Overexpression of an Orchid (Dendrobium nobile) SOC1/TM3-Like Ortholog, DnAGL19, in Arabidopsis Regulates HOS1-FT Expression

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Flowering in the appropriate season is critical for successful reproduction in angiosperms. The orchid species, Dendrobium nobile, requires vernalization to achieve flowering in the spring, but the underlying regulatory network has not been identified to date. The MADS-box transcription factor DnAGL19 was previously identified in a study of low-temperature treated D. nobile buds and was suggested to regulate vernalization-induced flowering. In this study, phylogenetic analysis of DnAGL9 and the MADS-box containing proteins showed that DnAGL19 is phylogenetically closely related to the SOC1-like protein from orchid Dendrobium Chao Parya Smile, DOSOC1. The orchid clade closed to but is not included into the SOC1-1/TM3 clades associated with either eudicots or monocots, suggesting that DnAGL19 is an SOC1-1/TM3-like ortholog. DnAGL19 was found to be highly expressed in pseudobulbs, leaves, roots, and axillary buds but rarely in flowers, and to be substantially upregulated in axillary buds by prolonged low-temperature treatments. Overexpression of DnAGL19 in Arabidopsis thaliana resulted in a small but significantly reduced time to bolting, suggesting that flowering time was slightly accelerated under normal growth conditions. Consistent with this, the A. thaliana APETELA1 (AP1) gene was expressed at an earlier stage in transgenic lines than in wild type plants, while the FLOWERING LOCUS T (FT) gene was suppressed, suggesting that altered regulations on these transcription factors caused the weak promotion of flowering. HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE 1 (HOS1) was slightly activated under the same conditions, suggesting that the HOS1-FT module may be involved in the DnAGL19-related network. Under vernalization conditions, FT expression was significantly upregulated, whereas HOS1 expression in the transgenic A. thaliana has a level similar to that in wild type. Taken together, these results suggest that DnAGL19 controls the action of the HOS1-FT module depending on temperature cues, which could contribute to regulation of D. nobile flowering time. These data provide insights into how flowering is fine-tuned in D. nobile to acclimate the plant to seasonal changes in temperature.

Keywords: Dendrobium, flowering, DnAGL19, vernalization, HOS1, FT, SOC1/TM3-like
INTRODUCTION

Proteins containing the conserved MADS-box typically function as transcription factors and are present in a wide range of organisms, from fungi, slime molds, and metazoans to land plants. Putative DNA-binding MADS domains have been found in bacteria (Mushegian and Koonin, 1996; Masiero et al., 2011; Smaczniak et al., 2012). In flowering plants, MADS-box proteins regulate diverse processes, including floral development, root growth, ovule and female gametophyte development, fruit ripening, and dehiscence (Zhang and Forde, 1998; Ng and Yanofsky, 2001; Giovannoni, 2004; Whipple et al., 2004; Colombo et al., 2008; Liu et al., 2009; Voogd et al., 2015). Two classes of MADS-domain proteins have been identified, based on the sequence of the MADS domain, and these have been named type I (SRF-like) and type II (MEF2-like; Masiero et al., 2011; Smaczniak et al., 2012).

SUPPRESSOR OF OVEREXPRESSION OF CO 1 (SOC1) and SOC1/TM3-like proteins are type II MADS-box proteins. They contain the highly conserved MADS-box, K domain, and a SOC1-motif at the C-terminus (Vandenbussche et al., 2003). In Arabidopsis thaliana, these proteins comprise a small subfamily that includes AGL20 (SOC1), AGL19, AGL14, AGL42, AGL71, and AGL72. A. thaliana SOC1 integrates the signals from photoperiodism, prolonged low-temperatures (vernalization), and the gibberellin and autonomous pathways, and controls the expression of the LEAFY (LFY) gene to promote floral initiation (Liu et al., 2008; Lee and Lee, 2010). It is also involved in prevention of a perennial-type lifestyle (Melzer et al., 2008), and plays a role in crosstalk between cold sensing and flowering (Seo et al., 2009). AGL19 also acts as a flowering activator, functioning in vernalization-related floral transition (Schönrock et al., 2006), while AGL42, AGL71, and AGL72 promote floral transition in axillary meristems (Dorca-Fornell et al., 2011). AGL14 was recently demonstrated to be involved in both shoot apical meristem transition and in the regulation of the AP1 and TERMINAL FLOWER 1 (TFL1) genes in A. thaliana (Perez-Ruiz et al., 2015). SOC1/TM3-like genes have been identified in a wide range of plant species, such as the TrcMADS1 gene from Trillium camtschatcense (Trilliaceae) (Nakamura et al., 2005), the GhSOC1 and the GhSOC2 gene from Gerbera hybridia (Ruokolainen, 2011), the PsSOC1 gene from Paeonia suffruticosa (tree peony) (Zhang et al., 2014) and the DOSOC1 gene from the orchid Dendrobium Chao Parya Smile (Ding et al., 2013). Most SOC1/TM3-like proteins play roles during the phase change from vegetative to reproductive development. For example, the rice SOC1 ortholog, OsMADS50, promotes flowering when overexpressed in either A. thaliana or rice, while loss of function of OsMADS50 in rice causes delayed flowering under long-day photoperiod (LD) conditions (Tadese et al., 2003; Lee et al., 2004; Ryu et al., 2009). In addition to flowering regulation, SOC1/TM3-like orthologs can play roles in other biological processes. For example, combined mutations at SOC1 and FUL loci in A. thaliana were reported to result in renewed growth at sites with dead cauline leaves, suggesting that SOC1 promotes the maintenance of an annual life-style (Melzer et al., 2008). In another study, constitutive expression in Gerbera of the A. thaliana AGL71 and AGL72 ortholog, GhSOC1, did not affect flowering time but led to partial loss of floral organ identity (Ruokolainen, 2011). In addition, FvSOC1 from the perennial short-day plant, woodland strawberry (Fragaria vesca) was shown to promote vegetative growth but not flowering (Mouhu et al., 2013).

