Arabidopsis JAGGED links floral organ patterning to tissue growth by repressing Kip-related cell cycle inhibitors

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Plant morphogenesis requires coordinated cytoplasmic growth, oriented cell wall extension, and cell cycle progression, but it is debated which of these processes are primary drivers for tissue growth and directly targeted by developmental genes. Here, we used ChIP high-throughput sequencing combined with transcriptome analysis to identify global target genes of the Arabidopsis transcription factor JAGGED (JAG), which promotes growth of the distal region of floral organs. Consistent with the roles of JAG during organ initiation and subsequent distal organ growth, we found that JAG directly repressed genes involved in meristem development, such as CLAVATA1 and HANABA TARANU, and genes involved in the development of the basal region of shoot organs, such as BLADE ON PETIOLE 2 and the GROWTH REGULATORY FACTOR pathway. At the same time, JAG regulated genes involved in tissue polarity, cell wall modification, and cell cycle progression. In particular, JAG directly repressed KIP RELATED PROTEIN 4 and KRP2, which control the transition to the DNA synthesis phase of the cell cycle. The krp2 and krp4 mutations suppressed jag defects in organ growth and in the morphology of petal epidermal cells, showing that the interaction between JAG and KRP genes is functionally relevant. Our work reveals that JAG is a direct mediator between genetic pathways involved in organ patterning and cellular functions required for tissue growth, and it shows that a regulatory gene shapes plant organs by releasing a constraint on S-phase entry.

Significance

Plant organs, such as leaves, petals, or fruits, are shaped by the behavior of their constituent cells: cell growth, oriented extension of cell walls, and cell division. However, we know little about how these processes are coordinated by regulatory genes that shape plant organs, such as JAGGED (JAG) in Arabidopsis. By identifying the genes bound by JAG throughout the genome and the consequent changes in gene expression, we reveal that JAG functions as a direct mediator between genes that control the identity of organs and tissues and the cellular activities required for organ growth. In particular, we show that JAG controls organ shape and size.

Results

To reveal the genome-wide JAG binding sites, we used anti-GFP antibodies to pull down JAG-bound DNA from jag-2 inflorescences supplemented with a genomic JAG-GFP fusion (JAG:JAG-GFP) (9). ChIP-Seq was performed and analyzed in triplicate, with WT antibody to pull down JAG-bound DNA from jag-2 inflorescences used as epitope-negative controls. As expected for the function of JAG as a transcription factor, binding sites were enriched within the 1.5-kb regions upstream and downstream of coding sequences (Table 1). A total of 1,634 genes contained binding peaks with a false discovery rate (FDR) of less than 1% in all three replicates, and these were selected as high-confidence JAG targets (Dataset S1).

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Database deposition: The ChIP-Seq data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE51537). Expression array data in this paper have been deposited in the Nottingham Arabidopsis Stock Centre NASCArrays database, http://affymetrix.arabidopsis.info (experiment ID NASCARRAYS-605).

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To resolve possible ambiguities in assigning gene models to ChIP-Seq peaks and to select functionally relevant direct JAG targets, we next overlapped the ChIP-Seq data with changes in gene expression detected with Affymetrix ATH1 oligonucleotide arrays (Fig. 1). In addition to comparing WT and jag-1 mutant buds, we looked for expression changes shortly after widespread JAG activation to facilitate detection of early JAG targets and of genes regulated by JAG in only a limited number of cells. For this, we used plants in which the constitutively expressed 35S promoter drove expression of a fusion between JAG and the rat glucocorticoid receptor (GR), which complemented the jag-2 mutant upon treatment with dexamethasone (9). In accordance with the suggestion that JAG functions as a transcriptional repressor (8), as discussed below, the overlap between genes repressed by JAG-GR and up-regulated in the mutant was higher than expected by chance, whereas genes activated by JAG-GR were not significantly enriched for lower expression in the mutant (Fig. S1 and Dataset S2). Surprisingly, there was also a significant enrichment for genes that responded in the same way to JAG-GR activation and to loss of endogenous JAG function (Fig. S1), although very few of these were directly bound by JAG (Dataset S3), suggesting that this overlap corresponded to indirect, downstream effects on gene expression. Especially in the case of genes repressed by JAG-GR but also down-regulated in the mutant, these indirect effects could result from inhibited growth of the relevant tissues in the jag mutant.

