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RESEARCH HIGHLIGHT

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A new regulation mechanism of bisexual flower development in cucurbitaceous sex determination

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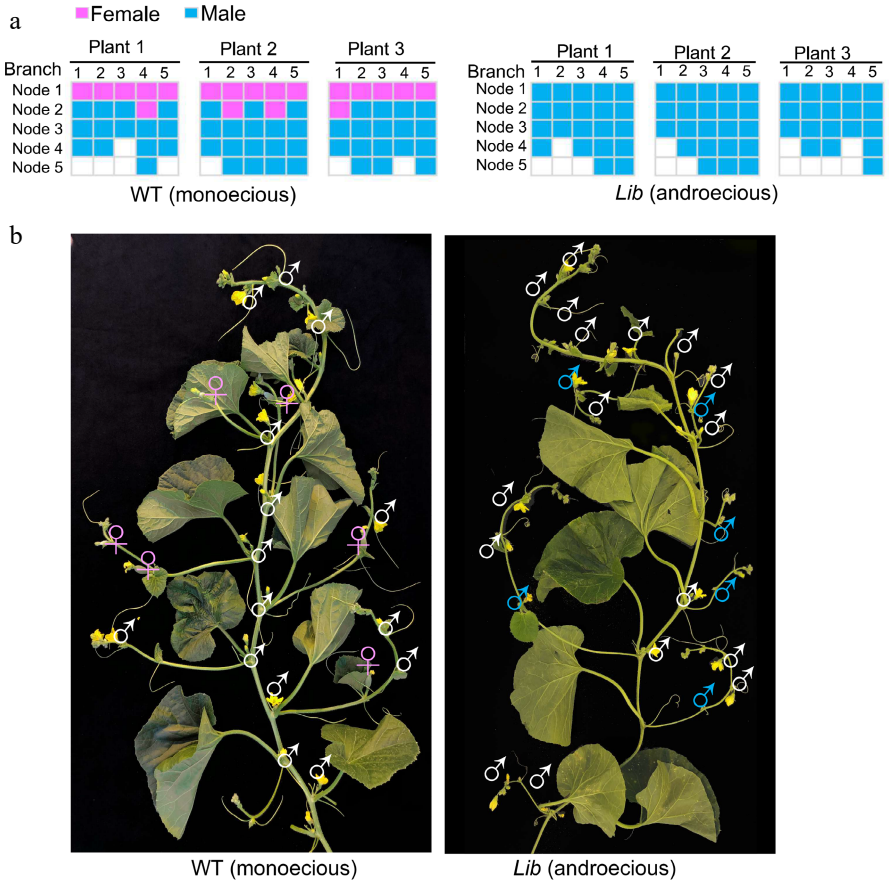
Angiosperms usually produce bisexual flowers. In order to increase the genetic diversity of offspring, plants have evolved several systems to prevent self-fertilization, such as sexual differentiation[1]. The plants with sex differentiation can produce different types, such as monoecious, dioecy and so on. The evolution from hermaphrodite to unisexual flowers is controlled by sex-determining genes[2,3]. Floral control is important for agricultural breeding and production, such as F_1 hybrid seed production and fruit production of dioecious crops.

There are a large number of monoecious or dioecious species in cucurbitaceous, so it is a good plant model for studying sexual differentiation[1]. In melon, the flowers are bisexual



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Parental lines/F2 sibling	Nb.	Monoecious	Androecious
WT	10	10	0
Little boy mutant	10	0	10
F2 sibling	141	104	37

Fig. 1 Sexual morphs observed in monoecious melon and androecious *little boy* mutant[7]. (a) Graphical presentation of flower sexual types observed in wild type monoecious melon (*Cucumis melo*) and *Lib* (*Little boy*) androecious mutant. (b) Phenotype of *Lib* mutant. Female flowers (in pink) were transformed into male flowers (in blue) in *Lib* mutant. The inflorescence structure in the Lib mutant is not affected. (c) Segregation analysis of androecious phenotype in F_2 populations. Nb, total number of plants.

during the early stages of development, and then the arrest of the stamen or carpel primordium leads to sex determination, resulting in unisexual flowers. In the process of sex determination, *andromonoecious* (*M*)[4], *Andromonoecious* (*A*)[5] and *gynoecious* (*G*)[6] encode *CmACS-7*, *CmACS11* and *CmWIP1*. The interaction of *M*, *A*, and *G* genes has resulted in different sex types[6], but the relationship between sex-determining genes and sexual flower development remains a mystery.

