The MED12-MED13 module of Mediator regulates the timing of embryo patterning in Arabidopsis

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SUMMARY
The Arabidopsis embryo becomes patterned into central and peripheral domains during the first few days after fertilization. A screen for mutants that affect this process identified two genes, GRAND CENTRAL (GCT) and CENTER CITY (CCT). Mutations in GCT and CCT delay the specification of central and peripheral identity and the globular-to-heart transition, but have little or no effect on the initial growth rate of the embryo. Mutant embryos eventually recover and undergo relatively normal patterning, albeit at an inappropriate size. GCT and CCT were identified as the Arabidopsis orthologs of MED13 and MED12 – evolutionarily conserved proteins that act in association with the Mediator complex to negatively regulate transcription. The predicted function of these proteins combined with the effect of gct and cct on embryo development suggests that MED13 and MED12 regulate pattern formation during Arabidopsis embryogenesis by transiently repressing a transcriptional program that interferes with this process. Their mutant phenotype reveals the existence of a previously unknown temporal regulatory mechanism in plant embryogenesis.

KEY WORDS: Arabidopsis, Embryo, Heterochrony, MEDIATOR, MED12, MED13, Polarity, KANADI

INTRODUCTION
Plant embryos exhibit both apicobasal and central-peripheral (radial) polarity. The shoot and root apical meristems (SAM and RAM, respectively) form at opposite ends of the apicobasal axis and contain stem cell populations that give rise to all postembryonic tissue. Lateral organs such as cotyledons arise at the boundary of the central and peripheral domains, and adopt the pattern present in the radial axis of the embryo. Thus, genes that specify central identity are also expressed on the adaxial (upper) side of developing cotyledon primordia, whereas genes that specify peripheral identity are expressed on the abaxial (lower) side. This continuity supports the conclusion that the adaxial surface of a lateral organ is a component of the central domain, whereas the abaxial side is a component of the peripheral domain (Kerstetter et al., 2001; McConnell et al., 2001).

Genes involved in the specification of radial polarity were initially identified in screens for mutations that affect the adaxial-abaxial polarity of leaves and floral organs. In Arabidopsis, these screens have produced mutations in three families of transcription factors. Members of the class III HD-ZIP family of transcription factors promote adaxial identity (McConnell et al., 2001; Emery et al., 2003), whereas KANADI (KAN) and YABBY (YAB) transcription factor families promote peripheral and abaxial identity (Kerstetter et al., 2001; Eshed et al., 2001; Siegfried et al., 1999). Evidence that these genes are also important for embryonic development is provided by the phenotype of gain-of-function alleles, and the phenotype of embryos lacking more than one member of these gene families. For example, mutations that cause the HD-ZIP III gene PHB to be ectopically expressed in the peripheral-abaxial domain produce SAMs (central structures) on the abaxial side of leaves (McConnell and Barton, 1998). Conversely, phb phv rev triple mutants lack a SAM (Emery et al., 2003). Ectopic expression of KAN1 in the central domain of the embryo blocks the development of the SAM, whereas kan1 kan2 kan4 triple mutant embryos have outgrowths on the abaxial surface of cotyledons and the periphery of the hypocotyl that have characteristics of leaf primordia (Kerstetter et al., 2001; Izhaki and Bowman, 2007).

The studies described above have begun to address the developmental role of KAN genes in embryonic and postembryonic development. However, the mechanism responsible for the initial establishment of radial polarity in embryogenesis remains a mystery. To identify factors in the polarity pathway that act upstream of KAN genes, we took advantage of an enhancer trap insertion in KAN2 that expresses green fluorescent protein (GFP) in the peripheral-abaxial domain of both embryonic and postembryonic organs. A screen for mutations that affect the expression of this reporter produced alleles of two genes, GRAND CENTRAL (GCT) and CENTER CITY (CCT), which we found encode the Arabidopsis homologs of MED13 and MED12.