Comparison of the ChIP-Seq and expression data showed that as expected for genes whose expression changed a short time after activating JAG function, the set of JAG-GR-responsive genes (Dataset S2) was strongly enriched for ChIP-Seq targets (Fig. 1A). This enrichment was significant for genes repressed by JAG-GR ($P = 3.90 \times 10^{-21}$, Fisher’s exact test) but not for activated genes ($P = 1.37 \times 10^{-1}$, Fisher’s exact test). Furthermore, the proportion of genes repressed by JAG-GR, but not the proportion of those activated, rose with increasing ChIP-Seq peak scores (Fig. 1B). Together, these data suggest that JAG functions preferentially as a transcriptional repressor, in accordance with the presence of the ethylene-responsive element binding factor-associated amphiphilic repression motif near its N terminus (8). However, we have also confirmed direct, positively regulated targets (Dataset S4). In contrast to the JAG-GR–responsive genes, and as expected for the presence of a large number of indirect effects, the sets of genes that were differentially expressed between jag-1 and the WT (Dataset S2) did not show significant enrichment for JAG-bound genes (Fig. 1A). Nevertheless, ChIP-Seq targets that were differentially expressed in the mutant vs. WT comparison, but did not respond to ectopic JAG-GR, may correspond to genes that are regulated by JAG in combination with cell type-specific factors; thus, the overlap with these differentially expressed genes was also included in the list of target genes.

The resulting set of 235 direct, transcriptionally responsive target genes (Dataset S3) showed a strong enrichment for gene ontology (GO) terms related to transcriptional control and hormone responses (Fig. 1C and Dataset S5), and included numerous known regulators of organ identity and growth. In addition to known direct target genes (9, 10), such as PELT1 LOSS (PTL) (11) and BELL1 (BEL1) (12), JAG directly repressed flowering patterns, such as CLAVATA1 (CLV1) (13) and HANABA TARANU (HAN) (14); genes involved in floral identity, such as LEAFY (LFY) (15) and its direct targets LATE MÉRISIEM IDENTITÉ 1 (LM11) (16) and LM12 (17); and BLADE ON PETIOLE 2 (BOP2), which regulates organ development along the proximodistal and adaxial-abaxial axes (18, 19). Consistent with the role of JAG in development of the distal region of floral organs, JAG interacted directly with AtMYB16, which has been implicated in the differentiation of conical cells.
that are characteristic of the petal lobes (20). Another regulatory network with multiple nodes targeted by JAG is the GROWTH REGULATING FACTORS (GRF) organ growth pathway, which preferentially promotes growth of the basal region of shoot organs (21, 22). JAG represses GRFS and its cofactor ANGUSTIFOLIA 3 (AN3) (23, 24) and activated TEOSINTE-BRANCHED 1, CYCLOIDEA, and Proliferating Cells 1 and 2 (TCP) 4, which, in turn, antagonizes GRF expression (25).

Apart from the gene regulatory networks mentioned above, JAG also targeted cellular functions required for tissue growth. Direct links to cell cycle control included repression of the KRP genes KRP4 and KRP2, which is analyzed in detail below, and of CYCLIN D3; (26). Target genes implicated in cell wall extension included ARABIDOPSIS H⁺-ATPase 2 (AHA2), which encodes a plasma membrane proton ATPase that participates in the acidification of the apoplast during the initial stages of wall extension (27, 28), and PROTEIN KINASE SOS2-LIKE 5 (PKS5), which controls AHA2 activity (29). Other JAG targets were homologous to genes implicated in cell wall synthesis or modifications, including XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 28 (XTH28) (30), TRICHOME BIREFRINGENCE-LIKE 37 (TBL37) (31), and CELLULOSE SYNTHASE LIKE A11 (CSLAI1) (32). Relevant to the role of JAG in promoting polarized tissue growth (9, 10), JAG activated UNICORN (UCN), which encodes a protein kinase implicated in oriented tissue growth (33), and inhibited PROLIFERATING CELL FACTORS 1 (34, 35). In summary, the combined ChIP-Seq and expression data showed that JAG is intimately connected with gene networks that control organ identity and organ patterning; at the same time, it directly controls genes required to execute tissue growth. To confirm differential expression in response to JAG independently, representative genes for different functional categories were also tested by quantitative RT-PCR (qRT-PCR) in an induced expression experiment (Dataset S4).

