Control of Flowering Time and Cold Response by a NAC-Domain Protein in Arabidopsis

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Background. Plants must integrate complex signals from environmental and endogenous cues to fine-tune the timing of flowering. Low temperature is one of the most common environmental stresses that affect flowering time; however, molecular mechanisms underlying the cold temperature regulation of flowering time are not fully understood. Methodology/Principal Findings. We report the identification of a novel regulator, LONG VEGETATIVE PHASE 1 (LOV1), that controls flowering time and cold response. An Arabidopsis mutant, long vegetative phase 1-1D (lov1-1D) showing the late-flowering phenotype, was isolated by activation tagging screening. Subsequent analyses demonstrated that the phenotype of the mutant resulted from the overexpression of a NAC-domain protein gene (At2g02450). Both gain- and loss-of-function alleles of LOV1 affected flowering time predominantly under long-day but not short-day conditions, suggesting that LOV1 may act within the photoperiod pathway. The expression of CONSTANS (CO), a floral promoter, was affected by LOV1 level, suggesting that LOV1 controls flowering time by negatively regulating CO expression. The epistatic relationship between CO and LOV1 was consistent with this proposed regulatory pathway. Physiological analyses to elucidate upstream signalling pathways revealed that LOV1 regulates the cold response in plants. Loss of LOV1 function resulted in hypersensitivity to cold temperature, whereas a gain-of-function allele conferred cold tolerance. The freezing tolerance was accompanied by upregulation of cold response genes, COLD-REGULATED 15A (COR15A) and COLD INDUCED 1 (KIN1) without affecting expression of the C-repeat-binding factor/dehydration responsive element-binding factor 1 (CBF/DREB1) family of genes. Conclusions. Our study shows that LOV1 functions as a floral repressor that negatively regulates CO expression under long-day conditions and acts as a common regulator of two intersecting pathways that regulate flowering time and the cold response, respectively. Our results suggest an overlapping pathway for controlling cold stress response and flowering time in plants.

INTRODUCTION

Timing of the developmental transition to the reproductive phase is very important for plants to ensure successful reproduction and requires the proper perception and processing of a variety of stimuli. It is therefore not unexpected that the integration of complex signals from environmental and endogenous cues is necessary to enable plants to time this transition at the most advantageous moment. In Arabidopsis, at least four major floral promotion pathways are known to mediate signalling from the different cues: the photoperiod, vernalization, autonomous and gibberellin (GA) pathways [1]. Of these pathways, the photoperiod pathway plays an important role in controlling the timing of the developmental transition to flowering in Arabidopsis.

CONSTANS (CO) is an important floral promoter that acts within the photoperiod pathway [2]. It encodes a nuclear protein containing a CCT motif and two B-Box-type zinc-finger domains. The mRNA expression level of CO is modulated by the circadian clock and by day-length, and exposure to light is required to activate CO protein function [3], suggesting that CO fulfills the role of a mediator between the photoperiod/circadian clock and the floral integrators. Several upstream regulators of CO in photoperiod and circadian clock signalling have been identified. GIGANTEA (GI) positively mediates signalling from the circadian clock oscillators to CO [4]. RED AND FAR RED INSENSITIVE 2 (RF2) [5], CYCLING DOF FACTOR 1 (CDF1) [6], and SUPPRESSOR OF PHYA-105 (SPA1) [7] are light signalling molecules controlling CO expression downstream of photoreceptors. Although it has been firmly established that CO expression is regulated by the photoperiod and circadian clock, the regulation of CO expression by other environmental stimuli is poorly understood.

NAC (NAM, ATAF1, -2, and CUC2)-domain proteins are a class of transcription factors known to control multiple processes in plants, including developmental programs and abiotic/biotic stress responses [8]. One of the first reported NAC genes, NO APICAL MERISTEM (NAM) from petunia, plays a critical role in meristem formation [9]. As NAC-domain transcription factors are found only in plants, it is highly likely that they are involved in various plant-specific functions. As such, it is not surprising that NAC-domain genes comprise one of the largest transcription factor families in the compact Arabidopsis genome [10]. However, only a small number of the NAC-domain proteins have been studied to date [8], and the functions and regulation of most Arabidopsis NAC-domain genes are still largely unknown.

