A shared cis-regulatory module activates transcription in the suspensor of plant embryos.
A shared cis-regulatory module activates transcription in the suspensor of plant embryos

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The mechanisms controlling the transcription of gene sets in specific regions of a plant embryo shortly after fertilization remain unknown. Previously, we showed that G564 mRNA, encoding a protein of unknown function, accumulates to high levels in the giant suspensor of both Scarlet Runner Bean (SRB) and Common Bean embryos, and a cis-regulatory module containing three unique DNA sequences, designated as the 10-bp, Region 2, and Fifth motifs, is required for G564 suspensor-specific transcription [Henry KF, et al. (2015) Plant Mol Biol 88:207–217; Kawashima T, et al. (2009) Proc Natl Acad Sci USA 106:3627–3632]. We tested the hypothesis that these motifs are also required for transcription of the SRB GA 20-oxidase gene, which encodes a gibberellic acid biosynthesis enzyme and is expressed with G564 at a high level in giant bean suspensors. We used deletion and gain-of-function experiments in transgenic tobacco embryos to show that two GA 20-oxidase DNA regions are required for suspensor-specific transcription, one in the 5′ UTR (+119 to +205) and another in the 5′ upstream region (−341 to −316). Mutagenesis of sequences in these two regions determined that the cis-regulatory motifs required for G564 suspensor transcription are also required for GA 20-oxidase transcription within the suspensor, although the motif arrangement differs. Our results demonstrate the flexibility of motif positioning within a cis-regulatory module that activates gene transcription within giant bean suspensors and suggest that G564 and GA 20-oxidase comprise part of a suspensor gene regulatory network.

plant embryos | Scarlet Runner Bean | cis-regulatory modules | suspensor | promoter analysis

In most higher plants, embryogenesis begins with the asymmetric division of the zygote to give rise to a small apical cell and a large basal cell (1). The apical and basal cells follow distinct pathways to differentiate into an embryo proper and suspensor, respectively (2, 3). Whereas the embryo proper undergoes many developmental and morphological changes to eventually become the mature embryo within the seed, the suspensor is a terminally differentiated embryo region that degenerates as the embryo matures. Several studies have shown that different genes are expressed in the embryo proper and suspensor (4–8), but how these genes are organized into regulatory networks (9) operating in the different embryo regions remains unknown.

Previously, we began to dissect the gene regulatory networks programming early embryo development by analyzing the activation of G564, a gene encoding a protein of unknown function that is active specifically in the giant suspensors of Scarlet Runner Bean (Phaseolus coccineus) and Common Bean (Phaseolus vulgaris) (Fig. 1 A–E) (10–12), which diverged ~2 Mya (13). G564 suspensor transcription is activated by five motifs: (i) three 10-bp motifs with the consensus 5′-GAAAGCCGAA-3′ that can tolerate up to three nonadjacent mismatches; (ii) a Region 2 motif, 5′-TTG(A/G)(A/G/T)AAT-3′; and (iii) a Fifth motif, 5′-(A/G)AGTTA-3′ (Fig. 2) (11, 14).

In this paper we test the hypothesis that genes with similar suspensor-specific expression patterns in giant bean suspensors utilize a shared cis-regulatory module (9) with common cis-control elements. We show that genes encoding enzymes for each step of the gibberellic acid (GA) biosynthesis pathway (15) are expressed at high levels in SRB and Common Bean globular-stage suspensors, similar to G564, suggesting that these genes are coregulated. We analyzed in detail the upstream region of one gene in the GA pathway, SRB GA 20-oxidase, and present experiments demonstrating that the GA 20-oxidase upstream region can activate suspensor transcription in globular-stage tobacco (Nicotiana tabacum) embryos. Deletion, gain-of-function (GOF), and mutation analyses in transgenic tobacco embryos showed that the GA 20-oxidase upstream region −341 to +238 is sufficient for suspensor-specific transcription and contains functional cis-regulatory elements that are also required for suspensor transcription of the SRB G564 gene (11, 14). Mutagenesis of the predicted suspensor cis-regulatory elements in the GA 20-oxidase upstream region showed that sequences similar to the 10-bp motif, Region 2 motif, and Fifth motif are required for GA 20-oxidase suspensor transcription. Our results demonstrate that the transcription of the G564 and GA 20-oxidase genes within the SRB suspensor is activated using a shared cis-regulatory module that differs in the number, spacing, and order of cis-motifs and that this cis-regulatory module may form part of a gene regulatory network that operates in giant bean suspensors shortly after fertilization.

Results

mRNAs Encoding GA Biosynthesis Enzymes Localize to the SRB and Common Bean Giant Suspensor. We carried out in situ hybridization analysis on SRB globular-stage seeds to determine the mRNA localization patterns for genes encoding enzymes in the plant embryo. We tested the hypothesis that these motifs are also required for transcription of the SRB GA 20-oxidase gene, which encodes a gibberellic acid (GA) hormone biosynthesis enzyme and is expressed specifically within the suspensor. We show that cis-regulatory motifs required for GA 20-oxidase transcription within the suspensor are the same as those required for suspensor-specific transcription of the SRB G564 gene, although motif number, spacing, and order differ. These cis-elements constitute a control module that is required to activate genes in the SRB suspensor and may form part of a suspensor regulatory network.

