LLM-Domain B-GATA Transcription Factors Promote Stomatal Development Downstream of Light Signaling Pathways in Arabidopsis thaliana Hypocotyls

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Stomata are pores that regulate the gas and water exchange between the environment and aboveground plant tissues, including hypocotyls, leaves, and stems. Here, we show that mutants of Arabidopsis thaliana LLM-domain B-GATA genes are defective in stomata formation in hypocotyls. Conversely, stomata formation is strongly promoted by overexpression of various LLM-domain B-class GATA genes, most strikingly in hypocotyls but also in cotyledons. Genetic analyses indicate that these B-GATAs act upstream of the stomata formation regulators SPEECHLESS (SPCH), MUTE, and SCREAM/SCREAM2 and downstream or independent of the patterning regulators TOO MANY MOUTHS and STOMATAL DENSITY AND DISTRIBUTION1. The effects of the GATAs on stomata formation are light dependent but can be induced in dark-grown seedlings by red, far-red, or blue light treatments. PHYTOCHROME INTERACTING FACTOR (PIF) mutants form stomata in the dark, and in this genetic background, GATA expression is sufficient to induce stomata formation in the dark. Since the expression of the LLM-domain B-GATAs GNC (GATA, NITRATE-INDUCIBLE, CARBON METABOLISM-INVOLVED) and GNC-LIKE/CYTOKININ-RESPONSIVE GATA FACTOR1 as well as that of SPCH is red light induced but the induction of SPCH is compromised in a GATA gene mutant background, we hypothesize that PIF- and light-regulated stomata formation in hypocotyls is critically dependent on LLM-domain B-GATA genes.

INTRODUCTION

Plants use stomata to control the exchange of oxygen, carbon dioxide, and water with their environment (Casson and Hetherington, 2010). Therefore, stomata are important for the efficiency of photosynthesis in plants and their activity contributes to the atmospheric environment (Dow and Bergmann, 2014). Stomatal pores are surrounded by guard cells in the epidermis of aboveground plant organs such as hypocotyls, cotyledons, stems, leaves, and floral organs, but they are absent from roots and only rarely found in the hypocotyls of dark-grown seedlings (Pillitteri and Torii, 2012; Wengier and Bergmann, 2012).

For stomatal development, protodermal cells enter the stomatal lineage in response to a number of input signals that include light and phytohormones (Casson et al., 2009; Casson and Hetherington, 2012, 2014; Kim et al., 2012; Wang et al., 2015). Stomatal development and patterning are initiated by signaling events that employ the plasma membrane-resident receptor-like proteins ERECTA (ER), ER-LIKE1 (ERL1), and ERL2 and the coreceptor TOO MANY MOUTHS (TMM), as well as the peptide ligands EPIDERMAL PATTERNING FACTOR1 (EPF1), EPF2, and the EPF-LIKE proteins (EPFLs), including EPFL9/STOMAGEN, EPFL6/CHALLAH (CHL), EPFL5/CHALLAH-LIKE1 (CLL1), and CLL2 (EPFL4/CLL2) (Yang and Sack, 1995; Shpak et al., 2005; Hara et al., 2007, 2009; Hunt and Gray, 2009; Sugano et al., 2010; Abrash et al., 2011; Lee et al., 2015). These peptide ligands act in part as activators and in part as repressors of stomatal development and, as has been recently demonstrated in studies with EPFL9/STOMAGEN and EPF2, promote and repress stomata formation through antagonistic interactions with the ER-TMM receptor pair (Lee et al., 2015). Interestingly, mutants of different members of the stomatal patterning genes may have differential defects in different tissues. This is most prominent in the tmn mutant, which forms more stomata than the wild type in cotyledons but lacks stomata in the hypocotyl and the stem (Yang and Sack, 1995; Nadeau and Sack, 2002).

Stomatal differentiation requires the consecutive activities of the basic helix-loop-helix (bHLH) transcription factors SPEECHLESS (SPCH), MUTE, and FAMA, which all interact with the functionally redundant bHLHs SCREAM/ICE1 and SCREAM2 (Ohashi-Ito and Bergmann, 2006; MacAlister et al., 2007; Pillitteri et al., 2007; Kanaoka et al., 2008). SPCH is selectively expressed in cells recruited to the stomatal lineage where it promotes the first asymmetric division in stomatal development that gives rise to a meristemoid. This meristemoid undergoes further amplifying asymmetric divisions, resulting in the formation of an oval-shaped guard mother cell. FAMA then promotes the differentiation of this oval-shaped cell into the guard cell pair of the fully differentiated stomatal pore (MacAlister et al., 2007). Following the initial SPCH-dependent divisions, a number of sister cells are produced that may also enter the stomatal lineage. It has been suggested that SPCH participates in the amplifying divisions of meristemoids (MacAlister et al., 2007) and, on the other side, that MUTE is required for the termination of the transient stem cell-like...
LLM-Domain B-GATAs Promote Stomata Formation

We previously observed that light-grown Arabidopsis seedlings of LLM-domain B-GATA gene overexpressors (GNCox, GNLox, GATA15ox, and GATA17ox) had longer hypocotyls than wild-type seedlings and accumulated more chlorophyll, most prominently at the base of the hypocotyl (Figure 1A; Behringer et al., 2014). In these overexpression lines, the effects on hypocotyl elongation and, at least in part also on greening, were dependent on the presence and absence of the LLM-domain and could not be observed in overexpression lines of the LLM-domain mutated GNC (GNC_LLM/AAAox or GNCΔCTox) of GATA23, which harbors a degenerate LLM-domain (GATA23ox), or of the HAN-domain B-GATA GATA19 (GATA19ox) (Behringer et al., 2014).