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Temperature is an important environmental stimulus that affects flowering time, and an abundance of studies have indicated that it is highly probable that plants use different mechanisms to control flowering time in response to different temperature ranges. The thermosensory pathway genes play a role in regulating flowering in response to ambient growth temperature (moderately elevated or decreased temperatures) [11–13]. In response to near-freezing temperatures, plants exhibit cold acclimation or the vernalization response [14]. The duration of cold exposure to establish these respective responses is different, such that a few days of cold exposure is sufficient to initiate cold acclimation [15], whereas several weeks are required for vernalization [16]. It is generally accepted that cold acclimation is necessary for a plant to deal successfully with sudden temperature changes and that vernalization is required to ensure that flowering is inhibited until spring [14]. Although the molecular mechanisms involved in the control of flowering time by vernalization are well understood [14,17], many questions have yet to be resolved in terms of cold temperature regulation of flowering time.

We report here a NAC-domain transcription factor, LOV1, which exerts its inhibitory effect on floral development by negatively regulating CO expression in a GI-independent manner. Mutations in LOV1 led to altered responses to freezing temperatures. The loss-of-function lov1 allele was hypersensitive to cold temperature, whereas a gain-of-function allele was tolerant to cold temperature. The freezing tolerance was attributed to the upregulation of cold response genes without altering expression of the C-repeat-binding factor/dehydration responsive element-binding factor 1 (CBF/DREB1) gene family. Based on our results, we propose that LOV1 plays an important role in the coordination of cold response and flowering time.

METHODS

Plant materials and growth conditions

All of the plants used in this study were Arabidopsis thaliana plants in the Columbia background, except for co-2 and gi-3, which were in the Ler background. gi-2 plants harbouring 35S::GI transgene were used for GI overexpressor plants (genotype: 35S::GI gi-2). The lov1-1D mutant was isolated from an activation-tagged mutant library that had been generated in our laboratory [18]. A loss-of-function allele of LOV1 that we used was a transposon insertion allele identified from the Exon Trapping Insert Consortium (EXOTIC) [19]. Since the allele was in the Landsberg background, we introgressed it four times into the Columbia background. The introgressed line was named lov1-4. Plants were grown in Sunshine Mix 5 (Quincy, Mich.) under long-day (16:8 h, light:dark) or short-day (8:16 h, light:dark) conditions at 23°C. The flowering time of the plants was measured by scoring the number of primary rosette and cauline leaves of at least 12 plants.

Recombinant plasmid construction

A LOV1 cDNA clone (C104984) was obtained from The Arabidopsis Information Resource (TAIR) and fused with the 35S promoter and nos terminator. The resulting construct, pST1004, is referred to as 35S::LOV1 and was used for an overexpression analysis. The 35S::LOV1::GFP (Green Fluorescence Protein) construct was generated by fusing a LOV1 cDNA to smGFP [20]. To generate a pLOV1::GUS (β-glucuronidase) construct, we amplified a fragment of the LOV1 promoter (-1,943 to -1, relative to a translational start) from the T16F16 BAC clone and cloned it into the pB1101.1 vector. The pLOV1::LOV1::HA transgene was generated by fusing the 1.9-kb LOV1 promoter with HA-tagged LOV1 cDNA in the pJH[A212B vector [21].

