We used an RNA interference screen to assay the function of 53 transcription factor messenger RNAs (mRNAs) that accumulate specifically within soybean (*Glycine max*) seed regions, subregions, and tissues during development. We show that basic helix-loop-helix (bHLH) transcription factor genes represented by Glyma04g41710 and its paralogs are required for the formation of stoma in leaves and stomatal precursor complexes in mature embryo cotyledons. Phylogenetic analysis indicates that these bHLH transcription factor genes are orthologous to Arabidopsis (*Arabidopsis thaliana*) SPEECHLESS (*SPCH*) that initiate asymmetric cell divisions in the leaf protoderm layer and establish stomatal cell lineages. Soybean *SPCH* (*GmSPCH*) mRNAs accumulate primarily in embryo, seedling, and leaf epidermal layers. Expression of Glyma04g41710 under the control of the *SPCH* promoter rescues the Arabidopsis *spch* mutant, indicating that Glyma04g41710 is a functional ortholog of *SPCH*. Developing soybean embryos do not form mature stoma, and stomatal differentiation is arrested at the guard mother cell stage. We analyzed the accumulation of *GmSPCH* mRNAs during soybean seed development and mRNAs orthologous to *MUTE*, *FAMA*, and *INDUCER OF C-REPEAT/DEHYDRATION RESPONSIVE ELEMENT-BINDING FACTOR EXPRESSION1/SCREAM2* that are required for stoma formation in Arabidopsis. The mRNA accumulation patterns provide a potential explanation for guard mother cell dormancy in soybean embryos. Our results suggest that variation in the timing of bHLH transcription factor gene expression can explain the diversity of stomatal forms observed during plant development.

Seeds are highly organized structures that consist of three main regions: the embryo, endosperm, and seed coat. Each region is genetically and ontogenetically distinct and has a unique physiological function (Le et al., 2007; Ohto et al., 2008). The seed coat is maternally derived from the ovule integuments that surround the embryo sac (Radchuk and Borisjuk, 2014). By contrast, the filial embryo and endosperm are derived from a double fertilization in which the egg cell and central cell each fuse with a sperm cell to give rise to the zygote and endosperm mother cell, respectively (Lau et al., 2012, 2014; Li and Berger, 2012). The endosperm mother cell undergoes syncytial development, with nuclei migrating to different domains of the endosperm cell before cellularization occurs. The zygote divides asymmetrically to give rise to the apical cell that will form the embryo proper and the basal cell that will give rise primarily to the suspensor. The body plan of the new sporophyte is established during embryogenesis with the formation of the embryonic axis, which is defined by the shoot and root apical meristems, and the cotyledons that serve as a storage organ for reserves that will nourish the germinated seedling.

Substantial information is available about the anatomy, physiology, and biochemistry of seed development; however, the network of transcription factors responsible for directing the differentiation and function of different seed regions and subregions remains poorly defined. Genome-wide mRNA profiling studies

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**Author Contributions**

J.D., E.M., A.Q.B., B.H.L., P.M., M.H., J.P.-L., M.C., and J.M.P. performed the experiments; J.D., D.A.S., R.B.G., and J.J.H. supervised the research; R.B.G. and J.J.H. conceived the project; J.D. wrote the first article draft; J.J.H. wrote the final article draft with significant editing by R.B.G.

**Revised**

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of whole seeds and specific seed regions from both model plants and agriculturally relevant crops have provided insights into the gene networks that operate in seeds and the transcription factors that anchor these networks (for review, see Palovaara et al., 2013; Sreenivasulu and Wobus, 2013; Zhou et al., 2013; Becker et al., 2014). In most cases, however, the functional roles of the transcription factors in these predicted gene networks remain to be confirmed.

In a directed screen to identify key regulators of seed development, we analyzed publically available mRNA transcriptome data sets to identify mRNAs encoding transcription factors that accumulate uniquely in specific soybean (Glycine max) seed regions and subregions at four different stages of development, from fertilization to maturation. We used RNA interference (RNAi) to down-regulate the expression of 53 genes encoding seed transcription factor genes and uncovered mutations that affect seed and vegetative plant development. Here, we report the characterization of a basic helix-loop-helix (bHLH) family of transcription factor genes, represented by Glyma04g41710, that shares significant homology with Arabidopsis (Arabidopsis thaliana) SPEECHLESS (SPCH). Arabidopsis SPCH is required to establish the stomata cell lineage in leaf epidermal tissue (MacAlister et al., 2007).