To examine the hypocotyl phenotype of these GATA overexpressors more closely, we analyzed seedling hypocotyls by scanning electron microscopy (Figure 1). Interestingly, we noted in the LLM-domain B-GATA overexpressors an up to 10-fold increase in the number of stomata in the nonprotruding cell files of hypocotyls that was accompanied by a strong increase in the number of cells in this cell file (Figures 1B to 1D). Whereas stomata formation in the wild-type hypocotyl is mainly restricted to the upper part of the hypocotyl (Berger et al., 1998), LLM-domain B-GATA-overexpressing seedlings displayed increased cell divisions and stomata numbers in the nonprotruding cell files along the entire hypocotyl (Figure 1B). Overexpressors of LLM-domain B-GATAs with mutations of the LLM-domain (GNC_LLM/AAAox or GNCΔCTox) were compromised in their ability to promote stomata formation but maintained the ability to promote cell divisions (Figures 1B and 1C). Overexpression lines of GATA23 (GATA23ox), which has a degenerate LLM-domain, as well as of the HAN-domain B-GATA GATA19 (GATA19ox) did not promote (GATA23ox) or only marginally promoted (GATA19ox) stomata formation and cell divisions in hypocotyls (Figures 1B and 1C). Thus, specifically overexpression of the four LLM-domain B-GATA genes led to a strong increase in stomata formation in hypocotyls. Importantly, there was no apparent correlation between the effects of the different GATAs on stomata formation and the expression of the respective transgenic proteins (Supplemental Figure 1).

To examine whether LLM-domain B-GATAs are required for stomata formation, we analyzed stomata abundance in mutants of GNC, GNCL, GATA17, and GATA17L (Ranftl et al., 2016). We detected reductions in stomata numbers in the hypocotyls of GATA gene single mutants, in the gnc gnl and gata17 gata17l double mutants, and even more pronounced effects in the gnc gnl gata17l triple and the gnc gnl gata17 gata17l (gataq) quadruple mutants (Figures 1A to 1C and 1B). Furthermore, we found that the number of nonstomatall cells in the nonprotruding cell file was not altered between the wild type and the gataq mutant. On the other side, we also did not find an increase in the number of meristemoids and...
thus concluded that stomata formation in the hypocotyl was at least in part dependent on the presence of these four GATA genes.

We next examined stomatal abundance at the abaxial side of the cotyledons. There, we again detected significant increases in the number of stomata formed in the GATA overexpression lines (Supplemental Figure 2). In this case, the relative increases were less pronounced than in hypocotyls (1.5- to 2-fold compared with up to 10-fold), and they could be observed in all GATA overexpression lines tested. GNCox variants with LLM-domain mutations (GNC_LLM/AAAox or GNCΔCTox) were again compromised in their ability to induce stomata formation when compared with GNCox wild-type overexpressors (Supplemental Figure 2). Conversely, we also detected a weak but significant decrease in the number of stomata in the cotyledons of the gataq quadruple mutant when compared with the wild type (Supplemental Figure 2). In summary, we concluded that overexpression of the four LLM-domain B-GATAs can promote stomata formation in the hypocotyl and in cotyledons, that the four

Figure 1. LLM-Domain B-GATAs Promote Stomata Formation in the Hypocotyl.

(A) Representative photographs of 8-d-old light-grown seedlings with the genotypes as specified in the figure. Bar = 1 mm. (B) and (C) Scanning electron micrographs (B) and magnification of a representative area from these micrographs (C) of seedlings with the genotypes as specified in (A). In (C), all stomata of the selected area are colored pink, cells of one selected protruding cell file are colored dark-green, and cells of one selected nonprotruding cell file are colored light-green. Bars = 250 µm. (D) and (E) Averages and standard errors of stomata number counts per visible surface in the hypocotyls of 8-d-old light-grown seedlings. The family membership of LLM- and HAN-domain B-GATAs is also specified. GATA23ox is marked with an asterisk (*GATA23ox) since it represents an LLM-domain B-GATA with a degenerate LLM-domain that behaves differently from canonical LLM-domain B-GATAs (Behringer et al., 2014). n = 8. Student’s t test or Mann-Whitney U test if the normality test failed in comparison to the wild-type sample: **P ≤ 0.01 and ***P ≤ 0.001; n.s., not significant.
LLM-domain B-GATAs participate in stomata formation in wild-type hypocotyls, and that their LLM-domain contributes to their ability to promote stomata formation. Since we noted in some genotypes (GNC, LLM/AAAox or GNCΔCTox) that the increases in cell numbers in the nonprotruding cell file were not coupled to increases in stomata numbers, we reasoned that the two processes may not be linked.

To determine whether the effects of B-GATA overexpression on stomata formation in the hypocotyl were cell-autonomous, we micrografted scions of wild-type and GNCox seedlings onto stocks of GNCox and wild-type seedlings, respectively. In both cases, we found that the phenotypes of the GNCox seedlings were not graft-transmittable and therefore likely cell autonomous (Supplemental Figure 3).

Overexpression of GATA genes, which we had chosen as representatives for the family, were expressed in the hypocotyls and cotyledons of light-grown seedlings (Supplemental Figure 4). Since the strong staining in the hypocotyls precluded a cell type-specific analysis, we also analyzed the GFP signal of pGNC:GUS:GFP and pGATA17:GUS:GFP. In the case of pGATA17:GUS:GFP, expression of the reporter was observed in stomatal lineage ground cells and stomatal meristemoids of hypocotyls and cotyledons as well as in cells of the hypocotyl cortex and the mesophyll (Figure 2A), pGNC:GUS:GFP, in turn, was mainly detected in the hypocotyl cortex and in the cotyledon mesophyll but only rarely in isolated cells of the epidermis (Figure 2B). Thus, at least GATA17 but possibly also GNL are directly expressed in cells of the stomatal lineage.

