Expression of Arabidopsis *SHORT INTERNODES/STYLISH* Family Genes in Auxin Biosynthesis Zones of Aerial Organs Is Dependent on a GCC Box-Like Regulatory Element[1][C][W]

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Auxin/indole-3-acetic acid (IAA) biosynthesis in Arabidopsis (*Arabidopsis thaliana*) plays a major role in growth responses to developmental and genetic signals as well as to environmental stimuli. Knowledge of its regulation, however, remains rudimentary, and few proteins acting as transcriptional modulators of auxin biosynthesis have been identified. We have previously shown that alteration in the expression level of the *SHORT INTERNODES/STYLISH* (*SHI/STY*) family member *STY1* affects IAA biosynthesis rates and IAA levels and that STY1 acts as a transcriptional activator of genes encoding auxin biosynthesis enzymes. Here, we have analyzed the upstream regulation of *SHI/STY* family members to gain further insight into transcriptional regulation of auxin biosynthesis. We attempted to modulate the normal expression pattern of *STY1* by mutating a putative regulatory element, a GCC box, located in the proximal promoter region and conserved in most *SHI/STY* genes in Arabidopsis. Mutations in the GCC box abolish expression in aerial organs of the adult plant. We also show that induction of the transcriptional activator DORNRÖSCHEN-LIKE (DRNL) activates the transcription of *STY1* and other *SHI/STY* family members and that this activation is dependent on a functional GCC box. Additionally, *STY1* expression in the strong *drnl-2* mutant or the *drn drnl-1 puchi-1* triple mutant, carrying knockdown mutations in both *DRNL* and its close paralogue *DRN* as well as one of their closest homologs, *PUCHI*, was significantly reduced, suggesting that *DRNL* regulates *STY1* during normal plant development and that several other genes might have redundant functions.

The key elements in auxin-mediated development in Arabidopsis (*Arabidopsis thaliana*) are auxin biosynthesis and active polar transport, which are required to produce and maintain auxin gradients and maxima (for review, see Feraru and Friml, 2008; Chandler, 2009; Zhao, 2010). In a recent model for explaining pattern formation and morphogenesis in roots, Grieneisen et al. (2007) suggested that auxin transport overrides the effects of changes in auxin biosynthesis. However, mutants with deficiencies in auxin biosynthesis show severe defects in vegetative and reproductive development (Cheng et al., 2006, 2007; Stepanova et al., 2008; Tao et al., 2008), indicating that not only auxin redistribution but also local auxin biosynthesis has a major impact on plant growth and development.

Several plant enzymes are rate limiting in auxin biosynthesis, each enzyme regulating one of the first steps in what is thought to be different Trp-dependent pathways, ultimately leading to the formation of indole-3-acetic acid (IAA). The cytochrome P450 family members CYP79B2 and CYP79B3 have been shown to convert Trp to indole-3-acetaldoxime (Zhao et al., 2002; Ljung et al., 2005), an important metabolite for glucosinolate as well as IAA biosynthesis, TRP AMINOTRANSFERASE OF ARABIDOPSIS1 (TAA1) and its two homologs TRP AMINOTRANSFERASE RELATED1 (TAR1) and TAR2 convert Trp to indole-3-pyruvic acid (Stepanova et al., 2008; Tao et al., 2008), and the YUCCA (YUC) family of flavin monooxygenases is reported to convert tryptamine to *N*-hydroxyl tryptamine (Zhao et al., 2001). The finding that other IAA biosynthesis pathways (e.g. YUC) cannot compensate for the loss of TAA1 suggests that these proteins are active at different spatial and temporal sites during the plant life cycle (Tao et al., 2008). Interestingly, recent reports suggest that *TAA/TAR* and *YUC* genes function in the same auxin biosynthetic

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pathway (Strader and Bartel, 2008; Phillips et al., 2011) and also question the biochemical function of YUC in the tryptamine pathway (Tivendale et al., 2010; Nonhebel et al., 2011), highlighting how little we actually know regarding these pathways.

We recently showed that *SHORT INTERNODES/STYLISH* (*SHI/STY*) family members are important throughout plant development and directly regulate *YUC4*-mediated auxin biosynthesis in Arabidopsis (Sohlberg et al., 2006; Ståldal et al., 2008; Eklund et al., 2010a), and we could show that transcription of *YUC8* was activated by STY1 (Eklund et al., 2010a). This suggests that the temporal and spatial regulation of *SHI/STY* family members may be crucial for the developmental regulation of auxin production. Only limited information regarding upstream regulators directly controlling the activity of *SHI/STY* family genes is present, and although genetic data have indicated that the transcriptional corepressor LEUNIG may participate in the transcriptional regulation of *SHI/STY* family members (Kuusk et al., 2006; Ståldal et al., 2008), the molecular connections still await verification. Also, *SWIRM domain PAO protein1/Lysine-Specific Demethylase1-LIKE1* has been suggested to fine-tune root elongation via transcriptional regulation of the *SHI/STY* family member *LATERAL ROOT PRIMORDIUM1* (*LRP1*; Krichevsky et al., 2009). Other upstream regulators could be genes known to affect auxin homeostasis and/or organ formation. Furthermore, *SHI-RELATED SEQUENCE5* (*SRS5*) has been shown to be activated by pathogen attack (Barcala et al., 2010), suggesting *SHI/STY* genes to have a function in stress responses.

Here, we have searched for putative upstream regulators by screening for common promoter elements in the highly redundant *SHI/STY* gene family members in Arabidopsis. We identified a putative GCC box (Ohme-Takagi and Shinshi, 1990) located within the promoter region 500 bp upstream of the translational start site in all but one family member in Arabidopsis. The putative GCC box is inverted and part of a 14- or 15-bp conserved region in five of the *SHI/STY* family promoters, strongly suggesting a conserved function for this element. The APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) domain is generally considered to be a GCC box-binding domain and is unique to members of the AP2/ERF superfamily, consisting of 147 putative transcription factors in Arabidopsis (Nakano et al., 2006). It has been shown that the N terminus of the AP2/ERF domain binds in a sequence-specific manner to GCC box elements (Hao et al., 1998). Our data indicate that a functional GCC box is required for the expression of *SHI/STY* family members in aerial IAA biosynthesis zones (i.e. in *YUC* gene expression domains). The *SHI/STY* family expression at other sites, such as the lateral root primordia, stem, and proximal part of cotyledons and mature leaves, is not affected by mutations in the GCC box and therefore is most likely regulated by other, yet unknown, mechanisms. We can also show that ectopic expression of the AP2/ERF family member DORNROSCHEN-LIKE (DRNL) activates the transcription of *STY1* in a GCC box-dependent manner and that *STY1* is down-regulated in the *drnl-2* mutant as well as in the *drn-1 drnl-1 puchi* triple mutant, suggesting that several AP2/ERFs redundantly regulate *STY1* during plant development.

RESULTS

The Arabidopsis *SHI/STY* Family Members Contain a Conserved Element Similar to a GCC Box

In order to identify conserved promoter motifs affecting the transcriptional activity of *SHI/STY* genes, we analyzed promoters, 5′ untranslated regions (UTRs), and intron sequences of the nine active *SHI/STY* family members in Arabidopsis (*SHI*, *STY1* and *-2*, *LRP1*, and *SRS3* to *-7*). Using the MEME (for Multiple Em for Motif Elicitation) software (Bailey and Elkan, 1994), we identified a short conserved promoter/5′ UTR element located only a few hundred bp upstream of the start codon of each of the *STY1*, *STY2*, *SHI*, *SRS5*, and *SRS7* genes (Table I). The identified element with the core GGCGGC is similar to an inverted ethylene-responsive element (TAAGAGCCGCC; Ohme-Takagi and Shinshi, 1990), usually referred to as a GCC box. The GCC box has been predicted to be a target for ethylene signaling pathways, because mutations in this element eliminated the ethylene responsiveness of a tobacco (*Nicotiana tabacum*) chitinase gene (Shinshi et al., 1995). Later reports define the ERF-binding element of the GCC box as an (A)GCCGCC core in which nucleotides G-1, G-4, and C-6 exhibit the highest binding specificity to the ERFs (Hao et al., 1998; Fujimoto et al., 2000).

