### Title

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### Authors

Hagelthorn, Lynne
Monfared, Mona M
Talo, Anthony
et al.



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ORIGINAL RESEARCH

# Unique and overlapping functions for the transcriptional regulators *KANADI1* and *ULTRAPETALA1* in Arabidopsis gynoecium and stamen gene regulation

Lynne Hagelthorn[1,2] | Mona M. Monfared[3] | Anthony Talo[4] | Frank G. Harmon[1,2] | Jennifer C. Fletcher[1,2]

[1]Plant Gene Expression Center, United States Department of Agriculture-Agricultural Research Service, Albany, California, USA

[2]Department of Plant and Microbial Biology, University of California, Berkeley, Berkeley, California, USA

[3]Department of Molecular and Cellular Biology, University of California, Davis, Davis, California, USA

[4]Biology Department, St. Mary's College of California, Moraga, California, USA

**Correspondence**
Jennifer C. Fletcher, Plant Gene Expression Center, 800 Buchanan Street, Albany, CA 94710 USA.
Email: jfletcher@berkeley.edu

**Present address**
Mona M. Monfared, Department of Molecular and Cellular Biology, University of California, Davis, Davis, California, USA.

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## Abstract

Plants generate their reproductive organs, the stamens and the carpels, de novo within the flowers that form when the plant reaches maturity. The carpels comprise the female reproductive organ, the gynoecium, a complex organ that develops along several axes of polarity and is crucial for plant reproduction, fruit formation, and seed dispersal. The epigenetic trithorax group (trxG) protein ULTRAPETALA1 (ULT1) and the GARP domain transcription factor KANADI1 (KAN1) act cooperatively to regulate *Arabidopsis thaliana* gynoecium patterning along the apical–basal polarity axis; however, the molecular pathways through which this patterning activity is achieved remain to be explored. In this study, we used transcriptomics to identify genome-wide ULT1 and KAN1 target genes during reproductive development. We discovered 278 genes in developing flowers that are regulated by ULT1, KAN1, or both factors together. Genes involved in developmental and reproductive processes are overrepresented among ULT1 and/or KAN1 target genes, along with genes involved in biotic or abiotic stress responses. Consistent with their function in regulating gynoecium patterning, a number of the downstream target genes are expressed in the developing gynoecium, including a unique subset restricted to the stigmatic tissue. Further, we also uncovered a number of KAN1- and ULT1-induced genes that are transcribed predominantly or exclusively in developing stamens. These findings reveal a potential cooperative role for ULT1 and KAN1 in male as well as female reproductive development that can be investigated with future genetic and molecular experiments.

**KEYWORDS**
Arabidopsis, gynoecium, KAN1, reproduction, stamen, ULT1

## 1 | INTRODUCTION

A unique characteristic of flowering plants is that they generate their reproductive tissues de novo at maturity, rather than during embryo development as do animals. Plant reproductive structures, the stamens and the gynoecium, form within the outer perianth organs of the flower. The stamens consist of an anther and subtending filament and contain the pollen, the male gametophytes, whereas the central

gynoecium is composed of one or more fused or unfused carpels and contains the embryo sacs, the female gametophytes. After fertilization, the gynoecium matures into a fruit that nourishes the developing embryos until seed dispersal (Sundberg & Ferrandiz, 2009). The mature fruits of many plants are harvested and their fleshy parts and/or seeds are consumed by animals.

The Arabidopsis gynoecium develops from two congenitally fused carpels, and its patterning occurs along multiple developmental axes. The adaxial–abaxial axis forms early during gynoecium development and distinguishes the outer (abaxial) valve and replum tissues from the inner (adaxial) ovules, placenta, septum, and transmitting tract (Larsson et al., 2013). The apical–basal axis becomes specified shortly thereafter as the organ grows vertically into a tube-shaped organ (Sundberg & Ferrandiz, 2009). Along this axis, the apical stigmatic tissue sits atop the gynoecium as the site of pollen deposition, the underlying style supports growth of the pollen tubes into the central ovary, and the basal gynophore connects the gynoecium to the rest of the flower. A third axis, the mediolateral axis, distinguishes the lateral valve and valve margin from the medial replum tissues (Gonzalez-Reig et al., 2012). Gynoecium development occurs over the course of days (Smyth et al., 1990) in a strictly coordinated fashion and requires the activity of intricately regulated networks of genes and hormones (Zuniga-Mayo et al., 2019).

The GARP domain-containing transcription factor KANADI1 (KAN1) is a key regulator of adaxial–abaxial axis formation in the Arabidopsis gynoecium. A member of a small family of MYB-like transcription factors, *KAN1* acts to specify abaxial cell identity in developing leaves and floral organs, including the gynoecium (Eshed et al., 1999, 2001; Kerstetter et al., 2001). In flowers, *KAN1* expression is initially detected on the abaxial side of the initiating carpel primordia at stage 6 but then switches to an adaxial-specific expression pattern at stage 8 and becomes restricted to the ovule primordia at stage 9 (Kerstetter et al., 2001; Pires et al., 2014). *kan1* gynoecia display a partial abaxial to adaxial cell fate transformation in which ectopic ovules form at the base of the valves, a phenotype that is enhanced by mutations in either the *KAN2* or *CRABS CLAW* genes (Eshed et al., 2001). KAN1 acts predominantly as a transcriptional repressor (Merelo et al., 2013; Wu et al., 2008) and can physically interact with the transcriptional corepressor TOPLESS (Causier et al., 2012). Downstream targets of KAN1 in seedlings and inflorescence meristems include genes involved in organ patterning, shoot development, and auxin response and transport (Merelo et al., 2013; Ram et al., 2020). Although a handful of genes with functions in floral organ specification and growth were identified as KAN1 targets in inflorescence apices (Ram et al., 2020), genome-wide KAN1 targets in reproductive tissues remain to be discovered.

Apical–basal gynoecium patterning is regulated by a handful of factors including the closely related *ULTRAPETALA1* (*ULT1*) and *ULT2* genes. The two genes encode SAND domain-containing proteins that are required to restrict shoot and floral stem cell accumulation (Carles et al., 2005; Fletcher, 2001). *ULT1* and *ULT2* are also expressed in the adaxial domain of the developing gynoecium (Carles et al., 2005), where they function redundantly to pattern the apical–basal polarity axis by promoting basal cell identity along the replum (Monfared et al., 2013). ULT1 acts genetically as a trithorax group (trxG) factor (Carles & Fletcher, 2009) that modulates gene transcription via epigenetic chromatin remodeling processes in a complex and stage-specific fashion. During early flower development, ULT1 physically associates with the H3K4me3 methyltransferase protein ARABIDOPSIS HOMOLOG OF TRITHORAX1 (ATX1) and antagonizes the ability of the Polycomb Group (PcG) PRC2 complex to deposit repressive histone marks at key target gene loci (Carles & Fletcher, 2009). However, during seed germination, both ULT1 and ATX1 physically associate with the PcG factor EMBRYONIC FLOWER1 (EMF1) to facilitate vegetative development by preventing widespread seed gene transcription after germination (Xu et al., 2018). ULT1 therefore has the capacity to positively or negatively regulate target gene transcription in a context-dependent manner. Consistent with this, whole-genome transcription profiling of ULT1 target genes in vegetative and inflorescence tissues identified hundreds of upregulated as well as downregulated genes (Tyler et al., 2019).

Molecular genetic analysis revealed a complex regulatory relationship between the KAN and ULT factors during gynoecium development (Pires et al., 2014). At stage 6 of flower formation, just after carpel initiation, the *KAN* and *ULT* genes are expressed in mutually exclusive domains within the developing gynoecium and function antagonistically to pattern the adaxial–abaxial polarity axis: *KAN1* and *KAN2* confer abaxial cell identity, whereas *ULT1* and *ULT2* counteract their activity in the adaxial region. At stage 7, *KAN1* expression switches to the adaxial domain of the developing gynoecium, coincident with *ULT1* and *ULT2*, and the *KAN* and *ULT* genes act in a dose-dependent manner to pattern the apical–basal polarity axis by promoting basal cell identity. In addition to forming homodimers as well as heterodimers (Carles & Fletcher, 2009; Monfared et al., 2013), the ULT1 and ULT2 proteins also physically associate with the KAN1 and KAN2 proteins in vivo (Kivivirta et al., 2021; Pires et al., 2014). A key downstream target of ULT and KAN regulation during apical–basal patterning is the *SPATULA* (*SPT*) gene, which encodes a basic helix–loop–helix transcription factor (Heisler et al., 2001) that affects stigma, style, and ovary formation by regulating cytokinin signaling as well as auxin biosynthesis and transport (Moubayidin & Ostergaard, 2014; Reyes-Olalde et al., 2017). ULT1 and KAN1 together prevent ectopic *SPT* expression in the abaxial and basal domains of the developing gynoecium, permitting proper axial patterning (Pires et al., 2014). However, the identity of other genes acting downstream of ULT1 and KAN1 during reproductive development is unknown.

Given the dynamic complexity of the gene regulatory networks that guide male and female reproductive development (Alves-Ferreira et al., 2007; Chavez Montes et al., 2015; Kivivirta et al., 2021), we sought to identify genome-wide downstream targets of KAN1 and ULT1 during the early stages of reproductive development by performing whole-genome expression profiling using RNA-sequencing (RNA-seq). Our analysis uncovered 278 genes that are regulated by either ULT1, KAN1, or both. These include a number of novel downstream target genes of ULT1 and/or KAN1 that are expressed in the

developing gynoecium, including a small subset expressed exclusively in the stigmatic tissue. Consistent with their role in regulating gynoecium patterning, genes involved in transcription regulation, development, and reproductive processes were identified as being enriched among ULT1 and KAN1 target genes, along with genes involved in abiotic or biotic stress responses. In addition, we determined that ULT1 and KAN1 act together to promote the transcription of genes expressed in developing stamens and pollen. These results indicate a potential role for ULT1 and KAN1 in regulating the development of male as well as female reproductive tissues.

## 2 | MATERIALS AND METHODS

### 2.1 | Plant materials

The three *Arabidopsis thaliana* lines are in the Landsberg *erecta* (*Ler*) background and have been previously described (Carles et al., 2005; Kerstetter et al., 2001). Arabidopsis seeds were sown in soil and stratified for 5 days at 4°C before being transferred to a growth chamber under constant light conditions (~150 μmol $m^{-2}$ $s^{-1}$ light intensity) at 21°C and 50% relative humidity. Following germination, the plants were fertilized daily with a dilute mixture of Miracle Grow 20–20–20 fertilizer until flowering (Carles et al., 2005).