In yeasts and animals, Med13 and Med12 act as transcriptional repressors by inhibiting core Mediator, a multisubunit complex that allows transcription factors bound at upstream enhancer elements to activate RNA polymerase II. Med13 and Med12 have identical mutant phenotypes in each organism (Samuelsen et al., 2003; Yoda et al., 2005; Janody et al., 2003), indicating that they have similar biological functions. In humans, MED13 negatively regulates transcription by causing an allosteric change in core Mediator that prevents its association with RNA polymerase II (Knuesel et al., 2009), whereas MED12 recruits the histone methyltransferase G9a to core Mediator, promoting epigenetic silencing of target genes via methylation of chromatin H3K9 (Ding et al., 2008). In yeasts and Drosophila, Med13 and Med12 physically interact with Cyclin-dependent kinase 8 (Cdk8) and Cyclin C (CycC) to form a complex known as the Cdk8 module of Mediator (Samuelsen et al., 2003; Loncle et al., 2007). However, the observation that in Drosophila Cdk8 and CycC do not have the same mutant phenotype as Med13 (skd – FlyBase) and Med12 (kto – FlyBase), implies that these
components are functionally distinct (Loncle et al., 2007). In both *Drosophila* and *Caenorhabditis elegans*, Med13 and Med12 specify cell identity by regulating downstream targets of the Wnt signaling pathway (Janody et al., 2003; Yoda et al., 2005; Carrera et al., 2008). In particular, the *Drosophila Med13* and *Med12* genes *skald* and *kohtalo* control the cell affinities that maintain boundaries between anteroposterior and dorsoventral compartment boundaries of the wing disc (Janody et al., 2003; Loncle et al., 2007). The developmentally specific phenotypes of mutations in *Med13* and *Med12* in *Drosophila* and *C. elegans* are consistent with a microarray analysis of these genes in yeast, which indicates that they regulate a relatively small number of genes (Samuelsen et al., 2003). This is in contrast to core Mediator, which is required for nearly all transcription (Kornberg, 2005).

The core Mediator complex was recently purified from *Arabidopsis* suspension culture cells (Bäckström et al., 2007). The purified complex contained most of the components present in the core complex in other organisms, but was missing proteins in the Cdk8 module, consistent with the limited and transient interactions observed between these proteins and core Mediator in other systems (Andrau et al., 2006). The phenotype of mutations in two components of the core complex (PFT1/MED25 and SWP/MED14) has been described in *Arabidopsis*. Mutations of PFT1/MED25 delay flowering under suboptimal light conditions (Cerdán and Chory, 2003; Bäckström et al., 2007), whereas mutations of *SWP/MED14* cause a premature arrest in cell proliferation during vegetative growth, resulting in extreme dwarfing (Aturan et al., 2002). By contrast, mutations in the *Arabidopsis* Cdk8 homolog *HEN3* have a mild reduction in cell expansion in leaves, and only show more severe phenotypes in combination with mutations that affect pre-mRNA processing (Wang and Chen, 2004).

Here we show that *MED13* and *MED12* regulate developmental patterning in *Arabidopsis*. We found that *MED13*, corresponding to the *GCT* gene, and *MED12*, corresponding to the *CCT* gene, are absolutely required for *KAN* expression during early embryogenesis, and promote *KAN* expression during postembryonic development as well. Analysis of markers for the SAM and vascular tissue showed that *ctt* and *cct* mutations have a unique effect on embryo patterning: the initiation of a number of patterning genes is delayed absolutely required for *KAN* (Andrau et al., 2006). The phenotype of mutations in two *KAN* lines (Alonso et al., 2003) and SAIL T-DNA insertion lines (Sessions et al., 2002) were obtained from the Arabidopsis Stock Center (ABRC) at www.arabidopsis.org. Seed for gct-2, cct-1, E2023, E2331 and gPHB-GUS have been deposited in the Arabidopsis Stock Center (ABRC).

E2023 seeds were mutagenized as follows: 100 mg of seed was suspended in 25 ml of water with 3 μl Tween-20 for 15 minutes, rinsed and then soaked overnight in water at 4°C on a rotator. Seeds were then suspended in 10 ml of 0.4% ethyl methane sulfonate in 100 mM sodium phosphate buffer (pH 7) for 9 hours on a rotator at room temperature, and subsequently rinsed 10 times with 25 ml of water. Embryos from three consecutive silks on the main inflorescence of 2500 M1 plants were examined for changes in E2023 expression and embryo morphology. The main inflorescence of M1 plants was consistently found to have two or three sectors, and thus our screen comprised 5000-7500 individual mutagenized sectors. Mutant lines were backcrossed three times before analysis.

**Molecular biology**

RNA in situ hybridization was performed using a protocol obtained from Jeff Long (www.its.caltech.edu/~plantlab/protocols/insitu.pdf), with the following minor modifications. The probe was hybridized to slides at a temperature of 60-65°C overnight. The blocking and antibody dilution solutions were produced using maleic acid instead of Tris-HCl. For the *STM, GCT* and *CCT* probes, partial cDNA sequences were amplified and transcribed using the following primers: STM-T7, 5′-CAAGCTTATACGACTCCTAGTATAGGAAAGATGTTGCCCAAGACAT-3′; STM-Sp6, 5′-CTCTAGATTTAGGTGACACTATAGGATCCATCAATC-3′; GCT-Sp6, 5′-ATTATGTTGACACTATAGAAGGCCC-CAATTGTTCCTACAATGGG-3′; CCT-T7, 5′-TAAATACGACTCACTATAGGGAGACCTTCCT-CAATCCTTCCC-3′.

