Cross-Repressive Interactions between SOC1 and the GATAs GNC and GNL/CGA1 in the Control of Greening, Cold Tolerance, and Flowering Time in Arabidopsis[1][W][OPEN]

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The paralogous and functionally redundant GATA transcription factors GNC (for GATA, NITRATE-INDUCIBLE, CARBON-METABOLISM INVOLVED) and GNL/CGA1 (for GNC-LIKE/CYTOKININ-RESPONSIVE GATA FACTOR1) from Arabidopsis (*Arabidopsis thaliana*) promote greening and repress flowering downstream from the phytohormone gibberellin. The target genes of GNC and GNL with regard to flowering time control have not been identified as yet. Here, we show by genetic and molecular analysis that the two GATA factors act upstream from the flowering time regulator *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*) to directly repress *SOC1* expression and thereby repress flowering. Interestingly, this analysis inversely also reveals that the MADS box transcription factor SOC1 directly represses *GNC* and *GNL* expression to control cold tolerance and greening, two further physiological processes that are under the control of SOC1. In summary, these findings support the case of a cross-repressive interaction between the GATA factors GNC and GNL and the MADS box transcription factor SOC1 in flowering time control on the one side and greening and cold tolerance on the other that may be governed by the various signaling inputs that are integrated at the level of *SOC1* expression.

Throughout evolution, plants have acquired the ability to adapt their growth and flowering to their environmental conditions to guarantee optimal reproductive success in their individual growth environments. Various signaling pathways are known that are integrated to control flowering time in response to light quality, daylength, temperature, and nutrient availability (Parcy, 2005; Franks et al., 2007; Izawa, 2007; Turck et al., 2008). In Arabidopsis (*Arabidopsis thaliana*), genetic studies have identified the transcription factors *FLOWERING LOCUS T* (*FT*), *LEAFY*, and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*) as major integrators of flowering (Nilsson et al., 1998; Samach et al., 2000). FT protein was shown to act as the florigen that moves from the leaves in inductive conditions to the shoot apical meristem, where it promotes the transition from vegetative to reproductive meristem identity by forming a complex with the bZIP transcription factor FLOWERING LOCUS D (Corbesier et al., 2007; Mathieu et al., 2007; Taoka et al., 2011).

Under inductive long days, expression of the MADS box transcription factor *SOC1* is essential for floral induction (Samach et al., 2000; Yoo et al., 2005). Under short days, SOC1 appears also to be the major integrator of flowering time stimulation in response to the phytohormone GA (Blázquez and Weigel, 1999; Moon et al., 2003). *SOC1* expression is furthermore repressed by the MADS box transcription factor FLOWERING LOCUS C, which controls flowering in response to long cold periods (vernalization) and acts together with the MADS box transcription factor SHORT VEGETATIVE PHASE (Hartmann et al., 2000; Li et al., 2008; Tao et al., 2012). Additionally, *SOC1* expression is controlled by an age-dependent regulatory system that involves SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) transcription factors and their antagonistic microRNA regulator miRNA156 (Wang et al., 2009).

SOC1 expression and flowering are also repressed in response to short periods of cold that plants experience, such as during a cold spring season (Seo et al., 2009). During such cold periods, *SOC1* expression is reduced and the concomitant delay in flowering correlates with an increase in the expression of cold-regulated genes such as *C-REPEAT/DROUGHT-RESPONSIVE ELEMENT-BINDING FACTOR* (*CBF*) and cold-response marker genes such as *COLD-REGULATED15a* (*COR15a*) and *COR15b*. Inversely, *SOC1* overexpression results in the inverse regulation of cold-regulated genes, and this supports experimental data that indicate that SOC1 is a direct regulator of cold-regulated genes (Seo et al., 2009).

GAs have been implicated in a variety of growth responses in plants, including the control of flowering

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time (Hisamatsu and King, 2008; Itoh et al., 2008; Schwechheimer, 2011). GAs are perceived by the GIBBERELLIC ACID-INSENSITIVE DWARF1 GA receptors, which in turn bind to and induce the degradation of DELLA repressors such as GIBBERELLIC ACID INSENSITIVE (GAI) and REPRESSOR OF *ga1-3* (RGA) from Arabidopsis (Ueguchi-Tanaka et al., 2005; Griffiths et al., 2006; Willige et al., 2007). DELLA proteins repress a broad range of different transcription factor activities, including that of the PHYTOCHROME INTERACTING FACTORS (PIFs), basic helix-loop-helix transcription factors that integrate GA as well as light signaling by interacting with DELLAs, and the phytochrome light receptors (de Lucas et al., 2008; Feng et al., 2008; Gallego-Bartolomé et al., 2010). Inversely, GA responses appear completely derepressed in mutants deficient in the function of the GlcNAc transferase SPINDLY (SPY; Jacobsen and Olszewski, 1993; Wilson and Somerville, 1995; Silverstone et al., 2007).

In long-day-grown wild-type plants, flowering is typically only moderately promoted by GA, because growth conditions in laboratory experiments are adjusted to guarantee a short generation time (Reeves and Coupland, 2001; Galvão et al., 2012). *DELLA* gene loss-of-function mutants with a constitutive GA response or *spy* mutants do not flower much faster than the wild type when grown under long days (Achard et al., 2003; Tseng et al., 2004). Based on the observation that the Landsberg *erecta* (L*er*) allele of the GA biosynthesis mutant *ga1-3* displays only a comparatively minor delay in flowering under long-day conditions, GA has for a long time been thought to be of minor importance for floral induction under long days. In contrast, *ga1-3* mutants failed to flower even after 5 to 6 months in short-day conditions, and this has given rise to the long-standing hypothesis that, in Arabidopsis, GA is essential for flowering only under short days (Wilson et al., 1992; Reeves and Coupland, 2001). Several recent studies, however, report a strong flowering time delay also under long days in *ga1* mutants and other Arabidopsis GA pathway mutants when examined in the Columbia ecotype (Willige et al., 2007; Hisamatsu and King, 2008; Galvão et al., 2012; Porri et al., 2012).

Plants use GAs also to control plant growth in response to abiotic stress such as cold, salt, and oxidative stress as well as biotic stress caused by pathogens (Achard et al., 2006, 2007, 2008a, 2008b; Navarro et al., 2008). The growth constraint resulting from the exposure to cold temperature correlates with and can be explained by a cold-induced stabilization of the DELLAs and results in increased cold tolerance in Arabidopsis seedlings (Achard et al., 2008a). The identities of the genes that confer cold tolerance downstream of the DELLAs have not been revealed as yet.

We have previously identified the two functionally paralogous GATA family transcription factors *GNC* (for *GATA, NITRATE-INDUCIBLE, CARBON-METABOLISM INVOLVED*) and *GNL/CGA1* (for *GNC-LIKE/CYTOKININ-RESPONSIVE GATA FACTOR1*) from Arabidopsis as critical transcription targets downstream from GA, DELLAs, and PIFs (Bi et al., 2005; Naito et al., 2007; Richter et al., 2010). *GNC* and *GNL* expression is repressed in response to GA and increased in mutants with a block in GA signaling, such as *ga1* and *gid1abc*. In agreement with the repressive activity of GAs on *GNC* and *GNL* expression, *GNC* and *GNL* overexpression plants, where *GNC* and *GNL* regulation is uncoupled from GA control, resemble *ga1* or *gid1abc* mutants at the phenotypic and global gene expression levels. Similar to the GA pathway mutants, *GNC* and *GNL* overexpressors are dark-green dwarfs with a delay in germination and flowering (Richter et al., 2010). PIF3 is at least one member of the PIF family of transcription factors that controls *GNC* and *GNL* expression. As recently reported, the greening phenotype of *gnc* and *gnl* mutants may be explained by their role in directly and indirectly regulating the expression of chlorophyll biosynthetic genes and chloroplast development, growth, and division (Hudson et al., 2011; Chiang et al., 2012). *GNC* and *GNL* have also been identified as transcription repression targets of the floral homeotic genes APETALA3 and PISTILLATA (Mara and Irish, 2008). In the context of the role of GNC and GNL in regulating chlorophyll biosynthesis, it has been proposed that repression of the two GATAs is at least in part responsible for the nongreening of petals and stamens (Mara and Irish, 2008).