The SOC1 gene regulatory network (GRN) has been extensively studied in A. thaliana, where SOC1 is regulated by prolonged low-temperature (vernalization) through the FLOWERING LOCUS C (FLC)-dependent pathway and transcription of SOC1 is enhanced due to the suppression of FLC gene (Lee and Lee, 2010). SOC1 expression is also up-regulated by the zinc finger transcription factor CONSTANS (CO; Lee and Lee, 2010). LFY is a downstream target of SOC1 (Liu et al., 2008; Lee and Lee, 2010), and is activated by the SOC1-AGL24 complex to up-regulate the expression of the API gene. The SOC1 protein not only directly targets to its own gene, but also to those of other flowering time regulators, some of which act upstream of SOC1 (Immink et al., 2012). For example, SOC1 physically interacts with API to form a higher order complex and suppress its own transcription (Immink et al., 2012).

In addition to controlling flowering, SOC1 also regulates other processes, such as the activation CBF or COR genes upon cold stress, suggesting that SOC1 serves as a hub for both cold-induced responses and floral development (Seo et al., 2009). It is known that cold affects flowering and that vernalization accelerates the flowering of A. thaliana and other temperate species (Sung and Amasino, 2005; Kim et al., 2009). In contrast, low ambient temperature (e.g., 16°C) or intermittent cold exposure (e.g., 4°C for 6 h/day) delays flowering (Kim et al., 2004; Seo et al., 2009). This has been associated with a ubiquitin E3 ligase protein, encoded by HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE 1 (HOS1), which mediates the degradation of CO during cold conditions, resulting in transcriptional suppression of FT (Jung et al., 2012; Lazaro et al., 2012). HOS1 can also act as a competitor to dissociate the histone deacetylase HDA6 from MULTICOPY SUPPRESSOR OF IRA1 4 protein (MS14, also known as FVE), thereby releasing FLC from transcriptional inhibition (Jung et al., 2013). The loss-of-function HOS1 mutant, hos1-1, flowers early even under normal conditions, and some floral regulators, such as FT, are expressed at higher levels in this mutant, demonstrating that the HOS1-FT module contributes to flowering regulation (Ishitani et al., 1998; Jung et al., 2012; Lazaro et al., 2012).

Dendrobium is a genus in the Orchidaceae family with more than 1,200 species (Adam, 2011), many of which are valued for their use in herbal medicine and ornamental gardening. Cold temperatures have different effects on flowering in different Dendrobium orchids. Generally, the Nobile-type Dendrobium can adapt to long-term low-temperatures in winter and bloom along the cane at each node in spring (Siam Orchid Culture Co., Ltd.), while the Cane-type Dendrobium species bloom in autumn before the weather turns cold. SOC1/TM3-like genes were identified from Dendrobium orchids previously. DOSOC1 is a SOC1 ortholog from Dendrobium Chao Parya

1http://www.siamnobile.com/aboutnobile.html
Smile. It is predominantly expressed in reproductive organs and promotes flowering when overexpressed in either *A. thaliana* or *Dendrobium* Chao Parya Smile. Additionally, *DOSOC1* affects flower morphogenesis by abolishing the development of flower buds in transgenic *Dendrobium* Chao Parya Smile plants (Ding et al., 2013). *DnAGL19* was identified from the Noble-type orchid *Dendrobium nobile* Lindl. It is closely related to members of *A. thaliana* SOC1/TM3-like subfamily and was proposed to function in flowering regulation network by acting upstream of the *D. nobile* API ortholog(s) under prolonged low temperature (Liang et al., 2012). It is not clear by now whether and how these orchid SOC1/TM3-like proteins play roles in cold acclimation and the cold-associated flowering control. In this current study, we investigated the roles of *DnAGL19* in flowering regulation under normal growth condition and in response to vernalization, aiming at exploring the genetic link between *DnAGL19* and other components of the regulatory network and the mechanism by which *DnAGL19* controls flowering.