Significance

Little is known about how genes are expressed in different plant embryo regions. We tested the hypothesis that shared cis-regulatory motifs control the transcription of genes specifically in the suspensor. We carried out functional studies with the Scarlet Runner Bean (SRB) GA 20-oxidase gene that encodes a gibberellic acid (GA) hormone biosynthesis enzyme and is expressed specifically within the suspensor. We show that cis-regulatory motifs required for GA 20-oxidase transcription within the suspensor are the same as those required for suspensor-specific transcription of the SRB G564 gene, although motif number, spacing, and order differ. These cis-elements constitute a control module that is required to activate genes in the SRB suspensor and may form part of a suspensor regulatory network.

Author contributions: K.F.H., A.Q.B., T.K. and R.B.G. designed research; K.F.H., A.Q.B. and T.K. performed research; K.F.H., A.Q.B., T.K. and R.B.G. analyzed data; and K.F.H. and R.B.G. wrote the paper.

Reviewers: Z.J.C., University of Texas at Austin; and T.T., Texas A&M University.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1805802115/-/DCSupplemental.

Published online June 4, 2018.
GA biosynthesis pathway (Fig. 1F–K). We observed that mRNAs encoding six enzymes leading to the synthesis of bioactive GA (ent-kaurene synthase A, ent-kaurene synthase B, ent-kaurene oxidase, ent-kaurenioic acid hydroxylase, GA 20-oxidase, and GA 3-oxidase) accumulated primarily in the giant suspensor region (Fig. 1F–K). This extends previous studies that showed that SRB suspensors are a rich source of GA (16), synthesize GA in cell-free extracts (17, 18), and contain GA 3-oxidase mRNA (6, 19).

We confirmed our SRB GA mRNA localization studies and expanded them to the closely related Common Bean by using (i) laser-capture microdissection technology to collect SRB and Common Bean globular-stage embryo proper and suspensor regions, (ii) RNA-sequencing (RNA-seq) for transcriptome profiling, and (iii) the Common Bean as a reference genome [Gene Expression Omnibus (GEO) accession no. GSE57537](Fig. 1L–Q) (20). The genome browser view illustrates that the up-regulation of GA biosynthesis enzyme mRNAs in the giant suspensor relative to the embryo proper was conserved in both these bean species. By contrast, GA 2-oxidase mRNA accumulated to very low, or nondetectable, levels in SRB and Common Bean globular-stage suspensor and embryo proper regions. RNA-seq data were taken from GEO accession no. GSE57537. Numbers indicate average reads per kilobase per million of two biological replicates. Each panel depicts an 8-kb window including the gene structure. Black boxes represent exons. Black lines represent UTRs and introns. Arrows indicate the transcription start site. bc, basal cells; ep, embryo proper; h, hypophysis region; Pc, P. coccineus; Pv, P. vulgaris; s, suspensor. (Scale bars: 50 μm.) The images in B and D are reproduced from ref. 10. The image in E is reproduced from ref. 12. Copyright American Society of Plant Biologists, www.plantcell.org.

Fig. 2. Consensus sequences for suspensor 10-bp motif, Region 2 motif, and Fifth motif. Consensus sequences were generated from G564 DNA sequences shown to be required for transcription within the suspensor (11, 14).
indicate that the synthesis of bioactive GA within giant bean suspensors (16) is primarily due to the spatially restricted accumulation of GA biosynthesis enzyme mRNAs.

We examined the temporal mRNA accumulation pattern of GA 20-oxidase during early SRB embryo development (Fig. 3 A–E). GA 20-oxidase mRNA was first detected in the basal cell of a two-cell embryo shortly after fertilization and then accumulated to high levels in the suspensor from the preglobular stage to the heart stage (Fig. 3 B–E), similar to our observations for G564 mRNA (12). Later, GA 20-oxidase mRNA accumulated within the epidermis of the heart-stage embryo proper (Fig. 3 E).

The temporal accumulation pattern of GA 3-oxidase mRNA in SRB embryos (Fig. 4) was indistinguishable from that of GA 20-oxidase mRNA (Fig. 3 B–E) and G564 mRNA (12). These results suggest that genes encoding GA biosynthesis enzymes are regulated by the same cis-regulatory elements as G564 and form part of a SRB suspensor gene regulatory network that is activated shortly after fertilization. To test this hypothesis, we used transgenic tobacco embryos to search for cis-regulatory elements required for GA 20-oxidase transcription within the SRB suspensor, similar to the approach that we used for G564 (11, 12, 14).

**SRB GA 20-Oxidase mRNA Accumulates Within the Suspensor of Transgenic Tobacco Embryos.** We transformed tobacco (Fig. 3 F) with a 7,271-kb SRB GA 20-oxidase genomic fragment (Fig. 3 G) and localized GA 20-oxidase mRNA in transgenic globular-stage embryos using in situ hybridization (Fig. 3 H and I). The GA 20-oxidase 5′ and 3′ regions were 4,509 and 596 bp in length, respectively, and did not contain similarity to any known genes. SRB GA 20-oxidase mRNA localized within the tobacco suspensor and embryo proper protodermal cells, the precursors to heart-stage epidermal cells (Fig. 3 H and I), similar to the pattern of GA 20-oxidase mRNA accumulation in SRB embryos (Fig. 3 E). These results indicate that the pattern of GA 20-oxidase mRNA accumulation is conserved during early embryo development in both tobacco and SRB.