On November 4th, 2022, Institute of Plant Sciences Paris-Saclay (IPS2), University of Paris-Saclay (Paris, France), Abdelhafid Benda mane's team published a research paper entitled 'The control of carpel determinacy pathway leads to sex determination in cucurbits' in the international authoritative academic journal *Science* (with Siqi Zhang and Fengquan Tan as co-first authors), shedding light on the mysteries of sex determination and the regulation of bisexual flower development[7].

The researchers isolated a female-to-male sex transition mutant in melon and identified the mutation as the carpel development gene *CRABS CLAW* (*CRC*) through genetic mapping (Fig. 1). The *CRC* was validated by virus-induced gene silencing (VIGS) and NGS-TILLING (targeted induced local genomic lesions based on second-generation sequencing) techniques. *In situ* hybridization and real-time quantitative PCR showed that the expression of *CRC* was inhibited by the sex-determined female flower gene *WIP1*, and genetic double mutant analysis showed that *WIP1* acted upstream and is epistatic against *CRC* in the sex determination pathway[7].

To investigate whether WIP1 is a direct suppressor of *CRC* expression, the authors used ampDAPseq to map genome-wide DNA binding of *WIP1*. The promoter of *CmCRC* was found to have a binding peak of CmWIP1 with a conserved WIP motif, but the *CmCRC* promoter could not be bound by *WIP1* mutant proteins G242R and S306F (Fig. 2a). Electrophoretic mobility change analysis (EMSA), ChIP-qPCR, luciferase reporting system were also used to further verify that CmWIP1 binds to the promoter of *CRC* and its binding inhibits the expression of *CRC* (Fig. 2b & c).

To uncover the molecular mechanism of WIP1-mediated *CRC* transcriptional suppression, IPS2 screened chaperone protein of WIP1 using a yeast two-hybrid system and found that WIP1 binds to the co-suppressor protein TPL. Further study showed that the physical interaction involves the N-terminal domain of CmWIP1 and the LisH, CTLH and CRA domains of TPL. The WIP1 N-terminal domain contains two conserved motifs N1 and N2. The three-dimensional structural model predicts that N1 and N2 may form a stem-ring structure, which is related to the interaction of TPL recruitment. The authors found that single N1 and N2 mutants partially affected the interaction, while N1-N2 double mutants completely lost the interaction. The interaction between WIP1 and TPL was further verified by transient expression and co-immunoprecipitation (Co-IP) in melon protoplasts. At the same time, it was found that the activity of *pCRC:LUC* reporter gene was significantly recovered when the N1 or N2 mutant proteins of WIP1 were expressed, indicating that WIP1 inhibited *CRC* expression through the binding of N1 and N2 motifs with TPL (Fig. 2e).