### 2.2 | Whole transcriptome sequencing and gene ontology analysis

Inflorescence apices and unopened flower buds were harvested from plants of each genotype when the main stem reached 1 cm in height. Tissue collected from at least 20 randomly chosen plants of each genotype was pooled and immediately flash-frozen in liquid nitrogen and then stored at −80°C until RNA extraction. Dyna-Bead extraction of RNA was performed using the TrueSeq RNA Library Prep Kit v2 (Illumina). For each genotype, samples from three independent biological replicates were collected in triplicate (for three technical replicates). Paired-end reads of 150 nucleotides were obtained using an Illumina HiSeq1000 sequencer at the UC Berkeley QB3 Genomic Sequencing Laboratory. Analysis of the next-generation sequencing data was performed (Trapnell et al., 2012). The quality of the raw expression data was determined using FastQC and the reads aligned to the TAIR 10 annotated reference genome using TopHat version 2.1.0 (Trapnell et al., 2009). Mapped reads were used to reconstruct the transcripts using Cufflinks version 2.2 (Roberts et al., 2011) and the reference genome annotation. The normalized expression of each transcript was calculated as fragments per kilobase of transcript per million mapped fragments for each sample. Genes with a ≥1.5-fold expression change and an adjusted *p*-value ≤ 0.01 compared with the wild type identified using edgeR software were considered to have significantly different expression levels. Principal component analysis was applied to raw fragments per kilobase of exon per million mapped fragments using the dplyr 1.10 and ggplot2 3.4.0 packages in R version 4.2.2. The sequence data are available from the National Center for Biotechnology Short Read Archive under BioProject PRJNA882916.

Gene ontology (GO) term enrichment analysis was performed using the agriGO v2.0 online platform Singular Enrichment Analysis tool (Tian et al., 2017), with enrichment calculated relative to the TAIR 10 reference genome using a hypergeometric test followed by Benjamin–Yekutieli false discovery rate (FDR) correction. GO enrichment analysis was conducted using the complete list of plant GO categories; however, the plant GO slim gene ontology analysis option was used to generate the *ULT1*-induced differentially expressed gene (DEG) hierarchical tree graph to reduce the volume of GO subcategories visualized from among a total of 67 overrepresented terms. Venn diagrams were generated using the Venny 2.0 interactive online tool. Gene expression patterns were generated using the Kleptikova Arabidopsis Atlas eFP Browser (Klepikova et al., 2016) online tool set to default values. Transcription factors were organized into families and subfamilies using the Plant Transcription Factor Database v5.0. Heatmaps were generated using the gplots package in R version 3.1.3. Fold changes were log2 transformed to calculate log2FC, and then, the heatmaps generated using the gplots heatmap.2 function with log2FC values using default parameters (Wickham, 2016).

### 2.3 | Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from inflorescence apices and unopened flower buds using Invitrogen TRIzol reagent (Thermo Fisher Scientific) according to the manufacturer's recommendations. Contaminating genomic DNA was removed from 10 μg of RNA by treatment with 2 units of DNase I (New England Biolabs) followed by phenol: chloroform extraction and ethanol precipitation. RNA was quantified with the Qubit RNA BR Assay Kit (Thermo Fisher Scientific) according to the manufacturer's recommendations. cDNA was synthesized from 2.5 μg of DNase I-treated RNA using the Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) with oligo $(dT)_{18}$ according to the manufacturer's recommendations. Before use, the final cDNA product was diluted with 4 volumes of Milli-Q water (EMD Millipore). The diluted cDNA samples served as template for quantitative polymerase chain reaction (qPCR) with the primers listed in Table S6. Six biological and two technical replicate reactions were performed as previously described (Bendix et al., 2013). $C_q$ values were calculated with the regression function for each primer set in the Bio-Rad CFX Manager Software. Values of relative transcript levels were calculated as $2^{\wedge}(C_q^{\text{normalizer}} - C_q^{\text{experimental}})$, where $C_q^{\text{normalizer}}$ is the mean $C_q$ value for the *IPP2* primer set. Individual relative transcript level values were normalized to the average of values from all genotypes and treatments (Bendix et al., 2013). Statistical analysis of relative transcript levels between genotypes employed t-testing implemented in Prism (GraphPad), at a significance level of $p < 0.05$.

Accession numbers are as follows:

- KANADI1, At5g16560.
- KANADI2, At1g32240.
- ULTRAPETALA1, At4g28190.
- ULTRAPETALA2, At2g20825.

## 3 | RESULTS

### 3.1 | Genome-wide expression analyses of ULT1 and KAN1 target genes in developing flowers

The mature gynoecium of a wild-type Landsberg *erecta* (L*er*) flower consists of a squat basal gynophore subtending two fused values separated by a thin strip of replum cells and topped with apical style and stigmatic tissue (Figure 1a). *ult1–2* gynoecia resemble wild-type gynoecia but frequently consist of three or four valves (Figure 1b) (Carles et al., 2004; Fletcher, 2001). *kan1–12* gynoecia form ectopic ovules at the base of the valves as well as outgrowths of ectopic style tissue along the abaxial replum (Figure 1c), demonstrating a partial transformation of abaxial to adaxial and basal to apical cell identity (Eshed et al., 2001; Kerstetter et al., 2001; Pires et al., 2014). In contrast, *ult1–2 kan1–12* gynoecia form supernumerary valves that are surrounded by long ectopic outgrowths of style with prominent stigmatic tissue along the replum from apex to base (Figure 1d). This dramatically enhanced phenotype indicated that *ULT1* and *KAN1* act together to promote basal cell identity during gynoecium development (Pires et al., 2014).

To identify downstream targets of *ULT1* and *KAN1* regulation during Arabidopsis reproductive development, we performed whole-genome transcription profiling of wild-type L*er*, *ult1–2*, *kan-12*, and *ult1–2 kan1–12* plants. The *ult1–2* allele is an ethyl methanesulfonate (EMS) allele identified in the L*er* background that replaces a serine residue with a phenylalanine residue in the conserved SAND domain (Carles et al., 2005). The *kan1–12* null allele is also in the L*er* background and consists of a 12.1-kilobase deletion of the *KAN1* promoter and first two exons (Kerstetter et al., 2001). To enrich for the transcripts present in developing male (stamen) and female (carpel) reproductive tissue, we collected inflorescence apices and unopened flower buds from reproductive-stage plants of each genotype when the main stem reached 1 cm in height. These reproductive "flower cluster" tissues (Figure 2a) consisted of floral buds from stages 1 to 12, which follows the formation of the stigmatic papillae at the apical end of the gynoecium (Smyth et al., 1990), and thus were enriched in developing male and female reproductive tissues prior to sexual maturity.

The flower cluster tissues were subjected to RNA extraction followed by whole-genome RNA-Seq expression analysis. Genes with a ≥1.5-fold expression change and an adjusted *p*-value ≤0.01 compared with the wild type were considered to have significantly different expression levels. DEGs identified between wild-type L*er* and the *ult1–2*, *kan-12*, and *ult1–2 kan1–12* mutant genotypes are listed in Tables S1–S3. Because transcriptomics analysis does not distinguish between primary and secondary effects on gene transcription, these DEGs represent both direct and indirect targets of ULT1 and/or KAN1.

Our whole-genome transcription profiling study identified 278 genes that were differentially expressed in at least one of the genotypes analyzed. Investigation of the ULT1 reproductive transcriptome revealed a total of 157 DEGs in *ult1–2* flower clusters compared with wild-type L*er* flower clusters (Figure 2a and Table S1). Among these, 104 were downregulated, indicating they are directly or indirectly induced by ULT1 during flower development, and 53 were upregulated, indicating they are repressed by ULT1 (Table S1). Therefore, nearly twice as many genes in this dataset were induced by ULT1 as were repressed. A total of 78 genes were differentially expressed in *kan1–12* flower clusters compared with wild-type flower clusters (Figure 2a and Table S2). Among these, 39 genes were downregulated, indicating their transcription is directly or indirectly induced by KAN1. The other 39 genes were upregulated, indicating that they are repressed by KAN1 (Table S2). Finally, a total of 43 genes were differentially expressed in *ult1–2 kan1–12* flower clusters compared with wild-type flower clusters (Figure 2a and Table S3). In this case, 35 genes were downregulated and thus were cooperatively induced by ULT1 and KAN1, whereas seven genes were upregulated and thus were cooperatively repressed by ULT1 and KAN1 (Table S3).

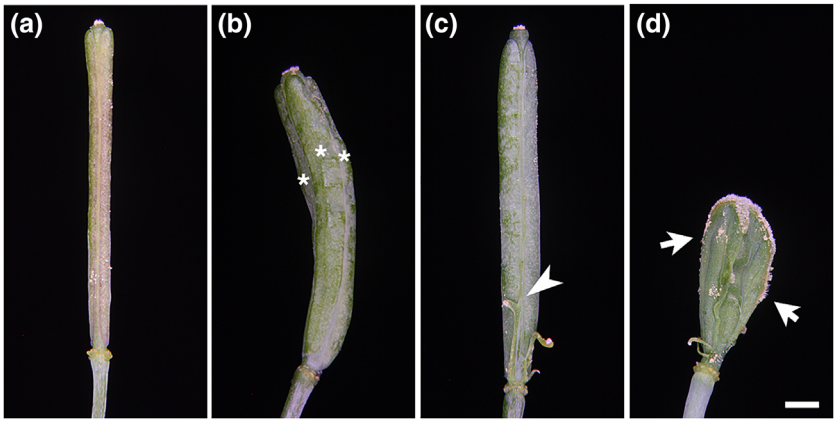


**FIGURE 1** *ULT1* and *KAN1* cooperatively regulate gynoecium patterning. (a) Wild-type L*er* gynoecium. (b) *ult1–2* gynoecium consisting of three fused carpels (asterisks). (c) *kan1–12* gynoecium with basal ectopic outgrowth (arrowhead). (d) *ult1–2 kan1–12* gynoecium with ectopic outgrowths of style and stigmatic tissue expanding basally between each valve (arrows). Scale bar, 5 mm.