**TMM and SPCH Expression Are Restricted to Stomatal Lineage Cells in GNCox Lines**

To establish whether the increased number of cells in the nonprotruding cell file of LLM-domain B-GATA overexpressors was a direct consequence of the activation of the stomatal lineage pathway, we next examined the expression of the stomatal lineage markers pTMM:GUS:GFP (TMM promoter fused to GUS:GFP) and pSPCH:nRFP (SPCH promoter fused to nucleus-targeted RFP) in the LLM-domain B-GATA overexpression line GNCox as well as in the gataq mutant (Figure 3) (Nadeau and Sack, 2002; Gudesblat et al., 2012). Because we had previously established that the LLM-domain B-GATAs had redundant biochemical functions (Behringer et al., 2014) (Figure 1; Supplemental Figure 2), we restricted this and further genetic analyses to the GNC overexpression lines GNCox, and in relevant cases also to GNC_LLM/AAAox (Richter et al., 2010; Behringer et al., 2014). In this analysis, we detected an increased number of pTMM:GUS:GFP- and pSPCH:nRFP-expressing cells in hypocotyls of GNCox and GNC_LLM/AAAox (pTMM:GUS:GFP tested only) (Figure 3). However, we found that most cells originating from cell divisions in the nonprotruding cell file did not express these markers, indicating that the increased number of cells in the nonprotruding cell file was not a consequence of a general activation of the stomatal lineage (Figure 3).
when we generated \textit{tmm} GNCox and \textit{tmm} GNC\_LLM/AAAox lines and compared their phenotypes with \textit{tmm} chal cll1, we noted differences in the distribution of the stomata on the hypocotyl. Whereas the distribution of stomata along the \textit{tmm} chal cll1 hypocotyl resembled that of the wild type, where the majority of stomata are formed in the upper part of the hypocotyl, stomata distribution in \textit{tmm} GNCox or \textit{tmm} GNC\_LLM/AAAox resembled that of GNCox and GNC\_LLM/AAAox in the wild-type background, where stomata formed along the entire hypocotyl (Figure 4B). Furthermore, while \textit{tmm} chal cll1 formed stomatal clusters, clusters were only occasionally observed in \textit{tmm} GNCox or \textit{tmm} GNC\_LLM/AAAox hypocotyls (Figure 4B). By contrast, the abundance of such clusters was increased in cotyledons of \textit{tmm} GNCox or \textit{tmm} GNC\_LLM/AAAox when compared with \textit{tmm}, while no clusters were observed in GNCox or GNC\_LLM/AAAox in the wild-type background (Figure 4C). We thus concluded that overexpression of the LLM-domain B-GATA GNC promoted stomata formation in hypocotyls downstream of \textit{TMM} and affected stomatal patterning in cotyledons.

Since \textit{CHAL/CLL} mutations suppress the \textit{tmm} phenotype, we were interested in examining whether \textit{CHAL/CLL} mutations could suppress the stomata formation defect of the \textit{gataq} (\textit{gnc gnl gata17 gata17i}) quadruple mutant. To this end, we generated \textit{gataq} chal cll1. However, \textit{gataq} chal cll1 failed to suppress the \textit{gataq} phenotype, suggesting that the LLM-domain B-GATAs promoted stomata formation downstream of or independently of \textit{TMM} and \textit{CHAL/CLL} (Figure 4D).

**Interaction between LLM-Domain B-GATAs and \textit{SDD1}**

\textit{sdd1} mutants produce more stomata in the cotyledons than does the wild type and these frequently occur in stomatal clusters

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**Figure 3.** Expression Analysis of pTMM:GUS:GFP and pSPCH:nRFP. Representative confocal microscopy images of hypocotyl epidermis cells of 5-d-old light-grown seedlings expressing pTMM:GUS:GFP (A) or pSPCH:nRFP (B) and (C). Arrowheads mark several meristemoids expressing the pTMM:GUS:GFP or pSPCH:nRFP reporters. In (A) and (B), cell walls were stained with propidium iodide; in (C), the propidium iodide staining was omitted to show that the nuclear signal originates from the pSPCH:nRFP reporter rather than from the propidium iodide cell wall stain. Bars = 50 µm.

**Figure 4.** GNC Overexpression Suppresses the Stomata Formation Defects in \textit{tmm} Mutant Hypocotyls. (A) and (D) Averages and standard errors of stomata number counts per visible surface in the hypocotyls of 8-d-old light-grown seedlings. \( n \geq 8 \). Student’s \( t \) test or Mann-Whitney \( U \) test if normality test failed: **\( P \leq 0.01 \) and ***\( P \leq 0.001 \); n.s., not significant. (B) and (C) Scanning electron micrographs of seedling hypocotyls (B) and abaxial cotyledon surfaces (C). In (B), cells colored in pink are all stomata of the selected area. Note the differential distribution of stomata in the hypocotyls between \textit{tmm} GNCox (along the entire hypocotyl) and \textit{tmm} chal cll1 (upper part of the hypocotyl only). Also note the increased formation of stomatal clusters in cotyledons of \textit{tmm} GNCox, \textit{tmm} GNC\_LLM/AAAox, and \textit{tmm} chal cll1. Bars = 100 µm (B) and 50 µm (C).
Whereas differentiated stomata were absent in GNCox well as in the cotyledons (Figure 6; Supplemental Figure 5). We observed that stomata formation was blocked in the hypocotyl as higher than that observed in gataq, we hypothesized that SDD1 may repress stomata formation through the GATAs, but also through one or several other mechanisms that act in parallel.

B-GATAs Act Upstream of SPCH, MUTE, and SCRM SCRM2

Stomata formation requires the consecutive activities of the three bHLH transcription factors SPCH, MUTE, and FAMA, which function together with the interacting bHLH proteins SCRM and SCRM2 (MacAlister et al., 2007; Pillitteri et al., 2007; Kanaoka et al., 2008). To examine whether stomatal differentiation downstream of the B-GATAs was dependent on these bHLH factors, we introduced GNCox into spch, mute, and scrm scrm2 loss-of-function mutants. In all three of the resulting genetic combinations, namely, GNCox spch, GNCox mute, and GNCox scrm scrm2, we observed that stomata formation was blocked in the hypocotyl as well as in the cotyledons (Figure 6; Supplemental Figure 5). Whereas differentiated stomata were absent in GNCox mute, this background accumulated an increased number of meristemoids, which were recognizable as small cells originating from asymmetric cell divisions (Figures 6D to 6G). This indicated that stomatal differentiation had been initiated but could not proceed in the mute loss-of-function mutant. Importantly, the general increases in cell numbers in the nonprotruding cell that were typical of GNCox lines could also be observed in GNCox spch, GNCox mute, and GNCox scrm scrm2 (Figures 6C and 6G; Supplemental Figure 5B). Additionally, the strong effects of GNCox on greening, that are particularly prominent at the hypocotyl base, were maintained in the different GNCox genotypes (Figures 6B and 6F; Supplemental Figure 5A). This suggested again that the effects of B-GATA overexpression in promoting greening and cell divisions in the nonprotruding cell files of hypocotyls were not linked to their role in promoting stomata formation.

In a complementary experiment, we evaluated the importance of the LLM-domain B-GATAs in the scrm-D gain-of-function mutant, which produces an excessive number of stomata (Kanaoka et al., 2008). Importantly, a scrm-D gataq mutant displayed a significant reduction of stomata formed in the hypocotyl when compared with scrm-D, whereas the number of stomata formed in the cotyledons were comparable between these two genotypes (Supplemental Figures 5C to 5E). This showed that the activity of the gain-of-function SCRM-D was compromised in the absence of the four GATA genes mutated in gataq.