Among the genes directly repressed by JAG, the two genes with the highest ChIP-Seq scores were KRP4 and KRP2 (Dataset S3). KRP2 and KRP4 encode cyclin-dependent kinase (CDK) inhibitors, which play a key role in controlling the transition to the S-phase of the cell cycle (36). Repression of KRP genes is consistent with the role of JAG in promoting cell proliferation (7, 8) and with the premature entry into the S-phase induced by ectopic JAG (9); thus, we focused next on the interaction between JAG and KRP genes. Both KRP2 and KRP4 showed strong ChIP-Seq peaks in all replicates, particularly in their 3′ regions (Fig. 2 A and B), and significant binding to the regions corresponding to the ChIP-Seq peaks was confirmed independently by ChIP-quantitative PCR (qPCR) using both the JAG-GFP fusion and anti-GFP antibodies (Fig. 2C) and JAG-GR with anti-GR antibodies (Fig. S2). To test differential expression independent of the array experiments, we used qRT-PCR to measure how each KRP family member responded to changes in JAG function (Fig. 2D). Of the seven family members, only KRP4 and KRP2 were repressed upon JAG-GR activation. In the jag mutant compared with the WT, both the arrays and qRT-PCR showed increased expression of KRP2 but not KRP4. This suggested that KRP4 could be widely expressed and repressed by endogenous JAG only in specific tissues or that there could be feedback regulation of KRP4 in the jag mutant. We could not test these possibilities by in situ hybridization or reporter genes, which were not sensitive enough to detect KRP4 expression reliably in floral buds. However, the phenotypic effects of krp4 mutations, which are described below, imply that KRP4 is expressed during floral organogenesis.

To test whether repression of KRP4 or KRP2 was required to promote floral organ growth, we compared mature organ sizes in plants with different combinations of the jag-1, krp-2, and krp-4 mutations with the WT Columbia-0 (Col-0) control. As reported previously (37), the krp2 and krp4 single mutants did not show any obvious defects or significant differences in organ size compared with the Col-0 control (Fig. 3 E–G). In the jag-1 mutant, sepals and petals were shorter and narrower than in the WT (Fig. 3 A and G). Both phenotypes were partially suppressed in the jag-1

![Figure 2](image_url)

**Fig. 2.** KRP4 and KRP2 are directly targeted by JAG in floral buds. ChiP-Seq peaks detected in each replicate within 3 kb upstream or downstream of the coding sequences for KRP4 (A) and KRP2 (B) are shown. (C) Binding of JAG-GFP to the regions of KRP4 and KRP2 confirmed by ChiP-qPCR. The numbers on the horizontal axis below the bars correspond to the left border of the amplified region (average amplicon size = 100 bp) relative to the coding sequence, and bars indicate means and SDs; asterisks indicate significant difference to the negative control (*, *P* < 0.01, Student t test). (D) Expression of all seven KRP genes in JAG-GR inflorescence apices 4 h after treatment with dexamethasone (red) or mock treatment (blue) or in WT apices (green) compared with jag-2 (yellow). Bars indicate means and SDs, and asterisks indicate a significant difference from the negative control (**, **P* < 0.01, Student t test).
ANOVA; jag-1 krp2-3 krp4-1 and mutants. (Scale bars: 1 mm.) Distribution of sepal area (partial recovery of growth in the double and triple mutants was statistically significant \((P < 0.05, \text{Student 't test)}\) (Fig. 3 H and I).

