Arabidopsis AGAMOUS-LIKE16 and SUPPRESSOR OF CONSTANS1 regulate genome-wide expression and flowering time

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Short title: AGL16-SOC1 collaborate to time flowering

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Author contributions

J-Y H and X D conceptualized and coordinated the research; L-P Z performed the ChIP and transient expression experiments and collected the RNA samples with help from X-D J; Y-H T, J-Y H, and D-M Y carried out the protein interaction assays; X D, J-Y H and L-P Z created the genetic materials and did the genetic analyses; X D analyzed and visualized all the data; Y-H T, F C, Y-X D, X-D J, and F-M Q did the other analyses; J-Y H and X D wrote the paper with help from the other authors. All authors read and approved the manuscript.

Abstract

Flowering transition is tightly coordinated by complex gene regulatory networks, in which AGAMOUS-LIKE 16 (AGL16) plays important roles. Here, we identified the molecular function and binding properties of AGL16 and demonstrated its partial dependency on SUPPRESSOR OF CONSTANS 1 (SOC1) function in regulating flowering. AGL16 bound to promoters of more than 2000 genes via CArG-box motifs with high similarity to that of SOC1 in Arabidopsis (*Arabidopsis thaliana*). Approximately seventy flowering genes involved in multiple pathways were potential targets of AGL16. AGL16 formed a protein complex with SOC1 and shared a common set of targets. Intriguingly, only a limited number of genes were differentially expressed in the *agl16-1* loss-of-function mutant. However, in the *soc1-2* knockout background, AGL16 repressed and activated the expression of 375 and 182 genes, respectively, with more than a quarter bound by AGL16. Corroborating these findings, AGL16 repressed the flowering time more strongly in *soc1-2* than in the Col-0 background. These data identify a partial inter-dependency between AGL16 and SOC1 in regulating genome-wide gene expression and flowering time, while AGL16 provides a feedback regulation on *SOC1* expression. Our study sheds light on the complex background dependency of AGL16 in flowering regulation, thus providing additional insights into the molecular coordination of development and environmental adaptation.

Introduction

Timely transitions from vegetative to reproductive growth (floral transition) and from dormant to germinating seeds determine the capacity of plant adaptation to changing environments, thus are under tight control by complex interactions between endogenous signals and exogenous environmental factors (Michaels, 2009; Andres and Coupland, 2012; Nee et al., 2017). The gene-regulatory-network (GRN) controlling floral transition converges at several floral integrator genes like *SUPPRESSOR OF CONSTANS1* (*SOC1*) and *FLOWERING LOCUS T* (*FT*). These genes often encode transcription regulators controlling the transcription of their downstream targets by binding to specific *cis*-motifs, for example CArG-boxes (Michaels, 2009; Fornara et al., 2010; Andres and Coupland, 2012). CArG-box motifs are binding sites specific for MADS-box transcription factors (TFs) like SOC1, FLOWERING LOCUS C (FLC), SHORT VEGETATIVE PHASE (SVP) and SEPALLATA 3 (SEP3) (Immink et al., 2009; Kaufmann et al., 2009; Kaufmann et al., 2010; Deng et al., 2011; Immink et al., 2012; Tao et al., 2012; Gregis et al., 2013; Mateos et al., 2015; Mateos et al., 2017; Aerts et al., 2018). These MADS-box TFs often form homo- and/or hetero- protein complexes that act in concert and bind to the CArG-box motifs in promoters of more than hundreds of downstream genes to regulate flowering time and other developmental processes in Arabidopsis (*Arabidopsis thaliana*).

SOC1 is one key flowering promoter integrating signals from photoperiod, temperature, hormones and age-related pathways (Lee and Lee, 2010). SOC1 forms protein complex with AGL24 to activate *LFY* and *AP1* to initiate and maintain flower meristem identity but represses *SEP3* to prevent premature differentiation of floral meristem (Lee et al., 2008). SOC1 activates the expression of *TARGET OF FLC AND SVP1* (*TFS1*) via recruiting histone demethylase RELATED TO EARLY FLOWERING 6 (REF6) and chromatin remodeler BRAHMA (BRM), and cooperates with SQUAMOSAL PROMOTER BINDING PROTEIN-LIKE 15 (SPL15) to modulate their targets expression thereby regulating flowering time (Hyun et al., 2016; Richter et al., 2019).

SOC1 forms a set of heterologous complexes with other MADS-box TFs (de Folter et al., 2005; Immink et al., 2009). Furthermore, *SOC1* times flowering downstream of several hormone signaling pathways including GA, ABA and BRs (Jung et al., 2012; Li et al., 2017; Hwang et al., 2019) and of nutrient status (Liu et al., 2013; Olas et al., 2019; Yan et al., 2021). Interestingly, profiling of SOC1 targets also identifies genes involved in the signaling processes of these hormones and nutrients (Immink et al., 2012; Tao et al., 2012). Via protein complexing with many TFs and binding to its own promoter, SOC1 regulates its own expression with auto-regulatory feedback loops (Immink et al., 2012). However, the biological importance of these molecular interactions remains to be explored further.

AGL16 is a floral repressor with dependency on genetic background, photoperiod of growth conditions, and gene dosages (Hu et al., 2014). Only under the inductive long-day conditions loss-of-function mutants for *AGL16* show early flowering especially in the functional *FRI-FLC* background (Johanson et al., 2000; Michaels and Amasino, 2001; Hu et al., 2014). *AGL16* expression can be modulated by the level of the Brassicaceae-specific *miR824*, for which natural variation has been identified (Rajagopalan et al., 2006; Fahlgren et al., 2007; Kutter et al., 2007; de Meaux et al., 2008; Hu et al., 2014). Change in *miR824* expression results in a significant modification of the plant flowering (Hu et al., 2014). *AGL16* acts in flowering time regulation via transcriptional regulation of *FT*, whose expression is also regulated by TFs like SVP and FLC and many others (Aukerman and Sakai, 2003; Searle et al., 2006; Jung et al., 2007; Castillejo and Pelaz, 2008; Li et al., 2008; Mathieu et al., 2009). AGL16 forms complexes with SVP and FLC, and mildly represses their expression (Hu et al., 2014). *AGL16* is a direct downstream target of both FLC and SVP, but the expression of *AGL16* changes only weakly in loss-of-function mutants of both genes (Deng et al., 2011; Gregis et al., 2013; Mateos et al., 2015). Yeast-two-hybrids assays suggest that AGL16 interacts with SOC1 and other MADS-box TFs and is hypothesized to modulate the *SOC1* expression (de Folter et al., 2005; Immink et al., 2009; Immink et al., 2012). Besides its roles in development (Kutter et al., 2007; Hu et al., 2014), AGL16 represses plant responses to salt stress and drought resistance via

binding to a specific set of downstream genes (Zhao et al., 2020; Zhao et al., 2021). The *miR824-AGL16* module participates also in heat stress adaptation (Szaker et al., 2019). However, the exact AGL16 target spectra and the impacts of interactions between AGL16 and its partners remain under-explored.

In this study, we profiled the target spectra of AGL16 and demonstrated the molecular and genetic links between AGL16 and SOC1 played important roles in flowering time regulation. We found that, in contrast to its weak effects in flowering time regulation in Col-0 background, AGL16 could bind to more than 2000 target genes that were involved in regulation of flowering time and many other biological processes. We showed that the regulatory roles of AGL16 on genome-wide gene expression and flowering time depended partially on the SOC1 activity.

Results

AGL16 binds to a large set of genomic segments with CArG boxes

We profiled AGL16 binding sites by a ChIP-seq approach (chromatin immuno-precipitation followed by sequencing). We used a line expressing *AGL16* fused to a combined Yellow Fluorescent Protein (YFP) -HA epitope tag under the control of the Cauliflower Mosaic Virus 35S promoter (*AGL16OX*), which restores the early flowering of *agl16-1* to wild type Col-0 level (Fig. S1) (Hu et al., 2014). In two independent trials, we identified respectively 5463 and 3294 DNA segments statistically enriched for AGL16 binding, of which 3086 were shared (Table S1, S2). Most of the peaks were around 150-500 bp in both trials (Fig. S2). To test whether these segments were real binding sites for AGL16, we carried out ChIP-qPCR assays with two independent chromatin preparations for 20 peaks identified by ChIP-seq. These efforts confirmed 12 regions bound by AGL16-YFP-HA with a minimum two-fold enrichment in the *AGL16OX* line compared to *agl16-1* background (Fig. 1). Besides these, four known targets including *HKT1;1* (AT4G10310), *HsfA6a* (AT5G43840),

148 *MYB102* (AT4G21440), and *CYP707A3* (AT5G45340) were also among the
149 list (Zhao et al., 2020; Zhao et al., 2021). Hence, a majority proportion of peaks
150 detected via ChIP-seq method were reproducibly enriched.