The GCC box-like elements found in *SHI/STY* genes are conserved at positions G-1, G-4, and C-6, suggesting that they are bona fide GCC boxes, possibly recognized by proteins of the AP2/ERF family.

The annotated transcriptional start site (TSS) of *STY1* and *STY2* is located just downstream of the GCC box, while the annotated TSS in *SHI* is found upstream, indicating that the element is located in the UTR of *SHI*. There is no annotated TSS in *SRS5* or *SRS7*. Shorter sequences with striking similarity to the GGCGGC component of the conserved sequence were found in the promoter or 5′ UTR of *LRP1*, *SRS4*, and *SRS6* but not in *SRS3* (Table I).

A phylogenetic analysis of conserved coding regions from all nine *SHI/STY* family genes in Arabidopsis showed that *SHI/STY* family members are separated into two major clades, one containing *STY1*, *STY2*, *SHI*, *SRS3* to *-5*, and *SRS7*, whereas the other includes *LRP1* and *SRS6* (Kuusk et al., 2006). The three *Selaginella moelendorffii* and the two *Physcomitrella patens SHI/STY* homologs cluster with the *LRP1/SRS6* genes (Eklund et al., 2010b). Several *SHI/STY* family members in Arabidopsis form evolutionarily closely related pairs,

Table I. *GCC-box-like sequences in SHI/STY family promoters*

Gene	Sequence	Strand	Position[a]
SHI	T**GGCGGC**GTTGCAG	+	−390
STY1	T**GGCGGC**GTTGCAG	+	−340
STY2	T**GGCGGC**GTTGCAG	+	−361
SRS5	T**GGCGGC**GTTTGCAG	+	−150
SRS7	T**GGCGGC**GTTTGCAG	+	−244
OsSRS4	T**GGCGGC**GTTTGCAG	+	−644
LRP1	C**GGCGGC**GACGGAG	+	−14
SRS6	C**GGCGGC**GACGGAG	−	−40
OsSRS1	**GGCGGC**GTCGG	+	−91
OsSRS2	C**GGCGGC**GGCGGA	+	−89
SRS4	C**GGCCGC**GTTGC	−	−97
OsSRS3	C**GGCGGCGGCGGC**	+	−146
SRS3	No match		
OsSRS5	No match		

[a]First nucleotide of the element in relation to the translational start site (ATG). Bold nucleotides represent the conserved GCCGCC component.

most likely originating from the last genome duplication event (Kuusk et al., 2006). *SHI* and *STY1* form one such pair and *SRS5* and *SRS7* form another pair, closely related to the *STY1/SHI* pair. Thus, it is not surprising to find the conserved GCC box in the regulatory regions of these four genes. However, *STY2* and *SRS4* also form a pair, and the conserved element in *STY2* is identical to that of *SHI* and *STY1*, while the element in *SRS4* is inverted and rearranged (Table I). *LRP1*, *SRS3*, and *SRS6* do not form any pairs (Kuusk et al., 2006); therefore, it is interesting that the 14-bp element in *LRP1* is identical to that found inverted in *SRS6* (Table I).

The spatial and temporal activities of *STY1*, *STY2*, *SHI*, and *SRS5* during plant development have been studied in detail (Fridborg et al., 2001; Kuusk et al., 2002, 2006) and were found to be largely overlapping, with some minor exceptions. Using an enhancer/promoter trap approach, Smith and Fedoroff (1995) suggested that *LRP1* expression is restricted to lateral root primordia, whereas phenotypic characterizations of multiple *SHI/STY* mutants carrying a mutation also in *LRP1*, together with real-time (RT)-PCR data, revealed that *LRP1* is expressed at similar sites as *STY1*, *STY2*, *SHI*, and *SRS5* also in Arabidopsis aerial tissues (Kuusk et al., 2006). Here, we have studied the expression of *SRS4* using a two-component GUS-reporter approach (Fig. 1). $SRS4_{pro}$>>*GUS* is expressed in cotyledon tips, leaf primordia, hydathodes, stipules, and lateral root primordia and weakly at the edges of petals and sepals, demonstrating that its activity largely overlaps with that of the *SHI/STY* genes studied previously. Kuusk et al. (2006) showed that mutations in *SRS4* enhanced the leaf phenotype of *sty1-1 sty2-1* and, to a limited extent, that of gynoecia, confirming that *SRS4* is active in leaves and buds. Additionally, transcriptome analysis (Hruz et al., 2008) reveals low levels of *SRS4* transcripts in floral organs. Because we have no detailed expression data for the remaining genes, we can only extrapolate their expression patterns from other data. Since mutation of *LRP1* enhanced the gynoecium and leaf defects of *sty1-1 sty2-1* (Kuusk et al., 2006), *LRP1* appears to act redundantly at certain developmental stages with other *SHI/STY* family members and thus should have at least partially overlapping expression patterns with *STY1*, *STY2*, *SHI*, and *SRS5* also in aerial organs. The identified mutations in *SRS6* and *SRS7* did not cause a complete loss of gene activity (Kuusk et al., 2006), which is why their developmental roles have been hard to elucidate. Coexpression analysis of *SHI/STY* family genes in different microarray experiments using ATTED-II (http://atted.jp) suggest that *SHI*, *STY2*, *SRS5*, and *LRP1* are partly coregulated, whereas *SRS4* and *SRS6* are less tightly coexpressed with other *SHI/STY* genes. *STY1*, *SRS3*, and *SRS7* were not

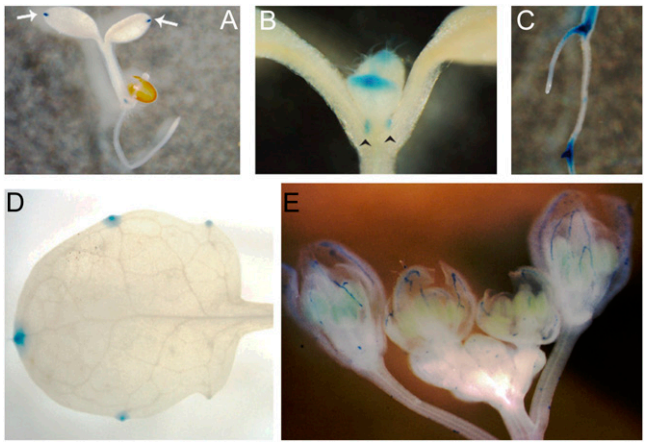

Figure 1. Expression of $SRS4_{pro}$>>*GUS* largely overlaps with the expression of other *SHI/STY* family members. $SRS4_{pro}$>>*GUS* is expressed in cotyledon tips (A; arrows), leaf primordium tips and stipules (B; arrowheads), lateral root primordia (C) as well as in the base of lateral roots and in the root vasculature, but not in the root tips, in hydathodes (D), and at the edges of petals and sepals (E).

included in the microarray analysis experiments. In summary, the available data from genetic and expression studies indicate that several *SHI/STY* family members could be partially coregulated, most likely by the same transcription factor or family of transcription factors.

