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(Original title: The Function of the Early Trichomes Gene in Arabidopsis and Maize)

New title: Genetic regulation of adaxial-abaxial polarity in Arabidopsis

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As presented in the original grant application, we identified the GCT gene and found that it encodes a protein with regions of similarity to MED13 proteins from yeast and animals. MED13 is one of four proteins in a cyclin dependent kinase module (also known as the SRB8-11 module) that, in conjunction with the main Mediator complex, negatively regulates a small number of genes. Studies in yeast and animals suggest that the role of the SRB8-11 module is to integrate cell- and tissue specific signals with transcriptional control of target genes. The identity of GCT as a negative regulator of transcription, as well as the enlarged shoot apical meristem and decreased expression of the peripheral/abaxial enhancer trap E2023 in *gct* mutants led us to propose that these mutants represent a 'centralized' phenotype.

Identification of the *CCT* gene Mutations in the *CCT* gene confer a phenotype almost identical to loss of function mutations in the *GCT* gene, with the exception that the phenotype of *cct* mutants is slightly weaker. We identified the *CCT* gene based on its map position, and found that *CCT* encodes a protein with regions of similarity to the MED12 component of the SRB8-11 module. Since we isolated a single EMS-induced allele of *CCT*, the identity of the *CCT* gene was verified by allelism tests with two T-DNA insertions in the gene At4g00450, confirming this as the *CCT* gene. This result was satisfying because mutations in MED12 and MED13 confer similar phenotypes in Drosophila and C. elegans, the other multicellular organisms in which they have been studied. Although the biological processes that the SRB8-11 module regulates in each organism are different, our results indicate that the SRB8-11 module itself is conserved in plants in addition to yeast and animals.

Double mutant analysis of *GCT* and *CCT* The similar phenotype of mutations in *GCT* and *CCT*, in addition to the molecular identity of the genes, suggested that *GCT* and *CCT* would interact genetically. To test this hypothesis, we created a plant that was heterozygous for *gct-2* and *cct-1*, and used PCR-based markers to check the progeny of this plant for *gct cct* double mutants. The reference alleles *gct-2* and *cct-1* (as well as all other alleles) show a similar range of phenotypes. In 10d old seedlings, the phenotypes range from seedlings with shortened petioles of cotyledons and leaves (weak), to cotyledons of variable shape (intermediate), to seedlings with an enlarged SAM and little or no development of cotyledons (strong). In contrast to the range of phenotypes seen in single mutants, we found that *gct cct* double mutants exclusively showed the strong phenotype. This enhanced phenotype of the double mutant could formally be interpreted as evidence that *GCT* and *CCT* act in parallel pathways that are required for a common process. However, in view of the fact that *gct* and *cct* mutants have an almost identical phenotype, and that they encode proteins that are predicted to be part of a conserved complex, we suggest that *GCT* and *CCT* participate in the same molecular function in the (predicted) SRB8-11 complex in Arabidopsis. In the absence of one of these genes, the complex retains some activity (which may explain the variable expressivity of the *gct* and *cct* phenotypes), but this stochastic activity is abolished in the absence of both proteins.

Expression of polarity genes in *gct* and *cct* To characterize the role of *GCT* and *CCT* in regulating genes involved in polarity of the shoot and lateral organs, we have begun examining the expression of transcriptional and translational fusions for various polarity and meristem genes. As a marker for the shoot apical meristem, we have seen that a pSTM::GUS transcrip-

tional reporter is highly overexpressed in the SAM of *gct* and *cct* embryos. In addition, we constructed a pPHB::PHB-GUS translation fusion (this genomic clone contains the entire 5' upstream region between PHB and the previous gene, as well as all exons and introns up to the stop codon) as a reporter for the central/adaxial gene *PHABULOSA*. We have seen that this transgene is overexpressed in *cct* mutants, and will soon determine its expression in *gct* mutants. As a marker of abaxial cell fate, we have examined the expression pattern of YAB3::GUS in *gct*. Expression of this marker is decreased. We will also soon be able to look at the expression of pKANADI::GUS, pREVOLUTA::REV-YFP, and pFIL::RFP in *gct* and *cct* mutants. The expression pattern of the anonymous enhancer trap E4097, which is expressed at the leaf margin and at the adaxial-abaxial junction of petioles (the two ridges at the top of petioles that are continuous with the leaf margin) has also been determined. This enhancer trap is variably expressed in leaf and cotyledon margins of *gct* and *cct* seedlings, suggesting defects in leaf margin identity in these mutants. In summary, the expression of markers of SAM fate, central/adaxial fate, abaxial fate, and leaf margin identity is consistent with our interpretation of the *gct* and *cct* phenotypes as being perturbed in the balance of central/adaxial and peripheral/abaxial genes.