**GA 20-Oxidase Expression Within the Suspensor Is Under Transcriptional Control.** We introduced a chimeric SRB GA 20-oxidase/β-glucuronidase (GUS) gene into tobacco (Fig. 3 J) and localized GUS enzyme activity in transgenic embryos to study GA 20-oxidase transcriptional regulation (Fig. 3 K–M). The GA 20-oxidase region −4,509/+238 fused to GUS (D-4509) (Fig. 3 J) first programmed GUS enzyme activity within the basal region of the two-cell tobacco embryo, followed by the entire suspensor at the preglobular stage, and then to the globular-stage embryo proper (Fig. 3 K–M). The GUS activity pattern was consistent with the localization of GA 20-oxidase mRNA in tobacco embryos driven by the entire GA 20-oxidase gene (Fig. 3 H and I), as well as that seen during SRB embryo development (Fig. 3 B–E). These results indicate that (i) the temporal and spatial expression pattern of GA 20-oxidase is controlled primarily at the transcriptional level by sequences within the −4,509/+238 region (Fig. 3 J) and (ii) the

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**Fig. 3.** GA 20-oxidase mRNA localization in SRB embryos and GA 20-oxidase transcriptional activity during tobacco embryogenesis. (A) SRB flower (image also in Fig. 1 A). (B–E) Localization of GA 20-oxidase mRNA in developing SRB embryos: two-cell stage (B), preglobular stage (C), globular stage (D) (image also in Fig. 1 J), and heart stage (E). (F) Tobacco flower. (G) Conceptual representation of the SRB GA 20-oxidase transgene introduced into tobacco. Dark-blue boxes represent exons. Light-blue boxes represent UTRs and introns. Numbers indicate positions relative to the transcription start site (+1). (H and I) Hybridization of SRB GA 20-oxidase antisense (H) and sense (I) probes to globular-stage transgenic tobacco embryos. (J) Conceptual representation of the GA 20-oxidase/GUS transgene introduced into tobacco. (K–M) GUS activity in transgenic tobacco embryos: two-cell stage (K), preglobular stage (L), and globular stage (M). Photographs were taken after 24-h GUS incubation for the two-cell and preglobular stages and after 1-h incubation for the globular stage. a, apical cell; b, basal cell; ep, embryo proper; s, suspensor; sy, synergid. (Scale bars: 50 μm.)
regulatory apparatus that controls GA 20-oxidase gene activity during early embryo development is conserved between SRB and tobacco.

The GA 20-Oxidase Upstream Region Contains Separate Embryo Proper and Suspensor cis-Regulatory Regions. We generated 5’ deletions of the GA 20-oxidase −4,509 to +238 region and analyzed GUS activity in transgenic tobacco embryos to identify the sequences required for transcription in the globular embryo (Fig. 5). Progressive 5’ deletion sequences from −4,509 to −275 first caused a loss of GUS activity in the embryo proper followed by loss of GUS activity in the suspensor (Fig. 5), indicating that there are separate embryo proper (−2,000 to −1,500) and suspensor (−450 to +238) cis-regulatory regions. The −450 to +238 GA 20-oxidase region appears to contain all the sequences required for transcription within the suspensor. Deletion to −275 reduced suspensor GUS activity to a barely detectable level, similar to that observed in the GUS-only negative control, which may be due to a low level of cryptic transcripts initiating in the vector sequence and reading through the reporter gene, as has been demonstrated for other plasmids (21). We conclude that sequences in the 175-bp region from −450 to −275 are required for suspensor transcription, although additional downstream sequences to +238 might also be required.

G564 Suspressor cis-Control Motifs Are Present in the GA 20 Oxidase −450 to +238 Region. Because GA 20-oxidase has the same suspensor-specific expression pattern as G564 (Fig. 1 E and J) (12), we searched the GA 20-oxidase −450 to +238 region for the presence of known suspensor cis-regulatory elements that activate G564 transcription: (i) the 10-bp motif (5′-GAAA-AGCGAA-3′ with up to three nonadjacent mismatches), (ii) the Region 2 motif [5′-TTG(A/G)(A/G/T)AAT-3′], and (iii) the Fifth motif [5′-(A/G)GTAGTTA-3′] (14). Within the −450 to +238 region we identified eight predicted 10-bp motifs, three predicted Fifth motifs, and six Region 2 motifs, allowing for one mismatch [except that the third nucleotide was not an A, as this nucleotide inactivates the Region 2 motif (11)] (Fig. 6A).