The gPHB-GUS translational fusion construct was made by cloning 1044 bp upstream of the *PHB* stop codon in front of the GUS+ gene in the pCAMBIA3300 vector, with an NAAIRS linker replacing the PHB stop codon. This DNA fragment contains all sequences from the previous gene on the 5′ end of *PHB* until the PHB stop codon, including all introns and the miR165/166 target sequence. Plants homozygous for the gPHB-GUS construct show mild adaxialized phenotypes, indicating that the PHB-GUS fusion protein is biologically active.

**Microscopy and histology**

The E2023 genetic screen was conducted using a Leica MZFLIII fluorescence microscope equipped with a long pass GFP filter. To detect GUS activity, embryos were stained at 37°C for 1h (p*KAN::GUS, pSTM::GUS*) or overnight (gPHB-GUS) according to Donnelly et al. (Donnelly et al., 1999). Embryos were cleared in Hoyer’s solution (bent codylen stage embryos) or mounted in 5% glycerol (globular to torpedo stage) and examined with DIC optics. Confocal images were obtained using a Leica TCS SL confocal microscope with a 20× water immersion lens. Embryos were removed from ovules using DuMont #5 forceps and mounted in 1×MS salts plus 500 mM glucose. Globular and heart stage embryos were counterstained by adding Nile Red to MS-glucose to a final concentration of 3 μg/ml (Jenik et al., 2005). For camera lucida drawings, wild-type and mutant leaves were vacuum infiltrated with 1% Tween 20 in water, and manually sketched using an Olympus BX40 microscope equipped with a U-DA light tube and mirror.

**RESULTS**

A genetic screen to identify upstream regulators of peripheral-abaxial polarity

The *KAN* family of transcription factors specify peripheral-abaxial identity beginning in early embryogenesis (Kerstetter et al., 2001; Eshed et al., 2004). To identify genes that act upstream of *KAN* in the embryolar pathway, we took advantage of the enhancer trap line E2023, which contains a GAL4-UAS::GFP insertion (Haseloff, 1999) that disrupts the *KAN2* gene (see Materials and methods). This line expresses GFP strongly and constitutively
within the peripheral-abaxial domain of embryos, the abaxial vascularization of leaves and the abaxial domain of carpels (Fig. 1A-F), an expression pattern that is essentially identical to that of KAN2 (Emery et al., 2003; Eshed et al., 2004). It has no obvious morphological defect beyond the production of a small longitudinal ridge of tissue below the first bract, and an occasional flower with five petals (data not shown).

Examination of approximately 6000 mutagenized M1 sectors (see Materials and methods) yielded more than 50 mutant lines with a heritable effect on E2023 expression. Five of these lines segregated embryos with a nearly identical recessive mutant phenotype, consisting of a decrease in the intensity of E2023 expression (Fig. 1G-J,M-P), and an increase in size of the SAM (see below). Complementation analysis revealed that these mutations represented two genes, which were named GRAND CENTRAL (GCT) (four alleles) and CENTER CITY (CCT) (one allele), to reflect the increased size of the SAM and the decrease in expression of the peripheral-abaxial enhancer trap E2023. No significant phenotypic differences were noted between the various alleles of gct; gct-2 and cct-1 (hereafter referred to as gct and cct) were used for all of the experiments presented here. Homozygous plants produce very few seeds, and thus gct and cct embryos were obtained from heterozygous plants, allowing us to stage the development of mutant embryos by observing wild-type embryos segregating in the same silique. Thus, for all analyses in this manuscript, the developmental stage of mutant embryos was inferred from wild-type siblings of the same chronological age.

GCT and CCT also regulate the expression of E2023 during postembryonic development, and affect the timing of a variety of developmental transitions including the embryo-to-seedling transition, vegetative phase change and flowering time. This paper focuses primarily on their role in embryogenesis.

GCT and CCT are absolutely required for E2023 expression at the heart stage of embryogenesis, and promote the correct level of expression later in embryo development (Fig. 1G-J,M-P). E2023 is undetectable in gct embryos (Fig. 1G-I) until the bent cotyledon

**Fig. 1. Mutations in GRAND CENTRAL (GCT) and CENTER CITY (CCT) cause decreased expression of the peripheral-abaxial enhancer trap E2023.** (A-F) E2023 is a KAN2 reporter expressed in the peripheral-abaxial domain of wild-type plants. Early heart (A), late heart (B), torpedo (C) and bent cotyledon (D) embryos; inset in D shows embryo cross section. Abaxial view of a 10-day-old seedling (E) reveals that E2023 is expressed in abaxial vascular tissue of expanded cotyledons and leaves, and throughout the abaxial side of leaf primordia (arrowhead). Carpels of wild-type flower (F). gct (G-L) and cct (M-R) delay and reduce the expression of E2023. Early heart (G,M), late heart (H,N), torpedo (I,O) and bent cotyledon (J,P) stage embryos. Ten-day-old gct (K) and cct (Q) seedlings (insets in K and Q; 2 X magnification of leaves). gct (L) and cct (R) carpels. Scale bars: 20 \( \mu m \) in A-D,G-J,M-P; 1 mm in E-F,K-L,Q-R.