**MATERIALS AND METHODS**

**Plant Material and Growth Conditions**

*Dendrobium nobile* plants were grown in a greenhouse under natural conditions without regulation of photoperiod at the Orchid Research Center at South China Normal University. For vernalization treatments, adult plants were exposed to low temperatures for 0, 5, 10, 20, or 30 days at 16°C/10°C (day/night). Axillary buds were collected from each node of the pseudobulbs and pooled. Each independent biological sample was comprised of axillary buds from four to five plants and at least three biological replicates for each treatment were analyzed. Untreated control plant material was collected in parallel. Samples for detection of organ specific expression were collected from control plant material was collected in parallel. Samples for measurement of Flowering Time

**RT-PCR and Real-Time Quantitative PCR**

Total RNA was extracted from *D. nobile* and *A. thaliana* seedlings using the E.A.N.A. Plant RNA Kit (OMEGA BIO-TEK, USA) and reversely transcribed to generate cDNA using the PrimeScript™ Reverse Transcriptase (TaKaRa, Dalian, China) according to the manufacturer's instructions. RT-PCR was used to detect organ specific expression patterns and real-time quantitative PCR was used for a time-course analysis of expression following vernalization in *D. nobile*. *D. nobile* 18S rRNA was used as a reference control. Gene-specific primer pairs are shown in Supplementary Table S1. The qPCR reactions and thermal program are as described below.

Real-time qPCR was used to quantify the expression levels of flowering associated genes in transgenic *A. thaliana*. A 25 µl qPCR reaction contains cDNA that reversely transcribed from 25 ng total RNA, specific primers with final concentration of 200 nM for each and 12.5 µl THUNDERBIRD SYBR qPCR Mix (TOYOBO, Japan) that contains the Taq DNA polymerase and the florescent dye SYBR Green. Real-time qPCR was run in an ABI PRISM 7500 system (Applied Biosystems, USA) following the program of a pre-denaturation for 1 min at 95°C and 40 cycles of 15 s at 95°C and 45 s at 60°C. Specific primers are listed in Supplementary Table S1. Relative expression levels for a given gene under the indicated condition were calculated using the $2^{-\Delta\Delta Ct}$ method by normalizing to the *A. thaliana ACTIN2/7* gene and calibrating to the wild type sample. Three biological samples and triplicate qPCR reactions for each combination of primers and sample were analyzed.

**Sequence Analysis**

Sequences used in the alignment and phylogenetic analysis were retrieved from the databases at Phytozome v10.22 or NCBI3, based on BlastP searches using the deduced peptide sequence of *DnAGL19* as a query. Databases incorporating

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2http://phytozome.jgi.doe.gov/pz/portal.html

3http://www.ncbi.nlm.nih.gov/
of DnAGL19 species by BlastP searches using the deduced amino acid sequence homologs, protein sequences were retrieved from a wide range of elucidate the evolutionary relationship between DnAGL19 and its WebLoGo5 was used to generate the consensus sequences of the we identified one protein sequence from C. reinhardtii a unique MADS domain at the N-terminus, nineteen protein related to the A. lyrata a total of 22 species were searched at Phytozome v10.2, including: (1) the dicotyledons A. lyrata (Aly), A. thaliana (At), Aquilegia coerulea Goldsmith (Aquca), Boechera stricta (Bostr), Brassica rapa FPsc (Brara), Capsella grandiflora (Cagra), Capsella rubella (Carubv), Cucumis sativus (Cusca), Medicago truncatula (Medtr), Citrus sinensis (Orange), Solanum tuberosum (PGSC), Populus trichocarpa (Potri), Theobroma cacao (Thecc) and Eutrema salsugineum (Thhalv); (2) the monocotyledonous grasses Orzya sativa (Os), Brachypodium distachyon (Bradi), Zea mays (GRMZM), Panicum virgatum (Pavir) and Sorghum bicolor (Sobic); (3) the Pteridophyta species Selaginella moellendorffii; (4) the moss Physcomitrella patens; and (5) the Chlorophyte Chlamydomonas reinhardtii. The phylogenetic relationships of all mentioned species are shown in Supplementary Figure S1 (adopted from Phytozome v10.2). The DOSOC1 (AGK07583) sequence was retrieved from NCBI. Alignment and phylogenetic analysis, as well as tree editing, was carried out using MEGA6 (Tamura et al., 2013) or ClustalX2 (Larkin et al., 2007). Conserved sites in the aligned sequences, defined as those with more than 70% similarity, were highlighted using BOXSHADER 3.214 and WebLoGo6 was used to generate the consensus sequences of the MADS-box, K-domain, and the SOC1-motif.