The GA 20-Oxidase Upstream Region −341 to −316 Is Required for Suspensor Transcription. To determine which of the predicted G564 motifs might be required for GA 20-oxidase suspensor transcription, we performed additional 5’ deletions within a GOF construct containing the GA 20-oxidase −450 to +238 upstream region fused to a Cauliflower Mosaic Virus (CaMV) 35S minimal promoter and GUS (GOF1) (Fig. 6A). This construct programmed high levels of GUS activity specifically within the suspensor (Fig. 6A), as predicted from our initial 5’ deletion analysis (Fig. 5), confirming that all the sequences required for GA 20-oxidase suspensor transcription are present within the −450 to +238 region. Deletions to −360 (GOF2) and −341 (GOF3) did not affect GUS activity, whereas deletion to −316 (GOF4) decreased GUS activity significantly within the suspensor (Fig. 6A). Together, these data show that the region −341 to −316 is required for full transcriptional activity in the suspensor and contains two predicted Fifth motifs, which may be functional.

The GA 20-Oxidase Upstream Region +119 to +238 Is Required for Suspensor Transcription. To determine whether sequences downstream of −341 to −316 are also required for GA 20-oxidase suspensor transcription, we performed 3’ deletions of the −450 to +238 GOF1 construct (Fig. 6B). A 3’ deletion to +119 or half of the 5’ UTR (GOF6) resulted in a significant decrease in GUS activity (Fig. 6B), indicating that sequences in the +119 to +238 GA 20-oxidase region were required for suspensor transcription. Further 3’ deletion (GOF7), did not significantly affect GA 20-oxidase suspensor transcription (Fig. 6B). We conclude that one or more suspensor cis-regulatory elements are present within the +119 to +238 region, which includes three predicted 10-bp motifs and one predicted Region 2 motif (compare GOF5 and GOF6 in Fig. 6).

The 10-bp and Region 2 Motifs Are Required for GA 20-Oxidase Suspensor Transcription. We carried out site-directed mutagenesis and 3’-deletion experiments within the GOF2 construct to determine which of the predicted 10-bp and Region 2 motifs in the +119 to +238 region were functional (Fig. 7). Mutation of the predicted Region 2 motif in this region (M1) caused a significant
Fig. 6. GUS activity in transgenic tobacco embryos containing 5′-(A) and 3′-(B) deletions of the GA 20-oxidase upstream region fused to the CaMV 35S minimal promoter/GUS gene. Conceptual representations of the constructs are shown to the left of each embryo. Yellow and green arrows, purple ovals, and blue ovals represent the 10-bp motif, Region 2 motif, and Fifth motif, respectively, with their sequences defined in the key. Dark-blue arrows represent the GA 20-oxidase 5′ UTR. Green boxes represent the CaMV 35S minimal promoter. Numbers indicate positions relative to the GA 20-oxidase transcription start site (+1). Expression levels were categorized as previously described (11, 14). +++ in the Expression column indicates that suspensor GUS activity was strong; that is, the majority of the suspensors with GUS activity at 24 h were GUS-positive at 2 h. ++/− in the Expression column indicates that suspensor GUS activity was weak; that is, the majority of the suspensors with GUS activity at 24 h were GUS-negative at 2 h. A minus sign (−) in the Expression column indicates no detectable suspensor GUS activity at 24 h. Numbers in the Lines column indicate the number of individual transformants displaying suspensor GUS activity over the total number of individual transformants analyzed. Numbers in the Suspensors column indicate the number of individual transformants displaying suspensor GUS activity at 24 h incubation over the total number of embryos analyzed. Photographs were taken after GUS incubation for 24 h. (Scale bars: 50 μm.)

Because the GA 20-oxidase −minimal promoter requires three copies of the 10-bp motif (14), we asked whether any predicted 10-bp motifs in the region upstream of +129 was also required for GA 20-oxidase suspensor transcription. Mutating the remaining five predicted 10-bp motifs (M4) had no effect on suspensor GUS activity (Fig. 7), indicating that probably both 10-bp motifs in the +129 to +205 UTR region are required for GA 20-oxidase suspensor transcription in addition to the Region 2 motif.

The Fifth Motif and an Additional Region 2 Motif Are Required for GA 20-oxidase Suspensor Transcription. Two Fifth motifs were predicted in the −341 to −316 GA 20-oxidase upstream region, which was also required for suspensor transcription (compare GOF3 and GOF4) (Figs. 6A and 8A). Site-directed mutagenesis was performed within the GOF2 construct to determine whether these predicted Fifth motifs at −336 to −341 and −329 to −324 were functional (Fig. 8B). Mutation of both predicted Fifth motifs in this region by either transversional mutagenesis (M5) or adenine substitution (M6) did not affect suspensor GUS activity (Fig. 8B). Because (i) the −341 to −316 region was required for suspensor transcription (compare GOF3 and GOF4) (Figs. 6A and 8A) and (ii) a Fifth motif is essential for G564 suspensor transcription (14), we searched the −341 to −316 region again for a Fifth motif allowing for one mismatch at any position in the consensus sequence (Fig. 2). We identified an additional Fifth motif that overlapped a predicted Region 2 motif in the opposite orientation (marked by an asterisk in GOF2 at −317 to −312 in Fig. 8B). Previously, we showed that G564 motif orientation did not affect suspensor transcription (11).
To determine whether the Fifth motif at −317 to −312 was required for GA 20-oxidase suspensor transcription, site-directed mutagenesis was performed on the GO2F construct (M7) (Fig. 8B). To mutate the Fifth motif and leave the overlapping Region 2 motif intact, we had to replace the predicted Region 2 motif at −309 to −315 (5′-TTTGAAT-3′) with the functional Region 2 motif from the 5′ UTR (5′-TTTATAT-3′), which had a slightly different sequence (M7) (Fig. 8B). This caused a mutation (red nucleotides in Fig. 8F) in the overlapping predicted Fifth motif while keeping the Region 2 motif intact (M7) (Fig. 8B). Mutation of all three predicted Fifth motifs in the −341 to −316 region (M7) resulted in a significant decrease in suspensor transcription (Fig. 8F), consistent with the results of GO4 that deleted the −341 to −316 region, including the first nucleotide of the −317 to −312 Fifth motif (Figs. 6A and 8A). Because mutating the two predicted Fifth motifs at −336 to −341 and −329 to −324 had no effect on suspensor GUS activity (M5 and M6) (Fig. 8B), the Fifth motif at −317 to −312 is required for suspensor transcription.

