The Ste20-like FvM4K1 Regulates Organ Size via Hippo Pathway in Strawberry (*Fragaria vesca*)

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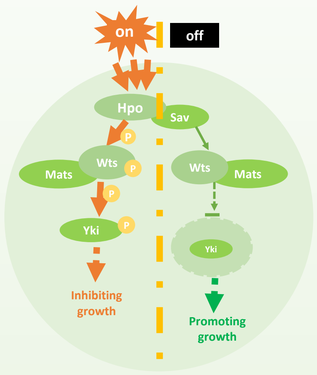
ABSTRACT

One of the main marketing preferences of strawberry (*Fragaria vesca*) is its fruit size, the bigger the better. Organ size control is complicated yet highly coordinated involving various signalling molecules and pathways. The Hippo signalling pathway is discovered to be the key regulator of organ size in fruit fly. This pathway was also identified from the budding yeast where the main component Ste20 plays vital role in cell division. Here, we report the identification of an Ste20-like protein, FvM4K1 from wild strawberry plant. FvM4K1 partially rescued the growth defect of yeast mutant *ste20Δ*, indicating FvM4K1 is most likely the homolog of Ste20. FvM4K1 interacts with the two FvMOB1s, the Mob1/Mats homologs in the Hippo pathway in yeast. The interactions between FvM4K1 and FvMOB1s were further confirmed by bimolecular fluorescence complementation where the N-terminal domain of FvM4K1 was shown to be essential for this interaction. Down regulation of FvM4K1 by RNAi results in dwarfed plants with much smaller vegetative and reproductive organs. These are caused by much reduced cell numbers and size as observed in the leaves and petals of the RNAi plants. Further, the RNAis seedlings were sucrose dependent and failed to grow in sucrose lacking media. Taken together, strawberry FvM4K1 functions as the main component of the Hippo pathway. Via the interaction with FvMOB1s and perhaps sugar signalling pathway FvM4K1 plays essential roles in organ size control in strawberry. These findings could contribute to our understanding towards the Hippo pathway in higher plants as virtually nothing known at present.

BACKGROUND

The Hippo pathway is discovered and identified as a key regulator of organ size. This pathway is conserved between drosophila and mammals. In drosophila, five core components were found in this pathway and these are Ste20-like kinase Hippo (Hpo), its associated scaffold protein Salvador (Sav), the NDR family kinase Warts (Wts) and its adaptor Mats, and the transcriptional co-activator Yokie (Yki). Hpo, in conjunction with Sav, phosphorylates hence activates the Mats and Wts complex. The activated Wts then phosphorylates Yki, leading to its cytoplasmic sequestration and inactivation, thus suppressing cell proliferation and inducing apoptosis. At the absence of Yki repression by Hippo pathway, activated Yki targets into the nucleus where it induces the expression of genes related to cell proliferation and anti-apoptosis by binding and activating a transcription factor.



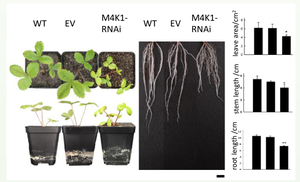
Recent study in the model plant Arabidopsis found that the Hpo and Mats homologs play important roles in cell proliferation. However, further studies in Arabidopsis and indeed a different higher plant(s) are required to clarify the role of Hippo pathway in organ size control so that this important pathway in higher plants could be further verified and constructed.

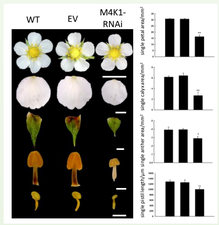
RESULTS

FvM4K1 Regulates Organ Size in Strawberry

RNAi-M4K1 strawberry plants have reduced height with smaller organs

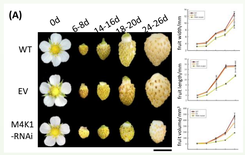
To explore the biological function of FvM4K1 in strawberry, M4K1-RNAi plants were generated. The transcript level of *FvM4K1* were reduced by 90 % in these plants. The 6-week-old plants of Hawaii 4 (WT), transgenic plants containing empty vector (EV) and M4K1-RNAi construct (M4K1-RNAi) were observed, showing that the M4K1-RNAi plants have **smaller leaves, shorter stems and roots**.

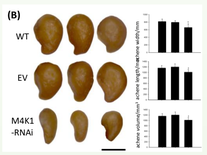




The floral organs of the RNAi strawberry were also observed. This showed that the petals, calyxes, anthers and pistils were all significantly smaller than those of WT and EV plants. The average size of petals of the M4K1-RNAi plants was 16.3 mm^2 compared to 30.74 mm^2 in the control petals, i.e., nearly reduced by 50%.

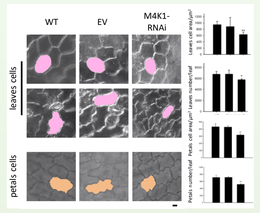
The sizes of the fruits were observed and recorded during different developmental stages. It was found that the most remarkable difference was observed 24-26 days after flowering where the RNAi fruits were only half the size of the control fruits (A). The seeds (achene) of the RNAi fruits were also much smaller than the controls (B).





M4K1-RNAi plants have less and smaller cells

The cell number and cell size of the leaves and petals of 4-week-seedlings were observed under confocal microscope. The epidermal cells of M4K1-RNAi leaves were smaller (630 μm^2 on average) compared to those of the controls (900 μm^2 on average). The number of pavement cells in **the M4K1-RNAi leaves was reduced** to be 85% of that of the WT (cells shaded in pink, top and middle panel). Similarly, the cell size and cell number in the **M4K1-RNAi petals were significantly reduced** (cells shaded in orange, bottom panel).