In this study, we show that GNL, as a representative GATA factor for the two functionally homologous GATAs GNC and GNL, represses flowering downstream from GA signaling and upstream from *SOC1*. We further show that *GNL* overexpression results in a decrease in *SOC1* expression and that both GATAs can directly bind to the *SOC1* promoter, suggesting that GNL may be a direct transcriptional repressor of *SOC1* expression. Interestingly, we also find that GNC and GNL promote two other SOC1-dependent physiological responses downstream from SOC1, greening and cold tolerance, and that SOC1 can directly bind to the promoters of both GATAs. We thereby present a case for a cross-repressive interaction of GNC and GNL on the one side and SOC1 on the other in the control of flowering time, greening, and cold tolerance.

RESULTS

GNC and *GNL* Repress Flowering Downstream from GA Signaling

We have previously established that the GATA transcription factors GNC and GNL repress GA responses downstream from GA, DELLAs, and PIFs in Arabidopsis (Richter et al., 2010). The role for GNC and GNL in the control of flowering time is already suggested by a subtle acceleration in flowering in long-day-grown as well as short-day-grown *gnc gnl* mutants when compared with the wild type (Fig. 1, A and B; Supplemental Fig; S1; Richter et al., 2010). The repressive role of the two GATAs, however, is much more apparent in the GA-deficient *ga1* background,

where the loss of *GNC* and *GNL* in *ga1 gnc gnl* resulted in the partial suppression of the flowering time delay of the *ga1* mutant (Fig. 1, D–F; Richter et al., 2010). Here, we found that *ga1 gnc gnl* mutants (99.7 ± 7.1 leaves; 79.8 ± 5.7 d) flower about 1 month earlier than *ga1* (118.9 ± 9.1 leaves; 118.7 ± 10 d) when grown under long-day growth conditions. Since *ga1 gnc gnl* flowers still much later than the wild type (12.3 ± 1.1 leaves; 23.1 ± 1.1 d) or *ga1* mutants after GA treatment (Fig. 1, D, E, and G), these findings suggest that other regulators in addition to GNC and GNL must repress flowering in the absence of GA. One the one side, such regulators could be functionally homologous GATA factors closely related to GNC and GNL or, on the other side, other unrelated proteins (Richter et al., 2010).

In line with the proposed role of the two GATAs as repressors of flowering, we found that the overexpression of either *GNC* (*GNC*:*GFP*) or *GNL* (*YELLOW FLUORESCENT PROTEIN* [*YFP*]:*GNL*) results in a strong delay in flowering in the wild type (Fig. 1, A and B; Richter et al., 2010). In order to understand at which stage of flowering time regulation the *GATA* transgenes are active, we introduced *YFP*:*GNL*, which in our hands is the genetically more stable of the two GATA transgenes, into the *spy* and the *rga gai* DELLA gene loss-of-function backgrounds. Also in the early flowering *spy* mutant, which mimics the phenotype of a constitutive GA response mutant, we observed a strong delay in flowering in the presence of *YFP*:*GNL* (Fig. 1, H and I; Jacobsen and Olszewski, 1993). Flowering time was also strongly delayed in the *rga gai* loss-of-function mutant that normally flowers early, specifically in short-day conditions (Supplemental Fig. S2, A and B; Dill and Sun, 2001; Cheng et al., 2004). Since our previous analysis of *GNC* and *GNL* regulation had shown that the two *GATAs* are targets of PIF3, we also analyzed *GNL* overexpression in a *PIF3*:*MYC* overexpression line. Also in this background, flowering was strongly delayed, indicating that *GNL* represses flowering downstream from its transcriptional regulator PIF3 (Supplemental Fig. S2, C and D). In summary, these findings confirm the role of *GNL* as a repressor of flowering downstream from GA, DELLA, and PIF3. Since all our experiments at present suggest that GNC and GNL are functionally redundant, we speculate that these observations also hold true for *GNC*.

SOC1 Is an Essential Flowering Time Integrator Downstream from GNC and GNL

Different flowering time pathways converge on the regulation of the two positive flowering time regulators *FT* and *SOC1*. To understand the contribution of *FT* and *SOC1* expression to the flowering time phenotypes of the genotypes described above, we performed quantitative real-time (qRT)-PCR using 10-d-old seedlings to assess their transcript abundance in the presence of the *GATA* overexpression constructs (Fig. 1C). While the abundance of *FT* is not significantly altered by the presence or absence of the GATAs, the abundance of *SOC1* is strongly repressed when either *GNC* or *GNL* is overexpressed. Furthermore, the reduced transcript abundance of *SOC1* also correlates with the late-flowering phenotype of the *spy YFP*:*GNL* and *PIF3*:*MYC YFP*:*GNL* genotypes (Fig. 1J; Supplemental Fig. S2D). Inversely, *SOC1* transcript levels are increased in *ga1 gnc gnl* when compared with *ga1* (Fig. 1F).

Since these findings invited the hypothesis that GNC and GNL may be regulators of *SOC1* expression, we became interested in examining the relationship between *SOC1* and the GATAs in more detail. To this end, we introduced the *YFP*:*GNL* overexpression transgene into a *SOC1*:*MYC* overexpression background. Flowering is strongly delayed in *soc1* loss-of-function mutants (32.7 ± 2.8 leaves) and slightly accelerated in a *SOC1*:*MYC* overexpression line (10.2 ± 0.7 leaves) when compared with the wild type (12.7 ± 1.0 leaves; Fig. 2, A and B). Interestingly, and unlike what we had observed when we introduced *YFP*:*GNL* into the GA pathway mutants (Fig. 1), *YFP*:*GNL* overexpression does not delay flowering in the *SOC1*:*MYC* background, where SOC1 expression is under the control of the 35S cauliflower mosaic virus promoter and thus uncoupled from its native transcriptional regulation (10.6 ± 0.9 leaves; Fig. 2, A and B). This suggests that *SOC1*:*MYC* promotes flowering downstream from *GNL*. Using promoter-GUS lines for *SOC1* ($SOC1_{Pro}$:*GUS*), *GNC* (GNC_{Pro}:*GUS*), and *GNL* (GNL_{Pro}:*GUS*), we could show in subsequent experiments that the three genes are coexpressed in the leaves of 10-d-old seedlings and that the overexpression of *YFP*:*GNL* represses *SOC1*:*GUS* expression in this tissue (Fig. 2C).

Since we could identify various GATA boxes in the promoter of *SOC1*, we reasoned that GNL and possibly also GNC may regulate *SOC1* expression directly (Fig. 2D). To test this hypothesis, we performed chromatin immunoprecipitation (ChIP) for GNC:GFP and YFP:GNL and, indeed, detected a strong binding of both GATA factors to two GATA boxes (boxes I and II) in the first intron and exon of the *SOC1* promoter (Fig. 2, E and F). In support of a model where the decrease in *SOC1* transcript abundance in the *GNC*:*GFP* or *YFP*:*GNL* overexpressors is the result of transcriptional repression, we further detected decreased abundance of K9-acetylated histone 3 (H3K9Ac), a marker for active chromatin, and increased abundance of K9-dimethylated histone 3 (H3K9me2), a marker for inactive chromatin, at two promoter regions, Hb and Hc, that are proximal to the ATG start codon of *SOC1* (Fig. 2, G and H). At the same time, binding of the two histone H3 variants to a more remote site, Ha, was unaltered (Fig. 2, G and H). A similar H3-variant chromatin-binding preference was observed when we tested H3K9Ac and H3K9me2 binding to the *SOC1* promoter in the GA-insensitive *gai-1D* mutant, where *GNC* and *GNL* expression is increased as a consequence of DELLA protein stabilization (Fig. 2, G and H; Supplemental Fig. S3; Richter et al., 2010). Since this analysis may suffer from the criticism that the observed promoter

