleted *HSP90* mRNA levels delay or even abolish flowering, cause radical flower deformations, and induce variation in the expression of flowering-related genes under normal growth conditions (Margaritopoulou et al., 2016). Here, we demonstrate that loss of *HSP101* delays flowering time at 22°C (Figs. 2-3). Together, these lines of data indicate a significant positive relationship between HSPs and flowering time under nonstress conditions. Given that these HSPs are different in structure and size but are common in terms of their chaperone activity, chaperone function may therefore play a positive role in flowering under nonstress conditions.

HSPs play roles in assisting protein refolding, preventing protein aggregation, promoting protein trafficking, and stabilizing nonnative proteins (Wang et al., 2004). These molecular behaviors act on numerous and various proteins in cells and can occur in different cellular compartments (Wang et al., 2004). They essentially occur via a multiple-gene mechanism. For example, HSP90 can associate with a wide range of proteins with unrelated amino acid sequences and functions in animal cells (Taipale et al., 2010). HSP90 acts as a molecular scaffold to arrange and organize flowering-related genes in plant cells (Margaritopoulou et al., 2016). In the present case, HSP101 displays this multiple-gene mechanism by diversely regulating the expression of flowering-related genes (Figure 4). These lines of evidence further support our hypothesis that HSP101 regulates flowering time via its multiple-gene regulation mechanism under nonstress conditions.

The diverse regulatory effects of HSP101 on flowering-related genes could yield two kinds of effects through flowering pathways on flowering time. First, for example, the effects from individual genes could conduct a contrary effect within a pathway, such as displayed in the photoperiod pathway by *CCA1* and *TOC1* (Figure 4; Ito et al., 2008; Lu et al., 2012); second, the effects of individual genes could play a role in the same direction within a pathway, such as shown within the age pathway by *MIR156A* and *MIR172B* (Figure 4; Wu et al., 2009). The effects together could be incorporated into integrators of the flowering network (Figure 5) and eventually promote flowering. The effects of gene expression alternation on flowering time were tested at the physiological level (Figure 7 and Supplemental Table S2). Furthermore, the effects of the dominant contributors regulated by HSP101 (*SVP* and *FLC*) in terms of flowering time were strongly demonstrated by both transcriptional and genetic data (Figure 4, Figure 6, and Supplemental Table S1). Notably, we investigated any potential physical interactions between HSP101 and SVP, between HSP101 and FLC, and between HSP101 and FCA (the upstream regulator of *FLC*) with both yeast two-hybrid and bimolecular fluorescence complementation assays but found no interaction (Supplemental Figure S6). This suggests that additional efforts are necessary to explore this interesting but complicated relationship. For example, the exploration of interactions between HSP101 and upstream regulators of *FLC* and *SVP*. Also, there might be direct interactions between HSP101 and gene regulatory regions, which could be explored using Yeast one-hybrid and EMSA assays in further studies.

Materials and methods

Plant material and growth conditions

Arabidopsis (*Arabidopsis thaliana* (L.) Heynh.) plants with mutations in *HSP101* and flowering repressors (*FLC*, *SVP*) were obtained from the Arabidopsis Biological Resource Center (ABRC) and the research groups of Dr. Jinyong Hu and Dr. Xiangyang Hu. The Arabidopsis ecotype Columbia (Col-0) was used as the wild type in this study. The mutant *flc-3* was in the Col-FRI background, and other mutants were in the Col-0 background. The *HSP101* T-DNA insertion mutants *hot1-1* (SALK_066374) and *hot1-3* (CS16284) and the other mutants used have been previously described (Hong and Vierling, 2001; Michaels and Amasino, 1999; Hartmann et al., 2000; Zhang et al., 2010). The *flc-31* mutant was isolated from the progeny of a cross between *flc-3* (in Col-*FRI*) and wild type Col-0. The double mutants *flc-31/hot1-1* and *flc-31/hot1-3* were isolated from the progeny of crosses between *hot1-1*, *hot1-3*, and *flc-31*, and the double mutants *svp-41/hot1-1* and *svp-41/hot1-3* were isolated from the progeny of crosses between *hot1-1*, *hot1-3*, and *svp-41*. Homozygosity was determined by PCR using a combination of gene-specific primers and a T-DNA-specific primer (Supplemental Table S5).

