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Mystery in genetics: PUB4 gives a clue to the complex mechanism of CLV signaling pathway in the shoot apical meristem

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Postembryonic growth and development in higher plants are ultimately reliant on the activity of meristems, where the cells divide frequently to provide source cells for new organs and tissues while in part maintain their pluripotent nature as stem cells. The shoot apical meristem (SAM) is maintained throughout the life of plants and responsible for the development of all areal tissues. In *Arabidopsis thaliana*, the size of SAM is controlled by a peptide ligand, CLAVATA3 (CLV3). Previously, genetic studies have identified several genes that function downstream of CLV3, many of which, intriguingly, encode receptors. Recently we identified an E3 ubiquitin ligase, PLANT U-BOX 4 (PUB4), as a key regulatory component of root meristem maintenance that functions downstream of an exogenous synthetic CLV3 peptide. Here, we report an additional function of *PUB4* in the SAM.

CLV3 is a peptide ligand that is secreted by stem cells and regulates the expression of *WUSCHEL* (*WUS*), a homeodomain transcription factor that is required for specifying stem cell identity. CLV1 is the major receptor-like kinase for CLV3 signaling and is shown to interact specifically with CLV3 *in vitro*.[1-3] CLV2, a receptor-like protein, associates with CORYNE (CRN)/SUPPRESSOR OF LLP1 2 (SOL2), a membrane associated protein pseudo-kinase with a short extracellular domain.[4-6] RECEPTOR-LIKE PROTEIN KINASE 2 (RPK2) is another receptor-like kinase that is essential for CLV3 signaling.[7] Genetic approach has demonstrated that these 3 receptor complexes function downstream of CLV3 basically, independently of each other, however, it remains unclear whether they are functionally redundant or each has distinct functions.[6-8,15] Previously, we have shown that a synthetic CLV3 peptide, MCLV3 could be a useful tool for investigating downstream components of the CLV signaling pathway.[7,9] Here we report the defects of SAM in a recently identified MCLV3 resistant mutant, *pub4*,[10] and discuss the possible relationship of its causal gene with the CLV signaling pathway.

PUB4 functions in the SAM

MCLV3 was able to mimic the *CLV3* over-expression phenotype when treated to wild type plants and induces SAM termination[9]. On the other hand, *pub4* mutants were able to maintain SAM activity and to induce bolting on the media containing MCLV3 (**Fig. 1A and B**). Furthermore, the size of SAM in *pub4* was slightly larger than that in wild type in the absence of MCLV3 (**Fig. 1C–E**). These results suggest that *PUB4* functions not only in the RM but also in the SAM. To examine whether *PUB4* regulates stem cell proliferation in the SAM, we observed the expression of the stem cell marker gene, *CLV3*. Histological analysis revealed that the expression region of CLV3 was enlarged in *pub4* (**Fig. 1F and G**). This observation suggests that *PUB4* regulates stem cell pool in the SAM, in a similar fashion to the known *CLV*-related genes.

pub4 enhances *clv* phenotype

In order to examine the genetic relationship between *PUB4* and known *CLV*-related genes, we constructed a series of double mutants using null alleles of *clv1, clv2, clv3* and *pub4*, and a point mutation allele of *rpk2*. Unexpectedly, *pub4* enhanced the phenotype in all genotypes, as *clv1 pub4, clv2 pub4, rpk2 pub4* and *clv3 pub4* showed larger SAM than each single mutant (**Fig. 2A–J**). Notably, the size of SAM was dramatically increased in *clv2 pub4* and *clv3 pub4*, and the expression regions of *CLV3* or *WUS* were enlarged in the double mutants (**Fig. 2H, I, K, L**). In order to quantify the phenotype, we evaluated the number of carpels, which is known to reflect the relative size of the flower meristem and has been used as an indicator for the *clv* phenotype. While wild type flowers exhibited invariantly 2 carpels per flower, *pub4* developed an increased number of carpels, especially in the flowers produced in the later developmental stage (**Fig. 2M**). In consistent with the phenotype in the SAM, the number of carpels is increased in the double mutants to the greatest extent in *clv2 pub4* and *clv3 pub4* (**Fig. 2N**). Moreover, *clv2 pub4* and *clv3 pub4* displayed