Stomata are specialized pores in the leaf epidermis that open and close to regulate gas exchange for photosynthesis while modulating water loss. Differentiating stomata undergo three main phases of development (Fig. 1; for review, see Lau and Bergmann, 2012; Pillitteri and Torii, 2012; Pillitteri and Dong, 2013). Initially, a protodermal cell in the leaf epidermis called the meristemoid mother cell divides asymmetrically to produce a meristemoid and a new epidermal cell. The meristemoid undergoes several additional rounds of amplifying divisions, resulting in epidermal daughter cells surrounding the differentiated meristemoid. The meristemoid next transitions into a guard mother cell that then terminally differentiates into a pair of symmetrical guard cells that form the mature stomata complex. As shown diagrammatically in Figure 1, three subgroup la bHLH transcription factors, SPCH, MUTE, and FAMA, successively regulate the three cell-state transitions that define the stomata differentiation pathway in Arabidopsis. SPCH initiates the stomata cell lineage by inducing the first asymmetric division that gives rise to the meristemoid (MacAlister et al., 2007). MUTE and FAMA, respectively, direct the meristemoid to differentiate into the guard mother cell and, subsequently, into guard cells (Ohashi-Ito and Bergmann, 2006; Pillitteri et al., 2007). INDUCER OF C-REPEAT/DEHYDRATION RESPONSIVE ELEMENT-BINDING FACTOR EXPRESSION1 (ICE1) and SCREAM2, two partially redundant, subgroup IIb bHLH transcription factors, interact physically with SPCH, MUTE, and FAMA, and they are collectively required to establish stomatal cell lineage (Kanaoka et al., 2008).

In this paper, we describe bHLH transcription factors that are orthologs of Arabidopsis SPCH and that are essential for stomatal formation in embryo and leaf epidermal layers. Down-regulation of GmSPCH using RNAi has a significant impact on soybean postgerminative growth and development. GmSPCH is active in the epidermal layers of developing wild-type embryos, and it is required to establish stomatal precursor cells during seed development. Stomatal precursor cells are formed during soybean embryo development, but mature stomata are formed only after germination in seedlings, a phenomenon termed guard mother cell dormancy in soybean (Chou and Liu, 1992). We present evidence to suggest that differential expression of the bHLH transcription factor genes required for stomatal development, GmSPCH, GmMUTE-LIKE, and GmFAMA-LIKE, provides a potential explanation for guard mother cell dormancy during soybean seed development.

RESULTS

Functional Analysis of Transcription Factor mRNAs That Accumulate in Specific Soybean Seed Regions and Subregions

We identified genes encoding transcription factors that accumulate specifically in different soybean seed regions and subregions. RNAi was used to down-regulate the expression of these genes, and the effects of down-regulation on seed and postgerminative development were analyzed.

Identification of Seed mRNAs Encoding Region- and Subregion-Specific Transcription Factors

To identify seed region- and subregion-specific transcription factors at different developmental stages, we queried the Goldberg-Harada Soybean Seed Laser Capture Microdissection (LCM) Microarray Transcriptome Dataset (http://seedgenenetwork.net; Gene Expression Omnibus [GEO] series accessions GSE6414, GSE7511, GSE7881, and GSE8112). These data sets contain the mRNA profiles of every soybean seed...
region (i.e. embryo, endosperm, seed coat), subregion (e.g. embryo proper, suspensor, inner integument), and tissue (e.g. vascular bundle) at four developmental stages, globular, heart, cotyledon, and early maturation, that were obtained in Affymetrix GeneChip hybridization experiments. We parsed the data sets to identify mRNAs that accumulated specifically in subregions of the embryo, endosperm, and seed coat at these stages of development. Among the subregion-specific mRNAs, we focused on those encoding transcription factors using an annotation of the soybean IVT GeneChip (http://seedgenenetwork.net/annotate#soybeanIVT) that was derived using Nucleotide Basic Local Alignment Search Tool (BLASTN) analysis to associate probe sets on the array with soybean gene models (Glyma version 1.01; Schmutzer et al., 2010). Based on these analyses, 53 candidate genes were nominated for our RNAi screen (Supplemental Table S1).

**RNAi-Mediated Down-Regulation of Gene Expression in Developing Soybean Seeds**

We used RNAi to determine if the candidate transcription factors were required for seed development. Each RNAi construct was designed using a 150- to 200-bp inverted-repeat element consisting of a sense and antisense arm that corresponded to DNA sequences from protein coding regions, or 3′ untranslated regions of the target transcription factor gene separated by a short spacer element (see Supplemental Figure S1). RNAi transgenes driven by the *Cauliflower mosaic virus* 35S gene promoter were transferred to soybean using *Agrobacterium tumefaciens*-mediated transformation (see “Materials and Methods”), and R0 lines containing a single copy of the RNAi transgene were obtained and grown to maturity in the greenhouse. Developing R1 and R2 seeds and seedlings were screened for abnormalities in seed and vegetative plant morphologies.