GCC Boxes Are Only Found in *SHI/STY* Family Promoters of Angiosperms

We also used MEME to search for the conserved promoter/UTR elements in the two moss (*P. patens*), three lycophyte (*S. moelendorffii*), and five rice (*Oryza sativa*) *SHI/STY* homologs. Interestingly, promoters of the two *P. patens SHI/STY* orthologs *PpSHI1* and *PpSHI2* (Eklund et al., 2010b) were not found to possess GCC box-like sequences. Likewise, GCC box-like elements could not be found in the lycophyte *SHI/STY* genes (data not shown). Of the five rice *SHI/STY* genes *OsSRS1* to *OsSRS5* (Kuusk et al., 2006), *OsSRS1*, *OsSRS2*, and *OsSRS4* cluster with *STY1/2*, *SHI*, and *SRS3-5/7* (Kuusk et al., 2006; Hong et al., 2010) and carry GCC box-like elements (Table I), although only *OsSRS4* has the conserved 15-bp element found in *SRS5/7* (Table I). Since *OsSRS1*, *OsSRS2*, and *OsSRS4* form a clade in the phylogram, and thus appear more closely related to each other than to any of the *SHI/STY* genes from Arabidopsis, it is quite likely that the 15-bp element has been rearranged in *OsSRS1* and *OsSRS2*. *OsSRS3* and *OsSRS5* form a clade with *LRP1* and *SRS6* (Kuusk et al., 2006), although none of them has a GCC box similar to *LRP1/SRS6*. *OsSRS3* appears to have a GCC repeat that potentially could function as a GCC box, whereas *OsSRS5* does not contain a GCC box (Table I). Interestingly, *OsSRS1* has a GCC box-like element similar to that of *LRP1/SRS6*.

This suggests that the GCC box-like element was present before the split of dicots and monocots and that this type of element may only be present in *SHI/STY* homologs of angiosperms.

The GCC Box-Containing Elements of *STY1*, *SHI*, *STY2*, *SRS5*, and *SRS7* Are Unique to *SHI/STY* Genes

To analyze the genome-wide distribution of the GCC box-containing elements found in *SHI/STY* family promoters, we performed a Patmatch search in all Arabidopsis genes using 3-kb regions located upstream of the predicted TSS as well as coding regions, UTRs, and introns. The entire 14- to 15-bp element present in *SHI/STY1/STY2/SRS5/SRS7* could not be detected in any other Arabidopsis gene. However, the 14-bp element of *SRS6/LRP1* was found in six additional sites in the genome (Supplemental Table S1). None of the genes possibly regulated by the *SRS6/LRP1*-like element appear to have functions directly related to those of *SHI/STY* genes, or hormonal homeostasis in general, although the full range of biological functions controlled by *SHI/STY* genes has not yet been established.

The Putative GCC Box Is Important for the Regulation of *SHI/STY* Expression in Aerial Tissues during Plant Development

The conservation, position, and base pair composition of the putative GCC box in the *SHI/STY* promoters strongly suggest that it could be important for the regulation of *SHI/STY* gene activity. Therefore, we mutated the core sequence GGCGGC to AAAAAA in the $STY1_{pro}$:*GUS* construct (Kuusk et al., 2002), creating a $STY1mut_{pro}$:*GUS* fusion that was introduced into the accession Columbia (Col). All four independent transformants investigated showed the same spatial and temporal expression pattern.

The expression pattern of $STY1_{pro}$:*GUS* has previously been described in detail (Kuusk et al., 2002). In summary, *STY1* is expressed in hypocotyls and cotyledons of young seedlings (Fig. 2, A and E), leaf primordia (Fig. 2, C and E), stipules, hydathodes, root tips, and lateral root primordia (Kuusk et al., 2002). $STY1_{pro}$:*GUS* is also expressed in floral buds, sepals, styles, ovules, and receptacles (Fig. 2G). The expression of other *SHI/STY* family members coincides with that of *STY1* in cotyledon tips, leaf primordia, lateral root primordia, receptacles, styles/stigmas, and hydathodes (Fridborg et al., 2001; Kuusk et al., 2002, 2006).

The GCC box mutation completely eliminated the strong *STY1* expression in the distal parts of the cotyledon, including the cotyledon tip (Fig. 2, B and F), leaf primordia (Fig. 2, D and F), apical end of young gynoecia, style, stigma, ovule, and receptacle (Fig. 2H), suggesting that the GCC box is important for the majority of *STY1* expression sites. However, signals were found in the hypocotyl, petiole, and proximal part of the cotyledon (Fig. 2, B, D, and F) as well as in lateral root primordia (Fig. 2I); therefore, *STY1* expression in these tissues is considered to be GCC box independent.

STY1 Transcription Is Not Regulated by IAA or 1-Aminocyclopropane-1-Carboxylic Acid Levels

Because GCC boxes have been shown to be regulated by ERF proteins, we were interested in analyzing if the GCC box in *SHI/STY* genes responds to ethylene signaling. In microarray experiments in seedlings, only *SRS4*, but no other *SHI/STY* gene, was slightly upregulated by 1-aminocyclopropane-1-carboxylic acid (ACC) treatment (Genevestigator; Hruz et al., 2008). This indicates that at least *SHI*, *STY2*, *LRP1*, *SRS5*, and *SRS6*, which are spotted on the arrays, are not very sensitive to ethylene. In accordance, 4-d-old $STY1_{pro}$:*GUS* seedlings treated with 10 μM ACC showed no altered GUS activity compared with nontreated seedlings (data not shown).

The transcription of some *SHI/STY* family members appears to be affected by exogenous auxin treatment in certain backgrounds (Genevestigator; Hruz et al., 2008), and in order to establish if the transcription of *STY1* is controlled by auxin, we measured *STY1* mRNA levels in Col seedlings treated with 5 μM IAA