**Fig. 2. gct enhances the polarity phenotypes of kanadi mutants.** (A-D) Wild-type (A), kan1 kan2 (B), gct (C) and gct kan1 kan2 (D) embryos at the early bent cotyledon stage of development. (E-H) Four-week-old wild-type (E) and kan1 (F) plants with inflorescences removed, and 5-week-old gct (G) and gct kan1 (H) plants. (I-L) Camera lucida drawings of adaxial and abaxial mesophyll tissue of leaf 1 of wild-type (I), kan1 (J), gct (K) and gct kan1 (L). (M-P) Wild-type (M), kan1 (N), gct (O) and gct kan1 (P) flowers. The arrowheads in D and P point to sites of ectopic outgrowths. The dotted lines in M-P underline stigmatic tissue. The leaves in E, F and G are numbered according to the order of their initiation. Scale bars: 20 \( \mu m \) in A-D; 1 cm in E-H; 2 mm in M-P.
stage (Fig. 1J), and is weakly expressed beginning at the torpedo stage in cct embryos (Fig. 1O). At the bent cotyledon stage, its expression is confined to patches on the abaxial side of the cotyledons in both mutants (Fig. 1J,P). After germination, gct and cct express E2023 in leaves in the same spatial pattern as wild-type plants, but at a reduced level (Fig. 1K,Q). E2023 expression was not detectable in carpels of gct and cct plants (Fig. 1L,R). Plants heterozygous for both gct and cct segregated the same range of embryo phenotypes as plants heterozygous for gct or cct only, implying that double mutant embryos have essentially the same phenotype as single mutants. Although we did not attempt to identify double mutants during embryogenesis, plants homozygous for both gct and cct were identified after germination using molecular markers, and supported this conclusion. This result suggests that GCT and CCT have closely related functions and may act in the same pathway.

gct enhances polarity defects of kanadi mutants

KAN1, KAN2 and KAN4 have overlapping functions in the specification of abaxial identity, and produce strong effects on embryo polarity only when all three genes are absent (Izhaki and Bowman, 2007). The observation that gct and cct reduce the expression of E2023 (KAN2) but do not have a polarity phenotype as severe as the kan1 kan2 kan4 triple mutant, suggests that GCT and CCT may not completely abolish KAN gene expression during embryogenesis, or may affect the expression of some KAN genes but not others. Reasoning that this effect might be visible only in a sensitized genetic background, we characterized the phenotype of gct in combination with kan1 and kan1 kan2.

gct enhanced the phenotype of kan mutations at every stage of plant development we examined. The embryonic phenotype of kan1 kan2 double mutants is similar to that of gct (Fig. 2A-C). Like gct (Fig. 2C), kan1 kan2 embryos (Fig. 2B) display a slight delay in morphogenesis, and have cotyledons that splay outward, a phenotype typical of a loss of abaxial identity. gct kan1 kan2 triple mutants resemble gct, but differ from gct in having ectopic growths on both the cotyledons and hypocotyl (arrowheads, Fig. 2D). These ectopic growths have previously been observed only in kan1 kan2 kan4 triple mutants (Izhaki and Bowman, 2007).

gct also enhanced kan1 postembryonic phenotypes. Whereas the leaves of kan1 and gct are held horizontally (Fig. 2E-G), the leaves of gct kan1 double mutants stand upright, curl upwards and are irregularly expanded (Fig. 2H). This effect on leaf morphology is associated with a change in polarity of mesophyll cells. Adaxial (palisade) mesophyll cells are typically round and densely packed, whereas abaxial (spongy) mesophyll cells are irregular in shape and are separated by large air spaces (Fig. 2I). kan1 (Fig. 2J), and to a lesser extent gct (Fig. 2K), cause spongy mesophyll cells to be less convoluted and more highly packed than normal. The spongy mesophyll of gct kan1 plants (Fig. 2L) shows an enhanced loss of abaxial identity, consisting of more densely packed, less branched cells that have a morphology intermediate between wild-type adaxial and abaxial mesophyll cells. Although kan1 and gct do not have a major effect on carpel development (Fig. 2M-O), gct kan1 flowers (Fig. 2P) have an enlarged septum with increased stigmatic tissue, less valve tissue and abaxial projections of carpel and papillary tissue, and are essentially identical to the flowers of kan1 kan2/+ plants (Pekker et al., 2005). These results are consistent with the decreased expression of E2023 in gct, and suggest that GCT contributes to the specification of peripheral-abaxial identity by promoting the expression of KAN2.
T-DNA insertion in At1g55325 (SAIL_1169_H11) has a phenotype essentially normal. Thus, the decrease in cotyledons, the timing and overall GUS pattern in these mutants was reduced gPHB-GUS expression on the adaxial side of cct (Eshed et al., 2004). To determine if the decrease in (McConnell et al., 2001) (Fig. 3A-D). Interestingly, although gct and cct did not affect the expression of pFIL::dsRed-N7, a reporter for the abaxially expressed gene YABBY1/FILAMENTOUS FLOWER (FIL) (Heisler et al., 2005) (Fig. 3M-O; see Fig. S1 in the supplementary material).