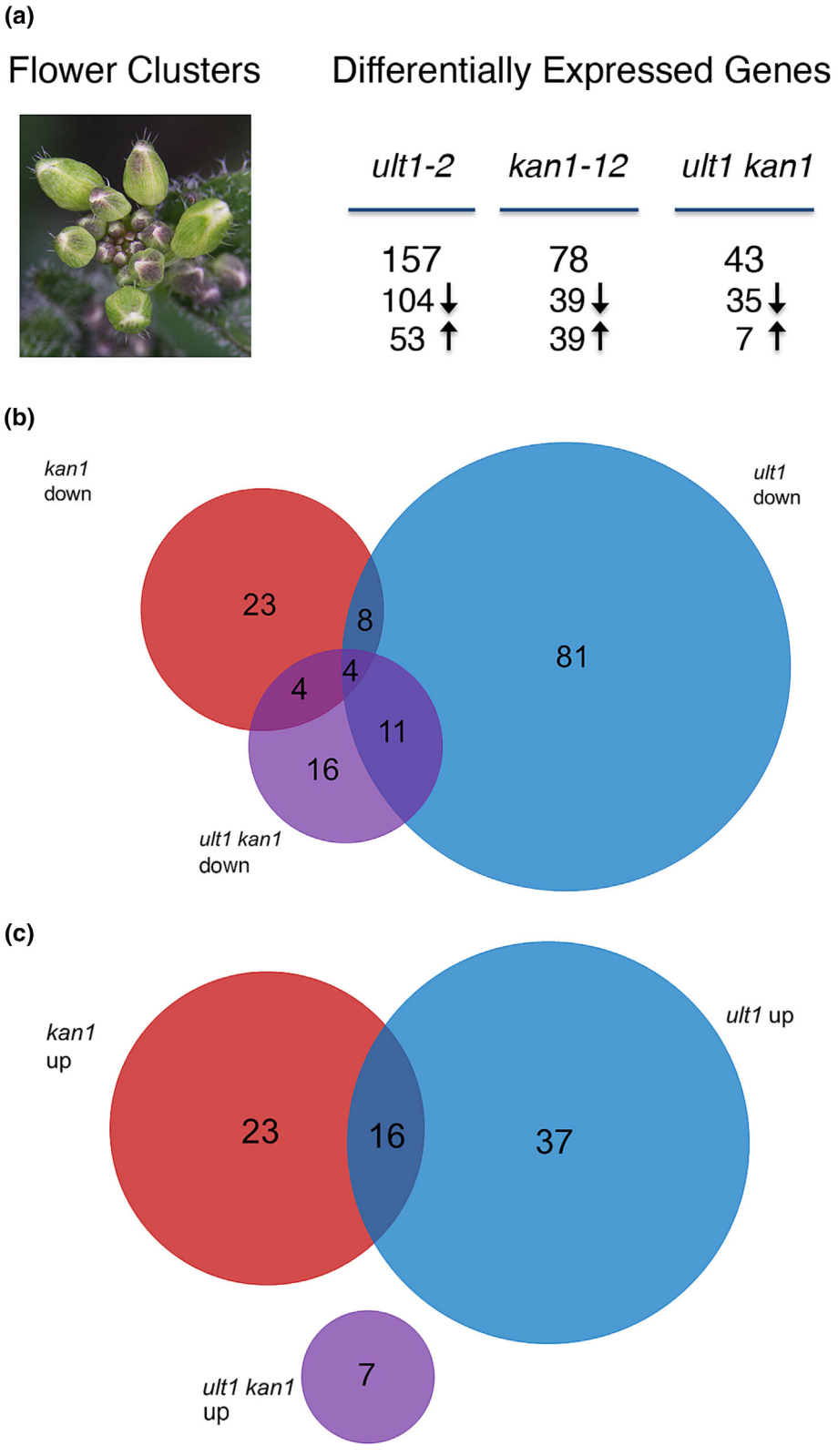


**FIGURE 2** Whole genome profiling of *ULT1* and *KAN1* target genes in reproductive tissues. (a) L*er* flower cluster tissue and number of differentially expressed genes in *ult1–2*, *kan1–2*, and *ult1–2 kan1–12* flower clusters ($p \leq 0.05$ and FC ≥ 1.5). Down arrows indicate downregulated genes and up arrows indicate upregulated genes. (b) Venn diagram showing overlap between downregulated DEGs in *ult1–2*, *kan1–12*, and *ult1–2 kan1–12* flower clusters. (c) Venn diagram showing overlap between upregulated DEGs in *ult1–2*, *kan1–12*, and *ult1–2 kan1–12* flower clusters.

Comparative analysis showed a modest degree of overlap in the regulation of the DEGs between the flower cluster datasets. Among the DEGs that were downregulated in one or more of the genotypes, eight genes were shared between the *ult1* and *kan1* datasets, 11 were shared between the *ult1* and *ult1 kan1* datasets, and four were shared between the *kan1* and *ult1 kan1* datasets (Figure 2b). Four additional genes were downregulated in all three genotypes. Among the smaller set of DEGs that were upregulated in one or more of the genotypes, 16 were shared between the *ult1* and *kan1* datasets (Figure 2c). This represents 30.2% of the total ULT1-repressed genes and 41% of the

total KAN1-repressed genes. None of the seven DEGs upregulated in *ult1 kan1* flower clusters were identified in either the *ult1* or *kan1* datasets, indicating that these DEGs constitute a unique subset of target genes cooperatively repressed by both ULT1 and KAN1.

## 3.2 | Functional categorization of KAN1 and ULT1 target genes

Differences in gene expression underlie different biological functions, so we used gene ontology (GO) term enrichment analysis to elucidate the functions of the DEGs. We utilized the agriGO web application (Tian et al., 2017) to assess the overrepresentation of GO categories in networks of biological processes for downregulated and upregulated genes among the three different genotypes. The resulting GO distribution datasets were visualized as hierarchical tree graphs using Singular Enrichment Analysis, with enrichment calculated relative to the Arabidopsis TAIR 10 reference genome using a hypergeometric test followed by Benjamin–Yekutieli FDR correction. GO terms with adjusted *p*-values less than 0.05 were considered to be significantly overrepresented.

The resulting GO distribution networks distinguished distinct as well as overlapping functional categories among the downregulated and upregulated genes in *ult1–2*, *kan1–12*, and *kan1–12 ult1–2* flower clusters. The most significant GO terms overrepresented among the 53 genes downregulated in *ult1–2* flower clusters fell into three main categories: *response to stimulus*, *regulation of biological process*, and *developmental process* (Figure 3a). The full list of significantly enriched GO terms among DEGs downregulated in *ult1–2* flower clusters is presented in Figure S1. Within the *response to stimulus* category, genes categorized as responding to endogenous stimulus, abiotic stimulus, and stress were significantly enriched. Within the *regulation of biological process* category, genes involved in cellular biosynthetic processes, carbohydrate metabolic processes, and the regulation of gene expression were overrepresented. The latter included a number of transcription factors, which will be described in more detail later. *ULT1*-induced genes significantly enriched within the *developmental process* category were those that play roles in reproductive processes and, specifically, in reproductive structure development. This finding indicates that *ULT1* promotes the transcription of genes that play roles in stamen and/or carpel formation.

The *response to stimulus* and *developmental process* GO functional categories were likewise overrepresented among the 104 genes upregulated in *ult1–2* flower clusters (Figure S2). Within this dataset, the *response to stimulus* category was enriched for genes involved in responses to chemicals such as acids, oxygen-containing compounds, and organic substances. *ULT1*-repressed genes enriched in the *developmental process* GO category were those associated single organism developmental processes, defined as biological processes whose specific outcomes are the progression of an integrated living unit, either an anatomical structure or an organism, over time. ULT1 therefore appears to negatively as well as positively regulate developmental gene expression during the reproductive phase.

Gene ontology analysis revealed fewer GO terms enriched among the smaller numbers of downregulated and upregulated genes in *kan1–12* and *kan1–12 ult1–2* flower clusters. A single GO category was overrepresented among the 39 genes downregulated in *kan1–12* flower clusters, that of *response to light stimulus* (Figure 4a). Genes in this pathway were also enriched among downregulated genes in *ult1–2* flower clusters (Figure 4b). No GO terms were overrepresented among the 39 genes upregulated in *kan1–12* flower clusters. Likewise, the set of seven genes upregulated in *ult1–2 kan1–12* flower clusters was too small to return any overrepresented GO terms. However, two major GO categories were enriched among the 35 genes downregulated in *ult1–2 kan1–12* flower clusters: *response to stimulus* and *single-organism metabolic processes* (Figure S3). Within the *response to stimulus* category, genes involved in responses to abiotic stimulus such as hormones, lipids, and chemicals, oxygen-containing compounds and inorganic substances were overrepresented, as were genes in biotic response pathways. In sum, our findings reveal that genes regulated by ULT1 and/or KAN1 during the reproductive phase function predominantly in pathways that affect development, responses to abiotic and biotic stimuli, including hormones, and plant metabolic processes.

## 3.3 | Identification of transcription factor genes among DEGs

Genes involved in the regulation of transcription were significantly overrepresented among ULT1-induced genes in reproductive-stage tissues. Twenty ULT1-induced DEGs were enriched in the *regulation of gene expression* GO category, including 17 canonical transcription factors plus additional transcriptional coregulators and regulatory cofactors (Table 1). Genes from a wide variety of transcription factor families were present among the DEGs, with no significant enrichment for particular families observed. Genes from several different AP2/ERF transcription factor subfamilies, which are regulators of abiotic stress responses (Xie et al., 2019), were represented; genes from multiple zinc finger transcription factor and homeodomain subfamilies were also represented (Table 1). *KAN1* itself was also on the list of genes upregulated in *ult1* flower clusters, reflecting our previous observation that *ULT1* acts in opposition to *KAN1* during the early stages of gynoecium formation (Pires et al., 2014). The overrepresentation of transcriptional regulatory genes among ULT1-induced DEGs is consistent with a significant role for ULT1 in transcription modulation (Tyler et al., 2019).

