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CRABS CLAW and SUPERMAN Coordinate Hormone-, Stress-, and Metabolic-Related Gene Expression During *Arabidopsis* Stamen Development

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The appropriate timing of the termination of floral meristem activity (FM determinacy) determines the number of floral organs. In *Arabidopsis*, two transcription factors, CRABS CRAW (CRC) and SUPERMAN (SUP), play key roles in FM determinacy. CRC belongs to the YABBY transcription factor family, whose members contain a zinc finger and a helix-loop-helix domain. The *crc* mutation causes the formation of unfused carpels and leads to an increase in carpel number in sensitized backgrounds. The *SUP* gene encodes a C2H2-type zinc-finger protein, and *sup* mutants produce extra carpels and stamens. However, the genetic interaction between *CRC* and *SUP* is not fully understood. Here, we show that these two transcription factors regulate multiple common downstream genes during stamen development. The *crc sup* double mutant had significantly more stamens and carpels than the parental lines and an enlarged floral meristem. Transcriptome data have implicated several cytokinin- and auxin-related genes as well as stress- and metabolic-related genes to function downstream of CRC and SUP during stamen development. The regulation of common downstream genes of CRC and SUP might contribute to the initiation of an appropriate number of stamens and to subsequent growth and development.

Keywords: *Arabidopsis*, CRABS CLAW, cytokinin, floral meristem, flower, SUPERMAN

INTRODUCTION

Plant development is dependent on the persistent activity of pluripotent meristematic cells that are responsible for organ formation (Laux et al., 1996). In angiosperms, flower development initiates from the floral stem-cell pool that is located in the center of the floral meristem (FM). The FMs produce an appropriate population of cells to initiate a fixed number of floral organs before FM activity terminates (FM determinacy) (Lenhard et al., 2011). In *Arabidopsis*, the FM is maintained by activity of the WUSCHEL (WUS) homeodomain protein and CLAVATA (CLV) ligand–receptor system (Laux et al., 1996; Brand et al., 2000; Schoof et al., 2000).

The WUS–CLV negative feedback loop controls the balance between stem-cell renewal and organ formation and determines the size and number of floral organs, which are sepals, petals, stamens, and carpels (Schoof et al., 2000; Sun et al., 2009). Loss-of-function mutants of any of the *CLV* genes results in enlarged FMs and consequently leads to an increase in floral organ number (Clark et al., 1993, 1995; Kayes and Clark, 1998). Conversely, *wus* mutants have fewer floral organs. Thus, the WUS–CLV negative feedback loop is essential to control FM activity.

The homeotic gene *AGAMOUS* (*AG*) determines when FM activity ceases and limits the size of the FM (Yanofsky et al., 1990; Lohmann et al., 2001; Lenhard et al., 2011). In loss-of-function *ag* mutants, the FM overproliferates, resulting in an increase in floral organ number or in a "flower within flower" phenotype, due to the failure of FM termination (Yanofsky et al., 1990). The AG protein binds to the *WUS* promoter and represses *WUS* expression via the deposition of repressive histone marks (Liu et al., 2011; Guo et al., 2018). During FM termination, AG represses *WUS* expression by directly inducing two key downstream targets, *KNUCKLES* (*KNU*) and *CRABS CLAW* (*CRC*) (Alvarez and Smyth, 1999; Bowman and Smyth, 1999; Payne et al., 2004; Gomez-Mena et al., 2005; Lee et al., 2005; Sun et al., 2014; Bollier et al., 2018). Previous studies have demonstrated that the *crc knu* double mutant shows prolonged *WUS* expression and produces flowers with reiterated floral organs, such as stamens and carpels (Breuil-Broyer et al., 2016; Yamaguchi et al., 2017). The *KNU* gene encodes a C2H2 zinc-finger protein with a conserved transcriptional repressor motif (Payne et al., 2004; Sun et al., 2009, 2014, 2019; Bollier et al., 2018). The KNU transcriptional repressor interacts with Polycomb group proteins and deposits repressive histone marks at the *WUS* locus (Sun et al., 2019). Alternatively, CRC belongs to the YABBY family of transcription factors, members of which contain a zinc finger and a helix-loop-helix domain (Bowman and Smyth, 1999; Baum et al., 2001; Lee et al., 2005). We previously identified two CRC direct and auxin-related targets, which are responsible for FM termination (Yamaguchi et al., 2017, 2018): *TORNADO2* (*TRN2*) encodes a transmembrane protein of the tetraspanin family and controls auxin homeostasis (Cnops et al., 2006; Chiu et al., 2007) and *YUCCA4* (*YUC4*) is one of the 11 *YUCCA* genes that encode flavin monooxygenases involved in tryptophan-dependent auxin biosynthesis (Cheng et al., 2006, 2007). The downregulation of *TRN2* and upregulation of *YUC4* by CRC terminates FM proliferation and triggers floral organ formation (Yamaguchi et al., 2017, 2018).

Other factors have been reported to be involved in FM termination (Uemura et al., 2017): *SUPERMAN* (*SUP*) encodes a C2H2 zinc-finger protein, which has been proposed to act as a boundary gene that specifies the separation between floral whorls 3 and 4 (Bowman et al., 1992; Sakai et al., 1995). The loss-of-function *sup* mutation leads to the formation of supernumerary stamens at the expense of carpel tissues (Sakai et al., 1995, 2000; Prunet et al., 2017). Ectopic *SUP* expression causes altered auxin and cytokinin phenotypes (Nibau et al., 2010). SUP interacts with CURLY LEAF, one of the Polycomb group (PcG) protein components (Xu et al., 2018). Then, the SUP-PcG complex represses two auxin biosynthesis genes, *YUCCA1* (*YUC1*) and *YUC4*, to limit stamen number through deposition of H3K27me3 (Zhao et al., 2001; Cheng et al., 2006, 2007; Sassi and Vernoux, 2013; Xu et al., 2018).

Organ formation is controlled by a complex network of interactions between plant hormones. One simple model of hormone interaction is via antagonism. In antagonistic interactions, the components of one plant hormone signaling pathway interact with those of another hormone. For example, auxin inhibits cytokinin signaling. One cytokinin signaling component, *ARABIDOPISIS HISTIDINE PHOSPHOTRANSFER PROTEIN6* (*AHP6*), is functionally important in auxin–cytokinin crosstalk (Mähönen et al., 2006; Moreira et al., 2013; Besnard et al., 2014a,b). Auxin signaling downstream of *AHP6* inhibits cytokinin signaling to regulate the positioning of floral primordia (Moreira et al., 2013; Besnard et al., 2014a,b). Although it is generally reported that auxin and cytokinin function antagonistically, recent studies have revealed that they also act synergistically during gynoecium formation (Wolters and Jurgens, 2009; El-Showk et al., 2013; Schaller et al., 2015; Müller et al., 2017; Reyes-Olalde et al., 2017a,b). Although these findings have demonstrated that the interplay between auxin and cytokinin via *AHP6* is important for floral primordium positioning and gynoecium formation, the role of *AHP6* in FM determinacy and floral organ initiation remains unclear.