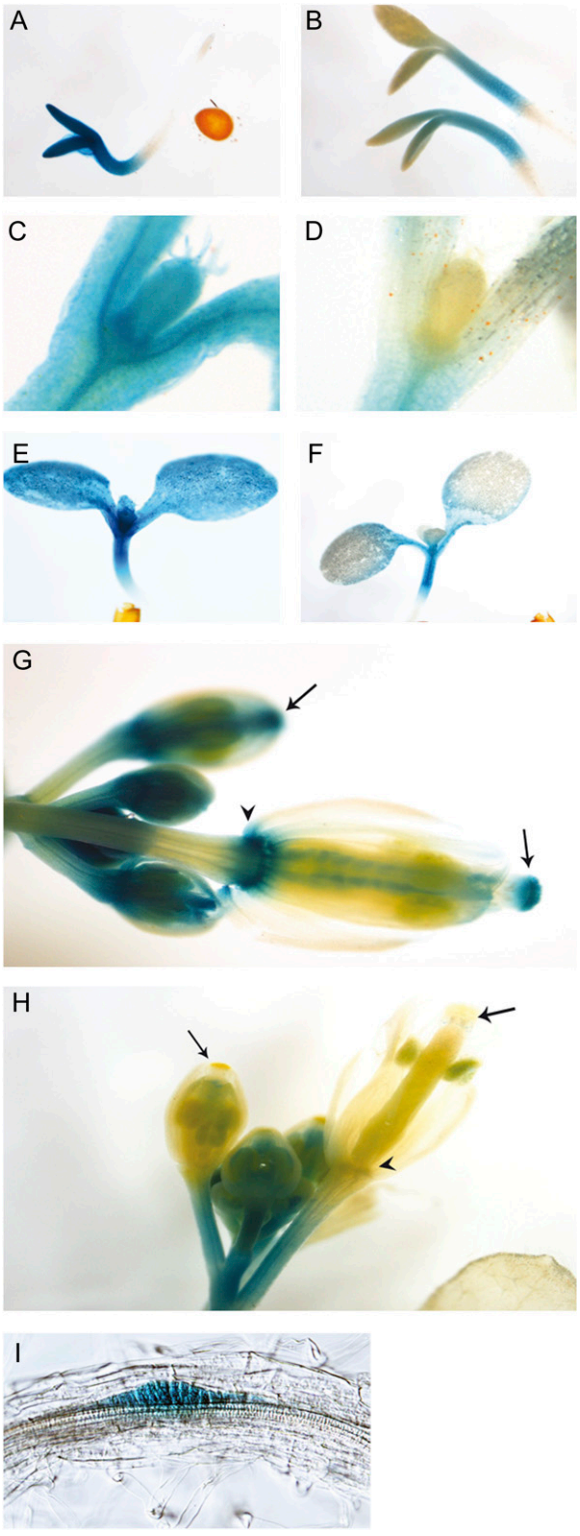

Figure 2. *STY1mut*$_{pro}$*:GUS* shows restriction in spatial activity compared with *STY1*$_{pro}$*:GUS*. A and B, In 3-d-old seedlings, *STY1*$_{pro}$*:GUS* (A) is active in hypocotyls and throughout the cotyledons, whereas *STY1mut*$_{pro}$*:GUS* (B) shows residual activity only in hypocotyls and the proximal part of cotyledons. C to F, In 9- and 7-d-old-seedlings, *STY1mut*$_{pro}$*:GUS* (D and F) activity remains in the hypocotyl and cotyledon petioles, whereas no *STY1mut*$_{pro}$*:GUS* activity was found in leaf primordia although *STY1*$_{pro}$*:GUS* is expressed at those sites (C and E). G and H, *STY1mut*$_{pro}$*:GUS* is active in inflorescence stems of 28-d-old plants (H) but not in the apical tips of developing gynoecia (arrows), receptacles (arrowhead), or ovules, sites of very strong *STY1*$_{pro}$*:GUS* activity (G). I, Lateral root primordium of *STY1mut*$_{pro}$*:GUS*.

for 10 min to 48 h. This showed that *STY1* expression in seedlings was not dramatically altered by exogenous IAA (Fig. 3) compared with the auxin-inducible gene *GRETCHEN HAGEN3.3* (*GH3.3*; Hagen and Guilfoyle, 2002). However, *STY1* was modestly but significantly (Student's t test, $P < 0.05$) down-regulated at 2 h of IAA treatment (Fig. 3). This indicates that if auxin affects *STY1* transcription, it is most likely as a repressor signal.

DRNL Can Activate the Transcription of *SHI/STY* Genes

Because several members of the AP2/ERF family have been shown to regulate GCC box-containing genes, we searched the literature for array experiments performed with the goal to identify downstream targets of individual AP2/ERF proteins. Interestingly, Ikeda et al. (2006) showed that *SHI* was significantly up-regulated (3.3 mean fold change) in root explants 1 h after the induction of constitutive expression of the AP2/ERF protein ENHANCER OF SHOOT REGENERATION2 (ESR2), previously named DRNL because of its high sequence identity to the Arabidopsis DRN protein (Kirch et al., 2003). As ESR2/DRNL was induced in the presence of cycloheximide (CHX), an inhibitor of protein synthesis, *SHI* was suggested to be a direct target of ESR2/DRNL (Ikeda et al., 2006). Marsch-Martinez et al. (2006) also studied downstream targets of ESR2/DRNL, although they called the protein BOLITA (BOL), and in a comparison of global gene expression in leaves of an *ESR2/DRNL/BOL* overexpressor line and the wild type, a 1.9- to 3-fold up-regulation of four of the nine *SHI/STY* family genes (*SHI*, *STY2*, *SRS4*, and *LRP1*) was revealed. *ESR2/DRNL/BOL* belongs to group VIII of the ERF/B subfamily in the AP2/ERF superfamily (Nakano et al., 2006). The ESR2/DRNL/BOL paralogue, DRN, has a very similar DNA-binding domain to that of ESR2/DRNL/BOL and has been shown to specifically bind GCC motif sequences in vitro (Banno et al., 2006). Furthermore, DRN was recently shown to target a GCC box in a transient in vivo assay (Matsuo and Banno, 2008), making ESR2/DRNL/BOL an interesting candidate to potentially regulate *SHI/STY* gene activities via their GCC box. Independent studies on this protein performed by different research groups have resulted in several names; consequently, ESR2/DRNL/BOL is also known as SUPPRESSOR OF PHYB-2 (SOB2; Ward et al., 2006). *ESR2/DRNL/BOL/SOB2* will hereafter be referred to only as *DRNL*.

Previous studies have demonstrated that *DRNL* has functions in flower organ initiation and outgrowth, particularly as an enhancer of the *pistillata* mutant with

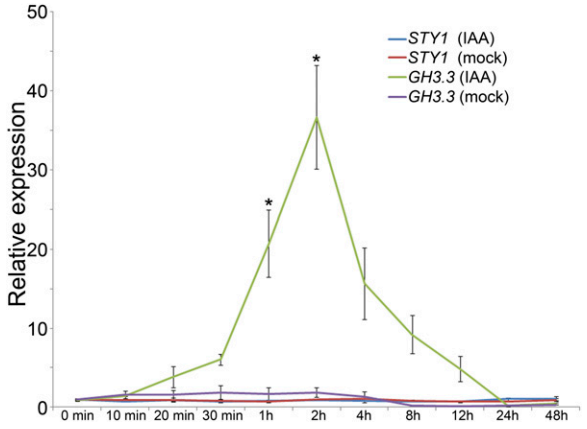

Figure 3. *STY1* gene activity in seedlings is not affected by exogenous IAA. qRT-PCR analysis of *STY1* transcripts in wild-type (Col) seedlings after various incubation times of mock treatment and 5 μM IAA. The graph shows mean values of two biological replicates, and error bars indicate SE. The *GH3-3* gene served as a control for the IAA treatment. Stars denote significantly increased expression in IAA compared with mock treatments (Student's *t* test, $P < 0.05$).

roles in stamen development (Nag et al., 2007). *DRNL* and its closest homolog, *DRN*, have been shown to act upstream of auxin transport and responses during embryo development and to have redundant roles during embryonic patterning and cotyledon organogenesis, likely in the same pathway as *CUP-SHAPED COTYLEDON* (Chandler et al., 2007, 2011a, 2011b).

To verify that DRNL can activate *SHI/STY* genes, we analyzed the ability of DRNL to activate the transcription of *STY1* and *LRP1* in $35S_{pro}$:*DRNL-ER* seedlings after β-estradiol (EST)-mediated nuclear import of the constitutively expressed DRNL-ER fusion protein (Ikeda et al., 2006). CHX was added to inhibit translation and thus to eliminate secondary effects. DRNL-ER activation resulted in a significant up-regulation of transcript levels of *STY1* and *LRP1* detected by quantitative (q)RT-PCR (Fig. 4), further suggesting that DRNL can activate several *SHI/STY* genes. We also analyzed the ability of DRNL-ER to activate the $STY1_{pro}$:*GUS* construct. An increased GUS signal intensity in EST-induced $35S_{pro}$:*DRNL-ER* $STY1_{pro}$:*GUS* seedlings compared with mock-treated seedlings (Fig. 5, A and C) confirmed the DRNL-ER-dependent activation of the *STY1* promoter.