Two experiments were used to assess the effectiveness of RNAi-mediated gene silencing during soybean seed development. First, we targeted a stably integrated and constitutively expressed GUS transgene for a siencing. An RNAi construct targeting the GUS gene, designated RNAi(GUS), was transferred into a line containing the 35S:GUS transgene. Four independent lines containing single-copy insertions of RNAi(GUS) were identified, and two lines that were homozygous for both the 35S:GUS and RNAi(GUS) transgenes and exhibited strong silencing were characterized in R2 seeds and R1 trifoliate leaves. Figure 2A shows that GUS activity in two lines containing the RNAi(GUS) transgene, lines 60 and 64, was reduced compared with that of plants without the RNAi construct in (1) cotyledon stage whole seeds [1.8% and 7.6% of GUS activity in lines without RNAi(GUS), respectively]; (2) early maturation stage whole seeds (15% and 9.4%); (3) early maturation stage embryo cotyledons (23% and 24%), axes (26% and 25%), and seed coats (2.7% and 1.1%); and (4) trifoliate leaves (0.42% and 0.079%).

Second, we used an RNAi transgene, RNAi(FIE), to silence orthologs of the Arabidopsis Polycomb group protein gene, FIE (Ohad et al., 1999). Mutations of the Arabidopsis FIE gene cause embryo lethality following double fertilization of female gametophytes containing maternally derived mutant alleles, and endosperm and seed coat development occur in ovules in the absence of fertilization (Ohad et al., 1996; Chaudhury et al., 1997). We targeted two of the four soybean genes most closely related to Arabidopsis FIE (Fig. 2B). Glyma10g02690 and Glyma02g17110 share 100% and 96.7% nucleotide sequence identity with the RNAi...
inverted repeat segment, respectively, whereas Glyma12g34240 and Glyma13g36310 shared no significant sequence similarity. Pods from R1 lines homozygous for the RNAi(GlymA4g41710) transgene were approximately 75% of the length of those on nontransgenic plants. R2 seeds were smaller relative to nontransgenic seeds, severely wrinkled, and inviable (Fig. 2, D and E), consistent with the observation in Arabidopsis that fie mutant seeds display embryo lethality (Ohad et al., 1996; Chaudhury et al., 1997). Moreover, R0 and R1 plants exhibited a range of vegetative mutant phenotypes, including short stature, extended internodes, and small trifoliate leaves as compared with nontransgenic plants. fie mutant Arabidopsis plants have been previously reported to display defects in vegetative development (Kinosita et al., 2001). Together, these RNAi silencing experiments targeting a reporter transgene and an endogenous seed development gene demonstrated that constitutively expressed RNAi transgenes effectively inhibited gene activity in both seeds and leaves of vegetative soybean plants.

We used RNAi to investigate the effects of down-regulating the expression of 53 different seed subregion-specific transcription factor genes (Supplemental Table). Eight to 10 independent R0 lines carrying a single copy of each RNAi transgene were generated, and five R1 progeny from homozygous R0 lines were analyzed. Three lines showing either defective seed or vegetative plant phenotypes that segregated with the RNAi transgene across several generations were uncovered from the 53 target transcription factor genes tested. One of these RNAi lines targeted a gene (Glyma01g38360) homologous to the Arabidopsis HAIRY MERISTEM4 gene (Engstrom et al., 2011) and produced a high frequency of hard seeds that failed to imbibe and germinate properly. Another RNAi construct targeted homologs of the Arabidopsis SCARECROW-LIKE gene (Glyma18g45220 and Glyma09g40620; Pysh et al., 1999) and exhibited an unusual inverted petiole branch growth. The third RNAi target, Glyma04g41710, encoded a gene homologous to an Arabidopsis bHLH transcription factor family that is involved in initiating stomatal development, and was subject to dosage effects.

To determine the extent to which the RNAi transgene down-regulated Glyma04g41710 gene expression, we measured Glyma04g41710 mRNA levels in newly emerged trifoliate leaves that had not yet displayed morphological defects from six independent R1 lines (lines 69, 73, 75, 78, 81, and 85) using quantitative reverse transcriptase-PCR. As shown in Figure 3B, Glyma04g41710 mRNA was detected in the RNAi (Glyma04g41710) transgenic lines at levels ranging between 7% and 68% of those in nontransgenic plants, and only one sibling had Glyma04g41710 mRNA levels that were similar to those of nontransgenic plants. A similar reduction of Glyma04g41710 mRNA was observed in an RNAi line at the early maturation stage (Fig. 3C). These results are consistent with the hypothesis that reduced Glyma04g41710 mRNA levels caused the observed mutant phenotype.

**Glyma04g41710 Is Functionally Equivalent to Arabidopsis SPCH**

Phylogenetic analyses, summarized in Figure 4A, indicated that Glyma04g41710 encodes a protein that is homologous with an Arabidopsis subgroup Ia family of bHLH transcription factors involved in stomatal development (Ohashi-Ito and Bergmann, 2006; MacAlister et al., 2007; Pillitteri et al., 2007). The predicted Glyma04g41710 protein, and three other paralogs, share highest similarity with the Arabidopsis SPCH gene that is required to establish the stomatal cell lineage in the leaf epidermis (Ran et al., 2013). Loss-of-function *spch* mutants lack stomata on their leaf epidermal surfaces (MacAlister et al., 2007).