Subsequent floral organ growth and development might also be affected by CRC and/or SUP, potentially via the transcriptional regulation of downstream genes. These might include, for example, the auxin-responsive gene *AUX/IAA19*, which regulates stamen elongation (Tashiro et al., 2009; Ghelli et al., 2018), and *REPRODUCTIVE MERISTEM* (*REM*) genes, which are potentially involved in the early stages of flower development and are often transcriptionally regulated by well-known key floral regulators (Mantegazza et al., 2014). Despite the importance of many genes involved in the regulation of floral organ growth and development, little is known concerning their regulation.

Here, we report the genetic interaction between *SUP* and *CRC*. Molecular genetic analysis revealed that *SUP* and *CRC* cooperatively fine-tune hormone signaling and various stress or metabolic events to regulate stamen formation during flower development.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The *Arabidopsis thaliana* mutants *crc-1* and *sup-5* used in this study were in the Landsberg *erecta* (L*er*) ecotype background. The *crc-1*, and *sup-5* lines were described previously (Gaiser et al., 1995; Bowman and Smyth, 1999; Jacobsen et al., 2000; Sakai et al., 2000; Yamaguchi et al., 2017, 2018). The double mutants were generated by genetic crossing and genotyped by PCR in subsequent generations. Seeds were sown on soil and stratified at 4°C for 3–7 days. Plants were grown at 22°C under 24 h of continuous light. Plants to be directly compared were grown side-by-side to minimize environmental differences within the growth chamber.

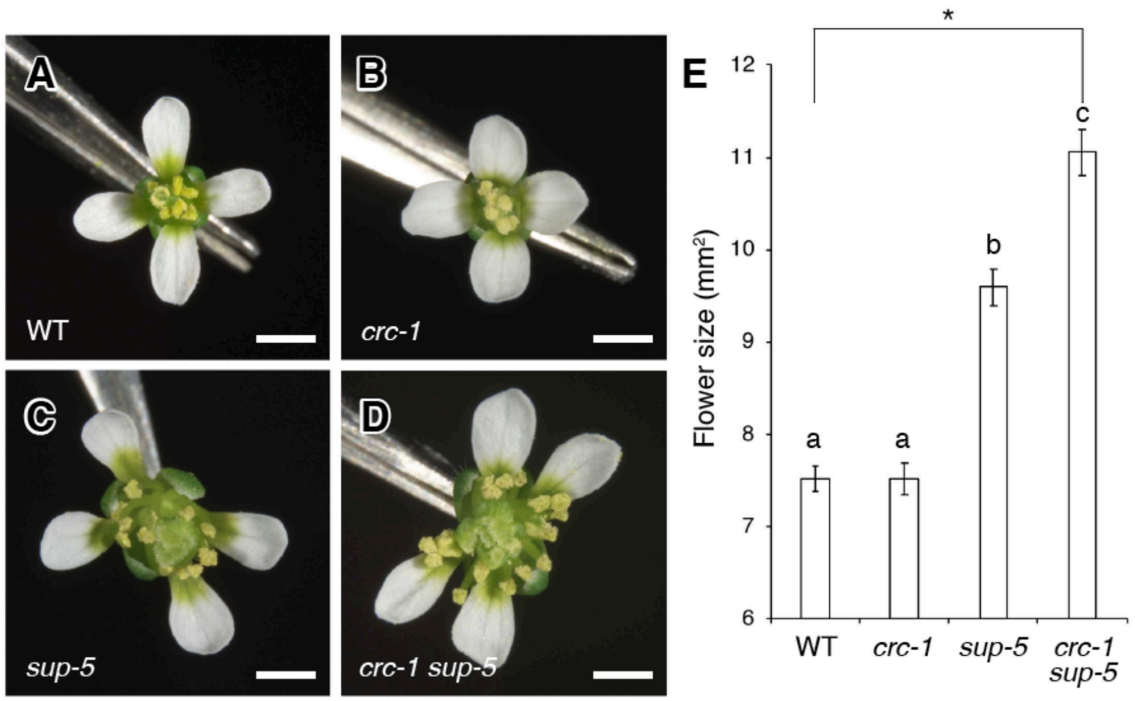

FIGURE 1 | Comparison of flower size among the wild type, *crc-1*, *sup-5*, and *crc-1 sup-5* at floral stage 13. **(A–D)** Top view of flowers. **(A)** The wild type (WT), **(B)** *crc-1*, **(C)** *sup-5*, and **(D)** *crc-1 sup-5*. Scale bars represent 500 μm. **(E)** Quantification of flower size. Mean ± SEM are shown. The asterisk indicates significant difference based on one-way ANOVA. The same letters indicate non-significant differences, whereas different letters indicate significant differences based on the *post-hoc* Tukey HSD test ($p < 0.01$).

Phenotyping Open Flowers

The first 5–10 flowers at developmental stage 13 (according to Smyth et al., 1990) were harvested for phenotyping open flowers (**Figures 1**, **2**). To measure flower size, flowers of L*er*, *crc-1*, *sup-5*, and *crc-1 sup-5* were removed with forceps and fixed onto agar, and photos were taken from above. Flower size was measured using Image J (http://imagej.nih.gov/ij/) software. Thirty flowers (five flowers each from six individual plants) from each genotype were measured. The number of floral organs (sepals, petals, stamen, and carpels) in the wild type (L*er*), *crc-1*, *sup-5*, and *crc-1 sup-5* of stage 13 flowers was counted under a dissecting microscope. Forty flowers (five each from eight individual plants) were counted for each genotype. To test for statistical significance, one-way ANOVA was followed by the *post-hoc* Tukey HSD test.

Measurement of Floral Meristem Size

To measure the size of the FM, inflorescences 1–3 cm tall were harvested immediately after bolting. Inflorescences were fixed with FAA overnight. The resulting inflorescences were dehydrated in an ethanol series (50, 60, 70, 80, 90, 95, and 100%; not <20 min each). The fixed samples were then removed from 100% ethanol and placed in Technovit 7100 resin (Heraeus) before overnight incubation for polymerization. Eight, 10-μm thick sections were prepared using a RM2255 microtome (Leica Microsystems) for each genotype or floral developmental stage. Significance was tested using the Student's *t*-test and one-way ANOVA followed by the *post-hoc* Tukey HSD test.

RNA-Seq

For RNA extraction, floral buds up to floral stage 8 from inflorescences 1–3 cm tall were harvested. Five biological replicates were harvested from wild-type (L*er*), *crc-1*, *sup-5*, and *crc-1 sup-5* backgrounds. Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen), and genomic DNA was removed using an RNase-Free DNase Set (Qiagen). Library preparation and sequencing were performed as described previously (Uemura et al., 2017; Ichihashi et al., 2018). The created libraries were sequenced by next-generation sequencing (Illumina), and the produced bcl files were then converted into fastq files by bcl2fastq (Illumina). Mapping of sequences to the *Arabidopsis* TAIR10 genome was performed using Bowtie with the following options ("—all—best—strata—trim5 8"). The number of reads for each reference was then counted, and the false discovery rate (FDR), log concentration (Conc) and log fold-change (FC) were obtained using the edge R package (Robinson et al., 2010). To determine DEGs, FDR with adjusted $p < 0.05$ was used. The data were deposited into the DNA Data Bank of Japan (DRA008874).