DRNL-Mediated Activation of *STY1* in Aerial Parts Is GCC Box Dependent

To test whether a functional GCC box is required for DRNL-mediated activation of *SHI/STY* genes in planta, we crossed the $35S_{pro}$:*DRNL-ER* line with the $STY1mut_{pro}$:*GUS* line. We compared GUS expression in $STY1_{pro}$:*GUS* $35S_{pro}$:*DRNL-ER* and $STY1mut_{pro}$:*GUS* $35S_{pro}$:*DRNL-ER* seedlings after growth on mock or EST-supplemented medium (Fig. 5). As mentioned above, we observed an elevated constitutive GUS expression in aerial parts, including newly formed leaves of EST-treated $STY1_{pro}$:*GUS* $35S_{pro}$:*DRNL-ER* seedlings (Fig. 5, A and C). EST-treated $STY1mut_{pro}$:*GUS* $35S_{pro}$:*DRNL-ER* seedlings, in contrast, did not show elevated or ectopic GUS activity (Fig. 5, B and D), suggesting that the GCC box indeed is required for DRNL-mediated activation of the *STY1* promoter.

The Phenotypic Effects of Constitutive DRNL Activity Are Suppressed in the *SHI/STY* Family Multiple Mutant Background

Ectopic expression of *DRNL* results in phenotypic alterations almost identical to those of $35S_{pro}$:*SHI/STY1/STY2/LRP1* plants (Fridborg et al., 1999; Kuusk et al., 2002; Kirch et al., 2003; Ikeda et al., 2006; Marsch-Martinez et al., 2006; Nag et al., 2007). The most striking phenotypes are epinastic leaves, stunted misshaped siliques, short internodes and hypocotyls, and small pointed cotyledons, indicating that the cotyledon disc has not expanded properly. This suggests that the phenotypes caused by ectopic *35S* promoter-driven *DRNL* expression might largely be mediated by DRNL-induced ectopic activity of *SHI/STY* family members. Therefore, we introduced the $35S_{pro}$:*DRNL-ER* construct into a *SHI/STY* multiple mutant background by crossing the $35S_{pro}$:*DRNL-ER* line with the *SHI/STY* quintuple mutant (*sty1-1 sty2-1 shi-3 lrp1 srs5-1*; Kuusk et al., 2006). Progeny of plants homozygous for $35S_{pro}$:*DRNL-ER* and with the severe *SHI/STY* family multiple mutant phenotype were EST or mock treated. Notably, EST treatment did not induce phenotypic changes in the *SHI/STY* family mutant seedlings to the same extent as in the wild-type background (Fig. 6), suggesting that the abnormalities induced by ectopic DRNL requires functional *SHI/STY* family members. These

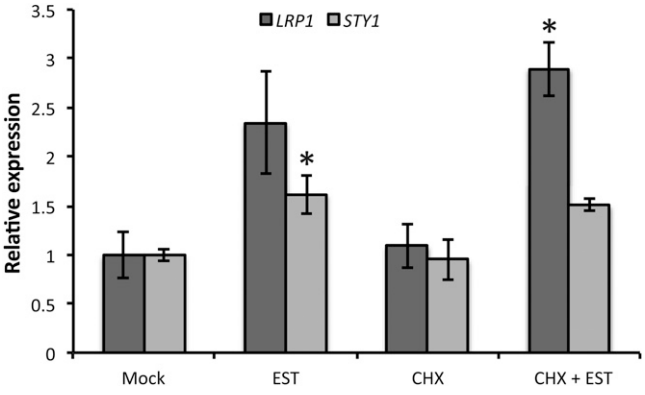

Figure 4. DRNL can activate *SHI/STY* genes. Transcript levels of *SHI/STY* family members *STY1* and *LRP1* in mock-, EST-, CHX-, and EST + CHX-treated 8- or 10-d-old $35S_{pro}$:*DRNL-ER* seedlings were measured using qRT-PCR. Graphs show mean values of three biological replicates (three technical replicates per biological sample). Error bars represent SE of three biological replicates. The asterisks for *STY1* and *LRP1* denote significant (Student's *t* test, $P < 0.05$) up-regulation compared with mock and CHX treatments, respectively.

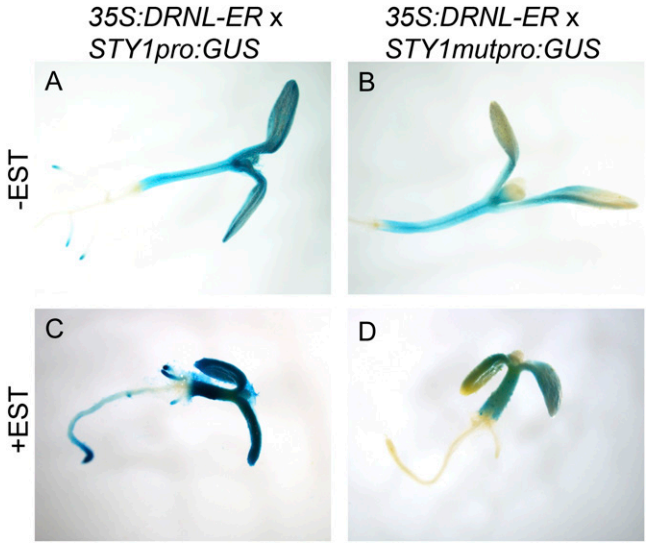

Figure 5. DRNL requires a functional GCC box to activate *STY1* in planta. A and C, Mock-treated (A) and EST-treated (C) $STY1_{pro}$:*GUS* $35S_{pro}$:*DRNL-ER*. B and D, Mock-treated (B) and EST-treated (D) $STY1mut_{pro}$:*GUS* $35S_{pro}$:*DRNL-ER*. Activation of DRNL by EST treatment results in thickening of the root and hypocotyls as well as a delay in cotyledon opening (C and D).

findings were supported by the inability of DRNL-ER to affect the development of seedlings in a $35S_{pro}$:*STY1-SRDX* background (Supplemental Fig. S1).

Loss of *DRN*, *DRNL*, and *PUCHI* Functions Results in Reduced *STY1* Expression

To test whether DRN and DRNL also regulate *STY1* activity in their normal expression domains, we analyzed the *STY1* transcript level in buds and seedlings of the *drn-1 drnl-1* double mutant line but found no statistically significant reduction (data not shown). However, in seedlings carrying the stronger *drnl-2* allele (Nag et al., 2007), the *STY1* transcript level was significantly reduced compared with the wild-type level (Fig. 7A). Furthermore, $STY1_{pro}$:*GUS* activity was reduced in cotyledons, shoot apices, and ovules of *drnl-2* plants. In 1-d-old *drnl-2* seedlings, GUS staining (2 h) was much reduced compared with wild-type seedlings (Fig. 7, B and C). Furthermore, we could not detect any GUS staining in 3-d-old *drnl-2* seedlings after 1 h of incubation with the substrate (Fig. 7, D and E), whereas some staining was detected in the cotyledons after overnight incubation (data not shown). The GUS activity in ovules of stage 12 flowers incubated in GUS substrate for 6 h or overnight was dramatically reduced in *drnl-2* plants compared with the wild type (Fig. 7, F–I). These tissues largely correspond to those losing *STY1* promoter activity when the GCC box is mutated (Fig. 2), suggesting that DRNL activates *STY1* transcription via the GCC box also in wild-type plants. In addition, when *drnl-1* was combined with *drn-1* as well as a mutation in *PUCHI* (Hirota et al., 2007), the closest homolog to the *DRN/DRNL* genes (Nakano et al., 2006), the *STY1* mRNA level in seedlings was significantly reduced compared with both Col and Landsberg *erecta* (L*er*; Fig. 7), further suggesting that not only DRNL, but also related proteins, play a role in the activation of *STY1* transcription.