Expression analysis

RNA expression levels were measured by semi-quantitative reverse transcriptase (RT)-PCR followed by Southern hybridization [22]. Oligonucleotide sequences used to detect the mRNA of the genes studied are listed in Table S1. Seedlings for RNA extraction were harvested at the indicated Zeitgeber (ZT) times. Either the UBQ10 or tubulin gene was used as an internal positive control. For the diurnal expression analyses, plants were entrained under 12:12 h (light:dark) conditions for 10 days, then grown under continuous light conditions. The subcellular localization of LOV1 was determined by using the 35S::LOV1:GFP reporter gene. The 35S::LOV1:GFP construct was introduced into onion (Allium cepa L.) epidermal cells by means of a particle gun (PDS-1000/He; Bio-Rad, Hercules, Calif.) using tungsten particles coated with plasmid DNA. The bombarded cells were then incubated at 22°C for 12 h, followed by staining with 4′,6-diamidino-2-phenylindole (DAPI; Sigma, St. Louis, Mo.); the GFP fluorescence was observed under a fluorescence microscope (model Axioskop 2 plus; Carl Zeiss, Germany) and photographed with AxiosCam HRc (Carl Zeiss). For the histochemical GUS assay, whole seedlings were stained according to the procedure of Sessions et al. [23].

Freezing-tolerance assay

lov1, co-2, and wild-type plant seeds were planted on soil and grown under long-day conditions. For the freezing treatment, the seedlings were placed in a controlled temperature chamber (CryMed® Freezer; ThermoForma, Marietta, Ohio) and subjected to freezing at -8°C for 2 h with or without cold acclimation. The plants were then transferred to a cold room (4°C) under white light and incubated overnight. Following thawing overnight, the plants were moved to a climate chamber maintained at 23°C and grown for 1 week under long-day conditions. For cold acclimation, 2-week-old seedlings were transferred to a cold room (4°C) and grown for 4 days prior to the freezing treatment.

RESULTS

Isolation of lov1-1D mutants that show a late-flowering phenotype

A mutant that displayed delayed flowering under long-day conditions was isolated from an activation tagging library [18]. This mutant, which we denoted as long vegetative phase 1-1D (lov1-1D), flowered with 26.6 leaves under long days, whereas wild-type plants flowered with 15.5 leaves (Figure 1A). A plasmid rescue experiment [24] revealed that a T-DNA had been inserted into the last exon of At2g02440 in chromosome 2. Despite the insertion of this T-DNA into the coding sequence of At2g02440, we concluded that the late-flowering phenotype was not associated with any disruption of the At2g02440 gene structure because (1) the late-flowering phenotype was dominant and (2) At2g02440 was a hypothetical protein whose cDNA was not detected in the known expressed sequence tag (EST) libraries. The next closest gene to the 35S enhancers in the T-DNA was At2g02450, which was located 8.9 kb from the right border of the T-DNA. Despite this long distance, the expression of At2g02450 was significantly upregulated in lov1-1D mutants (Figure 1B). Genomic Southern hybridization using the BAR gene as a probe revealed a single T-DNA insertion in the lov1-1D mutants (Figure 1C). Taken together, these results strongly indicated that the late-flowering phenotype in lov1-1D plants was closely associated with the transcriptional activation of At2g02450.

We carried out a recapitulation experiment to confirm that overexpression of At2g02450 caused the late-flowering phenotype.
We expressed At2g02450 cDNA under the control of the 35S promoter and chose transgenic lines that expressed At2g02450 at high levels (Figure 1D). These transgenic plants showed late flowering (average leaf number: 23.7 ± 5.1) under long-day conditions (Figure 1A), indicating that overexpression of At2g02450 conferred the late-flowering phenotype observed in lov1-1D mutants. The overall phenotypes of the lov1-1D mutants and those of the transgenic plants that overexpressed At2g02450 cDNA were similar. We therefore designated At2g02450, which encodes a NAC-domain transcription factor, as LOV1.

Alteration of LOV1 activity affects flowering time mainly under long-day conditions

We identified a transposon allele in the Landsberg background which had a Ds transposon inserted into the second intron of LOV1 (Figure 2A). Changes in flowering time in this line were very weak under long-day conditions (Figure 2B), possibly because Landsberg is a rapid-cycling accession. We introgressed this mutation into the Columbia background in order to facilitate its genetic analysis with other flowering time mutants that are in the Columbia background and then used this introgressed line (lov1-4) to investigate LOV1 function. Semi-quantitative RT-PCR did not detect any LOV1 expression in the mutants (Figure 2C), which suggested that lov1-4 was most likely an RNA null allele.