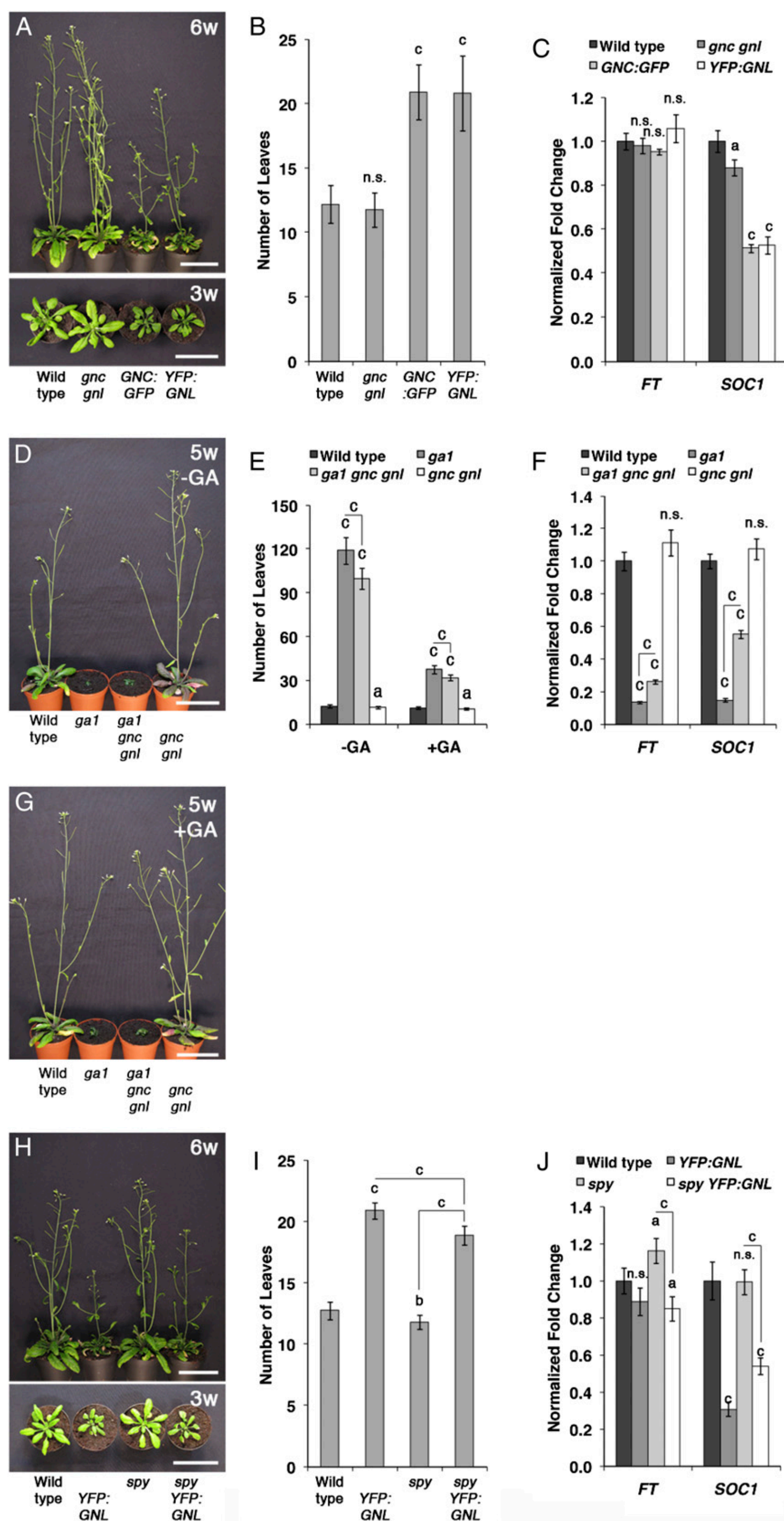

Figure 1. GNC and GNL repress flowering downstream from the GA signaling pathway. A, D, G, and H, Representative photographs of Arabidopsis plants grown for 5 or 6 weeks (w) under long-day conditions. Plants shown in G were watered twice per week with 1 μM GA_3. B, E, and I, Flowering time analysis (total rosette leaf number) of long-day-grown plants shown in A, D, G, and H. C, F, and J, Results of qRT-PCR analyses for the flowering time regulators *FT* and *SOC1* performed with 10-d-old seedlings. Fold change was calculated relative to wild-type levels. Student's *t* tests were performed in comparison with the wild type unless indicated otherwise: a = $P \leq 0.05$, b = $P \leq 0.01$, c = $P \leq 0.001$; n.s., not significant. Bars = 5 cm.

binding is the result of an off-target amplification of the overexpressed *YFP*:*GNL*, we established also a GNL_{Pro}:*GNL*:*HA* transgene that complemented the *gnc gnl* mutant phenotype (Supplemental Fig. S4, A and B). We then tested binding of the hemagglutinin (HA)-tagged GNL protein to promoter boxes IV (negative