To determine if Glyma04g41710 is functionally equivalent to *SPCH*, we tested its ability to genetically suppress the Arabidopsis *spch3* mutation. *spch3* mutants arrest their development as small and pale seedlings that fail to develop stomata in the leaf epidermis and eventually die as seedlings (Fig. 4C; Supplemental Fig. S2; MacAlister et al., 2007). We constructed a transgene consisting of the Glyma04g41710 complementary DNA (cDNA) fused with the Arabidopsis *SPCH* gene promoter, designated as *SPCH:Glyma04g41710*. As a control, we used the Arabidopsis *SPCH* cDNA under the

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**Down-Regulating Glyma04g41710 Expression Causes Defects in Leaf Development**

We analyzed the phenotype of lines containing an RNAi construct, RNAi(Glyma04g41710), that targeted an embryo proper-specific transcription factor mRNA that accumulated at the globular, heart, cotyledon, and early maturation stages of development (Supplemental Table). Nine independent R0 lines carrying one copy of the RNAi(Glyma04g41710) transgene were generated. Although no obvious morphological defects were observed in the R0 lines, the trifoliate leaves of R1 lines segregating with the RNAi transgene developed lesions, yellowed rapidly, and underwent premature senescence within 1 to 2 weeks after leaf expansion (Fig. 3A). Defects in leaf development continued after the initiation of flowering, and pod and seed development were severely compromised in the R1 and R2 plants of all nine lines. We consistently observed that plants homozygous for the RNAi(Glyma04g41710) transgene exhibited more severe vegetative defects than heterozygous plants among R1 and R2 siblings (Fig. 3A), and that heterozygous lines frequently died before setting pods. Nontransgenic, heterozygous, and homozygous plants produced approximately 62, 10, and zero to two pods per plant, respectively. These findings suggested that RNAi-mediated silencing of Glyma04g41710 compromised leaf development, severely impaired plant development, and was subject to dosage effects.
control of its own promoter, designated as \(\text{SPCH:SPCH}\) (Lampard et al., 2008). Both constructs were transferred into \(\text{spch}3\) mutants, and the resulting T2 seedlings were screened for lethality. Of transgenic seedlings that were homozygous for the \(\text{spch}3\) mutation, 84% of the seedlings containing \(\text{SPCH:Glyma04g41710}\) produced viable plants \((n = 56; \text{Fig. 4C})\), although the transgenic seedlings were often smaller than wild-type seedlings. Rescued transgenic seedlings possessed functional stomata, although stomatal spacing differed from that of wild-type seedlings (Supplemental Fig. S2). One hundred percent of \(\text{spch}3\) seedlings containing \(\text{SPCH:SPCH}\) were viable \((n = 15)\), but aberrant stomatal spacing was also observed with the Arabidopsis \(\text{SPCH:SPCH}\) gene (Supplemental Fig. 3).
Fig. S2), possibly indicating that the SPCH promoter in the transgene does not recapitulate the expression pattern of the endogenous gene. We conclude that Glyma04g41710 is a functional ortholog of Arabidopsis SPCH, because it can genetically suppress the spch3 mutation, although with reduced penetrance and expressivity.

**Down-Regulating GmSPCH Causes Defects in Stomatal Development**

We asked whether plants with the RNAi(Glyma04g41710) transgene displayed defects in stoma formation. We compared epidermal cell morphology in young trifoliate leaves of plants with and without the RNAi(Glyma04g41710) transgene. As shown in Figures 5, B, C, and G and summarized in Figure 5J, neither mature stomata nor meristemoid complexes were detected in trifoliate leaves of plants either heterozygous or homozygous for the RNAi construct, similar to Arabidopsis spch plants. By contrast, nontransgenic plants contained normal, mature stomata (Fig. 5, A and J).

We determined which of the SPCH paralogs in soybean were targeted by the RNAi transgene. Glyma06g13081, Glyma13g08740, and Glyma14g31385 have 98%, 85%, and 83% sequence identity, respectively, with the nucleotide sequence used to create the inverted repeat of the RNAi(Glyma04g41710) transgene (see Fig. 4A and Supplemental Table). Quantitative reverse transcriptase-PCR experiments with leaf mRNA revealed that all four SPCH paralogs were down-regulated by the RNAi transgene (Supplemental Fig. S3). We designated the four paralogs, Glyma04g41710, Glyma06g13081, Glyma13g08740, and Glyma14g31385, as GmSPCH1 to GmSPCH4, respectively, consistent with published nomenclature (Ran et al., 2013). Because at least one of the soybean SPCH paralogs has SPCH function, we collectively reference these genes as GmSPCH.