151 Peaks bound by AGL16 were annotated using Arabidopsis TAIR10 data to
152 identify their distribution and genomic features (Fig. 2). The peaks from both
153 trials were centered to the 3 Kb regions around transcriptional start sites (TSS;
154 Fig. 2B). Around 60% of peaks located in the 1 Kb regions surrounding TSS
155 (Fig. 2C; Table S2). About 10% of peaks were in the 1-2 kb promoter regions
156 upstream of TSS, while 10-12% of peaks were in exons/introns. Thus, AGL16
157 bound to DNA fragments close to TSS of a large set of genes. The 2339 genes
158 with peaks mapped to gene body or up to 2 Kb upstream of their TSS were
159 taken as AGL16 targets (Table S2).

160 We next searched for potential cis-motifs in the common peaks bound by
161 AGL16 using HOMER, which could predict new motifs and identify known
162 motifs (Heinz et al., 2010). This analysis reported a *de novo* CArG-box motif
163 CCATTTTTGG for AGL16 in 707 peaks (24.2% of all common peaks; *Fisher*
164 *P*=1e-340, in comparison to 3.8% at genome level; Fig. 2D, Table S2). Ten
165 other CArG-box motifs were also significantly enriched, and matched to the
166 known motifs of SVP, SOC1, SEP3, TAGL1, AGL63, and other MADS-box TFs,
167 most of which could potentially interact with AGL16 (Fig. 2D; Fig. S3; Table S2).
168 The *de novo* and the ten significantly enriched CArG-box motifs were all
169 distributed around the peak center, indicating that AGL16 bound to its targets
170 via the cluster of CArG-box motifs, just like SOC1 and other MADS-box
171 proteins (Deng et al., 2011; Immink et al., 2012; Tao et al., 2012). There were
172 also other motifs significantly enriched in the AGL16 bound peaks, such as
173 those bound by TCPs (321 peaks), bHLHs (1131), C2C2 DOFs (2524),
174 WRKYs (1039). However, these motifs were not in the peaks center. Since
175 AGL16 modulated significantly the flowering time in *Arabidopsis* (Hu et al.,
176 2014), we next asked which flowering time genes could be targeted by AGL16.

177 **AGL16 targets flowering time genes in multiple pathways**

The Arabidopsis genome contains ~400 flowering time genes, among which around 70 were targeted by AGL16 (Fig. 3; Table S2). This number was significantly larger than randomly expected (*Yates' Chi-square test*, p<0.0001). Consistent with the described photoperiod dependency for *AGL16*-mediated flowering regulation (Hu et al., 2014), 37 genes (for example *AGAMOUS LIKE 15/16/18* (*AGL15/AGL16/AGL18*), *CONSTANS LIKE 1/3/4/5* (*COL1/3/4/5*), *TWIN SISTER OF FT* (*TSF*) and *MOTHER OF FT* (*MFT*), etc.) were related to photoperiod and circadian clock pathways (Bouche et al., 2016). Ten genes (like *AGL19* and *SVP*, etc.) were in the vernalization and ambient temperature pathway, seven genes were involved in the gibberellin acid (GA) pathway, and nine genes are integrators or related to meristem response and developmental process. Four genes bound by AGL16 were not clearly defined for the flowering pathways (Boxall et al., 2005; Xiao et al., 2009; Zhao et al., 2011). Taken together, AGL16 might impact several flowering pathways, and the alteration of flowering time in mutants of *AGL16* could be a net effect of multiple flowering pathways.

AGL16 binds to *SOC1* promoter and modifies its expression

The floral integrator gene *SOC1* was one of the targets bound by AGL16 (Fig. 4A; Table S2). AGL16 interacted with three DNA segments (peaks 1389, 1390 and 1391) in the promoter region of *SOC1* that harbored several CArG-motifs，consistent with a previous observation that the whole intergenic region is required for proper SOC1 expression (Hepworth et al., 2002). Peak 1390 overlapped with a region bound by SOC1 itself (SOC1 binding region 1) (Tao et al., 2012), while peak 1389 overlapped with regions previously shown to be targeted by SVP (Tao et al., 2012; Mateos et al., 2015) or FLC (Deng et al., 2011; Mateos et al., 2015). An independent ChIP-qPCR assay confirmed AGL16 binding on all three peaks with the binding on peaks 1389 and 1391 relatively stronger than on peak 1390 (Fig. 4B). The second segment bound by SOC1 itself (SOC1 binding region 2 or fragment 7) was not targeted by AGL16. As AGL16 forms protein complexes with SVP and FLC (Hu et al., 2014), it is likely that AGL16 binds target regions together with these MADS-box TFs.

However, *SOC1* transcription was weakly upregulated by loss-of-function of *AGL16* in both Col-0 (Fig. 4C) and Col-*FRI* backgrounds (Fig. S4), a pattern likely caused by the very complex regulation of *SOC1* expression (Hepworth et al., 2002; Immink et al., 2012). A transient luciferase assay with the 1.7 Kb SOC1 promoter in *N. benthamiana* leaves demonstrated that the co-transfection of AGL16 significantly repressed the *pSOC1* expression compared to the co-transfection control with an empty vector (Fig. 4D; S5). In contrast, when the CArG box(es) bound by MADS-box TFs were mutated (m3, m456, and m3456), *pSOC1* expression increased remarkedly, and this increase became even more prominent when AGL16 was co-transfected additionally. These data together suggest that AGL16 represses directly the *SOC1* expression.

AGL16 forms protein complex with SOC1

AGL16 dimers likely with SOC1 (Fig. S3) (de Folter et al., 2005; Immink et al., 2009). We verified this interaction with Yeast-2-Hybrid (Y2H) and bimolecular fluorescence complementation (BiFC) techniques. Y2H assays confirmed interactions between SOC1 and AGL16 (Fig. 5A), which was as strong as the previously reported direct interaction between AGL16 and SVP, in clear contrast to the negative interaction between AGL16 and LHP1 (Hu et al., 2014). BiFC assays by fusing the N-terminal half of yellow fluorescent protein (nYFP) with AGL16 (*35S:AGL16-nYFP*) and the C-terminal of YFP with SOC1 (*35S:SOC1-cYFP*) detected an interaction of AGL16 with SOC1 in the nuclei of *Agrobacterium*-infiltrated *Nicotiana benthamiana* leaves (Fig. 5B). A further co-immunoprecipitation (co-IP) assay with AGL16 fused to a YFP tag and SOC1 fused with a Flag tag in Arabidopsis cells also confirmed the physical interaction between these two TFs (Fig. 5C). Hence AGL16 and SOC1 form hetero-protein-complexes.

AGL16 and SOC1 co-target a common set of genes

We next examined whether AGL16 and SOC1 had common targets. For this

aim, the previously generated binding profiles for SOC1 were used to identify shared targets with AGL16 (Immink et al., 2012; Tao et al., 2012). We applied the same annotation procedure for both AGL16 and SOC1 binding profiles in order to identify common genes. There were 193 AGL16-bound segments that overlapped with 240 SOC1 peaks (Table S3). These peaks were in the +/-2 Kb vicinity of 223 genes (five without annotation information), which were then taken as AGL16 and SOC1 common targets (Fig. 6A). Most of these common peaks were in the 1 kb region surrounding TSS with AGL16 peaks a bit more proximal (Fig. 6B). We further identified 211 CArG-box motifs in 144 common peaks (400 bp surrounding peak centers; 74.6% of all overlapped peaks) with MEME-ChIP. Eighty-seven peaks harbored one CArG-box (DCCAAAAAWGGAAAR; 60.4%), while the rest featured two (49 or 34%) or three (6 peaks or 4.2%) or more (2 peaks; Fig. S6A). The distances between the CArG-box motifs were spaced with 20-40 bases (Fig. S6B). Among these common targets, genes involved in floral organ development (or reproductive growth) and responses to hormone stimulus including ethylene and ABA were significantly enriched (Fig. 6C; Table S3). Eight genes of the photoperiod and circadian clock related pathways (*AGL15*, *AGL18*, *ATC*, *PHYA*, *RAV2*, *SMZ*, *SNZ* and *TOE3*), three genes of the temperature-related pathways (*CBF1*, *CBF2* and *SVP*), and *SOC1* itself were involved in flowering (Fig. 3), suggesting that AGL16 and SOC1 act likely together to time floral transition in *Arabidopsis*.

AGL16 regulates genome-wide gene expression depending partially on SOC1 function

We next determined to what extent the gene expression at the genome-wide level could be affected by the AGL16-SOC1 module (Table S1). For this, we carried out a comparative transcriptomics analysis using the single and double mutants between the *agl16-1* and *soc1-2* lines. In contrast to the very broad binding spectrum of AGL16, we only detected very small number of genes showing differential expression (DEGs) in *agl16-1* single mutant (9 up and 12 down) compared to Col-0 (Fig. 7A; Table S4). The *soc1-2* single (155 up and 285 down) and the *agl16 soc1* double (49 up and 353 down) mutants had

similar number of DEGs but *soc1-2* featured more up and less down DEGs (*Yate's chi-square test*, p<0.001; Fig. 7A), indicating that AGL16 either countered SOC1's repressive or inductive role on gene expression. A heatmap analysis of DEGs in the *soc1-2* vs Col-0 revealed that absence of *agl16* mostly reverted the differential gene expression observed in *soc1-2* to wild type levels (Fig. 7B). Genes down-regulated in the *agl16 soc1* mutants showed also down-regulation in *soc1-2* (Fig. 7C). In contrast, genes up-regulated in *agl16 soc1* were barely affected by either single mutation, suggesting that for these genes, AGL16 and SOC1 contribute redundantly to the repression. Accordingly, only 83 *soc1-2* DEGs (in total 155 up and 285 down; ~18.9%) overlapped with the *agl16 soc1* DEGs (375 up and 182 down; ~14.9%; Fig. 7D). Therefore, AGL16 has potential in regulating gene expression at the genome-wide level, but apparently depends on its genetic background, i.e. here the *SOC1* activity.