Class III HD-ZIP genes interact antagonistically with KAN genes (Eshed et al., 2004). To determine if the decrease in KAN1 and KAN2 expression in gct and cct embryos is the result of an increase in HD-ZIPIII expression, we examined their effect on expression of the HD-ZIPIII gene PHB. Because PHB is regulated post-transcriptionally by miR165/miR166, this experiment was conducted using a reporter in which GUS was fused to the C-terminal end of a PHB genomic clone (gPHB-GUS). This construct has an expression pattern identical to that reported for PHB mRNA (McConnell et al., 2001) (Fig. 3A-D). Interestingly, although gct and cct reduced gPHB-GUS expression on the adaxial side of cotyledons, the timing and overall GUS pattern in these mutants was essentially normal. Thus, the decrease in KAN expression in gct and cct is not attributable to an increase in the size of the PHB expression domain, or to an increase in the level of PHB expression.

**GCT and CCT encode MED13 and MED12, predicted transcriptional repressors conserved in all eukaryotes**

The molecular identities of GCT and CCT were determined using a map-based approach (Lukowitz et al., 2000). GCT is located on the lower arm of chromosome 4 and corresponds to At4g00450; cct-1 is a missense mutation in At4g00450, and two T-DNA insertions in At4g00450 (SALK_108241 and SALK_124276) have a phenotype identical to cct-1 and failed to complement this point mutation (Fig. 4B).

GCT and CCT are single copy genes in Arabidopsis. The predicted GCT protein has 1921 amino acids (AA), and contains a serine-rich region between AA 407 and 426. The predicted GCT protein is 16% identical and 19% similar to human GCT. KAN2 as they have on the expression of KAN1. KAN1 and KAN2 are single copy genes in Arabidopsis and are dynamically expressed during embryogenesis. The predicted GCT protein is 12% identical and 21% similar to human KAN1 and KAN2. The genomic clone (gPHB-GUS). This construct has an expression pattern identical to that reported for PHB mRNA (Fig. 3A-D). Interestingly, although gct and cct reduced gPHB-GUS expression on the adaxial side of cotyledons, the timing and overall GUS pattern in these mutants was essentially normal. Thus, the decrease in KAN expression in gct and cct is not attributable to an increase in the size of the PHB expression domain, or to an increase in the level of PHB expression.

**GCT and CCT are dynamically expressed during embryo development**

The expression of GCT and CCT during embryogenesis was characterized by RNA in situ hybridization. GCT mRNA was detected in both the apical and basal cell at the one-cell embryo stage (Fig. 4C), whereas CCT was first detected at the dermatogen stage of embryogenesis (Fig. 4L). Both genes are expressed in all cells of the embryo proper and suspensor through the globular stage of embryogenesis (Fig. 4C-F,L-M), but their expression begins to

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**Fig. 4. GCT and CCT encode the Arabidopsis homologs of the transcriptional regulators MED13 and MED12, and are dynamically expressed during embryogenesis.**

(A) GCT corresponds to At1g55325. The location and the nature of mutant alleles are shown. Serine-rich region (AA 407-426) shown in dark gray. Region of 42% identity (P-value 9e-11) to pfam06333 GCT domain shown in light gray (AA 1088-1179). (B) CCT corresponds to At4g00450. Region of 37% identity (P-value 5e-12) to pfam09497 CCT domain (AA 69-127) is shown in light gray. (C) mRNA in situ hybridization analysis of GCT expression in (C) one-cell, (D) octant, (E) dermatogen, (F) globular, (G) heart, (H) torpedo, (I) bent cotyledon, (J) SAM of bent cotyledon, and (K) RAM of bent cotyledon stage embryos. (L-R) mRNA in situ hybridization analysis of CCT expression in (L) dermatogen, (M) globular, (N) heart, (O) torpedo, (P) bent cotyledon, (Q) SAM of bent cotyledon and (R) RAM of bent cotyledon stage embryos. (S) GCT sense probe; (T) CCT sense probe. Scale bar: 10 μm.
In wild-type embryogenesis, the onset of the heart stage of development is marked by the initiation of cotyledon primordia on the upper flanks of the embryo, and the appearance of elongated vascular cells (Fig. 5F).