RESULTS

DnAGL19 Encodes a SOC1/TM3-Like Ortholog

We previously proposed that DnAGL19 encodes a protein closely related to the A. thaliana AGL19 (Liang et al., 2012). To further elucidate the evolutionary relationship between DnAGL19 and its homologs, protein sequences were retrieved from a wide range of species by BlastP searches using the deduced amino acid sequence of DnAGL19 as a query and an E-value cut-off of <10\(^{-45}\). This yielded a total of 151 sequences from nineteen representative angiosperm species. Initially, no homologs were found for the Pteridophyta species S. moellendorffii, the moss P. patens and the Chlorophyte C. reinhardtii; however, when less stringent criteria were used to re-search the databases (only the amino acids 1–70 of the MADS domain of DnAGL19 and an E-value of <10\(^{-10}\)) we identified one protein sequence from C. reinhardtii containing a unique MADS domain at the N-terminus, nineteen protein sequences from P. patens and ten from S. moellendorffii. The top matched entries from these three species were used for further analysis.

The complete sequences of DnAGL19, DOSOC1, and all 154 homologs were aligned and subjected to a phylogenetic analysis. Using the MADS-containing protein sequence from C. reinhardtii as the out-group, a neighbor joining (NJ) tree was constructed using 1,000 bootstrap replications (Supplementary Figure S2). This indicated that the P. patens and S. moellendorffii sequences, as well as the angiosperm homologs of OsMADS6, a protein similar to A. thaliana AGL6, clustered in a clade that was independent of the SOC1/TM3-like clade (Supplementary Figure S2), suggesting that the SOC1/TM3-like proteins from all the tested angiosperm species originated from a common ancestor and are closely related to the AGL6-like proteins.

Another NJ tree was generated with the sequences from the same species removed if they grouped together with bootstrap value of >99% in the preliminary tree. This simplified tree had a similar topological structure to the preliminary tree, and we then focused on the subtree containing the SOC1/TM3-like subfamily (Figure 1). Three angiosperm SOC1/TM3-like protein branches were clearly recognized, with the orchid SOC1/TM3-like proteins, DnAGL19 and DOSOC1, clustered together into a branch that was independent from both the eudicot and the monocot branches (Figure 1). An alignment of DnAGL19 and DOSOC1 with orthologous angiosperm proteins showed that DnAGL19 contains the conserved MADS-box, K-domain, and SOC1-1 motif, with overall identities of 78, 59, and 73% to the consensus sequences, respectively (Supplementary Figure S3). In addition, amino acid substitutions were observed between DnAGL19 and DOSOC1 at two positions within the MADS-box (R5K, E40D), three within the K-domain (A103V, I131L, Q133E) and thirteen scattered in the intervals between the conserved domains (Figure 2, Supplementary Table S3). All substitutions within the MADS and K domains of DnAGL19 were rarely presented on the sequences of the tested SOC1/TM3-like proteins (Figure 2, Supplementary Table S3). For example, the Arg at the fifth position from the N-end of MADS-box in DnAGL19 was only shared with ~9% of the tested SOC1/TM3-like proteins. Variations were also found at the final three positions of the SOC-motif. In comparing with the consensus sequence, the DnAGL19 had an insertion of “Asp (D)” before the highly conserved “Gly (G)” at the antepenult position and a deletion of the conserved “Pro (P)” at the final position in the SOC1-motif, while DOSOC1 showed the “Trp (W)” substitue for the “Leu (L)” at the penultimate position (Figure 2). As a result, “DGL” was presented at the final three positions of this motif on DnAGL19 instead of “GWP” on DOSOC1 (Figure 2D).

DnAGL19 Expression In Planta

To determine the DnAGL19 expression pattern in D. nobile, total RNA was extracted from various organs, including roots, pseudobulbs, leaves, flowers, and axillary buds. RT-PCR was performed using 18S rRNA as the endogenous control, and DnAGL19 expressed at higher levels in roots, pseudobulbs, leaves and axillary buds than in flowers (Figure 3A). DnAGL19 transcript levels were also evaluated in vernalized D. nobile axillary buds that had been exposed to low temperatures. The cold treatment was performed in three consecutive years, and samples were harvested after 5, 10, 20, and 30 days of treatment. The qPCR analysis indicated that DnAGL19 expression was induced by 5 days treatment and peaked after 10 or 20 days. Although slight differences were observed between biological replicate samples from different years, the trends over the time course were similar (Figure 3B). These results are consistent with a previous report (Liang et al., 2012), confirming that the DnAGL19 gene expression is induced in axillary buds by prolonged low temperature treatments.
Phenotypes of DnAGL19 Overexpressing A. thaliana

Phylogenetic analysis indicated that DnAGL19 is the ortholog of DOSOC1 and A. thaliana SOC1/TM3-like proteins (Figure 1) and so we next investigated whether and how DnAGL19 is involved in the regulation of flowering time. Transgenic A. thaliana plants harboring a 35S::DnAGL19-Myc fusion construct were generated and the DnAGL19 insertions were verified for 13 selected T1 plants by PCR (Figure 4). Of these,
six lines were identified as harboring single-copy insertions (Supplementary Table S2), and four homozygous lines were finally obtained. Expression of DnAGL19 in these homozygotes was detected using RT-PCR and we observed that lines OX-6myc # 1, 4, and 9 accumulated high levels of DnAGL19 transcripts (Figure 4). Lines #4 and #9 were used for subsequent phenotypic analysis.