Seeds of Col-0, the mutants, and the transgenic lines were sown in soil under controlled long days (LDs; 16 h light/8 h dark), moderate days (MDs; 12 h light/12 h dark), or short days (SDs; 8 h light/16 h dark) and then placed in a controlled growth chamber maintained at 22°C and 60% relative humidity. For physiological experiments, different conditions are described below. The seeds were stratified for 2 d at 4°C before sowing, and the plants were grown on Murashige and Skoog media to investigate the subcellular localization of HSP101.

Generation of transgenic and rescued lines

The 2736 bp coding sequence region of *HSP101* (At1g74310) was amplified from the genomic cDNA of 2-week-old Arabidopsis Col-0 plants using Phanta® HS Super-Fidelity DNA Polymerase (Vazyme, Nanjing, China). The PCR fragment was digested with *Eco*RI and *Bam*HI and then inserted into a pEGAD vector to generate a *35S::HSP101-GFP* construct (Cutler et al., 2000). The construct was subsequently transformed into *Agrobacterium tumefaciens* strain *GV3101*, which was then infiltrated into Col-0 plants and *HSP101* loss-of-function mutants using the floral dip method, and Basta-tolerant plants were selected using a previously described method (Clough and Bent, 1998). Homozygous T_3

transgenic plants were used for subsequent experiments. The primer sequences used for vector construction are listed in Supplemental Table S5.

RNA extraction and gene expression analysis

Two-week-old seedlings grown in soil were sampled, and a HiPure Plant RNA Mini Kit (Magen, Shanghai, China) was used to isolate total RNA. The cDNA templates were synthesized by reverse transcription of 1 μg of total RNA quantified by a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) using HiScript II Q Select RT SuperMix for qPCR (+gDNA wiper; Vazyme, Nanjing, China).

Reverse transcription-quantitative PCR (RT-qPCR) amplifications were monitored using EvaGreen 2X qPCR MasterMix-Low ROX (ABM, Richmond, BC, Canada). The presence of a single PCR product was verified by dissociation analysis in all amplifications. The comparative threshold cycle method ($\Delta\Delta C_t$) was used to calculate the relative amount of gene expression, which was normalized using the C_t values derived for the *ACTIN2* (*ACT2*) gene. All quantifications were undertaken for three biological replicates, with three technical replicates each. The primers used in this experiment are listed in Supplemental Table S5.

Physiological experiments and measurements of flowering time

Arabidopsis plants of different genotypes (*35S::HSP101-GFP*, Col-0, and *hot1-1*) were grown in soil. The flowering time was recorded after a series of physiological treatments. For the photoperiod treatment, seedlings were grown under LDs, MDs, and SDs, and for the light intensity treatment, seedlings were grown under different light intensities—60, 80, or 100 μmol m^{-2} s^{-1}. For the vernalization treatment, 1-week-old seedlings were grown at 4°C for 4 weeks and then returned to 22°C. For the GA_3 treatment, 2-week-old seedlings were sprayed with 50 μM GA_3 twice per week until flowering. For the ambient temperature treatment, 10-d-old seedlings were grown at 16, 22, or 27°C until flowering, and for the sucrose treatment, 2-week-old seedlings were irrigated with 25 mM sucrose solution twice per week until flowering. The flowering time was represented by the number of rosette leaves when the first flower opened. The numbers of rosette leaves recorded are listed in Supplemental Table S2.

Genetic hybridization experiments

Parental plants for hybridization were grown, and the genotypes were reconfirmed before crossing. Two to four unopened buds on the female parental plants were selected, and the stamens were removed. The remaining flowers, fruit, and inflorescence apex were removed so that only the emasculated buds were retained. Male parental plants were selected, and flowers that were fully open with mature pollen grains were chosen. Pollen from the male parental plants was lightly placed on the stigma of the emasculated buds. Following stigma pollination, after a certain period (approximately 10 hours), the stigmas generally begin to wither, and the siliques begin to develop after 3 d. Mature siliques were harvested individually after they turned yellow. Homozygous F_3 double mutant plants were used for subsequent experiments. The numbers of rosette leaves at the onset of flowering are listed in Supplemental Table S1.