We next examined to what extent these DEGs associated with AGL16 targeting. Among the 557 *agl16 soc1* DEGs, AGL16 bound to 98 genes (~22.2%), in which only 23 (~4.1%) were also targeted by SOC1 (Fig. 7D). About 13.6% or 60 *soc1-2* DEGs were likely the AGL16 targets (*Yate's chi-square test*, p=2e-8, in comparison to genome-wide level of AGL16 binding). However, we noticed that only nine *soc1-2* DEGs (~2% among 440) were potential targets of SOC1, a pattern similar to a previous report, in which 52 SOC1 targets were among the 1186 DEGs (Tao et al., 2012). There were six targets (~28.6%) showing differential expression in the 21 *agl16-1* DEGs. Moreover, we identified more than a quarter of up-regulated DEGs in the *agl16 soc1* line (77 among 286) being AGL16 targets in contrast to about 13.3% of up-regulated DEGs in the *soc1-2* mutant (29 among 218; Yate's chi-square test, p=0.0035; Fig. 7E). Among the 67 up-regulated DEGs shared between the *soc1-2* and *agl16 soc1* mutants, 18 (26.9%) were potentially AGL16 targets. In contrast, less than 8% of down-regulated DEGs in both mutants were targeted by AGL16. Together, these data suggest that AGL16 may act mainly as a transcriptional repressor and exert an antagonistic role against SOC1 regulation on target gene expression.

AGL16-SOC1 module is important for flowering time regulation

Among the DEGs between *agl16 soc1* and *soc1-2* plants, we identified 17 known genes involved in floral regulation with seven being targeted by AGL16 (*NF-YA2*, *TCP21*, *RHL41*, *AGL16*, three *AP2-like* genes *RAV1*, *RAV2/TEM2*, and *SNZ*; Fig. 7F; Table S4). Expression of *FT* was significantly enhanced in the *agl16 soc1* double mutant. In line with this, the double mutant *agl16 soc1* flowered significantly earlier (~20 rosette leaves) than the *soc1-2* single mutant (~25.6 rosette leaves; about 21.6% reduction in rosette leaf number) but still later than both *agl16-1* (~11.1 rosette leaves; ~13.6% reduction) and wild type Col-0 (~12.9 rosette leaves) plants (Fig. 8). This indicated that *AGL16* could counteract the *SOC1* regulation on flowering, while the regulatory role of *AGL16* in floral transition depends on *SOC1*, a pattern like the genetic dependency of *AGL16* on *FLC* (Hu et al., 2014).

Discussion

AGL16 acts in the hubs of GRN related to various biological processes

The MADS-box TF AGL16 is an important regulator in flowering time (Hu et al., 2014), stomata development (Kutter et al., 2007), heat stress adaptation (Szaker et al., 2019), drought resistance (Zhao et al., 2020) and salt stress adaptation (Zhao et al., 2021), suggesting that it might have very broad spectra of downstream targets. In this study, indeed, our ChIP-seq assay demonstrated that AGL16 could target more than 2000 genes featuring characteristic CArG-box motifs (Figs. 1-3). These genes were involved not only plant development but also various hormone signaling processes (Table S2) including some targets in the ABA signaling pathway that previously identified (Zhao et al., 2020; Zhao et al., 2021). These broad spectra are not rare, however, especially for MADS-box TFs. Two such examples would be SVP and SEP3, both of which can bind to thousands of downstream targets involved in a very broad set of biological processes (Kaufmann et al., 2009; Gregis et al., 2013; Mateos et al., 2015). Intriguingly, both SVP and SEP3 can

or potentially form hetero-protein-complexes with AGL16 (de Folter et al., 2005; Hu et al., 2014), indicating that they may work together to fine-tune plant developmental programs in responding to ever-changing environments, a hypothesis awaits for further investigation.

Interestingly, expression of AGL16 responds to ABA treatment as well as multiple stresses (Szaker et al., 2019; Zhao et al., 2021), thus revealing a very complex role of AGL16 (and its potential partners) in abiotic adaptation. Since both AGL16 and SOC1 play important roles in stomata development and movement (Kutter et al., 2007; Zhao et al., 2020), it's likely that the hetero-protein-complexes formed between AGL16 and its partners may be the key molecule that functions in these abiotic adaptations. Indeed, both AGL16 and SOC1 can directly bind and regulate the expression of a shared set of genes involved in ABA signaling and abiotic stresses (Fig. 6) (Immink et al., 2012; Tao et al., 2012). Considering the essential roles of ABA in seed dormancy and germination regulation, the fact that several AGL16 targets encode for ABA receptors may invoke us to further examine the regulatory roles of AGL16 and its related protein complexes in seed dormancy and germination (Table S2, S3). Corroborating with this, *AGL16* expression drops substantially during seed germination (Das et al., 2018).

AGL16 regulates multiple floral pathways

AGL16 might exert its regulation potential in several pathways controlling flowering time (Fig. 3). Being congruent with its photoperiod dependency in regulation of flowering time, AGL16 targets 37 genes (including *AGL16* itself) related to photoperiod and circadian clock pathways. Though under the tested environmental conditions *agl16-1* still shows a normal vernalization response (Hu et al., 2014), several genes related to temperature responses are directly targeted by AGL16. FLC, SVP and SOC1 might be partners of AGL16 in this respect as all three proteins target also directly on some of these temperature-related genes (Deng et al., 2011; Immink et al., 2012; Tao et al., 2012; Mateos et al., 2015). The binding of AGL16 may cause both positive and

negative influences on the transcription of these targets (Fig. 7), which encompass both repressors and promoters of the floral transition. Indeed, several of the flowering time genes targeted by AGL16 show an enhanced or decreased expression when *AGL16* activity is modified in the *soc1-2* background (Fig. 3 and 7; Table S3, S4). Therefore, the early flowering phenotypes present in *AGL16* loss-of-function mutants might be a net-effect or balanced regulation on different pathways (Fig. 8) (Hu et al., 2014).

It should be noted that AGL16 also targets and represses the expression of *MYC2*, which is previously claimed to modulate flowering time (Kazan and Manners, 2013; Zhai et al., 2015; Wang et al., 2017; Bao et al., 2019). However, our recent efforts have demonstrated, partially based on the data generated from this study, that the MYC2-family TFs only play very limited roles in timing floral transition, because it's the hidden mutation of *COP1*, not the *MYC* mutations, causing early flowering observed in the original *jin1-2* mutant (Yu et al., 2022). Whether the *AGL16-MYC2* interaction regulates flowering time upon different stress conditions needs to be tested later.

AGL16 and SOC1 collaborate in regulation of genome-wide gene expression

The formation of AGL16-SOC1 complex identifies the collaborative potential in targeting and regulation of genome-wide gene expression like other MADS-box TFs (Fig. 5) (de Folter et al., 2005; Lee et al., 2008; Immink et al., 2009; Kaufmann et al., 2009; Kaufmann et al., 2010; Deng et al., 2011; Immink et al., 2012; Tao et al., 2012; Mateos et al., 2015). AGL16 binds more than 2000 genes, which is in line with its very broad expression in many tissues and organs (Alvarez-Buylla et al., 2000), but affects the expression of a very limited number of genes in the background of Col-0 (Fig. 7). When *SOC1* becomes non-functional (*soc1-2*), AGL16 modulates the expression of more than 550 genes and acts both as a transcriptional repressor and activator. In the *soc1-2* background, AGL16 seems mainly act as a transcriptional repressor as more than a quarter of the up-regulated DEGs, in contrast to the less than 8.5% of

the down-regulated DEGs, are potential targets of AGL16. Hence AGL16's activity in gene expression regulation requires partially SOC1, and corroborating with this, both *AGL16* and *SOC1* expression can be detected in the shoot apex (Corbesier et al., 2007; Immink et al., 2012; Hu et al., 2014). On the other hand, SOC1 also needs *AGL16* as SOC1's repressive activity substantially drops (from 155 to 49 genes) but the promoting activity increases (from 285 to 353 genes) when *AGL16* has no function. Many *soc1-2* DEGs are not differentially expressed any more in *agl16 soc1* mutant (Fig. 7). Indeed, AGL16 and SOC1 co-bind a common set of targets and regulate the expression of many known flowering time genes (Fig. 6, 7). As expected, these two TFs collaborate in regulation of flowering time (Fig. 8). The *agl16 soc1* double mutant flowered significantly earlier than the *soc1-2* single mutant, on the other hand, still later than both *agl16-1* and wild type plants. *AGL16* could counteract *SOC1* effects in flowering time regulation, and vice versa, similar to the genetic dependency of *AGL16* on *FLC* (Hu et al., 2014). It's possible that these TFs may form higher-order protein complexes to regulate downstream genes, for example *FT* expression, which should be tested further.