Flowering phenotypes were recorded for the DnAGL19 overexpressing lines grown under normal LD conditions or following vernalization (4°C for 4 weeks), with wild type A. thaliana serving as the control. As indicated in Table 1, the number of rosette leaves (RL) in the transgenic lines #4 and #9 was not statistically different from the wild type under normal conditions. However, the number of days from sowing to bolting (DTB), was significantly reduced by 2.0 and 1.4 days for line #4 and #9, respectively (Table 1, Figure 5), indicating a small acceleration of flowering in the DnAGL19 overexpressing lines. We observed that the vernalization (V)
counteracted this promotion to some extent, since the transgenic lines required a longer time to bolt, resulting in no statistical differences in DTB between transgenic lines and wild type plants (Table 1). These observations indicate that DnAGL19 expression suppressed floral development as a consequence of vernalization in the transgenic lines. Flowering time was also evaluated at T2 generation for a population of DnAGL19 overexpressing plants and the observations were similar to those for the homozygous lines #4 and #9 (Supplementary Figure S4).

Expression of Flowering-Associated Genes in DnAGL19 Overexpressing Lines Under LD Conditions

To determine the cause of the phenotypic differences between the transgenic lines and the wild type plants, the transcript levels of LFY, AP1, SOC1, FT, and FLC were evaluated in line #4. We observed that the AP1 transcript abundance increased substantially in 21-day-old transgenic lines, which was earlier than in the wild type (Figure 5B). Real-time qPCR analysis demonstrated that this activation of AP1 expression occurred even earlier, in 7-day-old plants (Supplementary Figure S6). However, the expression of LFY and FT, regulatory genes that act upstream of AP1 (Wigge et al., 2005), did not correlate with the transcriptional activation of AP1. Transcription of LFY was not altered by overexpression of DnAGL19 (Figure 5B, Supplementary Figure S6), indicating that AP1 activation could not be attributed to LFY transcription. Furthermore, FT was down-regulated by 2.3-fold, on average, which was significantly different from the wild type (Figure 5), indicating that FT did not contribute to the activation of AP1. We therefore concluded that DnAGL19 operates in an alternative pathway to activate AP1 and promote flowering in A. thaliana. In addition, the expression levels of SOC1 and FLC showed no dramatic differences between line #4 and wild type under LD conditions.

Expression of Flowering Associated Genes in DnAGL19-Overexpression Lines in Response to Vernalization

Although the RL numbers in the DnAGL19 overexpressing plants did not differ from those of the wild type, the significant difference of DTB under normal LD conditions disappeared after vernalization treatment (Table 1). In addition, the suppression of FT by overexpression of DnAGL19 under non-vernalization conditions was not apparent following vernalization. The abundance of FT transcript returned to a level similar to that in

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**FIGURE 3** | In planta expression of DnAGL19. (A) Organ-specific expression of DnAGL19 is shown. R, root; Sa: adult pseudobulbs; Sy: juvenile pseudobulbs; La: adult leaves; Ly: juvenile leaves; F: flowers; Ab: axillary buds. Total RNA was extracted from the indicated organs and RT-PCR was performed. 18s rRNA was used as the endogenous control. (B) Time-course dynamics of the expression of DnAGL19 during vernalization. Total RNA was extracted from axillary buds of vernalized D. nobile. The treating time duration is shown as days post start of vernalization. Three technical and biological replicates were analyzed by qPCR. Values are shown as mean ± SD. 18s rRNA was used as the endogenous control and the "0"-day sample was used to calibrate the other samples. **"** indicates t-test p-value < 0.01.

**FIGURE 4** | Screening for homozygous DnAGL19 overexpressing Arabidopsis thaliana lines. (A) Detection of the DnAGL19 insertion in putative transgenic lines. Genomic DNA was extracted from wild type A. thaliana and putative transgenic lines marked #1 to #15. The DnAGL19 sequence (639 bp) was amplified using genomic DNA as a template. (B) Growth of identified homozygous plants on a MS plate containing 50 mg/L kanamycin. Lines # 1, # 3, # 4, # 9 and wild type are shown. (C) Expression of DnAGL19 in transgenic lines. Total RNA was extracted from lines # 1, # 3, # 4, # 9 and wild type seedlings and reversed transcribed. RT-PCR was performed to detect the expression level of DnAGL19.
TABLE 1 | Flowering phenotypes of DnAGL19 overexpressing Arabidopsis thaliana.

| Genotype         | Long-day (LD) | Vernalization (V, 4°C, 4 weeks) |
|------------------|---------------|---------------------------------|
|                  | DTB           | RL                             | DTB               | Diff. | RL               |
| Wild type        | 31.5 ± 1.3    | 8.9 ± 1.1                      | 45.5 ± 1.7        | 14.0  | 7.3 ± 1.3        |
| OX-6myc #4       | 29.5 ± 0.8**  | 9.3 ± 0.7                      | 45.7 ± 1.1        | 16.2  | 7.8 ± 1.1        |
| OX-6myc #9       | 30.1 ± 0.6**  | 9.5 ± 0.5                      | 46.4 ± 1.4        | 16.3  | 8.0 ± 1.0        |

DTB, days from sowing to bolting; the 4-weeks of vernalization are included in DTB; RL, number of rosette leaves; Diff. = (DTB after vernalization) – (DTB under LD condition). Differences between overexpression lines and wild type were evaluated using t-tests, with ** indicating p < 0.01.