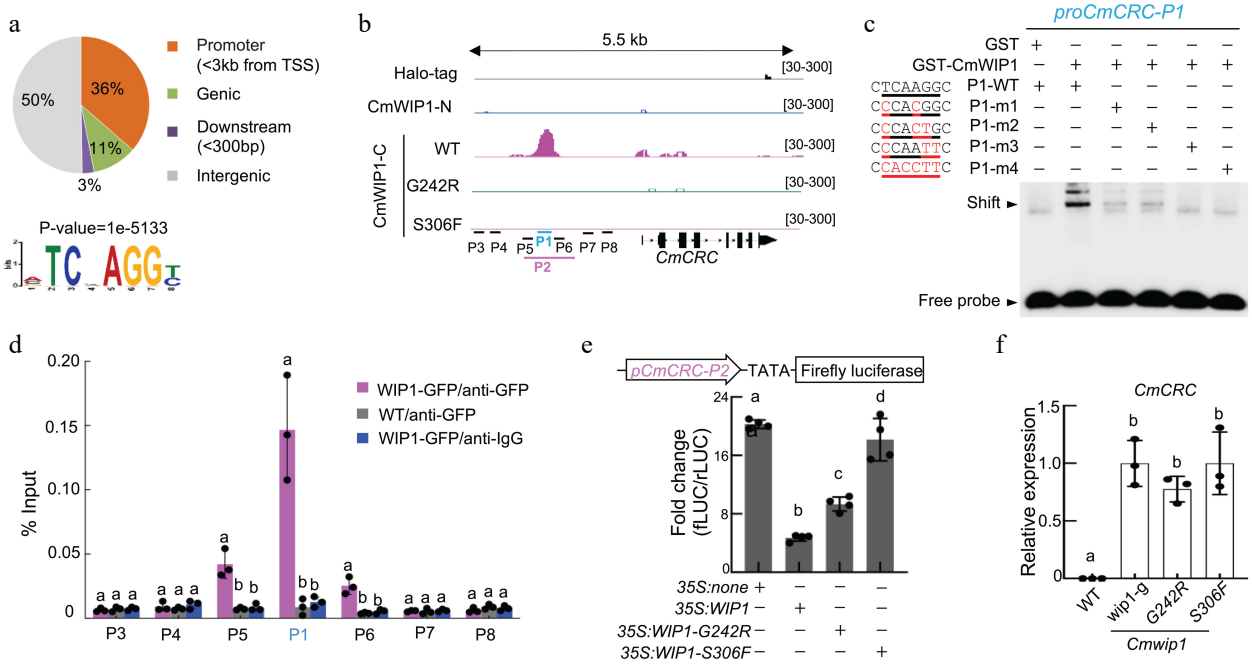

Fig. 2 CmWIP1 binds to the *CRC* promoter[7]. (a) Distribution of CmWIP1 DAP-seq peaks and CmWIP1 binding motif. (b) CmWIP1 binding at the *CmCRC* locus. Halo-tag, negative control; -N, N terminus; -C, C terminus; P1 to P8, promoter sequences used in EMSA, ChIP, and promoter activity assays. G242R and S306F mutations in WIP1 DNA binding domain. (c) EMSA assay. A5-μM GST or WIP1-GST protein and a 10-nM DNA probe were used. P1-m1 to P1-m4, mutant probes. GST, Glutathione S-transferase. (d) ChIP-qPCR assay in melon protoplast transformed with *35S:WIP1-GFP*. Results are expressed as a percentage of the INPUT fraction. Wild-type protoplasts and anti-IgG antibody were used as negative controls. (e) Promoter activity assays. Empty vector (*35S: none*), *35S:WIP1* or variants were co-transformed into melon protoplasts along with *pCmCRC-P2: fLUC*. Bars represent means ± SD (n = 4). (f) qRT-PCR analysis of *CmCRC*. Flower buds at stage 6 were used. *wip1* (*g*), a natural epigenetic mutant; G242R and S306F, TILLING mutants (14). In (d)–(f), bars represent mean ± SD (n = 3) and different letters denote significant differences ($p < 0.05$, one-way ANOVA with Tukey's post hoc test).

In order to verify the biological relevance of WIP1-TPL interaction in *CRC* inhibition, the male flowers of plants subjected to VIGS of TPL were partially feminized and even completely changed from male to female. Mutants with complete loss of TPL function screened using NGS-TILLING were unable to flower. Therefore, the authors performed phenotypic analysis of the single amino acid missense mutants and found that four mutants resulted in 30 to 40 percent female flowers developing where male flowers should have been. The WIP1-TPL interaction involved the N1 and N2 motifs of WIP1, and the authors also screened TILLING mutants in these two motifs and observed that they exhibited feminized characteristics, such as stigmas and ovule-bearing ovaries in the flowers that were male. Moreover, inhibition of *CRC* expression was removed in WIP1 N1 and N2 mutants. Since it has been previously reported that TPL recruits histone deacetylases to inhibit the expression of target genes, the authors analyzed the histone acetylation levels of *CRC* in male and female flowers, and the *CRC* region *H3K9ac* was significantly reduced in male flowers compared with female flowers. Thus, the WIP1-TPL complex binds to the promoter of *CRC* and deacetylates its gene region histone to inhibit its gene expression, thereby promoting male flower development (Fig. 3).