Transcriptome Analysis

To identify common differentially expressed genes (DEGs), online software (bioinformatics.psb.ugent.be/webtools/Venn/) was used to calculate the overlap between DEGs lists in all mutant backgrounds. Furthermore, Gene Ontology (GO) term analysis was performed using online software agriGO v2.0

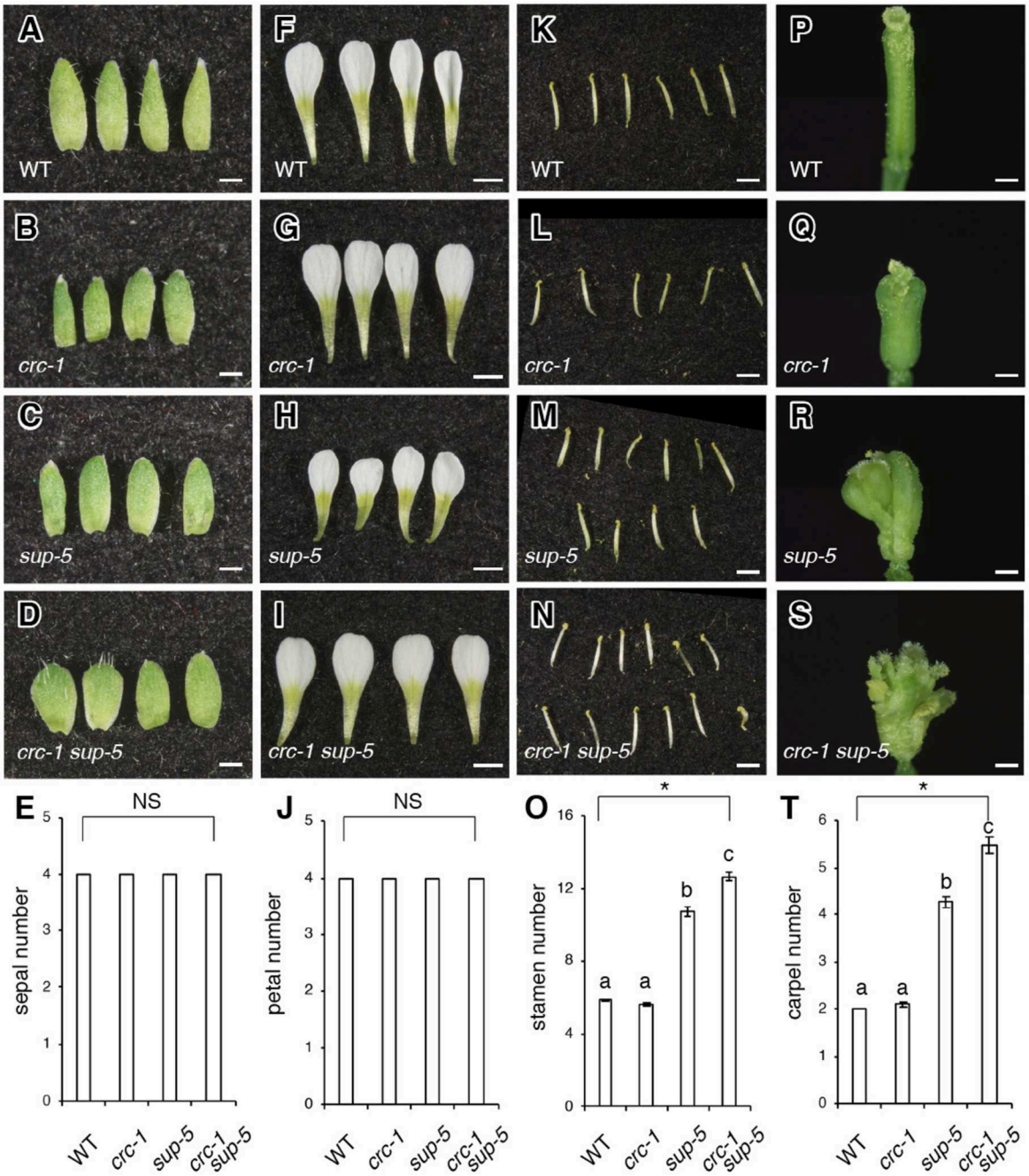

FIGURE 2 | Comparison of floral organ number among the wild type, *crc-1*, *sup-5*, and *sup-5 crc-1* at floral stage 13. **(A–D)** Side view of sepals. **(A)** The wild type (WT), **(B)** *crc-1*, **(C)** *sup-5*, and **(D)** *crc-1 sup-5*. Scale bars represent 500 μm. **(E)** Quantification of sepal number. **(F–I)** Side view of petals. **(F)** The wild type, **(G)** *crc-1*, **(H)** *sup-5*, and **(I)** *crc-1 sup-5*. Scale bars represent 1 mm. **(J)** Quantification of petal number. **(K–N)** Side view of stamens. **(K)** Wild type, **(L)** *crc-1*, **(M)** *sup-5*, and **(N)** *crc-1 sup-5*. Scale bars represent 1 mm. **(O)** Quantification of stamen number. **(P–S)** Side view of carpels. **(P)** The wild type, **(Q)** *crc-1*, **(R)** *sup-5*, and **(S)** *crc-1 sup-5*. Scale bars represent 500 μm. **(T)** Quantification of carpel number. Mean ± SEM are shown. Scale bars represent 500 μm. Asterisks indicate significant differences based on one-way ANOVA. The same letters indicate non-significant differences, whereas different letters indicate significant differences based on the *post-hoc* Tukey HSD test ($p < 0.01$).

(systemsbiology.cau.edu.cn/agriGOvs/) (Tian et al., 2017), followed by REVIGO (Reduced + Visualize Gene Ontology; revigo.irb.hr/) (Supek et al., 2011) to reduce the redundant GO terms.

RT-qPCR

For RNA extraction, floral buds up to floral stage 8 from inflorescences 1- to 3-cm tall were harvested. Plants from mutant backgrounds (*crc-1*, *sup-5*, and *crc-1 sup-5*) and

the controls [wild-type (L*er*)] were grown side-by-side. Approximately 100 mg of floral bud tissue was prepared and frozen immediately after trimming, without fixation. Tissues were kept at −80°C until use (<5 months). RNA extraction was performed using the RNeasy Plant Mini Kit (Qiagen). Genomic DNA was then removed using an RNase-Free DNase Set (Qiagen) to minimize contamination by genomic DNA. The RNA concentration was determined with an IMPLEN NanoPhotometer P-Class spectrophotometer. Synthesis of cDNA was performed with a PrimeScript first-strand cDNA Synthesis Kit (Takara) using <5 μg total RNA, 50 μM oligo dT primer and 200 U PrimeScript RTase with RNase Inhibitor, at 42°C for 30 min. The resulting cDNA was quantified by a LightCycler 480 (Roche) using FastSmart Essential DNA Green Master Mix (Roche) and C_q values were obtained. The expression levels of *AHP6* (*AT1G80100*), *IAA19* (*AT3G15540*), *REM25* (*AT5G09780*), and *TPPI* (*AT5G10100*) were quantified; *EIF4A-1* (*AT3G13920*) was used for the normalization of signals. Five biological replicates were performed and similar results were obtained. The primers used in this study were as follows: AHP6-FW, 5′-CAGCTGGAGCAGCAGAGAAT-3′; AHP6-RV, 5′-TTTCGCTTCGGTAGCTTATAACACA-3′; IAA19-FW, 5′-GATCTAGCCTTTGCTCTTGATAAGC-3′; IAA19-RV, 5′-ATGACTCTAGAAACATCCCCCAAG-3′; REM25-FW, 5′-CTTGGGAGACCACGAGTTTCTTA-3′; REM25-RV, 5′-TTTTGACACGACTAGAAGAAGCGAA-3′; TPPI-FW, 5′-TACAG GTTCGGTCGGTATTAAAGAA-3′; TPPI-RV, 5′-TTGTTAGTGTTCCCAAATCCAAGTG-3′; EIF4-FW, 5′-ACCAGGCGTAAGGTTGATTG-3′; EIF4-RV, 5′-GGTCCATGTCTCCGTGAGTT-3′.