FIGURE 5 | Phenotypes of Arabidopsis thaliana lines overexpressing DnAGL19. (A) Growth of 25-day old wild type (WT) and DnAGL19 overexpressing lines (35S::DnAGL19-6myc #9 and #4) under normal LD condition. (B) Expression of LFY and AP1 at different developmental stages in wild type and DnAGL19 overexpressing lines (#1 and #4). Total RNA was extracted from seedlings of wild type and the overexpressing lines growing under LD conditions. RT-PCR was carried out. The developmental stages are shown as days after sowing. (C) Expression of flowering time associated genes (HOS1, FT, FLC, and SOC1) in wild type and DnAGL19 overexpressing seedlings before and after vernalization. “N” indicates the non-vernalized plants and “V” indicates the vernalized ones. Total RNA was extracted from 10-day old seedlings. Real-time qPCR was carried out in triplicates for each sample/gene combination, and data are shown as mean ± SD, with *p < 0.05 and **p < 0.01.

wild type plants (Figure 5, Supplementary Figure S7); however, FLC expression was also 1.88-fold higher, significantly different from that in wild type plants. Together, these results suggested that the combined effects of vernalization and DnAGL19 expression modulated the expression of both floral activator (e.g., FT) and inhibitor (e.g., FLC) genes.

Expression of HOS1 in DnAGL19 Overexpressing A. thaliana

Our results indicated that overexpression of DnAGL19 suppressed the expression of FT under normal growth condition. We therefore investigated the expression of the known upstream that targets the CO-FT module. As mentioned above, HOS1 encodes an E3 ligase that is involved in cold-associated flowering regulation via the action of the CO-FT module (Ishitani et al., 1998; Jung et al., 2012; Lazaro et al., 2012). Interestingly, we observed that even under normal LD conditions the transcription of HOS1 was substantially elevated in 9-day-old DnAGL19 overexpressing plants compared to wild type, suggesting that a high level of DnAGL19 promotes the expression of HOS1 (Figure 5, Supplementary Figure S7). After vernalization, the accumulation of the HOS1 transcript in DnAGL19 overexpressing lines returned to a level similar to that in wild type plants (Figure 5, Supplementary Figure S7).

DISCUSSION

SOC1/TM3-like homologs have previously been identified from diverse species, and as mentioned in the Introduction section,
these proteins are functionally divergent among paralogs and across species. To date there have been few reports of SOC1/TM3-like proteins in orchid species. Ding et al. (2013) identified a SOC1 ortholog, named DOSOC1, from Dendrobium Chao Parya Smile and demonstrated that it functioned as a flowering activator and also played a role in floral organ identity (Mandel etal., 1992). However, it should be noted that the acceleration of flowering in the overexpression lines was weak (<2 days earlier flowering, no decrease in RL number; Table 1). It is known that in A. thaliana, SOC1 upregulates API expression via a pathway mediated by LFY or FT (Liu

DnAGL19 Regulates Flowering via Pathway Mediated by AP1 Ortholog

The expression of the A. thaliana API gene was significantly activated at an earlier time point in the DnAGL19 overexpressing lines than in wild type plants (Figure 5, Supplementary Figure S6). This would tend to promote the transition from vegetative to reproductive development and/or the establishment of floral organ identity (Mandel et al., 1992). However, it should be noted that the acceleration of flowering in the overexpression lines was weak (<2 days earlier flowering, no decrease in RL number; Table 1). It is known that in A. thaliana, SOC1 upregulates API expression via a pathway mediated by LFY or FT (Liu

DnAGL19 may have Functionally Diverged from DOSOC1 and Other Orthologous Proteins

To characterize the evolution of the D. nobile DnAGL19 protein, an alignment and phylogenetic analysis was performed using DnAGL19, DOSOC1, and homologous protein sequences from species for which a whole genome sequence was available. Two lines of evidence from these analyses indicated that DnAGL19 is orthologous to members of SOC1/TM3-like subfamily in eudicot and monocot grass. First, the DnAGL19 protein contains the typical organization of the MADS-Box, K domains, and importantly, the characteristic SOC1-motif at the C-terminus, confirming that DnAGL19 is a SOC1/TM3-like protein (Figure 2, Supplementary Figure S3) (Vandenbussche et al., 2003). Second, DnAGL19 was grouped in a cluster with other members of SOC1/TM3-like subfamily from angiosperm plants, which is distant from the sister cluster of AGL6-like proteins in the NJ tree (Supplementary Figure S2), indicating that DnAGL19 has the common recent ancestor(s) with the SOC1/TM3-like proteins. However, the NJ tree further indicated that DnAGL19 and DOSOC1 clustered together as an orchid-specific branch that is independent from the eudicot and monocot grass branches (Figure 1), suggesting functional divergence of orchid SOC1/TM3-like proteins from their orthologs.