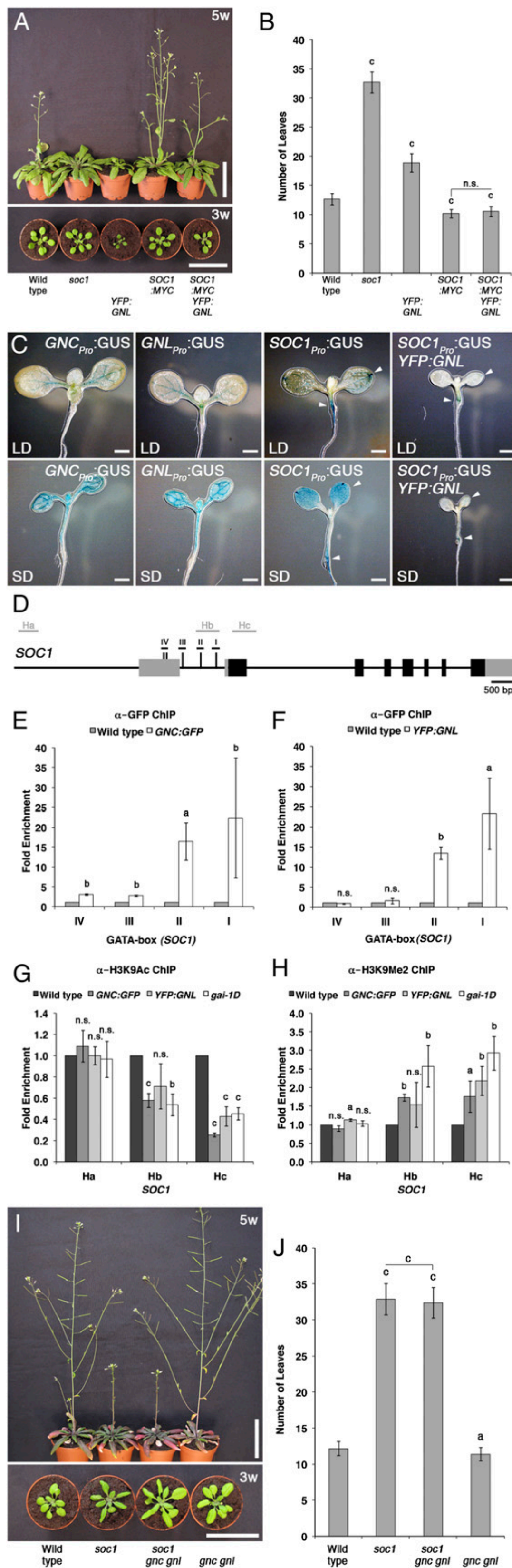

Figure 2. GNC and GNL act upstream of *SOC1*. A, Representative photographs of 3- and 5-week-old (w) Arabidopsis plants. B, Flowering time analysis (total rosette leaf number) of long-day-grown plants shown in A. C, GUS expression in the first pair of true leaves in 10-d-old long-day-grown (LD) and short-day-grown (SD) Arabidopsis seedlings expressing GNC_{Pro}:*GUS*, GNL_{Pro}:*GUS*, and $SOC1_{Pro}$:*GUS* in the wild type and the *YFP*:*GNL* overexpression background. Arrows indicate expression differences between the *GUS* transgenes. Note that *YFP*:*GNL* overexpression results in a dwarfed seedling phenotype (Richter et al., 2010). D, Schematic representation of the *SOC1* genomic locus. Black boxes, exons; gray boxes, untranslated regions; underlined roman numbers, promoter regions containing GATA boxes (vertical lines); gray lines, predicted nucleosome-binding regions. E and F, Fold enrichment (*GNC*:*GFP*/wild type [E] and *YFP*:*GNL*/wild type [F]) of promoter fragment amplification after ChIP-PCR with anti-GFP. G and H, Fold enrichment after ChIP with anti-H3K9Ac and anti-H3K9me2 and promoter fragment amplification by PCR when compared with the wild type. I, Representative photographs of 6-week-old Arabidopsis plants. J, Flowering time analysis (total rosette leaf number) of long-day-grown plants shown in I. Student's *t* tests were performed in comparison with the wild type unless indicated otherwise: a = $P \leq 0.05$, b = $P \leq 0.01$, c = $P \leq 0.001$; n.s., not significant. Bars = 5 cm.

control) and I (positive control) and could in both cases confirm the negative (IV) as well as the positive (I) binding of GNL:HA to the predicted target sites in the *SOC1* promoter (Supplemental Fig. S4C).

Since our experiments indicated that GNC and GNL are direct transcriptional repressors upstream from *SOC1*, we also expected that the late-flowering phenotype of *soc1*, unlike the late-flowering phenotype of the *ga1* mutant, is not suppressed in a *soc1 gnc gnl* triple mutant. Indeed, we found no significant difference in the late flowering of *soc1 gnc gnl* (32.4 ± 2.1 leaves) when compared with the *soc1* single mutant (32.9 ± 2.2 leaves). Therefore, we concluded that *SOC1* is critical for flowering time control downstream from *GNC* and *GNL* (Fig. 2, I and J). In line with a role of GA upstream of *SOC1* in the control of flowering, we also noted that the flowering time delay of the *soc1* mutant cannot be suppressed by GA treatments that are sufficient to accelerate flowering in the wild type, suggesting that SOC1 is essential to promote flowering downstream from GA (Supplemental Fig. S5, A and B). In summary, these findings suggest that GNC and GNL are direct repressors of *SOC1* and that the *GNC*- and *GNL*-dependent repression of *SOC1* transcript abundance correlates with activating and repressive chromatin changes at the *SOC1* promoter and with the late-flowering phenotype of *GNC* and *GNL* overexpressors. Thus, *SOC1* is the essential flowering time integrator downstream from *GNC* and *GNL*.

GNC and *GNL* Promote Greening Downstream from *SOC1*

A reduction in chlorophyll content had been the original phenotype associated with the *gnc* mutant in Arabidopsis (Bi et al., 2005). Both *GNC* and *GNL* have recently been characterized as positive regulators of chlorophyll biosynthesis and chloroplast division (Richter et al., 2010; Hudson et al., 2011; Chiang et al.,

2012). *gnc gnl* loss-of-function mutants are light green; conversely, *GNC* and *GNL* overexpression lines show increased greening and chlorophyll accumulation. Interestingly, we observed that the *soc1 gnc gnl* triple mutant is visibly less green and contains less chlorophyll than the dark-green *soc1* single mutant (Fig. 3A; Supplemental Fig. S6). When compared with *soc1* and the wild type, this reduction in chlorophyll accumulation of the *soc1 gnc gnl* triple mutant also correlated with a decrease in the transcript abundance of the three *PROTOCHLOROPHYLLIDE OXYDOREDUCTASE* (*POR*) genes *PORA*, *PORB*, and *PORC*, the gene products of which control a critical step in chlorophyll biosynthesis (Fig. 3B; Thomas, 1997). While our analysis of flowering time control had suggested that *GNC* and *GNL* function upstream of *SOC1*, the greening phenotype suggested a role of *GNC* and *GNL* downstream from *SOC1* in the control of chlorophyll biosynthesis. This antagonism in the genetic interaction between the *GATAs* and *SOC1* with regard to greening and flowering time control, respectively, was further supported by the greening phenotype of the *YFP*:*GNL* *SOC1*:*MYC* background. While we had observed that the *YFP*:*GNL* overexpressor cannot delay flowering in the early-flowering *SOC1*:*MYC* background, we found that the overexpression of *YFP*:*GNL* resulted in a stronger chlorophyll accumulation as well as increased *POR* transcript levels in *SOC1*:*MYC* (Fig. 3, C and D). In this regard, the *YFP*:*GNL* overexpressor when introduced into *SOC1*:*MYC* behaved in a similar manner as it did in the *PIF3*:*MYC* or *spy* background, and this finding suggested that *YFP*:*GNL* is downstream of SOC1 in the control of greening (Fig. 3, E–H). Furthermore, while GA treatments had not been sufficient to promote flowering in *soc1* (Supplemental Fig. S5, A and B), indicating that *SOC1* acts downstream of GA signaling in the control of flowering, we found that GA treatments of *soc1* are sufficient to suppress the *soc1* mutant's greening phenotype, clearly demonstrating that *SOC1* is upstream of GA in the control of greening (Supplemental Fig. S5C). In summary, these findings suggested a role for *YFP*:*GNL* downstream from the GA pathway and *SOC1* in the control of chlorophyll biosynthesis and greening.

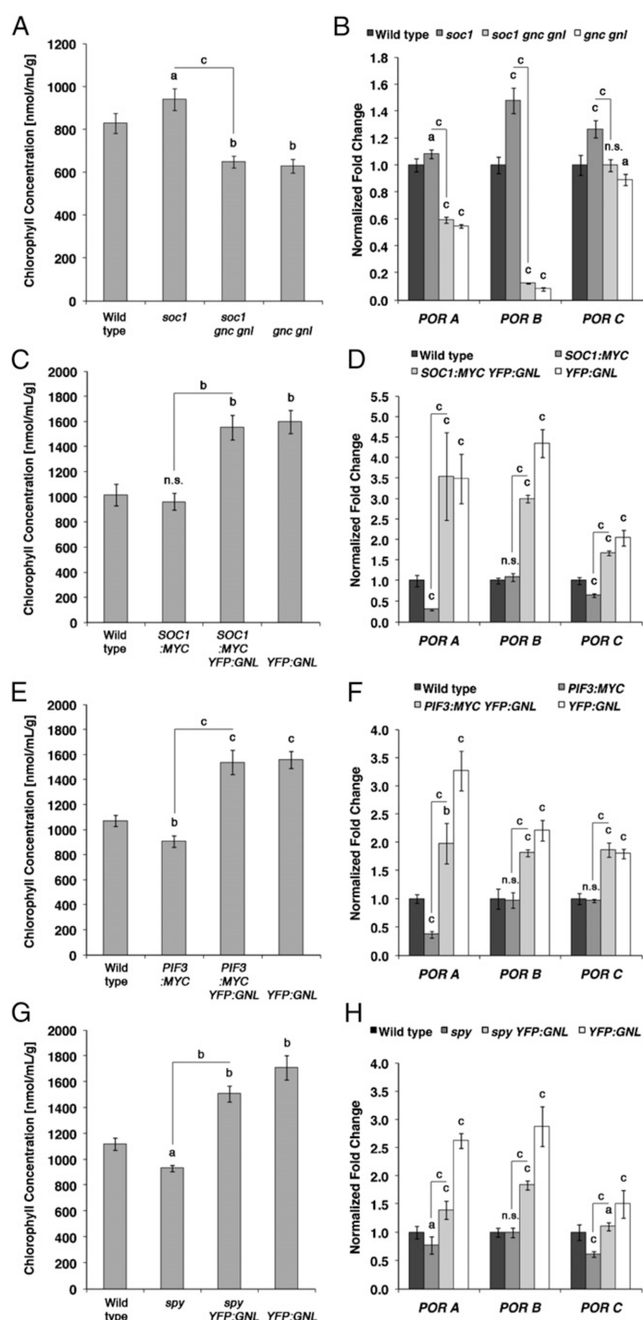

Figure 3. GNC and GNL promote greening downstream from *SOC1*. A, C, E, and G, Chlorophyll concentration. B, D, F, and H, qRT-PCR analyses of *POR* gene expression from 10-d-old seedlings as shown in A, C, E, and G. Student's *t* tests were performed in comparison with the wild type unless indicated otherwise: a = $P \leq 0.05$, b = $P \leq 0.01$, c = $P \leq 0.001$; n.s., not significant.