The Effects of B-GATAs on Stomatal Development Are Light Dependent and PIF Dependent

Stomata formation in wild-type seedlings is at least partially dependent on the activation of the phyB phytochrome receptor by red light and the consequent inactivation of PIF transcription factors, which are targeted for protein degradation in the light (Casson et al., 2009; Casson and Hetherington, 2014). We noted that the effects of B-GATA overexpression on stomata formation and cell divisions in the hypocotyl were strictly light dependent (Figure 7). While dark-grown wild-type seedlings as well as GNCox overexpressors did not form stomata in the hypocotyl, stomata formed in seedlings grown in white light as well as red, far-red, and blue light (Figure 7). Interestingly, light-dependent stomata formation was compromised in the gataq mutants and, conversely, enhanced in GNCox (Figure 7). This indicated that the four GATA genes mutated in gataq were required and rate-limiting for stomata formation in response to light and that GATA dosage was also limiting for stomata formation in the wild type (Figure 7).

The phyB and PIF-dependent repression of stomata formation in the dark is suppressed in pif1 pif3 pif4 pif5 (pifq) quadruple mutant seedlings where the requirement for the light inactivation of the respective PIF proteins no longer exists (Martinez-Garcia et al., 2000; Leivar et al., 2008). Since dark-grown pifq mutants are partially etiolated, we were interested in examining whether...
The Expression of LLM-Domain B-GATAs Is Light Regulated

LLM-domain B-GATA overexpression was sufficient to induce stomata formation in this background in the dark. To this end, we analyzed pifq GNLox, an overexpression line of the GNC paralog GNL that we had generated in the context of earlier work (Richter et al., 2010). Indeed, expression of the GNLox transgene in pifq resulted in increases in stomata formation in dark-grown seedling hypocotyls that exceeded the number of stomata formed in pifq (Figure 8D). Importantly, GNLox and pifq GNLox seedlings, when grown in the light, produced comparable numbers of stomata in the hypocotyl (Figure 8E). Taken together, these findings suggested, on the one hand, that LLM-domain B-GATAs are rate-limiting factors for stomata formation in hypocotyls downstream of PIFs and, on the other, that the GATAs are not sufficient to induce stomata formation in hypocotyls, but require other factors downstream of PIFs (or the PIFs themselves) in this process.

The Expression of LLM-Domain B-GATAs Is Light Regulated

GNL had originally been identified as a red light-induced gene, but the functional role of this regulation had remained unexplored (Naito et al., 2007). We examined the regulation of the four LLM-domain B-GATA genes, GNC, GNL, GATA17, and GATA17L, in response to red, far-red, and blue light and found that, most prominently, the expression of GNL but to some extent also of GNC was induced in all three light conditions (Figures 9A and 9B; Supplemental Figure 6). At the same time, the expression of GATA17 and GATA17L was slightly but significantly repressed after any of the respective light treatments in the wild type (Supplemental Figure 6). These transcriptional light responses were compromised in the respective phyA phyB or cry1 cry2 receptor mutants and, in the case of the red light-responsive transcription, also in the pifq mutant (Figures 9A and 9B; Supplemental Figure 6).

We had previously shown that GNC and GNL were repressed by PIFs and that the expression particularly of GNL but also of GNC was increased in light-grown pif mutant seedlings (Richter et al., 2010). Since increased expression of the GATA genes may account for stomata formation in dark-grown pifq seedlings, we examined the expression of the four GATAs (GNC, GNL, GATA17, and GATA17L) by qRT-PCR (Figure 9C). This analysis revealed a strong upregulation particularly of GNL, but also of GNC, in pifq, suggesting that the upregulation of these GATAs may be causative for the induction of stomata formation in dark-grown pifq seedlings. Since our analyses of GATA mutant phenotypes had been concentrated on the gataq quadruple mutant, but GNL was particularly strongly upregulated after red light treatment, we hypothesized that gnl single mutants may already be compromised in the light-dependent induction of stomata formation. However, gnl single mutants were indistinguishable from the wild type with regard to stomata formation in the hypocotyls of red light-shifted dark-grown seedlings (Figure 9D). The impairment of stomata formation observed in gataq mutants grown in the same conditions further substantiated our conclusions phrased above (Figure 9D) and from our earlier studies showing that the genes encoding LLM-domain B-GATAs act in a functionally redundant manner (Bi et al., 2005; Richter et al., 2010; Behringer et al., 2014; Ranftl et al., 2016).

Since the light-dependent effects of LLM-domain B-GATA overexpression in GNCox (or GNLox) may potentially be the result of the differential degradation or accumulation of the transgenic...
effects were the result of differences in protein abundance (Supplemental Figure 7A). We also found that the expression of the pTMM:GUS:GFP marker in the hypocotyls and cotyledons of dark-grown seedlings was restricted to a comparable number of meristemoids in the wild-type, gataq, and GNCox seedlings, while their number varied in light-grown seedlings in correlation with the numbers of stomata formed in these genotypes (Supplemental Figures 7B and 7C).

Figure 7. Red, Far-Red, and Blue Light Promote Stomata Formation through LLM-Domain B-GATAs in Hypocotyls. Representations of electron micrographs of hypocotyls from 8-d-old seedlings grown in the dark (A), in far-red light (0.35 µmol m⁻² s⁻¹) (B), red light (7.5 µmol m⁻² s⁻¹) (C), blue light (4.25 µmol m⁻² s⁻¹) (D), or white light (150 µmol m⁻² s⁻¹) (E) of the genotypes specified in the figure. Bar = 100 µm.

proteins, we examined the abundance of the HA-tagged GNC protein in GNCox by immunoblot analysis in dark- and light-grown seedlings as well as in dark-grown seedlings that had been shifted to the light for one day. Since we detected similar levels of GNCox in all light conditions, we excluded the possibility that the observed

Figure 8. LLM-Domain B-GATAs Promote Stomata Formation Downstream of PIFs.