Divergence of DnAGL19 from its orchid ortholog, DOSOC1, was supported by the fact that amino acid substitutions comprised the major variation between these two proteins (Figure 2, Supplementary Table S3), and that the substitutions were rare in DOSOC1 and other orthologs. This suggests that DnAGL19 has a diverged biological function from DOSOC1, such as regulating flowering, probably including other development processes, via distinct pathways. Indeed, overexpression of DnAGL19 did not perturb the expression of LFY in transgenic A. thaliana, which differs from the observation that DOSOC1 promotes flowering in DOSOC1 expressing A. thaliana by upregulating LFY and AGL24 (Ding et al., 2013). As shown in Supplementary Table S3, expression of FT and HOS1 were differentially regulated in the DnAGL19 overexpressing lines depending on temperature cues. However, it is not known whether these two genes are influenced by DOSOC1, and future studies will address whether the differences between DnAGL19 and DOSOC1 contribute to variation in flowering and cold-tolerance in different Dendrobium species.

DnAGL19 Orthologs

The DnAGL19 protein contains the typical organization of the MADS-Box, K domains, and importantly, the characteristic SOC1-motif at the C-terminus, confirming that DnAGL19 is a SOC1/TM3-like protein (Figure 2, Supplementary Figure S3) (Vandenbussche et al., 2003). Second, DnAGL19 was grouped in a cluster with other members of SOC1/TM3-like subfamily from angiosperm plants, which is distant from the sister cluster of AGL6-like proteins in the NJ tree (Supplementary Figure S2), indicating that DnAGL19 has the common recent ancestor(s) with the SOC1/TM3-like proteins. However, the NJ tree further indicated that DnAGL19 and DOSOC1 clustered together as an orchid-specific branch that is independent from the eudicot and monocot grass branches (Figure 1), suggesting functional divergence of orchid SOC1/TM3-like proteins from their orthologs.

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DnAGL19 may have Functionally Diverged from DOSOC1 and Other Orthologous Proteins

To characterize the evolution of the D. nobile DnAGL19 protein, an alignment and phylogenetic analysis was performed using DnAGL19, DOSOC1, and homologous protein sequences from species for which a whole genome sequence was available. Two lines of evidence from these analyses indicated that DnAGL19 is orthologous to members of SOC1/TM3-like subfamily in eudicot and monocot grass. First, the DnAGL19 protein contains the typical organization of the MADS-Box, K domains, and importantly, the characteristic SOC1-motif at the C-terminus, confirming that DnAGL19 is a SOC1/TM3-like protein (Figure 2, Supplementary Figure S3) (Vandenbussche et al., 2003). Second, DnAGL19 was grouped in a cluster with other members of SOC1/TM3-like subfamily from angiosperm plants, which is distant from the sister cluster of AGL6-like proteins in the NJ tree (Supplementary Figure S2), indicating that DnAGL19 has the common recent ancestor(s) with the SOC1/TM3-like proteins. However, the NJ tree further indicated that DnAGL19 and DOSOC1 clustered together as an orchid-specific branch that is independent from the eudicot and monocot grass branches (Figure 1), suggesting functional divergence of orchid SOC1/TM3-like proteins from their orthologs.

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et al., 2008; Kaufmann et al., 2010; Lee and Lee, 2010; Benlloch et al., 2011). However, the expression of LFY was not altered by overexpression of DnAGL19 (Figure 5, Supplementary Figure S6), indicating that LFY is not a component of the DnAGL19-API module. The expression of FT was down-regulated in the DnAGL19 overexpressing lines (Figure 5), indicating that suppression of FT by DnAGL19 did not cause the API activation. On the contrary, it is possible that the suppression of FT expression in combination with DnAGL19 overexpression may have had the reverse effect on flowering and weakened the consequences of API activation. Thus, we conclude that overexpression of DnAGL19 in A. thaliana had opposite effects on API and FT expression which counteracts each other and results in weak promotion of flowering.

For the purpose of discovering the regulation of the downstream targets of DnAGL19 in D. nobile in the future, the D. nobile API ortholog was identified (data not shown). Overexpression of the D. nobile API ortholog in A. thaliana not only promoted floral transition but also altered leaf and petal shape (Supplementary Figure S5), indicating that this API-like protein has conserved functions to its A. thaliana ortholog (Mandel et al., 1992). This information will be helpful in identifying a genetic link between DnAGL19 and the D. nobile API ortholog.

