RGC Ref. No.:
UGC/FDS25/M05/15
(please insert ref. above)

RESEARCH GRANTS COUNCIL
COMPETITIVE RESEARCH FUNDING SCHEMES FOR
THE LOCAL SELF-FINANCING DEGREE SECTOR

FACULTY DEVELOPMENT SCHEME (FDS)

Completion Report
(for completed projects only)

Submission Deadlines:
1. *Auditor's report with unspent balance, if any: within **six** months of the approved project completion date.*
2. *Completion report: within **12** months of the approved project completion date.*

Part A: The Project and Investigator(s)

1. Project Title

Molecular Characterization of Endocytic Recycling in Plant Cells

2. Investigator(s) and Academic Department(s) / Unit(s) Involved

Research Team	**Name / Post**	**Unit / Department / Institution**
Principal Investigator	Dr. Angus LAW / Teaching Fellow I	School of General Education and Languages / Technological and Higher Education Institute of Hong Kong
Co-Investigator(s)	NIL	NIL
Others	NIL	NIL

3. Project Duration

	Original	**Revised**	**Date of RGC / Institution Approval** *(must be quoted)*
Project Start Date	01/01/2016	NIL	NIL
Project Completion Date	3/12/2018	30/06/2019	6/11/2018
Duration *(in month)*	36	42	6/11/2018
Deadline for Submission of Completion Report	31/12/2019	30/06/2020	6/11/2018

Part B: The Final Report

5. Project Objectives

5.1 Objectives as per original application

1. To study the subcellular localization of Arabidopsis EHD in plants

2. To characterize the biochemistry and dynamics of EHD-positive organelles in plants
3. To study the molecular mechanism of Arabidopsis EHD in endocytic recycling of PM receptor
4. To study the function of selective Arabidopsis EHD in Arabidopsis plants

5.2 Revised objectives

Date of approval from the RGC: NIL

Reasons for the change: NIL

5.3 Realisation of the objectives

Objective 1: To study the subcellular localization of Arabidopsis EHD in plants (100% achieved)

Constructs for transient and stable expression in plants have been generated by fusion of the full-length coding sequence of Arabidopsis EHD1 (AtEHD1) with fluorescent protein at N- and C-termini, which subsequently used for subcellular localization study in tobacco and Arabidopsis cell lines and seedlings with the use of confocal imaging on live samples, confocal immunofluorescent labeling and TEM techniques. Endogenous localization of EHD1 was studied with the use of highly specific anti-EHD1 antibodies, raised by purified anti-sera extracted from rabbit and rat.

With a combination of confocal imaging on live samples, confocal immunofluorescent labeling and immuno-EM, we have identified that EHD1 localized at an organelle distinct from Golgi, *trans*-Golgi network (TGN), prevacuolar compartment (PVC) and GNOM-positive recycling compartment. Under TEM, EHD1-positive organelle is morphological similar to, but biochemically distinct from TGN (see objective 2).

Objective 2: To characterize the biochemistry and dynamics of EHD-positive organelles in plants (100% achieved)

Pharmacological studies have been carried out on cell lines and Arabidopsis transgenic plants stably expressing fluorescent protein-fused AtEHD1 generated under objective 1. With the use of endocytic tracker FM4-64, we have shown that EHD1-positive organelles resided on the endocytic pathway. Furthermore, with the use of protein trafficking inhibitors such as brefeldin A (BFA), concanamycin A (ConcA) and wortmannin (Wort), we have shown that unlike TGN, the EHD-positive organelles resided on a BFA-sensitive but ConcA- and Wort-insensitive pathway.

By capturing time-lapse videos of Arabidopsis seedlings stably expressing EHD1 and TGN marker in double transgenic Arabidopsis lines under spinning disk microscope, and analysed the dynamics of the puncta with imaging software, we have further confirmed that the EHD1-positive organelle and TGN are distinct in terms of localization and dynamics.

Objective 3: To study the molecular mechanism of Arabidopsis EHD in endocytic recycling of PM receptor (80% achieved)

Dominant negative (DN) mutants of Arabidopsis EHD1 with mutations in the key resides of the EH domain and NTP-binding G domain were generated in order to investigate the effects of EHD1 on protein trafficking. Results indicated that some of the DN mutants have defects in cytokinesis. Proteomic studies on subcellular fractions enriched with EHD1-positive organelle have also identified a few endocytic/cytokinetic Rab proteins, further confirm the involvement of EHD1-positive organelle in these processes. With a probable cargo in late cytokinesis identified (see objective 4), further works could be done on elucidating the molecular partners of EHD1, and the effects of the DN mutants in trafficking of the cytokinesis-specific syntaxin.