The identification of three DNA fragments bound by AGL16 in the upstream ~4 Kb intergenic region raises a possibility that the *SOC1* expression regulation might be more complicated than we have expected (Fig. 4) (Hepworth et al., 2002; Immink et al., 2012; Jung et al., 2012; Liu et al., 2013; Li et al., 2017; Hwang et al., 2019; Olas et al., 2019; Yan et al., 2021). Though AGL16 can repress the SOC1 expression in planta and when the frequently used ~1.7 Kb promoter was included in transient assays (Fig. 4), this short fragment may not be enough for full mechanistic understanding the regulation of *SOC1* expression. Whether higher order 3D chromatin loop presents for *SOC1*, like the ones for *FT* and *FLC* (Crevillen et al., 2013; Liu et al., 2014), and whether AGL16 has a role in the loop formation need further investigation.

Together, as a master regulator in GRNs connecting multiple pathways, AGL16's function has a partial inter-dependency with SOC1. AGL16 might act as a glue molecule, like other MADS-box TFs do, to micro-tune the expression of downstream genes at proper stages and environmental conditions (Immink

et al., 2009; Kaufmann et al., 2010; Pajoro et al., 2014; Richter et al., 2019). It will be important to address these further to understand their precise roles and mechanisms in balancing development and environmental adaptation.

Materials and Methods

Plant materials, growth conditions, and phenotype assays

Arabidopsis thaliana plants including wild-type Col-0, *agl16-1*, *35S:AGL16-YFP-HA* in *agl16-1* background, Col-*FRI*, *agl16-1* Col-*FRI*, and *m3* have been described previously (Kutter et al., 2007; Hu et al., 2014). The *soc1-2* mutant in Col-0 background (Torti et al., 2012) was kindly provided by Prof. George Coupland. To test the genetic interactions between *AGL16* and *SOC1*, *agl16-1* and *soc1-2* were crossed and double mutant was screened with gene-specific primers (Table S5) (Kutter et al., 2007; Torti et al., 2012; Hu et al., 2014).

Arabidopsis seeds were stratified in distilled water at 4°C for 72 h and sown in soil and grown under LD conditions (16-h light at 21°C and 8-h night at 18°C). Seedlings for phenotyping were planted either in growth rooms or chambers, while materials for gene expression analysis and ChIP assays were sown on Murashige and Skoog medium plates (Hu et al., 2014).

Flowering time assays were carried out according to previous report (Hu et al., 2014). Four independent trials were applied and each gave similar pattern. Phenotype comparisons were performed with Student's *t-test* with *Bonferroni-correction*.

RNA Isolation, RT-qPCR, and RNA-seq assays

Total RNA was extracted with TRI Reagent® (Molecular Research Center, Inc. Cincinati, USA). Ten days old seedlings were used for quantification of relative expression of selected genes with *PP2A* as reference (Hu et al., 2014).

Reverse transcription was carried out with the HiScript® II Q RT SuperMix for RT-qPCR (+gDNA wiper) and quantification PCRs were performed with ChamQ™ SYBR qPCR Master Mix (both from Vazyme Biotech co. ltd, Nanjing) on QuantStudio™ 7 Flex Real-Time PCR System (ThermoFisher). Three to four biological replicates from each of two to three independent trials were applied for each experiment. A similar protocol was developed for monitoring relative enrichment of DNA fragments in ChIP-qPCR experiments. All the primers used in this study are included in Table S1.

For RNA-seq, materials were collected from three independent biological replicates for each genotype, and DNA-free total RNA was generated as described above. Illumina True-seq library preparation was performed from 3 μg DNA-free total RNA and sequenced by the Biomarker Technologies Corporation, Beijing, China. Quality trimmed pair-end RNA-seq reads were mapped to the Arabidopsis TAIR10 annotation using the *HISAT2* v2.1.0 (Kim et al., 2019). The *featureCounts* included in *subread* v1.6.4 package was applied to calculate reads counts on each gene (Liao et al., 2013; Liao et al., 2014). *DESeq2* v1.14.1 was used to detect differentially expressed genes (DEGs; fold change above 1.5 and p.adj<0.1). Only uniquely mapped reads were used for downstream analysis. Transcriptional clustering analysis was performed using the *heatmap.2* function in *R*. GO analysis was performed with *PANTHER* in TAIR web-tool (https://www.arabidopsis.org/tools/go_term_enrichment.jsp) or *agriGO* pipeline (Mi et al., 2017; Tian et al., 2017).

ChIP-seq, ChIP-qPCR assays and data analysis

ChIP experiments were carried out following protocols described (Reimer and Turck, 2010; Zhou et al., 2016). Chromatin for both *agl16-1* and *agl16-1 AGL16OX* plants was extracted from ten-day-old seedlings grown under LD conditions at ZT14, and precipitated with antibody against GFP (Abcam, Ab290). For ChIP-seq, the immuno-precipitations from two independent trials were used for NGS library preparation with NEBNext® Ultra™ II DNA Library Prep Kit for Illumina® (E7645, New England BioLabs Inc.) and high-throughput

sequencing with HiSeq2000 platform. ChIP-seq reads were mapped to the TAIR10 assembly of *A. thaliana* using *BWA-MEM* (v0.7.17-r1188) (Li, 2013). Reads with mapping quality below 30 were discarded with *SAMtools* v1.7 (Li et al., 2009). Duplicated reads were removed using *Picard MarkDuplicates* v1.119. The resulted *.bam* file was used as input to call AGL16 enriched regions with MACS v2.2.7.1 (Zhang et al., 2008). Enriched regions were generated by the comparison of immune-precipitated products to input for *AGL16OX* and then compared against *agl16-1*. For annotation of AGL16 targets, the *R* package *ChIPseeker* was used (Yu et al., 2015). The position and strand information of nearest genes were reported with the distance from peak to the TSS of its closest gene identified. As annotations might overlap, we use 'promoter' definition in *ChIPseeker* as the highest priority for annotation. Each binding site was assigned to only one gene. IGV was used for data visualization of the binding profiles for targets (Thorvaldsdottir et al., 2013). Enriched motifs in AGL16 binding peaks were identified using *Homer* suite with *findMotifsGenome.pl* function (Heinz et al., 2010). Motifs in AGL16-SOC1 co-targeted regions were analyzed with *MEME-ChIP* tools (Machanick and Bailey, 2011), and the spacing between primary and secondary motifs was analyzed with *SpaMo* (*spamo -dumpseqs -bin 20 -verbosity 1 -oc spamo_out_1 -bgfile./background -keepprimary -primary DCCAAAAAWGGAAAR*). We compared the AGL16 targets to SOC1 targets from both Immink (2012) and Tao (2012) with the same annotation procedures for AGL16 (Immink et al., 2012; Tao et al., 2012). In an earlier independent trial, we pooled the immuno-precipitations from two biological replicates and sequenced the products. This pooled sequencing results gave similar pattern of AGL16 targets profile but with a lower coverage. *Yate's chi-square test*s were performed online (http://www.quantpsy.org/chisq/chisq.htm). The ~400 flowering time genes were downloaded from https://www.mpipz.mpg.de (Bouche et al., 2016) with self-curations. Reads data for RNA-seq and ChIP-seq experiments were accessible at NCBI under accession code SUB5067038.

Yeast two-hybrid and biomolecular fluorescence complementation (BiFC) experiments

Yeast two-hybrid and the BiFC assays were carried out to test the physical interaction between AGL16 and SOC1 proteins according to previous report (Hu et al., 2014). In yeast two-hybrid assay, interactions between AGL16-SVP and AGL16-AGL16 were applied as positive controls while the AGL16-LHP1, AGL16-BD, SOC1-BD, AD-AGL16, and AD-SOC1 were applied as negative controls together with empty vectors. For BiFC assay in *Nicotiana benthamiana* plants, *35S:SOC1-cYFP* construct was built by cloning the full-length encoding-region without stop codon of *SOC1* (from Col-0) into *pDONR221* entry vector first and later transferred into *RfA-sYFPc-pBatTL-B* vector. The interactions between AGL16 and SVP, between AGL16 and LHP1, were used as positive and negative controls, respectively.