Protoplast extraction and subcellular localization

For subcellular localization of the HSP101-GFP fusion protein, protoplasts were isolated from 2-week-old homozygous *35S::HSP101-GFP* Arabidopsis seedlings as described previously (Yoo et al., 2007) and then mounted onto a glass slide. The protoplasts were stained with Hoechst 33342 nuclear dye as a reference for nuclear localization before the green fluorescent protein (GFP) signal was observed with a 60 × oil objective by Confocal Laser Scanning Microscopy using an Olympus FV 1000 system equipped with argon as an excitation source. GFP fluorescence was excited at 488 nm and collected with a 520-550 nm filter, Hoechst 33342 was excited at 405 nm and collected with a 460-480 nm filter, chlorophyll autofluorescence was excited at 633 nm and collected with a 650-670 nm filter.

In addition, GFP signals were also observed in the abaxial leaf epidermis of *Nicotiana benthamiana* leaves injected with *Agrobacterium tumefaciens* strain *GV3101* transformed with the 35S::HSP101-GFP fusion protein, using the same method as mentioned above. At least three independent experiments were conducted, and representative patterns were observed.

Statistical analysis

For gene expression analysis and flowering time measurement, the significance of differences between genotypes was evaluated statistically with IBM SPSS Statistics 19 software using analysis of variance, followed by Fisher's least significant difference (LSD) test, at the 0.01 probability level.

Accession numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers:*HSP101* (AT1G74310), *CCA1* (AT2G46830), *TOC1* (AT2G46830), *CO* (AT5G15840), *VRN1* (AT3G18990), *FLC* (AT5G10140), *FLM* (AT1G77080), *SVP* (AT2G22540), *GID1A* (AT3G05120), *GID1B* (AT3G63010), *GID1C* (AT5G27320), *MIR156A* (AT2G25095), *MIR156C* (AT4G31877), *MIR172B* (AT5G04275), *FCA* (AT4G16280), *FVE* (AT2G19520), *LD* (AT4G02560), *FLD* (AT3G10390), *ELF7* (AT1G79730), *PIE1* (AT3G12810), *CLF* (AT2G23380), *FT* (AT1G65480), *SOC1* (AT2G45660), *LFY* (AT5G61850), *AGL24* (AT4G24540) and *ACT2* (AT3G18780)

Supplemental data

The following materials are available in the online version of this article.

Supplemental Figure S1. Homozygosity of *hot1-1* and *hot1-3* mutants.

Supplemental Figure S2. Phenotypes of *35S::HSP101-GFP*, wild-type (Col-0), and *hot1-1* plants grown under long days.

Supplemental Figure S3. A repeat of relative transcript levels of flowering time-related genes presented in Figure 4.

Supplemental Figure S4. Rhythmic expression of *CCA1* and *TOC1* in Arabidopsis Col-0 and *hot1-1* mutant seedlings.

Supplemental Figure S5. A repeat of relative transcript levels of floral integrators presented in Figure 5.

Supplemental Figure S6. No interactions were found between HSP101 and FCA, FLC, SVP in YEAST and *Nicotiana benthamiana*.

Supplemental Table S1. Number of rosette leaves of single and double mutants at flowering under long days.

Supplemental Table S2. Number of rosette leaves at flowering under different physiological treatments under moderate days.

Supplemental Table S3. Detailed changes of rosette leaf numbers at flowering under different physiological treatments.

Supplemental Table S4. Subcellular localization of several proteins encoded by flowering-related genes.

Supplemental Table S5. List of primers used.

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Abbreviations

HSP101	Heat shock protein 101
CCA1	Circadian clock associated1
TOC1	Timing of cab expression1
CO	Constans
VRN1	Vernalization1
FLC	Flowering locus C
FLM	Flowering locus M
SVP	Short vegetative phase
GID1A	GA insensitive dwarf1a
GID1B	GA insensitive dwarf1b
GID1C	GA insensitive dwarf1c
MIR156A	MicroRNA156A
MIR156C	MicroRNA156C
MIR172B	MicroRNA172B
FCA	Flowering control locus A
FVE	Flowering locus VE
LD	Luminidependens
FLD	Flowering locus D
ELF7	Early flowering7
PIE1	Photoperiod-independent early flowering1
CLF	Curly leaf
FT	Flowering locus T
SOC1	Suppressor of overexpression of CO1
LFY	Leafy
AGL24	Agamous-like24
GFP	Green fluorescent protein
GA	Gibberellin
LDs	Long days
MDs	Moderate days
SDs	Short days

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