Under long-day conditions, lov1-4 plants flowered with 11.9 ± 1.5 leaves (wild-type plants = 15.4 ± 1.3 leaves), indicating that loss of LOV1 function resulted in a slightly early-flowering phenotype (Figures 2D and 2E). However, no significant changes in flowering time were seen under short-day conditions. 35S::LOV1 plants and lov1-1D plants flowered with 51.5 ± 6.0 and 55.1 ± 3.0 leaves under short-day conditions (Figure 2E), respectively, whereas wild-type plants flowered with 48.1 ± 3.0 leaves under the same conditions. In addition, the flowering time of lov1-4 plants was also similar to that of the wild-type plants under short-day conditions (average leaf number: 55.7 ± 4.6 vs. 57.3 ± 5.0, respectively) (Figure 2E). These flowering time measurements indicated that both gain- and loss-of-function alleles of LOV1 (hereinafter lov1 mutants) exhibited altered flowering time under long-day conditions, but not under short-day conditions.

35S::LOV1 and lov1-4 plants resembled the wild-type plants in their response to GA treatment, vernalization, and different light qualities (Figure S1). These suggested that LOV1 may not be involved in the genetic pathways that mediate these floral promoting signals. These data indicated that LOV1 affected flowering time primarily under long-day conditions, which is characteristic of the photoperiod pathway mutants [25]. Taking into consideration the fact that the absence of LOV1 function caused early flowering, our data suggest that LOV1 may act as a floral repressor within the photoperiod pathway.

Complementation analysis of lov1-4 mutants

Since we introgressed lov1-4 mutation into the Columbia background, it was still possible that the early flowering phenotype seen in the introgressed line resulted from a linked quantitative trait locus (QTL). To confirm that a mutation in LOV1 caused the flowering time change in lov1-4 mutants, we introduced the LOV1 gene into lov1-4 mutants by crossing pLOV1::LOV1:HA and lov1-4 plants and then determined whether the early flowering defect was rescued. LOV1:HA protein expression in the transgenic plants was confirmed by means of Western blot analysis using HA antibodies (Figure 2F). pLOV1::LOV1:HA lov1-4 plants flowered with 13.1 leaves under long-day conditions, whereas wild-type Columbia and lov1-4 plants flowered with 13.4 and 11.2 leaves, respectively (Figure 2G). This indicated that LOV1:HA can functionally complement the lov1-4 mutation and further suggested that the early flowering phenotype seen in lov1-4 mutants resulted from the presence of the Ds transposon and, consequently, from the disruption of the LOV1 gene structure.

Expression patterns of LOV1 in wild-type plants

A time-course analysis of LOV1 expression in wild-type plants showed that LOV1 was highly expressed in the early stages of seedling development but that its transcript levels subsequently gradually decreased (Figure 3A). The expression of APETALA1...
(AP1) [26], a molecular marker of floral transition, was also measured to identify the growth stages of the seedlings that we had harvested. AP1 expression levels began to increase at day 10, suggesting that wild-type plants are in transition to flowering around this time point. This observation indicated that LOV1 expression decreased during flower development, which is in good agreement with our proposal that LOV1 acts as a floral repressor. We then measured circadian expression levels of LOV1 to determine whether LOV1 expression was controlled under the circadian clock [27]. The level of LOV1 mRNA displayed
a circadian oscillation with a peak at dawn under continuous light conditions (Figure 3B). Measuring the expression pattern of LOV1 protein using HA-tagged LOV1 protein, we found that the expression level of the LOV1-HA fusion protein also oscillated under continuous light conditions, with a broader peak around dusk and lower expression levels around dawn. These observations suggest that LOV1 protein began to accumulate after LOV1 transcript levels decreased and that the mRNA and protein levels were regulated differentially. They also raised the possibility that LOV1 protein expression is under the control of the circadian clock or that LOV1 function may be closely associated with circadian clock-controlled genes. An analysis of the tissue-specific expression patterns of LOV1 in wild-type plants revealed that the LOV1 transcript was detectable in all vegetative tissues except for the root (Figure 3C). This spatial expression pattern was also confirmed by the GUS reporter assay. LOV1 promoter-driven GUS expression was detectable mainly in the above-ground parts of the seedlings (Figure 3D). A subcellular localization analysis of LOV1 using LOV1:GFP protein revealed that LOV1 is predominantly localized in the nucleus in transiently transformed onion epidermal cells, whereas free smGFP was detected throughout the cell, thereby suggesting that LOV1 is a nuclear protein (Figure 3E).