In addition to rescuing organ growth partially in jag-1, the krp2-3 and krp4-1 mutations restored cell morphology in the petal epidermis. The characteristic conical epidermal cells seen in the distal region of WT petals are replaced in jag-1 by elongated epidermal cells resembling those found near the petal base in the WT \((7, 8)\) (Fig. 4A and B). In contrast, the krp2-3 jag-1, krp4-1 jag-1 double mutants and the krp2-3 krp4-1 jag-1 triple mutant developed WT conical cells in the petal lobe, although the area occupied by these cells was smaller than in the WT \((Fig. 4 C–K)\). The restoration of conical cells in the petals of the jag-1 krp2-3 and jag-1 krp4-1 double mutants is consistent with a recovery of growth specifically in the distal region of petals, which is preferentially inhibited in jag mutants.

**Discussion**

Our data reveal direct links between JAG and other genetic pathways that control organ patterning and growth. Examples include repression of meristem development genes, such as CLV1 and HAN, corroborating the previous finding that one of the roles of JAG is to antagonize meristem genes in cells that are changing from meristem to organ primordium identity \((9)\). Another example is LFY, which activates floral homeotic genes that, in turn, activate JAG \((38–40)\); our results suggest that JAG, in turn, antagonizes LFY activity, in line with the observation that ectopic JAG expression in the jag-5D mutant converts flowers into leafy shoots \((7)\). Connected to LFY function, BOP2 is expressed in the basal region of lateral organs, regulates organ development along the proximodistal and adaxial-abaxial axes, and antagonizes JAG \((18, 41)\). We found that JAG directly repressed BOP2, suggesting that mutual antagonism between JAG and BOP2 is involved in establishing proximodistal organ polarity.

The direct links between JAG and cellular effectors of growth also give insight into what cellular processes are directly targeted by regulatory genes to shape plant organs. Cytoplasmic growth, cell wall extension, and cell division are interdependent processes required for plant organ growth, but it is not clear how they are coordinated \((42)\); at present, no plant equivalent is known of the well-studied Myc and Hippo pathways that coordinate growth-related cellular activities in animals \((43)\). It has been debated which cellular processes are primary drivers for plant

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**Fig. 3.** Suppression of jag organ growth defects by krp2 and krp4 mutations. Representative mature flowers of jag-1 (A), jag-1 krp2-3 (B), jag-1 krp4-1 (C), jag-1 krp2-3 krp4-1 (D), krp2-3 (E), krp4-1 (F), and WT Col-0 (G) are shown; note the defective sepal and petal growth in jag-1 and the partial recovery of growth in the jag-1 krp2-3 and jag-1 krp4-1 double mutants. (Scale bars: 1 mm.) Distribution of sepal area \((H)\) and petal area \((I)\) for the same genotypes shown in A–G (indicated in italics on the horizontal axis); box plots show median (thick line) second to third quartiles (box), minimum and maximum ranges (dashed line), and outliers (single points). Asterisks indicate significantly different means for jag-1 krp2-3, jag-1 krp4-1, and jag-1 krp2-3 krp4-1 \((B–D)\) compared with jag-1 (A) \((P < 0.05, \text{one-way ANOVA}；n = 40)\). The means of the single krp2-3 (E) and krp4-1 \((F)\) mutants were not significantly different from the Col-0 control \((G)\) \((\text{one-way ANOVA}; n = 40)\). sq. mm, square millimeters.

**Fig. 4.** The krp2 and krp4 mutations recover epidermal cell morphology in jag petals. Scanning electron micrographs of the epidermis of the distal region of mature petals from WT Col \((A), jag-1 (B), jag-1 krp2-3 (C), jag-1 krp4-1 (D), and jag-1 krp2-3 krp4-1 (E)) note the characteristic conical cells in A and C–E. Scanning electron micrographs of mature petals from WT Col \((F), jag-1 (G), jag-1 krp2-3 (H), jag-1 krp4-1 (I), and jag-1 krp2-3 krp4-1 (J)) the black lines across the distal region of the petals show the boundary of the distal petal lobe, where conical epidermal cells are seen. (Scale bars: A–E, 20 μm; F–I, 500 μm.) \((K)\) Area of the distal petal lobe for the genotypes indicated. Box plots show median (thick line) second to third quartiles (box), minimum and maximum ranges (dashed line), and outliers (single points). Asterisks indicate that the mean is significantly different from jag-1 \((P < 0.05, \text{Student 't test)}\). wt, wild type.
organ growth and directly targeted by developmental genes and which are likely to be subordinate; tissue mechanics is a current focus in plant morphogenesis (44), and it has been disputed whether control of cell cycle progression can drive plant organ growth (45). The interaction between JAG and KRP2/KRP2 shows that direct developmental control of regulators of S-phase entry is important for growth and morphogenesis, consistent with the observation that meristem growth is rapidly inhibited by suppressing DNA synthesis but continues when mitosis is inhibited (46). The results are also consistent with the idea that the growth of plant tissues is actively restrained below the levels that would be physiologically possible.