Leaf identity phenotype of *gct* and *cct* mutants Post-embryonically, *gct* and *cct* mutants have a strong effect on leaf identity, as well as a late flowering phenotype. In wild-type Arabidopsis, the first two leaves are round, while each successive leaf becomes more elongated, where the length/width ratio of the 1st leaf is approximately 1, and the length/width ratio of leaf 10 is approximately 2.5. The number of serrations is also correlated with leaf identity: juvenile leaves lack serrations, while the number of serrations increases steadily on adult leaves: leaf 10 has approximately 10 serrations. Similar to wt, the first leaves of *gct* and *cct* are round, with a length/width ratio of about 1. However, unlike wt, *gct* and *cct* leaves do not exceed a length/width ratio of 1.5 until after leaf 12. Concomitant with this, *gct* and *cct* plants are extremely delayed in the production of serrations: leaf 12 of a *gct* or *cct* plant typically has 2-3 serrations. Because we had previously shown that GCT and CCT are required for the control of polarity genes, we were intrigued that an alteration of the expression of polarity genes such as *REV* and *YAB3* might explain the changes in leaf shape and serrations in *gct* and *cct* mutants. Due to our long standing interest in leaf identity, we have elected to first characterize the role of *GCT* and *CCT* in leaf development, and then investigate further their role in radial patterning during embryogenesis.

Genetic analysis of *GCT* and polarity genes To determine the genetic relationships between *GCT* and the genes that specify central/adaxial and peripheral/abaxial cell fates, we have analyzed double and triple mutants of *GCT* and polarity genes. For simplicity, we chose to study genetic interactions with *GCT* only, since *GCT* and *CCT* have nearly identical phenotypes and our genetic analysis suggests that they act at the same regulatory level. Single mutations in *YAB3* or *FIL* cause all vegetative leaves to be more round than wt leaves, and *yab3 fil* double mutants exaggerate this phenotype so that all leaves have a length/width ratio of about 1. *gct yab3* double mutants have an additive phenotype, as do *gct fil* double mutants. However, the leaf shape phenotype of *yab3fil* double mutants is almost identical to the phenotype of *gct yab3 fil* triple mutants. The additive phenotypes of *gct yab3* and *gct fil* suggest that *GCT* acts in a separate pathway from *YAB3* and *FIL*. However, the triple mutant phenotype suggests that *YAB3* and *FIL* are downstream of *GCT* in the determination of leaf shape, though this interaction may be indirect.

The indirect interation of *GCT* with *YAB3* and *FIL* in determining leaf shape may be explained by the results of our analysis of *gct rev* double mutants. While *gct* produces round leaves, *rev* mutants have elongated leaves. *gct rev* double mutants have elongated leaves, similar to *rev*, suggesting that *REV* is epistatic to *GCT*. Thus, if *GCT* acts upstream of *REV* to control its expression domain, this could explain the effect of *gct* mutations on the expression of polarity genes: loss of *REV* repression would lead to and increase of *REV* expression, and a resulting decrease in expression of peripheral/abaxial genes such as *KAN* and YAB3. The epistasis we observed with *REV* and *GCT* supports a role for *GCT* in repressing central genes over a role for *GCT* in promoting peripheral genes.