GNC and *GNL* Promote Cold Tolerance Downstream from *SOC1*

Besides its prominent role in the regulation of flowering time, SOC1 was previously also shown to interfere with cold tolerance (Seo et al., 2009). Interestingly, a qRT-PCR analysis of mock- and cold-treated 10-d-old seedlings revealed that *GNC* and *GNL* are regulated by cold temperatures, pointing at a putative role of the two genes in cold response (Fig. 4A). Indeed, when we tested cold tolerance in nonacclimated and cold-acclimated *gnc gnl* loss-of-function mutants and the *GNC*:*GFP* or *YFP*:*GNL* overexpression lines, we found that seedling survival in the *GNC* or *GNL* overexpressors is approximately twice as high as in the wild type, regardless of prior acclimation (Fig. 4B). At the molecular level, the increased survival correlated with an increase in the expression of *CBF2* and the two cold-response marker genes *COR15a* and *COR15b* (Fig. 4, C and D; Medina et al., 1999).

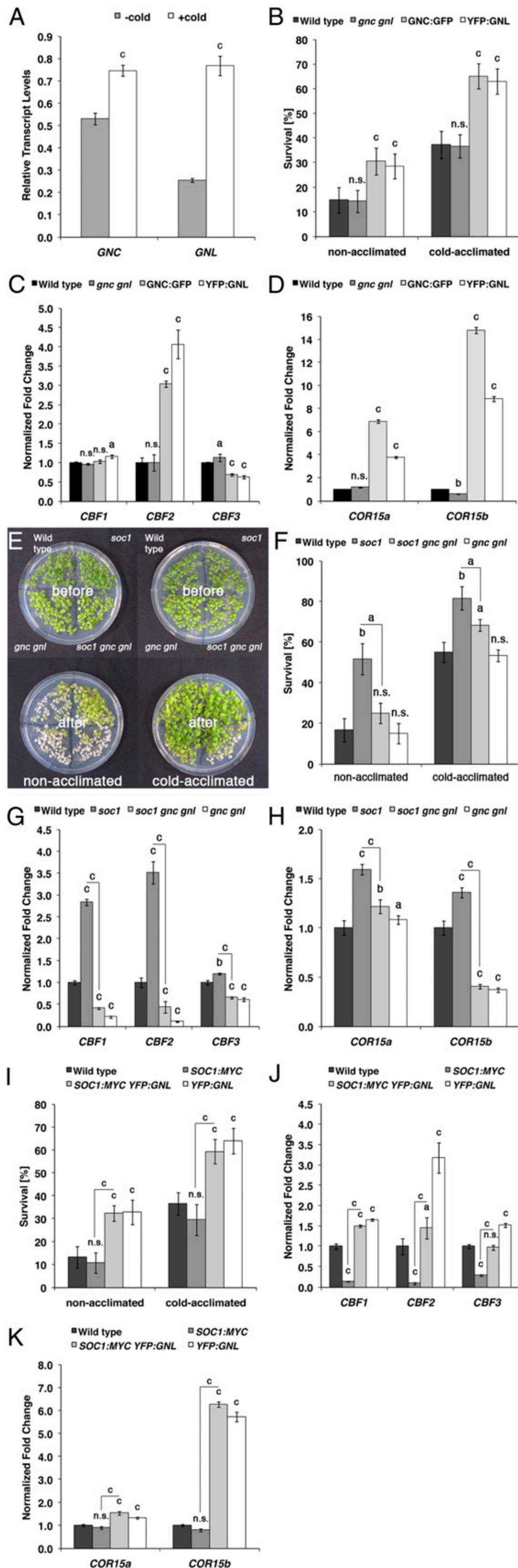

Figure 4. *GNC* and *GNL* promote cold tolerance downstream from *SOC1*. A, qRT-PCR analyses of *GNC* and *GNL* expression of 10-d-old seedlings in response to a 9-h cold treatment (4°C). B, F, and I, Seedling survival of nonacclimated and cold-acclimated 10-d-old seedlings after cold treatment. C, D, G, H, J, and K, qRT-PCR analyses of *CBF* (C, G, and J) and *COR15* (D, H, and K) gene expression in 10-d-old seedlings. E, Representative photograph of nonacclimated and cold-acclimated 10-d-old seedlings before (top panels) and after (bottom panels) cold treatment. Student's *t* tests were performed in comparison with the wild type unless indicated otherwise: a = $P \leq 0.05$, b = $P \leq 0.01$, c = $P \leq 0.001$; n.s., not significant.

As we will show later, we also detected strongly increased *GNC* and *GNL* transcript levels in the *soc1* mutant (Fig. 6A). Since increased cold tolerance is also a phenotype of *soc1* mutants (Seo et al., 2009), we reasoned that the elevated *GNC* and *GNL* transcript levels in *soc1* may be causative for the cold tolerance phenotype of this mutant. In support of this hypothesis, we found that the increased cold tolerance phenotype of *soc1* is strongly suppressed in the *soc1 gnc gnl* triple mutant (Fig. 4, E and F). This genetic suppression of the *soc1* cold tolerance phenotype was mirrored by a decrease in the abundance of the three *CBF* genes as well as that of *COR15a* and *COR15b* in *soc1 gnc gnl* when compared with the *soc1* single mutant (Fig. 4, G and H). Along the same lines, and in contrast to the dominant effect of *SOC1*:*MYC* overexpression over *YFP*:*GNL* overexpression with regard to the control of flowering, we observed that the overexpression of *YFP*:*GNL* is dominant over the overexpression of *SOC1*:*MYC* with regard to the cold tolerance of non-acclimated and cold-acclimated seedlings, *CBF* expression, as well as *COR* expression (Fig. 4, I–K). Taken together, these findings suggest that the cold tolerance phenotype of *soc1* is at least partially mediated by the increase in *GNC* and *GNL* transcript abundance and that *GNC* and *GNL* function downstream from *SOC1* in the control of cold tolerance.

It was previously established that cold-induced growth arrest and cold tolerance in Arabidopsis are promoted by DELLA repressors and that the exposure to cold temperatures correlates with an increase in DELLA protein abundance (Achard et al., 2008a). In line with a cold-induced stabilization of the DELLAs, we also detected increased levels of the DELLA protein RGA in 10-d-old cold-treated wild-type and *gnc gnl* seedlings (Fig. 5A). Interestingly, however, we also found that RGA levels are increased already at ambient temperature in *GNC* and *GNL* overexpressors of the same age (Fig. 5A). Thus, the *GNC* and *GNL* overexpressors mimic the DELLA accumulation phenotype of cold-treated wild-type seedlings. Since we had previously established that DELLA protein abundance correlates with *GNC* and *GNL* transcript abundance (Richter et al., 2010), we reasoned that the increased transcript abundance of the *GATAs* may be the molecular cause for the cold tolerance phenotype of *ga1*. However, when we compared the cold tolerance phenotype of *ga1 gnc gnl* with that of *ga1*, we detected only a mild, and in our conditions only in

cold-acclimated conditions statistically significant, suppression of the cold tolerance phenotype of *ga1* when examining *ga1 gnc gnl* (Fig. 5B). Surprisingly, the molecular analysis performed in parallel revealed a strong reduction in *CBF* and *COR15* gene expression when compared with *ga1* that we would have expected to correlate with a strong decrease in cold tolerance (Fig. 5, C and D). The absence of a strong suppression of the *ga1* cold tolerance phenotype and the concomitant presence of a molecular suppression of *CBF* and *COR* gene expression suggests that the accumulation of *GNC* and *GNL* transcript alone is not causative for the cold tolerance observed in the GA-deficient *ga1* mutant, that other genes downstream from GA and DELLA promote cold tolerance in the absence of GA, and that the reduction of *CBF* and *COR* transcript abundance is not sufficient to predict cold tolerance in a *ga1* mutant background.