Only a few transcription factor genes were differentially expressed in *kan1* and *ult1 kan1* flower clusters (Table 1), which based on KAN1 transcriptome studies in other tissues (Merelo et al., 2013; Ram et al., 2020; Reinhart et al., 2013) may be an underrepresentation of the total. Several MYB domain transcription factor genes are present among KAN1-regulated transcription factor genes, with *MYB24* and *MYB108* being repressed by KAN1 and *MYB28*, *CIRCADIAN CLOCK ASSOCIATED1* (*CCA1*) and *LATE ELONGATED HYPOCOTYL* (*LHY*) being induced by KAN1. CCA1 and LHY encode core

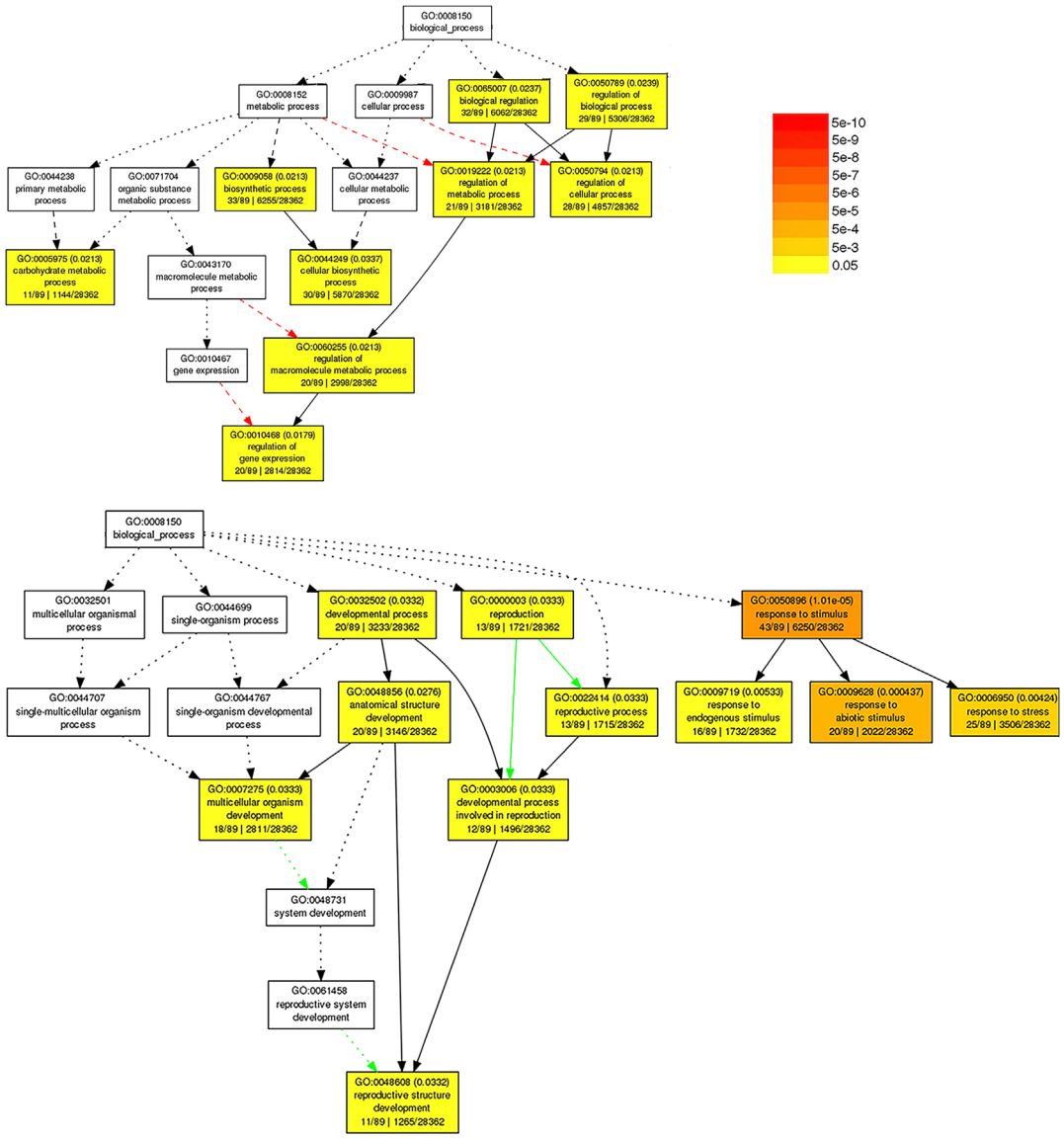


**FIGURE 3** Hierarchical tree graphs of significantly enriched GO terms among differentially expressed genes in *ult1–2* flower cluster tissues showing enrichment for transcription regulation, reproductive development, and abiotic stimulus among downregulated genes in *ult1–2* flower clusters. Nonsignificant GO terms are shown in white boxes and significant GO terms are shown in colored boxes, with the color scale indicating the false discovery rate-adjusted *p*-values from yellow ($p < 0.05$) to dark red ($p < 5e{-}10$). Solid, dashed, and dotted lines represent two, one, and zero enriched terms at the ends connected by the lines, respectively. The information inside each colored box describes the following: GO term number, adjusted *p*-value, GO description, number of terms in the query list that map to the GO term/total number of items in the background that map to the GO term, and total number of items in the query list/total number of items in the background.

components of the circadian clock and are expressed in all tissues of the plant (Alabadi et al., 2002; Mizoguchi et al., 2002), indicating that KAN1 can activate master clock genes in reproductive tissues. Finally, three transcription factor genes are differentially regulated in *ult1 kan1* flower clusters (Table 1). Among these, expression of the *ERF104* and *ATH1* genes is induced by ULT1 in the presence or absence of KAN1. The third DEG, the *ASYMMETRIC LEAVES2* (*AS2*) gene encoding a LOB domain (LBD) transcription factor, is induced cooperatively by ULT1 and KAN1, but not by either ULT1 or KAN1 alone.

## 3.4 | Regulation of gynoecium gene expression by ULT1 and KAN1

Among the DEGs in the *ult1*, *kan1*, and *ult1 kan1* genotypes, we identified a total of 29 genes expressed in the developing gynoecium. We performed heatmap analysis to compare the transcription profiles of these genes in the three genetic backgrounds and found that ULT1 and/or KAN1 positively regulate some gynoecium-expressed genes and negatively regulate others (Figure 5). More gynoecium genes were differentially expressed in the *kan1* background than in the *ult1*

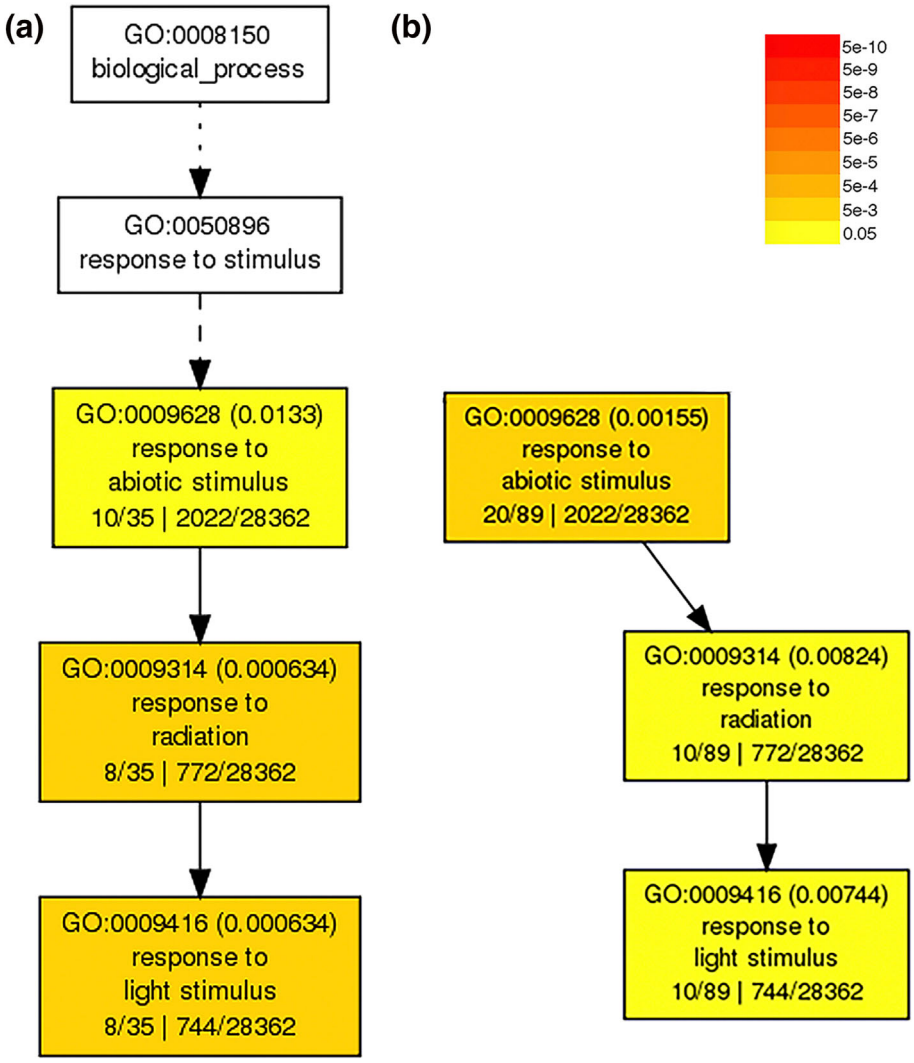


**FIGURE 4** Hierarchical tree graphs of significantly enriched GO terms among differentially expressed genes in *ult1–2* and *kan1–12*, flower cluster tissues. (a, b) Enrichment for response to light stimulus pathway genes among downregulated genes in *kan1–12* (a) and *ult1–2* flower clusters (b).

or *ult1 kan1* background, whereas some were differentially regulated by ULT1 and KAN1 cooperatively but not alone (Figure 5).

Interestingly, all seven of the genes upregulated in *ult1 kan1* flower clusters were preferentially expressed in the gynoecium (Table S4), according to data from the Arabidopsis eFP Browser (Klepikova et al., 2016). *KAN1* itself was predominantly expressed in transmitting tract tissues, as well as in other floral organs (Figure 6a). Transcripts from the *ABCG39* locus were predominantly found in ovules but were also detected in the stigmatic tissue (Figure 6b). The other five genes, *MLO6* (Figure 6c), *SLR1* (Figure 6d), *PER39* (Figure S4A), *E6L1* (Figure S4B), and At5g53710 encoding a hypothetical protein (Figure S4C), were highly expressed in wild-type stigmatic tissue and were upregulated three- to four-fold in the *ult1 kan1* background. Each of these five genes had a stigma-specific mRNA transcription pattern with no detectable expression outside the stigmatic tissue (Klepikova et al., 2016). Among these, *E6L1* is a stigmatic factor known to function in pollen–stigma interactions (Doucet et al., 2019). The S-locus related glycoprotein SLR1 is also likely to be involved in pollen recognition. Our data suggest that the other three genes may also have activities that are specific to stigmatic tissue development and/or pollen–stigma interactions.