To determine whether the overlapping predicted Region 2 motif at −309 to −316 was also required for GA 20-oxidase suspensor transcription, we mutated this motif within the GO2F construct by changing the third nucleotide (−311) from G to A, leaving the Fifth motif intact (M8) (Fig. 8F). Previously, we demonstrated that this mutation renders the Region 2 motif nonfunctional in the G564 suspensor cis-regulatory module (11). Similar to G564, mutating a single nucleotide in the Region 2 motif at −309 to −316 (M8) abolished suspensor GUS activity (Fig. 8F). Thus, the Region 2 motif at −309 to −316, in addition to the Region 2 motif within the 5′ UTR, is required for suspensor transcription. Together, these results indicate that both the Fifth motif and the Region 2 motif in the −317 to −309 GA 20-oxidase upstream region are essential for transcription within the suspensor and explain the loss of suspensor GUS activity using the −275 deletion (D-275) and GO2F constructs (Figs. 5, 6A, and 8A).

**Functional Suspensor Motifs Are Conserved in the Common Bean GA 20-Oxidase Gene.** We examined the Common Bean GA 20-oxidase gene region for suspensor motifs because it is nearly identical to SRB GA 20-oxidase in (i) structure (SI Appendix, Fig. S1A), (ii) sequence (SI Appendix, Fig. S1B), and (iii) expression pattern (Fig. 1P). We found sequences identical to the functional SRB GA 20-oxidase suspensor motifs in the Common Bean GA 20-oxidase gene region (SI Appendix, Fig. S1). The sequence, order, orientation, and spacing (with the exception of small indels) of the functional motifs were identical in the bean species, suggesting that the Common Bean GA 20-oxidase gene also utilizes the 10-bp, Region 2, and Fifth motifs for transcription within its giant suspensor.

**Discussion**

The giant SRB suspensor (Fig. 1) has been used for over four decades as a model system for investigating the physiological and cellular events that occur in this unique embryonic region and the role it plays in early embryo development (5, 10, 22, 23). We have been using SRB suspensors to dissect the regulatory processes required for the region-specific transcription of genes within the embryo shortly after fertilization (6, 11, 12, 14). We identified a large number of genes, including G564, which are expressed specifically within the SRB suspensor using a variety of genomic approaches (6, 12). Genes encoding all major enzymes of the GA biosynthetic pathway (ent-kaurene synthase A, ent-kaurene synthase B, ent-kaurenoic acid hydroxylase, GA 20-oxidase, and GA 3-oxidase) are also expressed specifically within giant SRB suspensors (Fig. 1); and, at least two (GA 20-oxidase and GA 3-oxidase)—and probably all—are activated in the basal region of the postfertilized embryo, similar to G564 (Figs. 3B and C and 4B) (12). Our results suggest that genes encoding GA biosynthesis enzymes, and others such as G564, are organized into a genetic regulatory network (9) that (i) is activated after the division of the SRB embryo into the apical and basal regions and (ii) functions exclusively within the suspensor. This model predicts that genes operating within the suspensor genetic regulatory network share a common cis-regulatory module that is responsible for activating genes within the suspensor. The results presented in this paper support this model, showing that G564 and GA 20-oxidase genes require the same
cis-regulatory motifs to be activated within the suspensor region (Fig. 9).

The SRB GA 20-Oxidase Upstream Region Is Organized into Different Modules That Direct Expression to the Suspensor and Embryo Proper. GA 20-oxidase contains two discrete regions that activate transcription in the suspensor and embryo proper during embryogenesis (Figs. 5 and 9). One region (–341 to +238) activates transcription within the suspensor following fertilization (Fig. 6A). The second region (–2,000 to −1,500) activates transcription later in embryo development within embryo proper epithelial cells (Fig. 5). This bimodal organization of suspensor and embryo proper control regions is similar to that of G564, except that the spatial distribution of these modules differs (Fig. 9) (11, 12).