Mutations in *DRNL*, *SHI/STY*, and *YUC* Genes Results in Similar Phenotypic Defects during Gynoecium Development

The carpel valve length is significantly reduced in plants with a reduced level of *SHI/STY* gene activity, such as the *SHI/STY* quintuple mutant, and in plants expressing a STY1 protein transformed from a transcriptional activator to a repressor by the addition of a C-terminal SRDX tag (Kuusk et al., 2006; Eklund et al., 2010a). Interestingly, it was recently demonstrated that around 25% of the *drn-1 drnl-1* flowers have one or two shortened carpel valves and/or are missing one valve (Chandler et al., 2011b; Table II). Here, we can show that this gynoecium defect was more severe in *drn-1 drnl-1 puchi-1* triple mutant plants, where 75% of the flowers had gynoecia with valve defects (Table II). In the triple mutant, some of the flowers also had valveless gynoecia with a protrusion of meristem-like tissue surrounded by a ring of stigma (Fig. 8), to our knowl-

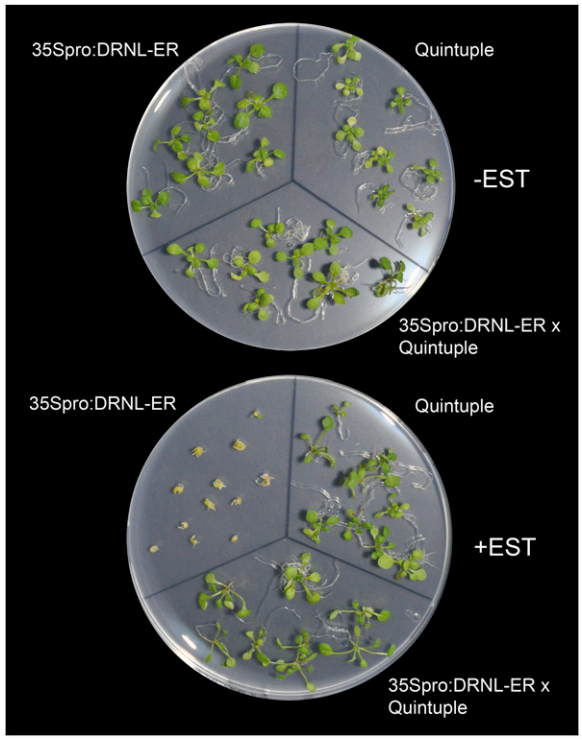

Figure 6. The phenotypes of seedlings constitutively expressing *DRNL* are mediated via *SHI/STY* genes. Plantlets of $35S_{pro}$:*DRNL-ER*, *SHI/STY* quintuple mutant (*sty1-1 sty2-1 shi-3 lrp1 srs5-1*), and $35S_{pro}$:*DRNL-ER SHI/STY* family multiple mutant lines, grown on mock treatment (top plate) or 10 μM EST (top plate) for 17 d, are shown.

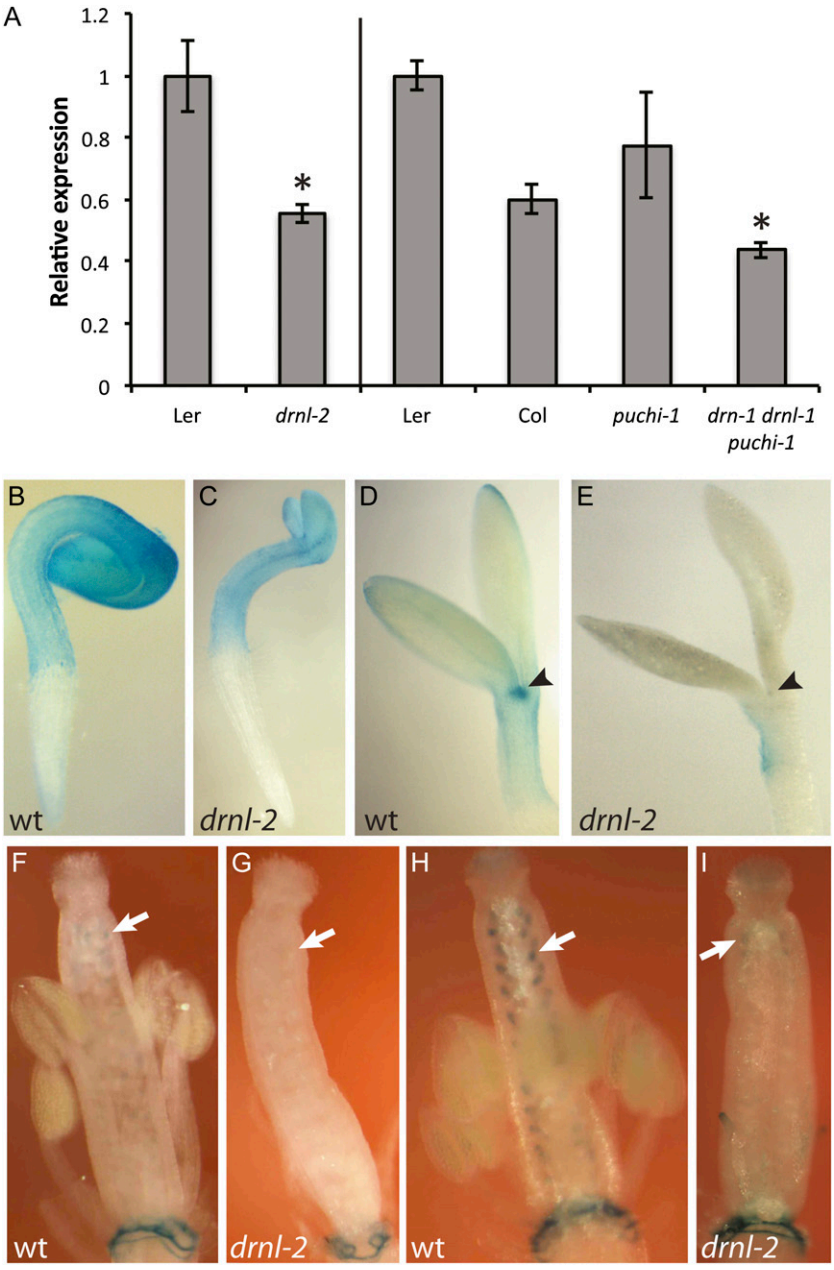

Figure 7. Expression of *STY1* is reduced in the *drnl-2* and *drn-1 drnl-1 puchi-1* mutant backgrounds. A, qRT-PCR-detected expression of *STY1* was reduced at 10 d after germination in seedlings of *drnl-2* and *drn-1 drnl-1 puchi-1* mutant lines. Shown are averages of three biological replicates (three technical replicates per biological sample). Error bars represent SE. The asterisk for *drnl-2* denotes a significant reduction in *STY1* expression compared with that of L*er* (Student's *t* test, $P < 0.05$); the asterisk for *drn-1 drnl-1 puchi-1* denotes a significantly reduced expression level compared with both L*er* and Col (Student's *t* test, $P < 0.05$). Both ecotypes were used for comparison, as *drn-1* and *puchi-1* are in the Col background, whereas *drnl-1* is in the L*er* ecotype. B to I, $STY1_{pro}$:*GUS* expression is reduced in *drnl-2* seedlings and flowers. B and C, GUS staining (2 h of incubation) is reduced in cotyledons of 1-d-old *drnl-2* seedlings compared with wild-type (wt) seedlings. D and E, No GUS staining was detected in cotyledons and the shoot apex (arrowheads) of 3-d-old *drnl-2* seedlings after 2 h of incubation, whereas STY_{pro}:*GUS* expression was strong at these sites in wild-type seedlings. F to I, STY_{pro}:*GUS* activity was strongly reduced in *drnl-2* ovules (arrows) compared with the wild type both after 6 h (F and G) and 24 h (H and I) of incubation.