RESULTS

The *sup crc* Double Mutant Has Significantly Larger Flowers and Supernumerary Stamens and Carpels

To analyze the genetic interaction between *SUP* and *CRC*, we generated a *crc-1 sup-5* double mutant and compared the size of flowers between the wild type, *crc-1*, *sup-5*, and *crc-1 sup-5* (**Figures 1A–E**). A wild-type flower consists of four sepals, four petals, six stamens, and two carpels (**Figure 1A**). The mean size of wild-type flowers was 7.5 ± 0.1 mm^2 (**Figure 1E**). Similar to the wild type, the *crc-1* single mutant had a fixed number of four types of floral organs (**Figure 1B**). The size of *crc-1* flowers was 7.5 ± 0.2 mm^2 (**Figure 1E**), which was not significantly different from wild-type and *crc-1* flowers ($p > 0.01$) (**Figure 1E**). As reported previously (Uemura et al., 2017), *sup-5* plants produce significantly larger flowers (9.6 ± 0.2 mm^2) than the wild type ($p < 0.01$) (**Figures 1C,E**). An increase in the size of *sup-5* mutant flowers was accompanied by the presence of extra whorls of stamens, due to sustained floral stem-cell activity (**Figure 1C**) (Xu et al., 2018). In *crc-1 sup-5* double mutants, a large number of stamens and carpels arose from whorls 3 and 4 (**Figure 1C**). The mean size of *crc-1 sup-5* flowers was 11.1 ± 0.2 mm^2 (**Figures 1D,E**) and was thus significantly larger than that of the wild type or either single mutant ($p < 0.01$) (**Figure 1E**).

Combination of *crc* With *sup* Results in a Synergistic Increase in the Number of Stamens and Carpels

To investigate the genetic interaction between *CRC* and *SUP* further, the number of organs was counted in wild-type, *crc-1*, *sup-5*, and *crc-1 sup-5* double mutant flowers (**Figures 2A–T**). Wild-type flowers had four sepals, four petals, six stamens, and two carpels (**Figures 2A,E,F,J,K,O,P,T**). Similarly, the *crc* mutant produced four sepals, four petals, and six stamens (**Figures 2B,E,G,J,L,O,Q,T**). Although *crc-1* mutants produced three or even occasionally four carpels, the mean carpel number for *crc-1* was 2.1 ± 0.0 (**Figure 2T**), which did not differ significantly from the wild type. Indeed, there was no significant difference in the number of all four floral organs between the wild type and *crc-1* mutants ($p > 0.01$) (**Figures 2E,J,O,T**). No significant difference was observed in the number of sepals and petals between wild-type and *sup-5* flowers ($p > 0.01$) (**Figures 2A,C,E,F,H,J**). However, the mean numbers of stamens and carpels in *sup-5* were 10.7 ± 0.3 and 4.3 ± 0.1, respectively (**Figures 2K,M,O,P,R,T**), significantly higher than those of the wild type ($p < 0.01$) (**Figures 2O,T**). Similar to the wild type or the parental lines, *crc-1 sup-5* double mutant flowers also produced four sepals and four petals ($p > 0.01$) (**Figures 2A–J**) but produced significantly more stamens and carpels than the wild type or either single mutant ($p < 0.01$) (**Figures 2K–T**). Thus, the combination of *crc* with *sup* enhanced the *sup* phenotype.

Combining *crc* and *sup* Enhances the FM Width Phenotype of *sup*

Next, we aimed to determine whether the changes in the size of flowers and/or the number of floral organs correlated with changes in FM height and width. Therefore, we quantified the height (from the groove between sepal primordia and the FM to the top of the floral meristem) and the width (between the two grooves along the lateral axis) of the FM by sectioning (**Figures 3A–O**). In wild-type plants, mean FM height and width at stage 3 were 12.0 ± 0.5 and 58.0 ± 1.1 μm, respectively (**Figures 3A,E**), and were 29.2 ± 0.9 and 75.2 ± 2.0 μm, respectively, at stage 5 (**Figures 3A,E**). In *crc-1* mutants, no significant difference in FM height and width at stage 3 or stage 5 was observed compared to wild-type plants ($p > 0.01$) (**Figures 3A,B,E–G,J**). Similarly, no significant difference in FM height and width was observed between wild-type and *sup-5* FMs at stage 3 ($p > 0.01$). Although FM height in the wild type and *sup-5* mutants did not differ significantly ($p > 0.01$) at stage 5, there was a significant difference in FM width ($p < 0.01$) (**Figures 3F,J**). In *crc-1 sup-5*, FM height and width were similar to in *sup-5* by stage 5 (**Figures 3C–E,H–J**). A significant difference in FM width was observed between *sup-5* and *crc-1 sup-5* FMs at stage 6, but no difference in height ($p < 0.01$, Student's *t*-test). Therefore, combining the *crc* mutation with *sup* enhanced the *sup* mutant FM width phenotype.