### 3.5 | Regulation of stamen gene expression by ULT1 and KAN1

Unexpectedly, 31 of the 35 DEGs downregulated in *ult1 kan1* flower clusters were expressed in developing stamens (Figure 7). Arabidopsis eFP Browser data indicated that 10 of these 31 DEGs were expressed exclusively in stamen tissues within developing flowers, nine genes were expressed predominantly in stamen tissues, and the remaining 12 genes were expressed at low but detectable levels in stamen tissues but at higher levels in other floral tissues, such as sepals or carpels (Table S5). Genes exclusively expressed in stamens include the MYB transcription factor gene *MYB99* (Figure 8a), *ACA5* (Figure 8b), *ABCG29* (Figure 8c), and *RHS14* (Figure S5A). Genes that were preferentially expressed in stamens but were also expressed at lower levels in other floral tissues include the LBD transcription factor gene *AS2* (Figure S5B), whereas genes that were expressed at low levels in

**TABLE 1** ULT1- and KAN1-regulated transcription factor genes.

| TF family | Subfamily | TF locus ID | Gene name | logFC | *p*-value |
|---|---|---|---|---|---|
| **ULT1 induced** | | | | | |
| AP2/ERF | ERF | AT5G61600 | ERF104 | −1.83 | 0.00005 |
| | RAV | AT1G25560 | TEM1/EDF1 | −1.46 | 0.0005 |
| ARF | | AT1G77850 | ARF17 | −.70 | 0.00005 |
| B3 | | AT4G31610 | REM1/REM34 | −.75 | 0.00055 |
| | | AT4G34400 | TFS1 | −.78 | 0.00005 |
| CO-like | B-box | AT3G02380 | COL2 | −1.36 | 0.00005 |
| GATA | | AT5G56860 | GNC/GATA21 | −1.19 | 0.0007 |
| GRF | | AT3G13960 | GRF5 | −.93 | 0.00005 |
| Homeodomain | TALE | AT4G32980 | ATH1 | −.80 | 0.00005 |
| | WOX | AT2G33880 | WOX9/HB-3 | −.61 | 0.0005 |
| MADS | | AT1G77080 | AGL27 | −1.42 | 0.00005 |
| MYB | | AT1G01060 | LHY | −.65 | 0.00005 |
| WRKY | | AT3G56400 | WRKY70 | −1.33 | 0.00005 |
| Zn finger | C2H2 | AT5G59820 | ZAT2 | −1.19 | 0.0001 |
| | CCCH | AT3G55980 | SZF1 | −.93 | 0.00005 |
| | HD | AT1g75240 | ZHD5/HB-33 | −.66 | 0.00005 |
| | RING-H2 | AT4G00335 | RHB1A | −.91 | 0.0007 |
| **ULT1 repressed** | | | | | |
| AP2/ERF | ERF | AT2G20880 | ERF53 | 4.00 | 0.00015 |
| bHLH | | AT5G50010 | SACL2/bHLH145 | 6.35 | 0.00005 |
| G2-like | | AT5G16560 | KAN1 | 1.71 | 0.00005 |
| GATA | | AT2G45050 | GATA2 | 1.55 | 0.0003 |
| MYB | | AT5G40350 | MYB24 | 1.90 | 0.00005 |
| NAC | | AT4G27410 | RD26/NAC072 | 2.83 | 0.00005 |
| | | AT1G77450 | NAC032 | 2.31 | 0.00005 |
| WRKY | | AT4G31800 | WRKY18 | 1.62 | 0.00005 |
| Zn finger | C2H2 | AT5G04340 | ZAT6 | 1.59 | 0.00035 |
| KAN1 induced | | | | | |
| AP2/ERF | ERF | AT5G61600 | ERF104 | −1.09 | 0.00035 |
| MYB | | AT5G61420 | MYB28 | −.69 | 0.0004 |
| | | AT2G46830 | CCA1 | −.94 | 0.00005 |
| | | AT1G01060 | LHY | −.73 | 0.00005 |
| KAN1 repressed | | | | | |
| bHLH | | AT5G50010 | SACL2/bHLH145 | 5.22 | 0.00005 |
| MYB | | AT5G40350 | MYB24 | 1.70 | 0.00005 |
| | | AT3G06490 | MYB108 | 2.62 | 0.00005 |
| ULT1 and KAN1 induced | | | | | |
| AP2/ERF | ERF | AT5G61600 | ERF104 | −1.12 | 0.0006 |
| Homeodomain | TALE | AT4G32980 | ATH1 | −.61 | 0.00085 |
| LBD | | AT1G65620 | AS2 | −1.15 | 0.00055 |
| MYB | AT5G62320 | MYB99 | | −1.06 | 0.00055 |
| | AT5G02840 | LCL1/RVE4 | | −.60 | 0.00015 |
| WRKY | AT3G56400 | WRKY70 | | −.86 | 0.00005 |
| ULT1 and KAN1 repressed | | | | | |
| G2-like | AT5G16560 | KAN1 | | 1.93 | 0.00005 |

Abbreviation: TF, transcription factor.

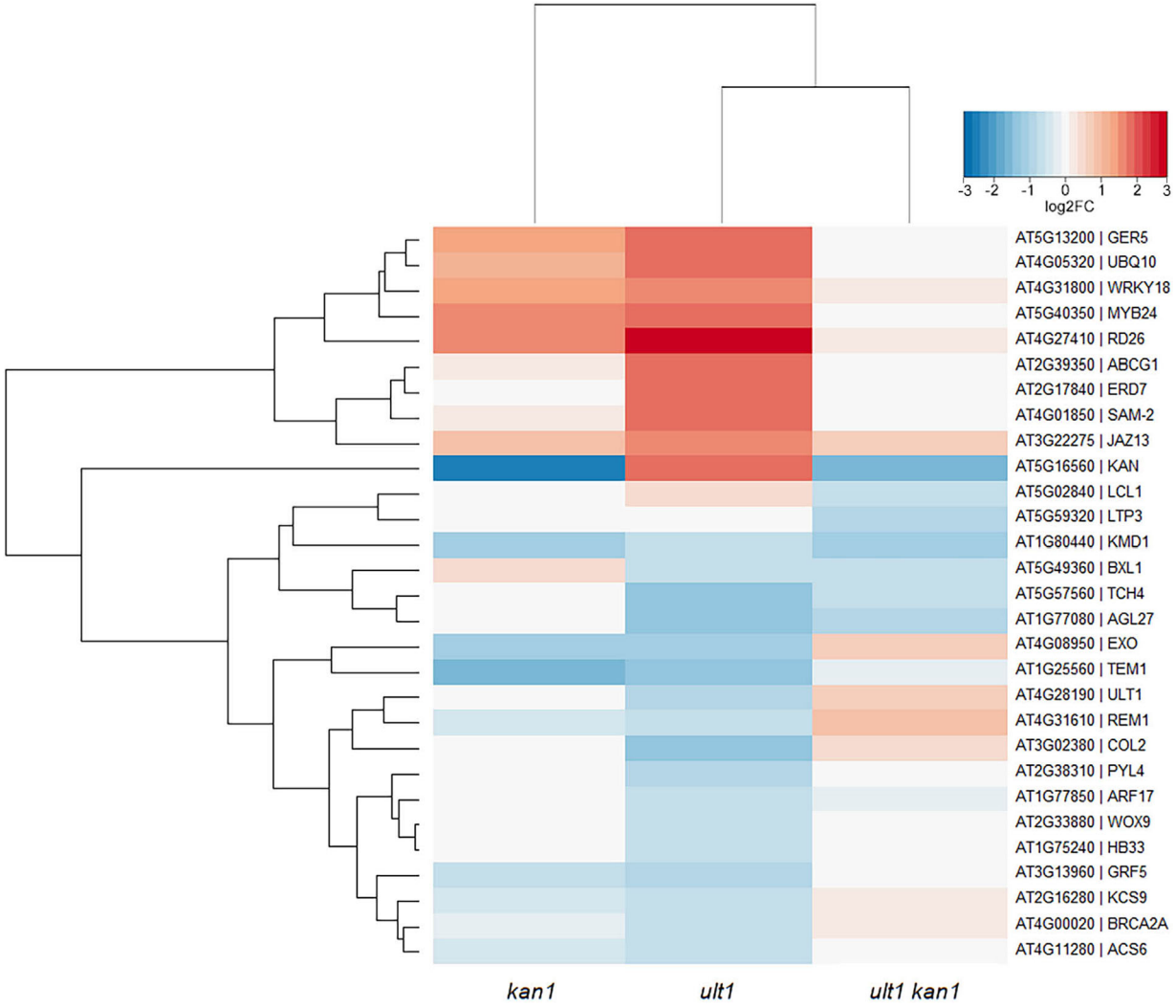


**FIGURE 5** Summary of gynoecium-expressed genes regulated by ULT1 and KAN1. Heatmap showing transcriptional profile comparisons of differentially expressed genes related to gynoecium development in *ult1–2*, *kan1–12*, and *ult1–2 kan1–12* flower clusters.

stamen tissues and at higher levels in other floral tissues include the AP2/ERF family transcription factor gene *ERF104* (Figure S5C). Sixty-one percent of the stamen-expressed DEGs (19 of 31) were expressed in wild-type mature pollen, which contains the mature male gametophytes generated within the anthers (Table S5). Thus, *ULT1* and *KAN1* act together to promote stamen and pollen gene expression. These findings suggest a heretofore unsuspected cooperative role for ULT1 and KAN1 in regulating stamen and male gametophyte development, perhaps together with other *ULT* and *KAN* gene family members.

## 3.6 | Validation of selected DEGs

To validate the RNA-seq results, we examined the mRNA levels of selected ULT1 and KAN1 target genes using reverse transcription-qPCR (RT-qPCR). We selected genes from two groups of DEGs, those expressed in the gynoecium and those expressed in the stamens and quantified their expression levels in flower cluster tissue from L*er*, *ult1–2*, *kan1–12*, and *ult1–2 kan1–12* plants grown under the same experimental conditions used for the RNA-seq analysis. Overall, the quantitative gene expression results (Figure 9) reflected the trend of regulation observed in the RNA-seq experiment.