The GA 20-oxidase suspensor module activates transcription uniformly over cells of the entire suspensor, in contrast with the cell-specific embryo proper module (Fig. 5). Our deletion and mutagenesis experiments with the –341 to +238 suspensor control region (Figs. 5–8) did not uncover subregions required for transcription within specific suspensor cell types, such as (i) the hypophysis adjacent to the embryo proper that is derived from the embryo apical cell and (ii) the enlarged basal cell that forms connections with the seed coat and is derived from the embryo basal cell. This suggests that the GA 20-oxidase gene is activated within suspensor cells using the same regulatory processes, irrespective of position or cell lineage.

The GA 20-oxidase suspensor control region activates transcription within the basal cell of the two-cell embryo (Fig. 3B and K) and in all suspensor cells by the late globular stage of development (Fig. 3B–D and K–M). We did not observe any shift in this temporal sequence of transcriptional events in our deletion and mutagenesis experiments, nor did we observe any ectopic activation of transcription within the globular-stage embryo proper region by deleting or mutating parts of the suspensor control module (Figs. 5–8). These results suggest the absence of both temporal and negative cis-regulatory elements within the suspensor regulatory region. Thus, the regulation of GA 20-oxidase within the SRB suspensor is controlled by positive elements that activate transcription shortly after fertilization and within daughter suspensor cells as they form during embryogenesis.

The organization of regulatory sequences within the GA 20-oxidase suspensor control region is relatively simple compared with those that activate the transcription of storage protein genes within the mature embryo proper. Storage protein gene embryo-proper control regions contain (i) territory-specific modules regulating transcription within embryo-proper subregions, such as the axis and cotyledons [e.g., Kti3 (2) and β-Phaseolin (24)], (ii) temporal cis-regulatory elements [e.g., β-Phaseolin (24) and β-conglycinin (25)], and (iii) repressor elements [e.g., Glycinin (26) and β-Phaseolin (24)]. This difference in regulatory architecture most likely results from the suspensor being a highly specialized, terminally differentiated embryonic region with few distinct cell types that degenerates later in development, in contrast with the more complex embryo proper that contains many functionally distinct embryonic territories that give rise to the mature plant following seed germination.

At Least Five cis-Regulatory Elements Are Required to Activate GA 20-Oxidase Suspensor Transcription. Within the SRB GA 20-oxidase –341 to +238 suspensor control region, we identified 17 short sequences similar to the motifs that are required to activate G564 in the suspensor: the 10-bp motif, the Region 2 motif, and the Fifth motif (Figs. 2 and 6) (11, 14). Deletion and site-directed mutagenesis experiments demonstrated that only five of these sequences are functional and required for GA 20-oxidase suspensor transcription—two Region 2 motifs, a Fifth motif, and two 10-bp motifs—demonstrating, in support of our original hypothesis, that both G564 and GA 20-oxidase utilize the same suspensor cis-control elements (Figs. 7 and 8). All five of these motifs are conserved at similar positions within the Common Bean GA 20-oxidase gene region (SI Appendix, Fig. S1). The remaining candidate motifs are not required for suspensor transcription (Figs. 7 and 8), illustrating the need to functionally dissect cis-regulatory modules to...
understand how they operate rather than relying solely on motif similarity from computer predictions.

The Fifth motif and Region 2 motif in the GA 20-oxidase –317 to –309 upstream region overlap (Figs. 8B and 9), and mutating either motif without disrupting the other leads to a decrease or complete loss of suspensor transcriptional activity (Fig. 8B). This type of regulatory element organization is not without precedent, as functional overlapping motifs have been identified in several animal enhancer regions (27–29). By contrast, two 10-bp motifs and a second Region 2 motif are located in the GA 20-oxidase 5′ UTR and do not overlap (Fig. 9). Other plant genes contain positive transcriptional control elements in their 5′ UTRs (30, 31). Together, these data indicate that transcription factors must bind to motifs that reside both upstream and downstream of the transcription start site within the regulatory module to activate GA 20-oxidase transcription in the suspensor.

**Suspensor-Specific Gene Transcription Is Generated by a Flexible Arrangement of cis-Regulatory Motifs.** The results presented here showing the organization of the GA 20-oxidase suspensor cis-regulatory module and those carried out previously with G564 (11, 14) provide a unique opportunity to compare the architecture of two suspensor cis-regulatory modules. The G564 suspensor module is composed of three 10-bp motifs, a Region 2 motif, and a Fifth motif, all tightly clustered within a 47-bp DNA region with little spacing between motifs (Fig. 9) (14). The G564 suspensor cis-regulatory module is repeated five times in the G564 upstream region, and each repeat is able to function individually, except for repeat five, as it lacks an intact Region 2 motif (Fig. 9) (11, 14).

By contrast, the single GA 20-oxidase suspensor module is larger, 579 bp in length, and is composed of two 10-bp motifs, two Region 2 motifs, and a Fifth motif divided between the upstream and 5′ UTR regions (Fig. 9). Thus, the number, order, and spacing of the suspensor motifs differ between the G564 and GA 20-oxidase cis-regulatory modules even though they lead to the same output—suspressor-specific transcription within the early plant embryo. This suggests that the suspensor cis-regulatory module most closely resembles a billboard-type model of control-element organization in which motif positions can vary among genes that program transcription to the same developmental state, compared with an enhanceosome-type model that requires fixed motif positions for similarly upstream and 5′ UTR overlap (Figs. 8–9) (11, 14).