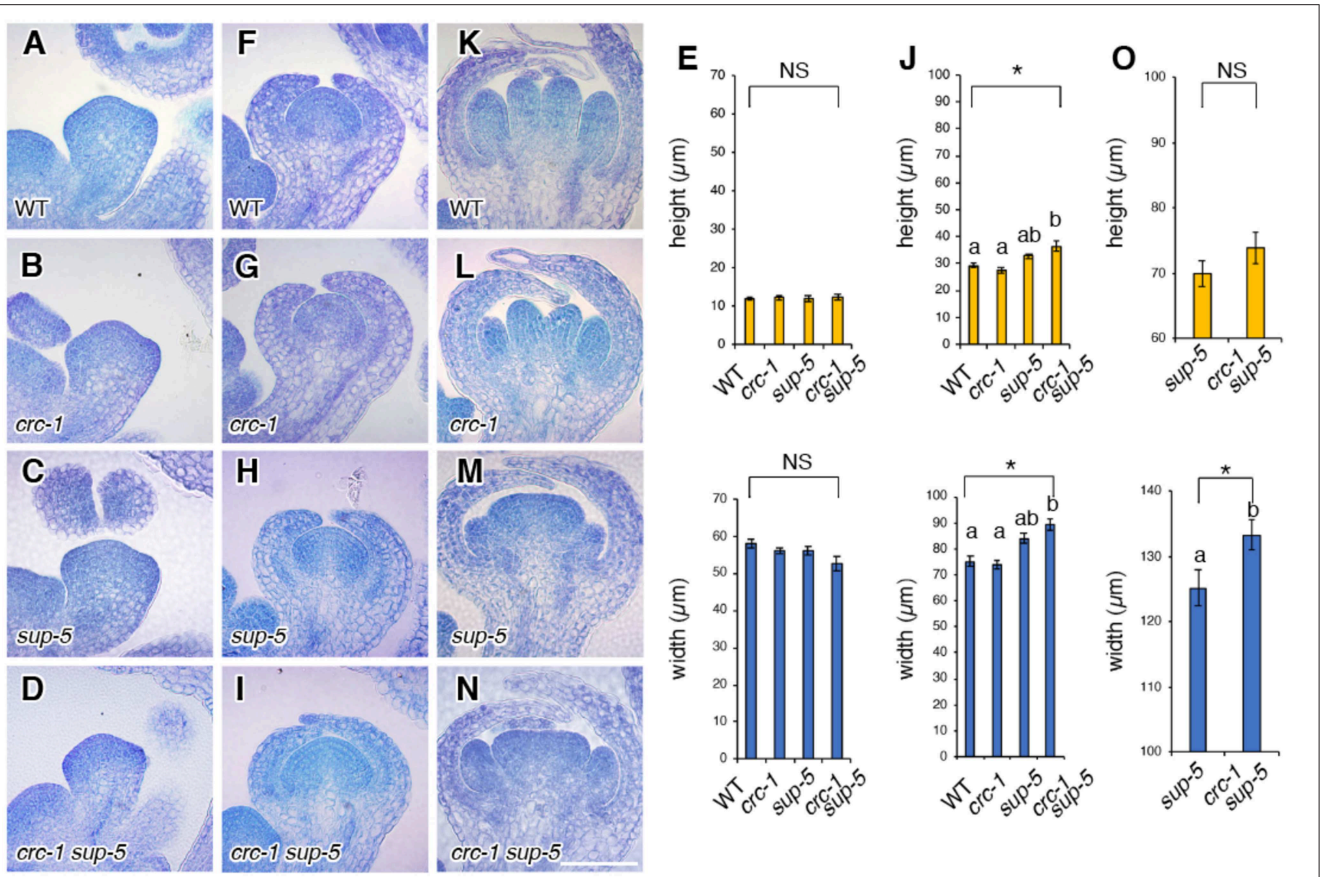

FIGURE 3 | Comparison of floral meristem height and width among the wild type, *crc-1*, *sup-5*, and *sup-5 crc-1* at different floral stages. **(A–D)** Vertical cross sections of stage 3 floral meristems. **(A)** The wild type, **(B)** *crc-1*, **(C)** *sup-5*, and **(D)** *crc-1 sup-5*. **(E)** Quantification of floral meristem height and width at stage 3. **(F–J)** Vertical cross sections of floral meristems at stage 5. **(F)** The wild type, **(G)** *crc-1*, **(H)** *sup-5*, and **(I)** *crc-1 sup-5*. **(J)** Quantification of floral meristem height and width at stage 5. **(K–O)** Vertical cross sections of floral meristems at stage 6. **(K)** The wild type, **(L)** *crc-1*, **(M)** *sup-5*, and **(N)** *crc-1 sup-5*. **(O)** Quantification of floral meristem height and width at stage 6. Mean ± SEM are shown. Scale bars represent 50 μm. For multiple comparisons, asterisks indicate significant differences based on one-way ANOVA. The same letters indicate non-significant differences, whereas different letters indicate significant differences based on the *post-hoc* Tukey HSD test ($p < 0.01$). For single comparisons, the *p*-value was determined using the Student's *t*-test (*$p < 0.01$).

Differentially Expressed Genes in *crc sup*

RNA-seq was performed to examine the genetic interaction at molecular levels in *crc*, *sup*, and *crc sup* (DRA008874). Approximately 10 M reads were sequenced per sample, which were then mapped onto the *Arabidopsis* TAIR 10 genome and differentially expressed genes (DEGs) were identified. In *crc-1* mutants, 263 genes were differentially expressed compared to the wild type (FDR < 0.05) (**Figure 4A**; **Tables S1**, **S2**). Similarly, 65 and 281 genes in total were differentially expressed in *sup* and *crc sup* mutants, respectively (FDR < 0.05) (**Figures 4B,C**; **Tables S1**, **S2**).

To identify genes involved in the enhancement of the *sup* phenotype in the *crc sup* double mutant, we focused on three different categories of DEGs, containing either 20 genes, 11 genes, or 216 genes (**Figures 4B–G**). The "20 genes" category contained the DEGs common to all *crc-1*, *sup-1*, and *crc-1 sup-5* backgrounds (**Figure 4A**). This category contained genes that were differentially expressed in single mutants and whose expression was further altered in the double mutant. To examine the probable functions of these 20 genes, GO term enrichment analysis was performed using agriGO v2.0 online software (Tian et al., 2017). Stimulus-related GO terms such as "response to hormone stimulus," "response to hormone stimulus," and "response to stimulus" were identified (**Figure 4B**; **Table S3**). A further reduction of redundant GO terms by REVIGO categorized ∼70% of the GO terms as "response to endogenous stimulus" (**Figure 4E**; **Table S4**). The "11 genes" category contained the DEGs shared by *sup-5* and *crc-1 sup-5* mutants. This category consisted of the downstream genes of SUP, whose expression was affected by combination with the *crc* mutation. In addition to stimulus-related terms, this category of GO terms also contained development-related GO terms, such as "developmental process" and "anatomical structure development" (**Figures 4C,F**; **Tables S3, S4**). The "216 genes" category contained genes that were differentially expressed only in *crc-1 sup-5*. The GO terms in this category included "response to chemical stimulus," "response to stimulus," and "response to hormone stimulus," which were also present in the "20 genes" and