Stem cell termination in flower organs is a prerequisite for carpel development. Expression of *WUS* in male and female flowers during sex transition was detected by *in situ* hybridization. No *WUS* mRNA was detected in female flowers at stage 6, but the level of *WUS* mRNA in the carpel primordium of male flowers at the same stage was very high. It was found that the expression of *WUS* in the carpel primordium of male and female flowers increased by 3 times. In *Arabidopsis*, *CRC* inhibits *WUS* expression by up-regulating *YUC4* and down-regulating *TRN2*. Similarly, three *YUC* genes were up-regulated and *TRN2* was down-regulated in the micro dissected carpels of female

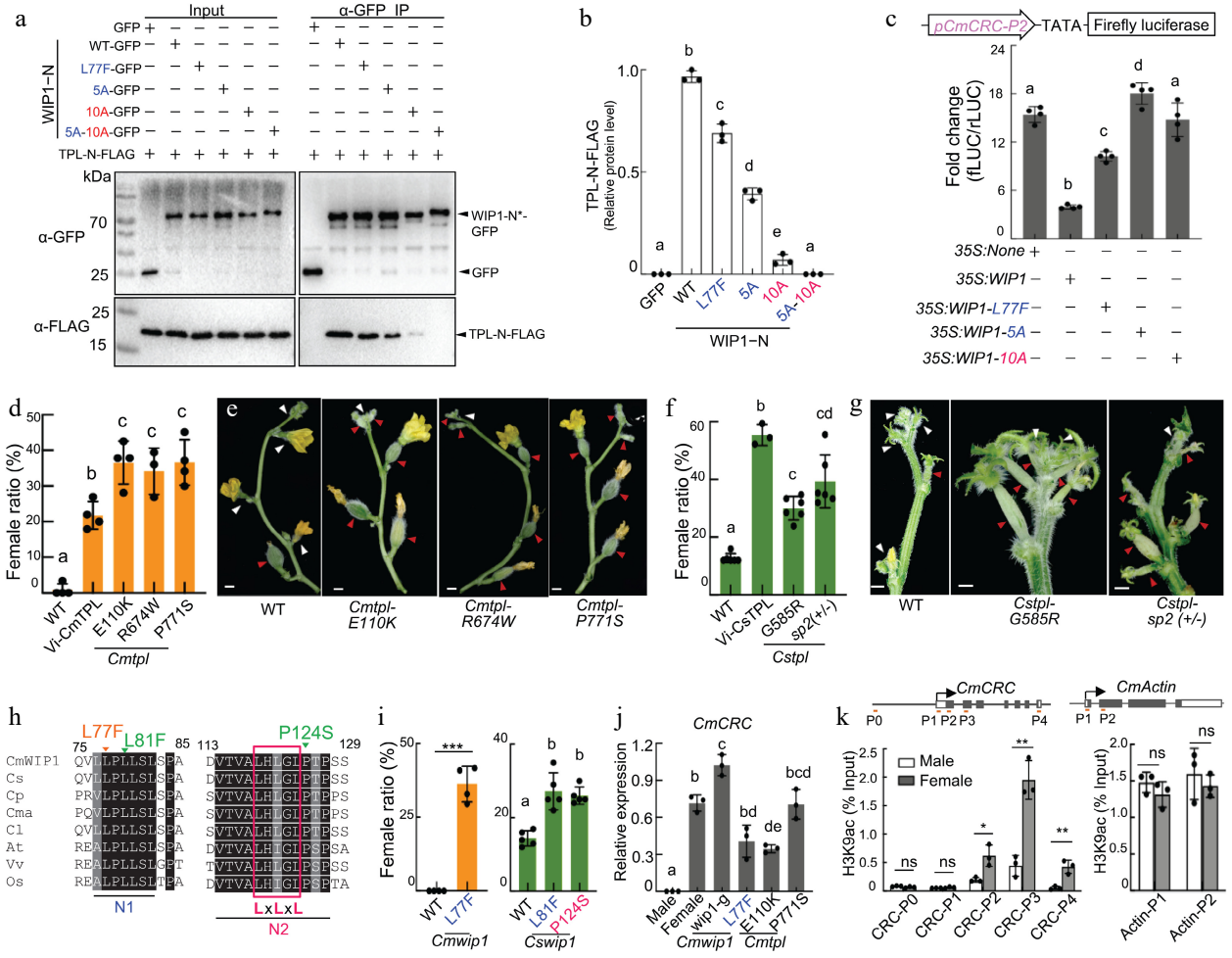

Fig. 3 WIP1 recruits Topless to repress *CRC*[7]. (a) Co-IP assays. GFP-tagged WIP1−N or variants (asterisk) were co-transformed into melon protoplasts along with FLAG-tagged TPL-N. (b) Quantification of FLAG-tagged TPL-N from Co-IP assays in (a). (c) Promoter activity assay. Empty vector (*35S: none*), *35S:WIP1* or variants were co-transformed into melon protoplasts along with *pCmCRC-P2*: *fLUC*. Statistical analysis of female flower ratio in monoecious (WT), VIGS-*CmTPL* (Vi-*CmTPL*) and *tpl* mutants in (d) melon and (f) cucumber. Flower phenotypes of tpl mutants in (e) melon and (g) cucumber; white and red triangles denote male and female flowers, respectively; scale bars, 5 mm. (h) Sequence alignment of WIP1 and homologous proteins showing N1 and N2 motifs. Initials of species are indicated. LxLxL, EAR motif. Induced mutations in melon (orange) and cucumber (green) are shown. (i) The female ratio [females/(females + males)], in percent, of nodes 2 to 10 of lateral branches (melon) and nodes 1 to 20 of main stem (cucumber). qRT-PCR analysis of (j) *CmCRC* and ChIP-qPCR analysis of (k) H3K9ac at stage 6 flower buds. *CmACTIN1*, control. Bars represent means ± SD (n = 3–5); different letters denote significant differences $p < 0.05$, one-way ANOVA with Tukey's post hoc test). * $p < 0.05$, ** $p < 0.01$ (Student's t test).

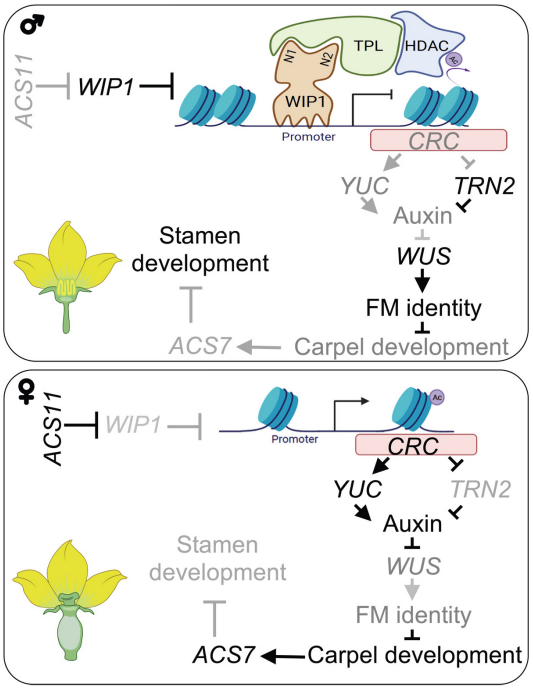

Fig. 4 Model explaining how WIP1-TPL controls the expression of *CRC* to lead to male flower development[7].