(A) Representative areas from scanning electron micrographs of hypocotyls from 8-d-old dark-grown seedlings. All stomata and all meristemoids are colored in pink and blue, respectively; meristemoids are also highlighted by asterisks. Cells of one selected protruding and non-protruding cell file are colored in dark-green and green. Note the relative increase in meristemoids in pifq GNLox (asterisks). Bar = 50 µm.

(B) and (C) Representative photographs of 8-d-old dark-grown (B) and light-grown seedlings (C). Bar = 1 mm.

(D) and (E) Averages and standard errors of counts of stomata and meristemoids per hypocotyl area (800 µm²) of 8-d-old dark-grown (D) and per hypocotyl of light-grown seedlings (E). n = 8. Student’s t test or Mann-Whitney U test if normality test failed: **P ≤ 0.01 and ***P ≤ 0.001; n.s., not significant.
SPCH Is a Strongly Light-Regulated Gene

To determine whether the expression of any of the critical regulators of stomata development was light regulated, we examined the expression of key regulators of stomata formation, namely, SPCH, MUTE, FAMA, SCRM, SCRM2, STOMAGEN, and TMM, in dark-grown seedlings and following short-term red light treatments by qRT-PCR (Figure 10A). These analyses identified SPCH as the only gene that was already strongly induced after a 30-min red light treatment (Figure 10A). Only after 120 min did we detect significant increases in the expression of other regulatory genes, namely, MUTE, SCRM2, and TMM (Figure 10B). Since SPCH expression in the dark could potentially be repressed by PIFs and relieved from repression in red light, we examined the levels of SPCH in the dark-grown pifq mutants as well as the kinetics of SPCH expression in pifq mutants. There, we detected increases in the expression of SPCH in dark-grown seedlings and a comparatively faster induction of SPCH expression in comparison to the wild type when seedlings were shifted to red light (Figures 10C and 10D). This suggested that PIFs may indeed suppress SPCH expression in the dark and that the pifq mutant, where four of the at least seven red light-sensitive PIF proteins from Arabidopsis are mutated (Leivar and Monte, 2014), was sensitized to the red light treatment.

Inversely, we observed that SPCH expression in dark-grown gataq seedlings was comparable to that of wild-type seedlings and that the red light-dependent induction of SPCH was delayed in the gataq mutant (Figures 10C and 10D). This suggested that the GATAs may positively regulate SPCH expression in the red light signaling cascade.

Since the experiments described above indicated that the LLM-domain B-GATAs may directly regulate SPCH expression, we were interested in identifying GNL target genes. To this end, we made use of a transgenic line expressing the LLM-domain B-GATA GNL fused to YFP, an HA-tag, and the hormone binding domain of the rat glucocorticoid receptor (GR) in a gnc gnl background. Such a GR fusion allows the cytoplasm-to-nucleus shuttling of the GNL:YFP:HA-GR protein after treatment with the glucocorticoid dexamethasone (Lloyd et al., 1994). We then performed chromatin immunoprecipitation of GNL:YFP:HA-GR followed by next-generation sequencing (ChIP-seq) using 10-d-old light-grown seedlings that had been treated with 10 µM dexamethasone or a mock control for 4 h. After mapping the reads to the Arabidopsis genome, we inspected all known loci with a role in stomatal development and identified, as a single gene hit, a peak located 1479 bp upstream of the SPCH start codon that was absent in the control sample (Figure 10D). This peak aligned with a TGATAA promoter element, which was thus a predicted promoter element for GATA transcription factors (Figure 10D). We then confirmed the binding to an amplicon comprising this promoter element (set 1) by qPCR after an independent ChIP with a transgenic line expressing HA-tagged GNL from a GNC promoter in the gnc gnl mutant background (ProGNL:GNL:HA; Figure 10E).

When we tested the neighboring region (set 2) that had not been identified in the ChIP-seq experiment, we did not find such an enrichment (Figure 10F). When taken together with the findings that the red light-dependent induction of SPCH was impaired in gataq mutants, this finding suggested that GNL was a good candidate for a protein that promotes SPCH expression and consequently stomata formation.

Figure 9. GNL and GNC Are Light-Regulated Genes and Differentially Expressed in pifq.

(A) and (B) Averages and standard errors of normalized fold changes from qRT-PCR analyses of GNL (A) and GNC (B) obtained from 5-d-old dark-grown seedlings of the specified genotypes after 60 min red, far-red, or blue light exposure. For each genotype, fold changes were calculated relative to the values obtained from dark-grown seedlings of the respective genotypes. GATA17 and GATA17L expression was not light activated (Supplemental Figure 6).

(C) Averages and standard deviations of normalized fold changes from qRT-PCR analyses of GNC, GNL, GATA17, and GATA17L expression in dark-grown wild-type and pifq seedlings. For each gene, fold changes were calculated relative to the values obtained with the respective dark-grown wild-type seedlings.

(D) Averages and standard errors of stomata numbers in the upper 500-µm section of the imaged side of hypocotyls grown for 3 d in the dark and an additional 4 d in red light. n ≥ 8. Student’s t test or Mann-Whitney U test if normality test failed: *P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.001; in all other cases, differences are not significant.
DISCUSSION

We identified the redundantly acting LLM-domain B-GATA transcription factors GNC, GNL, GATA17, and GATA17L as critical regulators of light-dependent stomata formation in the hypocotyl of Arabidopsis (Figures 1 and 7). We show that these LLM-domain B-GATA genes act upstream of SPCH and other bHLH transcription factors required for stomatal differentiation (Figure 6) and downstream of far-red, red, and blue light signaling pathways (Figure 7). Furthermore, we find that GATA overexpression can suppress the loss of stomata formation phenotype in tmm hypocotyls and that the loss of the GATAs can partially impair increased stomata formation as observed in the sdd1 mutant (Figures 4 and 5).

While the overexpression of these GATA factors strongly promotes stomata formation in light-grown hypocotyls, their overexpression does not show an effect in hypocotyls of dark-grown wild-type seedlings (Figure 7). In pifq mutants, four of the at least seven light-sensitive PIFs from Arabidopsis are mutated (Leivar and Monte, 2014). Our observation that GATA

Figure 10. SPCH Is a Red Light-Regulated Gene.

(A) Averages and standard errors of normalized fold changes from qRT-PCR analyses of genes involved in stomata formation after red light exposure for the duration specified in the figure. For each gene, fold changes were calculated relative to the values obtained with the respective dark-grown seedlings.