In both gct and cct the first division of the apical cell is transverse to the long axis of the embryo, instead of parallel as in wild type (arrows, Fig. 5A,B); furthermore, cells of the embryo proper (brackets) are abnormally elongated, and in this respect resemble suspensor cells (marked by the dashed line). gct (Fig. 5D) and cct (Fig. 5E) globular embryos are more elongated than wild type (Fig. 5C), and have more suspensor cells. The more severely affected embryos also lack the lens-shaped cell produced by asymmetric division of the uppermost cell of the suspensor (asterisks in Fig. 5D,E), and have one or more extra tiers of cells between the suspensor and the lower tier of the embryo (“x” in Fig. 5D,E). These extra tiers probably result from the abnormal first division plane of the apical cell shown in Fig. 5A,B. Although the percentage of phenotypically mutant gct and cct embryos segregating in siliques of heterozygous plants is quite low at the octant, dermatogen and globular stages (approximately 5%), these phenotypes were never observed in octant (n=227), dermatogen (n=215) or globular stage (n=344) embryos from wild-type plants.

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In wild-type embryogenesis, the onset of the heart stage of development is marked by the initiation of cotyledon primordia on the upper flanks of the embryo, and the appearance of elongated vascular cells (Fig. 5F). gct and cct delay both of these patterning events (Fig. 5G,H). The most strongly affected mutants decrease in the periphery of the hypocotyl and on the abaxial side of cotyledons starting at the heart stage (Fig. 4G,N). This trend continues through the torpedo stage (Fig. 4H,O), and by the bent cotyledon stage pKAN1::GUS expression is restricted to the vascular tissue and SAM and RAM (Fig. 4I-K,P-R). This pattern is consistent with the temporal effect of gct and cct mutations on the expression of E2023 and pKAN1::GUS (Figs 1 and 3), and suggests that these genes are important in the peripheral domain of the embryo only early in embryogenesis. Postembryonically, public microarray expression data indicate that GCT and CCT are expressed in most tissue types, with highest expression in the shoot apex (Winter et al., 2007).

**gct and cct uncouple cell division, pattern formation, and morphogenesis**

Our analysis of GCT and CCT expression demonstrated that their mRNAs are present in wild-type embryos before the initiation of KAN expression. To explore additional roles for GCT and CCT in early embryogenesis, the histological phenotype of wild-type, gct and cct embryos was characterized from the earliest stages of development. We found that pattern formation and morphogenesis are delayed in gct and cct embryos compared with wild type (Fig. 5A-K).

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**Fig. 5. gct and cct mutants uncouple pattern formation, growth and morphogenesis in early embryos.** (A,B) Wild-type and gct embryos at the octant stage (A), and wild-type and cct embryos at the dermatogen stage (B). The plane of the first division is marked with an arrow, the embryo is marked with a bracket and the suspensor is marked with a dashed line. (C) Wild-type globular stage embryo. (D) Weak (left) and strong (right) globular stage gct embryos. (E) Globular stage cct embryos show similar defects. In B-E, the cells of the upper tier and their derivatives (u), the lower tier and their derivatives (l), extra tiers in gct and cct (x) and the uppermost cell of the suspensor and its derivatives (asterisks) are labeled. (F) Wild-type early heart stage embryo. (G) Early heart stage gct embryos with two (left) or one (middle) small cotyledon primordia, or with random divisions throughout the embryo (right). (H) Similar phenotypes are seen in early heart stage cct embryos. (I-K) At the wild-type late heart stage (I), gct (J, left) and cct (K, left) embryos with two cotyledon primordia, and gct (J, right) and cct (K, right) embryos with random divisions throughout the embryo. In F-K, the shoot apical meristem, root apical meristem, and vascular primordia (vp) are indicated with lines, and the cotyledon primordia with arrowheads. (L) Cell numbers in medial sections of wild-type, gct and cct embryos at the early globular, late globular, early heart and late heart stages. Standard deviation is shown at top of bars in L (n≥8). All images shown at equal magnification. Scale bar: 10 μm.
The frequency of this latter class is similar to the frequency of rudimentary cotyledons and completely lack a SAM (Fig. 6C,H,E,J). This class probably represents a subsequent stage in the development of the severely affected globular embryos illustrated (right, Fig. 5G,H) display a disorganized cell division pattern. (A-E) Bent cotyledon stage wild-type, gct and cct embryos: 86% (n=151) of late stage gct embryos have an enlarged SAM (B); 14% (n=151) of late stage gct embryos lack the small vacuolated cells and layered tissue organization characteristic of the SAM (C); 94% (n=171) of late stage cct embryos have an enlarged SAM (D); 6% (n=171) of late stage cct embryos lack a SAM (E). (F) Medial section of wild-type SAM contains an average of 25±1 cells, (n=15). (G) Medial section of enlarged gct SAM contains an average of 68±5 cells (n=15). (H) gct embryo with large vacuolated cells in place of the SAM. (I) Medial section of enlarged cct SAM contains an average of 66±8 cells (n=15). (J) cct embryo with large vacuolated cells in place of the SAM. (K) Medial optical section of a wild-type RAM. (L) Of gct embryos, 86% had a slightly disorganized root cap and extra cells in RAM (n=84). (M) In addition, 14% of gct embryos lacked most cells of the root cap (n=84). (N) Of cct embryos, 100% (n=81) had a slightly disorganized root cap and extra cells in RAM. (O-Q) Hypocotyl of wild-type (O), gct (P) and cct (Q) embryos. Percentages in B-E refer to mutant embryos only, which are easily distinguished at late stages by their meristem phenotype. In A-E, the location of the SAM and RAM are shown in boxes. Original cleared images and tracings of the SAM, RAM or hypocotyl are shown in F-Q. The SAM was defined as the region bounded by the vascular tissue. A-E and F-Q shown at equal magnification. Scale bars: 40 μm in A; 10 μm in F. Asterisks indicate quiescent center cells of root meristem. co, cortex; en, endodermis; ep, epidermis; irc, lateral root cap; p, pericycle; v, vascular tissue; x, extra RAM cells.