**Negative regulation of CO expression by LOV1**

Based on our result that the main effect of LOV1 on flowering time was seen under long-day conditions, we analyzed the expression levels of flowering time genes that act within the photoperiod pathway. Due to the oscillation of the transcript and protein levels of LOV1, we first monitored the expression of the circadian clock genes and found that circadian expression levels of CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) [28] and LONG HYPOCOTYL (LHY) [29], the key regulators in the circadian clock function, were not altered by the overexpression of LOV1 (Figure 4A). No significant differences in the peak patterns, period length, and amplitude of expression of these genes between 35S::LOV1 and wild-type plants were observed, although the peak expression of LHY appeared to shift slightly at day 10. Furthermore, the circadian oscillation of CCA1 and LHY expression was not changed in lov1-4 mutants. Therefore, it appeared that alterations in LOV1 activity did not affect transcript levels of the circadian clock genes. This suggested that LOV1 may act independently of the central oscillators of the circadian clock to regulate flowering time under long-day conditions.

An analysis of the time-course expression of genes acting downstream of the circadian clock showed that the expression level
of GI, which may participate in a feedback loop of the plant circadian system [30], remained unaffected in 35S::LOV1 plants (Figure 4B). However, the expression levels of CO, which is an important floral promoter and known to act downstream of GI [4], were significantly downregulated in 35S::LOV1 plants (Figure 4B). Consistent with this result, the expression of FT [22,31], a floral
integrator gene acting downstream of CO, was also downregulated in 35S::LOV1 plants. These results indicated that downregulation of CO expression by LOV1 overexpression may be the main regulatory factor explaining why LOV1 overexpressor plants exhibited a late-flowering phenotype.

An expression analysis of the genes in the lov1-4 mutants was conducted to confirm that LOV1 affects the expressions of CO and FT but not GI. The result showed that GI expression was not altered in lov1-4 mutants (Figure 4C). In contrast, CO expression was slightly upregulated and the expression of FT was also upregulated, which is consistent with the expression data obtained in 35S::LOV1 plants (Figure 4B). Furthermore, the expression of AP1 [26] was precociously upregulated in lov1-4 mutants (Figure 4C), which can be explained by the upregulation of CO and FT in the mutants.

Because CO expression levels oscillate in wild-type plants [3], we measured circadian expression levels of CO in lov1 mutants to further confirm the negative regulation of CO expression by LOV1. The expression levels of CO were changed, such that overall expression levels of CO were lower in 35S::LOV1 plants but higher in lov1-4 mutants (Figure 4D), although peak patterns and period length remained unaltered. It was notable that the peaks of CO expression were broader in lov1-4 mutants. This observation suggested that more CO transcripts were present in lov1-4 mutants under light conditions and that this increased level may subsequently activate FT expression and ultimately induce flowering.

In contrast, GI transcript levels were largely unaffected by changes in LOV1 activity. These data suggest that LOV1 negatively regulates CO expression and that the de-repression of CO expression in the absence of LOV1 function causes early flowering in lov1-4 mutants.

Genetic interaction studies of LOV1: overexpression of CO is epistatic to LOV1 overexpression