We analyzed the transcription levels of five target genes that were expressed predominantly or exclusively in the gynoecium (Figures 6 and S4). In correspondence with the RNA-seq data, *KAN1* transcripts were not detected in *kan1–12* flower clusters and were upregulated in *ult1–2* flower clusters (Figure 9a), confirming that *KAN1* transcription was repressed by ULT1 activity. Expression of the stigma-specific *SLR1*, *At5g53710*, and *E6L1* genes was also upregulated in ult1 kan1 flower clusters (Figure 9b-d), although the relative expression levels of the latter two genes did not quite reach the threshold for statistical significance, likely due to the reduced sensitivity of RT-qPCR relative to RNA-seq. We additionally assayed the transcript levels of the transcription factor gene *ERF104*, which was expressed in stamens as well as other floral tissues (Figure S5C). Consistent with the RNA-seq data, we found that *ERF104* mRNA levels

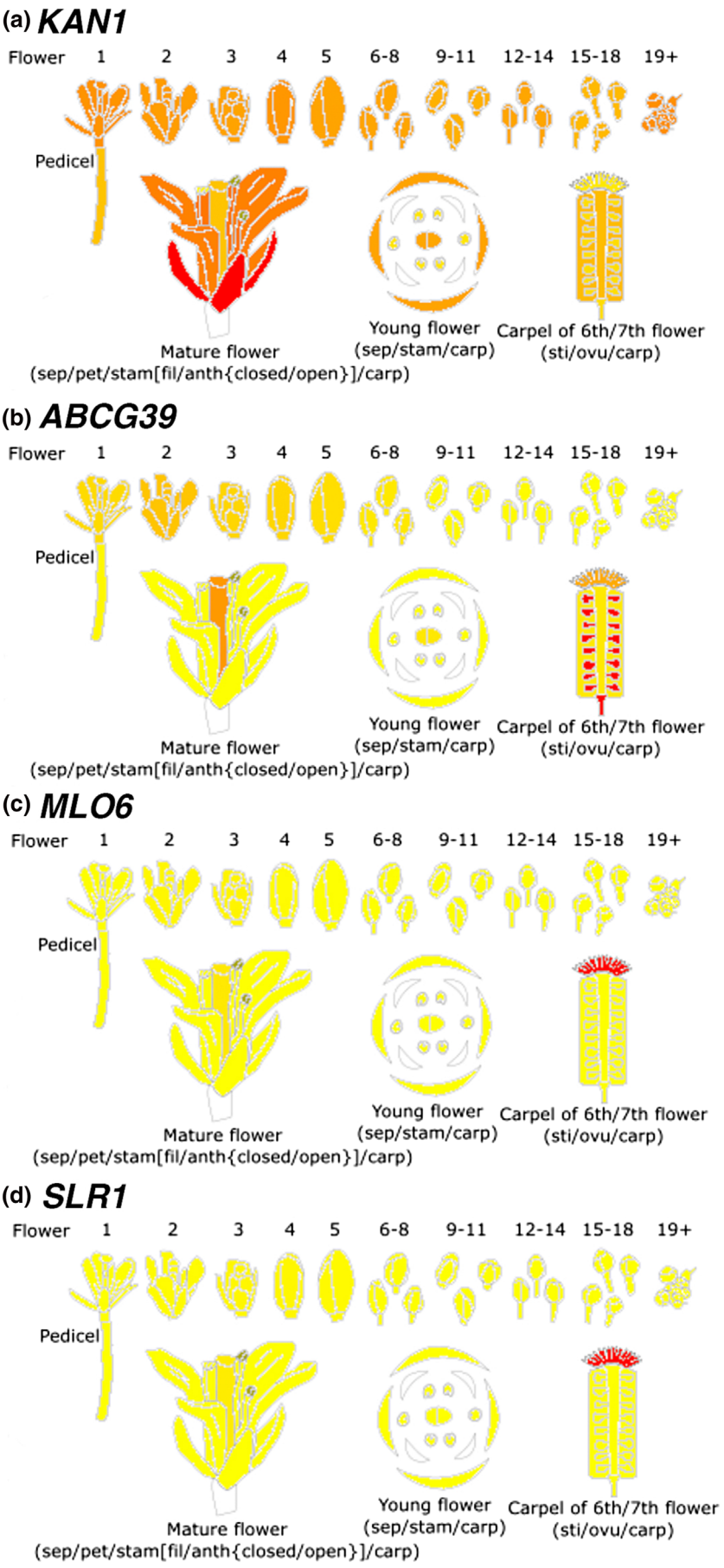


**FIGURE 6** Gynoecium-expressed genes cooperatively repressed by ULT1 and KAN1. (a–d) Expression patterns of the (a) *KAN1*, (b) *ABCG39*, (c) *MOL6*, and (d) *SLR1* genes in developing wild-type flowers. A gradation of apricot to orange to red color indicates increasing levels of gene expression.

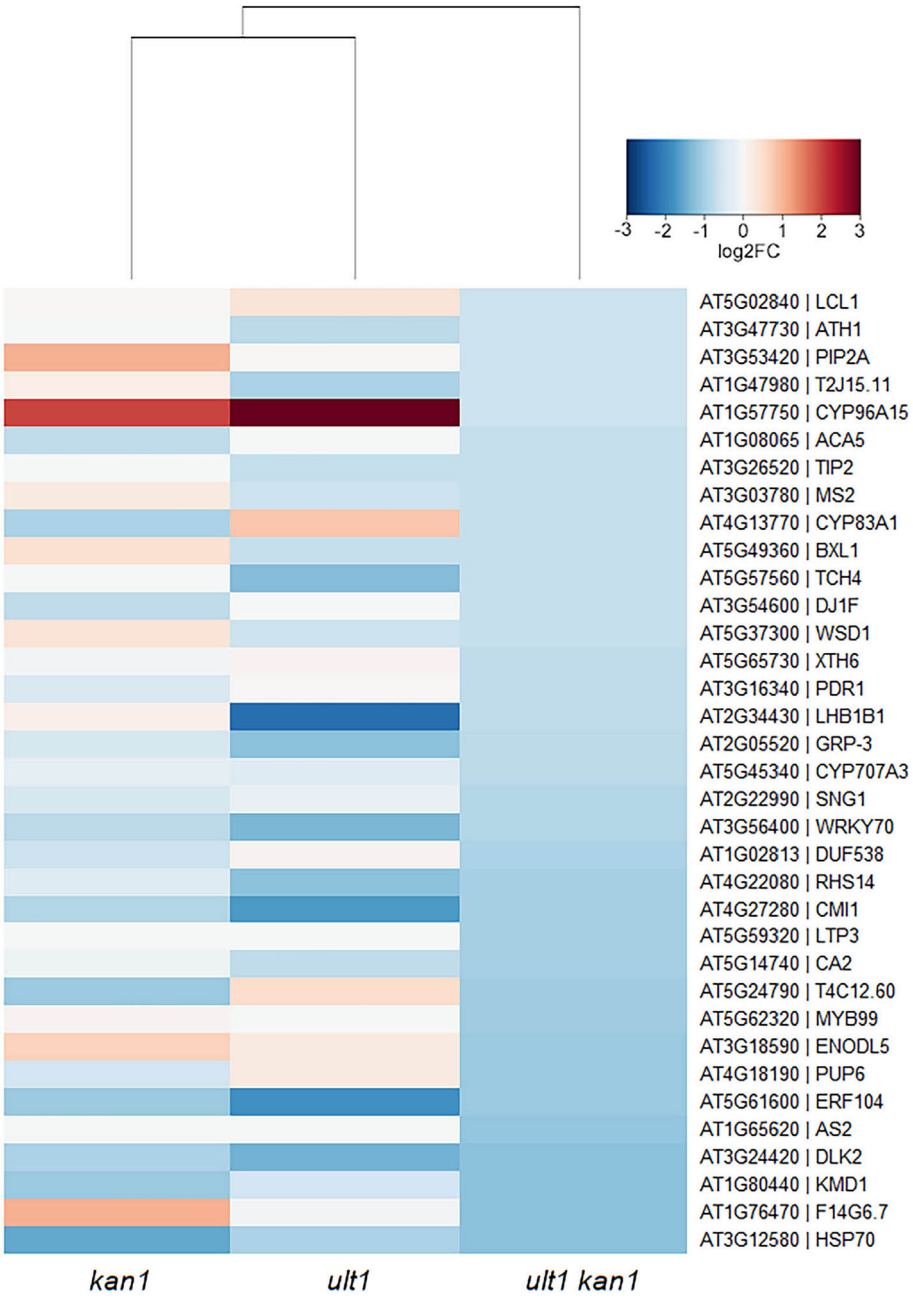


**FIGURE 7** Summary of stamen-expressed genes regulated by ULT1 and KAN1. Heatmap showing transcriptional profile comparisons of differentially expressed genes related to stamen development in *ult1–2 kan1–12* flower clusters.

were reduced in *kan1* and *ult1 kan1* flower clusters (Figure 9e). In contrast, RT-qPCR analysis of stamen-specific genes such as *MYB99* and *At1g76460* did not yield detectable differences in mRNA expression levels between genotypes.

## 4 | DISCUSSION

### 4.1 | Regulation of gene transcription by KAN1 and ULT1 in reproductive tissues

The KAN1 and ULT1 proteins play important roles in regulating multiple developmental processes in Arabidopsis. The KAN1 transcription factor promotes abaxial cell identity in leaf primordia (Eshed et al., 2001, 2004; Kerstetter et al., 2001) and developing reproductive organs (Eshed et al., 1999). The ULT1 protein functions as an epigenetic regulatory factor that controls shoot and floral meristem maintenance (Carles et al., 2005; Carles & Fletcher, 2009; Fletcher, 2001) and acts redundantly with ULT2 to pattern the apical–basal axis of the fruit (Monfared et al., 2013). Additionally, KAN1 and ULT1 function in a dose-dependent manner to promote basal cell identity in the developing gynoecium (Figure 1c) (Pires et al., 2014). Given the impact of these two transcriptional regulators on plant development, multiple investigations of KAN1 (Huang et al., 2014; Merelo et al., 2013; Ram et al., 2020; Reinhart et al., 2013) and ULT1 (Pu et al., 2013; Tyler et al., 2019; Xu et al., 2018) direct and indirect target genes have been carried out. However, these studies were conducted using seedling or inflorescence meristem tissues, meaning that