(B) Averages and standard errors of stomata numbers in the upper 500-µm section of the imaged side of hypocotyls grown for 3 d in the dark and an additional 4 d in red light. n ≥ 8. P values in parentheses refer to tests of the same time points between different genotypes.

(C) Averages and standard errors of normalized fold changes from qRT-PCR analyses of SPCH expression in dark-grown wild-type, pifq, and gataq seedlings. Fold changes were calculated relative to the values obtained with the wild-type control.

(D) Graphic displays of the CSAR enrichment scores after mapping of sequencing reads from two ChIP replicate samples of GNL:YFP:HA:GR after dexamethasone induction (sample 1 and sample 2) and two mock controls (mock 1 and mock 2) over the genomic region around SPCH. Set 1 represents a region identified by ChIP-seq of GNL:YFP:HA:GR that contains a GATA motif and that was subsequently tested for binding by ChIP-qPCR; set 2 represents a neighboring control region. The SPCH gene model is displayed, where boxes are exonic regions and lines represent introns. Colored triangles identify four different types of GATA sequence motifs, putative GATA transcription factor binding sites that can be found in the represented genomic sequence.

(E) and (F) Results from independent ChIP-qPCR analyses after immunoprecipitation of GNL:HA from a ProGNL:GNL:HA gnc gnl transgenic line and a gnc gnl negative control over an amplicon from the SPCH promoter containing a TGATAA sequence (set 1; [E]) and a neighboring amplicon as a negative control (set 2; [E]). Student’s t test or Mann-Whitney U test if normality test failed: *P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.001; n.s., not significant.
overexpression can promote stomata formation in the hypocotyls of dark-grown *pifq* mutants suggests that GATAs act together with other factors downstream from PIFs, or with the PIFs themselves, to promote stomata formation (Figure 8). Conversely, stomata formation in the hypocotyl is impaired in red light and, thus, in light conditions where PIFs are inactivated by proteolytic degradation. This indicates that the GATAs are critical regulators of light-dependent stomata formation in the hypocotyl. GATA overexpression results in stomata formation along the entire hypocotyl (Figures 1 and 4), whereas stomata formation in the wild type is generally restricted to the upper part of the hypocotyl (Berger et al., 1998). This may be taken as an indication that GATA expression levels are limiting in this process in wild-type hypocotyls.

We previously provided evidence for a direct regulation and transcriptional repression of the B-GATAs GNC and GNL by PIFs, and we also showed that GNC and GNL transcript abundance is upregulated in light-grown *pifq* quadruple mutants (Richter et al., 2010). We now show that the LLM-domain B-GATA factor GNL, and to some extent also GNC, are transcriptionally activated after red light treatment of dark-grown seedlings and that their expression is increased in dark-grown *pifq* mutants (Figure 9). This derepression of GATA gene expression in the *pifq* mutants and their light-dependent expression is in line with a model where the GATAs are downstream from PIF transcription factors in the regulation of light-regulated stomata formation.

Interestingly, our examination of SPCH expression following red light treatment of dark-grown seedlings indicated that SPCH was, among several stomatal development regulators tested, the only gene rapidly induced in response to red light (Figure 10). SPCH may therefore represent the critical light-regulated target in the context of light-dependent stomata formation. The recently reported findings that SPCH directly regulates a broad range of genes with a role in stomata formation and patterning strengthens the predicted critical role of SPCH in regulating stomatal development, in general and here specifically downstream from the light signaling pathway (Lau et al., 2014).

The strong red light-induced expression of SPCH suggests that SPCH functions downstream of phytochrome and PIFs. In line with the notion that the red-labile PIFs act as repressors of SPCH expression, red light-induced SPCH expression is stronger in *pifq* mutants than in the wild type, suggesting that the *pifq* mutants, in which four but not all red light-regulated PIFs are mutated, are sensitized to red light (Figures 10B and 10C). Inversely, we observed a delayed induction of SPCH expression in the *gataq* mutants, suggesting that the LLM-domain B-GATAs may be activators of SPCH expression (Figures 10B and 10C). Such a model finds further support in our observation from a ChIP analysis with the LLM-domain B-GATA GNL that identified a GATA motif-containing element in the *SPCH* promoter (Figures 10D to 10F). These ChIP-seq data were obtained from light-grown seedlings, and future experiments will have to explore the biological relevance of this binding for SPCH expression and light-dependent stomata formation.

Here, we show that the effects of the gain- and loss-of-LLM-domain B-GATA function on stomata formation are most prominent in the hypocotyl. While the positive effects of LLM-domain B-GATA overexpression on stomata formation can still be observed in cotyledons, the negative effects of the loss of LLM-domain B-GATA expression in the *gataq* quadruple mutant in cotyledons are significant but comparatively subtle. This may be explained through the activities of the other two LLM-domain B-GATAs, GATA15 and GATA16, that are still active in the *gataq* mutant or possibly also the compensatory upregulation of LLM-domain B-GATA gene expression in the *gataq* mutant background, as suggested already by other studies of this gene family (Richter et al., 2010, 2013a, 2013b; Behringer et al., 2014; Ranftl et al., 2016). Alternatively, and specifically with regard to the weak effects of the loss of GATAs on stomata formation in cotyledons (Supplemental Figure 2), it is likely that other input pathways exist that regulate stomata formation in addition to the LLM-domain B-GATAs. For example, brassinosteroids were shown to regulate SPCH by posttranslational modification of the SPCH protein and brassinosteroid signaling could thus act in parallel and downstream of the B-GATAs in the regulation of stomatal development (Casson and Hetherington, 2012; Gudesblat et al., 2012; Kim et al., 2012). Furthermore, it may be possible that the other B-GATA genes also participate in the regulation of stomata formation in this tissue, since we observed noteworthy effects of their overexpression in cotyledons, but not in hypocotyls (Supplemental Figure 2).

The presence of the C-terminal LLM-domain defines LLM-domain B-GATA factors, but the biochemical function of the LLM-domain is unclear. Here, we show that the overexpression of LLM-domain B-GATAs with a mutation in the LLM-domain compromises the ability of the B-GATAs to promote stomata formation (Figure 1; Supplemental Figure 2). However, LLM-domain mutated B-GATAs are not fully impaired in this activity, as most obviously shown in the *tmm* mutant, where overexpression of LLM-domain mutated GATAs efficiently promoted stomata formation (Figure 4). Furthermore, our study shows that the promotion of stomata formation is not restricted to LLM-domain-containing B-GATAs, since the other B-GATAs also promote stomata formation, at least to some extent, in hypocotyls and cotyledons (Figure 1; Supplemental Figure 2). A better understanding of the biochemical function of the LLM-domain and of the target gene specificity of the different GATAs will help unravel how this domain contributes to the activation of the stomata formation pathway.