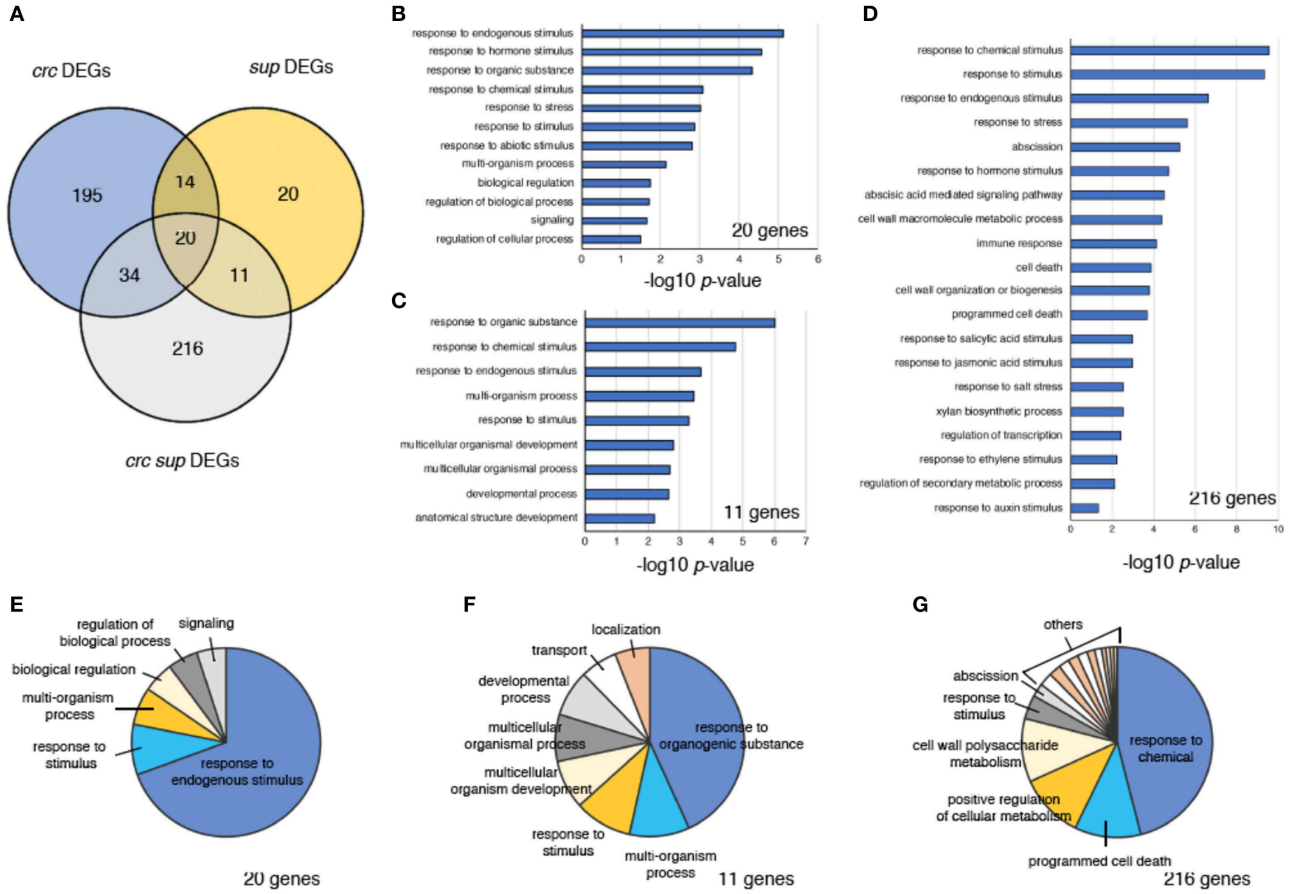

FIGURE 4 | Characterization of DEGs in *crc sup*. **(A)** Venn diagram showing the number of DEGs in *crc*, *sup*, and *crc sup* mutants. **(B–D)** GO term enrichment analysis of categories containing 20 genes **(B)**, 11 genes **(C)**, and 216 genes **(D)**. Selected terms determined by their $-\log_{10}$-adjusted *p*-values are shown. **(E–G)** Pie charts showing summarized GO terms by REVIGO in categories of 20 genes **(E)**, 11 genes **(F)**, and 216 genes **(G)** within the GO term category "biological process".

"11 genes" categories (**Figures 4B–D**; **Tables S3, S4**). In addition, unique GO terms such as "abscission," "programmed cell death," "immune response," and "cell wall organization or biogenesis" were present (**Figure 4D**; **Tables S3, S4**).

Identification of DEGs Related to Stamen Development

To identify the genes involved in supernumerary stamen initiation in *crc-1 sup-5* double mutants, we computationally identified genes involved in stamen development using a published transcriptome dataset (Alves-Ferreira et al., 2007; GSE8864). Previous studies have determined the global expression profile of *Arabidopsis* stamen development using *ap3*, *spl/nzz*, and *ms1* mutants. Among 247 DEGs identified in **Figure 4**, 47 genes were predicted to be expressed at early, intermediate, and late stages of stamen development (**Figure 5A**; **Table S5**). Out of 47 identified genes, 37 were differentially expressed in *ap3* (early stage) (**Figure 5A**). This is consistent with the functions of *CRC* or *SUP*, since both genes are highly expressed from early stages of flower development. Furthermore, an additional 21 and 8 identified genes were differentially expressed in *nzz/spl* (intermediate stage) and *ms1* (late stage), respectively (**Figure 5A**).

To understand the function of the 47 genes involved in stamen development, GO term analysis was performed (**Figure 5B**). The enriched terms included "response to organic substance," "response to chemical stimulus," "cell wall organization or biogenesis," "response to stimulus," "response to hormone," "multicellular organismal development," and "reproductive developmental process" (**Figure 5B**; **Table S6**). The interactive graph view of 47 genes generated by REVIGO identified a cluster of GO terms that contain five different terms: "response to organic substance," "response to hormone," "response to chitin," "response to carbohydrate," and "response to water deprivation" (**Figure 5C**). After removing redundant GO terms, ∼70% of GO terms were involved in "response to chemical" (**Figure 5D**; **Table S7**).

Genes Potentially Involved in Stamen Development

Based on RNA-seq, *SUP* was expressed at normal levels in *crc* and, similarly, *CRC* expression was unaffected in *sup*