**GmSPCH Is Required to Initiate Stomatal Development during Seed Development**

We queried the Goldberg-Harada Soybean RNA-Seq Dataset containing whole seed mRNA profiles at different developmental stages (GEO accession GSE29163) to investigate GmSPCH mRNA levels. As shown in Figure 6B, GmSPCH1 and GmSPCH2 mRNAs were down-regulated in the RNAi(Glyma04g41710) transgene, whereas GmSPCH3 and GmSPCH4 mRNAs were less affected. We observed that GmSPCH1 and GmSPCH2 mRNAs were significantly reduced in the RNAi(Glyma04g41710) transgene, whereas GmSPCH3 and GmSPCH4 mRNAs were less affected. We determined that all four SPCH paralogs were down-regulated by the RNAi transgene (Supplemental Fig. S3). We designated the four paralogs, Glyma04g41710, Glyma06g13081, Glyma13g08740, and Glyma14g31385, as GmSPCH1 to GmSPCH4, respectively, consistent with published nomenclature (Ran et al., 2013). Because at least one of the soybean SPCH paralogs has SPCH function, we collectively reference these genes as GmSPCH.
detected at the highest levels in early maturation stage seeds, and they were prevalent in both seedlings and trifoliate leaves. By contrast, GmSPCH3 mRNA was 0.2% to 6% of GmSPCH1 and GmSPCH2 mRNA levels, and GmSPCH4 mRNA was not detected at any seed stage.

We used the Harada-Goldberg Soybean Early-Maturation Stage Seed LCM RNA-Seq Dataset (GEO accession GSE46096) that contains the mRNA transcriptomes of 16 different seed regions, subregions, and tissues to determine where GmSPCH mRNAs are localized within the early maturation stage seed (Fig. 6A). These data indicated that GmSPCH genes are expressed specifically in the embryo during seed development, in agreement with the seed microarray data set. GmSPCH1 and GmSPCH2 mRNAs were detected at the highest levels in the epidermal cell layers of embryo subregions and tissues that contained an epidermis (e.g. cotyledons, shoot apical meristem, plumule, root tip; Fig. 6B), consistent with a role in stomatal development. The only exception was that GmSPCH1 mRNA also accumulated in the stele of the embryonic axis, a precursor of the vascular system.

We analyzed developing embryo cotyledons to determine if the stomatal cell lineage was established during seed development, given the robust expression of GmSPCH1 and GmSPCH2 in the abaxial and adaxial epidermal layers (Fig. 6, A and B) and the fact that soybean cotyledons possess stomata following germination. We examined the adaxial cotyledon epidermis from nontransgenic embryos at the midmaturation stage and observed regularly spaced meristemoid complexes consisting of a meristemoid surrounded by stomatal lineage ground cells (Fig. 5, D and H). No mature stomata were detected in cotyledons at the midmaturation stage of seed development, nor in mature seeds imbibed for 3 d. These observations indicate that stomatal development is initiated during seed development, and that mature stoma terminally differentiate after germination.

To determine if the formation of meristemoid complexes in developing cotyledons is dependent on GmSPCH expression, we analyzed the epidermis of cotyledons from plants containing the RNAi (Glyma04g41710) transgene in which the expression of GmSPCH was down-regulated. Figure 5, F, I, and J, and Supplemental Figure S4 show that meristemoid complexes were not detected in the cotyledon epidermal layers of lines homozygous for the RNAi(Glyma04g41710) transgene at the midmaturation seed development (top) and the indicated early maturation stage seed subregions (bottom). C, mRNA levels of soybean ICE1/SCREAM2-LIKE paralogs. RNA-seq data were taken from GEO series GSE29163 and GSE46096. cot, Cotyledon; dry, dry seed; em, early maturation; glob, globular; hrt, heart; lm, late maturation; mm, mid maturation; sdlg, seedling 6 d after imbibition; abepd, abaxial epidermis; abpy, abaxial parenchyma; adepd, adaxial epidermis; adpy, adaxial parenchyma; epd, epidermis; es, endosperm; hi, hilum; hg, hourglass; pl, plumule; ps, palisade; py, parenchyma; rt, root tip; sam, shoot apex; st, stele; vs, vasculature.
stage or at 6 d after imbibition. The absence of stomata was consistent with our finding that GmSpCH1 mRNA levels were only 3% to 5% of those present in nontransgenic seeds (Fig. 3C), and that all GmSpCH paralogs are targeted by the RNAi construct (Supplemental Fig. S3). In lines heterozygous for the RNAi construct, meristemoid complexes were observed in the cotyledon epidermis during seed development and stomata were detected in cotyledons of seedlings, although their number was reduced relative to the wild type (Fig. 5, E and J; Supplemental Fig. S4). Thus, GmSpCH is required to initiate stomatal development in developing cotyledons.