The observation that gct and cct delay the expression of KAN1 and KAN2 as well as provascular and cotyledon differentiation indicates that these genes are a key role in temporal regulation of embryo pattern formation. To more broadly characterize the function of GCT and CCT in pattern formation, we examined the expression of molecular markers for the SAM (pSTM::GUS) (Kirch et al., 2003), RAM (pWOX5::GFP) (Xu et al., 2006) and vascular tissue (E2331) in wild-type and mutant embryos. In wild type, the expression of pSTM::GUS is first detected at the transition stage of embryogenesis, just as cotyledon primordia emerge on the flanks of the SAM (compare Fig. 7A and 7B). The size of the STM expression domain then increases, reaching its maximum extent at the bent cotyledon stage (Fig. 7C-E). In situ hybridization with an STM probe demonstrated that the SAM of mature gct and cct embryos is approximately 2.5 times larger than normal (Fig. 7E,I,M). Although pSTM::GUS is expressed much later in mutant embryos than in their wild-type siblings, in both genotypes pSTM::GUS expression is first detected at the morphological heart stage (Fig. 7H,L). Thus, gct and cct delay pSTM::GUS expression in absolute time, but not with...
respect to developmental stage. This suggests that the delay in STM expression is a consequence of delayed patterning. By contrast, gct and cct do not affect the timing of pWOX5::GFP. This RAM marker was expressed in all but the most phenotypically severe gct and cct embryos at both the wild-type heart and bent cotyledon stages (see Fig. S4 in the supplementary material).

The enhancer trap E2331 expresses GFP brightly in vascular tissue starting at the early globular stage and throughout the rest of embryonic development (Fig. 7N-Q), and is therefore an excellent marker for vascular patterning. gct and cct embryos do not express E2331 at the globular or heart stages (Fig. 7R,S,V,W). E2331 expression begins in the basal part of the hypocotyl of torpedo stage gct and cct embryos (Fig. 7T,X), and by the bent cotyledon stage, it is expressed throughout the vascular tissue of mutant embryos (Fig. 7U,Y); at this stage, the expression pattern of E2331 in mutant embryos is essentially normal (Fig. 7Q). Thus, gct and cct delay the expression of E2331 both in absolute time and relative to the morphogenetic stage of the embryo, suggesting that they play a direct role in vascular patterning.

**DISCUSSION**

Pattern formation is a result of temporally and spatially regulated changes in gene expression. Although considerable attention has been paid to the spatial component of this problem, the temporal regulation of developmental patterning is an open question. Here we show that growth and pattern formation can be dissociated during embryogenesis in Arabidopsis, and demonstrate a role for GCT/MED13 and CCT/MED12 in the temporal regulation of radial polarity (summarized in Fig. 8).

**GCT and CCT regulate the timing of genes required for peripheral-abaxial identity**

Peripheral-abaxial identity is specified by the KANADI family of transcription factors. gct and cct mutations delay the onset of KAN1 and KAN2 expression, implying that GCT and CCT directly or indirectly promote the expression of these genes early in embryogenesis. Interestingly, the absence of KAN1 and KAN2 expression early in development is not accompanied by a change in...
the expression of at least two other genes involved in the specification of adaxial-abaxial polarity – the abaxial gene, FIL, and the adaxial gene, PHB. This result provides an exception to the antagonism that is generally observed between KAN and PHB gene expression: typically, a decrease in KAN1 and KAN2 expression in leaves is associated with an increase in the PHB expression domain (Eshed et al., 2004). This antagonistic relationship has also been observed in early embryogenesis (Grigg et al., 2009). One explanation for the lack of an increased PHB expression domain in get and cct is that at the heart stage there are residual levels of KAN1 and KAN2 gene expression that cannot be detected by our reporter constructs. Another possibility is that GCT and CCT are required to mediate the antagonistic interactions between PHB and KAN. If this is the case, further studies on the role of GCT and CCT may lead to an understanding of the molecular nature of PHB-KAN antagonistic interactions.