This idea was initially proposed as an adaptive response to environmental stress, during which growth is restrained by transcriptional repressors of the DELLA family (25). In this respect, it is interesting that DELLA proteins have also been shown to regulate KRP2 expression, although the functional relevance of this interaction has not been demonstrated (47). Our results suggest that in addition to a potential role in modulating growth in response to environmental conditions, localized release of a growth restraint imposed by the KRP CDK inhibitors can be used to generate the differential tissue growth required for morphogenesis.

The suppression of the jags growth defects by the krp mutations was not complete; therefore, additional targets of JAG are required for full organ growth. Plausible candidates include genes implicated in cell wall modification and cell wall extension. In addition, JAG controls not only growth rates but also polarized tissue growth (9, 10). The molecular targets of JAG provide a starting point to address the molecular mechanisms behind these other key aspects of plant tissue growth.

Materials and Methods

Plant Material. Arabidopsis thaliana Landsberg-erecta (L-er) and Col were used as WTs; jag-1 (T), jag-2 (R), and krp-2-3 (48) have been described. The krp4-1 (SALK 102417, Col background with a T-DNA insertion in the second exon) was obtained from the Netherlands Arabidopsis Stock Centre (NASC); loss of KRP4 expression in krp4-1 (37) was confirmed by RT-PCR (Fig. S3). SSS:JAG-GR and pJAG-JAG-GFP have been described (9). Plants were grown under long-day conditions (16 h light and 8 h dark, 80% humidity) in John Innes Centre Arabidopsis Soil Mix (Levington F2 compost with Inteript and grit at a 6:1 ratio), at 18 °C for Chip and array experiments and at 22 °C for floral organ measurements.

ChIP. pJAG-JAG-GFP, jag-2 and WT L-er control plants were used. Fluorescence aces [1,300–1,500 mg (fresh weight) per sample] were fixed in 3% formaldehyde for 15 min. Tissue was rinsed twice with 100 μL and twice with 200 μL washes with sterile water, the tissue was blotted dry and frozen in liquid nitrogen. Nuclei were purified as described (49) and resuspended in 1 mL of sonication buffer containing 500 mM Hepes, 150 mM NaCl, 5 mM MgCl2, 0.5% SDS. One hundred microliters of TE buffer and 9 μL of PCR-grade water (Roche), purified using a PCR purification Kit (catalog no. 18104; Qiagen), and stored at −20 °C.

ChIP-Seq and Data Analysis. Six Illumina TrueSeq ChIP-Seq libraries (three pJAG-JAG-GFP replicates and three WT controls) were produced as described (39) and sequenced (50-bp single-end reads) using an Illumina Hiseq 2500 (Run Mode) as described by the manufacturer (Illumina). Sequence reads that passed the Consensus Assessment of Sequence and Variation (CASAVA) sequencing quality filter were mapped to the unmasked Arabidopsis genome (The Arabidopsis Information Resource (TAIR); ftp://ftp.arabidopsis.org/ using the Short Oligonucleotide Analysis Package (SOAPaligner, version 2) (50), allowing a maximum of two mismatches and no gaps. Reads mapping in multiple genomic locations, to the chloroplast, or to the mitochondrial genome were discarded. The primary ChIP-Seq data were deposited at the Gene Expression Omnibus database (accession no. GSE51537).