Besides stomata formation, the overexpression of the LLM-domain B-GATAs also promoted greening and cell divisions in the nonprotruding cell file of seedling hypocotyls (Figure 1). Here, we did not investigate the molecular basis of this GATA-dependent activity but made several observations that allowed us to rule out the possibility that the effects of GATA overexpression on greening, cell division, and stomata formation are interrelated. First, we observed that not all cells in the nonprotruding cell file express the stomatal lineage reporters for TMM and SPCH, indicating that not all cells in this cell file have acquired stomatal lineage fate (Figure 3). Thus, the supernumerary cells in the nonprotruding cell file are not undifferentiated stomatal lineage cells. Second, we observed increased cell numbers in the nonprotruding cell file in the GNCox *spch* and GNCox *mute* backgrounds as well as in the GNCox *scrm scrm2* background, which do not form stomata (Figure 6; Supplemental Figure 5). Thus, the increased cell divisions as observed in the GATA overexpressors are not a consequence of increased stomata formation.
Loss-of-function mutants of the LLM-domain B-GATA genes are impaired in greening and GATA overexpressors accumulate more chlorophyll than the wild type (Bi et al., 2005; Richter et al., 2010; Hudson et al., 2011; Chiang et al., 2012; Behringer et al., 2014; Ranftl et al., 2016). Although it is intriguing that both greening and stomata formation are related to the efficiency of photosynthesis, we exclude, based on our data, a causal link between the stomata formation and greening phenotypes of the loss-of-function and overexpression backgrounds. One observation that supports this conclusion is the fact that defective stomata formation in the hypocotyl as observed in GNCox spch, GNCox mute, as well as in GNCox scrm scrm2 does not impair hypocotyl greening (Figure 6; Supplemental Figure 5). This observation is most prominent in the GNCox scrm scrm2 background (Supplemental Figure 5) and less prominent in the GNCox spch and GNCox mute backgrounds because the spch and mute mutants themselves have greening defects (Figure 6). Thus, our genetic data suggest that the role of the LLM-domain B-GATAs in promoting stomatal development can be uncoupled from their apparent role in promoting greening.

The molecular mechanisms that control greening in an LLM-domain B-GATA-dependent manner have, at least to some extent, already been elucidated (Bi et al., 2005; Hudson et al., 2011; Chiang et al., 2012). However, how the LLM-domain B-GATAs promote cell divisions and how this is achieved in a cell type-specific manner, remains unclear (Figure 1). We noted in the literature two previously published reports that may be relevant in this context. First, it was shown that the overexpression of the E2F-DP cell cycle repressors induces, similarly to the GATA overexpressers described here, cell divisions specifically in the nonprotruding cell file (De Veylder et al., 2002). Second, it was reported that mutants of the D-type cyclin CYCD4 have a reduced number of cells and stomata in the nonprotruding cell file of hypocotyls and, conversely, that CYCD4 overexpression gives rise to an increased number of cells and stomata in this cell file (Kono et al., 2007). Thus, the GATA factors may act upstream or downstream of E2F-DP and CYCD4. Our existing ChIP-seq data set does not give any indications that the GATAs examined here actively regulate cell cycle genes. How cell cycle regulation, stomatal differentiation, and cell file specificity of these regulatory mechanisms function together will be an interesting topic for future research.

**METHODS**

**Biological Material**

All experiments were performed in the Arabidopsis thaliana ecotype Columbia (Col). The following B-GATA gene-related and transgenic lines were used in this study: gnc (SALK_001778), gnl (SALK_003995), gata17 (SALK_101994), gata17 (SALK_026798), gataq, GNCox, GNC LLM/AAox, GNC DtCox, GATA17ox, GATA19box, GATA23ox, pGNL:GUS, and pGNL:GNL:HA gnc gnl (Richter et al., 2010, 2013a; Behringer et al., 2014; Ranftl et al., 2016). The experiment with pitf1 GNLox and GNLox was performed with the overexpression construct described by Richter et al. (2010), and all other experiments with GNLox overexpression were performed with GNLox as described by Behringer et al. (2014).

The following mutants were obtained from NASC (Nottingham Arabidopsis Stock Center): phyA phyB phytochromeA-211 phyB-9 (Reed et al., 1993), trmm (too many mouths-1) (Nadeau and Sack, 2002), trmm chal chl1 (trmm-1 chalah-2 chalah-like-1-1), and chal chl1 (Abrash et al., 2011). The following previously published mutants and reporter lines were used in this study and generously provided by Dominique Bergmann (Stanford, CA), Keiko Torii (Seattle, WA), or Eugenia Russinova (Ghent, Belgium): pTMM-GUS:GFP (Nadeau and Sack, 2002), pSPCh:nRFP (Gudesblat et al., 2012), spch (speechless-3) (MacAlister et al., 2007), mute (mute) (Pil litteri et al., 2007), scrm scrm2 (ice1-2 scream2-1), scrm-D (scream-D) (Kanaoka et al., 2008), and sdd1 (stomata density and distribution 1) (Berger and Allmann, 2000). pitf1 pitf3 pitf4 pitf5 pif1 pif3 pif4 pif5 (phytochrome interacting factor quadruple); pif1-1 pif3-3 pif4-2 pif5-3) was generously provided by Peter Quail (Berkeley, CA) (Leivar et al., 2008) and the cry1 cry2 mutant by Chentai Lin (Los Angeles, CA) (Mockler et al., 1999). Primers for genotyping are listed in Supplemental Data Set 1.

**Cloning Procedures**

The GATA17Lox transgenic line was generated as previously described using the primers listed in Supplemental Data Set 1 (Behringer et al., 2014). The pGNC:GUS:GFP and pGATA17:GUS:GFP constructs were generated using Gateway technology (Life Technologies) with pDONR201 and the destination vector pFAST-G04 (Shimada et al., 2010).