Co-immunoprecipitation (co-IP) assay

To test the AGL16 and SOC1 interactions, coding sequences of *AGL16* and *SOC1* were amplified from the wild type cDNA with Phanta Max Super-Fidelity DNA Polymerase (P505, Vazyme). All sequences were cloned into the *pDONR201* entry vector and verified via Sanger sequencing. The resulting destination vectors containing N-terminal tagged *pENSG-YFP-AGL16* and *pICH47811-SOC1* were used to transfect protoplasts prepared from leaves of wild type seedlings (Yoo et al., 2007). The transfected protoplasts were incubated at room temperature for 16 hr and used for co-IP assays as described previously (Cui et al., 2018). In brief, the protoplasts were lysed in immunoprecipitation buffer (50 mM Tris pH7.5, 150 mM NaCl, 10 % (v/v) glycerol, 2 mM EDTA, 5 mM DTT, protease inhibitor, 0.1% Triton). Lysates were centrifuged at 14000 × *g* for 15 min at 4 °C with aliquots of supernatants as input controls. Immunoprecipitations (IPs) were performed by incubating the supernatants with 15 μL GFP-Trap beads (gta-10, ChromoTek) for 2 h at 4 °C. After centrifugation at 1000 × *g* and washing four times with extraction buffer, beads were eluted with 2× Laemmli loading buffer. The proteins were then separated with SDS-PAGE and analyzed by immuno-blotting with antibodies against GFP (ab290, Abcam) and FLAG (ab49763, Abcam).

Transient transactivation assay

To test the regulatory effects of AGL16 on *SOC1* expression, the coding region of *AGL16* was inserted into the *pOCA30* vector to generate the effector, while the 1.7 Kb promoter and its mutated versions of *SOC1* was fused with a *pZP* vector to generate the reporter constructs (Chen et al., 2021). Equal amounts of the effector and reporter constructs in *Agrobacterium tumefaciens* strain GV3101 were used to co-infiltrate *N. benthamiana* leaves with at least 15 biological replicates that were randomly distributed. After two days of infiltration, the luciferase intensity was collected and quantified with a low-light cooled CCD imaging apparatus. Experiments were triplicated with each containing at least 15 replicates. Relative expression was examined for statistical significance using ANOVA followed by Dunnett's test.

Accession Numbers

Sequence data from this article can be found in the CNSA database (https://db.cngb.org/) under project number CNP0003940.

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Conflict of interest statement

The authors declare no competing interests.

Data and materials availability

All data and materials needed to evaluate the conclusions in the paper are present in the paper and the Supplementary materials.

Figure legends

Fig. 1 Validation of the AGL16 binding on target DNA fragments.

A. Binding profiles for selected target genes. The TAIR10 annotation of the genomic locus was shown at the bottom of each box. For each panel, the profiles for two trials (R1 and R2) in *agl16-1* background line were shown in the upper panel, while the profiles for *agl16-1 35S:AGL16-YFP-HA* (*AGL16OX;* two trials) were shown in the middle panel of each box. All the genes were from 5'-end to 3'-end with scale bars indicating sequence lengths of 500 bp. Note that data range for each gene in *agl16-1* and *AGL16OX* was the same scale, but different genes could have different scale. Red lines marked the binding regions tested via ChIP-qPCR assays (**B**).

B. ChIP-qPCR validation of AGL16 binding on 20 DNA segments. Significant enrichment (red bars) was defined with the following criteria: mean enrichment must be at least two-fold higher than negative control *ACT7*, the enrichment for *AGL16OX* (in *agl16-1* background) than *agl16-1* must be higher than two-fold change, and the amplification C_T number of IP samples must be at least two cycles less than no-antibody controls. This experiment was repeated with another independent trial, which gave similar pattern. Statistics was carried out with *Student's t-test* with Bonferroni correction. ***, P<0.001; **, p<0.01; *, p<0.05.

Fig. 2 Genome-wide identification of AGL16 target genes via ChIP-seq.

A. Venn diagram of AGL16 targets identified in two independent trials.

B. Distribution of AGL16 binding sites for two trials surrounding the

transcriptional starting site (TSS).

C. Location distribution in relative to nearby genes for AGL16 binding sites of trial 1. Peaks within the 3 Kb promoter region were taken as AGL16 targets.

D. CArG type of motifs over-represented in the AGL16 binding peaks. AGL16 new, which was highly similar to known SOC1 type, showed the *de novo* motif predicted for AGL16. Frequency gave the percentage for each motif presented in the binding peaks.

E. Distribution of new (orange) and known (gray; shown in **D**) CArG type of motifs around AGL16 peaks center.

Fig. 3 Molecular pathways (indicated with different color boxes) targeted by AGL16. Genes with names in bold were common targets for AGL16 and SOC1, while those in red were differentially expressed between the *agl16 soc1* and *soc1-2* mutants.

Fig. 4 AGL16 targets *SOC1* and represses its expression.

A. Schematic representation of the *SOC1* locus. Filled bars indicated exons and gray bars marked the 5'- and 3'-UTR regions while the line indicated the non-coding region of *SOC1*. Arrows downward labelled the putative CArG-boxes potentially bound by MADS-box proteins. The dark purple lines indicated the three peaks (P1389, P1390 and P1391) bound by AGL16. Orange, blue and black thick lines marked the known regions targeted by FLC, SVP and SOC1, respectively. Note that two sites in the regulatory region of *SOC1* were bound by itself (SOC1 binding R1 and R2; see ref. Tao et al. 2012). Red lines (1 to 7) showed the regions tested for AGL16-YFP-HA binding on *SOC1* chromatin. Horizontal arrows marked the position of primers used for quantification of CDS regions. The lower panel showed the ChIP-seq profile at *SOC1*.

B. Relative enrichment of AGL16 on *SOC1* chromatin tested with ChIP-qPCR. Mean fold change values with signficant enrichment was labelled above bars together with standard deviation. *ACT7* was taken as a negative enrichment control.

C. Relative expression of *SOC1* against *PP2A* in Col-0 and *agl16-1* plants.

655 Mean relative expression was given with standard deviation and the significant
656 difference was examined using Student's *t*-test.
657 **D**. Quantitative luciferase assay showing that AGL16 regulated the expression
658 of 1.7 Kb promoter of *SOC1* via *cis*-motifs 3,4,5,6 described in **A**. Box plots
659 mark the 25% to 75% quartiles with the line in box representing the median.
660 The lines extending from each box marked the minimum (5%) and maximum
661 (95%) values of the dataset. Circles showed the outliers. WT marked the 1.7
662 Kb promoter without any sequence modification, while the m3 indicated the
663 mutation of a CCW_6GG-box in WT background. The m3456 and m456 showed
664 the relative expression level for the WT promoter with *cis*-motifs 3,4,5,6 and
665 4,5,6 mutated, respectively. At least 15 randomly selected fields each from one
666 individual *N. benthamiana* leaf per treatment were used for measuring with +
667 and – labeling the presence and absence of *35S:AGL16*, respectively.
668 Different letters above the boxes represented the signficant differences among
669 treatments using one-way ANOVA Donnett's test ($P < 0.05$). This experiment
670 was triplicated and each trial gave similar results.
671
672 **Fig. 5 AGL16 forms protein complex with SOC1.**
673 **A.** Yeast two-hybrid assay revealed a direct interaction between AGL16 and
674 SOC1. Each protein was fused to either the activation domain (AD) as prey or
675 the DNA-binding domain (BD) as bait. Serial dilutions (10^0 x to 10^{-3} x) of J69-4A
676 cells containing different construct combinations indicated on the left were
677 grown on control (left) and selective (right) medium. The AGL16-SVP and the
678 AGL16-LHP1/empty vector combinations provided positive and negative
679 controls, respectively.
680 **B**. BiFC assay evidenced the formation of AGL16-SOC1 complex in nucleus of
681 *Nicotiana benthamiana* leaf epidermis. The interaction was tested with
682 constructs *35S:SOC1-cYFP* and *35S:AGL16-nYFP*. A negative interaction
683 between AGL16 and LHP1 and a positive interaction between AGL16 and SVP
684 were tested as well. Bars = 10 μm.
685 **C**. Co-immunoprecipitation (co-IP) assay confirmed the AGL16-SOC1
686 interaction in Arabidopsis protoplast. SOC1 was FLAG-tagged while the
687 AGL16 was fused with a YFP tag. Total protein of the transfected wild type

protoplasts was immuno-precipitated with antibody against GFP (anti-GFP) first, and further analyzed by Western blot using antibody against FLAG (anti-FLAG).

Fig. 6 AGL16 and SOC1 share a common set of target genes involved in multiple functions.

A. Venn diagram showing that 223/171 genes (Immink et al. 2012 / Tao et al. 2012) were co-bound potentially by both AGL16 and SOC1.

B. Binding intensities for AGL16 (red) and SOC1 (blue) peaks surrounding transcription starting sites (TSS). Regions 3kb upstream and downstream of TSS were plotted.

C. Selected significantly-enriched GO terms for the common targets.

Fig. 7 The *AGL16-SOC1* module collaborates on regulation of genome-wide gene expression.

A. The number of differentially expressed genes (DEGs) in three mutants. The exact number of up (red) or down (blue) regulated DEGs were given on each cone.