ChIP-Seq peaks were detected using ChIP-Seq Analysis in R (CSAR) (51) with default parameter values except for “backg,” which was set to 20. Each JAG-GFP library was analyzed independently in comparison to a single negative control with all three WT libraries combined. Mapped reads were extended directionally to 300 bp, and the distribution of the number of extended mapped reads overlapping each nucleotide in the JAG-GFP library and in the negative control was normalized to have the same mean and variance. Enrichment relative to control was calculated as the ratio of normalized extended reads between JAG-GFP and the control sample. Regions having less than 20 reads mapped in the control were set to zero (parameter backg = 20 in CSAR) to avoid false-positive results due to the low coverage of the control in some regions. FDR thresholds were estimated by permutation of reads between samples and controls using CSAR for each biological replicate independently. Candidate JAG target genes were defined as genes containing a significant (FDR < 0.01) binding event in all three replicates in the region between 3 kb upstream of the beginning and 1.5 kb downstream of the annotated gene. Because multiple copies of the JAG promoter are present in our pJAG-JAG-GFP plant in contrast to the unique copy in our WT plants, JAG was excluded from the Chip-Seq target list.

GO Analysis. GO term enrichment analysis was performed with the module BinGO (52) from Cytoscape (53). Plots of GO term enrichment vs. ChiP-Seq score threshold were produced in R (www.r-project.org). The GO database (GO.db, version 2.9.0) and the mapping of Arabidopsis genes to GO terms (org.ATAir.db, version 2.9.0) were downloaded from Bioconductor (www.bioconductor.org). The ChiP-Seq score attributed to each gene was the minimum value for the ChiP-Seq score of each of the three biological replicates.

Global Expression Analysis. For JAG-GR activation, inflorescences were treated for 5 h with 10 μM dexamethasone or mock-treated, and RNA was extracted as described (9) from 12 inflorescence apices per sample in three biological replicates per treatment. Probe synthesis and hybridization to Affymetrix gene chip ATH1 were performed at the NASC; the raw data and metadata are available at http://affymetrix.arabidopsis.info/ (experiment ID NASCARRAYS-605).

To select differentially expressed genes, raw expression values obtained from each hybridized chip were imported in an R session (www.r-project.org). The probe set to gene annotation ath1121501cd was downloaded from Bioconductor (www.bioconductor.org). Data were normalized using the padma package (54), and expression data were stored in an R data frame (org.At.tair.db). The FDR, based on the Benjamini and Hochberg method (55), was calculated using the Bioconductor package multtest. Probe sets targeting more than one TAIR10 gene and genes associated with multiple probe sets were discarded from the analysis. Because of the confounding effect of JSS:JAG-GR transcripts, JAG was also excluded. A gene was considered differentially expressed when FDR < 0.01 and the absolute value of the log2 ratio was larger than 0.5.

qPCR. For ChiP-qPCR of individual genes, 1 μL of the immunoprecipitated DNA and 1 μL of the purified input sample were used per 10-μL PCR reaction to perform qPCR in technical triplicates with the LightCycler 480 System (Roche) and SYBR Green I (Roche), as well as the primers listed in Table S1. Enrichment from cycle threshold (ΔCt) values ( Ct immunoprecipitated DNA – Ct input DNA) was evaluated using the 2−ΔΔCt method as described (56). qRT-PCR was performed as published (9) using the LightCycler System as described above. Data were normalized to TUBULIN alpha 4 chain (TUB4) expression amplified with primers TUB4-RT_1-F and TUB4-RT_1-R (Table S1) as described by Livak and Schmittgen (57). For each ChIP-qPCR and qRT-PCR, unpaired two-sample Student t tests were used to test for statistical significance of differences between treatments.

Imaging. To measure mature organs, flowers at full anthesis were detached from the inflorescences and dehydrated in a 15%, 30%, 50%, and 70% (v/vol) ethanol series. Sepals and petals were dissected in 70% (v/vol)
ethanol, imaged with a Leica DM6000 microscope, and measured using Fiji (57). Petal epidermal cells were imaged by cryoscanning EM using a Zeiss Supra 55 VP field emission gun scanning electron microscope (Zeiss SMT). Images of single flowers at anthesis were taken with a Leica 205A stereomicroscope.

For statistical analysis of petal and sepal measurements, the RCommander package (www.rcommander.org) was used for box plots and to test for normal distribution using the “Shapiro–Wilk” test for normality. Subsequently, one-way ANOVA with “Multiple Comparisons of means” using “Tukey Contrasts” was used.


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