To confirm the results obtained from the expression analysis, we investigated the genetic interaction between LOV1 and genes that act downstream of the circadian clock in the photoperiod pathway by crossing gain- or loss-of-function alleles of LOV1 with mutants in such genes. The result showed that the early-flowering phenotype of 35S::GI plants was suppressed by LOV1 overexpression (Figure 5A). Under long-day conditions, 35S::GI plants flowered with 11.2 ± 0.8 leaves, whereas plants overexpressing both GI and LOV1 flowered late, with an average of 26.0 (±5.6) leaves, which was comparable to the late-flowering time of 35S::LOV1 plants (24.9 ± 4.8 leaves). This result suggested that overexpression of LOV1 masked the early-flowering phenotype of 35S::GI and that LOV1 may act downstream of GI or be independent of GI. 35S::CO plants flowered with 7.2 leaves, whereas lov1-1D 35S::CO plants flowered with 8.4 ± 1.2 leaves, indicating that increased CO activity was able to suppress the late-flowering phenotype of 35S::LOV1 plants. Consistent with this, the overexpression of FT was also epistatic to LOV1 overexpression. The flowering of FT and LOV1 double-overexpressor plants resembled that of FT single overexpressor plants (4.1 ± 0.3 vs. 4.0 ± 0.1 leaves, respectively). These data suggested that the late-flowering phenotype caused by the overexpression of LOV1 was largely suppressed by the overexpression of CO or FT, but not by overexpression of GI. Thus, the results of the genetic analysis were consistent with the expression analysis (Figure 4), which further supports the hypothesis that LOV1 acts upstream of CO and may act downstream or independent of GI.

Genetic crosses with lov1-4 mutants were carried out to confirm the results of genetic studies performed with the gain-of-function alleles (Figure 5B). Both gi-3 lov1-4 double mutants and gi-3 single mutants showed a similar flowering time (average leaf number: 26.7 ± 1.3 vs. 27.0 ± 2.2, respectively), indicating that lov1-4 mutation failed to suppress the late flowering of gi-3 mutants and that de-repression of CO expression in the absence of LOV1 function would not be sufficient to overcome the effect of gi-3 mutations. This result suggested that LOV1 may not be simply downstream of GI, co-2 lov1-4 double mutants flowered similarly to co-2 single mutants (average leaf number: 22.8 ± 2.6 vs. 23.6 ± 3.4, respectively), indicating that co-2 mutation largely suppressed early flowering of lov1-4 mutants. This result is consistent with the epistatic relationship between LOV1 and CO. soc1-2 lov1-4 double mutants flowered with 17.6 leaves, whereas lov1-4 and soc1-2 single mutants flowered with 11.0 and 20.4 leaves, respectively, indicating that soc1-2 mutation largely suppressed the early flowering of lov1-4 mutants. Taking into consideration that SOC1 is a direct downstream target of CO [32], this observation also supports the notion that LOV1 acts upstream of CO and may act downstream or independently with GI.
lov1-4 plants are hypersensitive to freezing treatment

In order to identify the upstream signalling pathway regulating LOV1 activity, the responses of lov1 mutants to various stimuli, including hormones and abiotic stresses, were examined. lov1 mutants displayed normal responses to phytohormones and most of the abiotic stresses (data not shown). However, lov1-4 plants were hypersensitive to freezing temperature (–8°C), whereas 35S::LOV1 plants were tolerant (Figure 6A). Under our experimental conditions, 54% of the non-acclimated wild-type plants survived. In contrast, although only 24% of the lov1-4 plants survived, the survival rate of 35S::LOV1 plants was 88%. When plants were cold-acclimated prior to the freezing treatment, the majority (92%) of wild-type plants became tolerant to the freezing temperature; in comparison, 74% of the lov1-4 plants also became tolerant to the freezing treatment, suggesting that lov1-4 plants are sensitive to freezing temperatures but are able to acclimate to the cold. Following a cold acclimation period, 35S::LOV1 plants were largely insensitive to the freezing treatment, and 95% of the plants survived the exposure to freezing at –8°C. These data suggested that LOV1 may regulate the cold response in plants. As cold acclimation and constitutive freezing tolerance are under independent genetic control [33,34], LOV1 would appear to primarily control constitutive freezing tolerance in Arabidopsis.

Interestingly, we observed that a mutation in CO also led to freezing tolerance (Figure 6B). Of the co-2 plants tested, 55% of the non-acclimated and 95% of the acclimated plants showed freezing tolerance; in comparison, 35% and 66% of the non-acclimated and acclimated wild-type Ler plants showed freezing tolerance. Given our result that LOV1 negatively regulates CO expression, this freezing-tolerant phenotype seen in co-2 mutants is consistent with the phenotype of the LOV1 overexpressor plants. Thus, our results suggest that both LOV1 and CO, a well-known flowering time gene in the photoperiod pathway, may be involved in freezing tolerance.