flowers compared with those of male flowers. Together, these results suggest that *CmWIP1* promotes male flower development by inhibiting *CRC* expression, thereby interfering with floral meristem determination in the carpel primordium (Fig. 4).

Following the 2009 discovery[5] that *CmWIP1* is a female determining gene, this study reveals for the first time the transcriptional and epigenetic mechanisms of *CmWIP1*, and answers the outstanding questions about the relationship between sex determination and classical bisexual flower development. Together with the *ACS ethylene synthesis* gene pathway to regulate stamen development, the gene regulatory network for sex determination in Cucurbitaceae crops was improved.

Dr. Abdel Bendahmane, senior researcher at the French National Institute for Agri-Food and Environment (INRAE), the French National Center for Scientific Research (CNRS), and the Paris-Sacre Botanical Institute of the University of Paris Sacre, is the corresponding author of the paper, and postdoctoral fellows Zhang Siqi and Tan Fengquan are co-first authors of the paper. Professor Moussa BENHAMED of the University of Paris Sacre also took part in the study. The research was supported by the European Research Council (ERC) and the French National Research Agency (ANR).

It is worth mentioning that Abdel Bendahmane's team have been engaged in related research on plant sex determination for a long time, and have identified and solved the key genes of cucurbit crop sex determination *CmACS-7*, *CmWIP1* and *CmACS11*, etc., and have achieved a series of important research results, and are experts in the field of plant development[4–7].

Author contributions

The authors confirm contribution to the paper as follows: performed experiments, and finished the writing of the manuscript: Zhang K; study conception and design: Ma G; data collection: Lin T; draft manuscript preparation: Li J. All authors reviewed the results and approved the final version of the manuscript.

Data availability

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

Conflict of interest

The authors declare that they have no conflict of interest.

Dates

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