When we subsequently explored the relationship between *YFP*:*GNL* and the *rga gai* loss-of-function mutant with regard to cold tolerance, we found that *YFP*:*GNL* expression can induce cold tolerance also in this background, indicating that cold tolerance is promoted by *GNL* downstream from GA and DELLAs and, thus, that accumulation of the DELLAs as observed in *YFP*:*GNL* is not causative for promoting cold tolerance (Fig. 5E). In summary, these findings suggest that GNC and GNL as well as other factors are required to confer cold tolerance in the absence of GA. In this respect, the partial suppression of the cold tolerance phenotype of *ga1* in *ga1 gnc gnl* is reminiscent of the partial suppression of the flowering time defect of *ga1* in *ga1 gnc gnl*. As proposed above for the control of flowering time in the *ga1 gnc gnl* mutant, the cold response phenotype of *ga1* also may be regulated by other GATA transcription factors or by unrelated regulators in addition to GNC and GNL.

SOC1 Represses *GNC* and *GNL* Transcription

The suppression of the *soc1* greening and cold tolerance phenotypes in *soc1 gnc gnl* suggested a regulation of *GNC* and *GNL* gene expression by SOC1. Indeed, and as already mentioned before, we detected strongly increased *GNC* and *GNL* transcript levels in the *soc1* mutant when compared with the wild type (Fig. 6A). Since this may be the consequence of a direct repressive activity of SOC1 on the promoters of the two *GATA* genes, we tested the binding of SOC1:MYC to CArG boxes in the *GNC* and *GNL* promoters using ChIP analysis. Indeed, we found binding of SOC1 to two promoter regions that span four CArG boxes in each gene promoter (boxes I and II in *GNC* and boxes I and III in *GNL*; Fig. 6, B–D). In both promoters, we further found increased binding of the open chromatin marker H3K9Ac in the *soc1* mutant at a nucleosome-binding site proximal to their ATG start codons (Hb), while binding to a distal site (Ha) was unaffected (Fig. 6, E and F). Although the effect of *SOC1*:*MYC* overexpression on the transcript abundance of *GNC* and *GNL* was not strong when we examined whole seedlings using qRT-PCR, the repressive action of SOC1 on *GNL* became apparent when we examined GNL_{Pro}:*GUS* expression in the wild type and the *SOC1*:*MYC* background. Here, a strong reduction of GNL_{Pro}:*GUS* expression was detectable (e.g. in the upper region of the hypocotyl and the shoot meristem of long-day-grown seedlings; Fig. 6G). Taken together, these observations suggest that SOC1 is a direct transcriptional repressor of *GNC* and *GNL* transcription. There is thus a cross-repressive interaction between *SOC1* and the two GATA factor genes. Their respective gene products can reciprocally repress their transcription and thereby promote or repress SOC1-dependent flowering or GNC- and GNL-dependent greening and cold tolerance, respectively.

DISCUSSION AND CONCLUSION

In this study, we analyze the role of the functionally paralogous GATA factors GNC and GNL in flowering time control, greening, and cold tolerance. This analysis was stimulated by our observations that GNC and GNL repress flowering in the GA-deficient *ga1* mutant, on the one side, and that overexpression of the GATA factors is sufficient to delay flowering in the wild type, on the other (Fig. 1, D and E; Richter et al., 2010). Furthermore, we found here that overexpression of *GNL*, as a representative for the two GATAs, can delay flowering in a range of early-flowering GA pathway mutants. These findings are thus in line with a role of the two GATAs as repressors of flowering downstream from the GA signaling pathway. Our further observations that GNC and GNL repress flowering in wild-type plants only to a minor extent, under long-day as well as short-day conditions, and that GNC and GNL only partially repress flowering in *ga1* suggest that other regulators in addition to GNC and GNL repress flowering in the absence of the two GATAs. Several possibilities can be envisioned that can serve to explain this phenotype. First, we know from our previous analyses and the analysis of others that the *gnl* allele (SALK_003995) used in our study has reduced *GNL* expression but is not a null allele (Mara and Irish, 2008). Unfortunately, we and others have been unable in repeated attempts to reisolate a presumed *gnl* null allele (SALK_021362; Bi et al., 2005; Mara and Irish, 2008). Thus, the residual flowering time repression in *ga1 gnc gnl* could potentially be the result of repression by residual GNL. Second, it may be that other related GATA factors repress flowering in addition to GNC and GNL. Arabidopsis has at least four GATA factors that are closely related to GNC and GNL and that may act redundantly in the repression of flowering in the absence of *ga1* (Richter et al., 2010). Third, it may be that other unrelated proteins repress flowering in addition to GNC and GNL in the absence of GA. In this regard, it is interesting that the protein SPL9 was recently identified as a DELLA-controlled flowering time

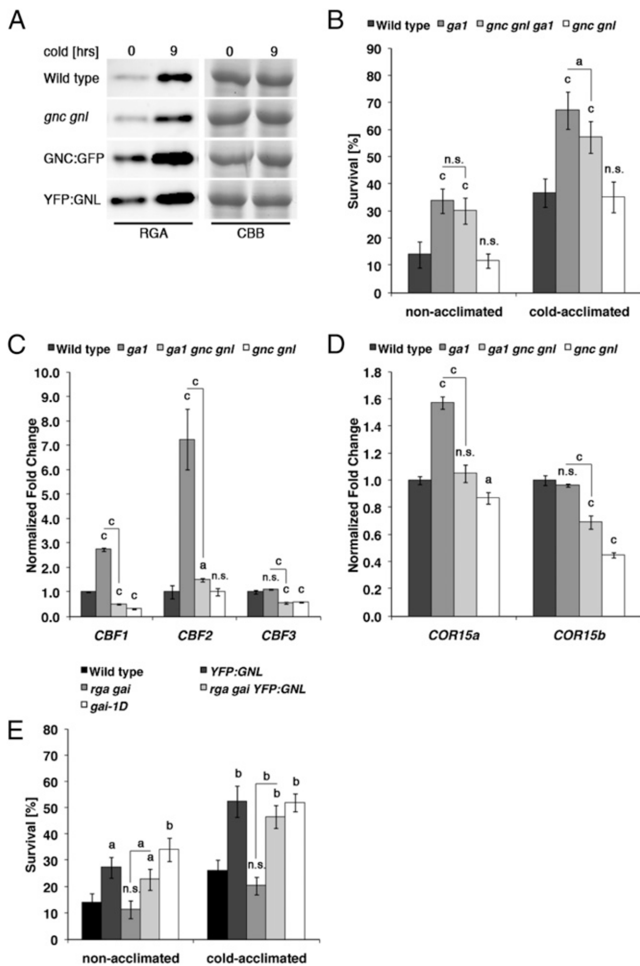

Figure 5. Analysis of GNC- and GNL-mediated cold tolerance in GA pathway mutants. A, Immunoblot of RGA protein abundance in 10-d-old Arabidopsis seedlings grown before and after a 9-h cold treatment (4°C). CBB, Coomassie Brilliant Blue as a loading control. B and E, Seedling survival of nonacclimated and cold-acclimated 10-d-old seedlings after cold treatment. C and D, qRT-PCR analyses of *CBF* (C) and *COR15* (D) gene expression in 10-d-old seedlings. Student's *t* tests were performed in comparison with the wild type unless indicated otherwise: a = $P \leq 0.05$, b = $P \leq 0.01$, c = $P \leq 0.001$; n.s., not significant.

regulator in Arabidopsis and that GA was recently described to promote the expression of the *SPL* genes *SPL3*, *SPL4*, and *SPL5* in a *SOC1*-dependent manner (Wu et al., 2009; Jung et al., 2012; Yu et al., 2012). Thus, DELLA repression of *SPL* gene expression and SPL protein activity could potentially delay flowering in *ga1* in parallel to GNC and GNL.