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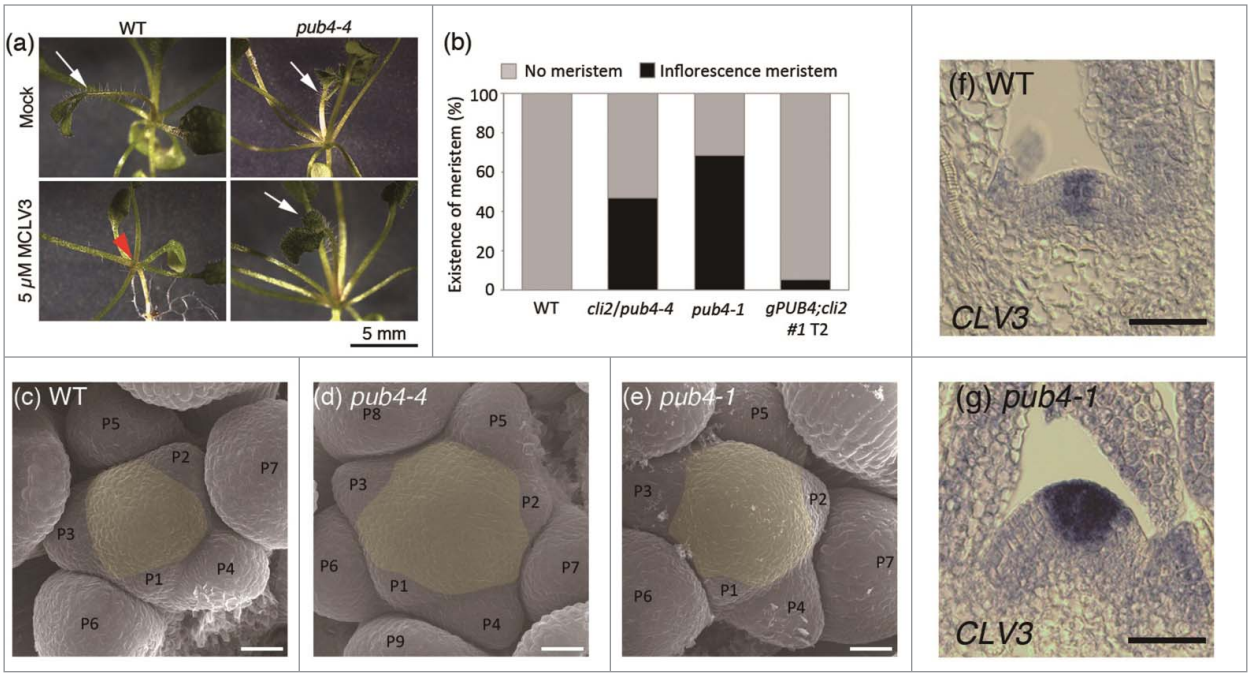

Figure 1. Phenotypes of pub4 mutants in the presence and absence f exogenous MCLV3 peptides (**A**) Twenty-day-old seedlings of wild-type and *pub4-4* grown on the media with or without 5 μM MCLV3. Arrows, inflorescence stems; arrowhead, terminated SAM. (**B**) Ratios of plants that produced inflorescence meristem in the presence of 5 μM MCLV3. (**C-E**) Scanning electron micrographs (SEM) of wild type (**C**), *pub4-4* (**D**) and *pub4-1* (**E**). The areas of inflorescence meristems are colored with yellow. (**F, G**) RNA *in situ* hybridization of wild type and *pub4-1* SAM performed using the CLV3 antisense probe. Scale bars, 5 mm (**A**), 20 μm (**C-E**), 50 μm (**F, G**). MCLV3 treatment, SEM analysis and in situ mRNA hybridization are performed as described previously.[7]

drastic defects in the architecture of mature plants, including increased number of rosette leaves, delayed flowering time, longer pedicel length and short inflorescence stems (**Fig. 2O–R**). These genetic evidences suggested the existence of a complicated regulatory mechanism that controls the homeostasis of SAM by *CLV*-related genes.