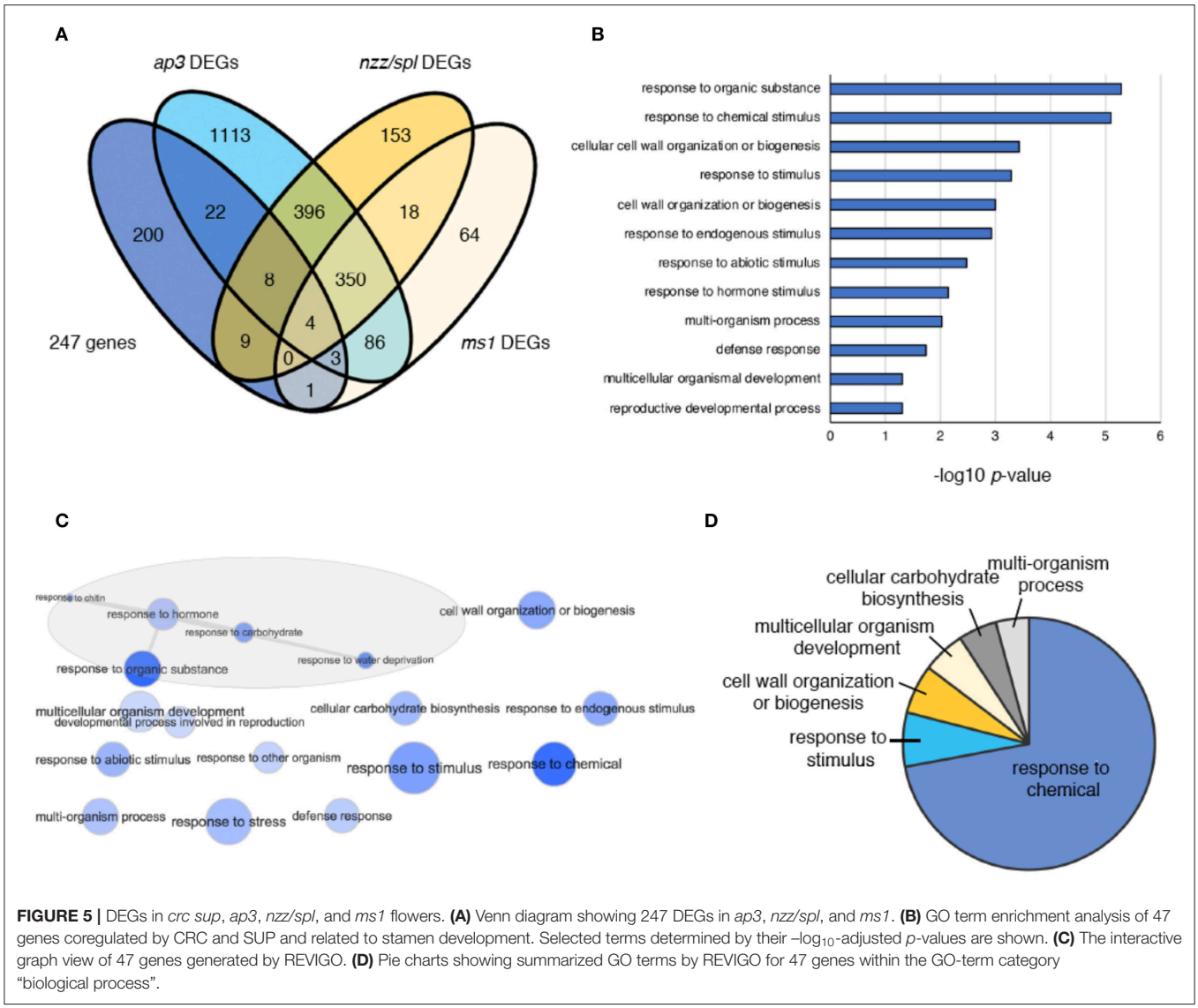

FIGURE 5 | DEGs in *crc sup*, *ap3*, *nzz/spl*, and *ms1* flowers. **(A)** Venn diagram showing 247 DEGs in *ap3*, *nzz/spl*, and *ms1*. **(B)** GO term enrichment analysis of 47 genes coregulated by CRC and SUP and related to stamen development. Selected terms determined by their $-\log_{10}$-adjusted *p*-values are shown. **(C)** The interactive graph view of 47 genes generated by REVIGO. **(D)** Pie charts showing summarized GO terms by REVIGO for 47 genes within the GO-term category "biological process".

(FDR < 0.05). Thus, it is unlikely that either transcription factor transcriptionally regulates the expression of the other (**Figure S1**). All of the 47 DEGs in *crc-1 sup-5* that overlapped with the list of DEGs from the stamen transcriptome dataset were categorized into two different clusters by K-means clustering (**Figure 6A**). Cluster 1 contained 28 genes, which were highly expressed in all mutant backgrounds, whereas cluster 2 contained genes that were downregulated in all mutant backgrounds (**Figures 6A,B**). Compared to the subtle difference in differential gene expression observed in either *crc-1* or *sup-5* single mutants, the expression levels of 47 genes were greatly affected in *crc-1 sup-5* double mutants.

The upregulated genes identified in *crc-1 sup-5* might be direct targets of SUP because SUP is a transcriptional repressor (**Figures 6C–J**). These genes might also be directly regulated by CRC, since CRC can act as a bifunctional transcription factor (Yamaguchi et al., 2017, 2018; Gross et al., 2018). Consistent with the GO term analysis, hormone-related genes such as *AHP6* and *IAA19* were highly expressed in the single mutants (**Figures 6C,D**) (Nakamura et al., 2003; Tatematsu et al., 2004; Mähönen et al., 2006; Besnard et al., 2014a,b). These two genes were also more highly expressed in *crc sup* than in either single mutant. The expression levels of *CELLULOSE SYNTHASE8* (*CESA8*), *REPRODUCTIVE MERISTEM25* (*REM25*), and *AT1G12080* (Turner and Somerville, 1997; Chen et al., 2005; Mantegazza et al., 2014) were also higher in *crc-1 sup-5* (**Figures 6E–G**). Among the potential downstream genes, the roles of *CESA8* and *REM25* have been well studied during cell wall organization and reproductive development, respectively.

By contrast, some of the genes related to hormonal regulation, such as *SMALL AUXIN UPREGULATED36* (*SAUR36*) and *DEHYDRATION-RESPONSIVE ELEMENT BINDING PROTEIN2A* (*DREB2A*) (Sakuma et al., 2006; Kim et al., 2011;

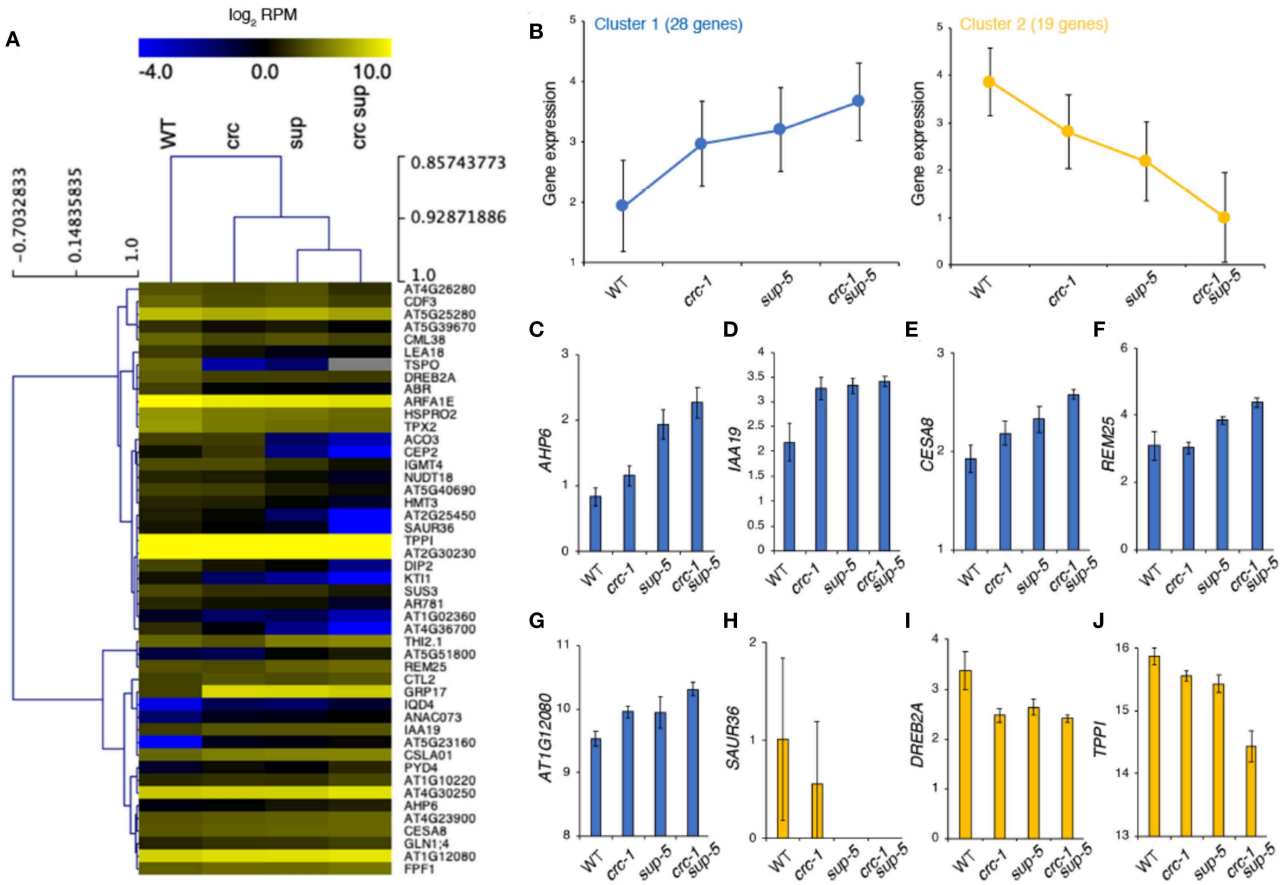

FIGURE 6 | Clustering of high-confidence downstream targets regulated by CRC and SUP. **(A)** Clustering of 47 genes during stamen formation. **(B)** Gene expression from two different clusters: cluster 1 (left) and cluster 2 (right). **(C–J)** Expression level of genes according to RNA-seq. *AHP6* **(C)**, *IAA19* **(D)**, *CESA8* **(E)**, *REM25* **(F)**, *AT1G12080* **(G)**, *SAUR36* **(H)**, *DREB2A* **(I)**, and *TPPI* **(J)**. Mean ± SEM (log_2 of reads per million mapped reads [RPM]) are shown.