That the suppression of the phenotypes caused by the GA deficiency of *ga1* must be genetically complex is already suggested by the results of several *ga1* suppressor screens that had been conducted in the past. In summary, only three genetic loci were identified as genetic suppressors of *ga1*, *RGA*, *SPY*, as well as a gain-of-function allele of the F-box protein subunit SLEEPY1 that promotes DELLA protein degradation (Wilson and Somerville, 1995; Silverstone et al., 1997; Fu et al., 2004). In fact, although it is known to date that all five Arabidopsis DELLA proteins contribute to the strong phenotype of the *ga1* mutant, only the suppression of *ga1* by the *RGA* loss-of-function allele significantly suppressed the *ga1* mutant (Silverstone et al., 1997; Cheng et al., 2004). In turn, the founding member of the DELLA gene family, *GAI*, had been identified based on the gain-of-function phenotype of the *gai-1* mutant, which, as we understand now, encodes for a GA-insensitive stabilized variant of GAI, and the reversion from this gain-of-function phenotype in the intragenic *gai-t6* suppressor mutation (Peng et al., 1997). Here, we see some parallels between the suppression of *ga1* by the loss of multiple DELLA genes that ultimately fully suppressed the *ga1* phenotype and the partial suppression of *ga1* in *ga1 gnc gnl* that could potentially be enhanced by the loss of other GATA factors or other regulators. Along the same lines, we see parallels in the genetics of the *DELLA* and the *GNC* or *GNL* loss-of-function mutants. In both cases, the repressive function is not obvious in the wild-type background but only apparent in GA deficiency or when protein content is increased due to a stabilization of the DELLA protein or due to an overexpression of the GATA factor.

While *GNL* overexpression delays flowering in the wild type, we further found that *GNL* overexpression cannot delay flowering when *SOC1* is overexpressed; its expression is thus uncoupled from its normal transcriptional control in the wild type. Since the strong delay in flowering in our *GNL* overexpression studies correlated with the repression of SOC1, we hypothesized and subsequently tested successfully by ChIP-PCR that GNC and GNL directly bind to *SOC1* to mediate its transcriptional repression. Using a complementing *GNL* promoter-driven *GNL* transgene, we could further substantiate our findings of *SOC1* as a direct target of the GATAs and thus eliminate the possible criticism that the binding observed with ChIP-PCR using GNC:GFP and YFP:GNL is an artifactual off-target binding and amplification resulting from their overexpression. Taken together, our data strongly support a model whereby GNC and GNL are transcriptional repressors of *SOC1*.

Interestingly, our genetic interaction studies revealed an inverse relationship between SOC1 and the GATAs in the control of greening and cold tolerance. The regulatory role of SOC1 on the expression of *GNC* and *GNL* became apparent due to the visible suppression of the enhanced greening phenotype of *soc1* in a *soc1 gnc gnl* mutant. Since SOC1 has been implicated in the control of cold tolerance, a finding that was initially based on the observation that cold-regulated genes are expressed in *soc1* mutants even at ambient temperature, we also tested the contribution of *GNC* and *GNL* to the enhanced cold tolerance of the *soc1* mutant as well as the cold tolerance phenotype of the late-flowering *GNC* and *GNL* overexpression lines. Here, we observed a suppression of the increased cold tolerance of *soc1* in the absence of *GNC* and *GNL* as well as an increase in cold tolerance when the two *GATAs* are overexpressed. It

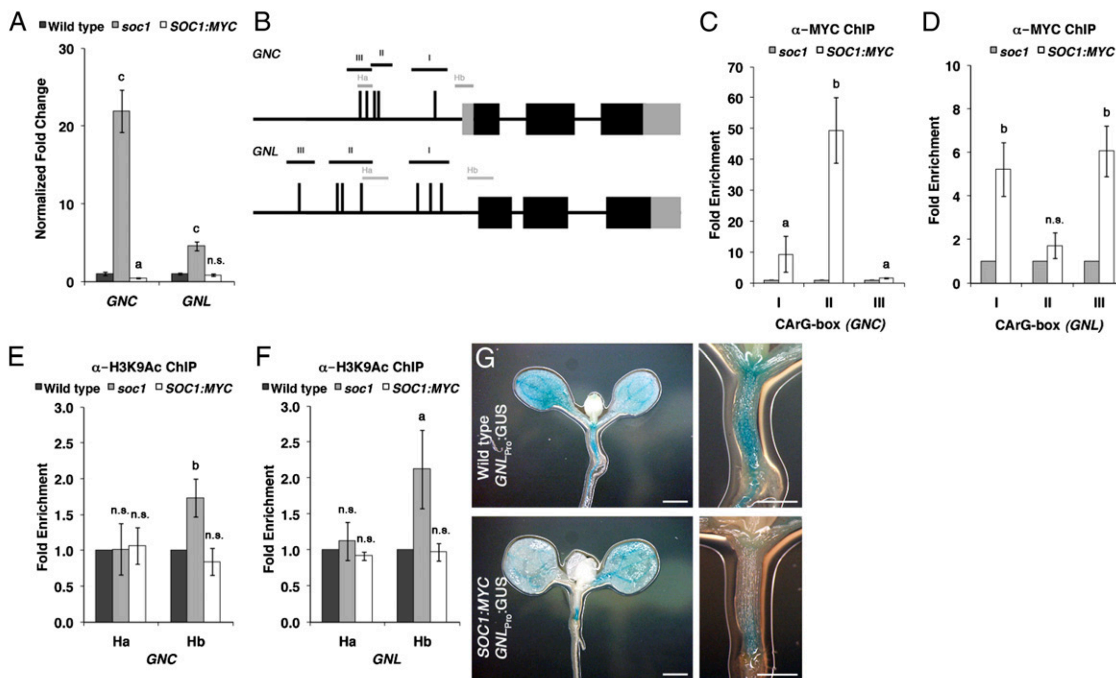

Figure 6. SOC1 is a direct repressor of *GNC* and *GNL* transcription. A, qRT-PCR analysis of *GNC* and *GNL* transcript abundance in *soc1* and *SOC1*:*MYC*. B, Schematic representation of the *GNC* and *GNL* genomic loci. Black boxes, exons; gray boxes, untranslated regions; underlined roman numbers, promoter regions containing CArG boxes (vertical lines); gray lines, predicted nucleosome-binding regions Ha and Hb. Note that there is no known 5′ untranslated region for *GNL*. C and D, Fold enrichment (*SOC1*:*MYC/soc1*) after PCR amplifications of *GNC* (C) and *GNL* (D) promoter fragments following ChIP with anti-MYC. E and F, Fold enrichment after ChIP with anti-H3K9Ac and PCR amplification of *GNC* (E) and *GNL* (F) promoter fragments in the wild type, *soc1*, and *SOC1*:*MYC*. G, Representative GUS staining of 10-d-old seedlings expressing *GNL*:*GUS* in the wild type and the *SOC1*:*MYC* background. Student's *t* tests were performed in comparison with the wild type unless indicated otherwise: a = $P \leq 0.05$, b = $P \leq 0.01$, c = $P \leq 0.001$; n.s., not significant. Scale bars = 0.5 mm.

was proposed that the antagonistic regulation of cold tolerance and flowering time by SOC1 may be relevant during short periods of cold temperature as they can be experienced by plants during cold periods in spring (Seo et al., 2009). Two studies have recently reported the genome-wide identification of direct SOC1 targets using ChIP (Immink et al., 2012; Tao et al., 2012). Interestingly, neither analysis has resulted in the identification of *GNC* or *GNL* as a direct SOC1 target. Since the different approaches employed make use of different parameters for data analysis, the differences in the results with regard to *GNC* and *GNL* promoter binding may simply be due to differences in the data analysis and stringency in data analysis.