**Mature Stomata Formation following Seed Germination Coincides with GmFAMA-LIKE Expression**

We analyzed the expression of other transcription factor genes that are key regulators of Arabidopsis stomatal development, MUTE, FAMA, ICE1, and SCREAM2, to understand what causes mature stomata not to form during seed development. Arabidopsis stomatal differentiation progresses through three transitional cell states (meristemoid mother cell to meristemoid, conversion of meristemoid to guard mother cell, and guard mother cell to stoma) that are regulated sequentially by SPCH, MUTE, and FAMA transcription factors, respectively (Fig. 1; for review, see Lau and Bergmann, 2012; Pillitteri and Toriz, 2012; Pillitteri and Dong, 2013). ICE1 and SCREAM2 are required to form functional heterodimers with SPCH, MUTE, and FAMA (Kanaoka et al., 2008). We queried the soybean seed mRNA databases to determine the mRNA accumulation patterns of putative MUTE, FAMA, ICE1, and SCREAM2 orthologs identified by phylogenetic analyses (Fig. 4, A and B) throughout development. As shown in Figure 6B, GmSpCH1, 2, and 3 mRNA levels peaked at the early maturation stage of seed development, whereas the two GmMUTE-LIKE mRNAs reached their highest levels later at the mid-maturation stage. All GmSpCH and GmMUTE-LIKE mRNAs accumulated in seedlings and leaves (Fig. 6B). By contrast, GmFAMA-LIKE mRNAs did not accumulate appreciably in developing seeds but became prevalent in seedlings and leaves (Fig. 6B). Two ICE1/SCREAM2-LIKE mRNAs were present throughout seed development, as well as in seedlings and leaves (Fig. 6C). Together, these results are consistent with the hypothesis that the low levels of GmFAMA-LIKE mRNAs in developing embryos account for the absence of mature stomata on cotyledon epidermal layers during seed development.

**DISCUSSION**

**RNAi Screen of Soybean Seed Subregion-Specific Transcription Factor Genes Identifies Developmental Regulators**

The directed gene silencing strategy that we carried out in this study focused on soybean transcription factor genes that are expressed in specific regions and subregions of developing seeds. We used RNAi to down-regulate the expression of 33 transcription factor genes and isolated three lines with mutant phenotypes that were linked to the RNAi transgene. Thus, 5.7% of the genes targeted with RNAi transgenes yielded plants with mutant phenotypes. The low number of lines with mutant phenotypes likely reflects incomplete penetrance of RNAi in down-regulating gene expression, duplication of targeted transcription factor genes, or both. For example, RNAi was estimated to effectively silence approximately 35% of maize (Zea mays) lines containing RNAi constructs (McGinnis et al., 2007). In addition, genome-wide RNAi screens of Caenorhabditis elegans showed that 10% to 25% of the genes targeted for silencing yielded mutant phenotypes (Sugimoto, 2004). Because soybean is a polyploid with approximately 75% of genes present in multiple copies (Schmutz et al., 2010), the phenotype discovery rate is predicted to be lower than that of C. elegans. For example, studies showed that approximately 2% of soybean lines mutagenized with fast neutron irradiation displayed altered phenotypes, even though each line was estimated to possess mutations in approximately 33 genes (Bolon et al., 2011). Despite the low mutant discovery rate of our RNAi screen, we were able to identify GmSpCH, a transcription factor that regulates cell fate and plays a critical role in initiating stomatal development during seed development. Our demonstration that the RNAi (Glyma04g41710) transgene silenced all four GmSpCH paralogs likely facilitated detection of the mutant phenotype (Supplemental Fig. S3). Thus, transgenes that target several paralogs may offer distinct advantages for RNAi screens.

**Soybean SpCH Is Required for Stoma Formation in Embryonic Cotyledons**

Several lines of evidence suggest that the subgroup Ia bHLH transcription factors represented by Glyma04g41710 are functionally equivalent to Arabidopsis SPCH and are required for the initiation of stoma formation in developing embryos. First, the GmSpCH genes are most closely related to Arabidopsis SPCH (Fig. 4; Pires and Dolan, 2010; Ran et al., 2013). Second, the GmSpCH representative, Glyma04g41710, at least partially suppresses the Arabidopsis spch3 mutation (Fig. 4; Supplemental Fig. S2). The ability of GmSpCH1 to rescue an Arabidopsis spch mutant is consistent with reports that the sequence and function of bHLH transcription factors involved in stomatal formation are highly conserved in land plants (Peterson et al., 2010; MacAlister and Bergmann, 2011; Rudall et al., 2013). Others have shown that a Physcomitrella patens subgroup Ia bHLH transcription factor can partially suppress mute and fama but not spch mutations in Arabidopsis (MacAlister and Bergmann, 2011). Therefore, our results strongly suggest that GmSpCH shares strong functional similarities with
Expression of Subgroup Ia bHLH Transcription Factor Genes Offers a Potential Explanation for the Absence of Mature Stomata in Seeds