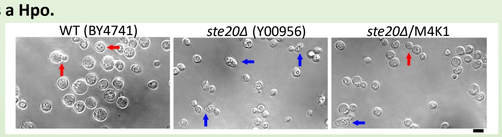


RESULTS

FvM4K1 Functions as Hpo

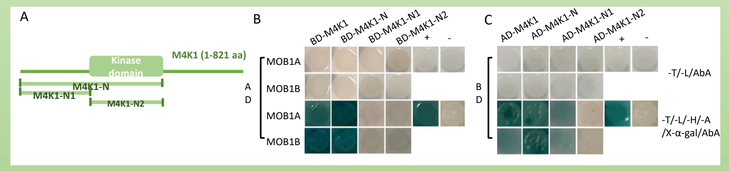
FvM4K1 rescues yeast *ste20Δ* mutant

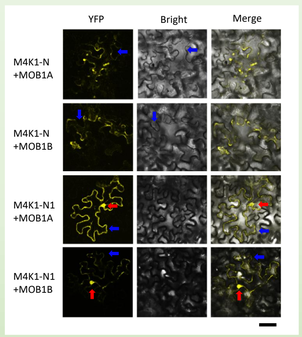
In order to see if FvM4K1 is an ortholog of yeast Ste20p complementation assay was carried out. FvM4K1 was introduced into a Ste20p lacking mutant strain *ste20Δ*. As shown below the mutant cells exhibited defects in the budding site selection (blue arrows). In contrast, 70% of yeast cells from FvM4K1 expressing *ste20Δ* cells (*ste20Δ*/M4K1) were normal in budding site selection like the WT (red arrows). Therefore, FvM4K1 can partially restore the abnormal budding phenotype of *ste20Δ,* **hence functions most likely as a Hpo.**



FvM4K1 interacts with FvMOB1s, the core component in the Hippo pathway

AtMOB1s were identified as the only other Hippo pathway components in Arabidopsis. To see if strawberry also has FvMOB1s yeasts two-hybrid screen was carried out. As shown in the figure below, the full length FvM4K1 was indeed to interact with both FvMOB1A and FvMOB1B. Further, the N-terminus containing the kinase domain of M4K1-N (amino acids 1–494) still interacted with FvMOB1s no matter they were fused to AD or BD. However, only AD-M4K1-N1 (amino acids 1-237) interacted with BD-MOB1A and BD-MOB1B while no interactions were found between BD-M4K1-N1 and AD-MOB1s. The kinase domain M2K1-N2 did not interact with MOB1s. Therefore, **N-terminal domain of FvM4K1 is important for its interaction with FvMOB1s which does not rely on the kinase domain.**

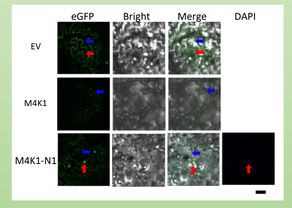




In order to further confirm the importance of N-terminus of FvM4K1 for interaction between FvM4K1 and FvMOB1s bimolecular fluorescence complementation (BiFC) analysis was carried out in tobacco. As seen in the figure, YFP florescent signals were detected at the plasma membrane (PM, blue arrows) when M4K1-N and MOB1s were co-expressed, indicating they are indeed interact, and the sites of action are at the PM. Intriguingly, when M4K1-N1 co-expressed with MOB1s, there were strong signals found at the PM as well as the nucleus (red arrows), confirming **the interaction between FvM4K1 and FvMOB1s and the importance of the N-terminus for nucleus targeting (N1).**

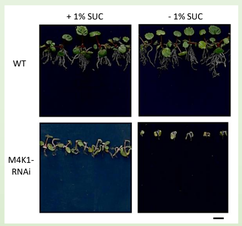
Subcellular localization of FvM4K1

Subcellular localization of M4K1 in tobacco was consistent with BiFC assay above. The full length M4K1-GFP was localized at the PM while the M4K1-N1-GFP signal was detected both at the PM (blue arrows) and in the nucleus (red arrows)



RESULTS

RNAi Plants Are Hypersensitive to Sucrose



The growth of M4K1-RNAi was severely supressed when sucrose was absent in the media. Seeds were germinated on ½ MS media with or without 1% sucrose. While the WT strawberry seedlings grew well on both sucrose containing and lacking media with green well developed true leaves the RNAi seedlings of strawberry only grew in sucrose containing media with smaller but developed true leaves. The RNAi seedlings on sucrose lacking media had only developed small cotyledons which were yellow, and no true leaves were observed. The roots were also affected where the RNAi plants had hardly any roots when grown on sucrose lacking media while the WT have well developed primary and lateral roots. 42-day-old seedling were shown.

SUMMARY

- FvM4K is a Ste20-like1 protein involved in Hippo pathway
- FvM4K1 interacts with other Hippo pathway components, FvMOB1A and 1B
- Reduced expression of *FvM4K1* by RNAi results in much reduced organ size in both vegetative and reproductive tissues
- Sugar may play an essential role in the function of FvM4K1 in the regulation of organ size in strawberry plant

FUTURE WORK

- The kinase activity of FvM4K1 will be confirmed to further elucidate its function in organ size control

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