Among genetic interactions, "additive" effects can be seen when the genes act in parallel, or independently.[11] Thus, the

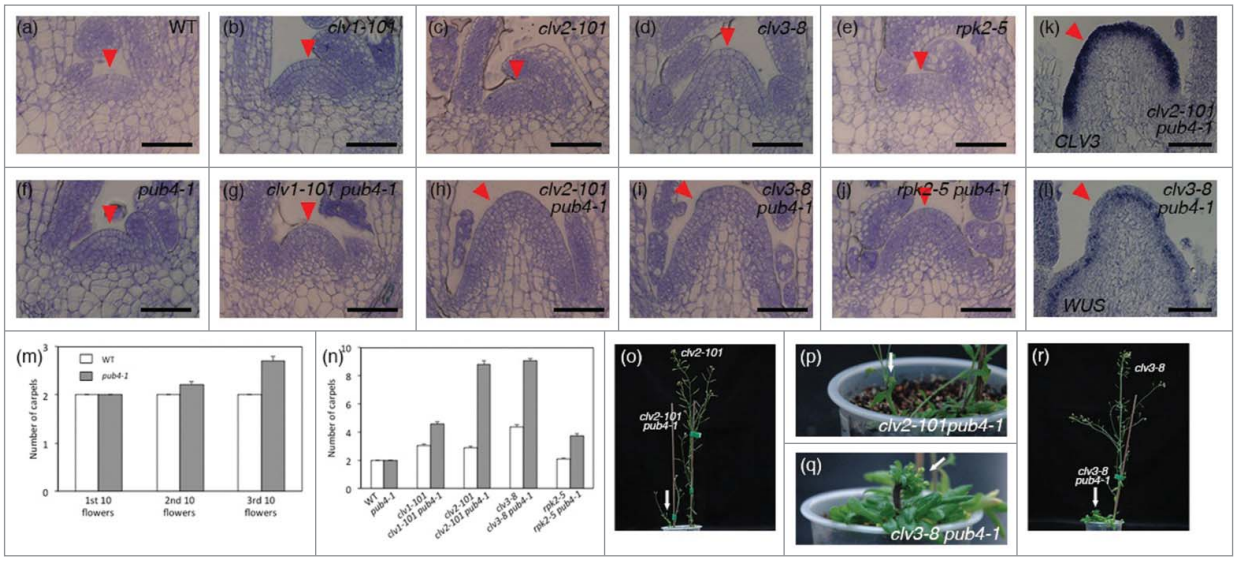

Figure 2. Genetic interactions of PUB4 and CLV-related genes. (**A-J**) SAMs for 7-day-old seedlings of each genotype. (**K, L**) RNA *in situ* hybridizations of *clv2-101 pub4-1* (**K**) and *clv3-8 pub4-1* (**L**), performed using the *CLV3* and *WUS* antisense probe, respectively. (**M**) The number of carpels per flower in wild type and *pub4-1*. The data was obtained by averaging the number of carpels per flower of each 10 flowers from first 30 flowers. (**N**) The number of carpels in single- and double- mutants. The first 10 flowers are used for the measurement. (**O-R**) Mature plants of *clv2-101 pub4-1* (**O, P**) and *clv3-pub4-1* (**Q, R**). Scale bars, 50 μm (**A-L**). Arrowheads, SAM (**A-L**); allows, main inflorescence stem (**O-R**). Plant materials and growth condition are used as described previously.[7,10,13]

enhancement of *clv*-phenotype by *pub4* mutation can be interpreted as a sign for the absence of functional relationships between *PUB4* and components of CLV signaling pathway. In this case, the resistance of *pub4* to MCLV3 peptide might be just a side effect, which is resulted from a defect in another pathway that regulates stem cell in SAM. However, our observation suggests that the effect of *pub4* on *clv* mutations is rather "synergistic" than "additive." Moreover, the "synergistic" effect of *pub4* was prominent when combined either with *clv2* or *clv3*. These results lead us to speculate that *PUB4* is not fully independent of CLV pathway, and that *pub4* mutation could shed some light on the potential difference and/or diversity of CLV components for the function in stem cell maintenance. Similar gene-specific interactions have been reported for the genes encoding BAM (BARELY ANY MERISTEM) receptors: *bam* mutations suppress *clv3* phenotypes, have no effect on *clv2* phenotypes, and synergistically enhance *clv1* phenotypes.[12] Interestingly, a recent report has demonstrated that this genetic interaction is result from a transcriptional regulation of BAM genes by CLV1 signaling.[14] Although further detailed analyses is needed to determine how *PUB4* interacts with CLV signaling pathway, we suggest that sharing such intriguing genetic data will be useful for our understanding of the complex mechanism that control homeostasis in SAM.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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