In light of the importance of the phytohormone auxin in apicobasal and radial patterning of Arabidopsis embryos (Nawy et al., 2008), it is interesting to note that both the abnormal orientation of the first division of the apical cell in get and cct and the resultant double octant embryos are also observed in monopterados and bodenlos auxin response mutants (Berleth and Jürgens, 1993; Hamann et al., 1999). The phenotypic similarity of these four mutants points to a possible role for GCT and CCT in promoting auxin transcriptional responses in early embryogenesis. The relationship between GCT, CCT and auxin is an exciting area for future research.

GCT and CCT are required for temporal coordination of growth and pattern formation during embryonic development

Mature get and cct embryos typically possess an enlarged SAM and abnormally shaped cotyledons. The basis for the phenotype became apparent when we examined the effect of these mutations on the rate of cell division and the timing of various events in embryo development. Whereas get and cct had little or no effect on the rate of cell division early in embryo development, they delayed key morphogenetic events, including production of provascular tissue, cotyledon initiation and the initiation of the SAM. We suspect that this delay explains the effect of these mutations on meristem size, because the effect does not persist after germination, as is the case for mutations in meristem maintenance genes such as STM, PHB and FIL – were expressed later than normal, but at the correct morphological stage. Others – specifically KAN1, KAN2 and the provascular marker E2331 – were delayed in time, with respect to the morphology of the embryo, and also relative to genes with which their expression is normally synchronized.

These results demonstrate the existence of a mechanism for temporally coordinating events in plant embryogenesis, and indicate that GCT and CCT are important components of this mechanism. Interestingly, among the Arabidopsis Mediator genes that have been functionally characterized, this role in temporal regulation of development is apparently specific to GCT/MED13 and CCT/MED12. For example, mutations in the Arabidopsis core Mediator components SWP (MED14) and MED21 result in severely dwarfed and lethal phenotypes (respectively) (Autran et al., 2002; Dhawan et al., 2009). By contrast, loss-of-function mutations in Arabidopsis HEN3/CDK8, a protein that in Drosophila physically interacts with MED12 and MED13, have a subtle phenotype that is apparently unrelated to developmental timing (Wang and Chen, 2004). This divergence in function between MED12/13 and CDK8 parallels studies in Drosophila, where, despite their physical interaction, mutations in Med12 and Med13 condition different phenotypes from Cdk8 mutants (Loncle et al., 2007).

Transient requirement for Arabidopsis MED13 and MED12 in embryonic patterning

With rare exceptions (Carrera et al., 2008), yeast and animal Med13 and Med12 proteins act as transcriptional repressors (Samuelson et al., 2003; Ding et al., 2008; Knuese et al., 2009). With this in mind, one attractive hypothesis is that GCT and CCT regulate the maternal-to-zygotic transition in early embryogenesis, perhaps by repressing zygotic transcription during the window of early embryogenesis when development is thought to be predominantly under maternal control (Pagnussat et al., 2005; Grimanelli et al., 2005) (reviewed by Baroux et al., 2008). In this scenario, the delayed pattern formation and morphogenesis seen in early get and cct embryos would be attributed to a confusion in cell and tissue identity caused by a heterochronic overlap of normally distinct maternal and zygotic patterning programs. This overlap would be relieved after the first few days of embryogenesis, when zygotic transcription normally predominates, and would explain the delayed initiation of radial patterning in get and cct embryos. Regardless of the mechanism, identification of the direct targets of GCT and CCT is a high priority for future research, and should illuminate the role that these genes play in temporal control of radial patterning in early embryogenesis.

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Author contributions

C.S.G. designed and performed all experiments with the exception of in situ hybridizations (M.Y.P.), and molecular and genetic characterization of the E2023 line (M.R.S., R.P. and R.A.K.). C.S.G. and R.S.P. analyzed data and wrote the manuscript.

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/lookup/suppl?doi=10.1242/dev.043174/-/DC1

References


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highly conserved residues (same color) (:) 358/1921 = 19%

semi-conserved residues (.) 353/1921 = 18%

Alignment made with Clustal W [http://www.ebi.ac.uk/clustalw/](http://www.ebi.ac.uk/clustalw/)

Colors:
- **Red** = Small and hydrophobic residues (AVFPMILW)
- **Blue** = Acidic residues (DE)
- **Magenta** = Basic residues (RK)
- **Green** = Hydroxyl + Amine (STYHCNGQ)
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highly conserved residues (same color) (:) 452/2144 = 21%

semi-conserved residues (.) 357/2144 = 17%

Alignment made with Clustal W  http://www.ebi.ac.uk/clustalw/

Colors:
Red = Small and hydrophobic residues (AVFPMILW)
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A  pWOX5 ::GFP

wt

gct

cct