B and **C**. Heatmaps showing the normalized relative expression of *soc1-2* (**B**) and *agl16 soc1* (**C**) DEGs in all four lines. The boxplots in the middle gave the data distribution pattern for each cluster. Box plots mark the 25% to 75% quartiles with the line in box representing the median. The lines extending from each box marked the minimum (5%) and maximum (95%) values of the dataset. Circles showed the outliers.

D. Venn diagram demonstrating the overlap between DEGs and the AGL16 targets profile.

E. A detailed comparison between the DEGs in soc1-2 and agl16 soc1 mutants with the AGL16 binding profile. Bold numbers in brackets showed the number of DEGs bound by AGL16.

F. A heatmap showing the normalized relative expression of the DEGs related to flowering time regulation in the *soc1-2* and *agl16 soc1* mutants.

Fig. 8 AGL16 and SOC1 regulate additively flowering time.

A. Overall flowering behaviors of LD-growing wild type Col-0, *agl16-1*, *soc1-2* and *agl16 soc1* mutants.

B. Leaf number production upon flowering under LD conditions. Mean rosette (filled bars, RLN) and cauline (open bars, CLN) leaves were shown with standard deviation. Numbers in percentage showed the earlier flowering level of *agl16-1* and *agl16 soc1* comparing to Col-0 and *soc1-2*, respectively. Analyses were triplicated and all had similar patterns. Statistical comparisons were performed with *Wilcoxon rank sum test* in *R*.

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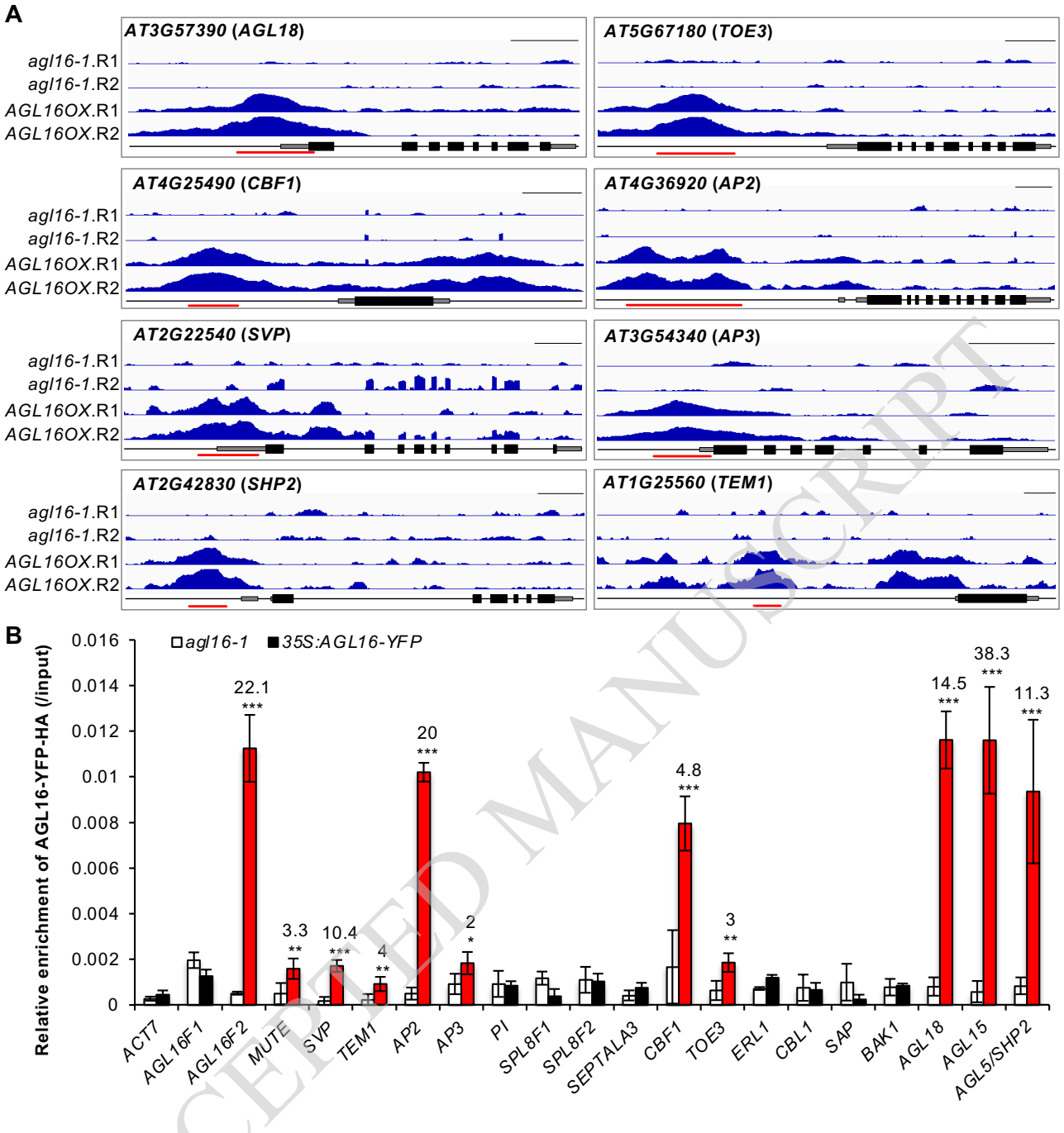

Fig. 1 Validation of the AGL16 binding on target DNA fragments.

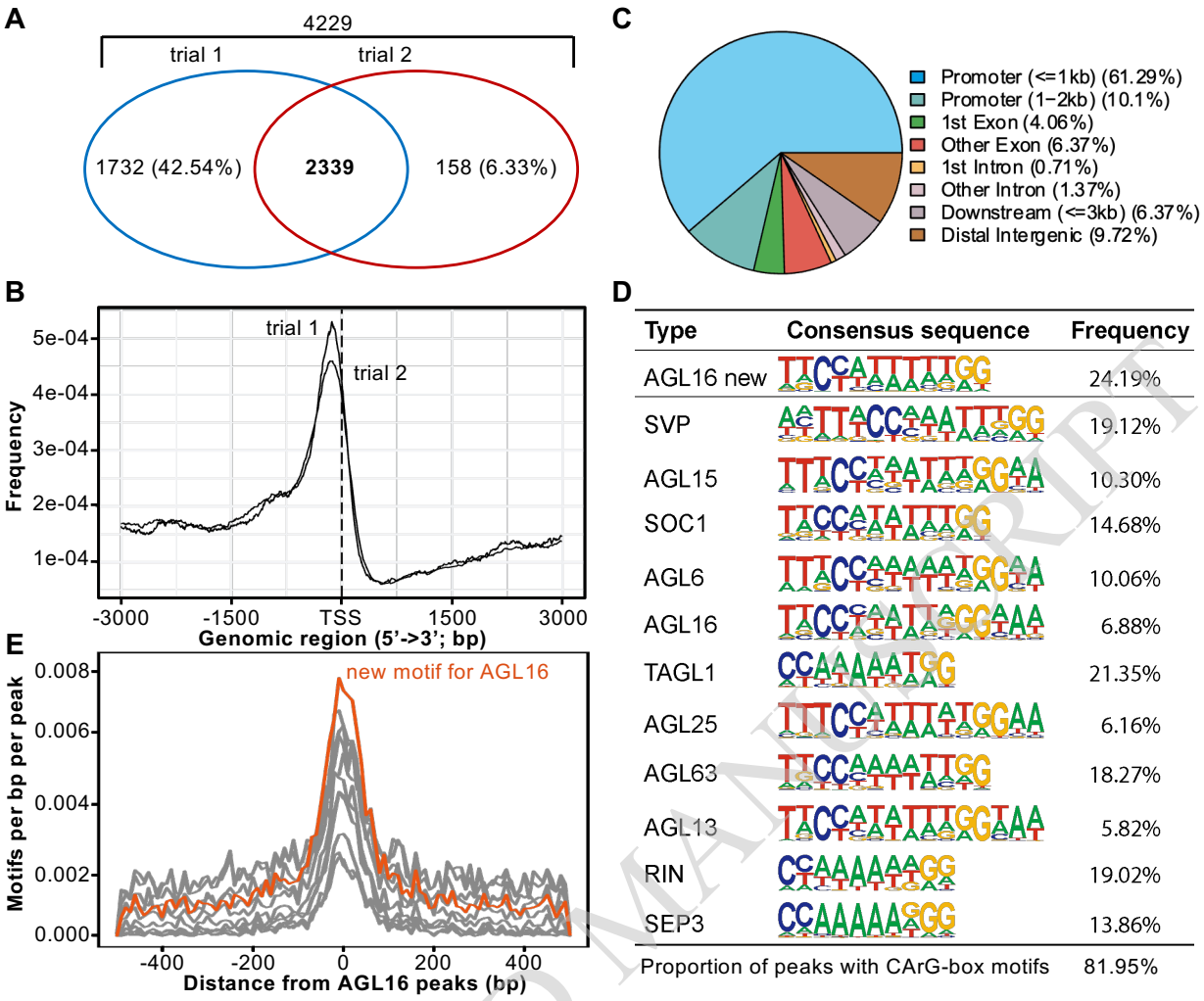

Fig. 2 Genome-wide identification of AGL16 target genes via ChIP-seq.