Plants exhibit a diversity of stomatal forms, and much of the variation has been attributed to differences in the timing of expression of the subgroup Ia bHLH transcription factors (Pillitteri and Torii, 2007; Peterson et al., 2010; Rudall et al., 2013). The extent of stomatal development that occurs during embryogenesis is a reflection of this variability. In only rare cases have mature stomata been observed on mature embryos; for instance, on the primary root axis of *Ceratonia siliqua* and occasionally on Arabidopsis cotyledons (Christodoulakis et al., 2002; Geisler and Sack, 2002). Instead of having embryos with mature stomata, most plant embryos are characterized by either the presence or absence of stomatal precursor complexes (Lovell and Moore, 1970; Marshall and Kozlowski, 1977; Chou and Liu, 1992). For example, Arabidopsis generally possesses stomatal precursor complexes on dry seed embryos, although their number varies greatly (Bougourd et al., 2000; Geisler and Sack, 2002), whereas stomatal precursor complexes are not detected in pea (*Pisum sativum*; Blackwell, 1914).

We have shown that soybeans possess meristemoid complexes on the adaxial surface of embryonic cotyledons, and that *GmSPCH* is required for meristemoid complex formation (Fig. 5). Consistent with our observations, others have reported the presence of guard mother cells on the cotyledon epidermal layers of soybean dry seeds, and designated this observation as guard mother cell dormancy (Chou and Liu, 1992). The expression patterns of subgroup Ia bHLH transcription factor genes offer a potential explanation for this phenomenon. First, *GmSPCH* mRNA is the subgroup Ia bHLH transcription factor mRNA that is first detected in developing embryos, consistent with its role in Arabidopsis in promoting the asymmetric division of the meristemoid mother cell (MacAlister et al., 2007; Pillitteri et al., 2007) and maintaining meristemoid self-renewal activity (Robinson et al., 2011). Second, *GmMUTE-LIKE* mRNA levels increase while *GmSPCH* mRNAs decline at the midmaturation stage. Arabidopsis MUTE acts to repress the self-renewal activity of meristemoids and induces guard mother cell formation, consistent with the presence of guard mother cells on embryonic cotyledons of dry seeds (Pillitteri et al., 2007). *GmMUTE-LIKE* mRNA levels decline by the late maturation stage and are not detected in dry seeds. Third, *GmFAMA-LIKE* mRNA is not detected during seed development, but is present in postgermination plants. FAMA is required to induce guard mother cells to divide and form guard cells in Arabidopsis (Ohashi-Ito and Bergmann, 2006). The functional roles of *GmMUTE-LIKE* and *GmFAMA-LIKE* in soybean stomatal development remain to be determined. However, studies with rice (*Oryza sativa*), which is more distantly related to Arabidopsis than soybean, showed that OsMUTE is involved in the transition of meristemoids into guard mother cells and that OsFAMA controls the terminal differentiation of guard cells (Liu et al., 2009). Thus, the sequential accumulation of *GmSPCH*, *GmMUTE-LIKE*, and *GmFAMA-LIKE* mRNA throughout seed and seedling development is consistent with the observations that guard mother cells are present in mature soybean embryos and that mature stomata are present only in seedlings. Thus, our findings offer a plausible explanation for guard mother cell dormancy.

MATERIALS AND METHODS
RNAi Construct Design

The sense and antisense arms of the inverted repeat element were designed using 150 to 320 bp of coding and/or 3'-untranslated region sequence corresponding to each target gene based on small interfering RNAi efficacy predictions using the best Reynolds score values (Khvorova et al., 2003; Reynolds et al., 2004). The sense and antisense arms were separated by the universal spacer sequence described by Hauge et al. (2009) to produce the inverted repeat element. A subset of RNAi constructs were designed to target the coding region of two or three genes simultaneously (Supplemental Table). In these multitarget vectors, the antisense and sense arms for each target gene were concatenated together end to end to produce one continuous antisense and sense arm (Supplemental Fig. S1). Inverted repeat elements were cloned using high-throughput ligase independent cloning methodology described by Hauge et al. (2009). Each RNAi element was cloned into a ligase independent cloning acceptor site of the binary transformation vector, pMON78277, downstream of the enhanced *Caulliflower mosaic virus* 35S constitutive promoter (Kay et al., 1987) and stably transformed into the soybean (*Glycine max*) genome.