To generate 35S:GNL:YFP:HA:GR, an AscI restriction site was introduced after the HA-tag in GNLox as described (Behringer et al., 2014). The GR domain was amplified from the vector pTA7002 (Aoyama and Chua, 1997) as a fragment flanked by AscI sites and inserted into the modified GNLox. The resulting construct was transformed into the gnc gnl/ double mutant using the floral dip method (Clough and Bent, 1998). The functionality of the transgene in the transgenic lines was verified by complementation and confocal analysis of GNLox:YFP:HA distribution. Primers used for cloning are listed in Supplemental Data Set 1.

**Microscopy**

Fluorescence microscopy was performed with an Olympus FV1000 confocal laser scanning microscope. Scanning electron microscopy was performed with a TM-3000 table-top scanning electron microscope (Hitachi).

**Physiological Experiments**

All plants were cultivated on sterile 0.5× Murashige and Skoog medium without sugar under continuous white light (150 μmol m⁻² s⁻¹). The experiments were repeated three times with comparable results and the result of one representative experiment is shown. For experiments with different light qualities, seedlings were grown in CLF FloraLED chambers using red (7.2 μmol m⁻² s⁻¹), far-red (0.35 μmol m⁻² s⁻¹), or blue light (4.25 μmol m⁻² s⁻¹).

**Immunoblotting**

To assay for protein expression in transgenic plants, total protein extracts were prepared from 8-d-old light-grown seedlings in an extraction buffer containing 50 mM Tris-HCl, pH 7.0, 150 mM NaCl, 0.5% (v/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and Protease Inhibitor Cocktail (Roche). Total protein (45 μg) was separated on a 10% (v/v) SDS-PAGE gel and detected with anti-HA-horseradish peroxidase (Roche) or anti-rabbit-peroxidase (Sigma-Aldrich). A part of a Coomassie Brilliant Blue-stained gel corresponding to the 50-kD molecular mass proteins was used as a loading control.

**qRT-PCR**

To measure the light-dependent induction of gene expression by qRT-PCR, 5-d-old dark-grown seedlings were transferred to CLF FloraLED...
chambers with constant red (7.2 µmol m⁻² s⁻¹), far-red (0.35 µmol m⁻² s⁻¹), or blue (4.25 µmol m⁻² s⁻¹) light. Total RNA was extracted with an RNaseasy Plant Mini Kit (Qiagen). Then, 2 µg total RNA was reverse transcribed with an oligo(dT) primer and M-MuLV reverse transcriptase (Fermentas). Transcript levels were determined using iQ SYBR Green Supermix (Bio-Rad) in a CFX96 real-time system cycler. The results were normalized to ACT2 (AT1G13320) in red and far-red light and to ACT2 (ACTIN2; AT3G18780) in blue light. Expression in the dark samples was set to one. Primers for qRT-PCR analyses are listed in Supplemental Data Set 1.

**ChIP**

For ChIP-seq analyses, 10-d-old 3SS:GNL-YFP:HA; GR gnc gnl seedlings were grown on GM medium under constant white light and then induced for 4 h with 10 µM dexamethasone or with a corresponding mock solution. Three biological replicate samples (2 g tissue) were fixed for 20 min in 1% formaldehyde, processed using GFP-TRAP_A (Chromotek) and prepared for DNA sequencing using Illumina MiSeq sequencing as previously described (Kaufmann et al., 2010). Illumina sequencing reads were then mapped to the Arabidopsis genome (TAIR10) using SOAPv1 (Li et al., 2008) with the following settings: three mismatches, mapping to unique positions. The data from this analysis are available as PRJNA291959 at NCBI-SRA (http://www.ncbi.nlm.nih.gov/sra/). To verify the binding of GNL to the SPCr promoter, ChIP-PCR was performed with pGNL:GNL:HA gnc gnl as well as gnc gnl seedlings grown on GM agar plates for 10 d under long-day conditions (16 h light/8 h dark). Plant tissue was subsequently fixed for 20 min in a solution containing 1% formaldehyde and the remainder of the ChIP experiment was performed as previously described using ChIP-grade anti-HA tag antibodies (Abcam) (Kaufmann et al., 2010). Primer sequences are listed in Supplemental Data Set 1.

**Accession Numbers**

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: ACT2 (AT3G18780); CHL (AT2G30370); CLL1 (AT3G32280); CLL2 (AT4G14723); cryptochrome1 (cry1; AT4G08920); cry2 (AT1G04400); FAMA (AT3G24140); GATA17 (AT3G16870); GATA17L (AT4G16141); GATA19 (AT4G36620); GATA23 (AT4G26930); GNC (AT5G58680); GNL/cGA1 (AT4G26150); MUTE (AT3G06120); phyA (AT1G09570); phyB (AT2G18790); PIF1 (AT3G20180); PIF3 (AT1G09530); PIF4 (AT2G43010); PIF5 (AT3G59060); PROTEIN PHOSPHATASE2 (PP2; AT1G13320); STOMATA DENSITY AND DIFFERENTIATION1 (SDD1; AT1G04110); SCREAMICE1 (AT3G26744); SCRAM2 (AT1G12860); SPCr (AT5G35210); STOMAGEN (AT4G12970); and TMM (AT1G08080).

**Supplemental Data**

**Supplemental Figure 1.** Immunoblot analysis confirms transgene expression in B-GATA overexpression lines.

**Supplemental Figure 2.** B-GATAs promote stomata formation in cotyledons.

**Supplemental Figure 3.** Micrografting experiments are indicative for a cell-autonomous effect of LLM-domain B-GATA overexpression on stomata formation and greening.

**Supplemental Figure 4.** GUS reporter gene analysis reveals broad expression patterns of LLM-domain B-GATAs in light-grown seedlings.

**Supplemental Figure 5.** LLM-domain B-class GATAs function downstream from SCREAM and SCREAM2.

**Supplemental Figure 6.** Expression of LLM-domain B-GATAs is light regulated.

**Supplemental Figure 7.** The promoting effects of B-GATAs on stomata formation are light dependent.

**Supplemental Data Set 1.** Primers used in this study.

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**AUTHOR CONTRIBUTIONS**

C.K., Q.L.R., and C.S. designed and performed research. J.D. performed research. R.R., Q.L.R., and E.B. provided important research material or data. C.K., E.B., and C.S. analyzed data. C.K. and C.S. wrote the manuscript.

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