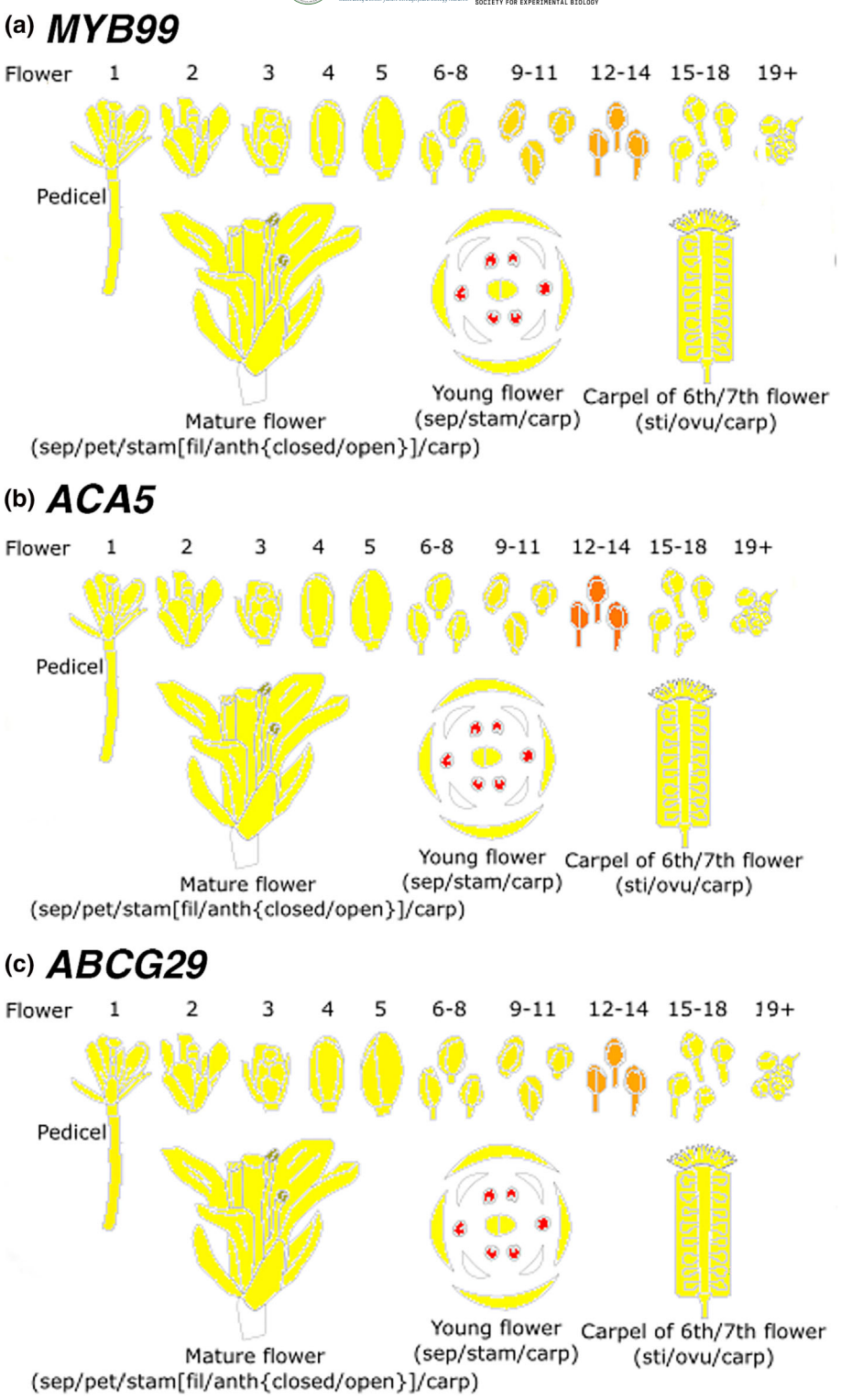


**FIGURE 8** Stamen-expressed genes cooperatively induced by ULT1 and KAN1. (a–c) Expression patterns of the (a) *MYB99*, (b) *ACA5*, and (c) *ABCG29* genes in developing flowers. A gradation of apricot to orange to red color indicates increasing levels of gene expression.

the downstream targets of ULT1 and KAN1 in reproductive tissues are largely unknown. To fill this gap, we performed a whole-genome transcriptional profiling of ULT1 and KAN1-regulated genes in flower clusters and identified several hundred target loci whose expression is significantly altered in the absence of one or both genes.

Gene ontology analysis of the DEGs uncovered functional categories for genes that are overrepresented in *ult1*, *kan1*, or *ult1 kan1* flowers clusters. Genes with functions related to inorganic stimulus as well as genes with functions related to abiotic and biotic stimulus response were enriched among ULT1-regulated genes during the reproductive phase, as has been previously observed during vegetative development (Pu et al., 2013; Tyler et al., 2019; Xu et al., 2018). Genes that function as transcriptional regulators were also enriched among ULT1-induced DEGs (Figure 3 and Table 1), although with the

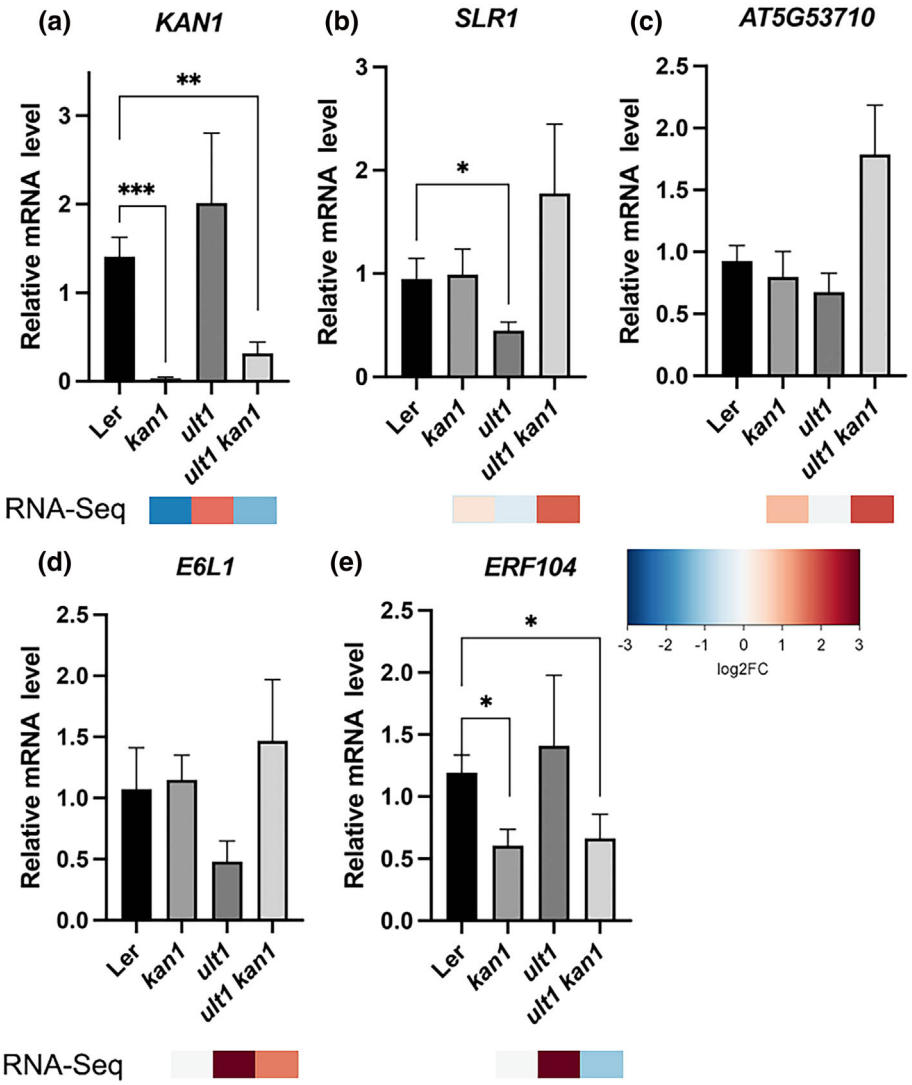


**FIGURE 9** Validation of selected differentially expressed genes expressed in gynoecia or anthers using reverse transcription-quantitative polymerase chain reaction. Relative mRNA expression levels (mean ± SE) of the (a) *KAN1*, (b) *SLR1*, (c) *At5g53710*, (d) *E6L1*, and (e) *ERF104* genes. Asterisks indicate a significant difference from the wild-type mean (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Heatmaps showing the transcriptional profile comparisons of each gene from the RNA-sequencing data are shown below the corresponding graph.

exception of the AP2/ERF transcription factor gene *ERF104*, there was little overlap between transcription factor genes regulated by ULT1 in seedlings/inflorescences (Tyler et al., 2019) and those in flower clusters. This is consistent with previous studies showing that ULT1 has highly stage/tissue-specific regulatory interactions and biological functions (Fletcher, 2001; Monfared et al., 2013; Ornelas-Ayala et al., 2020; Pu et al., 2013; Xu et al., 2018). In addition, the *developmental process* GO functional category was overrepresented among both downregulated and upregulated DEGs in *ult1–2* flower clusters (Figure S2). Thus, ULT1 both positively and negatively regulated developmental gene expression during reproduction. ULT1-induced genes significantly enriched within the *developmental process* category resolve to those that function in reproductive structure development, reflecting the role of *ULT1* and its paralog *ULT2* in regulating the patterning of the apical–basal axis of the gynoecium (Monfared et al., 2013).

Previous transcriptomics studies performed using vegetative tissues associated KAN1 with the regulation of genes in multiple hormone response pathways, particularly those involved in auxin biosynthesis and response (Huang et al., 2014; Merelo et al., 2013; Ram et al., 2020; Reinhart et al., 2013). Auxin mediates many aspects of organ development, and in the gynoecium, it plays a key role in establishing apical–basal polarity and in subsequent tissue differentiation (Moubayidin & Østergaard, 2017; Nemhauser et al., 2000; Zuniga-Mayo et al., 2019). Although auxin-response genes such as *CA2+-DEPENDENT MODULATOR OF ICR1* (*CMI1*) and *CYP83A1* were represented in the *kan1* flower cluster DEG dataset (Table S2), they were not significantly overrepresented among the DEGs. Likely this is because the *kan1* polarity phenotype in developing floral organs (Figure 1c) is mild compared with the dramatic phenotype observed in leaves (Eshed et al., 2001). However, the GO category *response to light stimulus* was overrepresented among the genes downregulated in *kan1* and also in *ult1* flower clusters (Figure 4a,b), which indicated KAN1 and ULT1 independently induced the expression of light signaling-related transcripts during reproduction. The expression of photosynthesis-related genes in particular increases during the late stages of carpel development (Kivivirta et al., 2021), suggesting a potential novel role for KAN1 and ULT1 as part of a regulatory network controlling this process. Future studies will assess whether this regulatory interaction is related to the regulation by KAN1 and ULT1

of *SPT* transcription (Pires et al., 2014), as the *SPT* gene encodes a member of the PIF family of phytochrome interacting factors and can induce the expression of light-regulated genes such as *HAT1*, several of which also mediate carpel margin development (Reymond et al., 2012).