Agrobacterium spp. Preparation and Plant Transformation

Binary transformation vectors containing each RNAi construct and the *aminoglycoside-3'-adenyltransferase* selectable marker gene were transformed into soybean var A3525 meristem explants using the *Agrobacterium tumefaciens* (strain AB30)-mediated transformation method as described by Ye et al. (2008). Transgenic plants were obtained using spectinomycin selection (Martellin et al., 2013).

Molecular Analysis of Transgene Copy Number

The transgene copy number for each transgenic line was determined using AGBio Services, Invader Technologies (Hologic). DNA was isolated from 8-mm leaf disc tissue samples and assayed for transgene copy number as described by the manufacturer using an Invader Technologies probe designed with a construct-specific sequence tag (GCCCTTGGGTTAATTTC) common to the 3'-untranslated region of all RNAi cassettes.

Quantitative Fluorimetric GUS Assay

Tissue samples were isolated from trifoliate leaves, cotyledon stage, and early maturation stage seeds. Lysophilized tissue was mechanically disrupted using metal beads, and total soluble protein was extracted in 800 μL of protein extraction buffer (0.1 M potassium phosphate, pH 7.4, 1 mM EDTA, 0.1% [w/v] lauryl sarcosine, 0.1% [v/v] Triton X-100, 2% [w/v] polyvinylpyrrolidone, 0.05% [v/v] L of protein extraction buffer (0.1 M potassium phosphate, pH 7.4, 1 mM EDTA, 0.1% [w/v] lauryl sarcosine, 0.1% [v/v] Triton X-100, 2% [w/v] polyvinylpyrrolidone, 0.05% [v/v]
glycerol, 10 mM β-mercaptoethanol, and 0.2 mM phenylmethylsulfonyl fluoride). Activity assays were performed using the 4-methylumbelliferoneβ-D-glucuronide substrate (Bradford, 1976; Jefferson et al., 1987; Russell and Fromm, 1997).

In Silico Analysis of Soybean Genes and Their Expression

The following data sets were used to identify subregion-specific mRNAs encoding transcription factors and to analyze mRNA accumulation during seed development: (1) Goldberg-Harada Soybean Seed LCM Microarray Transcriptome Dataset (GEO series GSE6414, GSE7511, GSE7881, and GSE8132), (2) Goldberg-Harada Soybean Whole Seed RNA-Seq Dataset (GEO series GSE29163), and (3) Harada-Goldberg Soybean Early Maturation Stage Seed LCM RNA-Seq Dataset (GEO series GSE46096). Data sets are also available (http://seedgenenetwork.net/).

Soybean homologs of Arabidopsis (Arabidopsis thaliana) transcription factors that regulate stomata development were identified using Translated BLAST (BLASTX) analyses (Camacho et al., 2009) coupled with phylogenetic analyses carried out using the bootstrap neighbor-joining algorithm in ClustalX2.1 (Larkin et al., 2007).

Genetic Suppression in Arabidopsis

The Arabidopsis SPCH promoter, SPCH cDNA clone, and pMDCl07 transformation vector were provided by the Bergmann Laboratory (MacAlister et al., 2007). The SPCH:SPCH construct was made in the pENTr/TOPO vector. The SPCH:Glyma04g41710 construct was made by cloning the amplified Glyma04g41710 cDNA into the pENTR/D-TOPO vector, and fusing it with the Arabidopsis SPCH promoter. Both constructs were cloned into pMCD107, transformed into A. tumefaciens strain GV3101, and transferred by Agrobacterium tumefaciens it with the Arabidopsis SPCH into wild-type Columbia-0 and spch3103 Plant Physiol. Vol. 168, 2015 heterozygous plants. Transgenic T1 seeds were selected on germination media and transferred to soil.

Confocal Microscopy

The seed coat was removed from the developing early maturation and mid-maturation stage seeds, and the cotyledons were carefully dissected to expose the adaxial surfaces. Epidermal tissue was hand sectioned from the adaxial and abaxial cotyledon surfaces using a scalpel and placed in a 0.02 mg mL−1 dilution of propidium iodide solution for imaging as described earlier.

Quantitative Real-Time PCR

Tissue was harvested from 1-cm² leaf punches of newly unfurled trilobate leaves or whole seeds removed from the pods at the appropriate developmental stage. All tissue was frozen in liquid nitrogen and stored at −80°C until processing. Isolation of total mRNA and quantitative real-time PCR was performed as described by Le et al. (2010) using gene-specific primers.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Diagrammatic representation of RNAi transgenes.

Supplemental Figure S2. Glyma04g41710 rescues the stomatal formation defect of the Arabidopsis spch-3 mutant.

Supplemental Figure S3. RNAi(Glyma04g41710) down-regulates all four GmSPEECHLESS paralogs.

Supplemental Figure S4. Effect of RNAi(Glyma04g41710) on seedling cotyledons.

Supplemental Table S1. Subregion-specific transcription factors targeted in the RNAi screen.

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