Objective 4: To study the function of selective Arabidopsis EHD in Arabidopsis plants (100% achieved)

We have generated a hairpin RNAi mutant which successfully knockdown expression of AtEHD1 under the control of dexamethasone (DEX)-inducible promoter (*dex::RNAi-ehd1*). When triggered with DEX, the mutant has shown mild dwarfism phenotype, with defects in cell division in root meristem. At late cytokinesis, we have found the accumulation of a cytokinesis-specific syntaxin, but not other plasma membrane proteins, in the EHD1-positive organelle, hinted that EHD1 participated in the endocytic recycling of the syntaxin in the late step of cytokinesis.

5.4 Summary of objectives addressed to date

Objectives *(as per 5.1/5.2 above)*	**Addressed** *(please tick)*	**Percentage Achieved** *(please estimate)*
1. *To study the subcellular localization of Arabidopsis EHD in plants*	✓	100
2. *To characterize the biochemistry and dynamics of EHD-positive organelles in plants*	✓	100
3. *To study the molecular mechanism of Arabidopsis EHD in endocytic recycling of PM receptor*	✓	80
4. *To study the function of selective Arabidopsis EHD in Arabidopsis plants*	✓	100

6. Research Outcome

6.1 Major findings and research outcome
(Maximum 1 page; please make reference to Part C where necessary)

Major findings

In this research, we have provided functional and morphological evidences to demonstrate that EHD1-positive endocytic PM-recycling vesicles contributed to cell plate formation and root development in Arabidopsis.

With the use of confocal live imaging and immunolabeling, we have demonstrated that the organelles marked by Arabidopsis EHD1 (AtEHD1) is distinct from Golgi, *trans*-Golgi network (TGN), prevacuolar compartment (PVC) and GNOM-positive recycling compartment. Immunogold labeling of thin sections cut from high-pressure frozen/freeze-substituted samples of tobacco BY-2 cell or Arabidopsis with EHD1 antibodies revealed the ultrastructure of EHD1-positive organelles. Specific labeling in both cell types can be found on vesicular structures in close proximity to the TGN, as cluster of vesicles, but occasionally as a single discrete vesicle. Specific labeling could also be found on budding/fusion profiles of cell plate. We have clearly demonstrated the distinct localization of EHD1.

We have utilized endocytic tracer FM4-64 and a number of protein trafficking inhibitors to understand the nature of protein trafficking pathway marked by EHD1-positive organelles. Results indicated the EHD1-positive organelles lie on the endocytic recycling pathway and function in the recycling of plasma membrane proteins from TGN, the early endosome in plant cells. Dynamic analysis of puncta between EHD1 and TGN marker SYP61 further confirmed that the organelles are distinct, yet probable fission between the puncta could also be found.

Functionally, we have identified the role of EHD1 in accumulating a cell plate-specific syntaxin in late cytokinesis. Interestingly, other plasma membrane-recycling cargoes such as PIN2 and SCAMP1 are not affected in the dexamethasone (DEX)-inducible RNAi mutants (*dex::RNAi-ehd1*), while the mutants have displayed mild dwarfism phenotype and defects in cell division, suggesting the uniqueness of EHD1 in specific cargo recycling and cell plate formation during late cytokinesis.

Taken together, we have provided evidences that EHD1 functions in endocytic recycling of specific cargo to the cell plate, and are functionally required for proper root growth, highlighting their contributions to the biogenesis of cell plate and cytokinesis. DEX-inducible mutational analysis suggested that the EHD1-marked recycling pathway is selective for specific cargo as PIN2 and SCAMP1 trafficking are unaffected in the deficiency of EHD proteins, rendering it an ideal marker for a specific endocytic recycling pathway. Morphological analysis using confocal microscopy, TEM and immuno-EM indicates that EHD1 marks a novel population of vesicles in close proximity to the TGN but are distinct from the parts of TGN labelled by SCAMP1 and VHAa1. Pharmacological analysis confirmed the distinction between the EHD1-marked vesicles and other parts of the TGN/EE. In short, we have provided functional and morphological evidence that EHD1 lies on an endocytic recycling pathway for specific cargo to the cell plate.

Research outcome

The research has yielded one manuscript for submission (currently under review, Law et al.) and three presentations in the following conferences:

1. International Conference in Arabidopsis Research 2016
2. Plant Biology 2017
3. Japan-Taiwan Plant Biology 2019

6.2 Potential for further development of the research and the proposed course of action *(Maximum half a page)*

With our understanding on the involvement of EHD1 in accumulating a cell plate-specific syntaxin during late cytokinesis, further research could be directed on understanding the mechanism and requirement of molecular machinery for endocytic recycling vesicles formation in this process. High-resolution time-lapse imaging and dynamics study on the developing cell plate at the background of knockdown expression of EHD1 will further shed lights on the involvement of the pathway in cell plate formation.

7. Layman's Summary

(Describe in layman's language the nature, significance and value of the research project, in no more than 200 words)

Over the decade, the field has gained better understanding on the existence and mechanism of endocytosis in plant cells, which are crucial for regulating the signaling receptor density on cell surface and the composition of plasma membrane (PM). On the contrary, recycling of endocytosed proteins and the nature and identity of PM-recycling carriers in plants are much less characterized and has remained elusive.