**Candidate Transcription Factors Have Been Identified That Bind to Specific Motifs Within the Suspressor Control Module.** The G564 and GA 20-oxidase suspensor cis-regulatory module can activate transcription within the suspensor of divergent plant embryos, including SRB, Common Bean, tobacco, and Arabidopsis (Figs. 1 and 3) (5, 11, 14). This suggests that the suspensor cis-regulatory module operates within a highly conserved regulatory network that utilizes a set of transcription factors that is shared by the suspensors of these plant species.

What transcription factors bind to the 10-bp, Region 2, and Fifth motifs that are required for the suspensor control module to function (Fig. 9)? We previously showed that the G564 Fifth motif resembles the canonical sequence of a MYB transcription factor-binding site, and yeast one-hybrid experiments with Arabidopsis transcription factors showed that several MYB transcription factors bind to the G564 suspensor control module to activate transcription within yeast cells (14, 33). Although we have yet to identify the specific MYB transcription factor that binds to the Fifth motif, there are a number of SRB MYB transcription factors that are encoded by suspensor-specific mRNAs (RNA-seq dataset GEO accession no. GSE57537) that are ideal candidates.

We searched the Arabidopsis DNA affinity purification sequence (DAP-seq) database (34) and identified an Arabidopsis C2H2-type zinc finger transcription factor (AT2G41835) that binds to the sequence 5′-GTGA(A/G)AA-3′, which is nearly identical to the Region 2 sequence 5′-TG(A/G)(A/G/T)AAAT-3′ (Fig. 2). Our yeast one-hybrid screen with Arabidopsis transcription factors showed that zinc finger transcription factors could activate the G564 cis-regulatory module, although AT2G41835 was not represented in the library (14, 33). We searched the Common Bean genome database (20) and identified a gene (Phvul.007G253000) that (i) encodes a protein that closely resembles the Arabidopsis AT2G41835 C2H2-type zinc finger transcription factor, (ii) has 99% identity to a SRB genomic sequence contig017197_00035 (GenBank ID QBDZ0118989.1), (iii) is represented by SRB suspensor ESTs (GenBank IDs GD428417.1 and GD420985.1), and (iv) is expressed in both the embryo proper and suspensor of Common Bean and SRB globular-stage embryos, although to an elevated level within the suspensor (RNA-seq dataset GEO accession no. GSE57537). This suggests that the Phvul.007G253000 C2H2-type zinc finger transcription factor is an excellent candidate for interacting with the Region 2 motif (Fig. 9).

Interestingly, a close relative of the Phvul.007G253000 C2H2-type zinc finger transcription factor is represented in maize egg cell and zygoe DNA populations (35), suggesting that it might be present before fertilization in SRB and Common Bean as well.

**Materials and Methods**

**Plant Materials.** Plants of the day-neutral SRB cultivar “Hammond’s Dwarf Red Flower” (Vermont Bean Seed Company) were grown in a greenhouse as described previously (12). Open flowers were pollinated by hand using a watercolor brush. Hand-pollinated flowers were tagged, and seeds were harvested 2–8 d after pollination (DAP), as described previously (12). Common Bean seeds (Andean common bean inbred landrace accession G19833) were obtained from Phillip E. McClean at North Dakota State University, Fargo, ND. Common Bean and SRB plants were obtained under the same conditions as SRB plants for 1 mo and then were moved to a growth chamber with an 8-h/16-h light/dark cycle to induce flowering. Seeds 1.6–2.0 mm in length were collected at 5–6 DAP.

**Radioactive in Situ Hybridization Analysis.** Radioactive in situ hybridization studies were performed as described previously (12). Briefly, SRB seeds were harvested 2–8 DAP, and seeds were cut at their chalazal ends before fixing to enhance penetration of the fixative. SRB seeds were fixed overnight at 4 °C in 1% glutaraldehyde, 0.1 M sodium phosphate buffer (pH 7.0), and 0.1% Triton X-100. Fixed seeds were dehydrated, cleared, and embedded in paraffin. Eight-micrometer sections were hybridized with 32P-labeled anti-sense RNA probes. Probes were generated from cDNA clones made from microdissected 6-DAP suspensor regions of globular-stage SRB embryos (12). These cDNA clones corresponded to Common Bean GA biosynthesis enzyme genes: ent-kaurene synthase A (Phvul.001G152100), ent-kaurene synthase B (Phvul.005G048500), ent-kaurene oxidase (Phvul.005G183600), ent-kaurenoic acid hydroxylase (Phvul.006G123500), GA 20-oxidase (Phvul.001G087500), and GA 3-oxidase (Phvul.009G07100). After hybridization and emulsion development, sections were stained with 0.05% toluidine blue in 0.05% borate solution. Photographs were taken using dark-field illumination with a compound microscope (Olympus BH2, Olympus Corp.). The photographs were digitized, adjusted for optimum silver grain resolution using the KPT-Equalizer program (Metacreations Corp.), and assembled in Adobe Photoshop CS5.1. Probe sequences are listed in SI Appendix, Table S2.
Nonradioactive in Situ Hybridization Analysis. Nonradioactive in situ hybridization with transgenic tobacco embryos was carried out using digoxigenin-labeled riboprobes (36). Briefly, transgenic tobacco seeds were harvested at 10 DAP and were fixed overnight at 4 °C in 10% formalin/5% acetic acid/50% ethanol (37). Fixed seeds were dehydrated, cleared, and embedded in paraffin using a Leica ASP300S Tissue Processor. Six-micrometer sections were hybridized to sense or antisense digoxigenin-labeled riboprobes overnight. Probes were generated from a GA 20-oxidase cDNA clone containing the region +182 to +2,166, which was isolated from microdissected suspensor regions of 6-DAP globular-stage SRB embryos (12). Photographs were taken using bright-field illumination with a compound microscope (Leica 5000 B).