The variability in differential gene expression we observed within and between the RNA-seq and RT-qPCR experiments may be attributable to several factors. One is slight variability in growth conditions between experiments, as principal component analysis of the RNA-Seq samples showed substantial variance in the *ult* and *ult1 kan1* biological replicates (Figure S2). This is not unexpected because *ult1* plants are strongly susceptible to environmental cues (Pu et al., 2013; Tyler et al., 2019). A second complicating factor may be the strong tissue specificity of many of the target genes, which can result in small but significant changes in expression levels becoming masked by the abundance of tissues in which they are not expressed.

## 4.2 | Roles for KAN1 and ULT1 in gynoecium development

A recent report of laser capture microdissection coupled RNA-Seq of four stages of gynoecium development, from carpel initiation at stage 5 of flower formation (Smyth et al., 1990) to stage 12 taking place between female meiosis and anthesis, identified *ULT1* and *KAN1* as genes expressed across all four stages (Kivivirta et al., 2021). This study confirmed the expression of both genes early in carpel formation, when the patterning of the reproductive structure takes place, and is consistent with the known function of *KAN1* and *ULT1* in regulating polar gynoecium patterning (Eshed et al., 1999; Monfared et al., 2013; Pires et al., 2014). It also suggested potential roles for the two genes during later stages of gynoecium development.

Although our previous work showed that mis-expression of *SPT* played a key role in conditioning the apical–basal patterning phenotype of *ult1 kan1* gynoecia (Pires et al., 2014), we did not detect significant differential expression of *SPT* in our *ult1 kan1* flower cluster dataset. This is likely because mis-expression of *SPT* occurs only in small number of abaxial gynoecium cells during stages 8–11 and thus became diluted when all reproductive tissues and stages were collected together for whole genome transcript profiling in this study. This is unsurprising, because RNA-seq experiments are designed to detect >1.5-fold changes in overall gene transcription levels rather than small differences in expression patterns. A combinatorial approach capturing both the expression domains and levels of key biological regulators will be required for a comprehensive understanding of the gene regulatory networks that drive plant reproductive development.

Our transcriptomics experiment did, however, uncover other DEGs that may have potential biologically relevant functions in ULT1 and/or KAN1 gynoecium regulatory networks. Ten out of the 11 ULT1-induced genes comprising the *reproductive structure development* GO category were expressed in the developing gynoecium. These include the *FLOWERING LOCUS M* (*FLM*) and *TEMPERANILLO1* (*TEM1*) transcription factor genes that currently are only known to play roles in the floral transition (Castillejo & Pelaz, 2008; Scortecci et al., 2001), as well as the *WUSCHEL-LIKE HOMEOBOX9/STIMPY* (*WOX9/STIP*) transcription factor gene that mediates apical–basal patterning during embryo development (Breuninger et al., 2008). An additional potentially biologically relevant target induced by KAN1 and ULT1 together was *AS2*, which encodes a regulator of lateral organ polarity that functions in a complex with AS1 to promote adaxial cell identity (Iwakawa et al., 2002; Lin et al., 2003). The AS1–AS2 complex also plays a role in patterning the medio–lateral axis of the gynoecium by repressing class I *KNOX* gene expression in the developing valves (Alonso-Cantabrana et al., 2007; Gonzalez-Reig et al., 2012). Whether the reduction in *AS2* transcript levels in *ult1 kan1* flower clusters is a cause or a consequence of the reduced valve tissue in *ult1 kan1* gynoecia (Pires et al., 2014) remains to be assessed.

Finally, seven DEGs were significantly upregulated in *ult1 kan1* flower clusters but not in either *ult1* or *kan1* flower clusters, indicating that these DEGs represent a unique subset of target genes that require both ULT1 and KAN1 for their repression. All seven genes were expressed in the developing gynoecium, and five of the seven were exclusively expressed in stigmatic tissue (Figures 6 and S4). The upregulation of stigma-expressed genes was consistent with the *ult1 kan1* gynoecium phenotype, in which apical stigma and style tissues expand into the basal region of the fruit (Figure 1). Additional experiments will be needed to determine whether the transcript levels of these genes are elevated due to an expansion of their normal expression domains or whether the loci are direct targets of ULT1 and KAN1 repression.

Among the stigma-expressed target genes, the *E6L1* gene is known to function in early pollen–stigma interactions (Doucet et al., 2019). The S-LOCUS RELATED PROTEIN1 (SLR1) protein encoded by locus At3g12000 can also be predicted to act in pollen recognition based on its sequence similarity to S-locus glycoproteins that are expressed in the stigma and function in the self-incompatibility response (Takayama & Isogai, 2005). *SLR1* and the putative peroxidase-encoding gene *PER39* also appear among papillar cell-specific genes identified in a whole-genome transcriptional profile of Arabidopsis stigmas and ovaries (Tung et al., 2005). Given that the other two ULT1 and KAN1-repressed genes *MLO6* and At53710 were likewise exclusively transcribed in stigmatic tissue (Figures 6 and S4), they too may function in stigma–pollen interactions and/or in stigmatic tissue formation. Our study therefore expands the cohort of genes that are likely to play functional roles in these critical aspects of Arabidopsis female reproduction.

## 4.3 | Potential novel roles for KAN1 and ULT1 in stamen development

Our data uncover a novel role for ULT1 and KAN1 in regulating the transcription of genes expressed in stamens (Figures 7, 8, and S5). *ULT1* is expressed in developing stamen cells from their inception in the third whorl of stage 5 flowers (Carles et al., 2005). As

development proceeds, *ULT1* transcripts become detected predominantly in the gametophytic tissues by stage 8 and subsequently are found in the tapetal cells (Carles et al., 2005). However, *ult1* and *ult1 ult2* plants are fully fertile, and to date, no stamen or male gametophyte-related phenotypes have been detected (Fletcher, 2001; Monfared et al., 2013).

Here, we identify among the ULT1-induced genes *AUXIN RESPONSE FACTOR17* (*ARF17*), a transcription factor gene that regulates anther dehiscence (Xu et al., 2019), the type III lipid transfer protein gene *ANTHER7* involved in microspore exine formation (Huang et al., 2013) and five *STP* genes from a family of 14 sugar transport proteins that are associated with pollen tube growth (Rottmann et al., 2018; Schneidereit et al., 2003). All five of the ULT1-induced *STP* genes are expressed in pollen, two of them exclusively so.

By contrast, *KAN1* and *KAN2* are known to play redundant roles in regulating stamen patterning. *KAN1* is expressed on the abaxial side of developing stamen primordia in stage 7 flowers (Kerstetter et al., 2001). Although *kan1* flowers have no discernable stamen phenotype, *kan1 kan2* anthers are often reversed in orientation, with the locules facing the petals (Eshed et al., 2001). Stamens are also occasionally observed that consist of a single locule-like disc radially topped with connective tissue, which is normally limited to the abaxial side, rather than a polarized structure (Eshed et al., 2001). However, our data suggest a potential role for KAN1 in regulating stamen development that extends beyond adaxial–abaxial patterning alone.

Our genome-wide study of KAN1 target genes in reproductive development revealed that 89% of the DEGS downregulated in *ult1 kan1* flower clusters were expressed in developing stamens (Table S5). That the differential expression of some of these genes may be associated with the *kan1* stamen polarity defect is possible but unlikely, because *ult1* mutations reverse all of the other *kan1* adaxial/abaxial polarity defects in the leaves and gynoecium (Pires et al., 2014, 2015). Rather, transcripts from over half of the DEGs were detectable in mature pollen, suggesting that KAN1 and ULT1 together induced the transcription of a cohort of genes involved in male reproductive processes. Among these, the *MYB99* gene is known to regulate pollen cell wall architecture as well as tapetal cell metabolism (Alves-Ferreira et al., 2007; Battat et al., 2019). *ENODL5* encodes a GPI-anchored protein that has been proposed to play a role in pollen tube growth (Lalanne et al., 2004). *KMD1* gene expression is repressed by the SDG4 histone methylation factor that promotes pollen tube growth (Cartagena et al., 2008), whereas the At1g47980 locus encoding a desiccation-like protein was highly expressed in pollen and repressed by the MALE GAMETOGENESIS IMPAIRED ANTHERS (MIA) ATPase protein that regulates pollen grain development (Jakobsen et al., 2005). The roles of the other ULT1- and KAN1-regulated stamen and pollen-expressed genes are as yet unknown, indicating much work remains to develop a complete picture of the molecular mechanisms that mediate male gametophyte development in Arabidopsis.

Several other studies also suggest a potential role for KAN1 in regulating pollen development. KAN1 can recognize part of a motif found upstream of the *ACA7* locus (Hoffmann et al., 2016), which displays pollen-specific expression and encodes a plasma membrane $Ca^{2+}$ P-type ATPase that is essential for proper pollen formation (Lucca & Leon, 2012). KAN1 protein also binds to the promoter of the enolase gene *AtENO2*, which affects the formation of the intine layer of the pollen wall (Ma et al., 2021). Although neither of these loci was detected among our stamen-expressed DEGs, the data collectively suggest that KAN1 and ULT1 may together play a hitherto unknown role in stamens and particularly in pollen development and/or pollen tube growth.

The goal of this study was to identify genome-wide downstream target genes of the Arabidopsis regulatory proteins KAN1 and ULT1 during reproductive development. We found that several hundred genes were regulated by ULT1, KAN1, or both factors together, including genes involved in reproductive processes as well as biotic or abiotic stress responses. Our results show that KAN1 and ULT1 regulate both gynoecium and stamen gene expression, which reveals potential roles for the two factors in female as well as male reproductive development. Future genetic and molecular experiments, including detailed phenotypic analysis of *ult1* and *kan1* stamen development and target gene transcription patterns, should shed additional light on the contribution of these two transcriptional regulators to male reproductive development.

## AUTHOR CONTRIBUTIONS

M.M., F.H., and J.F. designed the experiments. L.H., M.M., and F.H. performed the experiments. L.H., M.M., F.H., A.T., and J.F. analyzed the data. J.F. wrote the manuscript with input from the other authors. All authors have read and given approval to the final version of the manuscript.

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## CONFLICT OF INTEREST STATEMENT

The authors have declared no conflict of interest.

## DATA AVAILABILITY STATEMENT

RNA-seq datasets have been deposted at the National Center for Biotechnology Short Read Archive under BioProject PRJNA882916.

## ORCID

*Lynne Hagelthorn* https://orcid.org/0000-0002-6994-0346
*Anthony Talo* https://orcid.org/0000-0001-8895-8309
*Frank G. Harmon* https://orcid.org/0000-0001-7017-5373
*Jennifer C. Fletcher* https://orcid.org/0000-0003-1834-6213

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## SUPPORTING INFORMATION

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