edge a new phenotype not seen in the *drnl-2* single mutant, the *drn-1 drnl-1* double mutant, or the *puchi-1* single mutant. This indicates that *DRN*, *DRNL*, and *PUCHI* have redundant functions in gynoecium development. Furthermore, as similar gynoecium defects also are seen in the *yuc1 yuc4* double mutant (Cheng et al., 2006), our data suggest a possible link between *DRNL* and related genes, *STY1* and the STY1 downstream target *YUC4*, in gynoecium development.

DISCUSSION

Although auxins act in many diverse developmental processes, surprisingly little is known about auxin biosynthetic enzymes, intermediates, and pathways, and even less is known about the transcription factors that regulate genes involved in auxin biosynthesis (for review, see Chandler, 2009). Here, we have focused on the regulation of *SHI/STY* members to establish further their role in IAA-mediated plant development.

A GCC Box in Upstream Regulatory Regions of *SHI/STY* Members Is Essential for Expression in Most Aerial Organs

When searching for conserved upstream regulatory elements in *SHI/STY* family members, we found putative GCC boxes in all genes except for *SRS3*. However, the protein or proteins recognizing the element in the *STY1* promoter could still potentially recognize a

Table II. *The frequency of gynoecium defects is increased in drn-1 drnl-1 puchi-1 triple mutant flowers*

Values shown are percentages of flowers having different gynoecium defects. A total of 28 to 74 flowers were examined per genotype.

Genotype	Shortened Valves	Missing One Valve	Missing Both Valves	No Defects
puchi-1	0	0	0	100
drn-1 drnl-1	14	1	0	85
drn-1 drnl-1 puchi-1	13	35	27	25

rearranged element in *SRS3* not found in our analysis. Consequently, it remains to be analyzed whether the apparent lack of a GCC box in *SRS3* has resulted in major differences in its expression pattern compared with that of other *SHI/STY* genes. Although we have RT-PCR data suggesting that there are no major spatial differences in expression pattern among *SHI/STY* members in Arabidopsis, except that *SRS3* is not expressed in leaves, we lack the resolution of a $SRS3_{pro}$:*GUS* line or in situ hybridization data.

As shown previously, *SHI/STY* family members form two clades supported by high bootstrap values (Kuusk et al., 2006; Eklund et al., 2010b). We identified two main types of conserved GCC box-containing elements in *SHI/STY* members of Arabidopsis. Interestingly, one type was restricted to members of the *LRP1/SRS6* clade and the other type to members of the *STY1/SHI* clade. Apparent similarities between members of the two clades could mean that the GGCGGC part of the conserved element is important for shaping expression patterns of *SHI/STY* members of both clades. However, the split into two different regulatory elements could have contributed to subfunctionalization within the *SHI/STY* family, by subjecting *SHI/STY* family members to new regulatory mechanisms.

The GCC Box Is Essential for STY1-Mediated Auxin Biosynthesis

We have previously shown that STY1 induces the transcription of *YUC4* and *YUC8*, which are directly involved in the biosynthesis of IAA precursors (Sohlberg et al., 2006; Eklund et al., 2010a). Expression of $STY1mut_{pro}$:*GUS* resulted in the loss of GUS signal in the cotyledon tip and style/stigma, where the *SHI/STY* downstream target *YUC4* is expressed (Cheng et al., 2006, 2007), while $STY1mut_{pro}$:*GUS* expression remained in regions where auxin biosynthesis is not reported to occur: in stems, petiole margins, and the proximal part of the cotyledon. This suggests that the GCC box is essential for the regulation of *STY1*-mediated auxin biosynthesis in the shoot. *STY1*, however, does not appear to be strongly regulated by changes in auxin levels (Fig. 3).

$STY1mut_{pro}$:*GUS* Transformants Provide an Explanation for the Misleading GUS Signal in the *lrp1* Mutant

The *lrp1* mutant line, first described by Smith and Fedoroff (1995), was created by insertional mutagenesis with a gene trap transposon carrying a promoterless *GUS* gene. The insertion of the transposon, a DNA fragment of approximately 4 kb, is immediately in front of the GCC box-like regulatory region close to the TSS of *LRP1*. Hence, any cis-regulatory element in the 5′ UTR of *LRP1* is unlikely to contribute to the regulation of GUS expression. In this line, GUS signal was detected only in lateral root primordia, and the corresponding gene, *LRP1*, was suggested to be a lateral root primordium-specific gene (Smith and Fedoroff, 1995). However, Kuusk et al. (2006) showed that the putative null mutant *lrp1* enhances the gynoecium phenotype of the *sty1-1* mutant and also that *LRP1* transcripts are found by PCR-based methods in floral buds. Our results, implicating the strong lateral root primordium expression of *STY1* to be GCC box-independent, explain the spatially limited GUS expression of the *lrp1* mutant by suggesting that the GCC box in the 5′ UTR of *LRP1* does not contribute to activating GUS expression in *lrp1*.

Figure 8. *drn-1 drnl-1 puchi-1* triple mutants produce valveless gynoecia. At left is a wild-type (Col) gynoecium. At right is the valveless gynoecium of a *drn drnl-1 puchi-1* triple mutant, with a protrusion of meristem-like tissue surrounded by a ring of stigma. [See online article for color version of this figure.]

Ectopic Expression of *DRNL* Requires the GCC Box for the Activation of *SHI/STY* Genes

Our data clearly suggest that induction of the AP2/ERF protein DRNL activates the *STY1* and *LRP1* genes and that the expression of *SHI/STY* family members can explain some of the phenotypic effects of constitutive DRNL activity. First, we could show that the induction of constitutive DRNL expression results in activation of the *STY1* and *LRP1* promoters and that, at least for *STY1*, this activation required only a 1.3-kb upstream regulatory sequence present in the $STY1_{pro}$:*GUS* construct. These data suggest that DRNL may participate in the transcriptional complex regulating *SHI/STY* gene activity. Second, we could also show that the growth defects induced by ectopic DRNL expression are dependent on the activity of *SHI/STY* family members, as organs developed more normally in lines constitutively expressing DRNL in a background carrying multiple knockdowns of *SHI/STY* genes or the dominant negative repressor construct $35S_{pro}$:*STY1-SRDX*. Our data also reveal that the DRNL-mediated activation of ectopic $STY1_{pro}$:*GUS* activity in cotyledons is dependent on the GCC box, as DRNL failed to induce ectopic activity of the $STY1mut_{pro}$:*GUS* line. Our conclusion is that ectopic DRNL activates ectopic *SHI/STY* expression and that the GCC box is required for this activation.