LOV1 positively regulates COR15A and KIN1 expression for the cold response

To obtain empirical evidence of the mechanism by which LOV1 affects cold response, we first determined the expression levels of CBF/DREB1 family genes [35], the most important of the known transcription factors involved in the cold response, in lov1 mutants. CBF1 expression was not detectable at 23°C in wild-type plants, whereas CBF2 and CBF3 gene expressions were weakly detected (Figure 7A). When the wild-type plants were cold-treated at 4°C, the expressions of the CBF/DREB1 genes were rapidly induced. These induction patterns of CBF genes at 4°C were also observed with 35S::LOV1 and lov1-4 plants, indicating that CBF expression was not affected by LOV1 expression.

We also monitored the expression of several cold-regulated genes in lov1 mutants. We found that COR15A [36] and KIN1 [37] expressions were upregulated in 35S::LOV1 plants, whereas the expressions levels of RD29A [38] and KIN2 [39] were unaltered (Figure 7B). In lov1-4 mutants, COR15A expression was down-regulated (Figure 7C), which is consistent with the data obtained from 35S::LOV1 plants. However, KIN1 expression levels remained unaltered in lov1-4 mutants, suggesting that LOV1 may not be essential for the expression of KIN1. These results suggest that the changed sensitivity of lov1 mutants to cold temperature is, at least partially, conferred by the altered expression of COR15A and KIN1 genes and may be regulated by means of one of several CBF/DREB1-independent signalling pathways in the cold response [40]. Considering that CBF/DREB1 family genes do not have CRT/DRE motifs, a major co-acting element in cold stress response [41], but that COR15A and KIN1 do contain the motifs within their promoters, it is possible that LOV1 may bind to the motifs to regulate its target genes in the cold response.
DISCUSSION

An activation tagging screen resulted in the identification of LOV1, a member of the plant-specific NAC-domain transcription factors. Our data suggest that LOV1 regulates flowering time by negatively regulating CO expression and that it also regulates the cold response by regulating the expressions of COR15A and KIN1. We propose that LOV1 may play a pivotal role in coordinating flowering time and cold response.

Since LOV1 negatively regulates CO expression, an important question is whether LOV1 directly binds to a cis-acting element within the CO sequence. This possibility of such a binding is supported by the observation that the oscillation patterns of LOV1 protein levels and CO mRNA levels were largely in reverse phase to each other (Figures 2 and 3). However, an electrophoretic mobility shift assay that we performed did not identify the specific binding sites of LOV1 protein within the CO promoter sequence; rather, it appeared that LOV1 protein binds non-specifically to the promoter region of CO (S.Y.Y. and J.H.A., unpublished results). This suggests that LOV1 may not directly regulate CO expression and that LOV1 may require an additional downstream gene that mediates signalling from LOV1 to CO. However, we cannot exclude the possibility that non-specific binding to the CO promoter is required for changes in the chromatin structures of the CO locus, thereby leading to downregulation of CO, as seen in plant homeodomain finger proteins [42].

LOV1 appears to control CO expression in a pathway that might be distinct from those of the photoperiod pathway and the circadian clock. CO is known to integrate the circadian clock and photoreceptor signalling processes in flower development [2-4,34,44]. In this study, however, we have shown that alterations in flowering time associated with changes in LOV1 activity appear to be independent of G1 and other circadian clock oscillators (Figure 4) and that lov1 mutants did not show any significant changes in hypocotyl lengths under different light qualities (Figure S1). These results suggest that LOV1 may function in a pathway that does not require the photoreceptor and circadian clock function. This concept is further supported by the observations that LOV1's pattern of regulation of CO expression is different from those of previously identified regulators of CO, such as the g mutation, which is epistatic to the sfi2 mutation [5], the post-transcriptional regulation of CO by SPA1, which is likely short-day specific [7,45], and CDF1, which directly binds to a cis-acting motif with the CO promoter [6]. Based on our results, LOV1 is likely an additional upstream regulator that may mediate different signalling pathways to CO (Figures 3 and 4). Given the fact that CO plays a central role in photoperiodic flowering and that the determination of the timing of flowering is critical for plants' successful reproduction, it is not particularly surprising that multiple independent regulators control the expression of CO in its function as a regulator of flowering.