DnAGL19 Regulates the Activity of the HOS1-FT Module

Flowering is mediated by complex GRNs, with floral regulators modulating each other’s expression through various feedback-loops (Pose et al., 2012). A. thaliana SOC1 plays a central role during flowering regulation, controlling the activity of a large number of downstream genes (Lee and Lee, 2010; Immink et al., 2012). FT was previously proposed to form a feedback loop with FT previously been demonstrated to mediate the degradation of the CO protein, thereby repressing FT transcription (Jung et al., 2012; Lazar et al., 2012). It is reasonable to conclude that DnAGL19 is involved in the HOS1-CO-FT module to regulate the flowering time in the transgenic A. thaliana lines. Although API expression was activated earlier in the DnAGL19 overexpressing lines (Figure 5), this was not sufficient to overcome the negative regulation of flowering by the up-regulation of HOS1.

FLOWERING LOCUS C gene is also a regulation target of HOS1 protein. HOS1 acts as a competitor of HDA6, binding to FLC chromatin and removing HDA6 to activate FLC in a FVE-dependent manner (Jung et al., 2013; Jung and Park, 2014). But the FLC expression was barely altered in DnAGL19 overexpressing lines. On the other hand, a FLC ortholog has not been identified from D. nobile or other phylogenetically closely related orchid species until now (Liang et al., 2012; Cai et al., 2015). We thus propose that the DnAGL19-HOS1-FT module may be the main GRN in D. nobile. Transcriptional suppression of DnFT was previously observed to occur in parallel with an increase in DnAGL19 transcript levels in D. nobile axillary buds during vernalization (Li et al., 2012; Liang et al., 2012), and this is in agreement with the co-expression of DnAGL19 and A. thaliana FT in the present study. This suggests a link between DnAGL19 and the FT ortholog, at least in the axillary buds of adult D. nobile, and the HOS1 homolog may act as an intermediary component, as discussed above. Furthermore, an API ortholog has also been shown to be co-activated with DnAGL19 in axillary buds (Liang et al., 2012). Thus, DnAGL19-mediated repression of DnFT and activation of DnAPI may contribute to the regulation of D. nobile floral bud development (Figure 6).

Vernalization Regulates Flowering in Combination with DnAGL19

It is well-known that vernalization promote flowering in A. thaliana, wheat, and other species. SOCI/TM3-like proteins, such as A. thaliana SOC1 and AGL19, function in vernalization-related pathways (Alexandre and Hennig, 2007; Lee and Lee, 2010). It has also been reported that intermittent cold temperatures delay flowering in A. thaliana, which is associated with HOS1-mediated repression of FT and activation of FLC (Jung et al., 2012; Lazar et al., 2012; Jung and Park, 2014). Vernalization has no effect on HOS1 expression in wild type plants (Lee, 2012). In our study, however, the prolonged low-temperature treatment (i.e., vernalization for 4 weeks at 4°C) imposed on the DnAGL19 overexpressing plants resulted in a down-regulation of HOS1 expression (Figure 5). This suggests that high level of DnAGL19 has negative effects on HOS1 expression by combination with vernalization. Under the same conditions, FT transcription was activated (Figure 5), which may be attributed to the down-regulation of HOS1. Although elevated expression of DnFT as a consequence of vernalization has previously been observed in D. nobile leaves (Li et al., 2012), its possible co-expression with DnAGL19, or other SOCI/TM3-like orthologs, following vernalization has not been established. We propose that high levels of DnAGL19 expression in leaves (Figure 3) may explain the DnFT activation in leaves under these conditions. We also suggest that DnAGL19 may couple the long-term low-temperature signal to the inhibition of HOS1 expression, resulting in an up-regulation of FT expression via the HOS1-CO-FT module (Figure 6). However, additional studies of the genetic linkage between DnAGL19 and DnFT are needed to verify this hypothesis.
Our results suggest the existence of a GRN in which the SOCI/TM3-like ortholog, DnAGL19, regulates FT expression through the intermediary protein HOS1 (Figure 6). The DnAGL19-HOS1-FT module is likely differentially regulated, depending on whether or not the plant is experiencing vernalization conditions. Under long-term low temperature such as during winter, high level of DnAGL19 might repress the expression of the HOS1 ortholog, allowing the activation of FT and the initiation of floral transition. Subsequently, in the spring, DnAGL19 would then up-regulate HOS1 expression to repress FT in the new warmer conditions and API is activated at the same time, promoting flower development. Although additional evidence is needed to confirm this hypothesis, this GRN would explain the observed features of flowering and adaption in D. nobilis. The results presented here, shed light on the mechanisms underlying flowering control in Nobile-type Dendrobium.

AUTHOR CONTRIBUTIONS
X-RL, TP and W-QL generated the DnAGL19-overexpressed Arabidopsis and worked on phenotypic analysis and transcription detection, LG contributed to the generation and phenotypic analysis of DnAPI1-overexpressing Arabidopsis. X-JW and H-QL contributed to experimental design and manuscript revision. SL designed the experiments, performed data analysis and wrote the manuscript.

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SUPPLEMENTARY MATERIAL
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