In this project, we have utilized Arabidopsis EHD1 (AtEHD1) proteins as molecular marker to identify recycling compartment in plant cells. With a combination of the state-of-the-art cellular, molecular, biochemical and cell imaging techniques, we have revealed that AtEHD1 localized on a previously uncharacterized compartment, which is distinct from other endocytic compartments in terms of morphology, dynamics and biochemistry. Genetic approach has been used to knockdown expression of EHD1, which has resulted in mild dwarfism phenotype and defects in cytokinesis and cell division in the mutants, potentially via regulating endocytic recycling of a crucial cell plate syntaxin during late cytokinesis.

The project has shed light on the role of endocytic recycling in regulating cell plate formation. Further research on the pathway will further reveal the molecular machinery in building the recycling vesicles.

Part C: Research Output

8. Peer-Reviewed Journal Publication(s) Arising Directly From This Research Project
(Please attach a copy of the publication and/or the letter of acceptance if not yet submitted in the previous progress report(s). All listed publications must acknowledge RGC's funding support by quoting the specific grant reference.)

The Latest Status of Publications				Author(s) *(denote the corresponding author with an asterisk*)*	Title and Journal / Book *(with the volume, pages and other necessary publishing details specified)*	Submitted to RGC *(indicate the year ending of the relevant progress report)*	Attached to this Report *(Yes or No)*	Acknowledged the Support of RGC *(Yes or No)*	Accessible from the Institutional Repository *(Yes or No)*
Year of Publication	**Year of Acceptance** *(For paper accepted but not yet published)*	**Under Review**	**Under Preparation** *(optional)*						
		✓		**Angus Ho Yin Law*, Yonglun Zeng, Hao Wang, Wing Hang Wu, Liwen Jiang***	**EHD1-positive Endocytic PM-Recycling Vesicles Contribute to Cell Plate Formation and Root Development in Arabidopsis** **Submitted to Plant Physiology**	**No**	**No**	**Yes**	**No**

9. Recognized International Conference(s) In Which Paper(s) Related To This Research Project Was / Were Delivered
(Please attach a copy of each conference abstract)

Month / Year / Place	Title	Conference Name	Submitted to RGC *(indicate the year ending of the relevant progress report)*	Attached to this Report *(Yes or No)*	Acknowledged the Support of RGC *(Yes or No)*	Accessible from the Institutional Repository *(Yes or No)*
June / 2016 / GyeongJu, South Korean	EHD1-positive Endocytic PM-Recycling Vesicles Contribute to Cell Plate Formation and Root Development in Arabidopsis	27th International Conference on Arabidopsis Research, 2016	Yes, in interim report (reporting to 31/3/2017)	Yes	Yes	Yes
June / 2017 / Hawaii, U.S.A.	Endocytic Recycling Vesicles Function in Root Development	Plant Biology 2017	No	Yes	Yes	Yes

March / 2019 / Nagoya, Japan	EHD1 Define Endocytic Recycling Pathway for Cell Plate Formation and Root Development in Arabidopsis	Japan-Taiwan Plant Biology 2019	No	Yes	Yes	Yes

10. Whether Research Experience And New Knowledge Has Been Transferred / Has Contributed To Teaching And Learning
(Please elaborate)

Research experience of the PI and new knowledge generated on protein trafficking pathway in plant cells, for example, the contribution on the endocytic recycling pathway to cytokinesis and root development, have been incorporated into the topics of "protein trafficking in plant cell", "plant physiology & development" of a General Education elective module titled "Plants & Human Civilisations", which the PI acted as the module convenor.

Students were further engaged into the topic of protein trafficking with the laboratory session on observing different types of plant cells and organelles under light microscope. With these inputs provided, students from a diverse background have a better understanding on the impacts of protein trafficking in plant development and subsequently onto issues which would affect mankind (e.g. crop yield, resistance to environmental stress and food production).

11. Student(s) Trained
(Please attach a copy of the title page of the thesis)

Name	**Degree Registered for**	**Date of Registration**	**Date of Thesis Submission / Graduation**
NIL	NIL	NIL	NIL

12. Other Impact
(e.g. award of patents or prizes, collaboration with other research institutions, technology transfer, teaching enhancement, etc.)

The research project is a collaboration between Technological and Higher Education Institute of Hong Kong and The Chinese University of Hong Kong.

The work on protein trafficking has enhanced the teaching of a General Education elective module titled "Plants & Human Civilisations" (please refer to Q10).

13. Statistics on Research Outputs

	Peer-reviewed Journal Publications	Conference Papers	Scholarly Books, Monographs and Chapters	Patents Awarded	Other Research Outputs (please specify)	
No. of outputs arising directly from this research project	1 (under review)	3	0	0	Type	No.
					NIL	NIL

14. Public Access Of Completion Report

(Please specify the information, if any, that cannot be provided for public access and give the reasons.)

Information that Cannot Be Provided for Public Access	Reasons
NIL	NIL