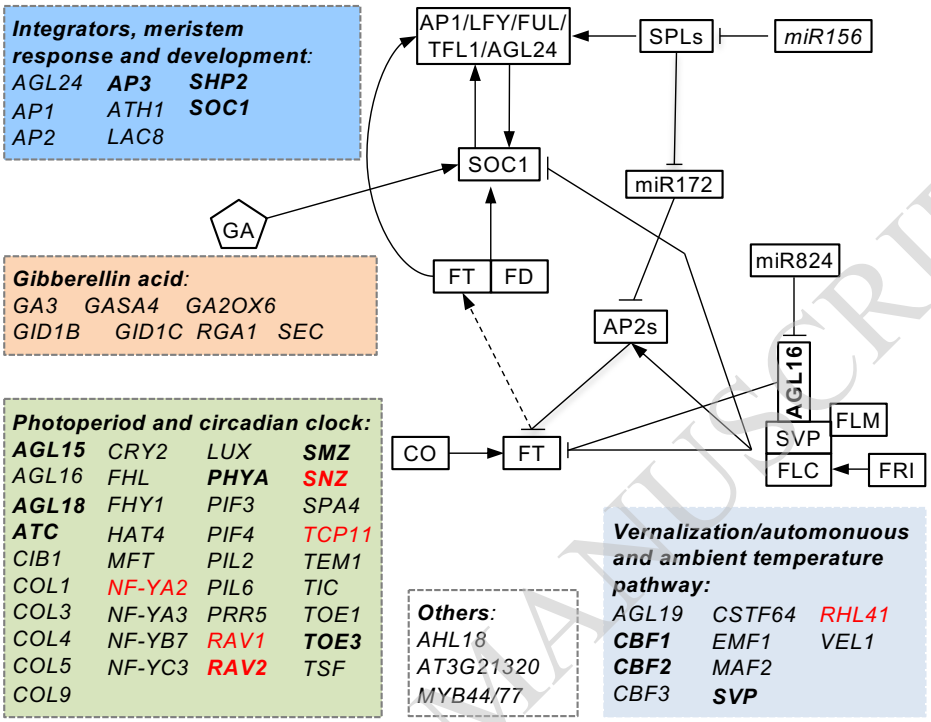

Fig. 3 Molecular pathways (indicated with different color boxes) targeted by AGL16.

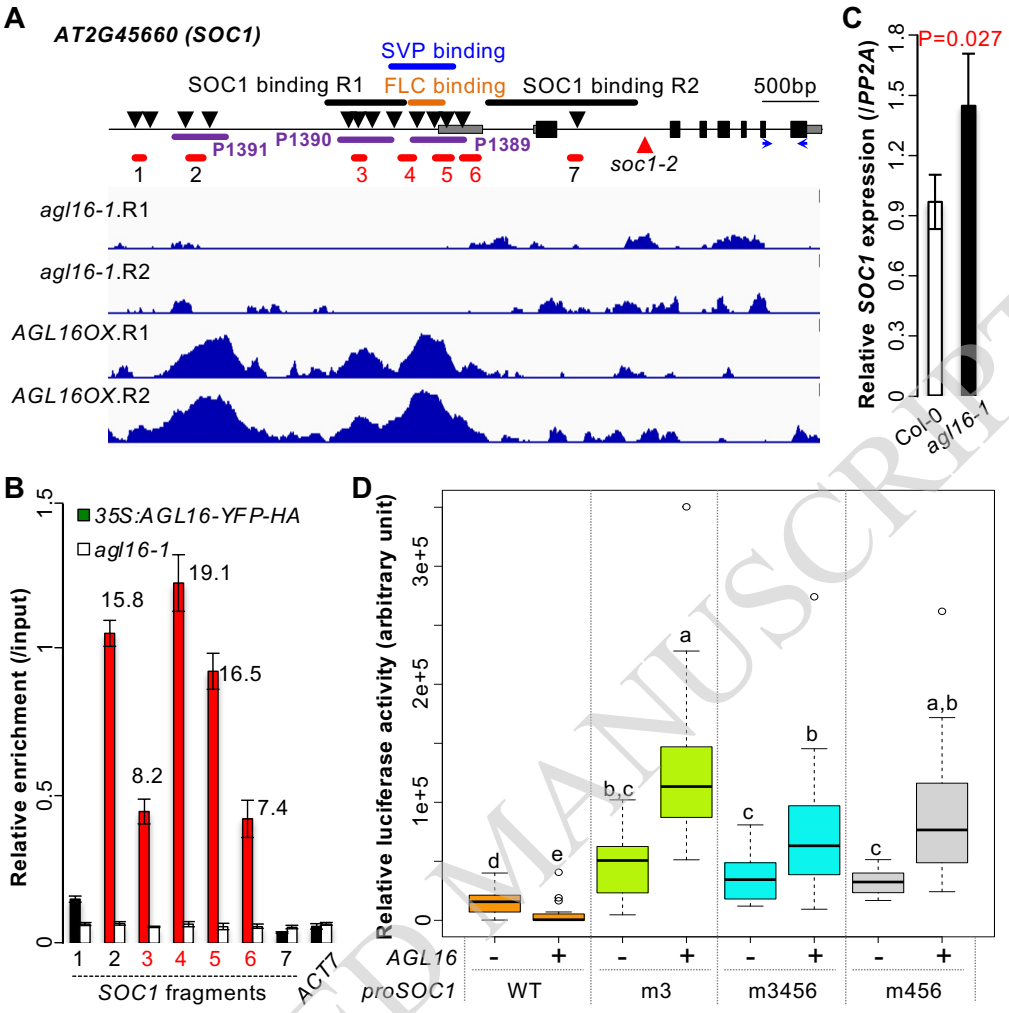

Fig. 4 AGL16 targets *SOC1* and represses its expression.

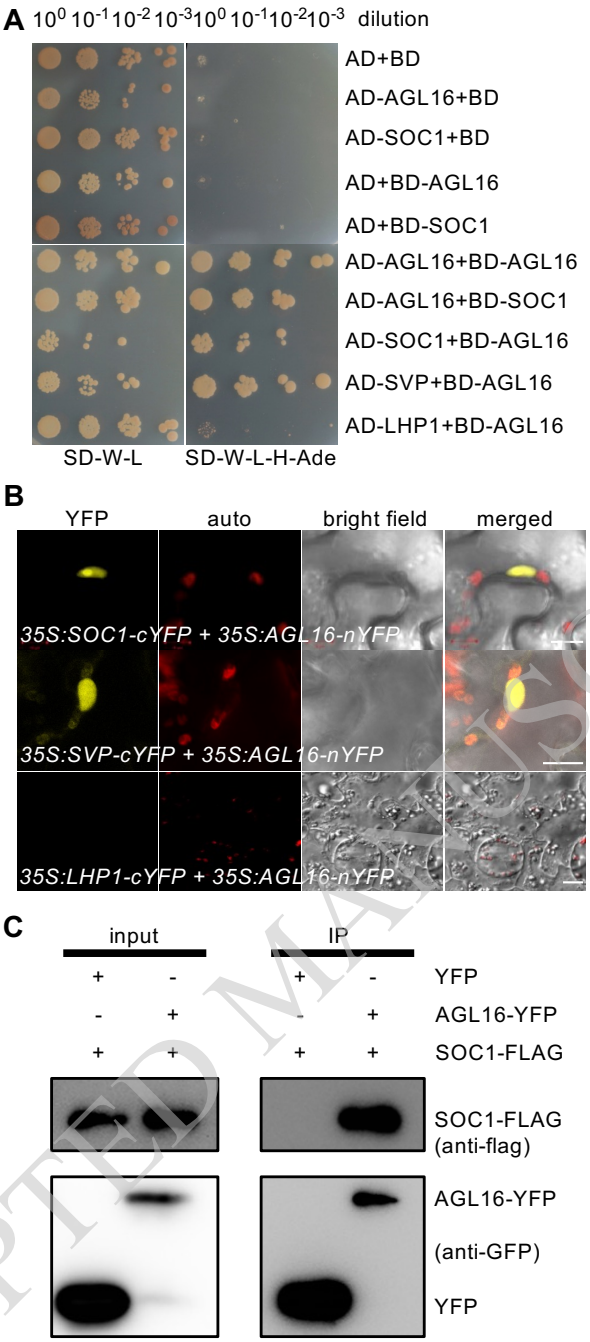

Fig. 5 AGL16 forms protein complex with SOC1.