One very interesting observation was that the downregulation of CO, a flowering time gene, also led to a tolerance to cold temperature, as this would suggest that CO could be an important regulator integrating developmental regulation and the cold stress response. The enhanced tolerance seen in co mutants is consistent with the finding that 35S::LOV1 plants are tolerant to cold temperature. Given our result that the overexpression of LOV1 caused a decrease in CO transcript level, it is expected that LOV1 overexpressor plants and a loss-of-function allele of CO exhibit a similar cold response (Figure 6). This implies that a subset of flowering time genes may play an additional role in the cold response. Consistent with this hypothesis is a recent finding that GI, an upstream regulator of CO, is involved in the cold stress response [46]. GI positively regulates tolerance to freezing temperature in a CBF-independent pathway, as observed in lov1 mutants. Thus, it is probable that the canonical photoperiod pathway genes in flower development may play a dual role in flowering time control and the cold response. This may explain, at least partially, the delay or inhibition in the flowering of wild-type Arabidopsis plants that are exposed to intermittent cold stress.

Our proposal that LOV1 may regulate the cold response is also supported by the observation that lov1 mutants showed responses similar to those of los9 and sfi6 mutants that are known to involve in cold signalling. los9 and sfi6 mutants display altered sensitivities to freezing stress without affecting CBF/DREB1 gene expression [47,48], as also seen in lov1 mutants. An interesting result is that los9 mutants also exhibit a flowering time phenotype [47,48]; however, the precise mechanism by which LOS9 regulates flowering time is unknown. In sfi6 mutants, expressions of KIN1 and COR15A, which contain the CRT/DRE motif in their promoters, were upregulated, as also seen in 35S::LOV1 plants. It is worth noting that SFR6 may be a positive regulator of LOV1 in the cold response since the level of LOV1 transcripts was significantly decreased in sfi6 mutants [49]. However, the SFR6 gene has not yet been cloned. Future elucidation of the identity of SFR6 will facilitate studies aimed at determining whether SFR6 and LOV1 act in the same pathway to control cold tolerance or whether they interact with each other to mediate the cold response.

In summary, our results suggest that the LOV1 encodes a NAC-domain transcription factor that plays a pivotal function in flowering time regulation and cold response. LOV1 acts as a floral effector by negatively regulating the transcript level of CO, a central regulator of the photoperiod pathway. It also regulates the response of plants to freezing temperature by controlling a subset of cold response genes via a CBF/DREB1-independent pathway. Our results suggest a shared mechanism for controlling cold stress response and flowering time in plants and that the regulation of CO via LOV1 may be common for these two distinct responses. Future investigations to determine the precise mechanism by which LOV1 acts in two intersecting pathways will provide a better understanding of the modulation of reproductive development in plants under continuously changing temperature conditions.

SUPPORTING INFORMATION

Figure S1 Physiological responses in los9 mutants and wild-type plants. (A) Effect of vernalization on flowering time of los9 mutants. Hydrated seeds were treated with (+Ver) or without (-Ver) vernalization for 4 weeks at 4°C in a cold room under dark conditions. (B) Effect of GA treatment on flowering time of los9 mutants. Flowering time was measured under short-day conditions. 20 µM of GA was sprayed onto the entire aerial part of the plants until the floral bud was emerged. (C) Light effects on the elongation of the hypocotyls of los9 mutants. Note that the length of the hypocotyl was not affected by the quality of the monochromatic light.

Table S1 Oligonucleotides used for RT-PCR

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Author Contributions

Conceived and designed the experiments: JA JL. Performed the experiments: SY YK. Analyzed the data: JA SY SK JL. Wrote the paper: JA SY SK.
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