DRNL and *DRN* Act Redundantly with Other AP2/ERF Proteins in the Regulation of *SHI/STY* Activity

Because *DRN/DRNL* and *SHI/STY* family members have overlapping expression patterns in the globular embryo, in the tips of cotyledon primordia in the embryo, in the leaf primordia and the distal tip of young leaves, in hydathodes, and in the stipules, ovules, and carpels (Fridborg et al., 2001; Kuusk et al., 2002, 2006; Kirch et al., 2003; Ikeda et al., 2006; Nag et al., 2007; Supplemental Fig. S2), and because DRNL clearly can induce *SHI/STY* gene activity, it appeared possible that DRNL could be involved in regulating the spatial and temporal activity of *SHI/STY* genes during plant development. However, the lack of DRNL activity in the style and receptacle suggests that other proteins may also activate *STY1*. *DRN* and *DRNL* have been suggested to have a highly redundant function in embryonic patterning and cotyledon formation in the same pathway as *MONOPTEROS* (Chandler et al., 2007, 2011a; Cole et al., 2009), suggesting that DRN could act as an upstream regulator of *SHI/STY* promoters as well. Although we were unable to detect alterations in *STY1* mRNA levels or STY_{pro}:*GUS*/SHI_{pro}:*GUS* expression in the *drn-1 drnl-1* double mutant (data not shown), a significant reduction of *STY1* mRNA levels was found in seedlings of the stronger *drnl-2* mutant allele (Nag et al., 2007) as well as of the *drn-1 drnl-1 puchi-1* triple mutant (Fig. 7). Furthermore, $STY1_{pro}$:*GUS* activity was reduced in *drnl-2* seedlings and gynoecia. Hence, DRNL does activate *SHI/STY* during plant development, but additional factors such as DRN and PUCHI appear to act redundantly with DRNL. This hypothesis is strongly supported by the enhancement of the *drnl-1* cotyledon defect in the *drn drnl* mutant (Chandler et al., 2011a) and of the *drn-1 drnl-1* valve defects in the *drn-1 drnl-1 puchi-1* triple mutant (this study) as well as the carpel valve phenotype in the single *drnl-2* mutant (Chandler et al., 2011b). Because the valve lengths also are affected in *SHI/STY* multiple mutants, it is likely that *DRN*, *DRNL*, and *PUCHI* mediate their control of valve development via the *SHI/STY* genes. Interestingly, constitutive expression of *LEP*, another *AP2/ERF* gene belonging to subgroup VIII, results in phenotypes resembling those of constitutive *DRNL* or *STY1* activity (Ward et al., 2006), suggesting that there could be additional upstream regulators of *SHI/STY1* among the AP2/ERF proteins. In addition, as *DRNL* is not expressed in the style, *STY* expression in this tissue is most likely mediated by another protein acting via the GCC box, potentially some of the other subgroup VIII proteins.

MATERIALS AND METHODS

Bioinformatic Analysis

Conserved motifs in upstream regulatory regions were investigated by MEME 4.0.0 (Bailey and Elkan, 1994; http://meme.sdsc.edu/meme/intro.html). Genome-wide searches for motifs in Arabidopsis (*Arabidopsis thaliana*) were performed using Patmatch version 1.1 (http://www.arabidopsis.org/cgi-bin/patmatch/nph-patmatch.pl).

Generation of Transgenic Lines

Plasmid pSRSGUS#7 (Kuusk et al., 2002) was mutated (GGCGGC to AAAAAA) with the QuickChange site-directed mutagenesis kit (Stratagene) using primers STY1sdmF and STY1sdmR (Supplemental Table S2). The resulting plasmid, $pSTY1mut_{pro}$:GUS, was transformed into *Agrobacterium tumefaciens* strain GV3101, containing the helper plasmid pMP90, and introduced into Arabidopsis Col by *A. tumefaciens*-mediated transformation. Selected T3 lines were crossed to $35S_{pro}$:*DRNL-ER* (Ikeda et al., 2006).

An *SRS4* promoter fragment was PCR amplified using primers SRS4P1SalI and SRS4P11BamHI (Supplemental Table S2). The fragment was cloned using the pCR-blunt II-TOPO cloning vector (Invitrogen), creating plasmid pSRS4.3. The promoter fragment was released by *Sal*I/*Bam*HI digestion and subsequently inserted into the two-component vector Bin-LhG4 (Craft et al., 2005), to create an $SRS4_{pro}$:LhG4 transcriptional fusion. Transformation of *A. tumefaciens* and Arabidopsis was performed as described above. Selected T3 lines were crossed with a transgenic line carrying the pOp:GUS construct, allowing the *SRS4* promoter to drive the expression of GUS. F1 SRS4pro>> GUS plants were analyzed for GUS expression.

A *SHI/STY* quintuple mutant (*sty1-1 sty2-1 shi-3 lrp1 srs5-1*; Kuusk et al., 2006) and a $35S_{pro}$:*STY1-SRDX* line (Eklund et al., 2010a) were crossed with $35S_{pro}$:*DRNL-ER* (Ikeda et al., 2006). The crosses resulted eventually in an F7 line homozygous for $35S_{pro}$:*DRNL-ER* with a distinct multiple *SHI/STY* mutant phenotype and an F3 line homozygous for $35S_{pro}$:*DRNL-ER* and $35S_{pro}$:*STY1-SRDX*.

ACC Treatment

Four-day-old etiolated $STY1_{pro}$:*GUS* seedlings were mock treated or treated with 10 μM ACC for 24 h.

Gene Expression Analysis

qRT-PCR was performed as described previously (Sohlberg et al., 2006) using primers targeting *ACTIN7* (*ACT7*) for normalization. Eight- to 10-d-old

35S$_{pro}$:DRNL-ER seedlings, grown on liquid medium, were mock treated or treated with EST (10 μM; Sigma E8875) or CHX (10 μM) at 45 or 75 min or 2 h. Seven-day-old light-grown or etiolated Col seedlings were mock treated or treated with 5 μM IAA for 10, 20, and 30 min or 1, 2, 4, 8, 12, 24, and 48 h. *drnl-2*, *puchi-1*, *drn-1 drnl-1 puchi-1*, L*er*, and Col seedlings were grown for 10 d. Primers targeting *ACT7* are described by Sohlberg et al. (2006). Primers targeting *STY1*, *LRP1*, and *GH3.3* are found in Supplemental Table S2.

Histochemical staining for GUS activity was performed using 5-bromo-4-chloroindolyl β-D-glucuronide as a chromogenic substrate according to Jefferson (1987). Plant tissues were incubated in GUS staining solution for 1, 2, 6, or 24 h and were destained in 70% ethanol. Samples were viewed in 50% ethanol and 50% glycerol and photographed using a stereo dissecting microscope (Nikon SMZ1500) with a Nikon DS-Fi1 camera and NIS-Elements D2.30 imaging software.

The *DRN$_{pro}$:GUS* line in this study was made by Kirch et al. (2003). The *DRNL$_{pro}$:GUS* line was made by Ikeda et al. (2006). Genotyping of the *drn-1* and *drnl-1* mutant alleles is described by Chandler et al. (2007), and the *drnl-2* mutant allele is described by Nag et al. (2007). The *PUCHI* mutant allele used was *puchi-1* (Hirota et al., 2007). *puchi-1* was genotyped using primers PUCHI_mark_F and PUCHI_mark_R (Supplemental Table S2).

Supplemental Data

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

Supplemental Figure S1. Suppression of SHI/STY function by SRDX-induced conversion of STY1 from a transcriptional activator to a transcriptional repressor prevents the induction of developmental changes induced by ectopic DRNL expression.

Supplemental Figure S2. Expression of *DRN*, *DRNL*, and *STY1* overlaps.

Supplemental Table S1. Localization of the additional six *LRP1/SRS6*-like GCC box elements.

Supplemental Table S2. PCR primers.

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