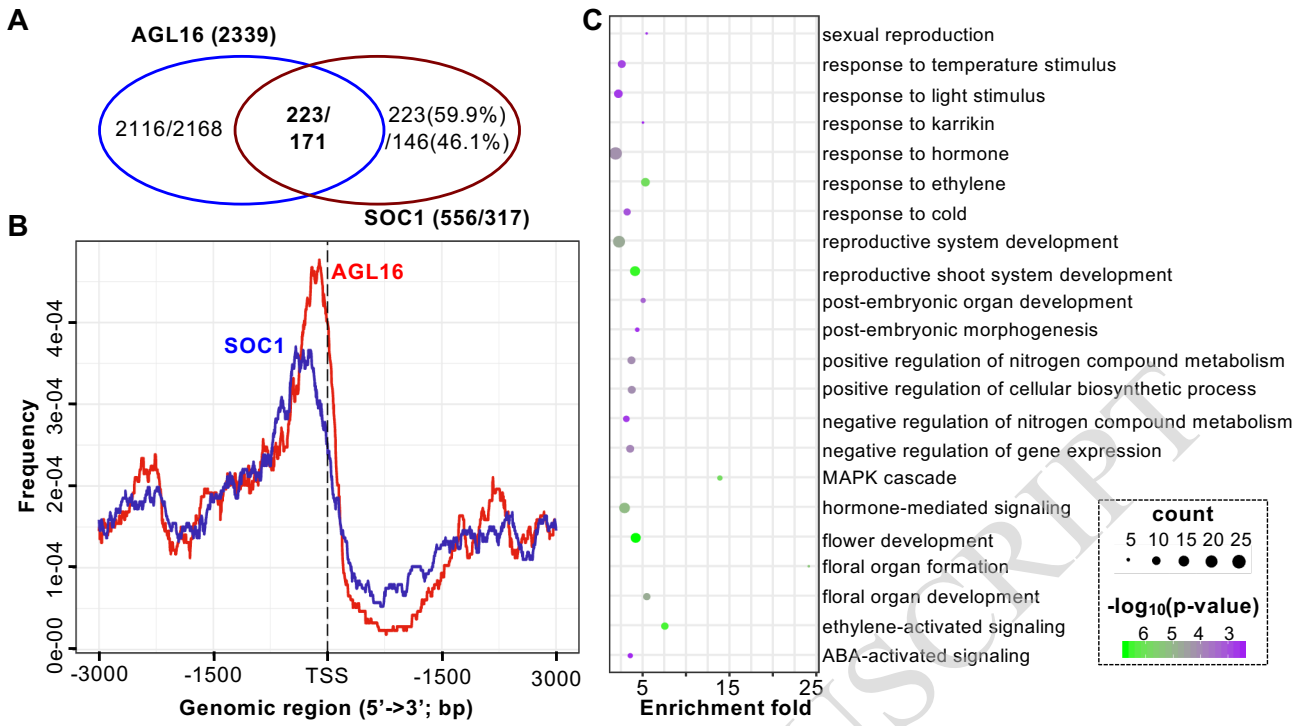

Fig. 6 AGL16 and SOC1 share a common set of target genes involved in multiple functions.

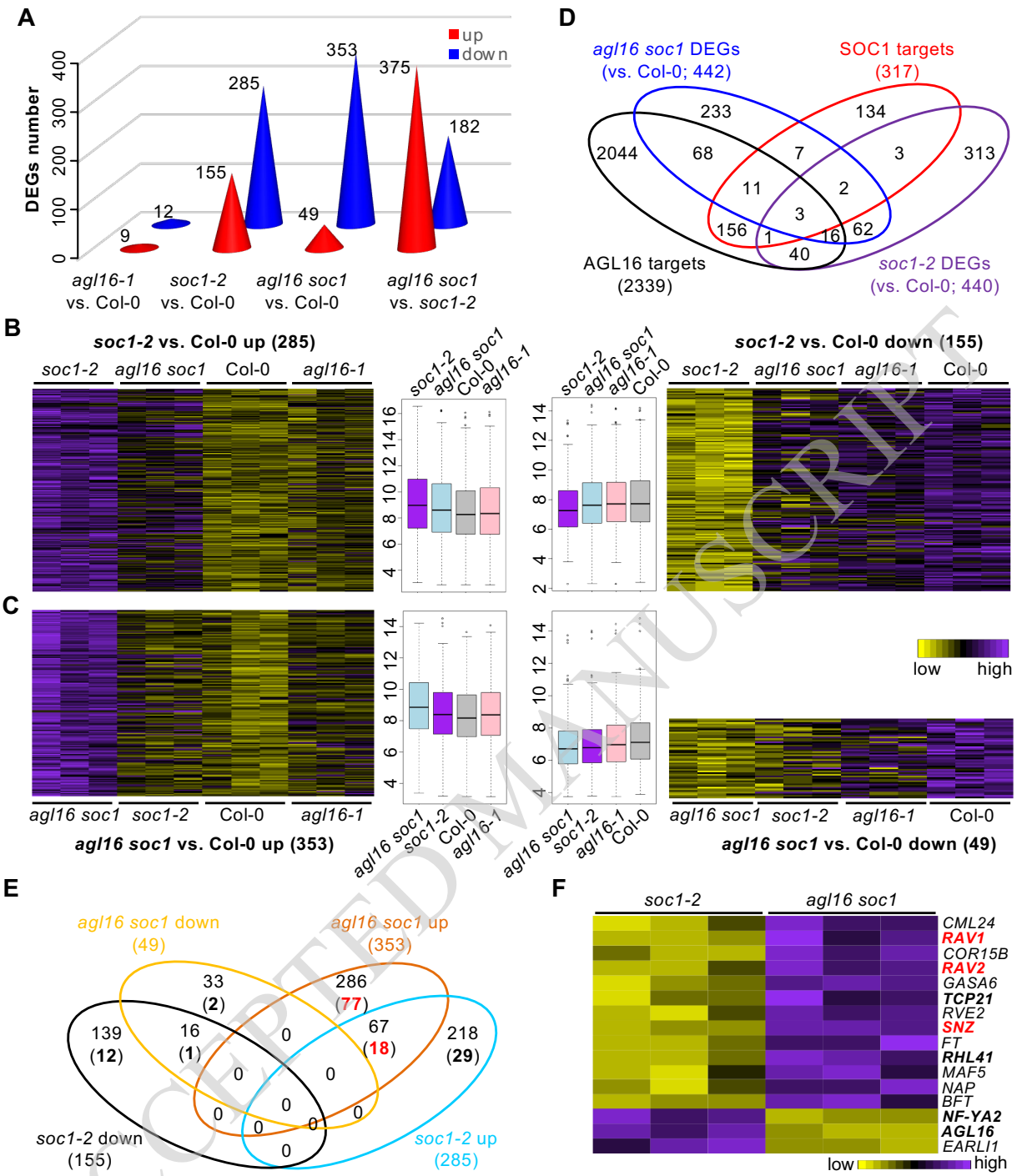

Fig. 7 The *AGL16-SOC1* module collaborates on regulation of genome-wide gene expression.

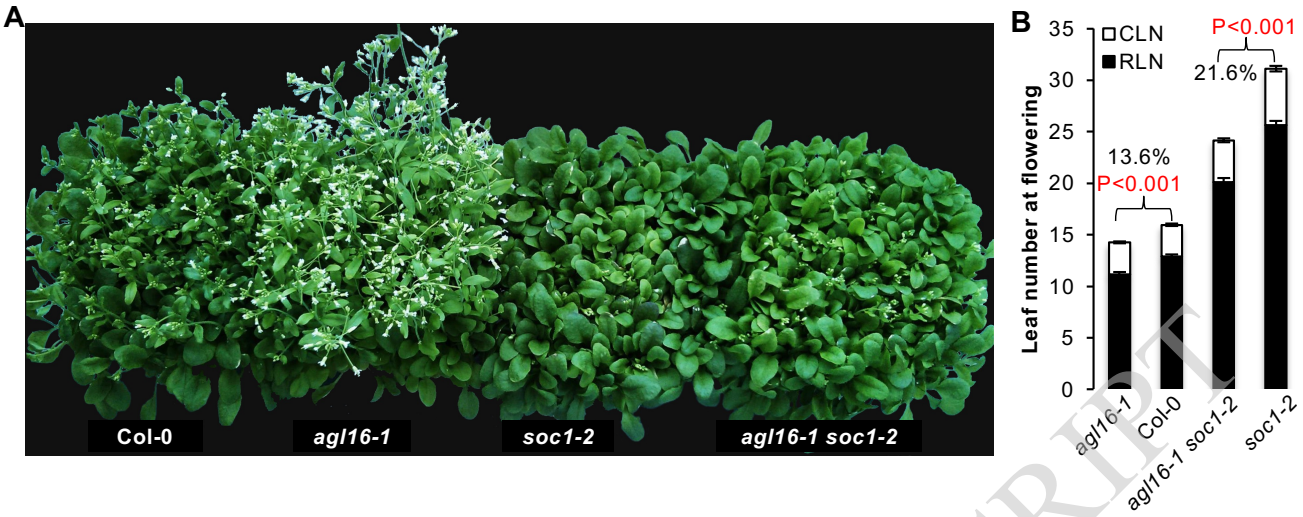

Fig. 8 AGL16 and SOC1 regulate additively flowering time.

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