Hou et al., 2013; Stamm and Kumar, 2013), were downregulated (**Figures 6H–J**). One sugar metabolism regulator, TREHALOSE-6-PHOSPHATE PHOSPHATASE I (TPPI) (Schluepmann et al., 2004), has also been identified as the downregulated target in the mutant backgrounds. The RNA-seq data were confirmed by RT-qPCR (**Figure S2**).

DISCUSSION

CRC and *SUP* Interact Genetically During Floral Meristem and Stamen Development

Floral meristem determinacy and meristematic cell differentiation are two critical steps in flower development (Sablowski, 2015; Bommert and Whipple, 2017; Xu et al., 2019). Disruption in either or both of these processes caused by the misexpression of FM regulators eventually results in abnormal floral phenotypes (Lohmann et al., 2001; Ma, 2005). Both *SUP* and *CRC* are highly conserved genes in Angiosperms and function as FM activity and identity genes in distinct spatio-temporal manners (Sun and Ito, 2015). Compared to *sup* mutants, *crc* mutants do not have altered floral organ number, despite the occasional presence of more than two carpels (Alvarez and Smyth, 1999; Bowman and Smyth, 1999). However, our data show that combination of the *crc* mutation with *sup-5* resulted in the formation of sterile flowers with significantly more stamens and carpels. The *crc* mutation enhanced FM indeterminacy in *sup-5* mutants, leading to an increase in FM size. Because the increased number of stamens in *sup* mutants is at the expense of carpel tissues due to the expansion of the *APETALA3* gene expression domain into the fourth whorl, an increase in FM size, leading to the formation of more carpels, might increase the rate of floral organ identity conversion, coupled with hormonal changes (Prunet et al., 2017). The sterility of *crc sup* double mutant flowers was probably due to a failure in establishing carpel polarity caused by *crc*. Polarity changes further enhance the carpel defect phenotypes in *sup* single mutants, which produce apically open carpels with abnormal ovule integument development (Gaiser et al., 1995; Baker et al., 1997; Eshed et al., 1999; Breuil-Broyer et al., 2016).

Compared to the *Arabidopsis CRC* gene, its orthologs in other species play broader and prominent roles during plant growth and development (Nagasawa et al., 2003; Yamaguchi et al., 2004;

Li et al., 2011). No clear defects in FM determinacy and no homeotic defects are observed in *crc* single mutants (Alvarez and Smyth, 1999; Bowman and Smyth, 1999; Baum et al., 2001; Nagasawa et al., 2003). By contrast, mutation of *CRC* orthologs in other plant species causes multiple phenotypic defects during the vegetative and reproductive stages. For example, one well-characterized *CRC* ortholog is *DROOPING LEAF* (*DL*) in rice (*Oryza sativa*). Molecular studies have identified the gene functions that specify carpel identity and regulate midrib leaf formation in rice (Yamaguchi et al., 2004). To date, no *crc* alleles that show floral homeotic or leaf defects as strong as those in *dl* mutants have been identified in *Arabidopsis*. When combined with mutants of other key regulators of floral meristem activity or development, these higher-order *crc* multiple mutants show synergistic effects and produce flowers with extra floral whorls or floral organs with abnormal phenotypes (Eshed et al., 2001; Prunet et al., 2008; Zuniga-Mayo et al., 2012). In *sup crc* double mutants, aberrant FM indeterminacy and floral organ identity were observed. Although *CRC* is only expressed in the abaxial region of carpels prior to FM termination, the *crc* mutation enhances not only FM determinacy, but also the initiation of floral organs such as stamens or carpels. Thus, it is conceivable that phytohormones are involved in the genetic interaction between *CRC* and *SUP* because phytohormones exert multiple roles in a non-cell-autonomous manner. Recent studies have identified roles for *CRC* and *SUP* in hormone homeostasis (Yamaguchi et al., 2017, 2018; Xu et al., 2018). Hormonal regulation by CRC might also explain why its orthologs have various functions in different plant species.

CRC and SUP Regulate Common Downstream Targets Involved in Stamen Development

Based on RNA-seq, *SUP* was expressed at normal levels in *crc* and similarly, *CRC* expression was unaffected in *sup* ($FDR < 0.05$). Thus, it is unlikely that either transcription factor transcriptionally regulates the expression of the other (**Figure S1**).

Cytokinins maintain meristem activity by controlling cell division (Riou-Khamlichi et al., 1999; Werner et al., 2001; Yang et al., 2002; Zhang et al., 2013). Consistent with this finding, we identified *AHP6* as a common downstream gene of CRC and SUP. It has been reported that supernumerary stamen primordia in *sup* are formed at stage 7 (Prunet et al., 2017). Considering that *CRC* is expressed from floral stage 6 onwards (Bowman and Smyth, 1999; Lee et al., 2005), the effect of the *crc* mutation in the *sup* background and *AHP6* misexpression was observed after stage 6. Stage-specific gene expression and binding tests using a synchronized system, together with the expression of multiple marker genes, will provide greater insight into how stamen number is defined by CRC and SUP via *AHP6*.

IAA19, which plays a key role in controlling stamen elongation (Tashiro et al., 2009; Ghelli et al., 2018), was also differentially expressed in *crc-1 sup-5* plants. This suggests that SUP might be important not only for early stamen formation, but also for subsequent stamen growth. The *crc* mutation affects the expression of *IAA19*, which is expressed in stamens, even though *CRC* is not expressed in these organs. It is unclear how this regulation occurs, but might involve non-cell-autonomous effects.

The *REM25* and *TPPI* genes were also differentially expressed in *crc-1 sup-5* (Mantegazza et al., 2014). Based on previous *in situ* hybridization data, *REM25* is highly expressed in stamen and carpel primordia at floral stage 6. Since *SUP*, *CRC*, and *REM25* have overlapping expression domains (Sakai et al., 1995; Bowman and Smyth, 1999; Lee et al., 2005), SUP and CRC might be upstream regulators of *REM25*. Genetic redundancy and the physical linkage of *REM* loci hamper functional studies of *REM* family genes (Mantegazza et al., 2014); therefore, the generation of mutants via CRISPR/Cas9 might contribute to understanding the function of genes within this family. *TPPI* is required for the appropriate establishment of organ boundaries (Lor, 2014), which is consistent with the regulation of organ boundary genes, such as *CUC2* (Xu et al., 2018), by SUP. However, the exact function of *TPPI* during flower development is largely unknown and it is relevant to study the molecular function of *TPPI* during stamen/carpel boundary specification.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the DRA008874.

AUTHOR CONTRIBUTIONS

ZL, YT, NY, and TI conceived and designed the experiments. ZL and YT performed the phenotypic experiments. ZL extracted RNA. YI, AS, and KS generated RNA-seq libraries. TS performed sequencing. NY conducted transcriptome analyses and wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2019.00437/full#supplementary-material

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