Besides positioning the GATA factors and SOC1 within their respective pathways, our observation of a mutual repression of the GATAs and SOC1 with regard to flowering time control, on the one side, and greening and cold tolerance, on the other, is particularly intriguing. In Arabidopsis, vernalization, temperature, photoperiod, as well as light quality and also GA biosynthesis are integrated at the level of *SOC1* expression to ultimately promote flowering (Fig. 7). Therefore, it is interesting to speculate that, at least in Arabidopsis, the repression of the pathways for cold tolerance and greening following *SOC1* activation and the transition to reproductive growth may ensure that sufficient resources can be allocated to flowering and thereby guarantee the plant's reproductive success (Fig. 7). Inversely, activation of *GNC* and *GNL* expression during unfavorable growth conditions, such as cold stress, that will lead to increased DELLA protein

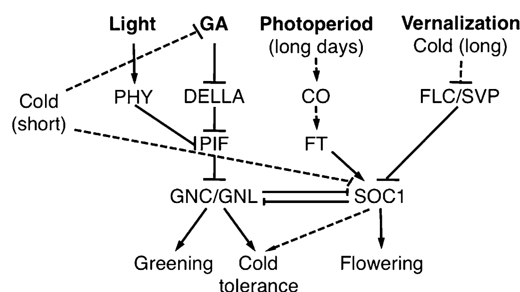

Figure 7. Model of the interactions of the GATAs GNC and GNL and the MADS box transcription factor SOC1 in the control of greening, cold tolerance, and flowering with their respective regulators pathways. CO, CONSTANS; FLC, FLOWERING LOCUS C; PHY, phytochrome; SVP, SHORT VEGETATIVE PHASE.

levels in the Arabidopsis wild type, would result in increased *GNC* and *GNL* expression and shift the balance toward the cold tolerance-promoting GNC and GNL pathway (Fig. 7).

MATERIALS AND METHODS

Biological Material

The following mutants and transgenic lines were used in this study: *ga1* (Salk_109115; Willige et al., 2007); *gai-1D* (Peng et al., 1997); *gnc* (SALK_001778), *gnl* (SALK_003995), *GNC:GFP* (*35S:GNC:GFP*), GNC_{Pro}*:GUS*, *YFP:GNL* (*35S:YFP:GNL*), and GNL_{Pro}*:GUS* (Richter et al., 2010); *PIF3:MYC* (Clack et al., 2009); *rga-24 gai-t6* (King et al., 2001); *soc1-2*, *SOC1:MYC(9x)*, and $SOC1_{Pro}$*:GUS* (Liu et al., 2008); and *spy-3* (Jacobsen and Olszewski, 1993). With the exception of *rga-24 gai-t6* and *gai-1D* (L*er*), all mutants and transgenic lines are in the Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia. *YFP:GNL* was introduced into the different genetic backgrounds by genetic crosses from a stably expressing *YFP:GNL* line in the Columbia or L*er* background.

GNL_{Pro}:GNL

HA was obtained by insertion of a genomic fragment obtained by PCR amplification into the Gateway system-compatible cloning vector pEarleyGate 301 (Earley et al., 2006). The resulting T-DNA construct was directly transformed into *gnc gnl* mutants using the floral dip transformation method (Clough and Bent, 1998). For a list of relevant primers, see Supplemental Table S1.

Physiological Experiments

For flowering time analyses, plants were randomly arranged and grown in 150 μmol m^{-2} s^{-1} white light in MobyLux GroBanks (CLF Plant Climatics) under long-day (16 h of light/8 h of dark, 21°C/18°C) or short-day (8 h of light/16 h of dark, 21°C/18°C) conditions. The time of bolting was scored from at least 18 plants by counting the number of rosette leaves (Richter et al., 2010). For GA treatments, plants were watered twice per week. Cold tolerance experiments were performed as described previously (Achard et al., 2008a) with the following modification: 14-d-old seedlings that had or had not been cold acclimated for 2 d at 4°C were transferred to −20°C and kept at this temperature until the agar or soil temperature reached −6°C. Subsequently, seedlings were transferred to ambient temperature for an additional 4 d before seedling survival was quantified. For chlorophyll quantification, chlorophyll was extracted and quantified from 10-d-old seedlings (three independent replicates) as described previously (Inskeep and Bloom, 1985).

qRT-PCR

Total RNA for qRT-PCR was isolated with a NucleoSpin RNA plant kit from 10-d-old whole seedlings (Macherey-Nagel). DNA was removed by an on-column treatment with rDNase (Macherey-Nagel), and 2 μg of total RNA was subsequently reverse transcribed with Moloney murine leukemia virus reverse transcriptase (Fermentas) using an oligo(dT) primer. The complementary DNA equivalent of 60 to 80 ng of total RNA was used in a 10-μL PCR in a CFX96 Real-Time System Cycler (Bio-Rad) with iQ SYBR Green Supermix (Bio-Rad) in a 40-cycle two-step amplification protocol (10 s at 95°C, 25 s at 60°C). Relevant primers are listed in Supplemental Table S1.

GUS Staining

GUS staining was performed according to previously published methods (Dohmann et al., 2008).

Immunoblots and ChIP

Immunoblotting was performed with the SuperSignal Femto West substrate as described previously (Thermo Fisher Scientific) using the anti-RGA antibody (Willige et al., 2007; Richter et al., 2010). Immunoblots were imaged using a LAS-4000 Mini image analyzer (FUJIFILM). ChIP and the subsequent quantitative PCRs were performed as described by others using antibodies against H3K9Ac (ab10812; Abcam) and H3K9me2 (ab1220; Abcam), with the GFP Vector Fusion Aid kit (Axorra) for GNC:GFP and YFP:GNL, anti-c-Myc agarose (Sigma) for SOC1:MYC, and an anti-HA antibody (3F10; Roche) together with A/Gplus-agarose beads (Santa Cruz Biotechnology) for GNL:HA (Fode and Gatz, 2009; Oh et al., 2009).

Arabidopsis Genome Initiative locus identifiers for the genes mentioned in this article are as follows: *CBF1* (AT4G25490), *CBF2* (AT4G25470), *CBF3* (AT4G25480), *COR15A* (AT2G42540), *COR15B* (AT2G42530), *FT* (AT1G65480), *GA1* (AT4G02780), *GAI* (AT1G14920), *GID1A* (AT3G05120), *GID1B* (AT3G63010), *GID1C* (AT5G27320), *GNC* (AT5G56860), *GNL/CGA1* (AT4G26150), *PIF3* (AT1G09530), *PORA* (AT5G54190), *PORB* (AT4G27440), *PORC* (AT1G03630), *RGA* (AT2G01570), *SOC1* (AT2G45660), and *SPY* (AT3G11540).

Supplemental Data

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

Supplemental Figure S1. Contributions of GNC and GNL to the repression of flowering in short-day conditions.

Supplemental Figure S2. GNC and GNL repress flowering downstream from DELLAs and PIF3.

Supplemental Figure S3. *GNC* and *GNL* transcript levels are increased in *gai-1D* when compared with the wild type.

Supplemental Figure S4. Transgenic GNL expressed from its own promoter binds to the *SOC1* promoter.

Supplemental Figure S5. Differential GA sensitivity of *soc1* mutants with regard to flowering time but not with regard to chlorophyll accumulation.

Supplemental Figure S6. Suppression of the *soc1* chlorophyll accumulation phenotype in *soc1 gnc gnl*.

Supplemental Table S1. List of primers used in this study.

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