Expression analysis of *GCT* To determine the expression domain of GCT, transcriptional and translational fusions to GUS+ were constructed. The pGCT::GUS+ transcriptional fusion includes the entire 5' region upstream of the translational start, until the previous gene (1kb). pGCT::GCT-GUS+ translational fusion include this 5' upstream region as well as the entire genomic clone, up to the stop codon, which was replaced by an NAAIRS linker region between the *GCT* gene and GUS+. pGCT::GUS+ and pGCT::GCT-GUS+ are expressed in similar tissues, but with different kinetics. Both constructs were expressed in vascular tissue of the hypocotyl, root and in lateral organs. Neither construct was expressed in the peripheral region of the hypocotyl and root. Both constructs were also expressed throughout young leaf primordia (less than ~1mm), and in older leaves the expression domain was progressively restricted to the base of the leaf (where most cell division occurs) and then to vascular tissues. Both constructs were also expressed in stomatal cells and trichomes.

Despite being expressed in similar tissues, the kinetics of expression of the two constructs were significantly different. The pGCT::GUS+ construct was expressed most highly at sites of auxin maxima, such as hydathodes and the tips of cotyledons. Expression of pGCT::GUS+ was also very high in trichomes and stomatal cells. In other cells, this construct was expressed intermittently, for example in only some cells of the vascular tissue of leaves. This expression may reflect an influence of the cell cycle on the GCT promoter region. In contrast to the transcriptional fusion, the pGCT::GCT-GUS+ translational fusion showed a consistent expression pattern within a given tissue. For example, pGCT::GCT-GUS+ was expressed in all cells of the vascular tissue, throughout young leaf primordia, and in all cells of the shoot meristem. Dynamic differences in the transcriptional and translational fusions raise the interesting possibility that transcription of the GCT gene is regulated by the cell cycle, while the GCT protein itself is stable.

However, these results must be interpreted in light of the fact that the pGCT::GCT-GUS+ construct was not able to complement the *gct-2* mutation. In addition, GUS+ expression in this transgenic line was localized to the nucleus and cytoplasm. We anticipate that the failure of this construct to complement the *gct-2* mutation is due to the large size of the GUS+ protein. We have made pGCT::GCT-eGFP constructs in the hope that these will be able to rescue the mutant phenotype, and will allow us to obtain a more robust reporter for cell and tissue-specific expression of GCT. In addition, we have constructed a pCCT::CCT-mCherry reporter construct to determine the cell and tissue-specific expression of the CCT protein.

If the expression of the pGCT::GCT-GUS+ construct reflects the expression of the native protein at the tissue level, one possibility is that the GCT protein controls leaf shape in a cell cycle dependent manner through its expression in dividing cells of the leaf. This idea is consistent with the prediction that the GCT CCT complex should contain a cyclin-dependent kinase and a cyclin C protein. Perhaps GCT and CCT integrate cell cycle cues with spatial cues from polarity genes to control leaf shape.

KANADI is a transcriptional repressor of AS2. We identified a dominant mutation whose effect on leaf polarity strongly resembled *kanadi1*, the gene that was the original focus of this grant. Genetic mapping revealed that this mutation was located in the vicinity of the LOB domain gene, ASYMMETRIC LEAF2 (AS2). Sequencing of the genomic region surrounding AS2 demonstrated that this new mutation, which we have named as2-d, is a point mutation in the promoter of AS2. Realizing that the phenotype of as2-d strongly resembled the phenotype of 35S::AS2 plants, we predicted that this mutation interferes with the binding of a negative regulator of AS2. We communicated this result to Randy Kerstetter because his lab has defined the binding site for KAN1, and has shown that KAN1 functions as a negative regulator (unpublished results). As we had hoped, the as2-d mutation resides in the predicted binding site of KAN1. In collaboration with Patty Springer (who has generated AS2::GUS reporter plants), we have shown that the as2-d mutation causes misexpression of AS2 in the abaxial domain of the leaf. Furthermore, Randy Kerstetter has shown using chromatin immunoprecipitation that KAN1 binds to the AS2 promoter, and that the as2-d mutation interferes with this binding. These results provide the first evidence for a direct interaction between transcription factors regulating abaxial (KAN1) and adaxial (AS2) polarity. Over-expression studies indicate that AS2 is capable of repressing the expression of KAN1, suggesting that mutual repression by KAN1 and AS2 plays a major role in establishing leaf polarity.

Publications

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