Bright-Field Microscopy. SRB and Common Bean seeds were fixed overnight at 4 °C in 1% glutaraldehyde, 0.1 M sodium phosphate buffer (pH 7.0), and 0.1% Triton X-100. Fixed seeds were dehydrated and cleared. SRB seeds were embedded in Spurr’s plastic resin (Polysciences) (38). One-micrometer sections were stained for 18-20 min at 42 °C with 0.05% toluidine blue in 0.05% borate solution. Photographs were taken using bright-field illumination with a compound microscope (Leica 5000 B). Common bean seeds were embedded in paraffin. Six-micrometer sections were stained for 1.5 min at 42 °C with 0.1% toluidine blue. Photographs were taken using bright-field illumination with a compound microscope (Leica 5000 B).

Plant Transformation. Tobacco (Nicotiana tabacum cultivar SR1) plants were transformed and regenerated using the leaf disk procedure (39). Each individual transformant was checked for transfer DNA (T-DNA) insertion by PCR and/or sequencing analysis. At least six independent transformants were generated for each construct. A total of 27 different constructs and 193 individual tobacco transformants were generated to carry out this study.

GUS Histochemical Assay. Transgenic tobacco seeds were harvested at 8 DAP. Globular-stage embryos were hand-dissected from seeds and assayed for GUS activity after 1, 2, and 24 h at 37 °C, as described previously (11). Embryos were photographed under bright-field illumination using a compound microscope (Leica 5000 B). T1 seeds from GUS-negative lines were tested for kanamycin-resistant segregation after selfing to confirm that the T-DNA was not silenced. In total, 2,326 individual globular-stage embryos were assayed for GUS activity to generate the results reported in this study.

5’ Deletion Constructs. A GA 20-oxidase genomic clone (GenBank accession no. FJ535441) was digested with EcoRI and HindIII, and a 5.5-kb fragment containing the upstream sequence and the first exon was cloned into ECoRI and HindIII-digested pBlueScript (Stratagene), generating plasmid pHtSII_5.5kb. To isolate the GA 20-oxidase upstream region, PCR was performed using the pHtSII_5.5kb EH 5.5 kb plasmid as a template with a forward primer containing AatII and SmaI sites and a reverse primer containing the region 182 to 30. The PCR fragments were sequenced to confirm that they contained the correct mutated bases. Primer sequences are listed in Table S3.

Site-Directed Mutagenesis Constructs. Predicted motifs were mutated according to the strategy we previously used in our laboratory (11). Transversal mutagenesis was used unless this process created a new predicted motif, in which case adenine substitution was used. For M1 and M2, Splicing by Overlap Extension (SOEing) PCR (43) was used to generate fragments containing the mutated motifs using the GOF2 plasmid as a template. The amplified fragments were digested with EcoRI and Xmal and were ligated into the EcoRI- and Xmal-digested GOF2 plasmid to create the mutated constructs. For M3 and M5-M8, the GOF2 plasmid was used as a PCR template with primers containing the desired mutation and EcoRI or Xmal restriction sites. The amplified fragments were digested with EcoRI and Xmal and were ligated to the EcoRI- and Xmal-digested GOF2 plasmid. A fragment of M4 flanked by EcoRI and Xmal restriction sites was made by gene synthesis (Genewiz). This fragment was digested with EcoRI and Xmal and was ligated to EcoRI- and Xmal-digested GOF2, generating plasmid M4. The GA 20-oxidase upstream region was sequenced to confirm that they contained the correct mutated bases. Primer sequences are listed in Table S3.

ACKNOWLEDGMENTS. We thank present and past members of our laboratory for discussion and advice with this project, especially Min Chen and Xiaomeg Wu for generating SRB and Common Bean embryo transcriptions; Professor John Harada for insightful comments on our suspension cell element research; and Professor Jeff Long for assistance with nonradioactive in situ hybridization. We dedicate this paper to the memory of our close friend and mentor, Professor Eric Davidson, who was a visionary in the field of developmental biology, and to the organization of eukaryotic gene regulatory networks, and who provided us with perceptive advice on cis-regulatory module identification. This work was funded by a grant from the National Science Foundation Plant Genome Program.

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